PROTECTIVE EFFECTS OF SELECTIVE ADENOSINE-TRIPHOSPHATE SENSITIVE POTASSIUM CHANNEL MODULATORS ON ENDOTHELIAL CELLS AGAINST ISCHEMIA-REPERFUSION INJURY

SUCHADA MEEYOS

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in Cardio-Thoracic Technology - (Type A2)
2023

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Thesis entitled "Protective effects of selective adenosine-triphosphate sensitive potassium channel modulators on endothelial cells against ischemia-reperfusion injury"

By Suchada Meeyos

has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Science in Cardio-Thoracic Technology - (Type A2) of Naresuan University

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ABSTRACT

Background and objectives: Increasing evidence suggested the roles of endothelial cells and ATP-sensitive potassium channels (K_{ATP} channels) in cardioprotection during cardiac surgery. However, there are limited data demonstrating the effects of endothelial K_{ATP} activators on endothelial cell viability and NO production in the absence and the presence of hyperkalemic cardioplegia (CPG) against ischemia-reperfusion injury (IRI). The aims of this study were to investigate the early and late effects of IRI with or without selective K_{ATP} channel activators on endothelial cell viability and nitric oxide production, in normal conditions and after exposure to hyperkalemic cardioplegia.

Method: Human umbilical vein endothelial cell line (EA.hy926) were cultured in a standard condition for 24 hr and were subsequently subjected to ischemia-reperfusion with or without K_{ATP} channel modulators, pinacidil, diazoxide, nicorandil and ZD0947. Cell viability and NO production were assessed after 10 min and 24-hr of reperfusion. To test the effects of hyperkalemic cardioplegia, the cells were subjected to ischemia and reperfusion of CPG alone or K_{ATP} channel modulator -added CPG. To test the effects of nicorandil and ZD0947 following CPG administration, the cells were treated with openers after

exposure to CPG. Cell viability and NO level were investigated after 30 minutes and 24 hours reperfusion.

Result: IRI- induced endothelial cell death was decreased significantly in the presence of K_{ATP} openers, pinacidil, diazoxide, nicorandil and ZD0947 both at 10 minute and 24 hr reperfusion. After 30 minutes of reperfusion, the NO production was also significantly lower in pinacidil, diazoxide and nicorandil groups when compared to control, IRI and ZD0947 groups. In the late response, it was shown that NO production of nicorandil and ZD0947 treated group was significantly higher than that of the IRI group. Pinacidil or diazoxide- added cardioplegia could improve cell viability against hyperkalemia with IRI, whereas nicorandil and ZD failed. However, adding nicorandil or ZD0947 at the reperfusion period showed the protective effect against IRI with CPG. Nicorandil and ZD0947 added-reperfusion solution reduced endothelial cells death in the early phase with relative cell viability of 74.17 ± 1.65 ($p = 0.913$ vs. I-CPGR) and 74.14 ± 1.62% ($p = 0.913$ vs. I-CPGR), respectively. However, in late response, cell viability of nicorandil and ZD0947 did not differ from that of I-CPGR group. ZD0947 and nicorandil showed the protective effect on endothelial cell function by increasing NO level in IRI in combination with CPGat the early phase. However, NO levels of all groups returned to the normal level after 24 hr reperfusion.

Conclusion: Ischemia-reperfusion jeopardized endothelial cell viability and function in normokalemia condition and in hyperkalemic CPG. KATP channel openers; pinacidil, diazoxide, nicorandil and ZD0947 ameliorated cell death from IRI. In the presence of cardioplegia, ZD0947 and nicorandil showed the protective effect on endothelial cells function by increasing and maintaining NO production against IRI.

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Suchada Meeyos

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CHAPTER I

INTRODUCTION

Background

Ischemia-reperfusion injury (IRI), an insult from the restoration of blood flow to ischemic tissue, is associated with cellular dysfunction and cell death [1]. The mechanism is initiated by ischemia-induced intracellular acidosis and disruption of mitochondrial functions and calcium homeostasis, leading to cell death [2]. Restoration of blood flow or reperfusion is the key step to limiting injury. However, reperfusion is also deleterious to the cells since it can trigger reactive oxygen species (ROS) generation. Furthermore, increased ROS activates the release of calcium from the om sarcoplasmic reticulum (SR), resulting in increased intracellular calcium levels. Correction of intracellular pH together with increased ROS generation lead to irreversible mitochondrial permeability transition pore (mPTP) opening which leads cells death [3-7].

IRI plays a major role in the pathophysiology of the cardiovascular system, especially, in the treatment of coronary artery disease, i.e., medical, intervention or surgical revascularization [3]. The other prominent role of IRI is indicated in cardiac surgery. Open heart surgery is associated with ischemia-reperfusion injury due to aortic cross-clamping (AoX) and intermittent cardioplegia administration. Aortic cross-clamping induces immediate global ischemia, therefore, subsequent perfusion of oxygenated-cardioplegic solution can cause IRI. Moreover, de-clamping at the final step of cardiac surgery also brings a rapid perfusion of oxygenated blood again, contributes to IRI [8, 9]. Ischemia-reperfusion occurs in 25%-45% of patients who underwent coronary artery bypass graft (CABG) with cardiopulmonary bypass

demonstrated endothelial cell dysfunction, damage myocardial cell, which is co-related to arrhythmias and myocardial infarction [10-12]. Therefore, myocardial preservation during cardiac surgery is a pivotal part of cardiac surgery, and prevention of IRI is also concern. One main strategy for myocardial protection is cardioplegic administration aiming to stop cardiac contraction, reduce oxygen demand and to provide necessary substrates to the heart. The improvement of cardioplegia recipe and method of administration has been aggressively studied in the past decades [13-15]. Studies have shown that endothelial cell dysfunction is linked to myocardial pathophysiology, highlighting the role of endothelial cells in myocardial protection during cardiac surgery [16-18].

Endothelial dysfunction during cardiac surgery was demonstrated by endothelial hyperpermeability, increased neutrophil and pro-inflammation cytokine migration as well as the impairment of coagulation cascade, resulting in microvascular obstruction [19, 20]. Moreover, it was demonstrated that decreased nitric oxide production was related to post-operative morbidity and mortality [10, 20, 21]. There are also supporting documents showing the reduced coronary blood flow and vasoconstriction during cardioplegic administration [18, 22].

The link between myocardial cells and endothelial cells is thought to be via nitric oxide (NO) [23, 24]. Endothelial NO release depends on the changes in membrane potential.[25, 26], which is regulated, at least partly, by ion channels and one of the ion channels that plays a role in NO synthesis is the ATP-sensitive potassium channel (K_{ATP}) [27, 28].

The ATP-dependent potassium channel (K_{ATP} channels) is an octameric complex of 4 pore-forming subunits of the KIR 6.0 family (Kir6.1, Kir6.2) and 4

sulfonylurea receptors (SUR1, SUR2A, SUR2B), linking cellular metabolism and electroactivity of the cells, especially in stress response. The composition and distribution of subunits vary between tissues. It was accepted that Kir6.2/SUR2A is cardiac sarcolemma type (sarcK_{ATP}) whereas Kir6.1 is a subunit of mitochondrial KATP channel (mitoK_{ATP}) [29, 30]. K_{ATP} channels found in endothelial/vascular smooth muscle cells are composed of Kir6.1 and SUR2B subunit. In normal conditions, the channels are predominantly close, and will be open in response to decreased ATP levels. In cardiomyocytes, the opening of K_{ATP} channels shortens the action potential duration, reduces calcium influx and decreases ROS production, resulting in reduced infarct size, reduced arrhythmia, and improved heart function [27, 31-33]. ATP-sensitive potassium channel openers (KCOs), pinacidil, and diazoxide, could reduce calcium overload in cardiomyocyte and mitochondrial matrix, leading to optimum ROS production and inhibition of MPTP opening in rat heart model [34-36]. Adding pinacidil (a non-selective K_{ATP} channel activator) to cardioplegia solution could increase vascular smooth muscle cell relaxation, but the effect on endothelial cells has not been evaluated [35, 37]. Moreover, Makepeace et al., (2018) and Suarez-Pierre et al., (2020) suggested that the addition of a K_{ATP} channel activator, diazoxide, in cardioplegic solution could improve cardiac functions in rat and swine model [38, 39].

In endothelial cells, K_{ATP} channels regulate resting membrane potential and are involved in NO synthesis [22, 24]. Opening of K_{ATP} channels causes hyperpolarization, leading to the activation of calcium channels, leading to Ca^{2+} influx [27, 28]. Increased intracellular Ca^{2+} enhances interaction of calcium and calmodulin (CaM). Calcium/Calmodulin binding activates endothelial nitric oxide synthase (eNOS), and eNOS catalyzes the conversion of L-arginine (L-arg) into L-citrulline and NO [28, 31,

32]. Li, Y., et al., (2020) found that endothelial-specific Kir6.1 knockout mice (eKO) exhibit high atherosclerotic plaque density more than that of their littermate controls when treated with a high-fat diet for 12 weeks [40]. Gao Shan (2019) reported that antihypertrophic effect of Kir6.1/SUR2B is via endothelin (ET)/NO production balance, by increasing the release of NO and inhibiting ET-1 system in pressure overload heart failure in rat model [41].

Hyperkalemic cardioplegic solution is widely used as a standard myocardial protection during open heart surgery. It induces depolarized- cardiac arrest and supplies some substrates to the heart [42, 43]. However, there are growing evidence suggesting that hyperkalemic solution might have detrimental effects to the cells, participating in membrane potential alterations and ionic imbalance, especially, intracellular sodium levels and calcium overload [42]. The effects of hyperkalemic cardioplegia were demonstrated in in-vitro, in-vivo and clinical study. It was found that hyperkalemic cardioplegia was associated with cell apoptosis and dysfunction [44]. In vitro, Yeh, C. H. (2010) reported that cardioplegia amplified hypoxia/reoxygenated injury and led to cardiomyocyte apoptosis [45]. There are evidence supporting that cardioplegia stimulates apoptosis signaling pathway in myocardial endothelium and cardiomyocytes, and induces mitochondrial permeability transition pore opening in animal model [46, 47]. Yeh, C. H. (2003) found that perfusion of hyperkalemic cardioplegia to dog's hearts during on cardiopulmonary bypass (CPB) jeopardized endothelial cells, associating with the activation of apoptosis signaling pathway [48]. Clinical studies reported that cardioplegia led to myocardial injury, the increased cardiac enzyme in patients undergoing open-heart surgery [49-51]. There are evidences showing that endothelial NO affected to coronary blood flow during cardiac surgery

[18]. Nakanishi, K. (1994) showed that endothelial cell dysfunction from cardioplegia induced IRI lowered NO release and impaired vasorelaxation [52]. This data suggests the possible roles of endothelial K_{ATP} channels, especially Kir6.1 containing channels, in myocardial protection.

Taken together, it remains unclear that endothelial K_{ATP} channel activators, including Kir6.1/SUR2B modulator, can preserve endothelial cells in IRI. Moreover, the effects of hyperkalemic cardioplegia and KCOs on cell viability and NO production in response to IR has not been investigated. Therefore, the objectives of this study are to evaluate the effects of endothelial KCOs on endothelial cell viability and NO production in response to IR in normokalemic conditions, as well as in hyperkalemic conditions.

Objectives

1. To study the effects of selective Kir6.1/SUR2B ATP potassium channel modulators on endothelial cell viability and NO production in response to ischemia-reperfusion.

2. To study the effects of hyperkalemic cardioplegia concomitant with selective Kir6.1/SUR2B ATP potassium channel modulators on endothelial cell viability and NO production in response to ischemia-reperfusion

3. To study the effects of ATP-potassium channel modulators on endothelial cell viability and NO production in response to ischemia-reperfusion in normokalemia and in presence of cardioplegia

Hypothesis

1. Kir6.1/SUR2B openers provides cardioprotective effect on endothelial cell in terms of cell viability and NO production in response to ischemia reperfusion.

2. Kir6.1/SUR2B openers concomitant with hyperkalemic cardioplegia provides cardioprotective effect on endothelial cell in terms of cell viability and NO production in response to ischemia reperfusion.

3. ATP-potassium channel modulators provides cardioprotective effect on endothelial cell in terms of cell viability and NO production in response to ischemia-reperfusion in normokalemia and in presence of cardioplegia

Scope of research

This study is an experimental study to assess the effects of K_{ATP} channel openers on endothelial cells, using endothelial cell line, EA.hy926 cells. To investigate the beneficial effects of K_{ATP} channel modulators, diazoxide, pinacidil, nicorandil, ZD0947 and glibenclamide were used in the presence or absence of high potassium cardioplegia solution.

Definitions

-K_{ATP} channel modulators refers to ATP-dependent potassium channel openers or blockers, which are ZD0947, pinacidil, diazoxide, nicorandil and glibenclamide.

-Simulated ischemia refers to ischemia produced by metabolic stress solution by replacing of D- glucose with deoxy-D-glucose.

CHAPTER II

REVIEW LITERATURE

Cell death

Cellular homeostasis disruption leads to the depletion of ATP, altered membrane permeability, destruction of biochemical pathways, and DNA damage [53]. The response to injury (i.e., ischemia) of the cells depends on the types, the nature, severity, and duration of the injury. The process of cell injury begins with reversible injury. Reversible injury is characterized by swelling of the cell and its organelles. Irreversible injury is characterized by increased cell swelling, severe organelles and mitochondria disruption, and nuclear changes. Laminated structures or myelin figures are derived from injured cell membranes and appear during reversible injury, and become more pronounced in irreversibly injured cells. a process follows injury are designed to enhance cell survival or remove irreparably injured cell or cell death [53-55].The mechanism of cell injury is summarized in Figure II-1.

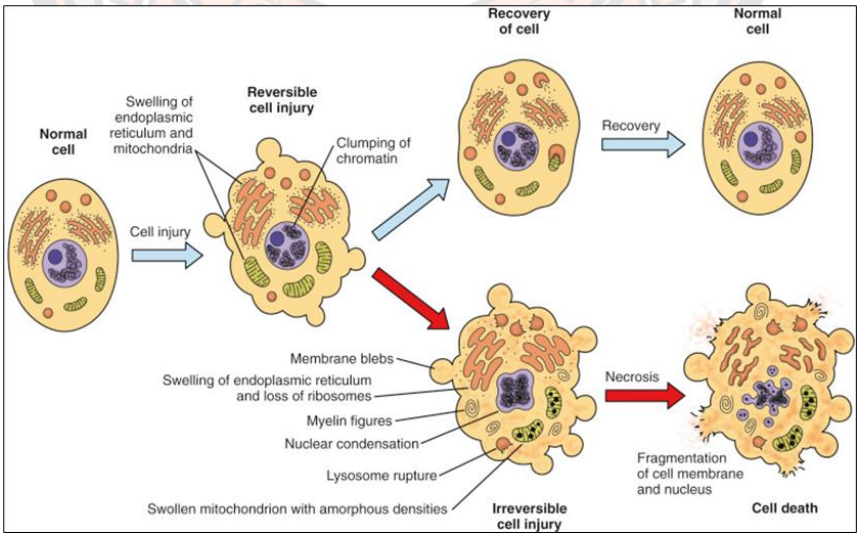


Figure 0-1 The process of cell injury.

(From Miller, M. A. and Zachary, J. F.,2017 [53]).

In 2005, the Nomenclature Committee on Cell Death (NCCD) guideline proposed that cell death can be classified into 4 types, according to its morphological appearance (apoptotic, necrotic, autophagic or associated with mitosis), enzymological criteria (caspases, calpains, cathepsins, and transglutaminases), functional aspects (programmed or accidental, physiological or pathological death) and immunological characteristics (immunogenic or non-immunogenic) [56]. Subsequently, the NCCD has formulated guidelines for the definition and interpretation of cell death from macroscopic morphological alterations, biochemical, and functional perspectives along with the mechanism of cell death [57]. The classification of cell death is described as follows.

1) Type I cell death or apoptosis

The cells are presented with cytoplasmic shrinkage, chromatin condensation, nuclear fragmentation, plasma membrane blebbing, and culminating with the formation of apparently intact small vesicles (apoptosis body) which subsequently triggers phagocytic activity and are finally degraded within lysosomes [57].

2) Type II cell death or autophagy

Autophagy, a self-eating program, is triggered by some types of stress. It is initiated with the activation of adenosine monophosphate-activated protein kinase (AMPK), which subsequently activates Unc-51 like autophagy activating kinase 1 (ULK1) complex and inhibits the Target of Rapamycin protein kinase (mTOR), allowing UKL1 activation. The nucleation of the cells is affected by the activation of P13K, a result of UKL1 activation, leading to cleation and maturation of the autophagosome for the fusion with the lysosome. The cellular components are then degraded and recycled as macromolecule components. [57, 58].

1) Type III cell death or necrosis

Necrosis cell usually displays increased cell volume or oncosis, cell and organelles swelling, plasma membrane rupture and leading to subsequent loss of intracellular contents. There are some evidences suggesting that the necrotic cell death may be regulated by a set of signal transduction pathways and catabolic mechanisms [56].

The summary of cell death classification was shown in Figure II-2.

Ischemia

Ischemia is a condition whereas the blood supply to the tissue or organ is limited. Limited blood supply to tissue results in decreased delivery of oxygen (hypoxia), reduced glucose and other nutrients to the cell leading to decreased intracellular ATP levels. Lack of ATP results in decreased ability of metabolic wastes removal, elevated intracellular hydrogen ion (H^+) and decreased intracellular pH. To eliminate H^+, the Na^+/H^+ exchanger is activated resulting in increased intracellular Na^+ concentration. The reduced activity of Na^+/K^+-ATPase due to acidic environment and insufficient ATP also promotes intracellular Na^+ accumulation, stimulating sarcolemmal Na^+/Ca^{2+} exchangers, resulting in an influx of intracellular calcium. An intracellular calcium overload can lead to cell apoptosis, autophagy or necrosis [59, 60]. The summary of cell swelling from hypoxia is described in Figure II-3.

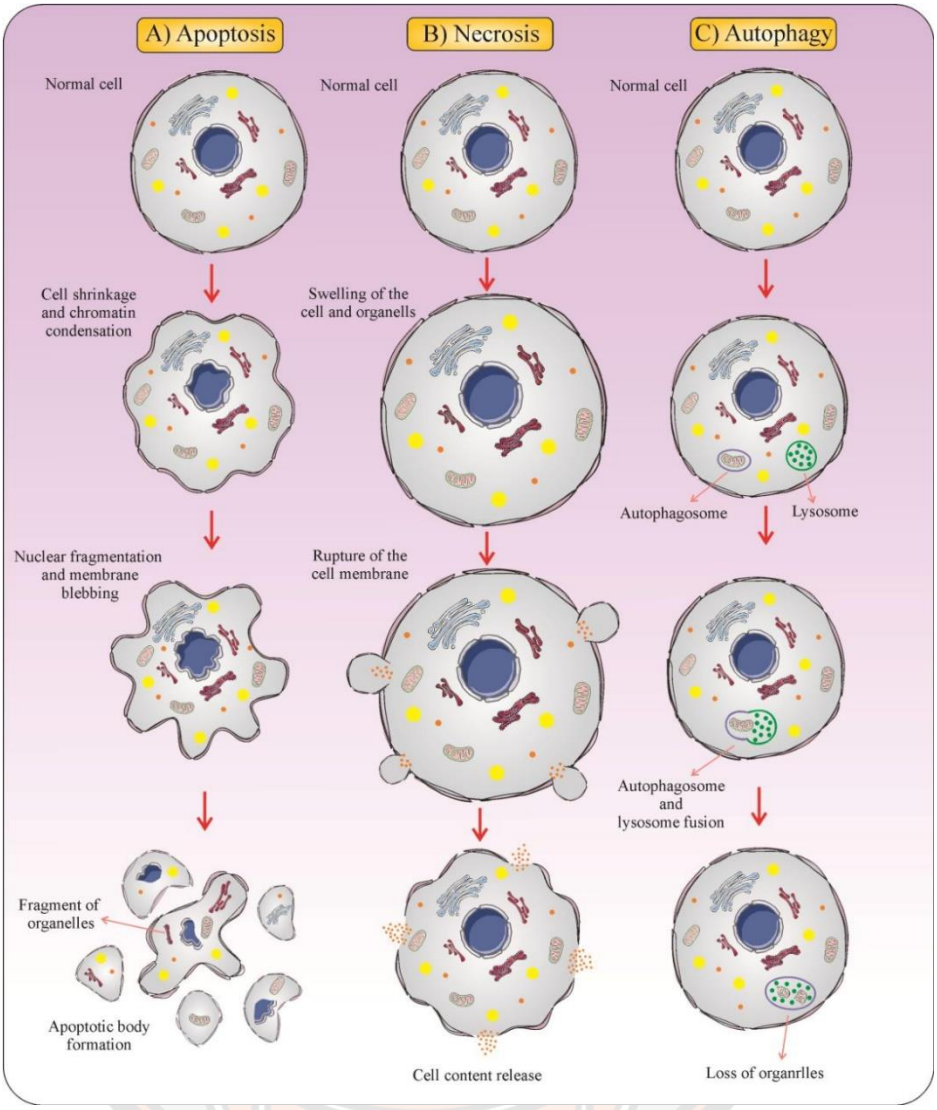


Figure 0-2 The classification of cell death

The classification of cell death, A) Apoptosis, B) Necrosis and C) Autophagy. (From Asadzadeh Z., et al. w,(2020) [61].)

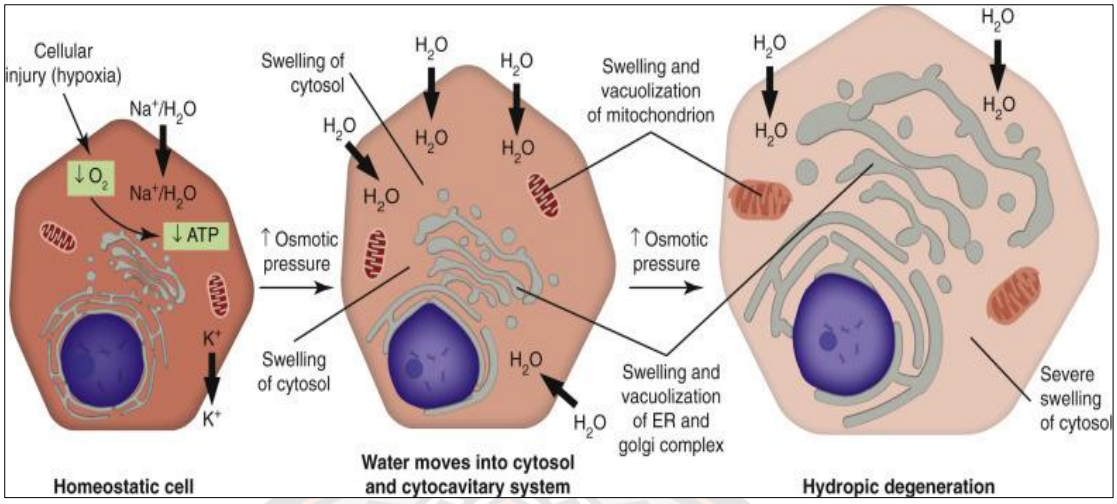


Figure 0-3 The summary of cell swelling from hypoxia.

K^+; potassium ions, Na;$^+$; sodium ions, O_2; oxygen, H_2O;chemical formula of water. (From Miller, M. A. and Zachary, J. F.,(2017) [53].)

Ischemia-reperfusion injury in cardiovascular system.

Ischemia-Reperfusion injury (I/R injury) is a paradoxical exacerbation of cellular dysfunction and death, resulting from restoration of blood flow to ischemic cell [1]. The aims of the treatment of myocardial infarction are to reduce myocardial injury and limit infarct size. Restoration of blood flow, by medications, intervention or surgery, is a crucial step to preserve myocardial cells. However, reperfusion can be harmful to cardiomyocytes, known as I/R injury. Therefore, prevention of IRI during reperfusion is of interest [2].

Following reperfusion, re-oxygenated blood stimulates respiratory activity, mitochondrial oxidative phosphorylation, leading to restoration of ATP synthesis and intracellular pH. Restoration of pH activates ryanodine receptor (RyR) on sarcoplasmic, Ca^{2+} dependent protease, calpain and increased intracellular Ca^{2+}level [59, 62]. Increasing of intracellular Ca^{2+} resulting in myofibrillar hypercontraction and opening

of mitochondrial permeability transition pore (mPTP). Calcium overload resulting from I/R injury also leads to increased calcium uptake by mitochondria, increased mitochondrial dehydrogenase, resulting in decreased ATP production and increased ROS [5]. Irreversible mPTP opening results in mitochondrial matrix swelling and outer membrane damage, bringing about the releasing of cytochrome-c into cytosol. Intracellular cytochrome-c activates the caspase cascade, which is a common cause of the programmed cell death. The opening of mPTP also leads to generation of reactive oxygen species (ROS). The ROS from mitochondria and from neutrophils further triggers intracellular Ca^{2+} and increased mPTP opening, contributing to cell death [59, 63]. Florea S and Blatter L. (2010) demonstrated that calcium overload can lead mitochondria dysfunction and arrhythmia [6]. Ruiz-Meana M. (2009) reported that calcium overload is associated with mPTP opening and cell death [7].

Mitochondria permeability transition pore (mPTP) opening plays a major role in cell death during I/R injury. The mPTP can be activated by altered calcium homeostasis and ROS [64]. It is primarily triggered by cyclophilin D (CypD). An osmotic alteration and mitochondrial swelling are also the causes of mitochondria dysfunction. Resulting of MPTP opening and mitochondrial injury are apoptosis substrates releasing, including cytochrome c (cyt-c), which is leads to cell death [65]. Ruiz-Meana M. (2007) showed that mPTP opening could induce ATP-dependent hypercontraction during calcium overload in cardiomyocyte [66].

Reactive oxygen species (ROS), including the superoxide anion, hydroxyl radical, and hydrogen peroxide, are key factors in I/R injury. Changes in respiratory rate or mitochondrial inner membrane potential can be a cause of damaged respiratory chain and lead to increase ROS production. The increased ROS reduces mitochondria

redox potential. ROS is associated with mitochondrial DNA (mtDNA) damage [67]. Increased ROS induces mitochondrial-damage associated with molecular patterns (mt-DAMPs) and vein graft failure in patient undergoing CABG [68]. The mechanism of IRI is shown in Figure II-4.

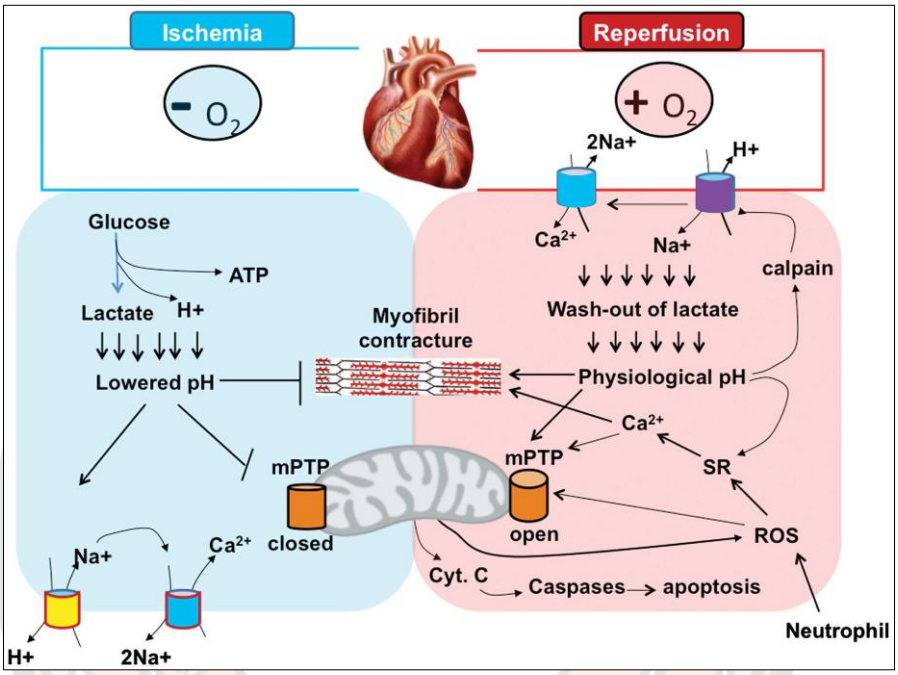


Figure 0-4 The mechanism of Ischemia-reperfusion injury.

Na^+; sodium ions, O_2; oxygen, ATP; Adenosine triphosphate, Ca^{2+}; calcium ions, H^+; hydrogen ions, SR; sarcoplasmic reticulum, ROS; reactive oxygen species, Cyt.C; cytochrome C, (From Li, X., et al., 2016 [59]).

Revascularization to resuscitate the ischemic myocardium, including thrombolysis, angioplasty and cardiac surgery (especially, coronary artery bypass graft surgery (CABG) surgery), are involved in I/R injury. It is associated with arrhythmias, cardiac stunning, microvascular obstruction, endothelial cell dysfunction and cell death [3]. The effects of IRI is depicted in Figure II-5.

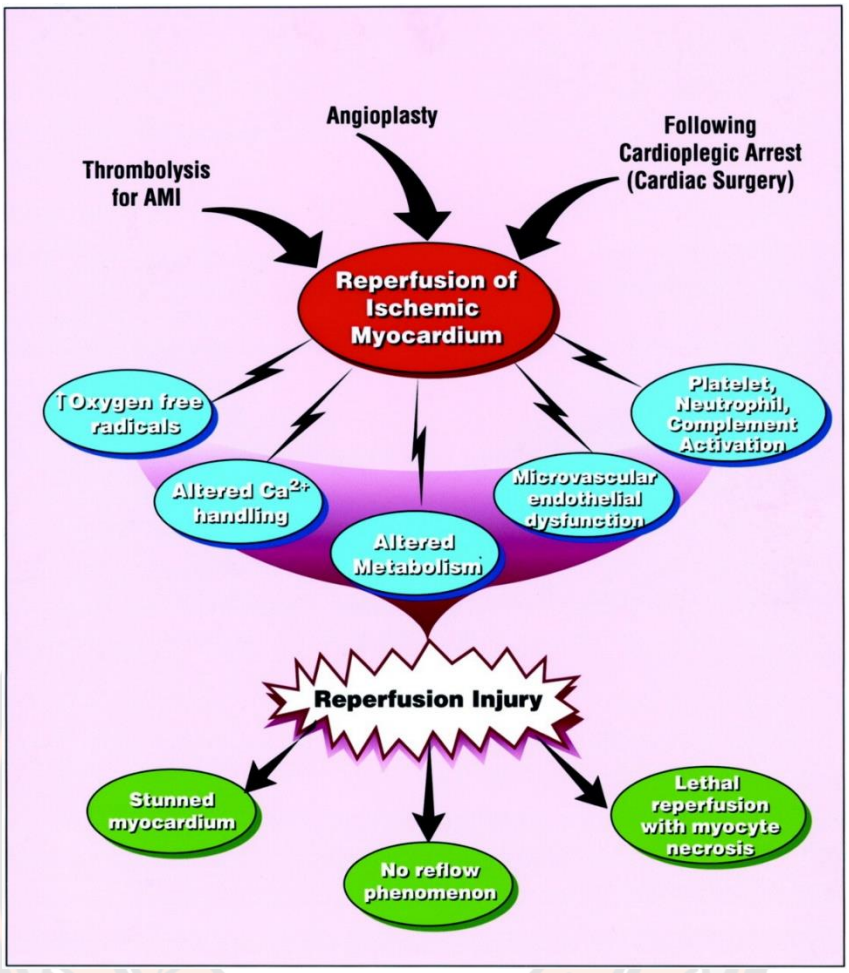


Figure 0-5 The effects of revascularization on myocardial cells.

