T.R.
BOLU ABANT İZZET BAYSAL UNIVERSITY
INSTITUTE OF GRADUATE STUDIES
Department of Biology

THE ACUTE COMBİNED EFFECT OF PİNACİDİL AND GLİMEPİRİDE ON ISCHEMİA AND REPERFUSİON İNDUCED ARRHYTHMİA.

"THE ROLE OF ATP- DEPENDENT POTASSİUM CHANNEL"

MASTER OF SCİENCE

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BOLU, MAYIS - 2023

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The acute combined effect of Pinacidil and Glimepiride on Ischemia and Reperfusion induced arrhythmia. submitted by **Mariam DAHER KHATIB** and defended before the Examining Committe Members listed below in partial fulfillment of the requirements for the degree of **Master of Science** in **Department of Biology, Institute of Graduate Studies of Bolu Abant Izzet Baysal University in 5.05.2023**

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Mariam DAHER KHATIB

ABSTRACT

THE ACUTE COMBİNED EFFECT OF PİNACİDİL AND GLİMEPİRİDE ON ISCHEMİA AND REPERFUSİON İNDUCED ARRHYTHMİA. "THE ROLE OF ATP- DEPENDENT POTASSİUM CHANNEL"

MSC THESIS
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DEPARTMENT OF BIOLOGY

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(xiv + 63)

This study was aimed to highlight the role of ATP-dependent-potassium channel on ischemia/reperfusion induced arrhythmia during the acute phase, with the application of Pinacidil as KATP channel opener and Glimepiride as a channel blocker. In this study among 4 groups, 58 male Sprague-Dawley rats 6-7 months old were used. The surgical procedure started with tracheostomy, then femoral cannulation was performed to measure blood pressure during the operation. By using silk thread, coronary artery was ligated and 6 min. ischemia had been performed, and following 6 min. for reperfusion produced by removing the silk. In control group just drugs solvents were administrated at 2 min of ischemia and at the beginning of reperfusion Time of drugs administration among groups did not change. In Pinacidil group, Pinacidil drug were given with Glimepiride solvent, and the same were applied in Glimepiride group with Pinacidil solvent. In combined group both Pinacidil and Glimepiride drugs were given. The type of arrhythmia, the incidence and arrhythmic score were statistically comprised between groups using one way-ANOVA and independent t-test. Pinacidil alone was anti-arrhythmic in reperfusion period, while Glimepiride was pro-arrhythmic. The protective effect of Pinacidil was abolished by Glimepiride in combination group. In conclusion, the expected protective results against I/R injury induce arrhythmia through using channel activator and blocker were not observed. This result might be increasing heterogeneity in action potential duration due to the different effect of two modulators on action potential duration. The protection generated by Pinacidil alone might be depend on its effects on action potential duration instead of its hypotensive effect.

KEYWORDS: Myocardial ischemia, reperfusion, arrhythmia, Pinacidil, Glimepiride.

ÖZET

PİNASİDİL VE GLİMEPİRİD'İN BİRLİKTE İSKEMİ VE REPERFÜZYON İLE UYARILAN ARİTMİLER ÜZERİNE AKUT ETKİSİ, "ATP BAĞIMLI POTASYUM KANALININ ROLÜ"

YÜKSEK LİSANS TEZİ

Mariam DAHER KHATIB

BOLU ABANT İZZET BAYSAL ÜNİVERSİTESİ
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Bu çalışmada, akut iskemi-reperfüzyon ile uyarılan aritmiler sırasında, K-ATP kanal açıcı olarak Pinasidil ve kanal bloker olarak Glimepirid uygulaması ile, ATP bağımlı potasyum kanalının rolünü vurgulamak amaçlanmıştır. Çalışmada 4 grup olarak 6-7 aylık 58 adet erkek Sprague-Dawley türü sıçan kullanılmıştır. Cerrahi işleme trakeostomi ile başlanmıştır, ardından operasyon sırasında kan basıncını ölçmek için, sağ femoral arterden kanülasyon yapılmıştır. İpek iplikle koroner arter bağlanmıştır ve bu şekilde 6 dakika kadar iskemi oluşturulmuştur, bu ip gevşetilerek 6 dakika reperfüzyon yapılmıştır. Kontrol grubunda, iskeminin ikinci dakikasında ve reperfüzyon başlangıcında sadece ilaç çözücüleri uygulanmıştır. Gruplar arasında ilaç uygulama zamanı değiştirilmemiştir. Pinasidil grubunda, Pinasidil ilacı Glimepirid solventi ile uygulanmıştır ve aynı şekilde Glimepirid grubunda Pinasidil solventi uygulandı. Kombinasyon grubunda hem Pinasidil hem de Glimepirid ilaçları birlikte verilmiştir. Aritmi tipleri, süreleri ve aritmi skoru, tek yönlü ANOVA ve bağımsız t-testi kullanılarak yapılmıştır. Pinasidil tek başına reperfüzyon döneminde antiaritmik etki göstermiş, ama Glimepirid proaritmik etkili olmuştur. Kombinasyon durumunda Pinasidil'in koruyucu etkisi Glimepirid ile ortadan kalkmıştır. Sonuç olarak, kanal açıcı ve bloker kullanımı birlikte aritmiye karşı beklenen koruyucu etkiyi göstermemiştir. Bunun nedeninin iki ilacın aksiyon potansiyeli süresindeki farklı etkisiyle, normal ve iskemik hücreler arasındaki heterojenliğin artmasından kaynaklanabileceği düşünülmüştür. Pinasidilin tek başına aritmiye azaltıcı etkisi, ilacın hipotansif etkisiyle değil, onun aksiyon potansiyeli üzerine etkisiyle olabilir.

ANAHTAR KELİMELER: myocardial ischemia, reperfusion, arrhythmia, Pinacidil, Glimepride

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LIST OF ABBREVIATIONS AND SYMBOLS

ATP : Adenosine triphosphate

ADP : Adenosine Diphosphate

AMP : Adenosine Monophosphate

KATP : ATP dependent potassium

MI : Myocardial infarction

I/R : ischemic/reperfusion

AP : Action potential

APD : Action potential duration

CCS : conductive system

SA : Sinoatrial

AV : Atrioventricular

O2 : Oxygen

NO : Nitric Oxide

mPTP : mitochondrial permeability transient pore

ROS : reactive oxygen specious

SR : Sarcoplasmic reticulum

SERCA : sarco- endo plasmic reticulum Ca2+-ATPase

Na/K-ATP : Sodium potassium ATP

NCX : Sodium Calcium exchanger

NHE : Sodium Hydrogen exchanger

SarcKATP : Sarcolemmal potassium ATP

mitoKATP : mitochondrial potassium ATP

KCO : Potassium Channel Opener

IPC : Ischemic Preconditioning

IPO : Ischemic Postconditioning

A1, A2 : Alpha Adrenergic

PK : Protien Kinase

Pina : Pinacidil

Glime : Glimepiride

Combi : Combination

BL : Before ligation

BP : Blood Pressure

HR	**:** Heart Rate
ECG	**:** Electrocadiogram
LCA	**:** left coronary artery
Arr	**:** Arrhythmia
VF	**:** Ventricular Fibrillation
VPC	**:** Ventricular Premature Contraction
VT	**:** Ventricular Tachycardia
BRAI	**:** Bradycardia in ischemic period
BRAR	**:** Bradycardia in Reperfusion period
^{O}C	**:** Celcius degree
g	**:** Gram
kg	**:** Kilogram
SE	**:** Standard Error
min	**:** Minutes
mg	**:** Milligram
mL	**:** Milliliter
mmHg	**:** Millimeter Mercury

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1. INTRODUCTION

Ischemia can be defined as a lack of oxygen and nutrients in the cell; due to lack of blood flow, which causes inability to produce ATP.

It's usually looks like the cause of sudden death in many cases, and it is believed that when this problem is treated by reperfusion and blood reflow to the affected tissue again, the cell will recover and return all its functions properly.

In fact, the cause of sudden death -if not due to cell death during ischemia- is due to process of therapy "Reperfusion" and what happens during and after it.

As it is known, reperfusion consists in removing the cause of ischemic or the hypoxic condition either it was thrombus or ligation to bring the blood back loaded with oxygen then ATP level and conversion from anaerobic to the aerobic respiration again (1).

All previous research works has been shown what happened during these two events -whether it is ischemia or reperfusion- and what happens to ionic currents in normal cell functions comprised with ischemic/reperfused cell and how any changing in N+, K+ and Ca2+ effect cell excitability -through action potential durations- and how these changes induce the mechanism of arrhythmia. (1–5) Therefrom, it can be understood easily that there must be an intervention by drug therapy for two related cases; firstly, for ischemia due to lack of oxygen and secondly for acute myocardial infarction which is caused by ventricle tachycardia (VT) and ventricle fibrillation (VF) due to reperfusion respectively (6).

In the beginning according to ischemia, it is necessary to obtain perfusion and regulate coronary flow to restore oxygen supply. This could occur by using a drug which works as vasodilator like Pinacidil drug (7). Pinacidil as a KATP channel opener, causes membrane hyperpolarization and vasodilation (8–10). These protective events of channel opening by Pinacidil infusion, unfortunately had been appeared to be the main causes of ventricular tachycardia by shortening the action potential duration (11).

The ATP sensitive channel in myocardial cells was studied and it was found it is formed from (Kir6x) subunits and sulfonylurea receptors (11–13). The sulphonylurea receptors is the main target of hypoglycemic drugs like Glimepiride, which is also known to have an antiarrhythmic effect (14).

Owing to the dual effect of channel activator (15) Pinacidil -as it is pro-arrhythmic drug by it is role in shorting action potential duration (16) and its role in anti-arrhythmic activity (17,18) by induced and enhanced relaxation of smooth muscle and reduce contractions (15,19)- with effect of blockers based on the ability to prevent ischemia induced arrhythmia (20,21), we suggest that combination of these two modulators at the same time may give the expected result in decreasing arrythmia by keeping the vasorelaxation effect of Pinacidil with maintain the normal action potential duration and reducing the Ca2+ loading through the effect of the blocker.

1.1 HEART ANATOMY, PHISYIOLOGY AND HISTOLOGY:

1.1.1 Anatomy and Physiology:

Heart pump the blood to all body organs through the pulmonary and systemic circulation. In the thoracic cavity the heart is located in the slightly left side of the body between the lungs, and it is separated from the abdominal cavity by the diaphragm.

The heart consists of four chambers, two chambers for receiving blood from the organs to the heart and lungs which is known the atria, and the other two chambers for pumping blood from the heart to the body and the lung again which is the ventricles.

The two atria are located in the upper part and the two ventricles are located in the lower part, that make on each side atrium in the base, and one ventricle in the apex of the heart. The left and right sides of the heart are completely separated by atrio-ventricular septum. However, even the chambers in each part are separated by septa and valves but the chambers themselves work together, which the 2 atria and the 2 ventricles contract together simultaneously (22).

There are two types of blood circulate in the body through these chambers; first one is oxygenated blood, which is pumped to the aorta from the left ventricle to the body organs after passing through the lungs by the pulmonary artery to gain the oxygen and the other one is deoxygenated blood, which comes from the body organs loaded with waste and carbon dioxide and enter the right atrium by the superior and inferior vena cava (22). Like all organs, the heart tissue is required a supply of oxygen and nutrients, even its chambers are full of blood; myocardium does not receive any nourishment from this blood, instead of that, the cardiac muscle take its own supply of blood from a network of arteries, called the coronary arteries that feed with their branches all parts of the heart muscle.

These coronaries supplies to each side of the heart from; the right coronary artery supplies the right atrium and right ventricle with blood, and the left main coronary artery which supplies the left atrium and left ventricle (22).

1.1.2 Histology:

At the histological level, there is three distinct layers that formed the heart wall which is from outside to inside respectively: epicardium, myocardium and endocardium.

Epicardium is the outermost layer which is actually a part of the heart wall, and myocardium is the muscle tissue of the heart and the thickest layer of the heart wall, and it is the layer that actually contracts, while the endocardium is the innermost layer of the heart that lines the heart chambers (22). Myocardium is the muscle layer between these two layers.

1.1.3 Heart Electrophysiology:

The electrical impulse that stimulates the heart chambers to contract is called the action potential (AP); and its spreads through special conductive system composed from bundles of fibers (22).

The sinoatrial node (SA node) -Pacemaker cells- is the normal source of the AP initiation and it’s located in the upper part of the right atrium, these electrical impulse travels within the conductive system (CCS) -non-Pacemaker cells- to AV

node that work to slows down the incoming impulses for a short time during its passage through Bundle of His branching to either ventricles (22) (Figure 1-1).

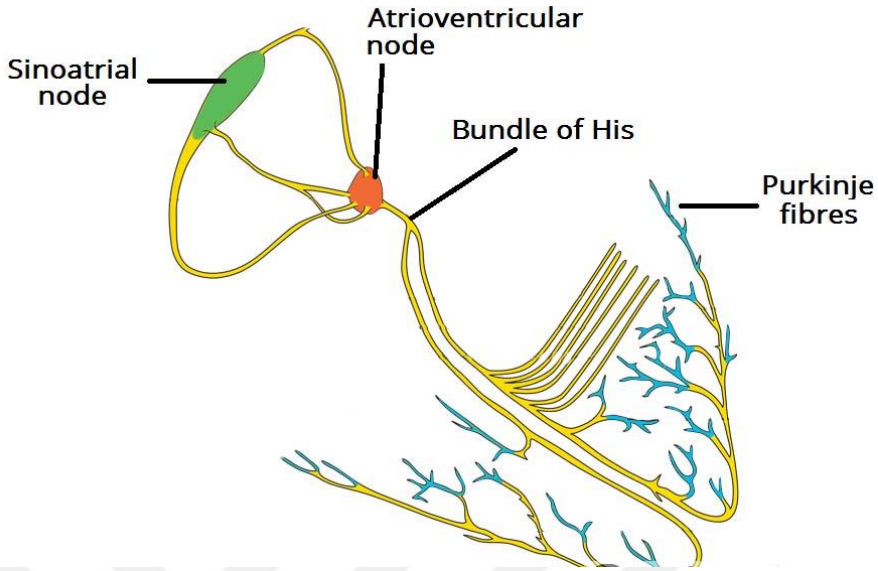


Figure 1-1. Impulse conductive system (23).

