

**ABANT İZZET BAYSAL UNIVERSITY
THE GRADUATE SCHOOL OF NATURAL AND APPLIED
SCIENCES**



**THE EFFECT OF RESVERATROL, BERBERINE AND
GLIBENCLAMIDE COMBINATION ON
ISCHEMIA/REPERFUSION INDUCED ARRHYTHMIAS IN
EXPERIMENTAL SHORT AND LONG TERM DIABETES;
THE ROLE OF ATP DEPENDENT POTASSIUM CHANNELS**

DOCTOR OF PHILOSOPHY

SALİH TUNÇ KAYA

BOLU, OCTOBER 2016

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DEPARTMENT OF BIOLOGY



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APPROVAL OF THE THESIS

The effect of resveratrol, berberine and glibenclamide combinations on ischemia / reperfusion induced arrhythmias in experimental short and long term diabetes; the role of ATP dependent potassium channels submitted by **Salih Tunç Kaya** in partial fulfillment of the requirements for the degree of Doctor of Philosophy in **Department of Biology, Abant İzzet Baysal University** by,

Examining Committee Members

Signature

Supervisor
Prof. Dr. Ömer BOZDOĞAN

.....

Member
Prof. Dr. Mustafa ÜNAL

.....

Member
Prof. Dr. Özge UZUN

.....

Member
Prof. Dr. Cihan DEMİRCİ TANSEL

.....

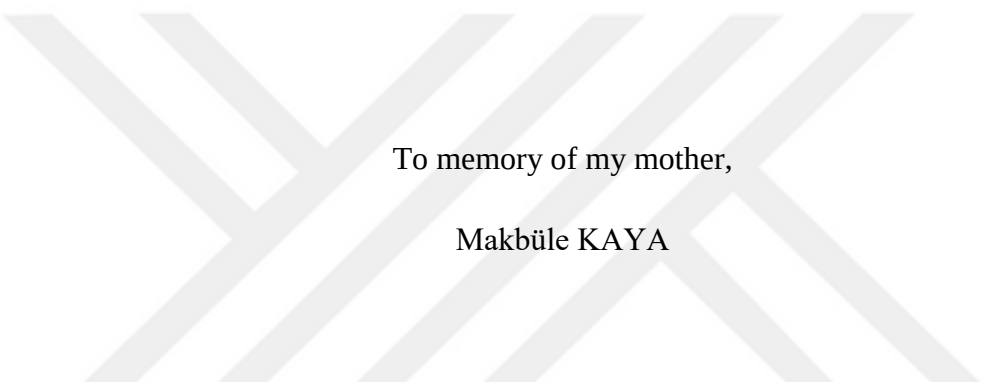
Member
Prof. Dr. Abdullah Tuncay DEMİRYÜREK

.....

October 14, 2016

Prof. Dr. Duran KARAKAS

Director, Graduate School of Natural and Applied Sciences



To memory of my mother,

Makbüle KAYA

DECLARATION

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Salih Tunç KAYA

ABSTRACT

THE EFFECT OF RESVERATROL, BERBERINE AND GLIBENCLAMIDE COMBINATION ON ISCHEMIA/REPERFUSION INDUCED ARRHYTHMIAS IN EXPERIMENTAL SHORT AND LONG TERM DIABETES; THE ROLE OF ATP DEPENDENT POTASSIUM CHANNELS

PHD THESIS

SALİH TUNÇ KAYA

ABANT İZZET BAYSAL UNIVERSITY GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

DEPARTMENT OF BIOLOGY

(SUPERVISOR: PROF. DR. ÖMER BOZDOĞAN)

BOLU, OCTOBER 2016

In the present study, it was aimed to investigate the effect of resveratrol, berberine and glibenclamide combination on ischemia/reperfusion induced arrhythmias and in relation with K_{ATP} channel in streptozotocin-induced diabetic rats. In this study, 210 male Sprague Dawley rats, 5-6 months age, were used. Diabetes was induced by intraperitoneal injection of streptozotocin (65 mg/kg). Resveratrol (5 mg/kg), berberine (10 mg/kg) and glibenclamide (5 mg/kg) were freshly prepared and daily administrated for 2 and 6 weeks. Six minutes ischemia and six minutes reperfusion were performed for evaluating arrhythmias. Short-term diabetes significantly decreased the incidence of other types of arrhythmias (Ventricular premature contraction, salvo, bigeminy) induced by reperfusion. However, combination of berberine and glibenclamide significantly increased the incidence of other types of arrhythmias. In long-term diabetes, resveratrol alone and its combination with glibenclamide significantly decreased the arrhythmia score, arrhythmic period, duration and incidence of other types of arrhythmia during reperfusion. Short-term diabetes did not change protein expression level of Kir 6.2 subunit; however, long-term diabetes significantly decreased protein expression level of Kir 6.2 subunit. Resveratrol, berberine and glibenclamide combinations significantly increased protein expression of Kir 6.2 subunits in long-term diabetes. In conclusion, short-term diabetes increased the resistance of diabetic hearts against reperfusion-induced arrhythmias. As the duration of diabetes increased, the resistance against the arrhythmias disappeared. Resveratrol alone and its combination with glibenclamide were effective in decreasing reperfusion-induced arrhythmias. Long-term diabetes resulted in decreased protein expression of Kir 6.2 subunit, which might be a reason for decreased resistance of diabetic heart to arrhythmias in long-term diabetes. Upstream and downstream signaling pathways for the effect of resveratrol and diabetes mellitus on myocardial K_{ATP} channel and arrhythmias should be determined in future studies.

KEYWORDS: Arrhythmia, Diabetes, Resveratrol, Berberine, Glibenclamide

ÖZET

KISA VE UZUN DÖNEM DENEYSEL DİYABETTE İSKEMİ VE REPERFÜZYON ARİTMİLERİ ÜZERİNE RESVERATROL, BERBERİN VE GLİBENKLAMİD KOMBİNASYONUNUN ETKİSİ; ATP BAĞIMLI POTASYUM KANALININ ROLÜ

DOKTORA TEZİ

SALİH TUNÇ KAYA

**ABANT İZZET BAYSAL ÜNİVERSİTESİ FEN BİLİMLERİ ENSTİTÜSÜ
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Bu çalışmada streptozosin ile diyabet oluşturulmuş sıçanlarda resveratrol, berberin ve glibenklamid kombinasyonunun iskemi ve reperfüzyon ile uyarılan aritmiler üzerine etkisinin ve K_{ATP} kanalları ile ilişkisinin araştırılması amaçlandı. Bu çalışmada, 5-6 aylık 210 erkek Sprague Dawley sıçan kullanıldı. Diyabet streptozosin'in (65 mg/kg) intraperitoneal enjeksiyonu ile oluşturuldu. Resveratrol (5 mg/kg), berberin (10 mg/kg) ve glibenklamid (5 mg/kg) uygulamadan hemen önce hazırlandı, 2 ve 6 hafta boyunca günlük uygulandı. Aritmilerin değerlendirilmesi için altı dakika iskemi ve altı dakika reperfüzyon gerçekleştirildi. Kısa dönem diyabet reperfüzyon ile uyarılan diğer tip aritmi (Ventriküler prematüre kasılma, salvo, bigemini) yoğunluğunu anlamlı şekilde azalttı. Fakat berberin ve glibenklamid kombinasyonu diğer tip aritmi yoğunluğunu anlamlı şekilde arttırdı. Uzun dönem diyabette, resveratrol tek başına ve glibenklamid ile birlikte reperfüzyonda aritmi skorunu, aritmik periyodu, diğer tip aritmilerin süresini ve sıklığını anlamlı şekilde azalttı. Kısa dönem diyabet Kir 6.2 alt birim protein anlatımını değiştirmede, fakat uzun dönem diyabet Kir 6.2 alt birim protein anlatımını anlamlı şekilde azalttı. Uzun dönem diyabette, resveratrol, berberin ve glibenklamid kombinasyonu Kir 6.2 alt birim protein anlatımını anlamlı şekilde arttırdı. Sonuç olarak, kısa dönem diyabet reperfüzyon ile uyarılan aritmilere karşı diyabetik kalplerin direncini arttırdı. Diyabet süresi uzadıkça aritmilere karşı direnç kayboldu. Resveratrol tek başına ve glibenklamid ile kombinasyonu reperfüzyon ile uyarılan aritmilerin azaltılmasında etkiliydi. Uzun dönem diyabet Kir 6.2 alt birim protein anlatımını azalttı, bu durum uzun dönem diyabette, diyabetik kalbin aritmilere karşı azalan direncinin bir nedeni olabilir. Resveratrol ve diabetes mellitus'un miyokardial K_{ATP} kanalı ve aritmiler üzerindeki etkisinde yukarı ve aşağı sinyal yolları gelecekteki çalışmalarda tespit edilmelidir.

ANAHTAR KELİMELEER: Aritmi, Diyabet, Resveratrol, Berberin, Glibenklamid

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LIST OF ABBREVIATIONS AND SYMBOLS

2C	: non-diabetic rats for 2 weeks
2D	: Non-treated diabetic rats for 2 weeks
2DB	: Diabetic rats treated with berberine for 2 weeks
2DBG	: Diabetic rats treated with combination of berberine and glibenclamide for 2 weeks
2DG	: Diabetic rats treated with glibenclamide for 2 weeks
2DR	: Diabetic rats treated with resveratrol for 2 weeks
2DRG	: Diabetic rats treated with combination of resveratrol and glibenclamide for 2 weeks
4-HNE	: 4-hydroxynoneal
6C	: non-diabetic rats for 6 weeks
6D	: Non-treated diabetic rats for 6 weeks
6DB	: Diabetic rats treated with berberine for 6 weeks
6DBG	: Diabetic rats treated with combination of berberine and glibenclamide for 6 weeks
6DG	: Diabetic rats treated with glibenclamide for 6 weeks
6DR	: Diabetic rats treated with resveratrol for 6 weeks
6DRG	: Diabetic rats treated with combination of resveratrol and glibenclamide for 6 weeks
ADP	: Adenosine Diphosphate
AGE	: Advanced Glycation End-products
AMP	: Adenosine Monophosphate
AMPK	: Adenosine Monophosphate-Activated Protein Kinase
ANOVA	: Analysis of variance
APD	: Action potential duration
ATP	: Adenosine Triphosphate
BB	: Bio-breeding rat
Bcl-2	: B-cell lymphoma 2
BSA	: Bovine serum albumin
Ca²⁺	: Calcium ion
CFTR	: Cystic Fibrosis Transmembrane Conductance Regulator
CK	: Creatine Kinases

Cl⁻	: Chloride ion
CSB	: Cystathionine β synthase
DAG	: Diacylglycerol
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic acid
ECG	: Electrocardiogram
EDTA	: Ethylenediaminetetraacetic acid
ELISA	: Enzyme-Linked Immunosorbent Assay
eNOS	: Endothelial Nitric Oxide Synthase
ERK	: Extracellular Signal-Regulated Kinases
ET-1	: Endothelin 1
g	: Gram
GDP	: Guanosine DiPhosphate
GFAT	: Glutamine: Fructose-6-Phosphate Amidotransferase
GLUT2	: Glucose Transporter 2
GTP	: Guanosine Triphosphate
H⁺	: Hydrogen Ion
HRP	: Horseradish Peroxidase
HSP	: Heat Shock Protein
I	: Ischemia
I/R	: Ischemia/Reperfusion
IL-1β	: Interleukin-1 beta
IL-6	: Interleukin-6
IP	: Intraperitoneally
IPC	: Ischemic Preconditioning
IU	: International Unit
IV	: Intravenous
K⁺	: Potassium Ion
K_{ATP}	: ATP Dependent Potassium Channel
KDP	: The Komeda Diabetes-Prone rats
Kir 6.x	: Inwardly Rectifying K ⁺ Channel Core
LAD	: Left Anterior Descending
LCA	: Left Coronary Artery
LDH	: Lactate Dehydrogenase

LETL	: Long Evans Tokushima lean rats
MAD	: Malondialdehyde
MAPK	: Mitogen-Activated Protein Kinases
MDA	: Malondialdehyde
mg	: Milligram
min	: Minute
mIU/L	: Milli-International Units Per Liter
mitoK_{ATP}	: Mitochondrial ATP Dependent Potassium
mmHg	: Millimeter Mercury
MnSOD	: Manganese Superoxide Dismutase
mPTP	: Mitochondrial Permeability Transition Pore
mRNA	: Messenger RNA
Na⁺	: Sodium Ion
Na⁺/K⁺ ATPase	: Sodium-Potassium Adenosine Triphosphatase
NaCl	: Sodium Chloride
NADH	: Nicotinamide Adenine Dinucleotide
NADPH	: Nicotinamide Adenine Dinucleotide Phosphate
NF-κB	: Nuclear Factor kappa B
Ng/ml	: Nano gram per Milliliter
NO	: Nitric Oxide
NOD	: Non-Obese Diabetic Mouse
NOS	: Nitric Oxide Synthase
O₂⁻	: Superoxide Anion
OH⁻	: Hydroxyl Radicals
PAI-1	: Plasminogen Activator-1
PBS	: Phosphate Buffered Saline
PBS-T	: Phosphate Buffered Saline-Tween 20
PI3K	: Phosphatidylinositol-3-kinases
PIP2	: Phosphatidylinositol 4,5-bisphosphate
PKC	: Protein kinase C
PMSF	: Phenylmethylsulfonyl Fluoride
PVDF	: Polyvinylidene Difluoride
R	: Reperfusion
ROS	: Reactive Oxygen Specious

R.P.M	: Revolutions per minute
SarcK_{ATP}	: Sarcolemmal ATP Dependent Potassium Channel
SC	: Subcutaneous
SDS-PAGE	: Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis
SE	: Standard Error
Sec	: Seconds
SERCA 2a	: Sarcoendoplasmic Reticulum Ca ⁺² -ATPase 2a
SOD	: Superoxide Dismutase
SPSS	: Statistical Package for the Social Sciences
STZ	: Streptozotocin
SUR	: Sulfonyurea Receptor
T3	: Triiodothyronine
T4	: Thyroxine
TGF-α	: Transforming Growth Factor Alpha
TGF-β1	: Transforming Growth Factor Beta 1
Thr-172	: Threonine residue
U/L	: Units per Liter
UDP-GalNAc:	UDP-Nacetylgalactosamine
UDP-GlcNAc:	UDP-Nacetylglucosamine
VF	: Ventricular Fibrillation
VPC	: Ventricular Premature Contraction
VT	: Ventricular Tachycardia
α	: Alpha
β	: Beta
γ	: Gama
δ	: Delta
ϵ	: Epsilon
ζ	: Zeta
θ	: Theta
μ	: Mu

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1. INTRODUCTION

Diabetes is generally defined as a chronic disease in which body cannot sufficiently control blood glucose level. It is mainly divided into two types: Type I and Type II diabetes. Type I diabetes is characterized by insufficient or no secretion of insulin hormone from pancreatic β (beta) cells whereas Type II diabetes is known as increased resistance to insulin hormone (Whittington et al., 2012).

Increases in the intensity of diabetes around the world is getting a worrisome level. In an announcement by International Diabetes Federation in 2013, it is estimated that diabetes will affect approximately 592 million people until 2035 (International Diabetes Federation, 2013). The increase in heart diseases associated with diabetes in developing countries has been observed as a significant burden (Bakalmur and Sharma, 2012). Diabetic patients become more prone to cardiovascular disorders. Coronary artery diseases, hypertension, atherosclerosis, cardiomyopathies and ischemia/reperfusion (I/R) injury are frequently observed dysfunctions among diabetics (De Angelis et al., 2002; Whittington et al., 2012). In diabetic patients, the main result of ischemic heart disease is the observation of more acute I/R injury (Whittington et al., 2012). However, exact causes of increased myocardial damage after reperfusion have not been fully understood (Ansley and Wang, 2013).

Myocardial ischemia is defined as decrease or prevention of blood flow to myocardium as a result of obstruction of coronary artery by a clot or mechanically whereas the re-opening of obstructed coronary artery by pharmacological agents or mechanically is called as reperfusion. Myocardial tissues damage occurred firstly by ischemia and then reperfusion is known as acute I/R injury (Frank et al., 2012). Although the only way to rescue previously ischemic myocardium from necrosis is reperfusion, reperfusion can itself increase tissue damage and can lead to life-threatening ventricular arrhythmias (Piers et al., 2011). The most important causes of death among diabetic patients have been thought to be ventricular arrhythmias observed following ischemia and reperfusion. For this reason, development of new

strategies in decreasing cardiovascular complications, preventing lethal arrhythmias and myocardial injury depending on diabetes become a critical health problem (Nakou et al., 2012). Activation of myocardial ATP-dependent potassium channels (K_{ATP}) plays important role in cardioprotective effect in the conditions of myocardial ischemia and/or hypoxia (Cole et al., 1991). It has been shown in a previous study that opening of myocardial K_{ATP} channel during ischemia is protective against ischemia-induced arrhythmias (Bozdogan, 2005). It has been indicated that myocardial Kir 6.2 channel protein expression in diabetic heart decreased two-fold compared to control heart after 8 weeks diabetic duration (Chen et al., 2012). Above study shows that myocardial K_{ATP} channel can be affected by pathologic situations. In literature, up to present no more researches were found that investigate the role of myocardial K_{ATP} channel in diabetes. Clinical studies indicate that diabetic heart is more susceptible to cardiovascular diseases; however, experimental researches indicate conflicting results. The sensitivity of diabetic heart to I/R induced arrhythmias show differences depending on the duration of diabetes. In the short period of diabetes, diabetic heart is thought to show more resistance to arrhythmias although in long period of diabetes, heart losses its intrinsic protective mechanisms against arrhythmias (Bakalmur and Sharma, 2012). Also, several studies show that diabetic hearts can exert resistance to I/R injury during acute phase of diabetes; however, during late period of diabetes diabetic heart can show more susceptibility to I/R injury. In an *in vitro* study (30 min global ischemia and 30 min reperfusion), the incidence of ventricular fibrillation (VF) and tachycardia (VT) was found to be lower in diabetic heart in compared to non-diabetic control heart after 2 weeks diabetic period. Nevertheless, it was not observed in 4, 6 and 8 weeks diabetic groups (Tosaki et al., 1996). Similar results were also obtained in another study performed by Zhang et al. (2002). Although diabetic hearts show different response, the underlying mechanism responsible for such effects have been studied only and not clarified well.

It has been shown in various studies that resveratrol reduced infarct size, the arrhythmias induced by I/R, and prolonged lifespan in non-diabetics rats (Chen et al., 2008; Lin et al., 2008; Lam et al., 2013). In addition, it has been demonstrated that resveratrol has a powerful antioxidant capacity and protects the pancreatic β cells, lowers blood glucose levels and increases insulin hormone levels in diabetic animals (Szkudelski and Szkudelska, 2011). However, the effect of resveratrol on I/R injury,

arrhythmias and especially on death due to VF in diabetic rats have not been yet clarified. In diabetic heart, it was showed that resveratrol (2.5 mg/kg/15 day orally administration) decreased infarct size in diabetic heart after 2 weeks diabetic period in compared to control heart (Thirunavukkarasu et al., 2007). A conflicting result has been obtained by Huang et al. (2010). Resveratrol alone or in combination with insulin do not have a significant effect in decreasing infarct area and the incidence of arrhythmias. However, survival rate in diabetic rats have been found to decrease in contrast to non-diabetic rats. In diabetic heart, cardio protective effect of resveratrol and underlying mechanism of the protection have not been completely investigated. Recently in a research, it has been indicated that intraperitoneal injection of resveratrol just 60 minute before 30 minute of ischemia increase expression of myocardial K_{ATP} channel in non-diabetic heart (Du et al., 2014). Consequently, both the result of expression of myocardial K_{ATP} channel decreases in 8 weeks diabetic heart (Chen et al., 2012) and resveratrol increases myocardial K_{ATP} channel in non-diabetic heart (Du et al., 2014) give rise following questions: Can resveratrol treatment prevent the decreased resistance to I/R injury and be protective against I/R induced arrhythmias in the late period of diabetes?

More death and myocardial I/R injury due to the heart attack have been observed in patients taken glibenclamide as an anti-diabetic drug (Monami et al., 2006; Khalangot et al., 2009). In various studies and in our previous study, it has been observed that glibenclamide can decrease I/R induced arrhythmias (Billman et al., 1993; Leprán et al., 1996; Bozdogan et al., 2013). On the other hand, it has been also observed in previous studies that glibenclamide can increase risk of arrhythmias (Bernauer, 1997; del Valle et al., 2001). However, the effect of glibenclamide on I/R induced arrhythmias depending on short and long-term diabetes has not been experimentally investigated before.

Berberine exerts hypoglycemic effect by activating glycolysis and adenosine monophosphates activated kinase (AMPK). Also, it has been stated that berberine increases insulin hormone sensitivity and secretion of insulin. In addition, berberine have been thought to be effective in decreasing cardiovascular complications in diabetes due to its cholesterol lowering, anti-arrhythmic and NO release stimulatory effect (Yin et al., 2012). With such beneficial effects, it has been used as an anti-

diabetic agent in China approximately for 1400 years. It has been demonstrated that Berberine can decrease arrhythmia score, prolonged QTc interval, Ca^{2+} and K^{+} inflow (Wang et al., 2012). These two studies show that berberine only or combination with glibenclamide may be effective in decreasing or preventing I/R induced arrhythmias in diabetic hearts. Up to now, any study investigating the role of berberine and glibenclamide combinations on I/R induced arrhythmias in diabetic rats are not found.

1.1 Diabetes Mellitus

Diabetes mellitus is a chronic disorder that results from insulin deficiency or insulin resistance. Diabetes mellitus is characterized by high blood glucose level or hyperglycemia. Body cannot control blood glucose level due to lack of insulin hormone, insufficient insulin secretion or resistance to insulin hormone. Diabetes mellitus can be classified in three groups; Type I diabetes, Type II diabetes and Gestational diabetes (American Diabetes Association, 2004).

Type I diabetes mellitus is known as insulin dependent diabetes mellitus. Autoimmune destruction of β cell of pancreatic islet leads to type I diabetes mellitus. There are several factors which result in destruction of β cells. Genetic and environmental factors can be given as example. T-lymphocytes attack the β cell of pancreatic islet, resulting in the cell destruction. The islets become infiltrated by lymphocytes and become necrotic. This condition is called as insulinitis or inflammation of the islets. As a result of insulinitis, cytokines are locally produced and can damage β cells of pancreatic islet, inducing diabetes (Szablewski, 2014). However, the exact causes of cell destruction are not still clarified. The usage of insulin is inevitable for all patients with type I diabetes mellitus for survival.

Type II diabetes mellitus is also called as insulin independent diabetes mellitus. It is a heterogeneous abnormality which is characterized by both resistance to insulin hormone and relative insulin insufficiency. Thus, type II diabetic patients do not require insulin therapy for acute survival. One of the important characteristic of type II diabetes mellitus is that ketosis cannot be seen because insulin level is

sufficient to prevent ketosis, except in the condition of extreme stress (McCowen and Smith, 2013).

Gestational diabetes mellitus is another type of diabetes mellitus. It is defined as hyperglycemia during pregnancy. It is estimated that gestational diabetes mellitus can be observed in 2-5% of pregnant women (McCowen and Smith, 2013). There are many causes that lead to gestational diabetes mellitus, including changes in cortisol secretion, human placental lactogen secretion, insulinase secretion, growth hormone secretion, decreased exercise and increased caloric intake (Gilmartin et al., 2008).

1.1.1 Animal Model of Diabetes Mellitus

Animal model of diabetes mellitus has been used frequently in experimental researches. Diabetes mellitus in experimental animals have been performed by using chemical, surgical, genetic or other techniques such as modified diets (Chatzigeorgiou et al., 2009; King, 2012). Experimental studies carried out on diabetic animals are essential to understand underlying mechanism of physiological dysfunctions, especially in cardiovascular system related with diabetes mellitus. Thus, new therapeutic strategies and cure can be developed for the treatment of diabetes and diabetes related cardiovascular diseases.

Types I diabetes in experimental animals can be classified as chemically induced type I diabetes and spontaneous type I diabetes (Rees and Alcolado, 2005; Chatzigeorgiou et al., 2009; King, 2012). Spontaneous diabetes type I refers to autoimmune models of type I diabetes which then can be divided into 5 animal models: non-obese diabetic mouse (NOD), bio-breeding rat (BB), Long Evans Tokushima lean rats (LETL), the Komeda diabetes-prone rats (KDP), LEW.1AR1/Ztm-iddm rats (Table 1.1). Among them, NOD mouse and BB rats are widely used autoimmune models accepted by researchers (King, 2012). NOD mouse was developed and first reported in 1980 (Makino et al., 1980). In 1970s, BB rats, spontaneous hyperglycemia and ketoacidosis appeared, are stem from a Canadian colony of outbred Wistar rats (Mordes et al., 2004). LETL rats were discovered in 1982. They were characterized with sudden onset of polyuria, polyphagia, and hyperglycemia, and there were no sex differences in severity (Kawano et al., 1991).

KDP rats are also another spontaneous animal model of type I diabetes mellitus, characterized with rapid onset of diabetes without sex differences (Yokoi et al., 2003). LEW.1AR1/Ztm-iddm rats were also a model of spontaneous type I diabetes mellitus, which were characterized by hyperglycemia, glycosuria, ketonuria and polyuria without significant sex differences (Lenzen et al., 2001).

Table 1.1. Animal models of Type I diabetes

Animal models of Type I diabetes	
Spontaneous Type I diabetes	Chemically induced Type I diabetes
Non-obese diabetic (NOD)	Streptozotocin
Bio-breeding (BB)	Alloxan
Long Evans Tokushima lean (LETL)	
The Komeda diabetes-prone (KDP)	
LEW.1AR1/Ztm-iddm	

Chemically induced diabetes mellitus is used in experimental studies to mimic type I diabetes mellitus. Many chemical substances can be used to induce diabetes mellitus in experimental animals (Table 1.2). Most commonly used and accepted drugs mimicking diabetes mellitus are streptozotocin (STZ) and alloxan (Kumar et al., 2012).

Table 1.2. Chemical compounds inducing experimental diabetes mellitus

Chemical compounds inducing Type I diabetes in experimental animals	
Substances	References
Alloxan	Karthicka et al., 2014
Streptozotocin	Akbarzadeh et al., 2007
Vacor	Lee et al., 1988
Dithizone	Monago and Ugbomeh, 2004
8-hydroxyquinolone	Root and Chen, 1952

Streptozotocin is N-nitro derivative of glucosamine, which is isolated from soil *Streptomyces achromogenes* (Szkudelski, 2001). STZ is also a broad-spectrum antibiotic and is toxic to β cells of pancreatic islets (Deeds et al., 2011). It is clinically used for the treatment of cancers of the Langerhans Islets (Pavel et al., 2014). STZ is taken by cell via GLUT2 transporter, resulting in alkylation of DNA and β cell death. Toxic effect of STZ is not only restricted to β cells but also can damage other tissues such as liver and kidney (Deeds et al., 2011). STZ induced diabetes mellitus in experimental animals are more stable, simple and can be used in long-term experimental studies. On the other hand, mortality rate is relatively more (Kumar et al., 2012). Administration route, preparation and dosage of STZ to induce diabetes exert differences in literature. Intraperitoneal (Mahay et al., 2004), intravenous (Gajdosík et al., 1999), subcutaneous (Qinna and Badwan, 2015), intracardiac (Aomine et al., 1990) and intramuscular (Nakhoda and Wong, 1979) administration have been performed to induce diabetes mellitus. However, intraperitoneal and intravenous administrations of STZ are commonly referred delivery routes.

Induction of Type II diabetes mellitus by STZ and alloxan is widely used in experimental studies (Szkudelski, 2001). Injection of low dose STZ and high fat diet are used to induce type II diabetes mellitus; however, discussion is going on whether these drugs induce type I or type II diabetes mellitus. It has been thought that administration of these chemicals destroy the β cells of pancreas, thereby creating mostly type I diabetes rather than type II diabetes (Kumar et al., 2012). The effect of STZ on pancreatic β cell is dose-dependent. It was shown that less than 50 mg/kg STZ induced reversible diabetes mellitus whereas higher than 50 mg/kg STZ resulted in rapid and irreversible diabetes mellitus (Ar'Rajab and Ahren, 1993).

1.1.2 Cellular Alternations during Diabetes

All forms of diabetes mellitus are associated with micro and macro vascular complications due to chronic hyperglycemia. Microvascular complications of diabetes mellitus resulting from damage to small blood vessels include retinopathy, nephropathy and neuropathy. However, macrovascular complications mainly contain

cardiovascular diseases, which results in myocardial infarction, arrhythmia and cerebrovascular disease such as strokes (Brownlee, 2001; Forbes and Cooper, 2013). In addition, erectile dysfunctions (Phé and Rouprêt, 2012), dementias (Luchsinger, 2010), depression and anxiety (Degmecić et al., 2014) are commonly observed physiological complications in diabetic patients.

Underlying mechanisms of biochemical and cellular changes observed during diabetes mellitus can be mainly categorized as (1) increased polyol pathways, (2) increased activity of the hexosamine pathway, (3) increased activation of protein kinase C and (4) increased advanced glycation end-products (AGEs) formation.

Polyol pathways are also known as the sorbitol-aldose reductase pathway. In conditions of hyperglycemia, excess glucose enters polyol pathways in which aldose reductase reduces glucose to sorbitol with oxidizing NADPH to NADH⁺. Sorbitol is then oxidized to fructose by sorbitol dehydrogenase, with producing NADH from NAD⁺. Increased in polyol pathways flux results in an increase in cytosolic NADH/NAD⁺, a decrease in cytosolic NADPH, a decreased Na⁺/K⁺ ATPase activity and finally increased osmotic stress which lead to osmotic damage to cell (Brownlee, 2001). Decreases in NADPH also decrease the glutathione reductase and glutathione peroxidase system, resulting in attenuation of regenerating the reduced glutathione/oxidized glutathione ratio. Briefly, these reductions could induce intracellular oxidative stress (Brownlee, 2001; Rochette et al., 2014).

In hexosamine pathway, fructose-6-phosphate is converted to glucoseamine-6-phosphate. Glutamine: fructose-6-phosphate amidotransferase (GFAT) is rate-limiting enzyme which catalyzes the conversion of fructose-6-phosphate and glutamine to glucosamine-6-phosphate and glutamate. Then, glucosamine-6-phosphate is metabolized to UDP-Nacetylglucosamine (UDP-GlcNAc), UDP-Nacetylgalactosamine (UDP-GalNAc) and CMP-sialic acid. These are the essential building blocks of glycosylic side chains of glycoproteins, glycolipids, proteoglycans and gangliosides. Hyperglycemia induce hexosamine pathway by increasing activity of GFAT (Brownlee, 2001). Increasing activity of GFAT results in overexpression of transforming growth factor beta 1 (TGF-β1), transforming growth factor alpha (TGF-α), nuclear factor kappa B (NF-κB) dependent genes, plasminogen activator-1 (PAI-1) (Brownlee, 2001; James et al., 2002; Sharma and Sharma, 2013). Also, increased

hexosamine pathway flux results in decreased expression of sarcoendoplasmic reticulum Ca^{+2} -ATPase 2a (SERCA 2a) both at mRNA and protein level, attenuated SERCA2a promoter activity, increased O-GlcNAcylation of nuclear proteins (Clark et al., 2003).

