ABANT IZZET BAYSAL UNIVERSITY
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

THE EFFECTS OF PHYTOESTROGEN ON ISCHEMIA REPERFUSION INDUCED ARRHYTHMIAS IN OVARIECTOMIZED RATS

MASTER OF SCIENCE

FİRDEVS ERİM

BOLU, SEPTEMBER 2015

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APPROVAL OF THE THESIS

THE EFFECTS OF PHYTOESTROGEN ON ISCHEMIA REPERFUSION INDUCED ARRHYTHMIAS IN OVARIECTOMIZED RATS submitted by **FİRDEVS ERİM** in partial fulfillment of the requirements for the degree of Master of Science in **Department of Biology, Abant Izzet Baysal University** by,

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September 11, 2015

Prof. Dr. Duran KARAKAŞ ....

Director, **Graduate School of Natural and Applied Sciences**

To My Mother,
Makbule Erim

and

My Father,
İsmail Erim

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.

FİRDEVS ERİM

ABSTRACT

THE EFFECTS OF PHYTOESTROGEN ON ISCHEMIA REPERFUSION INDUCED ARRHYTHMIAS IN OVARIECTOMIZED RATS
MSC THESIS
FİRDEVS ERİM
ABANT IZZET BAYSAL UNIVERSITY GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF BIOLOGY
(SUPERVISOR: PROF. DR. ÖMER BOZDOĞAN)

BOLU, SEPTEMBER 2015

More than 50% of sudden death in human occurring due to heart attack is caused by occlusion of coronary arteries. Ventricular arrhythmias that occurs due to the myocardial ischemia is the main reason of the sudden death. In most of the experimental researches, studies focus on these lethal arrhythmias and many chemical agents have been researched for the prevention of these arrhythmias. According to the previous researches, arrhythmia generation has been observed in females less than males. This difference has been based on gender differences which results from endogenous estrogen. There are many experimental studies that show protective effects of estrogen on ischemia and reperfusion induced arrhythmias. In recent years, plant derived substances that have a similar structure and function with estrogen have been used to prevent menopausal symptom in human being. Phytoestrogens are one of the examples of these substances. They are soy-derived substances which have a similar molecular shape with the endogenous estrogen. Genistein is the most known type of phytoestrogen and it has been used in the many experimental and clinical studies. Due to its similarity with estrogen, it was aimed to be researched whether it has a protective effect on ischemia and reperfusion induced arrhythmias in this study.

Spraque-Dawley female rats weighing 225 - 290 g and in 6 - 7 months old were used. Myocardial ischemia were made by ligation of left coronary artery and reperfusion were made by releasing of this artery. Ovariectomy were made in two groups (ovx group). Genistein were given intraperitoneally in a dose of 100 µg / kg for 4 weeks in all groups except their control. Electrocardiogram (ECG) and arterial blood pressure were recorded during ischemia and reperfusion.

It was found that plasma estradiol level significantly decreased in ovx group than the other group ($p < 0,05$). In genistein-treated group, no significant changes was observed in plasma estradiol level. There were no significant effect of genistein found on the total duration of arrhythmia and the incidence of ventricular tachycardia, ventricular premature contraction or ventricular fibrillation and arrhythmia score in unovariectomized and ovariectomized rats during 6 minutes of reperfusion.

The effect of ovariectomy and genistein on the ischemia reperfusion induced arrhythmia was found similar as like previous studies. The nonsignificant different effect of genistein in the unovariectomised rats and ovariectomized rats was thought to be depend on the changes of its affinity to the estrogen receptor or the changes in the number of estrogen receptor after ovariectomy. More research is required to clarify the relation of genistein effect with the number of estrogen receptor and its affinity to the estrogen receptor.

KEYWORDS: Myocardial Ischemia, Reperfusion, Arrhythmia, Ovariectomy, Genistein

ÖZET

OVARİEKTOMİZE SIÇANLARDA BİTKİSEL ÖSTROJENİN İSKEMİ-REPERFÜZYON ARİTMİLERİ ÜZERİNE ETKİLERİ
YÜKSEK LİSANS TEZİ
FİRDEVS ERİM
ABANT İZZET BAYSAL ÜNİVERSİTESİ FEN BİLİMLERİ ENSTİTÜSÜ
BİYOLOJİ ANABİLİM DALI
(TEZ DANIŞMANI: PROF. DR. ÖMER BOZDOĞAN)

BOLU, EYLÜL - 2015

İnsanlarda ani ölümlerin % 50 den fazlası koroner damar tıkaması sonucu oluşan kalp krizinden kaynaklanmaktadır. Miyokardiyal iskemi sonucu oluşan ventriküler aritmiler ani ölümlerin ana sabebidir. Deneysel çalışmaların çoğunda bu aritmilere odaklanılmaktadır ve pek çok kimyasal ajan bu aritmilerin önlenebilmesi için araştırılmaktadır. Geçmişteki çalışmalarda, aritmi oluşumu kadınlarda erkeklere göre daha düşük görülmüştür. Bu farklılık endojen östrojenden kaynaklı cinsiyet farklılığına dayandırılmaktadır. Östrojenin iskemi ve reperfüzyon aritmileri üzerine koruyucu etkisini gösteren pek çok çalışma vardır. Son yıllarda, insanlarda menapoz sendromlarının önlenmesi için östrojenle yapısal ve fonksiyonel benzerlikleri olan bitki kaynaklı maddeler kullanılmaktadır. Fitoöstrojenler bu maddelere bir örnektir. Fitoöstrojenler soya kaynaklı, endojen östrojene benzer moleküler yapıya sahip maddelerdir. Deneysel ve klinik çalışmalarda kullanılan genistein fitoöstrojenlerin en bilinen örneğidir. Bu çalışmada da östrojenle benzerliğinden dolayı genisteinin iskemi ve reperfüzyon sonucu oluşan aritmilere karşı koruyucu etkisinin olup olmadığının araştırılması amaçlanmıştır.

Bu çalışmada, 225 - 290 gram ağırlığında 6 - 7 aylık Spraque-Dawley cinsi dişi sıçanlar kullanılmıştır. Koroner ligasyon, sol koroner arterin tıkanmasıyla oluşturulmuş ve bu damarın gevşetilmesiyle de reperfüzyon yapılmıştır. İki grupta ovariektomi (ovx grubu) yapılmıştır. Genistein 100 µg / kg dozda intraperitoneal olarak 4 hafta uygulanmıştır. Koroner ligasyon ve reperfüzyon süresince elektrokardiyogram (EKG) ve arteriyel kan basıncı kaydedilmiştir.

Plasma östrojen seviyesi ovx grubunda diğer gruplara göre istatistiksel olarak anlamlı şekilde düşük görülmüştür ($p < 0,05$). Genistein uygulanan gruplarda östrojen seviyesinde anlamlı bir farklılık görülmemiştir. Reperfüzyon boyunca genisteinin, toplam aritmi süresi, aritmi skoru ve ventriküler taşikardi, ventriküler prematüre atım, ventriküler fibrilasyon oluşum sıklığına anlamlı bir etkisi görülmemiştir.

Ovariektominin ve genisteinin iskemi ve reperfüzyon sonucu oluşan aritmiler üzerine etkisi, önceki çalışmalardakine benzer bulunmuştur. Genisteinin ovariektomili ve ovariektomi yapılmayan hayvanlar üzerindeki istatiksel olmasada farklı etkisi, östrojen reseptörlerine karşı farklı yatkınlık göstermesinden

yada ovariektomi sonrası östrojen reseptörlerinin sayısının değişmesinden kaynaklanabilir. Genisteinin östrojen reseptör sayısına etkisi ve östrojen reseptörlerine olan ilgisinin belirlenebilmesi için daha fazla çalışmaya ihtiyaç bulunmaktadır.

ANAHTAR KELİMELER: Miyokardiyal İskemi, Reperfüzyon, Aritmi, Ovariektomi, Genistein.

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LIST OF ABBREVIATIONS AND SYMBOLS

HRV	: Heart Rate Variability
OVX	: Ovariectomy
HRT	: Hormone Replacement Therapy
AV	: Atrioventricular Node
SA	: Sinoatrial Node
NADPH	: Nicotinamide Adenine Dinucleotide Phosphate
NOS	: Nitric Oxide Synthases
K_{ATP}	: ATP Dependent Potassium Channel
NADH	: Nicotinamide Adenine Dinucleotide (reduced form)
NAD^+	: Nicotinamide Adenine Dinucleotide (oxidized form)

ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisor Prof. Dr. Ömer Bozdoğan for his guidance, advice, criticism, encouragements and insight throughout the research.

I would also like to thank my committee members Prof. Dr. Cihan Demirci Tansel and Prof. Dr. Özge Uzun for their critical suggestions and comments.

I want to especially my deepest thank to my family for their endless patience and support during my master period. I also want to thank to my laboratory partners for their helps and supports during my experiment.

This study was supported by the AIBU Research Project Foundation, Grant No: 2013.03.01.598.

1. INTRODUCTION

The major cause of death in coronary artery disease is the lethal arrhythmias that induced following coronary occlusion or reperfusion. It has been known that lethal effect of arrhythmias and the rate of mortality and morbidity in myocardial infarction has been observed as high level in men than women (Zhai et al., 2000). This gender differences has not been clarified yet. This difference in death following coronary artery occlusion and reperfusion is more in men than premenapousal women are shown in previous studies (Hayward et al., 2000). Although there is not obvious evidence, this gender difference has been based on estrogen (Barret-Connor et al.1991). The cardioprotective effects of estrogen have been resulted from increasing antioxidant activity (McHugh et al., 1998), increasing plasma nitric oxide level (Squadrito et al., 2000) and opening of calcium-activated potassium channels (Node et al., 1997). According to a research, 17-β-estradiol application caused cardioprotective effect by the way of activation of ATP-dependent potassium channels in canine hearts (McHugh et al., 1995).

Epidemiologic studies have shown that appearence of coronary artery disease in women rises fastly after postmenopausal period (Hayward et al., 2000, Mendelsohn et al., 1999). This situation is attributed to decreasing estrogen level in developing postmenapousal period (Barret-Connor et al., 1991). According to the previous research, some syndromes that appear after postmenapousal period has not been observed more in women living in Asian cauntries in respect to the women living in Western countries (Adlercreutz et al., 1992). This difference has been attributed to soy-derived food intake (Hollman et al., 1996). It is known that soy-derived food consists of phytoestrogens. Phytoestrogen is a general name used to classify the classes of compounds which are plant-origin or derived. Phytoestrogens have a weak estrogenic activity, so they are called as plant (phyto) estrogens (Wang et al., 1996). Genistein is the most known phytoestrogen group which is an isoflavone derived from soybeans and legumes, has a high affinity for the estrogen receptor-β, but has a low affinity for estrogen receptor-α (Kuiper et al., 1997).

There are many studies focused on the pharmacological activities of genistein, especially its anticancer activities and cardiovascular diseases. Genistein is thought to be beneficial for cardiovascular system through directly and indirectly (Lissin et al., 2000). It has been known that cardiomyoctes have functional estrogen receptors (Grohe et al., 1997, Mendelsohn et al., 1999). It is known that arrhythmias and death following ischemia and reperfusion have high incidence in male than female (Gonca et al., 2004, Zhai et al., 2000, Kuhar et al., 2007). Less arrhythmia observed following myocardial infarction in women has been attributed to the effects of estrogen which inhibits L-type Ca^{2+} current (Li et al., 2000) and result in decreased Ca^{2+} overload during myocardial ischemia. The excess Ca^{2+} accumulation is one of the accelerating mechanism for onset of the arrhythmias (Crestanello et al., 2000). Furthermore, estrogen administration provides reducing infarct size (Zhai et al., 2000), inhibiting apoptosis (Kim et al., 1998) during ischemia-reperfusion period. Thus, estrogen has been reported as cardioprotective agent for myocardial ischemia-reperfusion injury (Sugishita et al., 2003). A significant protection and reducing infarct size had been observed with genistein administration as effective as estradiol (Tissier et al., 2007). However, there are some contradiction that genistein could also act as inhibitor for tyrosine-specific protein kinases activity (Akiyama et al., 1987, Imagawa et al., 1997). Tyrosine kinase activity provides protection by the way of decreasing calcium accumulation during ischemia and suppress arrhythmias and restore contractile function. So, when genistein has been used with at a high dose approximately up to 1 mg/kg, these protection was observed to be abolished (Deodata et al., 1999). In this study, the long term effect of genistein on the ischemia reperfusion induced arrhythmia was aimed to be researched.

1.1 General Anatomy and Function of the Heart

The heart is an organ which is surrounded by a fibrous membrane called as pericardium. The pericardium is composed of two layers. These layers are a visceral layer surrounding the inner side of heart and a parietal layer lining the outside surface of heart (Whitaker, 2014). The heart lies posteriorly to the sternum and its apex lies in the left fifth intercostal spaces (Ellis, 2012).

The heart walls basically consist of the heart muscle cells. This collected tissue form is called as myocardium. The inner surface of the heart chambers is coated with endothelial cells or a thin layer known as endothelium (Widmaier et al., 2008).