. (From Verma, S., et al., 2002 [3]).

Ischemia-reperfusion injury during cardiac surgery

There are 2 types of cardiac surgery 1) closed-heart surgery (beating heart surgery) and 2) open-heart surgery. Close-heart surgery is a surgical technique that necessary to access the heart through sternotomy or thoracotomy without arresting the heart or cardiopulmonary bypass machine. Beating heart operation usually deals with extracardiac defect surgeries including ductus arteriosus closure, Blalock-Taussig (BT) shunt or off pump-coronary artery bypass graft (OPCAB) [69]. Open-heart surgeries

operate in intracardiac defects, which requires to open cardiac chamber [69]. Open-heart surgery is facilitated by using cardiopulmonary bypass (CPB) with arresting heart. Open-heart operations consist of intracardiac and cardiothoracic aorta surgery, including cardiac valve repair or replacement, great artery and on-pump coronary artery bypass graft [69, 70]. Procedures during cardiac surgery entail general anesthesia, CPB circuit preparation, anticoagulation management, median sternotomy, conduction of CPB, management of cardioprotection and organ perfusion, and weaning and termination of CPB [71]. The common procedures in open heart surgery were described below.

1) Median sternotomy and cannulation

The median sternotomy is performed after general anesthesia, using 4 main components; hypnosis, analgesia, amnesia and muscle relaxation, through intravenous and inhalation [72], before pericardiotomy. After heparin injection, umbilical tapes and snares are placed around the superior vena cava (SVC) and inferior vena cava (IVC). The ascending aorta, SVC and IVC are cannulated for cardiopulmonary bypass.

2) Cardiopulmonary bypass

Components of CPB include roller or centrifugal pumps, venous reservoir, extracorporeal membrane oxygenator, cannula, tubing, heat exchanger and cardioplegic circuit [73], The circuit is ready to use after priming and thorough de-airing [71, 73].The preparation of the CPB circuit was shown in Figure II-6.

Surgical steps are start with skin incision and median sternotomy to expose the heart. Intravenous heparin 3 mg/kg is perfused and arterial-venous cannula, aortic root vent and cardioplegia canula were subsequently placed before the initiation of cardiopulmonary bypass. Blood circulation throughout the body was then controlled by

the CPB. An optimal blood flow rate is about 2.2–2.4 L/m^2/min to maintain mean arterial blood pressure at 50-70 mmHg [73, 74].

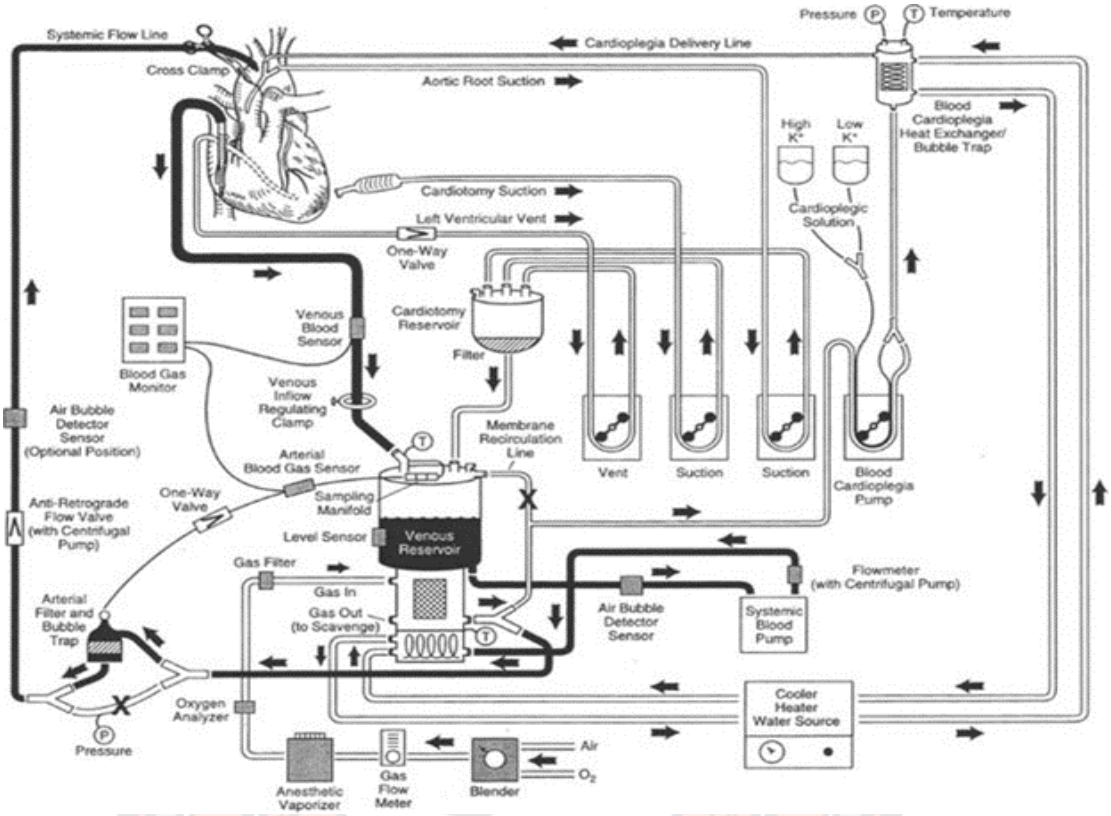


Figure 0-6 Cardiopulmonary bypass circuit during open heart surgery. (From Murphy, G. S., et al., 2009 [75]).

2) Aortic cross-clamping and cardioplegia administration

Cardioplegia cannula is cannulated to root of aorta. Aortic cross-clamping is performed by clamping at the ascending aorta and is followed by perfusion of cardioplegia to induce cardiac arrest with fully supported by using CPB [76].Aortic cross-clamping at the ascending aorta is necessary to stop the heart. At this process aortic cross clamping which renders the ischemic heart, so cardioplegic solution has been perfused to induce temporarily diastolic arrest and preserve the heart [76].

3) Correction of cardiac structure

There are common open heart surgery types as follows.

3.1) Coronary artery bypass grafting (CABG): CABG is a surgical operation to treat coronary artery patients. Venous and arterial conduits are harvested to make a bypass across blocked vessels [76]. The bypass graft restores blood and oxygen supply to ischemic myocardium. There are 2 different types of CABG. 1) On-pump CABG, which needs heart-lung machine to maintain the circulation during cardiac arrest. 2) Off-pump (OPCAB), which is a surgery that does not require cardiopulmonary bypass [77, 78].

3.2) Heart valve repair or replacement: valve surgery is the treatment that deals with stenosis and insufficiency heart valve pathologies. The diseases are commonly found on mitral and aortic valves [77]. The pathologic valve is usually replaced by mechanical bioprosthetic valve [78]. Valve repairment has been developed for the treatment of degenerative valve, valve annulus dilatation or chordae tendineae rupture, by using suture technique with or without annuloplasty ring [79, 80]

3.3) Aorta surgery: aorta surgery is a treatment to repair aortic aneurysms or dissection. Abnormalities of the aorta can involve all part of aorta, including aortic root, ascending aorta, aortic arch, descending aorta and abdominal aorta [81]. There are many types of aorta surgery techniques according to position of pathology, for example, Bentall's operation is suitable for ascending aortic aneurysm that be involved with aortic valve. Operation technique is replacing of aortic valve and ascending aorta with prosthetic valve and aortic graft, together with re-implantation of the coronary arteries [82].

3.4) Heart transplantation: cardiac transplantation is a therapeutic technique for end-stage heart failure patient, to transplant donor heart to the reciepient [83]. The Surgery starts with transecting a heart structure including the aorta, the main pulmonary artery and the superior and inferior vena cava, and dividing the left atrium, but the posterior wall of the left atrium and the pulmonary vein are placed. The donor heart are sewed with the native vena cava, aorta pulmonary artery and left atrium [84]. The summary of heart transplantation is shown in Figure II-7.

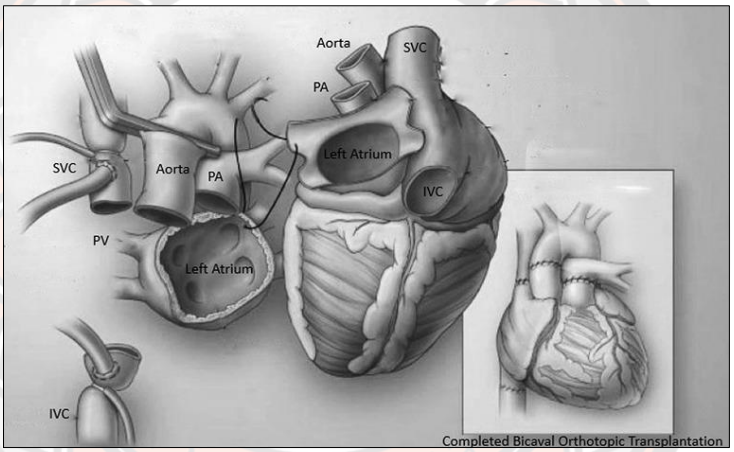


Figure 0-7 Heart transplantation.

PA; pulmonary artery, PV; pulmonary vein, SVC; superior vena cava and IVC; inferior vena caca. (From Cheng, A. and Slaughter, M. S.,2014 [84]).

4) Management of CPB

During correction of cardiac structure, the control of cardiopulmonary bypass (CPB) is necessary to achieve optimal organ perfusion [75]. The management of physiological variables during CPB is included 1) mean arterial blood pressure (MAP) around 50-70mmHg, 2) blood flow rates at least 2.2 L/m^2/min, 3) level of hematocrit and hemoglobin around 21-25% or 8g/dL, respectively, 4) systemic oxygen delivery

(DO_2) 350 – 450 mL/ min/ m^2, 5) temperature, which is according to surgical procedure, and 6) arterial blood gas-electrolyte management [73, 75].

5) Weaning and termination of CPB

Weaning from cardiopulmonary bypass (CPB) is a step that transition of blood circulation and respiration from CPB circulation to spontaneous heart circulation [85]. Weaning of CPB are requires several steps including rewarming, de-airing in cardiac chambers, de-clamping of aorta, management of patient's physiology and termination of CPB [73]. Rewarming is desired in hypothermic patient, by increase temperature to normothermia (core temperature > 36℃ , but not be exceeded than 38℃) [85]. The arterial blood gas-electrolytes should be within normal ranges, hematocrit (> 25%) or hemoglobin (> 7 g/dl) before de-clamping of aorta [73, 85]. After aortic de-clamping, hemodynamics should be return to normal range or similar to patient's baseline parameter, including spontaneous beating or sinus rhythm, blood pressure and cardiac contractility [86]. The inotropic and ventilation support are required during weaning off and separation of CPB, to support hemodynamic and cardiac output [87]. Weaning off CPB consist of gradually reducing venous return and blood flow support from CPB from 100% to 0%, and clamping the venous return line. The hemodynamic and vital sign parameters after terminate CPB should be in acceptable level with spontaneous beating. After successful termination of CPB, Aortic cannula, venous cannular, venting cannular and cardioplegia cannula are removed [88].

6) Closure of the chest

After termination of CPB, protamine is administered in a ratio of 1:1 to 1:3 through intravenous injection to achieve the protamine time at 10–15 min [73]. At the end of the operation, chest cavity and sternum closure are needed with traditional stainless steel wires or rigid sternal fixation and skin suturing is performed [89].

Cardioplegia

Cardioplegia is an essential pharmacological substance that is administered during cardiac surgery to stop the heart. Practically, the main component of cardioplegia is high potassium solution. The first hyperkalemic cardioplegia solution was created by Dr. Melrose in 1955, who identified that high levels of potassium citrate induced a reversible cardiac arrest [90]. Currently, there are some types of cardioplegia solution which contain different potassium levels, i.e., St. Thomas Custodial solution and Del Nido solution [76, 91]. The administration of cardioplegia is also varied, i.e., it can be delivered via coronary artery (antegrade) or coronary sinus (retrograde), as warm or cold solution, single or multiple doses.

Types and component of cardioplegia

There are 3 categories of cardioplegia solution including hyperkalemic cardioplegia (or St. thomas), Histidine-tryptophan-ketoglutarate (HTK or custodial) and Del Nido solution [76, 91]. High potassium cardioplegia or hyperkalemic cardioplegia solution is commonly used over the past decade, the solution includes potassium chloride at 15-35 mEq/L and other electrolytes such as Mg^{2+}, Ca^{2+}, Cl^{-}. Na^{+} [92]. Histidine-tryptophan-ketoglutarate (HTK, Custodiol) and Del Nido cardioplegia

are normally used as a single dose, which provides a long period of myocardial protection during cardiac surgery [93, 94]. Del Nido and HTK solution are suitable for long period-complex operation, minimally invasive and redo-cardiac operations[95], they offer at least 90 minutes and 120 minutes of preservation in single dose, respectively, which is resulting in avoid interruption of the surgical procedure [96]. Custodiol solution contains 9 mmol/l potassium chloride with Mg^{2+}, Ca^{2+}, Na^{+}, histidine, tryptophan, Ketoglutarate and mannitol [93]. Del Nido is 1:4 blood and crystalloid mixed formula, which can preserve myocardial by reducing calcium ion influx during and after ischemic arrest [94]. Del Nido is composed of potassium chloride 26 mEq/L, $Mg^{2+,}$ lidocaine, mannitol and sodium bicarbonate including in 1 L of Del Nido solution [94, 95].

The benefits and disadvantages of different cardioplegic types depend on the procedure, ischemic time, and patients age [97]. In pediatric patients, application of moderate potassium concentration cold blood cardioplegia (10mmol/L K^{+}) results in a better clinical outcome than that of high potassium concentration (20mmol/L K^{+}). Nowadays, some studies indicated that the moderate potassium concentration (10 mmol/L) cold blood cardioplegia is associated with a better myocardial protection when compared with conventional high potassium cardioplegia in pediatric patients [98].

Although custodial and Del Nido show effective cardioprotection in CABG and CABG with valve surgery, but it is still an issue [94]. Some studies reported that there was higher incidence of ventricular arrhythmias, especially ventricular fibrillation, during reperfusion in custodial [93]. It was found that Del Nido demonstrated inferior anti-inflammatory than high potassium cardioplegia [99]. In multi-vessel with left ventricular hypertrophy CABG patients, a study showed that single dose of Del Nido

cardioplegia may not be sufficient for myocardial protection resulting in post-operative myocardial dysfunction [95]. However, there is no difference in term of mortality rate [93, 100].

Some studies demonstrated that administration of some protective substances such as magnesium, monosodium phosphate and adenosine-lidocaine-magnesium (ALM) may benefit to myocardium [101-103]. Previous study reported that addition of magnesium to hyperkalemic cardioplegia could prevent increased intracellular lactate concentration [103]. A clinical study showed that Alpha-ketoglutarate (alpha-KG)-added cardioplegia could reduce cardiac enzyme and improves myocardial protection in patients underwent CABG surgery [104]. Diazoxide-added cardioplegia was also reported to prevent mitochondrial swelling, decrease N-terminal prohormone brain natriuretic peptide levels and improve myocardial protection during cardiac surgery [105]. Recently, the effects of diazoxide-added cardioplegia has been investigated in clinical trial phase 1. The summary of cardioplegic types is shown in Table II-1.

Table 0-1 The components of cardioplegia solutions.

Component (1L)	Crystalloid Hyperkalemic cardioplegia	Del Nido cardioplegia	HTK cardioplegia
K^+ (mM)	>15	26	9
Na^+ (mM)	110-147	142–144	15
Cl^- (mM)	153-160,203	13	50
Mg^{2+} (mM)	16-32	15	4
Ca^{2+} (mM)	2-2.4	0.22–0.27	0.015
HCO_3^- (mM)	10	-	-
Ketogluconate (mM)	-	-	1
Histidine (mM)	-	-	198
Tryptophan (mM)	-	-	2
Mannitol (mM)		18.13	30
Lidocain (g/L)	0.1-0.26	0.13	-

Modified from Sanetra, K., et al, (2022), Pizano, A., et al.,(2018), Bruyn, H., et al., (2014), Chocron, S., et al.,(2003) and Çayır, M. and A. Yüksel, (2019) [106-110].

Cardioplegia administration

There are 2 types of cardioplegia administration including antegrade and retrograde perfusion. Antegrade coronary perfusion techniques can be divided into 2 types, via root of aorta and direct coronary ostial. Retrograde coronary perfusion technique is an alternative perfusion, which perfuse via coronary sinus [111]. Cardioplegia can also be divided according to dose perfusion; 1) intermittent cardioplegia, 2) continuous cardioplegia, and 3) single dose cardioplegia. The

intermittent cardioplegia requires multi-doses giving of cardioplegic solution. While continuous cardioplegia allows to cardioplegic fluid to be delivered persistently [112]. A single dose cardioplegia could protects the myocardium for more than 90 minutes and 2 hours for Del Nido and histidine-tryptophan-ketoglutarate (HTK) solution, respectively [113, 114]. There are 2 temperature selected strategies for cardioplegia administration 1) warm cardioplegia (28°C to 37°C) and 2) cold cardioplegia (4°C to 15°C) [14]. The summary of cardioplegia administration is shown in Figure II-8.

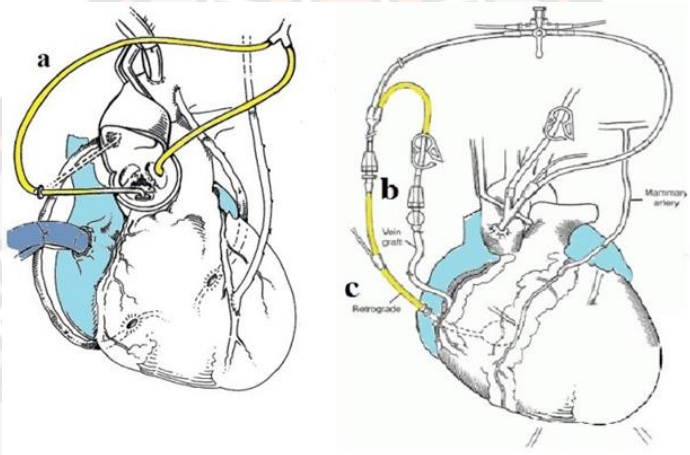


Figure 0-8 Cardioplegia administration.

(From Ismail, A. and Semien, G.,2020 [111]).

Effect of high potassium on cardiac cells

Potassium (K^+) ion plays a role in maintaining the resting membrane potential. Extracellular and intracellular potassium concentration are around 4 and 140 mmol/l, respectively. The potassium equilibrium potential (E_K), determined by the Nernst equation, is around -95 mV in resting cells. Potassium permeability and potassium equilibrium potential (EK) affects the resting membrane, especially in phase 4, which is participated by the inward rectifier potassium channels. [115]. In normal condition, current of outward K^+ through K^+ channels are restored by the Na^+-K^+ ATPase, which

exchange $3Na^+$ with $2K^+$ ions. Then, Na^+ concentration is recovered by influx Na^+ through Na^+ channels, and via Na^+-Ca^{2+} exchange (NCX) during diastolic phase. To balance Ca^{2+} in cell, Ca^{2+} entey to the cell via Ca^{2+} channels. Increasing intracellular Ca^{2+} activate calcium released from sarcoplasmic reticulum (SR). Intracellular calciu are balanced by re-uptake Ca^{2+} into SR via sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and release through ryanodine receptors (RyR). Calcium ions in cytoplasmic activate Ca^{2+}-calmodulin kinase (CaMK) signaling, which is a pathway that regulates ion channels [116, 117] as described in Figure II-9.

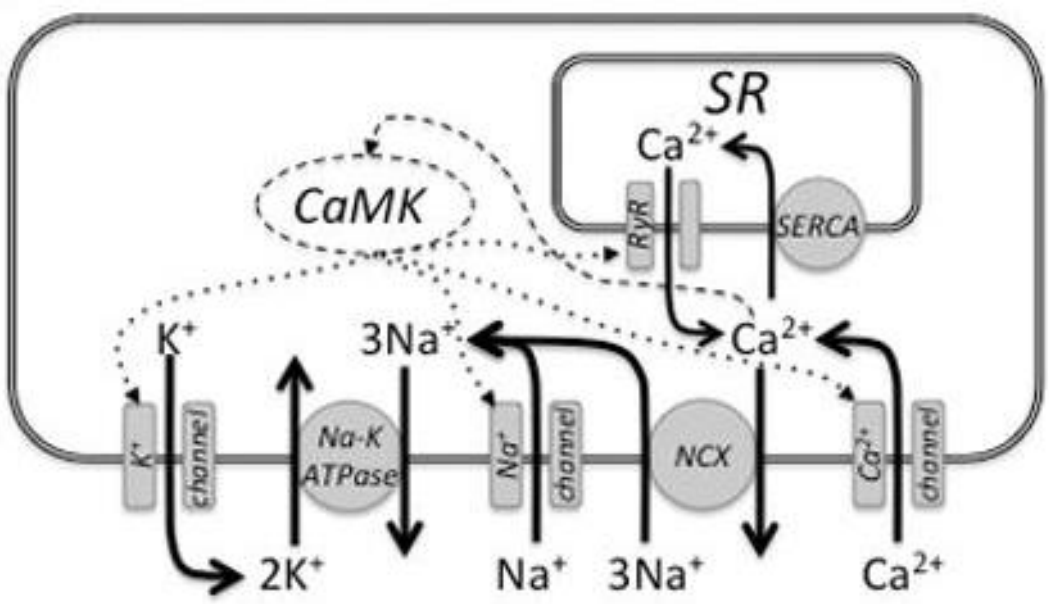


Figure 0-9 Interconnection of potassium, sodium and calcium balances in the cardiac myocyte.

Na^+; sodium ions, ATP; Adenosine triphosphate, Ca^{2+}; calcium ions, NCX; sodium-calcium exchanger, SR; sarcoplasmic reticulum, RyR; Ryanodine receptor, SERCA; sacoendoplasmic reticulum calcium ATPase (From Weiss, J. N., et al.,2017 [117]).

Hyperkalemia can be classified according to the serum potassium (K^+) level, that is: 1) mild hyperkalemia (K^+ = 5.5–6.5 mEq/l), 2) moderate (K^+ = 6.5–7.5 mEq/l) and 3) severe (K^+>7.5 mEq/l) [118]. The changing of potassium level provides fluctuation of membrane potential, according to the Nernst equation [117, 118].

$$Vm = -61.5\log[K^+]_i/[K^+]_o$$

There are documents reporting that severe hyperkalemia, from 15 mmol, can depolarize membrane potential from -80mv to -50mV, leading to inactivation of sodium channels stopping phase 0 action potential and therefore diastolic arrest [118, 119]. While, hyperkalemia (at least 15 mmol of potassium) elevates membrane potential to around -50 mV. Sodium fast channels are occurred, and sodium ions (Na^+) entry to intracellular. Sodium-calcium exchanger (Na^+-Ca^+ exchanger) expel $3Na^+$ and get $1Ca^{2+}$ into myocardial cell, which is leads to increase intracellular Ca^{2+} level [119]. A study found that membrane potential activity is associated with potassium concentration, for example Gelband H., et al.,(1972) showed that 10 mM increase resting membrane potential and 20 mM of potassium could inhibit action potential activity [120]. The summary of effect of hyperkalemia on cardiomyocyte is shown in Figure II-10.

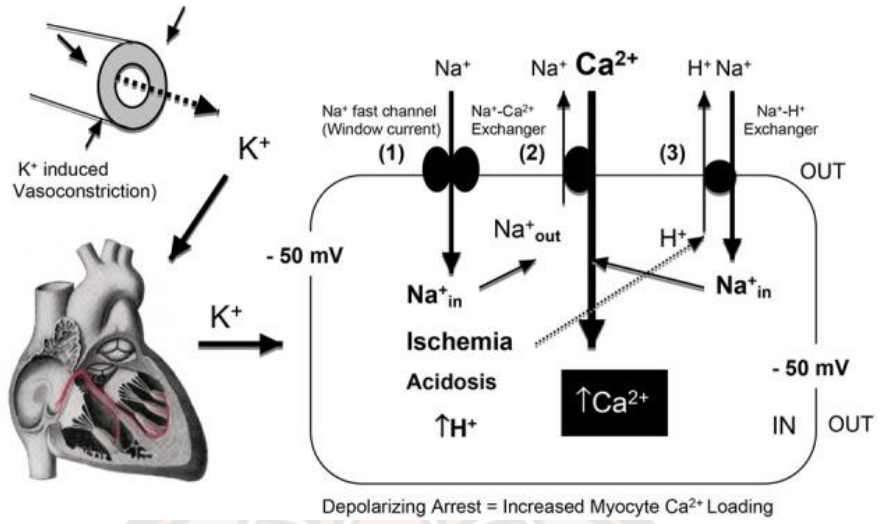


Figure 0-10 Effects of hyperkalemia on cardiac myocytes.

Na^+; sodium ions, Ca^{2+}; calcium ions, H^+; hydrogen ions (Figure from Dobson, G. P., et al, 2013 [119])

Myocardial protection

The aims of myocardial protection are to minimize infarct size and protect cardiomyocytes against IRI during cardiac surgery. Cardioprotection can be performed by using pharmacological and non-pharmacological strategies as described below [121].

Non-pharmacological cardioprotection

1. Ischemic preconditioning (IP)

Ischemic conditioning refers to application of sublethal ischemia before (ischemic preconditioning), after (ischemic postconditioning) long-deleterious ischemia which is believed to protect the target organ from subsequent lethal ischemia or reperfusion injury [122]. It includes remote ischemic preconditioning (RIPC), which

is an intrinsic process in preconditioning ischemia from a remote organ to protects the target organ against ischemia and I/R injury [123].

The effect of ischemic preconditioning (IP) is divided into 2 phases, early IP and late IP. Ealy IP occurs within minutes and disappears within 2-4 hours after the application, whereas late IP, second window effect, affects within 12-24 hours after ischemic preconditioning and can last for 3 days [124, 125].

Previous studies reported that preconditioning could protect against ischemia and IRI [126]. A study in dog's heart using 4 cycles of 5 minutes coronary occlusion/5 minutes reperfusion before a sustain 40 minutes coronary occlusion showed that IP could reduce infarct size from 29.4% to 7.3% of area at risk comparing with ischemia group [127]. A study demonstrated that IP, using hypoventilation/reoxygenation, induced decrease of ventricular arrhythmia threshold (VAT) but was effective only after three cycles of IP [128]. An experimental study in rabbit hearts from Mandel I. et al reported that IP could reduce damage of myocardium in comparison with non-conditioned area [129].

The mechanism of IP is thought to be involved in activation reperfusion injury salvage kinase (RISK) signaling pathway. The stimulation brings about Phosphoinositide 3-Kinase (PI3K) activation. The downstream of PI3 kinase are phosphorylation of a serine/threonine-protein kinase (Akt). The phosphorylated Akt subsequently activates endothelial nitric oxide synthase (eNOS) and protein kinase A. The activation results in activated PKC. On the other hand, RISK (reperfusion injury salvage kinase) signaling pathway also activates MEK1/2 and ERK1/2 and protein kinase C (PKC). PKC phosphorylation leads to activation of mitochondrial ATP-

dependent potassium channel, resulting in inhibition of mPTP opening which can reduce ROS production [125].

Ischemic postconditioning (IPo) activates two signaling pathways, RISK and Survivor Activating Factor Enhancement (SAFE) in response to reperfusion injury. SAFE signaling pathway activates tumor necrosis factor alpha (TNF α). TNF α binding with TNF α receptor. Then JAK-STAT3 are activated and resulted in transcription increased and inhibit mPTP opening [125]. The summary of the mechanism of ischemic conditioning is shown in Figure II-11.

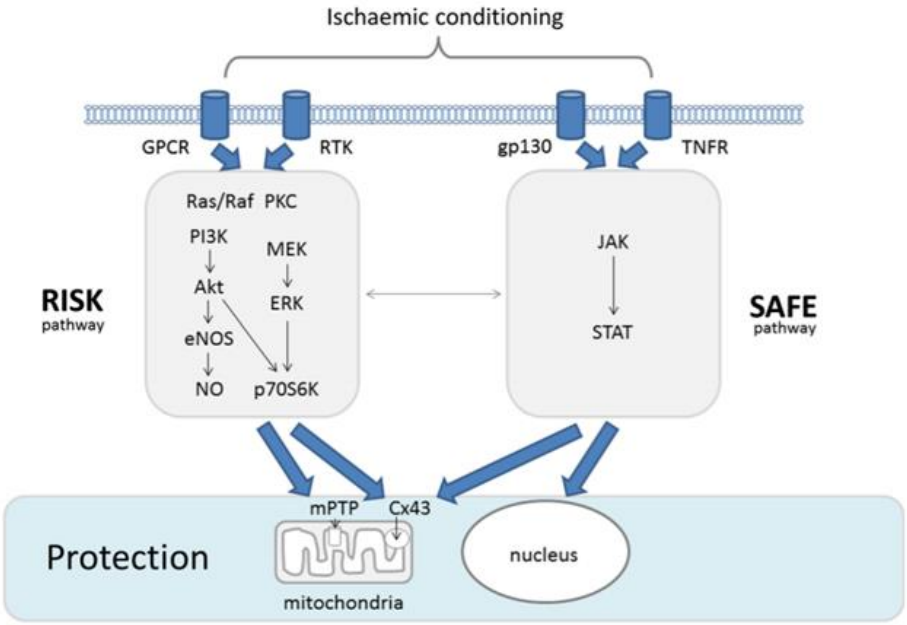


Figure 0-11 Mechanism of ischemia conditioning.