AP takes place through 5 phases (0, 1, 2, 3, 4 and 5) in non-pacemaker cells and through 3 phases (0, 3 and 4) in pacemaker cells, and each phase is responsible for a specific event in membrane stability through its own Ca2+, Na+ and K+ ion channels, pumps and exchangers (22,24).

These electrical communication between inside and outside the cell regulate the cell equilibrium then function of the cell through depolarization, repolarization, plateau and after depolarization phases, for example; Na+ channel when it is open, it is responsible for depolarization (which is phase 0 in non-pacemaker cells) that occur rapid Na+ ion influx, and the K+ ion efflux occurs in the initial repolarization state (phase 1) through opening K+ channel after Na+ channel closing, while Ca2+ channels opening manage the balance of plateau phase (phase 2), and following in the rapid repolarization phase (phase 3) which occurs by closing of Ca2+ and opening K+ channels. The last phase (phase 4) resting potential is controlled by K+ channels only (24).

Theses mechanical and electrical activity for each part in the heart appears as a wave on the ECG recording system.

1.1.4 Electrocardiograph (ECG):

ECG is a non-invasive technique (not requiring the introduction of instruments into the body) for record the time and the strength of electrical signals that produce a heartbeat from each part in the heart (atrial, ventricle contraction and relaxation), and it have no risk or complications to the body.

The ECG electrodes are used to measure the conductive current between two points (bipolar system), and they placed in a triangle between the skin of the arms and legs (6 limb leads) and the chest (which is also 6 leads). The recordings different a little bit between animals and human models; because in animals it's just needed for three leads I, II, and III (25).

As it is mentioned, heart performance appears as a wave on the device, which represent the rhythm and rate of each beat, and it can be said there is 5 waves appear together in one beat as:

P-wave: atrial depolarization (it should be mentioned that because of small mass of atrial muscle, the repolarization wave does not appear very clearly)

PR interval: the conductive impulses before ventricle depolarization (atrial repolarization)

QRS complex: ventricle depolarization

ST segment: reflect the plateau phases

T-wave: which is represent the rapid ventricular repolarization

It should be noted that when there are any abnormal changes in waves duration, intensity or rhythm, that maybe indicate type or subtype of arrythmia or ischemia (especially in ST segment for ischemia) (22,26).

1.2 CARDIAC ARRHYTHMIA:

In physiological abnormalities cases of heart automaticity (normal impulses initiate from sinoatrial (SA) node to atrioventricular (AV) node then to bundle of His), the SA node often become irritable either acquired or inherited (27), and the

pacemaker transfer to ectopic foci; which is one of the mechanisms of arrhythmia by enhanced abnormal impulse initiation (28).

Another mechanism of arrhythmia is called re-entrant mechanism. During single cardiac cycle, when the cardiac muscle become completely recovered; and extra depolarization point is available at the same time, it causes extra beat that is named as re-entrant or circus movement (28).

The third mechanism of arrhythmia is related to the action potential duration rapidity (especially after depolarization period) not the site of impulse generation; which is known as triggered arrhythmia (28) (Figure 1-2).

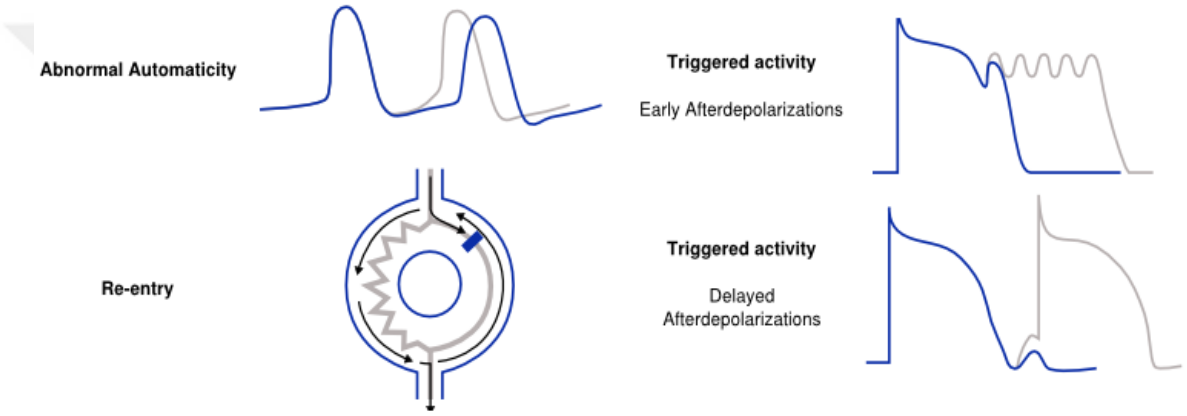


Figure 1-2. Arrhythmic mechanisms (29).

1.2.1 Type of Arrhythmia:

There are several types of arrhythmias that may occur due to the abnormal automaticity in pacemaker cells or non-pacemaker cells.

1.2.1.1 SINUS ARRHYTHMIA:

In general, the pacemaker activity of SA nodal cells is controlled by nervous system. The sympathetic activity is responsible for increasing firing level and heart rate by releasing norepinephrine, while the parasympathetic works against sympathetic activity and suppress heart rate and cardiac output by releasing acetylcholine.

Therefrom; when myocardium exposed to any stress conditions that stimulate or depressed nervous system -such as ischemia and myocardial infarction-, this leads to a change in sinus rhythm, either sinus tachycardia if it's too fast or sinus bradycardia if it's too slow (22,30).

1.2.1.2 Atrial Premature Contraction (APC):

Atrial contraction abnormalities appear from ectopic sites from atrial myocardial cells (outside the sinus node), it can be from single or multiple sites. The normal atrial contraction appears on the ECG recording as P wave; while the premature one appears as an earlier than expected P wave with an abnormal shape (22).

1.2.1.3 Atrial Flutter (AFL) and Fibrillation (AF):

Fibrillation is related to disorganized electrical stimulation in more than one focus of atrial muscle rather than stimulants single focus. The rate of conduction from atrial to ventricles is faster-than-normal, resulting as a reduction in a maximum blood pumping to ventricles.

Unlike AF, atrial flutter has a coordinate electrical impulse but with quite rapid rate, which doesn't allow all impulses to conduct to ventricles through AV-node. However, both AF and AFL are not fatal, because even in this rapid rates, the blood passes into ventricle decreases in small amounts (31).

1.2.1.4 Ventricular Arrhythmia:

Like atria, also ventricle with the same mechanisms can exposed to all type of arrhythmia and it could occur genetically or by various heart disease. When the heart beating start from the ventricles and the rate exceeds 500 beats per minute in rats is named as tachycardia (VT).

This type of arrhythmia abnormal QRS complex wave appear on ECG, and it may differ in the shape (wide or narrow) or frequency (flutter or fibrillation) to be monomorphic or polymorphic.

VT can consider as the most serious type of arrhythmia because it could be transfer to ventricular flutter or fibrillation, that mean the ventricles does not allow full filling so that cardiac output reduces (22,32).

1.2.2 Ischemia induced Arrhythmia:

When ischemia occurs either by thrombus or ligation (in experimental works), there will be a great difference in the ionic balance between the ischemic and non-ischemic tissues. These ionic changes affect the membrane stability and the action potential duration (depolarization and repolarization periods), and the ischemic cell become continuously in depolarized state which induce arrhythmogenesis in ventricles.

Probably, arrhythmia may appear either in early or delayed phases after ischemia, the early (acute) phases start roughly from 2-10 min after ligation and the delayed arrhythmia may start mainly from 10-30 min after ischemia (33). In rats, the arrhythmia starts at 4-5 minutes following ischemia and last about 12-15 minutes, then it begins to gradually decrease (34).

1.2.3 Reperfusion induced Arrhythmia:

As it is known, reperfusion provides to remove the cause of ischemic or the hypoxic condition either it was thrombus or ligation; to bring back the blood loaded with oxygen then ATP level and the conversion of myocardial cell metabolism from anaerobic to the aerobic respiration again (35).

At the first minutes of blood reflow to the injured tissue after a prolonged ischemia, it has been proposed that there will be an overproduction of free radicals (reactive oxygen specious ROS) which will later interact with membranes proteins and lipids causing cell necrosis (36). Due to this type of derangement of cellular metabolism, more severe ventricular arrhythmia appears during reperfusion

The rapid metabolic changes and the shortening in AP duration and heterogeneity between reperfused and non-reperfused regions; are more during reperfusion; more cellular injury and even lethal arrhythmia occur (37).

1.3 EXPEREMNTAL MYOCARDIAL INFARCTION

1.3.1 Animal Model of Experimental Myocardial Infarction

Experimental myocardial infarction models have been produced *in vivo* and *in vitro* conditions on several small and big mammalian models such as: rat and mice (34,38), pigs (39), dogs (40), rabbit (41) and sheep (42).

Experimental myocardial infarction in *in vitro* models is the Langendorff method which it is so suitable to small hearts size. In this technique the heart is extracted from the body, then after coronary ligation the heart is perfused by special solution in special conditions (43,44) as it shown (Figure1-3).

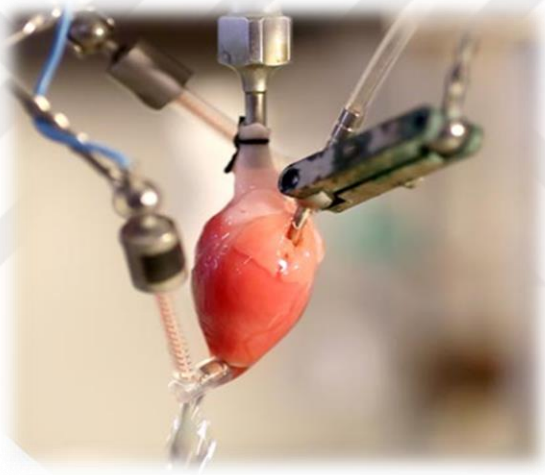


Figure 1-3. Isolated Langendorff heart preparation (45).

In vivo models, unlike *in vitro* models, the heart is not extracted, after applying the anesthesia; the left coronary artery (LCA) is ligated then the chest is closed again (34,46).

Whether *in vitro* or *in vivo* studies, researchers often prefer to use rodents as the ideal models. Because of their fast reproductive cycle, short life span and the low cost, the similar genetic characteristics with humans, their small size, ease of collecting samples and their harmlessness (47). However, sometimes some of these mentioned characteristics may make the use of rodents not ideal in some type of researches; for example, as the small size of the animal's body may make some

difficulties during handling or may make the sample size insufficient for the experiment (48).

1.3.2 Method to Produce Infarction

The I/R injuries or arrhythmia is induced by blocking the main coronaries or branch of them in both *in vivo* and *in vitro* models. Blocking coronaries occur in several methods (49) and here we will mention only the method of ligation by ligature.

Coronary ligation may be applied on smaller or larger branch of coronary artery. After the ligation of a small branch from the coronary, the severity of arrhythmia and the infarct size could be smaller.

1.4 ACUTE MYOCARDIAL INFARCTION (MI)

Myocardial infarction meaning cell necrosis or cell death after period of time from ATP level depleted (hypoxic condition) after coronary ligation (in animal models) or after sudden coronary blockage by thrombus (in human beings) (34,35,50).

MI is the main cause of sudden death through the complications after damaging the heart tissue by the lacking of oxygen. However, these tissue disfunction by MI will affect the function of myocardium like systole and diastole which make the animal model (or the patient) more susceptible to arrhythmia (31).

1.4.1 Cellular Events in Ischemic Condition

Across sarcolemmal cell membrane; all cellular activities are maintained by the most essential ions Na+, K+, Ca2+ through the membrane proteins like channels and ion exchangers which they are mainly depend on ATP molecule. Each normal cell must have its own ion concentration intra and extracellularly to ensure cell homeostasis (4,22).

As it's known, ion pumps and channels are so sensitive to O2+ level through electron transport chain in mitochondria (2). However; during ischemia there will be an insufficient blood supply and O2+ which result in depletion of ATP and decrease in pH levels and further, as ATP level decrease all ATP-dependent channels and pumps start to fail (35).

In the ischemic cell, Na+ ion accumulate by the impairing of Na+/K+-ATP pump and the K+ ion concentration increases extracellularly by the opening of sarcolemmal KATP channel. This Na imbalance will activate the Na+/Ca2+ exchanger leading to increasing in Ca2+ level intracellularly, which later caused mitochondrial dysfunction and failing in all contractile process (35,51).

During this stress condition and because Na+/H+ exchanger depends on intracellular Na+ concentration (after the failure of Na+/Ca2+ exchanger) and increase in the acidity; this exchanger will be activated to regulate H+ level in the cell so that it induces Na+ accumulation again (52) (Figure 1-4).