The heart is comprised of two parts and each of these parts include an atria and a ventricle. These two ventricles are separated with muscle wall called as interventricular septum. There are some valves between atria and ventricles called as atrioventricular (AV) valves. These valves are also known as tricuspid and bicuspid (mitral) valves. AV valves permit blood flow from atria to the ventricles, but they prevent passing of blood flow from ventricle to the atrium. This condition is controlled by the pressure differences between each side of the valves. When the atrial pressure is higher than the ventricular pressure, the valves open and the blood passes from atria to the ventricle. On the other hand, if the ventricular pressure reaches higher level than the atrial pressure, AV valve closes. The opening of the valves always takes place in one-way direction. There is also a specialized tissue called as chordae tendineae which is stretched by contraction of the pupillary muscles of the ventricles. This tissue strands provide keeping the valves closed even if the pressure differences is higher in the ventricles than the atria. By the way of this tissue strand, blood leakage from the ventricles to the atrium is prevented.

There are also pulmonary and aortic valves called as semilunar valves. These valves led to the blood flow from ventricle to the inside of the pulmonary arteries and the aorta during the ventricular contraction, but they prevent backflow of the blood during the ventricular relaxation. The movement of the semilunar valves is passive as well as the AV valves. The opening or closing of the semilunar valves depend on the pressure differences between the internal and external sides of the valves (Guyton et al., 2000).

The main function of the heart is to pump blood from ventricles to the pulmonaries and systemic circulation. This pumping system is supplied by branching arteries called as coronary arteries and blood flow in here is called as coronary blood flow. Coronary arteries are originated from the aorta and they are connected with arterioles, capillaries and venules. Moreover, coronary blood flow is the shortest circulation in the body. All the myocardial cells supply nutrients by the way of coronary arteries. They are branched as right and left arteries which provide nutrients to the myocardial cells. Right coronary artery supply to the right side, left coronary artery supply to the left side of the heart (Whitaker, 2014) (Figure 1.1).

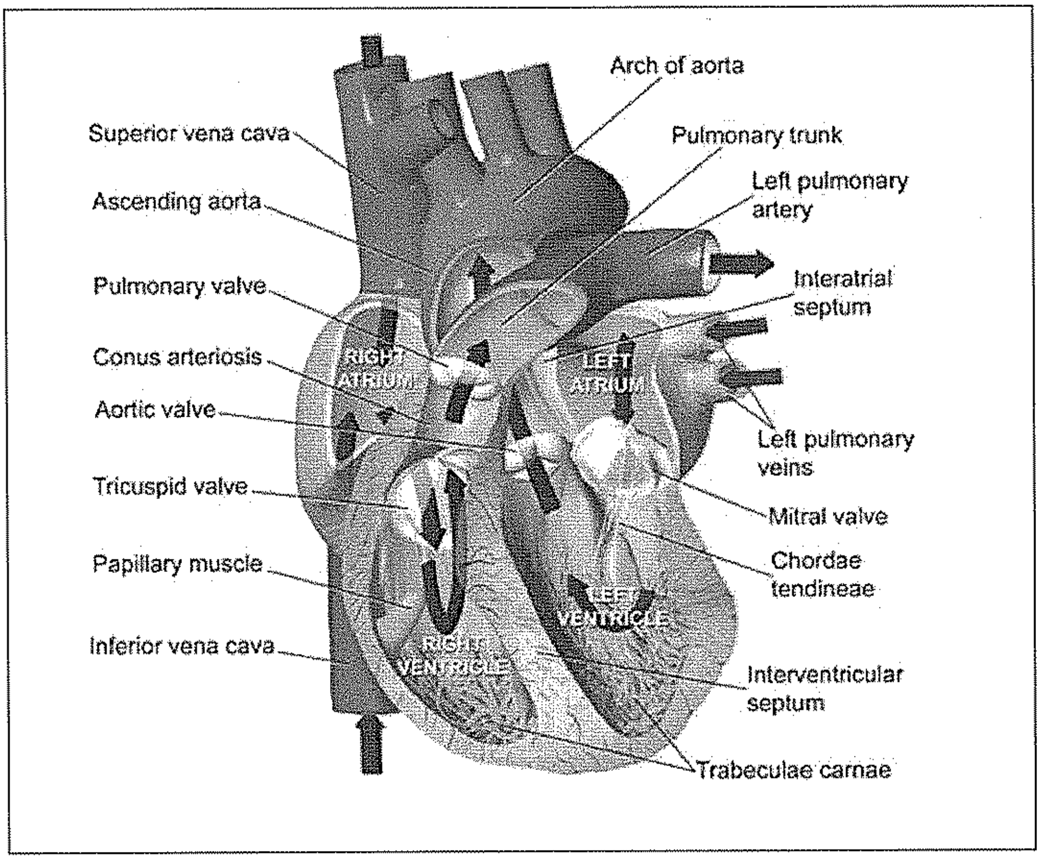

Figure 1.1 . Anatomy of the heart (taken Blausen Medical website)

1.2 Automaticity of the Heart

As it was mentioned before, the heart is a special organ to pump blood to the systemic circulation and pulmonary circulation and have specialized intrinsic and extrinsic conductive system for generating impulses to cause contraction of the heart muscle (Guyton et al., 2000). These impulses are conducted rapidly throughout the heart. Impulse conduction mainly occurs by the presence of gap junctions which is a passage gate for the impulses in contractile and autorhythmic myocardial cells. The specialized autorhythmic cells are localized in the sinoatrial node (SA), atrioventricular node (AV), atrioventricular bundle (bundle of His), right and left bundle branches and Purkinje fibers. All the impulses pass across the heart in this order (Bozdoğan, 2012).

The heart beat is generated in SA node which is a collection of specialized cells localized in the right arterial wall of the right atrium and just inferior to the opening of the superior vena cava. Due to the main place of generating impulse, SA node is called the pacemaker of the heart and determines heart rate (Marieb et al., 1998). Action potential which is generated in the SA node spreads via gap junctions to the whole myocardium. The right and left atrium contract synchronously with adequate speed of conduction and impulses pass to the AV node. At the AV node, the impulse transmission is delayed in order to allow the ventricle to be filled with blood from atria before the ventricular contraction. Then impulses passes to the specialized bundle of conducting fibers which is called bundle of His. AV bundles separate into two main branches as a right and left bundle branches. These branches constitute electrical connection between atria and ventricles. The bundle of His is connected with Purkinje fibers which are transmitter cells provide transmission of the impulses fastly. Purkinje fibers make connection with ventricular myocardial cells and so impulses spread to the rest of the ventricles entirely. This rapid conduction rate along the Purkinje fibers and wide distribution of these fibers induce synchronous depolarization of both the right and left ventricular cells. Finally, the impulse passes around each ventricular chamber and back to the base of the heart (Widmaier et al., 2008).

1.3 Electrophysiology of the Myocardial Cells

Autonomic cells of the heart have different electrophysiologic properties. Autonomic cells have an unstable resting membrane potential that continuously depolarizes. The action potential duration is much longer in cardiac cells than skeletal muscle cells or neural cells. There are two types of action potential: (1) Fast response current, (2) Slow response current. Fast response current is recorded in atrial, ventricular and the cells of purkinje fibers and it has a rapid upstroke velocity.

Slow response current is recorded in sinoatrial node and atrioventricular node and has a slow upstroke velocity.

In the fast response current, action potential duration is long. When the depolarization starts, Na^+ ions from the automatic cells pass through the gap junctions to the adjacent cells. These Na^+ ions constitute a small voltage change initiating depolarization. The opening of the fast Na^+ channels results in action potential generation. Four phase of action potential are recorded in heart. During phase "0", rapid Na^+ influx through rapid Na^+ channel occur and action potential reaches to peak level. After reaching the peak, action potential slowly decreases by the way of inward Cl^- currents. This phase is called Phase "1". After Phase 1, slow inward Ca^{2+} current and long depolarized state which is called plateau phase occurs. Slow Ca^{2+} channels close and K^+ channels open and causes repolarization at the end of the plateau phase. When the resting potential is occurred, Na^+ / K^+ pump works and ionic balance is regulated. Repolarization state continues until the new stimuli generate pacemaker potential.

In slow response current, action potential starts with a slow influx of Na^+ and Ca^{2+} ions and efflux of K^+ ions. Because of slow inward currents, cell membrane becomes more positive and the cells reach to threshold level. Reaching threshold level results in opening of fast Ca^{2+} and Na^+ channels. Thus, the action potential is induced. When the action potential reach to peak, K^+ channels open and rapid efflux of K^+ occurs. This K^+ efflux causes repolarization of the cell. During the repolarization, Na^+ / K^+ pump works and Na^+ and K^+ ions are replaced with each other. K^+ efflux which results from opening of K^+ channels causes hyperpolarization. This hyperpolarization triggers slow Na^+ and Ca^{2+} channels to open and results in generation of new stimuli (Guyton et al., 2000).

1.4 Myocardial Infarction and Reperfusion

Myocardial infarction occurs as a result of the restriction of blood flow by the blockage of the coronary arteries (Boersma et al., 2003). During the myocardial ischemia, blood flow to the myocardium is impeded and myocardial cells are not supported by enough oxygen and metabolic substrate such as nutrients and minerals.

As it is known that the left ventricule, which is responsible for forming the arterial blood pressure, utilizes most of oxygen delivered through the coronary system to maintain its normal function. Because of this, myocardial contraction is closely related with the coronary blood flow (Duncker et al., 2015). When the coronary blood flow is interrupted, oxygen delivery and the balance between oxygen supply and requirements change. This situation can cause abnormal myocardial contraction. Because of this abnormality, heart attack might be seen (Forfar et al., 1984).

Generation of the arrhythmias induced by the ischemia and reperfusion is the common cause of death. The clarification of alterations during the ischemia and reperfusion may improve the understanding of the mechanisms of arrhythmias and provide how it can be recovered.

1.4.1 Cellular Changes During Myocardial Ischemia

Myocardial ischemia is caused by blockage of the coronary blood flow and decreasing the oxygen and substrate supply to the myocardium (Ytrehus, 2000). Coronary ligation causes several biochemical and metabolic changes in the myocardial cells. Generation of free oxygen radicals and calcium overload are the most important ones of these cellular changes (Figure 1.2).

In the normal heart function, approximately 95% of ATP production using for energy source is supplied by mitochondrial oxidative phosphorylation (Stanley et al., 2005). During the ischemia, insufficient blood supply results in increasing NADH / NAD^+ ratio about 10-fold (Salem et al., 2002). As a result of this, oxidative phosphorylation changes toward glycolysis. In response to the accumulation of lactic acid from glycolysis results in acidosis (Robergs et al., 2004). Due to the fall in ATP levels, Na^+ / K^+ pump impairs and Na^+ ions accumulate in myocardial cells. Increasing intracellular Na^+ ions activates Na^+ / Ca^{2+} exchange channels and intracellular Ca^{2+} ions increases. Increasing cytosolic Ca^{2+} causes alterations in contractile proteins and decreases their sensitivity to Ca^{2+} ions (Buja, 2005). So, cardiac contractility reduces by interrupting the Ca^{2+} - Troponin C interaction and causes arrhythmias (Orchard et al., 1987). Myocytes may satisfy their oxygen demand by decreasing supply requirement and this results in reducing contractility and myocardial hibernation (Rahimtoola et al., 1993).