RISK; reperfusion injury salvage kinase SAFE; survivor activating factor enhancement, PI3K; phosphoinositide 3-kinase, MEK; Mitogen-activated protein kinase, PKC; protein kinase C, eNOS; endothelial nitric oxide synthase, ERK; Extracellular signal-regulated kinase, GPCR; G-protein-coupled receptors, RTK; Receptor tyrosine kinases, TNFR; Tumor necrosis factor receptor, JAK; The Janus kinase, STAT; Signal transducer and activator of transcription pathway, mPTP; Mitochondrial Permeability Transition Pore, Cx43; Connexin 43, (From Bell, R. M., et al., 2016) [130].

Clinically, IPo demonstrates protection effects both in PCI and cardiac surgery. A randomized- controlled trial investigating the effect of IPo in primary PCI after routine thrombus aspiration showed that IPo could reduce infarct size [131]. However, Mentias A. et al. (2017) demonstrated that PCI with IPo could not reduce risk of clinical adverse event and all-cause of mortality rate when compared with the conventional procedure [132].

Remote ischemic preconditioning (RIPC) is the phenomenon whereby a distant brief period-nonlethal ischemia can protect the target organ from the insults of subsequent sustained episode of ischemia. RIPC is suitable for patients undergoing elective CABG or PCI. A mechanism of RIPC is involved in humoral, neuronal and systemic immune system communication activation. In the heart, RIPC also activates survival signaling pathway [133]. RIPC has 2 phases of protection including early(< 4 hours) and late phase (24-72 hours) for protection against IRI [134].

Clinical application of RIPC has been reported. Lazaris A.et al, (2009) showed that RIPC by using subphrenic aortic cross-clamping could decrease renal ischemia reperfusion injury in rat model [135]. RIPC also reduces troponin T levels, preserves mitochondrial respiration, activates survival signaling pathway, in the protection of renal and myocardial cells in PCI and CABG patients [135-138]. However, it was found that application of four cycles upper-limb ischemia (5-minute blood-pressure cuff inflation to ≥200 mmHg, followed by 5-minute cuff deflation) before propofol-induced anesthesia did not affect all cause death, Infarct size, stroke and acute renal failure after cardiac surgery [139]. Similarly, a meta-analysis of randomized controlled trial of RIPC in patients underwent cardiac surgery showed that RIPC did not reduce morbidity or mortality rate but could reduce acute kidney injury [140, 141].

Hypothermia

Hypothermia is one in cardioprotective strategies during cardiac surgery. Generally, the patient's body temperature is kept below 35°C to reduce oxygen consumption during the surgery [142]. Khaliulin et. al, (2011) assessed the effects of different global ischemic temperatures at 7°C, 17°C, 26°C and 32°C in Langendorff-perfused on rat hearts. The results showed that 26°C is the optimal temperature of cardioprotection for hypothermic ischemia with or without polarized cardioplegia, resulting in maintain hemodynamic function and reduce LDH release during reperfusion [143]. It is documented that hypothermia, together with pre- or post-conditioning, improved hemodynamic parameters, reduced cardiac damaged, mortality and metabolic acidosis [144].

Endothelial cells

Endothelial cells are elongated and spindle-like shape cells in the inner of vessel They consist of four basic compartments including the glycocalyx, cell cortex, cytoplasm, mitochondrial and nucleus [145, 146], as shown in Figure II-12. Mitochondria content in endothelial cell compose 2–6% of the cell volume [146]. The function of mitochondria is to provide ATP for cells and communicate with other cellular organelle, for example, endoplasmic reticulum(ER) and nucleus [146]. In normal condition, endothelial cells play a role in regulating blood flow and suspending circulating leukocytes [147]. It plays a major role in regulation of vasomotor tone, homeostasis, angiogenesis, inflammation and signal mediation. [148].

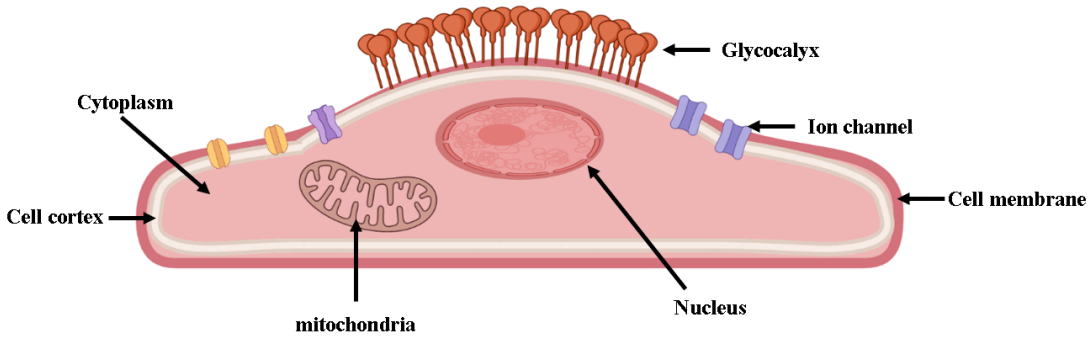


Figure 0-12 Endothelial cell structure.

Roles of endothelial cell in the cardiovascular system

Vascular Tone Regulation

Endothelial cells are associated with regulation of vascular tone, peripheral resistance, and tissue perfusion found [149, 150]. Furchgott and Zawadzki (1980) demonstrated that endothelial cells are involved in sheer stress induced-vasodilation of rabbit aorta by releasing acetylcholine (ACh) [151]. Subsequently, the different vasodilatory mediators including nitric oxide (NO), prostacyclin (PGI2) and endothelium derived hyperpolarizing factor (EDHF) has been found [149, 150]. In addition, endothelial cells also release vasoconstrictive factors such as thromboxane (TXA2) and endothelin-1 (ET-1). [152]. The vasoactive effects of vasodilatory/vasoconstricting mediators are mediated via vascular smooth muscle (VSM) cells, for example, nitric oxide (NO) was produced from reaction between endothelial-nitric oxide synthase (eNOS) and L-arginine. Increasing of intercellular Ca^{2+} in response to vasodilator agonists or shear stress are activate eNOS signaling. Production of nitric oxide (NO) from endothelial cells diffuse to vascular smooth muscle cell and bind to transmembrane receptor of guanylyl cyclase (GC). Reaction between GC and NO convert guanosine 5’ triphosphate (GTP) to cyclic guanosine 3’,

5’ monophosphate (cGMP), thereby increasing intracellular cGMP, cGMP acts as a second messenger of protein kinase G (PKG) activation [153]. Activation of protein kinase G causes opening of the large conductance Ca^{2+}-activated K^+ channel (BKca) on the cell membrane, leading to K^+ efflux and membrane hyperpolarization, which is a cause of closing voltage-dependent Ca^{2+} channels and decrease Ca^{2+} influx. PKG also contributes sarcoplasmic reticulum calcium ATPase (SERCA) pump to increase the Ca^{2+} re-uptake process into the sarcoplasmic reticulum (SR) [154]. Consequently, This NO-released from endothelial cell mechanism provides smooth muscle relaxation and vasodilation, as described in Figure II-13 [152-154].

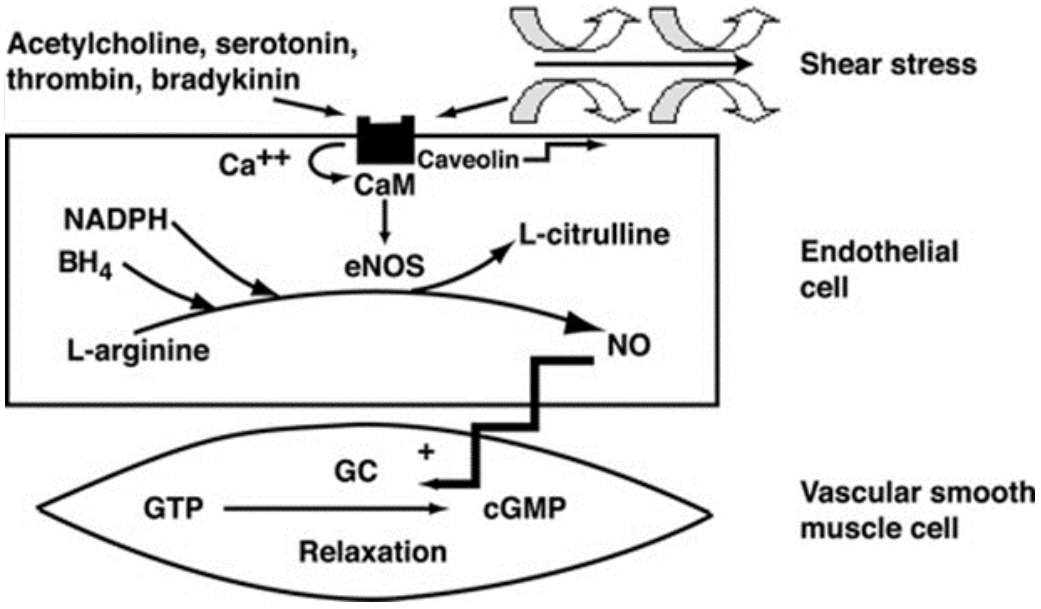


Figure 0-13 Production of nitric oxide (NO) by endothelial cells.

NADPH; nicotinamide adenine dinucleotide phosphate, Ca^{++}; calcium, CaM; calcium-calmodulin, BH_4; tetrahydrobiopterin, GTP; guanosine 5’ triphosphate, GC; guanylate cyclase, cGMP; cyclic guanosine 3’, 5’ monophosphate NO; nitric oxide, eNOS; endothelial nitric oxide synthase. (Figure from Davignon, J. and Ganz, P., 2004 [152]).

Farah C. et al., (2016) and Chen G. et al. (2020) reported that the crosstalk between cardiac microvascular endothelial cells (CMECs) - cardiomyocytes (CMs) and endothelial nitric oxide synthase (eNOS) were a key component of protection against ischemic reperfusion injury [155, 156]. Herrera-Zelada N. et al.,2021 suggested that endothelial cell is a novel therapeutic target for cardioprotection, which is related to nitric oxide, inflammatory response and pro-survival signal pathway [157]. It was found that IRI following coronary revascularization damaged endothelial cells, enhanced inflammatory response, increased ROS and decreased eNOS release [158]. It is also reported that endothelial conditioning can provide cardioprotective effects, probably via K_{ATP} channels [158].

Anti-inflammatory response

Endothelial cells are involved in inflammatory processes [147]. The roles of endothelial cell in inflammation involves acute inflammation (over a period of hours) and chronic inflammation [159]. Inflammation stimulates vasodilation and widening and increase vascular permeability, [160]. Endothelial cell dysfunction resulting in enhance expression of cell adhesion molecules (CAM) on membrane including vascular cell adhesion molecule 1 (VCAM-1), intracellular adhesion molecule 1 (ICAM-1) [161]. Their main function is to bind a rolling cell such as leukocyte and migrating leukocyte into damaged tissue [160, 161]. Endothelial cells also produce cytokine mediators during inflammation including tumor necrosis factor alpha or TNF-α, interleukin (IL-1, IL-6, IL-8, IL-11, IL-14, IL-15) and vascular endothelial growth factors (VEGF). Endothelial cells also play a role in leukocyte adhesion and transmigration o[161, 162].

Anticoagulation

Endothelial cells monolayer are lining in innermost of vessel as a barrier between the blood and the vascular structures, therefore endothelial cells prevent platelet activation and fibrin clot formation [163].

Endothelial cells produce surface proteins that regulates the balance of pro-thrombotic factors and anti-thrombotic factors, including thrombomodulin (TM), endothelial protein C receptor (EPCR), tissue factor pathway inhibitor (TFPI), and protein C (PC). These proteins are necessary for intrinsic and extrinsic coagulation pathways [164, 165]. Studies suggested that impaired endothelial function resulting in impaired fibrinolysis, reduce express nitric oxide (NO) and prostacyclin (PGI2), which is related to platelet aggregation and thrombus formation [166]. Endothelial cells (ECs) release both pro-thrombotic factors and anti-thrombotic factors. Verhamme, P. and Hoylaerts, M. F., (2006) demonstrated that the role of endothelial cells is associated with 1) An expression of nitric oxide (NO), prostacyclin (PGI2), and ectonucleoside triphosphate diphosphohydrolase-1 (E-NTPDase1). They inhibit platelet adhesion and aggregation. 2) Heparin-like molecules (Hep) expressed on endothelial surface work as a co-factor for antithrombin III (ATIII) to inactivating coagulation factors. 3) Releasing of tissue factor pathway inhibitor (TFPI) inhibits the action of tissue factor (TF), factors VII and X. 4) Thrombomodulin (TM) binding with thrombin resulting in activate protein C and degrades factor Va and VIIIa. And 5) Plasminogen activator inhibitor (PAI-1) inhibited endothelial tissue plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA), which lead to activate fibrinolysis [187]. The roles of endothelial cells in coagulation system are presented in Figure II-14.

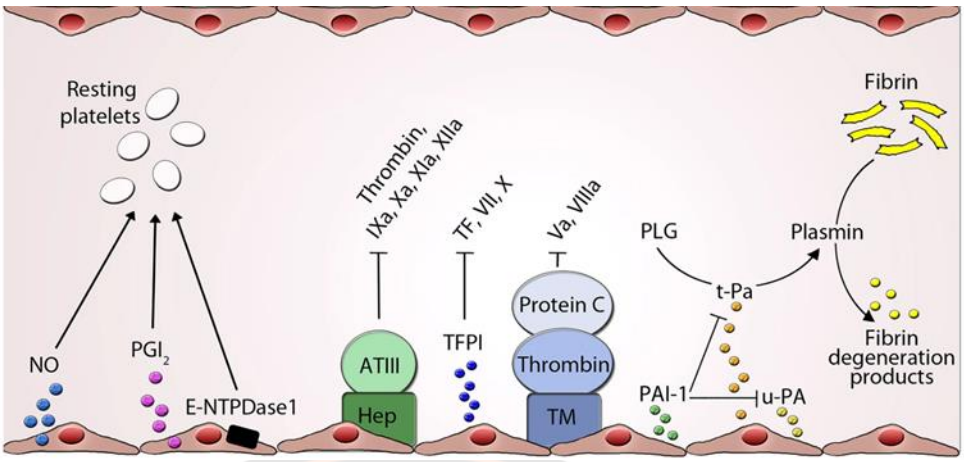


Figure 0-14 The roles of endothelial cells in coagulation system

NO; nitric oxide, PGI2; prostacyclin, E-NTPDase1; ectonucleoside triphosphate diphosphohydrolase-1, ATIII; antithrombin III, Hep; heparin-like molecules, TFPI; tissue factor pathway inhibitor, TM; thrombomodulin, t-PA; plasminogen activator, u-PA; urokinase-type plasminogen activator, PAI-1; plasminogen activator inhibitor. (Figure from Verhamme, P. and Hoylaerts, M. F.,2006 [166]).

Endothelial ion channel

The abundance of ion channels of endothelial cells contributes to their function and cell to cell communication (cell-cell communication) [167]. Ion channels in endothelial cell plasma membranes and in smooth endoplasmic reticulum are associated with intracellular calcium concentration and regulation of membrane potential [168]. Concomitantly, membrane potential also affects the electrochemical gradient and activate ion channels activation [167]. The illustration of ion channels of endothelial cells are shown in Figure II-15 and their functions are summarized in Table II-2.

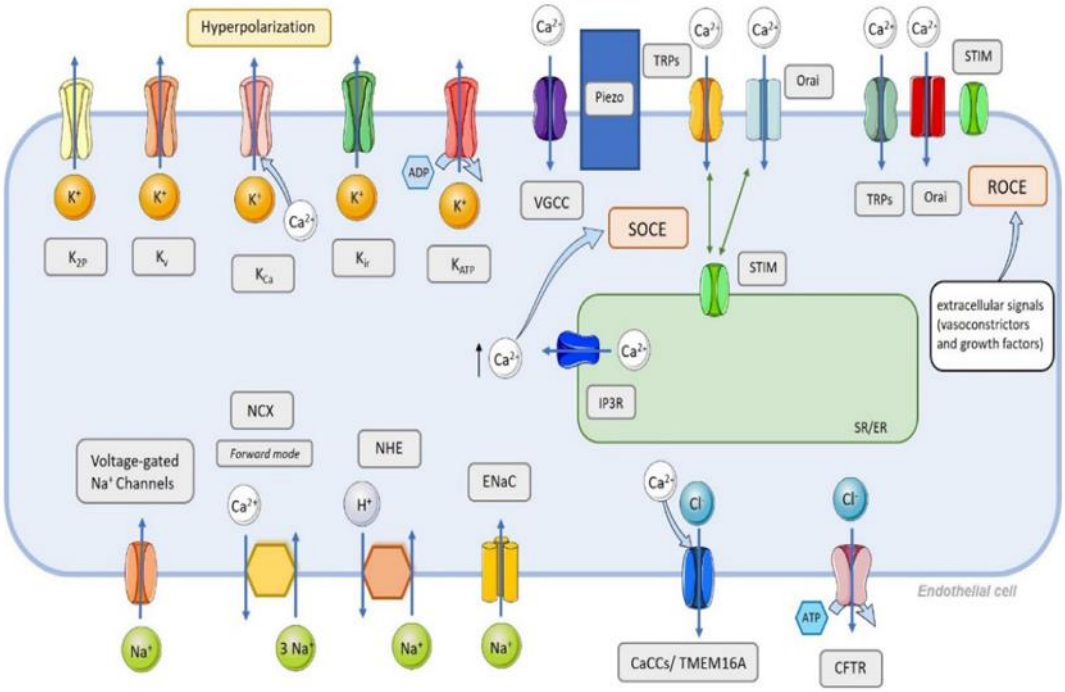


Figure 0-15 The illustration of ions channels of endothelial cells.

K_{ATP};ATP-sensitive K+ channel, CaCCs; Ca^{2+}-activated Cl^- channels, KCa; Ca^{2+}-activated K^+ channels, CFTR; cystic fibrosis transmembrane conductance regulator, SR/ER; sarcoplasmic/endoplasmic reticulum, ENaC; epithelial Na^+ channels,; IP3; inositol triphosphate; IP3R;inositol triphosphate receptor, STIM; interacting molecule, Kir; inward rectifier K^+ channels, NHE; Na^+-H^+ exchanger, NCX; Na^+/Ca^{2+} exchanger, ROCE; receptor-operated Ca^{2+} entry, ROCs; receptor-operated channels, SOCE; storage-operated Ca^{2+} entry, SOCs; store-operated channels, TRPs; transient receptor potential channels, K2P; two pore K^+ channels, VGCC; voltage-gated Ca^{2+} channels, Kv; voltage-gated K+ channels. (Figure from Santos-Gomes J., et al.,2022 [169]).

Table 0-2 Ion channels in endothelial cells

Channel	Channel subtype	Mechanism activator	Resulting of activation
Ca^{2+} Channels	Voltage-gated Ca2+ channels (VGCC)	Membrane depolarization	Calcium influx
	Non-voltage-dependent Ca^{2+} channels		
	1)Store-operated Ca^{2+} entry (SOCE);	Intracellular Ca^{2+} level	Refilling internal Ca^{2+} stores in the endoplasmic/sarcoplasmic reticulum (ER/SR) after Ca^{2+} depletion
	2)Store-independent Ca^{2+} entry (SICE), or Ca^{2+} entry (ROCE);	Binding of extracellular ligand signaling	Increasing intracellular Ca^{2+}
	3)Ca2+ stretch channels. (Piezo1 and Piezo2)	Membrane tension, Shear stress	Cations influx, including Na+, K+, $Ca^{2+,}$ and Mg^{2+}
Na+ Channels	Epithelial Na^+ Channels (ENaC)	Salt reabsorption, Mechanical stress, Blood pressure, Membrane depolarization	Reduced NO release and consequently vasoconstriction
	Voltage-Gated Na+ Channels	Membrane depolarization	Sodium influx [170]
	Sodium-Calcium Exchanger (NCX)	Intracellular Ca^{2+} levels	Cytosolic Ca^{2+} efflux and Na^+ influx ($1Ca^{2+}$-$2Na^+$)
	Na^+-H^+ exchanger (NHE)	Intra cellular H^+, pH	Exchanging of extracellular Na^+ for intracellular H^+, regulate pH homeostasis, cell volume, and transepithelial Na^+ uptake

Table II-2 Ion channels in endothelial cells(continue).

Channel	Channel subtype	Mechanism activator	Resulting of activation
Cl^- Channels	Ca^{2+}-activated Cl^- channels (CaCCs)	Intracellular Ca^{2+}	Cl^- efflux, membrane depolarization[171]
	Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)	ATP-dependent, adenosine monophosphate (cAMP)-dependent kinase and PKC phosphorylation	Cl^- efflux
K^+ Channels	Voltage-gated-K^+ channels (Kv)	Membrane depolarization	K^+ efflux, resulting in membrane repolarization at the resting membrane potential phase, associated with regulation of vascular tone and cell viability
	Ca^{2+}-Activated K^+ Channels (KCa)	Intracellular Ca^{2+} levels	EC membrane hyperpolarization, associated with NO release and NOS activation by regulating Ca^{2+} influx
	Two pore potassium channels (K2P)	pH variation, G-proteins, oxygen level, shear stress, membrane potential.	K^+ efflux, maintain membrane potential
	ATP-Sensitive K^+ Channel (K_{ATP})	Intracellular ATP concentration	Regulation of the resting membrane potential, K^+ efflux, hyperpolarization, associated with NO production
	Inward Rectifier Channel Family (Kir)	Sheer stress[172], membrane potential (hyperpolarization)	Increase outward K^+, regulation of K^+ equilibrium potential and resting membrane potential [173],essential for NO-dependent vasodilatation and eNOS phosphorylation

(Table modified from Santos-Gomes, J., et al., Role of Ion Channel Remodeling in Endothelial Dysfunction Induced by Pulmonary Arterial Hypertension. Biomolecules, 2022. 12(4) [169] and Gerhold, K.A. and M.A. Schwartz, Ion Channels in Endothelial Responses to Fluid Shear Stress. Physiology (Bethesda), 2016. 31(5): p. 359-69 [174]).

ATP-sensitive potassium (K_{ATP}) channels on endothelial cells

ATP sensitive potassium (K_{ATP}) channels play a role on endothelial cells function as a metabolic sensor. Depletion of adenosine tri-phosphate (ATP) or increasing of adenosine diphosphate (ADP) promote the opening of K_{ATP} channel [27]. Roles of K_{ATP} channels are regulation of the resting membrane potential by letting K^+ out of endothelial cell, which leads to hyperpolarization [169]. Katnik, C. and Adams, D. J. (1995) isolated endothelial cells from rabbit arteries and used whole-cell patch clamp technique to explore the effect of levcromakalim (K_{ATP} activator) on K_{ATP} channel and found that opening of K_{ATP} channels induced hyperpolarization [175].

ATP-sensitive potassium channel (K_{ATP})

K_{ATP} channels is octameric complex ion channels. consisting of 4 pore-forming subunits of KIR 6.0 family and 4 sulfonylurea receptors (SUR) (Figure II-16). Kir6.1 and Kir6.2 are potassium inward rectifying channel that were encoded by gene KCNJ8 and KCNJ11, respectively whereas SUR1 and SUR2 are ATP-binding cassette, encoded by ABCC8 and ABCC9 gene, respectively. The distribution of the channels varies across tissues. Kir6.2/SUR2A is prominently found in ventricular tissue, whereas Kir6.2/SUR1 and SUR2A are found in the atrium. In conduction system and endothelial

cells, Kir6.1 and Kir6.2 with SUR2B are present. These subunits are also found in smooth muscle cells [29].

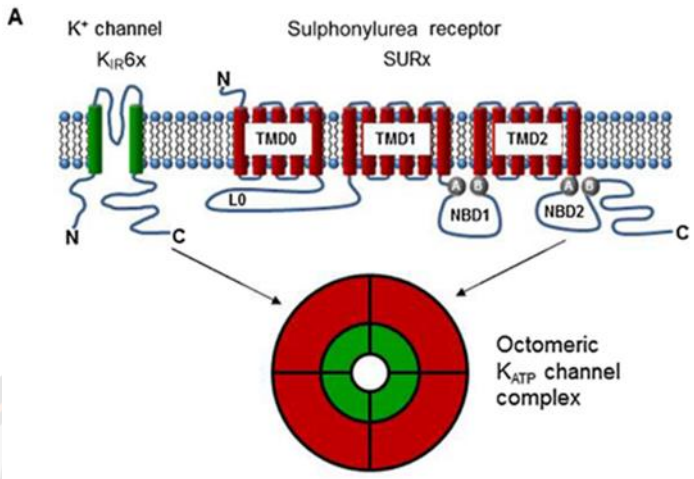


Figure 0-16 Molecular basis of the K_{ATP} channel.

K_{ATP}; ATP-Sensitive Potassium Channel, SUR; sulfonylurea receptor, Kir; inwardly rectifying potassium channels, and TMD; transmembrane domains. (Figure from Tinker, A., et al., 2014 [176]).

Sarcolemmal ATP-sensitive Potassium channel

Sarcolemmal ATP-sensitive Potassium Chanel (sarcK_{ATP}) is the complex of Kir6.0 family and SUR1, SUR2A or SUR 2B. SarcK_{ATP} are found in skeletal muscle and cardiac tissue. Ventricular sarcK_{ATP} are believed to compose of Kir6.2/SUR2A subunit. There is evidence supporting that sarcK_{ATP} are absent in Kir6.2 knockout mice [29, 177]. Some studies reported that Kir6.1 maybe a subunit of cardiac sarcoK_{ATP} [178, 179]. Chen S., et. al. (2014) found both Kir6.1 and Kir6.2 at the sarcolemmal membrane. It was also found that Kir6.1-like channel may play a role in regulation of membrane potential and action potential duration [30]. SarcK_{ATP} is responsible for myocardial protection in stress conditions such as hypoxia or ischemia [177, 180, 181]. The role of sarcK_{ATP} in response to stress is to promote potassium efflux, resulting in

action potential shortening and limiting calcium influx. A shorten action potential duration (APD) results in a longer diastolic interval and prevent depletion of cellular resources, therefore, It is hypothesized that sarcK_{ATP} of cardiomyocyte can act against cardiac injury and cardiac dysfunction [182].

Mitochondrial ATP-sensitive Potassium channel

Mitochondrial ATP-sensitive Potassium channel (mitoK_{ATP}) has gained attention in its cardioprotective effects. MitoK_{ATP} is an octameric complex of 4 pore-forming subunits of KIR 6.0 family and 4 sulfonylurea receptors (SUR) formed at inner membrane of mitochondria [178]. In normal condition, mitoK_{ATP} are closed and membrane potential is regulated by potassium/hydrogen antiporter (K^+/H^+ antiporter). The activation of mitoK_{ATP} in response to low ATP level leads to mild decrease membrane potential, responsible for a reduced uptake of Ca^{2+} into mitochondrial matrix through mitochondrial calcium uniporter (MCU), which regulates ROS production and inhibits MPTP opening irreversibly [34]. The distribution of K_{ATP} channels subunit in various tissues is shown in Table II-3.

ATP-sensitive potassium channel modulators

Potassium channel openers (KCOs), including diazoxide, cromakalim, nicorandil, pinacidil, BMS 191095 and Isoflurane can reduce infarct size, reduce arrhythmia, decrease superoxide production, prevent apoptosis and against ischemia-reperfusion injury in preconditioning, ischemic and reperfusion phase [34, 183-185].

Table 0-3 Distribution of K_{ATP} channels subunit in various tissues.

K_{ATP} subtypes family	**Distribution**			
	Atrium	Ventricle	Conduction system and Endothelial cell	Smooth muscle cell
Kir6.1/SUR2B		?	✓	✓
Kir6.2/SUR1	✓			
Kir6.2/SUR2A	✓	✓		
Kir6.2/SUR2B			✓	

Modified from: "KATP Channels in the Cardiovascular System" by Foster M. N., et al, 2016, Physiol Rev, 96(1): p. 177-252 [29].

Pinacidil

Pinacidil is a cyanoguanidine drug that can open non-selective ATP-sensitive potassium channels. It causes peripheral vasodilatation. The main effect of pinacidil is decreasing peripheral vascular resistance and thereby reducing blood pressure. In 1996, myocardial protection by pinacidil has been reported during global ischemia in rabbit heart. Pinacidil is an alternative to traditional hyperkalemic cardioplegia [37]. In the long-term global ischemia condition, i.e., heart transplant preservation, adding pinacidil (0.5 mM) into University of Wisconsin cardioplegia solution or St. Thomas cardioplegia solution is effective. This research reported that pinacidil or potassium channel opener maybe a novel strategy to improve donor heart preservation [35]. Pinacidil can activate both sarcoK_{ATP} and mitoK_{ATP}. A pharmacological effect of

pinacidil thought to be involved in limited ROS production and Ca^{2+} overload, leading to cardioprotection [186, 187]. The structure of pinacidil is shown in Figure II-17.

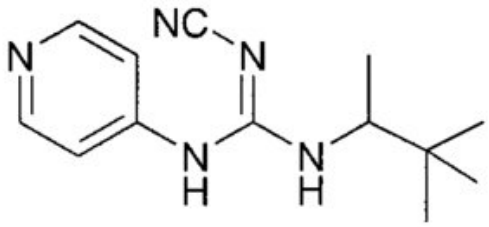


Figure 0-17 Structures of pinacidil.

(From Dabrowski, M., et al., 2002 [188]).

Diazoxide

Diazoxide is one of ATP-sensitive potassium channel openers (KCOs). There are many reports indicating cardioprotective effects of diazoxide. Facundo H. et al demonstrated that diazoxide increases mitoK_{ATP} activity and reduces mitochondrial ROS releasing in isolated rat hearts, resulting in ischemic protection [189]. Previous studies showed that diazoxide can reduce cell swelling and preserve contractility in isolated cardiomyocytes of wild type mice (WT) but not in Kir6.1(−/−) mice during stress [190, 191]. In a swine model, it was reported that diazoxide can preserve systolic and diastolic ventricular function in prolonged global myocardial ischemia when apply in conjunction with hyperkalemic cardioplegia solution [38]. Makepeace C. et al reported that adding diazoxide in hyperkalemic cardioplegia increased end-diastolic pressure but could not provide greater percent recovery of developed pressure and coronary flow in mice model [39]. Raphael J. et al., demonstrated that isoflurane can reduce infarct size, malonaldehyde level and myocardial apoptosis in rabbits but was ineffective in hyperglycemic rabbits. Diazoxide demonstrated effective

cardioprotection when conjunctively used with isoflurane [192]. The structures of diazoxide described in Figure II-18.