Protein kinase C (PKC) is a serine/threonine-related protein kinase, which plays a vital role in signal transduction system and cellular functions. PKC family comprises three subgroups and at least twelve isoforms. Classifications of PKC isoforms are made by determining whether their domains bind to Ca^{+2} or a lipid second messenger, diacylglycerol (DAG). Conventional PKC (α , $\beta 1$, $\beta 2$, and γ) contain domain for both Ca^{+2} and DAG; however, novel PKC (δ , ϵ , η , θ , μ) include domain for DAG, but not Ca^{+2} , and atypical PKC (ζ , ι/λ) binds neither of them (Meier and King, 2000; Noh and King, 2007). Hyperglycemia associated with diabetes results in production of excess amount of DAG via de novo synthesis of DAG from glycolytic intermediate dihydroxyacetone phosphate which is reduced to glycerol-3-phosphate. DAG directly activates nine of PKC isoforms. PKC families can also be activated by increased polyol pathway, oxidative stress and increased AGE. Cellular changes and functional disorders due to diabetes mellitus all seems to be resulted from increased production of superoxide ion (Giacco and Brownlee, 2010).

Endothelium dysfunction is defined as abnormalities in the vascular homeostasis and abnormalities in the function of endothelium. Initial dysfunction in endothelium of vascular system due to hyperglycemia is seem to be underlying causes of latter cardiovascular disorders such as atherosclerosis, coronary heart diseases, myocardial infarction, arrhythmias (Tabit et al., 2010). There are two important pathways regulating endothelial functions; PI3-K/Akt and MAPK/ERK pathways. PI3K/Akt pathways are dominated in healthy endothelium, which mainly enhance eNOS expression. However, in type II diabetes mellitus, MAPK/ERK pathways can dominate in the control of vasomotor activities, which promotes ET-1 expression (Roberts and Porter, 2013).

Diabetes mellitus induces a variety of changes in ion channels and transport mechanisms. Ca^{2+} -ATPase and sarcoplasmic reticulum Ca^{2+} pump activity decreases in myocardial cell of diabetic heart at 3 weeks after STZ-infusion (Takeda et al., 1996). In addition, it was reported in this study that $\text{Na}^+/\text{Ca}^{2+}$ exchange, Ca^{2+} channel density and α and β adrenoceptor density on sarcolemma were decreased in diabetic heart. Also, Ca^{2+} uptake in sarcoplasmic reticulum decreases as a result of depression in SERCA2a activity (Ganguly et al., 1983). In another study, it was indicated that Ca^{2+} pump activity in sarcolemma was suppressed, which resulted in changes in Ca^{2+} transport in 8 weeks STZ-induced diabetic heart (Heyliger et al., 1987). Another experimental study revealed that diabetes mellitus resulted in depressed Na^+ dependent Ca^{2+} uptake in heart sarcolemmal membrane, which revealed decrease in Ca^{2+} movement via $\text{Na}^+/\text{Ca}^{2+}$ exchanger and Ca^{2+} pump mechanisms, leading to Ca^{2+} overload (Makino et al., 1987). In alloxan-induced diabetic rats, Na^+/K^+ ATPase, $\text{Na}^+/\text{Ca}^{2+}$ exchanger and ATP dependent Ca^{2+} uptake decreased, which is suggested to induce Ca^{2+} overload (Golfman et al., 1998). Additionally, $\text{Na}^+/\text{Ca}^{2+}$ ATPase activity in sarcolemma of diabetic heart was found to be depressed (Pierce et al., 1983a). It was also reported by the same group that Ca^{2+} binding to heart sarcolemma decreased in diabetic heart from 8 weeks (Pierce et al., 1983b). Long-term diabetes mellitus resulted in decreased Ca^{2+} influx through voltage dependent Ca^{2+} channels due to reduction in ^3H -nitrendipine binding sites (Lee et al., 1992).

Another important alteration due to diabetes mellitus is cardiac autonomic neuropathy, which is called as abnormality in sympathetic and parasympathetic nervous system. Sympathetic activity and cardiac norepinephrine concentration in diabetic rats was found to increase at early stage of diabetes (Ganguly et al., 1986). Diabetes mellitus decreased the β adrenoceptor stimulation and arterial pressure reactivity to sympathetic stimulation, reducing systolic and diastolic arterial pressure (Gallego et al., 2002). In myocardial cell, adrenergic receptors density was found to be decreased in STZ-induced diabetic rat heart (Bitar et al., 1987). Response of myocardium to catecholamine decreases in diabetic rat heart due to decreased number of adrenergic receptors (Takeda et al., 1996). In a recent study, it was shown that 3 months diabetes resulted in myocardial parasympathetic denervation; however, 6 months diabetes caused sympathetic hyper-innervation and aggravated parasympathetic and sympathetic imbalance (Xuan et al., 2015).

1.1.3 Diabetes and Myocardial Infarction

Diabetic patients are highly associated with ischemic heart disease including myocardial infarction, which is one of the major causes of morbidity and mortality in developing countries (George and Linda et al., 2001; Hayat et al., 2004). Although hyperglycemia or diabetes mellitus is thought to be a primary factor clinically in cardiovascular dysfunction, investigations performed on experimental animal models of diabetes mellitus have indicated that no change or increased or decreased sensitivity of diabetic heart to myocardial ischemia are seen.

Several experimental studies have reported that diabetic heart shows less resistance to myocardial ischemia or having greater infarct size after I/R injury (Table 1.3). For example, Forrat et al. (1993) reported that infarct size after ligation of left descending coronary artery (LDA) for 2 hours in 75 days diabetic dogs was larger than that in control dogs. In diabetic rats at 8 days after STZ infusion, infarct size induced by 25 minute of ischemia was higher in diabetic hearts than in non-diabetic hearts (Marfella et al., 2002). In an another study, infarct size in diabetic hearts at 20 weeks after STZ administration was found to be higher in compared to control hearts (Xu et al., 2004). Ligation of coronary artery in 2 weeks diabetic rats results in infarct size elevation in contrast to age-matched control (Xiao et al., 2004). Additionally, it was reported by Di Filippo et al. (2005) that infarct size in *in vivo* acute I/R model after 8 days diabetes increased. Similarly, infarct size was found to be increased in 4 weeks diabetic rat heart in isolated heart model of I/R injury (Thirunavukkarasu et al., 2007). Another important study demonstrated in an *in vivo* I/R model that infarct size in STZ-induced diabetic rats at 3 weeks increased when compared to control rats (Annapurna et al., 2008). It was demonstrated that diabetic hearts were more sensitive to ischemic damage after both 4 weeks and 8 weeks diabetic durations (Shi-Thing et al., 2011). It was observed in that study that infarct size increased in both 4 weeks and 8 weeks diabetic hearts in comparison with their age-matched control groups. In addition, it was recently reported that infarct size was bigger in diabetic heart than in normal heart (Li et al., 2013). It was also indicated in an another recent study that 6 weeks diabetic duration cause infarct size to be bigger in diabetic heart than normal heart (Bhatta and Veeranjanyulu, 2014).

Table 1.3. Increased sensitivity of diabetic heart to myocardial I/R injury.

Animals	Chemical			Duration of diabetes	Time of I/R	Coronary artery ligated	References
	Type	Dosage	Route				
Increased infarct size or less resistance to ischemia							
Dog	STZ	-	-	75 days	120 min I/ 360 min R	LAD	Forrat et al., 1993
Rat	STZ	-	-	8 days	25 min I / 120 min R	LAD	Marfella et al., 2002
Rat	STZ	60 mg/kg	IP	20 weeks	30 min I /120 min R	LAD	Xu et al., 2004
Rat	STZ	65 mg/kg	IV	2 weeks	30 min I /120 min R	LAD	Xiao et al., 2004
Rat	STZ	70 mg/kg	IV	8 days	25 min I /120 min R	LAD	Di Filippo et al., 2005
Rat	STZ	65 mg/kg	IP	4 weeks	30 min I /120 min R	Global	Thirunavukkarasu et al., 2007
Rat	STZ	45 mg/kg	IV	3 weeks	30 min I /240 min R	LAD	Annapurna et al., 2008
Rat	STZ	60 mg/kg	IP	4 and 8 weeks	3 times 5 min I /5 min R	Global transient I/R	Shi-Thing et al., 2011
Rat	STZ	65 mg/kg	IP	4 weeks	45 min I /120-180 min R	LAD	Li et al., 2013
Rat	STZ	45 mg/kg	IP	6 weeks	30 min I /120 min R	LAD	Bhatta and Veeranjaneyulu, 2014

Abbreviations: STZ, Streptozotocin; I, Ischemia; R, Reperfusion; LAD, Left descending coronary artery; IP, Intraperitoneally; IV, Intravenous.

On the other hand, there are numerous studies that indicate diabetic hearts is more resistance to ischemic injury or it has less infarct size (Table 1.4) (Liu et al., 1993; Hadour et al., 1998; Ravingerova et al., 2003a; Xu et al., 2004; Ma et al., 2006; Galagudza et al., 2007).

Table 1.4. Decreased sensitivity of diabetic heart to myocardial I/R injury.

Animals	Chemical			Duration of diabetes	Time of I/R	Coronary artery ligated	References
	Type	Dosage	Route				
Decreased infarct size or more resistance to ischemia							
Rat	STZ	90 mg/kg	IP	44 weeks	30-45 min I/ 120 min R	LCA	Liu et al.,1993
Rabbit	Alloxan	100 mg/kg	IV	8 weeks	30 min I/ 180 min R	LCA	Hadour et al.,1998
Rat	STZ	45 mg/kg	IV	1 week	30 min I /240 min R	LDA	Ravingerova et al.,2003a
Rat	STZ	60 mg/kg	IP	4 weeks	30 min I /120 min R	LCA	Xu et al.,2004
Rat	STZ	50 mg/kg	IV	2 weeks	30 min I /120 min R	LDA	Ma et al., 2006
Rat	Alloxan	125 mg/kg	SC	6 weeks	30 min I /60 min R	LCA	Galagudza et al., 2007

Abbreviations: STZ, Streptozotocin; I, Ischemia; R, Reperfusion; LAD, Left descending coronary artery; LCD, Left coronary artery; IP, Intraperitoneally; IV, Intravenous; SC, Subcutaneous.

In contrast to studies demonstrating that diabetic hearts are more or less sensitivity to I/R injury, there are growing numbers of experimental study indicating no change in sensitivity of diabetic heart to I/R injury (Table 1.5). Kersten et al. (2001) showed that diabetes mellitus induced by combination of alloxan and STZ for 3 weeks did not change myocardial infarct size following 60 min ischemia and 180 min reperfusion in dogs. In addition, infarct size did not show significant difference between 8 weeks diabetic heart and age-matched control heart following 30 min ischemia and 240 min reperfusion (Ravingerova et al., 2003b). In a different duration of diabetes mellitus, it was obtained that myocardial infarct size in 6 weeks diabetic rat heart was not different from that in age-matched non-diabetic rat heart (Ma et al., 2006). Similarly, diabetes mellitus for 2 weeks did not result in a significant difference in myocardial infarct size followed by 30 min ischemia and 120 min reperfusion between diabetic and control group (Gross et al., 2007). Recently two similar *in vitro* experimental studies showed that after 4 weeks STZ administration, myocardial infarct size between diabetic and non-diabetic heart did not indicate any significant difference following 30 min ischemia/120 min reperfusion and 25 min ischemia/30 min reperfusion period (Okazaki et al., 2011; Ghaboura et al., 2011). Another important current study demonstrate that myocardial infarct size induced by 30 min ischemia and 120 or 180 min reperfusion in diabetic heart for 4 weeks did not significantly differ from that in control heart (Li et al., 2013).

Table 1.5. No change in sensitivity of diabetic heart to myocardial I/R injury

Animals	Chemical			Duration of diabetes	Time of I and R	Coronary artery ligated	References
	Type	Dosage	Route				
No change in infarct size or sensitivity to ischemia							
Dog	Alloxan +STZ	40 mg/kg +25 mg/kg	IV	3 weeks	60 min I /180 min R	LAD	Kersten et al., 2001
Rat	STZ	45 mg/kg	IV	8 weeks	30 min I /240 min R	LDA	Ravingerova et al.,2003
Rat	STZ	50 mg/kg	IV	6 weeks	30 min I /30 min R	LAD	Ma et al., 2006
Rat	STZ	65 mg/kg	IV	2 weeks	30 min I /120 min R	LAD	Gross et al., 2007
Rat	STZ	50 mg/kg	IP	4 weeks	30 min I /120 min R	Global	Okazaki et al., 2011
Rat	STZ	65mg/kg	IP	4 weeks	25 min I /30 min R	Global	Ghaboura et al., 2011
Rat	STZ	65 mg/kg	IV	4 weeks	30 min I /120 or 180 min R	LAD	Li et al., 2013

Abbreviations: STZ, Streptozotocin; I, Ischemia; R, Reperfusion; LAD, Left descending coronary artery; LCD, Left coronary artery; IP, Intraperitoneally; IV, Intravenous.

The discrepancy in results mentioned above may arise from duration and severity of diabetes, usage of different experimental animals or using different chemicals to induce diabetes. However, it has not been still completely clarified.

1.1.4 Diabetes and Arrhythmias

The underlying mechanism of arrhythmias induced by ischemia and reperfusion in diabetic heart is not completely understood. Therefore, it is difficult to develop effective strategies to prevent or decrease lethal ventricular arrhythmias in diabetic patients. In contrast to clinical studies, experimental data have shown paradoxical results about whether heart from diabetic animals has more, less or no protection against arrhythmias induced by I/R injury.

In an *in vitro* Langerdoff-perfused rat heart model, it was demonstrated that diabetic hearts were more resistant to I/R induced arrhythmias in acute phase of diabetes mellitus (Tosaki et al., 1996). In this study, 2 weeks diabetes after STZ administration, the incidence of VT and VF during 30 min global ischemia and reperfusion were significantly less than the non-diabetic heart. Similarly, one-week diabetic duration resulted in decreased incidence of VT and VF following 30 min of

LAD ligation in contrast to non-diabetic isolated rat heart and it was also showed that duration of arrhythmias was significantly less than in age-matched control group (Ravingerova et al., 2000). Another research demonstrated that the score of arrhythmias, the duration of arrhythmia and the incidence of VT and VF induced by 30 min ligation of LAD *in vivo* I/R model was found to be less in diabetic heart after 8 weeks diabetic duration in contrast to control (Ravingerova et al., 2001). It was also demonstrated in an another study that the arrhythmia score, the incidence of arrhythmias and total duration of VPC, VT and VF were significantly decreased in 8 weeks diabetic group in compared with control group (Ravingerova et al., 2003b). Furthermore, reperfusion-induced VF was not be observed in 8 weeks STZ-induced diabetic rat heart whereas VF was observed in all non-diabetic rat heart during 30 min reperfusion following 30 min ischemia (Zhang et al., 2002). Another important result of this study was that in 2 weeks diabetic group, the incidence of VF and VT during reperfusion were lower than that in control group. In addition, it was also observed that in 8 weeks diabetic group, the incidence of VT during ischemia was less than that in control. Another study revealed that 12 weeks diabetic duration decreases the incidence of VT and VF during 30 min global ischemia and reperfusion (Ooie et al., 2003). In a recent study it was shown that even one-week diabetes protect the heart against reperfusion-induced arrhythmia (Bozdogan et al., 2016). It was revealed in an another important study that reperfusion-induced arrhythmias and arrhythmia score decreased in 3 weeks diabetic heart, which indicates that diabetes resulted in increased resistance to reperfusion-induced arrhythmias (Chen et al., 2006). Similar to results of above studies, it was observed that appearances of ventricular arrhythmias decreased in diabetic heart at one-week after STZ-induction and also duration of VT and total number of VPC were found to be less than that in control group (Matejikova et al., 2008).

In contrast to mentioned previous studies indicating protective effect of diabetes mellitus against I/R induced arrhythmias, there have been also investigations showing no effect of diabetes mellitus on I/R induced arrhythmias. It was revealed that ventricular arrhythmias during ischemia and reperfusion were not significantly different in heart from 4, 6 and 8 weeks STZ-induced diabetic rats (Tosaki et al., 1995; Tosaki et al., 1996). Additionally, the score of arrhythmias, the incidence and the duration of arrhythmias during 30 min ischemia and 240 min reperfusion period

did not show any significant differences between one week diabetic and non-diabetic rat heart (Ravingerova et al., 2003b). In addition, the other studies confirmed that one-week diabetic duration did not result in significant changes in the incidence of VT and VF induced by ligation of LAD and reperfusion between diabetic and non-diabetic heart (Adameova et al, 2007a; Adameova et al, 2007b).

Several mechanisms have been suggested to clarify discrepancy in susceptibility of diabetic hearts to I/R-induced arrhythmias. For example, it was mainly proposed that differences in duration, severity and the types of diabetes mellitus might be reason for the discrepancy. Also, I/R protocol such as global ischemia, regional ischemia and the duration of ischemia and reperfusion all may be effective in variability of obtained results (Ravingerova et al., 2010). Increased resistance to I/R arrhythmias in diabetic heart in the acute phase may come from accumulation of less lactate in the ischemic region. It was reported that lactate was lower in myocardium of diabetic heart in compared to myocardium of non-diabetic heart (Ravingerova et al., 2000; Ravingerova et al., 2001). Furthermore, it was demonstrated that Ca^{2+} overload in diabetic myocardium during reperfusion is less than in non-diabetic myocardium, indicating increased resistance to reperfusion induced arrhythmias (Ziegelhöffer et al., 1999). It was suggested that hypothyroidism may play important role in resistance of diabetic myocardium against arrhythmias. Serum T_3 and T_4 levels in rat during 8 weeks diabetes was significantly less than the control (Zhang et al., 2002). It is thought that heart rate plays an important role in the generation, the incidence and the severity of ventricular arrhythmias induced by reperfusion. It was reported that rapid heart rate triggered reperfusion-induced arrhythmias whereas slow heart rate could be protective against reperfusion-induced arrhythmias (Murdock et al., 1980). It was also reported that basal heart rate was significantly lower in diabetic rats (Ravingerova et al., 2003b; Matejikova et al., 2008; Howarth et al., 2008). It is possible that increased resistance to reperfusion-induced arrhythmias in diabetic heart may result from slower heart rate. Another crucial factor affecting ventricular arrhythmias such as VT or VF is ionic concentration inside and outside of myocardium. Duration of ventricular arrhythmias during reperfusion is mainly affected by extracellular sodium concentration. Low sodium concentration can decrease the incidence of reperfusion-induced arrhythmias by preventing sodium and calcium entrance into myocardium and by leading to

myocardial potassium loss (Tosaki et al., 1989). For example, in 8 weeks diabetic rat heart, extracellular sodium and calcium concentration were found to be more than that in control rat heart while potassium ion concentration was less (Tosaki et al., 1996). Increased susceptibility to ventricular arrhythmias in the prolongation of diabetes could result from increased Na^+ and Ca^{+2} influx and decreased K^+ efflux. $\text{Na}^+/\text{Ca}^{2+}$ exchanger can be stimulated by an increase in Na^+ concentration, leading to Ca^{2+} overloading in the myocardium as the diabetes prolong (Khandoudi et al., 1990). In diabetic heart, produced neuropathy affects the sensory and autonomic nerves. In early period of neuropathy in diabetes mellitus, parasympathetic nerve activities are disturbed and sympathetic nerves activity become dominant. It has been thought that increased sympathetic nerve activity may induce resting tachycardia and coronary artery spasm, and also it may be a cause of arrhythmias leading to sudden dead in diabetic patients and bradycardia with increased neuropathy (Takeda, 2010).

1.2 Myocardial ATP Dependent Potassium Channel (K_{ATP} Channel)

Up to now, two types of K_{ATP} channels have been identified. First one is sarcolemmal ATP sensitive potassium channel ($\text{sarcK}_{\text{ATP}}$) which is expressed on sarcolemmal membranes and the other one is mitochondrial ATP sensitive potassium channel ($\text{mitoK}_{\text{ATP}}$) which is expressed on mitochondria.

$\text{SarcK}_{\text{ATP}}$ channels in cardiac cells were first discovered by Noma (1993). $\text{SarcK}_{\text{ATP}}$ channels are hetero-octamers which are composed of Kir 6.x subunits (Kir 6.1 or Kir 6.2, pore-forming subunits) and sulfonylurea receptor subunits (SUR1, SUR2A or SUR2B, regulatory subunits) (Inagaki et al., 1995). Inward rectifier Kir 6.x subunits constitute the channel pore and SUR subunits constitute the regulatory subunit. There are two Kir 6 genes; KCNJ8 for Kir 6.1 and KCNJ11 for Kir 6.2 and two SUR genes; ABCC8 for SUR1 and ABCC9 for SUR2 and KCNJ11 are located in chr11p15.1 whereas KCNJ8 and ABCC9 are located in chr12p12.1 (Nichols et al., 2013).

Different variations of subunits represent different physiological and pharmacological properties of sarcK_{ATP} channels in different organs, tissues or cells (Table 1.6). SUR2A/Kir 6.2 constitutes cardiac sarcK_{ATP} channels in ventricular cell (Inagaki et al., 1996); SUR1/Kir 6.2 in atria cell (Flagg et al., 2008); SUR2B/Kir 6.1-Kir 6.2 in cardiac conduction system (Bao et al., 2011b); SUR2B/Kir 6.1 in vascular smooth muscle (Yamada et al., 1997); SUR1/Kir 6.2 in pancreatic β cells (Inagaki et al., 1995); SUR2B/Kir 6.2 in smooth muscle (Isomoto et. al., 1996).

Table 1.6. K_{ATP} channels subunit variations

Subunit Variations	Cell Types	References
SUR2A/Kir 6.2	Ventricular cell	Inagaki et al., 1996
SUR1/Kir 6.2	Atria cell	Flagg et al., 2008
SUR2B/Kir 6.1/Kir 6.2	Conduction system	Bao et al., 2011
SUR2B/Kir 6.1	Vascular smooth muscle	Yamada et al., 1997
SUR1/Kir 6.2	Pancreatic β cells	Inagaki et al., 1995
SUR2B/Kir 6.2	Smooth muscle	Isomoto et. al., 1996

K_{ATP} channels are found in various tissues and exert different functions; insulin secretion in pancreatic β cells (Koster et al., 2005), cardioprotection against ischemia (Bozdogan et al., 2013), modulation of vascular smooth muscle tone (Wilson et al., 2000), control of transmitter release in nerve synapses (Rahamimoff et al., 1999).

Myocardial K_{ATP} channels have the unique ability to adjust cell excitability in response to changes in ATP and ADP levels. Reduction in ATP/ADP ratio that means increased cellular metabolic demand or reduced cellular ATP generation activate myocardial K_{ATP} channel. Activation of the channel allows K⁺ to efflux out of the cell. K⁺ efflux shortens cardiac action potential duration and limits the entry of Na⁺ and Ca²⁺ into interior of the cell. Thereby, opening of the channel leads to prolonged diastolic interval, decreased myocardial oxygen consumption, reduced energy requirement for ion transport and contraction (Weiss and Venkatesh, 1993; Zingman et al., 2011). Activation of 1% K_{ATP} channel in sarcolemma is enough to shorten action potential duration approximately 50%. Myocardial K_{ATP} channels plays critical role in energy metabolism under normal and disease conditions. Under hypoxic or ischemic conditions, vital role of K_{ATP} channels in physiological

adaptation have highly associated with their expression in myocardial cells (Zhu et al., 2011). In other words, increase in expression level of myocardial K_{ATP} channels may have great effect on energy balance and cardiac cell excitability. It has been shown that increase in myocardial K_{ATP} channel expression up to 50% by exercise results in less myocardial oxygen consumption and rapid action potential duration shorting an in response to increased heart rate whereas 80% reduction in expression of the channel lead to opposite effect (Zingman et al., 2011).

Kir subunits are sensitive to ATP, ADP and PIP_2 while the regulatory SUR subunits are sensitive to sulfonylureas. ATP with or without magnesium inhibit the channel by binding with Kir 6.2 subunits; however, ADP and PIP_2 activates Kir subunits. Sulfonylurea and channel openers exert their effect on the channel by interacting with SUR subunits (Nichols, 2006). Kir 6.2, ion-conducting subunits are indispensable for the channel activity and adapting to stress conditions. Absence of Kir 6.2 results in lethal arrhythmias and Ca^{2+} overload, and thereby impairing contraction and relaxation of cardiac cells (Zingman et al., 2002). Under normal physiological conditions, sarc K_{ATP} channels are also critical for regulating cellular energy metabolism. Experimental animals lacking sarcolemmal Kir 6.2 subunits have exhibited lower body weight or body mass index (Alekseev et al., 2010).

Mito K_{ATP} was first observed in rat liver mitochondrial inner membrane (Inoue et al., 1991) and then one year later in heart (Paucek et al., 1992). Molecular structures of mito K_{ATP} have not been definitely identified. It has been proposed that structure of mito K_{ATP} channels resemble sarc K_{ATP} channels, which might be composed of Kir 6.1 or Kir 6.2 subunits and in association with SUR1 or SUR2 subunit (O'Rourke, 2004; Testai et al., 2015). Although mito K_{ATP} channels show structural similarity with sarc K_{ATP} channels, they are regulated differently. Long-chain acyl-CoA esters, ATP and ADP in a magnesium-dependent manner inhibit whereas GTP and GDP activate mito K_{ATP} channels (Muntean et al., 2015). Mito K_{ATP} channels play important role in cardioprotection and apoptosis (O'Rourke, 2004). Mechanisms of protective effect of channel opening is not clear yet. However, it was proposed that Ca^{2+} entry into the mitochondria was restricted by the opening of mito K_{ATP} channels and also opening of the channels induced by ROS productions (Oldenburg et al., 2002).

1.2.1 Myocardial K_{ATP} Channels and Diabetes Mellitus

K_{ATP} channels are highly affected by metabolic changes in a cell. Limited data are there showing the relationship between diabetes mellitus or hyperglycemia and K_{ATP} channel expression in both mitochondria and sarcolemma. It has been shown that mRNA expression of SUR2A in heart and SUR2B in smooth muscle of aorta was significantly reduced during 3 months diabetic duration; however, no significant changes in expression of Kir 6.1 and Kir 6.2 in left ventricle of diabetic heart was obtained (Ren et al., 2003). On the other hand, in cardiac cells, 8 weeks diabetic duration has led to decrease in sarcK_{ATP} channel subunits; Kir 6.2 and SUR2A protein levels and gene expression (Chen et al., 2012). The protein level of channel subunits in diabetic rat heart have significantly found to decrease nearly 2-fold in compared to control rat heart. Injection of insulin for 4 days after 8 weeks diabetes reversed the decrease in mRNA and protein level of Kir 6.2 and SUR2A. Another important research performed on 5 weeks diabetic mice have demonstrated that diabetes mellitus decrease the expression of mitoK_{ATP} channel subunits; Kir 6.1 and SUR1 (Fancher et al., 2013). Another important result of that study is that the expression level of the channel proteins can show differences in subcellular region of mitochondria. In subsarcolemmal mitochondria, Kir 6.1 subunit expression reduced approximately 41% in contrast to age-matched control group; however, expression of SUR1 subunits has not been affected by diabetes. In the case of interfibrillar mitochondria, both Kir 6.1 and SUR1 expression have markedly decreased to 45% and 42% when compared to control group. Above papers show a close relationship between the metabolic status of a cell and K_{ATP} channels. In other words, diabetes mellitus can affect the physiological activity of K_{ATP} channels by changing expression of the subunits at both gene and protein levels. The most important question in this affect is how can hyperglycemia decrease the expression of these subunits in both sarcolemma and mitochondria? Underlying mechanism has not been exactly clarified.

1.2.2 Myocardial K_{ATP} channel and Ischemia/Reperfusion Arrhythmias

Growing evidences have shown that K_{ATP} channels play an important role in I/R-induced arrhythmias and infarct size in normal and diabetic heart (Chen et al., 2012; Bozdogan et al., 2013). That is why, experimental researches have been progressively carried out on the role of K_{ATP} channels in different physiological and pathological conditions.

The effect of sarcK_{ATP} channel activation on cardiac arrhythmias shows contradictory results. As mentioned earlier, activation of sarcK_{ATP} channels result in reduction in APD, slow conduction and increased K⁺ efflux from ischemic cells, which might lead to re-entry arrhythmias induced by I/R (Billman, 2008; Muntean et al., 2015). It was shown in previous studies that activation of sarcK_{ATP} channel resulted in increased K⁺ efflux and reduced APD, which was responsible for increased the incidence of ventricular arrhythmias during ischemia (Wilde et al., 1990; Wilde, 1993; Billman, 1994). In contrast, activation of sarcK_{ATP} channels caused the score of arrhythmia and VF to decrease by increasing extracellular K⁺ concentration (D'Alonzo et al., 1994). In an *in vivo* I/R model, it was demonstrated that activation of sarcK_{ATP} channel with pinacidil resulted in decreased arrhythmia score and duration of arrhythmia (Gonca and Bozdogan, 2010). Possible mechanism of protective effect of sarcK_{ATP} activation includes shorting of APD, which reduce the time for Ca²⁺ influx via L-type Ca²⁺ channels and increase the time for Ca²⁺ efflux via Na⁺/Ca²⁺ exchanger (Nakaya, 2014). On the other hand, it has been shown in various studies that sarcK_{ATP} channel blockers decrease the duration and the incidence of VF and re-entrant VPCs (Kantor et al., 1990; Pasnani and Ferrier, 1992; Dhein et al., 2000). There are also studies that sarcK_{ATP} channel blockers has no effect on cardiac arrhythmias (Smallwood et al., 1990; Rees and Curtis, 1995). There are many possible explanations for paradoxical effect of K_{ATP} opening and closing on cardiac arrhythmias. In a recent review by Baczkó et al. (2011), these mechanisms have been comprehensively explained. In brief, antiarrhythmic effect of K_{ATP} opening may include; (i) reduction of early and delayed after depolarization or reduced triggered activity by prolonging repolarization, (ii) hyperpolarization of resting membrane potential during reperfusion period, by this way Ca²⁺ overload is reduced via depressing reverse mode of Na⁺/Ca²⁺ exchanger and decreasing

extracellular K^+ accumulation. However, decrease in APD and refractoriness in normal cell due to K_{ATP} opening may cause re-entrant arrhythmias. In term of K_{ATP} blockage, re-entrant arrhythmias can be decreased via preventing excessive shorting in APD and refractoriness in repolarization (Baczkó et al., 2011; Tinker et al., 2014).

Mito K_{ATP} also plays an important role against cardiac arrhythmias. Opening of mito K_{ATP} results in membrane depolarization, mild matrix swelling, increased respiration and reduced Ca^{2+} overload (Gross and Fryer, 1999). During reperfusion, activation of mito K_{ATP} causes K^+ influx and reduced Ca^{2+} accumulation, thereby reducing probability of mitochondrial permeability transition pore (MPTP) opening (Testai et al., 2015). It was demonstrated in previous studies that opening of mito K_{ATP} was protective against I/R-induced arrhythmias. For example, in intact rabbit model of I/R injury, mito K_{ATP} opening was shown to decrease the incidence of life threatening arrhythmias such as VT and VF, infarct size and increase survival rate during both ischemia and reperfusion (Das et al, 2003; Das and Sarkar 2005). Similarly, in an *in vivo* rat model of I/R injury, the incidence of ventricular arrhythmias and the infarct size were decreased by activation of mito K_{ATP} (Matejikova et al., 2009). In addition, it was recently shown that activation of mito K_{ATP} resulted in reduced ventricular arrhythmias in anesthetized male rats but not in female rats (Gonca and Bozdogan, 2010).