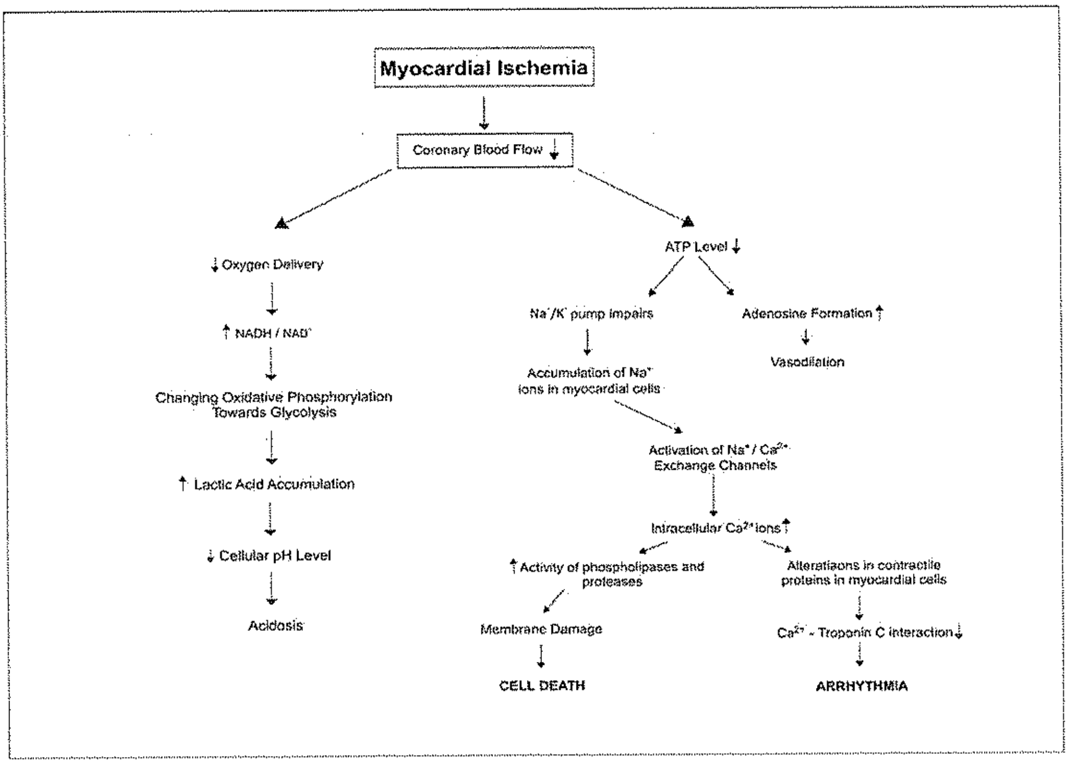

Figure 1.2. Cellular Changes during Myocardial Ischemia

1.5 CARDIAC ARRHYTHMIAS

Arrhythmias are mainly caused by abnormal cardiac rhythm (Podrid, 1997). Normal cardiac cycle begins in the SA node in which impulse generation occurs spontaneously. This normal impulse generation results in spontaneous heart rhythm called sinusal rhythm. Although arrhythmias are widely varied in their generation, there are three main mechanisms responsible for inducing abnormal cardiac rhythm (Gaztañaga et al., 2012). These are abnormal automaticity, triggered activity and re-entrant circuit. These mechanisms causes abnormalitiy in electrical activity of the heart (Hoffman et al., 1981). Various types of arrhythmias are classified induced by ischemia and reperfusion. These types of arrhythmias are sinusal bradycardia, sinusal tachycardia, atrial fibrillation (AF), bigeminal ventricular arrhythmias, ventricular premature contraction (VPC), ventricular tachycardia (VT) and ventricular fibrillation (VF). Ventricular arrhythmias are the most lethal ones of these types of arrhythmias (Henkel et al., 2006).

1.5.1 Ischemia and Reperfusion Induced Arrhythmias

In experimental study, there are two periods of arrhythmias. These are early and delayed arrhythmic period. Early arrhythmic period starts at 3-5 minutes and delayed arrhythmic period starts at 6-12 minutes after coronary ligation (Bozdoğan et al., 2001)

In the early phase of coronary ligation, the time of depolarization and repolarization changes. The myocyte is not completely damaged. In the ischemic zone, impulse transmission slows down or completely blocked. This results in the condition which is necessary for re-entrant arrhythmias (Hoffman et al., 1981). Moreover, K^+ efflux increases from ischemic region to the extracellular space that leads to the depolarization of myocardial cells in nonischemic region (Fisch, 1973).

In the late arrhythmic period, ischemic cells remain in depolarized state and generate ectopic stimuli for nonischemic region. Electrophysiology of the myocardium impairs and as a result of this cell death takes place. At the latest phase of ischemia, necrotic tissue occurs in ischemic zone. Arrhythmia generation generally decreases due to the necrotic tissue.

Reperfusion induced arrhythmias may occur at the second minutes of reperfusion. Reperfusion injury leads to more severe arrhythmias (Manning et al., 1984). Reperfusion induced arrhythmias are mainly caused by the generation of reactive oxygen species (ROS) which leads to membrane damage (Bernier et al., 1989). As a result of this damage, cytosolic Ca^{2+} accumulation occurs in cell membrane and causes heterogenity in myocardial cells which leads to more severe arrhythmias.

1.5.2 Types of Arrhythmias

1.5.2.1 Sinus bradycardia

Slow heart rate is called as bradycardia. Sinus bradycardia is caused by increasing vagal tone and decreasing sympathetic activity. In sinusal bradycardia P waves are present and followed by QRS complex. Normal PR interval and ST segments are observed (Podrid, 1997) (Figure 1.3).

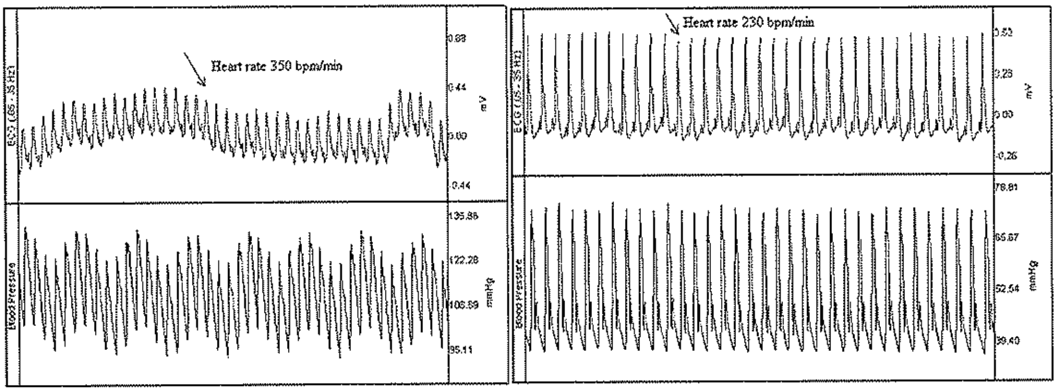

Figure 1.3. Sinus Bradycardia (from Control + Genistein group)

1.5.2.2 Sinus Tachycardia

Sinusal tachycardia occurs as a result of increasing autonomic stimuli from SA node. Sinus rate reaches to 450 beats/min in rats. In sinus tachycardia, P waves and ST segments are normal, but shortened or normal PR interval is observed (Figure 1.4).

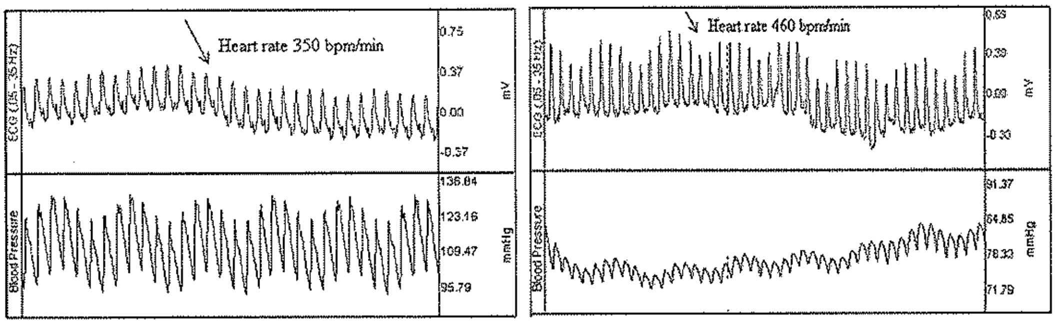

Figure 1.4. Sinus Tachycardia (from Control + Genistein group)

1.5.2.3 Atrial Fibrillation (AF)

Abnormal and irregular heart rhythm in atria is called as atrial fibrillation and electrical stimuli are generated randomly throughout the atria (Cavaliere et al., 2006). Atrial fibrillation is not fatal because of occurring normal blood flow passively through the atria to the ventricles. In atrial fibrillation, irregular heart rhythm with the absence of P waves is observed (Huang et al., 2007).

1.5.2.4 Ventricular Premature Contraction (VPC)

Ventricular premature contraction occurs in ectopic focus in the ventricles. Ectopic impulses result in stimulation of the heart before normal sinusal rhythm. As a result of this ventricles contract first and less blood is pumped to the circulation. Ventricular premature contraction is observed especially in myocardial ischemia and reperfusion. In VPC, QRS segment does not follow P wave and unusual shape and prolonged QRS is observed (Figure 1.5).

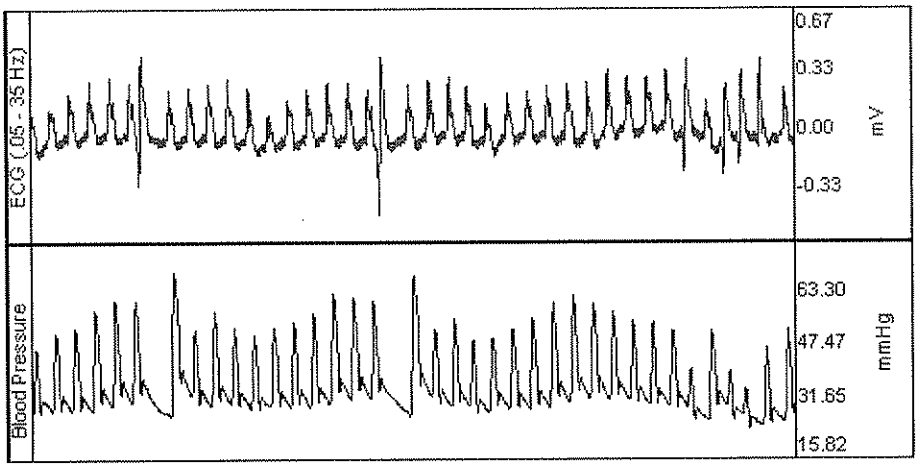

Figure 1.5. Ventricular Premature Contraction (from Control group)

1.5.2.5 Ventricular Tachycardia (VT)

Ventricular tachycardia is long lasting, sustained continuous ventricular beat. It occurs similar way with ventricular extra beat but the frequency and rate is higher in VT (Figure 1.6).

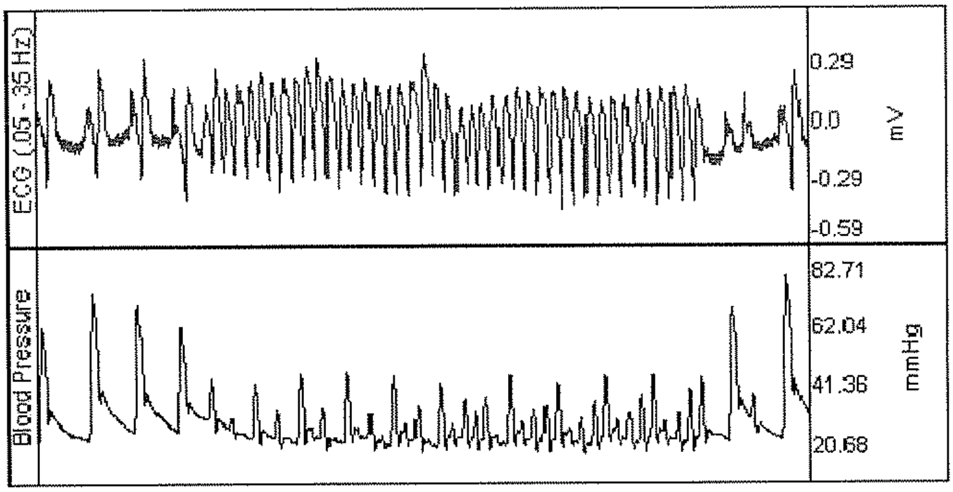

Figure 1.6. Ventricular tachycardia (from Control + Genistein group)

1.5.2.6 Ventricular Fibrillation (VF)

Long-lasting ventricular tachycardia generally results in ventricular fibrillation. Because ventricular tachycardia causes ischemia and this results in generation of ectopic focus. No coordinated contraction of myocardial cells are observed during ventricular fibrillation (Figure 1.7). During ventricular fibrillation, blood pressure decreases about 10 mmHg in rats. Irregular ECG waves are observed and QRS complex is not identified. When ventricular fibrillation continues more than 10 second, irreversible damage occurs in heart (Henkel et al., 2006).

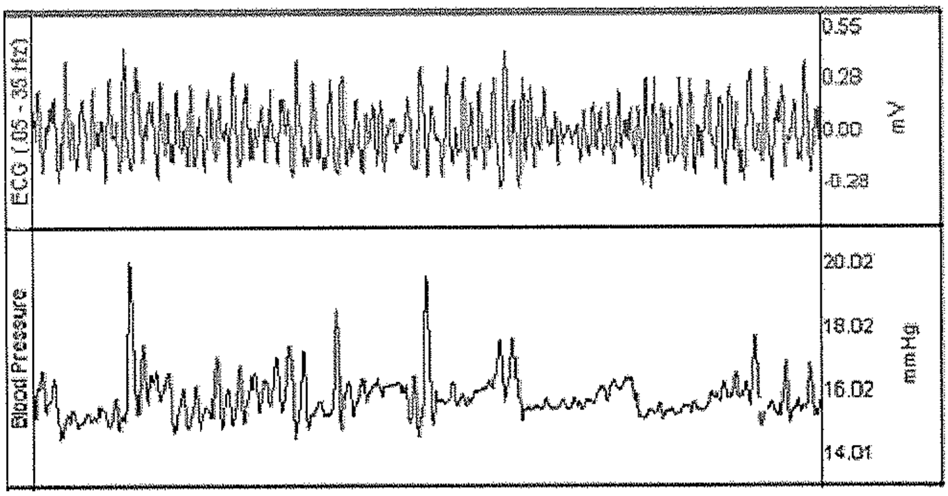

Figure 1.7. Ventricular fibrillation (from Ovx + Genistein group)

1.5.3 Underlying mechanism of cardiac arrhythmias

The normal heart rhythm is stimulated by spontaneous impulse generation from SA node. This impulse generation causes contraction of the heart and is called as sinusal rhythm. Arrhythmia is a general name and results from an abnormal cardiac rhythm which is initiated apart from SA node and abnormalities in electrical activity of the heart. The main causes of arrhythmias are divided into three major categories: (1) Abnormal automaticity, (2) Triggered activity and (3) Re-entrant circuits.

