The influence of ischemic pre-conditioning and inhibition of the ubiquitin proteasome system on cardiac ischemic injury in a murine model

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Dedicated to my parents, my wife and my son.

Contents

Abbreviations

$[Ca^{2+}]_I$	Cytosolic Ca2+ concentration
AAR	Area at risk
AAR	Area at risk
ang. p.	Angina pectoris
AP	Action potential
ATP	Adenosine triphosphate
AV	Atrial ventricular
bpm	Beats per minute
BZ	Bortezomib
CHD	Coronary heart disease
CO	Cardiac output
CPG-ODN	CpG oligodeoxynucleotides
CX3CR1	CX3C chemokine receptor 1
CXCL	Motiv Chemokin 8
DAMPs	Damage associated molecular patterns
DEPC	Diethylpyrocarbonate
ECG	Electrocardiogram
FTY720	Fingolimod
HF	Heart failure
HIF	Hypoxia inducible factor
HMGB-1	High-mobility group box-1
HOCM	Hypertrophic cardiomyopathy

HR	Herat rate
HSP	Heat shock proteins
I/R	Ischemia reperfusion injury
ICAM	Intercellular adhesion molecule
IL	Interleukin
IPC	Ischemic pre-conditioning
IPOC	Ischemic post-conditioning
IκB	Inhibitor of kappa B
LAD	Left anterior descending artery
MI	Myocardial infarction
MMPs	Matrix metalloproteinases
NDRG	N-myc downstream-regulated gene
NF-κB	Nuclear factor kappa B
POC	Post-conditioning
RIC	Remote ischemic pre-conditioning
ROS	Reactive oxygen species
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SR	Sarcoplasmic reticulum
STE	ST elevation
SV	Stroke volume
TGF-β	Transforming growth factor
TLR	Toll-like receptors
TNF	Tumor necrosis factor

TPR	Total peripheral resistance
TTC	Triphenyl tetrazolium chloride
UPS	Ubiquitin Proteasom System
VEGF	Vascular endothelial growth factor

1 Introduction

1.1 Physiology

1.1.1 Function of heart

In circulatory system, the function of the heart is to provide continuous blood flow to the whole body. This circulation consists of the systemic circulation to and from the body and the pulmonary circulation to and from the lungs. During the process of respiration, blood in the pulmonary circulation exchanges carbon dioxide for oxygen in the lungs (Fig. 1). After this, oxygen is transported to the body & carbon dioxide is returned by systemic circulation and relatively deoxygenated blood to the heart for transfer to the lungs (Betts et al., 2013).

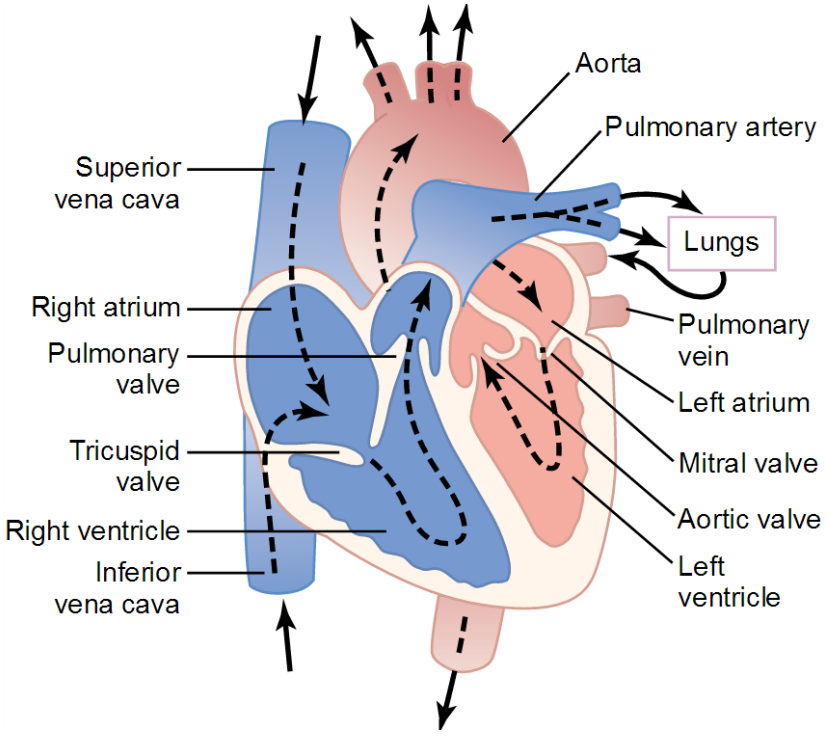

Fig. 1: Heart structure and blood flow course through the cardiac chambers and heart valves (Guyton and Hall, 2006)

The deoxygenated blood is collected by the right heart from two large veins, the superior and inferior vena cava. Blood accumulates in the right and left atrium continuously (Betts et al.,

2013*).* The superior vena cava receives blood from above the diaphragm and pours it into the upper back part of the right atrium. While the inferior vena cava receives the blood from below the diaphragm and pours it into the back part of the atrium below the opening for the superior vena cava. At the contraction of the right atrium, the blood is pumped through the tricuspid valve into the right ventricle. When the right ventricle contracts, the tricuspid valve closes and the blood is pumped into the pulmonary trunk via the pulmonary valve. The pulmonary trunk gives rise to pulmonary arteries and progressively smaller arteries throughout both lungs, until it reaches the capillaries. Carbon dioxide is exchanged with oxygen as these capillaries pass by alveoli in lungs. Oxygen diffuses from the lumen of the alveoli into the blood while diffusion of carbon dioxide acts in the opposite direction.

Oxygenated blood in the left heart is returned to the left atrium via the pulmonary veins. It is then, through the left ventricle, pumped into the aorta via the aortic valve for systemic circulation. The aorta is the largest artery that divides into many smaller arteries, arterioles, and ultimately capillaries. “In the capillaries, oxygen and nutrients from blood are supplied to body cells for metabolism, and exchanged for carbon dioxide and waste products” (Betts et al., 2013*).* The deoxygenated blood in the capillaries then travels into venules and veins and ultimately accumulates in the superior and inferior vena cava, and back into the right heart to restart the whole blood pumping cycle.

1.1.2 Cardiac cycle

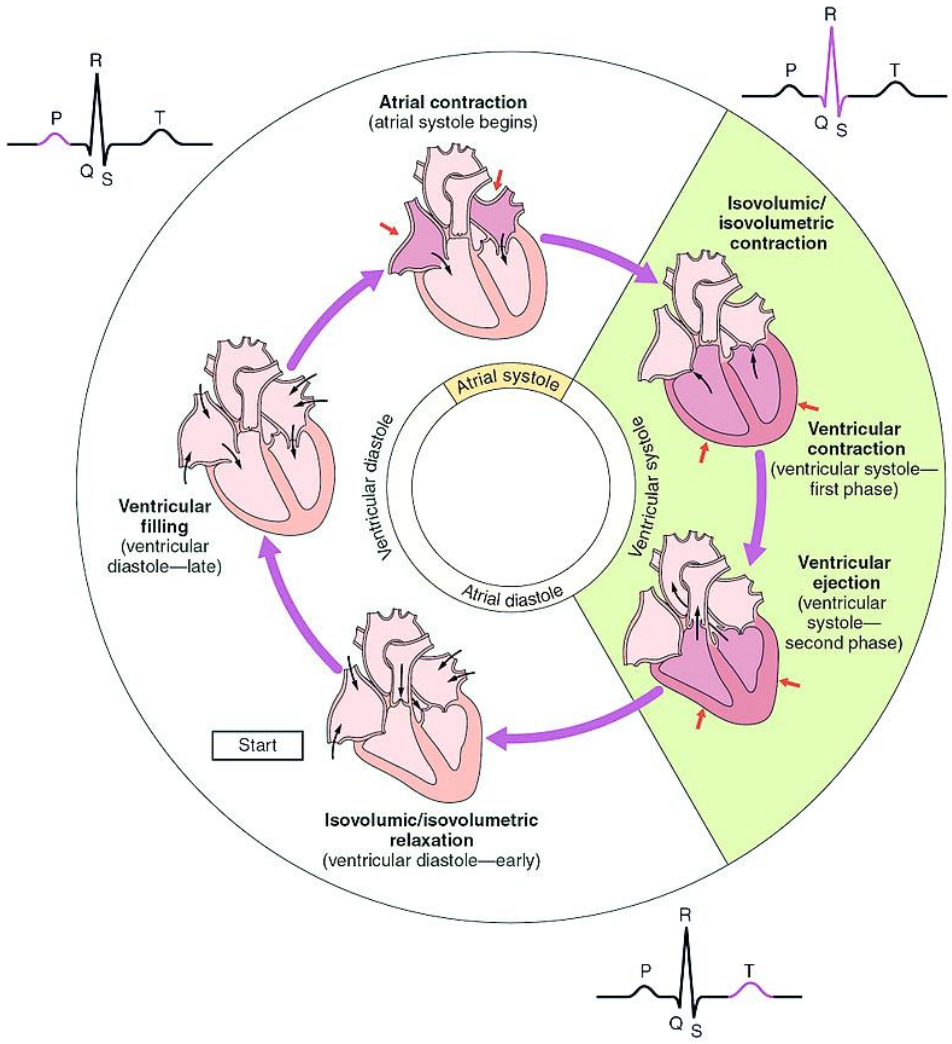

Fig. 2: The cardiac cycle. Different electrocardiogram (ECG) states are exhibited in relation to the cardiac cycle (Betts et al., 2013*).*

The heartbeat which includes systole and diastole and the intervening pause is called the cardiac cycle (Fig. 2), (Guyton and Hall, 2006). This cardiac cycle starts with the atrial contraction and ends with relaxation of the ventricles (Fig. 2). Systole means the contraction of the atria or ventricles of the heart. Diastole is the term used when the atria or ventricles relax and fill themselves with blood. The atria and ventricles work in a systematic cycle, i.e., each systole is followed by a diastole (Betts et al., 2013*).*

Both the atria and the ventricles are relaxed during the early diastole, at the onset of the cardiac cycle (isovolumic/isovolumetric relaxation). When the chambers are relaxed, blood will flow into the atria from the pulmonary veins and the coronary sinus. The pressure will rise as the atria begin to fill so that the blood moves from the atria into the ventricles. During late diastole the atria contract, pumping more and more blood into the ventricles. This will cause an increase in pressure inside the ventricles. As the ventricles reach systolic stage of cardiac cycle, the blood will be pumped into the aorta and pulmonary artery (isovolumic/isovolumic contraction) (Guyton and Hall, 2006) (Fig. 3).

When both of the ventricles undergo contraction, the pressure forces the aortic and pulmonary valves to open. As both ventricles undergo relaxation phase, the aortic and pulmonary valves will close because the pressure in the ventricles is lower in the pulmonary arteries and aorta. When the tricuspid and mitral valves (atrioventricular valves) are open, during blood flow to the ventricles, the aortic and pulmonary valves are shut closed to avoid backflow of the blood into the ventricles. When the ventricular pressure is higher than the atrial pressure the mitral and tricuspid valves will close (Guyton and Hall 2006).

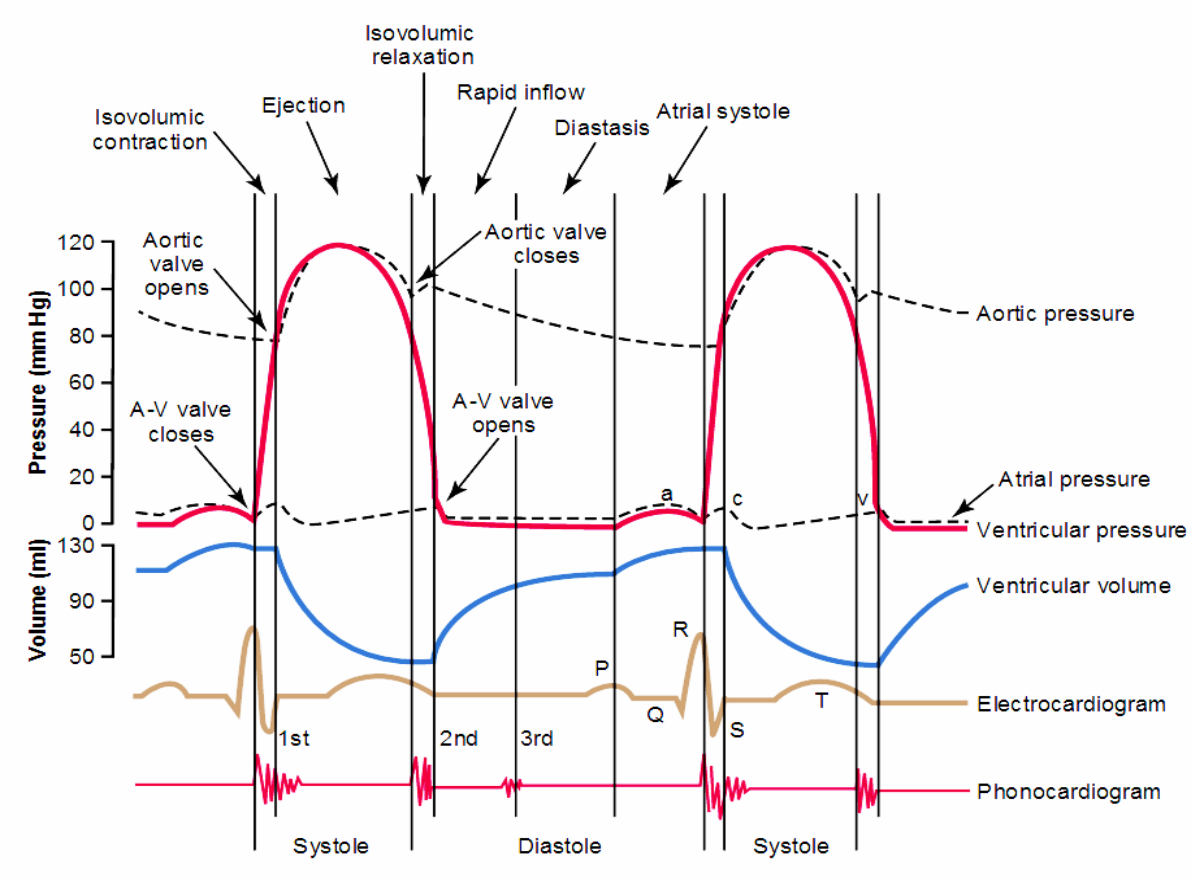

Fig. 3: Steps of the cardiac cycle for left ventricular function, showing changes in aortic pressure (black dotted line at top), the electrocardiogram (brown line), left atrial pressure (black dotted line in the middle), left ventricular pressure (red line), ventricular volume (blue line), and the phonocardiogram (pink line at the bottom) (Guyton and Hall, 2006).

1.1.3 Cardiac output

Cardiac output (CO) is a terminology used as measurement of the amount of blood pumped by each ventricle (stroke volume) in one minute. This is calculated by multiplying the stroke volume (SV) by the beats per minute of the heart rate (HR). So that: CO = SV x HR (Betts et al., 2013*).* The cardiac output is normalized to the body surface area and is called the cardiac index.

