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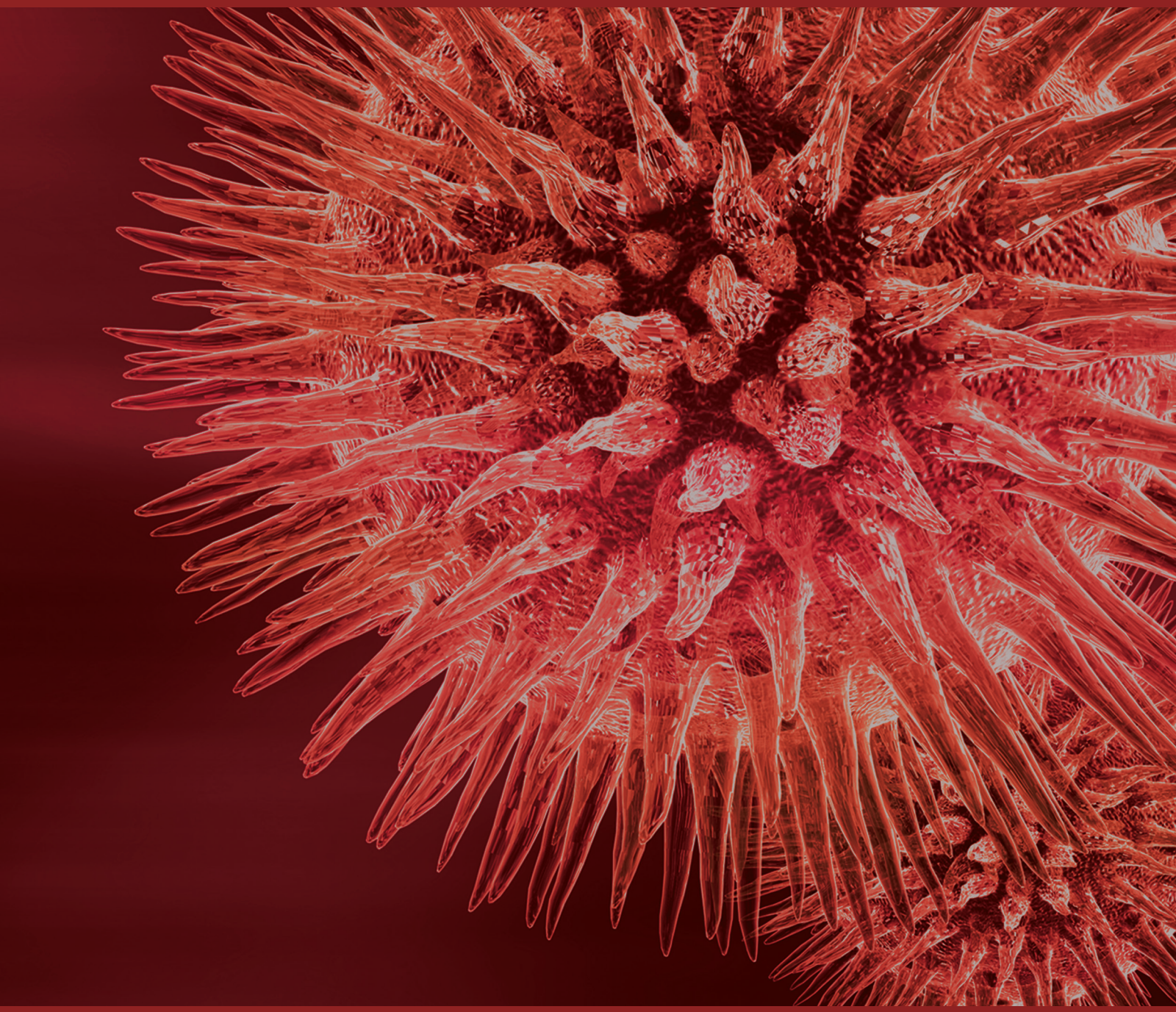
Guest Editors: M-Saadeh Suleiman, Malcolm Underwood, Hajime Imura, and Massimo Caputo



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Editorial

Cardioprotection during Adult and Pediatric Open Heart Surgery

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Myocardial reperfusion damage following cardioplegic ischemic arrest is a key determinant of postoperative organ functional recovery, morbidity, and mortality in adult and pediatric patients undergoing open heart surgery. The vulnerability of the diseased heart to ischemia and reperfusion is different for different pathologies or associated disease (e.g., coronary disease, hypertrophy, diabetes, etc.) and different age (e.g., neonate, infant, children, and adult). These differences and the changing nature of adult patients (e.g., aging population) present a major challenge in translating novel interventions. Thus far, hyperkalemic cardioplegic solutions, which by arresting the heart preserve substrates and delay the onset of the ischaemic insult, remain the corner stone for cardioprotection during open heart surgery. Ongoing strategies to improve myocardial protection include the inclusion of various additives that aim at reducing the damaging effects of ischemia and reperfusion (e.g., calcium overload, metabolic derangement, and accumulation of reactive oxygen species). Recent and novel strategies have also included gene and cell therapies.

In this special issue, several reviews and research articles have provided novel interpretations and data to help in the search for designing an optimal strategy to reduce myocardial injury during cardiac surgery and thus improve long term cardiac functional recovery. For example, a strong argument has been made for the potential role of inhibiting monoamine oxidases (MAOs) in cardioprotective strategies (O. M. Duicu et al.). The activity of this enzyme is linked to oxidative stress and the central role of mitochondria in disease and death. It is therefore recommended to test this in a prospective study in cardiac patients with and without diabetes undergoing heart surgery. In their review, A. Habertheuer et al. point out the importance of the changing characteristics of cardiac surgery patients and propose that better understanding of the associated molecular changes could offer new directions for the design of new more appropriate cardioprotective regimens.

N. Lakusic et al. address the very interesting topic linking changes in heart rate variability after coronary artery bypass grafting to postoperative morbidity. There is clearly an important role of the autonomic nervous system in the consequences of ischemia/reperfusion injury. They emphasize the fact that several studies have shown a reduction in heart rate variability after coronary artery bypass grafting surgery. They point out the need for a study investigating the link between decreased heart rate variability and the outcome of coronary artery bypass graft surgery patients. R. Wagner et al. focus on myocardial conditioning and its therapeutic cardioprotective potential. In this respect, they point out that despite the extensive experimental studies, almost all cardioprotective therapies have failed in the third phase of clinical trials. They propose that the evolutionary young cellular mechanisms of protection against oxygen handling are not very robust. An experimental study by T. Sato et al. suggests that insulin activated survival pathways facilitate preservation of cardiac

contractility during ischemia-reperfusion injury in the isolated rat heart in a way that could be similar to conditioning-induced protection.

E. W. Kuhn et al. present data from a pilot trial investigating the effect of cardioplegia temperature on endothelial injury in patients undergoing on-pump coronary artery bypass graft surgery. They demonstrate perioperative endothelial injury and showed that cold is better than warm blood cardioplegia. A relevant review by Q. Yang et al. points out that coronary endothelial dysfunction occurring during cardiac surgery could be due to functional alteration of endothelial channels and that these channels could be potential targets for endothelial protection during cardiac surgery.

The developing heart and myocardial protection during pediatric cardiac surgery is an area in need of more research. A. Mokhtari and M. Lewis address the very important issue of controlled reoxygenation in cyanotic paediatric patients undergoing open heart surgery. The finding that cardiopulmonary bypass triggers cardiac injury prior to cardioplegic arrest [1] highlights the need for controlling reoxygenation during cardiopulmonary bypass. Recent studies have successfully demonstrated the benefits of this approach [2]. M. Cherif et al. investigated the involvement of Gab1 (Grb2 associated binding protein 1), a protein required for fibroblast cell survival and in maintaining cardiac function. They showed that this protein was upregulated in hearts of cyanotic children possibly as part of survival signaling response to hypoxia. Finally, M. Shirakawa et al. have provided evidence showing that propofol at a clinically relevant concentration is cardioprotective in the immature heart. This anesthetic has already been shown to be protective in adult models when used in cardioplegia [3] and has been included in cardioplegic solutions during surgery in patients with isolated coronary artery bypass grafting or aortic valve replacement using cardiopulmonary bypass [4].

We hope that this special issue provides the readers with new insights into different approaches used to protect the adult and the pediatric heart against the damaging effects of ischemic and reperfusion injury. If anything, the work described emphasizes the need for a more comprehensive strategy taking into account pathologies and age.

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Massimo Caputo

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Review Article

Monoamine Oxidases as Potential Contributors to Oxidative Stress in Diabetes: Time for a Study in Patients Undergoing Heart Surgery

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Oxidative stress is a pathomechanism causally linked to the progression of chronic cardiovascular diseases and diabetes. Mitochondria have emerged as the most relevant source of reactive oxygen species, the major culprit being classically considered the respiratory chain at the inner mitochondrial membrane. In the past decade, several experimental studies unequivocally demonstrated the contribution of monoamine oxidases (MAOs) at the outer mitochondrial membrane to the maladaptative ventricular hypertrophy and endothelial dysfunction. This paper addresses the contribution of mitochondrial dysfunction to the pathogenesis of heart failure and diabetes together with the mounting evidence for an emerging role of MAO inhibition as putative cardioprotective strategy in both conditions.

1. Introduction

According to the World Health Organization, cardiovascular diseases represent the number one cause of death globally (WHO March 2013). In particular, coronary heart disease is a leading cause of mortality and morbidity due to heart failure (HF). With an increasingly aging population and improved survival after the onset of HF in elderly, the syndrome is recognized as a growing problem for the health-care systems worldwide due to its enormous financial burden [1]. Diabetes mellitus (DM), the most severe metabolic disease, is currently viewed as a serious threat to global health due to its increasing prevalence, especially in developing countries; it is predicted that 592 million people will have diabetes by 2035 [2]. The association of type 2 DM with increased cardiovascular morbidity and mortality is widely recognized [3] with both traditional and nontraditional risk factors being involved [4]. This is particularly true for the association between HF and diabetes, since according to the Framingham Study the frequency of HF was significantly higher in diabetic patients (mainly in women) as compared to the age-matched healthy subjects [5]. In the past two decades, mounting

epidemiological and clinical evidence suggests that DM increases the risk for the so-called "diabetic cardiomyopathy" that develops independently of other risk factors such as coronary disease and hypertension [6].

Oxidative stress is the common pathomechanism that greatly influences the progression of both cardiovascular and metabolic diseases. The difficulty to assess the redox pathophysiology is related to both its spatiotemporal heterogeneity and the existence of complex networks of redox signaling as well as the amplification of ROS generation that occur in pathological conditions. This latter condition, known as either "ROS-induced ROS release" [7, 8] or the "kindling radicals" concept [9, 10], refers to the situation in which extramitochondrial (or even mitochondrial) ROS are acting mainly as triggers for mitochondrial ROS production. From mechanistic point of view, this crosstalk to and from mitochondria [9] renders the complete characterization of a pathological entity in a particular model when using the causal reasoning difficult [11]. However, from therapeutic point of view, this crosstalk among several ROS generators appears to be advantageous since there is sound experimental evidence for the partial or even complete abrogation of oxidative stress (and of its deleterious consequences) by inhibiting a single source of ROS [12]. The complexity of the prooxidative status in patients with HF and DM is further contributed by the chronic low-grade inflammation with the induction of a vicious, self-perpetuating circle, responsible for the: (i) aggravation of the oxidative stress via ROS generation by the activated monocytes/macrophages [13–15] that can also interact with cardiac cells [16] and (ii) activation of the inflammasome and phagocytes by ROS originating in cardiac mitochondria [17–20].

The prominent sources of cardiovascular oxidative stress in HF and DM are mitochondria, uncoupled eNOS, and nicotinamide adenine dinucleotide phosphate (NADPH, Nox) oxidases (reviewed in [11, 21–25]). Whereas in case of Nox conflicting data have been reported in the literature, with both protective [26] and deleterious [27] roles of Nox4-derived ROS in the development of HF, the contribution of mitochondria and eNOS as major sources of intracellular oxidative stress is a widely accepted concept [10, 28–30]. However, in the past decade, the contribution of monoamine oxidases (MAOs), FAD-containing dehydrogenases located at the outer mitochondrial membrane, as novel sources of obligatory ROS generation in the cardiovascular pathology, has become evident [31].

Here, we briefly review the contribution of mitochondrial dysfunction to the pathogenesis of heart failure and diabetes, pointing out the commonalities between these two conditions. We will further refer to the beneficial effects of MAO inhibition in relation to cardiovascular pathology and experimental diabetes. Finally, we will emphasize the need for a translational approach, assessing the contribution of MAO-related oxidative stress to the pathogenesis of mitochondrial, endothelial, and contractile dysfunction in diabetic *versus* nondiabetic patients undergoing heart surgery.

2. Mitochondrial Dysfunction in Heart Failure

Mitochondria, the powerhouses of our cells that provide the main amount of energy required for normal cardiomyocyte function, have emerged in the past decades as the major sources and amplifiers of oxidative damage in the cardiovascular system [32].

Heart failure is a multietiological clinical syndrome that develops progressively as a consequence of a primary cause (acute or chronic) that impairs the systolic function (HF with reduced ejection fraction) and/or the diastolic one (HF with preserved ejection fraction). In the vast majority of cases, the primary event is represented by either a chronic hemodynamic (pressure or volume) overload or an acute coronary syndrome that triggers the pathological hypertrophy and ultimately the development of HF. Several experimental models (mainly mechanically or genetically induced hypertension and coronary artery ligation) have been used to mimic these conditions and shed light on the pathophysiology of the syndrome.

In the past decade, mitochondrial dysfunction and the subsequent disrupted redox signaling have been systematically reported to underlie both the development of pathological ventricular hypertrophy and its progression towards the overt cardiac failure (reviewed in [21, 22, 33, 34]). At variance from physiological (adaptive) hypertrophy, where mitochondrial function increases in order to maintain adequate cardiac function [35], pathological (maladaptive) hypertrophy and heart failure have been reported to share similar mitochondrial abnormalities [22] with respect to (i) substrate metabolism (decreased fatty acid oxidation plus increased/unchanged or decreased glucose oxidation in advanced stages of HF, responsible for the energetic deficiency [25]), (ii) calcium handling [36, 37], (iii) respiratory function (decreased in most of the cases; see below), and (iv) ROS production (variable degrees of oxidative stress [38]).

The contribution of metabolic impairment with the subsequent energetic dysfunction to the pathogenesis of HF and its therapeutic potential will not be addressed here (the reader is referred to several excellent reviews [39–43] of the field). Similarly, the role of impaired calcium uptake, release, and signaling in the development of cardiac dysfunction has been comprehensively characterized [36, 37, 44]. We will focus instead on the alteration of respiratory function and its consequence, oxidative stress.

Oxidative phosphorylation represents the ultimate source of aerobic ATP production and requires the coordinated activity of the electron transport chain (ETC) consisting of enzymatic complexes I–IV and complex V (ATP synthase) at the inner mitochondrial membrane. Impairment of the ETC activity is responsible on one side for the reduced ATP generation by ATP synthase (complex V) and on the other side for the increased superoxide production mainly at complexes I and III of the ETC due to partial reduction of oxygen [45–48]. However, it has to be mentioned that mitochondria are endowed with a robust ROS-detoxifying network comprising both enzymes and nonenzymatic antioxidants that are able to counteract even a significant oxidative burden in physiological conditions. Indeed, generation of superoxide, hydroxyl anions, and hydrogen peroxide by the ETC complexes becomes relevant only in pathological conditions [49]. The term oxidative stress refers to a persistent imbalance between ROS generation and detoxification; however, the vast

majority of studies have addressed the issue of ROS emission (defined as the difference between ROS production and ROS removal) without concomitant assessment of status of the antioxidant response [50].

The current evidence for ETC dysfunction and mitochondrial ROS production shows a broad variability in animal models of HF and humans with HF of different etiologies. The impairment of the ETC activity (in particular, of complexes I and III as the major sites for ROS production) in the failing myocardium has been reported in various models of HF. Ide et al. showed a decreased complex I activity with subsequent electron leakage and increased superoxide production in a model of HF induced in dogs by rapid ventricular pacing whereas the superoxide dismutase activity was not changed [51]. These authors further demonstrated in the same model a significant positive correlation between the cardiac production of superoxide and hydroxyl radicals (directly assessed by electron spin resonance spectroscopy) and the left ventricular contractile dysfunction [52]. The activities of complexes III and V have also been reported to decrease in the same experimental model of pacing-induced left ventricular failure in dogs; this paper also reported increased aldehyde levels in left failing ventricles as indirect measure of increased oxidative stress [53, 54].

In an elegant series of studies, the group of Torsten Doenst analyzed the occurrence of mitochondrial dysfunction in relation to the type of contractile abnormalities. In the rat model of HF induced by chronic pressure overload they reported a decline in complex I (but not in complex II [22]) supported respiration in isolated mitochondria that occurred in association with systolic dysfunction (diagnosed by impaired ejection fraction) 20 weeks after the induction of transverse aortic constriction (TAC) [55]. Of note, in this model, diastolic dysfunction occurred prior to the impairment of mitochondrial respiratory capacity. Interestingly, the same group recently also reported in the same experimental model (HF with systolic dysfunction 20 weeks after TAC) that the onset of diastolic dysfunction was coincident with the maximal ROS production; conversely, the occurrence of contractile dysfunction at 20 weeks was no longer related to the ROS production and was not reversed by the antioxidant interventions [56]. Similarly, in the rabbit model of pressure-overload induced HF, dysfunction of mitochondrial complexes I and II occurred during the transition from compensated left ventricular hypertrophy to overt failure and was also independent of ROS production [57]. In another experimental model of HF due to pressure overload, the spontaneous hypertensive rat, a defect in complex IV was demonstrated [58].

ETC defects were also associated with the murine model of HF induced by the coronary artery ligation. Ide et al. reported a decrease in enzymatic activity of the complexes I, III, and IV containing several mitochondrially encoded subunits (but not of the nuclear encoded complex II) and a parallel reduction in mtDNA-encoded gene transcripts, a significant increase in levels of hydroxyl radicals and lipid peroxides, changes that were associated with ventricular dilation and decreased contractility [59].

An important decrease in mitochondrial respiratory capacity was also found in a canine model of moderate HF induced by coronary microembolization in the presence of normal activities of ETC complexes, an effect that was assigned to the lack of assembly of complexes constituting the so-called respirasomes [60]. Rosca et al. considered the decrease in functional respirasomes in HF as the primary event responsible for the decreased oxidative phosphorylation and the increased ROS production leading to the progressive decline in cardiac performance [21, 61]. These authors also reported that, depending on the experimental model, mitochondrial subpopulations are differentially affected: whereas, in the canine model of intracoronary microembolization, both populations were equally affected, in the rapid ventricular pacing model, a significant decrease in oxidative phosphorylation was found in the interfibrillar mitochondria (but not in the subsarcolemmal population). Moreover, the isolation technique significantly accounts for the magnitude of the reported mitochondrial defect and explains the heterogeneity of the experimental and clinical data [21].

A great variability also characterizes the defects of ETC complexes reported to occur in the failing human heart. An important decrease of the respiratory capacity was reported in saponin-skinned muscle bundles obtained from myocardium of explanted human hearts with end-stage HF: in one study state 3 respiration was found to be significantly lower in endocardium *versus* the epicardium [62] and in the other the impairment of complex I-linked respiration was reported to occur early in the development of HF [63]. Similarly, Scheubel et al. reported a moderate decrease in complex I activity in left ventricular specimens harvested from explanted human hearts [64]; this decrease occurred in the absence of mtDNA damage, an observation that supports the hypothesis that the failing human heart is not irreversibly damaged [65]. Recently, Stride et al. reported a marked reduction in oxidative phosphorylation in left ventricle biopsies obtained from patients with chronic ischemia and systolic dysfunction (ejection fraction <45%) for complex II-supported respiration, an increased ROS production, and a tendency for decreased antioxidant defense in the ischemic tissue; however, the degree of coupling was comparable in mitochondria harvested from the ischemic and nonischemic tissue of the same heart [66]. We have previously reported that complex I- (but not complex II-) supported respiration is impaired in atrial appendages harvested from coronary patients with preserved systolic function (ejection fraction >50%) [67]. At variance from all the previous reports, in a recent study performed in freshly isolated mitochondria from failing ventricles, complex I-dependent respiration was reported to be coupled and enhanced in the failing hearts, whereas complex II-dependent succinate respiration was associated with greater uncoupling [68]. However, no major differences were found in the capacity of mitochondria to oxidize different substrates supplied *ex vivo*, a finding that reinforces the observation that reversible mitochondrial damage occurs in the failing hearts. Interestingly, these authors reported a reduced state 3 respiratory rate for succinate in the subgroup of diabetic

patients, an observation suggestive for an impairment of mitochondrial respiratory capacity in the failing hearts in the presence of diabetes.

3. Mitochondrial Dysfunction in Diabetes

The term diabetic cardiomyopathy refers to the association of left ventricular hypertrophy/remodeling with diastolic dysfunction that precedes the development of systolic dysfunction and may progress to heart failure [69]. Elucidation of the pathogenesis of diabetic cardiomyopathy is currently an active field of research. In particular, metabolic impairment and mitochondrial dysfunction have been systematically investigated in the past decades in both clinical and experimental settings (reviewed in [70–74]). We will further refer to the impairment of respiratory capacity and the subsequent redox imbalance in order to highlight commonalities with the aforementioned findings in HF. Early studies performed in rats with type 1 DM pharmacologically induced with streptozotocin firstly mentioned the contribution of mitochondria to the diastolic dysfunction [75] and reported the decrease in succinate-supported respiration and complex II activity; the latter change was attributed to the generation of an adduct of hydroxynonenal and complex II [76]. However, most of the knowledge of mitochondrial dysfunction was gained from genetically modified rodents that recapitulate the metabolic phenotype of humans with obesity and type 2 diabetes. In spite of some differences in pathophysiological mechanisms underlying cardiomyopathy in type 1 and type 2 of experimental diabetes, compromised mitochondrial energetics is a common feature in both types of diabetes [77]. Accordingly, depressed state 3 respiration was reported to occur in experimental models of type 1 [78, 79] and type 2 diabetes [80], and also in obesity with insulin resistance [81]. In the latter study, the decrease in oxidative phosphorylation capacity was associated with increased production of H_2O_2 and mitochondrial uncoupling, a process that decreases cardiac efficiency and may underlie the increased propensity of diabetic hearts to develop HF [82].

As in the case of HF, whether functional differences occur in cardiac mitochondrial subpopulations has been also investigated in a murine model of type 1 diabetes [83]. Complex II-supported respiration was decreased to a greater extent in interfibrillar mitochondria (as compared to subsarcolemmal ones). In the former population, a decrease in complex I respiration was also reported together with an increased production of superoxide and a decrease in cardiolipin. However, it is not clear if the active ADP-stimulated respiratory rate as indicator of maximal respiratory capacity was also depressed in this study.

Mitochondrial dysfunction has also been confirmed in the diabetic human heart. Neufer's group reported a decreased glutamate and fatty acid-supported respiration and an increased sensitivity to Ca^{2+}-induced permeability transition in permeabilized myofibers prepared from right atrial appendages harvested from coronary patients with type 2 diabetes; these authors also demonstrated the increase in oxidative stress as shown by a greater rate of H_2O_2 emission, glutathione depletion, and increased levels of hydroxynonenal and nitrotyrosine-modified proteins, respectively. Importantly, they also reported an inverse relationship between respiratory capacity and HbA1c [84, 85]. More recently, a decrease in complex I and fatty acid-mediated active respiration was found in subsarcolemmal (but not in interfibrillar) mitochondria isolated from atrial appendages of type 2 diabetic patients, regardless of the levels of HbA1c and hyperglycemia [86]. In another elegant study, the impairment in mitochondrial function and dynamics has been associated with contractile dysfunction in diabetic (but not in obese) patients; however, in this case, mitochondrial dysfunction correlated with the level of glycated haemoglobin [87].

The past decade of research provided convincing evidence that mitochondrial dysfunction is a central event in the pathogenesis of HF and DM. This concept extends far beyond the impairment of respiratory capacity and the generation of oxidative stress and includes several other pathomechanisms such as: impaired mitochondrial biogenesis, posttranslational modification of mitochondrial proteins, metabolic shifts and remodeling, and abnormal calcium handling that occur in both pathological conditions. Thus, it becomes more and more evident that the "common soil" hypothesis [88] proposed almost two decades ago (postulating that cardiovascular diseases and diabetes share common genetic and environmental risk factors) should be extended to include mitochondrial dysfunction as well.

4. Monoamine Oxidases as Novel Sources of Mitochondrial Oxidative Stress in Cardiovascular System

In the past decade, monoamine oxidases (MAOs) have emerged as another important mitochondrial source of oxidative stress in the cardiovascular system (please see [31] for a recent comprehensive review). MAOs are flavoproteins located in the outer mitochondrial membrane where they catalyze the oxidative breakdown of endogenous monoamines and dietary amines, with the constant generation of H_2O_2, aldehydes, and ammonia as byproducts. Two isoforms, MAO-A and MAO-B, with specific tissue distribution and substrate affinity, have been described [89]; in experimental settings, pharmacological criteria are useful to characterize the isoenzymes: MAO-A is selectively inhibited by low doses of clorgyline and MAO-B is blocked by low doses of deprenyl (selegiline) [90].

MAOs-related oxidative stress unequivocally contributes to acute myocardial ischemia/reperfusion injury [91] and to the mitochondrial dysfunction and pathologic hypertrophy elicited by pressure overload in a murine model of HF [92, 93]. Of note, MAO-A protein has been reported to be overexpressed in all the experimental models of HF induced in rat by hemodynamic overload (pressure and volume) and coronary ligation [31]. Also, MAO-A activity has been reported to increase in response to angiotensin II, an observation relevant for the clinical settings of heart failure and diabetes where the renin-angiotensin system is upregulated [94].

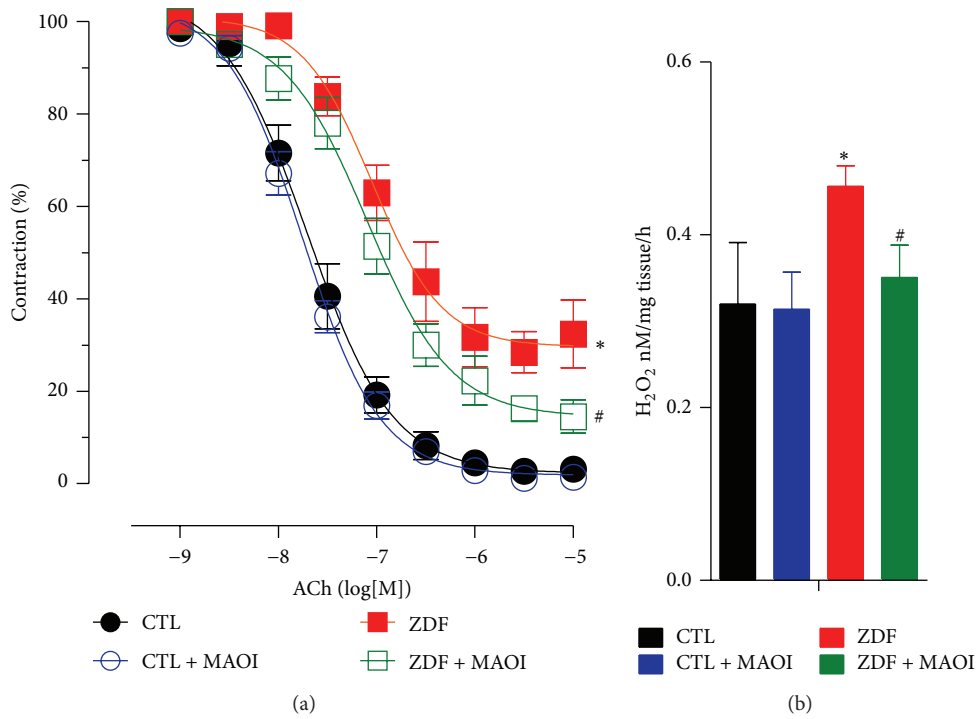

Figure 1: Effects of MAO-A inhibition on vascular function in isolated rat aortas. (a) Acetylcholine-induced endothelium-dependent relaxation in phenylephrine-preconstricted aortic segments ($n = 4$, $^{*}P < 0.05$ with and without diabetes; $^{\#}P < 0.05$ with and without MAO inhibitor, clorgyline, 10 μmol/L). (b) Assessment of H_2O_2 formation by ferrous oxidation xylenol orange (FOX) assay in the presence or absence of the MAO inhibitor ($n = 4$, $^{*}P < 0.05$ with and without diabetes; $^{\#}P < 0.05$ with and without clorgyline, 10 μmol/L).

Also, MAOs have emerged as mediators of experimental endothelial dysfunction via the excessive H_2O_2 production in two murine models of acute (induced with lipopolysaccharide, LPS) and chronic (induced with angiotensin II and A II) oxidative stress, respectively [12]. In this study, we demonstrated that exposure of mouse aortas isolated in organ bath to *exogenous* MAO elicited endothelial function via a ROS-dependent mechanism. Importantly, both the impairment of endothelial-dependent relaxation and H_2O_2 emission were partially reversible in the presence of pharmacological inhibition of MAO-A (with clorgyline and moclobemide) and MAO-B (with selegiline), respectively. Importantly, in this model, endogenous vascular catecholamines are sufficient to activate MAO to induce endothelial dysfunction (no exogenous substrate was added in the experiment). The mechanism was most probably related to the decreased vascular generation of nitric oxide since in a separate set of experiments MAO-A was found to limit the endothelial accumulation of cyclic guanosine monophosphate. We further investigated, in organ bath experiments, the contribution of *endogenous* MAO as mediator of endothelial dysfunction. We found that both MAO isoforms are expressed in the vascular system and induced in response to LPS and A II via the NF-κB and phosphatidylinositide 3-kinase signaling [95]. *In vivo* exposure to A II and LPS increased MAO expression in aortic rings and acute MAO inhibition partially restored normal endothelium-dependent relaxation in vessels harvested from A II and LPS treated animals; this effect was associated with a reduction in the vascular formation of H_2O_2 [12].

We also recently demonstrated that MAO-A inhibition corrects endothelial dysfunction in Zucker diabetic fatty rat (ZDF), a genetic model of type 2 diabetes [96]. In organ bath experiments, preincubation for 30 min with the MAO-A inhibitor, clorgyline, significantly improved the endothelium-dependent relaxation of the aortic rings isolated from ZDF rats and had no effect on vascular relaxation in control aortic rings. Also, vascular H_2O_2 generation was increased in diabetic vessels and significantly decreased in the presence of clorgyline (10 μmol/L, Figure 1).

Whether basic science's predictions on the role of MAO inhibition in the failing heart hold true in humans is not known. A pioneering study has recently reported that atrial activity of MAO assessed in right atrial appendages may serve as an independent predictor for postoperative atrial fibrillation in patients undergoing cardiac surgery [97].

Eugene Braunwald pointed out already back to 1997 that there are two emerging epidemics of cardiovascular disease, heart failure and atrial fibrillation [98]. MAOs contribution to both conditions has been documented. In line with our experimental data, it is conceivable to address the role of the enzyme in DM which together with obesity are the other two menacing pandemics of the 21st century. Accordingly, contribution of MAO-related oxidative stress to the pathogenesis of endothelial, mitochondrial, and contractile dysfunction in diabetic patients undergoing cardiac surgery should be thoroughly investigated.

Moreover, several studies reported the contribution of NADPH oxidase and eNOS uncoupling to the pathological production of vascular ROS after percutaneous coronary interventions (reviewed in [99]). In line with previously reported contribution of MAOs to the experimental endothelial dysfunction it is tempting to speculate that MAO-derived ROS may be involved in the postprocedural complications such as restenosis and stent thrombosis.

5. Conclusions

The past decade of research provided convincing evidence that mitochondrial dysfunction may be an important event in the development of pathological hypertrophy in both heart failure and diabetic cardiomyopathy. Not only mitochondrial but also endothelial dysfunction is a widely investigated mechanism in cardiometabolic diseases and a valuable therapeutic target. There is an unmet need for novel therapies tailored to reduce the risk of heart failure in patients with diabetes mellitus. Therefore, the design of a prospective study in cardiac patients with and without diabetes undergoing heart surgery aimed at providing further mechanistic insights into the role of MAO as an emerging mitochondrial therapeutic target for cardio- and vasculoprotection is strongly recommended.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Changes in Heart Rate Variability after Coronary Artery Bypass Grafting and Clinical Importance of These Findings

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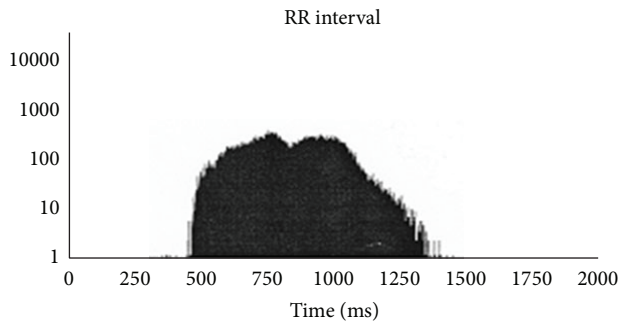
Heart rate variability is a physiological feature indicating the influence of the autonomic nervous system on the heart rate. Association of the reduced heart rate variability due to myocardial infarction and the increased postinfarction mortality was first described more than thirty years ago. Many studies have unequivocally demonstrated that coronary artery bypass grafting surgery generally leads to significant reduction in heart rate variability, which is even more pronounced than after myocardial infarction. Pathophysiologically, however, the mechanisms of heart rate variability reduction associated with acute myocardial infarction and coronary artery bypass grafting are different. Generally, heart rate variability gradually recovers to the preoperative values within six months of the procedure. Unlike the reduced heart rate variability in patients having sustained myocardial infarction, a finding of reduced heart rate variability after coronary artery bypass surgery is not considered relevant in predicting mortality. Current knowledge about changes in heart rate variability in coronary patients and clinical relevance of such a finding in patients undergoing coronary artery bypass grafting are presented.

1. Introduction

Sinus rate is neither constant nor uniform but is changing all the time under the influence of the sympathetic and parasympathetic systems. The impact of the autonomic nervous system on the occurrence and mortality of malignant arrhythmias was demonstrated on an experimental animal model as early as some thirty years ago. Decreased parasympathetic tone or increased sympathetic tone predisposes patients to the occurrence of malignant arrhythmias, even ventricular fibrillation. And *vice versa*, increased parasympathetic tone or decreased sympathetic tone reduces myocardial vulnerability and thus the occurrence of ventricular rhythm disturbances [1]. Such unambiguous experimental evidence has encouraged researchers to search for and develop a method to quantitatively measure autonomic nervous activity. Analysis of heart rate variability (HRV) is one of such indicators of the autonomic nervous system activity.

2. Heart Rate Variability: Basic Concept and Clinical Use

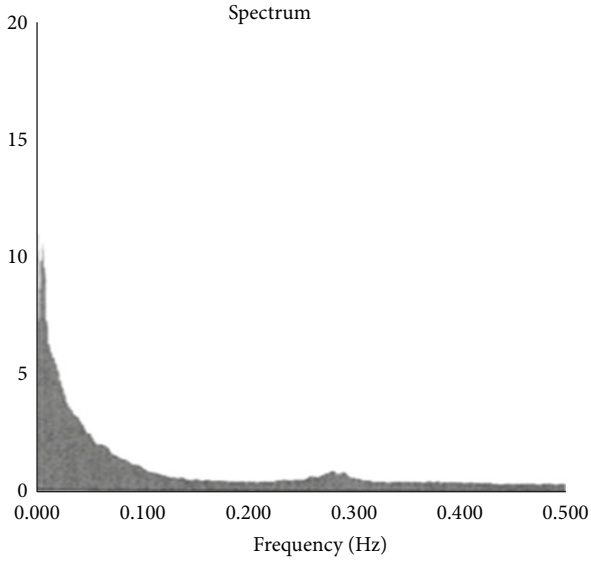
Heart rate variability is a physiological feature that indicates the effect of the autonomic nervous system on the heart action, that is, heart rate [2]. In 1996, the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology issued guidelines on HRV standards of measurement, physiological interpretation, and clinical use [3]. HRV implies two types of changes, that is, variability in the duration of consecutive R-R intervals of the respiratory sinus arrhythmia type and variable



Analysis results	(ms)	/mean
Mean	824.7	1.000
SDNN	159.6	0.194
SDNN-i	67.2	0.082
SDANN-i	138.7	0.168
r-MSSD	27.2	0.033
pNN50	5.659%	0.007

Intervals: 104760

(a) Time domain



	Range (Hz)	Power (ms·ms)
Overall band	0.0000–0.4000	1824.3
Band 1	0.0000–0.0033	235.9
Band 2	0.0033–0.0400	1278.7
Band 3	0.0400–0.1500	243.7
Band 4	0.1500–0.4000	66.0
Band 5	0.4000-0.4000	***
Band 6	0.4000-0.4000	***
Balance	(3/4)	3.7

(b) Frequency domain

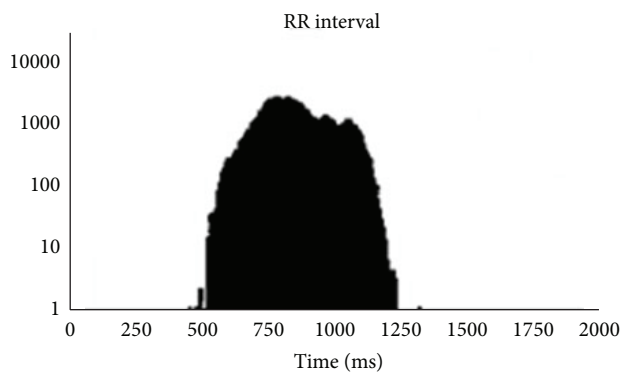
Figure 1: Normal heart rate variability and sympathovagal balance in healthy person (time and frequency domain).

heart rate such as sinus tachycardia oscillations on physical exertion, normal diurnal sinus rhythm, and nocturnal sinus bradycardia [3]. HRV is determined by commercial software from electrocardiograms (ECG) of variable duration, mostly 24-hour Holter ECG recording.

The measures used to express HRV have been obtained by analysis of the length of RR interval in the time domain and frequency domain. Only "normal," nonectopic impulses, that is, those produced by sinus node depolarization, are included in the HRV analysis. In daily clinical routine, standard deviation of all normal RR intervals (SDNN) and mean of R-R intervals for normal beats (Mean RR) are used for HRV measurement and basic analysis.