In pathological conditions, both sarc K_{ATP} and mito K_{ATP} were reported to be changed. For example, it was reported in a previous experimental model of diabetes mellitus that mRNA expression of SUR2A in heart and SUR2B in aortal smooth muscle decreased; however, Kir 2.1, Kir 3.1, Kir 6.1, and Kir 6.2 expression did not significantly changed (Ren et al., 2003). It was proposed that mRNA and protein expressions of Kir 6.2 and SUR2A decreased in both *in vivo* and *in vitro* model of diabetes mellitus (Chen et al., 2012). In diabetes mellitus, Kir 6.1 and SUR1 expression were reduced in cardiac interfibrillar mitochondria; however, only Kir 6.1 expression was decreased in subsarcolemmal mitochondria (Fancher et al., 2013). Although the changes in expression of K_{ATP} channels in diabetes mellitus has been demonstrated, relation of these observations with I/R-induced arrhythmia has not been investigated yet.

1.3 5' Adenosine Monophosphate-Activated Protein Kinase (AMPK)

5'-Adenosine monophosphate activated protein kinase (AMPK) is a highly conserved serine/threonine kinase in fungi, plants and animals (Hardie, 2007). AMPK plays a key role in sensing the energy status of a cell. Stress conditions such as heat shock, ischemia, hypoxia and low glucose lead to the activation of the kinase (Arad et al., 2007).

AMPK presents in all eukaryotic organisms as a heterotrimeric complex composed of a catalytic subunits (α) and two regulatory subunits (β and γ). Each subunit has multiple isoforms; $\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2$ and $\gamma 1$, $\gamma 2$, $\gamma 3$, which are encoded by distinct genes and when these are co-expressed in cells, they can give twelve possible heterotrimeric combinations. All subunits are present in 1:1:1 arrangement and all three subunits are necessary for functional AMPK activity.

In mammals, two genes, PRKAA1 and PRKAA2 in humans, encode the catalytic subunits of AMPK (Hardie et al., 2012). The catalytic subunits contain a kinase domain, auto-inhibitory domain and C-terminal domain. At the N terminus, both isoforms contain a protein kinase domain and a threonine residue (Thr-172). Activation of AMPK occurs via phosphorylation of Thr-172 by upstream kinases. The isoforms of α catalytic subunit exert differences in their expression and location throughout tissues. For example, $\alpha 1$ isoform is widely expressed whereas $\alpha 2$ is predominantly expressed in skeletal muscle, liver and heart (Stapleton et al., 1996; Quentin et al., 2011). The $\alpha 1$ isoforms account for 60-90% of total AMPK activity in liver, testis and brain while the $\alpha 2$ isoforms account for 70-80% of total AMPK activity in rat cardiac and skeletal muscle (Cheung et al., 2000). In addition, it is suggested that $\alpha 2$ may be more active than $\alpha 1$ for AMPK-regulating transcriptional factor (Oakhill et al., 2009).

The regulatory subunits ($\beta 1$ - $\beta 2$) are encoded by two genes; PRKAB1 and PRKAB2 in mammals (Hardie et al., 2012). Both isoforms are widely expressed. $\beta 2$ is mainly expressed in heart whereas $\beta 1$ is predominate isoform in rodent liver (Thorton et al., 1998). The regulatory subunits act as a scaffold for the other subunits. The β subunits act as a key for defining subcellular location of AMPK. Both subunits contain an N-terminal following by a carbohydrate-binding domain

and C-terminal (Oakhill et al., 2009). N-terminal sequences of β subunits are mainly important for mediating different function of each isoform. Also, N terminal is necessary for binding ability of AMP and ADP to enhance phosphorylation of Thr-172 (Oakhill et al., 2009; Hardie et al., 2012). Carbohydrate binding domain is highly conserved between species. This domain is also known as glycogen binding domain. Therefore, it is thought that the main physiological role of regulatory subunits is to control glycogen metabolism. C-terminal is well preserved and interact with α and γ subunits (McBride et al., 2009).

All three γ isoforms of the regulatory units contain repeated four-cystathionine β synthase (CSB) sequence in tandem pairs from N terminus to C-terminus. Single tandem pair forms a structure that is called as Bateman domains (Bateman, 1997). Bateman domains are regulatory structures for adenosine containing ligands such as AMP, ADP, ATP, and also CSB itself (Hardie et al., 2012). The four CSB domains are different from each other. CSB1 and CSB4 domains bind AMP or ATP for AMPK activation. However, CSB3 domain binds AMP, and also CSB2 domain binds no nucleotide (Xiao et al., 2007). γ 1, γ 2 and γ 3 are encoded by PRKAG1-3 in mammals. Expression pattern of all three isoforms differ from each other according to tissue types. γ 3 isoforms is highly specific to skeletal muscle while γ 1 and γ 2 isoforms are expressed in many tissues such as heart, brain, placenta and skeletal muscle (Cheung et al., 2000; Mahlapuu et al., 2004). It has been suggested that γ isoforms may be more important subunit for AMPK activity in regulation of cell activities since γ isoforms make the kinase to sense AMP/ATP levels (Moffat and Harper, 2010). Glycogen accumulation in myocytes, cardiomyopathy and cardiac arrhythmia such as Wolf-Parkinson-White syndrome are associated with mutation in γ 2 isoforms (Daniel and Carling, 2002; Arad et al., 2002; Blair et al., 2010).

As a result, increased AMP concentration due to decreased ATP production results in activation of AMPK. In addition, an increase in production of ROS and Ca^{2+} concentration can activate AMPK (Beauloye et al., 2011). Upon activation, AMPK leads to inhibition of ATP-consuming anabolic processes, and activation of ATP-producing catabolic pathways (Carling et al., 2011).

1.3.1 AMPK and Diabetes Mellitus

AMPK plays an important role for regulating body homeostasis, food intake, body weight, increasing glucose uptake, suppression of endogenous glucose and lipolysis. Taken into account, it has been regarded that AMPK will be one of the most promising target for developing a new drug to treat or decrease the diabetic complications such as cardiovascular disorders (Oakhill et al., 2009; Moussa and Li, 2012).

1.3.2 AMPK and Ischemia/Reperfusion arrhythmias

AMPK plays an essential role in protection to arrhythmogenesis. There are extensive evidences to show that AMPK activation may be protective against arrhythmias; however, information about whether activation of AMPK or not lead to changes in genesis of arrhythmias is restricted. Until now, no direct investigations are there about the effect of AMPK on I/R-induced arrhythmias both *in vitro* and *in vivo* conditions in the literature. AMPK plays essential role in various ion channels activity which is important for ionic regulations of cardiac excitability and therefore in cardiac arrhythmias (Harada et al., 2012).

It is known that Ca^{2+} homeostasis plays an important role in the maintenance of normal cardiac rhythm. Increased Ca^{2+} influx during I/R injury resulted in life-threatening arrhythmias (Bers, 2002). Reduction in AMPK $\alpha 2$ expression was found to decrease SERCA activity and caused increased intracellular Ca^{2+} levels (Dong et al., 2010). Based on this observation, it may be speculated that AMPK activation may decrease triggered arrhythmias. AMPK are also associated with myocardial K_{ATP} channel (Du et al., 2014; Sukhodub et al., 2007; Yoshida et al., 2012). AMPK activation may act as an opener of K_{ATP} . Depending on this observation, it can be said that AMPK related opening of K_{ATP} channel during ischemia and reperfusion may be cardioprotective against triggered activity; on the other hand, it may cause promotion of reentrant arrhythmia due to decreasing in APD (Harada et al., 2012). It is also important to emphasize the role of AMPK in oxidative stress. In mitochondria and cytoplasm, increased generation of ROS promotes myocardial cell death and cardiac arrhythmias (Jeong et al., 2012). It was proposed that NADPH oxidase activation is

suppressed by AMPK, and therefore ROS production is decreased (Eid et al., 2010). When it is taken into account, AMPK activation may exert anti-arrhythmic effect via suppression of ROS generation. In addition, it was indicated that NO production played essential role in cardiac contractility and suppression of cardiac arrhythmias (Kubota et al., 2000). Another important clue about AMPK activation may be suggested as anti-arrhythmic is that AMPK activation stimulates NO production in endothelial cell via phosphorylation and activation of eNOS (Morrow et al., 2003).

In langendorff-perfused heart, it was demonstrated that AMPK activation during the first 15 min of reperfusion caused myocardial infarct size to decrease via providing sufficient energy for cell survival (Paiva et al., 2010). It was reported in another study that pharmacological activation of AMPK before ischemia resulted in infarct size and apoptosis to be reduced in an *in vivo* model of I/R injury (Kim et al., 2011). In addition, it was reported that AMPK activation preserves impaired Ca^{2+} handling and contractility due to atrial fibrillation related metabolic dysfunction (Harada et al., 2015). It was also indicated that activation of AMPK during myocardial ischemia was effective to reduce myocardial infarct size by mediating inhibition of mPTP at reperfusion in both non-diabetic and diabetic heart (Paiva et al., 2011).

1.3.3 AMPK and Myocardial K_{ATP} Channel

There are few investigations to indicate that some ion channels are target of AMPK. For example, AMPK activation results in epithelial Na^+ channel to be inhibited (Carattino et al., 2005). Another study revealed that AMPK associated with the cystic fibrosis transmembrane conductance regulator (CFTR), an ATP-gated Cl^- channel (Hallows et al., 2000). In addition, there are few reports to show that cardiac K_{ATP} channels are affected by AMPK. The early study demonstrated that AMPK was shown to mediate ischemic preconditioning in cardiac cell via regulating activity of sarK_{ATP} without effecting mitochondrial membrane potential. Ischemic preconditioning induced stimulation of sarK_{ATP} channels requires AMPK $\alpha 2$ (Sukhodub et al., 2007). Another study revealed that cardiac K_{ATP} channels were directly regulated by activation of AMPK (Yoshida et al., 2012). Activation of

AMPK by increased cellular AMP may trigger and promote K_{ATP} channel opening. It was indirectly demonstrated in a recent study that prevention of I/R injury required interaction of AMPK with Kir 6.2/ K_{ATP} channel, which promotes opening of the channels. In this study, resveratrol in Kir 6.2 knockout mice did not show cardioprotective effect via AMPK. Cardioprotection by AMPK activation was disappeared in transgenic mice, which indicate that AMPK exerts its protective effect via K_{ATP} channel (Du et al., 2014).

1.4 Resveratrol

An epidemiological study performed in 1992 showed that the prevalence of cardiovascular disease was approximately 40% less in France than in the rest of European, that consume much more wine and a diet containing high saturated fat (Renaud and de Lorgeril, 1992). This paradoxical relationship is popularly known as 'French paradox'. Until the discovery of 'French paradox', little attention was paid to resveratrol. After that time, the great interest has been given to investigate biological and physiological role of resveratrol *in vivo* and *in vitro* studies.

Resveratrol was first described in the root of the white hellebore, *Veratrum grandiflorum* by M. Takaoka in 1939 (Vang, 2013). Resveratrol is one of the most studied polyphenols that belongs to subclass, stilbenes. Resveratrol is a phytoalexin produced by plants against fungal attack, ultraviolet radiations, injury and other environmental stresses such as cold temperatures (Roggero and Garciaparrilla, 1995; Soleas et al., 1997). Resveratrol is also present in a variety of plants such as in grapes, mulberries, peanuts, pines, soybeans, and bilberry (Catalgol et al., 2012).

Resveratrol exist in two isomeric form; cis- and trans- resveratrol, latter is biologically more active form (Figure 1.1). It is also named as 3, 4', 5-trihydroxystilbene with a chemical formula $C_{14}H_{12}O_3$ (Yu et al., 2012). In addition, this polyphenol can be produced by chemical and biotechnological way and is also sold as a nutritional supplement.

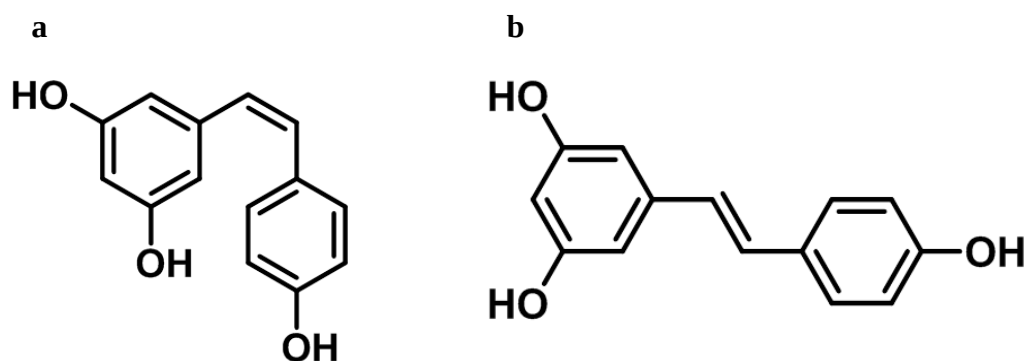


Figure 1.1. Chemical structures of resveratrol a-) cis-form, b-) trans-form

1.4.1 Resveratrol and Diabetes Mellitus

Diabetes mellitus is associated with many complications such as cardiovascular disorders, renal failure. It is important to find an effective cure to either prevent diabetes mellitus or decreasing such serious complications and thereby increasing quality of diabetic patient's life. Herbal compounds can be given as an alternative way to prevent or treat diabetes mellitus and its complications. Among them, resveratrol has a usage potential in treatment of both type I and type II diabetes mellitus.

Resveratrol has been mostly investigated in many studies as an anti-diabetic agent (Su et al., 2006; Ku et al., 2012; Zordoky et al., 2014). It has been reported that resveratrol treatment one week before STZ (70 mg/kg) administration prevents pancreatic β cell apoptosis and hyperglycemia (Ku et al., 2012). Similarly, administration of resveratrol following 15 days of STZ (65 mg/kg) shows significant anti-glycemic effect over 30 days (Thirunavukkarasu et al., 2007). This is consistent with the other reports that resveratrol has an insulin-like effect in STZ-induced diabetes (Su et al., 2006; Huang et al., 2010). Oral administration of resveratrol decreased blood glucose levels in STZ-induced diabetic rats and insulin resistance diabetic rats. Similarly, insulin released in normal and diabetic rats is stimulated by resveratrol (Chi et al., 2007). In addition, diabetic rats treated with resveratrol for one week was found to have lower blood glucose levels in contrast to non-treated diabetic rats (Chang et al., 2011).

Obesity, insulin resistance, disturbance in glucose homeostasis, polyphagia, polydipsia, weight loss and renal dysfunction are all characteristic features of diabetes mellitus. It is known that resveratrol usage may be beneficial against such dysfunctions (Catalgol et al., 2012). It has been shown in previous investigations that resveratrol is able to decrease blood glucose level and increase blood insulin level in animals with type I and type II diabetes mellitus (Chi et al., 2007; Ku et al., 2012).

Several mechanisms have been suggested that resveratrol decreases the increased blood glucose levels in diabetic animals. For example, increased blood insulin levels, suppression in hepatic glucose output and increased peripheral glucose utilization in diabetic animals have been thought to be underlying mechanisms of anti-glycemic effects of resveratrol (Szkudelski and Szkudelska, 2014).

1.4.2 Resveratrol and Oxidative Stress

The usage of herbal products with antioxidant properties can be more effective to decrease or prevent diabetic complications resulting from increased oxidative stress in diabetics. Among them, resveratrol has drawn intense attraction from scientists. There are different ways by which resveratrol shows its antioxidant activity such as scavenging ROS, increasing the activity of antioxidant enzymes or decreasing activity of oxidant enzymes (Carrizzo et al., 2013).

Oxidative stress is defined as imbalance between the systemic production of reactive oxygen species and antioxidant defense (Betteridge, 2000). Disturbance in the balance can lead to abnormalities in cell membrane, proteins, lipids, nucleic acids (DNA or RNA) and enzymes (Fidan and Dündar, 2008). Thus, oxidative stress can make a cell more vulnerable to apoptosis and necrosis. Hyperglycemia is thought to result in increasing oxidative stress in diabetes mellitus. Hyperglycemia leads to excess ROS production from many sources, including glucose auto-oxidation, lipid peroxidation, oxidative phosphorylation, NAD(P)H oxidase and nitric oxide synthase (Valko et al., 2007). In addition, SOD, catalase, glutathione reductase, glutathione peroxidase, glutathione levels, vitamins, lipid peroxidation and nitrite concentration are all changed due to hyperglycemia, which are called as oxidative stress biomarkers (Maritim et al., 2003).

Glucose oxidation is well known to be main source of free radicals. Glucose is auto-oxidized due to hyperglycemia, which then produce reactive ketoaldehydes and superoxide anion radicals. If these superoxide anions are degraded by catalase or glutathione peroxidase, glucose react with hydrogen peroxide to produce reactive hydroxyl radical in the presence of iron and copper ions (Maritim et al., 2003; Valko et al., 2007).

Lipid peroxidation is known as oxidation of lipid molecules. Free radicals such as $O_2^{\cdot-}$, $OH^{\cdot}$, $NO^{\cdot}$ convert non-radical lipids to radicals. These radicals remove hydrogen from lipids, producing an unpaired electron in lipids. Then, it goes free radical chain reaction (Arora et al., 2013). Malondialdehyde (MDA), 4-hydroxynoneal (4-HNE), lipid hydroperoxide and isoprostanes can be given as examples of end product of lipid peroxidation (Valko et al., 2007; Arora et al., 2013).

Mitochondria are an energy reservoir of living cells, which take important role in determining of a cell whether it goes to life or death. Oxidative phosphorylation is the metabolic pathways taking place in mitochondria. During this process, various free radicals are normally produced. For examples, under non-diabetic conditions superoxide are formed at site of complex I and the ubiquione-complex II interface in the mitochondrial membrane. However, in state of diabetes, complex II become the primary site for superoxide generation (Valko et al., 2007; Teshima et al., 2014). Taking into consideration of decreasing activity of antioxidant enzymes in diabetic organisms, mitochondria play a vital role in increasing oxidative stress and cell damage in diabetics.

It was shown that increased lipid peroxidation in diabetic liver and kidney was prevented in resveratrol treated diabetic rats in compared to control rats (Schmatz et al., 2012). Catalase and SOD activity in liver and kidney tissues decreased in diabetic rats. Resveratrol did significantly preserve this decrease in both enzymes activity (Schmatz et al., 2012). As well, resveratrol was found to be effective in decreasing enhanced level of malondialdehyde and increasing catalase levels in diabetic rats. Similarly, DNA fragmentation was markedly reduced by resveratrol in diabetic rats (Kumar et al., 2007). Protective effects of resveratrol on pancreatic β cells have been thought to be related with its strong antioxidant properties. It has been demonstrated that resveratrol administration prevents the

decrease in SOD, catalase, glutathione peroxidase, vitamin C, vitamin E and also reduced glutathione in pancreatic cells of diabetic rats (Palsamy and Subramanian, 2010).

1.4.3 Resveratrol and Ischemia/Reperfusion arrhythmias

Clinical and experimental data have shown that diabetic hearts are more prone to I/R injury, including cardiac arrhythmias and acute myocardial infarction (De Angelis et al., 2002; Whittington et al., 2012; Balakumar and Sharma, 2012). In addition, mortality and morbidity rate in diabetic patients are approximately doubled compared to non-diabetic individuals (Abbott et al., 1988). Hyperglycemia is suggested to be one of the reasons for increased I/R injury (Webster, 2008; Mapanga et al., 2014). That is why, it would be expected that reducing hyperglycemia in diabetic heart may be effective in diminishing I/R injury, and thereby cardiac arrhythmias. Within this context, resveratrol is a promising molecule with features of both lowering blood glucose level in diabetes and decreasing cardiovascular dysfunctions in non-diabetes by antioxidants properties.

The anti-arrhythmic effect of resveratrol has been well identified in non-diabetic heart (Hung et al., 2004; Kaga et al., 2005; Burstein et al., 2007; Raj et al., 2014). For instance, administration of resveratrol just before 30 minutes of ischemia resulted in decreasing the incidence of VT and VF in non-diabetic heart (Hung et al., 2000). However, in the case of diabetic heart, the effects of resveratrol on cardiac dysfunction, especially on arrhythmias have not been exactly clarified. Only limited data are available in this subject. A recent study has indicated that resveratrol treatment results in decreasing infarct size and myocytes apoptosis in diabetic rat heart following 30 min global ischemia and 2 h reperfusion via NO-mediated and increased antioxidant mechanism such as MnSOD activity (Thirunavukkarasu et al., 2007). However, the effect of resveratrol on arrhythmias induced by ischemia and reperfusion in diabetic rats were out of the scope in that study. It is generally accepted that severity of arrhythmias is proportional to infarct size (Yasar et al., 2015). From this point of view, resveratrol could decrease the severity and the incidence of arrhythmias in diabetic heart. However, resveratrol failed to decrease

arrhythmias. This was shown in another current study that resveratrol did not decrease both infarct size and severities of arrhythmias in diabetic animals treated with resveratrol in compared to diabetic animals only receiving the vehicle (Huang et al., 2010). In contrast, another important result of this study is that resveratrol was found to be effective in increasing survival rate, mean arterial pressure and heart rate in diabetic rats. Also, superoxide production due to hyperglycemia in diabetic heart was indicated to be decreased in resveratrol treatment. The reason why resveratrol could not be effective on severity of arrhythmias in previously mentioned studies may be related with the dosage, period and the route of resveratrol administration (1-2.5 mg/kg, 15 days and orally). In an important study, it has demonstrated that most effective dose on myocardial cells and administration route of resveratrol is minimally 5 mg/kg in dose and intraperitoneal (Du et al., 2014; Bresciani et al., 2014). In diabetic animals, heart to body weight ratios is suggested to be an important factor for indicating the damage of diabetes to heart (Tosaki et al., 1996). It has clearly shown that resveratrol administration preserves the decrease in heart and body weight in diabetic animals (Huang et al., 2010).

1.4.4 Resveratrol and K_{ATP} channels

It has been speculated that resveratrol may prevent arrhythmias by diminishing Ca²⁺ overload and acting as a K_{ATP} channel opener for inducing a pharmacological preconditioning in non-diabetic rat heart (Chen et al., 2008). In addition, it has been reported that the effect of resveratrol on I/R injury is blocked by glibenclamide in healthy rats (Shen et al., 2012). These findings are important for examining the relationship between resveratrol and myocardial K_{ATP}, and thereby mechanism of resveratrol action on cardiac arrhythmias.

1.5 Berberine

Berberine is a natural product, which has been used in Ayurvedic and Chinese medicine for many years due to having many pharmacological features. Berberine can be isolated from root, rhizome and stem of many plants such as *Coptis chinensis*, *Berberis aquifolium*, *Berberis vulgaris* (Kumar et al., 2015). Berberine is also chemically produced such as berberine hydrochloride, berberine sulfate and berberine citrate or phosphate (Figure 1.2). Several recent studies have showed that berberine may be used in the treatment of many different diseases, including cancer, cardiovascular dysfunction and diabetes mellitus (Lau et al, 2001; Zhang et al., 2010; Hong et al., 2015).

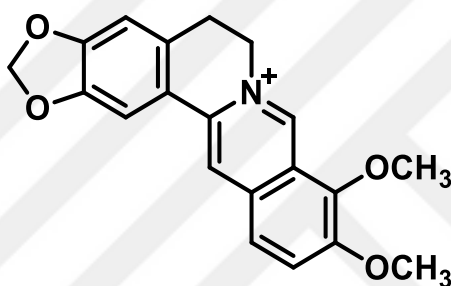


Figure 1.2. Chemical structure of berberine

1.5.1 Berberine and Diabetes Mellitus

In China, the hypoglycemic effect of berberine was first reported in 1986 (Jing et al., 2015). From that time, berberine has been commonly used in the treatment of diabetes mellitus in folk medicine.

The effect of berberine on blood glucose levels has been shown in several animal and clinical researches. For example, berberine could improve fasting blood glucose level in non-obese type I diabetic mice after 14 weeks administration without any toxic effects (Chueh and Lin, 2012) and increases cell number of islet and insulin level (Chueh and Lin, 2011). Another study revealed that berberine treatment could also lead to decrease blood glucose levels in alloxan-induced diabetic rats with a possible mechanism of increasing peripheral glucose utilization rather than stimulating insulin release from β cells (Tang et al., 2006). Conversely, berberine

was found not to be effective in reducing blood glucose levels in type I diabetes mellitus; however, it was effective in reducing blood glucose levels in type II diabetes via increasing expression of insulin receptor (Kong et al., 2009). It could be possible that increased expression of insulin receptor via protein kinase C might improve glucose consumption only when insulin is secreted from pancreatic cells (Kong et al., 2009).

Clinical studies have also shown anti-hyperglycemic effect of berberine in diabetic patients. For example, diabetic patients treated with berberine in a dose of 1 g/d for 2 months had lower blood glucose levels and berberine's effect in lowering blood glucose levels was found to be similar to those of metformin and rosiglitazone (Zhang et al., 2010). In addition, it was represented that berberine application in cultured human cell lines resulted in an increased insulin receptor expression and insulin signaling (Zhang et al., 2010). In a 3 months clinical trial, type II diabetic patients receiving berberine in 0.5 g dose, 3 times a day decreased fasting blood glucose levels (Yin et al., 2008). In another large clinical study, berberine treatment of diabetic patients for 8-12 weeks in a dose of 0.5-1.5 g/day resulted in decreased fasting blood glucose levels compared to placebo group (Dong et al., 2012).

1.5.2 Berberine and Oxidative Stress

Beneficial effects of berberine on oxidative stress have been shown in various studies, which exerts an action against abnormalities due to diabetes mellitus. In STZ-induced diabetic rats, administration of berberine in a dose of 200 mg/kg for 12 weeks decreased serum MDA and increased serum SOD levels compared to non-treated diabetic rats (Liu et al., 2008; Xie et al., 2013). In addition, it was revealed in alloxan-induced diabetic rats that berberine treatment in a dose of 100 and 200 mg/kg suppressed increased MDA level and increased total antioxidant capacity such as SOD and glutathione peroxidase in heart, indicating the free radical scavenger ability of berberine (Tang et al., 2006). In pancreases of diabetic rats, berberine administration caused SOD activity to decrease and MDA content to elevate when compared to non-diabetic control rats. Treatment of diabetic rats with berberine for 16 weeks resulted in increased SOD activity and decreased MDA content (Zhou et

al., 2009). It was also demonstrated in an another study that berberine administration to diabetic rats for 16 weeks significantly decreased MDA levels and increased antioxidant enzymes, including catalase, SOD, and glutathione peroxidase in both serum and liver tissue (Zhou and Zhou, 2011). In response to hepatic I/R injury, SOD activity decreased and MDA levels increased. Treatment of rat with berberine in a dose of 100 mg/kg/d for 14 days resulted in decreased MDA and increased SOD activity, which indicates anti-oxidative activity (Sheng et al., 2015). In cultured cardiomyocytes, it was represented that berberine application showed significant anti-oxidative capacity by suppressing ROS production and also berberine decreased the production of superoxide and MDA in I/R injured heart (Yu et al., 2016). In addition, a recent study shows that oxidative damage induced by myocardial I/R injury was depressed by berberine treatment, indicating decreased myocardial superoxide production and MDA levels and increased SOD activity (Zhao et al., 2016).

Underlying mechanism of antioxidant effect of berberine is related with multiple cellular signaling pathways and it is not completely clarified, which require further researches. Increased SOD expression via SIRT1/FOXO pathways, decreased expression levels of NADPH oxidase and increased expression of AMPK may be given as examples of cellular mechanisms of antioxidant effect of berberine (Li et al., 2014).

1.5.3 Berberine and Ischemia/Reperfusion arrhythmias

Beneficial effect of berberine in both clinical and experimental studies has been shown on cardiovascular dysfunctions. In addition, studies have shown that several molecules and signaling pathways involve in its protective effects. In 1996, it was suggested in an *in vitro* study that berberine might be blocker of myocardial K_{ATP} channels by which it could be protective against ischemia-induced arrhythmias (Wang et al., 1996). In a another study, berberine application resulted in prolonged action potential duration in ventricular myocytes in a dose dependent manner via inhibition of delayed rectifier K^+ currents and increase L-type Ca^{2+} currents (Wang and Zheng, 1997). It was represented in an *in vivo* model of I/R injury that berberine

pretreatment for 14 days to healthy rats decreased infarct size, occurrence and duration of VF, VT and premature beats via regulation of AMPK in ischemic and non-ischemic area (Chang et al., 2012). In non-ischemic area, AMPK upregulation might increase glucose uptake, glycolysis and fatty acid oxidation and in ischemic areas, AMPK downregulation might attenuate acidosis and apoptosis by decreasing glucose ends products such as lactate and proton from fatty acid oxidation (Chang et al., 2012). Recently, it has been shown that pretreatment of rat with berberine for 14 days before I/R resulted in decreased the arrhythmias score, duration and the incidence of VPC, VF and VT via suppression of PI3K/AKT signaling pathways which further decreased the expression of IL-6, IL-1 β and TNF- α (Qin-Wei and Yong-Guang, 2016). Berberine treatment also decreased myocardial infarct size, myocardial apoptosis and oxidative damage (Zhao et al., 2016). Similarly, berberine attenuated myocardial infarct size, apoptosis and decreased CK, LDH, upregulating Bcl-2 expression and down regulating Bax and caspase-3 expressions (Yu et al., 2016).

In a clinical study, 14 days berberine treatment in patients with heart failure was shown to decrease frequency and complexity of VPC without any side effect (Zeng and Zeng et al., 1999). In another clinical study, berberine treatment for 8 weeks was shown to decrease frequency of VPC and mortality in patients with chronic congestive heart failure without any adverse effects (Zeng et al., 2003).

In type II diabetic rats, berberine treatment in a dose of 180 mg/kg orally for 14 days resulted in decreased incidence of VT, VF and VPC and also it was found that the arrhythmia score in diabetic rats treated with berberine was significantly decreased when compared to non-treated diabetic rats (Wang et al., 2012). In that study, it was also represented that decreased protein expression of Kir 2.1 in diabetic rats was recovered by berberine administration, which recovered transient outward potassium current. In another study, administration of berberine in a dose of 100 mg/kg orally for 7 days to 8 weeks diabetic rats was shown to decrease the duration of arrhythmia and the arrhythmia score without having any effect on heart rate when compared to non-treated diabetic rats (Wang et al., 2012). It was also observed in that study that berberine increased the suppressed transient outward K⁺ current and

inward Ca^{2+} current in diabetic rat heart, which can be underlying mechanism of antiarrhythmic effect of berberine in experimental model of diabetes mellitus.

1.6 Glibenclamide

Glibenclamide is a K_{ATP} channels blocker. It has been commonly used as a hypoglycemic agent for treatment of type II diabetes mellitus (Rambiritch et al., 2014). Glibenclamide increases insulin secretion by blocking of K_{ATP} channels in the pancreatic β cells. It also blocks K_{ATP} channels in cardiac cells. However, controversies are going on whether glibenclamide usage increases or decreases cardiovascular risk in diabetics.

1.6.1 Glibenclamide and Diabetes Mellitus

Glibenclamide is a widely used as anti-diabetic agent. Glibenclamide binds to SUR-1 site of K_{ATP} channels in pancreatic β cells. Binding of glibenclamide leads to inhibition of K_{ATP} channels, which results in depolarization of the β cell membrane. Depolarization triggers the opening of voltage-gated Ca^{2+} channels, which increases Ca^{2+} influx. Increased Ca^{2+} influx causes increased insulin secretion. In addition to stimulating insulin secretion, glibenclamide has insulin-independent blood glucose decreasing action. Endogenous glucose production decreases and peripheral glucose utilization increases with glibenclamide administration (Overkamp et al., 2002). It also reduces hepatic glucose production, thereby decreasing blood glucose levels (Rendell et al., 2004).