1.5.3.1 Abnormal Automaticity

Abnormal automaticity is enhanced activities by the ectopic pacemakers or acquired automaticity in atrial and ventricular myocardial cells. In normal condition, atrial and ventricular cells do not have automatic activity, but during ischemia or myocardial injury they may generate abnormal impulses. When the impulse generation from ectopic foci is higher frequency than the SA node, impulses coming from SA node is blocked. Even though abnormal automaticity is not totally responsible for most arrhythmias, it can causes re-entrant arrhythmias or accelerate these arrhythmias (Braunwald et al., 2001).

1.5.3.2 Triggered Activity

Triggered activity means the abnormal action potentials initiated by a preceding action potentials. Triggered activity is accelerated by afterdepolarizations which are oscillatory depolarizations induced by one or more preceding action potentials. There are two types of afterdepolarizations: (1) Early and (2) Delayed afterdepolarizations.

While early afterdepolarizations (EAD) occur during phase 2 or 3 of the myocardial action potential (Boutjdir et al., 1994), delayed afterdepolarizations (DAD) occur after the completion of repolarization (Phase 4) (Gaztañaga et al., 2012) (Figure 1.8). Their mechanisms are poorly understood, but EAD is observed in cardiac tissues which are exposed to hypoxia, acidosis, catecholamines or QT-prolonging agents. It is also associated with high intracellular Ca^{2+} ions which results from L-type Ca^{2+} channel imbalance (Yamada et al., 2008) or low level of K^{+} current (Mitsunori et al., 2011). EAD-induced activity is the main cause of development of long QT syndromes and by the way of this it results in development of Torsades de Pointes.

The reason of DAD is similar with EAD which is caused by excess Ca^{2+} accumulation via inhibition of the Na^{+} / K^{+} pump which causes release of Ca^{2+} from the sarcoplasmic reticulum. Some of the toxic levels of cardiac glycosides such as digitalis results in these situation (Rosen et al., 1973). Idiopathic ventricular tachyarrhythmias and idioventricular rhythms are result from DADs (Gaztañaga et al., 2012).

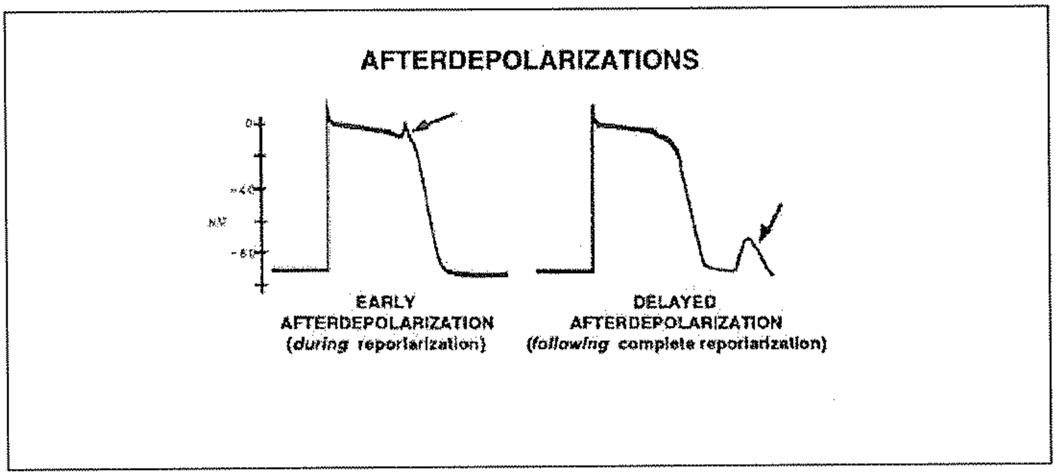

Figure 1.8. Early and Delayed Afterdepolarization

(from Pharmacology2000 website)

1.5.3.3 Re-Entrant Circuit

In normal condition, the cardiac cycle begins with impulse generation in the SA node and continues until the entire heart is stimulated. Second impulse from SA node or from ectopic focus can not stimulate these myocardial cell until they completely repolarized. However in abnormal electrical activity, some of the isolated fibers are not activated during the initial wave of depolarization or slow conduction occur here and they may recover their excitability and depolarize myocardial cells before the next sinusal impulse. This situation is called as reentry (Issa et al., 2009) (Figure 1.9).

Reentry is divided into 2 main groups: (1) Anatomical and (2) Functional reentry. Anatomical reentry is caused by an inexcitable anatomical obstacle which is surrounded by circular pathway. When the impulse encounters the obstacle, it turns around until excitatory impulses from blocked region exit to the myocardium and find it repolarized state, so initiates a reentrant circuit. Clinical examples of anatomical reentrant arrhythmias are AV reentrant tachycardia, AV nodal reentrant tachycardia and atrial flutter (Gaztañaga et al., 2012).

Unlike in anatomical reentry, in functional reentry the reentrant circuit is not generated by anatomical obstacles but it is initiated by spontaneous generation of abnormal impulse from anywhere of ventricles. Functional reentry is determined by dynamic heterogeneities in the electrophysiological properties of the tissue (Grant et al., 2007). Its location and size can change, but functional reentry is usually small and unstable (Gaztañaga et al., 2012). The rate of anatomic re-entry is slower than the functional re-entry.

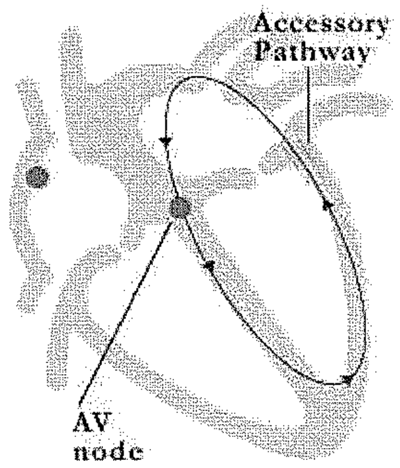

Figure 1.9. Re-entry Mechanism (from Brighamandwomen.org website)

1.6 The Effect of Estrogen to the Myocardial Cells

Ischemia-reperfusion induced arrhythmia is less in female than men was shown before (Gonca et al., 2004). Because of this, most of the researches focus on the underlying mechanism of this difference.

Less arrhythmogenic profile in female has been based on the effect of estrogen on the expression of some ion channels especially ATP-dependent potassium (K_{ATP}) channels (Ranki et al., 2001). K_{ATP} channels enhances protection to the myocardium during ischemia. These channels are inactive in the normally oxygenated myocardium. During ischemia, these channels opens as a result of decreasing ATP (Jilkina et al., 2002, Seino et al., 2003, Fujita et al., 2000).

The number of K_{ATP} channels are higher in female than male (Ranki et al., 2001). There are two types of K_{ATP} channel: mitochondrial K_{ATP} channel and sarcolemmal K_{ATP} channel. Endogenous estrogen affects especially sarcolemmal K_{ATP} channel (Ranki et al., 2002a). Also levels of SUR2A subunit expression which is one of the four subunits of K_{ATP} channel is increased by estrogen and resistance of the myocardium towards metabolic stress increases (Hayward et al., 2000). In one of the study it was shown that the effect of estrogen on decreasing infarct size during myocardial ischemia was prevented by 5HD which is a blocker of mitochondrial K_{ATP} channel (mitoK_{ATP}) (Lee et al., 2000). This result supports the effect of estrogen on K_{ATP} channel activity.

It is also known that endogenous estrogen induces coronary dilation and decreases myocardial injury by accelerating of NO synthases during ischemia and reperfusion (Kauser et al., 1997). The probable mechanisms of NO induced protection in myocardial injury are based on the inhibition of Ca^{2+} accumulation into the cell and reduces ROS generation by decreasing lipolysis (Gaudiot et al., 2000). NO production also results in increasing blood flow, producing negative inotropic effect and inhibition of catecholamine release (Node et al., 1997).

In smooth muscle cells, NO causes activation of guanylate cyclase which synthesizes cyclic CMP (cCMP) that is an agent of vasodilation. cCMP plays a role in the activation of protein kinase G. Protein kinase G activates Ca^{2+} channels, decreases cytosolic Ca^{2+} by activating sarcoplasmic reticulum Ca^{2+} uptake (Lau et al, 2003) and inhibits extracellular Ca^{2+} entry (Xiong et al., 1995). The low level of intracellular Ca^{2+} in smooth muscle cells reduces cellular contractility and provide relaxation. Decreasing contractility by increasing efflux of K^+ enhances preservation of ATP during ischemia (Escande et al., 1992). Also, as a result of this, action potential generation reduces and generation of reentrant arrhythmias are prevented (Wilde, 1994).

1.7 Phytoestrogens and Genistein

Phytoestrogens are substances called isoflavones which are produced by plants. The isoflavonoids have a limited distribution in the all plant kingdom. They are classified under Leguminosae family. The main functions of the isoflavonoids in plants is to provide the defence against microbial diseases (Dixon et al., 2002).

The main dietary sources of isoflavonoids are soy products (Wuttke et al., 2007). Alfalfa, red clover and flax seed also comprise isoflavonoids compounds. They are taken up with plant food and digested by intestinal bacteria then absorbed in the gastrointestinal tract and excreted in the urine (Wuttke et al., 2007). The most known and experimented group of the isoflavonoids is genistein. It has been reported that one gram of soy protein has nearly 250 μg of genistein (Dixon et al., 2002).

The main structural units of isoflavonoids are two benzene rings linked by a heterocyclic pyrone rings (Rimbach et al., 2008). Genistein have similar structures with the endogenous estrogen 17-β estradiol (Vaya et al., 2004) (Figure 1.10). Their phenolic ring and molecular distance between their 4'- and 7-hydroxyl groups are same in both. These similarities provide ability to bind estrogen receptors (Wang et al., 1996). It is also known that genistein binds either α and β estrogen receptor (Barnes et al., 2000). However, estrogen receptor binding affinity of the genistein is higher to estrogen receptor β than to estrogen receptor α (Harris et al., 2005). This affinity difference is important during the experiments which examining the results of phytoestrogen administration in animal models.

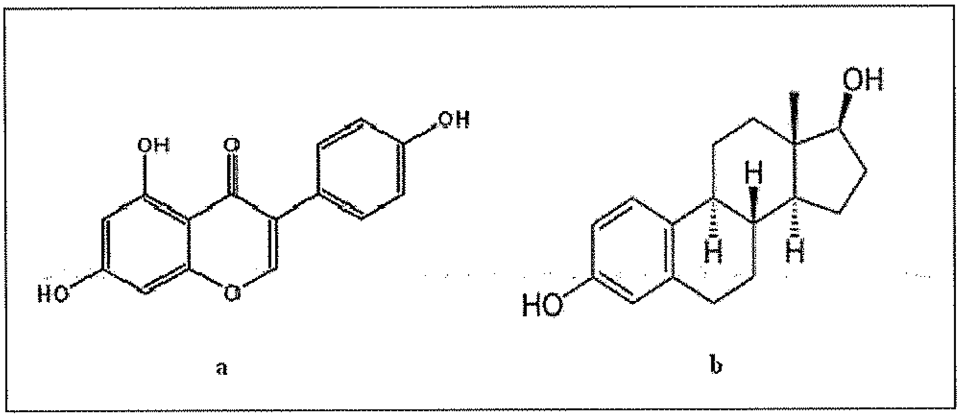

Figure 1.10. Molecular Shapes of Genistein (a) and 17-β Estradiol (b)

1.8 Cellular Effect of Phytoestrogen and the Relation of Phytoestrogens with the Arrhythmias

During the ischemia and reperfusion period, excess amount of reactive oxygen species (ROS) are excreted (Murphy et al., 2008, Zucchi et al., 2007). The most known protective mechanism of flavonoids including phytoestrogen genistein during ischemia and reperfusion is the scavenging of these reactive oxygen species. According to a study, flavonoids have been shown to reduce ischemia and reperfusion induced damage in myocardium by decreasing ROS products (Jovanovic et al., 2000). Scavenging of ROS products increases nitric oxide (NO) synthases and improve regulation of blood flow to the ischemic region.

The one of the most known protective mechanisms of flavonoids is vasodilatory effects in hypertensive rats (Bitto et al., 2009). This vasodilatory effects of flavonoids might be offered by inhibiting NADPH oxidase activity and increasing plasma endothelin-1 level (Holland et al., 2000, Jiménez et al., 2007). NADPH oxidases is an enzymes that catalyze transfer of one electron from NADPH to O_2 results in generation of O_2^- (Cave et al., 2005). NADPH oxidase in myocardial cells has been shown to increase during myocardial ischemia (Qin et al., 2008).