Other HRV measures used in time domain are standard deviation of the 5-minute means of R-R intervals (SDANNi); mean of the 5-minute standard deviations of RR intervals (SDNNi); square root of the mean of the squared successive differences in R-R intervals (rMSSD); and percentage of R-R intervals that are at least 50 ms different from the previous interval (pNN50). The following measures are used in frequency domain: Total Power (range of frequency 0.0–0.5 Hz)—variance of all RR intervals obtained by spectral analysis that corresponds to the SDNN variable in time domain; components of the ultralow frequency spectrum (ULF; 0.0–0.0033 Hz); very low frequency spectrum (VLF; 0.0033–0.04 Hz); low frequency spectrum (LF; 0.04–0.15 Hz); high frequency spectrum (HF; 0.15–0.4 Hz); and their ratio (LF/HF) (Figure 1), [3].

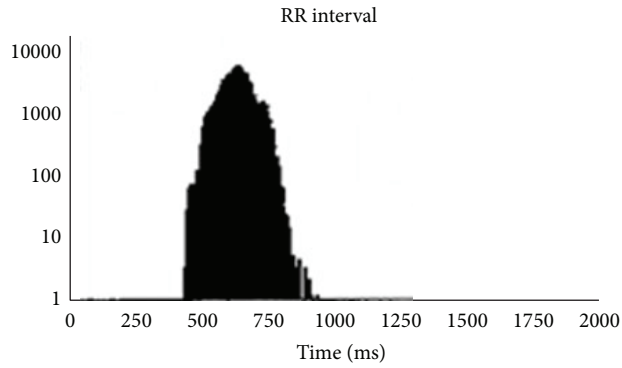
The LF component reflects the sympathetic (and vagal) activity, whereas the HF component along with the rMSSD and pNN50 measures in time domain reflects vagal activity in heart rate modulation. In healthy subjects, the ratio of low frequency and high frequency components (LF/HF) points to the sympathetic and vagal balance, whereas in patients with severely decreased HRV, the LF/HF ratio is very difficult to interpret and its clinical value remains obscure [4]. According to current recommendations [3], SDNN > 100 ms is considered as normal HRV. As the criteria distinguishing pathological from physiological HRV findings have not been clearly identified after release of the guidelines on HRV use [3], Miličević et al. [5] conducted a study on more than 2500 patients in an attempt to define the physiological, moderately decreased, and pathologically decreased HRV values in various groups of cardiac patients. The SDNN < 59 ms



Analysis results

	(ms)	/mean
Mean	837.9	1.000
SDNN	129.8	0.155
SDNN-i	52.0	0.062
SDANN-i	120.0	0.143
r-MSSD	18.3	0.022
pNN50	1.228%	0.001

Intervals: 102987



Analysis results

	(ms)	/mean
Mean	621.1	1.000
SDNN	59.2	0.095
SDNN-i	30.0	0.048
SDANN-i	49.3	0.079
r-MSSD	12.2	0.020
pNN50	0.344%	0.001

Intervals: 131567

Figure 2: Normal and significantly decreased heart rate variability (HRV) (time domain analysis); see SDNN and other measures.

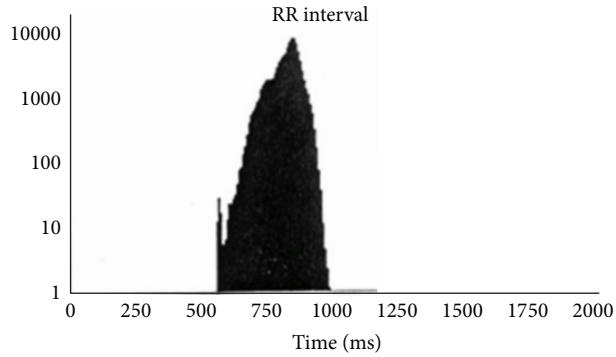
was identified as borderline of pathologically decreased HRV and 93 ms as borderline normal HRV, whereas SDNN values of 59–92 ms were found to indicate mildly to moderately decreased HRV in the "general cardiologic population" [5] (Figure 2). Figure 3 shows pathologically decreased HRV in a patient with subchronic myocardial infarction of the anterior wall and repetitive, nonsustained ventricular tachycardia.

In addition to the above, the researchers also used nonlinear analysis and indices of HRV [6].

3. Heart Rate Variability and Myocardial Infarction

Wolf et al. were the first to describe the association of HRV reduction and increased postinfarction mortality in 1978. Analyzing 1-minute ECG recording obtained in a patient with acute myocardial infarction immediately upon admission to coronary unit, they concluded that patients with sinus arrhythmia, that is, with more pronounced sinus impulse variability, had a lower mortality rate than patients with less pronounced variability of sinus impulses [7]. Acute myocardial infarction almost as a rule leads to considerable HRV reduction [8]. This is caused by ischaemia and partial myocardial necrosis. Noncontractile and necrotic left ventricular segments are known to enhance sympathetic afferent and efferent activity, which is manifested as HRV reduction and eventually leads to greater myocardial vulnerability and electrical instability, as well as to the risk of malignant arrhythmias. Furthermore, sympathetic excitation weakens or inhibits vagus influence on the sinus node, which also contributes to lesser heart rate oscillations and HRV reduction. Decreased HRV points to a reduced response of the heart as the target organ to neural modulation inputs or to the impact of sinus node oversaturation by the continuously high sympathetic tone [9, 10].

Bigger Jr. et al. found HRV to be significantly lower in patients having sustained myocardial infarction even a year after the acute coronary event as compared to healthy age-matched subjects [11]. Various other conditions such as heart failure, heart transplantation, stroke, multiple sclerosis, and cardiac surgery procedures can also entail HRV reduction [12–16]. In 1987, Kleiger et al. published their pioneer work demonstrating that patients with a history of myocardial infarction and a higher risk of sudden death could be identified by use of HRV. Analyzing mortality in patients included in the follow-up study, the authors found the patients with decreased HRV, that is, with SDNN < 50 ms, to be at 5.3-fold greater relative risk of death as those with SDNN > 100 ms [17]. This study was followed by a number of other studies that unanimously confirmed the results reported by Kleiger et al. and defined reduced HRV as a strong marker of rhythmogenic death [18–22].



Analysis results		
	(ms)	/mean
Mean	850.5	1.000
SDNN	53.4	0.063
SDNN-i	20.1	0.024
SDANN-i	47.3	0.056
r-MSSD	13.8	0.016
pNN50	0.078%	0.000

Intervals: 99335

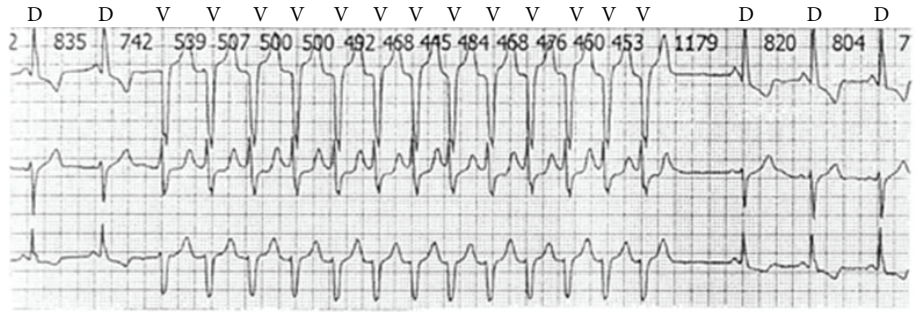


Figure 3: Severely decreased HRV in a patient with subchronic myocardial infarction (see SDNN) and repetitive, nonsustained ventricular tachycardia.

4. Heart Rate Variability and Coronary Artery Bypass Grafting

Many studies have invariably demonstrated that coronary artery bypass grafting (CABG) generally leads to significant HRV reduction, which is even more pronounced than after myocardial infarction [16, 23–29]. HRV reduction after cardiac surgery is not exclusively related to CABG, as it is also recorded in patients undergoing valve surgery [30]. Unlike myocardial infarction where the main reason for this is ischaemia and myocyte necrosis, the probable reasons for considerable HRV reduction immediately after CABG include a combined effect of surgical manipulation during operative procedure on the heart and adjacent anatomical structures, prolonged anaesthesia, cardioplegia, and extracorporeal circulation.

Analyzing HRV differences between patients operated on off-pump *versus* on-pump, Kalisnik et al. conclude that off-pump CABG is also followed by extensive adrenergic activation that is comparable to on-pump CABG [31]. Our results also suggested that there were no differences in HRV a few months after surgery between patients undergoing off-pump and patients undergoing on-pump CABG [32].

Generally, in most patients, HRV recovery to the values measured before CABG occurs gradually within six months of the operative procedure [16, 23]. There are reports indicating that a finding of reduced HRV after CABG is of no relevance in predicting mortality, unlike reduced HRV in patients having sustained myocardial infarction [33–35]. To put it more precisely, the authors of those studies conclude that, unlike the strong prognostic potential of HRV in postmyocardial infarction patients, HRV finding has no prognostic value in post-CABG patients. It is explained by revascularization of the ischaemic or viable myocardial tissue, which exceeds the significance of decreased HRV and autonomic dysfunction [34]. Also, Stein et al. conclude that excluding CABG and diabetic patients from HRV analysis significantly increases the relationship of reduced HRV and mortality rate [33, 35]. Contrary to the reports where decreased HRV after CABG had no significant prediction of mortality, the results of our study indicated that postoperative HRV decrease influenced mortality rate in patients after CABG [35]. Unlike some previous studies comparing mortality of patients having sustained myocardial infarction and CABG patients with reduced HRV [34], we analyzed mortality in the group of CABG patients with normal *versus* decreased postoperative HRV, which could at least in part explain differences in the results. In our study, one-third of patients had reduced and two-thirds had normal postoperative HRV, measured at a mean of 3.7 months after CABG, with the average 3-year follow-up after HRV analysis. In the follow-up period, 7.8% of adverse coronary events (death from diagnosed new myocardial infarction or sudden death) were recorded and the majority of patients had decreased HRV ($P = 0.001$) [36].

Accordingly, it is logical to ask why HRV reduction definitely is of prognostic value in one group of patients like those with myocardial infarction, whereas in another group

of patients like those undergoing CABG such a finding is at least dubious. HRV is decreased to a certain extent in various clinical conditions, but the underlying mechanisms of this reduction are different and that is why the finding of reduced HRV is of different prognostic relevance. In myocardial infarction, HRV reduction is caused by partial myocardial necrosis, in stroke by cerebral parenchymal necrosis, in hyperthyroidism by the effect of elevated thyroid hormone concentrations in the circulation, and in CABG mostly by surgical manipulation and all other instrumentation such as anaesthesia and cardioplegia. For example, treatment of hyperthyroidism results in decreased thyroid hormone concentration in the circulation, reduced heart rate, and consequently HRV normalization [37]. In addition, comorbidities in each individual patient should always be taken in consideration; in CABG patients, these may include diabetes mellitus, heart failure, and previous myocardial infarction. HRV should also be observed in relation to other relevant indicators available, such as left ventricle ejection fraction and patient functional capacity, and only then clinical conclusions can be made. Thus, while a decreased HRV may objectively be a poor prognostic sign in one patient, in another one it will be so to a much lesser extent.

Yet, reduced HRV persisting for months after CABG should raise suspicion in clinicians, in particular if accompanied by a reduced ejection fraction. As ejection fraction correlates well with HRV parameters, prolonged HRV reduction following CABG can also be perceived as a reflection of the level of ejection fraction damage [3, 38].

In conclusion, it is clear that, in the majority of patients, HRV decreases immediately after CABG, with gradual recovery within a few months of the operation. In our opinion, as a guideline for daily clinical practice, it is still unclear whether decreased postoperative HRV several months after CABG has prognostic relevance for the outcome of CABG patients. Correlation between postoperatively decreased HRV and outcome of CABG patients is controversial and additional studies are needed, the more so as the current guidelines on HRV analysis do not answer this question either [3]. It is necessary to conduct studies in a larger sample of patients, in order to acquire additional knowledge and make definitive conclusion on the prognostic value of post-CABG HRV.

According to the results of our previous study [36], we strongly believe that subgroup of patients with decreased HRV a few months after CABG require careful long-term monitoring, diagnostic evaluation, and wide usage of medications with a well-documented favourable effect on HRV and patient clinical outcome [39–42].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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Research Article

Gab1 Is Modulated by Chronic Hypoxia in Children with Cyanotic Congenital Heart Defect and Its Overexpression Reduces Apoptosis in Rat Neonatal Cardiomyocytes

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Gab1 (Grb2 associated binding protein 1) is a member of the scaffolding/docking proteins (Gab1, Gab2, and Gab3). It is required for fibroblast cell survival and maintaining cardiac function. Very little is known about human Gab1 expression in response to chronic hypoxia. The present study examined the hypothesis that hypoxia regulates Gab1 expression in human paediatric myocardium and cultured rat cardiomyocytes. Here we showed that Gab1 is expressed in myocardial tissue in acyanotic and cyanotic children with congenital heart defects. Gab1 protein was upregulated in cyanotic compared to acyanotic hearts suggesting that Gab1 upregulation is a component of the survival program initiated by hypoxia in cyanotic children. The expression of other Gab1 interacting partners was not affected by hypoxia and Gab1 regulation. Additionally, using an *in vitro* model, we demonstrated that overexpressing Gab1 in neonatal cardiomyocytes, under hypoxic condition, resulted in the reduction of apoptosis suggesting a role for this protein in cardiomyocyte survival. Altogether, our data provide strong evidence that Gab1 is important for heart cell survival following hypoxic stress.

1. Introduction

Heart malformation during embryonic development can cause congenital heart diseases (CHD). These affect one baby in 125 live births and tetralogy of Fallot (TOF) represents the most common form of the "blue baby syndrome." In UK, one baby in 3,600 is born with TOF malformation [1]. TOF malformation exhibits four abnormalities. These include a ventricular septal defect (VSD), right ventricular hypertrophy, overriding of the aorta, and pulmonary stenosis (PS) [2]. The causes that induce TOF are not fully understood but the aetiology is thought to be multifactorial. Some studies associated TOF with untreated maternal diabetes, phenylketonuria, and intake of retinoic acid. In addition, chromosomal abnormalities (such as trisomies 21, 18, and 13) have been shown to exhibit a higher TOF incidence [3]. The degree of stenosis varies between individuals with TOF and is the primary determinant of symptoms and severity. Indeed, TOF is divided into two categories: acyanotic (pink) and cyanotic (blue), depending on the blood oxygen saturation. Although successful corrective surgery of heart defects exists, there is an increased risk of morbidity and mortality in cyanotic children compared with acyanotic [4]. There is evidence that an unintended reoxygenation injury occurs in myocardium of cyanotic patients due to the delivery of high levels of oxygen during cardiopulmonary bypass (CPB) used in surgery, which does not match preoperative levels of oxygen in these children. Reoxygenation injury produces an increase in free radical production, which may result in cell damage [5].

In previous study, we have shown that chronic hypoxia in pediatric patients with TOF triggered the expression of network of genes associated with apoptosis and reduced the expression of genes involved in myocyte contractility and function [6]. This state of hypoxia in TOF children may be responsible for the susceptibility of cyanotic children to reoxygenation injury during and after surgery. We have also shown that concomitant with the initiation of the injurious program a protective program is triggered by cyanosis. Gab1, shown to significantly increase at messenger level in cyanotic compared to acyanotic patients [6], could be an important player in this protective program.

Grb2 associated binding protein 1 (Gab1) is a member of the scaffolding/docking proteins (Gab1, Gab2, and Gab3) [7, 8]. Gab1 knockout mice are not viable and display impaired development of heart, placenta, skin, and muscle [9]. In cultured cardiomyocytes, Gab1 is shown to interact with tyrosine phosphatase SHP2 and to promote cardiac hypertrophy [10]. There is evidence that Gab1 is essential for cardiac function in the postnatal heart *in vivo* [11]. In addition, Gab1 has been shown to exert an antiapoptotic role in mouse embryonic fibroblasts and is activated through tyrosine phosphorylation following oxidative treatment (H_2O_2) [12]. In their investigation, Holgado-Madruga and Wong identified Gab1 as an important component in oxidative stress signalling with an essential role in the activation of c-Jun NH(2)-terminal kinase (JNK) and the influencing of cell survival [12]. This Gab1 antiapoptotic role in fibroblasts following oxidative treatment [12] has led us to hypothesise that Gab1 may play similar role in cardiac tissue and cardiac myocytes subjected to hypoxia.

In this study, we investigated the effects of cyanosis on Gab1 in myocardium samples from paediatric patients suffering from TOF and we examined the effects of hypoxia in primary cultures of rat neonatal cardiomyocytes on Gab1 and its possible role in cell survival.

2. Materials and Methods

2.1. Reagents. All reagents were from Sigma (UK) except those stated otherwise. Gab1 antibody was from Millipore. Antibodies against SHP2 and p85 were from Cell Signalling Technology (UK). GAPDH antibody was from Research Diagnostics Inc. (UK).

2.2. Cardiac Biopsies. The collection of human right ventricle specimens used in this study was approved by the North Somerset and South Bristol Research Ethics Committee (REC reference 07/H0106/172), the National Research Ethics Service, England. Parental informed written consent was gained for all patients. Patients with a diagnosis of cyanotic (O_2 saturation $79.6 \pm 7.5\%$; age 10.6 ± 5.5 months) or acyanotic (O_2 saturation $94.2 \pm 3.5\%$; age 9.5 ± 2.3 months) tetralogy of Fallot undergoing surgical repair at the Bristol Royal Hospital for Children were studied.

Five ventricular biopsy specimens per group were collected from the right ventricle of acyanotic and cyanotic TOF patients by using "True-Cut" needle immediately after institution of cardiopulmonary bypass (CPB). Each specimen was immediately put in liquid nitrogen for protein extraction.

2.3. Immunohistochemistry. Right ventricular specimens were fixed in 4% paraformaldehyde, washed in PBS, and embedded in paraffin and 4 μm sections were obtained. Immunohistochemistry was performed using the ABC-Kit from Dakocytomation. Slides were observed with an Olympus B40 microscope. Pictures were taken using a Media Cybernetics camera (Bethesda, MD, USA) and analysed with proimage plus software (Bethesda, MD, USA).

2.4. Rat Neonatal Primary Culture. All the procedures involving laboratory animals conformed to the guidelines and regulations of the University of Bristol and the United Kingdom Home Office. Neonatal rat cardiomyocytes were cultured as previously described with minor modifications [13, 14]. One cell culture preparation was used for each experiment and each experiment was repeated three times using different cell cultures prepared at different time from pooled neonatal rat hearts. For each cell culture, 14–24 neonatal rats were used to harvest hearts. Hearts were quickly removed from one- to three-day-old Wistar rats and only ventricles were kept. They were washed with PBS three times and incubated with 0.05% trypsin and 0.02% EDTA for 30 minutes. They were then enzymatically digested six times for fifteen minutes in 0.1% trypsin and 0.02% EDTA in PBS. Digestion was stopped by addition of foetal calf serum at a final concentration of 30%. Cells were then centrifuged at 400 g for five minutes and resuspended in DMEM supplemented with 10% FBS and 1% P/S. Cells were then incubated one to two hours in a T75 flask to allow noncardiac myocytes (mainly cardiac fibroblasts) to adhere to plastic. They were then plated in gelatin-coated plates at a density of 1.28×10^5 cells per cm^2. After 40 hours of culture, the medium was changed in DMEM supplemented with 20% M-199 and 1% P/S. All treatments were performed on three-day-old cultures. Hypoxia was induced by placing the cells in a hypoxic chamber where oxygen levels could be monitored (Biospherix ProOx C21, Lacona, NY, USA). The ProOx is connected to a sensor, which monitors the oxygen concentration within the host chamber. The ProOx quickly infuses nitrogen (hypoxia) in the chamber and reaches a set point. In this study, the chamber was set to 0.2% O_2 and 5% CO_2 in a humid atmosphere for 24 hours.

2.5. Immunocytochemistry. Cells were grown on gelatin coverslips (Nunc, UK) at a density of 1.32×10^5 cells per cm^2. Immunocytochemistry was performed on normoxic and hypoxic (24 hours, 0.2% O_2, 5% CO_2) cells. Cells were washed twice with PBS, fixed with 4% paraformaldehyde for 15 minutes, washed 3 times with PBS, and washed with 0.1% Triton X-100 3 times for 5 minutes. They were then incubated with NH_4Cl for 10 minutes, washed three times with PBS, and blocked using 5% goat serum (Dakocytomation, Dako, UK) for 45–60 minutes at room temperature. Cells were incubated in Gab1 overnight at 4°C, washed three times, incubated in anti-rabbit conjugated with Alexa-488 (Invitrogen, UK) for 1 hour at room temperature, washed again, and incubated with mouse antisarcomeric actin (Dakocytomation,

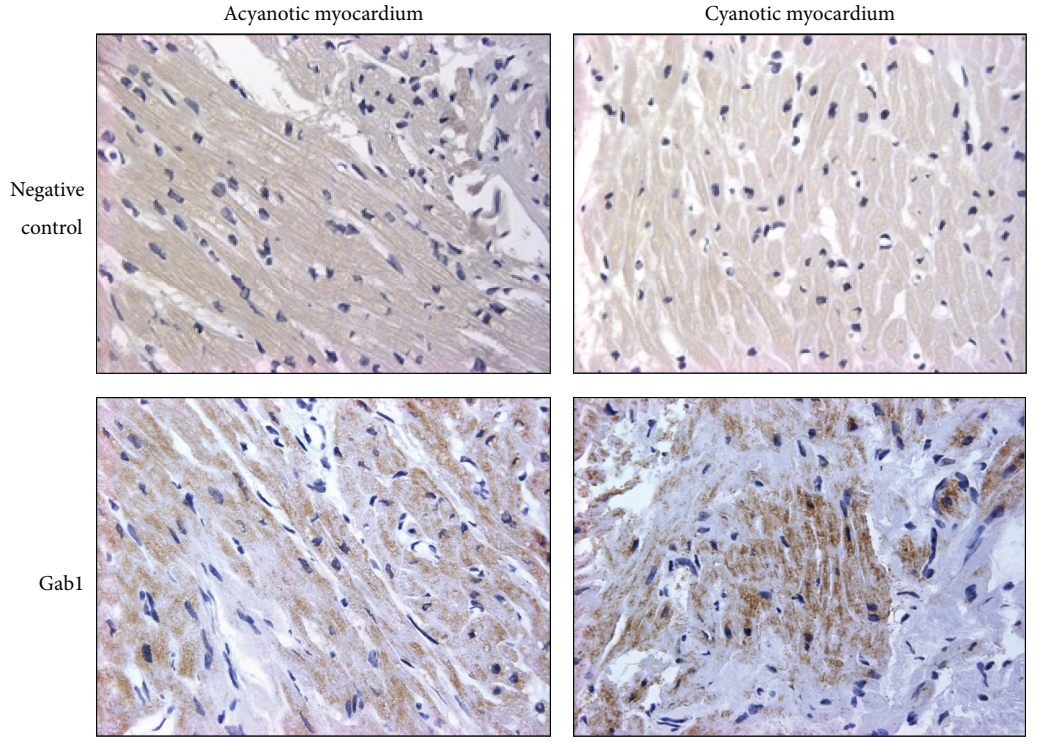

Figure 1: Expression of Gab1 in acyanotic and cyanotic paediatric myocardium tissues. Immunohistochemical analysis of paraffin embedded heart tissue from acyanotic and cyanotic patients using Gab1 specific antibody. Negative controls are section processed without using primary antibody. Magnification: ×200.

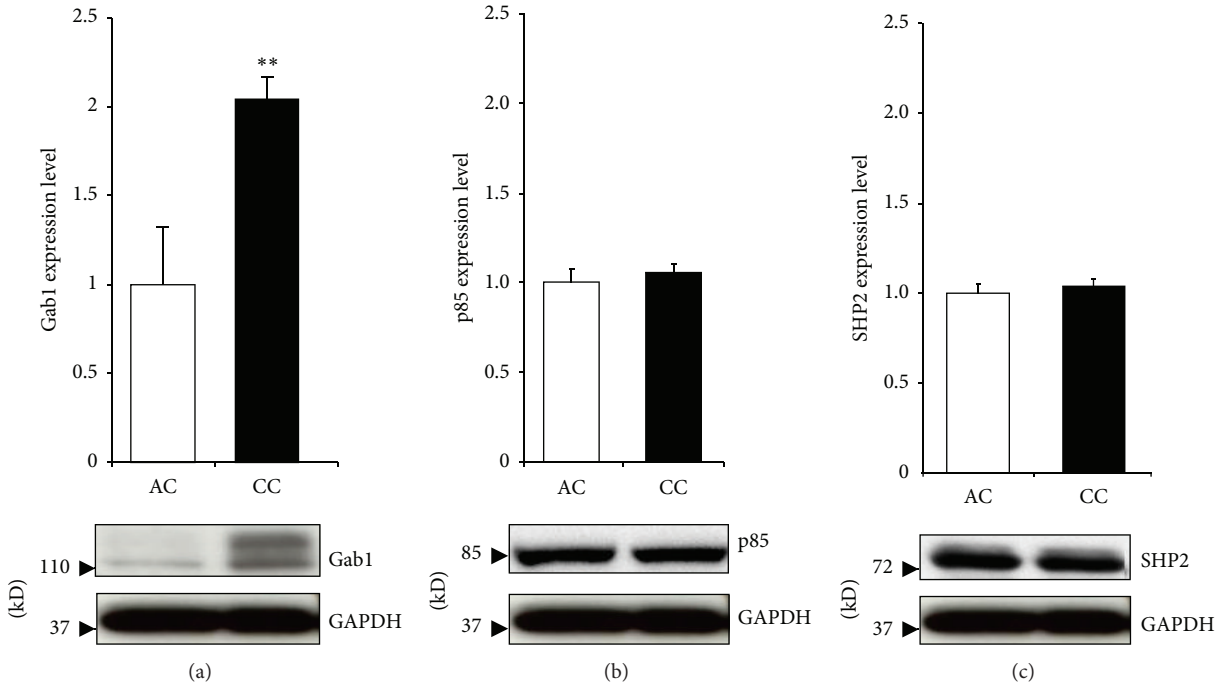

Figure 2: Gab1 protein expression in myocardium of cyanotic (CC) and acyanotic (AC) patients. Myocardium biopsies were homogenized to isolate protein content and western blot analysis performed using Gab1, p85, SHP2, and GAPDH antibodies. All results were normalised to GAPDH levels. Data are mean ± SEM. $^{**}P < 0.01$ ($n = 5$).

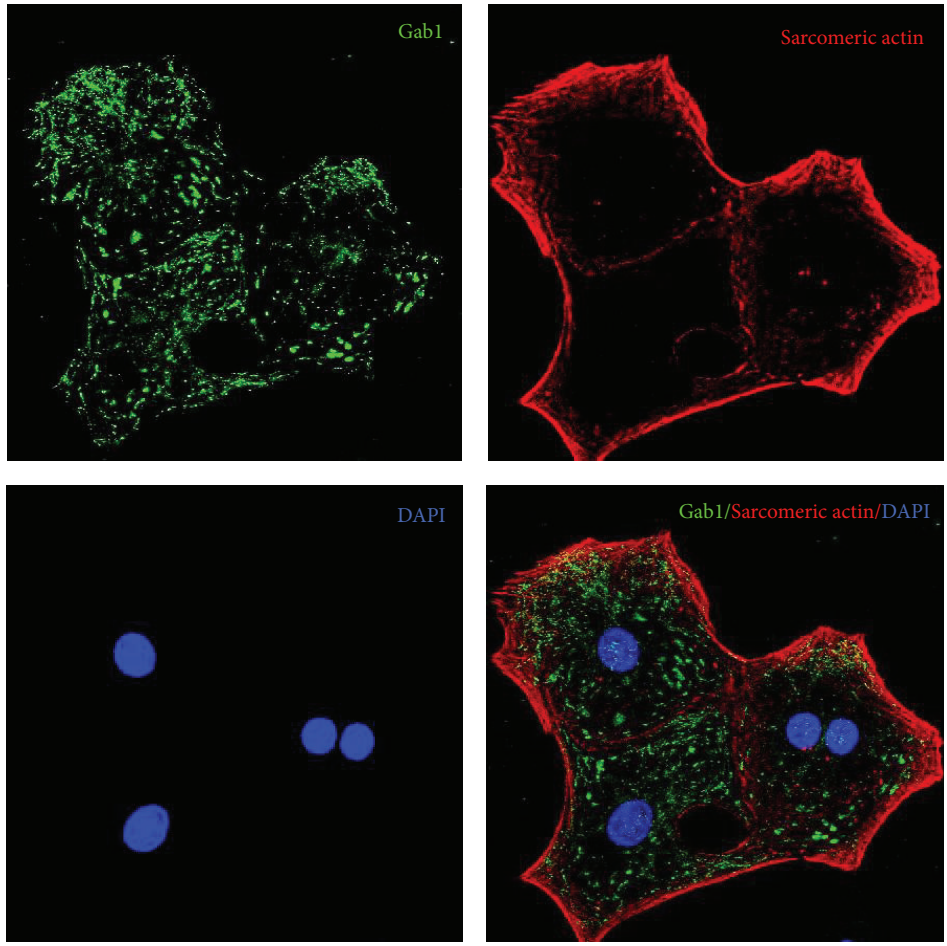

Figure 3: Localization of Gab1 in cultured rat cardiomyocytes. Cells were cultured for 5 days *in vitro* then fixed and stained with specific antibodies. Cardiomyocytes were stained for Gab1 (green) and sarcomeric actin (red) and then counterstained for nuclei with DAPI (blue). Magnification: ×1200.

Dako, UK) and then with anti-mouse conjugated with Texas Red (Vector Laboratories, UK). Cells were then mounted in VECTASHIELD and observed with a Leica AOBS SP2 confocal microscope (MRC Cell Imaging Facility, University of Bristol, UK), using excitation filters at 340–380 nm, 450–490 nm, and 515–560 nm for DAPI, Alexa-488, and Texas Red, respectively. The slides were observed on a 63x lens and pictures were taken using the Leica software (Leica, Bucks, UK).

2.6. Use of Adenoviruses. Adenoviruses expressing Gab1 wild type were previously used [10, 15]. Adenovirus expressing wild type Gab1 was referred to as Ad-Gab1-WT. Control adenovirus expressing Ad-β-galactosidase was a kind gift from Dr. Steve White (University of Bristol, UK). Cardiomyocytes were infected with adenoviruses diluted in DMEM supplemented with 20% M199, 1% FBS, and 1% P/S for 24 hours. Then, the medium was changed to DMEM supplemented with 20% M199 and 1% P/S. The infected cells were then subjected to normoxia or hypoxia for 24 hours.

2.7. Western Blotting. Five myocytes culture dishes (from the same cell preparation) per group (normoxia versus hypoxia) were used in the rat cell culture part. The *in vitro* experiment was repeated three times using new cell preparation each time. Total proteins were extracted from both clinical and rat samples and quantified. For electrophoresis, protein samples were prepared by adding 4x Laemmli buffer (0.24 M Tris pH 6.8, 6% SDS, 40% sucrose, 0.04% bromophenol blue, and 10% β-mercaptoethanol), heated to 95°C for 5 min, and loaded on a 8–10% SDS gel. Separated proteins were transferred to Hybond nitrocellulose membrane (Amersham, UK) which was subsequently blocked in 5% nonfat dry milk/TBS-T (TBS-T; 20 mM Tris pH 7.4, 1.37 M NaCl, 1% Tween) for 1 h and incubated in primary antibodies overnight at 4°C. Membranes were washed three times in TBS-T and then incubated in appropriate anti-rabbit or anti-mouse secondary antibody (Amersham, UK) for 1 h at room temperature. Membranes were washed 3 times in TBS-T, antibody bound HRP was detected using ECL (Amersham, UK), and membranes were exposed to Hyperfilm (Amersham, UK).

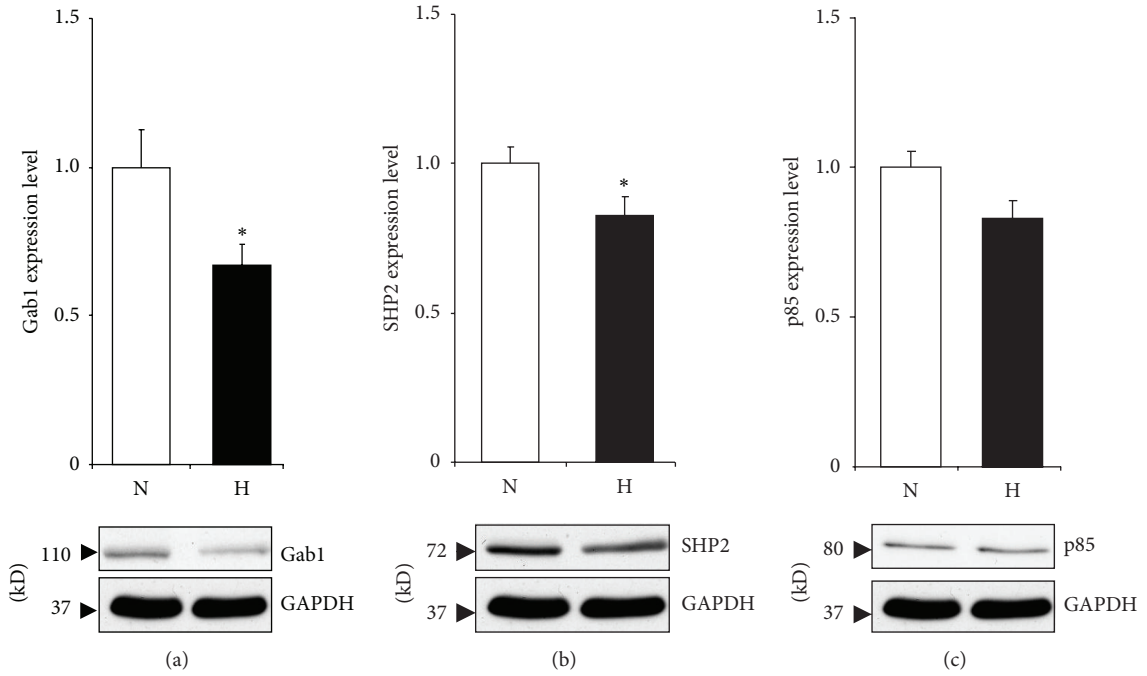

Figure 4: Gab1 protein expression in rat cardiomyocytes following 24 h of hypoxic treatment. Cells were treated with hypoxic conditions (H) by incubating in a chamber with 5% CO_2/0.2% O_2 or normoxia (N) by leaving cells in normal CO2 incubator. After 24 h cells were lysed and western blot analysis performed using GAB1 (a), SHP2 (b), and p85 (c) antibodies. All results were normalized to GAPDH levels. Data are mean ± SEM. $^*P < 0.05$ ($n = 5$).

Protein bands were quantified using NIH Image J software. The expression level of each protein was normalized to the loading control, GAPDH, and then the obtained values for experimental (cyanotic or hypoxic) were divided per control value (acyanotic or normoxia). Representative blots were presented in the western blotting figures.

2.8. Apoptosis Assay. Cell death was investigated with the "*in situ* cell death detection" kit from Roche. Rat neonatal cardiomyocytes were cultured in 4-well chamber slides (Nunc, UK) at a density of 1.5×10^5 cells per cm^2. Cells were washed twice with PBS and permeabilized for 1 hour at room temperature with 0.1% Triton (v/v) in 0.1% sodium citrate (v/v). They were then washed three times in PBS and incubated with the labelled enzyme for 1 hour at 37°C. They were washed twice, mounted in VECTASHIELD containing DAPI, and observed with an Olympus B40 microscope. Apoptosis was quantified by counting apoptotic cells in 25 different fields or 400 cells. Pictures were taken using a Media Cybernetics camera (Bethesda, MD, USA) and analysed with proimage plus software (Bethesda, MD, USA).

2.9. Statistical Analysis. All data were analyzed using the software Instat 3.1 (GraphPad). Results are expressed as ± standard error of mean (±SEM). Statistical significance was assessed by one-way ANOVA or Student's *t*-test. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Gab1 Protein Is Expressed in Human Heart Sections of Both Cyanotic and Acyanotic Congenital Heart Patients. The analysis of fixed paediatric heart biopsy sections by immunohistochemistry showed expression of GAB1 in heart tissue taken from both acyanotic and cyanotic patients undergoing corrective surgery for congenital heart defects (Figure 1).

3.2. Gab1 Protein Expression Is Upregulated in Cyanotic Patients Compared to Acyanotic. Western blot analysis of proteins extracted from children myocardium biopsies revealed a significant upregulation of Gab1 protein expression in cyanotic patients (Figure 2(a)). Additionally, we investigated the protein expression of Gab binding partners, SHP2 and the regulatory subunit of PI3K (p85). The levels of both Gab binding partners proteins were unaffected (Figure 2(b)).

3.3. Gab1 Shows a Cytoplasmic Localization in Neonatal Heart Cardiomyocytes. Gab1 appeared to be expressed in rat neonatal cardiomyocytes (Figure 3). It is interesting to note that Gab1 is located mainly in the cytoplasm of normoxic cells with some nuclear expression.