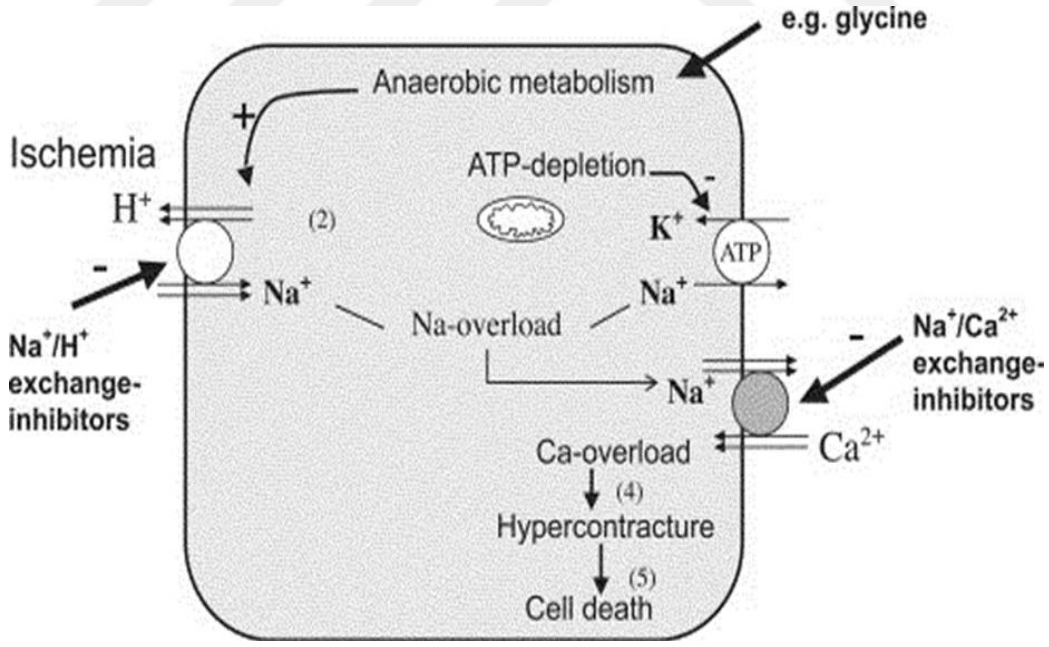


Figure 1-4. Channels and exchangers in ischemic condition (53).

1.4.2 Cellular Events in Reperfusion

After opening the occluded site and achieved the washout, almost of all the lethal cellular changes are suggested to be related to two pathological processes, first one is the Ca2+ overloading and the second one is the activation of reactive oxygen species (ROS) production (36,37).

Due to Ca2+ overload hypothesis, in the first few minutes of reperfusion period, the cell acidity is still high that means the Na+/H+ exchanger is still active and there is a Na+ accumulation intracellularly inside of the cell, thereby the Ca2+ overloading still continues. Because of that, it can be said the Ca2+ overloading in ischemic or in reperfusion periods could cause the cell to get irreversible injury (54).

On the other hand, according to ROS activation hypothesis, its known that there are two main sources of reactive oxygen species, the electron transport chain in mitochondria and the other one is the cellular xanthine oxidase system (55).

After the reloading of oxygen, it's thought to be that the combination between Ca2+ overloading and free radicals could cause the injury either directly by damaging the lipids and membrane proteins or indirectly by inducing the opening of mitochondrial permeability transition pore (mPTP) and the cellular pathways of cell necrosis (56).

1.5 FACTORS RELATED TO ARRHYTMIA AFTER EXPERIMENTAL CORONARY ARTERY LIGATION

ANIMAL MODEL

The incidence of arrhythmias after I/R injuries is always determined by several cellular components and parameters. The most important of these factors can be summarized in the changing in heart rate, infarct size or ischemic area, and finally the cellular activity in cardiac cells through its channels and pumps so that the pumps might be different in number and percentage due to the used animals (57).

1.5.1 Heart Rate:

After reduction in blood flow and oxygen consumption in ischemia; the increasing in heart rate (reflex tachycardia) in the early few minutes of reperfusion is considered as an autoregulatory mechanism through the activation of sympathetic nervous system and the releasing of catecholamines to enhance the blood flow (22,58).

Therefore, it can be said that the combination between electrical dysfunction and neural and humoral stimulation to the ischemic and non-ischemic areas could create the complexity of the mechanism of arrhythmia after I/R injuries (59).

1.5.2 Size of Infarct Zone:

It has been found that in MI, the heart parameter and electrical activity of heart is so related with the size of ischemia, because of the complex structure between injured and non-injured tissues that induces the reentrant mechanism in later times (60).

Some findings support the hypothesis of the incidence of arrhythmogenesis is inversely proportional to large size of infarct zone, which mean the severity of arrhythmia is more in the small area of ischemia (61). The most possible explanation for that supposition is the time for new excitable impulses (as reentrant mechanism) become prolonged in the large area and this reduces the rate of ventricular tachycardiac (62,63).

1.5.3 Channels:

Channels of sarcoplasmic reticulum and mitochondrial membranes in each cell have critical role in cellular metabolism, also their role had been found in pathophysiological conditions which causes myocardial dysfunction (64,65).

1.5.3.1 SODIUM CHANNELS

In normal AP generation process, the role of sodium channel appears in depolarization phase.

Na+ ion influx (inward current) in early depolarization after channel activation causes electrical stimulation of the heart through membrane potential in each single myocyte. During early ischemia or reperfusion periods, sodium channel dysfunction leads to ventricular arrhythmia probably by changing in electrical properties between active and inactive channels which later causes Na+ and Ca2+ overload (66).

1.5.3.2 CALCIUM CHANNELS

Calcium channels is a gated channel. It controls the concentration of Ca2+ ions inside and outside of the cells which leads to control the contraction and relaxation of myocardial cells through the plateau phase in action potential process in healthy heart.

Inside the cell, Ca2+ ions that is needed for heart contraction enter by the voltage-gated channel (L-type channel in atrial cells and T-type channel in ventricular cells) then stored in the sarcoplasmic reticulum (SR). Ca2+ return to the SR by the special pump named as SERCA (sarco/endoplasmic reticulum Ca2+-ATPase) and Ca2+ stored there in the relaxation state, while in the contraction state, SR allows Ca2+ releases by the stimulation of ryanodine receptors. Then Ca2+ binds later with troponin protein causing contraction for all myocytes at the same time (22). The imbalance in oxygen supplies in ischemic and reperfused cells lead to change in signaling pathway of Ca2+ ions that results in ventricular arrhythmia due to the Ca2+ overload intracellularly (54,67).

1.5.3.3 NA/K PUMP EXCHANGER

Na+/K+ pump is another type of membranes integral protein that play an important role in physiological and pathophysiological heart cell. This pump is ATP dependent pump, it works to keep and regulate cell volume and charge. It transports three sodium outside and two potassium ions toward the inside of the cell at the same time.

In the ischemic condition, the pump become in inhibited state which led to increase in K+ efflux and Na+ influx. The accumulation of Na+ intracellularly stimulates Ca2+ accumulation later, which subsequently induce the process of Ca2+ overload through the Na+/Ca+ exchanger (68).

1.5.3.4 NA/CA EXCHANGER (NCX)

Calcium ion is the main ion that regulate the excitation contraction coupling (ECC) process in normal muscle cell by the process of calcium-induced calcium release. In normal pathway, Ca2+ release from the L-type calcium channels trigger sarcoplasmic reticulum to increase Ca2+ level intracellularly, results in more contraction.

Following that contraction to relaxation take place, the Ca2+ start return to SR either by Ca2+/ATPase pump (SERCA) or by the Na+/Ca2+ exchanger (NCX) (67).

NCX is work in the same principle of Na+/K+ pump by transport 3 ion of Na in for 1 Ca2+ ion out. In ischemic cells after inhibition of Na+/K+ ATPase pump, this exchanger also start work in reverse direction causing Ca2+ overload after Na+ overload (69,70).

1.5.3.5 NA/H EXCHANGER (NHE)

Through Na+/H+ exchanger (NHE) in normal cells; one H+ ion is exchanged for one ion of Na+, but because of anaerobic respiration during ischemic

period, the acidity of the heart start to increase in early minutes as a result of increasing hydrogen ions in myocardial cells. In this conditions sodium is taken into the cell by the NHE.

The Na+ accumulation in this condition lead to rise in Ca2+ level inside the cell consequently, from that its clearly understood that NHE works to regulate pH level and prevent the injuries in ischemic and reperfusion periods (52).

1.5.3.6 POTASSIUM CHANNELS

Like other types of channels in cell membrane, potassium channels also have an important role in the regulation and the setting of the resting potential in many cells. In last decades, K+ channels become the main target for the pharmacological strategies especially ATP dependent potassium channel to overcome the I/R injury induced arrhythmia.

1.6 ATP DEPENDNET POTASSIUM CHANNEL

Since 1983 after Noma's discovery of the ATP-dependent potassium channel in mammalian hearts (71), these types of channels have been observed clearly in various organs of tissues (72). The function of this channel is highly depended on ATP molecule as understood from its name. However, in normal physiological state these channels are closed state, while in pathophysiological conditions is known to be opened, that may take their important role in ischemic myocardial cells when the ATP level fall considerably.

In cardiac myocyte, this channel is found in both inner and outer membranes of the cell. The channel in outer membrane is called as sarcolemmal KATP channel, while the inner one which located in mitochondrial membrane is named as mitochondrial KATP channel. These channels are consisting from the Kir subunits and sulfonulurea binding unit designated as SUR, that they mainly considered the main target for cardioprotective mechanisms (11–13,73).

In response to ischemia or hypoxia, KATP channels show a protective function in the open or active state through its role as vasodilator in almost all vascular beds. On the other hand, inhibition of these channels induces

vasoconstriction response and activate another metabolic process like insulin secretion in pancreatic cells and vasoconstriction in vascular smooth muscle (20).

1.6.1 Effect of Channel Opening on Arrhythmia

In ischemic cells due to anaerobic respiration, the ATP levels start to decline and the ADP start to increase, as a result, ATP dependent potassium channels open and concurrently it leads to many cellular changes like decreasing in pH level and increasing in lactic acid concentration. In these circumstances, the contractile state of atrial and ventricular cells is affected depending on the changes in ions concentration in either side of sarcollemal membrane through the activated channels and exchangers.

The dysfunction in ATP synthesis leads to Na+ accumulation and increasing in K efflux firstly because inactivation of Na+/K+ ATPase pump. Later, the high concentration of Na+ will activate NCX and as a result Ca2+ overload occurs in ischemic cells. This electrophysiologic changes makes the ischemic cell continuously depolarized, while the neighboring cell is still in normal electrophysiology which generate significant local heterogeneity between ischemic and non-ischemic cells in the same tissue.

The unstable cardiac signaling or the heterogeneity between the cells and tissue assumed to be one of the main causes of arrhythmia (28,33). Moreover, K+ currents determine the shape and the duration of the action potentials, so the K+ outward movement could hyperpolarize the cell membrane and shorten the action potential duration and close the Ca2+ channels.

As we mentioned before, both sarcKATP and mitoKATP channel would be targets for cardioprotection (20). Through the opening of mitochondrial potassium channels, K+ influx modulates the activity of Na+/H+ exchanger and increasing acidity. Increasing H+ ions in the intermembrane space induces Ca2+ entry in to the matrix region of mitochondria. These actions make the mitochondria to swell and respiratory chain is inhibited (74). However, the activation of these channels is coupled to each other and highly dependent on complex signaling pathways, since

ATP level is depleted and ROS start to increase rapidly in the mitochondria, this will translated as the changes in sarcolemmal membrane proteins levels (75) (Figure1-5).

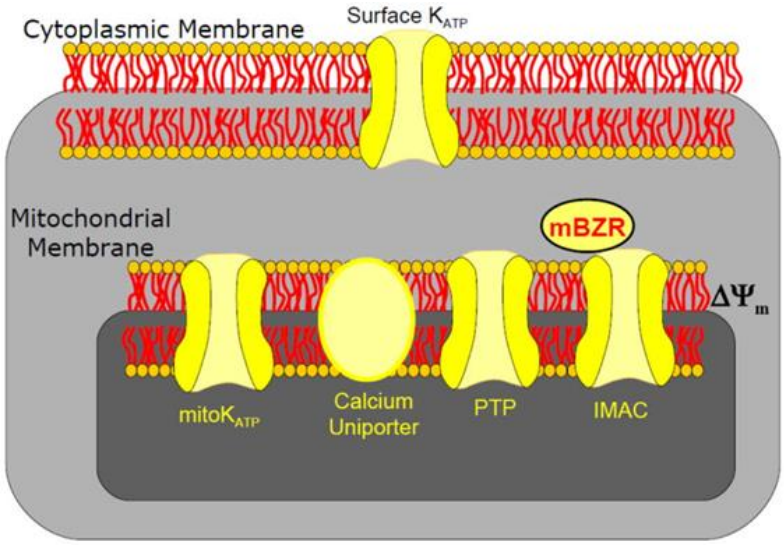


Figure 1-5. Schematic of key energy sensitive ion channels that can promote cell survival, death, or arrhythmias **(76)**.

PTP: Permeability transition pore, IMAC: Inner membrane anion channel.

1.6.2 Effect of Channel Openers (KCO) on Ischemia Induced Arrhythmia

Both cardiac and vascular smooth muscles are affected by the opening of this channel (8,11,13). Fundamentally, the main influence of ischemia is the rise in left ventricular end-diastolic pressure, reducing cardiac output, and the initiation of abnormal contractility (5,77).

A great number of studies discussed that KCO like Pinacidil, nicorandil, diazoxide and other pharmacological drug have been shown to preserve tissue against I/R injuries (9,14,15). These protective effects may come directly from their vasorelaxant influence by increasing K+ efflux and hyperpolarize the membrane that causes voltage-dependent Ca2+ channels closing and decreasing the end-diastolic pressure, or it could be indirectly by inducing membrane hyperpolarization through another endogenous pathways and cellular metabolites (7,8,13,73,78).