Furthermore, blockage of extracellular Ca^{2+} influx during the myocardial ischemia and reperfusion is the most important protective endothelium-independent effects of flavonoids (Akhlaghi et al., 2009). They also activates voltage-gated K^+ channels and induces arterial relaxation (Novakovic et al., 2006).

Phytoestrogen genistein might be a cause of tyrosine kinase inhibition and it was shown that 5 mg / kg genistein administration has prevented the protection of preconditioning against ischemia-reperfusion induced arrhythmias *in vivo* (Fatehi-Hassanabad et al., 1997). It has been claimed that this dosage accelerates tyrosine kinase inhibition and by the way of this decreases the effect of preconditioning (Deodato et al., 1999). As it is known that, tyrosine kinases are responsible in three main functions in preconditioning. These functions are decreasing of myocardial damage (Imagawa et al., 1997), suppression of arrhythmias and restoring of contractile function during ischemic period (Fatehi-Hassanabad et al., 1997). Moreover, in an another study, 100μM genistein in the Krebs solution has inhibited the increasing of MAP kinases and MAPKAP kinase 2 in the rat hearts. These kinases are normally activated in preconditioned heart (Maulik et al., 1996). In recent years, all of these inhibitory effects of genistein are still being debated.

As it was mentioned before, phytoestrogen genistein have many similarities between endogenous estrogen 17β-estradiol. It is known that 17β-estradiol regulates expression of K_{ATP} channels in heart (Ranki et al., 2002). In spite of molecular similarities, inhibitory effects of genistein on K_{ATP} channels regulation in rabbit portal vein smooth muscle, which is different from estradiol, has been shown (Ogata et al., 1997). The opening of K_{ATP} channels are the most effective in the suppression of the arrhythmias which occurred during ischemia and reperfusion (Koyuncu, 2008).

2. AIM AND SCOPE OF THE STUDY

The incidence of cardiovascular disorders and their mortality in human being approximately 50 % in all over the world (Myerburg et al., 1997). The main reason of death due to the cardiovascular diseases is lethal arrhythmias. In recent years, there are many studies focusing on the prevention of these arrhythmias. In order to find alternative protective methods, the plant-derived substances have been researched by most of the researchers in recent years. Phytoestrogens are the examples of these plant-derived substances. In many studies their protective effects have been shown especially on myocardial infarct size and ischemic tolerance. Unlike these studies, there are not enough studies which shows their protective effect on ischemia and reperfusion induced arrhythmias, although there are a few study on ischemia reperfusion induced arrhythmias that they are generally performed *in vitro*. That is why, the long term effect of genistein administration on ischemia reperfusion induced was aimed to be researched in this study. It was thought that this study might be a supportive as an experimental study and enlighten whether there is a protective effect of genistein on ischemia and reperfusion induced arrhythmias.

3. MATERIALS AND METHODS

3.1 Animals

In this study, 39 female Spraque-Dawley rats weighing 225-290 g and in 6-7 months old were used. All the animals were maintained under same standart laboratory conditions. They were separated randomly and caged into groups. The animals were fed with commercial rat pellet food and drunk tap water ad libitum. The animals were kept in 12 hours light/12 hours dark. All the animals were handled according to the protocol approved by the ethical committe for the protection of animal research of the Abant Izzet Baysal University, Bolu, Turkey. They were also treated in adherence to guiding principles in the care and use of animals with the recommendation from the Declaration of Helsinki.

3.2 Ovariectomy

Two groups of animals were ovariectomized to remove the ovaries bilaterally before coronary artery ligation and reperfusion (n: 14). In ovariectomized group, firstly the rats were anaesthesized with Ketamine (90mg / kg) and Ksilazine (10mg / kg) intraperitoneally. The skin was prepared and then lateral abdominal incision was made. The ovaries were isolated and removed along with the suture. The animals were controlled in the laboratory until completely awaked from anaesthesia and then put back to cages. Two weeks were waited for complete recovery of animals prior to the coronary ligation and reperfusion. In the sham-operated animals, as in the ovariectomized groups, all the operative procedure were same except that the ovaries were isolated but not removed.

3.3 Coronary Artery Ligation and Reperfusion

Animals were anaesthesized with Urethane (1,2 g / kg). The trachea was canulated for artificial respiration. The left carotid artery was canulated for measuring mean arterial blood pressure. The catheter for canulating carotid artery was filled with heparine diluted 1 / 10 with saline and then inserted into the left carotid artery for measuring blood pressure. The chest was opened in the fourth and fifth intercostal spaces. Pericardium that encircle the heart was removed from heart and then the heart was exposed. A loose loop of 5.0 silk suture was placed around the branch of left main coronary artery approximately 2 mm from its origin (Figure 3.1, Yaşar, 2009).

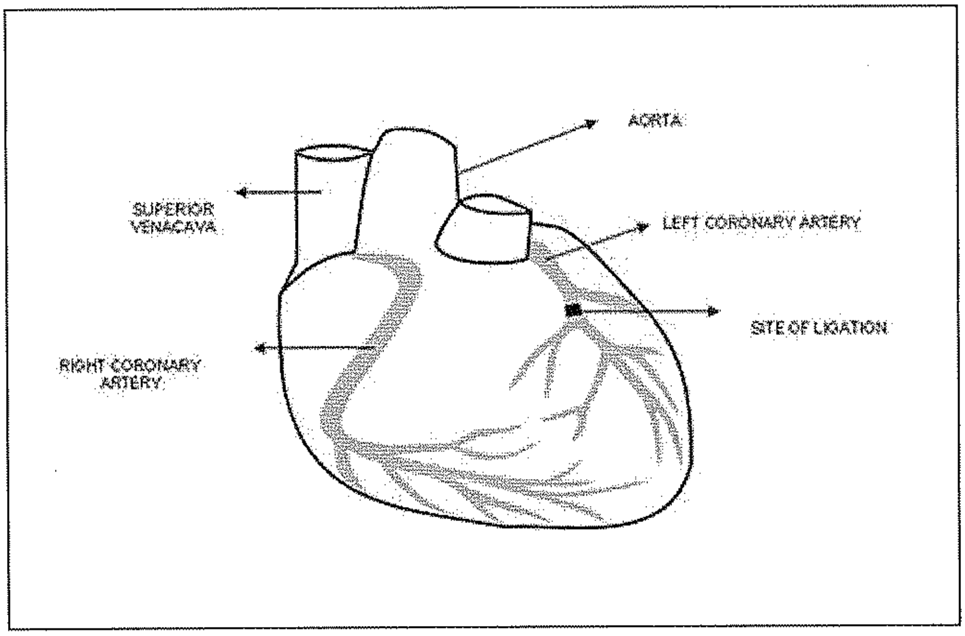

Figure 3. 1. Region of coronary ligation (from MSc thesis of Yaşar, 2009)

Then, the heart was set back in its place and artificial respiration was started using an animal respirator (Ugo Basile Rodent Ventilator, Italy) that set in air 0.9 ml / 100 g body weight at a rate of 60 stroke / minute. Subcutaneous needles were placed under the skin to record standart electrocardiogram. Then, it was waited 5 minutes for the stabilization of heart rate and blood pressure. The animals having arrhythmias or having mean arterial pressure below 70 mmHg was discarded from the experiment. Coronary ligation was made by tightening the silk by forming a bowknot to produce myocardial ischemia for 6 minutes. After that, reperfusion was made by loosening the bowknot for 6 minutes.

At the end of the coronary ligation and reperfusion, the heart was perfused by the way of the aorta with giving firstly sodium chloride (NaCI) and then ethanol to determine the risk of infarct zone. After that, the non-perfused area that seeing red color was separeted from the perfused area that seeing white color. The non-perfused area and then perfused area was weighted together with the non-perfused area. The percentage of non-perfused area in respect to total weight of ventricle was calculated. This calculation was called as the risk of infarct zone.

3.4 Drug Administration

Genistein was dissolved in dimethylsulphoxide (DMSO) and given (100μg / kg) intraperitoneally for four weeks prior to the coronary ligation and reperfusion.

3.5 Evaluation of the Arrhythmias

In all the groups, heart rate and blood pressure were recorded at 1, 3, 5 minute during ischemia and reperfusion. The incidence and duration of the arrhythmias during 6 minutes of ischemia and reperfusion were calculated from recorded ECG. The onset and offset of the arrhythmias were determined.The arrhythmias were classified as ventricular tachycardia (VT), ventricular fibrillation (VF) and the other types of arrhythmias including single ventricular extra beat, bigemini and salvos. The sinusal tachycardia is differentiated from ventricular tachycardia in terms of heart rate and blood pressure. The arrhythmias were designed as sinusal tachycardia when heart rate was between 500 and 600 / min and blood pressure was higher than 70 mmHg and as ventricular tachycardia when heart rate was above 500 / min and blood pressure was lover than 50 mmHg.

Arrhythmias were classified according to the Lambeth conventions (Walker et al., 1988). An arrhythmia score was calculated according to the incidence and duration of the arrhythmias for each animals as follows; 0 = no arrhythmias, 1 = < 10 second VT or other arrhythmias, 2 = 11-30 second VT or other arrhythmias, 3 = 31-90 second VT or other arrhythmias, 4 = 91–180 second VT or other arrhythmias and / or < 10 second reversible VF, 5 = >180 second VT or other arrhythmias and / or >10 second reversible VF, 6 = irreversible VF (Lepran et al., 1996).

3.6 Statistical Analysis

The mean and standart errors were calculated for all parameters including heart rate and blood pressure. The duration of arrhythmias and arrhythmia scores were compared by the analysis of variance with one-way ANOVA combined with the LSD post hoc. test. Furthermore, the survival rate and the incidence of arrhythmias were compared by chi-square test (Fisher exact test, two-tailed).

3.7 Hormonal Measurement

Plasma estradiol concentration level was measured in both control and operated rats using commercial ELISA kits according to the instruction given by the manifacturer.

4. RESULTS AND DISCUSSIONS

4.1 Results

ST segment elevation or QRS changes were observed following coronary artery ligation in all groups. Following coronary artery ligation, blood pressure decreased significantly in all groups (Figure 4.1). Basal blood pressure (means before ligation) was higher in sham + genistein group than the sham and control groups ($p < 0,05$). That means genistein increases blood pressure. In ovx group, basal blood pressure nonsignificantly increased ($p = 0,08$) (Figure 4.2) (Table 4.2).

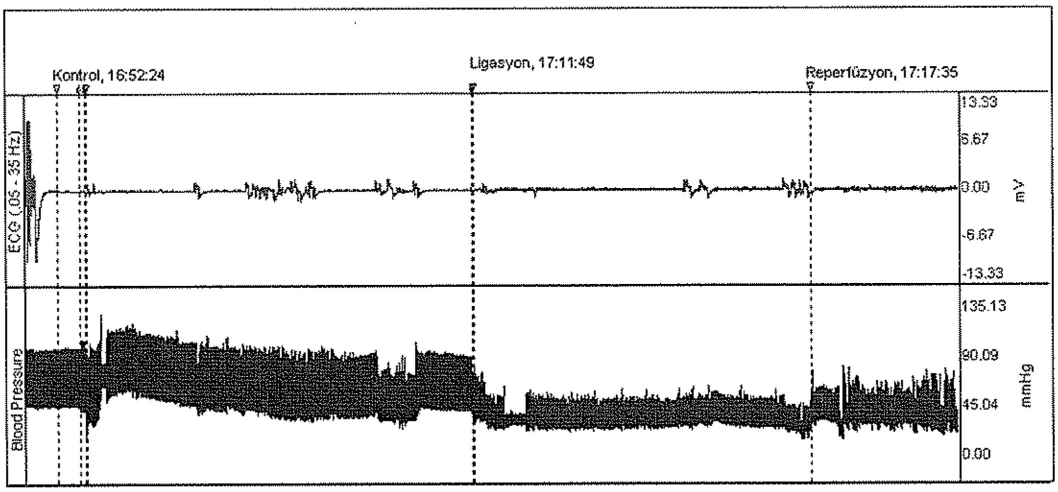

Figure 4.1. ECG Recording during 6 min of Ischemia and 6 min of Reperfusion

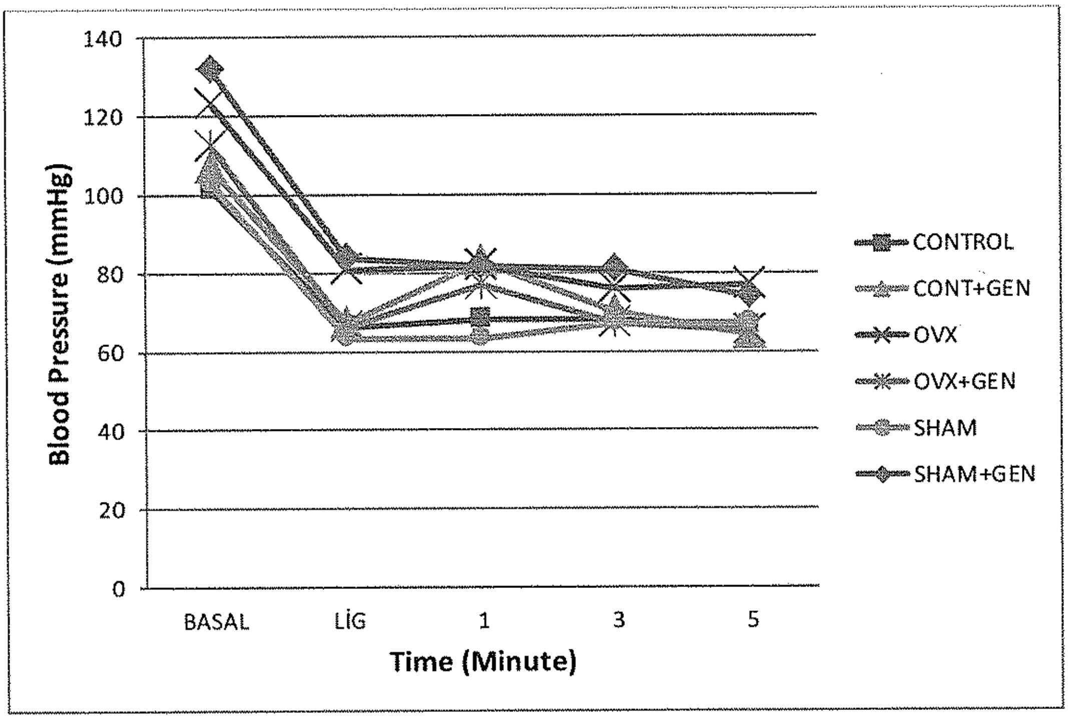

Figure 4.2. Blood Pressure during 6 min of Coronary Ligation

No significant differences were found in the heart rate between groups before and during 6 minutes of coronary artery ligation (Figure 4.3) (Table 4.2)

Following reperfusion, blood pressure immediately increased in all groups. There were no significant differences in blood pressure between groups during 6 minutes of reperfusion (Figure 4.4) (Table 4.3) .