"The average cardiac output, using an average stroke volume of about 70 mL, is 5.25 L/min, with a normal range of 4.0–8.0 L/min" (Betts et al., 2013*).* An echocardiogram is normally used

to measure the stroke volume and can vary by the size of the heart, sex, duration of contraction, physical and mental condition of the individual contractility, preload and afterload (Fig. 3) (Betts et al., 2013*).*

While preload is called the filling pressure of the atria at the end of diastole. A huge factor influencing the preload is how long it takes the ventricles to fill- the preload will be less if the ventricles contract faster and then there is less time to fill those ventricles (Betts et al., 2013). There is another state that can influence the preload-person's blood volume. “The force of each contraction of the heart muscle is proportional to the preload, described as the Frank-Starling mechanism. This states that the force of contraction is directly proportional to the initial length of muscle fiber, meaning a ventricle will contract more forcefully, the more it is stretched” (Betts et al., 2013; Guyton and Hall, 2006).

How much pressure the heart must generate to eject blood at systole is called afterload. Afterload is influenced by the total peripheral resistance (TPR). TPR can itself vary by narrowing of the heart valves (stenosis) or contraction or relaxation of the peripheral blood vessels (Betts et al., 2013*).*

The stroke volume is heavily dependent on strength with which the heart muscle contracts. The agents termed as inotropes can influence it in a positive or negative way (Berry et al., 2007*).* “These agents can be a result of changes within the body, or be given as drugs as part of treatment for a medical disorder, or as a form of life support, particularly in intensive care units”. Positive inotropes are the inotropes that increase the force of contraction of heart muscle, and include sympathetic agents such as adrenaline, noradrenaline and dopamine (Bersten, 2013). Negative inotropes reduce the contraction force of heart muscle and include calcium channel blockers (Berry et al., 2007*).*

1.1.4 Electrical pacemaking in the sinoatrial node and electromechanical coupling in the myocardium

The resting heart rate of an adult person ranges from 60 to 100 bpm. While the resting heart rate of a newborn baby can be 129 beats per minute (bpm) and this keeps on decreasing until maturity (Ostchega et al., 2011). In case of an athlete, the heart rate can be lower than 60 bpm. While during physical exertion-exercise the heart rate can be as high as 150 bpm with maximum rates reaching from 200 to as high as 220 bpm (Betts et al., 2013).

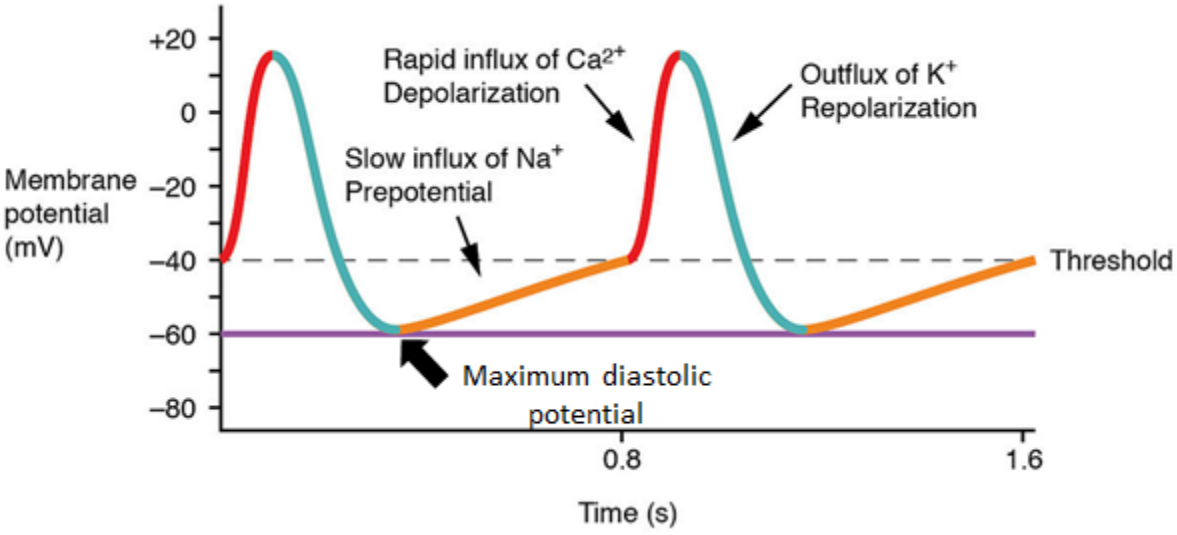

Fig. 4: Time course of the membrane potential during pacemaking in the sinoatrial node (Betts et al., 2013).

Sinus rhythm is the normal resting heart rate. It is created and sustained by a group of pace making cells found in the wall of the right atrium called the sinoatrial node. Sinus node pacemaker cells do not exhibit a stable resting potential. During diastole their membrane potential slowly depolarizes until the threshold of the L-type Ca^{2+}-current of -40 mV is reached. Then L-type Ca^{2+}-channels open and an action potential (AP) is elicited (Fig. 4). After reaching the peak of the AP around +20 mV the L-type Ca^{2+}-channels start to inactivate and K^{+}-channels slowly open. Increasing K^{+}-inward currents (I_{Kr}, I_{Ks}) together with the ceasing L-type Ca^{2+}-current lead to repolarization of the AP. Finally the maximal diastolic potential is reached and

the AP has ended. The phase of the slow diastolic depolarization depends on I_F and on decreasing K^+-conductances. I_F is a hyperpolarization activated cation current mostly carried by Na^+-inward current. These current systems are mainly responsible for the diastolic depolarization, which is terminated when the threshold of the L-type Ca^{2+}-current is reached (Betts et al., 2013).

Myocardial cells are electrically coupled by gap junctions; therefore the AP can be conducted from the sinus node cells into neighboring cells. Starting at the sinus node the AP travels over the atria to the atrial ventricular (AV) node and from there through the septum via the purkinje fibers to the ventricular wall. There the ventricular myocardial cells become depolarized and develop a ventricular AP (Betts et al., 2013).

Ventricular myocardial cells exhibit a stable resting membrane potential around -70 mV. When they are depolarized until around -50 mV, the threshold of the Na^+ channels is reached and the I_{Na} is elicited. The I_{Na} depolarizes the ventricular cells to +30 mV. During this process the L-type Ca^{2+}-current is activated. As long as the $I_{Ca,L}$ persists the membrane potential stays depolarized (plateau phase of the AP). When the $I_{Ca,L}$ inactivates and the K^+-currents activate the cell repolarizes and the AP ceases. The inward flow of Ca^+ during the plateau phase induces a Ca^+ release from the sarcoplasmic reticulum (SR) (Ca^{2+} induced Ca^{2+} release). This leads to an increase in $[Ca^{2+}]_i$ which induces the contraction cycle of the actin and myosin filaments in the sarcomeres. The contraction process ends when the cell repolarizes and Ca^{2+} is pumped back into the SR by the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Davis and Tikunova, 2008).

1.2 Epidemiology & pathophysiology of diseases of the cardiovascular system

Diseases of the cardiovascular system have long been the most common cause of death in Germany. In 2003 47% of deaths were attributed to cardiovascular diseases. Of these, 41% were due to ischemic heart disease. Myocardial infarction is, based on this data responsible for about 171,000 deaths in Germany each year (Löwel and Meisinger, 2006).

Myocardial infarction (MI) is a state when flow of the blood stops in a coronary artery or a succeeding vessel resulting in hypoxia leading to damage in the downstream cardiac tissue. Chest pain or discomfort is the most common symptom. The pain may travel into the shoulder, arm, back, neck, or jaw. This chest pain is mostly felt on the left side of the body and lasts for more than a few minutes. Moreover, atypical symptoms like shortness of breath, nausea, feeling faint, cold sweat, or feeling tiredness have also been reported by patients (Coventry et al., 2011*)*. About 30% of people have reported atypical symptoms of myocardial infarction (Steg et al., 2012*)*. Especially women have more atypical symptoms than their male counterparts (Coventry et al., 2011*)*. Out of these persons over 75 years old, about 5% have had an MI with little or no history of symptoms. MI may finally lead to an irregular heartbeat, heart failure, cardiogenic shock, or cardiac arrest (Valensi et al., 2011).

ECGs, blood tests like detection of troponin or creatine kinase, and coronary angiography are methods applied in early diagnosis of MI. An ST elevation (STE) during an ECG recording is taken as a sign for MI (Steg et al., 2012*)*. However, STE cannot be demonstrated in every case of MI. In 2015 in a cohort of 15.9 million of MI patients worldwide STE was detected in 3 million whereas it was not shown in 4 million. Thus missing STE does not guaranty the absence of MI.

Coronary artery diseases are the major causes of myocardial infarction. High blood pressure, high blood cholesterol, lack of exercise, smoking, diabetes, obesity, poor diet, and excessive alcohol intake are risk factors which promote myocardial infarction (Herold, 2003; Mehta et al., 2015; Mendis et al., 2011*)*. MI is often caused by complete blockage of a coronary artery due to a rupture of an atherosclerotic plaque. Coronary artery spasms due to cocaine abuse, significant emotional stress or extreme cold are more rarely responsible for MI (Devlin et al., 2008*)*.

As mentioned above the coronary heart disease (CHD) plays the main role in MI. CHD is a narrowing of the cardiac coronary vessels due to plaque formation. In contrast to MI, which is a sudden and permanent interruption of the blood flow, also transient discontinuations of blood flow can appear. This is referred to as angina pectoris (ang. p.). Ang. p. develops in arteries with pre-existing sclerosis due to CHD. In many cases the ang. p. serves as a pre-stage for myocardial infarction.

Heart failure (HF) is a state where the heart can't pump out enough blood to meet the requirements of the body. HF is generally a chronic condition which is associated with age and progresses gradually with time (Davidson, 2010). Right and left heart has the capacity to fail independently. Left HF can induce right HF by increasing strain on the right heart. “In a situation where heart cant not pump out sufficient amount of blood, it may accumulate throughout the body, causing breathlessness in the lungs (pulmonary congestion; pulmonary edema), swelling (edema) of the feet or other gravity-dependent areas, decrease exercise tolerance, or cause other clinical signs such as an enlarged liver, cardiac murmurs, or a raised jugular venous pressure”. The most common reported causes of HF are coronary artery disease, valve disorders and diseases of cardiac muscle (Davidson, 2010). But also MI especially in case of large necrotic areas may progress to HF.

The state in which heart muscle’s ability to contract is deteriorated is called cardiomyopathy and this can lead to heart failure. There are many types of cardiomyopathies and some are poorly understood. Some known reasons of cardiomyopathy include alcohol, toxins, systemic disease such as sarcoidosis, and congenital conditions such as hypertrophic cardiomyopathy (HOCM). The nomenclature of cardiomyopathies is described according to how they affect heart muscle. Cardiomyopathy can lead myocardial enlargement (hypertrophic cardiomyopathy), restriction of the outflow tracts (restrictive cardiomyopathy), or cause the dilation of the heart and thus affect the myocardial performance (dilated cardiomyopathy) (Davidson, 2010). “HOCM is often undiagnosed and can cause sudden death in young athletes” (Betts et al., 2013*;* Davidson, 2010).

Cardiac arrhythmias are the cardiac abnormalities of the normal sinus rhythm as well as of the AP conduction. They can prevent the heart from pumping the blood efficiently to the body. The abnormalities can be identified by ECG. These cardiac arrhythmias can appear during a regular heart rhythm, such as a rapid resting HR (tachycardia, faster than 100 bpm) or a slow resting HR (bradycardia, less than 60 bpm); or may occur as irregular heart rhythms. Atrial or ventricular fibrillations are other two types of cardiac arrhythmias with random or varying cardiac rhythms depending on the origination of the electrical activity either in the atria or the ventricles. In ventricular fibrillation there is no heart beat this is named asystole. Abnormal electric conduction in atria or ventricles has the potential to delay or alter contraction of the heart muscle. This delayed electric conduction can be a result of a disease process, such as a local block in the

myocardium or AV block, or congenital, such as Wolff-Parkinson-White syndrome (Davidson, 2010).

The congenital diseases may also affect the heart. Among these are ventricular or atrial septal defects, diseases of the heart valves (e.g. congenital aortic stenosis) or diseases relating to blood vessels or blood flow from the heart (such as a patent ductus arteriosus or aortic coarctation)" (Davidson, 2010; Kasper et al., 2011). Symptoms of congenital diseases appear at different ages. If a woman gives birth to a baby with noticeable blue skin, it may mean that unoxygenated blood is traveling directly from the right to the left side of the heart. This condition is called cyanotic and may affect the ability of the child to grow normally (Davidson, 2010). Also other congenital diseases can prevent normal growth of children. Up to 25% of the adults exhibit an insufficiently closed foramen ovale, which under specific conditions may allow blood flow from the right to the left atrium.

The pericardium connective tissue layer surrounding the heart can also be affected by diseases. Systemic disorders such as amyloidosis or sarcoidosis, tumors, high uric acid levels are known to be able to damage the pericardium. Infective diseases such as glandular fever, cytomegalovirus, coxsackievirus, tuberculosis or Q fever can attack the pericardium this is called pericarditis. Diseases of the pericardium can reduce the heart's ability to pump the blood, e.g. increased fluid between the pericardium and the heart can compress the myocardium and thus hamper the contraction (cardia tamponade). Pericardiocentesis is the treatment/process to remove this fluid out of pericardium using a syringe (Davidson, 2010).

1.3 Physiology and Pathophysiology of Ischemia Reperfusion

Occlusion of a coronary artery creates ischemic zones also called "area at risk" (AAR), this is the region without perfusion. The outer borders of the area at risk are sharply defined by a lack of communication between neighboring capillary beds (Berry et al., 2007; Sjoquist et al., 1984). After sufficient time cellular deterioration will take place in the AAR. During reperfusion additional stress will be exerted on the cardiomyocytes within the AAR, this leads to the so called reperfusion injury. Finally there will be a necrotic zone of dead cells within the AAR. The

detailed processes happening during the ischemic and the following reperfusion state will be explained in the next three chapters.