Against to that protective effect, some studies reported that the pro-arrhythmic impact of these drugs through the same mechanism. In ischemic cells, arrhythmia is associated with shorting of action potential due to Ca2+ channel inactivation, whereas KCO also induce shortening of APD appears in vasodilation and hypotensive effect, which may later induce tachycardia by activation of sympathetic system as a reflex mechanism (16,22,79). Because the severity of injury and the heterogeneity in myocardial fibers cannot be determined, it may not be clear how to estimate the efficacy and efficiency of channel opener.

1.6.3 Effect of Channel Blockage on Ischemia Induced Arrhythmia

In normal physiological conditions ATP potassium channels are closed state due to the presence of sufficient ATP molecule. In ischemic conditions, the channel can be blocked by pharmacological agents like Glibenclamide, Glyburide and Glimepiride.

Sulfonylurea receptors in KATP channels are known to be the main target for blockers. That is why channel blockers are named as sulfonylurea drugs. However, those antagonists known to be non-selective, it has also a non-cardiac effect, which elevates insulin release in β pancreatic cells and reduces blood flow in systemic vessel, especially coronaries.

ATP dependent channel blockers have a potent antiarrhythmic effect that was observed in many I/R experimental works (80–82). The effectiveness of drugs is probably due to the enhancing the refractoriness by increasing APD. The blockage of K+ outward current, seems that it may reduce the heterogeneity between ischemic and non-ischemic cells and suppress the arrhythmia especially occur by the re-entrant mechanism (14,21,83).

These modulators also possibly are proarrhythmic through excessive lengthening of APD and initiation early afterdepolarization period (84). In the same way, it could be said that these agents are proarrhythmic more than the antiarrhythmic because of their vasoconstrictor action on vascular smooth muscle and their hypoglycemic effect (85,86).

Therefore, the mode of action of KATP channel blocker is likely to be controversial, depending on the drug dose and channels selectivity (87,88).

1.7 ISCHEMIC PRECONDITIONING (IPC) AND POSTCONDITIONING (IPO)

Ischemic injuries are outcomes from the deterioration of blood supply to the myocardial cells. Before prolonged impairment of blood supply and prior necrosis due to this hypoxic state, the short period of ischemic episode called as ischemic preconditioning (IPC) modulates metabolic action of reperfusion injury and decrease the arrhythmia (89). The ischemic preconditioning has been produced by the applying of more than one short intervals of occlusion of the coronary vessel before long lasting occlusion period. However, the specific details of protective mode of action of the ischemic IPC are still controversial (90). Some studies have demonstrated that cardioprotection may be triggered supposedly by humoral pathways like as stimulating adenosine receptors (91) and nitric oxide releasing (92), protein kinase PKC activation (93) and prostacyclin (94), or either by neural component by reducing sympathetic activity (95).

Briefly non-lethal occlusions before prolonged ischemia is named as ischemic preconditioning, similarly the short occlusions after the reperfusion period is named as postconditioning (96). The evidence is extremely powerful that both of these cardioprotective adaptations are able to reduce the incidence of injuries induced arrhythmia and the same in minimizing the infarct size (97–99).

Concurrently, IPC and IPO could be triggered pharmacologically, and the modulation of KATP channels is often the target in this protection (100).

According to the opening KATP channels, the reducing and preserving of ATP molecule during ischemia could improve mitochondrial function and myocardial metabolism. The increasing in protein kinase activity within the intermembrane space of mitochondria is induced by K+ influx after the opening of mitoKATP channel, which stimulate also sarcKATP channel to open and shorten the APD and decrease Ca2+ influx (101). Also, free radicals or ROS production

after KATP channel opening, can be considered as one of protective pathway directly by activating protein kinases (101,102). The small amount of generated ROS proposed to inhibit mitochondrial permeability transient pores mPTP activation through several steps (101,103), which preserve mitochondrial function and prevent cell necrosis.

Applying pharmacological IPC and IPO treatments like KATP channel openers, were known to be effective in reducing I/R injuries induced arrhythmia by complex interactions (104). The antiarrhythmic effect of IPC have been shown to be abolished by blocking of ATP dependent potassium channel (105). It suggested that inner mitochondrial membrane depolarization and the limitation of Ca2+ influx is the protective effect against arrhythmia (106).

1.8 METABOLIC FACTORS IN ISCHEMIA AND REPERFUSION

1.8.1 Adenosine

In hypoxia, ATP, ADP and AMP hydrolysis, adenosine appears in high concentration. In every cell like myocytes, endothelial and vascular smooth muscles have a specific adenosine receptor to bind with. Cardioprotection by adenosine receptors in plasma membrane take place through A1 and A3 receptors, while A2 receptor mediate vasodilation in smooth muscle. Adenosine have the ability to normalize oxygen demand, which could result in reduction and depletion of ATP in ischemia and reperfusion periods respectively (107). Intracoronary adenosine administration had been known to act on endothelium and facilitate coronary dilation by inducing NO releasing (108). The biological effect and response of adenosine in physiological and pathological conditions could be complex because of interaction with G-proteins system, which they may induce other signaling pathways (109–111).

1.8.2 Reactive Oxygen Species (ROS)

Free radicals like hydroxyl radicals (-OH), hydrogen peroxide (H2O2), superoxide anion (-O2) and others, all of that species have a one or more unpaired electron which make them highly active with other membrane component especially under ischemia -reperfusion. In normal conditions, the level of free

radical is controlled by scavenging enzymes like catalase and glutathione peroxidase. The enzymatic system possibly is impaired due to cells necrosis after ischemia or reperfusion injures. In perfused healthy tissue, the main source of free radicals is xanthine oxidase (XO) and xanthine dehydrogenase (XDH) enzymes. These enzymes use NAD as an electron receptor in oxidation process, but instead of that after the ischemia, the reperfusion lead to the production of superoxide radicals by using oxygen as an electron receptor. By the increasing in Ca2+ concentration, phospholipase is also activated by increasing in Ca2+-dependent proteases, which later is converted the form of hydrogenase to oxidase. The rapid production of -O2 in reperfused tissue is coming from the presence of active XO and xanthine and hypoxanthine in high concentrations.

Reactive oxygen species could be directly toxic to cells by enhancing the release of lysosomal enzymes intercellularly, that promote another injury like atherosclerosis (112).

1.8.3 Alpha and Beta Adrenergic Stimulation

Alongside with coronary occlusion, the imbalance in adrenergic action can conceivably be able to cause lethal arrhythmias. Ischemia- reperfusion are known to activate sympathetic system and elevate catecholamines level (113). The high concentration of catecholamines and the accumulation of ADP in myocardial tissues in ischemic state leads probably to abnormal coronary reflexes.

In myocardial cells, the oxygen consumption, heart rate, inotropy and chronotropy increase as a result of β-adrenoreceptors stimulation by the circulating epinephrine, which increases peripheral atrial pressure. The effects of β-receptors activation could be suppressed through activation of α-receptors, which mediate the vasoconstriction in vascular smooth muscle. However, the effect of all types and subtypes of adrenergic receptors is highly dependent on the expression and distribution in the tissues (114).

1.8.4 Activation Protein Kinase

Protein kinase PK is a major enzyme in mediating signal transduction pathways and regulation of cell growth. The exact role of PK in cardioprotection is supposed to be occur by the activation of PK and then phosphorylation of another effector molecule (115). PK stimulation is crossbond with stimulation of G-proteins-coupled receptors, that could be activated by endogenous ligands such as adenosine and catecholamines.

Under metabolic stress, it had been demonstrated that PKC is the key modulator of KATP channel regulation (116). PKC activation have also various other effects, decreasing Ca2+ overload, suppression of diastolic dysfunction (117), reducing arrhythmia intensity during reperfusion period (118) and stimulate cell division and programming regeneration post-injury in myocardial cell (119).

The diverse protective role of PKC inhibitor or activator may differ in various animal species, because its crucially participate in mediating varied multiple signaling pathways *in vivo* and in *vitro* (115,120).

2. AIM AND SCOPE OF THE STUDY

In continuation to previous studies that discuss the importance of ischemic preconditions (IPC) against myocardial I/R injuries induced arrythmia through ATP dependent potassium channel, a combination drug treatment (combined between channel opener and channel blocker) will be applied to mimic the mechanism and action of the IPC especially in the acute phase in ischemia and reperfusion periods, and this will be as a first step by the ligation of the coronary artery in an ischemic heart model.

The compound drug will be a solution of the hypotensive drug Pinacidil which is known to had an anti-arrhythmic role during ischemia, in addition to the hypoglycemic drug Glimepiride, which is shown the similar effect of Pinacidil against arrhythmia with completely different way in mechanism of action.

The hypothesis of this study was that the combination of these Glimepiride given during reperfusion and Pinacidil during ischemia would be very effective to decrease the arrhythmia. This hypothesis was not proved with the obtained results in this study. But it was seen that the protective effect of Pinacidil against arrhythmia is abolished by Glimepiride. This result suggests that the protection against arrhythmia provided by Pinacidil possibly provided by the shortening of action potential in normal myocardial cell and by this way decreased heterogeneity in action potentials of myocardial cell in ischemic and nonischemic region.

This study provides to develop the efficacy and manufacturing of special drugs to decrease ischemia-reperfusion induced arrhythmia or the new method of clinical administration of drugs, and provides the answer to the question clearly that either the antiarrhythmic effect of channel activator is come from opening KATP channel and decrease heterogeneity of action potentials or from its action on blood pressure and decreased myocardial oxygen consumption.

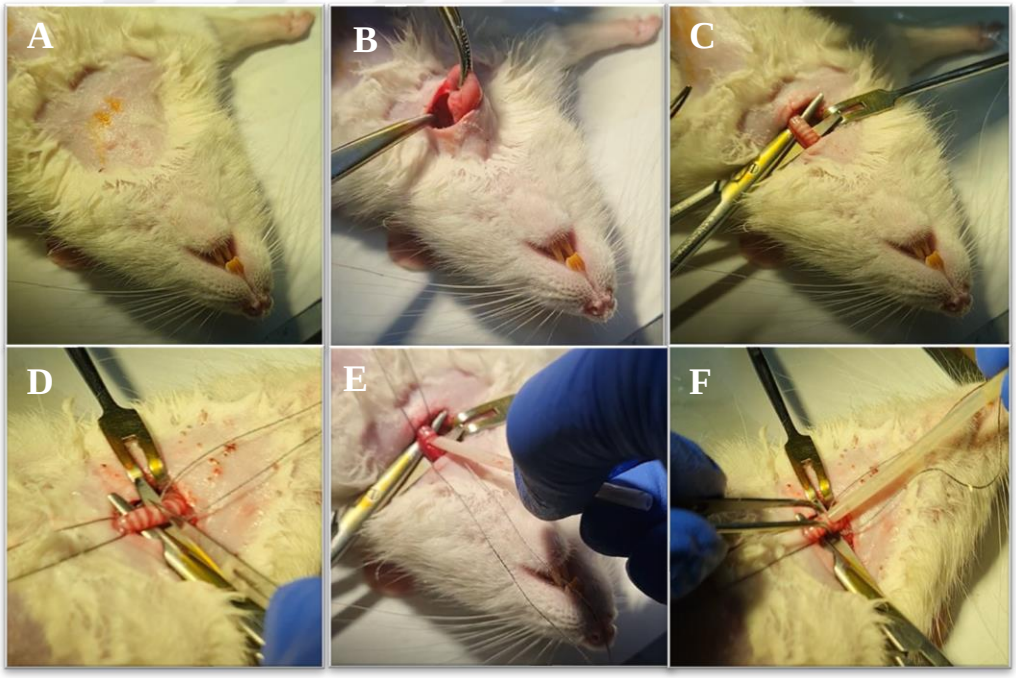
3. MATERIALS AND METHODS

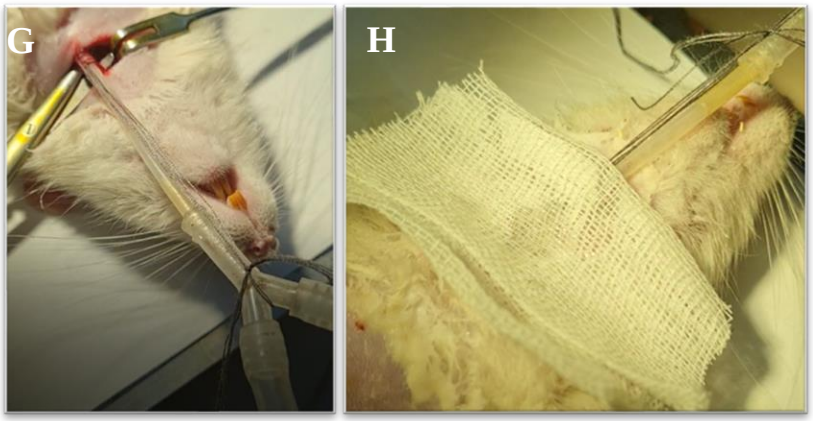
3.1 ANIMALS

In this study, 40 rats from male Sprague-Dawley rats, 6-7 months, between 290-420 g weight were used. Animals were allowed to fed with pellet -commercial rat food- and drink water. All animals were treated in commitment to the instruction principles in the use and care of animals together with the statement of ethical principle of Helsinki.