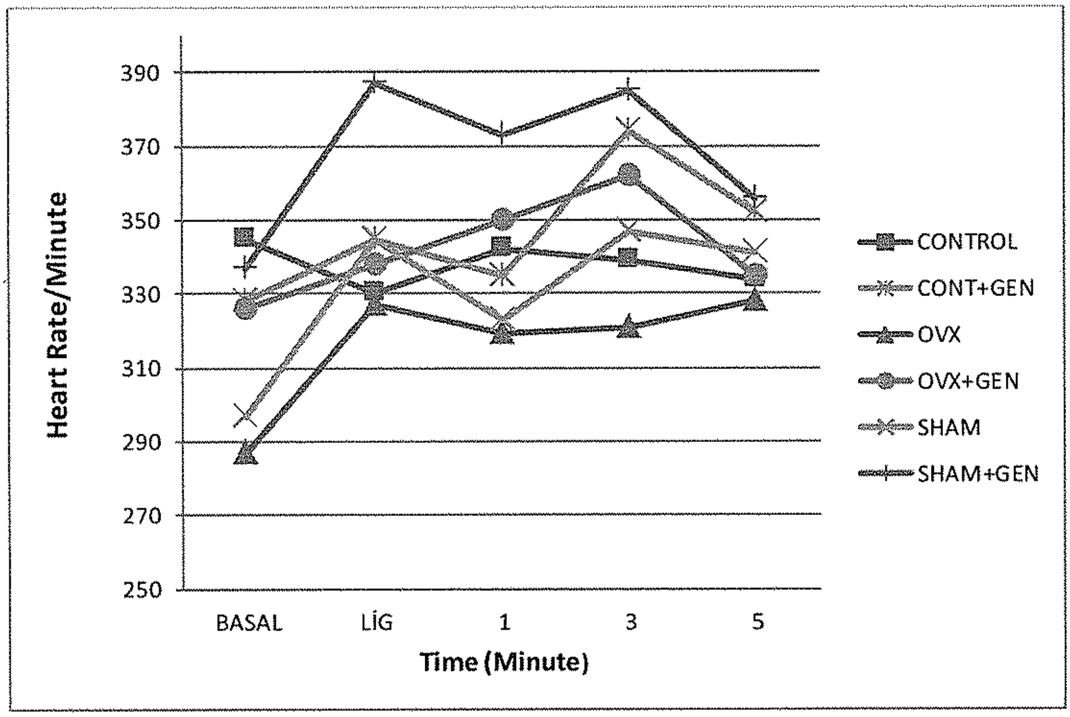

Figure 4.3. Heart Rate during 6 min of Coronary Artery Ligation

There were significant differences in heart rate during 6 minutes of reperfusion between groups. At the beginning of reperfusion heart rate was higher in sham + genistein group than ovx group ($p < 0,05$). At first minute of reperfusion, heart rate was higher in ovx + genistein and control+genistein groups than sham group ($p < 0,05$). At third minutes of reperfusion heart rate was higher in ovx + genistein group than ovx group ($p < 0,05$) (Figure 4.5) (Table 4.3).

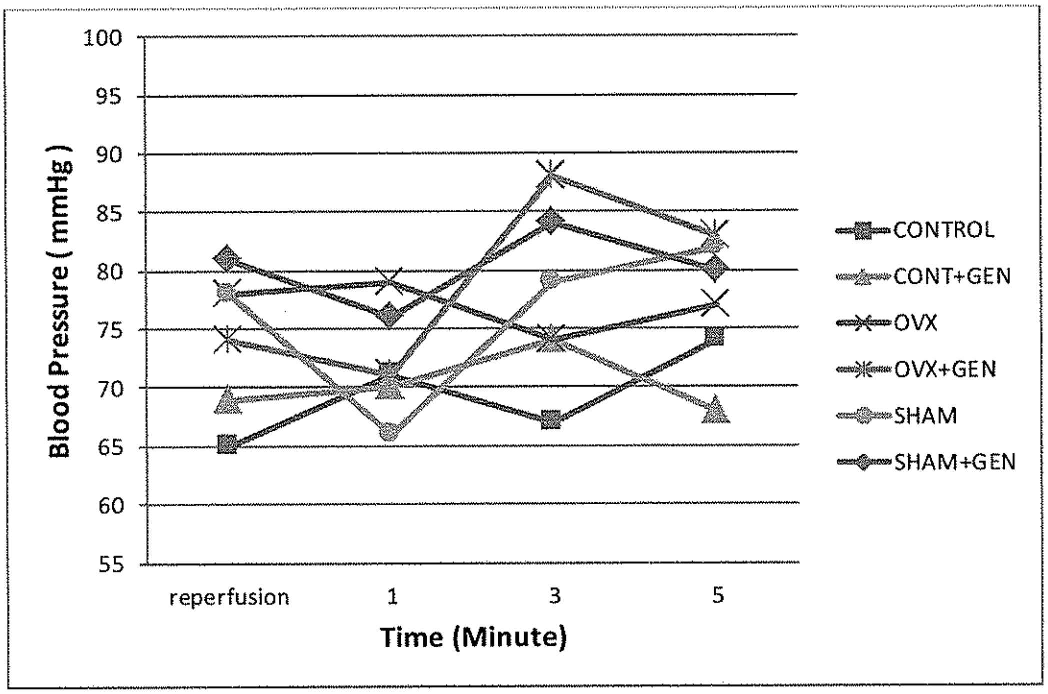

Figure 4.4. Blood Pressure during 6 min of Reperfusion

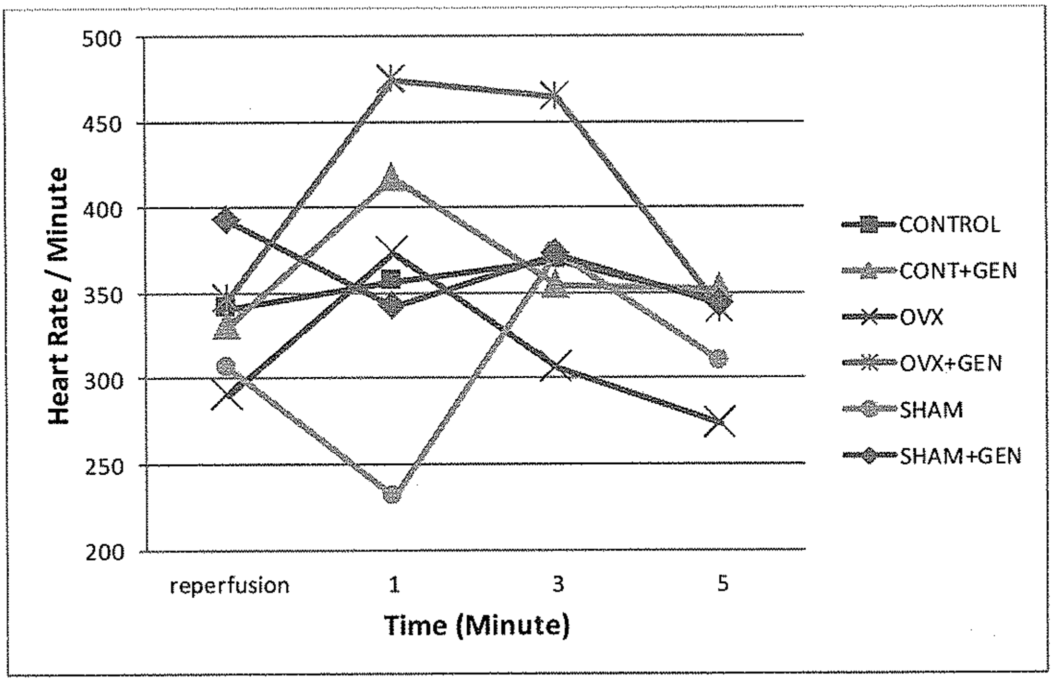

Figure 4.5. Heart Rate during 6 min of Reperfusion

The onset of arrhythmias during reperfusion was significantly lower in control + genistein group than control group ($p < 0,05$) (Figure 4.6) (Table 4.6). There were no significant differences in arrhythmic period between groups. But arrhythmic periods were nonsignificantly lower in control + genistein group than sham group ($p = 0,06$), control+genistein than ovx group ($p = 0,07$), control + genistein than sham group ($p = 0,06$) and sham + genistein group than sham group ($p < 0,06$). That means the arrhythmic period in sham group is tend to be more than the other groups. When the groups were compared for arrhythmic period among themselves, there were no significant differences between control and control + genistein, sham and sham + genistein, ovx and ovx + genistein, sham and ovx and sham + genistein and ovx + genistein (Figure 4.7) (Table 4.6).

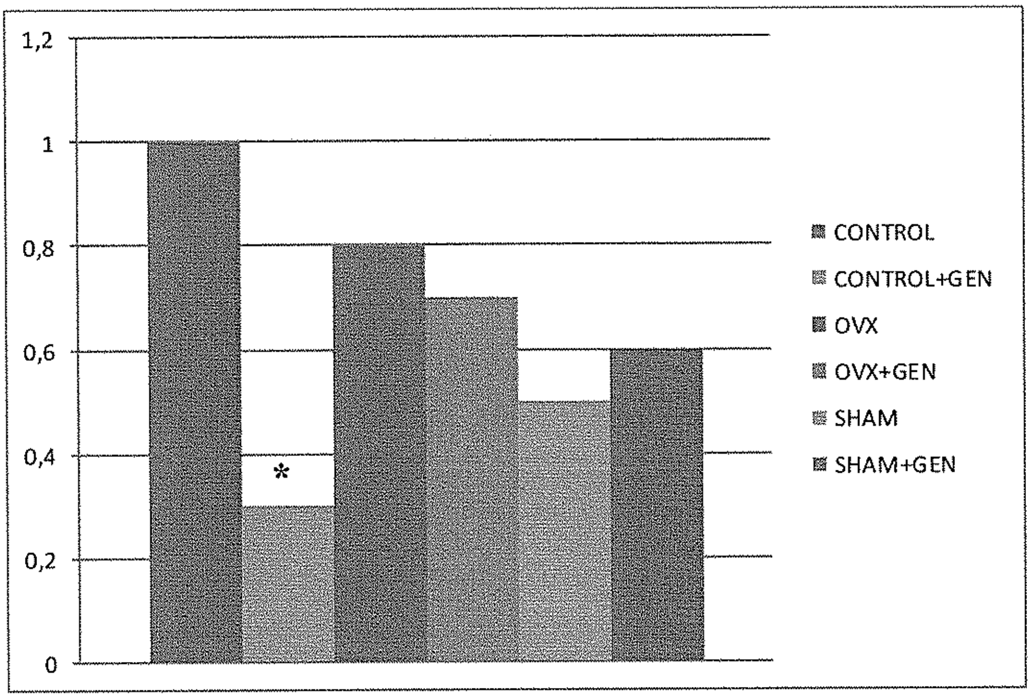

Figure 4.6. The Onset of Arrhythmias during 6 min of Reperfusion (* $p < 0,05$; According to the Control)