3.4. Hypoxia Produce a Downregulation of GAB1 in Cultured Rat Cardiomyocytes. As there is a limit to what can be done using human biopsies, an *in vitro* model of cyanosis would be advantageous. Both GAB1 and SHP2 were downregulated

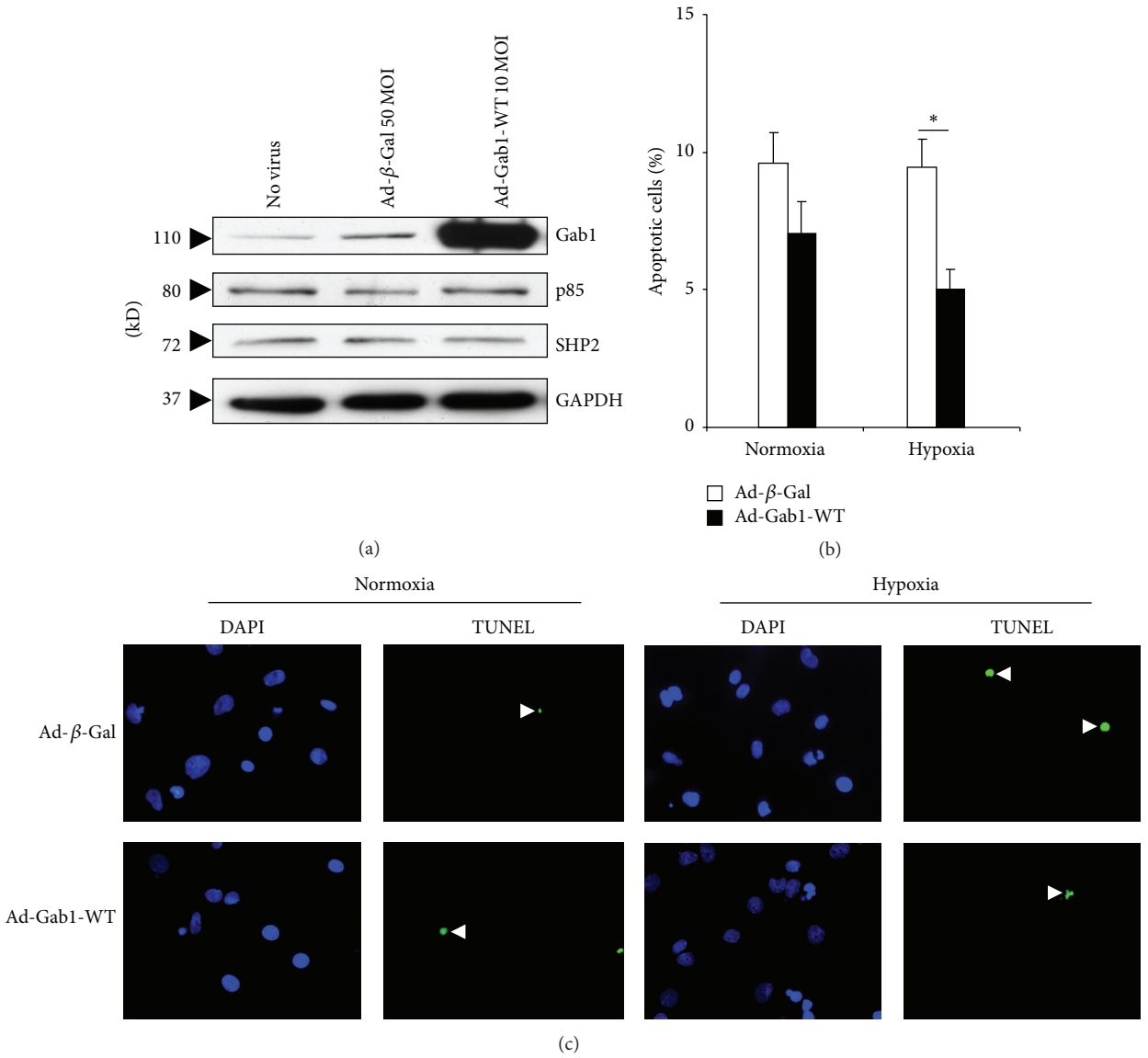

Figure 5: (a) Protein expression level of Gab1, p85, and SHP2 in infected rat neonatal cardiomyocytes. Cardiac myocytes were infected with Ad-β-Gal (50 MOI) and Ad-Gab1-WT (10 MOI), for 24 hours. GAPDH was used as a loading control. (b) Apoptosis quantification of infected rat neonatal cardiomyocytes subjected to normoxia and hypoxia. Cardiac myocytes were infected with Ad-β-galactosidase (Ad-β-Gal) and Ad-Gab1-WT, for 24 hours. Data are mean ± SEM. $^{*}P < 0.05$. (c) Representative pictures of the TUNEL assay performed on rat neonatal cardiomyocytes infected by Ad-β-Gal or Ad-Gab1-WT and subjected to normoxia or hypoxia. Arrows show apoptotic nuclei (green fluorescence). Cell nuclei were stained by DAPI (blue fluorescence). Magnification: ×400.

at the protein level following hypoxia when compared to the normoxic control (Figures 4(a) and 4(b)). However, hypoxia did not affect p85 protein expression (Figure 4(c)).

3.5. Gab1 Overexpression Reduces Apoptosis during Hypoxia in Rat Cardiomyocytes. We first examined the efficiency of cell infection by Ad-Gab1-WT (Figure 5(a)). Infection of rat neonatal cardiomyocytes with Ad-Gab1-WT was successful as demonstrated by the increase of Gab1 protein expression compared to cells infected with Ad-β-Gal (Figure 5(a)). In addition, the infection with both viruses did not affect the protein expression of p85, SHP2, and GAPDH.

During normoxia, the overexpression of Gab1 in cardiomyocytes did not alter the percentage of apoptotic cells significantly. However, during hypoxia Gab1 overexpression reduced significantly apoptosis in rat neonatal cardiomyocytes (Figures 5(b) and 5(c)).

4. Discussion

Our study revealed a significant upregulation of Gab1 protein expression in cyanotic TOF patients. This result confirmed our previous findings by microarray analysis [6]. The upregulation of Gab1 protein expression in cyanotic patients may

suggest an increase of survival signalling mediated through Gab1 in cyanotic patients, independently of SHP2 and p85. These data are the first to implicate Gab1 in cardioprotective signalling in cyanotic patients in response to chronic hypoxia stress.

Gab1 protein levels were downregulated during hypoxia in neonatal rat cardiomyocytes. These are also the first results to implicate Gab1 in the cardiomyocytes response to hypoxia. The difference between the *in vivo* and *in vitro* data regarding the response to hypoxia can be explained by the complexity of the *in vivo* situation in comparison to the relative simplicity of the *in vitro* model. Furthermore, it can be explained by the short time of hypoxia protocol (24 h) used for rat myocytes compared to the patients that stayed cyanotic for months before surgery. Additionally, this may be attributed to the difference between TOF patients' tissue specimens and neonatal rat ventricle. Neonatal rat ventricle includes LV and RV tissues, whereas TOF patients' tissue was only from RV. It is well known that there is a difference in cardiac tissue between the two ventricles [16].

We have previously shown that chronic hypoxia induces both cytoprotective and injury related transcriptomic reprogramming [6]. The protective program induces survival pathways and the deleterious program triggers cell death signaling [6]. Any imbalance between these two programs would result in either cell survival or death. Therefore, modulating the balance between these two programs offers the potential to develop strategies for cardioprotection. Our data suggest that, in cyanotic pediatric heart, the increase in Gab1 expression is part of the survival pathway. Additionally, the reduction of apoptosis observed following the overexpression of Gab1 suggests a critical and prosurvival role for Gab1 in rat neonatal cardiomyocytes. This is in line with previous reports describing an antiapoptotic function for Gab1 [12]. It has also been shown that SHP2 can help to promote cell survival by the activation of the Raf/MEK/ERK signalling pathway [17].

Gab1 has been shown to play an antiapoptotic role in oxidative condition [12]. It would be interesting to see how Gab1 expression levels would be following corrective surgery for cyanotic congenital heart disease. Similarly, it would be interesting to examine the removal of hypoxic stress in an experimental model. One can speculate that the removal of hypoxic stress would result in the return of Gab1 expression to normoxic condition levels. Additionally we would expect thatan abrupt reoxygenation could modulate the levels of this unique protein. Furthermore, the normal transition from foetal to neonatal circulation may affect Gab1 expression levels.

Gab1 expression changes following hypoxia may be controversial considering the observed difference between the *in vivo* and *in vitro* situations. However, it has previously been shown that oxygen availability can play a critical role in defining the cellular responses to stimuli [18]. Compared to the cell culture system, the *in vivo* situation adds another level of complexity. It is likely that other *in vivo* signalling pathways come into play that could result in a different response to hypoxia as compared to culture system. In the above-mentioned study, it has been shown that whereas IGF signaling promotes muscle cell differentiation under normoxia, it stimulates proliferation under hypoxia by differentially regulating multiple signaling pathways [18]. A possible mechanism involved in modulating Gab1 expression could be the HIF1 alpha-signaling pathway.

SHP2 and p85 expressions showed no difference between cyanotic and acyanotic TOF patients; however, they decreased, although it was not significant for p85, in hypoxic myocytes. A plausible explanation is the difference between RV tissue collected from TOF patients and the mixed cell population harvested from LV and RV of neonatal rats. There is evidence that cardiac tissue obtained from the two ventricles has different expression profile and can respond differently to stimuli [16].

Altogether, our data provide strong evidence that Gab1 is important for cardiomyocytes survival following hypoxic stress. Gab1 represents a potential target for cardioprotection.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Endothelial Injury Associated with Cold or Warm Blood Cardioplegia during Coronary Artery Bypass Graft Surgery

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The aim of this investigation was to analyze the impact of intermittent cold blood cardioplegia (ICC) and intermittent warm blood cardioplegia (IWC) on endothelial injury in patients referred to elective on-pump coronary artery bypass graft (CABG) surgery. Patients undergoing CABG procedures were randomized to either ICC or IWC. Myocardial injury was assessed by CK-MB and cardiac troponin T (cTnT). Endothelial injury was quantified by circulating endothelial cells (CECs), von Willebrand factor (vWF), and soluble thrombomodulin (sTM). Perioperative myocardial injury (PMI) and major adverse cardiac events (MACE) were recorded. Demographic data and preoperative risk profile of included patients (ICC: $n = 32$, IWC: $n = 36$) were comparable. No deaths, PMI, or MACE were observed. Levels of CK-MB and cTnT did not show intergroup differences. Concentrations of CECs peaked at 6 h postoperatively with significantly higher values for IWC-patients at 1 h (ICC: 10.1 ± 3.9/mL; IWC: 18.4 ± 4.1/mL; $P = 0.012$) and 6 h (ICC: 19.3 ± 6.2/mL; IWC: 29.2 ± 6.7/mL; $P < 0.001$). Concentrations of vWF (ICC: 178.4 ± 73.2 U/dL; IWC: 258.2 ± 89.7 U/dL; $P < 0.001$) and sTM (ICC: 3.2 ± 2.1 ng/mL; IWC: 5.2 ± 2.4 ng/mL; $P = 0.011$) were significantly elevated in IWC-group at 1 h postoperatively. This study shows that the use of IWC is associated with a higher extent of endothelial injury compared to ICC without differences in clinical endpoints.

1. Introduction

Cardiopulmonary bypass during cardiac surgical procedures is associated with myocardial and endothelial injury [1–3]. Myocardial protection in form of intermittent cold (ICC) and intermittent warm blood cardioplegic solutions (IWC) is still under investigation even though clinically introduced decades ago. Good clinical results were reported with ICC and IWC among other potential options for myocardial protection. The use of IWC was demonstrated to be favourable over ICC in low-risk patients referred to coronary artery bypass procedures, whereas ICC was shown to provide superior myocardial protection in high-risk populations requiring prolonged cross clamp times [4, 5].

Most trials investigating the impact of cardioplegic solutions failed to detect differences in patients' clinical outcome due to the limited direct impact of cardioplegia on hard clinical endpoints. However, the influence of myocardial protection techniques is well investigated on inflammatory processes [3]. Since inflammation correlates with the degree of endothelial injury we sought to investigate in the presented study endothelial function in patients undergoing coronary artery bypass graft (CABG) procedures using IWC or ICC. For the assessment of endothelial injury circulating endothelial cells (CECs), von Willebrand factor (vWF), and soluble thrombomodulin (sTM) were chosen as well established indicators since respective concentrations reflect the degree of endothelial injury associated with inflammation (vWF and sTM) and vascular damage (CECs) especially in patients with heart failure, diabetes, and various types of vasculitis [6–9]. In patients with acute myocardial infarction, recent data indicates that CEC counts may predict rupture of

atherosclerotic plaque [10]. However, CEC count has never been established for evaluation of IWC and ICC in coronary artery bypass graft (CABG) patients.

We therefore aimed in the present study to test the hypothesis if the use of ICC is associated with a reduced endothelial injury in routine CABG procedures what might in part explain the favourable results associated with IWC over ICC.

2. Materials and Methods

2.1. Study Design and Patients. A randomized and blinded single-center pilot trial was performed of patients ($n = 72$) scheduled for elective coronary artery bypass graft (CABG) surgery using cardiopulmonary bypass (CPB) at the Heart Center of the University of Cologne between July 2013 and December 2013. Exclusion criteria were ejection fraction <25%, age >80 years, acute myocardial infarction, atrial fibrillation, combined or redo procedures, history of diabetes mellitus, renal or hepatic dysfunction, vasculitis, cancer, or infectious diseases as well as intake of drugs that are known to directly interact with the endothelium (e.g., antioxidants). Eligible patients were randomized into two groups: (1) intermittent cold blood cardioplegia- (ICC-) group and (2) intermittent warm blood cardioplegia- (IWC-) group using a 1 : 1 web-based and open-access randomization plan (generated by http://www.randomization.com/). Sample acquisition was incomplete in 4 patients in the ICC-group; these patients were not included into analysis. All patients were blinded for group allocation and data analysis was carried out without knowledge of group allocation. Intraoperative blinding of surgeons and perfusionists was not carried out. The study was approved by the ethics committee of the University of Cologne (Cologne, Germany) and all participating patients gave written informed consent.

2.2. Surgical Technique and Cardioplegic Delivery. Induction of anesthesia was performed with 1 μg/kg sufentanil, 0.2 mg/kg etomidate, 0.04 mg/kg midazolam, and 0.15 mg/kg cisatracurium and maintained with sufentanil (1 μg/kg/mh) and midazolam (0.1 mg/kg/h). Additional sevofluran was administered until CPB.

Surgical technique and perioperative management were uniform for all patients. All operations were performed on CPB consisting of a centrifugal pump, a membrane oxygenator (Jostra Quadrox, Maquet Cardiopulmonary AG, Hirrlingen, Germany), and an in-line arterial filter. Mean arterial pressure was maintained between 50 and 70 mmHg and CPB flow was kept at 2.2–2.5 L/min per m^2. Core temperatures for patients in the IWC-group were kept at 37°C during CPB and moderate hypothermia (32–34°C) was applied for ICC-patients. Cardioplegia was chosen according to randomization.

In the ICC group, induction of cardiac arrest was achieved giving cold (4–6°C), nonsubstrate enriched Buckberg solution (Köhler Chemie GmbH, Alsbach-Hähnlein, Germany) for 4-5 min with perfusion pressures of 60 mmHg for antegrade cardioplegia delivery [2, 11]. The induction solution was supplemented with potassium to deliver a final concentration of ~20 mmol/L. In the IWC-group, warm (37°C) oxygenated blood was infused into the aortic root. A syringe pump containing a potassium and magnesium mixture (30 mL of 2 mmol/mL KCL; 10 mL of 2 mmol/mL $MgSO_4$) was connected to the cardioplegia circuit. Cardiac arrest was induced at a blood flow rate of 200–300 mL/min by continuous infusion of 150 mL/h of the syringe pump over a 2-3 min time period [12, 13]. For both IWC and ICC, maintenance of cardiac arrest was facilitated by antegrade reinfusions into the aortic root for 3-4 min every 15–20 min or after completion of an anastomosis (cardioplegic delivery via completed bypass grafts is not routinely performed at our institution). Independently of applied cardioplegic strategy, warm reperfusion was always obtained by release of the aortic clamp. Proximal anastomoses were completed on a beating heart using a partial aortic clamping technique. Retrograde delivery of warm cardioplegia was not applied.

2.3. Endothelial Markers and Circulating Endothelial Cells. Assessment of markers of myocardial (CK-MB and troponin T) and endothelial (vWF, sTM, and CEC) injury was performed as previously described preoperatively and at 1 hour, 6 hours, 12 hours, and 24 hours after CPB initiation, respectively [14]. Briefly, blood was drawn from the central venous catheter and collected in citrate tubes for determination of vWF and sTM and centrifugated at 1000 g for 20 minutes at 4°C (Allegra X-15R, Beckman Coulter), and supernatant was frozen at −80°C. Concentrations of vWF (AssayMax Human von Willebrand Factor ELISA Kit, Assaypro, St. Charles, USA) and sTM (Human CD141 ELISA Kit, Diaclone SAS, Besançon, France) were assessed in triplicate measurements by a quantitative sandwich enzyme immunoassay technique, for which commercial ELISA kits were used.

For CEC isolation, blood was collected in EDTA tubes and measurements were immediately carried out using M-450 Dynabeads, 4.5 μm diameter polystyrene beads coated with rat anti-mouse immunoglobulin-G1 (Dynal, Hamburg, Germany) coupled with murine anti-human CD146 antibody (Biocytex, Marseilles, France). Blood and phosphate-buffered saline containing 0.1% bovine serum albumin were mixed (1 mL each) with 20 μL FcR blocking agent (Miltenyi, Bergisch-Gladbach, Germany) and incubated with 100 μL anti-CD146-coated Dynabeads (8×10^6 beads/10 μL anti-CD146) for 60 minutes while agitating. Magnet separation (Dynal MPC) of cells bound to anti-CD146-coupled beads from blood was followed by washing with PBS-BSA-solution and incubation for 1 hour with 10 μL of rhodamine-labeled Ulex-Europaeus-Agglutinin-1 solution (1 : 10 dilution, Linaris, Wertheim, Germany) on an orbital shaker in darkness. After washing and resuspension in 100 μL of PBS-BSA, cells were counted in a Nageotte chamber (Brand, Wertheim, Germany) with a fluorescent microscope (Leica DMLB, Leica Microsystems GmbH, Wetzlar, Germany) using an excitation filter N2. CECs were identified and then counted as well-delineated round or oval rhodamine-labeled cells with the size of 10 to 40 μm with more than 4 beads attached.

Samples for CK-MB and troponin T analysis were assessed at the Central Core Laboratory of the University of Cologne using commercially available assays for CK-MB

Table 1: Patients' demographic and operative data.

Criteria	ICC-group	IWC-group	*P* value
Number (*n*)	32	36	
Age (years)	65.9 ± 11.3	63.9 ± 7.7	*0.392*
Male gender (%)	75	81	*0.486*
Preoperative EF (%)	62 ± 19	56 ± 23	*0.249*
NYHA-class	2.1 ± 0.6	2.2 ± 0.6	*0.495*
EuroSCORE	3.7 ± 2.3	4.2 ± 2.4	*0.385*
CPB time (min)	76.2 ± 16.4	68.7 ± 12.5	*0.037*
Cross clamp time (min)	39.7 ± 11.7	42.6 ± 14.6	*0.373*
Procedure time (min)	187.7 ± 39.2	197.5 ± 42.7	*0.330*
Number of grafts	3.1 ± 0.6	3.3 ± 0.6	*0.175*
Ventilation time (hours)	17.4 ± 6.2	14.7 ± 8.5	*0.144*
ICU stay (hours)	39.1 ± 17.6	52.3 ± 23.2	*0.011*
Atrial fibrillation (%)	23.1	28.6	*0.772*
PMI	0	0	—
MACE	0	0	—
Mortality	0	0	—

CPB: cardiopulmonary bypass; EF: ejection fraction; ICC: intermittent cold blood cardioplegia; ICU: intensive care unit; IWC: intermittent warm blood cardioplegia; MACE: major adverse cardiac event; NYHA: New York Heart Association; PMI: perioperative myocardial injury.

(CK-NAC method, Roche Diagnostics, Mannheim, Germany) and cardiac troponin T determination (Elecsys troponin T, Roche Diagnostics). PMI was defined as the combination of (1) creatine kinase- (CK-) MB level 5 times greater than the upper level of normal (>120 U/L) with a CK-MB fraction between 6 and 25% and (2) a cardiac troponin T elevation greater than 1.5 ng/mL during the first 72 h after surgery [15, 16]. Major adverse cardiac events (MACE) were recorded as a composite endpoint including cardiac cause of death, PMI, inotropic support with epinephrine >24 hours (h), postoperative need for IABP or ECMO, and severe ventricular arrhythmias. The components of this endpoint were chosen to ensure high sensitivity for detection of complicated postoperative course.

2.4. Statistical Analysis. For statistical analysis, SPSS statistical software (SPSS Inc., Chicago, IL, US) was used. Categorical variables of groups were compared with Fisher's exact test and expressed as percentages. Continuous variables, given as mean ± standard deviation, were compared between groups by using the unpaired *t*-test for normally distributed values; otherwise, the Mann-Whitney *U* test was used. Variables with nonnormal distribution were presented as median and interquartile range (IQR). Two-way analysis of variance (ANOVA) with post hoc Bonferroni test was used for repeated measurements. *P* values <0.05 were considered to be statistically significant.

3. Results

3.1. Group Characteristics. Table 1 shows patients' demographic and operative data. Patients in the IWC-group ($n = 36$) presented with higher EuroSCORE and stayed longer on the ICU compared to patients treated with ICC ($n = 32$), but groups did not differ in terms of age, gender, preoperative EF, and NYHA-class. Similarly, procedure-related variables such as cross clamp time, total procedure time, and number of grafts implanted as well as postoperative ventilation times were comparable among groups, whereas CPB times were longer for ICC-patients. Postoperative incidence of atrial fibrillation was comparable for patients in the ICC- (23.1%) and IWC-group (28.6%; $P = 0.772$), respectively. Outcome variables PMI, MACE, and mortality were not detected in both treatment arms.

3.2. Markers of Myocardial Injury. The course of CK-MB values for patients in the ICC- and IWC-group is depicted in Figure 1. For both groups, CK-MB concentrations rose uniformly from normal preoperative values to peak concentrations at 6 hours after CPB initiation (ICC: 58.3 ± 29.7 U/L; IWC: 52.3±27.5 U/L; $P = 0.392$). Although CK-MB concentration dropped after 12 hours in IWC-group, values were not significantly different.

Cardiac troponin T levels for ICC- and IWC-groups are displayed in Figure 2. Parallel to CK-MB, cTnT concentrations of both groups peaked at 6 hours (ICC: 0.72 ± 0.25 μg/L; IWC: 0.63 ± 0.29 μg/L; $P = 0.174$) and declined uniformly in both groups without significant difference.

3.3. Markers of Endothelial Injury. Preoperative CEC counts (cells per milliliter of blood) were comparable among treatment groups (ICC: 3.9 (IQR: 2.8–4.6)/mL; IWC: 2.5 (IQR: 0.9–3.6)/mL; $P = 0.488$). However, CEC counts augmented in both groups and peaked at 6 hours after CPB initiation and nearly approached preoperative levels after 24 hours. Of note, CEC counts were significantly higher at 1 hour (ICC: 10.1 ± 3.9/mL; IWC: 18.4 ± 4.1/mL; $P = 0.012$) and 6 hours (ICC: 19.3±6.2/mL; IWC: 29.2±6.7/mL; $P < 0.001$) after CPB initiation in IWC-treated patients compared to ICC-group (Figure 3).

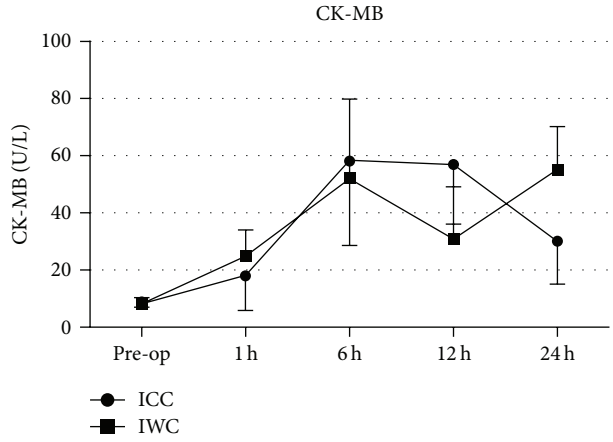

Figure 1: Course of values for CK-MB in the ICC-group and IWC-group preoperatively and at 1 h, 6 h, 12 h, and 24 h after the procedure.

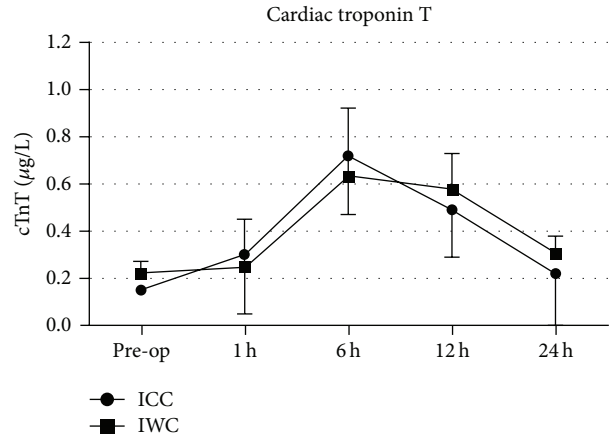

Figure 2: Course of values for cardiac troponin T (cTnT, μg/L) in the ICC-group and IWC-group preoperatively and at 1 h, 6 h, 12 h, and 24 h after the procedure.

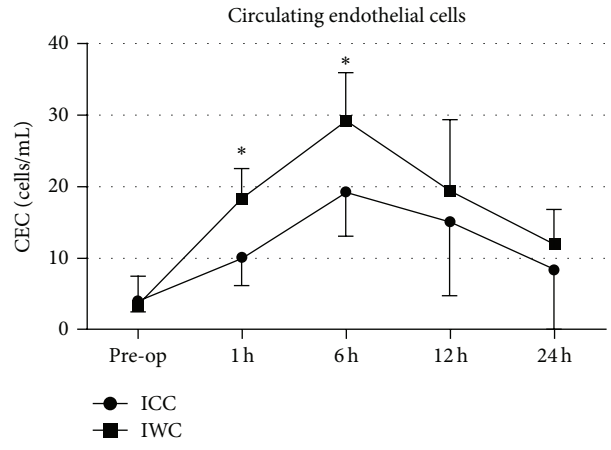

Figure 3: Number of circulating endothelial cells (CEC, cells per milliliter of blood) in the ICC-group and IWC-group preoperatively and at 1 h, 6 h, 12 h, and 24 h after the procedure.

Measurements of vWF-concentrations showed rising trends uniformly for both groups with significant higher values at 1 hour after CPB initiation for IWC-group compared to the ICC-group (ICC: 178.4 ± 73.2 U/dL; IWC: 258.2 ± 89.7 U/dL; $P < 0.001$; Figure 4).

Quantification of sTM concentration of patients in the ICC- and IWC-group revealed rising values with a peak at 6 hours after CPB initiation for IWC-patients and a peak at 12 hours for ICC-patients. Parallel to findings of vWF, values of patients in the IWC-group were significantly higher compared to ICC-patients at 1 hour after CPB initiation (ICC: 3.2 ± 2.1 ng/mL; IWC: 5.2 ± 2.4 ng/mL; $P = 0.011$; Figure 5).

4. Discussion

The present randomized and blinded single-center study assessed the impact of two routinely applied blood cardioplegic solutions on myocardial and endothelial injury in patients undergoing on-pump coronary artery bypass surgery. In this patient population with comparable myocardial damage as assessed by postoperative CK-MB and cardiac troponin T levels we demonstrate a slightly higher endothelial injury in patients treated with intermittent warm cardioplegia compared to intermittent cold cardioplegia. However, the detected differences do not allow preferring one solution over the other during elective CABG surgery in patients with average risk profile.

For determination of endothelial function in cardiac surgery patients, elevated numbers of circulating endothelial cells (CECs) and endothelium-specific plasma markers such as von Willebrand factor (vWF) and soluble thrombomodulin (sTM) have been shown to correlate with global vascular injury [9]. In patients undergoing on-pump cardiac procedures, Schmid et al. demonstrated the link of endothelial injury caused by cardiopulmonary bypass and blood levels of CECs since concentrations were significantly elevated 6 hours postoperatively compared to preoperative levels with a declining trend afterwards [17]. Similar to these findings, we generally detected rising concentrations of markers for endothelial injury with peak values of CECs at 6 hours postoperatively reflecting significant vascular damage triggered by the cardiac procedure. According to results of Skrabal et al. who assessed endothelial injury during on-pump CABG operations, the disturbance of the endothelium does not seem to be related to the mechanical manipulation of the heart itself, since levels of each of CECs, vWF, and sTM were unchanged 30 minutes after initiation of cardiopulmonary bypass but ascended in the postoperative period as a potential result of reperfusion injury [14]. This reperfusion injury comprises an activation of neutrophils, release of cytokines and proteases triggered by the contact of blood cells with artificial surfaces, and air during cardiopulmonary bypass finally interacting with the integrity of the endothelium [18].

Myocardial injury of included patients in the presented study did not differ between ICC- and IWC-group shown by similar levels of CK-MB and cardiac troponin T levels. Otherwise, endothelial injury was more pronounced in the IWC-group as we detected higher concentrations of markers for vascular damage compared to ICC-patients. Furthermore,

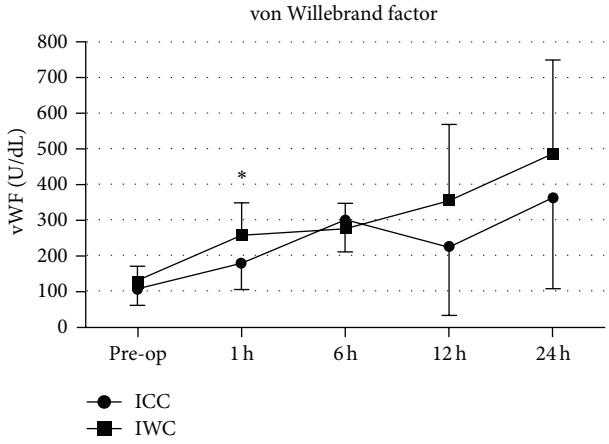

Figure 4: Concentration of von Willebrand factor (vWF, U/dL) in the ICC-group and IWC-group preoperatively and at 1 h, 6 h, 12 h, and 24 h after the procedure.

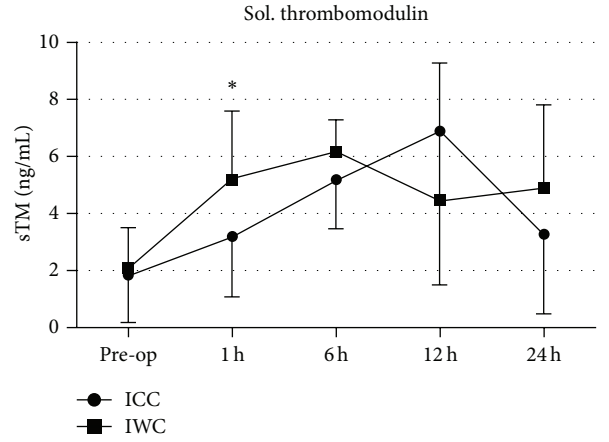

Figure 5: Concentration of soluble thrombomodulin (sTM, ng/mL) in the ICC-group and IWC-group preoperatively and at 1 h, 6 h, 12 h, and 24 h after the procedure.

significantly higher concentrations were uniformly detected of CECs, vWF, and sTM 1 hour after surgery for IWC-patients, while markers of myocardial damage were similar for both treatment groups. Therefore, the early rise of all markers for endothelial damage and the peak level of CECs 6 hours postoperatively might indicate a potential value of CECs as a super-sensitive marker for endothelial injury in cardiac surgical patients. In consequence, a lower endothelial damage in the ICC-group might indicate a superior myocardial protection when compared to IWC-use in this patient cohort undergoing CABG procedures, even though this can just be seen as a hypothesis-generating aspect. However, this finding would be in line with a study investigating the impact of ICC and IWC in high-risk patients subjected to a wide spectrum of different cardiac procedures with cross clamp times longer than 90 minutes. Use of IWC resulted in significantly higher cardiac mortality and in a more pronounced myocardial injury compared to ICC. Furthermore, application of IWC even turned out to be an independent predictor for 30-day all-cause mortality, cardiac death, and perioperative myocardial injury after multivariate analysis in this cohort [5]. It is obviously needed to underline the fact that the patient cohort in the presented study was different in terms of cross clamp time and risk profile. Otherwise, Franke et al. analyzed laboratory and clinical outcomes in a low-risk patient cohort undergoing exclusively CABG surgery. Markers of myocardial ischemia (CK-MB and cTnT) were significantly lower in patients treated with IWC when compared to ICC, whereas no differences in clinical endpoints were detected [4]. These contradicting results implicate the relevance of the patients' preoperative risk profile, the kind of underlying disease, and the influence of the cross clamp time since prolonged cross clamp time serves as an independent predictor for mortality in cardiac surgery patients [19].

Measuring endothelial injury during CABG procedures is naturally associated with uncontrollable covariates leading to limitations of our study and unanswered questions. Thus, we cannot explain the marked increase of CK-MB values in IWC-patients at 24 hours postoperatively. As we did not perform coronary angiography routinely in the postoperative period, we are unable to explain this phenomenon. Additionally, blood sample acquisition from the coronary sinus would have been desirable for determination of endothelial injury exclusively in the heart instead of using the central venous catheter. However, a sample collection via the coronary sinus would have been impossible after the end of the procedure for further measurements at 24 hours postoperatively.

Furthermore, it is important to underline that it is impossible to create two identical groups of patients even by randomization. Thus, both groups show differences in demographic and preoperative data that might have influenced the overall result. This fact might also be affected by the limited number of participating patients. Since this work presents a pilot trial, it is now possible to perform a sample size calculation for future studies on this topic.

Additionally, the presented investigation was not powered to detect significant differences in vascular damage associated with various myocardial protection techniques. This study reveals rather more the perioperative kinetics of markers for endothelia damage.

5. Conclusions

In summary, we demonstrate that perioperative endothelial injury can be assessed by measuring circulating endothelial cells, von Willebrand factor, and soluble thrombomodulin in patients undergoing elective, on-pump myocardial revascularization indicating a slight benefit of intermittent cold over warm blood cardioplegia in contrast to the primary hypothesis. However, this advantage is not reflected in differences in markers of myocardial injury or even clinical endpoints. The results of the presented pilot trial can serve as an argument for the safe use of intermittent cold blood cardioplegia in routine CABG surgery and as a basis for further investigations.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Elmar W. Kuhn and Yeong-Hoon Choi contributed equally to this work.

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Review Article

Normoxic and Hyperoxic Cardiopulmonary Bypass in Congenital Heart Disease

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Cyanotic congenital heart disease comprises a diverse spectrum of anatomical pathologies. Common to all, however, is chronic hypoxia before these lesions are operated upon when cardiopulmonary bypass is initiated. A range of functional and structural adaptations take place in the chronically hypoxic heart, which, whilst protective in the hypoxic state, are deleterious when the availability of oxygen to the myocardium is suddenly improved. Conventional cardiopulmonary bypass delivers hyperoxic perfusion to the myocardium and is associated with cardiac injury and systemic stress, whilst a normoxic perfusate protects against these insults.

1. Introduction

As a consequence of advances made in the fields of paediatric cardiac surgery, anaesthesia, and perfusion science, an increasing number of surgical repairs of congenital cardiac abnormalities are being carried out each year. In fact the field of paediatric cardiac surgery has progressed so far that virtually no lesion is considered "inoperable" [1]. These congenital cardiac abnormalities fall into two broad groups; those causing cyanosis through intra- or extracardiac right to left sided shunts, or those which are not acyanotic (Figure 1). The cyanotic type is, by definition, associated with chronic hypoxia, as well as with consequent malnutrition and growth failure [2], and the uncorrected has outcomes that are universally worse than the acyanotic group.

The cyanotic group may be further divided along many lines, but a significant classification from an operative perspective is into those patients with a physiologically functional double-ventricle circulation and those with only a single functioning ventricle. Evidence is emerging that those patients with univentricular pathologies are more vulnerable to deleterious systemic and myocardial effects from standard "hyperoxic" cardiopulmonary bypass (CPB) during and after operative treatment of these pathologies than those patients with biventricular pathologies [3]. However, both of these groups show evidence of end-organ injury as well as systemic inflammation and stress when they undergo conventional hyperoxic CPB [4–6].

Therefore, this review seeks to examine the mechanisms by which hearts in patients with cyanotic-type circulations adapt to chronic hypoxia, the mechanisms underlying so-called reoxygenation injury from animal studies, and also the current clinical evidence showing the existence of this phenomenon perioperatively.

2. Cardiac Metabolic and Adaptive Responses to Hypoxia

Hypoxia is an imbalance between tissue oxygen demand and oxygen supply in the context of normal tissue perfusion. A

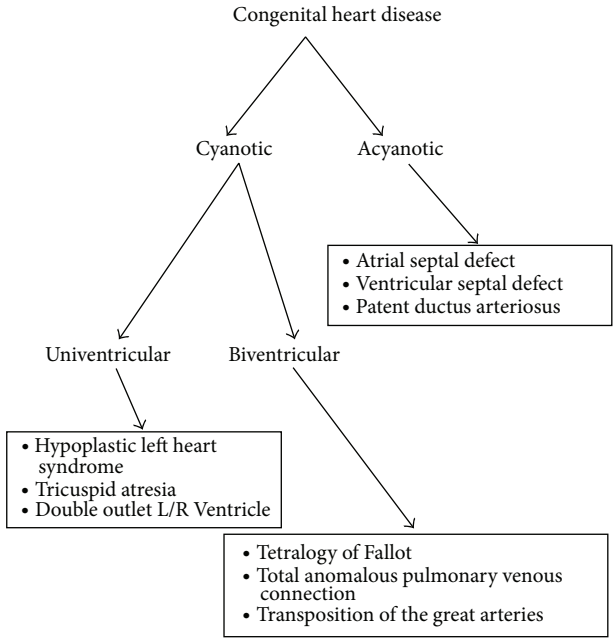

Figure 1: Schematic representation of a classification system for Congenital Heart Disease, with examples of typical lesions from each category. Acyanotic disease makes up the majority of cases of congenital abnormalities, and a large proportion goes silent and undiagnosed. The cyanotic lesions will undergo surgical repair.

number of processes, both adaptive and maladaptive, take place under hypoxic conditions, and these contribute to the risk of reoxygenation injury under hyperoxic conditions during CPB (Figure 2). These processes prime the myocyte for particular responses when perfusion with oxygen and respiratory fuel is reintroduced and so influence the eventual degree of reoxygenation injury.

2.1. Hypoxic Adaptations. Significant changes in the myocyte gene expression profile occur in the chronically hypoxic heart. Hypoxia inducible factor 1 (HIF-1) is a transcription factor whose significance during hypoxic states is increasingly evident [7]. During normoxia, constitutively expressed HIF-1α is degraded by HIF prolyl-hydroxylase. However in hypoxic conditions, HIF prolyl-hydroxylase is inhibited which results in HIF-1α accumulation in the cytoplasm and translocation to the nucleus [8, 9]. This leads to activation of many genes including, for instance, erythropoietin (EPO) and VEGF [10, 11]—these targets produce changes in the processes of angiogenesis, vascular remodelling, erythropoiesis, ROS production, and inflammation. In the chronically hypoxic heart, as might be expected, HIF-1α levels are significantly increased, to a level proportionate to the degree of hypoxia. In addition, under the influence of HIF-1α, phosphorylated p38MAPK levels as well as eNOS and VEGF levels increase as would be expected [12]. These are adaptive changes to chronic hypoxia and collectively produce a heart which at a genomic and ultrastructural level is profoundly altered.

Hypoxia also alters the intracellular distribution of protein kinase C (PKC) [13] isoforms, including α but also ε [14]. Under hypoxic conditions, these isoforms are more abundant in particulate fractions than soluble, signifying a translocation from cytosolic to membrane-bound compartments and activation of PKC activity. Mammalian PKC α is known to play important roles in the control of cell proliferation and exerts antiapoptotic properties [15, 16], whilst ε has for some time been closely associated with responses to ischaemia and protection from I/R injury.