Although glibenclamide is commonly used in the treatment of type II diabetes, its anti-hyperglycemic effect is also observed in STZ-induced diabetic rats. Glibenclamide administration orally once daily for 4 weeks resulted in decreased blood glucose levels in STZ-induced diabetic rats when compared to non-treated diabetic rats (Erejuwa et. al., 2011a; Erejuwa et. al., 2011b). Similarly, treatment of diabetic rats with glibenclamide in a dose 0.04 mg/ kg daily administered for 1, 2, 4, 8, 12 and 16 weeks in diabetic rats significantly decreased blood glucose levels in respect to non-treated diabetic rats (Akbar et al., 2012). In addition, glibenclamide

treatment in a dose of 0.25 mg/kg orally for 4 weeks was shown to decrease blood glucose levels in STZ-nicotinamide induced type 2 diabetic rats in contrast to non-treated diabetic rats (Pierre et al., 2012). On the other hands, there are many studies that glibenclamide do not change the blood glucose levels in diabetic rats. For example, in 8 weeks and 2 weeks diabetic rats, glibenclamide administration did not significantly change the blood glucose levels in contrast to non-treated diabetic rats (Tosaki et al., 1995). In addition, it was found in another study that glibenclamide did not resulted in a significant change in blood glucose levels between drug treated diabetic rats and control rats (Takahashi et al., 2003). In addition, it has been recently shown that oral administration of glibenclamide to diabetic mice in a dose of 5 mg/kg daily for 2 weeks did not change blood glucose levels as compared to non-treated diabetic mice (Cai et al., 2014).

1.6.2 Glibenclamide and Ischemia/Reperfusion arrhythmias

The effect of glibenclamide on I/R induced arrhythmias has been extensively studied in healthy rats (Bernauer, 1997; Bozdogan et al., 2013). Many experimental studies are present, showing the anti-arrhythmic effect of glibenclamide. For example, *in vivo* rat model of ischemia, glibenclamide was shown to decrease the number of VPC and duration of VF (Ballagi-Pordany et al., 1990). Additionally, it was shown that glibenclamide decreased the ischemic arrhythmias; however, it did not affect the reperfusion-induced arrhythmias (Bril et al., 1992). It was also demonstrated that glibenclamide decreased the ischemia induced VF and reperfusion induced arrhythmias, and also improved the survival rate during ischemia and reperfusion (Vajda et al., 2007). It was also shown in our previous study that the incidence of VT decreased in glibenclamide treated rats (Bozdogan et al., 2013). In contrary, there are also some studies showing that glibenclamide has pro-arrhythmic effect. Incidence of VF, VT and other type of arrhythmias increased in glibenclamide administrated healthy conscious sheep (del Valle et al., 2001). In addition, it was observed that glibenclamide increased the severity of arrhythmias induced by ischemia although it did not affect reperfusion-induced arrhythmias (Bernauer, 1997).

In contrast to studies presenting cardiac effect of glibenclamide in non-diabetic heart, limited investigations are present in the effect of glibenclamide on pathologic conditions such as diabetes mellitus exerting high risk from acute myocardial ischemia. Likewise, the effect of glibenclamide on the incidence of I/R induced arrhythmias in healthy heart, experimental studies in diabetic rats show controversial results about cardiovascular effect of glibenclamide. In an *in vitro* study, administration of glibenclamide in a dose of 0.1 mg/kg, intraperitoneally three times to 8 weeks diabetic rats was found to increase the incidence of reperfusion-induced VT when compared to control rats; however, the incidence of VF was not changed due to glibenclamide administration (Takahashi et al., 2003). In addition, it was observed in alloxan-induced diabetic sheep that glibenclamide administration in a dose of 0.1 mg/ kg was found to worsen mechanical recovery and the incidence of arrhythmias (Negroni et al., 2007). In a recent study, it was demonstrated that glibenclamide administration increased I/R injury and worsen left ventricular function in Goto-Kakizaki diabetic rat heart (Kristiansen et al., 2011). In contrast, treatment of 8 weeks diabetic rat heart with glibenclamide 10 min before ischemia and during reperfusion resulted in decreased incidence of reperfusion induced VF and VT when compared to non-treated diabetic rat heart. However, in 2 weeks diabetic rat heart, administration of glibenclamide 10 min before ischemia and during reperfusion did not cause a significant change in the incidence of arrhythmias (Tosaki et al., 1995). In 8 weeks diabetic rats, intracellular Na^+ and Ca^{2+} ion concentration in myocardium before ischemia was found to be higher than that in non-diabetic control rats and following reperfusion Na^+ and Ca^{2+} gain increased. Glibenclamide decreased Na^+ and Ca^{2+} overload and K^+ loose in diabetic heart, which was suggested as an antiarrhythmic mechanism of the drug in diabetic heart (Tosaki et al., 1995).

Glibenclamide has been used as an anti-hyperglycemic agent in diabetic patients for many years. However, debate about the safety of glibenclamide on cardiovascular function has continued. There are a few clinical studies that show no, beneficial or adverse cardiovascular effect of glibenclamide in diabetic patients. In type II diabetic patients, glibenclamide decreased the incidence of VPC and VT although it did not result in a change of the number, duration of ischemic attack and spontaneous ventricular arrhythmias (Cacciapuoti et al., 1991). Besides, the

incidence of VF and VT was found to be decreased in glibenclamide treated type II diabetic patients, the mortality rate decreased when compared to non-treated diabetic patients (Lomuscio et al., 1994; Lomuscio and Fiorentini, 1996). It was represented in another study that the incidence of non-sustained VT and the frequency of repetitive ventricular contraction significantly decreased in glibenclamide treated diabetic patients (Aronson et al., 2003). In contrast, it was shown in a recent clinical study that glibenclamide was found to increase ischemic area and possibility of malignant arrhythmias in type II diabetes mellitus (Xianghua et al., 2010). In another study, it was observed that glibenclamide treatment caused to increase mortality and morbidity in diabetic patients after myocardial infarction (Jørgensen et al., 2011). In addition to adverse and beneficial cardiovascular effect of glibenclamide in diabetes mellitus, studies indicating glibenclamide has no cardiovascular effect are also present. The rate of VF did not show significant difference between glibenclamide treated diabetic patients and non-treated diabetic patients (Davis et al., 1998). Hospital mortality rate was found to be higher in diabetic patients than non-diabetic patients (Klamann et al., 2000). It was also emphasized in that study that treatment of diabetic patients with or without glibenclamide did not influence myocardial necrosis and mortality rate. In addition, it was shown in another clinical study that usage of glibenclamide did not affect the long-term survival of diabetic patient from acute myocardial infarction (Meier et al., 2003).

2. AIM AND SCOPE OF THE STUDY

The aims of present study were to investigate (i) the response of diabetic heart to I/R-induced arrhythmias depending on duration of diabetes, (ii) the role of myocardial K_{ATP} channel in the susceptibility of diabetic heart to I/R-induced arrhythmias (iii) the effect of resveratrol, berberine and glibenclamide treatment combinations on I/R-induced arrhythmias, (iv) the role of myocardial K_{ATP} channel in the effect of these compound with the relation of I/R-induced arrhythmias. With mentioned aims, it was purposed to bring an innovative and original research to literature. Further, it was thought that the evaluation of whether anti-diabetic molecules like resveratrol and berberine have protective effect against acute I/R injury, especially cardiac arrhythmias. This study was also thought to provide important contribution to future investigations and clinical application.

3. MATERIALS AND METHODS

3.1 Animals

In this study, 210 Sprague-Dawley, 5-6 months old male rats, weighting between 300 and 350 g were used. The rats were maintained under standard laboratory conditions at a temperature of 22°C with 12 hours light/dark cycle and with free access to standard pellet food and water ad libitum. All study protocols were approved by Animal Research Local Ethical Committee of Abant İzzet Baysal University (protocol number; 2014/26), Bolu, Turkey (Appendix A) and all animals were treated in adherence to all of the guiding principles in the care and use of animals.

3.2 Induction of Diabetes

Diabetes was induced by intraperitoneal injection of a single dose of 65 mg/kg streptozotocin (Santa Cruz Biotechnology, sc-200719, U-9889) dissolved in 0.1 M citrate buffer (pH 4.5) and given at a volume of 0.1 ml/100 g rat weight. Age and weight matched control animals were injected with an equal volume of vehicle (citrate buffer) only. STZ was injected after overnight fasting and after 48 hours of STZ injection, animals having blood glucose level more than 250 mg/dl were considered as diabetic (Naskar et al., 2011; King, 2012). Blood glucose levels were measured from blood taken from tail vein with a glucometer (PlusMED Accuro pM1-300) before STZ injection, 48 hours after STZ injection and weekly during experimental periods. In diabetic groups, animals having blood glucose level lower than 250 mg/dl were excluded from the study.

3.3 Experimental Groups and Drug Administrations

In the present study, two main experimental groups were designed: 2 weeks short and 6 weeks long term diabetes. Each main groups were then consisted of seven subgroups. Experimental groups were given in Table 3.1.

Table 3.1. Experimental Groups

<i>Experimental Groups</i>
A) 2 weeks short term diabetes
a) Subgroup 1: Control (2C)
b) Subgroup 2: Diabetes (2D)
c) Subgroup 3: Diabetes + Resveratrol (2DR)
d) Subgroup 4: Diabetes + Resveratrol + Glibenclamide (2DRG)
e) Subgroup 5: Diabetes + Glibenclamide (2DG)
f) Subgroup 6: Diabetes + Berberine (2DB)
g) Subgroup 7: Diabetes + Berberine + Glibenclamide (2DBG)
B) 6 weeks long term diabetes
a) Subgroup 1: Control (6C)
b) Subgroup 2: Diabetes (6D)
c) Subgroup 3: Diabetes + Resveratrol (6DR)
d) Subgroup 4: Diabetes + Resveratrol + Glibenclamide (6DRG)
e) Subgroup 5: Diabetes + Glibenclamide (6DG)
f) Subgroup 6: Diabetes + Berberine (6DB)
g) Subgroup 7: Diabetes + Berberine + Glibenclamide (6DBG)

All drugs were freshly prepared in dimethyl sulfoxide (DMSO) and were administrated intraperitoneally between 10 and 12 a.m. once a day until the surgical operation for 2 and 6 weeks to diabetic animals. All drugs were kept in dark containers to protect drugs from light induced decomposition. Resveratrol (Santa Cruz Biotechnology, sc-200808A) was administered in a dosage of 5 mg/kg/day at a volume of 0.1 ml/100 g. Glibenclamide (Sigma-Aldrich, G0639) was administrated in a dosage of 5 mg/kg/day at a volume of 0.1 ml/100 g. Berberine chloride form (Sigma-Aldrich, B3251) was administrated in a dosage of 10 mg/kg/day at a volume of 0.1 ml/100 g. Dose and administration route of drugs used in the present study were chosen according to previous studies (Du et al., 2014; Bresciani et al., 2014). The drug doses were adjusted according to animal weight that weekly determined throughout the experiment.

3.4 Ischemia and Reperfusion Protocol

Experimental animals were anaesthetized with urethane (1.2 g/kg in a volume of 0.5 ml/100 g) administrated intraperitoneally and taken to operation table that heated automatically at 37°C. Then, tracheotomy was performed for artificial respiration. The right carotid artery was cannulated with polyethylene tubing (0.58 mm X 0.96 mm) to record the arterial blood pressure with a blood pressure transducer (Biopac System Inc. Goleta CA-USA, Turkey Distributor Commat-Ankara/Turkey). A standard limb lead I electrocardiogram (ECG) was recorded using subcutaneous needle electrodes. The chest was opened by left thoracotomy followed by sectioning of the fourth and fifth ribs. Artificial respiration was started immediately with room air using a volume of 0.9 ml/100 g body weight at a rate of 60 strokes/min (Ugo Basile Rodent Ventilator, Italy). After incision of pericardium, the heart was eased out of the chest by gentle pressure on the ribcage. A 5/0 silk suture was placed around the left coronary artery approximately 2 mm from its origin. The heart was then placed back into the chest cavity. The animal was allowed to recover for stabilization period of 5 minutes (Gonca, 2013). Any animal having arrhythmias or a sustained decrease in mean arterial blood pressure below 70 mmHg before ligation was discarded. Six minutes ischemia and six minutes reperfusion were performed and the arrhythmias recorded in these periods were evaluated (Bozdogan et al., 2005; Gonca and Darıcı, 2014).

3.5 Western Blotting

Western blot is used to separate and identify proteins. Depending on molecular weight, the protein is separated through gel electrophoresis. The sample is then transferred to a membrane, which produces a band for the protein. The membrane is then incubated with antibodies specific to the protein.

3.5.1 Tissue Collection

For western blotting, 3 rats without surgical operation were used in all experimental groups. Whole heart was perfused with cold PBS (Phosphate Buffered Saline) on ice containing petri dish to remove blood. Then, the left ventricle was taken and submersed into liquid nitrogen until frozen to stop all cellular process and preserve macromolecular structure. The frozen tissue samples were kept at -80°C until western blotting was performed.

3.5.2 Sample Preparation

Tissue samples were processed in order to obtain a pool of proteins that could be separated by SDS-PAGE. The powdered cardiac tissue previously obtained was homogenized using a glass teflon homogenizer in ten volumes of ice-cold RIPA buffer containing PMSF, sodium orthovanadate, proteinase inhibitors in a ratio of 50 mg tissue/250µl buffer kept at low temperatures at all times. The cold buffer was used to aim cell lysis and protein stabilization and also to prevent denaturation and/or degradation of proteins such as proteases and phosphatases. The homogenate obtained was centrifuged at 12.000 g for 10 min at 4°C (Eppendorff model 5417R, rotor 45-30-11). The resulting pellet, containing cell debris, membrane and organelles was discarded, and the supernatant containing the solubilized proteins transferred into a new tube.

3.5.3 Protein Quantification

The protein content in supernatant was determined using Bradford assay (Bio-Rad, Hercules, CA, USA). In this assay, cardiac protein samples were incubated with Bradford reagent, according to the manufacturer's instructions, at 37°C for 5 min. Upon color development, absorbance at 595 nm was measured using a spectrophotometer (6405 UV/Vis Jenway, UK). A standard absorbance versus protein concentration curve was done in parallel using bovine serum albumin (BSA) as standard protein.

3.5.4 Sample Denaturation

The supernatants were diluted 1:4 in sample buffer-Laemli lysis buffer from Bio-Rad (Sigma, UK) and then heated to 95°C for 5 min. After denaturation, the protein samples were immediately incubated on ice.

3.5.5 Gel Electrophoresis

Proteins samples were separated through 10% SDS-PAGE. For each sample, 100 µg of protein were loaded onto a SDS-PAGE gel as well as 5µl of standard protein marker (Bio-Rad, 161-0374). Proteins were run on gel electrophoreses (Invitrogen) at 120V for one hour which is approximately enough for that good protein separation of the protein marker bands was achieved.

3.5.6 Blotting

Proteins separated by the SDS-PAGE gel can be further transferred onto a membrane via hydrophobic and charged interactions between membrane and protein. In the presence of electrical current and adequate transfer buffer, negatively charged proteins will migrate from the gel matrix onto the hydrophobic membrane, which will mirror the protein separation obtained in the gel. Once proteins are transferred to a specific membrane, interaction with a primary antibody that recognizes a specific amino acid sequence of the target-protein is enhanced. Binding of the primary antibody to the targeted protein is achieved by incubating the membrane with the primary antibody. To aim in the visualization of the protein of interest, a secondary antibody is often used. Secondary antibodies are targeted at a species-specific sequence of the primary antibodies and are linked to a reporter enzyme, such as alkaline phosphatase, biotin or horseradish peroxidase (HRP) that will enhance the protein signal and facilitate its detection.

3.5.7 Protein Transfer onto PVDF Membrane

In order to make the proteins of interest accessible to antibody detection, cardiac proteins were transferred from the gel onto polyvinylidene difluoride (PVDF) membrane by using iBlot (Invitrogen, IB1001EU). A dry transfer system was performed as follows: preassembled stacks ready for protein transfer containing copper cathode-top buffer matrix-gel-membrane-bottom buffer matrix-copper anode. A roller was used for ensuring no air bubbles between the gel and the membrane. An electrical voltage was applied and the negatively charged proteins migrated from the gel towards the positive-charged electrode, maintaining the organization they had on the gel.

3.5.8 Probing with Primary and Secondary Antibodies

Before blocking, membrane was washed 5 times with distilled water because of using one signal enhancer kit for western blot (Invitrogen) and then were incubated with pretreatment solution for 15 min at room temperature. Then membrane was again washed with distilled water. Membrane were then incubated in blocking buffer including 5% BSA+PBS-T (PBS-Tween 20) for 60 min at room temperature, using orbital shaker to reduce non-specific binding between the membrane and proteins. After blocking, the membrane was incubated with primary antibodies overnight at 4°C, using an orbital shaker. Primary antibody was used in a dilution ratio in accordance with the manufacturer's instructions; 1:50 for Kir 6.2 (Alomone, APC-020). After overnight incubation of membrane with primary antibodies, the membrane was washed three times for 10 min with PBS-T to apply secondary antibody. Secondary antibody (anti-rabbit, 1:3000) was diluted with 5% BSA solution. The membrane was incubated with secondary antibodies for 60 min at room temperature, using an orbital shaker. Then membrane was washed with PBS-T three times for 10 min. The membrane proteins were visualized with Alkaline Phosphatase (BCIP/NBT; Invitrogen, WP20001).

3.5.9 Relative Quantification of Protein Expression

After seen bands, membranes were scanned and the relative densitometry of the bands was assessed using ImageJ Analysis System (NIH, Bethesda, MD, USA).

3.6 Biochemical Analysis

For biochemical analysis, 3 rats without surgical operation were used in all experimental groups. Animals were anesthetized with urethane and then 10 ml blood was directly collected by cardiac puncture. Blood samples were waited at room temperature for 20 minutes, and after that were centrifuged at 4°C with 2000 r.p.m for 20 minutes to separate serum. Serum samples were then stored at -20°C until analysis. Serum insulin, superoxide dismutase and lactate dehydrogenase levels were measured in all groups using a commercial ELISA kits according to manufacturer's instructions (Bioassay Technology laboratory, Shanghai Crystal Day Biotech Co., LTD., China).

3.7 Determination of Area at Risk of Infarct

At the end of experiment, the survived animals were heparinized with 1/10 heparin (25000 IU/100 ml) via tail vein for 1 ml/100 g body weight. Respirator was closed and the heart of the animal was getting out by cutting the aorta. After the retightening of the LAD, the heart was perfused through the aorta with solutions: first with 10 ml of NaCl and then with 2 ml of 96% ethanol for demarcation of the occluded and non-occluded myocardial regions. Following the perfusion of the heart, the non-perfused area (red color, area at risk of infarct) was separated from the well-perfused area (white color, non-ischemic myocardium mass) through a borderline (Figure 3.1). The wet weights of the area at risk of infarct and the non-ischemic myocardial mass were measured. The percentage of the non-perfused area in respect to the total weight of the ventricle, indicating area at risk of infarct, was calculated (Bozdogan et al., 2005).

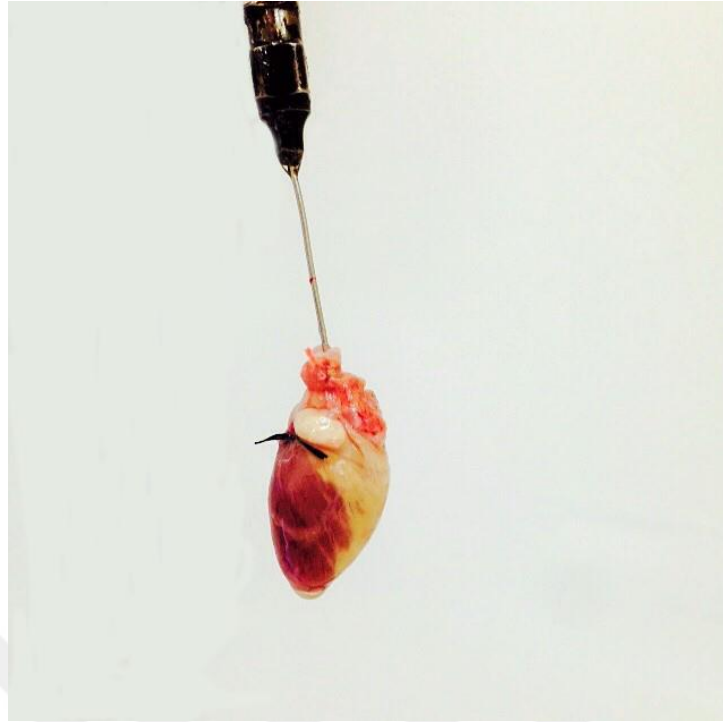


Figure 3.1. Area at risk of infarct

3.8 The Evaluation of Arrhythmias

The heart rate and blood pressure were determined from recorded ECGs at one minute before ischemia (basal), at the time point of ligation and 1st, 3rd, and 5th minutes following the ischemia and reperfusion. The incidence and total length of arrhythmias were calculated during 6 minute of ischemia and 6 minute of reperfusion. The arrhythmias were identified in accordance with the Lambeth Conventions (Walker et al., 1988) as VT, VF, and other types of arrhythmias including ventricular premature beat, bigeminy, and salvos (Figure 3.2). The arrhythmic period that includes the time interval between the onset and offset of arrhythmias was measured. An arrhythmia score was used to indicate the severity of arrhythmia based on the incidence and duration of arrhythmia by giving a grade to each animal as follows: 0 = No arrhythmias; 1 = < 10 sec VT or other arrhythmias; 2 = 11–30 sec VT or other arrhythmias; 3 = 31–90 sec VT or other arrhythmias; 4 = 91–180 sec VT or other arrhythmias and / or < 10 sec reversible VF; 5 = > 180 sec VT or other arrhythmias and / or > 10 sec reversible VF; 6 = irreversible VF (Lepran et al., 1983).

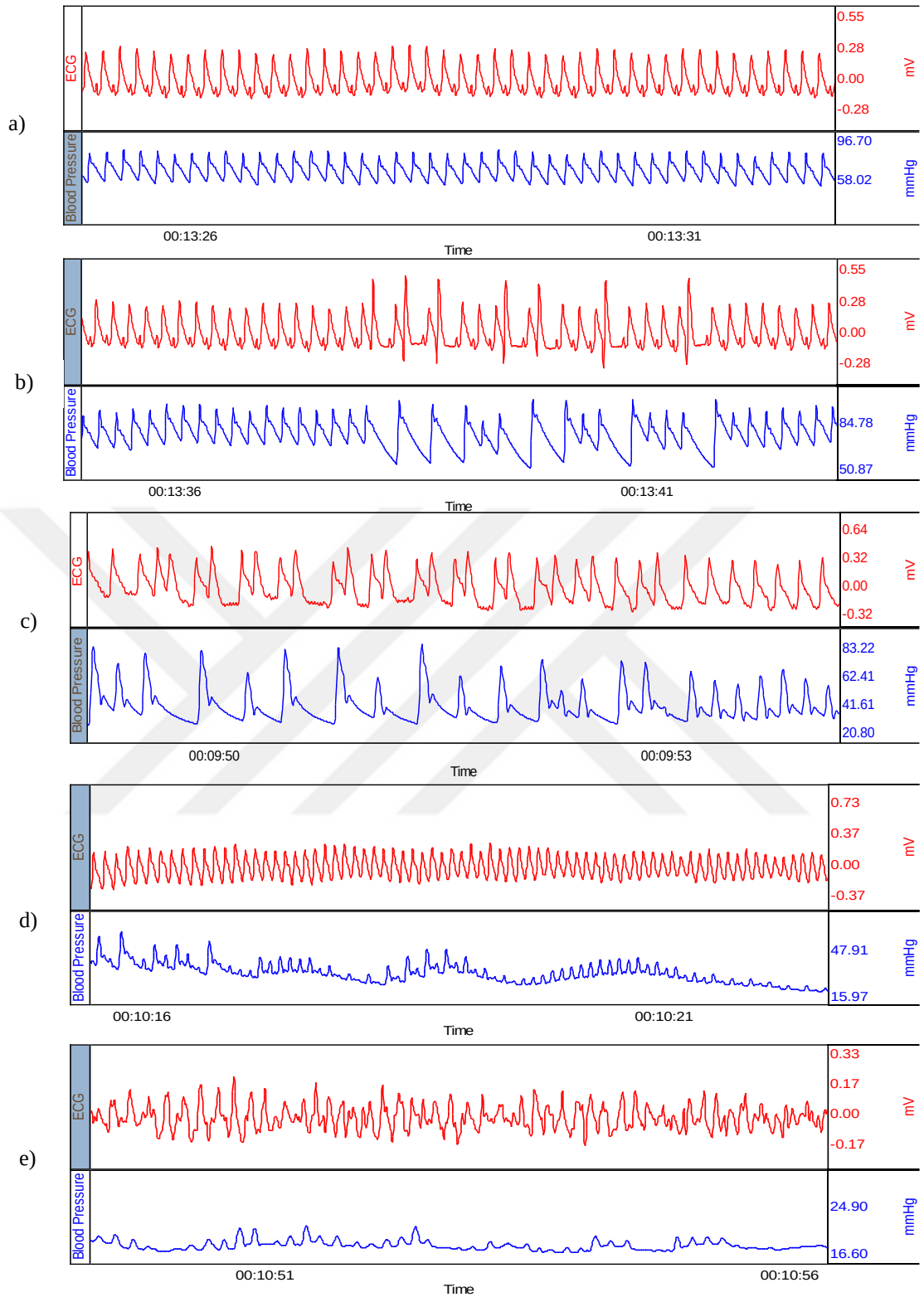


Figure 3.2. Arrhythmia types a) normal rhythm, b) ventricular premature beat, c) bigeminy, d) ventricular tachycardia, e) ventricular fibrillation

3.9 Statistical Analysis

Data were analyzed using SPSS (SPSS Statistical Software, SPSS Inc., USA, and Ver. 22.0). The mean and standard errors were determined for all parameters including heart rate, blood pressure, body weight, arrhythmia score, blood glucose levels, survival rate, SOD, LDH and insulin levels. Values were expressed as means $\pm$ standard errors (S.E). Data were analyzed within and between groups by one-way analysis of variance combined with the least significant difference post hoc test and Student's t-test. The incidences of arrhythmia were assessed by chi-square test (Fisher's exact test). Differences between means yielding a probability of $p < 0.05$ were considered to be statistically significant.

4. RESULTS AND DISCUSSIONS

4.1 Results

53 animals (approximately 33%) in short and long diabetic groups before surgical operations were death due to unknown reasons, and 17 animals (approximately 10%) did not occur diabetes despite STZ administration. In long-term diabetes mellitus, there were no animals in combination of berberine with glibenclamide groups due to excess death. Therefore, ischemia and reperfusion were not performed in this group. Only three animals were alive, which were used for molecular and biochemical analysis.

ST segment elevation and increased QRS amplitude were accepted as a sign of ischemia or successful ligation. ST segment elevation and increased QRS amplitude were observed in animals following successful coronary artery ligation (Figure 4.1). In control groups, animals having mean arterial blood pressure lower than 70 mmHg and arrhythmias before coronary artery ligation (10 animals) were excluded from the evaluation. After perfusion of coronary artery, 6 animals having lower area at risk of infarct than 40% and 7 animals having bleeding after ligation were also discarded.

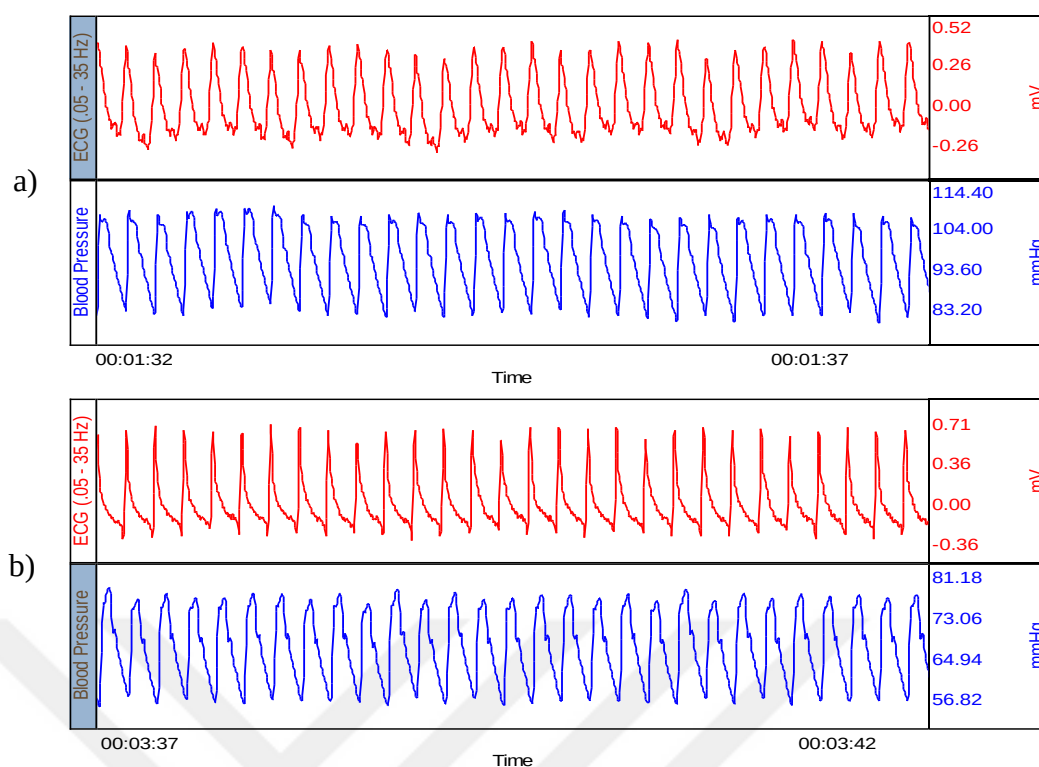


Figure 4.1. ECG and blood pressure recorded in control rat a) before ligation, b) 30 s after ligation

4.1.1 Blood Glucose Levels and Body Weight

Blood glucose levels between groups did not show any significant differences before STZ administration in short term diabetes (Figure 4.2). Two days after STZ induction, blood glucose levels significantly increased in all animals treated with STZ ($p < 0.05$). Two weeks later, resveratrol treatment did not significantly decrease blood glucose levels in diabetic rats when compared to non-treated diabetic rats. Treatment with combination of resveratrol and glibenclamide significantly decreased blood glucose levels in respect to non-treated diabetic rats ($p < 0.05$). Glibenclamide treatment alone also significantly resulted in decreased blood glucose levels when compared to non-treated diabetic rats ($p < 0.05$). Blood glucose levels in diabetic rats treated with berberine was found to significantly decrease as compared to non-treated diabetic rats ($p < 0.05$). However, berberine and glibenclamide administration to diabetic rats were not found to decrease blood glucose levels when compared to non-treated diabetic rats.