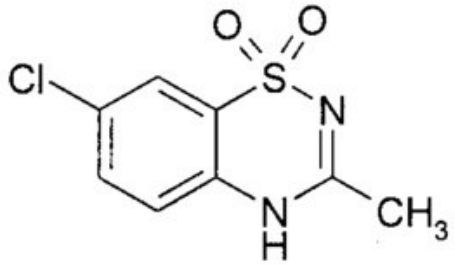


Figure 0-18 Structures of diazoxide.

(From Dabrowski, M., et al., 2002 [188]).

Nicorandil

Nicorandil is an anti-anginal agent, which consists of N-[2-hydroxyethyl])-nicotinamide nitrate as described in Figure II-19 [193]. Pharmacodynamic effects of nicorandil are opening of plasma membrane K_{ATP} channels and increasing NO production by via its nitrate [193, 194]. Lamping, Kathryn A. (1984) reported that nicorandil can reduce infarction size in heart dogs that subjected to ischemic condition [195]. Wu H., et al., (2015) demonstrated that nicorandil at 100 μM could maintain post-ischemic contractility, reduce myocardial infarction size on ischemic-reperfusion rat hearts and inhibit apoptotic cell death through PI3K/Akt pathway [196].

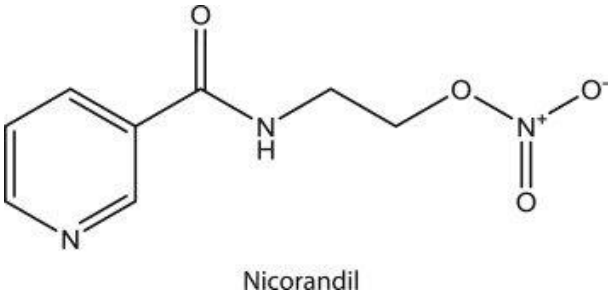


Figure 0-19 The structure of nicorandil.

(From Kim, Jung-Un, et al., 2014 [197]).

Kir6.1/SUR2B openers

Natakalim is a selective Kir6.1/SUR2B K_{ATP} channel opener. In 2009, Natakalim was pharmacologically investigated in cardioprotective effects in male Wistar rats. It was shown that at concentrations ranging from 0.01μmol/L to 100μmol/L, Natakalim had no effect in isolated rat heart. However, natakalim at 0.5, 2 and 8 mg/kg could affect endothelium. It could decrease blood pressure and at concentration 8 mg/kg, but also dramatically compromised cardiac function [198]. The study of Tang Y., et al (2010), showed that Natakalim had endothelium-dependent vasodilatory effects, indicated by the increase levels of nitric oxide and plasma prostacyclin. It was found that both atrial and B-type natriuretic peptide messenger RNA was downregulated in rats which were treated by natakalim. These results were relevant to the finding that Kir6.1/SUR2B can inhibit the progression of heart failure by lowering pressure overload [199].

Zhou H., et al. (2014,2016), demonstrated that Natakalim can counteract cardiac hypertrophy and prevent heart failure in ischemic rat hearts. It is suggested that Natakalim provided therapeutic effect via improvement of endothelial function. Natakalim is a candidate for a therapeutic drug in coronary artery disease and myocardial infarction-induced heart failure [200, 201].

Chen X., et al. (2015), has demonstrated that Natakalim-induced vasorelaxation was through increased Ca^{2+} and nitric oxide (NO) production at endothelial ATP-dependent potassium channel [28].

Jin F. (2016) reported that treatment with natakalim 3 mg/kg/day for 10 days can improve hemodynamics, cardiac functions and ventricular remodeling [202].

Natakalim was demonstrated as a new pharmacological approach for ameliorating chronic heart failure by activation SUR2B/Kir6.1 activity, enhanced miR-1-3p (an associated vascular remodeling gene) expression and downregulation the expression of endothelin-1 (ET-1) pathway in endothelial cell [203].

The other Kir6.1/SUR2B activator, ZD0947, is a potent ATP-sensitive potassium channel opener, acting mainly on vascular smooth muscle and detrusor smooth muscle myocytes [204]. In 2006, Aishima M., et al report that ZD0947 is the cause of detrusor relaxation through activation of detrusor smooth muscle myocytes K_{ATP} channels [205]. Yunoki T., et al. (2008) demonstrated that ZD0947 can reduced contraction of detrusor smooth muscles and induced hyperpolarizing on guinea pig detrusor and aortic myocytes [206]. In vitro study, there has been reported that ZD0947 was the cause of significant relaxation of both vascular and urinary bladder smooth muscle, because ZD0947 activates both Kir6.1/SUR2B on vascular smooth muscle cells and Kir6.2/SUR2B on smooth muscle cell with a similar potency [204]. Effects of ZD0947 in mouse portal vein has been demonstrated in 2017, the result showed that 3 μM of ZD0947 can induced relaxation and this effect was inhibited by 300 nM glibenclamide, that is functionally similar to Kir6.1/SUR2B channels stimulation [207].

The clinical study of ZD0947(25 mg/day) reported that these non-cholinergic pathways drug was safe with no adverse event in heart rate or blood pressure, but there was no significant improvement in clinical signs in overactive bladder patients. Further studies are suggested to advance the therapeutic effects of ZD0947 [208].The pharmacological action of ZD0947 is involved in increased potassium permeability, hyperpolarization, relaxation, reduced calcium influx leading to reduced calcium

overload on vascular smooth muscle cell [204-207]. The structure of ZD0947 described in Figure II-20.

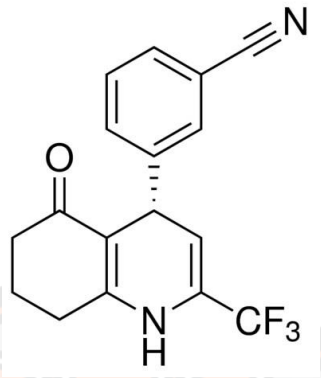


Figure 0-20 Structures of ZD0947.

(From Mori, K., et al., 2016 [204]).

K_{ATP} channel inhibitors

The most common K_{ATP} channel inhibitors are glibenclamide, tolbutamide and 5-Hydroxydecanoate (5-HD). The glibenclamide and tolbutamide inhibit K_{ATP} channels by affecting sulfonylureas subunits, whereas 5-Hydroxydecanoate (5-HD) inhibits K_{ATP} function mitochondrial K_{ATP} channels [29, 209]. K_{ATP} inhibitors can decrease mitochondrial inner membrane potential and inhibit KCOs effects, resulting in increased generation of superoxide-derived ROS and also inhibiting mitochondrial respiration [210-212].

Glibenclamide is an oral antihyperglycemic drug, for type II diabetes mellitus treatment [213]. The structure of glibenclamide is shown in Figure II-21. The mechanism of glibenclamide is involved in depolarizing β-cells, leading to Ca^{2+} influx and insulin secretion [214]. There are evidences reported that glibenclamide binds to sulfonylurea receptor and keeps the channel closed during ischemia or ATP depletion, resulting in increased the action potential duration (APD). The change increased Ca^{2+}

influx, contributing to Ca^{2+} overload in papillary muscle of pigs and sinoatrial node (SA node) of rabbits [215].

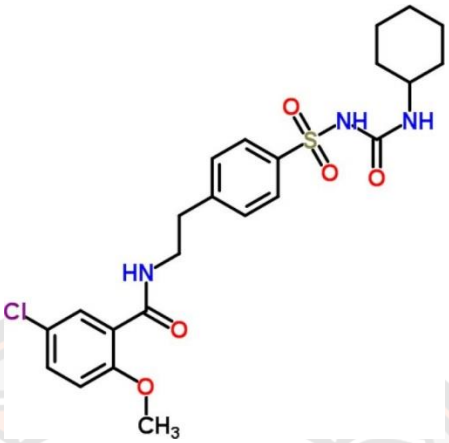


Figure 0-21 Structure of glibenclamide.

(From Haq, N., et al.,2014 [216]).

Role of ATP-sensitive potassium channels in regulation of nitric oxide synthesis in endothelial cells.

ATP-sensitive potassium (K_{ATP}) channels couple cellular metabolism with membrane excitability, calcium entry, and endothelial mediator release [217]. Their activity is activated by falling ATP and increasing ADP [176]. A study reported that opening K_{ATP} channels could amplifier membrane hyperpolarization and potentially promote calcium entry in endothelial cells [27]. The opening of K_{ATP} channels results in K^+ efflux leading to membrane hyperpolarization which subsequently activates transient receptor potential vanilloid 1 (TRPV1) channels, contributing to Ca^{2+} influx [27, 28]. Increasing of intracellular Ca^{2+} enhances interaction of calcium and calmodulin (CaM), Calcium/Calmodulin, binding, leading to activation of endothelial nitric oxide synthase (eNOS). eNOS catalyzes L-arginine (L-arg) into L-citrulline and, finally, into nitric oxide (NO). NO affects vascular smooth cells by stimulating soluble

guanylyl cyclase (sGC) and inducing formation of cyclic guanosine monophosphate (cGMP). The cyclic monophosphate activates protein kinase G (PKG) resulting in reduced intracellular calcium by inhibition voltage-dependent calcium channel (VDCC). The decrease of intracellular calcium inhibits calcium-induced calcium release from sarcoplasmic reticulum (SR), and stimulates reuptake of cytosolic calcium into the sarcoplasmic reticulum (SR) [28, 218]. Intracellular ^{Ca2}+ depletion inactivates myosin light chain kinase (MLCK) in smooth muscle cell, leading to vascular relaxation [218]. The mechanism is shown in Figure II-22.

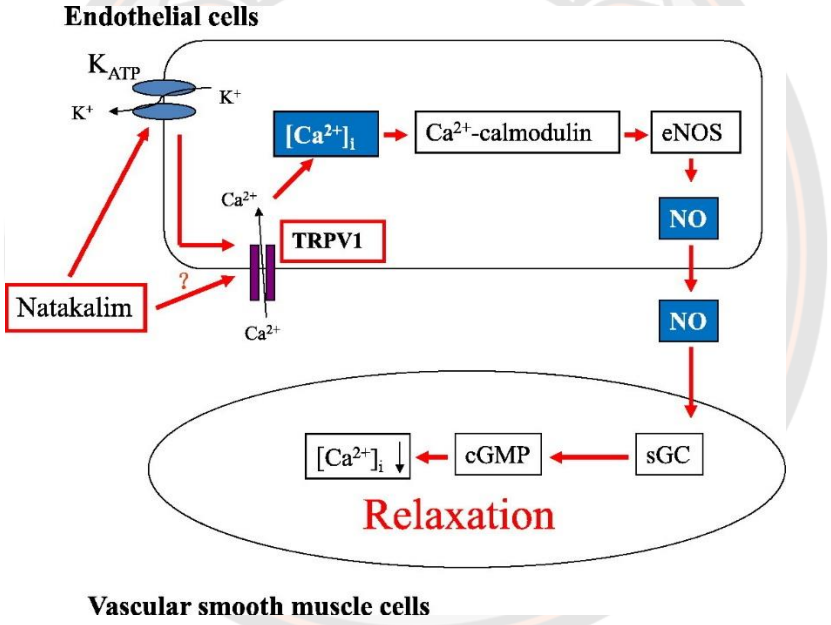


Figure 0-22 The role of ATP-sensitive potassium channel (natakalim) in endothelial nitric oxide synthesis.

eNOS; endothelial nitric oxide synthase, TRPV; transient receptor potential vanilloid 1, NO; nitric oxide, cGMP; cyclic guanosine monophosphate, sGC; soluble guanylyl cyclase. (Figure from Chen, X., et al.,2015 ; [28]).

CHAPTER III

RESEARCH METHODOLOGY

This study was approved by the biosafety committee of Naresuan University, project number: NUIBC OT 64-11-58.

1. Experimental design

1.1 Optimization

1.1.1 Optimization of ischemia-reperfusion model

Ischemia-reperfusion (IR) model was performed according to the protocol in the previous study [219]. Briefly, endothelial cell (EA.hy926) were seeded into 24 well plate at the density of 45,000 cells/well and were incubated overnight at 37°C in a humidified 5% CO_2-95% air mix environment. Ischemia was simulated by replacing 400 µl of completed Dulbecco's Modified Eagle's Medium (DMEM) with 400µl of simulated ischemic solution. Varied ischemic times (30, 45, 60, 90, 120 and 150 minutes) with a 10-minute reperfusion with 360 µl of DMEM was applied to optimize the IR model. The 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay was performed to assess relative cell viability. The absorbance of the samples was assessed by a microplate reader at the 570 nm wavelength. The protocol was shown as the diagram below.

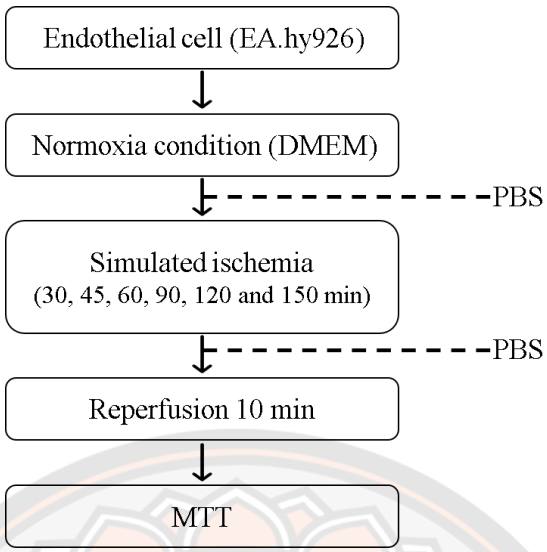


Figure 0-23 The diagram for optimization of ischemia-reperfusion model.

1.1.2 Optimization of K_{ATP} channels activators

To investigate the effects of IR and K_{ATP} channel activators (KCOs) on endothelial cell viability. Cell viability in response to different concentrations of KATP channel modulators, pinacidil (P), diazoxide (D), nicorandil (N), ZD0947 (Z) was assessed. The optimization was outlined as the diagram below:

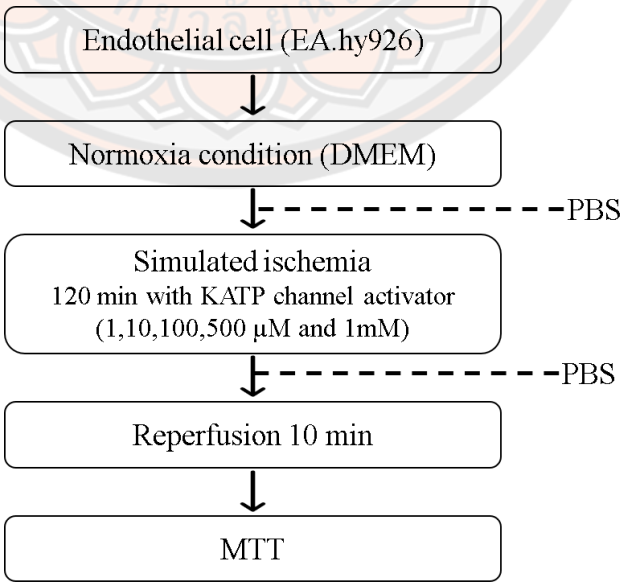


Figure 0-24 The diagram for optimization of K_{ATP} channels activators model.

1.2 Experimental design for assessing the effects of K_{ATP} channel modulators on endothelial cell viability and NO production in normokalemic condition.

To investigate the effects of IR and K_{ATP} channel activators on endothelial cell viability. Cell viability in response to different KATP channel modulators, 1 µM pinacidil (P), 1 µM diazoxide (D), 1 µM nicorandil (N1), 10 µM nicorandil (N10), 1 µM ZD0947 (Z) with and without 10 µM glibenclamide (G) was assessed. The experimental design for assessing the effects of KATP channel modulators on endothelial cell viability condition was outlined as the diagram below:

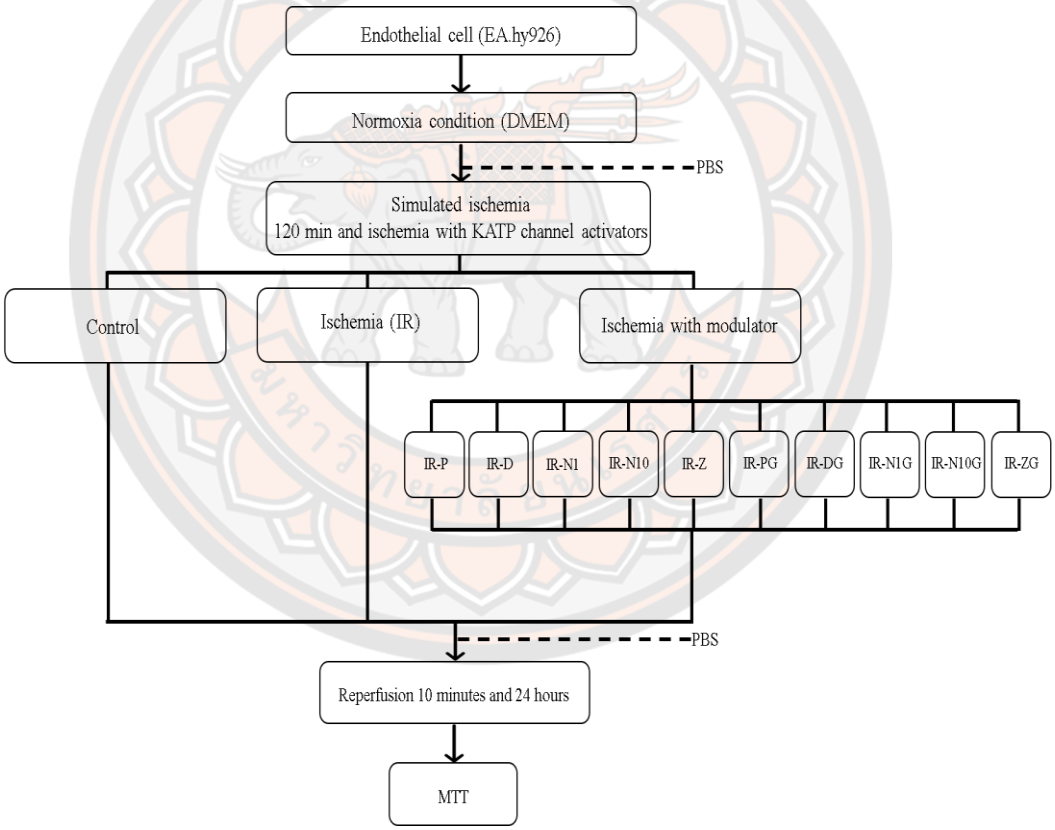


Figure 0-25 The diagram for effects of K_{ATP} modulators on endothelial cell viability model.

To investigate the effects of IR and KATP channel modulators on endothelial NO production in normokalemic condition, the cells were divided into 6 groups as the described 1) control, 2) ischemia reperfusion (IR), 3) IR with 1 µM of pinacidil (IR-P), 4) IR with 1 µM diazoxide, 5) IR with 10 µM nicorandil (IR-N10) and 6) IR with 1 µM ZD0947. The experimental design for assessing the effects of KATP channel modulators on endothelial cell NO production was outlined as the diagram below:

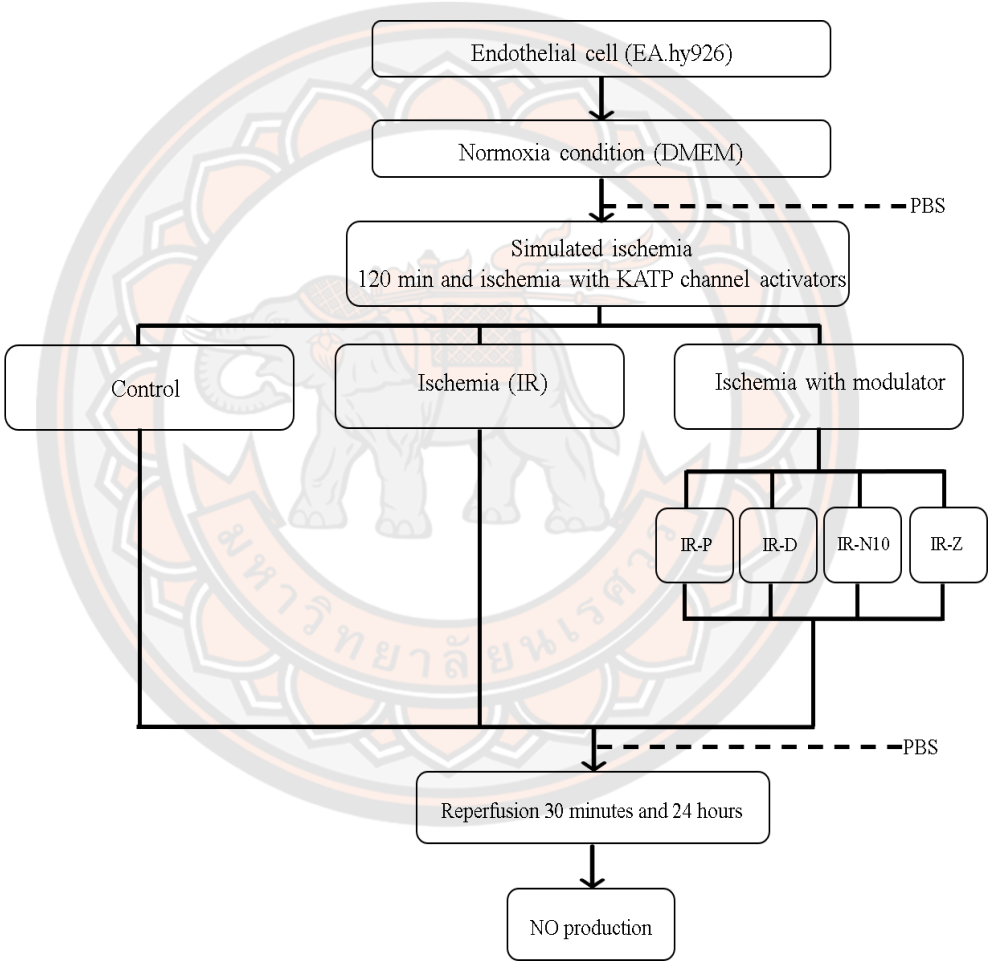


Figure 0-26 The diagram for effects of KATP channel modulators on endothelial cell NO production model

1.3. Experimental design for assessing the effects of hyperkalemic cardioplegia and KATP channel modulators on endothelial cell viability and NO production.

1.3.1 Model construction

To test the effect of IR and KCOs which may occur during cardioplegic application, we designed a short ischemia (10 min), which may be a result of aortic cross clamping, followed by application of oxygenated hyperkalemic cardioplegia with or without KCOs for 30 minutes, and subsequent reperfusion by DMEM for 10 minutes and 24 hr before the MTT test. The concentration of KCOs was optimized as follows; 1 µM and 100 µM of pinacidil, 1 µM and 100 µM of diazoxide, 1 µM and 100 µM of nicorandil, 1 µM and 100 µM of ZD0947, and 10 µM and 100 µM of glibenclamide. According to the study of Ducko, Christopher T., et al., (2000)[220], the effect of KATP channel modulators on endothelial cells viability was observed in two conditions, the effect of KCOs-added to CPG, and the effect of KCOs-added to reperfusion solution, the experimental design was summarized in Figure III-5.

The assessment of endothelial cell viability in response to IR in KCOs-added in hyperkalemic condition the cells were separated to various groups according to the treatments; 1) the control group , 2) ischemia reperfusion group (IR), 3) cardioplegia group (CPG); cells were treated with cold cardioplegia (4ºC) alone without ischemia and followed by DMEM reperfusion in the final step for 10 minutes, 4) ischemia reperfusion and cardioplegia group(I-CPGR); cells were treated with simulated ischemic solution for 10 minutes, followed by cardioplegia and DMEM reperfusion, respectively, 5) ischemia reperfusion and cardioplegia with K_{ATP} modulator groups, which were labelled as I-CPG-P, I-CPG-D, I-CPG-N, I-CPG-Z, and I-CPGR-G; cells

were treated with simulated ischemic solution, followed by cardioplegia with KATP modulator and DMEM reperfusion, and 6) ischemia reperfusion and cardioplegia with KCO and glibenclamide group(which were labelled as IR-CPG-KCO+G); cells were treated with simulated ischemic solution, followed by cardioplegia with KATP activator and 10μM of glibenclamide, and DMEM reperfusion, respectively. The experimental design for model 1 was outlined as the diagram in Figure III-5(A).

The assessment of endothelial cell viability in response to IR in KCOs-added to reperfusion solution condition, the cells were separated to various groups according to the treatments; 1) the control group, 2) ischemia reperfusion group (IR), 3) cardioplegia group (CPG), 4) ischemia reperfusion and cardioplegia group(I-CPGR), 5) ischemia and cardioplegia with KATP reperfusion groups (I-CPGR-N and I-CPGR-Z). The experimental design for model 1 was outlined as the diagram in Figure III-5(B).

According to the results from model of the effects of hyperkalemic cardioplegia and K_{ATP} channel modulators on endothelial cell viability which described above, we were created experimental protocol to test the effects of KCOs on cell viability and NO production by using the protocol which described in Figure III-5(B). Two potential KCOs, nicorandil and ZD0947, were used to evaluate endothelial cell viability and NO production in response to IR in hyperkalemic condition both in immediate phase (10-30 minutes after application) and delayed phase (24 hr). The cells were separated to 5 groups according to the treatments 1) the control group , 2) ischemia group (IR); cells were treated with simulated ischemic solution for 30 minutes, followed by DMEM reperfusion for 30 minutes, 3) cardioplegia group (CPG); cells were treated with cold cardioplegia (4ºC) for 30 minutes, 4) ischemia reperfusion and cardioplegia group(I-CPGR); cells were treated with simulated ischemic solution, followed by cardioplegia

and DMEM reperfusion, respectively, 4) ischemia reperfusion and cardioplegia with KCO groups, which were labelled as I-CPG-N or I-CPGR-Z; cells were treated with simulated ischemic solution, followed by cardioplegia, followed by DMEM reperfusion with 100 μM of nicorandil or ZD0947. A diagram for describe model 3 was showed in Figure III-6.

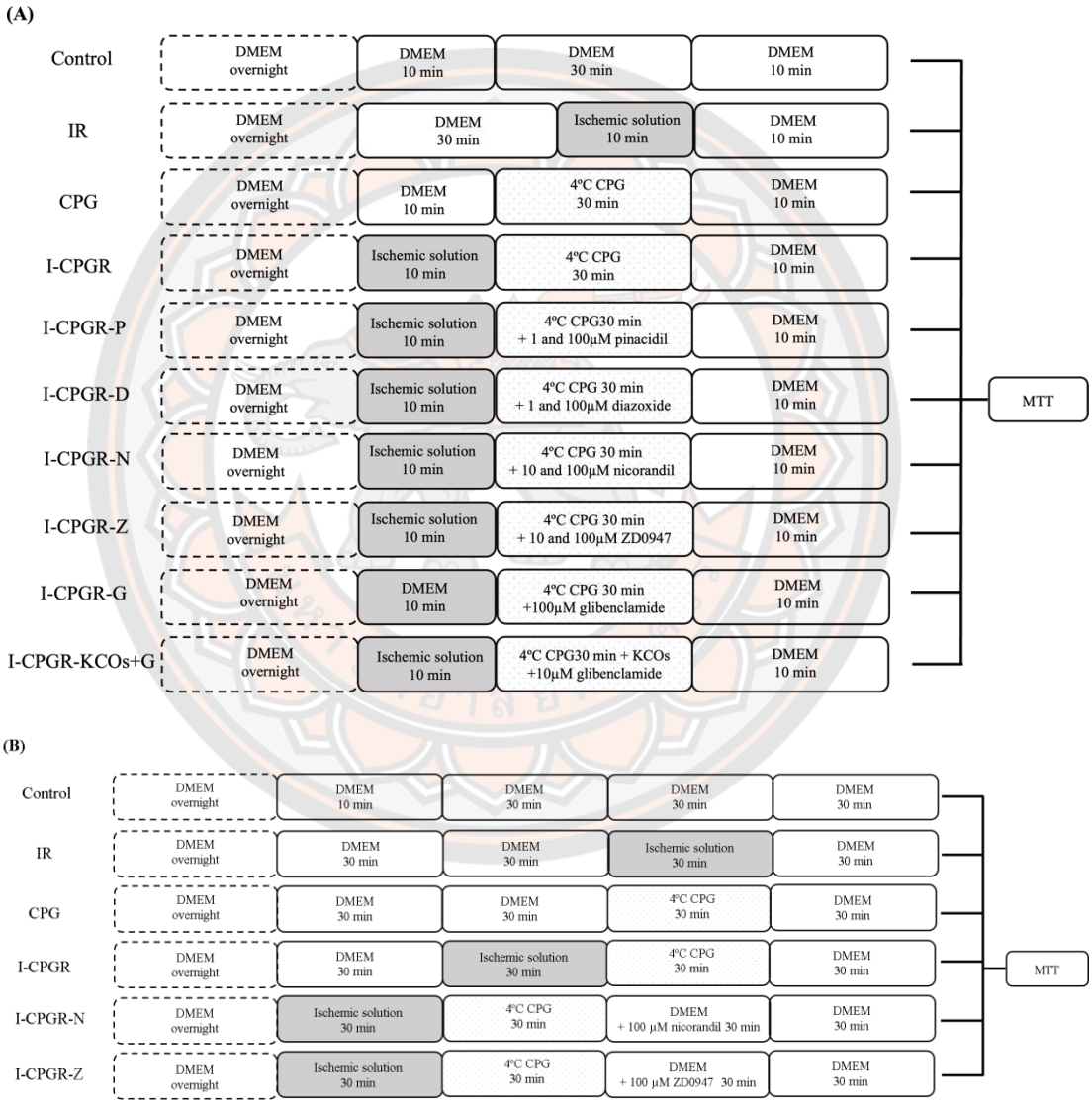


Figure 0-27 The diagrams for assessing the effects of hyperkalemic cardioplegia and KATP channel modulators on endothelial cell viability.

(A) Diagram for assess effect of KCOs-added to CPG. (B) Diagram for assess the effect of KCOs-added to reperfusion solution. DMEM; Dulbecco's Modified Eagle Medium, CPG; hyperkalemic cardioplegia, MTT; MTT assay.