1.3.1 Ischemic injury

MI results in an interrupted blood flow, which causes an abrupt decrease of O_2 supply. This hypoxia prevents the continuation of oxidative phosphorylation and thus of oxidative adenosine triphosphate (ATP) production. The cells will metabolize creatine phosphate and switch from aerobic to anaerobic metabolism, thereby an accumulation of lactic acid happens and the ATP production is much less effective. As a consequence the ATP level falls by 65% after 15 min and by 95% after 40 min of ischemia (Adams et al., 2007; Calise and Powell 2013). Furthermore, an intra-extracellular acidification develops due to increased lactic acid and CO_2. The lack of ATP and the acidification leads to reduced activity of ionic pumps such as Na^+/K^+-ATPase, Na^+/H^+-ATPase and SERCA. This in turn causes intracellular rise in Na^+ and Ca^+ as well as loss in K^+. Consequently the extracellular K^+ concentration rises. The cells depolarize and develop a moderate contracture. This contracture weakens the cellular cytoskeleton. If the ischemia lasts more than 30 min, the cardiomyocytes start to undergo necrosis. The longer the hypoxia lasts, the more cardiomyocytes will deteriorate. Therefore a fast reperfusion is the only way to limit the ischemic insult (Bopassa, 2012)

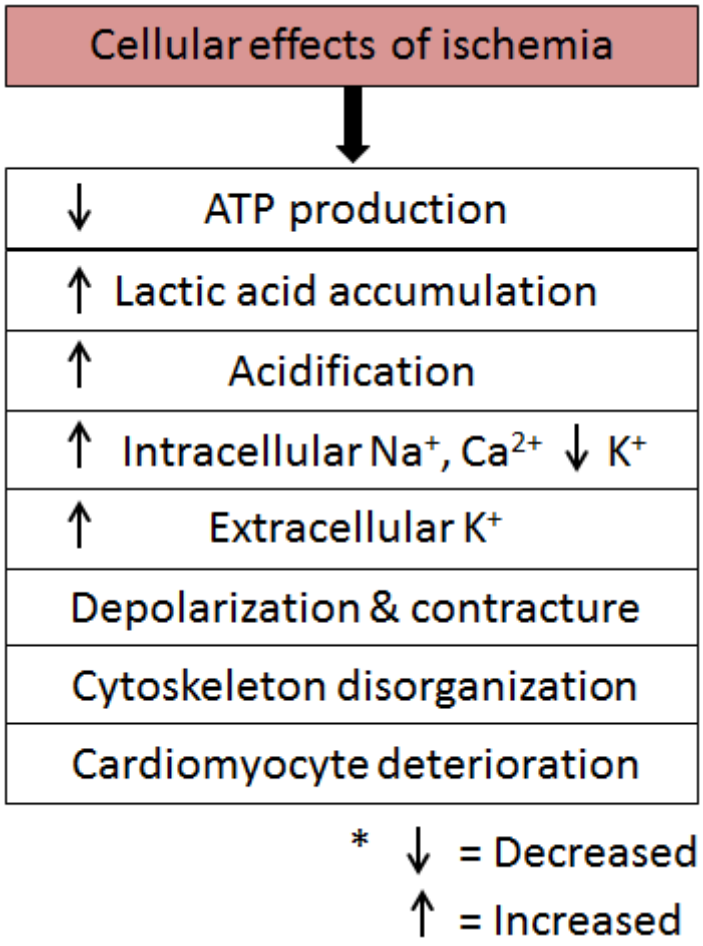

Fig. 5: Ischemia results in a variety of structural and metabolic changes in the cardiomyocytes.

1.3.2 Reperfusion Injury

Reperfusion, however, causes additional damage to the cardiac tissue. This damage is called "reperfusion injury". In reperfusion, the depolarized acidified cardiomyocytes with high intracellular Na^+ are again perfused with solution rich in O_2 and Na^+. In this situation Na^+/Ca^{2+} exchanger will transiently run reversely and Ca^{2+} will also enter the cardiomyocytes via L-type Ca^{2+} channels. This leads to an intracellular Ca^{2+} overload which can be counteracted by additional storage of Ca^{2+} in the SR. However, the SR starts to release Ca^{2+} spontaneously leading to Ca^{2+} oscillations. In this situation, mitochondria start to take up Ca^{2+} to rescue the cardiomyocytes. Furthermore, the increasing O_2 will promote the production of reactive oxygen species (ROS). Taken together, the cardiomyocytes weakened during ischemia may now

deteriorate completely due to the Ca^{2+} overload and the toxic effects of ROS. However, it is known that enzymes necessary for apoptosis are also activated during early reperfusion. The region of dead cells lies within the AAR. The processes described here occur within the first minutes after reperfusion. The amount of reperfusion injury depends highly on the duration of ischemia. The existence of dead cells and debris within the myocardium triggers a chain of inflammatory reactions (Yellon and Hausenloy 2007).

1.3.3 Post-ischemic inflammation

Following the ideas Frangogiannis and Nahrendorf and coworkers (Entman et al., 2000; Frangogiannis 2008; Frangogiannis et al., 2002; Leuschner et al., 2012; Nahrendorf et al., 2007), the inflammatory post-ischemic reactions can be divided into three different states:

1. Initiation of an inflammatory reaction

2. Invasion of immune cells and clearing of the necrotic zone

3. Repair and remodeling

1.3.3.1 Initiation of an inflammatory reaction

The reperfusion is accompanied by a vasodilation within the AAR, which also leads to increased permeability of the endothelium. This followed by a passage of blood plasm into the AAR mainly the necrotic zone, where an edema develops (Garcia et al., 2012). The release of intracellular contents called damage associated molecular patterns (DAMPs) from necrotic cells starts the inflammatory chain. Among the DAMPs are the high-mobility group box-1 (HMGB-1) protein, heat shock proteins (HSP), ATP and matrix fragments like fibronectin. Some of these like HMGB-1 and fibronectin bind to Toll-like receptors (TLR) and thus activate the innate immune system (Nahrendorf et al., 2010). TLRs have been shown to play an important role in the post-ischemic inflammatory reaction e.g. by activation of Nuclear factor (NF)-κB a transcription factor which leads to the expression of TNF-α and IL-1β as well as other cytokines. Interestingly, TLR4 deficiency attenuated the inflammatory reaction and was associated with reduced infarct sizes (Stapel et al., 2006).

As mentioned above ROS production starts already in the early reperfusion. ROS help to initiate inflammatory chain in different ways by stimulating the complement reaction and the cytokine and chemokine expression. The cells neighboring to the necrotic zone release pro-inflammatory cytokines as well as chemokines. Cytokines and chemokines are washed into the bloodstream and trigger an intensified inflammatory reaction in which cells of the immune system, e.g. macrophages and lymphocytes are recruited into the myocardium (Stumpe et al., 2001). It is important to mention, that the inflammatory reaction has to be limited in time and amount as an overwhelming inflammation will have deleterious consequences, especially in case of ROS. Thus the positive and the negative consequences of inflammation have to be balanced within narrow limits (Cadenas, 2018)

1.3.3.2 Invasion of immune cells and clearing of the necrotic zone

Activation of the complement system as well as expression of specific chemokines like CXCL8 and CXCL1 attracts neutrophils as well as Ly-6C^{high} monocytes, which both are potent phagocytotic and pro-inflammatory immune cells. Thus both will clear the cellular debris in the necrotic zone and keep the inflammatory reaction at a high level by secreting further pro-inflammatory cytokines. Furthermore, these cells secrete matrix metalloproteinases (MMPs). These enzymes cleave the components of the existing extracellular matrix thus helping the invasion of further immune cells and preparing the extracellular space for repair processes. At this state it is important to downregulate pro-inflammatory processes to initiate repair. Therefore the further invasion of pro-inflammatory cells has to be downregulated and reparative cells have to be attracted to the infarct zone possibly by the chemokine CX3CR1. Ly-6C^{low} monocytes have been characterized as specialized in repair and attenuation of inflammation (Nahrendorf et al., 2007; Swirski et al., 2009).

1.3.3.3 Repair and remodeling

Ly-6C^{low} monocytes are known to express transforming growth factor (TGF)-β, vascular endothelial growth factor (VEGF) and IL-10. IL-10 is a well-known anti-inflammatory cytokine

which has been shown to play an important role in I/R (Markowski et al., 2013). TGF-β also exhibits anti-inflammatory characteristics and stimulates myofibroblasts to synthesize and deposit collagen. VEGF induces vascular growth which enables blood supply to new tissue areas. As cardiomyocytes are unable to divide, a scar of connective tissue has to grow in the former ischemic region. This scar is mainly formed by myofibroblasts. Furthermore, other parts of the ventricular wall have to develop more power to take over contractile activity of the necrotic region. Development of the scar as well as the adaptation processes in the non-ischemic myocardium are called remodeling. Remodeling has to guarantee that the post-ischemic myocardium matches the requirements of the body. If the heart cannot suffice these requirements it will proceed to HF (Frangogiannis 2014)

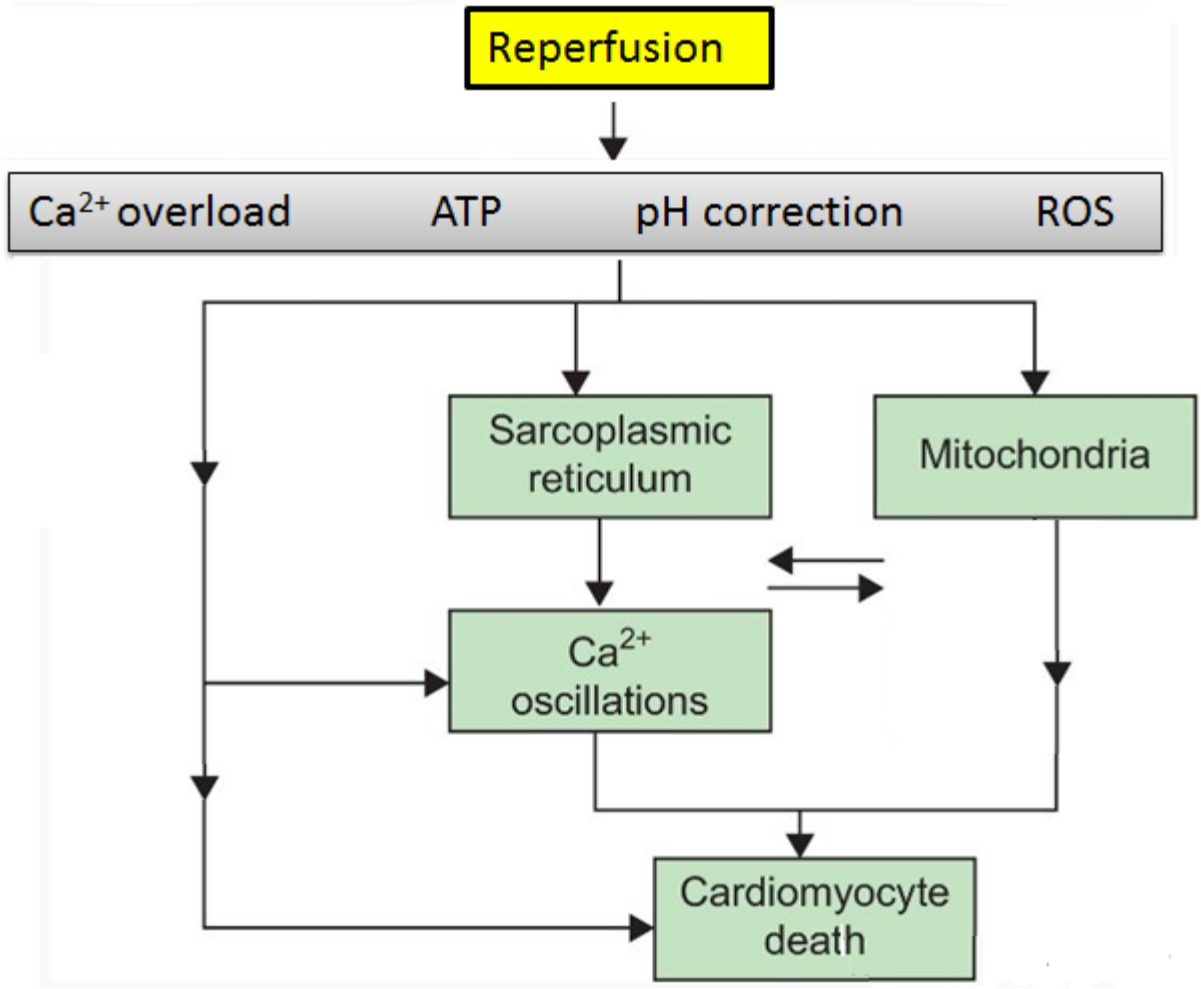

Fig. 6: Diagram showing the main mechanisms of cardiomyocyte death progressively during myocardial reperfusion.

1.4 Role of myocardial pre- and post-conditioning in cardiac remodeling

I/R injury in hearts vary even in the case of comparable ischemia times. The history of the heart seems to play a role in the extent of an ischemic injury. This led to the idea that pre-treatment of a heart may help to survive an ischemic period better (Murry et al., 1986). The observation that people with ang. p. seem to have smaller infarcts than formerly healthy persons supported this idea (Abete et al., 1997; Mladenovic et al., 2008). In experiments repetitive short periods of ischemia prior to an infarction have diminished infarct size (Riksen et al., 2004-a; 2004-b). This treatment was named ischemic pre-conditioning (IPC). Application of pharmacological

substances prior to an infarction was called pharmacological pre-conditioning. Every treatment which was applied prior to an infarct in order to reduce effects of ischemia has been called pre-conditioning (Demirehl et al., 2001; Markowski et al., 2013; Taylor et al., 1999).

Besides pre-conditioning also post-conditioning has been tried (Cohen et al., 2007; Zhao et al., 2003; Zhao et al., 2006). Post-conditioning (POC) means a treatment is applied after the ischemic period or at the end of the ischemic period before reperfusion. In the clinical setting the concept of POC is much more interesting because it allows treating a patient after the infarct has happened, i.e. after the patient has reached the clinic. Many experimental studies as well as some clinical studies on POC have been performed.

In general both pre- and post-conditioning aim to two different processes the ischemic and/or the reperfusion injury. Hypoxic pre-treatments may prepare the organism for the ischemic period. Murry et al. (1986) were the first to apply an IPC protocol. 4 periods of ischemia of 5 min duration and by 5 min of reperfusion each were followed 40 min of sustained ischemia. This reduced the infarct size by 75%. The explanation for the beneficial influence of IPC was rather wage in this early study. Meanwhile many molecules and pathways which help to reduce ischemic insult after IPC have been identified, for review see Heusch (2015). Even inducing an ischemia in another organ than the heart prior to MI can reduce the ischemic insult in the myocardium. This is called remote ischemic pre-conditioning (RIC). As RIC was successful in animal experiments it was also tested in multicenter phase III clinical trials, however without clear positive outcome (Heusch and Gersh, 2016).

An example for pharmacological pre-conditioning is a recent work from our group (Markowski et al., 2013). In this study a moderate inflammation was induced by i.p. injection of 0.2 mg/g of CPG-oligodeoxynucleotides (CPG-ODN) 16 h before a 60 min ischemia. This reduced the infarct size by 75% which was explained by a reduced inflammatory reaction due to an increased IL-10 expression caused by the pre-conditioning.

Zhao et al. (2003) invented the concept of ischemic post-conditioning (IPOC) in experiments on dogs. After a 45 min long ischemia at the start of reperfusion, three cycles of 30 s reperfusion and 30s (left anterior descending artery) LAD re-occlusion preceded the continuous reperfusion. They compared their post-conditioning results to a control group with ischemia and reperfusion

only and a group with hypoxic pre-conditioning. Infarct size was 25% of AAR in the control but only around 15% in the pre- and post-conditioned groups. As ischemic post-conditioning relies on brief iterative episodes of reperfusion before the continuous perfusion starts, the very early events during reperfusion which induce Ca^{2+}-overload of the SR and formation of ROS may be involved in this process. Recently a study underlined this by showing that Ca^{2+}-overload of the SR is reduced by IPOC (Inserte et al., 2014). Like pharmacological pre-conditioning also pharmacological post-conditioning has been tested successfully in experiments. A study of our group showed that i.p. injection of 1 mg/kg of the sphingosine-1-phosphate agonist FTY720 after 1 h of ischemia and before 24 hour of reperfusion reduced the infarct size by 61.26%. In this case invasion of Ly-$6C^{high}$ monocytes was reduced by the FTY720 treatment (Goltz et al., 2015).