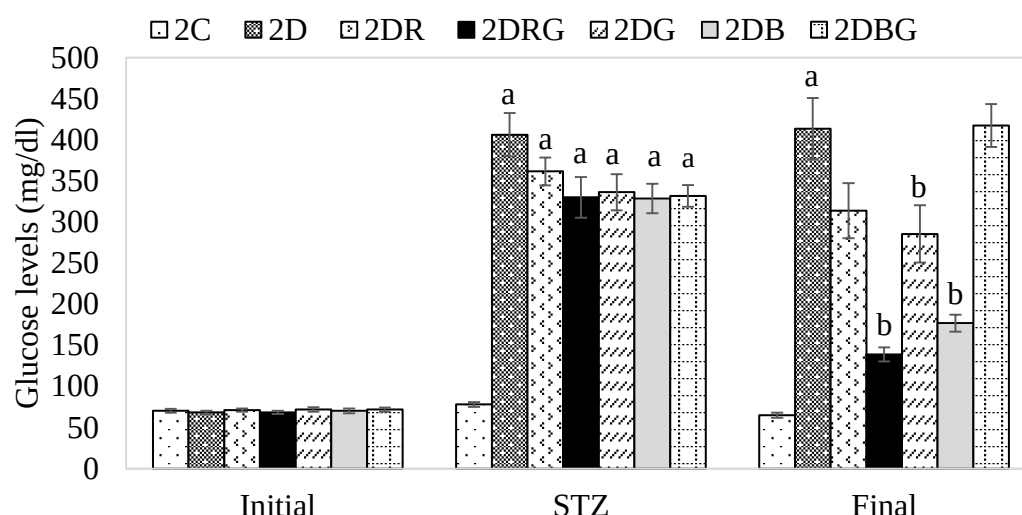


Figure 4.2. Blood glucose levels in 2 weeks diabetic rats

Abbreviations: 2C, non-diabetic rats (n:11); 2D, diabetic rats (n:12); 2DR, diabetic rats treated with resveratrol (n:11); 2DRG, diabetic rats treated with combination of resveratrol with glibenclamide (n:11); 2DG, diabetic rats treated with glibenclamide (n:12); 2DB, diabetic rats treated with berberine (n:10); 2DBG, diabetic rats treated with combination of berberine with glibenclamide (n:9). Results were expressed as mean $\pm$ standard error. ^a $p < 0.05$; different from 2C, ^b $p < 0.05$; different from 2D.

In short-term diabetes mellitus, there were no significant differences in initial body weight of all groups ($p < 0.05$, Figure 4.3). At the end of 2 weeks after STZ induction, body weight in non-treated diabetic rats was found to be significantly less than control rats ($p < 0.05$). Final body weight in diabetic rats treated with resveratrol, berberine and glibenclamide was not found to be different as compared to non-treated diabetic rats. However, final body weight in diabetic rats treated with combination of resveratrol and glibenclamide was significantly higher than non-treated diabetic rats ($p < 0.05$). Similarly, final body weight in diabetic rats treated with combination of berberine and glibenclamide was significantly found to be higher than non-treated diabetic rats ($p < 0.05$).

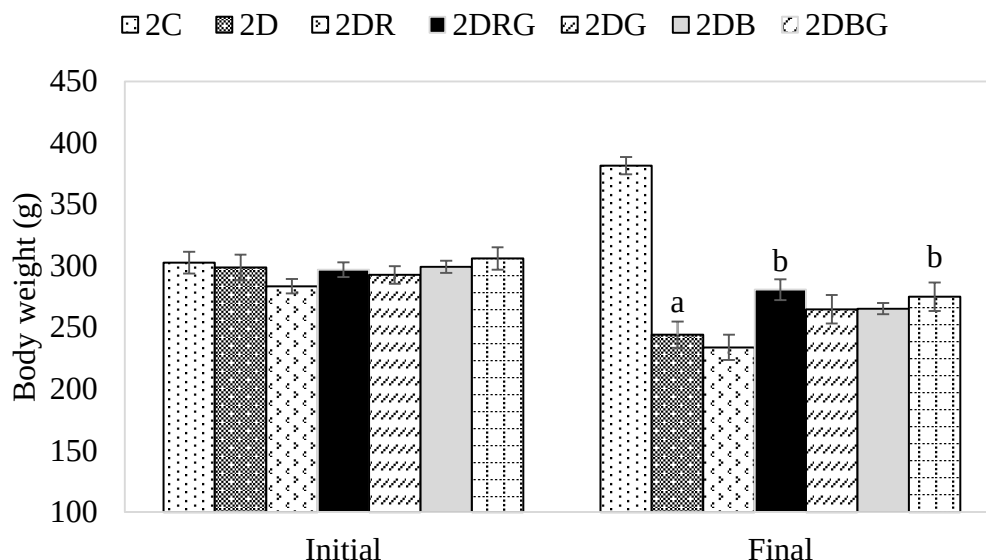


Figure 4.3. Body weight change in 2 weeks diabetic rats

Abbreviations: 2C, non-diabetic rats (n:11); 2D, diabetic rats (n:12); 2DR, diabetic rats treated with resveratrol (n:11); 2DRG, diabetic rats treated with combination of resveratrol with glibenclamide (n:11); 2DG, diabetic rats treated with glibenclamide (n:12); 2DB, diabetic rats treated with berberine (n:10); 2DBG, diabetic rats treated with combination of berberine with glibenclamide (n:9). Results were expressed as mean $\pm$ standard error. ^a $p < 0.05$; different from 2C, ^b $p < 0.05$; different from 2D.

In long-term diabetes mellitus, blood glucose levels before STZ administration in all groups were not significantly different (Figure 4.4). Two days after STZ treatment, blood glucose levels significantly increased in all diabetic rats in contrast to non-diabetic rats. Following 6 weeks, blood glucose levels in diabetic rats treated with resveratrol, glibenclamide and their combinations were not significantly different from the non-treated diabetic rats. Otherwise, blood glucose levels in diabetic rats treated with berberine alone and its combination with glibenclamide were found significantly lower than the non-treated diabetic rats ($p < 0.05$).

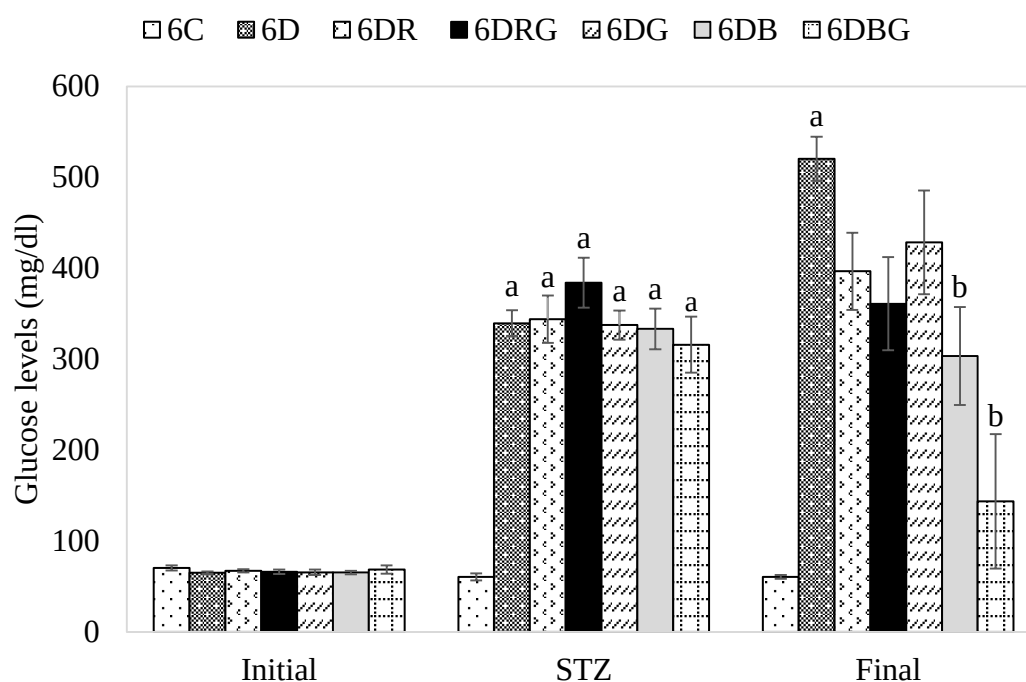


Figure 4.4. Blood glucose levels in 6 weeks diabetic rats

Abbreviations: 6C, non-diabetic rats (n:9); 6D, diabetic rats (n:12); 6DR, diabetic rats treated with resveratrol (n:13); 6DRG, diabetic rats treated with combination of resveratrol with glibenclamide (n:13); 6DG, diabetic rats treated with glibenclamide (n:9); 6DB, diabetic rats treated with berberine (n:11); 6DBG, diabetic rats treated with combination of berberine with glibenclamide (n:5). Results were expressed as mean $\pm$ standard error. ^a $p < 0.05$; different from 6C, ^b $p < 0.05$; different from 6D.

In long-term diabetes mellitus, initial body weight between groups did not show significant differences (Figure 4.5). At the end of 6 weeks after STZ induction, body weights in non-treated diabetic rats were found to be significantly less than control rats ($p < 0.05$). Final body weights in diabetic rats treated with resveratrol alone and glibenclamide alone were not significantly different from non-treated diabetic rats. However, final body weights in diabetic rats treated with combination of resveratrol and glibenclamide were found to be significantly higher than non-treated diabetic rats ($p < 0.05$). In addition, final body weights in diabetic rats treated with berberine alone and its combination with glibenclamide were significantly higher than the non-treated diabetic rats ($p < 0.05$).

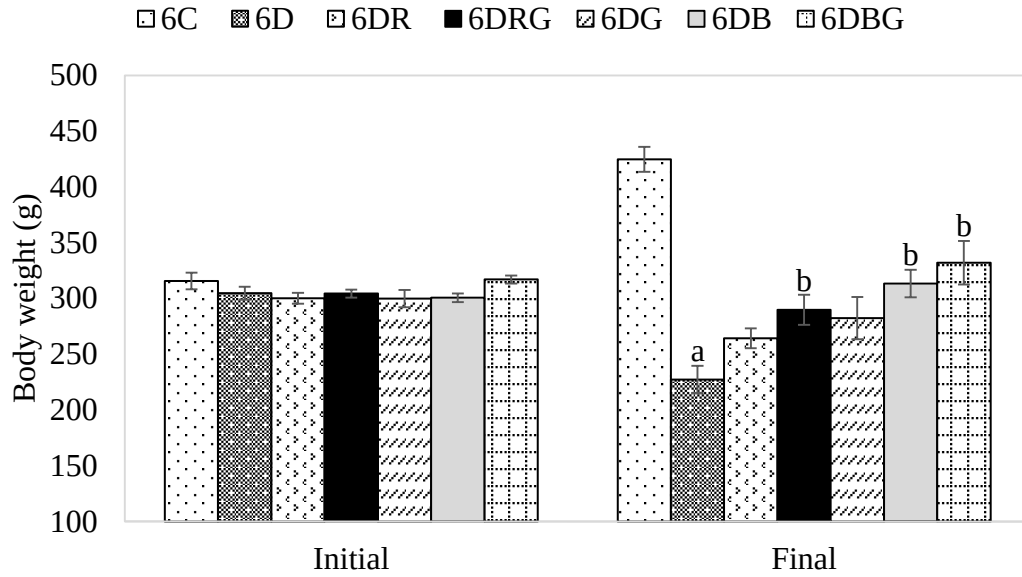


Figure 4.5. Body weight change in 6 weeks diabetic rats.

Abbreviations: 6C, non-diabetic rats (n:9); 6D, diabetic rats (n:12); 6DR, diabetic rats treated with resveratrol (n:13); 6DRG, diabetic rats treated with combination of resveratrol with glibenclamide (n:13); 6DG, diabetic rats treated with glibenclamide (n:9); 6DB, diabetic rats treated with berberine (n:11); 6DBG, diabetic rats treated with combination of berberine with glibenclamide (n:5). Results were expressed as mean $\pm$ standard error. ^a $p < 0.05$; different from 6C, ^b $p < 0.05$; different from 6D.

4.1.2 Heart Rate and Mean Arterial Blood Pressure

In short-term diabetes, there were no significant differences in basal heart rate between control rats and non-treated diabetic rats (Figure 4.6). However, heart rate in non-treated diabetic rats was found to be significantly lower than control rats at the time point of ligation ($p < 0.05$). In addition, at 1st, 3rd and 5th min following ligation, non-treated diabetic rats showed significantly lower heart rate when compared to control rats ($p < 0.05$).

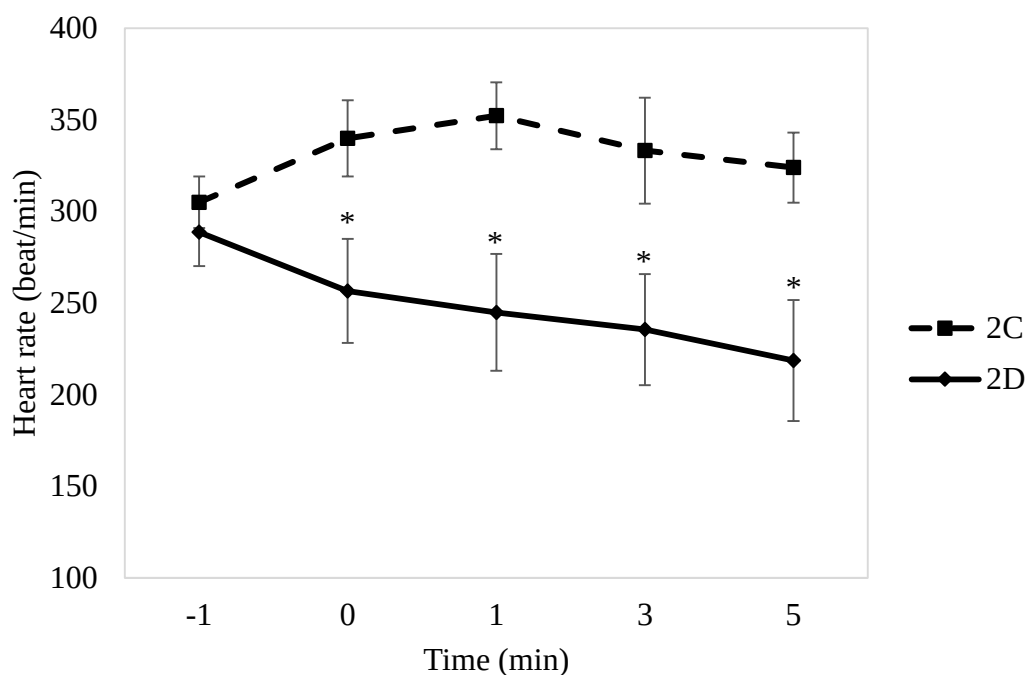


Figure 4.6. Heart rate during ischemia in short-term diabetes.

Abbreviations: 2C, non-diabetic rats (n:7); 2D, diabetic rats (n:8); -1 min, 1 minutes before ligation; 0 min, at the time point of ligation; 1 min, 1 minute after ligation; 3 min, 3 minutes after ligation; 5 min, 5 minutes after ligation. Results were expressed as mean $\pm$ standard error. * $p < 0.05$; different from 2C.

In ischemia, there was no significant difference in the heart rate between non-treated diabetic rats and the diabetic rats treated with resveratrol before ligation, at the time point of ligation and at 1st, 3rd and 5th min following ligation (Table 4.1); however, resveratrol treatment resulted in slightly higher heart rate than non-treated diabetic rats. Although basal heart rate in diabetic rats treated with combination of resveratrol and glibenclamide were not significantly different than non-treated diabetic rats, treatment of diabetic rats with combination of resveratrol and glibenclamide resulted in significantly higher heart rate in contrast to non-treated diabetic rats at the time point of ligation, 1st, 3rd and 5th min. of ligation ($p < 0.05$). However, glibenclamide treated diabetic rat heart showed non-significantly higher heart rate than that in non-treated diabetic rat heart before ligation, at the time point of ligation and at 1st min of ligation; however, glibenclamide treatment was found to increase heart rate significantly more than that in non-treated diabetic rat at 3rd and 5th min of ligation ($p < 0.05$). Similarly, treatment of diabetic rats with berberine resulted in non-significantly higher heart rate than that in non-treated diabetic rat heart at the time point of ligation and at 1st min of ligation, but berberine treatment

caused significantly higher heart rate at 3rd and 5th min of ligation ($p<0.05$). Treatment of diabetic rats with combination of berberine and glibenclamide were found to increase heart rate significantly more than the non-treated diabetic rats at 1st, 3rd and 5th min of ligation ($p<0.05$) whereas it increased heart rate non-significantly before ligation and at the time point of ligation.

Table 4.1. Heart rate in short-term diabetes during ischemia.

Groups	N	Heart rate (beat/minutes)				
		Basal	Ligation	1 st min	3 rd min	5 th min
2D _(a)	8	289±19	257±28	245±32	236±30	217±33
2DR _(b)	7	295±14	264±27	268±32	276±28	273±32
2DRG _(c)	8	282±12	324±11 ^a	316±11 ^a	313±9 ^a	305±11 ^a
2DG _(d)	7	271±20	300±24	298±19	292±17 ^a	289±17 ^a
2DB _(e)	6	267±11	284±20	297±16	306±12 ^a	305±15 ^a
2DBG _(f)	5	291±12	315±9	320±8 ^a	320±10 ^a	312±7 ^a

Abbreviations: 2D, diabetic rats; 2DR, diabetic rats treated with resveratrol; 2DRG, diabetic rats treated with combination of resveratrol with glibenclamide; 2DG, diabetic rats treated with glibenclamide; 2DB, diabetic rats treated with berberine; 2DBG, diabetic rats treated with combination of berberine with glibenclamide; basal, 1 minutes before ligation; ligation, at the point of ligation; 1st min, 1 minute after ligation; 3rd min, 3 minutes after ligation; 5th min, 5 minutes after ligation. N, number of animals. Results were expressed as mean ± standard error. ^a $p < 0.05$; different from 2D.

In short-term diabetes, mean basal arterial blood pressure did not show significant difference between non-treated diabetic rats and control rats (Figure 4.7). Similarly, mean arterial blood pressure at 1st, 3rd and 5th min of ligation was not significantly different between control rats and non-treated diabetic rats. However, mean arterial blood pressure at the time point of ligation was found to be significantly higher in control rats than non-treated diabetic rats ($p<0.05$).

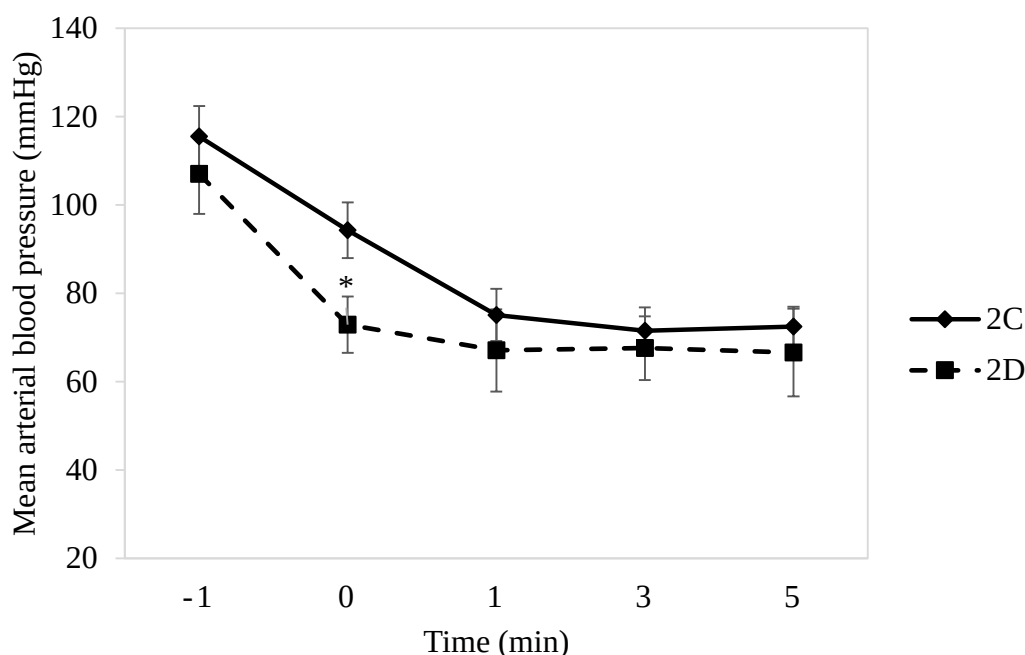


Figure 4.7. Mean arterial blood pressure during ischemia in short-term diabetes

Abbreviations: 2C, non-diabetic rats (n:7); 2D, diabetic rats (n:8); -1 min, 1 minutes before ligation; 0 min, at the point of ligation; 1 min, 1 minute after ligation; 3 min, 3 minutes after ligation; 5 min, 5 minutes after ligation. Results were expressed as mean $\pm$ standard error. * $p < 0.05$; different from 2C.

In short-term diabetes, there were no significant differences in mean arterial blood pressure before ligation, at the time point of ligation and at 1st, 3rd and 5th min following ligation between diabetic rats treated with resveratrol and non-treated diabetic rats (Table 4.2). Treatment of diabetic rats with combination of resveratrol and glibenclamide resulted in significantly higher blood pressure at the time point of ligation and at 1st min following ligation than non-treated diabetic rats ($p < 0.05$); however, before ligation and at 3rd and 5th min of ligation, the combination of resveratrol and glibenclamide non-significantly increased mean arterial blood pressure. Basal mean arterial blood pressure in diabetic rats treated with glibenclamide were non-significantly higher than non-treated diabetic rats, but glibenclamide treatment alone significantly increased mean arterial blood pressure at time point of ligation, 1st, 3rd and 5th min of ligation ($p < 0.05$). Berberine treatment resulted in non-significantly higher blood pressure before ligation, at the time point of ligation and at 3rd min of ligation than the non-treated diabetic rats ($p < 0.05$). Treatment of diabetic rats with the combination of glibenclamide and berberine did not show significant difference in mean arterial blood pressure when compared to non-treated diabetic rats.

Table 4.2. Mean arterial blood pressure during ischemia in short-term diabetes

Groups	N	Mean arterial blood pressure (mmHg)				
		Basal	Ligation	1 st min	3 rd min	5 th min
2D _(a)	8	107±9	73±6	67±9	68±7	67±10
2DR _(b)	7	112±7	82±5	76±4	76±5	76±5
2DRG _(c)	8	119±4	90±5 ^a	86±5 ^a	79±7	81±5
2DG _(d)	7	118±4	93±6 ^a	90±6 ^a	88±6 ^a	91±6 ^a
2DB _(e)	6	118±2	84±6	81±7	89±4 ^a	89±5 ^a
2DBG _(f)	5	120±3	86±7	69±8	69±9	62±10

Abbreviations: 2D, diabetic rats; 2DR, diabetic rats treated with resveratrol; 2DRG, diabetic rats treated with combination of resveratrol with glibenclamide; 2DG, diabetic rats treated with glibenclamide; 2DB, diabetic rats treated with berberine; 2DBG, diabetic rats treated with combination of berberine with glibenclamide; basal, 1 minutes before ligation; ligation, at the point of ligation; 1st min, 1 minute after ligation; 3rd min, 3 minutes after ligation; 5th min, 5 minutes after ligation. N, number of animals. Results were expressed as mean ± standard error. ^a $p < 0.05$; different from 2D.

In short-term diabetes, heart rate was found to be significantly lower in non-treated diabetic rats than control rats at 1st, 3rd and 5th min of reperfusion ($p < 0.05$, Figure 4.8). However, there was no significant difference in mean arterial blood pressure between non-treated diabetic rats and control rats (Table 4.3). Treatment of diabetic rats with resveratrol did not show significant differences in heart rate and mean arterial blood pressure when compared to non-treated diabetic rats. The combination of resveratrol with glibenclamide resulted in significantly higher heart rate in contrast to non-treated diabetic rats ($p < 0.05$); however, it did not cause a significant change in mean arterial blood pressure during reperfusion. Diabetic rats treated with glibenclamide showed significantly higher heart rate at 1st and 3rd min of reperfusion ($p < 0.05$). Mean arterial blood pressure, on the other hand, did not significantly different between glibenclamide treated diabetic rats and non-treated diabetic rats. Berberine treatment was found to increase heart rate significantly at 1st, 3rd and 5th min of reperfusion in respect to non-treated diabetic rats ($p < 0.05$). In addition, berberine treatment resulted in higher mean arterial blood pressure significantly at only 3rd min of reperfusion when compared to non-treated diabetic rats ($p < 0.05$). Treatment of diabetic rats with combination of berberine and glibenclamide was found to increase heart rate significantly at 3rd and 5th min of reperfusion in respect to non-treated diabetic rats ($p < 0.05$); however, there was no significant difference in mean arterial blood pressure during reperfusion.

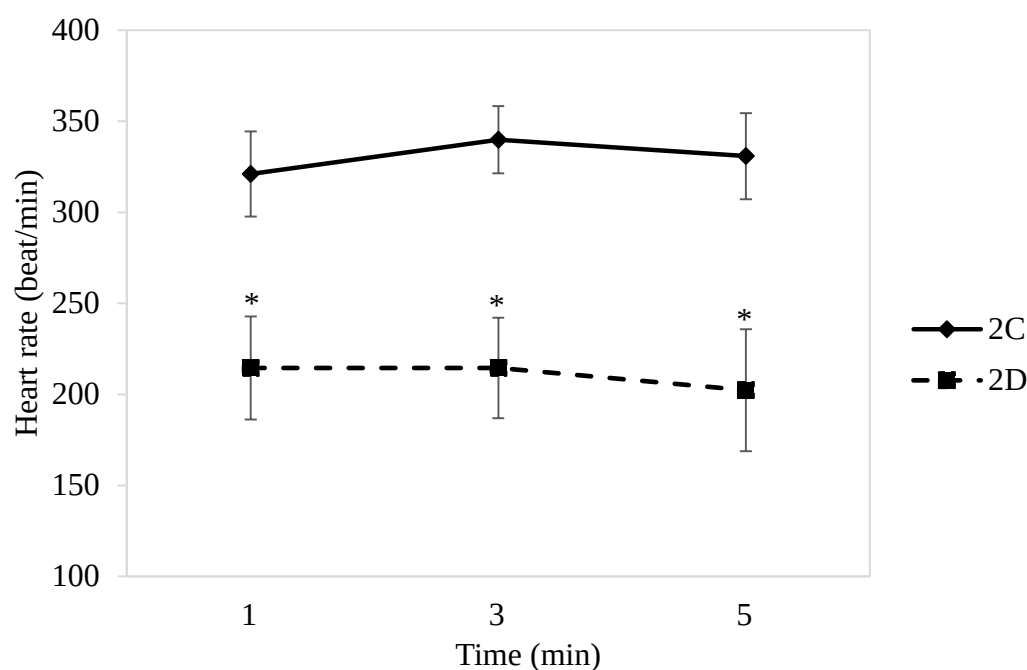


Figure 4.8. Heart rate during reperfusion in short-term diabetes

Abbreviations: 2C, non-diabetic rats (n:7); 2D, diabetic rats (n:8); 1 min, 1 minute after reperfusion; 3 min, 3 minutes after reperfusion; 5 min, 5 minutes after reperfusion. Results were expressed as mean $\pm$ standard error. * $p < 0.05$; different from 2C.

Table 4.3. Heart rate and mean blood pressure during reperfusion in short-term diabetes

Groups	N	Heart rate (beat / minute)			Mean arterial blood pressure (mmHg)		
		Following reperfusion					
		1 st min	3 rd min	5 th min	1 st min	3 rd min	5 th min
2C _(a)	7	321 $\pm$ 23	340 $\pm$ 19	331 $\pm$ 24	65 $\pm$ 8	75 $\pm$ 4	71 $\pm$ 3
2D _(b)	8	215 $\pm$ 28 ^a	215 $\pm$ 28 ^a	202 $\pm$ 34 ^a	74 $\pm$ 8	73 $\pm$ 9	76 $\pm$ 8
2DR _(c)	7	252 $\pm$ 30	261 $\pm$ 32	261 $\pm$ 26	81 $\pm$ 8	76 $\pm$ 11	84 $\pm$ 9
2DRG _(d)	8	297 $\pm$ 12 ^b	302 $\pm$ 13 ^b	284 $\pm$ 10 ^b	82 $\pm$ 7	86 $\pm$ 5	85 $\pm$ 4
2DG _(e)	7	285 $\pm$ 12 ^b	268 $\pm$ 11 ^b	247 $\pm$ 24	77 $\pm$ 10	75 $\pm$ 10	77 $\pm$ 9
2DB _(f)	6	286 $\pm$ 12 ^b	294 $\pm$ 11 ^b	296 $\pm$ 11 ^b	88 $\pm$ 6	94 $\pm$ 5 ^b	89 $\pm$ 8
2DBG _(g)	5	263 $\pm$ 45	299 $\pm$ 20 ^b	307 $\pm$ 26 ^b	78 $\pm$ 10	80 $\pm$ 3	77 $\pm$ 6

Abbreviations: 2C, non-diabetic rats; 2D, diabetic rats; 2DR, diabetic rats treated with resveratrol; 2DRG, diabetic rats treated with combination of resveratrol with glibenclamide; 2DG, diabetic rats treated with glibenclamide; 2DB, diabetic rats treated with berberine; 2DBG, diabetic rats treated with combination of berberine with glibenclamide; 1st min, 1 minutes after reperfusion; 3rd min, 3 minutes after reperfusion; 5th min, 5 minutes after reperfusion. N, number of animals. Results were expressed as mean $\pm$ standard error. ^a $p < 0.05$; different from 2C, ^b $p < 0.05$; different from 2D.

In long-term diabetes, basal heart rate was found to be significantly lower in non-treated diabetic rats than control rats ($p < 0.05$, Figure 4.9). In addition, heart rate was found to be significantly lower in non-treated diabetic rats than control rats at the time point of ligation and 1st, 3rd and 5th min following ligation ($p < 0.05$).

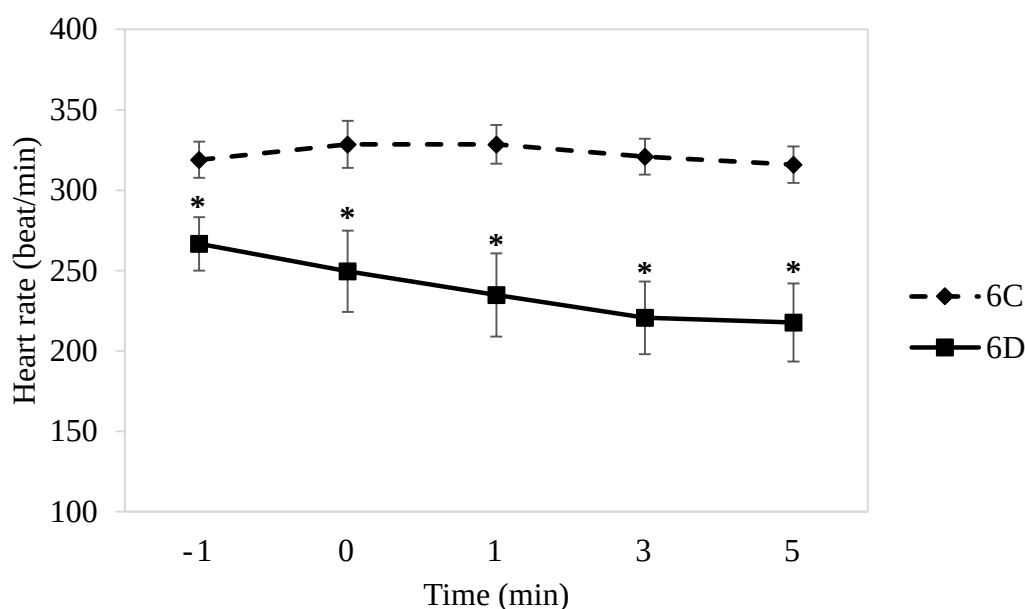


Figure 4.9. Heart rate during ischemia in long-term diabetes

Abbreviations: 6C, non-diabetic rats (n:8); 6D, diabetic rats (n:8); -1 min, 1 minutes before ligation; 0 min, at the point of ligation; 1 min, 1 minute after ligation; 3 min, 3 minutes after ligation; 5 min, 5 minutes after ligation. Results were expressed as mean $\pm$ standard error. * $p < 0.05$; different from 6C.