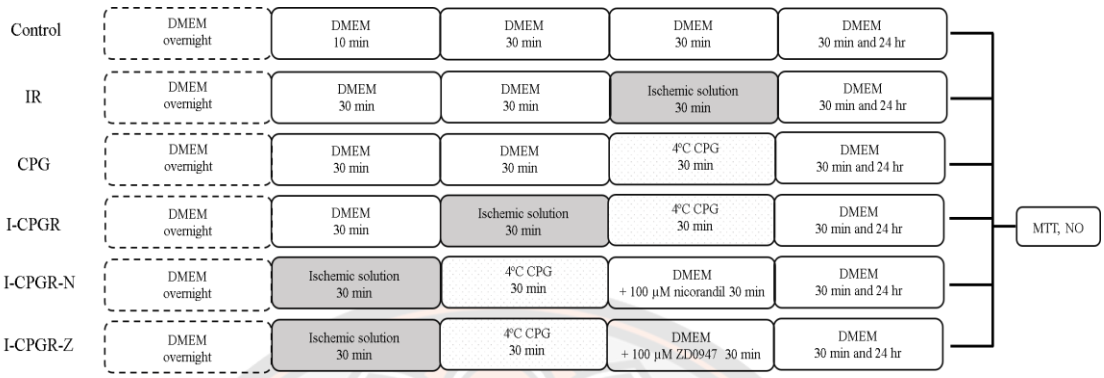


Figure 0-28 The diagram for assessing the effects of KCOs on cell viability and NO production.

DMEM; Dulbecco's Modified Eagle Medium, CPG; hyperkalemic cardioplegia, MTT; MTT assay, NO; nitric oxide production assay.

2. Methods

2.1 Cells

Immortalized Human Vascular Endothelial Cells line (EA.hy926) was purchased from Biomedia (Thailand) Co., Ltd. The frozen cells (EA.hy926) are thawed as a following protocol:

1) The frozen tube of endothelial cell line is briefly immersed in 37˚C water bath.
2) The cells are moved to 10 ml centrifugal tube containing completed Dulbecco's Modified Eagle's Medium (DMEM) and were subsequently centrifuged at 1,500 rpm for 5 minutes.
3) The supernatant was replaced with new completed DMEM 2 ml and was suspended gently.

4) Cells were seeded into T25 flask containing 8 ml of completed DMEM and were incubated overnight at 37°C in a humidified 5% CO_2-95% air mix environment.
5) After 24- hr incubation, the cells were washed with Phosphate-buffered saline (PBS) and were incubated in DMEM at for 7 days.
6) When the cells demonstrated 70-90% confluence, the cells were sub-cultured and were incubated at 37°C in a humidified 5% CO_2-95% air mix environment for further experiments.

2.2 The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test

The MTT assay is used to measure cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity [221]. A 5 mg/ml MTT solution was prepared by mixing MTT powder (from VWR life science®, United States of America) with DMEM freshly or within 24 hr before use. The cells were incubated with the MTT solution at 37 °C for 2 hours. After that, the supernatant was gently removed and 500 µL of dimethyl sulfoxide (DMSO) were applied to solubilize the purple formazan crystals. The mixing solution was mixed thoroughly, and was processed for measuring the absorbance using a microplate reader at the 570 nm wavelength. The relative cell viability of the sample is calculated as follow:

$$\text{Relative cell viability (\%)} = \left[\frac{(\mathbf{O.D.Treated}_{\mathbf{cell}}) - (\mathbf{O.D.Mean}_{\mathbf{blank}})}{(\mathbf{O.D.Mean}_{\mathbf{control}}) - (\mathbf{O.D.Mean}_{\mathbf{blank}})}\right] \times \mathbf{100}$$

2.3 Nitric oxide production measurement

The assessment of nitric oxide production was performed by using a direct and automation-ready procedure Nitric Oxide (NO) Assay Kit (ab272517, from Abcam Biotechnology company, United Kingdom). The measurement of NO production is based on the reduction of nitrate to nitrite, according to Griess method [222]. A premix 100 µM standard solution was freshly prepared by mixing 1 mM 50 µL Nitrite standard and 450 µL ultrapure water. Then, the standard was titrated in various dilutions as shown in Table III-1.

Table 0-4 Nitric oxide standard preparation.

Standard Number	Premix(µL)	H_2O	NO Concentration (µM)
1	**250**	**0**	**100**
2	**150**	**100**	**60**
3	**75**	**175**	**30**
4	**0**	**250**	**0**

Working reagent (WR) was freshly prepared, containing 100 µL reagent A, 4 µL reagent B and 100 µL reagent C. One hundred and fifty microliters of the medium of EA.hy.296 culture was de-proteinized by adding 8 µl $ZnSO_4$ to the sample, mixing and subsequent adding of 8 µl NaOH. The sample was mixed thoroughly and was subsequently centrifuged at 14,000 rpm for 10 minutes. After that, 100 µl clear supernatant was collected and 200 µl working reagent was added to the supernatant. The reaction was allowed for 60 minutes, at 37ºC. The sample tubes were centrifuged at 14,000 rpm for 10 minutes. Two hundred and seventy microliters of clear supernatant were collected. Ninety microliters of supernatant from each sample were transferred to 96-well plate and then was measured for the absorbance by using a microplate reader with an excitation wavelength of 540 nm. The standard curve was generated from the

standard test described in Table III. The assay with the R^2 value of < 0.95 was considered as unacceptable. The relative NO production (%) of the sample was calculated as follow:

$$\text{Relative NO production (\%)} = \left[\frac{(\text{O.D.Treated}_{\text{cell}}) - (\text{O.D.Mean}_{\text{blank}})}{(\text{O.D.Mean}_{\text{control}}) - (\text{O.D.Mean}_{\text{blank}})}\right] \times 100$$

3. Chemical reagent and solutions

3.1 Chemicals

ZD0947 (Made in India), pinacidil monohydrate (Made in India), nicorandil (Made in India) and glibenclamide (Made in India) were purchased from SIGMA-ALDRICH, Co., United States of America). Diazoxide (ab120266, from Abcam Biotechnology company, United Kingdom). The chemicals were prepared as 1 mM stock solution by dissolving in distilled water or dimethyl sulfoxide (DMSO). The final concentration of DMSO was less than 0.3% in all groups. St. Thomas cardioplegia was Siriraj hospital formula, containing 16 mM potassium chloride, 16 mM magnesium chloride and 1 mM procaine hydrochloride.

3.2 Solution preparation

Simulated ischemic solution

Simulated ischemic solution contains 140 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM HEPES, 10 mM 2-deoxy-d-glucose and 10 mM sodium dithionine.

High potassium crystalloid cardioplegia

High potassium crystalloid cardioplegia solution was prepared in Acetate Ringer's solution. Briefly, 20 ml of St. Thomas cardioplegia solution and 11 ml of 7.5% Sodium bicarbonate ($NaHCO^-_3$) were added to 1,000 ml of Acetate Ringer's solution,

giving the final potassium concentration of 20 mM. The solution was stored at 4°C until use. The components of solution are described in Table III-2.

Table 0-5 Chemicals and substances of hyperkalemic cardioplegia

Component	Volume (ml)
Acetate Ringer's solution (Containing Na^+ 130 mM, K^+ 4 mM, Ca^{2+} 2.7 mM and Cl^- 108.7 mM)	1,000
St. Thomas cardioplegia (Containing potassium chloride 16 mmol, magnesium chloride 16 mmol and procaine hydrochloride 1 mmol)	20
7.5%Sodium bicarbonate ($NaHCO^-_3$)	11

Phosphate buffered saline (PBS) solution

The PBS solution containing 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4 was prepared by using a commercial PBS tablet (Made in United Kingdom, purchased from Thermo Fisher Scientific, United States of America).

4. Statistical analysis

The data are expressed as percent and the mean ± standard error of mean (SEM). Analysis of Variance or ANOVA with LSD post hoc test were used to compare relative cell viability and related nitric oxide production. A p value less than 0.05 ($p<0.05$) was considered as statistical significance.

CHAPTER IV

RESULTS

1 Optimization

1.1 Optimization of the ischemia-reperfusion model

Ischemia-reperfusion model was optimized by varying ischemic time as described in Chapter III (Optimization of ischemia-reperfusion model). The result showed that the mean relative viability of endothelial cells dropped after the first 30 minute from the control. The cell viability decreased with time. However, cell viability was 1) 83.84 ± 4.24% in 30 min ($p = 0.037$ vs. control), 2) 81.24 ± 7.24% in 45 min ($p = 0.021$ vs. control), 3) 80.35 ± 3.31% in 60 min ($p = 0.009$ vs. control), 4) 64.72 ± 5.44% in 90 min ($p < 0.001$ vs. control), 5) 52.23 ± 5.94% in 120 min ($p < 0.001$ vs. control) and 6) 52.17 ± 6.55% in 150 min ($p < 0.001$ vs. control). The experimental performed triplicate (n = 3 x 3). The results were shown in Figure IV-1.

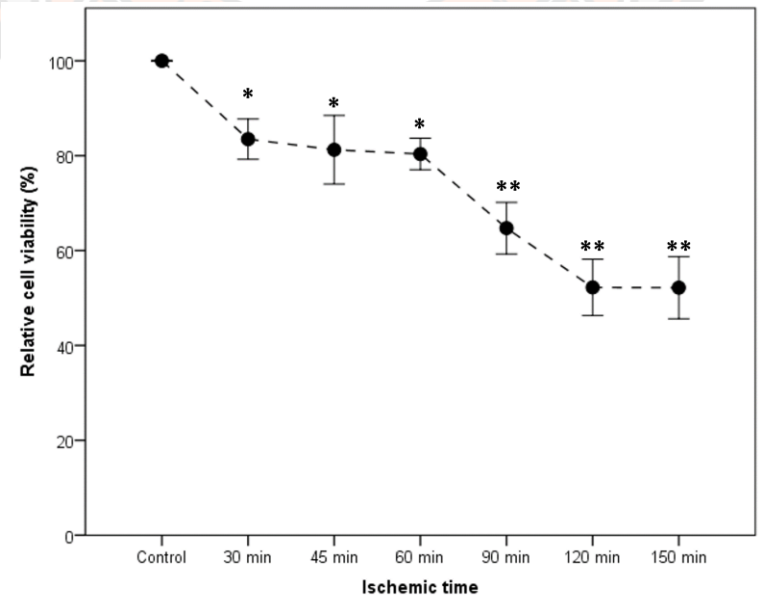


Figure 0-29 Effect of ischemic time on endothelial cell viability.

*; $p < 0.05$, **; $p < 0.001$ compared with control.

1.2 Optimization of potassium channel modulators

We optimized the concentration of four K_{ATP} channel modulators, including ZD0947, pinacidil, diazoxide, and nicorandil by using the protocol described in Figure III-2. Each experiment was performed triplicate (n = 3 x 3). The effect of each modulator is described below.

1.2.1) The optimization of ZD0947 concentration

ZD0947 at concentration 1 µM, 10 µM, 100 µM, 500 µM and 1 mM were used to observe their protective effect. The results showed that ZD0947 at the concentration of 1, 10 and 100 µM could increase cell viability significantly, $p < 0.001$, when compared to that of IR group. The protective effect was decreased in 500 µM groups ($p = 0.02$ vs. IR). The effect was lesser, not significant, in the 1 mM group ($p = 0.154$). However, ZD could not fully restored cell viability when compared to the control group.

The relative viability in IR group was 57.40 ± 3.20% ($p < 0.001$ vs. control) and relative cell viability in the treated groups were 1) 82.88 ± 5.17% for 1 µM ($p < 0.001$ vs. control and $p < 0.001$ vs. IR), 2) 79.35 ± 1.60% for 10 µM ($p < 0.001$ vs. control and $p < 0.001$ vs. IR), 3) 79.15 ± 1.37% in 100 µM ($p < 0.001$ vs. control and $p < 0.001$ vs. IR), 4) 67.08 ± 1.97% in 500 µM ($p < 0 .001$ vs. control and $p = 0.02$ vs. IR) and 5) 63.86 ± 2.47% in 1 mM ($p < 0.001$ vs. control and $p = 0.154$ vs. IR). Endothelial cells morphology and the effect of various ZD0947 concentrations were shown in Figure IV-2.

1.2.2) The optimization of pinacidil concentration

Pinacidil at concentration of 0.1 ,1, 10, 100, 500 µM and 1mM could increase cell viability significantly when compared to that of IR. Pinacidil at concentration 0.1-

500 µM increased cell viability dramatically, $p < 0.001$, when compared to the IR group. The protective effect was decreased in 1 mM groups.

The relative viability in IR group was 42.13 ± 1.66% ($p < 0.001$ vs. control) and relative cell viability in treatment groups were 62.71 ± 2.31% for 0.1 µM group ($p < 0.001$ vs. control, $p < 0.001$ vs. IR), 68.36 ± 3.18% for 1 µM group ($p < 0.001$ vs. control, $p < 0.001$ vs. IR), 71.81 ± 2.90% for 10 µM group ($p < 0.001$ vs. control, $p < 0.001$ vs. IR), 73.35 ± 3.84% for 100 µM group ($p < 0.001$ vs. control, $p < 0.001$ vs. IR) , 64.42 ± 2.84% for 500 µM ($p < 0.001$ vs. control, $p < 0.001$ vs. IR), and 58.32 ± 3.20% for 1mM ($p < 0.001$ vs. control, $p = 0.003$ vs. IR). Endothelial cells morphology and the effect of various pinacidil concentrations were shown in Figure IV-3.

1.2.3) The optimization of diazoxide concentration

Diazoxide at concentration of 0.1 µM,1 µM, 10 µM, 100 µM and 500 µM showed protective effects to the cells in response to IR. Diazoxide at concentration 1 - 100 µM increased cell viability dramatically, $p < 0.001$, when compared to the IR group ($p < 0.001$). The protective effect was decreased in 0.1 µM and 500 µM groups, $p = 0.006$ and $p = 0.003$, respectively.

The relative viability in IR group was 41.20 ± 6.65% ($p < 0.001$ vs. control). The relative cells viability affected by different concentrations was; 66.83 ± 5.60% for 0.1 µM ($p < 0.001$ vs. control, $p = 0.006$ vs. IR), 2) 79.11 ± 6.63% for 1 µM ($p = 0.008$ vs. control, $p < 0.001$ vs. IR), 3) 79.11 ± 6.63% for 10 µM ($p = 0.007$ vs. control, $p < 0.001$ vs. IR), 4) 77.25 ± 4.78% for 100 µM ($p = 0.003$ vs. control, $p < 0.001$ vs. IR), 5) 66.46 ± 5.80% for 500 µM ($p < 0.001$ vs. control, $p = 0.003$ vs. IR). Endothelial cells morphology and the effect of various diazoxide concentrations were shown in Figure IV-4.

1.2.4) The optimization of nicorandil concentrations

Nicorandil at 0.1 µM,1 µM, 10 µM, 100 µM and 500 µM could increase cell viability significantly when compared to that of IR. showed that nicorandil concentration ranging 0.1-100 µM could increase cell viability when compared to IR group. However, the cell viability tended to decrease, in 100 µM and 500 µM groups.

The relative viability in IR group was 49.32 ± 4.75% ($p < 0.001$ vs. control). And relative cell viability in IR with nicorandil groups were 1) 80.35 ± 3.83% for 0.1 µM ($p < 0.001$ vs. control, $p < 0.001$ vs. IR), 2) 74.96 ± 2.19% for 1 µM ($p < 0.001$ vs. control, $p < 0.001$ vs. IR), 3) 66.98 ± 2.19% for 10 µM ($p < 0.001$ vs. control, $p < 0.001$ vs. IR), 4) 66.21 ± 3.69% for 100 µM ($p<0.001$ vs. control, $p = 0.001$ vs. IR), 5) 43.95 ± 4.49% for 500 µM ($p < 0.001$ vs. control, $p = 0.270$ vs. IR). Endothelial cell morphology and the effect of various nicorandil concentrations were shown in Figure IV-5.

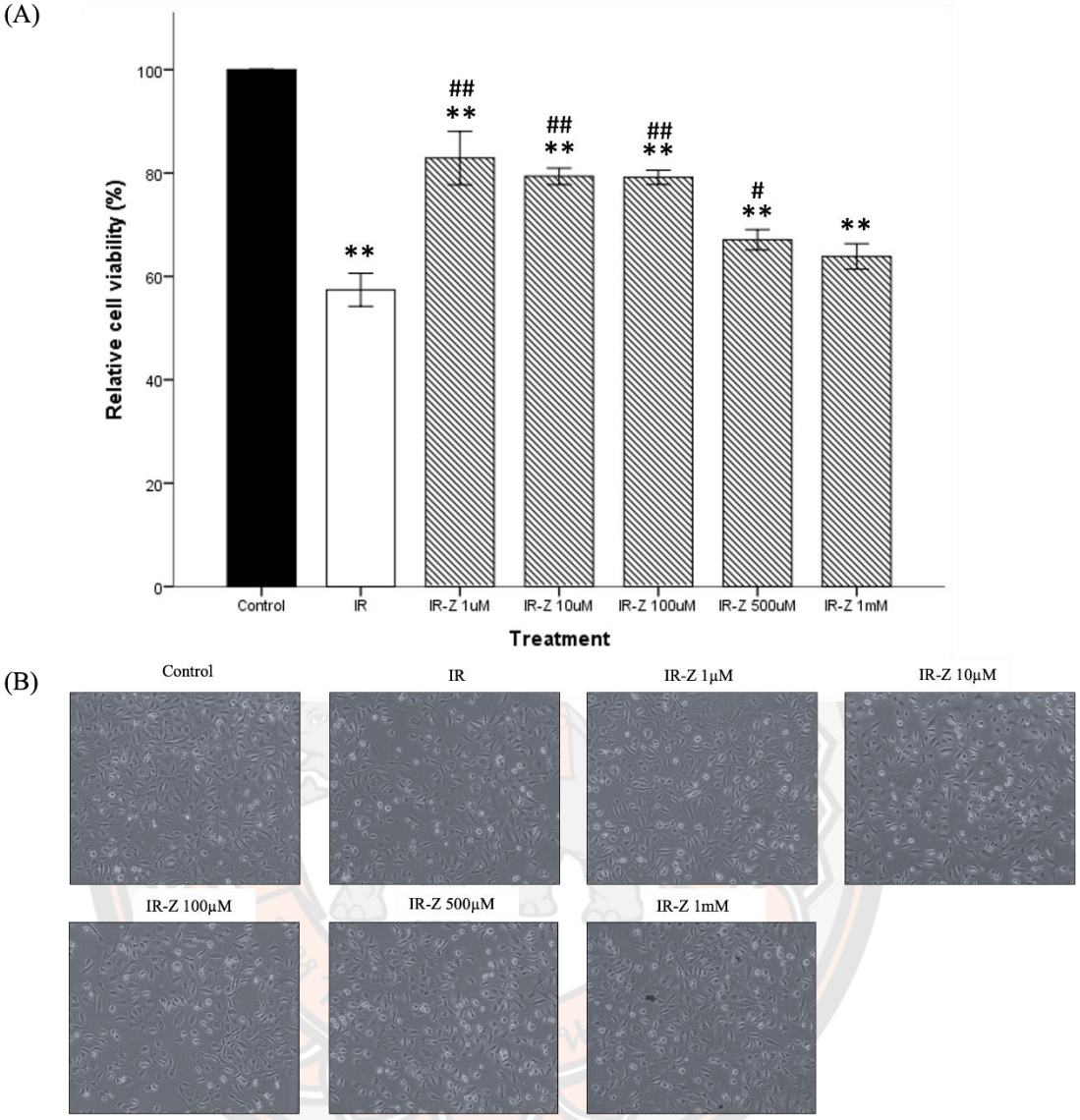


Figure 0-30 The effect of ZD0947 on cell viability and morphology according to its concentrations.

(A) Relative viability of endothelial cells after being treated with IR and ZD0947 in various concentrations. (B) Endothelial cell morphology after being treated with IR and IR with ZD0947. The images were taken under 10X magnification. *; $p < 0.05$ and **; $p < 0.001$ compared to control. #; $p < 0.05$ and ##; $p < 0.001$ compared to IR.

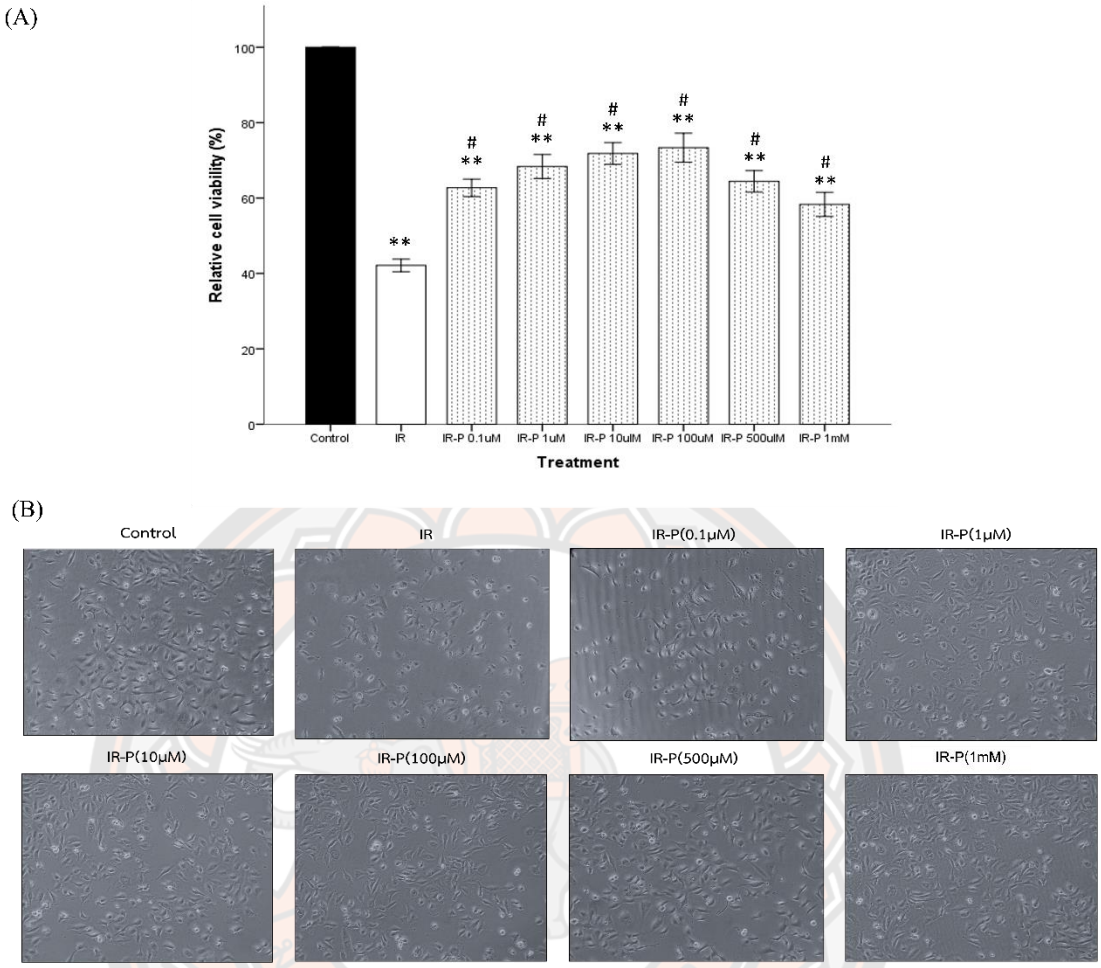


Figure 0-31 The effect of pinacidil on cell viability according to its concentrations.

(A) Relative viability of endothelial cells after being treated with IR and pinacidil in various concentrations. (B) Endothelial cell morphology after being treated with IR and IR with pinacidil. The images were taken under 10X magnification. *; $p < 0.05$ and **; $p < 0.001$ compared to control. #; $p < 0.05$ and ##; $p < 0.001$ compared to IR.

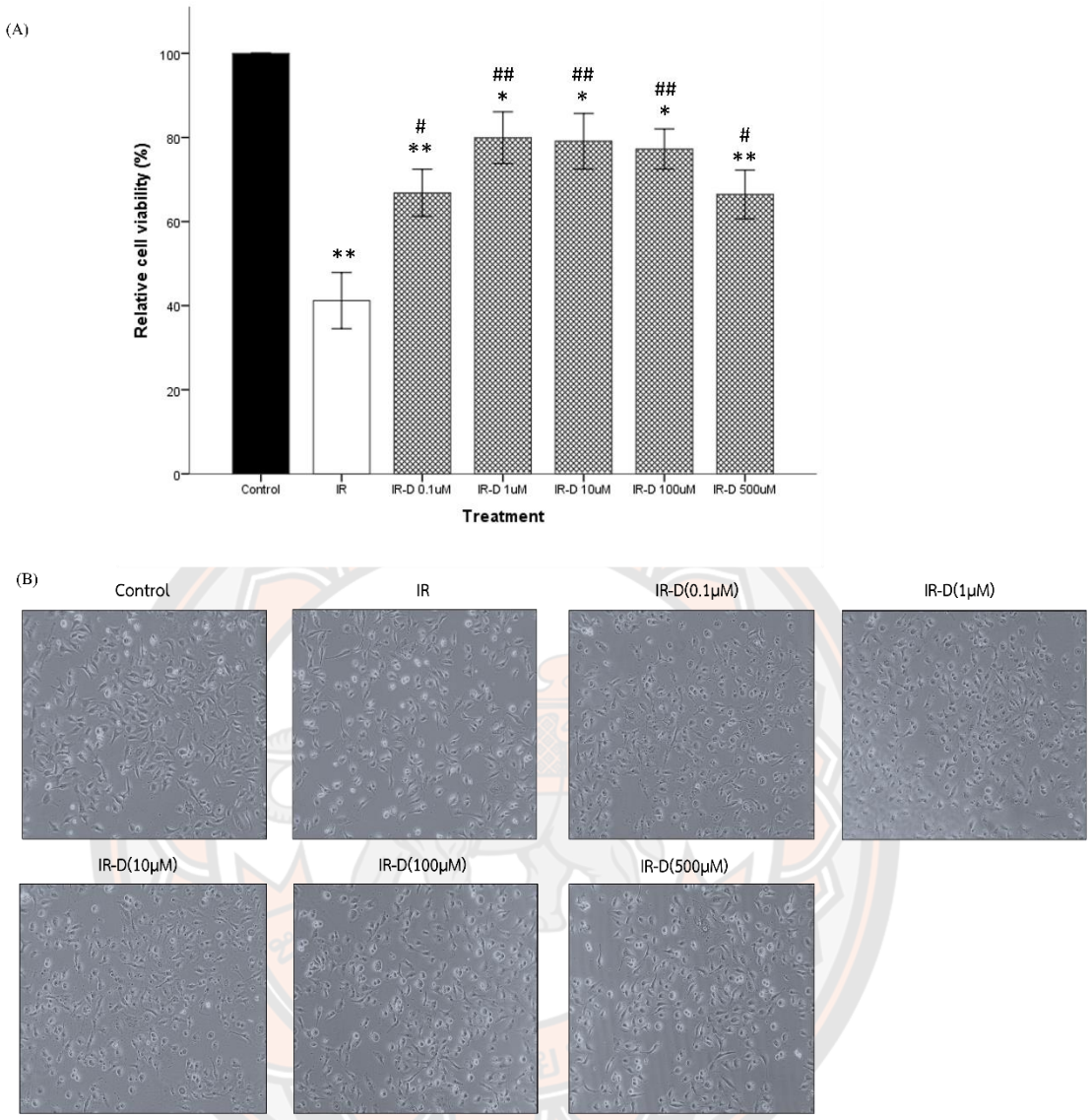


Figure 0-32 The effect of diazoxide on cell viability according to its concentrations.

(A) Relative viability of endothelial cells after being treated with IR and diazoxide in various concentrations. (B) Endothelial cell morphology after being treated with IR and IR with diazoxide. The images were taken under 10X magnification. *; $p < 0.05$ and **; $p < 0.001$ compared to control. #; $p < 0.05$ and ##; $p < 0.001$ compared to IR.

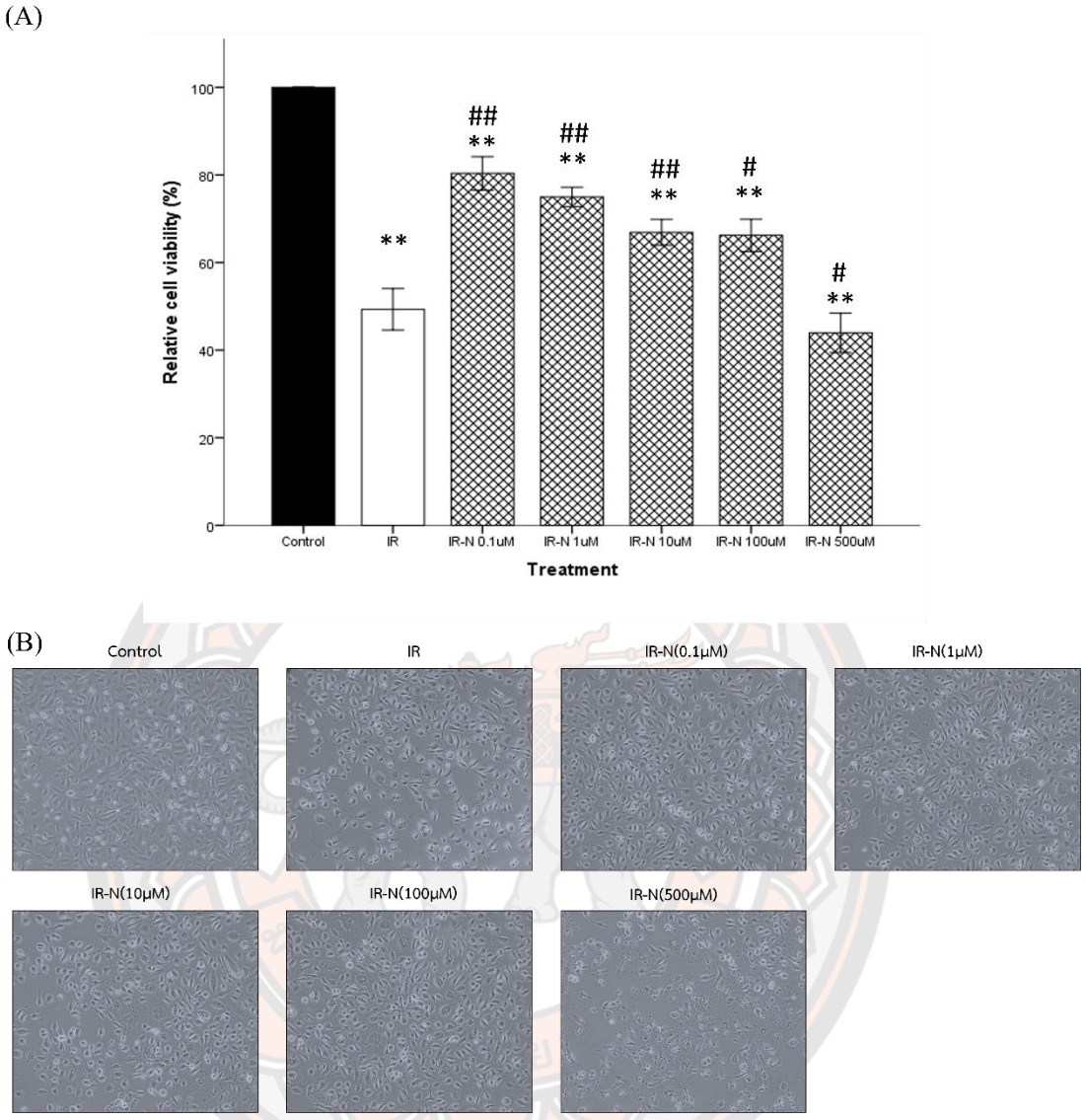


Figure 0-33 The effect of nicorandil on cell viability according to its concentrations.