1.5 Role of proteasome inhibition in I/R

As explained above NF-κB is a central transcription factor in initiation of the inflammatory cascade starting directly after reperfusion in the necrotic area. Activation of NF-κB depends on phosphorylation of IκB an inhibitor of NF-κB activation. After phosphorylation of IκB it separates from NF-κB and is degraded via the Ubiquitin Proteasome System (UPS). This can be prevented by proteasome inhibition and block of IκB degradation will as a consequence reduce NF-κB activation. Decreased NF-κB activation may reduce the intensity of the inflammatory cascade after I/R, which may have beneficial influence on the resulting infarct size and the outcome. This hypothesis was first proposed in a review of Yu and Kem (2010) and was supported by the results of a series of investigations, which demonstrated reduced cardiac infarct size after application of the proteasome inhibitors PR-39, PS-519 and bortezomib (Bao et al., 2001; Pye et al., 2002; Stansfield et al., 2007; Yu et al., 2005). However, in a later review Calise and Powell (2013) discussed that PS-519 may not develop its function by NF-κB inhibition. Therefore further investigations seemed to be necessary.

Another interesting aspect of the block of UPS is the interaction of proteasomes with hypoxia inducible factor (HIF). HIF-1α is transcription factor which is responsible for the cells to adapt the inflammatory and hypoxic conditions (Betts et al., 2013; Cramer et al., 2003; Frede et al.,

2007). In addition, several studies have documented different interactions between NF-κB and HIF (Frede et al., 2006; Frede et al., 2009; Görlach and Bonello, 2008).

It is important to know that HIF-1α is usually expressed constitutively and degraded constantly by the UPS. In normoxic conditions HIF-1α expression and degradation is in steady state, i.e. there is a low but constant level of HIF-1α present in the cytoplasm. In hypoxia UPS dependent HIF-1α degradation is inhibited, but the production is still stable, HIF-1α level in the cells will rise (Jaakkola et al., 2001). Thus pharmacological UPS inhibition will simultaneously increase the level of HIF-1α and decrease NF-κB in the cardiac cells (Fig. 7).

The first potent proteasome inhibitor, which was clinically available, is Bortezomib (Velcade ®), which is indicated for the treatment of multiple myeloma. This was chosen for the UPS inhibition in this study (Bedford et al., 2011).

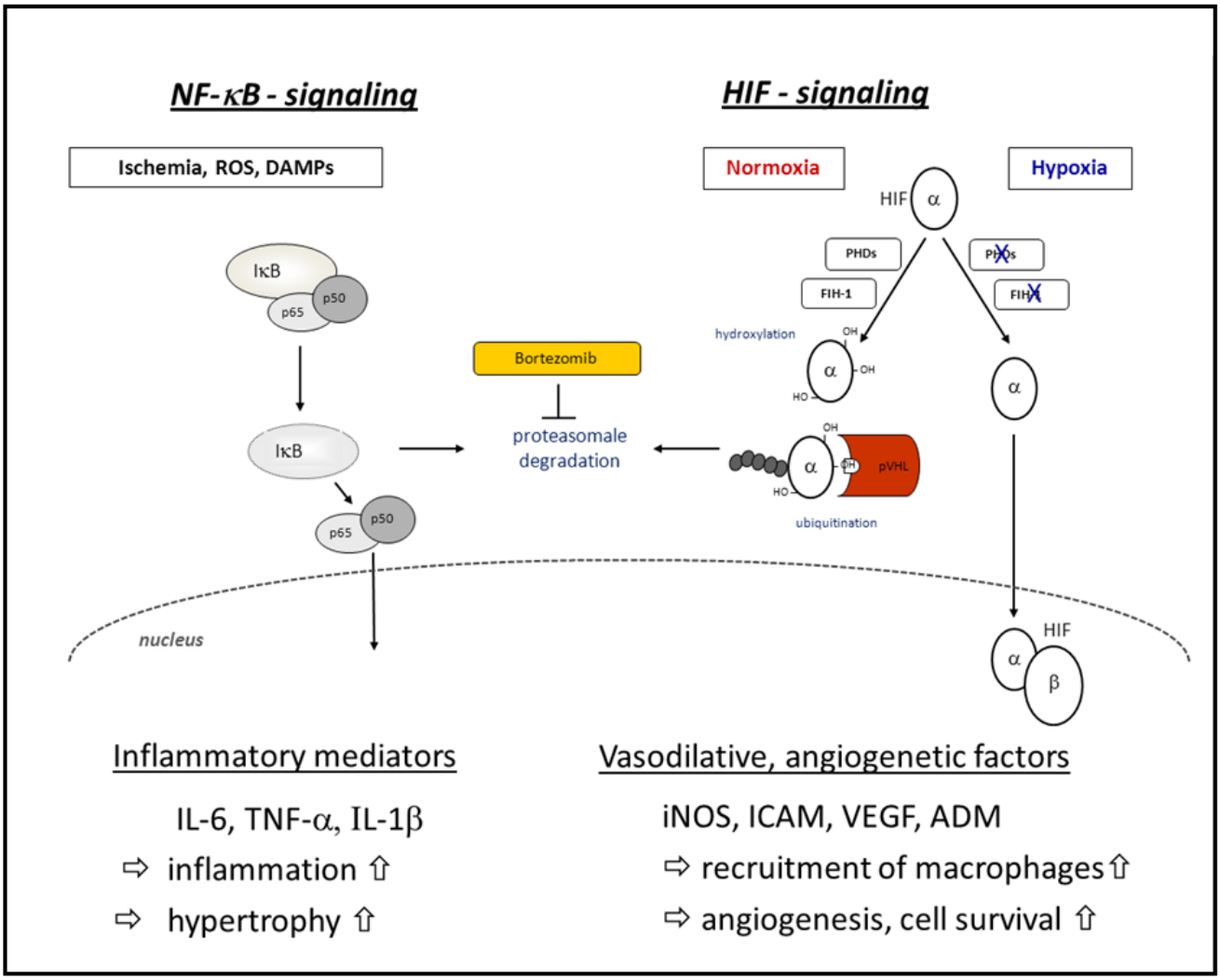

Fig. 7: Schematic explanation of interactions between nuclear factors NF-κ B and HIF and the proteasome system (Gölz et al., 2011).

1.6 Aim of the study

The aim of the study on the one hand is to suppress the ischemia induced inflammatory reaction and on the other hand to promote cardioprotective mechanisms. In this context, two hypotheses were formulated:

1. Repetitive cycles of ischemia and reperfusion will raise the cardiac HIF-levels and reduce the sensitivity of the myocardium to inflammation.

2. Inhibition of the UPS will raise the cardiac HIF-levels and attenuate NF-κB levels in the cardiomyocytes and thus decrease inflammation.

3. The different treatments will decrease ischemic injury and help to preserve cardiac function.

4. The different treatments will facilitate remodeling after I/R and thus allow better recovery of cardiac function.

To investigate these hypotheses IPC was applied to one group and pharmacological pre- and post-conditioning with bortezomib to inhibit UPS was applied to another group of mice.

2 Materials and Methods

2.1 Experimental animals

The female C57BL/6 experimental mice used were between 9-12 weeks old and were bought from Charles River Deutschland GmbH. The mice were kept in individually ventilated polycarbonate transparent pathogen free cages measuring 365X207X140 mm with bedding (ASBE-wood GmbH, Ahrensfelde, Germany). The room temperature was set between 20-22°C and at 50% humidity. The day and night cycle was set to 12 hours. The experimental animals were given free access to standard rodent chow (sniff Spezialdäten GmbH, Seost, Germany) and tap water. All mice were housed and kept according to the principles of laboratory animal care (NIH publication no: 85-23 revised 1996) and experimental procedures followed the rules of the German Protection of Animal Acts from 8th of May, 2006; changed on 7th August 2013 (Animal rights 18th May 2006, changed 7th August 2013). The experiments were approved by LANUV (Landsamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen). The tracking number of the project is: 84-02.04.2011.A358.

2.2 Experimental protocols

Table Nr. 1: The 9-12 weeks old experimental animals were divided into following experimental groups.

Nr.	Experimental Group	Treatment
1	Sham	No treatment
2	60 min ischemia	6 x 15 min sham ischemia + 1 x 60 min ischemia
3	Repetitive group	6 x 15 min ischemia + 1 x 60 min ischemia
4	Repetitive group + bortezomib	6 x 15 min + 1 x 60 min ischemia + bortezomib
5	60 min ischemia + bortezomib	6 x 15 min sham ischemia + 1 x 60 min ischemia + bortezomib

60 min groups (60 min Ischemia and 60 min Ischemia + Bortezomib) and repetitive groups (Repetitive group and Repetitive group + Bortezomib) underwent LAD artery ligation whereas the sham group was operated without LAD ligation. The purpose of LAD ligation is to induce experimental ischemia/reperfusion (I/R) injury to simulate myocardial infarction (MI) in mouse models. Details of LAD mouse model are explained in section 2.3. The experimental protocol of repetitive groups and 60 min groups is shown in figs. 8, 9, 10 and 11

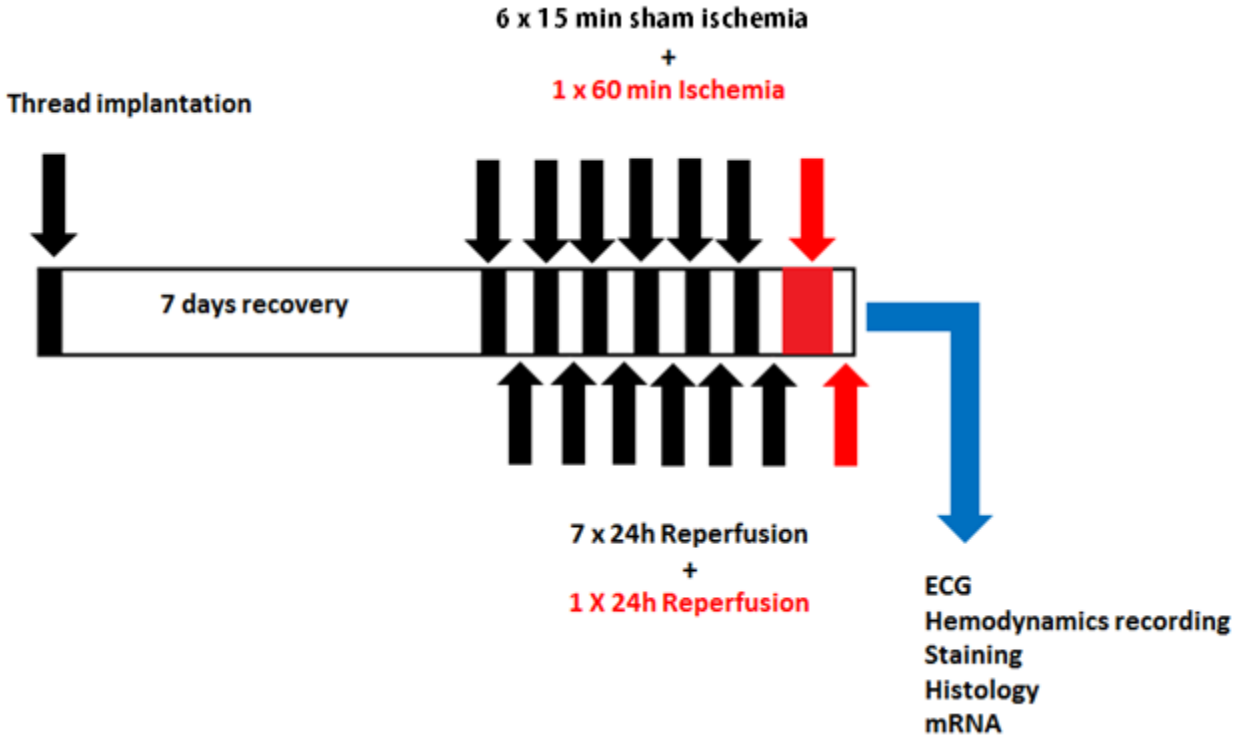

Fig. 8: A diagrammatic overview of the 60 min experimental protocol.

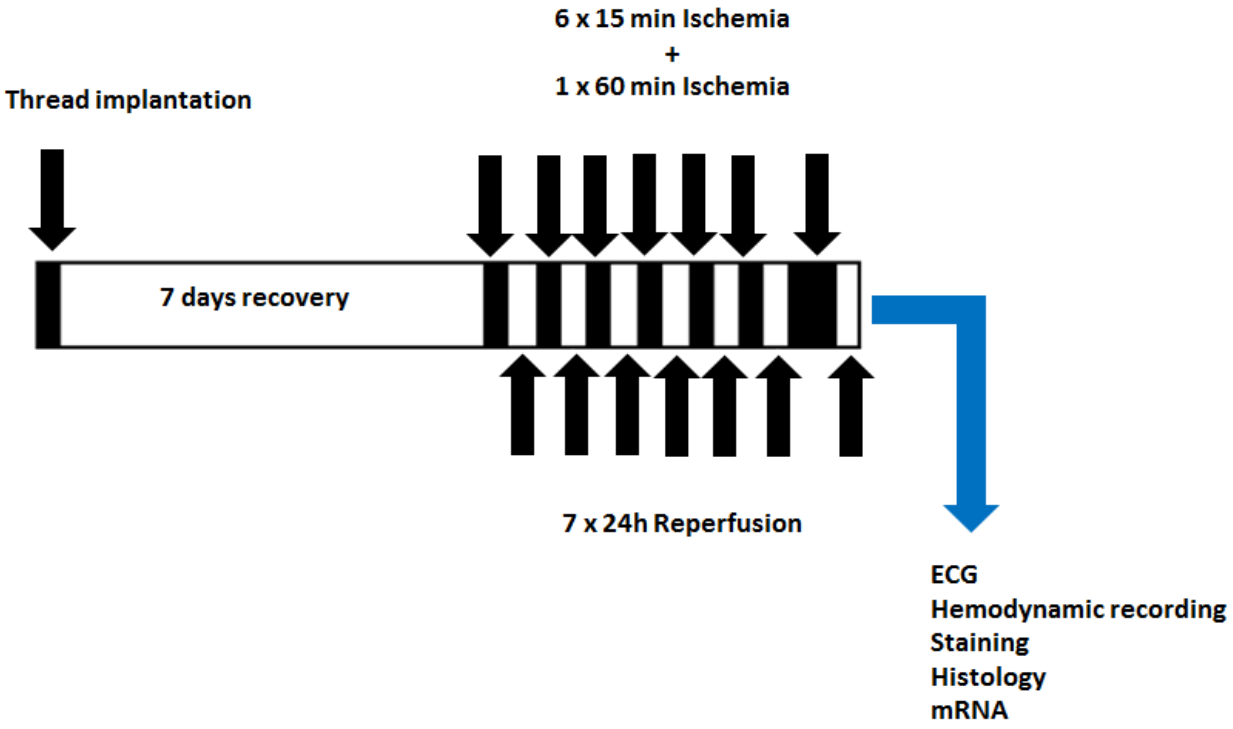

Fig. 9: A diagrammatic overview of the repetitive experimental protocol.