In long-term diabetes, basal heart rate between non-treated diabetic rats and resveratrol treated diabetic rats did not show a significant difference (Table 4.4). Resveratrol treatment caused heart rate to be higher than the non-treated diabetic rats at the time point of ligation, 1st and 5th min of ligation; however, it was significantly different at only 3rd min of ligation ($p < 0.05$). Treatment of diabetic rats with the combination of resveratrol and glibenclamide significantly resulted in higher heart rate at 1st, 3rd and 5th min of ligation when compared to non-treated diabetic rats ($p < 0.05$). There was no significant difference in heart rate before ligation, at the time of ligation, 1st and 5th min of ligation between glibenclamide treated diabetic rats and non-treated diabetic rats; however, at 3rd min of ligation, heart rate was found to be significantly higher than the non-treated diabetic rats ($p < 0.05$). Basal heart rate was non-significantly higher in berberine treated diabetic rats than the non-treated

diabetic rats although berberine treatment caused heart rate to be significantly higher during ischemia than the non-treated diabetic rats ($p < 0.05$).

Table 4.4. Heart rate during ischemia in long-term diabetes

Groups	N	Heart rate (beat / minute)				
		Basal	Ligation	1 st min	3 rd min	5 th min
6D _(a)	8	267±17	250±25	235±26	221±23	218±24
6DR _(b)	7	268±16	285±24	285±23	284±24 ^a	272±21
6DRG _(c)	7	284±13	302±18	306±17 ^a	302±21 ^a	294±23 ^a
6DG _(d)	5	264±20	281±15	284±16	284±17 ^a	267±10
6DB _(e)	7	286±30	334±22 ^a	349±20 ^a	320±20 ^a	307±27 ^a

Abbreviations: 6D, diabetic rats; 6DR, diabetic rats treated with resveratrol; 6DRG, diabetic rats treated with combination of resveratrol with glibenclamide; 6DG, diabetic rats treated with glibenclamide; 6DB, diabetic rats treated with berberine; basal, 1 minutes before ligation; ligation, at the time point of ischemia; 1st min, 1 minute after ligation; 3rd min, 3 minutes after ligation; 5th min, 5 minutes after ligation. N, number of animals. Results were expressed as mean ± standard error. ^b $p < 0.05$; different from 6D.

In long-term diabetes, mean arterial blood pressure before and during ischemia was not significantly different between control rats and non-treated diabetic rats (Table 4.5).

Table 4.5. Mean arterial blood pressure during ischemia in long-term diabetes

Groups	N	Mean arterial blood pressure (mmHg)				
		Basal	Ligation	1 st min	3 rd min	5 th min
6C _(a)	8	106±4	85±5	78±6	78±5	73±5
6D _(b)	8	100±9	70±6	67±10	61±10	58±9
6DR _(c)	7	113±5	86±7 ^b	85±9	85±8 ^b	85±7 ^b
6DRG _(d)	7	121±4 ^b	92±3 ^b	86±4 ^b	86±4 ^b	85±5 ^b
6DG _(e)	5	102±14	80±7	72±6	78±9	72±6
6DB _(f)	7	118±4	93±5 ^b	83±4	84±6 ^b	83±9 ^b

Abbreviations: 6C, non-diabetic rats; 6D, diabetic rats; 6DR, diabetic rats treated with resveratrol; 6DRG, diabetic rats treated with combination of resveratrol with glibenclamide; 6DG, diabetic rats treated with glibenclamide; 6DB, diabetic rats treated with berberine; basal, 1 minutes before ligation; ligation, at the time point of ischemia; 1st min, 1 minute after ligation; 3rd min, 3 minutes after ligation; 5th min, 5 minutes after ligation. N, number of animals. Results were expressed as mean ± standard error. ^b $p < 0.05$; different from 6D.

Resveratrol treatment caused basal mean arterial blood pressure to be slightly higher than the non-treated diabetic rats although it was significantly higher mean blood pressure at the time point of ligation, 3rd and 5th min following ligation ($p<0.05$). Treatment of diabetic rats with the combination of resveratrol and glibenclamide did show significantly higher mean arterial blood pressure before and during ischemia ($p<0.05$). On the other hand, glibenclamide treatment did not cause a significant change in mean arterial blood pressure during ischemia when compared to non-treated diabetic rats. Diabetic rats treated with berberine showed significantly higher mean arterial blood pressure at the time point of ligation, 3rd and 5th min following ligation than non-treated diabetic rats ($p<0.05$) although basal mean arterial blood pressure was not significantly higher in glibenclamide treated diabetic rats.

In long-term diabetes, heart rate during reperfusion was found to be significantly lower in non-treated diabetic rats than control rats ($p<0.05$, Figure 4.10).

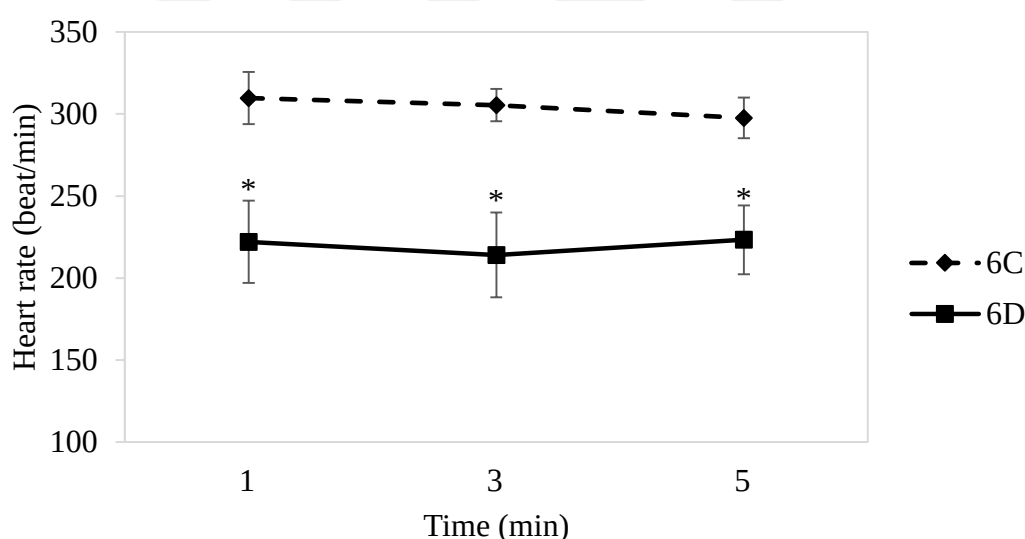


Figure 4.10. Heart rate during reperfusion in long-term diabetes

Abbreviations: 6C, non-diabetic rats (n: 8); 6D, diabetic rats (n: 8); 1st min, 1 minute after reperfusion; 3rd min, 3 minutes after reperfusion; 5th min, 5 minutes after reperfusion. Results were expressed as mean $\pm$ standard error. * $p < 0.05$; different from 6D.

However, mean arterial blood pressure during reperfusion did not show significant difference between control rats and non-treated diabetic rats (Table 4.6). Resveratrol non-significantly increased heart rate during reperfusion. However, diabetic rats treated with resveratrol showed significantly higher mean arterial blood pressure at 5th min of reperfusion when compared to non-treated diabetic rats ($p<0.05$). Although diabetic rats treated with the combination of resveratrol and glibenclamide showed non-significant increase in heart rate during reperfusion in respect to non-treated diabetic rats, the combination of resveratrol with glibenclamide resulted in significantly higher mean arterial blood pressure at 1st and 3rd min of reperfusion when it was compared to non-treated diabetic rats ($p<0.05$). On the other hand, glibenclamide treatment was found to increase heart rate and blood pressure non-significantly in respect to non-treated diabetic rats during reperfusion. Diabetic rats treated with berberine were found to have significantly higher heart rate at 3rd and 5th min of reperfusion than non-treated diabetic rats ($p<0.05$). In addition, mean arterial blood pressure during reperfusion was significantly higher in diabetic rats treated with berberine than the non-treated diabetic rats ($p<0.05$).

Table 4.6. Heart rate during reperfusion in long-term diabetes

Groups	N	Heart rate (beat/minute)			Mean arterial blood pressure (mmHg)		
		Following reperfusion					
		1 st min	3 rd min	5 th min	1 st min	3 rd min	5 th min
6C _(a)	8	310±16	305±10	298±12	72±7	71±6	73±6
6D _(b)	8	222±25 ^a	214±26 ^a	223±21 ^a	60±10	60±11	62±9
6DR _(c)	7	271±22	265±22	259±22	80±8	80±7	81±7 ^b
6DRG _(d)	7	277±19	267±17	255±12	86±5 ^b	82±5 ^b	81±4
6DG _(e)	5	258±13	255±14	230±31	71±5	68±3	66±3
6DB _(f)	7	285±21	295±20 ^b	292±25 ^b	89±8 ^b	87±5 ^b	89±8 ^b

Abbreviations: 6C, non-diabetic rats; 6D, diabetic rats; 6DR, diabetic rats treated with resveratrol; 6DRG, diabetic rats treated with combination of resveratrol with glibenclamide; 6DG, diabetic rats treated with glibenclamide; 6DB, diabetic rats treated with berberine; 1st min, 1 minute after reperfusion; 3rd min, 3 minutes after reperfusion; 5th min, 5 minutes after reperfusion. N, number of animals. Results were expressed as mean ± standard error. ^a $p < 0.05$; different from 6C, ^b $p < 0.05$; different from 6D.

4.1.3 Arrhythmias and Area at Risk of Infarct

The size of the occluded zone (area at risk of infarct) was evaluated to confirm correct ligation of coronary artery at the same site in each experimental animal. There were no significant differences in area at risk of infarct between groups in both short- and long-term diabetes (Figure 4.11).

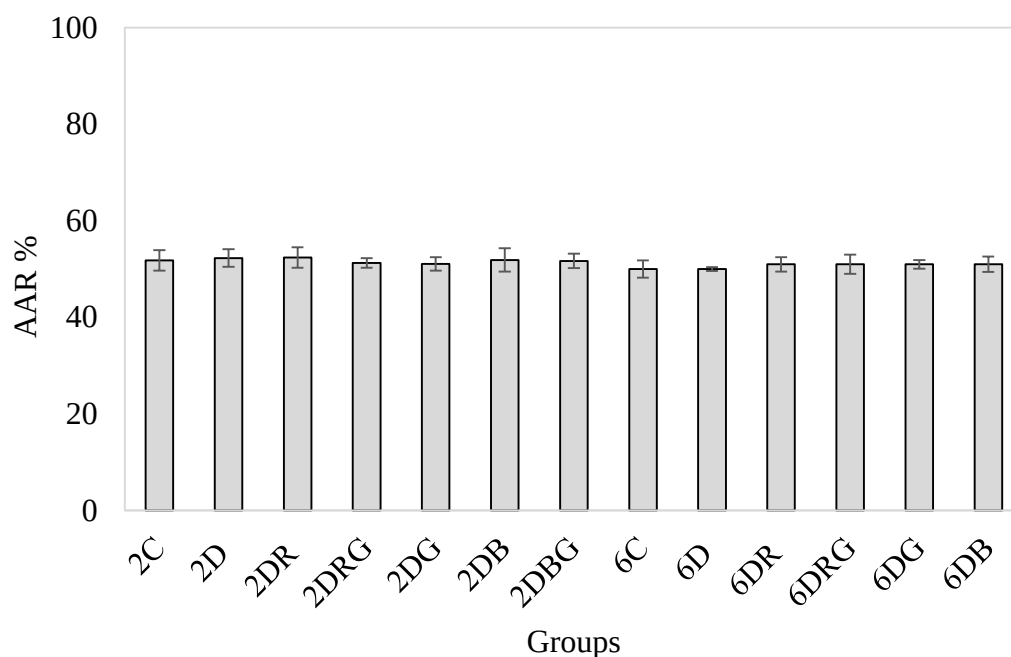


Figure 4.11. Area at risk of infarct

Abbreviations: 2C, non-diabetic rats (n:7); 2D, diabetic rats (n:8); 2DR, diabetic rats treated with resveratrol (n:7); 2DRG, diabetic rats treated with combination of resveratrol with glibenclamide (n:8); 2DG, diabetic rats treated with glibenclamide (n:7); 2DB, diabetic rats treated with berberine (n:6); 2DBG, diabetic rats treated with combination of berberine with glibenclamide (n:5); 6C, non-diabetic rats (n:8); 6D, diabetic rats (n:8); 6DR, diabetic rats treated with resveratrol (n: 7); 6DRG, diabetic rats treated with combination of resveratrol with glibenclamide (n:7); 6DG, diabetic rats treated with glibenclamide (n:5); 6DB, diabetic rats treated with berberine (n:7). AAR, area at risk of infarct. Results were expressed as mean $\pm$ standard error.

In short-term diabetes, arrhythmia score at ischemia was not significantly found to be different between groups (Figure 4.12).

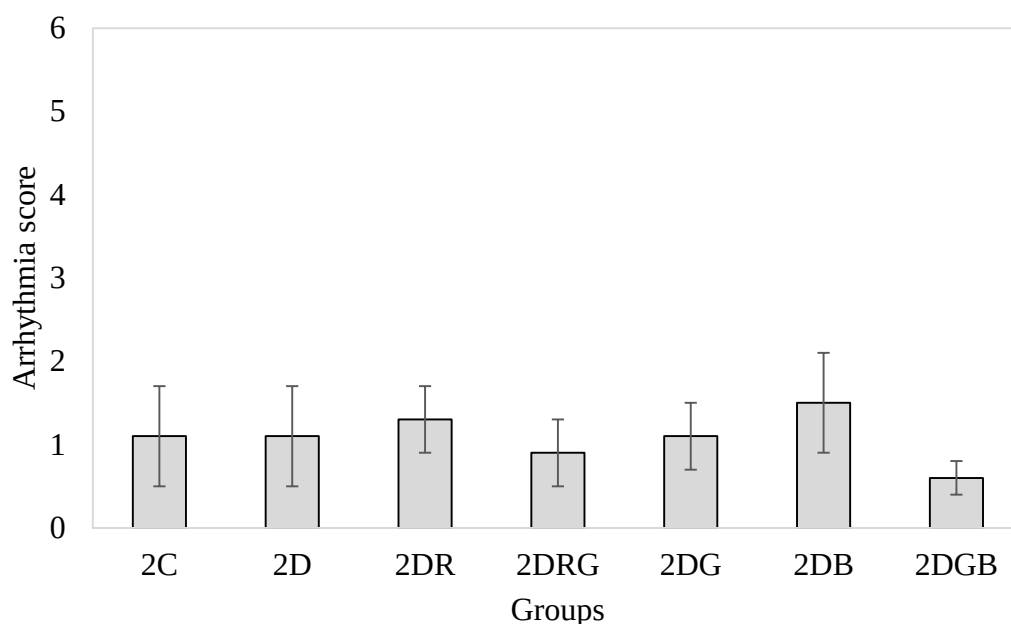


Figure 4.12. Arrhythmia score at ischemia in short-term diabetes

Abbreviations: 2C, non-diabetic rats (n:7); 2D, diabetic rats (n:8); 2DR, diabetic rats treated with resveratrol (n:7); 2DRG, diabetic rats treated with combination of resveratrol with glibenclamide (n:8); 2DG, diabetic rats treated with glibenclamide (n:7); 2DB, diabetic rats treated with berberine (n:6); 2DGB, diabetic rats treated with combination of berberine with glibenclamide (n:5). Results were expressed as mean $\pm$ standard error.

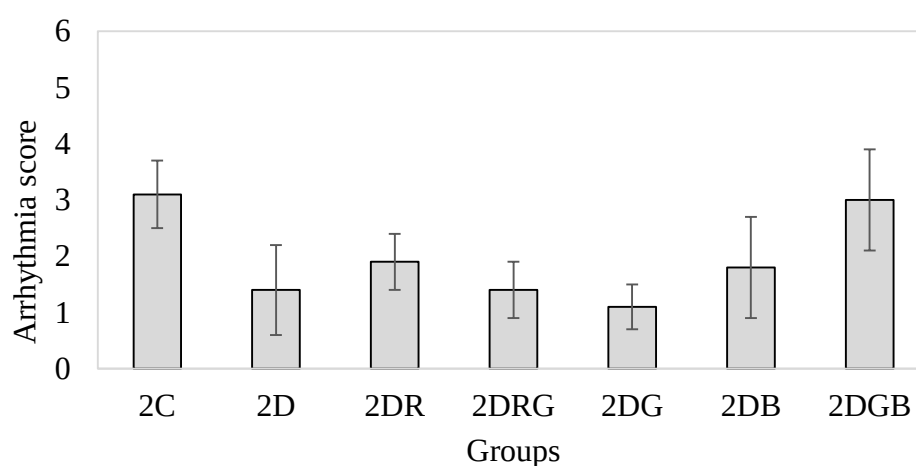
In short-term diabetes, the onset of arrhythmia, arrhythmic period and the duration of VF, VT and other types of arrhythmias during ischemia were not significantly different between groups (Table 4.7). However, arrhythmias non-significantly started earlier in non-treated diabetic rats in respect to control rats. When compared to non-treated diabetic rats, resveratrol non-significantly delayed the onset of arrhythmias. Similarly, the onset of arrhythmias was late in diabetic rats treated with the combination of berberine and glibenclamide. Interestingly, arrhythmic period in diabetic rats treated with berberine was non-significantly longer than the non-treated diabetic rats.

Table 4.7. Arrhythmia duration during ischemia in short-term diabetes

Groups	N	Start of arrhythmia (sec)	Arrhythmic period (sec)	Duration of arrhythmias (sec)			
				VF	VT	Others	Total
2C	7	51.9±31.3	84.7±45.9	0±0	0±0	28.9±14.9	25.9±14.9
2D	8	21.1±9.2	79.1±47.2	0±0	2.7±2.4	27.9±15.9	30.6±17.2
2DR	7	82.0±43.7	107.9±46.5	0±0	3.9±3.4	13.6±8.0	17.5±11.3
2DRG	8	43.6±40.2	130.1±60.8	0±0	0.2±0.2	11.5±7.9	11.7±8.1
2DG	7	46.0±35.5	86.0±45.4	0±0	0±0	9.0±4.6	9.0±4.6
2DB	6	10.8±4.1	207.8±67.6	0±0	1.1±0.9	25.8±18.7	26.9±18.6
2DGB	5	79.6±61.9	69.2±64.5	0±0	0±0	3.0±1.6	3.0±1.6

Abbreviations: 2C, non-diabetic rats; 2D, diabetic rats; 2DR, diabetic rats treated with resveratrol; 2DRG, diabetic rats treated with combination of resveratrol with glibenclamide; 2DG, diabetic rats treated with glibenclamide; 2DB, diabetic rats treated with berberine; 2DGB, diabetic rats treated with combination of berberine with glibenclamide; VF, ventricular fibrillation; VT, ventricular tachycardia; Others; ventricular premature contraction, bigeminy, salvo. N, number of animals. Results were expressed as mean ± standard error.

In short-term diabetes, arrhythmia score in non-treated diabetic rats at reperfusion was found to be non-significantly lower than control rats (p : 0.054, Figure 4.13). When compared to non-treated diabetic rats, treatment of diabetic rats with the combination of berberine and glibenclamide resulted in non-significantly increased in arrhythmias score.

**Figure 4.13.** Arrhythmia score at reperfusion in short-term diabetes

Abbreviations: 2C, non-diabetic rats (n:7); 2D, diabetic rats (n:8); 2DR, diabetic rats treated with resveratrol (n:7); 2DRG, diabetic rats treated with combination of resveratrol with glibenclamide (n:8); 2DG, diabetic rats treated with glibenclamide (n:7); 2DB, diabetic rats treated with berberine (n:6); 2DGB, diabetic rats treated with combination of berberine with glibenclamide (n:5). Results were expressed as mean ± standard error.

In short-term diabetes, the onset of arrhythmias, arrhythmic period and the duration of VF, VT and other types of arrhythmias during reperfusion did not show significant difference between non-treated diabetic rats and control rats (Table 4.8). In contrast to non-treated diabetic rats, treatment of diabetic with the combination of resveratrol and glibenclamide resulted in non-significant decrease in arrhythmic period and the duration of arrhythmias during reperfusion.

Table 4.8. Duration of arrhythmias during reperfusion in short-term diabetes

Groups	N	Start of arrhythmia (sec)	Arrhythmic period (sec)	Duration of arrhythmia (sec)			
				VF	VT	Others	Total
2C _(a)	7	48±29	154±35	28±22	34±14	34±8	94±38
2D _(b)	8	26±19	76±49	2±2	13±12	48±33	63±41
2DR _(c)	7	43±19	131±57	0±0	14±8	36±22	51±26
2DRG _(d)	8	7±5	42±23	1±1	9±5	4±2	13±6
2DG _(e)	7	12±4	70±46	0±0	2±1	14±10	16±12
2DB _(f)	6	12±9	85±56	0±0	23±13	45±42	68±43
2DBG _(g)	5	19±7	102±45	10±9	29±13	29±15	68±28

Abbreviations: 2C, non-diabetic rats; 2D, diabetic rats; 2DR, diabetic rats treated with resveratrol; 2DRG, diabetic rats treated with combination of resveratrol with glibenclamide; 2DG, diabetic rats treated with glibenclamide; 2DB, diabetic rats treated with berberine; 2DBG, diabetic rats treated with combination of berberine with glibenclamide; VF, ventricular fibrillation; VT, ventricular tachycardia; Others; ventricular premature contraction, bigeminy, salvo. N, number of animals. Results were expressed as mean ± standard error.

In short-term diabetes, survival rate and the incidence of VF, VT and other types of arrhythmias between groups did not show significant differences at the end of ischemia (Table 4.9). VF was observed in all groups during ischemia. Similarly, VT did not occur in diabetic rats treated with glibenclamide alone and in a combination group including glibenclamide and berberine together; however, two animals in non-treated diabetic rats showed VT during ischemia.

Table 4.9. The incidence of arrhythmia at the end of ischemia in short-term diabetes

Groups	N	Survival rate (n/%)	Incidence (n / %)		
			VF	VT	Others
2C _(a)	7	7/100	0/0	0/0	3/42.9
2D _(b)	8	8/100	0/0	2/25	3/37.5
2DR _(c)	7	7/100	0/0	1/14.2	5/71.4
2DRG _(d)	8	8/100	0/0	1/12.5	3/37.5
2DG _(e)	7	7/100	0/0	0/0	5/71.4
2DB _(f)	6	6/100	0/0	1/16.6	4/66.7
2DBG _(g)	5	5/100	0/0	0/0	3/60

Abbreviations: 2C, non-diabetic rats; 2D, diabetic rats; 2DR, diabetic rats treated with resveratrol; 2DRG, diabetic rats treated with combination of resveratrol with glibenclamide; 2DG, diabetic rats treated with glibenclamide; 2DB, diabetic rats treated with berberine; 2DBG, diabetic rats treated with combination of berberine with glibenclamide; N, number of animals; n, number of animals having arrhythmias; VF, ventricular fibrillation; VT, ventricular tachycardia; Others; ventricular premature contraction, bigeminy, salvo.

At the end of reperfusion, it was found that the incidence of other types of arrhythmias was significantly lower in non-treated diabetic rats in respect to control rats ($p < 0.05$, Table 4.10).

Table 4.10. The incidence of arrhythmia at the end of reperfusion in short-term diabetes

Groups	N	Survival rate (n/%)	Incidence (n / %)		
			VF	VT	Others
2C _(a)	7	7/100	2/28.5	5/71	7/100
2D _(b)	8	8/100	1/12.5	2/25	3/37.5 ^a
2DR _(c)	7	7/100	0/0	3/42.9	6/85.7
2DRG _(d)	8	8/100	1/12.5	3/37.5	5/62.5
2DG _(e)	7	7/100	0/0	2/28.5	5/71.4
2DB _(f)	6	6/100	0/0	3/50	3/50
2DBG _(g)	5	5/100	2/40	3/60	5/100 ^b

Abbreviations: 2C, non-diabetic rats; 2D, diabetic rats; 2DR, diabetic rats treated with resveratrol; 2DRG, diabetic rats treated with combination of resveratrol with glibenclamide; 2DG, diabetic rats treated with glibenclamide ; 2DB, diabetic rats treated with berberine ; 2DBG, diabetic rats treated with combination of berberine with glibenclamide ; N, number of animals; n, number of animals having arrhythmias; VF, ventricular fibrillation; VT, ventricular tachycardia; Others; ventricular premature contraction, bigeminy, salvo, ^a $p < 0.05$; different from 2C, ^b $p < 0.05$; different from 2D.

In addition, the incidence of VT non-significantly decreased in non-treated diabetic rats in respect to control rats. However, treatment of diabetic rats with the combination of glibenclamide and berberine significantly increased the incidence of other types of arrhythmias in respect to non-treated diabetic rats ($p<0.05$). Survival rate, the incidence of VF and VT did not show significant differences between groups. VF was not observed during reperfusion in diabetic rats treated with glibenclamide and berberine alone.

In long-term diabetes, the score of arrhythmia during ischemia was not significantly different among groups (Figure 4.14). However, the score of arrhythmias in non-treated diabetic rats was non-significantly higher than control rats. In addition, the treatment of diabetic rats with both resveratrol alone and its combination with glibenclamide caused the score of arrhythmias to decrease non-significantly when compared to non-treated diabetic rats.

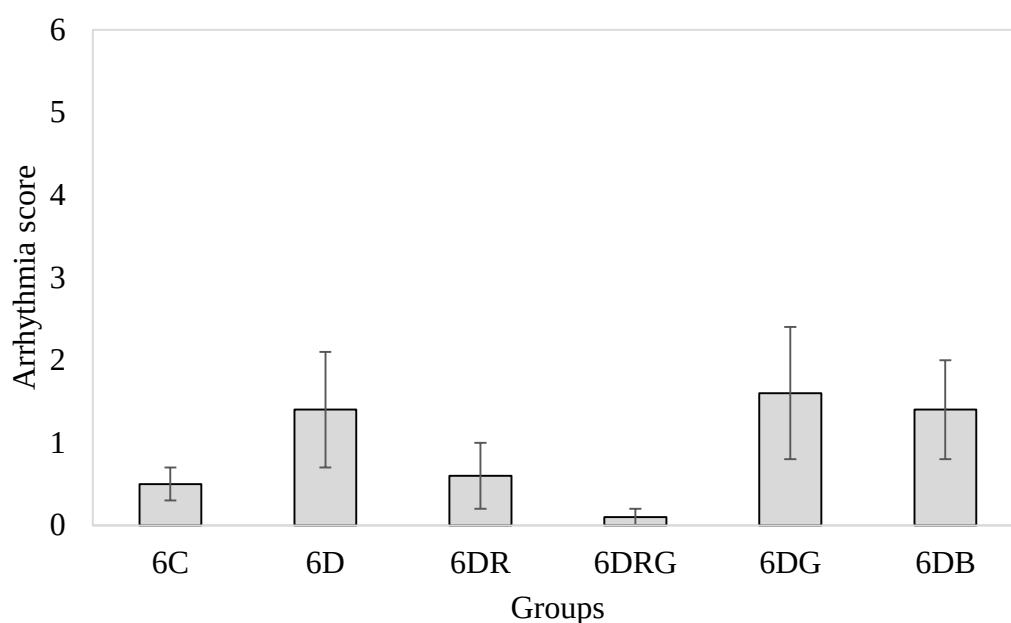


Figure 4.14. Arrhythmia score at ischemia in long-term diabetes

Abbreviations: 6C, non-diabetic rats (n:8); 6D, diabetic rats (n:8); 6DR, diabetic rats treated with resveratrol (n:7); 6DRG, diabetic rats treated with combination of resveratrol with glibenclamide (n:7); 6DG, diabetic rats treated with glibenclamide (n:5); 6DB, diabetic rats treated with berberine (n:7). Results were expressed as mean $\pm$ standard error.

In long-term diabetes, there were no significant differences between groups in the onset of arrhythmias, the arrhythmic period, the duration of VF, VT and other types of arrhythmias during ischemia (Table 4.11). However, the arrhythmic period and the duration of arrhythmias were non-significantly longer in non-treated diabetic rats than control rats.

Table 4.11. Duration of arrhythmia during ischemia in long-term diabetes

Groups	N	Start of arrhythmia (sec)	Arrhythmic period (sec)	Duration of arrhythmia (sec)			
				VF	VT	Others	Total
6C _(a)	8	21±11	18±11	0±0	0±0	2±1	2±1
6D _(b)	8	70±38	73±44	2±2	4±4	24±14	30±18
6DR _(c)	7	27±23	20±20	0±0	0±0	5±5	5±5
6DRG _(d)	7	2±2	18±18	0±0	0±0	0.4±0.4	0.4±0.4
6DG _(e)	5	64±37	48±39	2±2	2±2	9±7	13±8
6DB _(f)	7	89±49	112±54	0±0	4.2±3.3	22 ±16	26±17

Abbreviations: 6C, non-diabetic rats; 6D, diabetic rats; 6DR, diabetic rats treated with resveratrol; 6DRG, diabetic rats treated with combination of resveratrol with glibenclamide; 6DG, diabetic rats treated with glibenclamide; 6DB, diabetic rats treated with berberine; VF, ventricular fibrillation; VT, ventricular tachycardia; Others; ventricular premature contraction, bigeminy, salvo. N, number of animals. Results were expressed as mean ± standard error.

In long-term diabetes, there was no significant difference in arrhythmias score between control rats and non-treated diabetic rats at reperfusion (Figure 4.15). In addition, berberine and glibenclamide treatment alone did not significantly change the arrhythmia score. However, resveratrol treatment was found to decrease the score of arrhythmias significantly when it was compared to non-treated diabetic rats ($p<0.05$). Similarly, arrhythmias score was significantly lower in diabetic rats treated with the combination of resveratrol and glibenclamide than in non-treated diabetic rats ($p<0.05$).

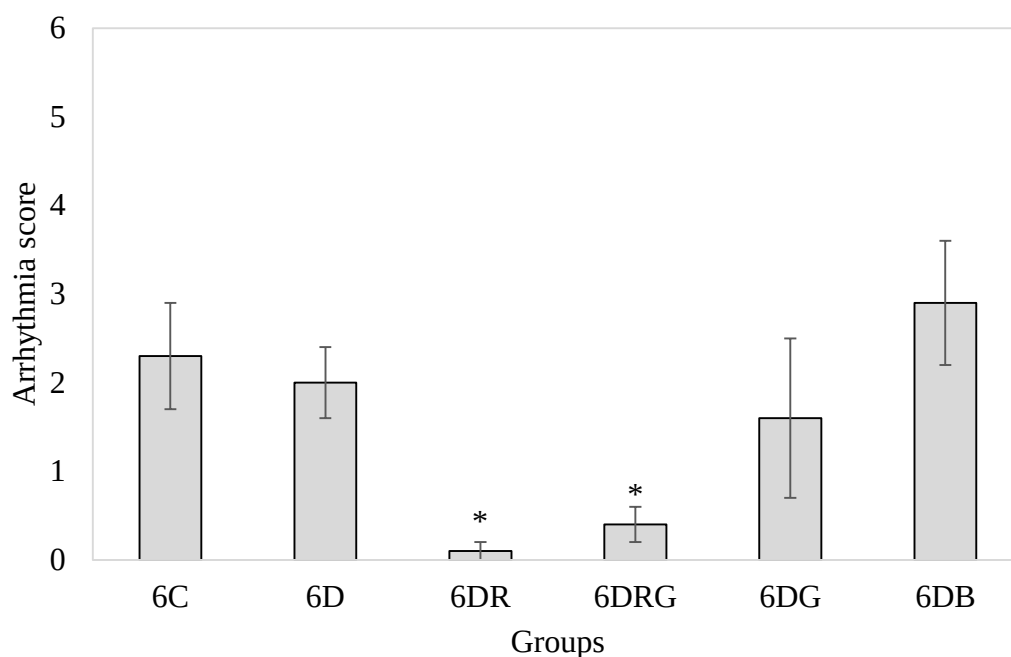


Figure 4.15. Arrhythmia score at reperfusion in long-term diabetes

Abbreviations: 6C, non-diabetic rats (n:8); 6D, diabetic rats (n:8); 6DR, diabetic rats treated with resveratrol (n:7); 6DRG, diabetic rats treated with combination of resveratrol with glibenclamide (n:7); 6DG, diabetic rats treated with glibenclamide (n:5); 6DB, diabetic rats treated with berberine (n:7). Results were expressed as mean $\pm$ standard error. * $p < 0.05$; different from 6D.