In normal physiological conditions most of the cardiomyocyte ATP production is from mitochondrial oxidative phosphorylation, which even under physiological conditions leads to a small amount of reactive oxygen species (ROS) production. However this form of ATP production is diminished during hypoxia due to reduced oxidation of fatty acid and carbohydrate [17, 18] and consequently processes that are ATP dependent such as ion exchange by the Na^+/K^+ ATPase are inhibited. Therefore cardiac metabolism switches partially to anaerobic metabolic pathways, which results in a metabolic acidosis as the degree of tissue hypoxia progresses. This acidosis leads to increased intracellular Na^+ via Na^+/H^+ exchanger (NHE) and, consequently, elevated levels of intracellular Ca^{2+} (through the Na^+/Ca^{2+} exchanger (NCX)) [18]. These processes are interesting, in the context of investigating the ideal way of treating paediatric cyanotic heart disease; however they are limited by the fact that these experimental studies examine a state of complete *anoxia*, rather than the pathophysiological state of hypoxia.

2.2. Reperfusion and Reoxygenation: Intracellular Events. ROS are intrinsic byproducts of the mitochondrion through oxidative phosphorylation. There are cellular mechanisms that counterbalance the physiological accumulation of small amounts of reactive oxygen species; these include enzymes such as catalase and glutathione peroxidase that ultimately reduce hydrogen peroxide and other organic peroxides into less reactive species. Superoxide dismutase (SOD) isoforms may also form part of this endogenous myocardial antioxidant mechanism, albeit upstream, since they facilitate hydrogen peroxide formation from superoxide [19]. However, in the hypoxic situation, whether acute or chronic, there is a lower antioxidant reserve due to increased ROS production. This imbalance leads to interaction of these ROS with many cellular constituents, including nucleic acids, lipid, and proteins, resulting in cell damage and death [20–22].

Other mechanisms for production of ROS have also been noted; the most significant of these is perhaps the Fenton pathway [23]. This describes a process where hydrogen peroxide undergoes catalytic oxidation by Fe^{2+} ions into a hydroxyl radical and hydroxide ion, and the resultant Fe^{3+} is then reoxidised back to ferrous iron by a further molecule of hydrogen peroxide, to leave byproducts of a superoxide radical and hydrogen ion. As a whole, the disproportionation with hydrogen peroxide results in two different reactive oxygen species being formed, which then go on to participate in secondary oxidation of cellular components. Additionally, Beckman et al. [24] described another possible mechanism

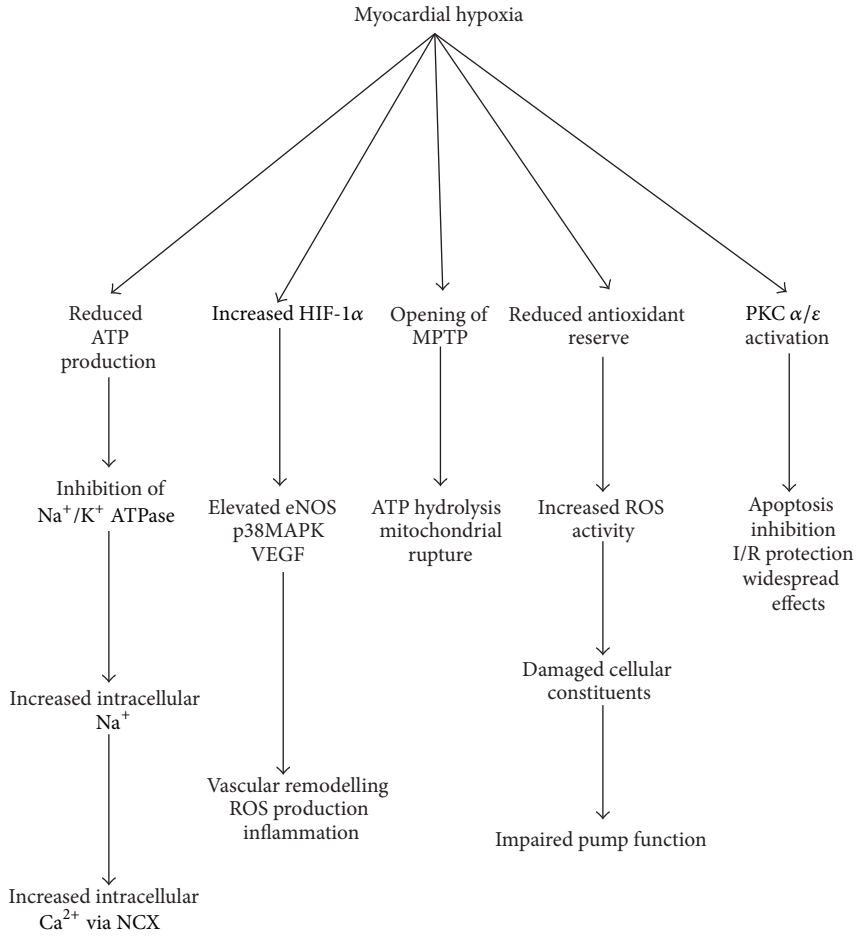

Figure 2: Schematic representation of selected consequences of hypoxia in the cardiomyocyte. Shown here are five different downstream chains of events initiated under hypoxic conditions in the cardiomyocyte. These pathways have significant horizontal interaction which synergises and amplify the outcome, and not all of these events occur synchronously or in all contexts. A reduction in ATP production and MPTP opening alongside PKC activation are early consequences of acute ischaemia, whilst the effectors of HIF-1 occur sometime later.

of oxidant injury when cytotoxic reactive oxygen species are formed as a result of interaction between superoxide anion ($O_2^{\bullet -}$) and nitric oxide (NO). In the experimental setting, with cyanosis and a reduction in endogenous myocardial antioxidants, free radical generation during reoxygenation is augmented [25]. In the cardiomyocyte, this alters ion channel flux leading to reductions in pump function and ultimately contractile impairment [20]. A further burst of ROS activity occurs immediately during reperfusion [26]. This effect, coupled with the initial ROS flux from the hypoxic period, causes both primary impairment and damage to the myocyte, as well as increasing the likelihood of mitochondrial pore opening.

Reperfusion and reoxygenation results in opening of the mitochondrial permeability transition pore (MPTP) [27]. Under conditions of reperfusion with attendant oxidative stress, or chronic congestive heart failure as might be seen in more advanced or severe forms of congenital heart disease, the levels of intracellular and mitochondrial Ca^{2+} rise. In this situation, this pore, a nonselective 1.5 kDa channel in the inner mitochondrial membrane, opens, which leads to swelling of the mitochondrion, metabolic uncoupling, ATP hydrolysis, and ultimately cell death. The probability of pore opening is enhanced by other conditions that are present in the myocyte made ischaemic and subsequently reperfused, adenine nucleotide depletion, high levels of inorganic phosphate, and high concentrations of ROS all synergise to stabilise the open pore state. Slightly confusing this picture, however, is the fact that in some animal studies, chronic hypoxia is protective against both MPTP opening and increased ROS flux on reoxygenation. The details of this are not well established, but it does mean that the effects of MPTP opening on reoxygenation injury are not straightforward.

It is believed that the results of surgical repair of cyanotic heart defects are complicated by both local myocardial and wider systemic multiorgan damage as a consequence in part of acute reoxygenation at the time of institution of cardiopulmonary bypass with elevated oxygen content, followed by myocardial ischaemia required to arrest the heart with the subsequent reperfusion [28]. In order to further investigate

these phenomena, a number of experimental and clinical studies have been performed to delineate their nature.

3. Reoxygenation and Reperfusion Injury: Animal and Clinical Studies

The existence of reoxygenation injury due to a high P_aO_2 in the field of resuscitation is well established [29]. There has been much interest in widening the scope of these findings, and there is now strong clinical and experimental evidence indicating that immature hearts have a marked increase in tolerance to ischaemia and a greater resistance against the damaging effects of ischaemia and reperfusion injury (I/R) than mature hearts [30–35]. However, some conflicting clinical and animal studies at intermediate age groups suggests that the postnatal, developing myocardium, is more susceptible to reperfusion injury compared to the adult heart [36–40].

These studies have tended to compare adult heart with *one selected* developmental age group. Differences in the choice of age have contributed to some of the conflicting results. This issue has been highlighted by Awad et al. [41] who investigated the vulnerability of the intact rat heart during different stages of development (4, 7, 14, and 21 days, and adult). Their work and later work from others [42] demonstrated that the recovery of developing rat heart following ischaemia and reperfusion changes during maturation and appears to follow a bell-shaped relationship. Clinical research in paediatric surgery also supports the view that vulnerability to I/R changes during postnatal development [43].

There are some proposed explanations for these ontogenetic changes such as lower energy demand in the neonatal group, higher glycogen levels and glycolytic flux [44, 45], a greater anaerobic capacity, and alterations in Ca^{2+} handling plus ROS production [46]. All of these processes accommodate the shift from a hypoxic in utero environ to a normoxic ex utero one. However, the precise mechanisms behind this varying resistance to oxygen deprivation in neonatal myocardium have still not been clearly defined [47, 48].

Cyanosis, chronic hypoxia, remains the most common preoperative physiological stress in paediatric cardiac patients [1, 49] as well as the single largest cause of mortality [47, 48]. In the clinical setting, it seems as though patients with cyanotic congenital heart defects have severely diminished myocardial protection [1], as well as persistently impaired ventricular function following corrective surgery and a higher rate of morbidity and mortality compared to nonhypoxaemic patients [50–52].

In comparison to the acyanotic population, cyanotic patients have an inferior protective cardioplegic protection effect even with comparatively shorter ischaemic intervals [53]. One possible explanation could be the fact that, in children with decreased pulmonary blood flow, that is, those with significant shunts producing cyanosis, there is an increased bronchial collateral drainage to the left heart that can noticeably compromise intraoperative myocardial protection [1]. Further, in cyanotic pathologies, even the degree of cyanosis can affect the cardiac metabolic reserve. For instance, Imura and coworkers reported worse reperfusion injury and clinical outcome in cyanotic patients when compared to acyanotic patients [54], whilst Najm and coworkers studied forty-eight patients who underwent a repair of Tetralogy of Fallot (TOF) and noticed a correlation between preoperative hypoxia and the degree of ventricular dysfunction [55]. They divided the patients into 3 groups. The first group consisted of 14 patients with preoperative arterial oxygen saturation of 90% to 100%; oxygen saturation of sixteen patients in a second group was between 80% and 89% and the remaining eighteen patients in the third group had an oxygen saturation of 79% or less. They reported that even before any surgical intervention the group with O_2 saturation of $\leq$79% had the most impaired ventricular function. They also reported lowest ATP levels preischaemic and 15 minutes following ischaemic arrest. They concluded that the degree of cyanosis has adverse effects on myocardial ATP levels as well as worse clinical outcome.

Further evidence confirming an alteration in oxidative metabolism in the clinical setting came from Del Nido and colleagues [51], this time looking at perioperative changes. This group obtained myocardial biopsies from 16 children undergoing surgical repair of the tetralogy of Fallot as well as 20 adults with coronary artery disease. They showed that TOF patients have a further reduction in adenosine triphosphate and increased lactate not only during ischaemia, but also during reperfusion. They therefore suggested that the limitation to oxidative metabolism exacerbates reperfusion injury as a mechanism of myocardial damage in the setting of cyanotic heart disease.

Changes to oxidative phosphorylation are not the only pathophysiological difference in patients with cyanotic heart disease. Teoh et al. [56] theorised that those patients with cyanotic disease are more susceptible to ROS induced injury. They compared myocardial biopsies of patients with the tetralogy of Fallot to acyanotic adults and reported that patients with tetralogy of Fallot are at higher risk of oxygen-derived free radical injury during cardiac surgery. Examining genetic influences on vulnerability, we [57] investigated myocardial gene profile from right ventricular biopsy of 20 TOF patients undergoing surgical correction. 11 of these patients were cyanotic, whilst 9 were acyanotic. We discovered that the cyanotic population had a widespread increase in gene expression associated with apoptosis and remodelling alongside reduced expression of genes associated with myocardial contractility. As might have been predicted from a theoretical approach to these conditions, those patients with cyanotic disease do demonstrate increased vulnerability both to hypoxia and to reperfusion injury; this seems to be multifactorial, with influences from alterations in oxidative phosphorylation, ROS flux and genomic/transcriptomic variation.

4. Experimental Evidence for Hyperoxic Injury

Numerous studies have been carried out to examine the effect of exposure to higher levels of oxygen in hypoxic animal models. Corno and his coworkers examined the effect of ischaemia/reperfusion in both cyanotic and normoxic

models in Langendorff-perfused male Sprague-Dawley rats and reported a worse outcome in the cyanotic group [58] which was ascribed to the sudden increase in oxygen tension in the coronary perfusate. Further, in order to examine the effect during CPB of hyperoxia on distant organs, Fujii et al. [59] examined inflammatory responses at high (400 mmHg) and normal P_aO_2 (100–150 mmHg) levels in a rat model of cardiopulmonary bypass. They found an increase in proinflammatory cytokines and total lung water in the hyperoxic group; responses that were suppressed in the normoxic arm.

However, another recent study [60] compared cardiac function in Sprague-Dawley rats following a 25-minute KCl induced cardiac arrest. The rats were resuscitated with 100% oxygen on CPB until 3 minutes after return of spontaneous circulation, at which point they received either 40–50% O_2 via CPB or 100% O_2. Postresuscitation haemodynamics, cardiac function, mitochondrial function, and immunostaining of 3-nitrotyrosine were then compared between the two different groups. They found that the hyperoxic group had significantly improved myocardial and mitochondrial function compared to the normoxic group. However, these rats were studied only at 1 hour following ischaemia, in an isolated heart model. Therefore, longer term deleterious effects, damage to end-organs as well as systemic stress, and the interaction of these effects with the myocardium cannot be commented upon.

Whilst suggestive, these studies were however only an ex vivo, small animal studies, so much of the recent work is performed in larger animals, especially swine. Work on large animals included experiments on piglet hearts of less than 4 days of age, perfusing them on cardiopulmonary bypass for 90 minutes with 40% oxygen saturated blood and then increased the saturation to 100% [61]. The workers compared these hearts to a control group, which were treated in an identical fashion, but oxygen saturation was maintained at 100%. They reported a decrease in myocardial function in hearts exposed to hypoxia and reoxygenation with no significant decrease in myocardial function in the control group. In doing so, these findings recapitulated the rodent work, again confirming that transient hypoxia prior to CPB is related to a poorer postoperative outcome. However, these investigations only examined preoperative transient hypoxia models then treated with hyperoxic cardiopulmonary bypass.

In order to address the questions around the most appropriate P_aO_2 to maintain on CPB, Ihnken et al. [22] subjected Duroc Yorkshire piglets to cardiopulmonary bypass and blood cardioplegia (BCP). They divided the piglets into control, treatment, and nontreatment groups. Piglets in the control group had 1 hour of CPB without hypoxia with 30 min of BCP arrest. Other piglets were subjected to 120 min of hypoxia (P_aO_2 = 20–30 mmHg) before the procedure. Piglets in the nontreatment group were reoxygenated on CPB for 5 min with high P_aO_2 (350–450 mmHg) followed by 30 min of BCP arrest and 25 min of reoxygenation/reperfusion on CPB with high P_aO_2 levels. Piglets in the treatment group had normoxic CPB (P_aO_2 100 mmHg) and BCP. They reported that hypoxic piglets with hyperoxic CPB developed worse LV function and reoxygenation injury compared to the group treated with normoxic CPB. In addition to this observation, they found that hyperoxic CPB led to a several-fold increase in lipid peroxidation and oxygen consumption as well as a decrease in antioxidant capacity compared to a normoxic group. Thus on both functional grounds and biochemical, they had demonstrated benefit to the myocardium of a normoxic approach to CPB.

Further pursuing the hypothesis that ROS were at least in part responsible for reoxygenation injury, Morita et al. [62] studied 40 immature Yorkshire Duroc piglets of 2 weeks of age. They analysed reduction of nitric oxide (an intermediate here of cytotoxic oxygen species) production with controlled cardiac reoxygenation in acutely hypoxic infantile hearts. They reported that CPB and blood cardioplegic arrest caused no functional or biochemical change in hearts maintained at normoxia. In piglets in which hypoxia was induced on a ventilator prior to surgery, a brief period of abrupt hyperoxic reoxygenation as short as 5 minutes after initiation of CPB, is enough to cause injury and that there was no myocardial protective effect even by controlling the P_aO_2 during cardioplegia delivery after this period. However, in a group in which the oxygen tension was slowly increased at the initiation of CPB, this large rise in NO production was not seen nor was there the previously seen elevation of dienes, and functionally the hearts were much closer to the normoxic group. This finding suggests that it is a sudden and abrupt change in the oxygen tension to which the myocardium is exposed, rather than a high P_aO_2 *per se*, which may be responsible for at least some of the reoxygenation injury.

Of course, the injurious effect of open heart surgery is not limited to the myocardium. To examine whether normoxic bypass had any effect upon the systemic stress endured perioperatively, Bandali et al. [63], using neonatal Yorkshire pigs, demonstrated that initiation of hyperoxia on CPB for period of 2 hours showed an increase in blood glucose levels by 40% during the first hour and a further increase by 12% in the second hour. Additionally they reported that following 2 hours of normoxic CPB, blood glucose levels remained unchanged. However with the induction of hyperoxic CPB, blood glucose levels raised by 46% and returned to normal following reestablishment of normoxia. So, broadly speaking, normoxic CPB is associated with significantly reduced elevation of blood glucose; this is indicative of reduced flux through the HPA endocrine axis and a decreased level of systemic stress.

Although these studies shed some light on the concept of hypoxia and hyperoxic reoxygenation injury, they had their limitations as the hypoxia was acute and induced by reducing F_iO_2, not by right to left sided shunts as would be seen in a cyanotic neonate. These patients will typically be operated upon some months after birth, so the ex utero hypoxic state is more chronic than the acute hypoxia in these models.

In order to address these criticisms and replicate an anatomical shunt causing cyanosis, Silverman and coworkers [64] used a canine model to anastomose left atrium proximal to a banded pulmonary artery to create cyanosis. They then compared this group to dogs with either pulmonary banding alone or no surgical intervention at all. They showed that after 3 months, there was a significant reduction in global biventricular function in the cyanotic group when

compared with the other two groups. They later subjected these animals to cardiopulmonary bypass and cardioplegic arrest. They reported that there was a significant reduction in ATP levels in cyanotic dogs, which was preserved in the other groups. They concluded that chronic hypoxia impairs global ventricular function and predisposes to the accelerated depletion of high-energy phosphates during cardioplegic arrest. These findings are similar to those performed earlier using animals exposed to hypoxic environments and confirm the validity of those conclusions.

However, a potentially valid criticism is that cyanosis in these models was interrupted with a period of normoxia after birth. This is clearly a large deviation from the pathophysiological state these models hope to duplicate and limits the applicability of the conclusions.

Addressing these complaints by attempting to produce a model of hypoxia from birth, Baker et al. [65] kept pregnant New Zealand white rabbits in a normoxic environment throughout their study (F_iO_2 of 0.21). Once the kits were born, following their first feed, they were immediately transferred to a hypoxic environment (F_iO_2 of 0.09). They maintained the oxygen level in the chamber at this level throughout the study. However they transferred the kits back to their mother twice a day for 20 min each to allow feeding. They have indicated that in a preliminary study they observed that mothers that were maintained at (F_iO_2 of 0.09) were unable to nurse their offspring. They reported that hearts of hypoxic from birth compared with normoxic controls had better protection against ischaemia in hypothermic conditions, undergoing hypothermia plus cardioplegia. This is surprising, and in conflict with what would be expected based upon both theoretical considerations and the available other clinical and experimental evidence. This model is not a complete replication of cyanotic congenital heart disease, however, as the kits were returned to 21% oxygen regularly for feeding, which is clearly not something which would happen in the human disease state.

In order to address the aberrant finding, Milano et al. [66] examined the effect of daily reoxygenation further to delineate where the apparent benefit was originating. They dubbed this model "chronic hypoxia with daily reoxygenation" (CHReox). In their animal model, they reported that daily reoxygenation during chronic hypoxia reduced ischaemia/reperfusion injury as well as improved recovery of ventricular performance. They concluded that it is not hypoxia in itself that results in protection in these models, but rather a daily reoxygenation associated with chronic hypoxia. This could be due to an increase of NO production in CHReox, which is involved in the ischaemic preconditioning, where short antecedent ischaemic periods render tissue more resistant to subsequent prolonged ischaemic insults. These findings then bring the work of Baker et al. [65] into consistency with other studies.

However, this study is limited by the fact that their animal models were made hypoxic at 5 weeks of age, whereas children with cyanotic congenital heart defects are cyanotic from birth and usually present with ventricular hypertrophy, pressure, and/or volume overload, heart failure, or a combination. Only the cyanotic element is modelled in the aforementioned investigations and none have avoided any normoxic period entirely.

To avoid episodes of normoxic exposure, Corno and coworkers [67] came up with a novel idea of storing animals in specially designed cages that remained hypoxic throughout even during and cleaning and feeding. They randomly allocated eighteen 5-week-old male Sprague-Dawley rats to be exposed to room air (F_iO_2 = 0.21) or chronic hypoxia (F_iO_2 = 0.10) for 2 weeks. They reported that uncontrolled reoxygenation of hypoxic hearts had negative systemic and cardiac effects. Although this was certainly a novel approach, an important drawback to this study, however, is that this design does not closely model the pathophysiology of clinical syndromes of cyanotic heart disease as the animals were already exposed to 5 weeks of normoxia before being subjected to hypoxia.

So although there are some well-performed studies in animal models, due to either flaws in design or inherent difficulties in reproducing the pathological state, the evidence they produce is only suggestive rather than a convincing demonstration for a deleterious effect from cyanosis and hyperoxic reoxygenation. However, evidence from clinical sources, taken together with the experimental data, strengthens the case.

5. Clinical Evidence for Hyperoxic Injury

Some of the earliest data to emerge on questions of comparisons of normoxic to hyperoxic cardiopulmonary bypass in the clinical setting was from Ihnken et al. [68]. They randomised 40 consecutive (adult, acyanotic) patients who were listed for coronary artery bypass graft surgery to either hyperoxic or normoxic CPB. Moderate hypothermia (28° to 32°C) was achieved during CPB in these patients. They reported higher release of creatine kinase, LDH, antioxidant levels, malondialdehyde, and polymorphonuclear elastase in the hyperoxic group. There were also extracardiac improvements in the normoxic group; the hyperoxic patients underwent a 57% longer duration of mechanical ventilation postoperatively, although this did not reach significance. While they reported lower vital capacity and forced vital capacity after CPB in both groups, normoxic patients had better vital capacity and forced vital capacity after CPB than the hyperoxic group. Maximal expiratory flow was also was reduced in hyperoxic patients with no changes in normoxic ones. They concluded that hyperoxia can contribute to multiorgan injury and failure and is unnecessary during established extracorporeal circulation. However, the applicability of this data to the paediatric and in particular the paediatric cyanotic population is clearly limited, although it does suggest that the basic and experimental data may be borne out in the clinical setting.

In the paediatric environ, Modi et al. [4] observed twenty-nine patients with congenital heart disease undergoing cardiac surgery. Of these, twenty were cyanotic and nine were acyanotic. All of these patients underwent at least 30 minutes of hyperoxic CPB before ischaemic cardioplegic arrest. They assessed plasma levels of troponin I (TnI, a marker of myocardial damage) at 1, 10, and 30 minutes of

CPB. They reported an increase in troponin I levels with time in both groups; however the rate of increase was greater in the cyanotic population by approximately threefold. Thus this effectively provides clinical evidence of myocardial reoxygenation injury as a direct result of hyperoxic CPB distinct from cardioplegic ischaemia/reperfusion insult; the rises were seen prior to the use of any cardioplegia, as samples were taken before aortic cross clamping. However, this study was purely observational, and so did not provide any further insights into mechanisms or changes in the underlying pathophysiology.

Attempting to take a closer look at the redox state and inflammatory mechanisms that take place in these scenarios, Bulutcu et al. [69] performed a prospective randomised trial where they allocated cyanotic patients to either an F_iO_2 on CPB of 1.0 (producing a measured P_aO_2 of 300–350 mmHg in their patients) or an F_iO_2 of 0.21 (P_aO_2: 90–110 mmHg). Furthermore, they also studied an acyanotic group that were at F_iO_2 of 1.0. They measured the antioxidant reserve capacity, as well as the levels of TNF-α and IL-6. As expected from the earlier discussion of ROS production in cyanosis, they found a lower antioxidant reserve capacity in cyanotic groups when compared to the acyanotic patients at baseline. Following initiation of CPB, TNF-α and IL-6 levels in the cyanotic groups were higher than for the acyanotic group. Cyanotic groups with hyperoxic CPB also had further reduced antioxidant reserve capacity compared to cyanotic group with normoxic CPB as well as higher TNF and IL-6 levels. Unfortunately it is not clear from their report what the P_aO_2 was during anaesthetic induction as a consequence of the supplemental oxygen administration which occurred, which could certainly have a confounding impact upon the normoxic group.

To further ascertain the underlying mechanisms, we [70] examined transcriptomic changes in a trial of paediatric patients with the tetralogy of Fallot causing cyanosis undergoing corrective cardiac surgery. Patients received either controlled reoxygenation (50–80 mmHg) or hyperoxic/standard CPB (150–180 mmHg). We found significant whole genome expression changes in the hyperoxic group that were not seen with controlled reoxygenation. Our findings suggested a decrease in the transcripts responsible for the adaptive and remodelling capacity of cyanotic hearts subjected to hyperoxia compared with controlled reoxygenation CPB. Our data also showed a reduction in global mRNA levels with hyperoxic CPB, suggesting a detrimental effect of hyperoxic CPB to the myocardium as a result of possible reduction of taurine, which can lead to cardiomyocyte atrophy, mitochondrial and myofibre damage, and cardiac dysfunction.

These studies, however, do not address the questions as to the wider systemic stress and end-organ injuries as outcome measures. Our group [5] performed a randomised trial which compared the effect of normoxic (50–80 mmHg) versus hyperoxic (150–180 mmHg) CPB on cyanotic children undergoing corrective cardiac surgery. We achieved a normoxic CPB by delivering medical nitrogen to the oxygenator and using an in-line PO_2 monitor to measure the PO_2 of the prime and matching it to the patient's own (cyanotic) P_aO_2 levels. Anaesthetic induction in the normoxic group was carried out with 21% oxygen, whilst in the hyperoxic group the F_iO_2 was 50%. We demonstrated that compared to hyperoxic CPB, normoxic CPB was associated with lower end-organ damage; cardiac, liver, and brain injuries were assessed with Tn-I, Alpha GST, and protein S100 and in all three cases were significantly reduced. In addition we reported lower levels of 8-isoprostane, indicative of a lesser degree of reoxygenation injury in the normoxic group. So not only does it seem that normoxic cardiopulmonary bypass is associated with an improved myocardial outcome, it is also correlated with a lesser degree of end-organ injury.

Some have wondered whether there is a third approach between the extremes of using either 100% or 21% oxygen for reoxygenation. Babu et al. [71] compared hyperoxic versus controlled oxygenation in a graded manner during initiation of cardiopulmonary bypass in cyanotic children undergoing cardiac surgery. They achieved controlled reoxygenation by initiating the CPB with an effective F_iO_2 of 0.21 and increased it by 0.1 per minute to ultimately achieve an equivalent of 0.6 (PO_2: 200–300 mmHg) at five minutes. The CPB in the hyperoxic group was started with F_iO_2 of 0.6 from the beginning. They reported lower CPK-MB levels and shorter ventilation time in the controlled reoxygenation group compared to hyperoxic CPB. It is noteworthy however that all patients during the anaesthetic induction were ventilated using F_iO_2 of 0.6, which may be confounding. In addition, the P_aO_2 was unclear from their report when they initiated the CPB with an F_iO_2 of 0.21. They indicated that to minimize costs, they opted to not use in-line PO_2 monitors for instantaneous PO_2 measurement and instead they increased oxygen levels over a 5-minute period [64, 71]. So there are significant limitations to this study; however, this novel approach may go some way to ameliorate concerns about the safety of purely normoxic CPB. It is also strongly reminiscent of the basic work of Morita et al. [62] who also took a graded approach to reoxygenation in animal models and found results similar to a normoxic group.

In support of these findings Joachimsson et al. [72] argue that in clinical practice, hyperoxic CPB is likely never needed, since a P_aO_2 of 400 to 500 mmHg produces only a trivial increase in oxygen content compared with a P_aO_2 of 100 to 150 mmHg, which is easily achieved using a modern oxygenator [73] and that in both instances the oxygen saturation is essentially 100%. Moreover, a CPB P_aO_2 of more than 180 mmHg has been associated with impairment of peripheral perfusion. Furthermore, an increased incidence of ventricular fibrillation has been reported [74] as well as some findings that hyperoxia may cause cerebral vasoconstriction that leads to nonhomogeneous oxygen distribution [75] and regional hypoperfusion.

Most of these studies from clinical practice have regarded cyanotic heart disease as one distinct entity. In reality, it consists of a large number of syndromes, many with cardiac and extracardiac manifestations. We have attempted to break down the benefits of normoxic cardiopulmonary bypass further by examining the effects differentially on patients with univentricular or biventricular circulations [76]. We examined markers of cardiac, hepatic, and cerebral injury, as well as long term functional outcome. We demonstrated a significant

improvement in both in markers of organ damage and in oxidative stress, a lower degree of systemic injury, as well as a significantly improved outcome at 7 years postoperatively in univentricular patients undergoing open heart surgery for congenital heart disease. There is however a paucity of evidence considering the univentricular circulation so it remains difficult to place this interesting and novel data into context. Additionally, we also showed a significant reduction in the level of postoperative cortisol and proinflammatory cytokines in both biventricular and univentricular circulations; the proinflammatory cascade consequent to CPB as well as the surgical systemic inflammatory response is accountable for end-organ dysfunction postoperatively. Therefore, any reduction in inflammatory mediators as a consequence of this intervention may lead to an improvement in postoperative systemic dysfunction and possibly overall outcome measures.

Considering contraindications to normoxic CPB, the only significant cautions have come from Nollert et al. [77]. They have provided data that show that normoxic CPB should not be considered during deep hypothermic circulatory arrest (DHCA) which can lead to cerebrals insult (assessed by protein S100 and histological evaluation), which showed cerebral insults in particular to neocortex and hippocampal region. In the converse situation, Fontes et al. [78] examined whether hyperoxia might contribute to cognitive decline following cardiopulmonary bypass; no association was found. These findings were confirmed experimentally by Wang and colleagues in 2012 [79], who found that hyperoxic management during DHCA demonstrated better cerebral protection than normoxic.

So DHCA remains the strongest indication for conventional oxygenation on CPB; for the remainder, there is strong experimental and clinical evidence of benefit to a controlled reoxygenation strategy.

6. Conclusion

It has been demonstrated that hyperoxic cardiopulmonary bypass predisposes towards direct myocardial injury and systemic stress, with no counterbalancing benefit. In fact not only is this unfavourable effect apparent in the cyanotic population, but there are also reports that even in acyanotic conditions; controlled reoxygenation can be advantageous. Normoxic cardiopulmonary bypass has been reported to be safe and easy to achieve, as long as the appropriate equipment is available, and the perfusionist and anaesthetist are both familiar with the technique. Despite many pieces of both clinical and experimental evidence that hyperoxic CPB can have a detrimental effect on the cyanotic population it seems that the use of controlled reoxygenation is not widely accepted. It is our hope that this presentation of the available evidence might prompt a reevaluation of the wisdom of this approach. Given the limited numbers of patients presenting to each centre, and the variation in current practice, it seems as though a large prospective randomised control trial would be the best way to obtain a definitive answer to the questions raised herein. Certainly, the potential benefits are likely to be significant.

Conflict of Interests

The authors have no conflicting interests with any of the subjects discussed in this paper.

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Review Article

Cardioprotection: A Review of Current Practice in Global Ischemia and Future Translational Perspective

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The idea of protecting the heart from ischemic insult during heart surgery to allow elective cardiac arrest is as old as the idea of cardiac surgery itself. The current gold standard in clinical routine is a high potassium regimen added either to crystalloid or blood cardioplegic solutions inducing depolarized arrest. Ongoing patient demographic changes with increasingly older, comorbidly ill patients and increasing case complexity with increasingly structurally abnormal hearts as morphological correlate paired with evolutions in pediatric cardiac surgery allowing more complex procedures than ever before *redefine requirements for cardioprotection*. Many, in part adversarial, regimens to protect the myocardium from ischemic insults have entered clinical routine; however, functional recovery of the heart is still often impaired due to perfusion injury. Myocardial reperfusion damage is a key determinant of postoperative organ functional recovery, morbidity, and mortality in adult and pediatric patients. There is a discrepancy between what current protective strategies are capable of and what they are expected to do in a rapidly changing cardiac surgery community. An increased understanding of the molecular players of ischemia reperfusion injury offers potential seeds for new cardioprotective regimens and may further displace boundaries of what is technically feasible.

1. Introduction

For the majority of cardiac surgical interventions arresting the heart is inevitable, with systemic arterial perfusion and oxygenation being transferred to a heart lung machine. Until the present day, cardioplegic arrest remains the gold standard of cardioprotection and requires a potassium rich solution sending the heart into a depolarized arrest [1]. Despite its almost universal usage, cardioplegia in its current form is associated with potential downsides rendering those cardioprotective regimens a less than optimal choice in certain clinical situations and certain patient collectives.

25% of the population over 75 years suffers from symptoms of cardiovascular disease [2], and as the elderly represent the fastest growing population demographic in industrialized nations, the proportion of elderly patients being evaluated for cardiac surgery is only expected to increase (the average age of cardiac surgical patients increased from 55.8 years to 68.8 years in the course of the last decade [3]). In general, the elderly represent a comorbidly ill patient population with a higher perioperative risk. Factors influencing operative risk include age >70, female sex, renal impairment, extracardiac arteriopathy, chronic lung disease, pulmonary hypertension, insulin dependent diabetes, NYHA III/IV, and ejection fraction <50%. This is especially important in the present light of change in the field of interventional cardiology offering catheter-guided approaches to an increasingly larger patient cohort causing a shift in cardiac surgery away from isolated "simple" procedure towards more complex interventions [4], sometimes in the very old and the severely ill [1, 3]. This increase in case complexity in a changing patient population is especially relevant for patients with impaired

Table 1: Composition of crystalloid (intracellular type: Custodiol, HTK, Bretschneider's, and extracellular type: Plegisol, St. Thomas solution) and blood based cardioplegic solutions.

Formulation ingredient	Crystalloid-based cardioplegia		Blood-based cardioplegia		Units
	Intracellular Custodiol, HTK, Bretschneider's	Extracellular Plegisol, St. Thomas solution	Blood cardioplegia induction 4 : 1	Blood cardioplegia maintenance 4 : 1	
Na^+	15	110	140	140	mmol/L
K^+	9	16	20	10	mmol/L
Mg^{2+}	4	16	13	9	mmol/L
Ca^{2+}	0.015	1.2	—	—	mmol/L
Histidine	198	—	—	—	mmol/L
Tryptophan	2	—	—	—	mmol/L
Ketoglutarate	1	—	—	—	mmol/L
Mannitol	30	—	—	—	mmol/L
Glucose	—	—	6	6	mmol/L
Lidocaine	—		260	—	mg/L
pH	7.02–7.20	7.8	7.2	7.4	$[H^+]$

ventricles associated with left ventricular hypertrophy (LVH) and heart failure where it is generally acknowledged that current methods of myocardial protection are inadequate. LVH increases myocardial workload and renders hypertrophic hearts more susceptible to ischemic injury [5] and impaired recovery postoperatively.

To develop new approaches towards innovative cardioprotective regimens it is increasingly important to understand the pathophysiologic and molecular players of ischemia as a two-fold phenomenon in which ischemic injury is only part of the truth and subsequent reperfusion injury has the potential to grossly outweigh the primary ischemic insult [6]. Myocardial reperfusion damage following cardioplegic ischemic arrest is a key determinant of postoperative organ functional recovery, morbidity, and mortality in adult and pediatric patients undergoing open-heart surgery and has the potential to cause protracted organ recovery, myocardial stunning, and acute myocardial infarction.

This paper reviews and compares current clinical regimens of cardioprotection via elective global ischemia induction and draws attention to potential avenues for innovative therapeutic approaches with the potential of translational application in future clinical trials, thereby highlighting management of ischemia reperfusion injury as a central dogma.

2. Electrophysiological Concepts: Induction of Arrest

Sending the heart into a diastolic flaccid arrest requires understanding of the underlying electrophysiology principles. Hyperkalemia (as the current clinical practice either via blood cardioplegia or crystalloid cardioplegic solution, Table 1) changes the cellular resting membrane potential (E_m) of cardiac myocytes towards a less negative value (i.e., closer to zero). The resting membrane potential is largely maintained via an adenosine triphosphate (ATP) driven primary active $3Na^+/2K^+$ exchange pump creating both chemical and electric gradients across the cellular membrane and via a passive K^+ outward flux. As the cardiac myocyte membrane is most permeable to K^+ ions but relatively impermeable to other ions, E_m potential is close to the K^+ equilibrium potential of −91 mV (Nernst equation, Figure 1) and approaches −85 mV (Goldman-Hodgkin-Katz voltage equation).