Two counter-groups of the repetitive group and the 60 min group were treated the same way but a proteasome blockade was initiated at the onset of the first, fourth and last reperfusion by injecting 0.05 mg/kg of bortezomib (Santa Cruz Biotechnology, Dallas, TX, USA) into the tail vein. The experimental protocol of these groups is explained in Fig. 10 and 11. The bortezomib concentration was chosen according to recently published studies (Lee et al., 2009; Marfella et al., 2009; Yu et al., 2005). For the study, bortezomib was dissolved in 0.9% NaCl. The control groups received equivalent doses of 0.9% NaCl.

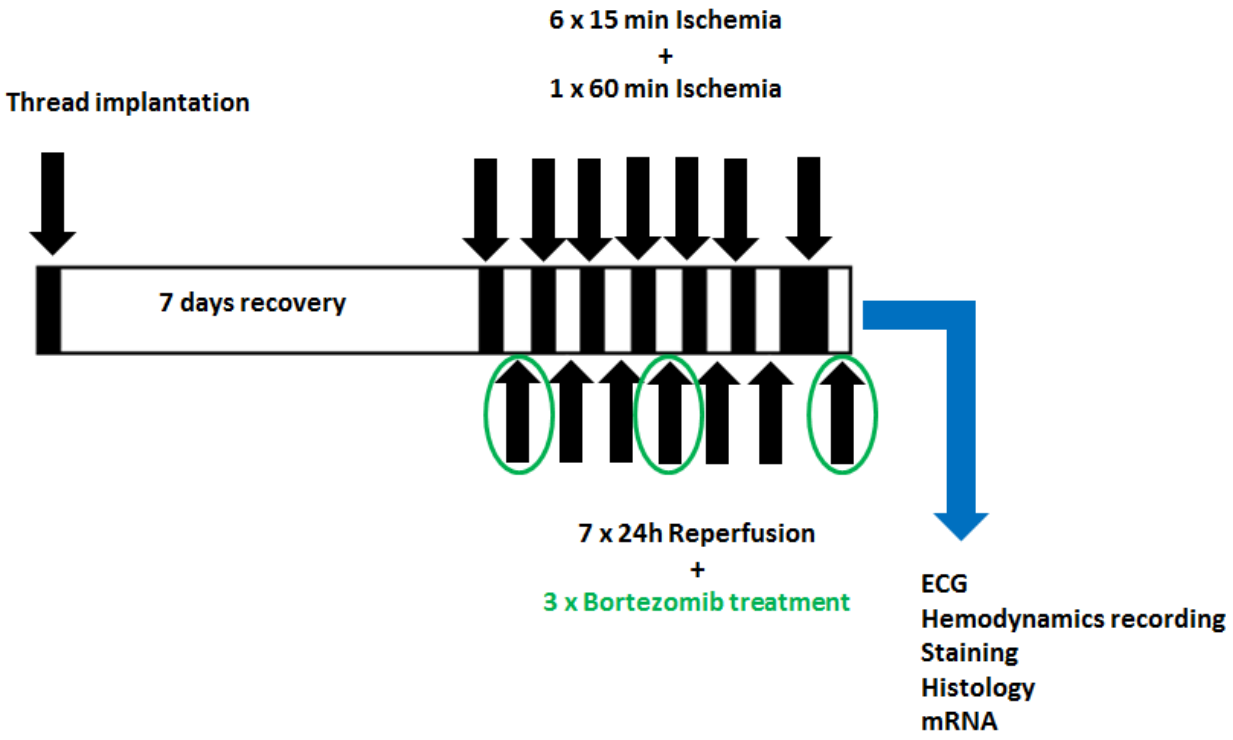

Fig. 10: A diagrammatic overview of the repetitive + bortezomib treatment experimental protocol.

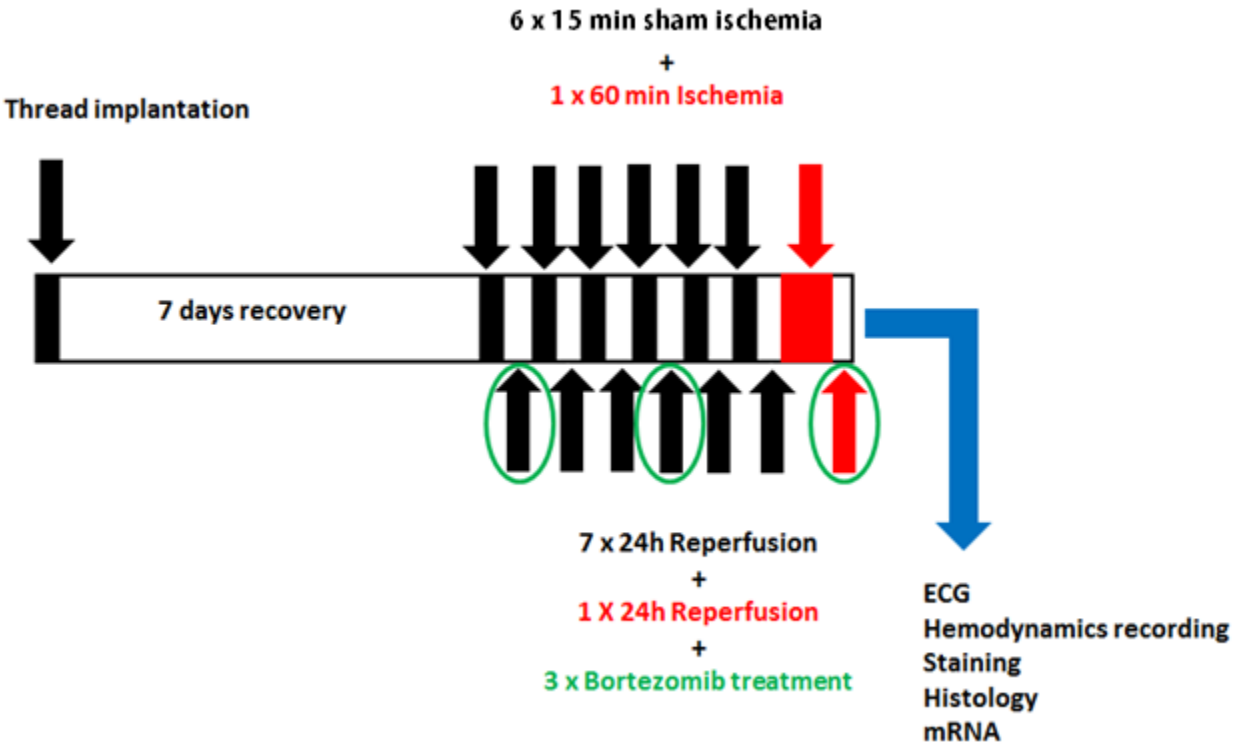

Fig. 11: A diagrammatic overview of the 60 min Ischemia and 60 min Ischemia + bortezomib treatment experimental protocol.

2.3 In *vivo* experiments

2.3.1 LAD ligation

LAD ligation as research model of infarction and myocardial ischemia is essential to investigate the acute and chronic pathophysiological processes in myocardial ischemia and reperfusion to develop and optimize future treatment.

2.3.1.1 Pre-surgical preparations

The operating table was disinfected using 70% ethanol. A water bath (Fig. 12-A) was connected to the operating field (Fig. 12-B) in order to circulate warm water under the under the mouse

operating field to maintain the body temperature at 37°C. It's of ample importance to maintain the normal body temperature during LAD ligation process to avoid a rapid fall in HR. Other pre-surgical preparations included the control check for the optimal function of the ventilator, isoflurane vaporizer, infrared heating lamp and oxygen flow.

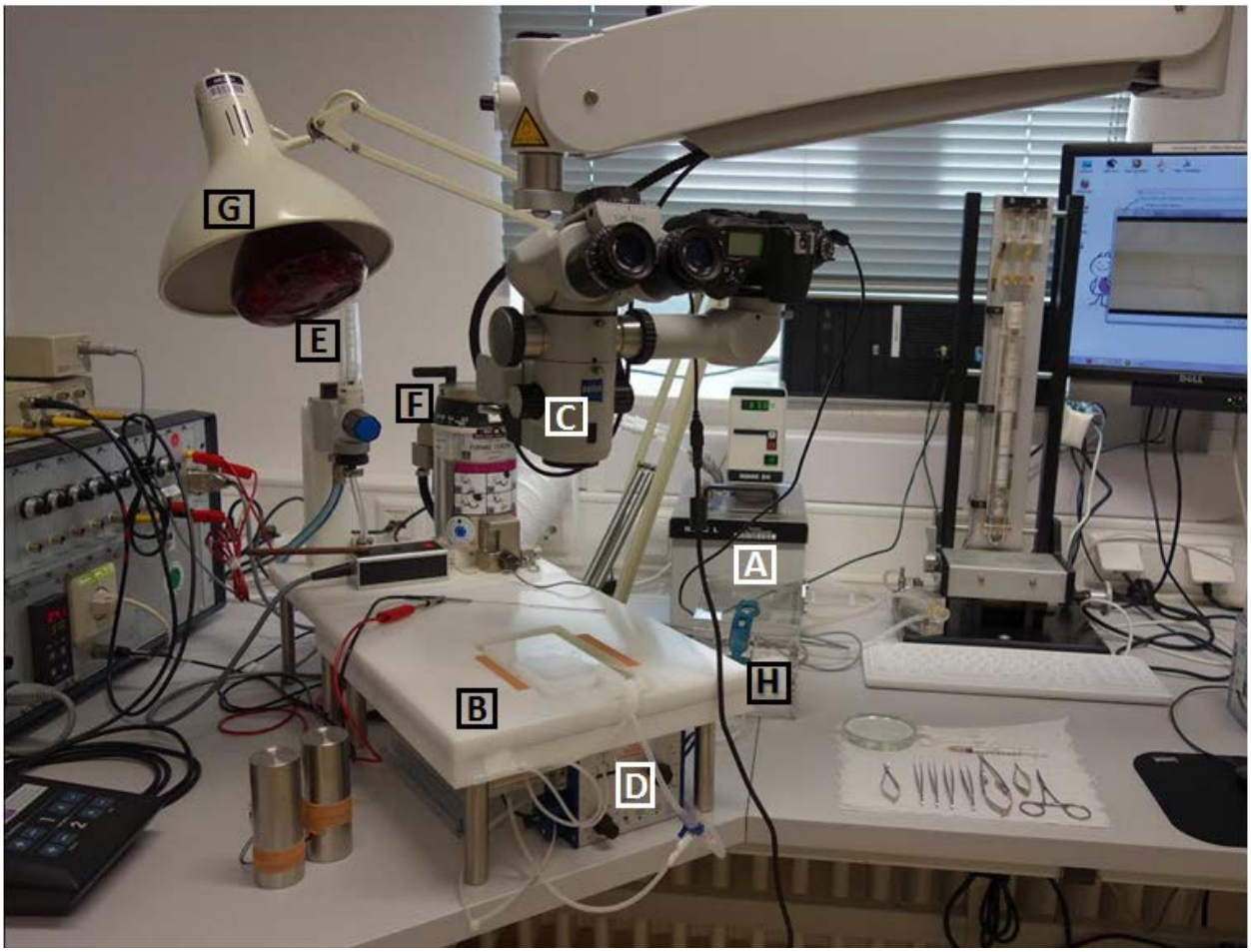

Fig. 12: An overview of the surgical setup. (A) Water bath, (B) operating table, (C) operating microscope with digital camera, (D) ventilator, (E) oxygen flow control meter, (F) isoflurane vaporizer, (G) infrared heating lamp, (H) anesthesia inducing acrylic glass chamber (Bualeong, 2015)

The surgical tools were sterilized by autoclaving. The instruments used in mouse surgeries included: curved scissors, fine curve forceps, mosquito clamps, chest retractors, needle holder and cubic curve forceps (Fig. 13).

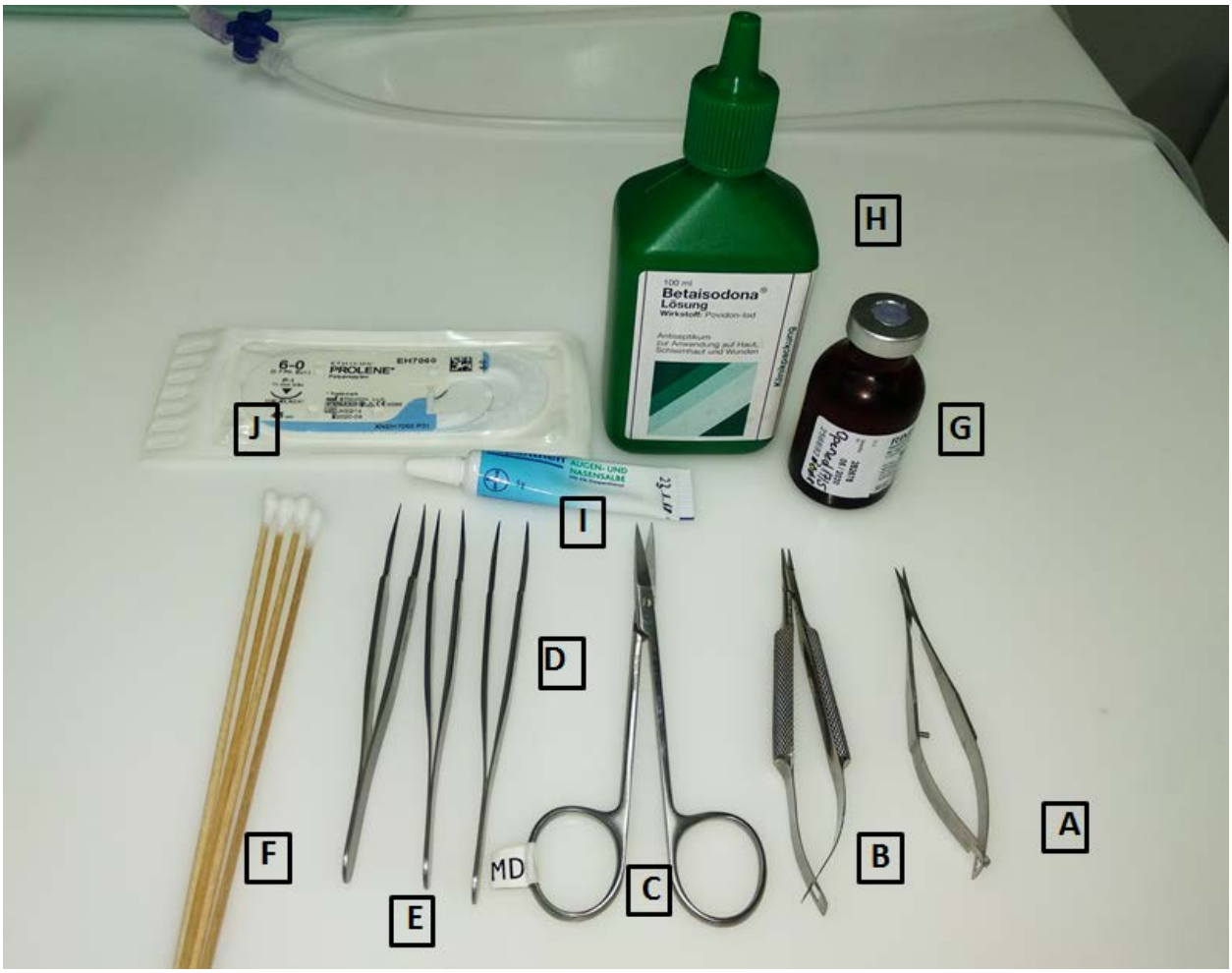

Fig. 13: The surgical tools used for the surgeries. (A) small scissors, (B) needle holder, (C) scissors, (D) forceps, (E) fine curve forceps, (F) cotton tips, (G) buprenophine Temgesic, (H) betaisodona skin disinfectant, (I) Bepanthen eye ointment, (J) prolene suture.