In long-term diabetes, arrhythmias during reperfusion appeared in non-treated diabetic rats significantly later than control rats ($p < 0.05$, Table 4.12).

Table 4.12. Duration of arrhythmia during reperfusion in long-term diabetes

Groups	N	Start of arrhythmia (sec)	Arrhythmic period (sec)	Duration of arrhythmia (sec)			
				VF	VT	Others	Total
6C _(a)	8	11 $\pm$ 8	132 $\pm$ 48	8 $\pm$ 7	21 $\pm$ 10	27 $\pm$ 11	56 $\pm$ 24
6D _(b)	8	76 $\pm$ 36 ^a	144 $\pm$ 41	0.4 $\pm$ 0.4	3 $\pm$ 2 ^a	36 $\pm$ 19	39 $\pm$ 20
6DR _(c)	7	20 $\pm$ 20	0.1 $\pm$ 0.1 ^b	0 $\pm$ 0	0 $\pm$ 0	0.2 $\pm$ 0.2	0.2 $\pm$ 0.2
6DRG _(d)	7	3 $\pm$ 2 ^b	4 $\pm$ 3 ^b	0 $\pm$ 0	0 $\pm$ 0	0.6 $\pm$ 0.3	0.6 $\pm$ 0.3
6DG _(e)	5	0.8 $\pm$ 0.5 ^b	147 $\pm$ 86	0 $\pm$ 0	0 $\pm$ 0	51 $\pm$ 46	51 $\pm$ 46
6DB _(f)	7	26 $\pm$ 12	62 $\pm$ 20	18 $\pm$ 17	20 $\pm$ 9 ^b	19 $\pm$ 14	57 $\pm$ 20

Abbreviations: 6C, non-diabetic rats; 6D, diabetic rats; 6DR, diabetic rats treated with resveratrol; 6DRG, diabetic rats treated with combination of resveratrol with glibenclamide; 6DG, diabetic rats treated with glibenclamide; 6DB, diabetic rats treated with berberine; VF, ventricular fibrillation; VT, ventricular tachycardia; Others; ventricular premature contraction, bigeminy, salvo. N, number of animals. Results were expressed as mean $\pm$ standard error. ^a $p < 0.05$; different from 6C, ^b $p < 0.05$; different from 6D.

However, there was no significant difference in arrhythmic period between control rats and non-treated diabetic rats. Although arrhythmia appeared non-significantly earlier in diabetic rats treated with resveratrol, arrhythmic period was found significantly shorter than the non-treated diabetic rats ($p<0.05$). Treatment of diabetic rats with the combination of resveratrol and glibenclamide significantly decreased the onset of arrhythmia and the arrhythmic period when it was compared to non-treated diabetic rats ($p<0.05$). Glibenclamide treatment alone also decreased the onset of arrhythmia in respect to the non-treated diabetic rats ($p<0.05$). In addition, arrhythmic period in diabetic rats treated with glibenclamide was non-significantly higher than the non-treated diabetic rats. When compared to control rats, the duration of VT was significantly less in non-treated diabetic rats ($p<0.05$). Also, the duration of VF and other types of arrhythmias was non-significantly lesser in non-treated diabetic rats than control rats. However, resveratrol alone and its combination with glibenclamide non-significantly decreased the duration of other types of arrhythmias compared to non-treated diabetic rats. Interestingly, berberine treatment was found to significantly increase the duration of VT when compared to non-treated diabetic rats ($p<0.05$).

In long-term diabetes, survival rate and the incidence of VF, VT and other types of arrhythmias determined at the end of ischemia did not show significant differences between groups (Table 4.13).

Table 4.13. The incidence of arrhythmia at the end of ischemia in long-term diabetes

Groups	N	Survival rate (n/%)	Incidence (n / %)		
			VF	VT	Others
6C _(a)	8	8/100	0/0	0/0	4/50
6D _(b)	8	8/100	1/12.5	1/12.5	4/50
6DR _(c)	7	7/100	0/0	0/0	2/25.8
6DRG _(d)	7	7/100	0/0	0/0	1/14.3
6DG _(e)	5	5/100	1/20	1/20	3/60
6DB _(f)	7	7/100	0/0	3/42.9	5/71.4

Abbreviations: 6C, non-diabetic rats; 6D, diabetic rats; 6DR, diabetic rats treated with resveratrol; 6DRG, diabetic rats treated with combination of resveratrol with glibenclamide; 6DG, diabetic rats treated with glibenclamide; 6DB, diabetic rats treated with berberine; N, number of animals; n, number of animals having arrhythmias; VF, ventricular fibrillation; VT, ventricular tachycardia; Others; ventricular premature contraction, bigeminy, salvo.

VF was observed only in non-treated diabetic rats and diabetic rats treated with glibenclamide. In addition, VT occurred in non-treated diabetic rats and diabetic rats treated with combination of glibenclamide and berberine. Berberine treatment non-significantly increased the incidence of VT in respect to non-treated diabetic rats. In all groups, other types of arrhythmias were observed. However, resveratrol alone and its combination with glibenclamide non-significantly decreased the incidence of other types of arrhythmias compared to non-treated diabetic rats.

In long-term diabetes, survival rate and the incidence of VT and VF at the end of reperfusion were not found to show significant difference between groups (Table 4.14). Also, there were no significant differences in the incidence of other types of arrhythmias between control rats and non-treated diabetic rats. However, treatment of diabetic rats with resveratrol alone and its combination with glibenclamide was found significantly to decrease the incidence of other types of arrhythmias when compared to non-treated diabetic rats ($p<0.05$).

Table 4.14. The incidence of arrhythmia at the end of reperfusion in long-term diabetes

Groups	N	Survival rate (n/%)	Incidence (n / %)		
			VF	VT	Others
6C _(a)	8	8/100	2/25	5/62.5	6/75
6D _(b)	8	8/100	1/12.5	3/37.5	8/100
6DR _(c)	7	7/100	0/0	0/0	1/14.3 ^b
6DRG _(d)	7	7/100	0/0	0/0	2/28.5 ^b
6DG _(e)	5	5/100	0/0	0/0	3/60
6DB _(f)	7	7/100	4/57	2/28.6	6/85.7

Abbreviations: 6C, non-diabetic rats; 6D, diabetic rats; 6DR, diabetic rats treated with resveratrol ; 6DRG, diabetic rats treated with combination of resveratrol with glibenclamide; 6DG, diabetic rats treated with glibenclamide; 6DB, diabetic rats treated with berberine; N, number of animals; n, number of animals having arrhythmias; VF, ventricular fibrillation; VT, ventricular tachycardia; Others; ventricular premature contraction, bigeminy, salvo. ^b $p<0.05$; different from 6D.

4.1.4 Biochemical Findings

In short-term diabetes, serum insulin levels were not significantly different between control rats and non-treated diabetic rats (Table 4.15). Drug administrations did not change the serum insulin levels in diabetic rats. In addition, serum SOD levels did not show significant difference between groups. There were no significant differences in serum LDH levels between control rats and non-treated diabetic rats. However, serum LDH levels in diabetic rats treated with combination of berberine and glibenclamide were found to be higher than non-treated diabetic rats ($p<0.05$).

Table 4.15. Insulin, superoxide dismutase and lactate dehydrogenase levels in short-term diabetes

Groups	N	Insulin (mIU/L)	SOD (ng/ml)	LDH (U/L)
2C _(a)	3	5.5±0.6	3.7±0.2	71.9±7.5
2D _(b)	3	5.6±0.5	3.5±0.1	56.4±10.4
2DR _(c)	3	7.4±2.3	3.8±0.2	57.7±8.1
2DRG _(d)	3	5.3±0.2	2.9±0.6	58.1±3.2
2DG _(e)	3	4.9±0.2	3.4±0.1	55.8±7.0
2DB _(f)	3	6.0±0.3	3.6±0.1	58.1±1.5
2DBG _(g)	3	5.9±0.3	3.4±0.7	75.4±2.1 ^b

Abbreviations: 2C, non-diabetic rats; 2D, diabetic rats; 2DR, diabetic rats treated with resveratrol; 2DRG, diabetic rats treated with combination of resveratrol with glibenclamide; 2DG, diabetic rats treated with glibenclamide; 2DB, diabetic rats treated with berberine; 2DBG, diabetic rats treated with combination of berberine with glibenclamide; N, number of animals. Results were expressed as mean ± standard error. ^b $p<0.05$; different from 2D.

In long-term diabetes, serum insulin levels did not show significant difference between control rats and non-treated diabetic rats (Table 4.16). Also, treatment of diabetic rats with resveratrol, berberine and their combination with glibenclamide did not change the serum insulin levels. Serum SOD levels did not show significant change between control rats and non-treated diabetic rats. However, serum SOD levels were found to be significantly lower in diabetic rats treated with resveratrol and glibenclamide than non-treated diabetic rats ($p<0.05$). All other treatments did not change the serum SOD levels. In addition, serum LDH levels did not show a significant change between groups.

Table 4.16. Insulin, superoxide dismutase and lactate dehydrogenase levels in long-term diabetes

Groups	N	Insulin (mIU/L)	SOD (ng/ml)	LDH (U/L)
6C _(a)	3	5.7±0.7	3.8±0.3	62.4±2.4
6D _(b)	3	5.7±0.1	3.7±0.2	52.8±5.4
6DR _(c)	3	5.5±0.5	3.6±0.3	53.5±3.2
6DRG _(d)	3	4.3±1.2	2.0±0.4 ^b	46.3±8.5
6DG _(e)	3	4.3±0.3	3.5±0.8	53.9±4.6
6DB _(f)	3	4.8±0.3	3.1±0.4	53.3±1.5
6DBG _(g)	3	4.7±0.2	3.5±0.1	43.9±2.7

Abbreviations: 6C, non-diabetic rats; 6D, diabetic rats; 6DR, diabetic rats treated with resveratrol; 6DRG, diabetic rats treated with combination of resveratrol with glibenclamide; 6DG, diabetic rats treated with glibenclamide; 6DB, diabetic rats treated with berberine; 6DBG, diabetic rats treated with combination of berberine with glibenclamide; N, number of animals. Results were expressed as mean ± standard error. ^b*p* < 0.05; different from 6D.

4.1.5 Molecular Findings

In short-term diabetes, there was no significant difference in protein expression level of Kir 6.2 subunit between non-treated diabetic rats and non-diabetic rats (Figure 4.16). Compared to non-treated diabetic rats, resveratrol, berberine and glibenclamide combination did not significantly change protein expression levels of Kir 6.2 subunits.

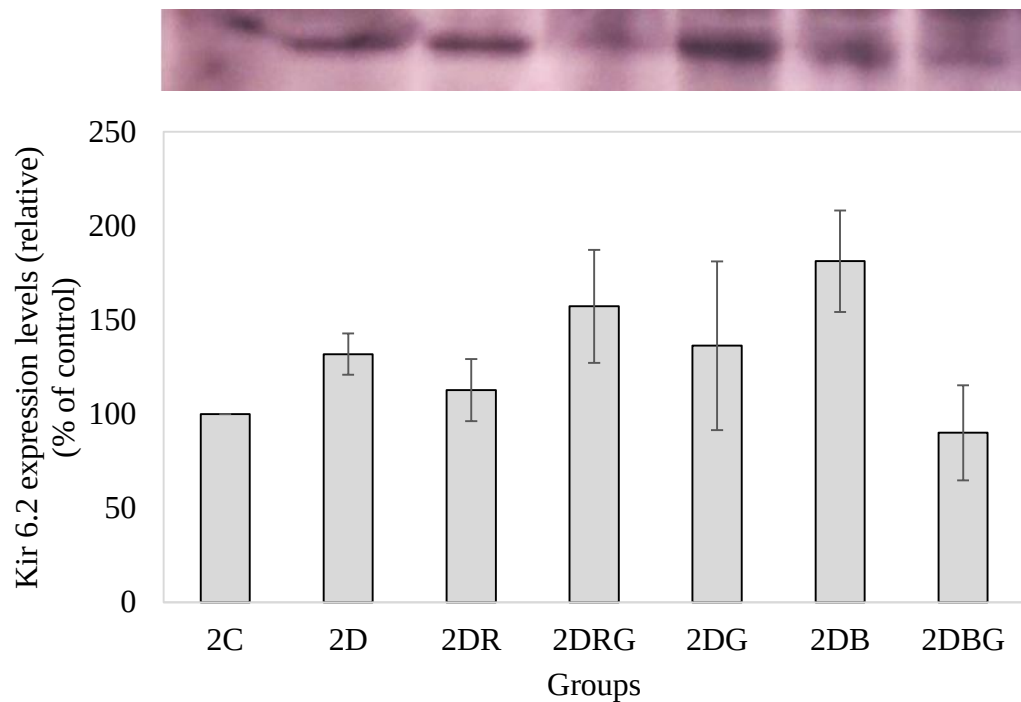


Figure 4.16. Expression levels of Kir 6.2 subunit in short-term diabetes

Abbreviations: 2C, non-diabetic rats (n:3); 2D, diabetic rats; 2DR, diabetic rats treated with resveratrol (n:3); 2DRG, diabetic rats treated with combination of resveratrol with glibenclamide (n:3); 2DG, diabetic rats treated with glibenclamide (n:3); 2DB, diabetic rats treated with berberine; 2DBG, diabetic rats treated with combination of berberine with glibenclamide (n:3). The blots were shown at the top and the quantified ratios were shown at the bottom. Results were expressed as mean $\pm$ standard error.

In long-term diabetes, protein expression level of Kir 6.2 subunit was found to significantly decrease in non-treated diabetic rats when compared to control rats ($p < 0.05$, Figure 4.17). All drug treatments significantly increased protein expression level of Kir 6.2 subunit compared to non-treated diabetic rats ($p < 0.05$).

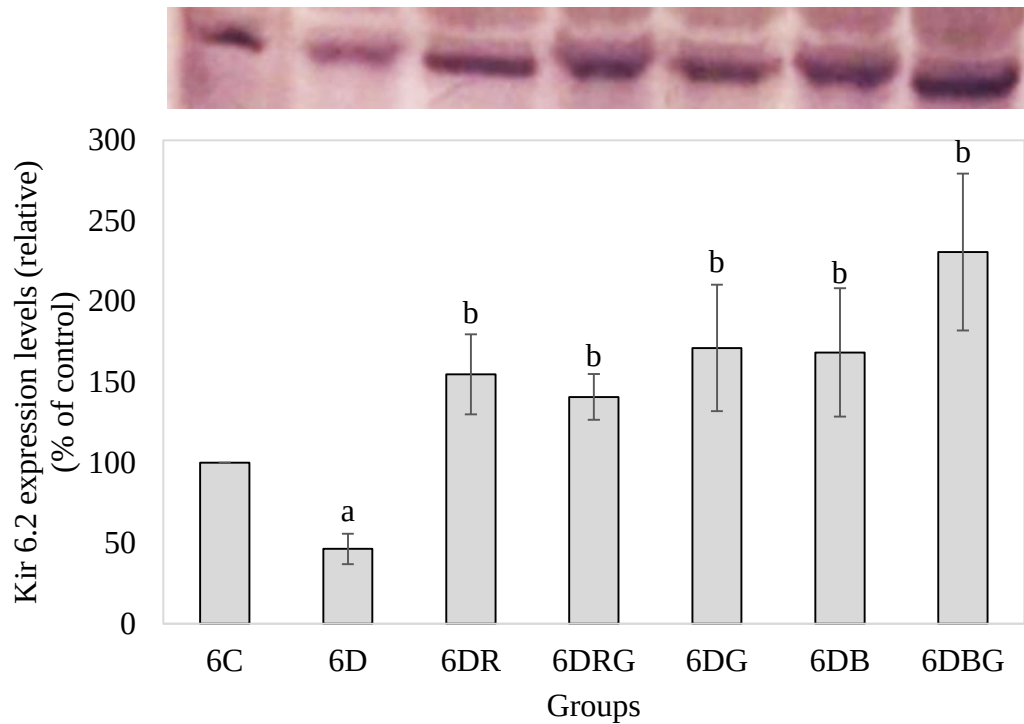


Figure 4.17. Expression levels of Kir 6.2 subunit in long-term diabetes

Abbreviations: 6C, non-diabetic rats (n: 3); 6D, diabetic rats; 6DR, diabetic rats treated with resveratrol (n: 3); 6DRG, diabetic rats treated with combination of resveratrol with glibenclamide (n: 3); 6DG, diabetic rats treated with glibenclamide (n: 3); 6DB, diabetic rats treated with berberine; 6DBG, diabetic rats treated with combination of berberine with glibenclamide (n: 3). The blots were shown at the top and the quantified ratios were shown at the bottom. Results were expressed as mean $\pm$ standard error. ^a $p < 0.05$; different from 6C, ^b $p < 0.05$; different from 6D.

4.2 Discussions

The present study revealed the effect of resveratrol, berberine and glibenclamide combination on I/R-induced arrhythmias in model of short- and long-term experimental diabetes and the role of K_{ATP} channels in these arrhythmias. The major findings of this study are: (i) the combination of resveratrol with glibenclamide and berberine alone decreased the blood glucose levels in both short- and long-term diabetes, (ii) heart rate decreased during ischemia and reperfusion in both long- and short-term diabetes, (iii) the combination of resveratrol with glibenclamide increased basal blood pressure in long-term diabetic rats, (iv) the incidence of reperfusion induced arrhythmias reduced in short-term diabetes, (v) the incidence of other types of arrhythmias increased in diabetic rats treated with combination of berberine and glibenclamide in short-term diabetes, (vi) in long-term diabetes, resveratrol alone and its combination with glibenclamide decreased the

arrhythmias score and the incidence of reperfusion induced arrhythmias, (vii) protein expression levels of Kir 6.2 subunits between non-diabetic rats and diabetic rats did not change in short-term diabetes (xi) protein expression level of Kir 6.2 subunit decreased in non-treated diabetic rats, and all drugs treatment increased protein expression levels of Kir 6.2 subunit in long-term diabetes.

4.2.1 Blood Glucose and Serum Insulin Levels

In this study, surgical operations were performed in experimental animals at the end of 2 and 6 weeks after STZ administration. During these periods, the drugs were applied daily. Diabetic rats had general characteristics of diabetes mellitus, including polyuria, increased blood glucose levels and body weight loss. In this case, STZ destroy or damage β cells of pancreas, which ultimately lead to decrease in insulin secretion or increase in blood glucose levels (Szkudelski, 2001). In both short- and long-term diabetes, blood glucose levels in non-treated diabetic rats were significantly higher than the control rats. It was consistent with the previous studies (Tosaki et al., 1996; Ravingerova et al., 2003a; Howarth et al., 2007). Despite a marked hyperglycemia, serum insulin levels between non-treated diabetic rats and control rats did not change, which is similar to the results of previous studies (Junod et al., 1969; Ar'Rajab and Ahrén, 1993; Pushparaj et al., 2007). It was suggested that no change in insulin levels despite high blood glucose levels might be explained by a certain degree of insulin insensitivity (Ar'Rajab and Ahrén, 1993).

Glibenclamide is mostly used as an oral hypoglycemic agent, which stimulates insulin secretion and reduces hepatic glucose production (Tayek, 1995). In addition, glibenclamide shows its hypoglycemic effects via increasing peripheral glucose uptake and decrease production of endogenous glucose production rather than stimulating insulin secretion (Overkamp et al., 2002). In short-term diabetes, glibenclamide treatment caused blood glucose levels to be lower than the non-treated diabetic rats. The hypoglycemic effect of glibenclamide in this study may be due to increased insulin sensitivity and/or increase in glucose transport and in glycolysis activity (Zueva et al., 2003). On the other hand, hypoglycemic effect of glibenclamide was not observed in long-term diabetes. There have been

controversies about the effect of glibenclamide on blood glucose levels. In previous studies, glibenclamide treatment did not have any effect on blood glucose levels (Tosaki et al., 1995; Takahashi et al., 2003; Cai et al., 2014) or decreased blood glucose levels in STZ-induced diabetic rats (Eliza et al., 2009; Erejuwa et al., 2011a; Erejuwa et al., 2011b; Akbar et al., 2012; Sokolovska et al., 2012). Oral administration of glibenclamide (5 mg/kg, 14 day) did not change blood glucose in diabetic mice (Cai et al., 2014); however, our results showed that glibenclamide treatment (5 mg/kg, ip, 14 days) resulted in decrease blood glucose levels in diabetic rats. In long-term diabetes mellitus, hypoglycemic effect of glibenclamide was not obtained, which is contrary to a previous study in that administration of glibenclamide (2 mg/kg) for 6 weeks resulted in decreased blood glucose levels in diabetic rats via increasing insulin secretion (Sokolovska et al., 2012). In both long- and short-term diabetes, glibenclamide treatment did not have a significant effect on serum insulin levels that rules out the possibility of glibenclamide having hypoglycemic effect by other than the increasing insulin secretion from pancreatic β cell. Moreover, the effect of glibenclamide on blood glucose levels in diabetic rats may change according to the duration of diabetes. The reasons of such different effect: as the duration of diabetes prolongs, cellular damage and oxidative stress in organs, especially in β cell of pancreases increases. Due to these damage, glibenclamide will not to be effective on β cells to increase insulin release so that this underlies why the insulin level do not change in response to glibenclamide in short- and long-term diabetes. If there is effectiveness of glibenclamide on serum insulin level, that means damage produced on the pancreatic β cell is less.

Berberine has been extensively used in the treatment of diabetes mellitus in China for more than 1400 years (Zhou et al., 2009). In addition, experimental studies have revealed that berberine has the hypoglycemic effect. For examples, berberine administration to diabetic animals induced by STZ and alloxan resulted in decreased blood glucose levels via increasing peripheral glucose utilization, increasing number of islet cell and insulin levels (Tang et al., 2006; Chueh and Lin, 2011; Chueh and Lin, 2012). The present study showed that treatment of diabetic rats with berberine in both short- and long-term diabetes mellitus resulted in decreased blood glucose levels, which is consistent with mentioned previous studies. However, serum insulin levels in diabetic rats treated with berberine in short- and long-term diabetes did not

change. This may be caused by berberine treatment resulted in decreased blood glucose levels via increasing peripheral glucose utilization.

Numerous studies revealed that resveratrol had blood glucose lowering effect in diabetic rats with different mechanisms, including increased glucose uptake, increased blood insulin levels and increased insulin sensitivity (Su et al., 2006; Chi et al., 2007; Mohamad Shahi et al., 2011; Szkudelski and Szkudelska, 2011; Ku et al., 2012). Contrary to investigations showing the anti-diabetic effect of resveratrol, it was also stated that resveratrol treatment failed to decrease blood glucose levels in diabetic rats (Ates et al., 2007; Schmatz et al., 2009a; Schmatz et al., 2009b). Similarly, in the present study, administration of resveratrol to diabetic rats in short-term diabetes did not change blood glucose; however, treatment of diabetic rats with resveratrol for 6 weeks exerted hypoglycemic effect. Despite the hypoglycemic effect of resveratrol was seen in our study, blood glucose levels did not decrease to the control levels in animals. One of previous studies that supports our results suggest that the treatment of diabetic rats with resveratrol for 6 weeks decreased glucose levels (Silan, 2008). The length of diabetes and the length of drug administration might be important in the effect of resveratrol. However, the underlying mechanism in this effect is not so clear. In addition, resveratrol treatment in long- and short-term diabetes did not change serum insulin levels. These results suggest that resveratrol may not show its hypoglycemic effect via increasing insulin secretion. Instead, resveratrol may decrease blood glucose levels in long-term diabetes by increasing glucose uptake or increasing insulin sensitivity. Interestingly, it was shown in a previous study that resveratrol treatment in STZ-nicotinamide induced diabetic rats resulted in reduced plasma insulin levels compared to non-treated diabetic rats, which suggested that metabolic effect of resveratrol do not rely on insulin secretion (Su et al., 2006). On the other hand, it was also reported in STZ-induced diabetic rats that resveratrol administration did not change serum insulin levels (Chi et al., 2007), which is consistent with the result of present study.

The combination of resveratrol with glibenclamide in short-term diabetes was effective in decreasing blood glucose levels although resveratrol treatment alone did not have significant effect on blood glucose levels. In addition, glibenclamide treatment alone decreased the blood glucose levels in short-term diabetes. Depending

on these results, it may be stated that the combination treatment could not generate additive hypoglycemic effects. It was also interesting that combination of berberine and glibenclamide treatment neither increase nor decrease blood glucose levels in diabetic rats although berberine and glibenclamide alone decreased the blood glucose levels in short-term diabetes. It may be stated that berberine and glibenclamide may act as an antagonist for each other because combination treatment disappeared the hypoglycemic effect of each drugs. However, exact reason why the combination treatment was ineffective in decreasing blood glucose levels in short-term diabetes is unclear. In long-term diabetes, combination treatment decreased the blood glucose level whereas glibenclamide treatment alone was not effective on blood glucose level. Further investigations are needed to clarify the effect of combination treatment and drug interaction on blood glucose levels. In both short- and long-term diabetes, serum insulin levels did not also change in diabetic rats treated with the combination treatment, which might indicate that the effect of drug interactions may not be related with insulin secretion.

4.2.2 Body Weight

In the present study, body weight loss occurred as a classical symptom of diabetes as like previous studies although high food and water intake were seen in experimental diabetes mellitus induced by STZ in both short-term and long-term diabetes (Ito et al., 2006; Howarth et al., 2008; Ozturk et al., 2013). Under hyperglycemia, glucose release from liver increases and glucose utilization in peripheral tissues decreases, thereby protein breakdown and lipolysis increases, which resulted in decrease body weight in diabetes mellitus.

In short-term diabetes mellitus, resveratrol treatment did not restore body weight. One study revealed that there was no significant difference in body weight between diabetic rats treated with resveratrol for 3 weeks and non-treated diabetic rats (Yazgan et al., 2015). This study confirms our results. In long-term diabetes mellitus, treatment of diabetic rats with resveratrol attenuated body weight loss. The result of present study is compatible with most of previous studies (Silan, 2008; Soufi et al., 2012; Carolo dos Santos et al., 2014; Gencoglu et al., 2015). It may be

suggested that energy metabolisms such as sugar and lipid metabolism in diabetic rats treated with resveratrol increases (Su et al., 2006). As mentioned previously, resveratrol did not change blood glucose levels in short-term diabetes; however, it decreased blood glucose levels in long-term diabetes, which was compatible with the effect of resveratrol on body weight.

Glibenclamide treatment did not significantly improve body weight compared to non-treated diabetic rats in short-term diabetes. In a previous study (Erejuwa et al., 2011a), treatment of diabetic rats with glibenclamide orally once a day for 4 weeks did not cause significant change in body weight. In addition, STZ-induced diabetic mice treated with glibenclamide for 2 weeks were not significantly different in body weight compared to non-treated diabetic mice (Cai et al., 2014). The results in mentioned studies are compatible with the results of present study. However, long-term treatment of diabetic rats with glibenclamide caused body weight to be higher than non-treated diabetic rats. It can be proposed that the effect of glibenclamide might be independent of glucose metabolism because glibenclamide decreased blood glucose levels without effecting body weight in short-term diabetes; however, it decreased blood glucose level with a higher body weight in long-term diabetes.

In short-term diabetes mellitus, berberine administration did not change body weight in compared to non-treated diabetic rats. The similar phenomenon was observed in an experimental study performed by Chen et al. (2011). On the other hand, chronic treatment of diabetic rats with berberine had higher body weight than non-treated diabetic rats, which is well in accordance with previous studies (Liu et al., 2013; Moghaddam et al., 2013; Ma et al., 2016). In short-term diabetes, the combination of glibenclamide with resveratrol or berberine resulted in higher body weight than the non-treated diabetic rats although resveratrol and berberine alone did not have significant effect on body weight. How drug combinations improved body weight in diabetic rats needs further investigation.

4.2.3 Myocardial K_{ATP}

Although clinical data indicated that diabetic heart was more susceptible to I/R injury, experimental studies have still shown inconsistent results. Several mechanisms have been proposed regarding the increased resistance of diabetic heart to I/R injury, including increased myocardial HSP90 (Chen et al., 2006), increased myocardial HSP27 (Chen et al., 2005; Gawłowski et al., 2009), reduced Ca²⁺ overload (Lee et al., 1992), enhanced NO formation (Adela et al., 2015), reduced oxidative stress (Ravingerova et al., 2000), activation of PKC (Ooie et al., 2003), increased osmolarity (Chen et al., 2006), increased phosphorylation of ERK (Xu et al., 2004). It was proposed that as the duration of diabetes prolong, such protective mechanisms disappear and susceptibility of diabetic heart to I/R injury increases (Balakumar and Sharma, 2012). However, the role of myocardial K_{ATP} channel in diabetic heart depending on diabetic duration is not exactly clarified.

In the present study, it was shown that protein expression levels of Kir 6.2 subunit did not change in short-term diabetic hearts when compared to non-diabetic hearts. It was reported that mRNA expression levels of SUR1, SUR2 and Kir 6.2 in STZ-induced diabetic rat hearts for 2 weeks did not differ from that in non-diabetic rats heart (Li et al., 2000), which supports the result of the present study. However, it should be said that changes in the expression at mRNA levels may not always mean changes in protein expression levels of subunits. On the other hand, expression of Kir 6.2 subunit in long-term diabetic heart significantly reduced in compared to non-diabetic heart. The similar phenomenon was observed in an experimental study performed by Chen et al. (2012). They found that both mRNA and protein expression of cardiac K_{ATP} channel decreased in 8 weeks diabetic rat heart. In addition, it was shown in another experimental study that diabetes mellitus reduced the expression of mitoK_{ATP} channel (Fancher et al., 2013). In the present, SarcK_{ATP} and mitoK_{ATP} were not separated by western blotting analysis that were performed for the whole protein of left ventricle. Therefore, future studies may be performed for evaluating channel protein in both mitochondria and sarcolemma.

Present study is the first study that shows the effect of resveratrol on myocardial K_{ATP} channels in diabetic rat hearts. In long-term diabetes mellitus,

resveratrol treatment resulted in increased protein expression of Kir 6.2 subunits. In a previous study, resveratrol administration just 60 min before ischemia induced a significant increase in expression of Kir 6.2 subunit in healthy mice, which indicate that Kir 6.2 subunit is responsible for cardioprotective effect of resveratrol (Du et al., 2014). In mentioned study, resveratrol administration increases Kir 6.2-AMPK association in healthy animals. In addition, AMPK activation directly regulated by cardiac K_{ATP} channels in non-diabetic rats (Yoshida et al., 2012). Therefore, it should be performed in further investigation to clarify the role of AMPK and its association with myocardial K_{ATP} channel in diabetic rat heart. By this way, it would be clear which signaling pathways is closely related with the expression of myocardial K_{ATP} channel in diabetic heart, and thereby pathway for the effect of resveratrol on the channel.