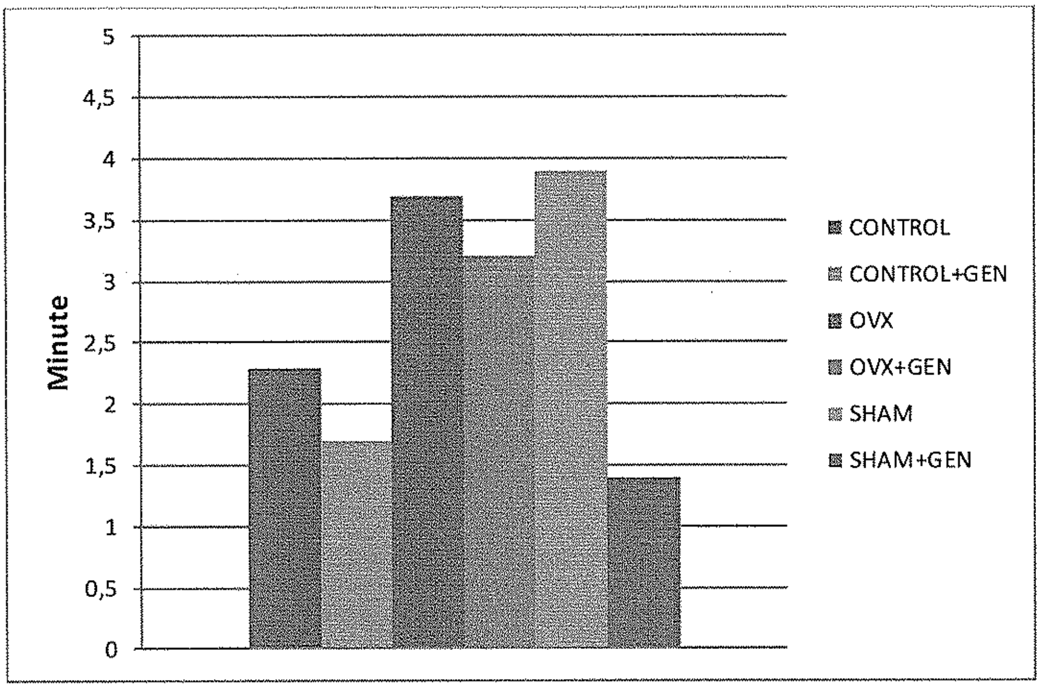

Figure 4.7. Arrhythmic Period during 6 min of Reperfusion

The total length of all type of arrhythmias were not significantly different between all groups (Figure 4.8) (Table 4.6). There were no significant differences in the length of ventricular fibrillation, ventricular tachycardia or ventricular premature beats. The arrhythmia score was significantly higher in sham group than the control + genistein group (Figure 4.9) (Table 4.7).

Plasma estradiol levels was significantly lower in ovx group than the other groups (Table 4.1). According to this data, it can be said that ovariectomy has decreased plasma estradiol level significantly. There were no significant differences in plasma estradiol levels between ovx and ovx + genistein, sham and sham + genistein groups.

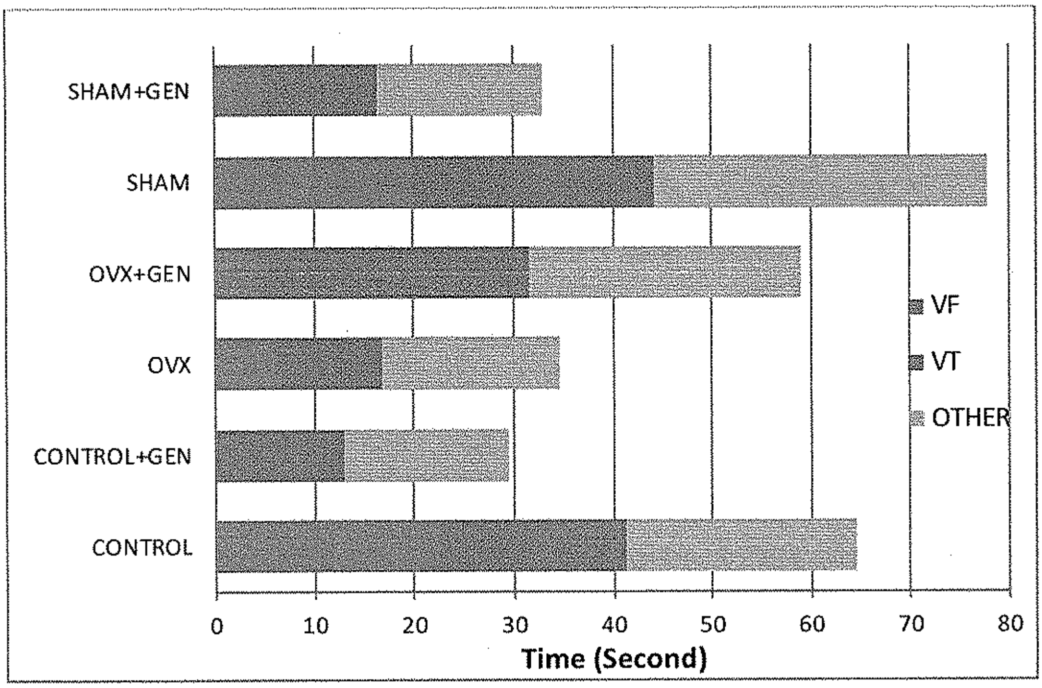

Figure 4.8. Total Length of Arrhythmias during 6 min of Reperfusion

(Other: Ventricular Premature Contraction (VPC), Ventricular gemini and Salvo)

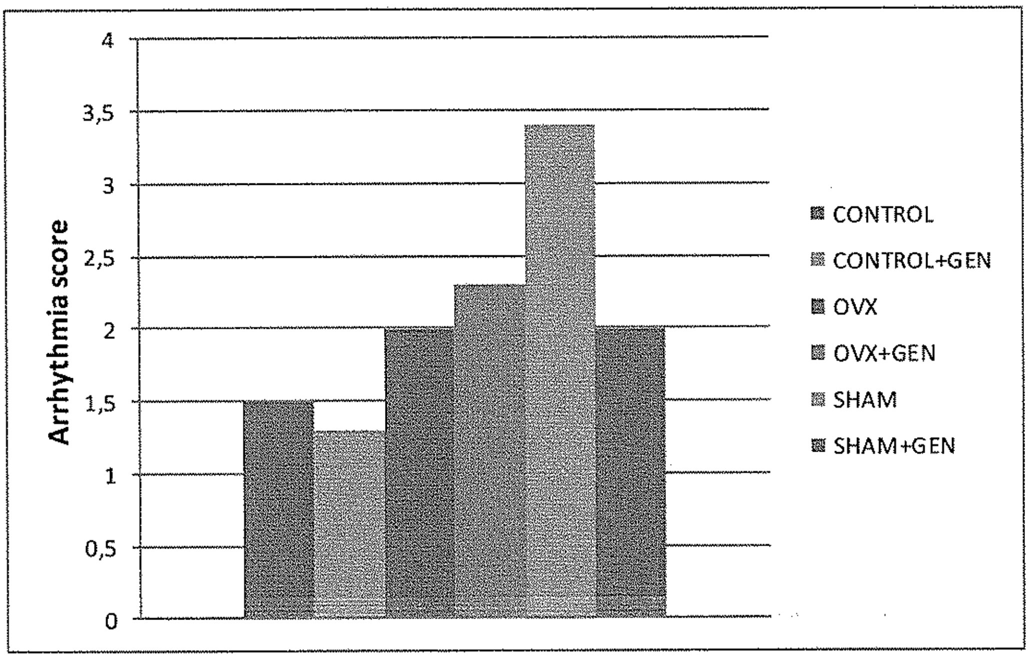

Figure 4.9. The Score of Arrhythmias during 6 min of Reperfusion

Table 4. 1. Plasma Estradiol Levels (ng/ml) *p<0,05

GROUPS	N	PLASMA ESTRADIOL LEVEL (ng/ml
Control	6	109 ± 3
Control + Genistein	6	95 ± 4
Ovx	6	68 ± 10 *
Ovx + Genistein	6	95 ± 4
Sham	6	99 ± 4
Sham + Genistein	6	97 ± 4

Table 4. 2. The Heart Rate (HR) and Mean Arterial Pressure (BP) during 6 min of Coronary Ligation (Data are Mean ±SE, *p<0,05)

GROUPS	N	Heart rate (Beat / min)					Blood pressure (mmHg)				
		BASAL	Lig	01	03	05	BASAL	Lig	01	03	05
CONTROL	8	345 ± 23	330 ± 29	342 ± 26	339 ± 28	334 ± 23	101 ± 5	66 ± 3	68 ± 5	68 ± 6	66 ± 6
CONTROL+GEN	7	328 ± 21	345 ± 28	335 ± 19	374 ± 30	352 ± 26	107 ± 8	67 ± 7	83 ± 5	70 ± 4	64 ± 5
OVX	7	287 ± 23	327 ± 26	319 ± 28	321 ± 30	328 ± 32	123 ± 15	81 ± 11	82 ± 11	76 ± 8	77 ± 10
OVX+GEN	7	326 ± 23	338 ± 40	350 ± 30	362 ± 17	335 ± 42	112 ± 4	66 ± 6	77 ± 4	67 ± 7	65 ± 8
SHAM	5	297 ± 12	345 ± 22	323 ± 41	347 ± 29	341 ± 30	103 ± 9	63 ± 5	63 ± 3	67 ± 6	67 ± 7
SHAM+GEN	5	337 ± 21	387 ± 20	373 ± 28	385 ± 24	356 ± 16	132 ± 5*	84 ± 3	82 ± 5	81 ± 7	74 ± 12

N: The number of animals used in the experiment

* : Different from control and sham groups

Table 4. 3. The Heart Rate (HR) and Mean Arterial Pressure (BP) during 6 min of Reperfusion (Data are Mean ±SE, $^*p < 0,05$, $^{\#}p < 0,05$)

GROUPS	N	Heart rate (Beat / min)				Blood pressure (mmHg)			
		REP	01	03	05	REP	01	03	05
CONTROL	8	341 ±24	357 ± 59	369 ± 51	345 ± 43	65 ± 7	71 ± 4	67 ± 8	74± 9
CONTROL+GEN	7	331 ± 24	417 ± 63#	354 ± 52	352 ± 39	69 ± 6	70 ± 5	74 ± 4	68 ± 3
OVX	7	290 ± 35	374 ± 52	306 ± 27	274 ± 22	78 ± 12	79 ± 13	74 ± 13	77 ± 12
OVX+GEN	7	346 ± 42	475 ± 71#	464 ± 61*	340 ± 58	74 ± 9	71 ± 11	88 ± 11	83 ± 10
SHAM	5	306 ± 20	231 ± 24	374 ± 68	309 ± 32	78 ± 9	66 ± 9	79 ± 13	82 ± 11
SHAM+GEN	5	393 ± 34*	342 ± 27	372 ± 19	343 ± 21	81 ± 8	76 ± 8	84 ± 3	80 ± 6

N: The number of animals used in the experiment

*: Different from Ovx group

#: Different from Sham group

Table 4.4 The Duration and Length of Arrhythmias during 6 min. of Coronary Ligation (Data are Mean ± SE, *p<0,05)

GROUPS	N	Onset of arrhythmia (minute)	Arrhythmic period (minute)	Length of arrhythmia (second)				
				VF	VT	Other	Total	Bradycardia
CONTROL	8	0,5 ± 0,3	0,3 ± 0,3	0 ± 0	0 ± 0	0,3± 0,1	0,2 ± 0,1	0 ± 0
CONTROL+GEN	7	0,7 ± 0,3	0 ± 0	0 ± 0	0 ± 0	0,1 ± 0,1	0,1 ± 0,1	0 ± 0
OVX	7	1,2 ± 0,7	0,1 ± 0,1	0 ± 0	0 ± 0	0,7 ± 0,5	0,7 ± 0,5	0 ± 0
OVX+GEN	7	0,7 ± 0,3	0,8 ± 0,8	0,7 ± 0,7	5,3 ± 5,3	1,8 ± 1,5	7,9 ± 7,6	0 ± 0
SHAM	5	0,5 ± 0,2	0,2 ± 0,1	0 ± 0	0 ± 0	2,2 ± 1,8	2,2 ± 1,8	0 ± 0
SHAM+GEN	5	1,5 ± 0,9	0,9 ± 0,8	0 ± 0	5,2 ± 5,2	2,1 ± 1,9	7,3 ± 7,1	0 ± 0

N: The number of animals used in the experiment

Other: Ventricular Premature Contraction (VPC), Ventricular gemini and Salvo

Total: Ventricular Fibrillation (VF) + Ventricular Tachycardia (VT) + Other types of arrhythmias including VPC, Ventricular gemini, AV nodal arrhythmias and Salvo

Table 4. 5. The Incidence of Arrhythmias during 6 min. of Coronary Ligation (Data are Mean ± SE, *p < 0,05)