2.3.1.2 Animal preparations

On the day of experiment, mice were weighed on an electronic balance (Precision balance, Kern EMB 500-1, Kern & Sohn, Balingen). The mouse was then transferred to a transparent anesthesia induction chamber (Fig.12-H). The anesthesia inducing chamber was ventilated with 2% isoflurane (Abbot GmbH, Wiesbaden) and 0.5- 1.0 l/min 100% O_2. Once a mouse is visibly

anesthetized, it was removed from anesthesia inducing box and was placed on the pre-warmed surgical table (Fig. 12-B) in spine position with its head pointing to the surgeon. The tail was straightened and the paws were taped to the warm surgical table. Once the animal was fixed to the operating table using an adhesive tape, a continuous dose of 2% isoflurane + 100% O_2 were kept flowing into the inhalation tube of the mouse to maintain anesthesia. An eye ointment (Bepanthen®, Bayer vital GmbH, Leverkusen, Germany) was applied into the both eyes to avoid dry eye burns and post-surgical eye irritations. The nose of the mouse was put into the wide side of a plastic tube that was connected to the Y-branch of the ventilator. The 2% isoflurane flow from the isoflurane vaporizer (Fig. 12-F) and combines with a flow of 100% O_2 (0.5% - 1.0% l/min) from an O_2 flow meter (Fig. 12-E) to provide a continuous supply of 2% isoflurane" (Bualeong, 2015). In order stabilize the body temperature of the mouse; a temperature probe was inserted into the rectum of the mouse. This temperature probe was connected to an automatic temperature control unit which switches on the heating infrared lamp (Fig. 12-G) once the body temperature of animal has fallen below 37°C to restore the normal body temperature. The neck of the animal was extended and fixed using a thread around front incisors.

2.3.1.3 Endotracheal intubation

Once the mouse is fixed to the operating field, neck and chest regions were disinfected using Betaisodona Fig. 13-H). Then hair from the neckline to middle of chest was removed using a readily available hair removing cream (Fig. 14). After this, the tongue of the mouse is gently taken out using a forceps. A longitudinal cut was made from the neckline to the middle of the chest and down to 5th intercostal space using a scissors. Trachea and larynx were exposed by separating the submandibular salivary glands and sternothyroid muscles using a pair of curved forceps. The larynx was fixed in the left hand using a curved forceps, the tongue was lifted, and an endotracheal tube was put into oral cavity and was advanced towards the larynx. By visualizing the endotracheal tube in the larynx, it was gently pushed into tracheal tube. At this step, the endotracheal tube was visible in the trachea of the mouse (Fig. 15). Without any delay, the mouse was attached to a rodent ventilator (MiniVent 845, Hugo Sachs Elektronik, March - Hugstetten, Germany) (Fig. 12-D) at a rate of 110 breaths per minute and at a tidal volume of 0.2

ml. The mouse was injected 0.1 mg/kg of buprenorphine (Buprenorphine hydrochloride 0.3 mg in 1 ml, Reckitt Benckiser Pharmaceuticals Inc, Richmond) intraperitoneally for analgesia. Meanwhile, 2.0% isoflurane with 0.5 – 1.0 l/min 100% O_2 was supplied to the animal via the inhalation tube to maintain anesthesia.

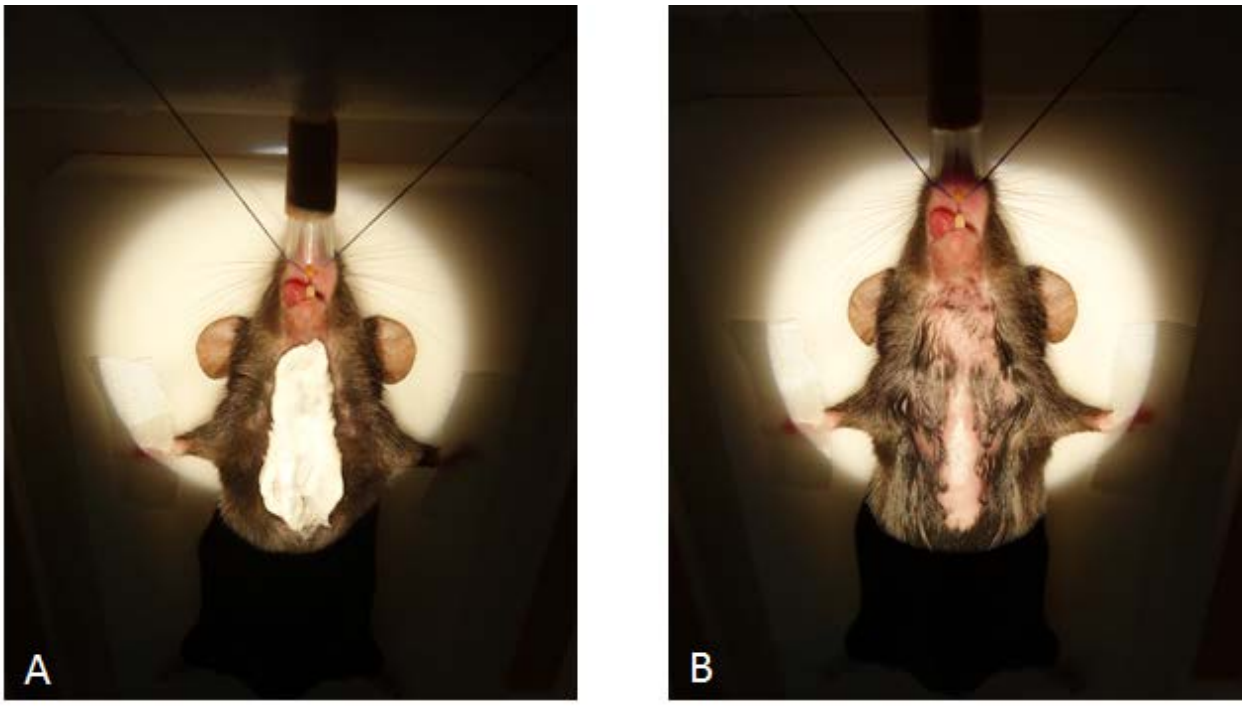

Fig. 14: Pre-surgical epilation using common hair removing cream. (A) Before. (B) After (Bualeong, 2015)

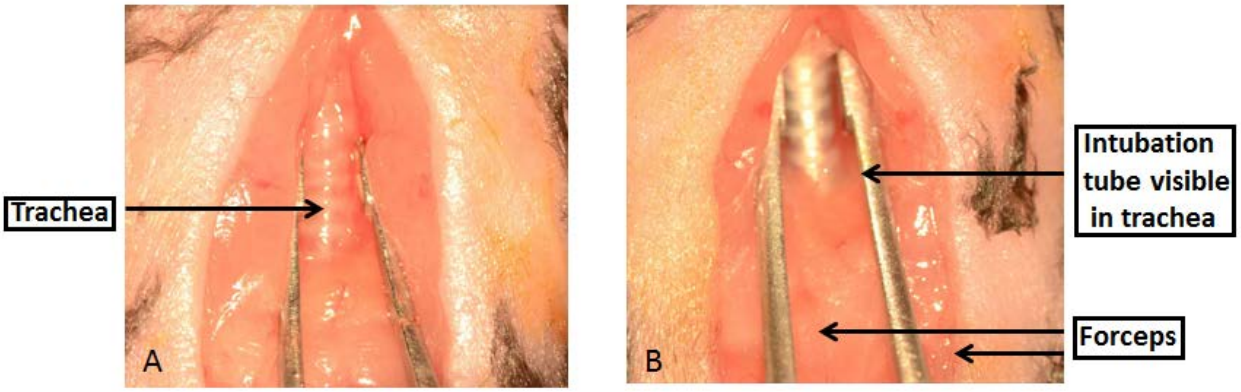

Fig. 15: Endotracheal intubation. (A) Before. (B) After, the intubation tube insertion in the trachea (Bualeong, 2015).

2.3.1.4 Left Anterior Descending artery ligation

In order to ligate the LAD, the ribs of the mouse were visualized; pectoral muscles were taken out of the way by forceps to make room for the place where thoracotomy was to be made (Fig. 16-A). Then the surgeon opened the thorax for about 2 mm lateral of the sternum with the help of forceps by making a hole through the intercostal space and pleura (Fig. 16-B). The intercostal space was enlarged by retracting the ribs using a pair of retractors (Fig. 16-C). Special care is necessary to avoid any possible injury to lungs during the intercostal space incision and retraction process. Pericardium was opened using a forceps. LAD was visualized by using high magnification lens (16 X) and if needed, by lifting the left auricle with a forceps. Then a curved needle which was attached to 8-0 prolene suture (Naht material 6-0 Prolene®, Ethicon GmbH, D-Norderstedt) was passed under the LAD at about 2-3 mm depth (Fig. 17-A). In the next step, both ends of the suture were passed through a 2 mm long polyethylene tube which serves as an occluder to close the LAD during ischemia induction (Fig. 17-B). Once both ends of the silk suture were passed through the polyethylene tube, both ends were drawn out through the intercostal muscles and to the outer muscles. Then both ends were knotted together to form a loop and this loop was placed under the skin of the mouse (Fig. 17-C). During the ischemia induction, this loop will be stretched by weight and will induce ischemia to the mouse heart. In the end, the retractors were removed, opened the intercostal space was stitched using a single stitch of 6-0 prolene suture (Fig. 18-A). The incised outer skin was also stitched with a continuous suture pattern using 6-0 prolene sutures (Fig. 18-B).

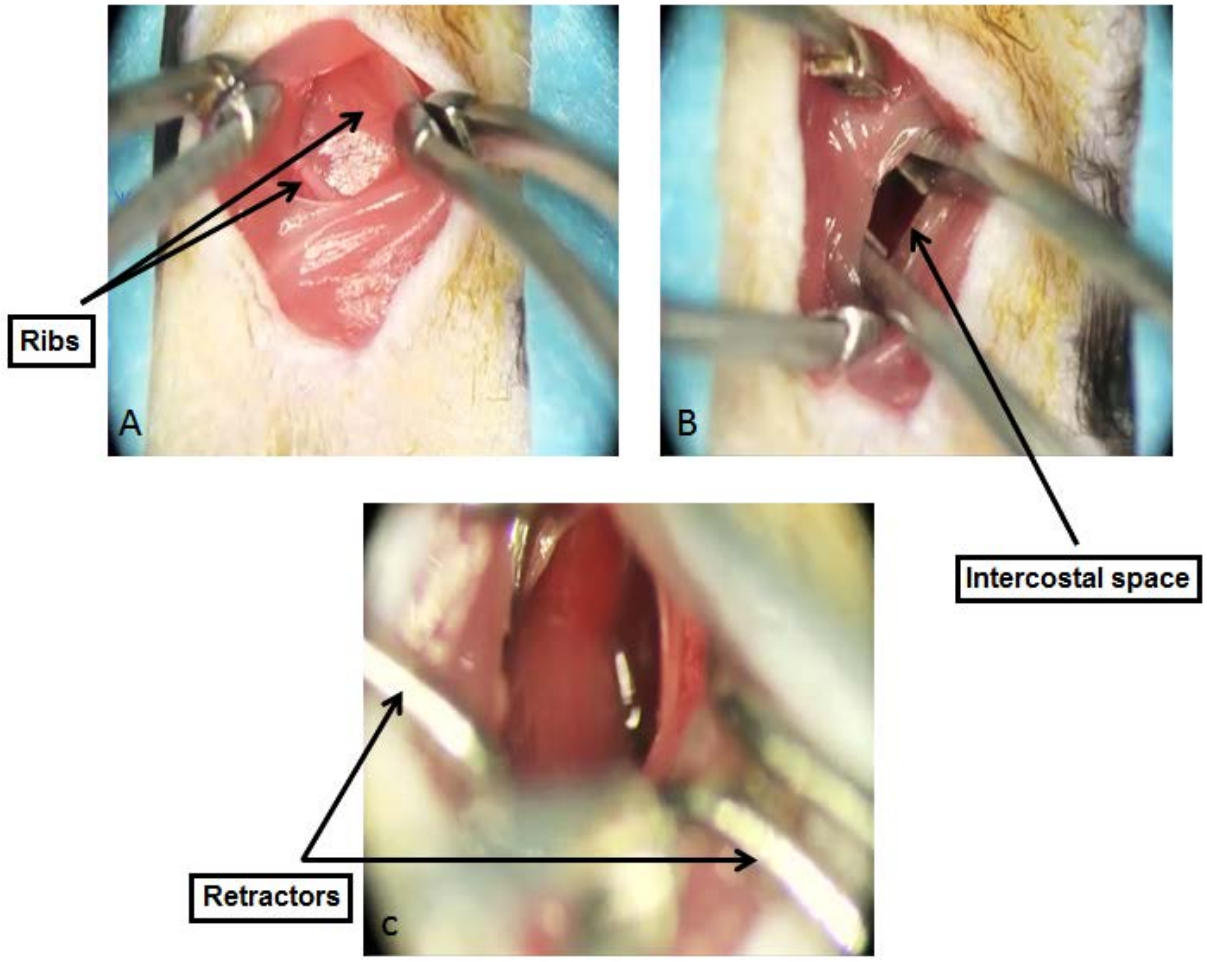

Fig. 16: Heart and LAD visualization preparations. (A) Exposing the rib cage by setting of the pectoral muscles, (B) making a hole through the intercostal space, and (C) enlarging the intercostal space using pair of retractors (Kim et al., 2012).