(A) Relative viability of endothelial cells after being treated with IR and nicorandil in various concentrations. (B) Endothelial cell morphology after being treated with IR and IR with nicorandil. The images were taken under 10X magnification. *; $p < 0.05$ and **; $p < 0.001$ compared to control. #; $p < 0.05$ and ##; $p < 0.001$ compared to IR.

2. Effect of KATP activators on endothelial cell viability and NO production in response to ischemia-reperfusion.

After optimization, the effect of KATP activators on endothelial cell viability and NO production was assessed by using KCOs at the optimal concentration for each modulator. One µM of pinacidil, 1 µM of diazoxide, 1 µM and 10 µM of nicorandil, and 1 µM of ZD0947 were used. Glibenclamide at 10 µM was added to the KCOs solution to observe its inhibitory effects [193, 223].

2.1 Effect of KATP activators on endothelial cell viability in response to ischemia-reperfusion after 10 minutes of reperfusion

It was shown that 1 µM pinacidil, 1 µM diazoxide, 1 µM and 10 µM nicorandil could increase endothelial cell viability from the IR group but could not restore the cell viability to the control level.

The relative cell viability in the IR group was 47.25 ± 1.64% ($p < 0.001$ vs. control). Cell viability in KCOs groups was 1) 72.17 ± 5.04% for the IR-P group ($p < 0.001$ vs. control, $p < 0.001$ vs. IR, $p = 0.045$ vs. IR-N1, $p = 0.001$ vs. IR-N10 and $p < 0.001$ vs. IR-Z), 2) 81.99 ± 4.20% for IR-D group ($p = 0.001$ vs. control, $p < 0.001$ vs. IR, $p = 0.036$ vs. IR-Z), 3) 82.21 ± 5.18% for IR-N1 group ($p = 0.001$ vs. control, $p < 0.001$ vs. IR and $p = 0.04$ vs. IR-Z), 4) 89.07 ± 4.13% for IR-N10 group ($p = 0.03$ vs. control, $p < 0.001$ vs. IR), and 5) 92.48 ± 3.69% for IR-Z group ($p = 0.141$ vs. control, $p < 0.001$ vs. IR, $p < 0.001$ vs. IR-P, $p = 0.036$ vs. IR-D, $p = 0.04$ vs. IR-N1, and $p = 0.494$ vs. IR-N10). Adding glibenclamide could reduce the protective effect of KCOs in response to IR. However, it did not block the effect completely. The percentage of cell viability in different groups is as follows, 1) 65.20 ± 2.01% for IR-PG ($p < 0.001$ vs. control, $p < 0.001$ vs. IR, $p = 1.62$ vs. IR-P), 2) 71.58 ± 3.04% for IR-DG ($p < 0.001$

vs. control, $p < 0.001$ vs. IR, $p = 0.038$ vs. IR-D), 3) 76.25 ± 1.99% for IR-N1G ($p < 0.001$ vs. control, $p < 0.001$ vs. IR, $p = 0.232$ vs. IR-N), 4)74.96 ± 2.51% for IR-N10G ($p < 0.001$ vs. control, $p < 0.001$ vs. IR, $p = 0.005$ vs. IR-N10), and 5) 72.31 ± 5.73% for IR-ZG ($p < 0.001$ vs. control, $p < 0.001$ vs. IR and $p < 0.001$ vs. IR-Z). The experiment was carried out 4 times (n = 4 x 3). The results were shown in IV-6.

2.2 Effect of KATP activators on endothelial cell viability in response to ischemia-reperfusion after 24 hours reperfusion.

To test the effect of KCOs in the late phase, cell viability affected by different KCOs was evaluated after 24 hour-reperfusion. It was shown that the effect of IR still persisted after 24 hr. KCOs, including pinacidil, diazoxide, nicorandil and ZD0947 could increase cell viability when compared to that of the IR group, but they failed to restore the percentage of cell viability to the control level. The detail is as follows, cell viability in IR group was 34.88 ± 2.1% ($p < 0.001$ vs. control) and was 48.50 ± 2.09% in IR-P ($p < 0.001$ vs. control, $p < 0001$ vs. IR), 51.79 ± 2.40% in IR-D ($p < 0.001$ vs. control, $p < 0.001$ vs. IR), 49.13 ± 2.84% in IR-N10 ($p < 0.001$ vs. control, $p < 0001$ vs. IR), and 46.96 ± 2.44% ($p < 0.001$ vs. control, $p < 0001$ vs. IR). The experiment was performed triplicate 4 times (n = 4 x 3). The result was shown in Figure IV-7.

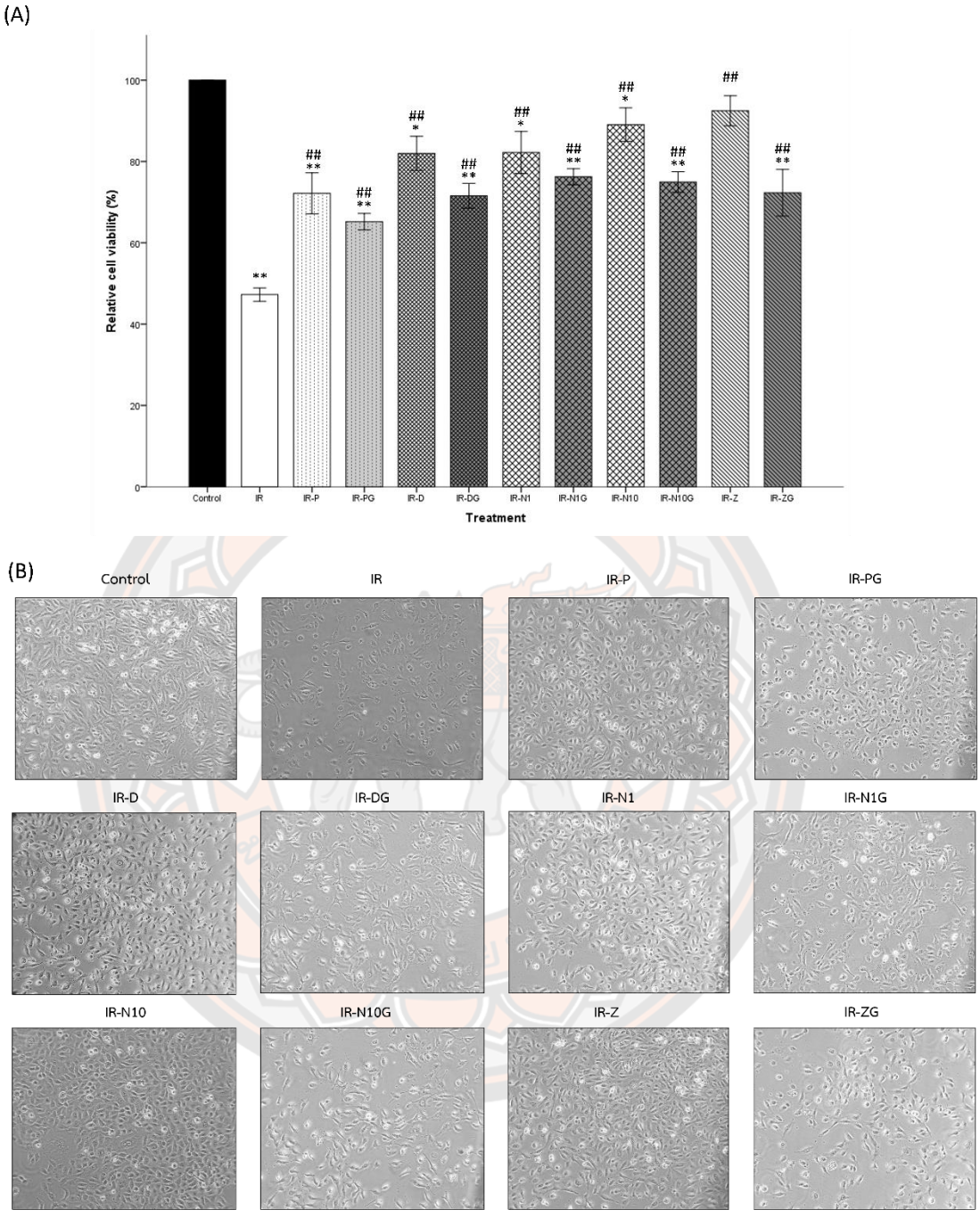


Figure 0-34 Effect of KATP activators on endothelial cell viability and cell morphology in response to ischemia-reperfusion.

(A) Relative viability of endothelial cells after being treated with IR and IR with KATP modulators. (B) Endothelial cell morphology after being treated with IR and IR with KATP modulators. The images were taken under 10X magnification. *; $p < 0.05$ and **; $p < 0.001$ compared to control. #; $p < 0.05$ and ##; $p < 0.001$ compared to IR.

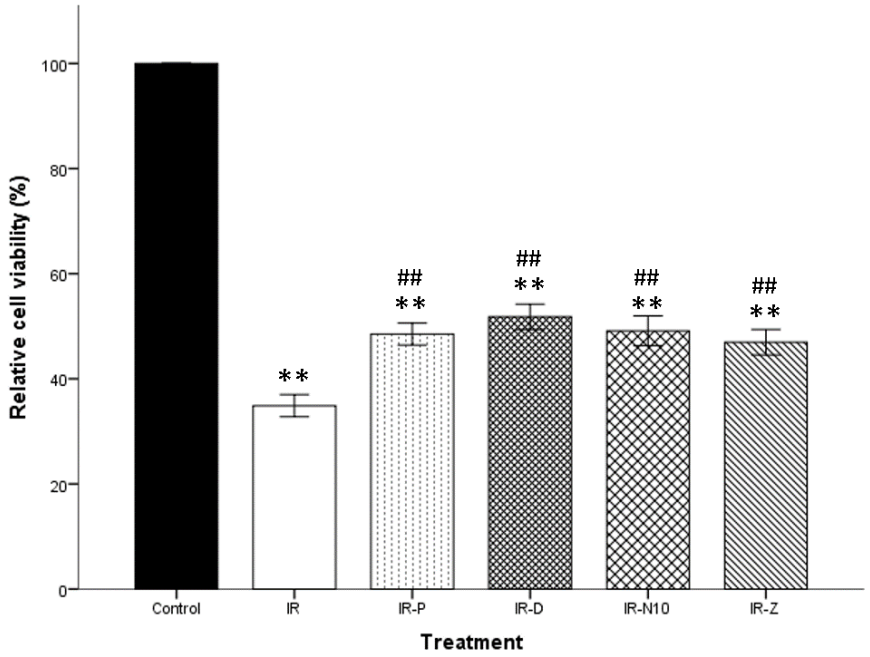


Figure 0-35 Effect of KATP activators on endothelial cell viability in response to ischemia-reperfusion after 24 hours reperfusion.

Relative viability of endothelial cells after being treated with IR and IR with KATP modulators. **; $p < 0.001$ compared to control. ##; $p < 0.001$ compared to IR.

2.3 Effects of KATP activators on endothelial cell-nitric oxide (NO) production in response to ischemia-reperfusion

2.3.1 Supernatant NO levels after 30 minutes of reperfusion

To assess the effect of KATP channel modulators on endothelial cell function, both in immediate and late response, supernatant NO level was measured after 30 -minute or 24 -hr reperfusion. It was found that the levels of supernatant NO were reduced in IR-P, IR-D, and IR-N10 groups, whereas the levels of IR and IR-Z groups did not significantly differ from that of the control after 30-minute reperfusion. The relative NO production of different groups is as below.

The NO levels of IR and IR-Z groups were 104.91 ± 3.98% and 104.67 ± 5.47%, respectively, and p = 0.33 and p = 0.35, compared to control, respectively). The relative nitric oxide concentration was 82.91 ± 2.92% in IR-P, 84.72 ± 3.84% in IR-D and 84.09 ± 3.39% in IR-N10 group. NO production in IR-P, IR-D and IR-N10 group were reduced significantly when compared to the control (p = 0.001, p = 0.005 and p = 0.002, respectively). The effect of KATP channel modulators on NO production was shown in Figure IV-8.

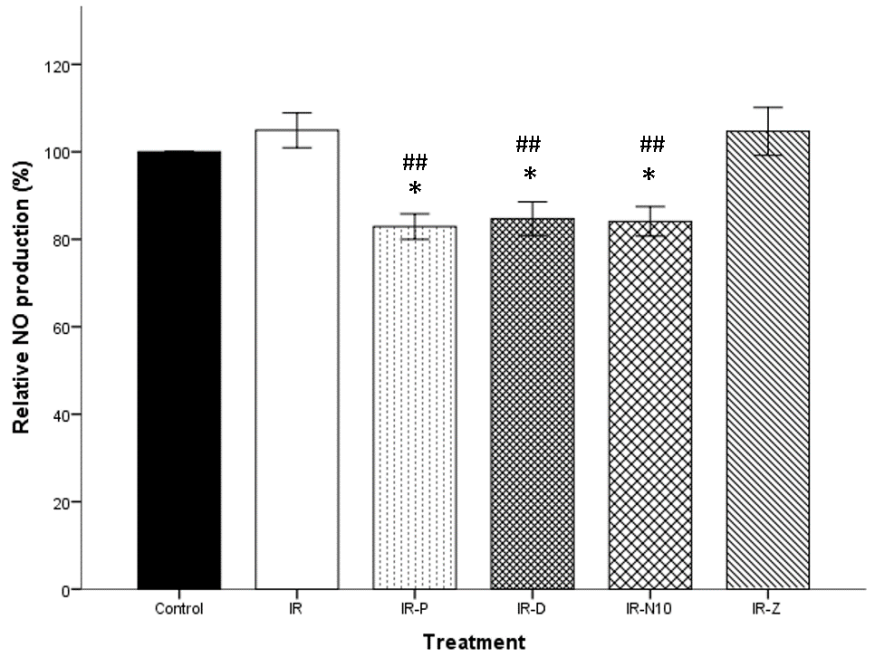


Figure 0-36 Effect of KATP activators on supernatant NO levels after 30-minute reperfusion.

Supernatant relative nitric oxide levels of control, IR, and KCO treated groups after 30 minutes of reperfusion. *; $p < 0.05$ compared to control and ##; $p < 0.001$ compared to IR.

2.3.2 Supernatant NO levels after 24 hours of reperfusion

The NO production was observed after 24 hours of reperfusion. It was found that the levels of supernatant NO were reduced in IR, IR-P, IR-D, and IR-Z groups, whereas the levels in IR-N10 groups did not significantly differ from that of the control. The results also showed that NO in IR-N10 and IR-Z groups were higher than that of the IR group significantly. The details are as below.

The relative NO concentrations in different groups were 65.67 ± 6.12%, 71.69 ± 4.82%, 76.75 ± 4.41%, 89.50 ± 5.42%, and 80.96 ± 2.60% in IR, IR-P, IR-D, IR-N10 and IR-Z, respectively. NO production in IR-P, IR-D and IR-Z groups was reduced when compared to that of the control ($p < 0.001$, $p < 0.001$ and $p = 0.002$, respectively). Nitric oxide levels in IR-N10 and IR-Z groups were reduced when compared to that of the IR group with $p < 0.001$ and $p = 0.002$, respectively. The effects of KATP channel modulators on NO production after 24 hours of reperfusion was shown in Figure IV-9.

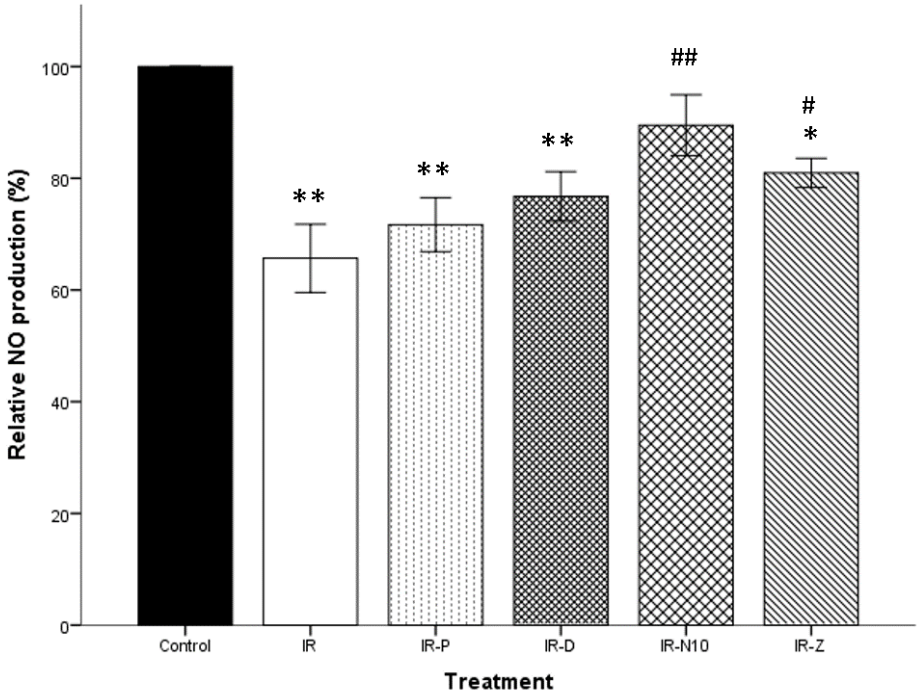


Figure 0-37 Effect of KATP activators on supernatant NO levels after 24 hours of reperfusion.

Relative nitric oxide levels of endothelial cell culture supernatant after 30 minutes reperfusion. *; $p < 0.05$ and **; $p < 0.001$ compared to control. #; $p < 0.05$ and ##; $p < 0.001$ compared to IR.

3. Effects of Kir6.1/SUR2B activators in high potassium cardioplegic condition on endothelial cells viability and endothelial NO production in response to ischemia-reperfusion

3.1 Effect of high potassium cardioplegia and KCOs-added cardioplegia

To investigate the effect of hyperkalemic cardioplegia on endothelial cells, cardioplegia administration was perfused to normoxia and post-ischemic endothelial cells. The results showed that cell viability in IR (82.78 ± 3.73%) and CPG (85.88 ± 1.39%) were decreased when compared to that of control ($p = 0.003$ and $p = 0.001$, respectively). The relative cell viability of the I-CPGR group (73.39 ± 1.63%)

dramatically decreased cell viability when compared to that of the control and CPG group ($p < 0.001$), but not different when compared to the IR group ($p = 0.571$).

To investigate the effect of high-potassium cardioplegia in the presence and absence of KATP activators. Different concentrations of KCOs were used. The relative cell viability in different groups of pinacidil was 1) 61.77 ± 4.51% for I-CPGR-P(1) groups ($p < 0.001$ vs. control, $p < 0.001$ vs. IR, $p < 0.001$ vs. CPG and $p = 0.006$ vs. I-CPGR), 2) 84.33 ± 3.81% for I-CPGR-P(100) group ($p = 0.001$ vs. control, $p = 0.795$ vs. IR, $p = 0.735$ vs. CPG and $p = 0.01$ vs. I-CPGR), and 3) 68.40 ± 5.35% for I-CPGR-P(1)G ($p < 0.001$ vs. control, $p = 0.19$ vs. IR, $p < 0.001$ vs. CPG and $p = 0.263$ vs. I-CPGR).

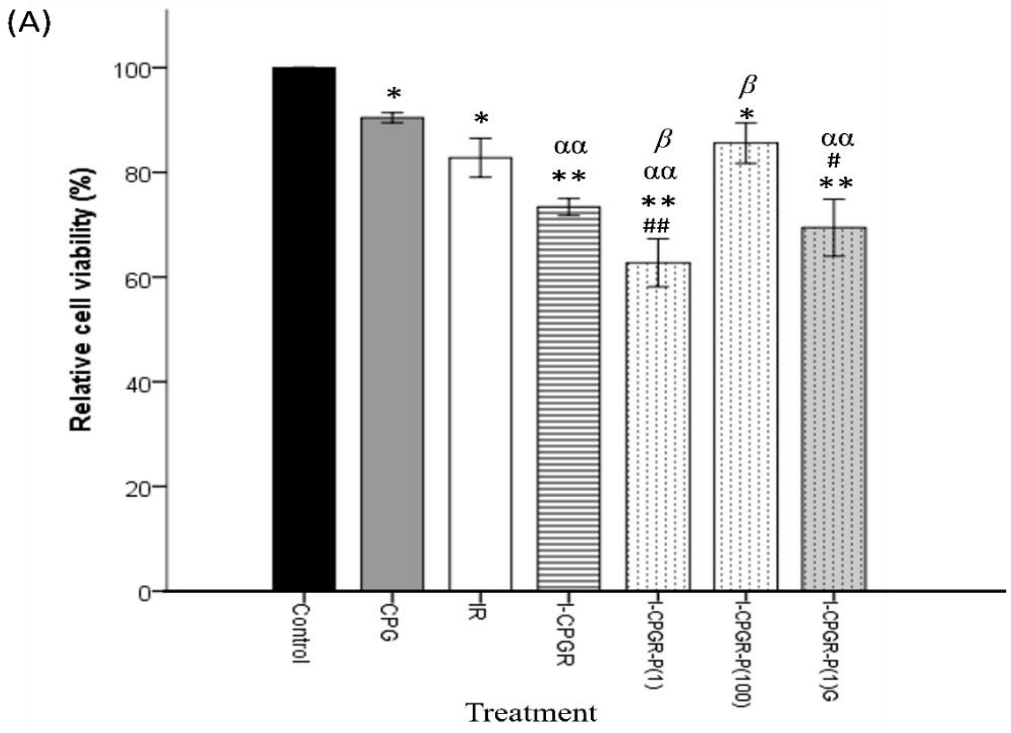
The relative cell viability in different groups of diazoxide was 1) 64.88 ± 4.32% for I-CPGR-D(1) group ($p < 0.001$ vs. control, $p = 0.003$ vs. IR, $p < 0.001$ vs. CPG, $p = 0.44$ vs. ICPGR), 2) 81.94 ± 3.84% for I-CPGR-D (100) group ($p < 0.001$ vs. control, $p = 0.89$ vs. IR, $p = 0.39$ vs. CPG and $p = 0.043$ vs. I-CPGR), and 3) 66.18 ± 4.85% for I-CPGR-D(1)G group ($p< 0.001$ vs. control, $p = 0.005$ vs. IR, $p < 0.001$ vs. CPG, $p = 0.09$ vs I-CPGR).

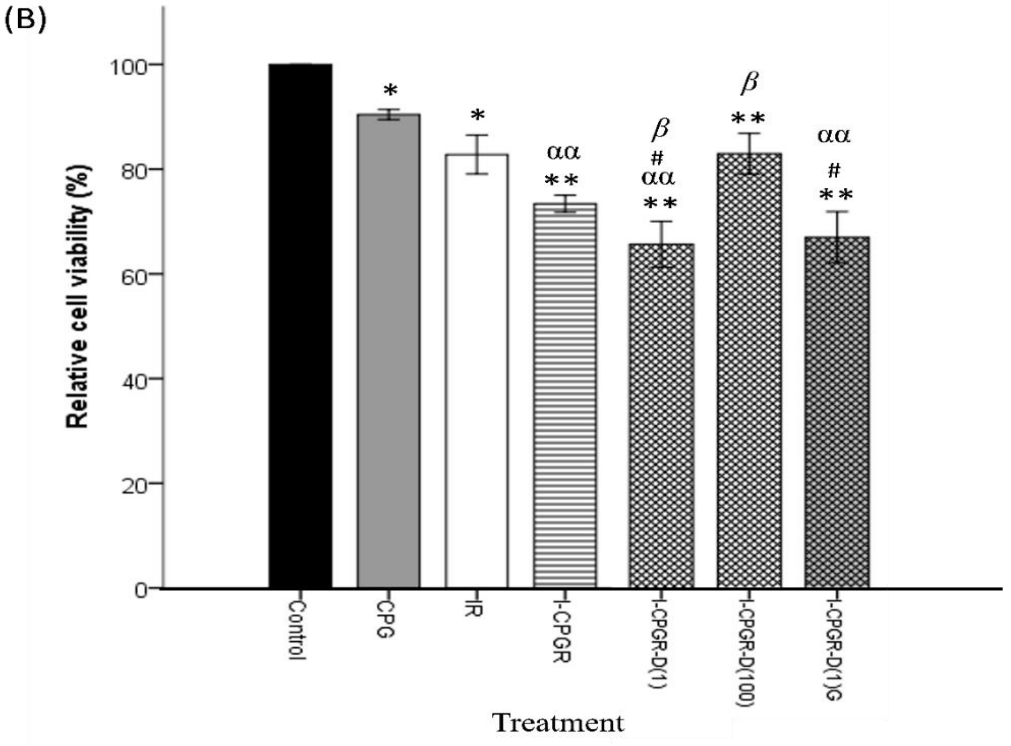
The relative cell viability in different groups of nicorandil was 1) 50.08 ± 4.43% for the I-CPGR-N(1) group ($p < 0.001$ vs. control, IR, CPG, and I-CPGR), 2) 55.71 ± 4.74% for I-CPGR-N(10) group ($p < 0.001$ vs. control, IR, CPG, and I-CPGR), 3) 77.86 ± 2.34% for I-CPGR-N(100) group ($p < 0.001$ vs. control, $p = 0.406$ vs. IR, $p = 0.08$ vs. CPG and $p = 0.29$ vs. I-CPGR), 4) 49.56 ± 4.45% for I-CPGR-N(1)G group ($p < 0.001$ vs. control, IR, CPG, and I-CPGR), and 5) 58.12 ± 4.57% for I-CPGR-N(10)G group ($p < 0.001$ vs. control, IR, CPG, and I-CPGR).

The relative cell viability in different groups of ZD0947 was 1)50.31 ± 3.72% for I-CPGR-Z(1) group ($p < 0.001$ vs. control, IR, CPG, and I-CPGR), 2) 73.33 ± 2.95% for I-CPGR-Z(100) group ($p < 0.001$ vs. control, $p = 0.11$ vs. IR, $p = 0.007$ vs. CPG and $p = 0.99$ vs. I-CPGR), and 3) 46.97 ± 4.46% for I-CPGR-Z(1)G group ($p < 0.001$ vs. control, IR, CPG, and I-CPGR).

The relative cell viability in different groups of glibenclamide 100 μM (I-CPGR-G(100) was 59.35 ± 1.44% ($p < 0.001$ vs. control, $p = 0.002$ vs. IR, $p < 0.001$ vs. CPG and $p = 0.02$ vs. I-CPGR).

The experiment was performed 5 times (n = 5 x 3). The results were shown in Figure IV-10.


(A)
Relative cell viability (%)
100
80
60
40
20
0
*
*
αα
**
β
αα
**
##
β
*
αα
#
**
Control
CPG
IR
I-CPGR
I-CPGR-P(1)
I-CPGR-P(100)
I-CPGR-P(1)G
Treatment


(B)
Relative cell viability (%)
100
80
60
40
20
0
*
*
αα
**
β
#
αα
**
β
**
αα
#
**
Control
CPG
IR
I-CPGR
I-CPGR-D(1)
I-CPGR-D(100)
I-CPGR-D(1)G
Treatment

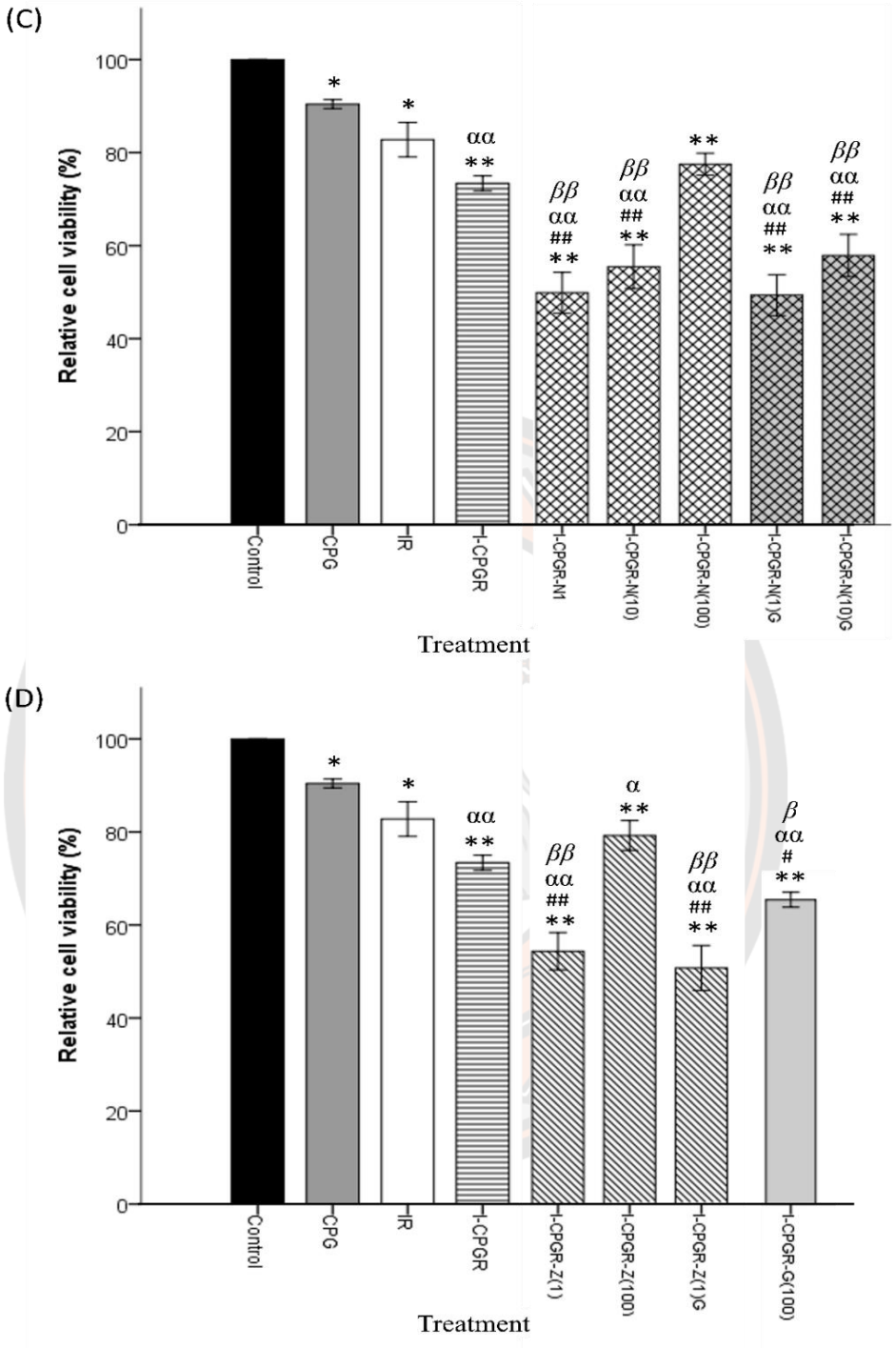


Figure 0-38 Effect of cardioplegia and KCOs-added in cardioplegia on endothelial cell viability.