3.2 CORONARY ARTERY LIGATION AND REPERFUSION

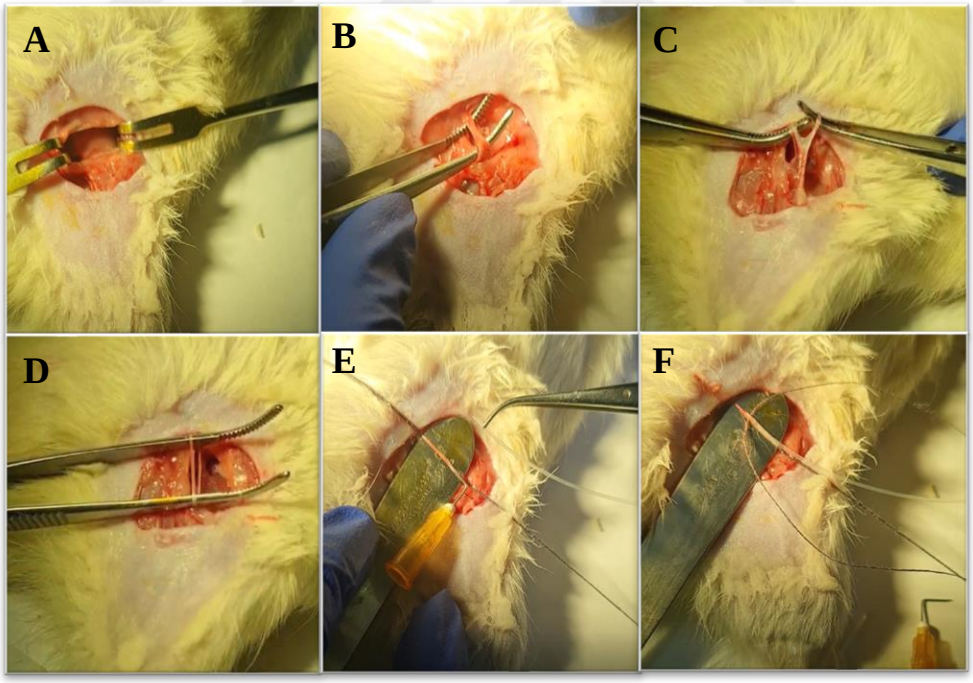
At the beginning of work the animals were anesthetized intraperitoneally with urethane (2g/kg) at a dose appropriate to body weight. After making sure that the anesthesia is safe and that it is completely done, the animal was taken to the operation table and the airway was opened with a simple incision through the trachea to connect the ventilator. This operation was shown in (Pictures1).

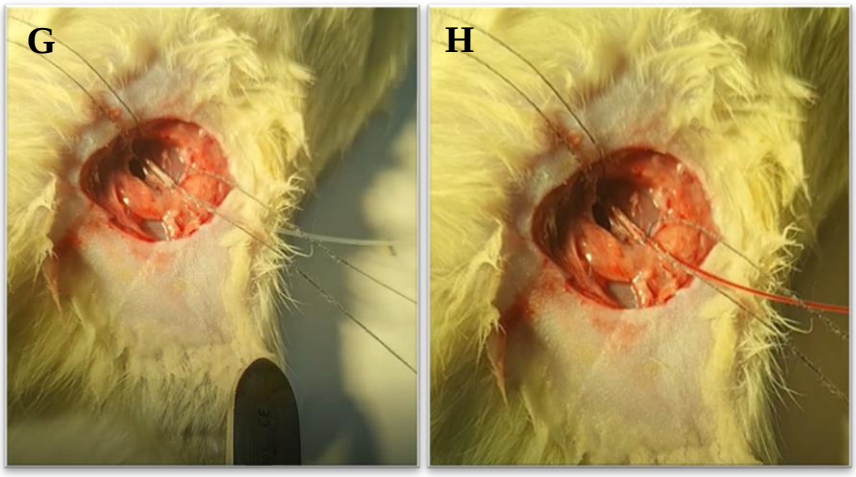




Picture 1. (A, B) Removing of body hair and Incision, (C- H) Tracheostomy.
Taken by Mariam DAHER KHATIB, 2022

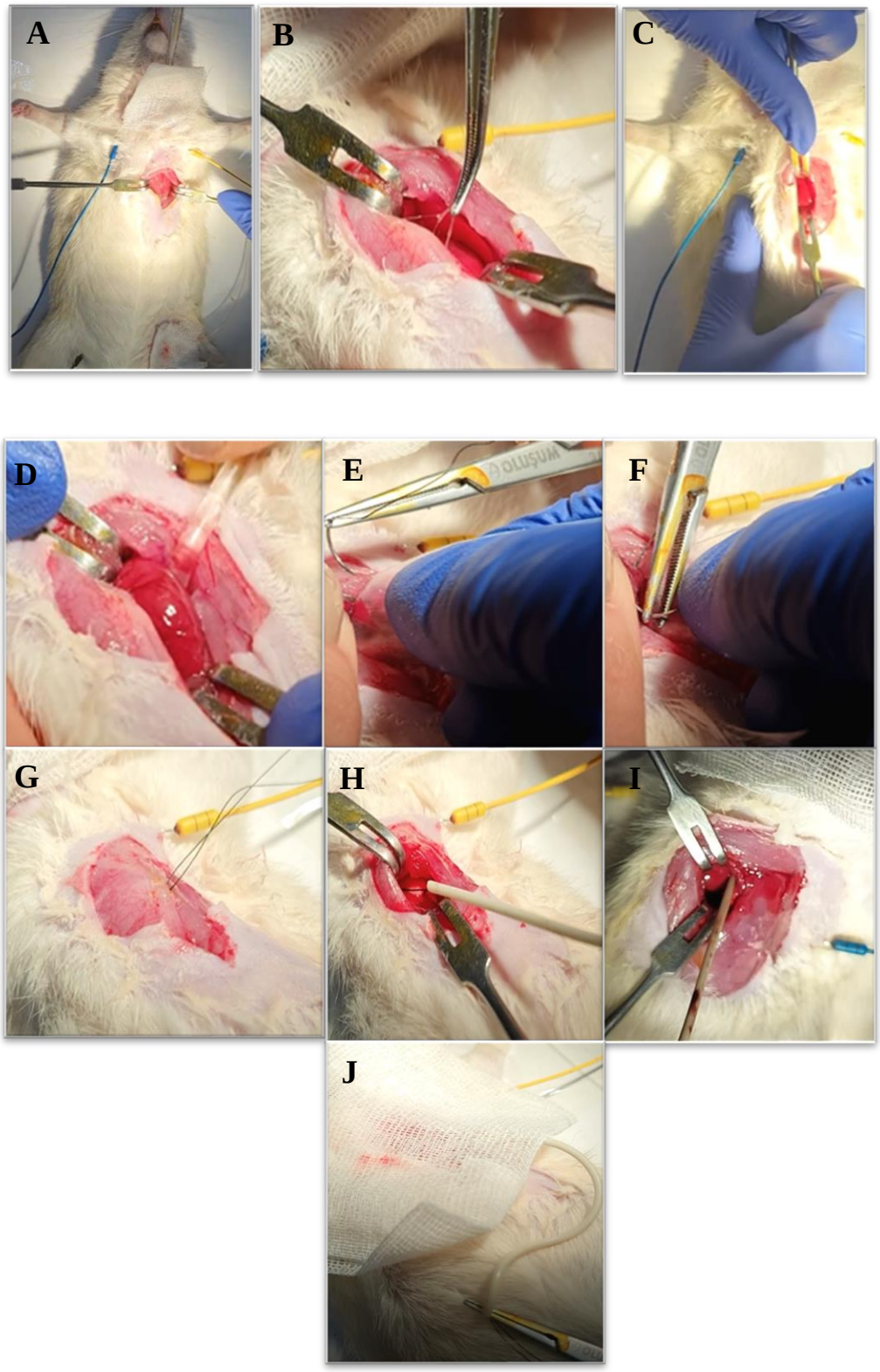
In femoral artery area, the artery had been separated from the nerve and tied, then the cannula was inserted and heparin was injected to prevent blood clots, then the cannula was connected to the blood pressure monitor to detect the changes in pressure, as shown below. (Picture 2).





Picture 2. (A) Incision, (B, C) separation of femoral artery and vein and vessels. (D, E, F) Femoral artery cannulation, and securation of the catheter, (G, H) Blood flow through cannulation. *Taken by Mariam DAHER KHATIB, 2022*

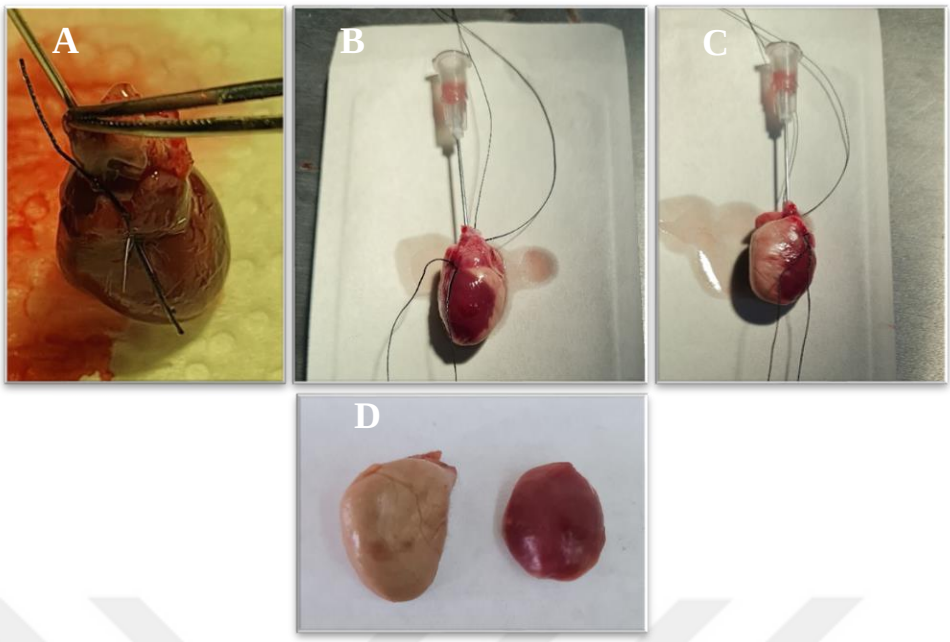
In the third step of the work, the chest cavity was opened at 3-4 intercostal ribs. The pericardium on the heart was removed when the heart still in thoracal cavity. Then respirator turned off and the heart was exposed gently by pressing the thorax in either side from right and left. Following exposure of the heart from left side, the left coronary artery was visualized clearly and the silk with needle 6/0 in size passed under the left branch of coronary artery at 2 mm away from its origin of aorta, and then the heart was returned back to the thorax. The opening of the thorax was closed by using a piece of gauze and the respirator turned on again. The needle electrodes were placed under the skin to record ECG. Following this operation 5 minutes were waited for stabilization of heart rate and blood pressure, and during this period the silk from coronaries was passed through a plastic tube for clamping of coronaries and the needle of the silk was cut out. (Picture 3).



Picture 3. (A, B, C) Thoracotomy and Pericardiotomy, (D) Exposure of heart (E, F) Placing the silk suture around the left coronary artery to ensure the ligation (G,H, I, J) Coronary ligation. *Taken by Mariam DAHER KHATIB, 2022*

In the fourth step at the end of 5 minutes, the left coronary artery was clamped by pressing the silk on the top of the tube by hemostatic pens and remain it for six minutes to produce ischemia. At the end of 6 minutes ischemia, the clamp on the silk was released by removing the hemostatic pens, and the reperfusion was produced for 6 minutes. After the observation was completed, the ventilator was stopped. 1ml/100 mg of heparin was given through catheter in carotid arteries to prevent blood clotting in coronaries, then ribs around of heart were cut and the heart was exposed with lung and aorta, then the aorta was clamped by hemostatic pens and cut on the clamp. The lung around of heart was separated and then heart was getting out with the clamped aorta. The heart then was getting into the beaker filled with physiologic saline solution

Perfusion needle was inserted in the open end of aorta and then firstly saline solutions and following ethanol in 2 ml was infused in to the aorta. After injection of ethanol, the unperfused area was seen in pinkish color but perfused area with ethanol was seen in white color. All other part of heart except ventricle were removed and the pale pink color area and white seen area was separated from each other by surgical scissors and weight separately. The percentage of the area seen in pinkish color (ischemic area) was calculated according to the whole ventricle that was the risk of infarct zone. (Picture 4).



Picture 4. (A) Tightening the knot of ligation, (B, C) Perfusion with serum fluid and alcohol, (D) Perfused area (white), non-perfused area (pink, area at risk). *Taken by Mariam DAHER KHATIB, 2022*

3.3 PREPERATION OF DRUGS

In this work, two different drugs were prepared, each of them in their own solution. The stock solution of Pinacidil was included 0,1 mg of drug powder dissolved with only 1 ml of isotonic solution. In similar way, 1 mg of Glimepiride powder was dissolved in 0,25 ml dimethyl sulfoxide mixed with same ratio of ethanol, then added to 0,5 ml of isotonic solution to make 1 ml of stock solution.

3.4 DRUG ADMINISTRATION PROTOCOL

In this study, four groups each including 10 animals have planned to form. In the control group, only the solvent of the drugs was given and ischemia-reperfusion was created. In Pinacidil drug group, Pinacidil were given at 2 minutes of ischemia and the solvent of Glimepiride at the beginning of reperfusion. In Glimepiride drug group, the Pinacidil solvent were given at 2 minutes of ischemia and Glimepiride drug at the beginning of reperfusion. In the combination group, Pinacidil (0,1mg/kg) was given at the second minutes of ischemia, and Glimepiride (1 mg/kg) was given the beginning of reperfusion. All administration were performed intravenously. Drug administration protocol were shown in (Figure 3-1).