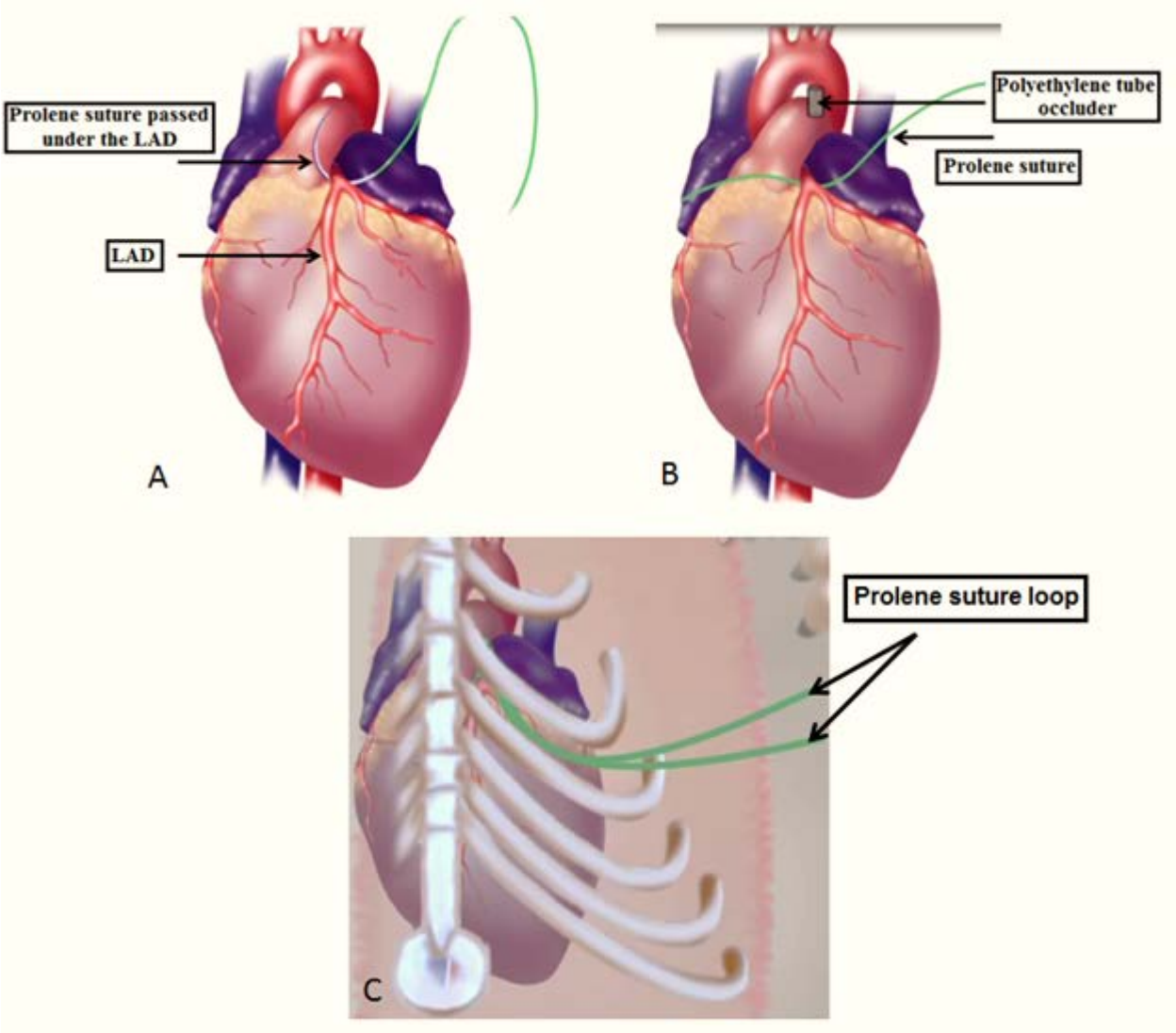

Fig. 17: Overview of LAD occlusion. (A) Curved needle passing under the LAD, (B) placement of an occluder, and (C) prolene suture loop formation and placement under the skin of the mouse (Kim et al., 2012).

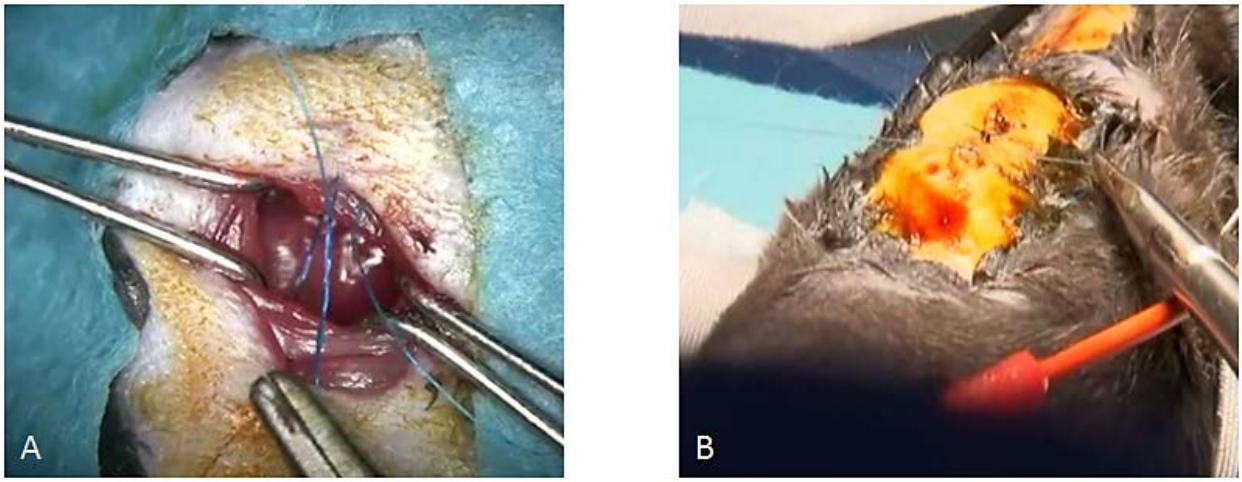

Fig. 18: Stitching of the animal. (A) Closure of the intercostal space and (B) the stitching of the outer skin (Kim et al., 2012).

2.3.1.5 Post-operative recovery

The exhalation tube of ventilator was closed with pinch for 2 seconds to inflate the lungs. Isoflurane flow to inhalation tube was gradually decreased to 1% for couple of minutes and then turned off. Upon spontaneous breathing of the animal, the endotracheal tube was removed and ventilator switched off. The mouse was put under the infrared lamp for few minutes in normal position and let recover. Once a mouse showed movements and had wakened up completely, it was transferred to a cage but still kept under infrared lamp to provide warmth until it started walking. Finally, the cage was transferred to the animal housing facility.

2.3.2 Ischemia reperfusion injury induction

The ischemia reperfusion (I/R) injury induction was started after 7 days of recovery from implanting the suture and the occluder. Before the induction of I/R, the experimental mouse obtained an intraperitoneal injection of 10 µl/g of analgesic. The detailed recipe of the used anesthesia is described in Tab. 2. After anesthesia induction, the mouse was fixed to the operative table exactly as described in 2.3.1.2. Then the neck and chest regions were disinfected using Betaisodona (Mundipharma GmbH, Limburg, Deutschland). ECG monitoring clips were

put into the place and the animal was intubated to supply constant fresh air flow during anesthesia. Again, a temperature probe was inserted into the rectum in order control the body temperature of the mouse. This temperature probe was part of a control loop which ensures the body temperature of 37°C and switches on the heating light as soon as the body temperature falls short of 37°C. Electrodes of the ECG were connected to the Power Lab Data Acquisition System (ADInstruments Ltd, Spechbach, Germany) which operated with Lab Chart 7 software (LabChart v6.0 for Windows) to record and save the ECG data (Einthoven type 1).

The scar from the initial surgery was opened from the neck and the thorax. The loop of the suture that we put under the skin of the mouse was visualized and a weight of about 2-3 g was hanged freely onto this suture loop. This free weight occluded the LAD of the heart (Fig. 19-A and B). This occlusion was testified by observing an elevation in the ST segments in the ECG signal (Fig. 19-C, D). The animals underwent 15 min or 60 min of cardiac ischemia depending on the experimental protocol. The ECG was monitored for the whole ischemia duration to make an effective myocardial infarction sure. After the ischemia, the weight on the loop was removed and the reperfusion was testified in ECG monitor. After this, the mouse was stitched again and wakened up as described in section 2.3.1.5 “post-operative recovery” to continue the experimental protocols.

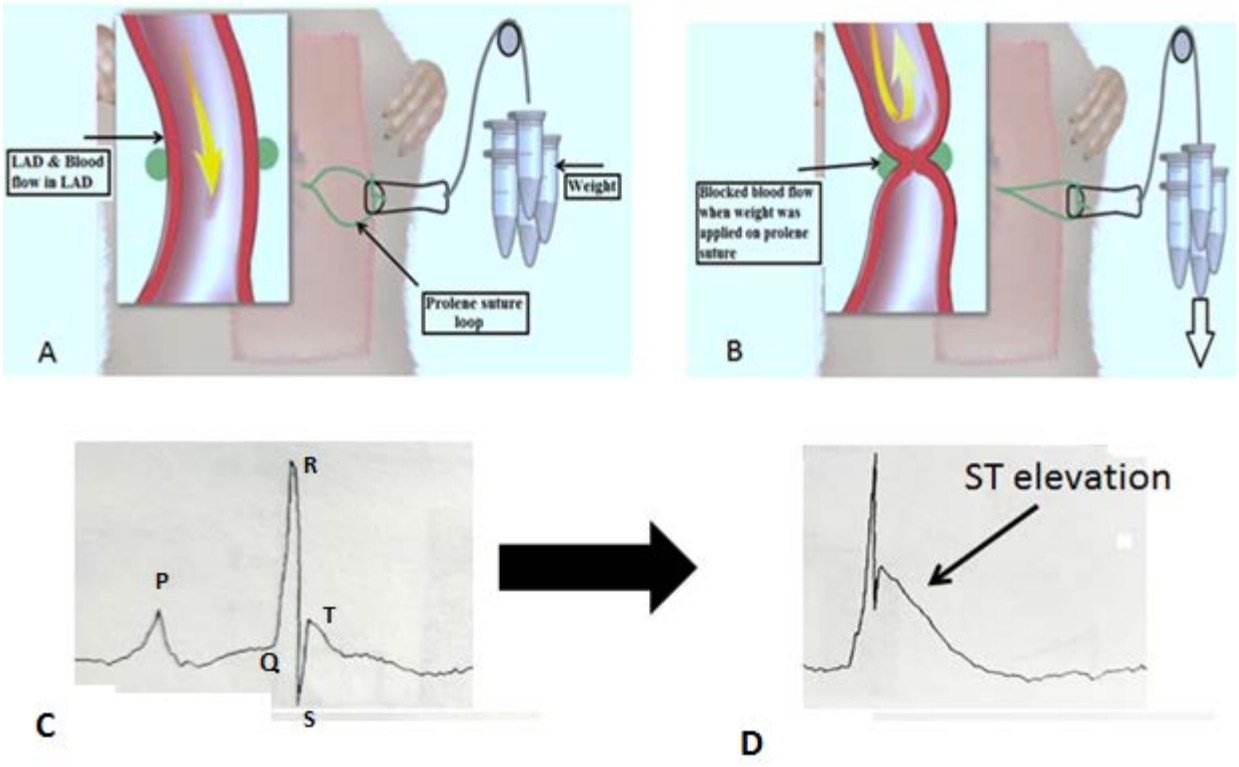

Fig. 19: Schematic sketch of ischemia induction. (A) Blood flow in LAD before the application of weight, (B) Blood flow in LAD after the application of weight, (C) murine ECG signal, before and (D) ST elevation after the weight application showing the occlusion of LAD after ischemia induction. P: P-wave; Q, R, S: QRS complex; T: T-wave (Kim et al., 2012).

Table Nr. 2: Composition of the anesthesia.

Concentration	Ingredient	Product #	Company
0.5 mg	Atropin	AC119C-85	B. Braun Meslungen AG, Germany
1 mL	Xylacin	96187-19	Ecupher GmbH, Greifswald, Germany
1 mL	Ketanest S	P58197/85	Pfizer, Germany
7 mL	NaCl 0.9%	12092409	B. Braun Meslungen AG, Germany
Total	10 mL		

2.3.2.1 Analysis of electrocardiogram (ECG)

To evaluate the changes of the electric stimulus conduction in the heart, the ECG patterns were monitored during the ischemia visible as ST elevation (Fig. 19-D). A special analysis software LabChart 7 was used to analyze and calculate the HR, PR interval, P duration as well as QRS complex and ST amplitude etc.

2.3.3 Hemodynamic measurements

Depending on the experimental protocol, hemodynamic parameters were measured after 24 hours or 21 days after the last reperfusion using a pressure volume Millar catheter system (ADInstruments; Millar Instruments Inc; Houston, TX; USA).

A pressure volume catheter has to be always calibrated before measurement of the hemodynamics. The pressure volume catheter was connected to Powerlab/ 8SP data acquisition system (ADInstruments Ltd, Spechbach, Germany) and a computer (Hewlett Packard GmbH, Böblingen, Germany). The labChart 7 software was run at 1 kHz. Catheter was then calibrated by setting 0 mmHg and 100 mmHg in unit conversion tool box of lab chart 7 software.

During the calibration process of the Millar catheter, the tip of the catheter was dipped into saline solution for at least 20 min in order to fully hydrate the sensors of the pressure volume catheter thereby to minimize the drift of the baseline pressure during the hemodynamic measurements. After these short calibration steps, the pressure volume catheter was ready to be advanced into the right carotid artery of the mouse and to start recording.

Once again, the mice were taken out of the cage, weighed, anesthetized with isoflurane and mounted onto the operative stage as described in section 2.3.1.2. The body temperature was constantly monitored and set to 37°C. The hair/fur at the frontal neck region were cut down using sharp scissors; salivary glands of the animal were separated very gently using pair of cube forceps. Animal was intubated as described and right carotid artery was visualized on the right side of the trachea using a cotton bud. 2 domestic use sutures were passed underneath the right carotid artery with the help of forceps (Fig. 20-A). Here it is very important not to disturb the

Vagus nerve as disturbing it will severely affect the hemodynamics of the animal mounted on the operative stage. One thread was tied completely around the proximal end of the right carotid artery while the second thread was used to tie a lose knot at the distal end and was fixed by a small curve clamp. After everything was set in place, and right common carotid artery fully stretched, a small incision was made at the middle of the artery using very sharp small scissors (Fig. 20-B). A pressure volume catheter was inserted through this incision (Fig. 20-C). Once the catheter was in the artery, it was advanced deep into the artery towards the distal end. Here the isoflurane concentration was removed from 2.5% to only 1% in order to keep the heartbeat of the animal above 500 bpm. Once the pressure was stabilized there, hemodynamic parameters were measured for about 2 min and catheter was advanced into the left ventricle. A significant change in the shape of the wave of ventricular pressure was used to testify the successful advancement of pressure catheter into the left ventricle. The hemodynamic parameters in the left ventricle were recorded for another 2 minutes. After this, the animal was euthanized by high dose of isoflurane and the heart was explanted to proceed with further experimental processes e.g., histology, molecular analysis etc.