Relative endothelial cell viability after being treated with IR, CPG, I-CPGR, and I-CPGR with KATP modulator condition. (A) pinacidil, (B) diazoxide, (C) nicorandil, (D) ZD0947 and glibenclamide. *; $p<0.05$ and **; $p<0.001$ compared to control. #; $p < 0.05$, ##; $p < 0.001$ compared to IR. α; $p < 0.05$ and αα; $p < 0.001$ compared to CPG. β; $p < 0.05$ and $\beta\beta < 0.001$ compared to I-CPGR.

3.2 The assessment of in Effect of ZD0947 and nicorandil on endothelial cell viability and nitric oxide level in response to IR and cardioplegia in KCOs-added to reperfusion solution condition.

To assess the protective effect of Kir6.1/SUR2B or ZD0947 100μM and nicorandil 100μM after ischemia and hyperkalemic cardioplegia, we used protocol that was described in Figure III-6.

3.2.1) Effect of ZD0947 and nicorandil on endothelial cell viability after ischemia and cardioplegia reperfusion

We simulated ischemia and cardioplegia condition, and reperfusion for 30 minutes and 24 hours. After reperfusion for 30 minutes, the results showed that cardioplegia (CPG) alone can preserve endothelial cell viability, 97.99 ± 1.75%, $p = 0.412$ when compared to that of control. Ischemia-reperfusion (IR) decreased relative viability to 82.55 ± 1.76% ($p < 0.001$ vs. control and CPG). Ischemia-reperfusion with cardioplegia (I-CPGR) injure endothelial cells, relative cell viability in the I-CPGR group was 67.38 ± 3.22% ($p < 0.001$ vs. control, CPG and IR). Endothelial cell viability in KCOs groups was 1) 74.17 ± 1.65 in I-CPGR-N group ($p < 0.001$ vs. control and IR, $p = 0.001$ vs. IR and $p = 0.013$ vs. I-CPGR) and 2) 74.11 ± 1.62% in I-CPGR-Z ($p < 0.001$ vs. control and IR, $p = 0.001$ vs. IR, $p = 0.013$ vs. ICPGR). It was shown that ZD0947 provided endothelial cell viability same level as the I-CPG-N group ($p = 0.994$). An experiment was performed 5 times ($n = 5 \times 3$). The results were shown in Figure IV-11.

After reperfusion for 24 hours, it was found that the cell viability of the CPG group did not different from the control. However, the cell viability of IR, I-CPGR, and ICPGR with modulators was lower than the control notably. The relative cell viability of all groups is as follows, 1) 97.82 ± 1.49% for CPG group ($p = 0.395$ vs. control and $p < 0.001$ vs. IR, I-CPGR, I-CPGR-N, and I-CPGR-Z), 2) 86.72 ± 1.77% for IR group ($p < 0.001$ vs. control and CPG, $p = 0.02$ vs. I-CPGR, $p = 0.366$ vs. I-CPGR-N and $p = 0.002$ vs. I- CPGR-Z), 3) 80.83 ± 2.20% for I-CPGR group ($p < 0.001$ vs. control and CPG, $p = 0.024$ vs. IR, $p = 0.18$ vs. I-CPGR-N, and $p = 0.353$ vs. I-CPGR-Z), 4) 84.35 ± 2.95% for I-CPGR-N group ($p < 0.001$ vs. control and CPG, $p = 0.366$ vs. IR, $p = 0.180$ vs. I-CPGR), and 5) 78.40 ± 1.27% in I-CPGR-Z ($p < 0.001$ vs. control and CPG, $p = 0.002$ vs. IR, $p = 0.353$ vs. I-CPGR and $p = 0.028$ vs. I-CPGR-N). An experiment was performed 5 times ($n = 5 \times 3$). The summary of the results was shown in Figure IV-12.

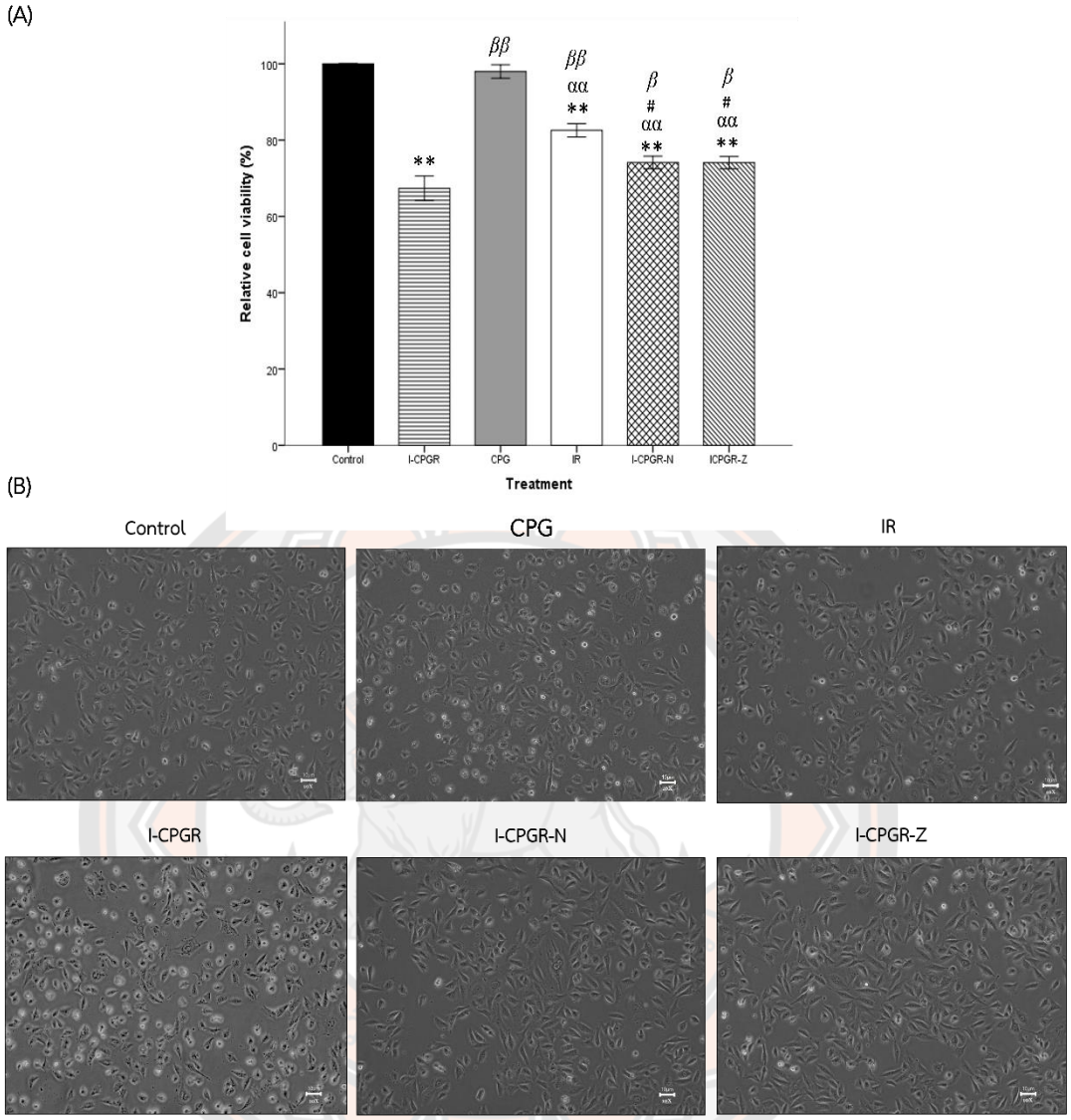


Figure 0-39 Effect of ZD0947 and nicorandil on endothelial cell viability in response to IR and cardioplegia in KCOs-added to reperfusion solution after 30 minutes of reperfusion.

(A) Relative endothelial cell viability after simulated ischemia-cardioplegia-reperfusion condition, after reperfusion for 30 minutes. (B) Endothelial cell morphology after treatment and reperfusion for 30 minutes. The images were taken under 10X magnification. **; $p < 0.001$ compared to control. #; $p < 0.05$ compared to IR. α; $p < 0.05$ and αα; $p < 0.001$ compared to CPG. β; $p < 0.05$ compared to I-CPGR.

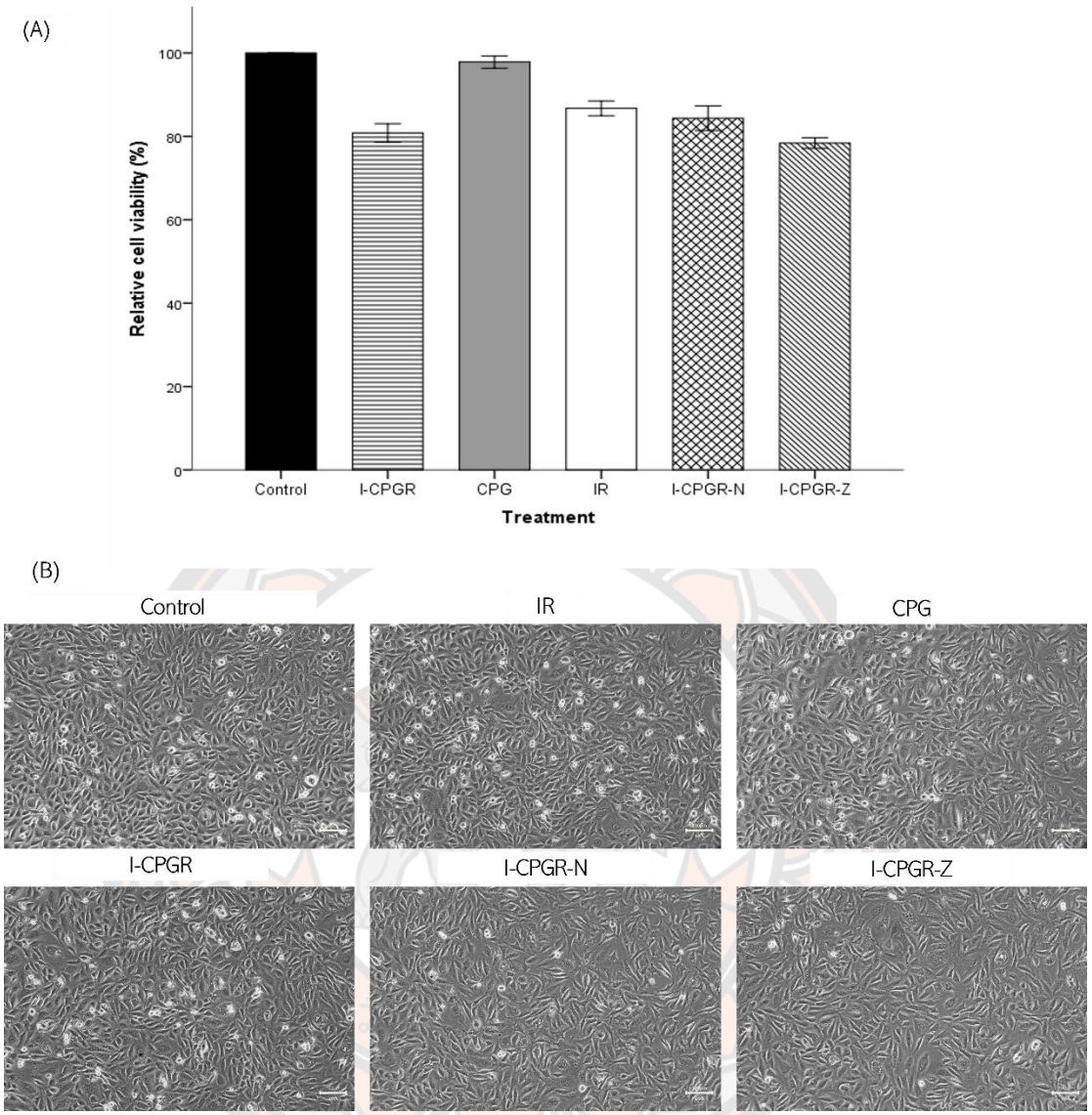


Figure 0-40 Effect of ZD0947 and nicorandil on endothelial cell viability in response to IR and cardioplegia in KCOs-added to reperfusion solution after 24 hours reperfusion.

(A) Relative endothelial cell viability after simulated ischemia-cardioplegia-reperfusion condition, after reperfusion for 24 hr. (B) Endothelial cell morphology after treatment and reperfusion for 24 hr. The images were taken under 10X magnification. **; $p < 0.001$ compared to control. #; $p < 0.05$ compared to IR. αα; $p < 0.001$ compared to CPG. β; $p < 0.05$ and $\beta\beta$; $p < 0.001$ compared to I-CPGR.

3.2.2) Effect of ZD0947 and nicorandil on supernatant nitric oxide levels in response to IR following cardioplegia administration

To assess the effect of ZD0947 and nicorandil on endothelial cell functions, the activators were applied after cardioplegia administration, and NO production was observed within 30 minutes and 24 hr after reperfusion.

It was found that after 30-minute reperfusion, the levels of supernatant NO in CPG, IR, and I-CPGR groups tended to be lower, but not statistically different when compared to control. Surprisingly, the nitric oxide levels in I-CPGR-N and I-CPGR-Z groups were increased when compared to control and I-CPGR ($p < 0.001$).

The NO level of the I-CPGR group was 97.86 ± 2.71% ($p = 0.73$ vs. control). The relative NO level of different group was 1) 90.83 ± 2.97% in the CPG group ($p = 0.143$ vs. control and $p = 0.257$ vs. I-CPGR), 2) 88.44 ± 6.49% in the IR group ($p = 0.068$ vs. control and $p = 0.133$ vs. I-CPGR, 3) 150.08 ± 4.44% in I-CPGR-N group ($p < 0.001$ vs. control and I-CPGR), and 4)140.97±4.61% in I-CPGR-Z group ($p < 0.001$ vs. control and I-CPGR). The results demonstrate that NO levels in I-CPGR-N and I-CPGR-Z were increased at the same level ($p = 0.112$). The results were shown in Figure IV-13(A).

The late effect of cardioplegia and KCOs was assessed 24 hr after reperfusion. It was found that the levels of supernatant NO in treatment groups were not different when compared to the control. The relative NO level after 24 hours in I-CPGR was 103.58 ± 3.28% ($p = 0.563$ vs. control). NO level in different groups was 1)94.58 ± 4.69% in CPG group ($p = 0.389$ vs. control and $p=0.154$ vs. I-CPGR), 2)92.72 ± 5.32% in IR group ($p = 0.241$ vs. control and $p=0.081$ vs. I-CPGR), 3)108.51 ± 4.66% in I-CPGR-N ($p = 0.171$ vs. control and $p = 0.427$ vs. I-CPGR), and 5)109.04 ± 5.95% in I-CPGR-Z group ($p = 0.152$ vs. control and $p = 0.385$ vs. I-CPGR). The results demonstrate that

NO levels in I-CPGR-N and I-CPGR-Z were increased at the same level (p = 0.932). The results were shown in Figure IV-13(B).

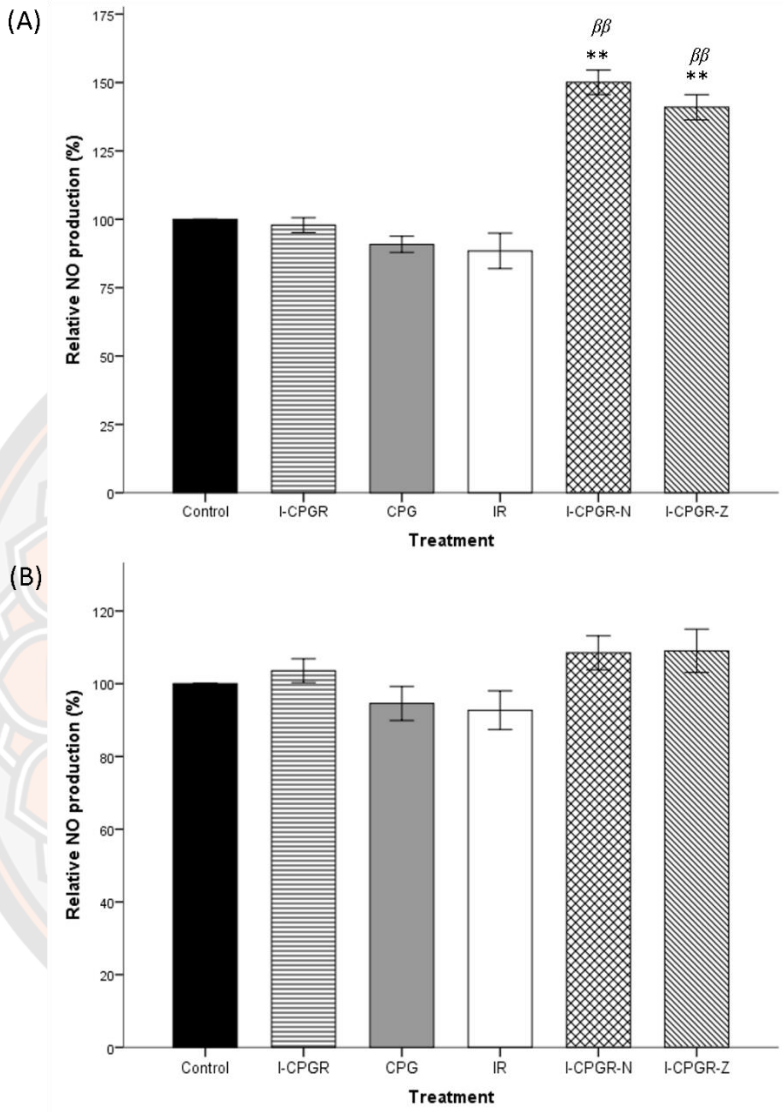


Figure 0-41 Effect of ZD0947 and nicorandil on supernatant nitric oxide level after IR with cardioplegia and minute reperfusion.

(A)Relative nitric oxide production after 30 minutes. (B) Relative nitric oxide production after 24 hours of reperfusion. **; $p < 0.001$ compared to control. $\beta\beta$; $p < 0.001$ compared to I-CPGR.

CHAPTER V

DISCUSSIONS AND CONCLUSIONS

Discussions

The objectives of this study are to investigate the effects of K_{ATP} activators, especially, Kir6.1/SUR2B KCO on endothelial cell viability and NO production in response to ischemia-reperfusion injury, in the presence and absence of hyperkalemic cardioplegia. The main findings of the present study are;

1. K_{ATP} activators including ZD0947, pinacidil, diazoxide, and nicorandil can preserve endothelial cell viability in response to IR both in short and long terms. One micromolar of pinacidil, diazoxide, and nicorandil improves cell viability against IRI, although not completely. Surprisingly, ZD0947 could restore endothelial cell viability to the same extent as the control. However, it was found that NO production in pinacidil, diazoxide, and nicorandil groups significantly decreased after 30 minute-reperfusion, whereas that in IR and ZD0947 groups was not altered. After 24 hr reperfusion, the effect of IR on cell viability remained. Pinacidil, diazoxide, nicorandil, and ZD0947 partially restored the viability whereas NO levels in the IR group dropped dramatically. Nicorandil and ZD0947 could increase NO production when compared with the IR group.

2. Hyperkalemic cardioplegia preserved endothelial cell viability and NO level to the same level as the control. However, cardioplegia perfusion following ischemia led to more endothelial cell death in both immediate and late response whereas NO levels did not change.

3. K_{ATP} modulators concordant with cardioplegia provided different effects on endothelial cells.

Our study suggested that IR led to endothelial cell death both in the early and late phases. Endothelial cell death may be involved in apoptosis and necrosis mechanism. Kalogeris T., et al., (2014) suggested that both necrosis and apoptosis are generally found in ischemia-reperfusion cells [224]. There are relevant studies that reported that apoptotic activity is an early programmed cell death which was rapidly generated on endothelial cells after ischemia -reperfusion [225, 226]. Scarabelli, T., et al., (2001) investigated the cell death mechanism from ischemic-reperfusion condition by using rat hearts which were subjected to 35 minutes of global ischemia followed by reperfusion for 5, 60, and 120 minutes, and found that apoptosis activities were detected; caspase 3 were positively stained on endothelial cells at 5 minutes after reperfusion. The number of endothelial cells positive for cleaved C3 peaked at 60 minutes after reperfusion [225]. In addition, Scarabelli TM., et al., (2002) investigated caspase-9, an indicator for mitochondrial damage, and showed that caspase-9 was increased in endothelial cells since initial reperfusion for 5 minutes and rose to the peak level after 60 minutes of reperfusion. It was reported that endothelial cell death was related to mitochondria injury and caspase-9 release, which contributed to apoptosis programmed cell death activation [226]. There is a report suggesting that caspase-3 and caspase-9 activation are common factors for apoptosis activation [227]. Increased intracellular calcium and ROS generation during ischemia-reperfusion induce mitochondrial permeability transition opening, leading to ATP depletion and cell swelling and bursting, plasma membrane damage, and subsequent necrosis [224, 228]. Although apoptosis can occur without inflammation apoptosis itself degrade DNA, which activates inflammation leading to necrosis activation [228]. Moreover, the concomitant of apoptosis and necrosis, necroptosis or necrapoptosis, was thought to be

involved in cell death. [224, 228]. Cytochrome-C was believed to be downstream of the caspases and other apoptosis mediators, which leads to apoptosis cell death. ATP reduction occurring during the downstream apoptotic signaling pathway blocks caspase activation, resulting in the opening of a glycine-sensitive organic anion channel which is associated with plasma membrane rupture and is the onset of necrosis cell death [228]. Our results demonstrated that the confluency of endothelial cells decreased dramatically in the late phase. It was hypothesized that apoptosis and necrosis continued even in the late phase. This result is relevant to previous studies showing that 120 minutes of ischemia followed by 24 hours of reperfusion could reduce the confluency of endothelial cells [229, 230]. Moreover, Wang, W. Z., (2008) reported that IRI could induce both necrosis and apoptosis on isolated skeletal muscle from rats in 24 hours of reperfusion. Noteworthy, apoptosis seems to be a predominant mechanism in this case [231].

The unchanged level of NO in response to IR in the early phase was thought to be involved in the inducible nitric oxide synthase/nitric oxide signaling pathway (iNOS/NO). The result is relevant to the previous studies indicating that NOS activities instantly increased in ischemic-isolated rabbit hearts and declined after prolonged reperfusion [232]. In addition, Taimor, G., et al.(2000) demonstrated that overexpression of NO production in ischemic-isolated cardiomyocytes led to apoptosis and necrosis [233]. Nitric oxide is an important mediator for numerous molecular targets, which can be generated by three different isoforms, that is; 1) neuronal nNOS (or NOS I), 2) inducible iNOS (or NOS II), and 3) endothelial eNOS (or NOS III) [234, 235]. nNOS is specific in neurons of the central nervous system [234]. Whereas eNOS plays a role in eNOS-derived NO from endothelial cells to smooth muscle [234].

Remarkably, studies reported that iNOS was associated with inflammation and IRI [234, 236]. Yu, X., et al., (2018) suggested that during IR, iNOS produced excessive NO, which is related to increased production of reactive oxygen species (ROS), peroxynitrite (OONO−), and superoxide, resulting in increased apoptosis and released pro-inflammatory response [237]. Weiser, M R., et al., (1996) suggested that inflammation following IR could bring about irreversible cell injury and necrosis on vascular endothelium [238]. Wu L., et al., (2020) suggested that NO^-release following IR caused lipid peroxidation generation, leading to inflammation and apoptosis [239]. The proposed mechanism of IR on endothelial cell death was shown in Figure V-1.

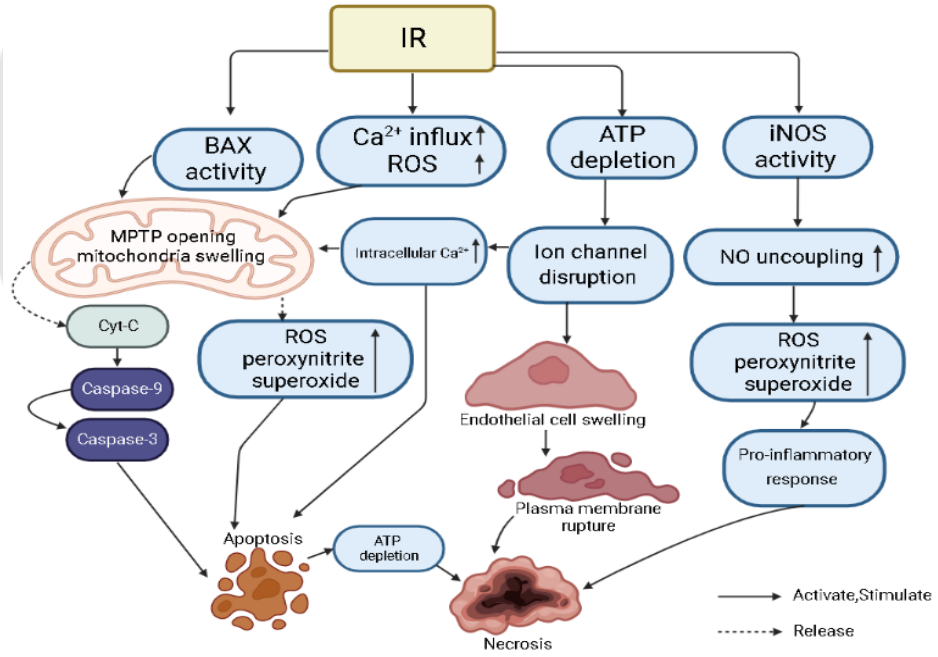


Figure 0-42 The effects of IR on endothelial cell

IR; ischemic-reperfusion, Cyt-C; Cytochrome c, Ca^{2+}; calcium ion, ROS; reactive oxygen species, BAX; B-cell lymphoma 2 associated X protein. Created by Biorender.com

There are relevant studies suggesting the effect of pinacidil on cardiomyocytes in response to IRI. Critz, S. D., et al., (1997) reported that pinacidil 0.1 µM induced K_{ATP}

channel activity but concentrations at 1, 10, 100 μM and 1 mM increase more K_{ATP} channels activation on rabbit cardiomyocytes in mild ischemic conditions, and induced more activity during severe ischemia [240]. Fifty micromolar of pinacidil can attenuate calcium overloading during ischemia and reperfusion in isolated rat heart [241, 242], and reduced rabbit cardiomyocyte cell death during ischemia [240]. One hundred micromolar of pinacidil reduced rabbit lung injury from IRI and decreased myeloperoxidase activity which is related to reduced ROS and inflammation [243]. However, there are a few studies investigating the effect of pinacidil on endothelial cell viability during IR. Therefore, our optimization could provide information about the effective doses of pinacidil.

These results demonstrated that pinacidil reduced the harm of IRI on endothelial cell viability. The recent study supports previous studies finding that pinacidil did not increase cyclic GMP or cyclic AMP levels in rat aorta [244, 245] and rabbit aorta [246], and pinacidil was not associated with the NO/cGMP/PKG signaling pathway and endothelium-derived nitric oxide (EDNO) [244-247]. Nakajima S., et al., (1989) demonstrated the effect of 100 μM of pinacidil on the thoracic artery of rabbits. They found that pinacidil induced vasorelaxation through decreased intracellular Ca^{2+} of smooth muscle cells, but was not associated with calcium-calmodulin (CaM) formation and guanosine 3,5-cyclic monophosphate (cGMP) [246]. Calcium-calmodulin is an essential activator that stimulates eNOS activity and leads to NO production from endothelial cells [152]. NO diffused to smooth muscle cells and bound guanylyl cyclase (GC), to convert guanosine 5’ triphosphate (GTP) to cGMP, resulting in PKG activation and membrane hyperpolarization and relaxation [153]. Therefore, we hypothesized that the effects of pinacidil does not increase NO level since the NO/cGMP/PKG signaling

pathway, CaM activation and endothelium-derived nitric oxide (EDNO) were not involved, which is correlated with the prior studies [244-247].

The mechanism of the protective effect of pinacidil on endothelial cell viability remains unclear. It was hypothesized that pinacidil, as a non-selective KCO, could activate both sarcK_{ATP} and mitoK_{ATP} channels, which led to the restoration of mitochondrial energy metabolism by closing the mitochondrial transition pore (MTP), stabilizing the mitochondrial membrane potential (MMP) and reducing ROS generation, decreased intracellular Ca^{2+} level [248, 249]. Choi Eun Mi., et al., (2014) reported that 0.01, 0.1, and 1 μM of pinacidil could protect osteoblasts cell death from antimycin-A cytotoxicity, and 0.01 and 0.1 μM of pinacidil activated the PI3K/Akt/CREB signaling pathway, a pro-survival signal. Moreover, Choi Eun Mi., et al., (2014) demonstrated that pinacidil at concentrations of 0.01 and 0.1 μM attenuated mitochondrial superoxide levels from the effect of antimycin A [250]. The PI3K/Akt/CREB signaling pathway is a signal transduction pathway, which is started with the activation of receptor tyrosine kinases (RTKs) on the cell membrane, leading to phosphoinositide 3 kinase (PI3K) activation and conversion of phosphatidylinositol (3,4)-bisphosphate (PIP2) lipids to phosphatidylinositol (3,4,5)-trisphosphate (PIP3), resulting in protein kinase B (Akt) activation [251]. The Akt inhibits caspase 9 activation and inactivates pro-apoptosis including GSK-3β [251, 252]. Moreover, Akt activates cAMP-response element binding protein (CREB), resulting in survival gene transcription and inhibiting apoptosis program [251-254]. A study reported that pinacidil activated the mitoK_{ATP} channel, induced mitochondrial membrane depolarization, and decreased transmembrane potential difference. The opening of mitoK_{ATP} lower Ca^{2+} influx to mitochondria [255]. In addition, the role of pinacidil in

the attenuation of ROS and Ca^{2+} overloading was demonstrated [249]. Brookes, P. S., et al., (2004) suggested that mitochondrial matrix Ca^{2+} overload is a cause of ROS generation, and was a trigger of the mitochondria permeability transition pore and cytochrome c release [256]. Taken together, we proposed that pinacidil might protect endothelial cells against IRI through the PI3K/Akt/CREB signaling pathway, together with reduced ROS and mitochondria damage. The proposed mechanism of the effects of pinacidil on endothelial cells in response to IRI was shown in Figure V-2.