GROUPS	N	Survival (%)	Incidence of arrhythmia (N / %)				Arrhythmia score
			VF	VT	Other	Bradycardia	
CONTROL	8	100	0/0	0/0	2/25	0	0,3 ± 0,2
CONTROL+GEN	7	100	0/0	0/0	3/43	0	0,4 ± 0,2
OVX	7	100	0/0	0/0	3/43	0	0,4 ± 0,2
OVX+GEN	7	100	1/14	1/14	4/57	0	0,9 ± 0,4
SHAM	5	100	0/0	0/0	3/60	0	0,6 ± 0,2
SHAM+GEN	5	100	1/20	1/20	3/60	0	0,8 ± 0,4

N: The number of animals used in the experiment

Table 4. 6. The Duration and Length of Arrhythmias during 6 min. of Reperfusion (Data are Mean ± SE, *p < 0,05)

GROUPS	N	Onset of arrhythmia (minute)	Arrhythmic period (minute)	Length of arrhythmia (second)				
				VF	VT	Other	Total	Bradycardia
CONTROL	8	1 ± 0,2	2,9 ± 0,7	22,5 ± 22,5	18,9 ± 9,1	23,3± 7,5	64,7 ± 29	0 ± 0
CONTROL+GEN	7	0,3 ± 0,1*	1,6 ± 0,8	0 ± 0	13 ± 6,7	16,6 ± 7,8	34,3 ± 16,6	0 ± 0
OVX	7	0,8 ± 0,3	3,7 ± 0,8	6,04 ± 6,04	10,8 ± 8,6	17,8 ± 7,8	34,7 ± 14,4	0 ± 0
OVX+GEN	7	0,7 ± 0,3	3,1 ± 0,7	4,4 ± 3,6	27,3 ± 12,4	27,4 ± 19,6	59,1 ± 27,5	0 ± 0
SHAM	5	0,5 ± 0,2	3,9 ± 1,1	20 ± 13,5	24,2 ± 9,8	33,7 ± 10,9	78 ± 10,3	0 ± 0
SHAM+GEN	5	0,6 ± 0,2	1,8 ± 0,7	4,8 ± 4,8	11,8 ± 6,4	16,5 ± 11,9	33,1 ± 16,7	0 ± 0

N: The number of animals used in the experiment,

*: different from control group

Table 4.7. The Incidence of Arrhythmias during 6 min. of Reperfusion (Data are Mean ± SE, *p<0,05)

GROUPS	N	Survival (%)	Incidence of arrhythmia (N / %)				Arrhythmia score
			VF	VT	Other	Bradycardia	
CONTROL	8	88	1/13	7/88	8/100	0	2,1 ± 0,6
CONTROL+GEN	7	100	0/0	3/43	5/71	0	1,3 ± 0,4
OVX	7	100	1/14	3/43	7/100	0	2 ± 0,6
OVX+GEN	7	86	2/29	3/43	7/100	0	2,3 ± 0,6
SHAM	5	100	3/60	5/100	5/100	0	3,4 ± 0,8
SHAM+GEN	5	100	1/20	3/60	5/100	0	2 ± 0,8

N: The number of animals used in the experiment

4.2 Discussion

Although there are many studies which have reported increases of arterial blood pressure in ovx rats (Thorin et al., 2003, Irigoyen et al., 2005, Souza et al., 2007), it was observed that post-ovariectomy in rats did not increase arterial blood pressure in the present study as similar as many other studies (Lam et al., 2002, Brandin et al., 2003, Tezini et al., 2008, Gonca, 2008). According to a clinical study, the changes in arterial blood pressure after menapouse has been told to take approximately 5 to 20 years (Burt et al., 1995). Despite of some study which has showed increases of arterial blood presure in post-menopausal period, it may be said that endogenous estrogen may not be only determining factor for the elevated arterial blood pressure in post-menopausal women and ovx rats. In rats increasing blood pressure after ovariectomy takes more than 2 - 4 weeks (Gonca, 2008). Similarly in our study, blood pressure were found higher in ovariectomized group than the control but it was not significant. Nonsignificance in our study might be depend on less number of animals used in the group. There were no significant changes in arterial blood pressure when genistein was administrated for 2-days or 2-week observed by some researcher (Nevala et al., 2002, Sbarouni et al., 2006, Deodato et al., 1999). However, in this study basal blood pressure before coronary artery ligation were found higher in sham group ($p < 0,05$) following genistein administration. Different findings in our result may be caused by species differences in experimental animals or method of the study which that was as *in vivo* or *in vitro*. Also, it was reported that genistein and endogenous estrogen bind with the same receptors and compete with each other for binding. Genistein could be competitive with endogenous estrogen for estrogen receptors and inhibits estrogenic activity or may inhibits the synthesis of estrogen-binding protein in the presence of endogenous estrogen (Lissin et al., 2000). When the genistein binds to the estrogen receptor, endogenous estrogen do not bind to the receptor. In this case, question is the result of estrogen and genistein binding to the estrogen receptor is same or not? Increasing blood pressure after genistein administration in this study might be generated by the prevention of estrogen binding to the estrogen receptor and different responce of myocardial cell to the genistein. This is the research point for further studies.

During the ischemia and reperfusion, no significant differences in heart rate was observed in sham and ovx groups. According to these data, it can be said that post-ovariectomy did not affect heart rate variability. In the various study, it has been shown that ovariectomy did not modify heart rate variability during myocardial ischemia and reperfusion (Al-Nakkash et al., 2009, Dias da Silva et al., 2009, Dhote et al., 2007). There are some genistein-based studies which has shown that genistein decreses heart rate in spontaneously hypertensive rats (Bitto et al., 2009, Al-Nakkash et al., 2010). However, genistein is known as positive inotropic agent that it increases Ca^{2+} transients and inhibits protein tyrosine kinase (Chu et al., 2005). In this study, it was observed that genistein increased heart rate nonsignificantly but in some period significantly during reperfusion. The dose of genistein used in the study is also important in the heart rate variability. In this study, 100µg / kg dose of genistein were used and the heart rate was observed to increase at this dose in ovx and sham group but not in control. When the heart rate is high, blood pressure tend to be less were observed in the same group. In previous research, the blood pressure increased in lower dose and decreases in higher doses of genistein (5 mg / kg or 250 mg / kg human equivalent dose) were shown (Fatehi-Hassanabad et al., 1997, Al-Nakkash et al., 2009). The dose used in the present study was lower and similarly increased the blood pressure. Estrogen-dependent studies focus on especially heart rate variability (HRV) and baroreflex sensitivity. These factors have importance for the generation of cardiac arrhythmias and their mortality (La Rovere et al., 2001). Genistein depending on used dose might be effective on the ischemia reperfusion induced arrhythmias as similar as endogenous estrogen.

The onset of arrhythmias was significantly lower in control + genistein group than control group during reperfusion ($p < 0,05$). In a study, genistein did not modify the onset of arrhythmias, duration of ventricular tachycardia or the arrhythmia score (Söylemez et al., 2003). No significant differences was found between sham and sham + genistein, ovx and ovx + genistein groups. But in genistein used groups, the arrhythmia score and total duration of all arrhythmia during reperfusion was nonsignificantly lower than their control. Because of this, this differences can not be compared definitely. If ovx - operated or sham - operated groups were compared, although plasma estrogen level decrease in ovx group, the incidence of arrhythmias did not increase or decrease with ovariectomy. This results might indicate that there is no direct relation between plasma estrogen level and cardioprotection against arrhythmias in female rats (Humphreys et al., 1999, Gonca, 2008). Protection against arrhythmias may result from sustained effect of estrogen even after removal of ovaries. It has been shown that sex - dependent resistance to myocardial ischemia reperfusion injury is correlated with sarcK_{ATP} channel activity during ischemia (Chicco et al., 2007). Total arrhythmia was found more in sham and control group of rats than ovx rats. This was opposite result of our expectation. Normally we thought that since estrogen level decrease in ovariectomized rats, the protection against the arrhythmia is to be expected decrease and more arrhythmia would be seen in this group than the control. This finding support the result of a previous study (Gonca, 2008). In that study, ovariectomy did not decrease or increase the ischemia reperfusion arrhythmia. Since we expect estrogen and genistein have a synergy on the myocardial protection and additive effect of both genistein and endogenous estrogen will appear on arrhythmia in genistein administered group. Genistein nonsignificantly decreased arrhythmia in control and sham group but not ovx group. The result of genistein decreased arrhythmia in unovariectomized female was also shown in previous study (Deodato et al., 1999). This may be derive from the other effect of genistein than the estrogen like effect or changes in the affinity of genistein to the estrogen receptor after ovariectomy. Long term administration of genistein may be lead to decreasing estrogen receptor and also decreasing the affinity of genistein to the estrogen receptor and also decreasing of estrogen in ovx rats might be involve in the generation of more arrhythmia in genistein administered ovx rats. It is known that as the hormone or analog of hormone increase, their receptor decrease (Mohamed et al., 2000, Bozdoğan, 2012). Since the estrogen receptor did not researched in this study, the relation of genistein and estrogen receptor expression can not be directly correlated. Again in this study the level of estrogen and the score of arrhythmia is not correlated. Since the arrhythmia did not change in ovx group than the control and sham group. Similar result was also shown in previous studies (Humphreys et al., 1999, Gonca, 2008). Although in most of study the protective effect of estradiol was observed against the arrhythmia, in this study no relation was shown between estrogen level and ischemia reperfusion induced arrhythmia. This means that ovariectomy and decreasing estrogen level is not enough for the potentiation of ischemia reperfusion arrhythmias and ovariectomy may not suitable model for menopause. Because in menopause period, beside increasing age, estrogen level decreases. Increasing age might be major determinant for inducing of more severe arrhythmia following coronary ligation (Bozdoğan et al., 2013). In a study, it was found that the ATP dependent potassium channel decreases in female but not male in increasing age (Ranki et al., 2002b). It is not known the ATP dependent potassium channel or estrogen receptor expression in myocardial cell decreases in ovx rat. This means that the clarification of why the arrhythmia did not increase in ovx rat and why the genistein was not effective in ovx rats to decrease arrhythmia requires further research.

Although genistein is known as a protective agent for cardiovascular disease, there are some reports that genistein also has inhibitory effect on tyrosine kinase activity (Fatehi-Hassanabad et al., 1997, Söylemez et al., 2003). Tyrosine kinase is a powerful mediator which enhances contractile recovery after myocardial ischemia and is triggered by preconditioning before complete coronary ligation and reperfusion (Maulik et al., 1998). Tyrosine kinase is also involved in the reduction of infarct size in rats (Imagawa et al., 1997). Genistein prevents cardioprotection following preconditioning by its inhibitor activity in tyrosine kinases (Fatehi-Hassanabad et al., 1997). No significant protective differences in arrhythmia generation or the length of arrhythmias might be depend on the possible inhibitory effect of genistein to the tyrosine kinase. In our study the increasing arrhythmia in ovx rats after long term genistein administration might be depend on such effect mentioned above. Moreover, the dose which was used in the present study (100 μg / kg) might be lower for protection or is enough to inhibit tyrosine kinase activity. As it was mentioned before, K_{ATP} channels are so effective in cardioprotection during ischemia and reperfusion. Genistein has been defined as an inhibitor for K_{ATP} channels with a dosage of 100 μg / kg and this blockage may be generated by the inhibition of tyrosine kinase activity through genistein (Ogata et al., 1997). In this study, it can not definitely explained whether there is an inhibition of K_{ATP} due to genistein. In order to determine this possibility, further research is required.

5. CONCLUSIONS AND RECOMMENDATIONS

Genistein nonsignificantly decreased the total lenght of arrhythmias with the existence of endogenous estrogen. However genistein was not found to be effective to decrease arrhythmia in ovariectomized rats. Nonsignificant effect of genistein on the ischemia reperfusion induced arrhythmia might be related with less number of animals used in this study. Because in this study and our previous studies there were seen more individual differences in response against ischemia reperfusion injury. That is why this study might be repeated with the higher number of experimental animals. Different effect of genistein on arrhythmias both in ovariectomized and unovariectomized animals might be result of changes in affinity of genistein to estrogen receptor. However the result of the present study is not enough to clarify it yet. Further research should be designed to clarify it. Although hormone replacement therapy with phytoestrogen was advised to decrease menopausal syndrome in postmenopausal women, it was seen in this study that long term adminastration of genistein was not found to be effective to decrease ischemia reperfusion induced arrhythmia. In the literature it was observed that genistein block the ATP dependent potassium channel and can potentiate the arrhythmia. But the effect of genistein on the ATP dependent potassium channel expression or activity was not shown in this study. In the later study, the effect of genistein on the activity and the expression of ATP dependent potassium channel can be researched. Another forthcoming study might be to research the relation of estrogen receptor expression with the ovariectomy and also with the long term genistein administration in myocardial cell. Nonsignificant decreasing can be also explained with the low dose of drug which was used as 100 µg / kg. In order to clarify whether there are dose dependent effect of genistein to the ischemia reperfusion induced arrhythmias further studies should be planned in future.

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List of Publications :

1. Orallar H., Kaya S.T.,Yaşar S., Erim F., Bozdoğan Ö. Does the food restriction affect the ischemia induced arrhythmia and infarct size after coronary occlusion in rats. Abstract. Acta Physiologica, 203 (Supplement 686): PC056, 2011.

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