The action potential (AP) is the result of an orchestrated activation of various voltage-gated channels located in the cell membrane (Figure 1). Upon stimulation by the sinuatrial node, voltage dependent Na^+ channels open and allow rapid sodium ion influx and depolarization of the cardiac myocyte cell membrane to about +20 mV. The threshold potential for fast Na^+ channel opening is about −65 mV. Na^+ channels become inactivated within a split second and E_m would return to normal unless L-type Ca^{2+} channels open and allow further influx of positively charged ions, characteristically prolonging the AP in a plateau-like fashion. For most parts of the plateau phase Ca^{2+} influx and K^+ outward directed back diffusion is balanced; however, as the membrane potential reaches more negative values, Ca^{2+} channels close and delayed K^+ rectifiers send E_m back to normal and terminate yet another AP. Electromechanical coupling and force generation evolves as Ca^{2+} influx via dihydropyridine receptors of the myocyte cell membrane causes cytosolic Ca^{2+} release from the sarcoplasmic reticulum via ryanodine receptors (calcium induced calcium release). In a physiologic state with extracellular K^+ levels of 3.5–5.0 mmol/L, E_m approaches −85 mV. One can easily calculate that an elevation of extracellular K^+ towards 16.2 mmol/L decreases E_m to about −60 mV (Figure 1) well beyond the Na^+ channel threshold, not allowing any further myocyte AP propagation generated by the sinuatrial node (SAN) because fast Na^+ channels remain inactivated. As this state does not allow any repolarisation either the current clinical practice of hyperkalemic cardiolegia induction is called depolarized arrest. The institution of cardioplegic arrest ensures that myocardial

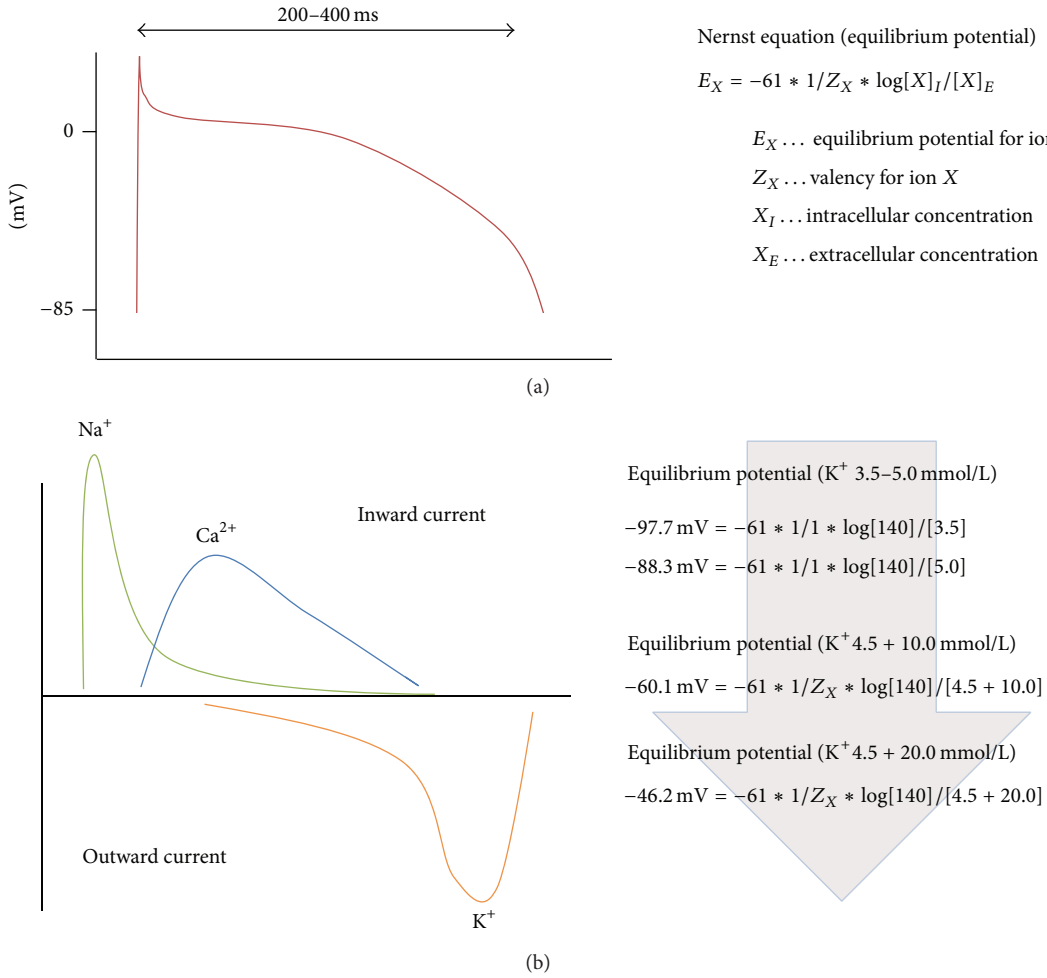

FIGURE 1: (a) Action potential for cardiac myocytes and (b) ion flux occurring during each cardiac myocyte action potential. Nernst equation on the right-hand side illustrating membrane potential changes upon modification of the extracellular K^+ ion concentration.

oxygen consumption (MVO2) is significantly reduced, as is the ATP depletion characteristic of severe ischemia [7–9].

3. Cardioprotection: A Strategic Comparison of Current Clinical Practice

There is no doubt that adequate myocardial protection plays a key role in achieving successful outcomes in cardiac surgery. Many methods to achieve and maintain sustained electromechanical quiescence have been advocated and obviously few institutions share identical protocols which makes comparison in large multicenter studies and meta-analysis difficult. Nevertheless, we provide a comprehensive review of the most important clinical trials in Table 3. The spectrum of strategies has led to the creation of, in part, adversarial positions. In general, blood-based or crystalloid-based solutions are used as potassium-containing transport medium. Blood cardioplegia is mixed in a ratio of 1 : 4 (1 part of crystalloid solution and 4 parts of blood); crystalloid solutions may be of intracellular type (Custadiol) or extracellular type (Plegisol). Specific details are given in Table 1. Modes of application range from antegrade versus retrograde versus antegrade plus retrograde, intermittent versus single shot, cold (with or without additional warm induction) versus warm. Thereby antegrade and retrograde refer to the route of application: antegrade application follows anatomical routes and normal coronary circulation via insertion of a cardioplegia line into the aortic root below the aortic cross-clamp, whereas retrograde perfusion is achieved via direct intubation of the coronary sinus.

All in situ hearts receive some noncoronary collateral flow so that intermittent replenishment of cardioplegia is needed to maintain the primary goals of hypothermia for cardiac myocyte metabolic demand reduction, washout of accumulated metabolites, counteraction of acidosis, and provision of a cardioprotective composition to lower perfusion injury before the next period of planned ischemia is initiated. So, what is better?

3.1. Blood versus Crystalloid? Several experimental studies favor the use of blood cardioplegia over crystalloid solutions when comparing release of cardiac enzymes and metabolic response [10]. To our knowledge, Øvrum et al. from the Oslo

Table 2: Definitions of low output syndrome (LOS) used in 34 trials. Adapted from [13].

Definitions of LOS
Requirement of inotropes for >30 minutes or IABP for maintenance of blood pressure >80 mm Hg
The need for inotropic and/or IABP assistance to maintain the systolic BP at a level >90 mm Hg for at least 30 minutes in the ICU
Requirement for inotropic agents or IABP for hypotension
Use of inotropic agents or IABP assistance for hypotension
CI <2.01 L/min/m^2 and the need for dopamine (>4 μg/min/kg), dobutamine, or adrenaline administration for >30 minutes or IABP
CI <2 L/m^2 despite PAWP ≥15 mm Hg, dopamine in a dose of 3–4.9 μg/kg/min, and, if required, adrenaline in a dose of 0.02–0.10 μg/kg/min
Unusual need of inotropic (>6 U dopamine) or mechanical (IABP/VAD) support to maintain the normal CO of the patient (normal CO 4–8 L/min)
CI <2.1 L/min/m^2
Systolic BP <90 mm Hg and mixed venous oxygen saturation <60% despite adequate preload and afterload

Heart Center conducted the largest prospectively randomized single center trial comparing postoperative outcomes of cold blood versus cold crystalloid cardioplegic regimens (1440 CABG patients [11] and 345 aortic valve patients [12]). All patients were gender, age, and perioperative risk matched and no statistical significant differences were seen regarding perioperative and postoperative parameters (Table 3). Even in patients with higher operative risk (female sex, age >70 years, unstable angina, diabetes, emergency operation, ejection fraction <50%, crossclamping time >50 minutes, and EuroSCORE II >5), no statistically significant differences could be demonstrated [11, 12].

A large meta-analysis by Guru et al. [13] from the Toronto University compared 34 trials with a total of 5,044 patients. 2,582 received blood cardioplegia and 2,462 received crystalloid cardioplegia (no differentiation between intracellular and extracellular type crystalloid solutions was made). The authors found no difference between groups regarding perioperative and postoperative myocardial infarction and death ($P = 0.19$ and $P = 0.44$, resp.); however, they did observe a significantly lower incidence of low output syndrome (LOS) immediately upon reperfusion with blood cardioplegia ($P = 0.006$). Various definitions of LOS exist in literature and are discussed in Table 2. Also, CKMB release at 24 h after surgery was considerably lower with blood cardioplegia ($P = 0.007$).

3.2. Antegrade versus Retrograde versus Antegrade/Retrograde? Retrograde cardioplegia is an established method of myocardial protection [14–16]. The rationale behind retrograde application is that distribution of antegrade delivered cardioplegia might be impaired due to ventricular hypertrophy or significant coronary artery stenosis and retrograde application, bypassing plagued vessels, might be of advantage. However, the problem with retrograde perfusion is purely anatomical. Many of our colleagues believe that Thebesian veins play a central role in the distribution of retrograde cardioplegia. Of course this cannot be true. By definition, Thebesian veins constitute venous anastomoses directly "escaping" into the atrial and ventricular cavities bypassing the microvasculature and diminishing nutrient flow. This is reflected in the clinical setting by higher perfusion volumes rather than higher perfusion pressures required to induce cardiac arrest. In studies conducted by Gates et al. from UCLA in Los Angeles on human freshly explanted hearts it became obvious that all regions of the heart can be homogenously perfused in a retrograde fashion [17]. However, when collecting the effluents using colored microspheres 67.2 ± 6.4% of retrograde delivered blood cardioplegia was found to exit Thebesian veins directly into heart cavities, whereas only 29.3 ± 6.3% and 3.5 ± 3.1% escaped from the left and right coronaries, respectively [17]. Although all areas of the ventricles are perfused by retrograde blood cardioplegia, the majority of flow is through the Thebesian system and, thus, is nonnutritive. Further, the nutritive capillary flow that does occur is heavily weighted between ventricles and the right side gets only 10% of the nutrient flow when compared to the left ventricle [17]. Vähäsilta et al. [18] used isolated pig hearts to compare antegrade versus retrograde *crystalloid cardioplegia* in a standardized perfusion model. He assessed for cardiomyocyte apoptosis via terminal transferase mediated ddUTP nick end-labeling (TUNEL) assay and immunohistochemical staining for caspase-3 (Table 3). Cardiomyocyte apoptosis was significantly elevated following reperfusion in both groups, suggesting that myocardial protection in general is suboptimal in antegrade and retrograde cardioplegia. In the right ventricle, however, retrograde cardioplegia was associated with a 3.4-fold higher amount of apoptotic cardiomyocytes as compared with antegrade cardioplegia (0.107% versus 0.032%, $P < 0.05$). A similar difference was also found in the left ventricle, although at a lower level (0.027% versus 0.012%, $P < 0.05$). A subsequent clinical study with 20 patients undergoing aortic valve surgery led by the same authors found that cardiomyocyte apoptosis is significantly increased in the left ventricle after the procedure in the retrograde, but not in the antegrade group (0.00% versus 0.092%, $P = 0.01$ and 0.00% versus 0.023%, $P = 0.14$) [19]. Regarding functional data the amplitude of longitudinal systolic motion of the lateral mitral annulus (an indicator of systolic contractile function) was lower after the operation than before in the retrograde ($P = 0.03$) but not in the antegrade group ($P = 0.78$) [19]. There were no differences in cardiac output, velocities of the E or A waves or E/A ratios in either antegrade or retrograde groups. Altogether, retrograde cardioplegia was associated with left better than right ventricular protection as evident by morphometric and functional analysis; however, this finding did not reflect on patient

Table 3: Best evidence papers.

Authors	Title	Year	Intervention	Patients included	Study design	Study endpoints	Reference
Øvrum et al.	A prospective randomized study of 1440 patients undergoing coronary artery bypass grafting	2004	Blood versus crystalloid	1.440*	Prospective randomized	Operative variables[1], inotropic support, ICU/hospital stay, arrhythmias, stroke, mortality	[11]
Øvrum et al.	A prospective randomised study of 345 aortic valve patients	2010		345*	Prospective randomised		[12]
Guru et al.	Is blood superior to crystalloid cardioplegia? A meta-analysis of randomized clinical trials	2006		5.044*	Meta-analysis	LOS, MI, CKMB at 7 h, 24 h, 48 h	[13]
Vhsilta et al.	Cardiomyocyte apoptosis after antegrade and retrograde cardioplegia during aortic valve surgery	2011	Antegrade versus retrograde	20*	Prospective randomised	Cardiomyocyte apoptosis (TUNEL assay, caspase 3, BCL-2, and BAX via ventricular biopsies upon reperfusion) ECHO	[19]
Lotto et al.	Myocardial protection with intermittent cold blood during aortic valve operation: antegrade versus retrograde delivery	2003		39*	Prospective randomised	Biopsies 20 min after cross-clamp removal, adenine nucleotide metabolites, lactate, troponin I	[20]
Radmehr et al.	Does combined antegrade-retrograde cardioplegia have any superiority over antegrade cardioplegia?	2008	Antegrade plus retrograde	87*	Prospective randomised	Inotropic support morbidity, ICU/hospital stay, mortality	[21]
Fan et al.	Does combined antegrade-retrograde cardioplegia have any superiority over antegrade cardioplegia?	2010	Warm versus cold	5.879*	Meta-analysis	LOS, inotropic support, MI, stroke, arrhythmias, cardiac index, Troponin, CKMB	[22]
Mallidi et al.	The short-term and long-term effects of cold or tepid cardiopelgia	2003		6.064*	Prospective cohort	MI, Mortality	[23]
Caputo et al.	Warm blood hyperkalaemic reperfusion (hot shot) prevents myocardial substrate derangement in patients undergoing coronary artery bypass surgery	1998	Cold-cold plus hot-shot	35*	Prospective randomised	Adenine nucleotide metabolites, alanine-glutamate ratio, lactate, troponin I: 5 min after begin of bypass, 30 min after arrest and 20 min after reperfusion	[25]

LOS, low output syndrome (requiring intotropic and/or intra-aortic balloon pump support); MI, myocardial infarction; CKMB, creatinine kinase MB; BCL-2, B-cell lymphoma 2 (antiapoptotic) and BAX protein (proapoptotic). [1]Operative variables include amount of cardioplegia used, spontaneous sinus rhythm after declamping, atrioventricular block, fluid excess. *7 prospective randomized studies as well as 2 large meta-analyses yielded a total number of **17.513 patients**. The meta-analysis performed by Guru et al. [13] included the initial Ovrum et al. [12] study and those patients were thus subtracted.

outcome. Findings of increased right ventricular stress with retrograde cardioplegia alone could be confirmed by Lotto et al. from the Bristol Heart Institute comparing 39 patients undergoing elective aortic valve replacement [20]. They also found that troponin I levels were significantlyelevated in both groups again suggesting that myocardialprotection in general is suboptimal [20]. However, both authorsdid not observe statistical differences in the postoperative patient outcome.

There is sufficient evidence, however, that combined antegrade and retrograde cardioplegia is of advantage. Radmehr et al. [21] compared antegrade versus antegrade/retrograde cardioplegia in 87 randomly assigned, age, gender, and perioperative risk matched patients undergoing CABG and found that 35.5% versus 19.0% of patients needed inotropic support while weaning from bypass ($P = 0.04$).

3.3. Cold versus Warm? Fan et al. [22] conducted a meta-analysis of warm versus cold cardioplegia identifying 41 randomized controlled trials with 5,879 patients. In-hospital mortality, length of stay, incident of stroke, and atrial fibrillation (Afib) and use of balloon pumps did not differ between groups. However, warm cardioplegia was associated

with significantly better postoperative cardiac index ($P < 0.00001$), lower troponin concentrations on day 0 ($P = 0.006$), and significantly lower peak CKMB concentrations ($P = 0.002$). Mallidi et al. [23] performed a prospective single center cohort study comparing patients receiving cold or tepid/warm cardioplegia during isolated CABG on early and late outcomes and found superior outcomes in the warm cardioplegia arm: perioperative death (1.6 versus 2.5%, $P = 0.027$) and myocardial infarction (2.4 versus 5.4%, $P < 0.0001$).

3.4. Terminal Warm Induction (Hot-Shot)? Intermittent antegrade cold-blood cardioplegia followed by terminal warm-blood cardioplegic reperfusion (hot-shot induction) is reported to reduce myocardial injury in the setting of coronary surgery [24]. Caputo et al. [25] from the Bristol Heart Institute compared 35 patients receiving cold blood cardioplegia with or without terminal warm induction prior to removal of the cross-clamp. Significant metabolic derangement occurs in the ischemic-reperfused hearts of patients with cold blood cardioplegia but not in the hot-shot group ($P < 0.05$) as evidenced by high ADP/ATP ratios and an increase in the alanine-glutamate ratio suggesting the occurrence of anaerobic metabolic activity (Table 3) [25]. Troponin I concentrations were consistently higher in the cold blood group, without reaching statistical significance.

A survey of practice in the UK [26] among cardiac consultants from 2004 found that, of the surgeons performing on-pump CABG, 56% use cold blood cardioplegia, 14% use warm blood cardioplegia, 14% use crystalloid cardioplegia, 21% use retrograde infusion, and 16% do not use any cardioplegia (cross-clamp fibrillation). This impressively highlights that there exists no consensus in the cardiac surgery community regarding the type and route of cardioplegia application.

4. Ischemia and Reperfusion on the Blueprint: A Double-Edged Sword?

Since the initial description of the phenomenon by Jennings et al. [27, 28] (see above) some 50 years ago, our understanding of the underlying mechanisms of ischemia reperfusion injury has grown significantly, yet molecular and cellular events underlying IR injury are complex, representing the confluence of divergent biologic pathways [29].

4.1. Ischemia-Microvascular Dysfunction. The microcirculation represents the major target site of ischemia reperfusion (IR) injury [30–33]. Response to ischemia, initially compensatory and adaptive in nature, progresses to structural changes that become self-perpetuating and pathogenic, when sustained for more than a couple of minutes. Ion influx and cellular swelling impair reperfusion (no-reflow phenomenon) as energetic imbalance characterized by both decreased oxidative phosphorylation and impaired cellular energy production causes tissue acidosis and reactive oxygen species (ROS) formation leading to toxic cell damage [34]. Thereby, ROS generated as the final electron acceptor (O_2) in the electron chain is insufficient in quantity and O_2 cannot be further reduced to H_2O (Figure 2) and the electron transport chain comes to a hold.

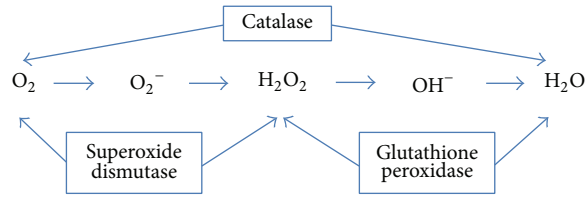

Figure 2: Rapid release of ROS overwhelms the cellular defense mechanisms and leads to toxic cell damage.

Reperfusion in turn, as a growing body of evidence unveils, triggers, an inflammatory response via leukocyte transmigration into tissue with concomitant activation and dysfunction of the vascular endothelial cell barrier and mediates both innate and adaptive immunogenic processes via a multitude of cascades [35, 36].

During ischemia, energy rich phosphates are depleted and the cellular active transmembrane ion transports (Na^+/K^+ATPase) are working at a lower pace with intracellular sodium buildup and ionic cellular swelling. High sodium concentrations, in turn, drive increases in intracellular Ca^{2+} via Na^+/Ca^{2+} exchange [37]. Further Ca^{2+} uptake via sarcolemmal L-type channels and impaired sarcoplasmic clearance via sarcoplasmic endoplasmic reticular calcium ATPase (SERCA) further drives a mechanism of self-perpetuating intracellular Ca^{2+} overload [38–41] eventually leading to ultrastructural changes, activation of apoptotic caspase pathways, autophagy-associated cell death, and necrosis [42].

4.2. Reperfusion: The Many Faces of Inflammation. Not only is IR limited to capillary events described above but also the postcapillary venules and their lining endothelial cells set an even bigger stage for a variety of molecular effectors to occur upon reperfusion [29, 35, 36, 43]. The central idea of reperfusion is injury-mediated activation of endothelial cells attracting polymorphonuclear leukocytes (PMNs) via upregulation of receptors and signal molecules on both leukocytes and endothelial cells [44] with subsequent transmigration and tissue invasion through a dysfunctional endothelial cell barrier and impaired cell-cell connections with concomitant increased vascular permeability, edema formation and inflammation [36]. The phenotype of inflammatory response to IR and to that observed during microbial infection share many similarities [42]. For example, ligand binding to pattern recognition molecules like TLRs (toll-like receptors) leads to downstream activation of nuclear factor kappa B (NFκB) and mitogen activated protein kinase (MAPK) pathways resulting in increased transcription of proinflammatory cytokines activating both endothelial cells and leukocytes. Surprisingly, TLRs can either be activated by microbial compounds or cellular debris in the context of IR injury [42, 45–47].

Activation of endothelial cells and PMNs results in upregulation of cellular receptors engaging leukocytes into a dance across endothelial cells (Figure 3) consisting of leucocyte

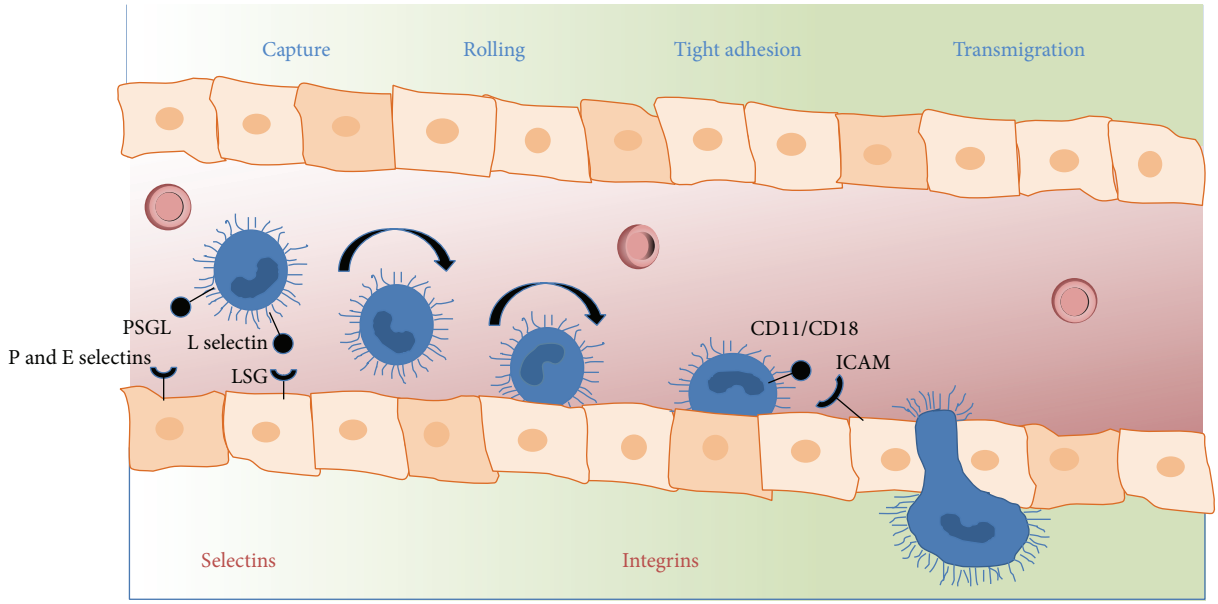

FIGURE 3: Dance of neutrophils. Leukocyte capture, rolling, adhesion, and transmigration through the endothelial cell barrier. PSGL, P selectin glycoprotein ligand; LSG, L selectin glycoprotein ligand; ICAM, intercellular adhesion molecule. Initial capture of leukocytes is mediated via selectin (endothelial P/E selectin binds PSGL); tight adhesion is mediated via integrins (neutrophil beta-2, leukocyte function associated antigen, CD11/CD18 binds ICAM).

tethering, activation, adhesion, and subsequent transmigration. The ability of PMNs to leave the blood stream and enter surrounding tissues is a critical feature of the immune response [36]. Each of these steps requires either upregulation or activation of distinct sets of adhesion molecules. Thereby, selectins initiate leukocyte attachment along vascular endothelium by mediating leukocyte rolling along activated endothelium (P selectin CD62P binds P selectin glycoprotein ligand 1) whereas integrins such as the neutrophil beta-2 integrin (CD11/CD18) bind endothelial intercellular adhesion molecules (ICAM) and play an important role in the subsequent steps of leukocyte migration into tissues (Figure 3).

Myocardial reperfusion not only saves the majority of the ischemic cells, but paradoxically has a downside called reperfusion injury with further myocardial injury and cardiomyocyte death, in part from microvascular (endothelial) injury and sterile inflammation. Reperfusion damage is a key determinant of postoperative organ functional recovery, morbidity, and mortality in adult and pediatric patients undergoing open-heart surgery. It is, therefore, important to understand the concept of ischemia with subsequent reperfusion as a two-edged sword.

5. Experimental Translational Approaches

When preparing our review we found a total of 17.513 patients being compared to the type of cardioplegia they received (Table 3) and there is little evidence that blood or crystalloid, antegrade or retrograde, cold (with or without additional warm induction) or warm have any major impact on clinical outcome. Manipulating the conditions of delivery are, essentially, cosmetic changes. Real changes require innovative steps in the concept of myocardial protection and treatment of ischemia reperfusion injury is the cornerstone of future cardioprotective regimens. The most promising and recent approaches are presented in Table 4.

5.1. Is Peptide Treatment a Trojan Horse? A competent endothelial cell barrier is the center structure integrating various proinflammatory signals and sets course for perfusion injury development. In terms of endothelial cell integrity and fluid extravasation VE cadherin is one of the key molecules integrating signals for opening and tightening of cell junctions [43, 48, 49] and is required for maintaining a restrictive endothelial cell barrier [44]. Barriers are largely formed by endothelial cell-cell contacts built up by VE-cadherin and are under the control of RhoGTPases [43]. VE cadherin has a binding site for the E1 fragment of the fibrin molecule [36]. Interaction of the fibrin β-chain and VE cadherin induces capillary tube formation, cell-cell contraction, and upregulation of ICAM-1 expression and induces leukocyte migration [50]. Cell-cell contraction and rupture of cell-cell contacts result in capillary leak. Blockage of the VE cadherin binding site for the fibrin β-chain proves promising in reduction of IR injury and leukocyte transmigration [43, 48, 49]. The molecular key for protection is a peptide from the N-terminus of the β-chain ($B\beta_{15-42}$) which dissociates the src kinase Fyn from VE-cadherin-containing junctions. Following exposure to $B\beta_{15-42}$, Fyn dissociates from VE-cadherin and associates with p190RhoGAP, a known antagonist of RhoA activation [43]. The activity of RhoGTPases is strictly regulated [51]. Rho GTPases cycle between an active GTP bound and an inactive GDP bound state. Rho GTPases can only interfere with their downstream effectors in the

Table 4: Examples of promising therapeutic approaches targeting ischemia reperfusion injury. Adapted from [42].

Intervention	Target	Potential downside	Stage	Reference
Fibrinogen split product Bβ_{15-42}	VE Cadherin	Unclear	Preclinical	[35, 36, 43, 48, 49, 53, 54, 66]
Cingulin derived sequence GRRPGGISGG	RhoAGTPase and VE Cadherin	Unclear	Preclinical	[35]
TAK-242	TLR4	Immunosuppression	Phase II clinical trial	[60, 67]
Cyclosporine	Cyclophilin/mPTP	Immunosuppression	Phase II clinical trial	[58]
PHD inhibitor	Oxygen sensing PHD enzyme, HIF stabilization	Unclear	Phase II clinical trial	[61, 63]
Ischemic preconditioning	Multiple	Unclear	Phase II clinical trial	[61, 68]
Ischemic postconditioning	Multiple	Unclear	Phase II clinical trial	[69]
Remote ischemic conditioning	Multiple	Unclear	Phase II clinical trial	[70]
Endothelin blockers	Endothelin A receptor/Na^+/H^+ exchange	Hypotension	Phase II clinical trial	[55, 71]

active GTP bound state. The activation of Rho GTPases is mediated by specific guanine nucleotide exchange factors (GEFs), which catalyse the exchange of GDP for GTP. The fibrin derived peptide drug Bβ_{15-42} has been proven to significantly reduce IR injury and leukocyte transmigration in experimental preclinical studies specifically designed with prolonged ischemia times [heart transplantation model in rodents [36, 49, 52], kidney transplantation model in rodents [48], animal models of Dengue shock syndrome [43], burn model in rodents [35], vascular interpolate models [53], and lung transplantation model in rodents (unpublished)] and to significantly decrease the necrotic core zone in acute ST elevation myocardial infarction in a clinical phase II study [54] and may constitute a Trojan horse in future IR injury treatment (Table 4).

5.2. Endothelin Receptor Blockers. The central role of the endothelial cell barrier in IR injury is not just confined to the receptors they express or the cytokines they upregulate but also the hormones they secrete [55]. Both circulating endothelin (ET-1) and endothelin receptors are significantly upregulated over ischemic myocardial tissue during IR and are associated with ischemic myocardial contracture [55]. This phenomenon is defined as a rise in resting tension and has attracted quite some attention since the recognition of the "stone heart" as a complication of cardiopulmonary bypass. On isolated rat hearts the ET_A selective antagonist PD 155080 (Table 4) reduces peak ischemic contracture (−49%), delays its time to onset (+56%), and improves recovery of reperfusion left ventricular developed pressure (LVDevP +12%), coronary flow (+16%), and diastolic relaxation (+50%) [55].

5.3. The Role of Mitochondrial Pores-Cyclosporine Rescue. Cardiac myocytes constitute both highly energy dependent and consuming cellular entities and as such not surprisingly host a high density of mitochondria for electromechanical coupling and force generation. Those cellular batteries can only guarantee function if the innermost mitochondrial membrane is maintained as an impermeable layer for buildup of an electrochemical gradient. Pores in this membrane lead to dissipation of the electric potential across the mitochondrial membrane resulting in "cellular suffocation" in part resembling uncoupling agents and enable mitochondrial-cytosolic escape of reactive oxygen species and induction of apoptosis. As part of this, the mitochondrial permeability transition pore (mPTP) has attracted attention recently as being Ca^{2+} and ROS inducible [56], which directly links mPTP to ischemic injury, and Cyclosporine A sensitive (nonspecific mPTP opening inhibitor) [29, 57]. mPTP seems to be the common effector of a series of upstream signals and Cyclosporine trials on perfusion injury reduction published in the New England Journal of Medicine proved promising [29, 58] (Table 4).

5.4. Toll-Like Receptors and TAK-242. Surprisingly, the phenotype of inflammatory response to IR and to that observed during microbial infection is very similar [42]. Ligand binding to pattern recognition molecules like TLRs (toll-like receptors) leads to downstream activation of nuclear factor kappa B (NFκB) and mitogen activated protein kinase (MAPK) pathways resulting in increased transcription of proinflammatory cytokines activating both endothelial cells and leukocytes. TLRs can either be activated by microbial compounds or cellular debris in the context of IR injury [42, 45–47]. One of the most widely investigated pattern

recognition molecules is TLR4 usually mediating inflammatory response to gram negative lipopolysaccharide particles (LPS); however, TLR4 activation is significantly enhanced by oxidative stress occurring during IR injury [59]. TAK-242, an inhibitor of TLR4, shows efficacy in reduction of IR injury in large animal trials [60] (Table 4).

5.5. The Potential of Endogenous Mechanisms and Prolylhydroxylase. Apart from the lab bench, the heart does have endogenous protective mechanisms to fight ischemia for a limited period of time and those mechanisms might be amenable for future clinical practice as well. One of those endogenous mechanisms can be activated by repeated cycles of brief ischemia. Ischemic preconditioning (IP) is defined as an experimental technique that renders tissues resistant to the deleterious effects of ischemia reperfusion by prior exposure to brief repetitive cycles of vascular occlusion [61]. Cardioprotection by IP involves alteration of the myocardial cell phenotype to become more resistant to subsequent ischemic challenges [61]. During ischemia, energy metabolism switches from fatty acid oxidation to more oxidation-efficient glycolysis, allowing tissues to tolerate ischemic insults for a longer period of time. Thereby, hypoxia inducible factor- (HIF-) 1 acts as a molecular switch [62]. The stability of HIF is actually regulated by the oxygen-sensing prolylhydroxylase (PHD) enzyme. Convincing evidence suggests hypoxia inducible factor- (HIF-) 1 to play a central role in cardioprotection during IP. HIF-1 resides in the cytosol and translocates into the nucleus for subsequent gene expression of protective pathways upon anoxic insults [61]. When oxygen is abundant, HIF-1 is degraded mediated by Van Hippel Lindau tumor suppressor and PHD [61]. Treatment with pharmacological PHD inhibitors results in increased ischemia tolerance of the kidneys [63] and in cardioprotection similar to that seen with ischemic preconditioning in the heart [61]. To date, PHD inhibitors seem to be well tolerated in humans [64] suggesting that they could be readily tested in larger clinical trials (Table 4).

Recent experiments suggests that, for activation of this self-protective mechanisms, it is not relevant whether conditioning ischemic cycles precede (preconditioning) or follow (postconditioning) the sustained myocardial ischemia or whether they occur in organs remote from the heart (remote conditioning) [65].

6. Conclusion

Taken together, this review shows that cardioprotection has evolved from pure application of potassium rich solution towards a complex field in which different and, in part, adversarial strategies exist: ranging from different routes of administration (antegrade/retrograde) via different preparations (crystalloid/blood) to different modes (warm/tepid/warm, single shot/intermittent). But despite this multitude of options, current clinical regimens might be less than optimal for certain patient collectives and certain clinical scenarios. Current cardioplegic regimens were originally developed in the setting of CABG procedures, with morphologically relatively unchanged hearts. But the field of cardiac surgery is continuously changing while the patient collective undergoing cardiac surgery is becoming more complex.

In an area in which current cardioprotective regimens are limited and no longer adequately meet the demands of a still growing and high-rising cardiothoracic surgery community's expectations, one possibly needs to redefine cardioprotective regimens.

This review highlights both improved understanding and improved management of ischemia reperfusion injury as the central dogma of future cardioprotective approaches and draws attention towards potential new clinical avenues with a considerable translational perspective. Chances are good that we find one of those promising approaches as part of future cardioprotective regimens.

Conflict of Interests

The authors declare that they have no competing interests.

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Research Article

Propofol Protects the Immature Rabbit Heart against Ischemia and Reperfusion Injury: Impact on Functional Recovery and Histopathological Changes

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The general anesthetic propofol protects the adult heart against ischemia and reperfusion injury; however, its efficacy has not been investigated in the immature heart. This work, for the first time, investigates the cardioprotective efficacy of propofol at clinically relevant concentrations in the immature heart. Langendorff perfused rabbit hearts (7–12 days old) were exposed to 30 minutes' global normothermic ischemia followed by 40 minutes' reperfusion. Left ventricular developed pressure (LVDP) and coronary flow were monitored throughout. Lactate release into coronary effluent was measured during reperfusion. Microscopic examinations of the myocardium were monitored at the end of reperfusion. Hearts were perfused with different propofol concentrations (1, 2, 4, and 10 μg/mL) or with cyclosporine A, prior to ischemic arrest and for 20 minutes during reperfusion. Propofol at 4 and 10 μg/mL caused a significant depression in LVDP prior to ischemia. Propofol at 2 μg/mL conferred significant and maximal protection with no protection at 10 μg/mL. This protection was associated with improved recovery in coronary flow, reduced lactate release, and preservation of cardiomyocyte ultrastructure. The efficacy of propofol at 2 μg/mL was similar to the effect of cyclosporine A. In conclusion, propofol at a clinically relevant concentration is cardioprotective in the immature heart.

1. Introduction

Generation of reactive oxygen species and Ca^{2+} overload are key triggers of ischemia and reperfusion (I/R) injury of the heart and it is now generally agreed that their effect is due to opening of the mitochondrial permeability transition pore (MPTP) [1, 2]. Further evidence in support of a role for MPTP comes from work showing that inhibition of MPTP opening (e.g., with cyclosporine A (CsA)) is cardioprotective in vitro [1, 3, 4] and in vivo [5].

Propofol is a general anesthetic that is widely used for induction and maintenance of anesthesia during cardiac surgery and in postoperative sedation [6–8]. It has been shown to protect hearts against cardiac insults in a variety of experimental models [9–12]. Propofol can act as a free radical scavenger [13], enhance tissue antioxidant capacity [14, 15], and also inhibit calcium channels [16, 17]. Its antioxidant property could be responsible for its inhibitory action on MPTP opening in the Langendorff perfused rat heart [11] and for its antiapoptotic properties [18]. Its cardioprotective properties have clinical relevance as shown in work involving a pig model of cardiopulmonary bypass and intermittent antegrade warm blood cardioplegic arrest [19]. However, these beneficial effects of propofol have not been shown in immature rabbit hearts.

Vulnerability of the immature heart to cardiac insults remains controversial. This is largely due to the fact that the extent of dysfunction and injury is directly related to disruption to cellular metabolic and ionic homeostasis, which both change during development [3]. In addition to issues relating to vulnerability, there are also controversies surrounding the efficacy of different cardioprotective strategies [20–22]. For

example, inhibiting MPTP can be protective in piglets [23] but not in neonatal 7-day-old rat hearts [24]. Thus, myocardial protection for immature hearts needs further investigation to catch up that of the mature heart.

To our best of knowledge we, for the first time, investigate the efficacy of propofol on immature hearts at clinically relevant concentrations in the present study. The aim of the current study was to test the hypothesis that, in addition to its reported ability to protect normal and diseased adult hearts, propofol would also protect immature hearts against I/R. To address this issue, we investigated the cardioprotective efficacy of different concentrations of propofol and compared it to inhibition of MPTP using CsA in an immature rabbit heart model.

2. Material and Methods

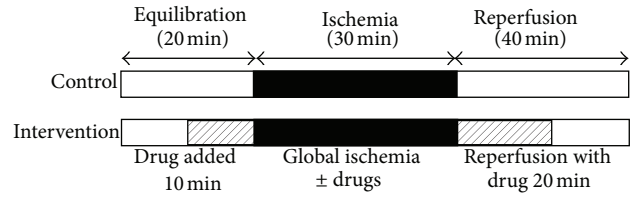
This study was approved by the Animal Ethics Committee of Nippon Medical School. All animals received care in compliance with the Principles of Laboratory Animal Care.

2.1. Animals and Heart Perfusion. Newborn rabbits (7–12 days old) were obtained from a commercial breeder (Saitama Experiment Animal, Saitama, Japan) and anesthetized with sodium pentobarbital (50 mg/kg) intraperitoneally. Hearts were removed and placed in cold (4°C) Krebs-Henseleit buffer solution (standard perfusion media). The composition of the Krebs-Henseleit buffer solution (in mM) is as follows: NaCl: 118.5, $NaHCO_3$: 25.0, KCl: 4.75, $MgSO_4 \cdot 7H_2O$: 1.19, KH_2PO_4: 1.18, Glucose: 11.0, pH: 7.4 (after gassed with 95% O_2 and 5% CO_2), and $CaCl_2 \cdot 2H_2O$: 1.8. The ascending aorta was then quickly cannulated (within 20 seconds) and the heart was perfused in the Langendorff mode at 39.0°C [25] and constant perfusion pressure of 43 mmHg (which is equivalent to the mean aortic pressure for the age of the rabbits at the time of study) [25].