Up to now, no study could be found in literature related with the effect of berberine on the expression of myocardial K_{ATP} channel in diabetic rats. Interestingly, berberine treatment in short-term diabetes did not significantly change protein expression levels of Kir 6.2 subunit; however, in long-term diabetes, it increased protein expression levels of Kir 6.2 subunit. The reason why berberine treatment has different effect on the channel subunit expression depending on the duration of diabetes is unknown. On the other hand, previous studies showed that berberine might act as a blocker of myocardial K_{ATP} channel in healthy rats (Wang et al., 1996) and increased the suppressed transient outward K^+ current and inward Ca^{2+} current in the diabetic rats (Wang et al., 2012). How berberine increased the channel subunit expression needs further investigation.

No published data have been found previously regarding the effect of long-term glibenclamide treatment increased protein expression of Kir 6.2 subunits in healthy and diabetic heart. Glibenclamide blocks myocardial K_{ATP} channel by binding to SUR subunits. In short-term diabetes mellitus, glibenclamide did not change protein expression of Kir 6.2 subunits in left ventricle of diabetic heart. It was consistent with a previous study in which mRNA expression of SUR 1, SUR 2 and Kir 6.2 were not different between glibenclamide treated diabetic heart and non-treated diabetic heart for 2 weeks (Li et al., 2000). In addition, glibenclamide combination with resveratrol and berberine in long-term diabetes resulted in

increased protein expression of Kir 6.2 subunits. However, the agonist effect of the drug with each other did not observed in the combination treatment. It is unknown how glibenclamide increased expression of Kir 6.2 subunits in long-term diabetic hearts. However, it is known that different variations of subunits represent different physiological and pharmacological properties of sarcK_{ATP} channels in different organs, tissues or cells. SUR2A/Kir 6.2 combinations forms the cardiac sarcK_{ATP} channels in ventricular cell (Inagaki et al., 1996). In addition, protein expression level of channel subunits can be changed depending on the duration of I/R (Akao et al., 1997), which might be a clue for that acute and chronic application might alter the channel function and variations of subunits. In the present study, left ventricle of diabetic heart was used for evaluating protein expression of K_{ATP} channels. SUR1/Kir 6.2 variations are present in atrial cell (Flagg et al., 2008); however, it was surprising that changes in protein expression of SUR1 in left ventricles of diabetic hearts were observed (data not shown). It might be said that long-term binding of glibenclamide to SUR subunits could change physiological and pharmacological properties of the channel. It was reported that STZ-induced diabetes decreased the sensitivity of myocardial K_{ATP} channel to glibenclamide administration at transcription levels (Li et al., 2000). SUR2A, a regulatory subunit of myocardial K_{ATP} channel, is suggested to have importance for cardioprotection against myocardial I/R injury. Increase in SUR2A protein levels can cause increase in the number of myocardial K_{ATP} channel, indicating earlier activation of the channel (Sukhodub et al., 2010; Jovanović et al., 2013). The combination of Kir 6.2/SUR has reported to have ~50–80 pS signal channel conductance by inhibition of micro molar ATP present; however, the combination of Kir 6.1 has reported to have smaller conductance (~30 pS) than Kir 6.2 (Flagg et al., 2010). K_{ATP} channel activity can change due to the change in the subunit of K_{ATP} channel protein. For example, one of recent study showed that the potency of Na⁺-azide at 3 mmol/L which is caused energy depletion in the cell, activates SUR/Kir 6.x channels in the following order: SUR1/Kir 6.2 > SUR2B/Kir 6.2 > SUR1/Kir 6.1 > SUR2B/Kir 6.1 > SUR2A/Kir 6.2 > SUR2A/Kir 6.1 (Li et al., 2016). It means that the sensitivity of SUR subunit is different from each other as following order SUR1 > SUR2B > SUR2A. Therefore, combination of channel subunits may be more important than only one subunit alteration. In addition, the responding of channel combination is ranked as SUR1/Kir 6.2 or SUR1/Kir 6.1 > SUR2B/Kir 6.2 or SUR2B/Kir 6.1 >

SUR2A/Kir 6.2 under cellular metabolic disorders. It was suggested the Kir 6.2/SUR1 combination is the most sensitive K_{ATP} combination, while Kir 6.1/SUR2A is insensitive to cellular metabolic disorder. Surprisingly Kir 6.2/SUR2A combination has just slightly sensitive to cellular metabolic disorders. Moreover, Kir 6.2 has intrinsic sensitivity against cellular metabolic disorders (Li et al., 2016). Therefore, it should be investigated the changes in protein and mRNA level of both Kir 6.x and Sur subunit of the channel and the reasons of different variations of subunits in treated and non-treated diabetic rats hearts in further studies.

4.2.4 Serum SOD and LDH levels

Reactive oxygen species play a vital role in the pathogenesis of diabetes related complications, including cardiovascular diseases (Maritim et al., 2003; Valko et al., 2007). SODs are the major antioxidant defense system against superoxide anion (O_2^-) and it catalyze the reaction in which superoxide anion is converted to hydrogen peroxide and oxygen (Fukai and Fukai, 2011). An increasing in free radical production or a decrease in endogenous antioxidant defenses induces oxidative stress (Rochette et al., 2014). Increase in SOD activity is a triggering activity for its ability to scavenge ROS and gives additional protective effect against oxidative stress and preventing cellular damage.

The activity of SOD in diabetes mellitus is variable depending on gender, animal species, the type of tissue and the duration of diabetes (Maritim et al., 2003). For example, SOD levels in liver decreased 3 or 4 weeks after STZ administration (Maritim et al., 2003). In addition, serum SOD levels were lower in STZ-induced diabetic rats (Liu et al., 2008; Sen et al., 2011; Xie et al., 2013). In the present study, serum SOD levels in STZ-induced diabetic rat were not changed in long- and short-term diabetes, which is consistent with the result of a previous study in which SOD activity did not significantly change between control and 3 weeks diabetic rats (Abdul Sani et al., 2014). In short- and long-term diabetes, resveratrol administration did not increase or decrease serum SOD levels. In contrast, several studies indicated that resveratrol treatment increased SOD levels (Li et al., 2012). It was also interesting that combination of resveratrol with glibenclamide decreased serum SOD

levels in long-term diabetes; however, it did not change in short-term diabetes. The effect of combination of resveratrol and glibenclamide treatment on the serum SOD levels could not be explained with the data of present study. It may be suggested that decreased SOD levels in the diabetic rats treated with resveratrol and its combination with glibenclamide may be due to the highest consumption of SOD in the convention process of O_2^- to H_2O_2 . In other words, as the activity of this enzyme increases, the enzyme may become depleted. In a previous study, it was indicated that an increase in the production of H_2O_2 caused the reduction of SOD (Bray et al., 1974). In this sense, other antioxidant enzymes such catalase and its relation with SOD levels may be important because SOD catalase converts H_2O_2 to O_2 and H_2O . It means that a decrease in the activity of catalase may result in an increase in the production of H_2O_2 , thereby decreasing the amount of SOD levels. That is why, the total antioxidant capacity or other antioxidant enzymes in the effect of resveratrol and glibenclamide combination should be studied. Another possible explanation may be related with the free radical scavenging effect of resveratrol and glibenclamide combinations. Antioxidant molecules removes free radicals, act as radical scavenger (Lobo et al., 2010). Therefore, resveratrol and its combination of glibenclamide may remove non-enzymatically free radicals in environment; by this way, SOD levels may decrease.

Glibenclamide increased SOD levels in STZ-induced diabetic rats after 4 weeks treatment (Bukan et al., 2004). In addition, glibenclamide administration increased serum SOD levels in alloxan-induced diabetic rats (Obi et al., 2016). Contrary, present study indicated that glibenclamide did not change serum SOD levels in both short- and long-term diabetes. Similarly, berberine treatment alone and in combination with glibenclamide did not have any effect on serum SOD levels. To date, no study examining serum SOD levels in diabetic rats treated with the combination of berberine and resveratrol with glibenclamide has been reported. Therefore, we were not able to compare our results with others.

LDH catalyzes the conversion of pyruvate to lactate, and then subsequently converted to glucose in gluconeogenic pathway. LDH is released during tissue damage; it can be used as a marker of common injuries and disease. The serum activity of marker enzymes, especially LDH which are distributed throughout the

body and have isoenzymes that are recognized as markers for lesion in liver and heart muscle. For examples, diabetes mellitus resulted in increased LDH activity. In addition, LDH activities increased in kidney of STZ-induced diabetic rats (Rajeswarareddy et al., 2012) and serum sample of diabetic rats (Adaramoye, 2012), which are disagreement with the result of the present study that there was no significant change in LDH levels after diabetes in rats. Glibenclamide treatment did not have effect on serum LDH levels in both short- and long-term diabetes. This is in agreement with a previous experimental study (Adaramoye, 2012). Resveratrol reduces increased serum LDH levels in exercised healthy rats, which have been shown before (Xiao, 2015) and also in cell culture of cardiac cell under hyperglycemic condition. It was shown that resveratrol administration preserved increased LDH levels (Uma and Sreepriya, 2016). In contrast, resveratrol administration alone in short- and long-term diabetes did not change serum LDH levels in the present study.

Berberine pretreatment decreased an increase in serum LDH levels after I/R in rats and cell culture (Wnag et al., 2015; Zhao et al., 2016). In our present study, it was shown that berberine administration did not change serum LDH levels in short- and long-term administration in diabetic rats. Unfortunately, there is no study about the effect of berberine on serum LDH levels in STZ-induced diabetic rats. In a previous study, it was indicated that berberine increased LDH levels in the kidney of diabetic rats (Punitha et al., 2005). It was interesting that serum LDH levels increased in short-term diabetic rats treated with the combination of berberine and glibenclamide although the serum LDH levels did not change in short-term diabetic control rats.

In the present study, serum samples were preferred to evaluate systemic effect of STZ-induced diabetic rats on serum SOD and LDH levels without I/R injury. However, the result of present study indicated that hyperglycemia did not affect the serum SOD and LDH levels. It should be stated that antioxidant and oxidant activity might be investigated in tissue specific manner such as in heart and pancreas.

4.2.5 Heart Rate and Blood Pressure

In short-term diabetes, basal heart rate and mean arterial blood pressure were not different between control and non-treated diabetic rats. However, in long-term diabetes, basal heart rate was significantly lower in non-treated diabetic rats, which is consisted with previous studies (Zhang et al., 2002; Ravingerova et al., 2003a; Howarth et al., 2009). Following ischemia and reperfusion, heart rate decreased in both short- and long-term non-treated diabetic rats compared to non-diabetic control rats. It can be suggested that reduction in heart rate due to the prolongation of diabetes may be related to development of cardiomyopathy (Ravingerova et al., 2001). In addition, decline in heart rate in STZ-induced diabetic rats may be caused by the alternations in autonomic nervous system, including increased vagal activities or decreased sympathetic activities (Hicks et al., 1998).

Resveratrol treatment did not have any effect on the heart rate and mean arterial blood pressure recorded during ischemia and reperfusion in short-term diabetes. In an experimental study, resveratrol treatment for 3 weeks resulted in increased mean arterial blood pressure without pronounced effect on heart rate in STZ-induced diabetic rats (Huang et al., 2010). Conversely, in another study it was shown that 2 weeks resveratrol treatment improved heart rate both at the baseline and at the end of reperfusion (Thirunavukkarasu et al., 2007). In the present study, long-term resveratrol administration in diabetic rats resulted in an increased heart rate and mean arterial blood pressure during ischemic period. As well, mean arterial pressure during reperfusion increased in diabetic rats receiving resveratrol; however, heart rate did not change. The mechanism of hemodynamic effect of resveratrol could not clearly be explained with the result of present study. A previous study showed that resveratrol prevents increased heart rate and contractile response by attenuating sympathetic nervous system in spontaneous type 2 diabetes (Mozafari et al., 2015). In contrast, it was indicated that resveratrol treatment did not affect the autonomic nervous system, and therefore heart rate in healthy mice (Mayers et al., 2008). However, the result of present study indicates that long-term administration of resveratrol improves decreased heart rate and blood pressure in STZ-induced diabetic rats.

Glibenclamide treatment did not change basal blood pressure and heart rate in both short- and long-term diabetes mellitus. This is similar to the results of a previous study (Akbar et al., 2012) in that glibenclamide administration to diabetic rats did not have a significant effect on basal mean arterial blood pressure in different period of diabetes. On the other hand, glibenclamide significantly increased mean arterial blood pressure and heart rate at the end of ischemia in short-term diabetes. In long-term diabetes, glibenclamide non-significantly increased mean arterial blood pressure and heart rate during ischemia. During reperfusion, glibenclamide treatment significantly increased heart rate in short-term diabetes and non-significantly increased mean arterial blood pressure and heart rate in long- and short-term diabetes. In a previous study, acute administration of glibenclamide to pancreatectomized dogs resulted in decreased heart rate and blood pressure (Pogfitsa and Dubecz, 1977), which is contradictory to the result of this study. Perhaps, this may be explained by different animal species and different model of diabetes mellitus.

Berberine treatment did not show significant effect on basal heart rate and mean arterial blood pressure in short-term and long-term diabetes. This is consistent with the previous study that oral administration of berberine to diabetic rats for 7 days did not affect the heart rate (Wang et al., 2012). On the other hand, berberine resulted in an increased mean arterial blood pressure and heart rate following ischemia and reperfusion in both short- and long-term diabetes mellitus. In a previous study that is performed in diabetic rats which low dose STZ used to produce diabetes, berberine treatment orally did not change heart rate during ischemia and reperfusion, which is inconsistent with the present study. The discrepancy may be related to the differences in the model of diabetes and/or dosage of berberine. Both in clinical and experimental studies, it has been shown that berberine could reduce blood pressure in type 2 diabetic and healthy subject (Ko and Lim, 1980; Pérez-Rubio et al., 2013; Lan et al., 2015). Contrary, the opposite observation was obtained in the present study. The discrepancy in this different response could not be explained. Berberine administration may block α_1 receptor of vascular smooth muscle and increase the effect of acetylcholine effect and increases NO release by which it can show its blood pressure lowering effect (Xia and Luo, 2015). In this study, the mechanisms by berberine show its effect on hemodynamic parameters

were not investigated. As well, in literature, there were no direct study by which the effects of berberine on mean arterial blood pressure and heart rate in STZ-induced diabetic rats were evaluated. Taken together, it should be stated that further studies are needed to investigate the mechanisms by which berberine increased mean arterial blood pressure and heart rate during ischemia and reperfusion in both short- and long-term diabetes.

4.2.6 Ischemia and Reperfusion-Induced Arrhythmias

Little information is present regarding the impact of diabetes mellitus on myocardial K_{ATP} channel and its relationship with arrhythmias induced by I/R. Kir 6.2 subunit is essential for cellular adaptation under stress condition and knockout of Kir 6.2 resulted in the disruption of membrane excitability and calcium handling, which could induce lethal arrhythmias (Zingman et al., 2002). In addition, increased expression of myocardial K_{ATP} channel resulted in less myocardial oxygen consumption and rapid shorting of APD; however, 80% reduction of the channel expression caused opposite effect (Zingman et al., 2011). Taken together, it could be stated that increased susceptibility of diabetic heart to I/R injury in long-term diabetes mellitus may be due to the reduction in protein expression levels of Kir 6.2 subunit. In short term diabetes mellitus, the arrhythmic period, the duration of arrhythmias, the incidence of VF, VT, other types of arrhythmia and the arrhythmia score during ischemia did not show significant differences between non-treated diabetic heart and control heart. These results are compatible with previous studies (Kusama et al., 1992; Adameova et al., 2007a). Kusama et al (1992) indicated that 3 weeks diabetes did not result in a significant change in the incidence of arrhythmias induced by 5 min ischemia *in vivo*. Similarly, a previous study in an *in vivo* model of I/R injury reported that the incidence of VF and VT, the arrhythmia score and the duration of arrhythmias induced by 30 min ischemia were not different between one week diabetic hearts and control hearts (Ravingerova et al., 2003a). However, it should be also mentioned that in isolated working rat heart, total number of arrhythmias induced by ischemia was lower in 2 weeks diabetic rats (Zhang et al., 2002). In addition, similar studies revealed that the incidence and the duration of arrhythmias induced by 30 min of ischemia in one week diabetic heart was found to

be lower (Ravingerova et al., 2000; Matejškova et al., 2008). The results of mentioned investigations are inconsistent with the result of present study. The different results may be due to the different experimental model of I/R injury. In the present study, short-term diabetes did not affect the arrhythmic period and the duration of arrhythmias induced by reperfusion. However, arrhythmia score during reperfusion was found to be lower in non-treated diabetic rats compared to control rats. Although the incidence of VT and VF in diabetic rats did not differ from control rats, the incidence of other types of arrhythmias was lower. The results are inconsistent with previous studies (Tosaki et al., 1996; Zhang et al., 2002; Huang et al., 2010; Bozdogan et al., 2016). The incidence of VF induced by reperfusion was shown to be lower in 2 weeks diabetic rat heart (Zhang et al., 2002). In an another *in vitro* study, it was indicated that the incidence of VF and VT was lower in 2 weeks diabetic rat heart (Tosaki et al., 1996), which is in agreement with study performed by Zhang et al. (2002). These data are not in accordance with the result of present study. In the mentioned above studies, several different factors such as I/R protocol, the duration of diabetes etc. might be involve in different results; hence, it is difficult to explain exact reasons for the discrepancy in the sensitivity of diabetic heart to I/R injury. The results of present study indicated that myocardial K_{ATP} channels may not be a major factor for the decrease in the incidence of other types of arrhythmias induced by reperfusion because protein expression level of Kir 6.2 subunits were not different in diabetic rats from non-diabetic rats. It should be stated that diabetes mellitus causes a variety of changes in ion channels and transport mechanisms depending on the duration of diabetes. For example, Ca^{2+} overload in diabetic myocardium during reperfusion is less than the non-diabetic myocardium that indicates increased resistance to reperfusion-induced arrhythmias (Ziegelhöffner et al., 1999). Therefore, ion channels such as L-type Ca^{2+} channels, Na^+/Ca^{2+} exchanger may play important role in the resistance to arrhythmias in short-term diabetic heart (Takeda et al., 1996; Ganguly et al., 1983; Heyliger et al., 1987; Makino et al., 1987; Golfman et al., 1998).

Arrhythmogenesis starts usually around 4th or 5th min of ischemia in open-chest rat model, and it lasts about 10 min. In our model, 6 min ischemia was induced; hence, the incidence of arrhythmia was low in all groups. Duration of preceding ischemia before reperfusion is one of the most important factors for determining the

severity of reperfusion induced arrhythmias (Manning and Hearse, 1984). Short 5-6 min ischemic periods resulted in high incidence of reperfusion arrhythmias (Kane et al., 1984). Therefore, the period of ischemia was referred to evaluate reperfusion-induced arrhythmias in this study. In an *in vivo* model of I/R injury, heart rate is an important factor to determine severity and the incidence of reperfusion arrhythmias. For example, rapid heart rate triggers more arrhythmias during reperfusion, especially VF occurring immediately following reperfusion while slow heart rate suppresses the arrhythmia (Murdock et al., 1980; Bernier et al., 1989; Ng et al., 2013). Our study showed that heart rate in short-term diabetes was lower than the control heart. It is possible that reduced heart rate could contribute the reduction in the incidence of other types of arrhythmias and the arrhythmias score in reperfusion. However, it was also observed in the present study that although the heart rate in long-term diabetes was significantly lower in diabetic rat heart than control rats, the arrhythmias during ischemia and reperfusion did not decrease. Therefore, it might be said that decreased susceptibility of short-term diabetic heart to reperfusion-induced arrhythmias was not primarily due to reduced heart rate.

Glibenclamide has been used as an oral hypoglycemic agent in diabetic patients for many years. However, the safety of glibenclamide on cardiovascular function are debated. Clinical studies showed that glibenclamide increased mortality and morbidity after myocardial infarction in addition to increased possibility of malignant arrhythmias and ischemic area (Xianghua et al., 2010; Jørgensen et al., 2011). In addition, experimental study shows that there is still inconsistency in the effect of glibenclamide on the arrhythmias in diabetic hearts. For examples, in langendorff-perfused heart, glibenclamide administration to diabetic rats 10 min before ischemia and reperfusion reduced the incidence of reperfusion-induced arrhythmias (Tosaki et al., 1996). Conversely, glibenclamide (0.1 mg/kg) administration to diabetic rats three times a week for 8 weeks in duration increased the incidence of reperfusion induced VT rather than VF (Takahashi et al., 2003). In the present study, glibenclamide treatment did not change the incidence of arrhythmias induced by ischemia and reperfusion and the arrhythmias score in both short- and long-term diabetes, which is inconsistent with the above mentioned published *in vitro* studies. The reasons for inconsistent results may include

differences in the dose of the drug, application period, and the duration of diabetes and the model of I/R.

Anti-arrhythmic effects of resveratrol in healthy heart is well documented (Hung et al., 2004; Kaga et al., 2005; Burstein et al., 2007; Raj et al., 2014). However, limited studies are present regarding its anti-arrhythmic effect in diabetic heart. A previous study reported that administration of resveratrol to 2 weeks diabetic rats once a day for five consecutive days did not alter infarct size, the incidence of VPC, the duration of VF and VT in one hour ischemia (Huang et al., 2010). This study is compatible with the results of present study in which short- and long-term administration of resveratrol to diabetic rats did not change the duration of arrhythmia, the arrhythmia score and the incidence of arrhythmias during ischemic period. Otherwise, it is found that in an *in vitro* study, the administration of resveratrol to 2 weeks diabetic rats for 15 days resulted in decreased infarct size and cardiomyocytes apoptosis (Thirunavukkarasu et al., 2007). However, the arrhythmias were not evaluated in the mentioned study even though resveratrol decreased infarct size in diabetic rats. Considering reperfusion-induced arrhythmias, resveratrol treatment failed to improve arrhythmogenesis in short-term diabetes; however, it reduced arrhythmias score and the incidences of other types of arrhythmias induced by reperfusion in long-term diabetes. The reason for the ineffectiveness of resveratrol in short-term diabetes may be related with the lower incidence of arrhythmias and arrhythmias score in short-term diabetes in respect to control rats. It could be said that the protective effect of resveratrol against reperfusion-induced arrhythmias in long-term diabetes might not be only related with myocardial K_{ATP} channel because resveratrol, berberine and their combination with glibenclamide in long-term diabetes, all increased protein expression levels of Kir 6.2 subunits; however, glibenclamide and berberine did not have anti-arrhythmic effect. In this case, it might be thought that other mechanisms should be involved in the protective effect of resveratrol. For examples, it was shown in healthy rats that resveratrol exerted anti-arrhythmic effect by inhibiting L-type calcium current or reducing intracellular Ca²⁺ overload (Chen et al., 2008). Still more studies are needed to explore the mechanisms whether resveratrol alone and in combination with glibenclamide decreased reperfusion-induced arrhythmias.

In short-term and long-term diabetes mellitus, berberine treatment did not show anti-arrhythmic effect in diabetic rats so that there was no change in the arrhythmic period, arrhythmias score and the incidence of arrhythmias during ischemia and reperfusion. It is interesting that berberine treatment resulted in increased duration of VT during reperfusion compared to non-treated diabetic rats in long-term diabetes. In addition, berberine non-significantly increased the arrhythmias score during reperfusion in long-term diabetes. This is inconsistent with a previous study that berberine treatment to STZ-induced diabetic rats decreased the arrhythmia score and the duration of arrhythmias induced by ischemia (Wang et al., 2012). It was suggested that diabetes mellitus disrupted resting membrane potential, which resulted in abnormal generation of depolarization leading to the inhibition of the conductivity and automaticity via depressing K^+ conductance. As a result, block and re-entry arrhythmias appeared. It was proposed that berberine exert protective effect against arrhythmias in type II diabetic rat via recovering depressed Kir 2.1, the main subunit of K^+ channels, and stabilizing resting membrane potential (Wang et al., 2011). In addition, in other study performed by same group (Wang et al., 2012), transient outward K^+ current, which is responsible for rapid phase 1 repolarization, and L-type Ca^{2+} current, which control the plateau phase of action potential, depressed in diabetic heart. Therefore, these alterations in ionic current resulted in a delay of electrical conduction and induced ischemic arrhythmias and berberine reduced ischemic arrhythmias via improving these channel activities. In the present study, the relation K^+ channel and L-type Ca^{2+} with the arrhythmias were not investigated, but it was found that long-term treatment of berberine to diabetic rats resulted in increased expression of Kir 6.2 subunit; however, in long-term diabetes berberine increased reperfusion-induced VT duration. The inconsistent results may be explained by the duration of diabetes, dosage of berberine and different model of I/R injury or the involvement of different effect of berberine apart from K_{ATP} channel on myocardial cell.

However, further studies are required to explain why berberine treatment increased reperfusion-induced VT duration and had no effect on ischemia-induced arrhythmias although berberine treatment preserved the depressed Kir 6.2 subunits in long-term diabetes. And may be the role of K_{ATP} channel in the production of ischemia and reperfusion-induced arrhythmia should be asked.

It was suggested that berberine might act as a blocker of myocardial K_{ATP} channels in healthy rats as like glibenclamide, and by this mechanism, it could be protective effect against ischemia-induced arrhythmias (Wang et al., 1996). And also berberine resulted in the prolongation of action potential by inhibiting delayed rectifier K^+ currents and increasing L-type Ca^{2+} currents (Wang and Zheng, 1997). In the present study, combination of berberine and glibenclamide in short-term diabetes increased the incidence of other types of arrhythmias induced by reperfusion. This may be due to prolongation of action potential and increased inward Ca^{2+} current. Unfortunately, the effect of berberine and glibenclamide on the ischemia and reperfusion-induced arrhythmias were not evaluated in long-term diabetic rats due to unexpected excessive death in this group.

5. CONCLUSIONS AND RECOMMENDATIONS

In present study, the effect of resveratrol, berberine and their combinations with glibenclamide on I/R induced arrhythmias via myocardial K_{ATP} channels was investigated in different term of STZ-induced diabetes.

The results of present study indicate that short-term diabetes increased the resistance against reperfusion-induced arrhythmias and this protection are not seen in the long-term diabetic rats. Another result of this study is that the combination of berberine with glibenclamide interestingly resulted in increases in the incidence of reperfusion-induced arrhythmias and increases in the arrhythmia score nearly to a normal level in short-term diabetes.

In the present study, our hypothesis was that increased susceptibility of long-term diabetic rats to cardiac arrhythmias might be due to depression of myocardial K_{ATP} channels. The results of present study support this hypothesis. Kir 6.2 subunit in short-term diabetes did not change; however, Kir 6.2 subunit decreased in long-term diabetes. It should be also emphasized that all drugs treatment in long-term diabetes increased expression of Kir 6.2 expression and did not have protective effect on I/R-induced arrhythmias, excepting resveratrol and its combination with glibenclamide. These results indicate that not only myocardial K_{ATP} channel but also other ion channels may be important in the protective effects of resveratrol and its combination with glibenclamide. Myocardial K_{ATP} consists of two subunits; Kir 6.x and SUR, which plays an important role in I/R-induced arrhythmias. As mentioned in discussion, variations of the channel subunits are important in the response of heart to I/R injury. Therefore, it is reasonable to suggest that all combination of K_{ATP} should be investigated by further studies. In addition, functional and pharmacological features of subunits variations in diabetic heart and its relation with I/R-induced arrhythmias should be explained. In addition, in the present study, sarc K_{ATP} and mito K_{ATP} channel were not separately studied. Therefore, there is a need to study the role of each channel individually on cardiac arrhythmias in diabetic heart and later time the relation of drugs used in this study.

In the present, the effect of diabetes on AMPK expression in heart and its relationship with cardiac arrhythmias, resveratrol and berberine could not be examined. However, it is necessary to state the importance of AMPK in diabetes-related cardiovascular complications. AMPK is important for various channel activity such as SERCA activity and SarcK_{ATP} channel is responsible for ionic regulation of cardiac excitability and also in I/R-induced arrhythmias. In diabetes mellitus, suppression of AMPK activity or increased activation of AMPK either physiologically or pharmacologically can ameliorate diabetic complications, especially cardiac dysfunction in diabetes. The effect of diabetes mellitus on AMPK activity and its associations with cardiac arrhythmias in diabetic heart should be investigated. By this way, it may be easy to explain which upstream and/or downstream pathways effect or highly related with myocardial K_{ATP} channel in diabetic heart.

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APPENDICES

6. APPENDICES

Appendix A



T.C.
ABANT İZZET BAYSAL ÜNİVERSİTESİ
Hayvan Araştırmaları Yerel Etik Kurulu

Sayı :B.30.2.ABÜ.0.05.05-050.01.04-52

10.09.2014

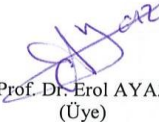
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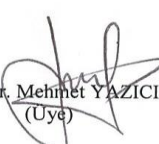
Sayın; Prof. Dr. Ömer BOZDOĞAN

A.İ.B.Ü Fen Edebiyat Fakültesi Biyoloji Bölümü Öğretim Üyesi

Hayvan Araştırmaları Yerel Etik Kurulu tarafından **2014/26 no.lu** "Kısa ve uzun dönem deneysel diyabette iskemi ve reperfüzyon aritmileri üzerine resveratrol, berberin ve glibenklamidin etkisi" isimli çalışmanız etik olarak uygun bulunmuştur.


Prof. Dr. Ayşe KÜKNER
(Başkan)


Prof. Dr. Erol AYAZ
(Üye)


Prof. Dr. Mehmet YAZICI
(Üye)

Prof. Dr. Neriman ŞENGÜL
(Üye)

Yrd. Doç. Dr. Serdar GÖZÜTOK
(Üye)

Prof. Dr. Hamit COŞKUN
(Üye)


Doç. Dr. Serkan ÇAKIR
(Üye)

Doç. Dr. Fatih DEMİRCİOĞLU
(Üye)

Yrd. Doç. Dr. Fatih ULAŞ
(Üye)

Yrd. Doç. Dr. Eray KEMAHİ
(Üye)

Yrd. Doç. Dr. Orçun TOPTAŞ
(Üye)

Av. Erol ALTINTAŞ
(Üye)

Orhan BULUT
(Üye)

Adres : Abant İzzet Baysal Üniversitesi Rektörlüğü Hayvan Araştırmaları Yerel Etik Kurulu 14280 Gölköy / BOLU
Telefon : 0374 253 45 68 Fax : 0374 253 45 59

7. CURRICULUM VITAE

Name SURNAME : Salih Tunç KAYA

Place and Date of Birth : Sivas / 10.09.1987

Universities

Bachelor's Degree : Abant Izzet Baysal University, Faculty of Arts and Science, Department of Biology

MSc Degree : Abant Izzet Baysal University, the Graduate School of Natural and Applied Science, Department of Biology

E-mail : kayasalihtunc@gmail.com

Address : Duzce University, Faculty of Arts and Sciences, Department of Biology, 81620, Duzce/ TURKEY

List of Publications :

1. Kaya ST and Bozdogan O. 2012. The effect of intermittent fasting and water restriction on myocardial ischemia/reperfusion-induced arrhythmia in rats. Turkish Journal of Medical Sciences, 42 (Sup.2): 1484-1489.
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