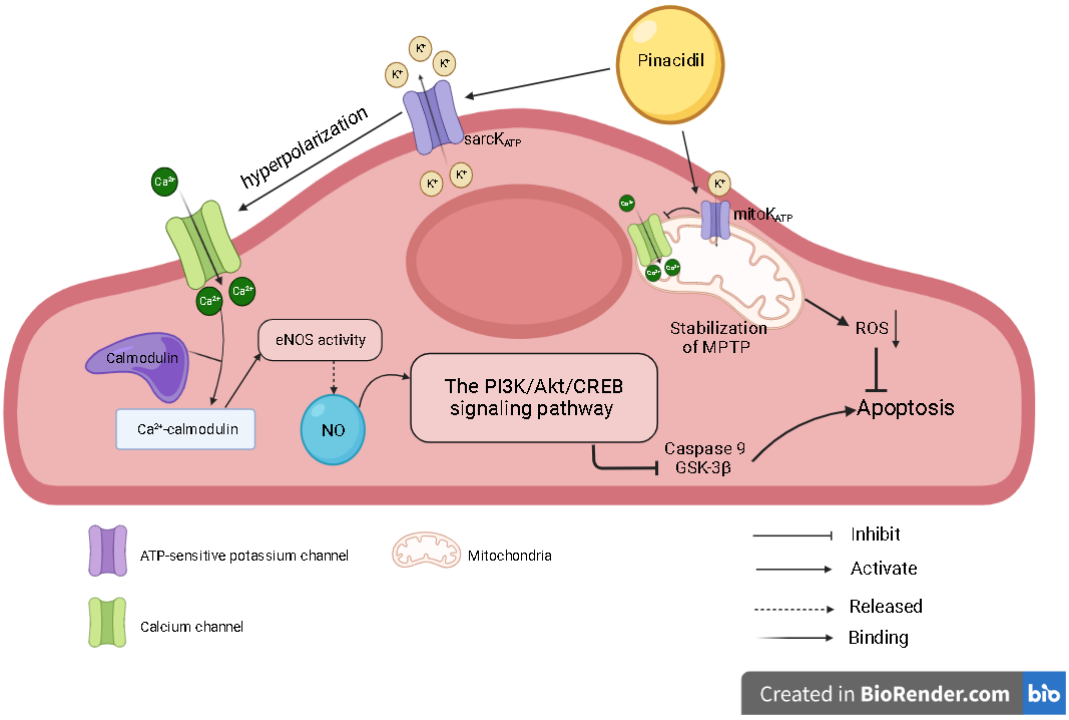


Figure 0-43 The proposed mechanism of effects of pinacidil on endothelial cells in response to IRI.

eNOS; endothelial nitric oxide synthase, PI3K; phosphoinositide 3 kinase, CREB; cAMP-response element binding protein, Akt; protein kinase B, MPTP; Mitochondrial permeability transition pore, ROS; reactive oxygen species. Created by Biorender.com

Diazoxide, a selective mitoK_{ATP} activator, attenuated ischemia-reperfusion injury in some organs including the heart, liver, intestinal and spinal cord, and lung [257-262]. Our results supported a previous study demonstrating the effects of diazoxide on hypoxic endothelial cells. It was found that diazoxide improved tndothelial function, ameliorated survival rate, reduced apoptosis rate, and maintained NO level [263]. There is a study suggesting that post-conditioning by 5withμM of diazoxide increased myocardial cell viability and inhibited apoptosis of cardiomyocytes [264]. Su C., et al., (2014) investigated the effect of 100 μM of diazoxide on rat myocardium microvascular endothelial cells (MMECs) subjected to 2 hours-hypoxic/2 hours-reoxygenation. It was reported that diazoxide reduced apoptosis cells, increased PI3K and Akt mRNA gene expression, and reduced p53 gene expression [265].

Our finding demonstrated that diazoxide maintained NO level. Zhang, L.C., et al., (2020) and Katakam, P.V.G., et al., (2016) indicated that diazoxide promotes nitric oxide synthase [263, 266]. This discrepancy may be from the different concentrations used between the two studies. [263].

It was documented that diazoxide reduced oxidative stress by scavenging the superoxide anions generated including reactive oxygen species (ROS) from mitochondria [267, 268]. In addition, there is evidence suggesting the direct effect of diazoxide on enhancing phosphorylation of extracellular signal-regulated kinases (ERKs), via the opening of mitoK_{ATP} channels, and inhibition of apoptosis of vascular endothelial cell by activation of PI3K/Akt pathway [269]. Similarly, Su C., et al., (2014) showed that diazoxide increased PI3K and Akt mRNA gene expression, but reduced p53 gene expression, which was associated with the regulation of cell growth and apoptosis [265]. The proposed mechanism of the protective effect of diazoxide is that

diazoxide opens mitoK$_{ATP}$ channels, regulates ROS generation and extracellular signal-regulated kinases (ERKs), and activates PI3K/Akt pathway. The proposed mechanism of the effect of diazoxide on endothelial cells in response to IRI was shown in Figure V-3.

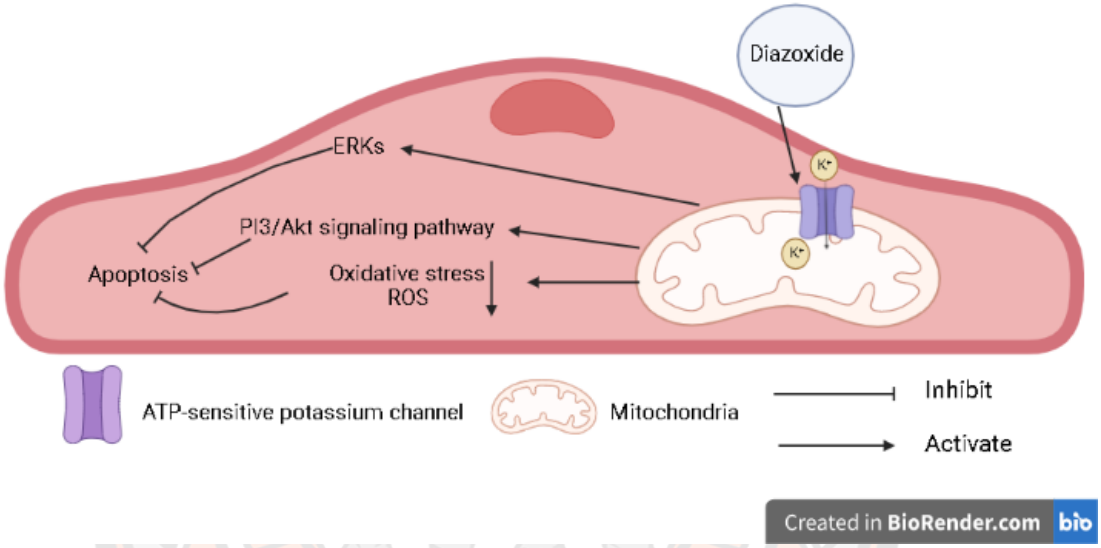


Figure 0-44 The effect of diazoxide on endothelial cells in response to IRI
ERKs; extracellular signal-regulated kinases, PI3K; phosphoinositide 3 kinase, Akt; protein kinase B, ROS; reactive oxygen species. Created by Biorender.com

1.4) Nicorandil

There is a supporting document showing that nicorandil provided a protective effect on myocardial cells during hypoxia-reoxygenation situation, and acted against cell apoptosis through IL33/ST2 signaling pathway [270]. Chen CC. et al., (2019) suggested that the effect of nicorandil is dose-dependent manner. It was demonstrated that 10 µM of nicorandil improved human umbilical vein endothelial cell (HUVEC) viability and decreased cytotoxicity [271]. In 2005, Date T., et al., demonstrated that

100 µM of nicorandil decreased human umbilical vein endothelial cells (HUVECs) death and the effect of nicorandil lasted for 48 hr. It was presented that nicorandil altered expressions of apoptosis regulatory proteins (BAX), involving MPTP opening and cytochrome C release, increased expression levels of Bcl-2, an antiapoptotic protein [272]. ShamsEldeen, A.M., et al., (2022) investigated the possible mechanism of nicorandil on ischemic-reperfusion pancreatic tissues and bone marrow mesenchymal stem cells of rats, showing that nicorandil enhanced PI3K/Akt/mTOR expression levels and increased cGMP, which is associated with cell survival and proliferation [273]. Moreover, ShamsEldeen, A.M., et al., (2022) also demonstrated that nicorandil reduced two apoptosis marker expressions, caspase 3 and Bax [273]. Nicorandil is also involved in ROS and NO production [274]. Nicorandil can act as a NO donor via its nitrate structure [194]. The pharmacodynamic effects of nicorandil are induction of membrane hyperpolarization and opening of plasma membrane K_{ATP} channels, resulting in Ca^{2+} influx, eNOS activation, and increased NO production [193, 194]. Serizawa, K., et al., (2011) reported that nicorandil at 100 µM did not increase eNOS expression in rat femoral artery. It was suggested that overexpression of eNOS promoted eNOS uncoupling and generating superoxide and ROS, which contributed to endothelial cell damage [274, 275].

The possible mechanism of nicorandil on endothelial cell viability and NO level was depicted in Figure V-4.

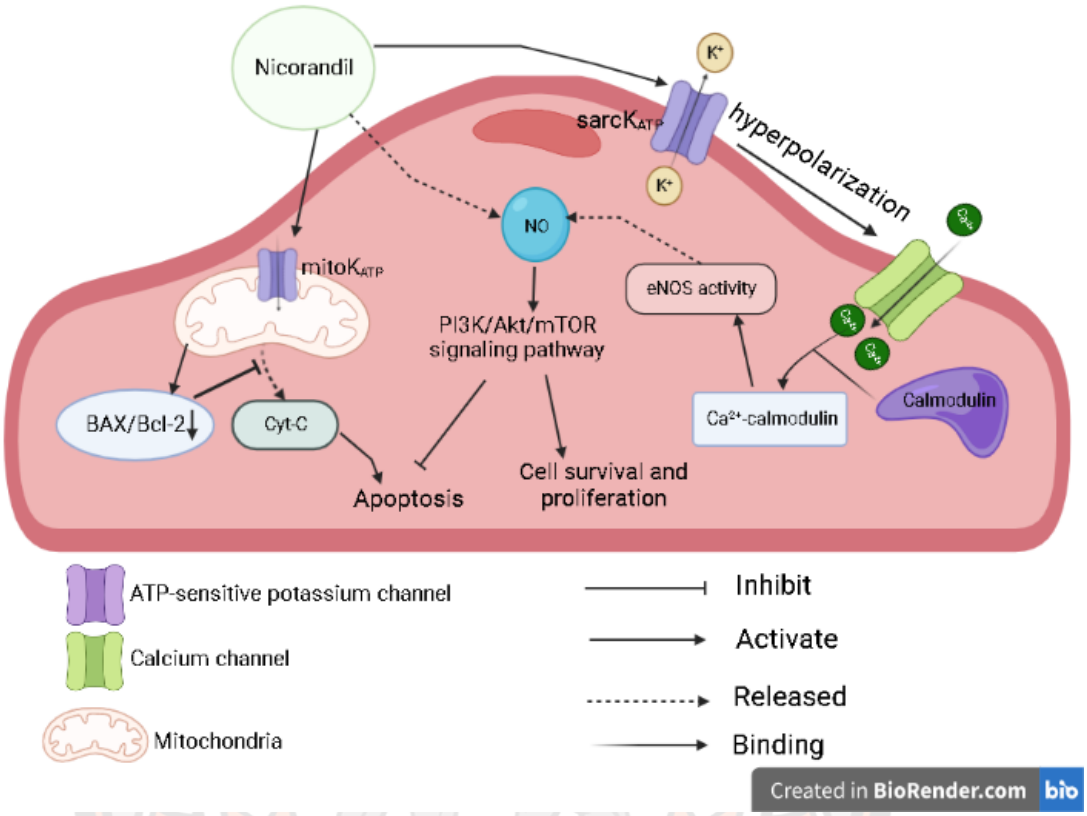


Figure 0-45 The possible mechanism of nicorandil on endothelial cell viability and NO level

Cyt-C; Cytochrome c, ROS; reactive oxygen species, Bcl-2; B-cell lymphoma 2 protein, BAX; B-cell lymphoma 2 associated X protein, eNOS; endothelial nitric oxide synthase, PI3K; phosphoinositide 3 kinase, CREB; cAMP-response element binding protein, Akt; protein kinase B, MPTP; mTOR; Mammalian target of rapamycin. Created by Biorender.com

1.5) ZD0944

Noteworthy, we demonstrated the effect of selective Kir6.1/SUR2B activator (ZD0947) against ischemia-reperfusion injury on endothelial cells. It was found that ZD0947 at concentrations of 1, 10, 100, and 500 µM protected endothelial cell injury as represented by increased cell viability after IR. ZD0947 could restore to the normal level after reperfusion for 30 minutes and 24 hr. There is no data concerning the effect

of ZD0947 on endothelial or vascular smooth muscle cell function. Kir6.1/SUR2B subunits are expressed in various tissues including endothelial cells, vascular smooth muscle cells, and cardiac muscle cells [30, 276] The evidence showing that the Kir6.1/SUR2B K_{ATP} channel might be involved in protective mechanism against IR injury on endothelial cell, by induction of hyperpolarization, induced Ca^{2+} entry into the endothelial cells, and resulting in regulated endothelial mediator release and associated with limiting infarction size in the mouse hearts [276]. We also hypothesized that the mechanism of ZD0947-increased endothelial cell viability might be involved in NO and PI3K/Akt/mTOR expression, and regulation of eNOS and iNOS protein expression. Studies are reporting that the NO/cGMP pathway could provide an antiapoptotic effect on endothelial cells and NO inhibits apoptosis cell death [277-279]. In addition, activation of Kir6.1/SUR2B induces membrane hyperpolarization, leading to calcium influx, and enhanced eNOS activity, leading to NO released [33]. Zhou, H. M., et al., (2014) reported that Kir6.1/SUR2B KCO could restore the concentration of NO production to normal levels in post-ischemic rat hearts, by increasing the expression of eNOS protein and decreasing the expression of iNOS protein [200]. The escalation of iNOS activity leads to excessive NO generation and induces apoptosis [280]. However, the exact mechanism of protective effects of ZD0947 on endothelial cells and NO production should be further investigated. The possible mechanism of the protective effect of ZD0947 on cell viability and NO production was shown in Figure V-5.

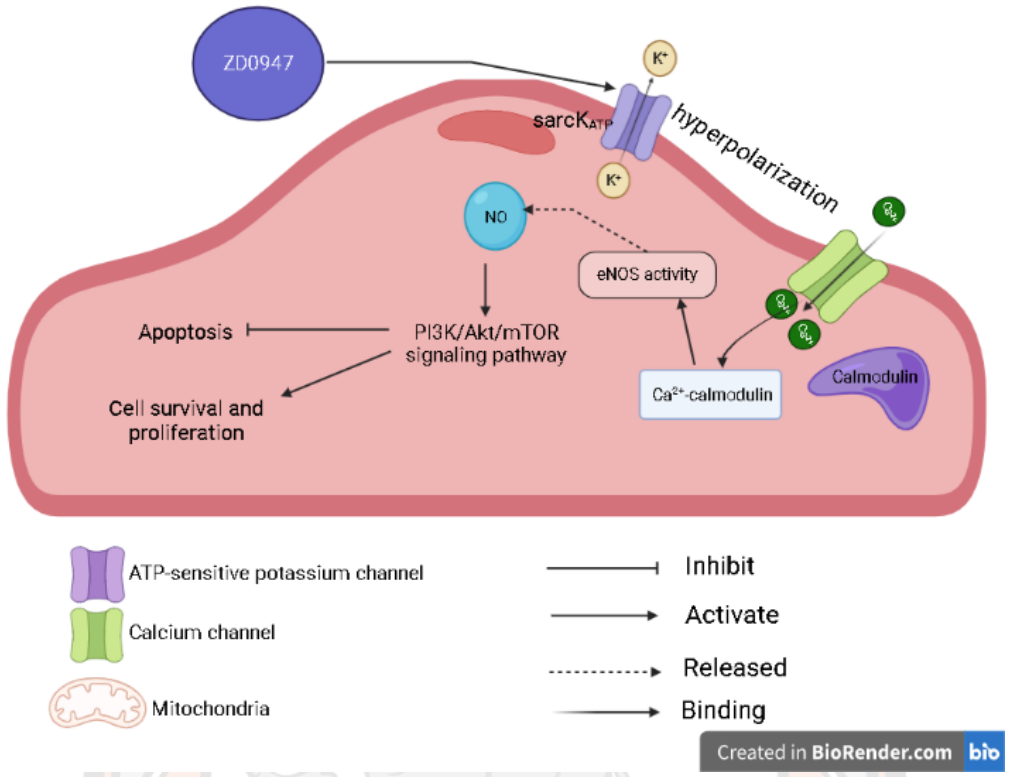


Figure 0-46 The possible mechanism of ZD0947 on endothelial cell and NO production

eNOS; endothelial nitric oxide synthase, PI3K; phosphoinositide 3 kinase, CREB; cAMP-response element binding protein, Akt; protein kinase B, MPTP; mTOR; Mammalian target of rapamycin. Created by Biorender.com

2. The effects of KCOs and hyperkalemic cardioplegia

It was demonstrated that hyperkalemic cardioplegia itself did not change endothelial cell viability and NO production. Our results are relevant to the report of He G. W., et al., (1994) and He G. W., et al., (1995), which demonstrated that high K^+ cardioplegia (K^+=16, 25, and 50 mM) could preserve endothelial cell relaxation function, which was related to endothelium-derived nitric oxide [281, 282]. Gunaydin S., et al., (2020) demonstrated that redosing of St. Thomas hyperkalemic cardioplegia (K^+=16mM) every 25 minutes maintained HUVECs viability via the inhibition of apoptosis after being perfused for 2 hours [283]. On the other hand, Gunaydin S., et al.,

(2020) reported that St. Thomas cardioplegia reduced viability of HUVECs and increased apoptosis cells after reperfusion for 6 and 24 hours, respectively [283]. A study found that hyperkalemia can be deleterious to endothelial cells at the concentration of 30 mM of potassium [92]. There are some studies reported that hyperkalemic cardioplegia induced endothelial membrane depolarization to around −50 mV, which possibly causes endothelial dysfunctions [119, 284, 285], decreased endothelium-derived hyperpolarizing factor (EDHF)-mediated relaxation [286-288]. A mechanism of hyperkalemia cardioplegia and endothelial cell death is possibly associated with depolarization arrest. Coleman et al., (2004) reported that membrane depolarization by high K^+ concentration decreased the driving force for Ca^{2+} entry into endothelial cells from Ca^{2+}-activated K^+ channels, this mechanism increased NADPH oxidases, endothelin and ROS, which possibly jeopardizes endothelial cells [119, 289]. The possible mechanism involving the negative effects of hyperkalemic cardioplegia is that hyperkalemia changes cell electrical activity, resting membrane potential and Ca^{2+} homeostasis which affects endothelial cell functions and viability [286, 287]. In addition, hyperkalemia-induced cell apoptosis should be a concern because the association between hyperkalemia and apoptosis has been found [44]. There are few studies investigating the effect of hyperkalemic cardioplegia on endothelial cell viability and NO levels, and the results are still controversial release [22, 119, 237, 286].

Furthermore, the effect of hyperkalemic cardioplegia on ischemic endothelial cells was assessed. It was found that IR together with hyperkalemic cardioplegia worsen the levels of NO production were identical to the control. There are studies suggesting deleterious effects of hyperkalemic cardioplegia on ischemic myocardial and

endothelial cells resulting in increased cytotoxicity, depletion of ATP, increase generation of ROS, and endothelial cell dysfunction, leading to cell apoptosis and necrosis [119]. A study found that circulating endothelial cells (CECs), von Willebrand factor (vWF), and soluble thrombomodulin (sTM) as endothelial cell injury markers in patients that underwent on-pump coronary artery bypass graft (CABG) surgery by using hyperkalemic cardioplegia occurred at 1 hour, 6 hours, 12 hours, and 24 hours after CPB initiation [290]. Jun Feng., et al., (2005) reported that crystalloid hyperkalemic cardioplegia (K^+ = 25mM) impaired endothelium-dependent relaxation factors and increased caspase 3 and related to apoptosis activity on rabbit hearts, which were subjected to cardioplegic-I/R condition [291]. It is known that endothelial cell death rapidly occurred after ischemia-reperfusion [225, 226], and hyperkalemic cardioplegia increased NADPH oxidases, endothelin, and ROS, and leads to jeopardizes endothelial cells [119, 289]. Therefore, we hypothesized that the reduction of endothelial cell viability could be influenced by IR and could be aggravated by hyperkalemic cardioplegia.

Although endothelial cell viability was diminished, level of NO production was equivalent to control. This result was similar to a study from Yang, Q., et al., (2005) demonstrating that hyperkalemic solution impaired endothelium-derived hyperpolarizing factor (EDHF)-mediated relaxation, but not NO release [22]. Some studies reported that hyperkalemic cardioplegia or St. Thomas cardioplegia impaired EDHF-mediated relaxation, but the direct effect of hyperkalemic cardioplegia on releasing NO from endothelial cells has not been reported [292-294]. A study reported that there were several endothelium-dependent relaxation factors, including endothelium-derived nitric oxide (EDNO), prostacyclin (PGIz), and endothelium-

derived hyperpolarizing factor (EDHF), changes during cardioplegia perfusion [286]. The mechanism of the effect of hyperkalemic cardioplegia on NO released from ischemic endothelial cells remains unclear and further investigation is required. We hypothesized that NO production in the ischemic-cardioplegia reperfusion group (I-CPGR) might be generated from eNOS and iNOS activity. According to the fall of cell viability, the NO production in the I-CPGR group was expected to be low. The unchanged level of NO may indicate the increased NO production when normalized with the number of viable cells. Overexpression of NO generated from iNOS could lead to increased apoptosis and necrosis cell death [237]. The possible mechanism of hyperkalemic cardioplegia on IR endothelial cells was shown in Figure V-6.

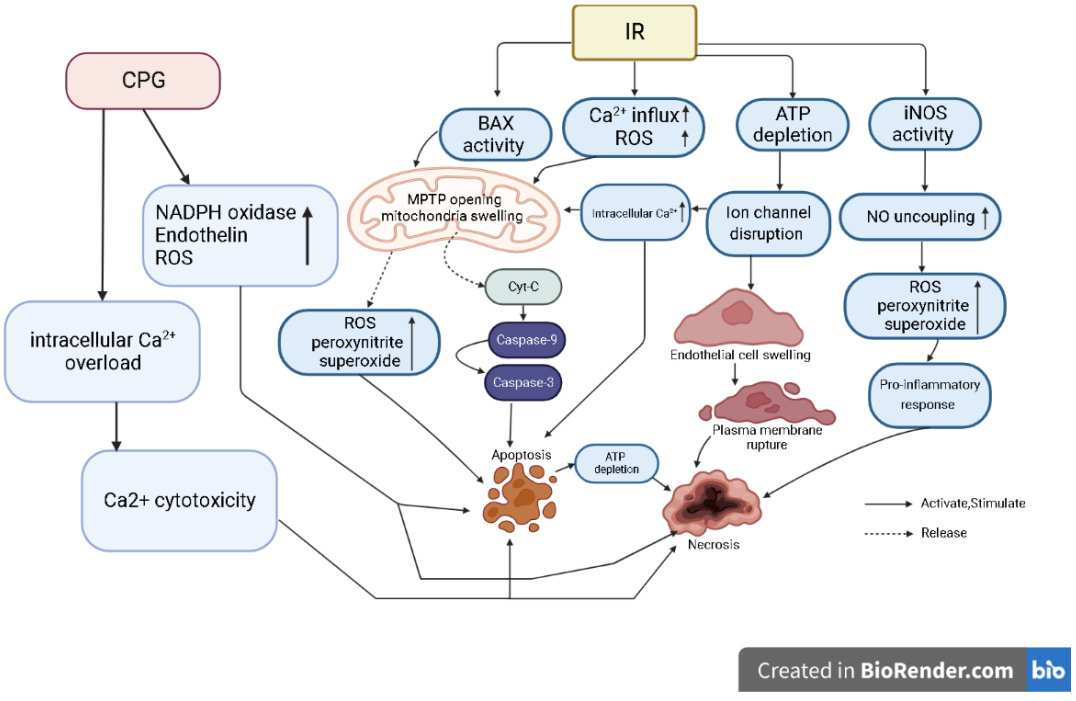


Figure 0-47 The possible mechanism of hyperkalemic cardioplegia on IR endothelial cell

CPG; hyperkalemic cardioplegia, IR; ischemic-reperfusion, Cyt-C; Cytochrome c, Ca^{2+}; calcium ion, NADPH; nicotinamide adenine dinucleotide phosphate, ROS; reactive oxygen species, BAX; B-cell lymphoma 2 associated X protein. Created by Biorender.com

We optimized concentration K_{ATP} activators perfusion and found that adding 100 µM of K_{ATP} activators to cardioplegia could reduce cell death when compared to that of the I-CPGR group. This is relevant to other studies showing a protective effect of KCO-added cardioplegia on cardiomyocytes and smooth muscle cells [37, 105, 295-298]. In addition, nicorandil-added cardioplegia protected myocardial function against hyperkalemic cardioplegia [299-301]. However, the study on endothelial cells was limited.

We chose a selective endothelial KCO, ZD0947, and nicorandil for further investigation. It was observed that both modulators could protect endothelial cell viability and maintain NO levels. It was thought that nicorandil may be involved in the inhibition of MPTP opening during reperfusion by altering the expressions of apoptosis regulatory proteins (BAX) and increased Bcl-2 expression, which controls the MPTP opening and resulting in inhibition of cytochrome C released [272]. Moreover, nicorandil may limit oxidative stress generation and activate Reperfusion Injury Salvage Kinase or "RISK" pathway, leading to compromised apoptosis cell death [302-304]. Nicorandil also enhanced PI3K/Akt/mTOR expression and increased cGMP, which promotes cell survival and proliferation [273].

Our results demonstrated that ZD0947 and nicorandil increased NO-released from endothelial cells after being reperfused for 30 minutes and restored NO to its control after 24 hr. Serizawa, K., et al., (2011) suggested that nicorandil can prevent endothelial dysfunction, by balancing ROS and NO generation [274]. One of the mechanisms that is associated with NO release is endothelial cell hyperpolarization. Studies reported that KCOs-induced endothelial cell hyperpolarization leads to Ca^{2+}

influx, and activation of eNOS, resulting in NO release from the endothelial cells [28, 29, 33].

There is limited data demonstrating the effect of ZD0947 on endothelial cells. As far as we know, this is the first study demonstrating a beneficial effect of ZD0947 against ischemic-cardioplegia reperfusion injury. It was found that ZD0947 maintained endothelial cell viability and NO production in the early phase in the same magnitude as nicorandil. A possible mechanism for the protective effect of ZD0947 is that ZD0947 might be associated with the NO/cGMP pathway, increased antiapoptotic mediators, and cell apoptosis inhibition via increased nitric oxide levels [277-279]. A study reported that Kir6.1/SUR2B subunit K_{ATP} channel activator is responsible for cell hyperpolarization, resulting in Ca^{2+} influx and activation of an eNOS [33]. Moreover, there are pieces of evidence showing that activation of endothelial Kir6.1/SUR2B results in activation of the eNOS pathway and NO release, giving a balanced nitric oxide/endothelin system [203, 305]. However, the mechanism of the effect of ZD0947 and cardioplegia on endothelial cells is unclear and needs further investigations.

Limitations

The limitations of this study were, 1) the use of human umbilical vein endothelial cell line (Ea.hy926), which may be different from primary human vascular endothelial cells (ECs). However, a study reported that Ea.hy926 and ECs have similar genes around 1,542 genes from 2,000 highly expressed genes [306]. Ahn, Kyunghye. ,et al., (1995) demonstrated that Ea.hy926 responded to chemicals and condition as same as ECs, it preserves ECs function and is useful for the in vitro study [307]. 2) simulated-ischemia created by glucose deprivation may not resemble the pathophysiology of

ischemia occurring in the human body [308]. However, it was found that this model is commonly used in in vitro studies [184-186]. 3) we mimicked the administration of single dose cardioplegia which may be different from most cardiac surgery which need multiple doses of cardioplegia. However, the benefits of single dose over multi-dose cardioplegia are controversial [91, 309]. It is suggested that the effect of multi-dose cardioplegia, with shear stress, should be performed. To clarify the effect of hyperkalemic cardioplegia, investigations with several doses, timing, types, and potassium concentrations are suggested.

Clinical applications

The results showed that K_{ATP} channel activators preserved endothelial cell viability and their function against IRI, both in the presence and absence of hyperkalemic cardioplegia. These findings will be fundamental for further studies for improving endothelial cell and myocardial cell protection during cardioplegic administration.

Further investigations

To elucidate the mechanisms of endothelial cell death, both in vitro/in vivo studies are required. The association between endothelial and myocardial functions affected by KCOs is suggested. The different types and doses of cardioplegia should be performed to clarify the effect of potassium concentration and types of administration on endothelial cells and myocardial cells.

Conclusion

Our results concluded that,

1. Pinacidil, diazoxide, nicorandil and ZD0947 decreased endothelial cell death in response to ischemia-reperfusion at 10 minutes and 24 hours reperfusion.

2. Exposure of the cells to ZD0947 during IR could restored NO production in response to ischemia-reperfusion at 30 minutes and nicorandil preserved NO levels on endothelial cells in response to ischemia-reperfusion at 24 hours reperfusion.

3. ZD0947 and nicorandil maintain endothelial cell viability in response to ischemia-reperfusion with cardioplegia at 30 minutes of reperfusion.

4. ZD0947 and nicorandil increased NO production in response to ischemia-reperfusion following cardioplegia exposure at 30 minutes of reperfusion, and maintain NO level at 24 hours reperfusion when compared to control.

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