2.2. Experimental Protocols. Details of the experimental protocols are shown in Scheme 1. After 10 minutes of stabilization, the heart was perfused for 10 minutes with normothermic standard perfusion media either with 2 μg/mL intralipid which is the propofol vehicle (Fresemius Kabi Japan Inc, Tokyo, Japan) or with various concentrations of propofol (1, 2, 4, and 10 μg/mL, $n = 6$ each). Diprivan (Astrazeneca Inc, Osaka, Japan) was the commercial formulation for propofol. After stabilization with or without the drug, the perfusion was stopped and normothermic global ischemia was commenced for 30 minutes. During this period, the hearts were immersed in buffer and maintained at 39.0°C in a temperature-controlled chamber. After the global ischemic period, the hearts were reperfused with the same preischemic media for 20 minutes after which the heart was perfused with buffer devoid of added propofol or intralipid.

In addition to investigating the protective effect of propofol, we also investigated the effect of the MPTP inhibitor, CsA. In these experiments, 0.2 μmol/L of CsA was used.

2.3. Hemodynamic Measurement. The left ventricular pressure was monitored continuously with a handmade water-filled vinyl balloon, which was inserted into the left ventricle via the left atrial appendage and connected to pressure transducer as described previously [26]. The balloon volume was set so that an initial left ventricular end diastolic pressure (LVEDP) between 4 and 8 mmHg was achieved. The left ventricular developed pressure (LVDP) was calculated as the difference between left ventricular end systolic pressure and LVEDP.



Scheme 1: Experimental protocols for control and for intervention groups.

2.4. Collection of Coronary Effluent. Coronary effluent was collected in an Eppendorf-tube before ischemia and at 0, 1, 3, and 5 minutes after reperfusion. The aliquots were frozen in liquid nitrogen and stored at −80°C. Lactate concentration in the coronary effluents was measured using Lactate Assay Kit (BioVision Research Products, CA, USA).

2.5. Collection and Histopathological Examination of Left Ventricular Myocardium. At the end of the protocols using propofol concentrations 0 (control), 2, and 10 μg/mL, the free wall of the left ventricle was resected and preserved in 2% glutaraldehyde solution for histopathological investigation. All samples were investigated by an experienced pathologist (blinded to the protocols) using light and electron microscopy and evaluated by a semiquantitative method. In the assessment, each sample was simply marked as "a" (2 μg/mL), "b" (control), or "c" (10 μg/mL) without details of the protocols. The pathologist investigated all the samples (6 hearts from each group) in the same way as his previous report [27] and gave us his final results for each group. The scale of 0 to 4 represents the degrees of each morphological change and corresponds with the grading in his previous study [27]: 0, normal (grade 0); 1, trivial (grade 1); 2, mild (grade 2); 3, moderate (grade 3); and 4, severe (grades 4 and 5).

2.6. Statistical Analysis. Statistical analyses were performed using SPSS 16.0J for Windows (SPSS Japan Inc, Tokyo, Japan). Data were expressed as the mean ± SE unless otherwise stated. ANOVA was used to compare different groups and considered significant at $P < 0.05$ tested using Fisher's PLSD. Area under the curve to estimate total lactate release during reperfusion was calculated using the trapezium rule (Microsoft Excel).

3. Results

3.1. Effect of Different Concentrations of Propofol on Basal Hemodynamic Parameters. Figure 1(a) shows the effect of perfusion for 10 minutes with different concentrations of

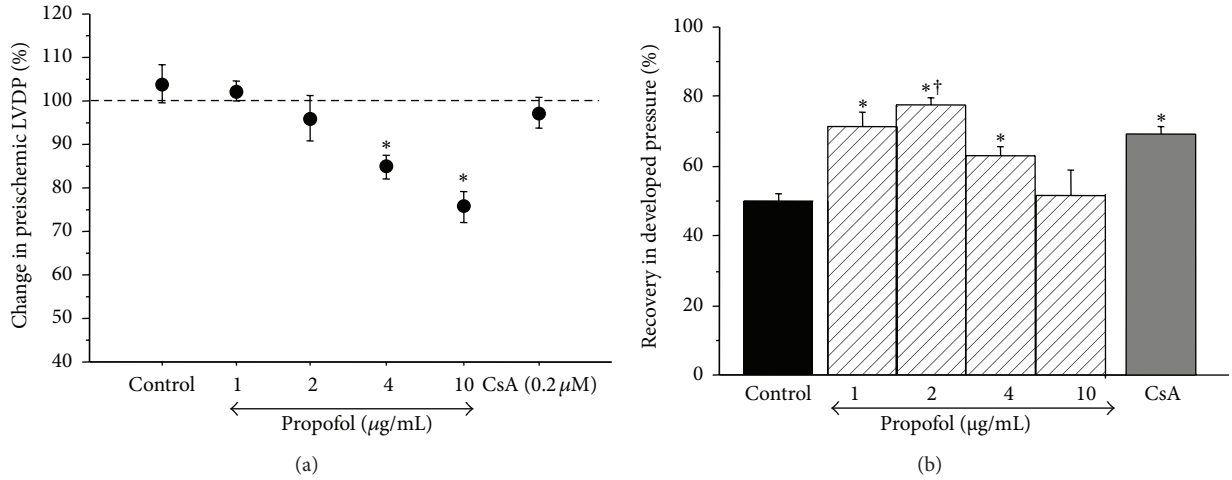


Figure 1: The effect of propofol on developed pressure. (a) Changes in left ventricular developed pressure prior to ischemia. The data shows the percentage change during 10 min perfusion with different concentrations of propofol or with cyclosporine A. Data are presented as mean ± SE. *Significant difference from basal level for the same intervention. (b) Recovery in developed pressure after ischemia and reperfusion. The data shows the change in LVDP at the end of protocol compared to basal line level. Data expressed as percentage of LVDP at the end of reperfusion/LVDP prior to ischemia. Data are presented as mean ± SE. $^{*}P < 0.05$ versus control and 10 μg/mL; $^{\dagger}P < 0.05$ versus 4 μg/mL.

Table 1: Changes in hemodynamic parameters.

(a) Changes in developed and end diastolic pressures

	Developed pressure (mmHg)		End diastolic pressure (mmHg)		
	Equilibrium	End of protocol	Equilibrium	End of ischemia	End of protocol
Control	88.4 ± 10.7	44.2 ± 6.3	4.3 ± 0.8	30.6 ± 3.2	16.3 ± 2.4
Propofol (μg/mL)					
1	83.6 ± 5.2	60.8 ± 6.8^{*1}	5.2 ± 0.7	28.9 ± 2.6	6.6 ± 1.6
2	72.2 ± 5.8	56.6 ± 6.0^{*2}	4.3 ± 1.2	38.7 ± 3.0	6.7 ± 4.3
4	75.6 ± 4.0	47.8 ± 3.9	5.0 ± 0.5	28.5 ± 3.3	7.4 ± 3.0
10	72.2 ± 4.7	36.5 ± 4.4	4.2 ± 1.0	31.3 ± 4.8	12.0 ± 3.6
Cyclosporine A	76.7 ± 6.7	52.9 ± 4.0^{*2}	4.0 ± 0.7	36.2 ± 3.8	9.1 ± 3.0

Data are presented as mean ± SE. $^{*1}P < 0.05$ versus control and 10 μg/mL of propofol; $^{*2}P < 0.05$ versus 10 μg/mL of propofol.

(b) Changes in coronary flow following ischemia and reperfusion

	Coronary flow (mL/min)		Recovery (%)
	Equilibrium	End of protocol	
Control	11.0 ± 0.8	5.7 ± 0.5	52.5 ± 2.9
Propofol (μg/mL)			
1	11.4 ± 0.9	7.8 ± 0.5	69.8 ± 4.1*
2	10.2 ± 0.7	7.0 ± 0.4	69.1 ± 1.8*
4	11.9 ± 0.5	6.9 ± 0.7	57.5 ± 5.5
10	10.3 ± 0.4	5.8 ± 0.5	57.0 ± 4.1
Cyclosporine A	11.3 ± 0.8	7.1 ± 0.5	61.6 ± 2.1^{a}

Data are presented as mean ± SE. $^{*}P < 0.05$ versus control; $^{a}P = 0.08$ versus control.

propofol or CsA on LVDP. Propofol at the higher concentrations of 4 or 10 μg/mL caused a significant drop in LVDP. Propofol at lower concentrations or the addition of CsA did not alter LVDP.

3.2. Effect of Propofol and Cyclosporine A on Functional Recovery following I/R. Table 1(a) shows the values for LVDP and LVEDP measured before ischemic arrest, at the end of ischemia, and at the end of reperfusion. It is evident that the extent of the changes in both parameters was lower in groups perfused with low propofol or CsA. The percentage change (recovery) in LVDP for all groups is shown in Figure 1(b). Propofol at 2 μg/mL showed the best recovery whilst 10 μg/mL was not protective. Recovery with CsA was similar to 1 and 2 μg/mL propofol.

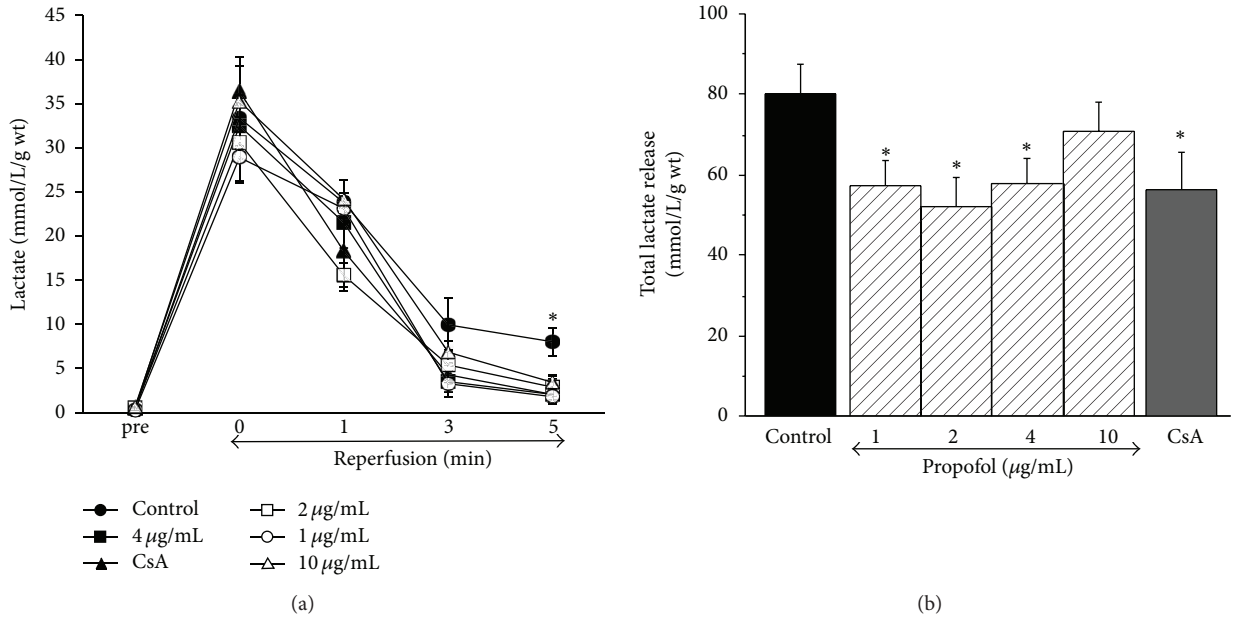


Figure 2: Lactate release during reperfusion. (a) Time-dependent lactate release. There were no significant differences between the groups at 0, 1, and 3 minutes after reperfusion ($P = 0.09$ at 3 minutes), whereas control group showed significantly higher lactate release than all other groups at 5 minutes of reperfusion. (b) Total lactate release during first 5 min of reperfusion. The data shows the total release of lactate as measured in the effluent during first 5 min of reperfusion using different interventions. Data are presented as mean ± SE. $^*P < 0.05$ versus control.

Table 2: Histological changes following ischemia and reperfusion.

	Light microscopy				Electron microscopy (Mitochondrial changes)		
	Clumping of nuclear chromatin	Intracellular edema	Interstitial edema	I-band in myocyte	Dense material deposit	Clarity of matrix	Deformation of cristae
Control	2	2	3	1	2	2	1
2 μg/mL Propofol	1	1	1	0	1	1	1
10 μg/mL Propofol	3	2	2	1	3	3	2

The scale of 0 to 4 represents the degrees of each morphological change: 0, normal; 1, trivial; 2, mild; 3, moderate; and 4, severe.

3.3. Effect of Propofol and Cyclosporine A on Recovery in Coronary Flow following I/R. Changes in coronary flow for all groups before ischemia and at the end of the protocol are shown in Table 1(b). Propofol at 1 and 2 μg/mL resulted in a significant improvement in coronary flow recovery compared to the control. On the other hand, both 4 and 10 μg/mL propofol did not improve recovery. Recovery with CsA was between low and high propofol concentrations but did not reach statistical significance against control ($P = 0.08$).

3.4. Effect of Propofol on Lactate Release during Reperfusion. Figure 2 shows time-dependent changes in lactate release and total lactate release as measured in coronary effluents during reperfusion for different interventions. All groups demonstrated similar changes with no significant differences for the first 3 minutes during reperfusion; however, control group showed significantly higher lactate release at 5 minutes compared to other groups (Figure 2(a)). Total lactate release was significantly lower than control for 1, 2, and 4 μg/mL propofol (Figure 2(b)). It was also significantly lower for CsA. There was no significant difference between control and 10 μg/mL propofol.

3.5. Histopathological Changes during I/R. The results for histopathological changes using light and electron microscopic studies are presented in Table 2. In summary, the following myocardial histological changes resulting from I/R were monitored: clumping of nuclear chromatin, intracellular and interstitial (extracellular) edema, appearance of abnormal I-band in myocytes (which are seen as white bands in myofibrils following ischemic damage), appearance of dense material deposit in mitochondria, clarity of mitochondrial matrix, and deformation of cristae [27].

3.5.1. Light Microscopy Examination. The extent of the changes in nuclear morphology and intracellular edema was evaluated and tended to be lower in 2 μg/mL of propofol compared to both control and 10 μg/mL of propofol (Figure 3).

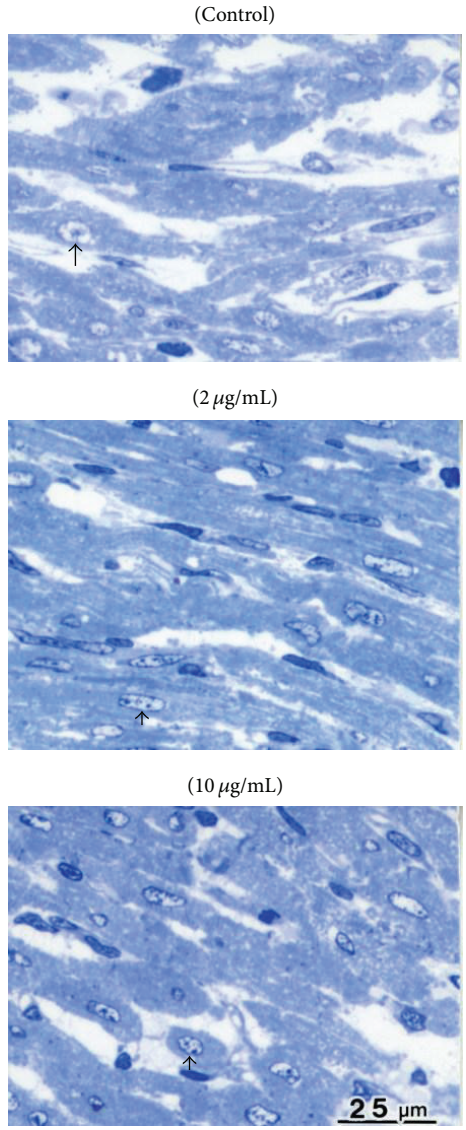


Figure 3: Histopathological assessment using light microscopy following ischemia and reperfusion. Light microscopy examination stained with toluidine blue (upper: control, middle: 2 μg/mL of propofol, and lower: 10 μg/mL of propofol). Myocardium with 2 μg/mL of propofol showed mild intracellular swelling and clumping of nuclear chromatin, whilst these changes were more evident in myocardium with 0 and 10 μg/mL of propofol. Interstitial (extracellular) edema was also less in 2 μg/mL than in 0 and 10 μg/mL.

While 2 μg/mL of propofol was accompanied with mild or less interstitial (extracellular) edema, myocardium with 0 and 10 μg/mL of propofol showed moderate or more severe interstitial (extracellular) edema after the protocols (Table 2).

3.5.2. Electron Microscopy Examination. Clumping of nuclear chromatin, appearance of I-bands in myocytes, and mitochondrial changes (swelling and dense material deposit) tended to be lower in 2 μg/mL propofol compared to control and to 10 μg/mL propofol (Figures 4(a) and 4(b)).

4. Discussion

Propofol is widely used for anesthesia in current clinical practice and its target blood concentration is between 1 and 5 μg/mL [28]. The cardioprotective potential of propofol against I/R injury in mature hearts is well established within this range in both clinical studies [29–31] and experimental models of normal [11, 19] and hypertrophic adult hearts [12]. It is known that the relevant blood concentration of propofol is similar for pediatric populations [32]; however, its cardioprotective effect has not been investigated. This study tested the hypothesis that propofol at a clinically relevant dose would enhance the functional recovery of the isolated immature heart following an ischemic insult. In addition, the protective potential of propofol in comparison to the MPTP inhibitor CsA was investigated. The results showed that after 30 minutes' global normothermic ischemia, propofol significantly improved functional recovery of LVDP and coronary flow (Figure 1(b) and Tables 1(a) and 1(b)). The

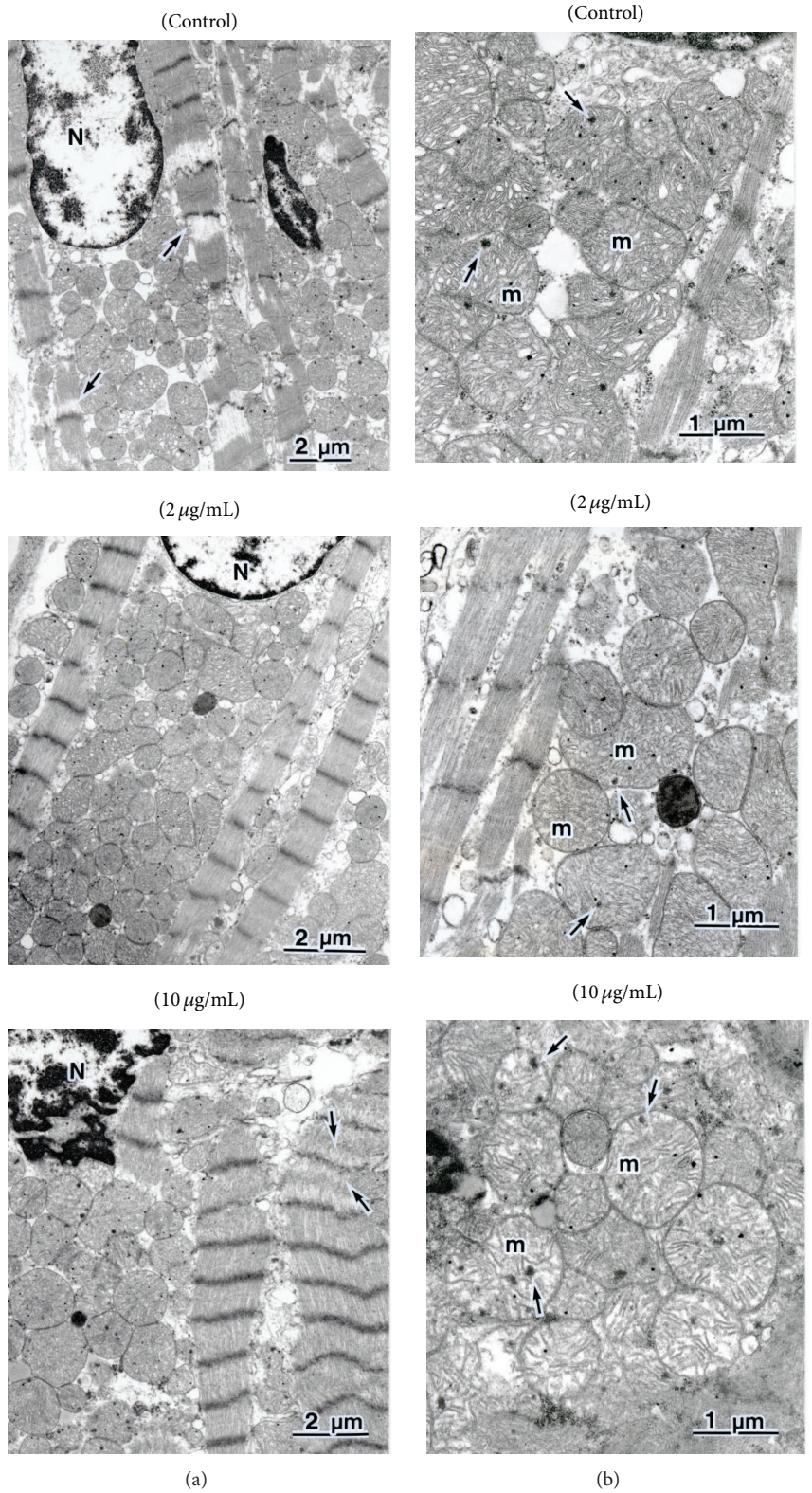


Figure 4: Electron micrographs following ischemia and reperfusion. (a) Electron micrographs of cardiomyocyte in each group (upper: control, middle: 2 μg/mL of propofol, and lower: 10 μg/mL of propofol). Many wide I-bands (arrows) appeared in the myocyte with 0 and 10 μg/mL of propofol, whereas the I-band was not observed in 2 μg/mL of propofol. (b) Dense material deposits (arrows) in mitochondria appeared in all groups (upper: control, middle: 2 μg/mL of propofol, and lower: 10 μg/mL of propofol). However, the size and number of these deposits were less in mitochondria with 2 μg/mL of propofol than the other 2 groups. Moreover, structure of mitochondrial cristae was well preserved in group of 2 μg/mL, whilst it was significantly deformed with separation in other 2 groups.

cardioprotective efficacy of propofol was dose dependent and 2 μg/mL of propofol showed the best functional recovery. Although 1 and 4 μg/mL of propofol significantly improved functional recovery their beneficial effects were less than that of 2 μg/mL. On the other hand, the functional recovery in the presence of 10 μg/mL was no different from control (Figure 1(b)). Recovery in coronary flow was also improved with 2 and 1 μg/mL propofol but not with higher concentrations. Finally, propofol at 2 μg/mL reduced ultrastructural disruptions of cardiomyocytes caused by I/R (Figures 3 and 4 and Table 2).

Addition of propofol under baseline conditions caused a dose-dependent depression of LVDP where doses at 4 and 10 μg/mL resulted in a significant fall compared to initial levels (Figure 1(a)). The depressive effects of propofol at high concentrations could be due to its known inhibitory effect of calcium channels [17, 33–35]. Taken together the current study supports a cardioprotective role for propofol in the immature heart exposed to I/R.

Several mechanisms have been proposed to explain the cardioprotective properties of propofol which include increased phosphorylation of phospho-glycogen-synthase kinase (GSK-3β) [36], inhibition of MPTP opening [11], reduced lipid peroxidation and lowered inflammation [37], and modulation of calcium channel activity [17]. The reduction in levels of cytosolic calcium is unlikely to be involved in cardioprotection in immature heart. This is because we found higher concentrations of propofol which depress function (presumably by inhibiting calcium channels) are not protective. On the contrary, 10 μg/mL propofol seemed to worsen structural damage (Figures 3 and 4) as shown by the number of dense material deposits in mitochondria and the degree of mitochondrial swelling [38]. The degree of clumping of nuclear chromatin, the degree of intracellular edema, and the degree of appearance of I-band in myocyte present a degree of cardiomyocyte damage (Table 2 and Figures 3 and 4).

Earlier work has reported that the cardioprotective effect in the adult heart is due to inhibition of MPTP [11]. This is consistent with our finding that inhibition of MPTP using CsA also protected the immature heart and this protection was similar to protection by propofol when used at 2 μg/mL. We previously reported that hypothermia and hyperkalemia protect the heart from I/R injury in mature guinea pig hearts, in which we observed that both hypothermia and hyperkalemia dramatically reduced lactate release as from immediately starting reperfusion [39]. In the present study, 2 μg/mL of propofol and CsA showed similar changes in lactate release without significant differences to control during the first 3 minutes and with significant decreases at 5 minutes after reperfusion. This finding may indicate a similar mechanism of protection between propofol and CsA and probably means a different mechanism compared to hypothermia and hyperkalemia. The observation of LVEDP at the end of ischemia without significant differences among control and 2 μg/mL of propofol and CsA may support this speculation because it was significantly reduced by hypothermia and hyperkalemia in our previous work. Since CsA and propofol are established as MPTP inhibitor by numerous previous works, we believe that the demonstrated myocardial protection by propofol in this study was at least partly due to inhibition of MPTP opening.

It is known that swelling or edema of mitochondria is an important finding of I/R injury in studies of myocardial protection. Previous studies determined the degree of swelling by evaluating the matrix clarity and separation of cristae in mitochondria, and we followed their way of assessment in this study. Although our protocol did not bring severe edema to mitochondria in control group, mitochondria with 2 μg/mL of propofol showed even less edema than control group at the end of the reperfusion period. Increase of dense material deposits (size and numbers), which is caused by calcium overloading to the mitochondria, is also an important marker of mitochondrial damage during I/R [38, 40]. The structure of mitochondria, including these deposits and cristae, was well preserved with 2 μg/mL of propofol at the end of protocol. The I-band is another finding of I/R injury, which increases in cardiomyocytes with moderate to severe damage by I/R [27]. In the present study, 2 μg/mL of propofol showed mild changes to the cardiomyocytes which are potentially reversible. Although cardiomyocytes in the other 2 groups were identified as moderately (not severely) damaged in histopathological examination, they were judged by an expert pathologist as irreversible changes.

Previous studies demonstrated negative inotropic effect of propofol with decreased intracellular calcium in the myocardium. The effect is known to depend on its concentration [41]. Our study showed that propofol at 10 μg/mL showed significant negative inotropic changes before ischemia and more importantly, it did not have protective effect against I/R injury, which was confirmed not only by functional recovery but also in histopathological examination. Our results indicate potentially harmful effects of high concentration of propofol for immature heart based on histopathological investigation. High concentrations of propofol can cause decreased production of ATP and inhibition of cardiac L-type calcium current (which causes low level of intracellular calcium) before, during, and immediately after ischemia [33, 42, 43] and may introduce an exaggerated calcium influx during the following period (same mechanism as calcium paradox). Excessively increased dense material deposit, which consists of calcium in mitochondria [44], seen in the heart treated with 10 μg/mL of propofol might reflect this mechanism. Thus, the potential mechanism of harmful effects of high concentration of propofol is speculated. MPTP inhibition is difficult under conditions of low adenine nucleotides and excessive calcium. Further investigations are necessary to elucidate the details.

5. Conclusion

This study shows that propofol at clinically relevant concentrations can protect immature hearts from I/R injury and that this effect is dose dependent where optimal protection is similar to what is seen when inhibiting the MPTP. In contrast to the beneficial effect of clinically relevant concentration of propofol, high dose of propofol can be harmful to the immature heart.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Adjuvant Cardioprotection in Cardiac Surgery: Update

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Cardiac surgery patients are now more risky in terms of age, comorbidities, and the need for complex procedures. It brings about reperfusion injury, which leads to dysfunction and/or loss of part of the myocardium. These groups of patients have a higher incidence of postoperative complications and mortality. One way of augmenting intraoperative myocardial protection is the phenomenon of myocardial conditioning, elicited with brief nonlethal episodes of ischaemia-reperfusion. In addition, drugs are being tested that mimic ischaemic conditioning. Such cardioprotective techniques are mainly focused on reperfusion injury, a complex response of the organism to the restoration of coronary blood flow in ischaemic tissue, which can lead to cell death. Extensive research over the last three decades has revealed the basic mechanisms of reperfusion injury and myocardial conditioning, suggesting its therapeutic potential. But despite the enormous efforts that have been expended in preclinical studies, almost all cardioprotective therapies have failed in the third phase of clinical trials. One reason is that evolutionary young cellular mechanisms of protection against oxygen handling are not very robust. Ischaemic conditioning, which is among these, is also limited by this. At present, the prevailing belief is that such options of treatment exist, but their full employment will not occur until subquestions and methodological issues with the transfer into clinical practice have been resolved.

1. Introduction

The spectrum of cardiac patients has recently shifted to groups exposed to a higher risk level in terms of age and comorbidities, as well as the type of treatments needed. This increases the need for emergency surgery in acute coronary syndromes with complications including acute heart failure [1]. Another growing group of patients comprises those with advanced chronic heart failure who require long-term, combined treatment. Similarly, a longer graft ischaemia is often needed in heart transplantations. These groups of patients have a higher incidence of postoperative complications (acute heart/renal failure, cerebral stroke) and ultimately a higher mortality. One factor to consider involves the current limits for perioperative myocardial protection [2, 3]. Some patients may be offered revascularisation on the beating heart, transcatheter implantation of heart valve prosthesis, or a mitral clip, but for the surgical field to be peaceful and bloodless, the majority of high-risk patients are operated on using the so-called ischaemic cardioplegic arrest. Here, the restoration of the coronary circulation is accompanied by acute ischaemia-reperfusion injury (IRI) with raised cardiac enzymes [4]. Some degree of cardiac necrosis is inherent in each cardiac surgery and, in addition to reperfusion injury, multiple

factors may be involved [5]. According to recent studies, the incidence of myocardial infarction (MI) after CABG ranges, depending on the definition, from 2% to 10% [6]. According to the latest revised definition, MI arising in connection with CABG ("Category 5") is arbitrarily determined by a 10-fold increase in cardiac-specific enzymes during the first 48 hours along with ECG signs of necrosis or displaying a coronary occlusion/contractility disorder [7, 8]. The term "perioperative myocardial injury" describes a condition that, although not fully achieving MI type 5, has health consequences even at this level of affection. A new retrospective study on 18,908 CABG patients has found that CK-MB/troponin elevations in the initial 24 hours were associated with increased mortality in the coming months to years [9]. Thus, it is obvious that the restriction of perioperative myocardial injury is important for the prognosis of the patient. The facts mentioned above open the door to finding other methods of perioperative myocardial protection in cardiac surgery.

2. Cardioplegia

The current gold standard of cardioplegia is a depolarisation myocardial arrest through perfusion of the coronary arteries using a hyperkalaemic solution. The time of reversible ischaemia that it provides is satisfactory for the surgery (up to 4 hours); plus, there is the restoration of function (a number of days) and low systemic toxicity [10]. Myocardial protection is expressed by delaying irreversible ischaemia, to which the arrest of electrical and mechanical activity is a contributing factor. The use of potassium, however, is not lacking in problems; in addition to the very narrow safety profile of extracellular potassium (10–30 mmol per litre), there is a calcium overload of the myocytes; plus, there are other types of ionic imbalance that lead to arrhythmias and depressed myocardial function persisting over several days [11]. Myocardial protection is reinforced using hypothermia, mixing the cardioplegic solution with the patient's blood and a number of additives: procaine, adenosine (augmented attenuation of electrical activity), calcium antagonists, magnesium (inhibition of calcium overloading), desensitisation of calcium channels (inhibition of calcium at the myofilament level), energy substrate (anaerobic ATP production), mannitol, Fe chelates (oedematous and oxidation control effect), and others. Additives are not part of every cardioplegic solution, because they are accompanied by side effects [12]. An alternative to the hyperkalaemic depolarisation arrest is the hyperpolarisation arrest induced by decreasing sodium and calcium from solutions such as Bretschneider solution and its later version, Custodiol HTK (histidine-tryptophan-ketoglutarate solution), but even this approach does not lead to a qualitative change in myocardial protection from reperfusion injury [2]. Hyperpolarisation can also be induced by agents that open membrane potassium channels (outflow of potassium from myocytes). This leads to the reduction in the calcium influx into myocytes (by reducing the action potential) without having to add other agents with adverse effects. Studies with aprikalim, pinacidil, or nicorandil showed a comparable or better cardioprotection level but failed in clinical practice due to a long elimination time and systemic hypotension [13].

3. Reperfusion Injury (RI) of Myocardium

Myocardial RI is a complex response of the organism to the restoration of coronary blood flow in the ischaemic tissue and is an important component of ischaemia-reperfusion injury [14, 15]. The flow restoration rescues viable myocytes, accelerating the formation of necrosis of irreversibly damaged cells [16, 17]. In another portion of reperfused myocardium, there are subtle changes such as cell swelling, enlargement of mitochondria, or loss of myofibrillar organisation. The exposed area may recover or reach up to the death of cells, the morphological correlates of which are, at the tissue level and organ levels, referred to as contraction band necrosis [18] and the no-reflow phenomenon [19], respectively. This observation led to the concept of lethal reperfusion injury as early as 1985 [20]. RI is mostly manifest in reversible changes such as ventricular arrhythmias [21] and contractile dysfunctions (stunning) [22, 23]. The restoration of coronary flow after cardioplegic arrest is also manifest in disorders of rhythm and contractility, with contraction band necrosis possibly found in the myocardium. These were detected in 26% of early deaths of CABG patients, whilst also being found in almost all those with synchronous myocardial infarction as a result of graft occlusion [24].

Although the no-reflow phenomenon was described as early as 1966, the aetiology is still unclear. There are a number of other events involved, including embolisation of debris from the site of the occlusion, the release of vasoconstrictor and thrombogenic factors, and inflammatory substances; plus there are considerations in respect of the structural collapse of the capillary bed [25]. Whether RI is an independent factor responsible for additional tissue necrosis or simply speeds up exiting of cells condemned to death from ischaemia has been the subject of debates, the process being most intense in the 1990s [26], since it is difficult to discern the death of myocytes that were viable at the end of ischaemia. However, it is now accepted that RI is an independent factor for the spread of infarction after myocardial ischaemia [27]. The evidence is, nonetheless, only indirect, relying on the positive impact of therapeutic interventions at the reperfusion stage. Cardiac surgery has long been aware of the positive effect of the modified reperfusion (temperature and composition) and staged reperfusion (a slow, 20-minute restoration of coronary flow) [28, 29]. Mainly, the 36% reduction in infarction in STEMI patients treated with primary PCI and randomized to ischaemic postconditioning (see below [30]) is indirect evidence of this.

3.1. The RI Mechanisms. Oxygen is the main factor of damage, that is, its acute lack in the phase of ischaemia and toxicity in the reperfusion phase. This paradox can be explained by the evolution of the relationship between organisms and oxygen on Earth. After the increase in concentration of oxygen in the atmosphere 2.4 billion years ago that nearly wiped out life on Earth (the great oxygenation event), aerobic

organisms entered the path of adaptation, eventually ending up on oxygen, an electron acceptor for their energy mechanisms. Multicellular organisms thus gained the opportunity of explosive development at the cost of shortening the lifespan of individuals. Although widespread, the evolutionary young cellular mechanisms of protection against oxygen handling are not very robust. To this day, eukaryotic cells are dealing with a difficult logistical task, that is, to bring enough oxygen to the cell for respiration whilst eliminating the pernicious consequences of its presence. The start of reperfusion is the moment at which cells are most vulnerable, and as for antioxidant enzymes to regenerate, available energy is needed [31, 32].

The closure of the coronary artery leads to a series of changes, which begins with dysfunction and ends with myocardial infarction. The changes mainly concern the reduction of energy production and consumption. From the onset of ischaemia in myocytes, there is a sharp ongoing decline in ATP, with anaerobic utilisation of energy with a subsequent decrease in pH. Acidosis leads to contractility arrest within minutes, and complete depletion of ATP over 15–30 minutes results in myocyte death, at least as part of experiments. Intracellular acidosis is mitigated by a sodium-hydrogen pump; this taking place at the cost of intracellular entry of sodium, then water, and then even calcium. Deficient membrane ATPases are at the beginning of the disorder of the control of intracellular calcium, which leads to the first calcium overload. The hypothesis of ATP depletion being a central cause of ischaemic death still applies [33]; it was, however, extended by subsequent damage in the reperfusion phase. The restored oxygen supply and energy production in a situation of abnormal cellular environment leads to further damage [34]. Endogenous defence mechanisms are also activated in the early phase of reperfusion; these designed to minimize further damage to the myocardium; more specifically, there is a decision-making process as to which myocytes will be repaired or eliminated by apoptosis. Interconnected pathophysiological processes are supportive of RI, such as rapid pH fluctuations (pH paradox), oxygen toxicity (oxygen paradox), calcium overload of cells (calcium paradox), and inflammation [35]. Reperfusion promptly washes off the low pericellular pH with the emergence of a large H^+ gradient on myocyte membranes. Activation of the Na/H pump follows, as well as the rapid entry of sodium into myocytes. This causes a passive reverse running of the membrane Na/Ca exchanger, the exchange of sodium for calcium causing the second intracellular calcium peak. The result of the rapid pH adjustment and calcium overload comprises an abolition of protease disinhibition (calpain, etc.), opening of mitochondrial transport channels (mPTP) and hypercontracture of the myofibrils (Figure 1). Reoxygenation starts aerobic ATP production, accompanied by explosive formation of the reactive oxygen species (ROS), the main sources of which involve calcium-activated xanthine oxidase and cytochrome of the respiratory chain. After overcoming the capacity of the main antioxidation enzymes SOD (superoxide dismutase) and catalase, the excess of ROS damages cell structures, especially membrane proteins and phospholipids. However, the initial amount of ROS during ischaemia alone and even

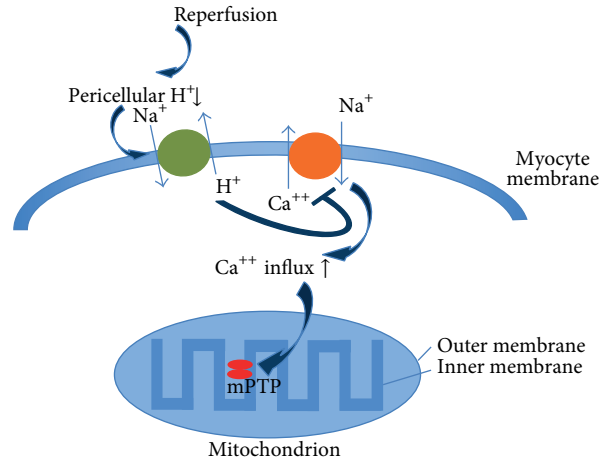

Figure 1: Role of mitochondrial permeability transition pore (mPTP) in myocardial reperfusion injury.

at the beginning of reperfusion is necessary as a signal that activates defence mechanisms. These processes, along with the activation of inflammation cascades and bioactive factors such as cytokines, may lead to death of myocytes [36]. In the best case, the gradual regulation of ionic and electrophysiological processes in membranes is accompanied by arrhythmias and contractile dysfunction [37].