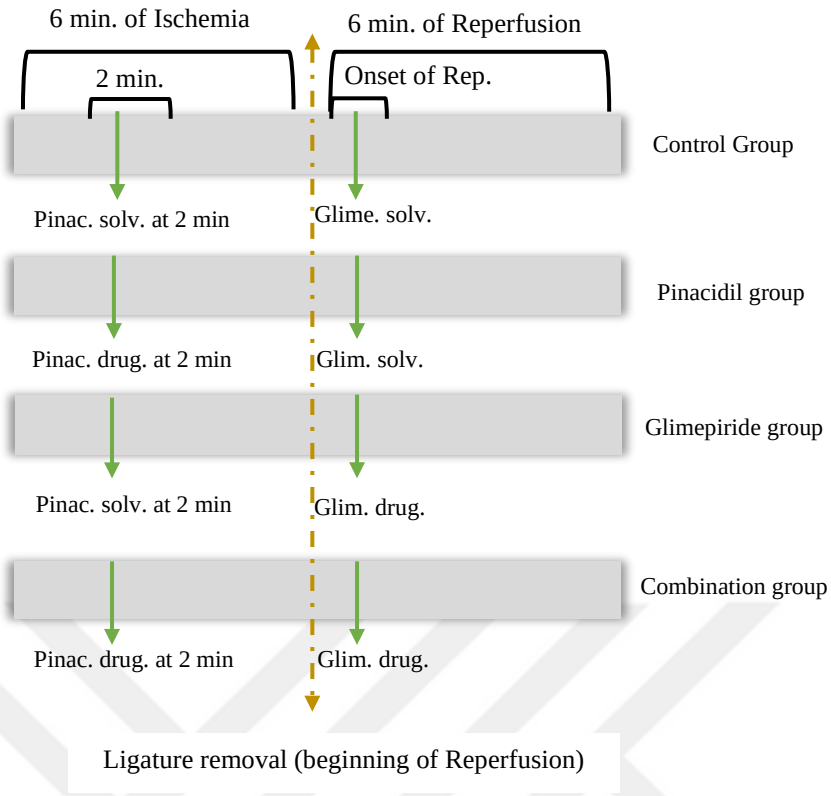


Figure 3-1. Drug administration protocol chart. Drawn by Mariam DAHER KHATIB

3.5 THE EVALUATION OF ARRHYTHMIA

The heart rate, blood pressure and other variables related to that were monitored on the computer screen, and the calculation and reading was performed by the software program (Pro 3.7 version of Biopac computerized recording system). Readings of blood pressure and heart rate were determined in the first, third, and fifth minutes of both the 6 minutes of ischemia and reperfusion periods.

According to the scoring system used in our laboratory, arrhythmia score was determined depending on the duration and the type of arrhythmia. Almost all arrhythmic types have been noted like ventricular tachycardia (VT), ventricular fibrillation (VF), and premature ventricular contractions (PVCs) as a single extra beat. The differentiation of arrhythmia were determined according to the heart rate and blood pressure changes observed on the recording. When the heart rate was between 500-600/min and the pressure was more than 70-80/mmHg it is called as sinusal tachycardia. Whereas more than these values and very low-level blood pressure below 30 mmHg were accepted as VT, and less than 40 beat and very low

blood pressure was called as ventricular escape rhythm or sinusal bradycardia if there is regular beat.

According to Lambeth conventions, the duration of arrhythmia and the incidence of arrhythmia was taken. The scores as follow: 0 = No arrhythmia, 1 = < 10 sec VT or other arrhythmia, 2 = < 11-30 sec VT or other arrhythmia, 3 = < 31-90 sec VT or other arrhythmia, 4 = < 91- 180 sec other arrhythmia, VT or reversible VF for less than 10 s, while 5 indicates arrhythmia duration longer than 180 s or reversible VF for more than 10 sec and 6 indicates irreversible VF or death of the animal.

3.6 STATISTICAL ANALYSES

The blood pressure, heart rate and the duration of arrhythmia were analyzed by both One- way ANOVA with LSD post hoc. tests, and double comparison by independent t-test. The mean and standard error were calculated for all data. The survival rate and the incidence of arrhythmia for all animals were compared by Chi-square test.

4. RESULTS

In this experiment, 40 animals were used, and 16 animals were unsuccessful and discarded because of death during operation. Latter, 18 rats were added to complete the work. To obtain meaningful statical analysis, the number of animals in each group like to be increased to at least 7 or 8 animals to minimize standard error in statistical analyses. The criteria for the animals to be accepted as successful and unsuccessful was the increased QRS amplitude, or ST-segment elevations just following the coronary ligation, not to have ventricular arrhythmia before coronary ligation, the clear reading with the blood pressure and the ECG recording at the same time. All of the successful and unsuccessful animals used in this study at the end of operation were indicated in table. 4-1.

Table 4-1. The number of successful and unsuccessful animals in each group.

Groups	**N**	**Successful animals (alive at the end of experiment)**	**Successful animals (dead at the end of experiment)**	**Unsuccessful animals**
Control (a)	21	6	0	15
Pinacidil (b)	12	5	0	7
Glimepiride (c)	10	7	0	3
Combination(d)	15	6	2	7

The doses of anesthetic drug (URETHANE) were calculated according to animal weight and the supplementary doses as it needed were given later time through the operation. The first does was given in the morning hours, second dose was after 2 hours from the first one. The third, fourth and fifth (if it was needed depending on animal response for the first two doses) doses respectively were given after 1- 1,5 hours after the previous ones. (**Table 4-2, Figure 4-1.)**

Figure 4-1. Total amount of urethane from stock solution (2 g/ml) were given.

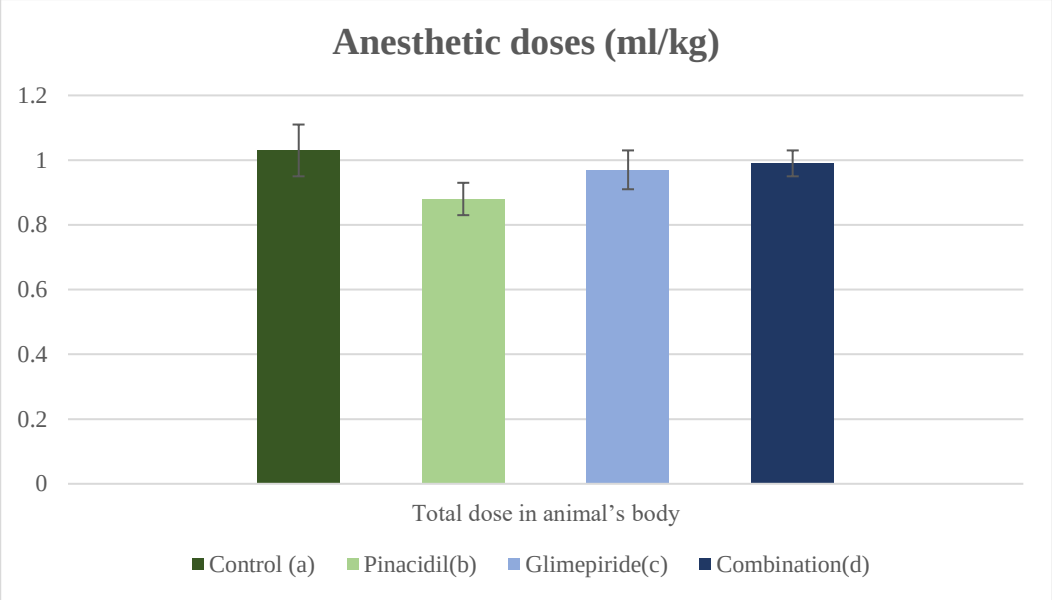


Table 4-2. The amount of anesthesia (urethane) given to animals depending on their weights. (Mean ± standard error).

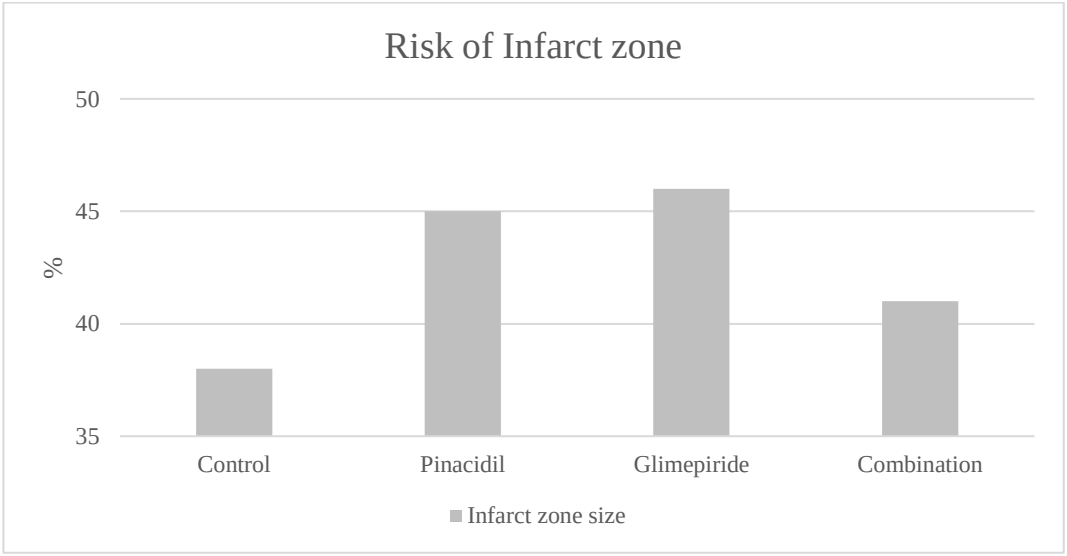
Groups	**N**	**BW (gr)**	**1st. dose (ml/kg)**	**2nd. dose (ml/kg)**	**3rd. dose (ml/kg)**	**4th. dose (ml/kg)**	**5th. dose (ml/kg)**	**Total dose in animal's body (ml/kg)**
Control (a)	6	417.50 ± 19.8	0.53 ± 0.03 **0.83 ± 0.03 gr.**	0.22± 0.03	0.17± 0.03	0.08± 0.03	0.01± 0.01	1.03± 0.08 **2.00 gr**
Pinacidil (b)	5	374.00 ± 13.8	0.48 ± 0.02 **0.74 ± 0.02 gr.**	0.22± 0.02	0.18± 0.03	0	0	0.88 ± 0.05 **1.76 gr**
Glimepiride (c)	7	408.71 ± 15.9	0.52 ± 0.02 **0.81 ± 0.03 gr.**	0.23± 0.03	0.15± 0.01	0.06± 0.02	0	0.97± 0.06 **1.94 gr**
Combination (d)	8	418.78 ± 13.5	0.55 ± 0.01 **0.83 ± 0.02 gr.**	0.21± 0.02	0.16± 0.02	0.05± 0.02	0	0.99± 0.04 **1.98 gr**

The risk of infarct zone was not significantly different among groups. This was evidence for the right point of coronary ligation, **(Table 4-3. Figure4-2)**

Table 4-3. Body weight and the risk of infarct zone in rats within 6 min. ischemia and 6 min. reperfusion. (Mean ± standard error).

Groups	N	Body wight (g)	Heart wight (g)	Risk of infarct zone size (g)	Risk of infarct zone size (%) / HW
Control (a)	6	417.50 ± 19.8	1.18 ± 0.03	0.46 ± 0.05	38
Pinacidil(b)	5	374.00 ± 13.8	1.05 ± 0.05	0.48 ± 0.05	45
Glimepiride(c)	7	408.71 ± 15.9	1.06 ± 0.06	0.49 ± 0.04	46
Combination(d)	8	418.78 ± 13.5	1.15 ± 0.04	0.49 ± 0.06	42

Figure 4-2 The risk of infarct zone.



4.1 Hemodynamic Parameters

Heart rate (HR) and mean arterial blood pressure was measured before coronary artery ligation (Basal), and following 1, 3, and 5 min of coronary ligation (Occlusion) and reperfusion. Heart rate in reperfusion period was only significantly different in Glimepiride drug group at 1 minutes of ischemia. The heart rate at 1,3, and 5 minutes of ischemia in the combination group were significantly lower in respect to Glimepiride drug group. The heart rate in Pinacidil group in ischemic period was not different in respect to control, (Table 4-4). In reperfusion period, the heart rate in the drug treated and, in the combination, group were not different from control. Similarly in the ischemic period, the heart rate was low level in combination drug group in respect to Glimepiride drug group.

In all groups, BP decreased instantly just after the ligation. Blood pressure before and during the ischemia were not different among the groups, (Table 4-6, Figure 4-3). Even it did not change in reperfusion period in respect to control, (Table 4-7, Figure 4-4).

Table 4-4. Heart rate before and during 6 min. of ischemia. (Mean ± Standard error), (*P<0.05, **P<0.01). *a. difference to control, *c difference to Glimepiride group

Groups	N	Heart Rate/ min.				
		Basal	**Before Ligation**	**1 min.**	**3 min.**	**5 min.**
Control (a)	6	346.50 ± 17.8	320.16 ± 29.5	310.55 ± 22.7	311.72 ± 24.2	319.50 ± 25.8
Pinacidil (b)	5	369.20 ± 18.3	371.40 ± 17.5	363.60 ± 23.9	348.40 ± 22.4	359.40 ± 24.4
Glimepiride(c)	7	361.57 ± 16.7	387.85 ± 13.5 *a P-value = 0.02	379.57 ± 9.1 **a P-value= 0.01	380.14 ± 11.2	381.42 ± 10.3
Combination (d)	8	322.00 ± 12.0	344.00 ± 16.8	326.00 ± 25.7 *c P-value= 0.04	293.00 ± 29.4 **c P-value= 0.01	277.12 ± 37.4 **c P-value= 0.01

Table 4-5. Blood Pressure before and during 6 min. of ischemia. (Mean ± Standard error), (*P<0.05, **P<0.01).