4. Myocardial Conditioning (MC)

One of the new ways of augmenting intraoperative myocardial protection is the activation of an innate defence mechanism to avoid reperfusion injury; one that was termed myocardial conditioning. Originally discovered in 1986 in the analysis of cumulative episodes of coronary occlusion and reperfusion, this principle was termed ischaemic preconditioning. Murry et al. described, on a canine model, a phenomenon in which four five-minute cycles of coronary occlusion and reperfusion prior to the sustained 40-minute occlusion reduce the extent of infarction by 25% [38]. This definition was further enhanced by the knowledge that ischaemic preconditioning also reduces dysrhythmias and myocardial dysfunction [39, 40]. The discovery was preceded by finding a warm-up phenomenon: patients with prodromal angina pectoris often show minor variations in the ST segment, less myocardial dysfunction, and an even lesser extent of necrosis [41]. Ischaemic preconditioning is underway in two stages, the early stage beginning immediately after ischaemia/reperfusion stimuli and lasting up to three hours, and is followed by a 12- to 24-hour interval after which there is the onset of the late stage; one that is weaker but lasts three days [42]. The early stage depends on the activation of available signalling molecules, receptors, and intracellular pathways, while the late stage requires the expression of defensive genes and de novo protein synthesis; see below. Myocardial conditioning evolved into multiple modalities that may be applied before (preconditioning), during (perconditioning) and immediately after the ischaemic insult

or at reperfusion (postconditioning). The stimulus can be applied directly to the myocardium or a remote tissue (remote conditioning).

4.1. Molecular Mechanisms of MC. Despite the accumulation of facts as regards the mechanism of inception, spreading, and implementation of the cardioprotective signal, there is now some sort of consensus as to the architecture of these; it can be divided into three levels (triggers, intracellular pathways, and end-effectors) [43]. Not only ischaemic, but also other types of stress (thermal, chemical), release triggers from the autocrine source (adenosine) or exogenous/paracrine sources (bradykinin, opioids). The triggers bind to receptors of the myocyte membrane, followed by a cascade activation of proteinases of multiple parallel pathways, with this ending at the effector (mitochondria, cytoskeleton).

Intracellular signal transmission takes place via at least three parallel channels: the first is activated by receptors coupled with G protein (GPCR) and proceeds via a nitric oxide (NO), cGMP, and PKG (protein kinase G). The second channel, also activated by GPCR and termed RISK (reperfusion injury salvage kinase), contains a number of kinases including PKB (protein kinase B), ERK (extracellular regulated kinase) and key GSK 3beta (glucose synthase kinase). The third channel is SAFE (survival activating factor enhancement), which is activated by TNF-alpha and includes JAK (Janus kinase signal transducer) and STAT3 (mitochondrial activator of transcription).

Mitochondria are considered key effectors of cardioprotection, with mPTP (mitochondrial permeability transition pore) being the primary end point. They are nonspecific channels in the inner mitochondrial membrane, their physiological role being not known in detail [44]. Ischaemic stress opens mPTP, penetration of ions and water leads to swelling as far as rupture of the outer membrane, the release of proteins including cytochrome c, caspase activation, and apoptosis of the cell. Inhibition of GSK 3beta is an integration point of activation of protein kinase pathways, and, along with Connexin 43 and activation of potassium ATP channels (KAPT), "holds" mPTP in the closed state during the critical phase of reperfusion. Mitochondria are also a source of cardioprotective signal. At the start of reperfusion, it is necessary to produce a certain amount of reactive oxygen species that amplify protective mechanisms. Important factors also include the presence of acidosis, which is involved in the closure of mPTP and inhibits excessive contractile activity in the presence of excess calcium ions. The immediate reperfusion is thus a crucial moment when protective mechanisms (kinase signalling systems, ROS, acidosis) can be not only activated spontaneously or enhanced by the ischaemic pre/postconditioning, but also violated by inappropriate interventions, for example, incorrect timing of alkalising substances and antioxidating agents. Figure 2 summarizes the molecular mechanisms of MC.

4.2. The Late Stage of Cardioprotection. The mechanisms of the acute and late stages of cardioprotection have many things in common. Some mediators of the intracellular signal transduction, however, induce gene transcription and synthesis of defensive proteins within 12–24 hours after the ischaemic stimulus [45]. These include transcription factors JAK-STAT 1/3 (Janus kinase/signal transducer and activator of transcription), PKC (protein kinase C), NF-kappa B (nuclear factor-kappa B), AP-1 (activator protein-1) and HIF-1 alpha (hypoxia inducible factor-1). This is followed by the synthesis of iNOS (induced nitric oxide synthases), Cox-2 (cyclooxygenase type 2), aldose reductase, mSOD (mitochondrial superoxide dismutase), and HSP (heat shock proteins). The products of these enzymes directly affect the mPTP as NO [46] and regulate the excessive production of ROS and aldehydes [47, 48]; plus, they protect the structure of proteins using HSP [49]. The detailed task of Cox-2 products (prostaglandins: PGE2, PGF1-alpha) is not yet known [50].

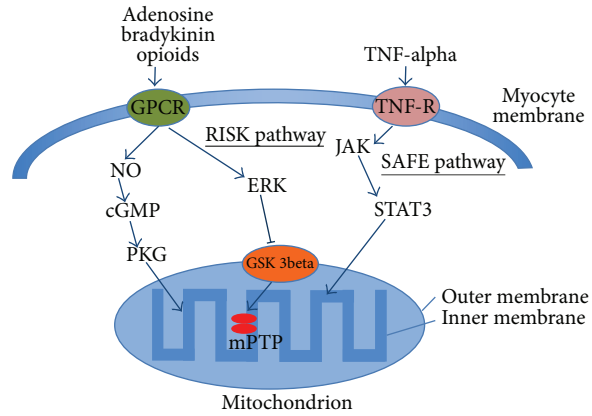

FIGURE 2: Molecular mechanism of myocardial conditioning. cGMP, cyclic guanosine monophosphate; ERK, extracellular regulatory kinase; GSK 3beta, glucose synthase kinase 3 beta; GPCR, G-protein-coupled receptor; JAK, Janus kinase signal transducer; mPTP, mitochondrial permeability transition pore; NO, nitric oxide; PKG, protein kinase G; RISK, reperfusion injury salvage kinase; SAFE, survival activating factor enhancement; STAT3, signal transducer and activator of transcription 3; TNF-alpha, tumour necrosis factor-alpha; TNF-R, TNF receptor.

4.3. Ischaemic Postconditioning. Another modality which can reduce the size of myocardial infarction involves the application of several ischaemia-reperfusion stimuli immediately (within the first minute) after the restoration of perfusion in the ischaemic region [51]. The reduction in infarction was observed in all species of tested animals [52] and also in a clinical trial [30]. The mechanism is, in many respects, the same as for the standard modality, both of them sharing the necessity of the reperfusion stage, but here the SAFE kinase pathway dominates over RISK. While ischaemic preconditioning may be used only for elective procedures (percutaneous or surgical), postconditioning can be applied even to patients during primary percutaneous coronary interventions (PPCI).

4.4. Remote Ischaemic Conditioning: RIPC. Remote ischaemic conditioning is a form of cardioprotection induced by

short cycles of ischaemia and reperfusion, which are applied to a distant tissue and/or organ. The phenomenon was originally observed intraorganally and it is interesting that it was predicted using a mathematical model [53]. In the original experiment, four cycles of five-minute occlusion-reperfusion in the circumflex branch of the left coronary artery led to a reduction of infarction in the area of ramus interventricularis anterior, which was subjected to 60-minute occlusion [54]. Subsequent studies showed that cardioprotection can be achieved even by applications to remote organs like kidney, intestine, brain, and skeletal muscle [55]. Remote pulses can be applied during an ongoing ischaemia (remote perconditioning), which can be advantageous prior to reception in STEMI patients [56] and also at the beginning of reperfusion (remote postconditioning) [57].

The mechanism of RIPC at the myocardial level is largely the same as in the basic application, but the transfer of the protective signal from a remote organ to the myocardium is not fully explained. Three channels of communication were designed and partially tested: the humoral blood channel, neuronal stimulation, and communication of immunity cells. The humoral mechanism was tested by a perfusate and dialysate from the ischaemic organ, which induced cardioprotection after being applied to the isolated myocardium [58]. The identity of humoral factors also remains speculative. Classic triggers (bradykinin, opioids), but also as yet undefined small hydrophobic molecules, are considered [59]. Neuronal stimulation was verified by stimulation of the femoral nerve with the conclusion that intact neural pathways are required for the release of humoral components [60]. Other studies focused on parasympathetic activity, concluding that RIPC is dependent on the activity of specific vagal preganglionic neurons [61]. Not surprisingly, many assume the necessary interplay of these components [62].

Its noninvasiveness and ease of application determined RIPC to be the most tested modality in clinical research. In terms of orders, dozens of "proof of concept" clinical studies have tested remote conditioning by an application of ischaemia/reperfusion on the upper/lower limb using a pressure cuff in elective operations in cardiac surgery and invasive cardiology [63–71]. Nonetheless, not every study has confirmed cardioprotection [72–74].

5. Clinical Studies

The basic type, ischaemic preconditioning (IPC), was first used in clinical testing on a small set of CABG patients in 1993, the stimulus comprising repeated aortic clamping and declamping prior to the cardioplegic arrest itself. The IPC group was observed to have an increased level of ATP in biopsy samples and lower levels of serum troponin I [75, 76]. Meta-analysis of 22 similar studies (937 patients) found fewer arrhythmias, a lower consumption of inotropes, and shorter stay in the ICU in the IPC group, but these were not the main parameters assessed [77]. This basic technique was, however, not developed any further due to its invasive nature (risk of thromboembolism in the handling of the aorta) and the need for extended surgery time.

Remote ischaemic conditioning (RIC) has regained interest in clinical testing. The first clinical trial was performed on a group of 37 children undergoing surgery for congenital heart defect. RIC stimulus comprised three five-minute inflations/deflations using a pressure cuff (200 mmHg) on the lower limb before connecting to extracorporeal circulation. Children randomized in the RIC group had a lower consumption of inotropes, lower inspiratory pressures, and lower serum troponin I concentrations for 24 hours after the surgery [78]. A series of similar studies followed, mainly in operations for CHD, but differences in the assessed parameters were not always found [72, 79, 80]. The reasons for the negative results could involve issues in transferring the experimental results into clinical practice in general (see below) and also differences in protocols (stimulus magnitude and timing), the selection of patients (age, comorbidities, and extent and type of operation), use of anaesthesia (IV versus inhaled anaesthetics), and others. A new meta-analysis of clinical studies on CABG patients, however, revealed that RIC reduced perioperative myocardial injury as measured by lower levels of serum troponin [81]. Currently ongoing large multicenter studies (ERICCA, RipHeart) are expected to resolve the issues [82].

Ischaemic postconditioning (IPostC) has been successfully tested in paediatric cardiac surgery. The IPostC stimulus comprised repeated 30-second aortic declamping and clamping before the definite myocardial reperfusion. Children randomized in the IPostC group had lower serum levels of CKMB and troponin T for two hours after the surgery [83]. Use in adults has the disadvantage of increased risk of thromboembolic complications in handling the aorta [84]. The children's cardiac surgery also tested the remote application of the stimulus (remote ischaemic postconditioning, again with a lower release of troponin [85]), but again there was a failure of clinical effect in a study on 1,280 patients [86]. The authors themselves admitted that the propofol (a scavenger of oxygen radicals) that was used could void the cardioprotective effect of ischaemic preconditioning as demonstrated in previous studies [87]. IPostC was also clinically tested in invasive cardiology in primary percutaneous interventions, and, as already mentioned, the positive results of this study became indirect proof of the existence of reperfusion injury [30]. Checking of the usefulness in this indication is foreseen in the ongoing large multicenter studies. The initial results suggest that IPostC and other techniques benefit mainly STEMI patients with anterior localisation as well as a greater extent of affection [56].

Compared to the previous modalities, the late phase of ischaemic preconditioning was clinically tested only in two studies. Our research group conducted a study based on some negative studies in CABG patients. At the start of surgery or, more specifically, prior to the cardioplegic cardiac arrest itself, there are stress stimuli that may also activate the phenomenon of the early stage of ischaemic preconditioning (skin incision, sternotomy, and cardiopulmonary bypass) and the IC stimulus applied, local or remote, already comes as an extra event [88]. In addition, the early IC stage lasts a maximum of three hours, which does not even cover the period of surgery, not to mention the initial hours

after the surgery, when there is the highest frequency of complications. In contrast, the late IC stage takes up to three days and offers protection from adverse events even in the early postoperative period. Our study was conducted on 60 CABG patients, the remote IC stimulus comprising three five-minute inflations/deflations using a pressure cuff applied to the upper limb 18 hours before the operation itself. Patients randomized in the L-RIPC group had a significantly lower serum level of troponin I in the eight hours after surgery [66]. While the second study published so far and conducted in children did not find differences in serum levels of troponin I, the L-RIPC group had lower levels of NT-BNP (N terminal pro-B-type natriuretic peptide) [89].

6. Pharmacological Cardioprotection

Revelation of the principles of ischaemia-reperfusion myocardial injury, on the one hand, and congenital defence mechanisms, on the other hand, offers the possibility of pharmacological intervention. Cardioprotective agents may be applied before application of the aortic clamp, added to the cardioplegic solution, or used in the reperfusion stage, or a combination of these may be an option. Just as with STEMI patients, drug administration may be considered during the acute myocardial ischaemia or in the reperfusion stage. The experiment successfully tested many substances and processes, clinical testing reducing the number of positive ones, and the most promising are now being tested in large sets of patients in multicenter studies. Recently, drugs are being tested (and attracting great attention) that mimic ischaemic conditioning (IC), at all of the three hierarchical levels, adenosine being amongst the first of such pharmaceuticals, the substance ranking among the IC triggers. For CABG patients, the administration of adenosine (intravenously or as part of cardioplegia) was associated with less myocardial injury and faster postoperative recovery [90, 91]; other studies were less conclusive [92, 93] and any further testing in cardiac surgery was paused due to hypotensive side effects. Similar side effects were exhibited by bradykinin [94]. Our research group tested tramadol on CABG patients, an opioid that also shows the serotonin effect, for which cardioprotective effects were demonstrated as well [95, 96]. Conversely, however, in this study tramadol increased postoperative serum levels of troponin I quite significantly. The explanation lies in a possible paradoxical serotonin response in patients with coronary artery disease. Serotonin dilates normal coronary arteries, while in atherosclerotic arteries it causes vasoconstriction [97]. Another substance that activates intracellular defensive pathways is atrial natriuretic peptide. Infusion of carperitide, the synthetic analogue, when started after primary PCI reduced infarction size in STEMI patients, as measured by lower levels of cardiac enzymes (15% reduction) and maintaining systolic function measured by EFLK [98]. Exenatide, a new antidiabetic drug with cardioprotective properties, appears to be more promising because it has demonstrated efficiency even over longer follow-up periods. The infusion of exenatide initiated 15 minutes prior to primary PCI in STEMI patients decreased infarction size by 23% as documented using CMRI (cardiac magnetic resonance imaging) 90 days after the intervention [99]. Inhaled anaesthetics are a group of agents which have shown cardioprotective effects by influencing multiple hierarchical pathways. Meta-analysis of 27 clinical trials in CABG patients described in the sevoflurane group a lower release of troponin, less inotropic support, and preserved ventricular function [100]. Sevoflurane testing is now underway in acute cardiology in STEMI patients subjected to reperfusion (SIAM trial: In terms of mechanism of action, great attention is paid to cyclosporine A, which inhibits opening of MPTP channels in the mitochondria in the post-ischaemia reperfusion stage). Complex beneficial effects of nitric oxide donors in cardioprotection were described, which may be summarized in three points: (1) a direct haemodynamic effect mediated through vasodilation of coronary arteries, (2) a direct effect on improving cardiac output, and (3) an increase in vascular sensitivity to sympathetic stimulation could lead to increased diastolic blood pressure [101]. NO can also directly modify sulfhydryl residues of proteins through S-nitrosylation, which has emerged as an important posttranslational protein modification. S-nitrosylation of critical protein thiols has been shown to protect them from further oxidative modification by reactive oxygen species. Recently it has been suggested that S-Nitrosylation could play important role in cardioprotection [102]. Cyclosporine thus intervenes at the end-effector, where the defensive signals of intracellular pathways converge. A small clinical study on 27 STEMI patients treated by PPCI demonstrated in the cyclosporine group a 20% reduction in infarction on CMRI [103]. This and another study, which observed a lasting effect even after six months on the CMRI, gave rise to a wider study of CIRCUS trial: NCT01502774, which is now underway [104]. Other more specific inhibitors of MPTP channels are now being tested in ongoing trials (MITOCARE: NCT01374321, EMBRACE: NCT01572909) [105, 106]. Further interventions and pharmaceuticals that appeared to be efficient in animal models are being tested in acute cardiology: mecasermin, insulin-like growth factor analogue (RESUS-AMI: NCT01438086) [107], mangafodipir, iron oxidation inhibitor and chelator (MANAMI: NCT00966563) [108], melatonin (MARIA: NCT00640094) [109], inhaled nitric oxide (NOMI: NCT01398384) [110], IV sodium nitrite (NIAMI: NCT01388504) [111], intracoronary sodium nitrite (NITRITE-AMI: NCT01584453) [112], thymosin beta-4, growth regulator (NCT00378352) [113], and metoprolol (METOCARD-CNIC: NCT01311700) [114]. Those ongoing studies are summarized in Table 1. On the other hand, a number of pharmaceuticals proved inefficient in laboratory trials. In CABG patients, failure was found in cariporide, sodium-hydrogen exchanger inhibitor (GUARDIAN and EXPEDITION trials) [115, 116], acadesine, adenosine precursor (RED-CABG trial) [117], pexelizumab and C5 complement inhibitor (PRIMO-CABG trial) [118], while in STEMI patients failure was found in trimetazidine, fatty acid oxidation inhibition (EMIP-FR 2000 trial) [119], eniporid, sodium-hydrogen exchanger inhibitor (ESCAMI trial) [120], delcasertib, protein kinase C inhibitor

Table 1: Overview of ongoing major clinical studies in pharmacological cardioprotection.

Agent	Property	Clinical trial	Reference
Cyclosporine	mPTP inhibitor	CIRCUS trial: NCT01502774	[103]
TRO40303	mPTP inhibitor	MITOCARE: NCT01374321	[104]
Bendavia	mPTP inhibitor	EMBRACE: NCT01572909	[105]
Mecasermin	IGF analogue	RESUS-AMI: NCT01438086	[106]
Mangafodipir	Iron oxidation inhibitor and chelator	MANAMI: NCT00966563	[107]
Melatonin	Multimodal effects	MARIA: NCT00640094	[108]
Inhaled Nitric Oxide	Vasodilator, mPTP inhibitor	NOMI: NCT01398384	[109]
IV sodium nitrite	Vasodilator, mPTP inhibitor	NIAMI: NCT01388504	[110]
Intracoronary sodium nitrite	Vasodilator, mPTP inhibitor	NITRITE-AMI: NCT01584453	[111]
Thymosin beta-4	Growth regulator	NCT00378352	[112]
Metoprolol	β-blocker	METOCARD-CNIC: NCT01311700	[113]

(PROTECTION-AMI) [121], atorvastatin (REPARATOR trial) [122], and also magnesium (MAGIC trial) [123] and glucose-insulin-potassium infusion (CREATE-ECLA) [124]. According to the results of the REVEAL study, in patients with STEMI who had successful reperfusion with primary or rescue PCI, a single intravenous bolus of epoetin alfa within four hours of PCI did not reduce infarct size and was associated with higher rates of adverse cardiovascular events [125]. Moreover, erythropoietin may increase clinical adverse events [125, 126]. FX06, a naturally occurring peptide derived from human fibrin, has been shown to reduce myocardial infarct size in animal models by mitigating reperfusion injury. On the other hand in the human FIRE Trial, FX06 reduced the necrotic core zone as one measure of infarct size on magnetic resonance imaging, while total late enhancement was not significantly different between groups [127].

7. Challenges and Perspectives in Translation to Clinical Outcomes

The above studies represent only a small part of the efforts and resources that have been expended in this field. In the US alone, it is estimated that over the last 40 years, hundreds of millions of dollars were spent in preclinical studies for so-called infarction life-saving therapies. This gave rise to hundreds of treatments that were identified as controlling myocardial infarction. Nonetheless, these enormous resources have failed to lead to clinical application due to a number of methodological shortcomings.

Mechanisms of cellular defensive response to ischaemic stress and pharmacological interventions were investigated using animal, tissue, and subcellular models, particularly in mice, rats, and rabbits, and, to the lesser extent, large animals. Thus, they cannot easily be transferred to human research, where differences may exist. In addition, little is known about the spatial and temporal organisation of these defence mechanisms. This may be one of the reasons for pharmacological cardioprotection failing in clinical trials [128]. Pharmacological influence of the reperfusion injury runs into the issue of targeted application in effective concentrations. Solutions may include the drug to be enclosed in liposome nanoparticles, since nanoparticles preferentially accumulate at sites with increased postischaemia vascular permeability. For example, adenosine encapsulated in nanoliposomes boosts local cardioprotective effects without evidence of systemic hypotension [129].

In 2003, a workshop took place in the US, initiated by NHLBI (National Heart, Lung, and Blood Institute), the event's title, "Transfer of Therapies to Protect the Myocardium from Ischemia," was self-explanatory and its main recommendations included continuation of the clinical testing of adenosine [130]. The AMISTAT 2 trial of 2005 compared infarction size in STEMI patients treated with PPCI with three-hour infusion of adenosine with dual concentration: 50 and 70 μg/kg/minute versus placebo. SPECT (single photon emission CT) revealed the smallest infarction in higher concentrations: 11%, 23% versus 27% for placebo [131]. However, the second NHLBI workshop in 2010 still identified a number of gaps existing in the knowledge of the key moments of acute reperfusion injury and defensive response of the organism. The mechanism of lethal reperfusion injury, as well as its possible influence, is still not explained. The same applies to the exact mechanism of microvascular obstruction (no-reflow phenomenon). There is also no determination regarding which cardioprotective therapy may be appropriate in which clinical situations. The so-called combination therapies, which could strengthen the efficiency of individual measures, were not tested to a great extent. As cardioprotective therapies fail in the patients who need them most, that is, older persons and persons with comorbidities (diabetes, hypertension, and dyslipoproteinaemia), it will therefore be necessary to find procedures that work especially in this regard. Finally, there is the necessity of identifying molecular markers that would indicate the presence of the cardioprotective state [132]. Challenges prevail over resolved issues; preclinical studies are, however, a good start. In 2011, the NHLBI awarded a five-year grant to the initiative called the Consortium for Preclinical Assessment of Cardioprotective. Therapies (CAESAR), which will test promising cardioprotective therapies through the application of standard and randomized protocols carried out by blinded researchers and analysed by blinded analysis, as with clinical studies. The main aim of this consortium is to

ensure repeatability of the results on relevant animal models, including conscious animals and comorbid models [133]. Optimism prevails in Europe as well. In 2013, a working group for cell biology of the heart, attached to the European Society of Cardiology, published a document entitled "Transferring Cardioprotection for the Benefit of the Patient," its main proposition comprising the belief that the failure was in the inability successfully to transfer promising therapies into procedures that improve clinical outcomes, rather than in a lack of potential cardioprotective therapies in preclinical research [134].

8. Conclusion

The fundamental discovery of cardioplegic myocardial protection in cardiac surgery in the 1970s and 1980s enabled effective surgical treatment bringing prolonged and better-quality life to the patient. Also, early reperfusion in STEMI patients remains the only effective treatment in cardiology. Both therapeutic strategies, however, are accompanied by reperfusion injury of the myocardium, which leads to dysfunction and/or loss of part of the myocardium. During the same period, another fundamental discovery was made: termed ischaemic preconditioning, it activates protection from ischaemia-reperfusion myocardial injury. The extensive research on this phenomenon in the past three decades has revealed basic mechanisms and suggested methods of use. Thus, the question is whether activation or augmentation of the defence mechanism may enhance myocardial protection in cardiac surgery or rescue another portion of the jeopardised myocardium after reperfusion therapy in STEMI patients. Although widespread, the evolutionary young cellular mechanisms of protection against oxygen handling are not very robust. Ischaemic conditioning, which is among them, is also thus limited and for myocardium, the organ with the highest oxygen turnover, it is of undeniable importance. At present, the prevailing belief is that such options of treatment exist for reperfusion myocardial injury, but the time for their full employment has not yet come, due to unresolved subquestions and methodological issues with the transfer into clinical practice.

Conflict of Interests

The authors declare no conflict of interests.

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Research Article

Insulin Preconditioning Elevates p-Akt and Cardiac Contractility after Reperfusion in the Isolated Ischemic Rat Heart

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Insulin induces cardioprotection partly via an antiapoptotic effect. However, the optimal timing of insulin administration for the best quality cardioprotection remains unclear. We tested the hypothesis that insulin administered prior to ischemia provides better cardioprotection than insulin administration after ischemia. Isolated rat hearts were prepared using Langendorff method and divided into three groups. The Pre-Ins group (Pre-Ins) received 0.5 U/L insulin prior to 15 min no-flow ischemia for 20 min followed by 20 min of reperfusion. The Post-Ins group (Post-Ins) received 0.5 U/L insulin during the reperfusion period only. The control group (Control) was perfused with KH buffer throughout. The maximum of left ventricular derivative of pressure development (dP/dt(max)) was recorded continuously. Measurements of TNF-α and p-Akt in each time point were assayed by ELISA. After reperfusion, dP/dt(max) in Pre-Ins was elevated, compared with Post-Ins at 10 minutes after reperfusion and Control at all-time points. TNF-α levels at 5 minutes after reperfusion in the Pre-Ins were lower than the others. After 5 minutes of reperfusion, p-Akt was elevated in Pre-Ins compared with the other groups. Insulin administration prior to ischemia provides better cardioprotection than insulin administration only at reperfusion. TNF-α suppression is possibly mediated via p-Akt leading to a reduction in contractile myocardial dysfunction.

1. Introduction

In 1986, Murry et al. introduced the concept of ischemic preconditioning (IPC) in which short, repetitive periods of ischemia protected the myocardium from a subsequent, prolonged, and otherwise lethal, ischemic assault [1]. It is also known that the application of certain pharmacological agents to the heart prior to an episode of myocardial ischemia and reperfusion has the capacity to reduce myocardial injury. For example, volatile anesthetics have been shown to induce pharmacological preconditioning (PPC) in a variety of experimental animal models and in humans [2–5].

Insulin is another agent that has been shown to reduce myocardial injury and improve cardiac function, especially when it is combined with normoglycemia [6, 7]. The cardioprotective effect of insulin is induced metabolically by optimizing cardiac metabolism [8, 9], and also nonmetabolically by promoting cardiomyocyte survival pathway [10, 11]. Insulin promotes cardiomyocyte survival by activation of Akt, phosphatidylinositol 3-kinase (PI3K), and p70s6 kinase [10, 12]. The PKB/Akt [13, 14] signaling pathway is implicated in inducing cardioprotection via an antiapoptotic effect [10, 15]. Timing of insulin therapy, related to ischemia, is a crucial factor for its cardioprotective effects. More recently, ischemic postconditioning (IPost) was introduced [16], which can be administered at the time of myocardial reperfusion, and is, therefore, more applicable to situations where myocardial ischemia is already present. The degree of myocardial salvage with IPost has been demonstrated to be comparable with IPC [16], and therefore postconditioning is often a favored strategy because of its applicability. Recent acute myocardial ischemia (AMI) model studies have demonstrated reduction

Table 1: Baseline measurements.

	Control	Post-Ins	Pre-Ins
Number (n)	12	12	12
Rat weight (g)	303 ± 5	301 ± 3	303 ± 5
Dry heart weight (g)	0.24 ± 0.05	0.23 ± 0.02	0.23 ± 0.04
Heart rate (bpm)	248 ± 29	236 ± 35	242 ± 32
dP/dt max (mm Hg/sec)	3197 ± 648	3141 ± 453	3215 ± 653
Coronary flow (mL/min)	12.8 ± 1.8	13.1 ± 2.0	12.8 ± 1.9

Data are mean ± SD. There are no significant differences among the groups by analysis of variance.
Baseline measurements are presented in absolute values as obtained after 10 min stabilization except for dry heart weight which was measured at the end of the experiment.
dP/dt max: maximum of left ventricular derivative of pressure development.

in infarct size in isolated rat heart when insulin was administered after ischemia [17]. On the other hand, our previous studies have shown that high-dose insulin administration prior to cardiopulmonary bypass (CPB) elicits cardioprotection in humans [6, 7]. Insulin improved cardiac contractility when it was administered preemptively before the "iatrogenic ischemia," caused by CPB. In most of the AMI scenario, conditioning the heart before onset of ischemia is rarely an option. On the contrary, in elective surgery, cardiac conditioning before or after CPB induced ischemia is entirely optional. Nevertheless, the optimal timing of insulin institution for the best quality cardioprotection is still unclear.

In this study, in isolated rat hearts, we tested the hypothesis that insulin administered prior to ischemia provides better cardioprotection than insulin administered after ischemia. The primary measure/outcome was myocardial contractility. We also assessed p-Akt and TNF-α in order to identify potential mechanisms underlying the effects of insulin.

2. Materials and Methods

2.1. Isolated Rat Heart Model. With the approval of the Committee on Animal Research at the Faculty of Medicine, University of Yamanashi, Male Wistar rats (weighing 300–320 g) were anesthetized using intraperitoneal injection of pentobarbital sodium (30 mg/kg body weight).

Their hearts were rapidly excised, immersed in cold saline solution (4°C), and mounted on the stainless-steel cannula of a modified Langendorff perfusion apparatus. Retrograde aortic perfusion was initiated at a perfusion pressure of 55 mm Hg with modified Krebs-Henseleit (KH) buffer solution of the following composition (mmol/liter): NaCl 118, KCl 4.7, CaCl-2H_2O 2.0, $MgSO_4$-7H_2O 1.2, KH_2PO_4 1.2, glucose 5.5 (99 mg/dL), and $NaHCO_3$ 25. The perfusate was bubbled continuously with 95% O_2 and 5% CO_2 and maintained at 37°C 1hroughout the experiment. A water-filled latex balloon containing a pressure transducer (TruWave, Edwards Lifesciences, CA, USA) was placed into the left ventricle (LV) through the left atrium to measure LV function. The heart was paced to 222 beats/min during the ischemic period with electronic stimulator (SEN-3201, Nihon Kohden, Tokyo, Japan).

2.2. Experimental Protocol. The animals were divided into three groups and the experimental protocols are shown in Figure 1. The Pre-Ins group received 0.5 U/L insulin in KH buffer prior to 15 min no-flow ischemia for 20 min and during 20 min of reperfusion. The Post-Ins group received 0.5 U/L insulin during the reperfusion period only. The control group was perfused with KH buffer throughout.

2.3. Measurements. Heart rate (bpm) and maximum left ventricular derivative of pressure development (LV dP/dt max) (mmHg/sec) were recorded continuously. Coronary flow (mL/min) was measured by timed collection of the perfusate (baseline, after 20 min of preconditioning, and after 5, 10, 15, and 20 minutes of reperfusion).

Coronary efferent fluid samples for TNF-α measurements were drawn after 20 min of preconditioning and after 1, 5, and 10 minutes of reperfusion. The concentrations of TNF-α were measured by the sandwich ELISA technique (Invitrogen rat TNF-α ELISA Kit, Life Technologies, CA, USA) and quantified photometrically (Spectra Max 340, Molecular Devices, CA, USA) at an absorbance of 450 nm. The values were expressed as pg/mL. Measurements of TNF-α in the coronary effluent were normalized to 1 min volume of coronary flow.

At the end of the perfusion (after 20 min of reperfusion), the whole heart was quickly frozen in liquid nitrogen and freeze-dried for six days. The myocardium was suspended in assay lysis buffer (Lysis Buffer 6, R&D Systems, MN, USA) containing phenylmethanesulfonyl fluoride (PMSF, 2 mM, Sigma-Aldrich, Inc., MO, USA) and protease inhibitor cocktail (Sigma-Aldrich, Inc., MO, USA). The samples were then homogenized using a microhomogenizing system (MicroSmash MS-100R, TOMY SEIKO Co., Ltd., Japan). The homogenates were centrifuged for 5 min at 2000 g and the supernatants were assayed for their p-Akt content by ELISA (Surveyor IC Human/Mouse/Rat Phospho-Akt (Pan) (S473) Immunoassay, R&D Systems, MN, USA). At the other time points (after 20 min of preconditioning and after 5 min of reperfusion), muscle samples of whole heart for p-Akt measurements were taken using different rats ($n = 12$ in each group) in the above same technique (Figure 1). The concentrations of p-Akt (ng/gram of dry heart weight) were measured by the sandwich ELISA technique and quantified

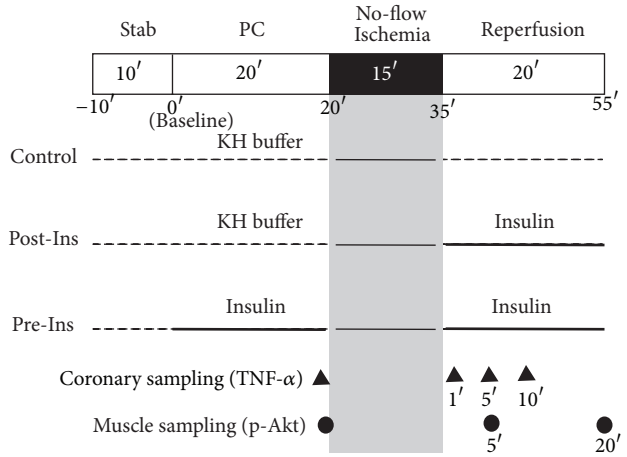

Figure 1: Experimental protocol. The Pre-Ins group received 0.5 U/L insulin in KH buffer prior to 15 min no-flow ischemia for 20 min followed by 20 min of reperfusion. The Post-Ins group received 0.5 U/L insulin during the reperfusion period only. The control group was perfused with KH buffer throughout.

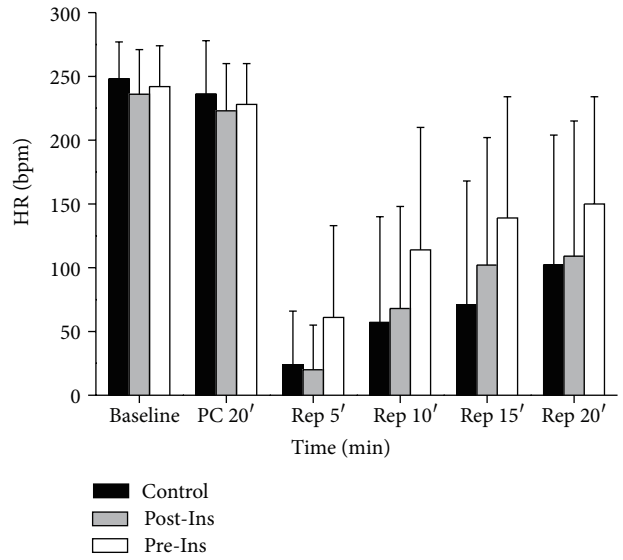

Figure 2: Time-course changes of heart rate before and after ischemia in the three groups ($n = 12$ in each group). The data are presented as means ± SD. HR: heart rate (bpm).

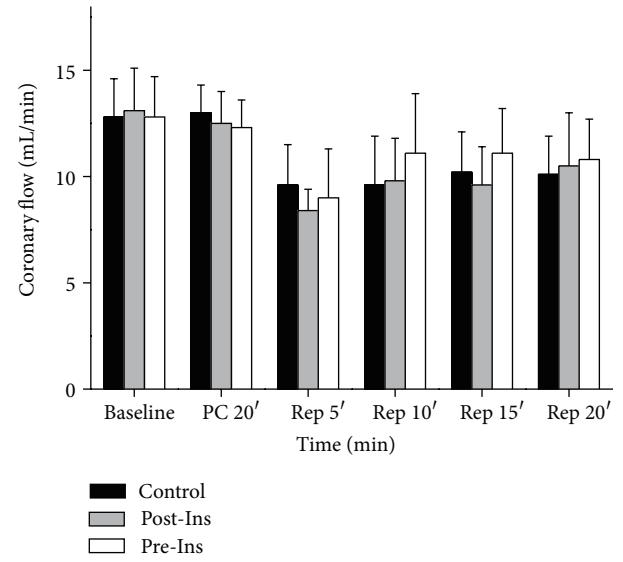

Figure 3: Time-course changes of coronary flow before and after ischemia in the three groups ($n = 12$ in each group). Coronary flow (mL/min) was measured by timed collection of the perfusate (baseline, after 20 min of preconditioning and after 5, 10, 15, and 20 minutes of reperfusion). The data are presented as means ± SD.

photometrically (Spectra Max 340, Molecular Devices, CA, USA) at an absorbance of 450 nm. The values were expressed as ng of p-Akt per gram of dry heart weight.

2.4. Statistical Analysis. The data are presented as means ± SD. Changes in hemodynamics and the concentrations of TNF-α and p-Akt were analyzed using two-way analysis of variance (ANOVA), followed by the Bonferroni post hoc test. Intergroup comparisons for baseline measurements, hemodynamic data, and the concentrations of TNF-α and p-Akt at each time point were made with one-way ANOVA followed by the Bonferroni post hoc test. Two-sided P values less than 0.05 were considered statistically significant.

Sample size calculation was based on the expected difference in the contractility (LV dP/dt max) at 20 min after reperfusion among groups. The results of a preliminary of our pilot study showed contractility of 1000 ± 800 (mmHg/sec) in the control group, 1500 ± 1000 in the Post-In group, and 3000 ± 1500 in the Pre-In group at 20 min after reperfusion. In order to achieve a power level of 80%, with an alpha error of 5%, at least 12 subjects were required in each group. All statistical analyses were performed using SPSS 21 for Windows (IBM, Chicago, IL) and PASS 11 (NCSS, Kaysville, UT).

3. Results and Discussion

There was no significant difference in baseline measurements between the groups (Table 1).

Heart rate increased gradually after reperfusion in all groups (Figure 2); however there were no differences between groups. Coronary flow was comparable among all experimental groups ($P = 0.58$) (Figure 3).

In Pre-Ins group, LV dP/dt max increased after administration of insulin prior to ischemia (after 20 min of preconditioning) compared to both Post-Ins and control groups (Figure 4). After reperfusion, LV dP/dt max in Pre-Ins group was significantly elevated, compared with Post-Ins groups at 10 minutes after reperfusion and control groups at all-time points (after 5, 10, 15, and 20 minutes of reperfusion).

The TNF-α concentrations, which were normalized to 1 min volume of coronary flow, were below the detectible range in all groups before ischemia. A significant increase in TNF-α level was observed in the control group at the beginning of reperfusion (Figure 5). Independent of timing, insulin

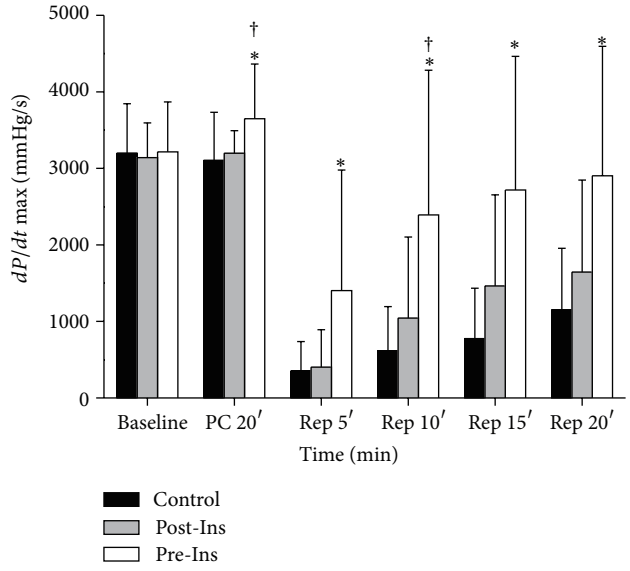

Figure 4: Time-course changes of LV dP/dt max before and after ischemia in the three groups ($n = 12$ in each group). The data are presented as means ± SD. $^{*}P < 0.05$ versus Control; $^{\dagger}P < 0.05$ versus Post-Ins. In Pre-Ins, LV dP/dt max increased after administration of insulin prior to ischemia (PC 20′) compared to both Post-Ins and Control. After reperfusion, dP/dt max in Pre-Ins was elevated, compared with Post-Ins at 10 minutes after reperfusion (Rep 10′) and Control at all-time points (Rep 5′, 10′, 15′, and 20′). dP/dt max (mmHg/sec): maximum of left ventricular derivative of pressure development.

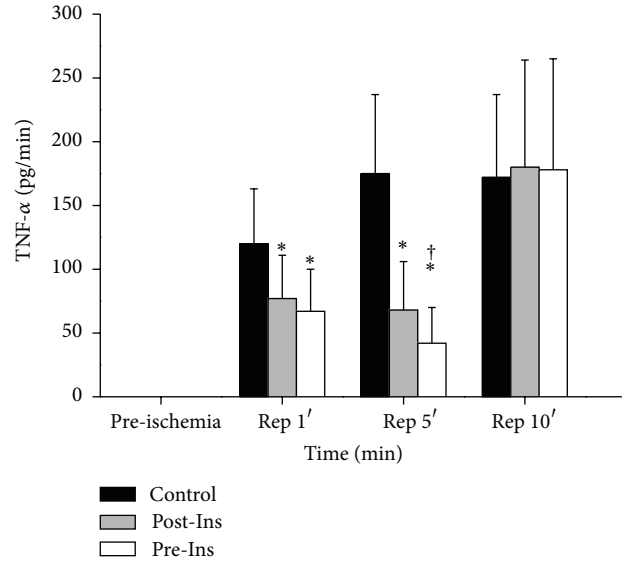

Figure 5: Time-course changes of TNF-α before and after ischemia in the three groups ($n = 12$ in each group). Coronary efferent fluid samples for TNF-α measurements were drawn after 20 min of preconditioning (preischemia) and after 1, 5, and 10 minutes of reperfusion. Measurements of TNF-α in the coronary effluent were normalized to 1 min volume of coronary flow (pg/mL). The data are presented as means ± SD. $^{*}P < 0.05$ versus Control; $^{\dagger}P < 0.05$ versus Post-Ins. A significant increase in TNF-α was observed in Control at the beginning of reperfusion (Rep 1′ and 5′). The TNF-α at 5 minutes after reperfusion (Rep 5′) in the Pre-Ins was lower than both Post-Ins and Control.

attenuated the increase in TNF-α at 1 and 5 minutes after reperfusion. The TNF-α levels at 5 minutes after reperfusion in the Pre-Ins group were significantly lower than both Post-Ins and control groups.

The levels of p-Akt increased after administration of insulin before ischemia (Figure 6). After 5 minutes of reperfusion, p-Akt was elevated in the Pre-Ins group when compared with values observed in the control and Post-Ins group. The p-Akt levels in the Pre-Ins group were still significantly higher than in Control after 20 minutes of reperfusion.

In present study, insulin administration prior to ischemia provides better cardiac contractility than insulin administered only at reperfusion. The myocardium p-Akt was increased and maintained at the highest level in the Pre-Ins group, where suppression of TNF-α and elevated cardiac contractility were also observed. This increase in p-Akt resulted in improved cardiac function at reperfusion suggesting that TNF-α suppression is possibly mediated via p-Akt leading to a reduction in contractile myocardial dysfunction.

The Akt plays an important role in IPC protecting the myocardium from prolonged ischemia [1, 18]. Targeting proteins in IPC induced prosurvival pathway, such as p-Akt, with pharmacological agents is shown to provide similar cardioprotection, which is termed "pharmacological pre- or postconditioning." Insulin is well known to activate the Akt signaling cascade with various effects. In the current study, insulin in the Pre-Ins group elevated p-Akt in cardiomyocytes prior to ischemia compared with the noninsulin perfused heart and was maintained at the highest level during reperfusion. It is also known that ischemia itself activates the cell survival p-Akt cascade [19], and all three groups showed increases in p-Akt above baseline after ischemia. The prosurvival kinase Akt is the common signaling cascade that both insulin and IPC share in reducing cardiac ischemia-reperfusion injury [20] and "conditioning" the heart. Applying both insulin and ischemia amplifies the degree of Akt phosphorylation as shown in Pre- and Post-Ins group and possibly augments the cardioprotective conditioning effect.

Although essential for tissue survival, oxygen can be injurious during reperfusion. The intracellular changes during ischemia and reperfusion lead to the formation of reactive oxygen species (ROS), which play an important role in ischemia-reperfusion injury [21]. The cytokine-mediated cascade is initiated by this nonspecific injury, which results in the production of TNF-α [22]. Excessive TNF-α expression directly induces contractile myocardial dysfunction and cell apoptosis [23]. The current study demonstrates that TNF-α was suppressed in Pre- and Post-Ins group, especially in the Pre-Ins group at 5-minute reperfusion. It has been suggested that TNF-α inhibition is partly regulated by the p-Akt pathway [24]. Thus, one may speculate that insulin infusion increased p-Akt and promoted TNF-α suppression, which then resulted in preserved cardiac contractility in Pre-Ins group.

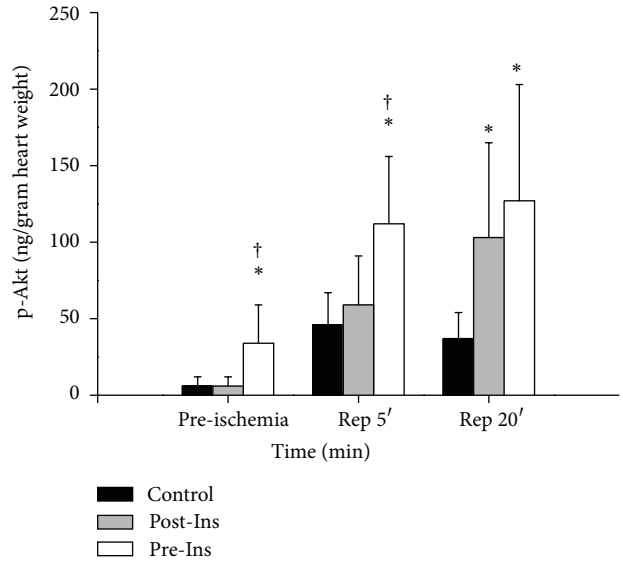

Figure 6: Time-course changes of p-Akt before and after ischemia in the three groups. Muscle samples for p-Akt concentrations (ng/gram of dry heart weight) were taken after 20 min of preconditioning (preischemia, $n = 12$ in each group) and after 5 min of reperfusion ($n = 12$ in each group) and 20 min of reperfusion ($n = 12$ in each group). The data are presented as means ± SD. $^{*}P < 0.05$ versus Control; $^{\dagger}P < 0.05$ versus Post-Ins. In Pre-Ins, p-Akt increased after administration of insulin before ischemia (preischemia). After 5 minutes of reperfusion, p-Akt was elevated in the Pre-Ins when compared with Control and Post-Ins (Rep 5′). The p-Akt levels in the Pre-Ins were still higher than Control after 20 minutes of reperfusion (Rep 20′).

Several studies emphasize the importance of the timing of insulin in relation to myocardial ischemia [10, 25]. It is not yet clear, however, whether preconditioning the heart with insulin provides the best quality protection or delayed insulin administration, that is, postconditioning, is equally effective. The current study results show an increase in p-Akt, TNF-α suppression, and a better preserved LV dP/dt max, when insulin was administered before ischemia compared to when it was started later. The results suggest that insulin preconditioning provides better cardioprotection than post-conditioning in ischemia-reperfusion injury. Interestingly, differences in p-Akt and TNF-α between Pre- and Post-Ins group are no longer significant at 20 minutes of reperfusion, where LV dp/dt remains the highest in Pre-Ins group. This can be explained by the possible existence of a threshold of Akt activation which is required to confer cardioprotective effect [26, 27]. Applying insulin prior to ischemia activates the cell survival p-Akt cascade early enough to protect the myocardium during reperfusion.

Recent studies demonstrated that the cardioprotective effect of insulin against myocardial infarction occurred when insulin was administered only during reperfusion, but not when started prior to ischemia [9, 10]. Jonnasen et al. studied isolated rat hearts applying 35 minutes regional myocardial ischemia. They showed that the administration of insulin at a high dose, for 10 minutes prior to ischemia, continued throughout ischemia and reperfusion, failed to reduce infarct size. Interestingly, administration of insulin only at the onset of reperfusion significantly reduced infarct size [10]. On the other hand, our current study showed that administration of insulin prior to ischemia preserved cardiac contractility better than insulin administration only during reperfusion. Discrepancies in cardioprotection related to the timing of insulin administration may be explained by differences in study protocol and measurements. Jonnasen's study measured infarct size with regional myocardial ischemia, whereas the present study assessed whole heart ischemia and cardiac function. They have suggested that insulin inhibits proapoptotic protein on outer mitochondrial membrane which resulted in reduction of infarct size, whereas in our study, insulin via pAkt inhibits TNF-α production putatively and thus also inhibits TNF-α effect directly on myocardial contractile dysfunction [28, 29]. The inotropic effect of insulin, while the myocardium is exposed to regional ischemia, possibly attenuates cell survival by aggravating the imbalance between myocardial oxygen supply and demand.

4. Conclusions

In conclusion, administration of insulin prior to ischemia protected cardiac contractility better than insulin administered during reperfusion. The p-Akt activity in cardiomyocyte was significantly higher when insulin was administered before ischemia supporting the contention that the p-Akt cell survival pathway contributes to "conditioning" effect of IPC and PPC. The current study suggests that insulin activated p-Akt also facilitates preservation of cardiac contractility during ischemia-reperfusion injury in the isolated rat heart.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

Protection of Coronary Endothelial Function during Cardiac Surgery: Potential of Targeting Endothelial Ion Channels in Cardioprotection

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Vascular endothelium plays a critical role in the control of blood flow by producing vasoactive factors to regulate vascular tone. Ion channels, in particular, K^+ channels and Ca^{2+}-permeable channels in endothelial cells, are essential to the production and function of endothelium-derived vasoactive factors. Impairment of coronary endothelial function occurs in open heart surgery that may result in reduction of coronary blood flow and thus in an inadequate myocardial perfusion. Hyperkalemic exposure and concurrent ischemia-reperfusion during cardioplegic intervention compromise NO and EDHF-mediated function and the impairment involves alterations of K^+ channels, that is, K_{ATP} and K_{Ca}, and Ca^{2+}-permeable TRP channels in endothelial cells. Pharmacological modulation of these channels during ischemia-reperfusion and hyperkalemic exposure show promising results on the preservation of NO and EDHF-mediated endothelial function, which suggests the potential of targeting endothelial K^+ and TRP channels for myocardial protection during cardiac surgery.

1. Introduction

Coronary circulation is of vital importance to myocardial perfusion. The vascular endothelium of coronary arteries has been identified as the important organ that locally regulates coronary perfusion and cardiac function by producing vasoactive substances. The compromised function of coronary endothelium during cardiac surgery contributes to the no- or low-reflow phenomenon that ultimately leads to myocardial dysfunction and jeopardizes postoperative cardiac performance. Ischemia-reperfusion (I-R) and the direct contact of coronary endothelium with hyperkalemic solutions during cardioplegic intervention both pose detrimental effects on coronary endothelial function. Ion channels, in particular, potassium (K^+) channels and calcium-(Ca^{2+}-) permeable channels in endothelial cells, are essential to the production and/or function of endothelium-derived vasoactive factors. This review addresses the role of K^+ and Ca^{2+}-permeable channels in endothelial function by focusing on the regulation of vascular tone and summarizes the findings of alterations of these channels under conditions related to cardiac surgery. The potential of targeting these channels for myocardial protection during cardiac surgery is also discussed from the viewpoint of endothelial protection.

2. Endothelial Dysfunction during Cardiac Surgery: Effect of I-R and Cardioplegic Exposure

Endothelium functions to counteract leukocyte adhesion and platelet aggregation to prevent inflammation and thrombosis and actively regulate vascular tone by producing vasoactive substances [1, 2]. During cardiac surgery and cardioplegic intervention, coronary vasculature is inevitably subjected to I-R and hyperkalemic exposure. A considerable body of research shows the susceptibility of vascular endothelium to I-R or hypoxia-reoxygenation (H-R) injury. I-R/H-R activates endothelial cells resulting in neutrophil-endothelium adhesion and inflammation [3]. Activation of endothelial contractile machinery during I-R due to cell reenergization disturbs endothelial barrier function [4]. Moreover, I-R disrupts the balance between endothelium-derived constricting and relaxing factors and thus interrupts blood flow and organ perfusion [5]. The association of I-R and endothelial cell injury in cardiovascular surgery has been discussed in a previous review article by Boyle Jr and colleagues [6].

Cardioplegic and organ preservation solutions were initially designed to protect cardiac myocytes from I-R injury in heart surgery including heart transplantation. However, since endothelial cells differ with myocytes in structure, function, and electrophysiological properties (nonexcitable versus excitable), use of these solutions may not be able to provide protection to coronary endothelium. In fact, although there were studies showing the preservative effect of crystalloid cardioplegic or organ preservation solutions on endothelial function [7, 8], accumulating evidence suggests endothelial damage after exposure to these solutions. Histological examination and cell culture studies showed that crystalloid hyperkalemic cardioplegia impairs vascular endothelium and reduces the replicating ability of coronary endothelial cells [9, 10].

3. Impact of Cardioplegic Intervention on Endothelium-Derived Vasoactive Factors

Cardioplegic intervention interrupts the balance between endothelium-derived constricting and relaxing factors. I-R/H-R increased the production of vasoconstrictors such as endothelin-1 [5]. A large number of studies have revealed the significance of reduction of endothelium-derived relaxing factors (EDRFs), in particular, nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF), in the disturbance of blood flow in cardiac surgery-related conditions. Mechanisms underlying the impairment of endothelium-dependent vasorelaxation include I-R-induced availability and functional changes of NO and EDHF [11–16] as well as EDHF alterations caused by hyperkalemic exposure [17–20].

3.1. Impact of Cardioplegic Intervention on NO: Role of I-R and Hyperkalemic Exposure. Endothelial function mediated by NO, the major EDRF [21], is impaired during cardiopulmonary surgery. After 1-hour crystalloid cardioplegic arrest, NO release decreased significantly in human coronary vasculature and further decreased upon reperfusion, evidenced by the reduction of NO end-products nitrite and nitrate [22]. Inhibition of NO release after infusion of University of Wisconsin (UW) solution is associated with an attenuated endothelium-dependent vasodilatation [23]. Downregulation of eNOS protein was reported to underlie the loss of NO production caused by cardioplegia-reperfusion [24] and the NO loss after cold ischemic storage in crystalloid cardioplegia could be recovered by chronic oral administration of the NO substrate *L*-arginine [25]. All these studies demonstrated the unfavorable effect of cardioplegic intervention on endothelial eNOS-NO function. Consistently, we observed NO loss-related endothelial dysfunction and protection of endothelial function provided by eNOS enhancement in porcine coronary arteries which underwent 1 h of hypoxic exposure in St. Thomas cardioplegic (ST) solution and 30 min reoxygenation [26].

3.1.1. I-R and NO. Endothelial I-R injury is closely associated with decrease of NO bioavailability. Myocardial I-R impairs endothelium-dependent NO-mediated relaxation in coronary arteries [27, 28]. Administration of *L*-arginine preserves postischemic endothelial function in both animals and humans [29, 30]. With measurement of NO using a NO microsensor, we provided direct evidence of NO reduction in coronary arteries in an *in vitro* I-R model [31].

In addition to the potent vasodilatory effect, NO inhibits platelet aggregation and leukocyte adhesion that is an important component of the endogenous defense mechanism against vascular injury, inflammation, and thrombosis. The loss of NO after myocardial I-R therefore poses profound deleterious effects on the coronary vasculature that further compromises myocardial function.

3.1.2. Hyperkalemic Exposure and NO. Compared to numerous studies of I-R, investigations regarding the impact of hyperkalemic exposure *per se* on NO function remain limited. We reported that after exposure to crystalloid cardioplegia containing 16–50 mmol/L K^+, endothelium-dependent vasorelaxations are well preserved in porcine epicardial coronary arteries [32] and neonatal rabbit aorta [33]. In these studies, the cardioplegic solutions were continuously oxygenated and the whole experiment was performed under well-oxygenated condition; therefore, the effect of I-R was completely excluded. It is worth mentioning that, in these studies, endothelium-dependent relaxation was studied in the presence of PGI_2 inhibitor indomethacin; thus, the relaxation involves both NO and EDHF components. However, considering the susceptibility of EDHF to hyperkalemic exposure [17–19], which is addressed in details in the next section, the preserved endothelium-dependent dilator response to a certain extent suggests that hyperkalemic cardioplegic solutions may barely impair NO-related function. Direct measurements of NO demonstrated that NO production in porcine coronary arteries was unaffected by 1 h of exposure to hyperkalemic solution containing 20 mmol/L K^+ [20]. Whether higher K^+ concentration and longer exposure

may affect endothelial NO production, however, remains unknown.

3.2. Impact of Cardioplegic Intervention on EDHF: Role of I-R and Hyperkalemic Exposure. The contribution of EDHF in vasodilatation increases as vessel size decreases [34, 35], which highlights the importance of this factor in blood flow regulation.

3.2.1. I-R and EDHF. I-R alters EDHF-mediated endothelial function. In a rat I-R model of 2 h occlusion of the middle cerebral artery followed by 24 h reperfusion, the EDHF-mediated dilatation was potentiated [36, 37]. Potentiation of the EDHF-type response was also observed in dog coronary arteries subjected to 1 h of ischemia and 2 h of reperfusion [38]. These findings support the "compensatory or back-up" theory of EDHF mechanism in conditions involving NO loss. However, contradictory evidence is also available showing the deleterious effect of I-R on EDHF-mediated function. For example, in porcine coronary arteries exposed to H-R, the EDHF-mediated relaxation and hyperpolarization were significantly attenuated [15, 39–41]. H-R also blunted the EDHF response in coronary microveins [16]. The reasons of the controversy remain unclear that may include differences in species and vascular beds and differences in experimental settings. The residual NO may also have influence on the result interpretation in studies that only used eNOS inhibitor with no further use of NO scavenger to completely abolish the NO component.

3.2.2. Hyperkalemic Exposure and EDHF. Our laboratory has contributed a great deal to the knowledge of the effect of hyperkalemia on EDHF pathway. With exclusion of the effect of I-R and elimination of the role of PGI_2 and NO, we have demonstrated that hyperkalemic solutions [17–19] and clinically used crystalloid cardioplegia/organ preservation solutions such as ST [42] and UW solutions [43] impair EDHF-mediated function in porcine or human coronary arteries and veins. The depolarizing effect of hyperkalemia on the membrane of vascular smooth muscle cells counteracts and restricts the hyperpolarizing effect of EDHF, which was suggested to be responsible for the attenuated EDHF-mediated vasorelaxation under hyperkalemic cardioplegic exposure [17, 44]. This mechanism also explains the superiority of hyperpolarizing cardioplegia to depolarizing cardioplegia in preservation of EDHF-mediated endothelial function [45].

4. Role of Endothelial Ion Channels in NO and EDHF Pathways

Calcium- (Ca^{2+}-) permeable and potassium (K^+) channels are essential to vascular endothelial function. The importance of Ca^{2+}-permeable channels is highlighted by the Ca^{2+}-dependency of the synthesis or action of vasoactive agents including relaxing factors. Enzymes responsible for the production of NO and PGI_2 requires an increase in endothelial $[Ca^{2+}]_i$ for activation [46, 47]. The classical EDHF response is initiated by opening of intermediate and small conductance Ca^{2+}-activated K^+ channels (IK_{Ca} and SK_{Ca}) on the plasma membrane of endothelial cells [48]. In some vasculatures, nonclassical EDHF response mediated by epoxyeicosatrienoic acids (EETs) may also exist. EETs not only activate endothelial IK_{Ca} and SK_{Ca} but also open myocyte large-conductance K_{Ca}(BK_{Ca}) to relax vessels [49]. The opening of IK_{Ca} and SK_{Ca} and the production of EETs also depend on $[Ca^{2+}]_i$ rise in endothelial cells [48, 50].

4.1. Ca^{2+}-Permeable TRP Channels. Transient receptor potential (TRP) channels are the most important Ca^{2+}-permeable channels in vascular endothelium [51–53]. TRP channels regulate $[Ca^{2+}]_i$ by directly acting as Ca^{2+} entry channels in the plasma membrane or by changing membrane potentials to modulate the driving force for Ca^{2+} entry [54]. Available evidence suggests that, among the six subfamilies of TRP (TRPA, TRPC, TRPV, TRPM, TRPP, and TRPML), canonical and vanilloid TRP (TRPC and TRPV) channels are more significantly involved in the vascular tone regulation. The role of TRPC1, C3, and C4 in Ca^{2+} influx in aortic endothelial cells has been implicated in the relaxant response of mouse aorta to agonists such as carbachol and acetylcholine [55, 56]. The partial involvement of TRPC6 in carbachol-induced relaxation was also observed in the mouse aorta [57]. TRPC3 forms channels endowed with significant constitutive activity [58] and native TRPC3 contributes to constitutive and ATP-dependent Ca^{2+} influx in human coronary artery endothelial cells [59]. Studies of various vascular beds including mesenteric [60], coronary [31, 59], and human internal mammary arteries, a widely used coronary arterial bypass graft [61], have revealed the significance of native TRPC3 in endothelial Ca^{2+} influx and vasorelaxation. Further mechanistic studies demonstrated that TRPC3-mediated Ca^{2+} influx in endothelial cells is necessary to the production of NO and the function of EDHF [31, 62, 63].

Participation of members of TRPV subfamily, in particular V4, was also reported in the control of vascular tone. In TRPV4-deficient mice, endothelium-dependent relaxations in response to acetylcholine and sheer stress were significantly attenuated in small resistance arteries, which was attributed to the inhibition of both NO and EDHF components [64, 65]. In fact, Ca^{2+} influx through TRPV4 has been proposed as a molecular mechanism sensing shear stress that significantly contributes to endothelial mechanotransduction [66]. Recently, in human coronary arterioles, Bubolz and colleagues demonstrated that TRPV4-mediated Ca^{2+} entry is involved in flow-induced vasodilatation. The TRPV4-dependent vasodilator response to arachidonic acid, a potentially important mediator of endothelium-derived hyperpolarizing-related vasodilation, was also reported [67, 68]. The findings that activation of merely a few TRPV4 channels enables local Ca^{2+} signals and causes maximal dilation through IK_{Ca} and SK_{Ca} activation further highlighted the functional significance of endothelial TRPV4 in vasorelaxation [69]. The physical interaction of TRPV4 with SK_{Ca}(K_{Ca}2.3) in endothelial cells provided a structural basis

for K_{Ca} activation by TRPV4-Ca^{2+} influx [70]. It is suggested that TRPV4-mediated $[Ca^{2+}]_i$ rise in endothelial cells may trigger both NO- and/or EDHF-dependent vasodilatation that seems to be vascular bed-dependent.

4.2. K^+ Channels. Vascular endothelium expresses a variety of K^+ channels including inwardly rectifying K^+ (Kir), ATP-sensitive K^+ (K_{ATP}), voltage-gated K^+ (K_V), and Ca^{2+}-activated K^+ (K_{Ca}) channels. The functional significance of Kv channels in endothelium physiology has not been established. Kir channels may serve as sensors for elevated extracellular K^+ and contribute to K^+-induced dilation [71] and act as amplifiers of hyperpolarization initiated by the opening of other K^+ channels, in particular, IK_{Ca} and SK_{Ca}, to conduct hyperpolarizing signals along endothelial cells [72]. In comparison with Kv and Kir, a considerable body of knowledge has been gained concerning the significance of K_{ATP} and K_{Ca} channels in endothelial function.

4.2.1. K_{ATP} Channel. Patch-clamp studies in knockout mice suggested that endothelial K_{ATP} channels are an important component of shear-sensing mechanism in pulmonary microvasculature [73]. Shear stress increases the expression and activity of K_{ATP} channels in pulmonary vascular endothelial cells [74]. Previous studies also implicated the functional role of endothelial K_{ATP} channels in coronary vasodilatation, although evidence gained largely relied on the effect of pharmacological tools (channel antagonist or/and agonists) that therefore might be indirect. Endothelial K_{ATP} channels participate in flow and shear stress-mediated vasodilation [75] and dilations induced by isoflurane [76] and hyperosmolarity [77] in coronary microvessels. In the heart, K_{ATP} channels also expressed in endothelial cells of aorta and capillaries [78, 79]. It is believed that hyperpolarization mediated by K_{ATP} activation may facilitate Ca^{2+} influx in endothelial cells and thus modulate the production of EDRFs.

4.2.2. K_{Ca} Channel. Endothelial IK_{Ca} and SK_{Ca} channels are of great importance in vascular tone regulation [80–83]. High or low SK_{Ca} expression in arteries of SK3 transgenic mouse, respectively, exaggerated or abolished the tonic endothelial dilating influence [84]. In IK_{Ca} knockout mice, disruption of the $K_{Ca}3.1$ gene reduced acetylcholine-induced hyperpolarization in vascular cells which was associated with impairment of vasodilatation and elevation of blood pressure [85]. Intraluminal application of inhibitors of IK_{Ca} and SK_{Ca} channels blocked EDHF-mediated vasorelaxation [86]. Endothelial membrane hyperpolarization resulting from IK_{Ca} and SK_{Ca} opening can be conducted along the endothelium via homocellular endothelial gap junctions and transmitted to smooth muscle cells through myoendothelial gap junctions to cause vasodilatation. IK_{Ca} predominantly resides in myoendothelial projections whereas SK_{Ca} preferentially is located the sites of homocellular endothelial gap junctions and caveolin-rich domains [87–90]. The difference between the subcellular localization of these two subtypes is believed to be a mechanism facilitating efficient signaling transduction within endothelium and between endothelium and smooth muscle. Activation of IK_{Ca} and SK_{Ca} channels may also induce K^+ efflux from endothelial cells that elicits hyperpolarization and relaxation of smooth muscle by activating Kir channels and Na^+-K^+-ATPase on the smooth muscle membrane [91]. In addition to the significant role in EDHF pathway [82, 83, 86–88, 92–94], activation of IK_{Ca} and SK_{Ca} channels may also amplify NO production in endothelial cells [95]. It was proposed that the channel opening-induced endothelial hyperpolarization enhances driving force for Ca^{2+} entry, resulting in elevation of $[Ca^{2+}]_i$ and amplification of NO production. The capacity of K_{Ca} channels in endothelial $[Ca^{2+}]_i$ modulation, therefore, further highlights the significance of these channels in the regulation of vascular tone.

5. Impact of Cardioplegic Intervention on K^+ and TRP Channels in Endothelial Cells: Role of I-R and Hyperkalemic Exposure

5.1. Effect of I-R and Hyperkalemic Exposure on K_{Ca} Channels. Inactivation of IK_{Ca} and/or SK_{Ca} channels may contribute to endothelial dysfunction related to cardioplegic arrest/cardiopulmonary bypass in human. Following cardioplegic arrest/cardiopulmonary bypass, coronary arterioles and skeletal muscle arterioles showed significant decreases in vasorelaxant responses to IK_{Ca}/SK_{Ca} activator and endothelium-dependent vasodilators [96, 97]. In these studies, the alteration of IK_{Ca} and SK_{Ca} occurred at channel activity level but not at expression level. Alteration of K_{Ca} channels under these circumstances may be attributable to both I-R injury and hyperkalemic cardioplegic exposure. Although there are controversial findings regarding the effect of I-R on K_{Ca} [98, 99], I-R-induced K_{Ca} inhibition was reported repeatedly. Vasorelaxant response to ADP significantly attenuated in goats underwent 1 h occlusion of the left middle cerebral artery followed by 1 h reperfusion and the loss of K_{Ca}-EDHF mediation in postischemic vessels was evidenced by the loss of inhibitory effect of IK_{Ca} and SK_{Ca} channel blockers [100]. Consistently, we showed that EDHF-mediated hyperpolarization and relaxation were impaired in both coronary arteries and veins after 1 h hypoxia and 30 min reoxygenation [15, 16]. Further patch-clamp experiments demonstrated that inhibition of IK_{Ca} and SK_{Ca} channels is responsible for the compromised EDHF responses after H-R [41].

The effect of hyperkalemia on K_{Ca} channels remains poorly studied. We previously demonstrated that the IK_{Ca} and SK_{Ca}-dependent EDHF responses are impaired by crystalloid hyperkalemic cardioplegia/organ preservation solutions in both coronary and pulmonary vasculatures [101–104]. Although these are not direct evidence of the inhibitory effect of hyperkalemia on endothelial K_{Ca} channel activity, given that K_{Ca} activation requires $[Ca^{2+}]_i$ rise and membrane depolarization in endothelial cells decreases the driving force for Ca^{2+} entry, suppression of K_{Ca} activity therefore may be expected when vascular endothelium is exposed to hyperkalemic cardioplegia/organ preservation solutions.

5.2. Effect of I-R and Hyperkalemic Exposure on K_{ATP} Channels. The findings of K_{ATP} mediation in the protection offered by preconditioning and postconditioning against endothelial I-R injury may suggest the association of K_{ATP} alterations and endothelial dysfunction [105–107]. Pharmacological inhibition of K_{ATP} channels reduced or completely blocked the protective effect of preconditioning and postconditioning on endothelium-dependent vasodilation. K_{ATP} channel blockade also abolished the protective effect of heat stress on endothelial function in ischemic heart [108]. K_{ATP} channel opener KRN_{4884} mimicked the protective effect of hypoxic preconditioning on the EDHF-mediated relaxation in coronary arteries [40], which may serve as an additional proof of I-R-induced functional disturbance of K_{ATP} channels. Further mechanistic investigations established the pivotal role of mitochondria K_{ATP} channels in cardioprotection. Opening of the mitochondrial K_{ATP} channel is thought to prevent the mitochondrial permeability transition pore from opening, leading to cellular protection from I-R injury in cardiac myocytes and vascular endothelial cells [109, 110].

Supplementation of K_{ATP} channel openers such as aprikalim and Nicorandil in hyperkalemic solutions protects EDHF-mediated function in coronary arteries [20, 111, 112]. Although the protection most likely results from the hyperpolarizing effect of K_{ATP} channel openers on smooth muscle membrane that counteracts hyperkalemia-induced depolarization thus facilitates EDHF responses, it cannot be ruled out that activation of K_{ATP} channels in endothelial cells might as well contribute to the protection of endothelial function. Endothelial K_{ATP} channel opening-induced membrane hyperpolarization may increase the driving force for Ca^{2+} influx thus promoting the production of EDRFs including EDHF.

6. Effect of I-R and Hyperkalemic Exposure on TRP Channels

I-R/H-R disturbs intracellular Ca^{2+} homeostasis and Ca^{2+} signaling in vascular endothelial cells. During ischemia, hypoxia/anoxia occurs in conjunction with acidosis and reperfusion is accompanied by increased formation of reactive oxygen species, which all have impact on $[Ca^{2+}]_i$. For example, $[Ca^{2+}]_i$ changes when extracellular acidosis occurs although the change may vary in different cells. Extracellular acidosis causes Ca^{2+} overload in anoxic endothelial cells from rat coronary artery [113], whereas in porcine aortic endothelial cells, extracellular acidification decreases $[Ca^{2+}]_i$ and inhibits agonist-stimulated production of EDRFs [114]. Suppression of store-operated Ca^{2+} entry via nonselective cation channels was found to be responsible for the decreased Ca^{2+} response in this study [114]. The modulation of store-operated Ca^{2+} entry was also reported in rat cardiac microvascular endothelial cells exposed to H-R [115]. Although these studies did not address by what molecular mechanisms store-operated Ca^{2+} entry is affected, they provided a logical basis for further investigation on the role of TRP channels in I-R-induced $[Ca^{2+}]_i$ disturbance given the significance of TRP channels in store-operated Ca^{2+} entry [52]. In fact, recent studies started to reveal the effects of I-R/H-R on TRP channels and associated vascular endothelial function. In patch-clamp studies of porcine coronary endothelial cells, we, for the first time, demonstrated that H-R suppresses TRPC3 channel activity through inhibiting the membrane translocation of this channel. The inhibition of TRPC3-mediated Ca^{2+} influx is an underlying mechanism of H-R-induced reduction of NO production and attenuation of endothelium-dependent relaxation in coronary arteries [31]. In a mice model of prolonged H-R, preconditioning amplifies EDHF-mediated relaxation with an association of an increase in TRPV4 expression in endothelial cells. Moreover, TRPV4 is involved in eNOS regulation in preconditioning, evidenced by an increase of phosphorylation on eNOS serine 1177 in wide type whereas an inhibition in TRPV4 knockout mice [116].

Changes of membrane potential affects $[Ca^{2+}]_i$. In endothelial cells depolarized by high K^+ concentration, reduction of Ca^{2+} influx was observed [117, 118]. However, the mechanism by which endothelial Ca^{2+} channel is affected by hyperkalemia remains poorly studied. Our recent attempt in exploring the impact of hyperkalemia on endothelial Ca^{2+} channels provided the first evidence of the alteration of TRPC3 channels and the functional significance of TRPC3 alteration in hyperkalemia-induced EDHF dysfunction. We demonstrated that Ca^{2+} influx via TRPC3 channels in endothelial cells is reduced by hyperkalemia and activation of TRPC3 restores Ca^{2+} influx and prevents EDHF dysfunction caused by hyperkalemic solutions and clinically used crystalloid cardioplegia/organ preservation solutions such as ST and UW solutions [62].

7. Potential of Targeting K^+ and TRP Channels for Endothelial Protection during Cardiac Surgery

Advances in understanding the mechanisms of endothelial dysfunction promote the development of strategies for endothelial cell protection. Better preservation of coronary endothelial and myocardial function can be achieved by supplementation of additives in cardioplegic or organ preservation solutions. Earlier studies have provided substantial evidence of the beneficial effect of supplementation of NO precursor *L*-arginine or NO donors, that is, nitroglycerin in cardioplegia on postischemic ventricular performance and endothelial function [119–121]. The EDHF component is well preserved in cardioplegic solutions containing $EET_{11,12}$, a possible chemical candidate of EDHF [102, 122].

Earlier attempts including K_{ATP} channel openers in hyperkalemic cardioplegia have set a successful example of targeting K^+ channels for endothelial protection. Hyperkalemia-induced impairment of EDHF function is ameliorated by addition of K_{ATP} channel openers such as nicorandil, aprikalim, and KRN_{4884} in cardioplegic solutions [15, 40, 112]. In addition, cardiac myocytes also benefit from the addition of K_{ATP} channel openers. Aprikalim inhibits Na^+-Ca^{2+} exchange enhanced by hyperkalemia thus

preventing $[Ca^{2+}]_i$ overload and improves the contractile function of ventricular myocytes [123]. Addition of the mitochondrial K_{ATP} opener diazoxide to heart preservation solution preserves diastolic function and reduces myocardial edema [124]. As to the benefit of K_{ATP} channel openers on endothelial function, although hyperpolarization of the smooth muscle cell membrane, the "effector" of EDHF responses is believed to underlie the preservation of EDHF pathway; the activation of K_{ATP} channels in endothelial cells might also be involved.

The unveiling of the key role of K_{Ca} and TRP channels in endothelial function together with the findings of the alterations of these channels during I-R and hyperkalemic exposure provided scientific basis for the potential of targeting these channels for endothelial protection during cardiac surgery. In an *in vitro* I-R model, the loss of endothelium-dependent relaxation of coronary arteries can be prevented by activation of IK_{Ca}/SK_{Ca} and TRPC channels [31, 41]. Pharmacological activation of IK_{Ca} and SK_{Ca} channels during H-R improves EDHF responses including relaxation and hyperpolarization [41]. A recent review article provided an update on the effectiveness of IK_{Ca}/SK_{Ca} activators in the treatment of cardiovascular disorders through the improvement of endothelium-derived hyperpolarizations [125]. Meanwhile, the therapeutic potential of activation of endothelial K_{Ca} channels to enhance NO availability in conditions associated with NO loss was reviewed by Kerr and colleagues [126].

Recent investigations started to show the potential of targeting endothelial TRP channels for cardiovascular protection, although so far the number of studies remains quite limited. In coronary arteries subjected to H-R, activation of TRPC3 channels in endothelial cells was observed to restore NO production [31]. Most recently, we demonstrated that supplementation of the TRPC3 activator in crystalloid cardioplegia such as ST and histidine-tryptophan-ketoglutarate (HTK) solution preserves TRPC3-mediated Ca^{2+} influx in endothelial cells and improves EDHF-mediated relaxation of coronary arteries [62]. The findings of protective effects of TRPC3 activation on coronary endothelium under H-R and hyperkalemic exposure may therefore shed light on future development of TRPC3-targeting strategies for myocardial protection during cardiac surgery.

Despite advances in the research of endothelial TRP channels, there are still many issues to be resolved, for example, the functional significance of interactions among different TRP isoforms. Whether a pathological condition that inhibits one isoform exerts the same effect on other isoforms or leads to "compensatory" enhancement of the function of others? Whether pharmacological modulation of one isoform has impact on others and changes their role in endothelial function? Answers to these questions are required to fully understand the potential of targeting TRP channels for endothelial cell protection.

In summary, Ca^{2+}-permeable TRP and K^+ channels in endothelial cells are essential to the regulation of vascular tone. Coronary endothelial dysfunction occurs in cardiac surgery that is attributable to I-R injury and hyperkalemic exposure. The functional alteration of endothelial TRP and K^+ channels during I-R and hyperkalemic exposure renders these channels potential targets for endothelial protection during cardiac surgery.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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