Groups	N	Blood Pressure (mmHg)				
		Basal	**Before Ligation**	**1 min.**	**3 min.**	**5 min.**
Control (a)	6	66.16 ± 6.5	44.16 ± 8.4	38.50 ± 9.8	46.33 ± 11.14	49.00 ± 10.2
Pinacidil (b)	5	63.60 ± 7.5	63.80 ± 3.2	51.40 ± 2.3	52.60 ± 5.3	52.20 ± 5.1
Glimepiride (c)	7	73.42 ± 6.4	57.00 ± 5.7	44.85 ± 3.2	55.85 ± 4.5	57.14 ± 5.4
Combination(d)	8	54.85 ± 5.9 *c p-value=0.04	55.87 ± 5.6	42.28 ± 5.6 N=7	46.00 ± 6.6 N=7	42.42 ± 7.0 N=7

Table 4-6. Heart rate during 6 min. of reperfusion. (Mean ± Standard error), (*P<0.05, **P<0.01) *c: difference to Glimepiride group.

Groups	N	Heart Rate/ min.			
		At the beginning of Rep.	**1 min.**	**3 min.**	**5 min.**
Control (a)	6	328.21 ± 26.9	315.16 ± 27.9	316.83 ± 23.9	317.00 ± 33.7
Pinacidil (b)	5	359.20 ± 23.6	351.60 ± 27.8	345.20 ± 22.7	347.00 ± 23.0
Glimepiride(c)	7	399.00 ± 24.9	366.28 ± 20.6	379.00 ± 13.4	374.42 ± 14.6
Combination(d)	8	295.87 ± 31.3 **c p-value=0.01	304.87 ± 29.7	289.00 ± 45.8 *c p-value=0.05	296.12 ± 41.5

Table 4-7. Blood Pressure during 6 min. of reperfusion. (Mean ± Standard error), (*P<0.05, **P<0.01)

Groups	N	Blood Pressure (mmHg)			
		At the beginning of Rep.	**1 min.**	**3 min.**	**5 min.**
Control (a)	6	49.16 ± 10.1	56.66 ± 10.7	63.83 ± 11.8	73.50 ± 7.5
Pinacidil (b)	5	54.60 ± 4.5	64.80 ± 5.6	62.00 ± 6.8	60.20 ± 6.2
Glimepiride (c)	7	55.14 ± 4.5	60.57 ± 8.2	69.71 ± 7.0	68.42 ± 5.0
Combination(d)	8	47.40 ± 5.1 N=5	55.60 ± 4.8 N=5	60.16 ± 4.5 N=6	60.00 ± 4.0 N=6

Figure 4-3. Heart Rate during ischemic and reperfusion periods.

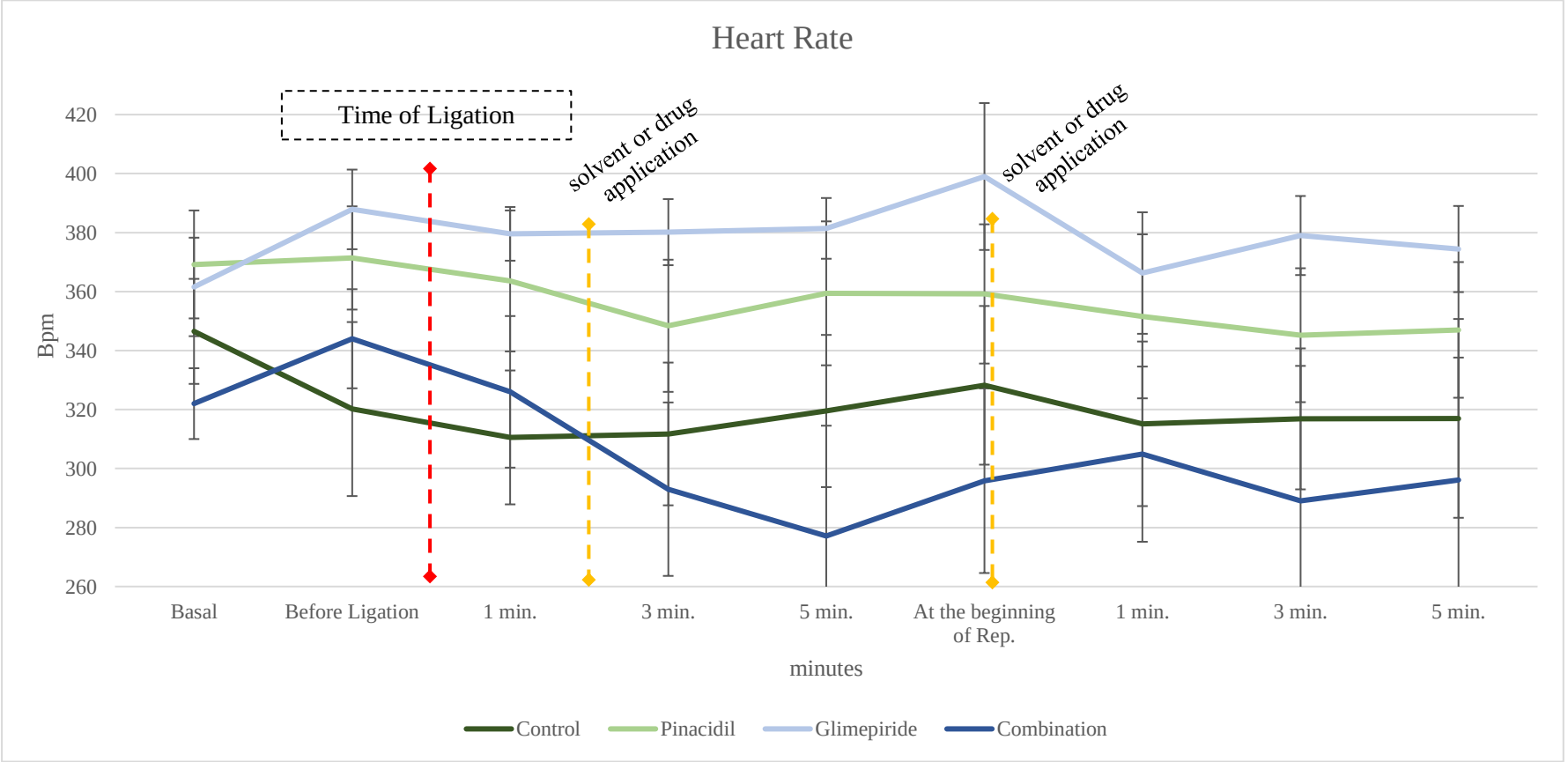
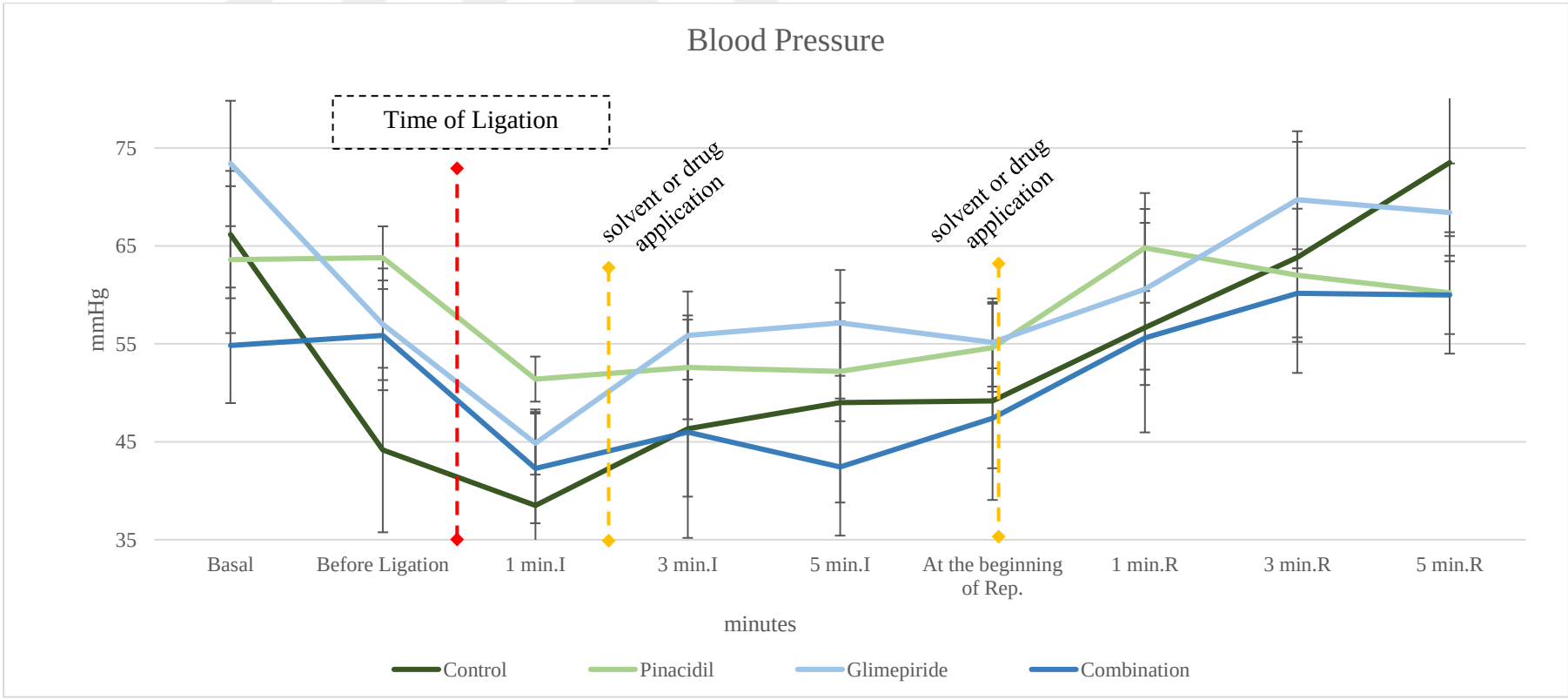


Figure 4-4. Blood Pressure during ischemic and reperfusion periods.



4.2 Arrhythmias during Coronary Artery Ligation and Reperfusion.

The incidence of arrhythmia in ischemic period was not significantly different between groups (Table 4-8). In combination group there was a bradycardia in one animal both in ischemia and reperfusion period. Ventricular fibrillation was observed only in one animal in Glimepiride group during reperfusion period. The other type of arrhythmia including ventricular premature contraction (VPC) like ventricular escape rhythm, AV nodal block and ventricular bradycardia were observed in all groups during ischemia and reperfusion period. Although highest incidence ventricular tachycardia (VT) reperfusion period was observed in Glimepiride drug group it was not significantly different from control group, (Table 4-9).

4-6. Arrhythmias incidence during 6 min. of ischemia and surviving number after 6 min. of ischemia. (*P<0.05, **P<0.01)

Groups	**N**	**Survival animals**	**Incidence of Arrhythmias (N/%)**				**Arr. Score**
			VPC	**VT**	**VF**	**BRAR**	
Control (a)	6	6	4/66	0	0	0	0.66 ± 0.2
Pinacidil (b)	5	5	5/100	0	0	0	1.20 ± 0.2
Glimepiride (c)	7	7	7/100	1/14	0	0	1.42 ± 0.2
Combination (d)	8	7	7/87	0	0	1/14	1.62 ± 0.6

Table 4-7. Arrhythmias incidence during 6 min. of reperfusion and surviving number after 6 min. of reperfusion. (*P<0.05, **P<0.01)

Groups	N	Survival animals	Incidence of Arrhythmias (N/%)				Arr. Score
			VPC	VT	VF	BRAR	
Control (a)	6	6	2/33	2/33	0	0	1.00 ± 0.3
Pinacidil (b)	5	5	1/20	1/20	0	0	0.20 ± 0.2 *a p-value=0.33
Glimepiride (c)	7	7	6/85	3/42	1/14	0	1.42 ± 0.3
Combination (d)	8	6	3/37	2/25	0	1/14	1.12 ± 0.8

Arrhythmia score was calculated from type and duration of arrhythmia in each phase. There was no significant difference among groups in both ischemia (Figure 4-5) and reperfusion period (Figure 4-6), but the arrhythmia score in reperfusion period was nonsignificant lesser in Pinacidil group (p= 0.33) in respect to control.

Figure 4-5. Arrhythmia score during 6 min. of ischemia.

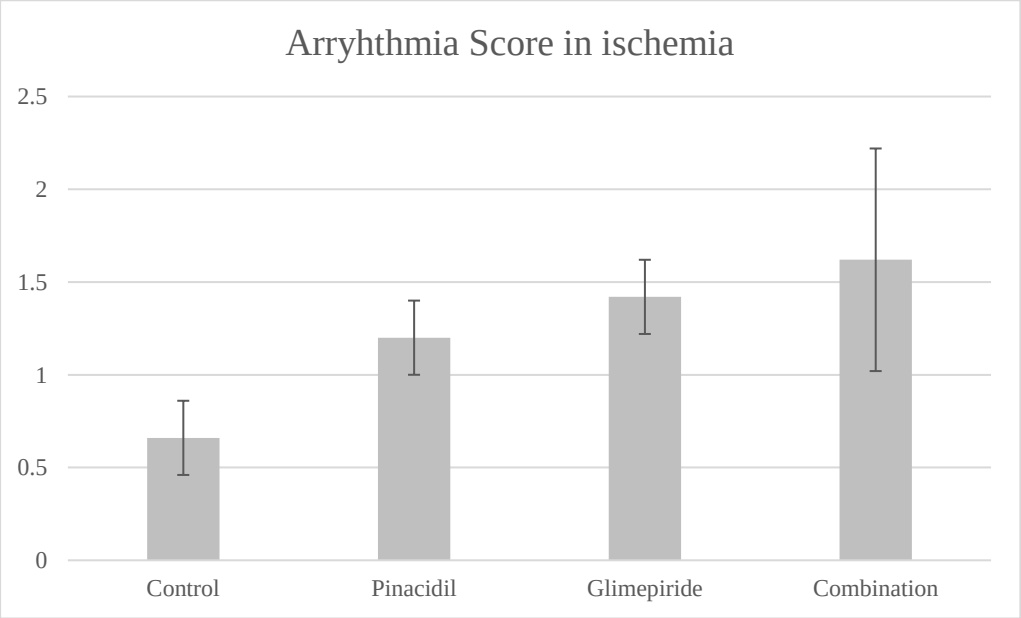
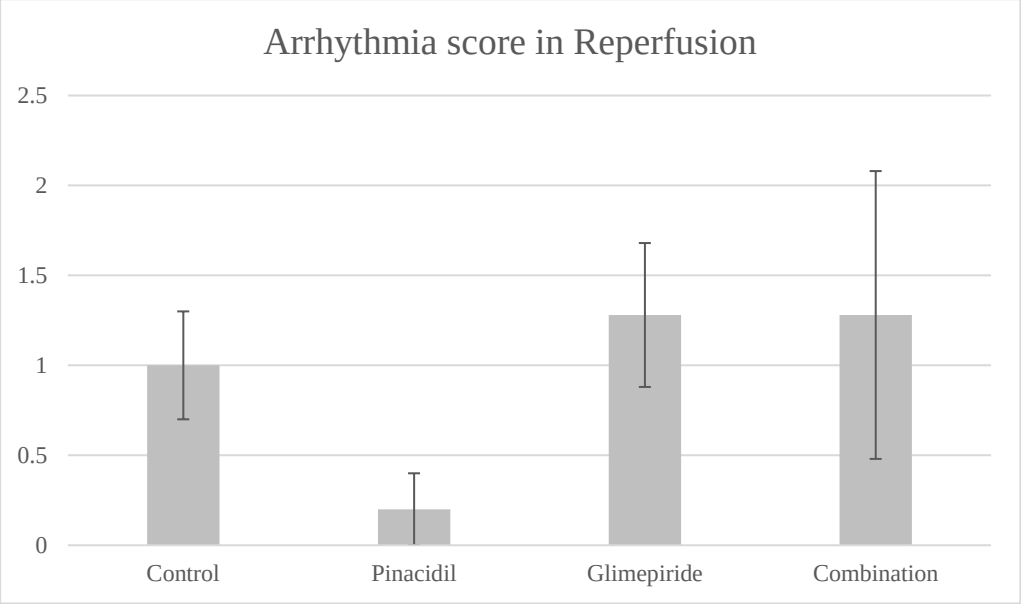


Figure 4-6. Arrhythmia score during 6 min. of reperfusion



The total length of arrhythmia in ischemic period was significantly and in the reperfusion period nonsignificantly higher in the glimepiride drug group in respect to control, (Table 4-10, Table 4-11). But the total length of arrhythmia in reperfusion period was observed non significantly lower than the control in Pinacidil drug group.

Table 4-8. The duration of arrhythmias during 6 min. of ischemia. (*P<0.05, **P<0.01). *a: difference to control.

Groups	N	Length of Arrhythmia (sec.)				
		VPC	VT	VF	Total arr.	Bradycardia
Control (a)	6	1.80 ± 0.9	0	0	1.80 ± 0.9	0
Pinacidil (b)	5	6.44 ± 3.2	0	0	6.44 ± 3.2	0
Glimepiride (c)	7	8.67 ± 2.9	0.80 ± 0.8	0	9.51 ± 2.7 *a p-value=0.029	0
Combination (d)	8	4.96 ± 1.7	0	0	4.70 ± 1.7	0.12 ± 0.1

Table 4-9. The duration of arrhythmias during 6 min. of reperfusion. (*P<0.05, **P<0.01). *a: difference to control

Groups	N	Length of Arrhythmia (sec.)				
		VPC	VT	VF	Total arr.	Bradycardia
Control (a)	6	2.79 ± 2.5	3.58 ± 2.3	0	6.37 ± 2.8	0
Pinacidil (b)	5	0.03 ± 0.03	1.21 ± 1.2	0	1.25 ± 1.2 *a p-value=0.40	0
Glimepiride (c)	7	6.43 ± 5.3	4.52 ± 2.2	0.81 ± 0.8	11.6 ± 6.5	0
Combination (d)	8	1.49 ± 0.8	1.01 ± 0.7	0	2.73 ± 1.0	0.55 ± 0.5

5. DISCUSSION

In recent and past decades, ATP-potassium channels and their modulators attracted the attention for many researchers through their protective role against ischemic-reperfusion myocardial injuries. In several studies, KATP channel modulators showed both pro-arrhythmic and anti-arrhythmic effects in both ischemic and reperfusion phases (14–18,21).

In most animal studies, they have been studied and evaluated the influence of drugs on I/R injuries induced arrhythmia. In earlier studies, it was suggested that the combination of these two channel modulators may have synergic effect (121).

This competitive study presented here indicated that there was no significant effect of Pinacidil and Glimepiride combination on the ischemia-reperfusion injury induced arrhythmia, but in paired comparison by independent t-test, Pinacidil alone was found to decrease reperfusion induced arrhythmia non significantly in respect to control. In previous acute studies it was found that Pinacidil administration decreased the arrhythmia in ischemic periods (122–126). ATP dependent potassium channel opener improved cardiac function in reperfusion period observed in this study supported to the results observed in previous study (127). The effect of channel blocker on the heart rate and cardiac function was also similar as observed in the previous study (128).

In some studies, the pro-arrhythmic effect of Pinacidil was observed in reperfusion period (16,18). Glimepiride effect was as like previous study. Applying of ATP channel blocker in reperfusion period was found pro-arrhythmic as like a previous study that Glimepiride was given before coronary ligation (80,129,130).

In this type of studies, usually the drugs were administered before ischemia and reperfusion, but the time of drug treatment application and the doses of drug given are also important for their arrhythmic or antiarrhythmic effect. In this study, 1mg/kg of Glimepiride was used, based on previous studies (131) which found that the effective dose of channel blocker Glimepiride on ischemia/reperfusion induced arrhythmia is lesser than the affective dose of Glibenclamide which is 5mg/kg. Even

though small doses of Glimepiride used in this study, Glimepiride was found pro-arrhythmic when it was given in reperfusion period. Otherwise Pinacidil given in a 0.1mg/kg dose as like used in previous study was found to be effective on the arrhythmia, even they are given during the ischemic period. But in previous study the Pinacidil was given before coronary ligation (14).

Since this experiment is acute study, the route of drug administration was important to illustrate the drug effect (132). Glimepiride showed an antiarrhythmic effect during the reperfusion period even though it had been introduced prior to the ischemic period (131). In that study the route of Glimepiride administration was also different from the present study, that was intraperitoneal instead of intravenous. Since the less absorption occur to the blood in intraperitoneal injection, the effect of Glimepiride might be more effective to decrease arrhythmia. In our study the drugs were given intravenously to observe immediate action and to ensure the higher concentration in blood within a few minutes, this kind of application procedure was thought to generate more effects to decrease arrhythmia, but it was found pro-arrhythmic in this case (133,134).

The previously mentioned results, which correlate with our results, confirmed that the channel antagonist Glimepiride is pro-arrhythmic and abolishes the anti-arrhythmic effect of channel agonist Pinacidil. This does not support our hypothesis that formed at the beginning of the study. Observed results was different from the expected results. The pro-arrhythmic effect of Glimepiride in reperfusion period was not abolished by the Pinacidil. But less arrhythmia observed during reperfusion in Pinacidil treated group did abolish by the treatment of Glimepiride. No similar or identical study found in the literature that the drugs applying both Pinacidil at ischemic period and Glimepiride at reperfusion. That is why, more study required to support the result of combination obtained in this study.

The hypotensive effect of Pinacidil was observed minimally during ischemia, and this is supposed because of the small dose of Pinacidil used in this work and other effects may be hypotension induced reflex sympathetic activity. In the reperfusion period, the blood pressure was restored to the baseline values in Pinacidil group. Glimepiride treated group showed the highest blood pressure, and heart rate in both periods. The possible explanation for the high blood pressure in ischemic period even there was no drug applied -just a serum fluid as a Pinacidil solvent given- could be related to the individual differences between the animals, or by systemic alteration after infusing of the normal saline as a solvent of pinacidil (135).

The blood pressure and heart rate in combination group in our study was observed similarly to the control. These data of combination group were relatively unpredicted, it was assumed that the result of blood pressure and heart rate in this group maybe the average value obtained from Pinacidil and Glimepiride groups. It is indicated in the previous research related with the competition and molecular docking field of studies that there might be competition between Pinacidil and Glimepiride to bind to the same receptor. It was expected that hypotensive effects of openers would be decreased by KATP potassium channel blocker (136).

It was found in that study, KATP channel blocker Glibenclamide have the predominant effect against the strong selective channel opener diazoxide and inhibit the K+ efflux. It seems that the I/R preconditioning was inhibited by the influence of Glibenclamide because of competition of both drugs for the binding site in the receptors. They explained that the disappearance of protective preconditioning effect of channel opener by the Glibenclamide was due to the specific shape of Glibenclamide molecule, which they have a specific portion named as Cyclohexyl group that considered to be the best target for receptors protein and not found in the channel opener (137).

Regarding to the possible mechanism behind the anti-arrhythmic effect of Pinacidil group alone observed in this study, it can be said that it would be due to the working of Pinacidil as a mediator of NO release. MitoKATP channels in many

studies had been known the most capable target or mediator of preconditioning, but because of Pinacidil is non selective channel opener and used dose was small, it is likely to be that Pinacidil through its vasorelaxant ability stimulates NO release and consequently NO induce the opening of mitoKATP channels indirectly.

The possibility that Glimepiride could indirectly attenuate the cardioprotective effects through it may related to Ca2+ overload mechanism. At beginning of reperfusion mitochondrial dysfunction which affect the cellular events leading to Ca2+ overload and membrane depolarization. Thus, by the opening of ATP dependent potassium channels may prevent Ca2+ overload and remove excess Ca2+ from the inner membrane of mitochondria, and reduces Ca2+ entry through voltage gated Ca2+ channel (138,139). In view of non-significant difference in blood pressure in combination group, hypotensive influence of Pinacidil may not be involved in antiarrhythmic effects, instead it may depend on its effect to the action potential shortening. Since our study do not include cellular data, this suggestion could remain uncertain.

The risk of infarct zone in this study was lesser especially in control group, in respect to others but it was not significant. Less risk of infarct zone in control group is conceivably because of the exposing of short periods of ischemia, and smaller area may generate lesser heterogeneity between ischemic and nonischemic region which may lead to lesser arrhythmia in ischemic period in control groups in respect to others (140). The risk of infarct zone was not found different among groups, but in drugs treated groups was a little bit high more than the control, these could be explained by drugs effects on heterogeneity and arrhythmia susceptibility between hypoxic and normoxic cardiac tissue due to increase or decrease K+ releasing rate (141). The different effect of Glimepiride and Pinacidil on reperfusion induced arrhythmia may be related to their vasoactive properties on peripheral resistance just like as their effect on cardiac output (130).

Another point of view is that the different properties and cardioprotection of channel agonist might be related with the absorption and effectiveness of Pinacidil drug (142). In that study it was observed that there is higher

bioavailability of Pinacidil because of rabid absorption, with less amount around 20% not absorbed and 80% remain in plasma. This may also be another envisage that explain why Pinacidil is very effective to decrease arrhythmia in reperfusion period, and the reason of why in combination with Glimepiride result in arrhythmic score close to Glimepiride treated group not Pinacidil. This might be due to the short periods for the drug effect and the small doses of Pinacidil that used.

In ischemic/reperfusion-induced arrhythmias mechanism, the anesthetic agent used in the operation should be also considered. It's known that almost all of anesthetic agents have a hypotensive influence and effect the function of cardiac and respiratory system (143). Some studies suggest that there are unfavorable effect of urethane in this type of studies (144–146). Urethane has been chosen in this type of research because of its long-lasting effect. In this type of studies, the operation lasts for 1-2 hours, and another reason for usage this anesthetic agent as well it was the only one available in our hand. To minimize the risk factor of urethane during the operation, the administrated doses -as had been discussed in result part with details- were calculated and given according to animals' body weights, which the unnecessary doses were avoided. It means none of the supposed cellular changes caused by urethane occurred, and these values and results showed here were due to the operation and the drugs that were applied in the study.

Since this study it's not a cellular study, the significant anti-arrhythmic or pro-arrhythmic mechanism of Pinacidil and Glimepiride respectively alone or in competition, remain unknown. These results may require further studies to explain more details.

6. CONCLUSIONS AND RECOMMENDATIONS

The hypothesis of this study was that the combination of ATP dependent potassium channel opener Pinacidil administrated during ischemia, Glimepiride in reperfusion may be effective to decrease arrhythmia. Because the action potential duration in normal cells close to ischemic cell by the Pinacidil and Glimepiride given reperfusion period closes the ischemic cells to normal cell. This combined effect was thought to decrease heterogeneity in action potential duration between ischemic and non-ischemic region and finally it would decrease the arrhythmia in reperfusion period. Another expectation of the result of this combination study was that the reflex induced sinusal tachycardia produced due to the hypotensive effect of Pinacidil might be decreased by Glimepiride application. But the data obtained in this study did not support these hypotheses. But similarly, as like the previous study, the Pinacidil was found antiarrhythmic. The protective effects of Pinacidil against to reperfusion induced arrhythmia disappear by the Glimepiride application. This result indicates that heterogeneity in action potential duration increases and more arrhythmias occur in combination. This study reveals that the Pinacidil was also effective to decrease reperfusion induced arrhythmia when it is given in acute stage of ischemia. At the end, it can be told that more study is required to research to clarify the acute effects of ATP dependent channel openers and blocker given alone or in combination, in different dose and route and with using more specific different channel opener and blocker. Another study requirements in future might be to find an answer to the question wither the antiarrhythmic effect of channel opener Pinacidil is derived from opening of KATP channel or from its hypotensive effect.

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Vancouver citation system was used in this thesis.

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