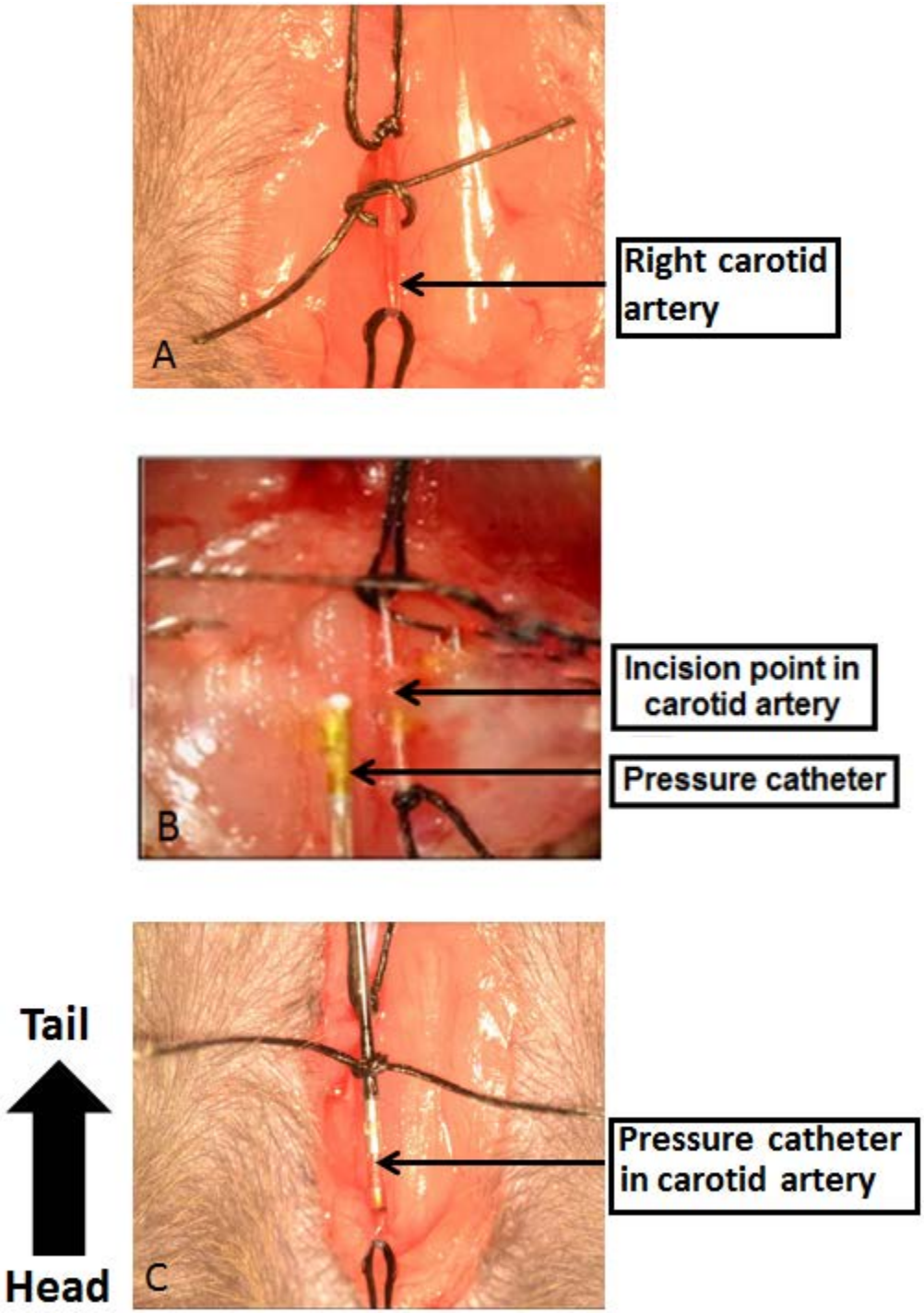

Fig. 20: Insertion of Millar catheter in right common carotid artery. (A) Right common carotid artery prepared for hemodynamic measurements. (B) Incision point at carotid artery where the Millar catheter is to be inserted. (C) Insertion of Millar catheter in carotid artery (Bualeong, 2015).

2.3.3.1 Hemodynamics evaluation

After the recordings of the hemodynamics, the measured data were transferred to computer via a computerized data acquisition system. Offline LabChart 7 software was used to analyze different hemodynamic parameters e.g., heart rate, peripheral and intraventricular pressure, ejection fraction, end-systolic pressure, end-diastolic pressure, cardiac output as well as maximal positive and negative first derivatives of pressure changes with respect to time ($+dP/dt_{max}$ and $-dP/dt_{min}$).

2.3.4 Triphenyl tetrazolium chloride staining

To quantify the myocardial ischemic injury, the infarct size and the area at risk (AAR) were measured. After 24 hours of the last reperfusion, a staining technique called Triphenyl tetrazolium chloride (TTC) staining was used for this purpose. TTC staining is a redox indicator staining and indicates the cellular respiration. The triphenyl tetrazolium chloride is a white compound and is reduced to 1,3,5-tripheylformazan which is red in color in metabolically active tissues due to the activity of different hydrogenases while Triphenyl tetrazolium chloride remains unreacted in the areas of no metabolic activities. In simple words, this technique is used to differentiate between metabolically active and dead cells/tissues (Kim et al., 2012).

The first part of the TTC is performed “in situ”; therefore the investigated mice were anaesthetized, analgesised and mounted on the operative table as described in section 2.3.1.2. The frontal part of the thorax was explanted to visualize the heart. The fat and the pericardium was removed to uncover the occluder (Fig. 21-A). The LAD was ligated by pressing a needle holder against the occluder. Simultaneously, a diluted blue stain, Toluidine blue (Dr. Franz Köhler Chemie GmbH, Bensheim, Germany) was injected into the left ventricle just above just above the area where the LAD was ligated until the whole heart turned significantly blue except the occluded area (Fig. 21-B). This perfused the whole animal with blue dye except the area occluded by LAD ligation (Fig. 21-C). The animal was euthanized by a high dose of isoflurane while the occluder was still pressed against the LAD to avoid the flow of the blue dye into the

supply area despite of the ligation. Then the intubation tube was removed and heart of the animal was explanted. The heart was washed in the DEPC water; occluder and proline suture were removed out of the heart. Additional tissue or lung parts were removed from the heart and it was cooled to -20°C for 30 min. Then the heart was sliced into 2 mm slices using a mechanical slicer made by the mechanical workshop of our institute (Fig. 22). Finally the slices were incubated in the TTC solution for 20 min at 37°C. In the end, 4% formalin was used to fixate the slices for 30 min at 4°C. Heart slices were put onto a glass slide in order and pictures were taken with Nikon D-7000 camera at 4X resolution of OPMI 1FR pro surgical microscope (Carl Zeiss Surgical GmbH, Oberkochen, Germany)

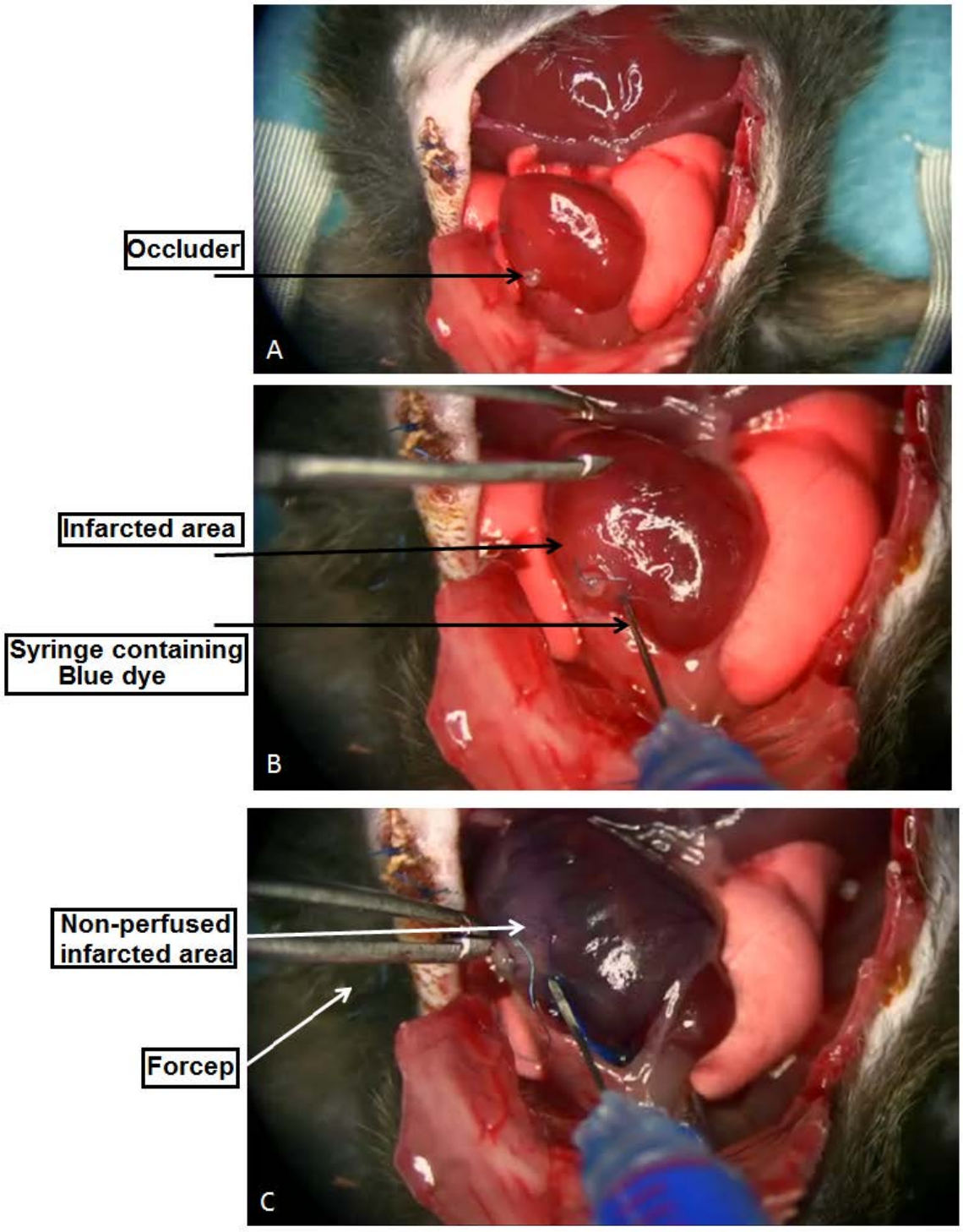

Fig. 21: Injecting the heart with blue dye. (A) Heart with the occluder. (B) Injecting the blue dye into the heart. (C) Heart turned completely blue after the blue dye injection except the occluded area (Kim et al., 2012).

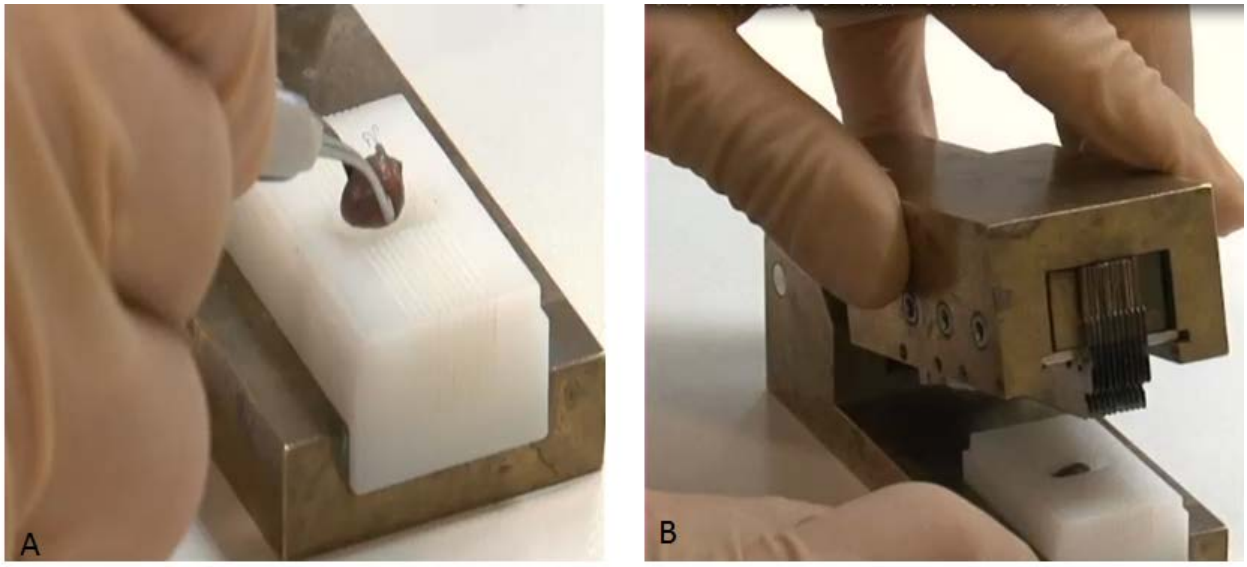

Fig. 22: Slicing of the perfused heart into 2 mm slices (Kim et al., 2012).

2.3.4.1 Assessment of myocardial injury

In the end of this set of experiments, AAR and infarct size were calculated using Image J-win32 software and the extent of myocardial damage was calculated as the percentage of the infarcted myocardium tissue from the AAR (Fig. 23).

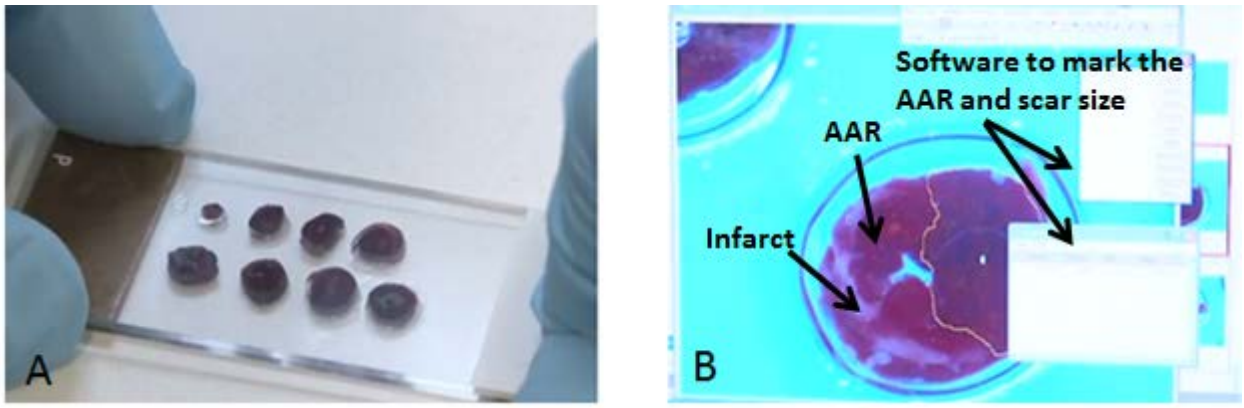

Fig. 23: (A) Placement of sliced heart onto the glass slide and (B) Assessment of myocardial injury (Kim et al., 2012).

2.3.5 Quantitative gene expression

Hearts used for the quantitative gene expression were instantly frozen in liquid nitrogen after explantation and then transported to -80°C freezer.

2.3.5.1 RNA isolation

These frozen samples were taken out and RNA was isolated out of them using TRIzol™ Reagent method. The whole procedure was performed at room temperature and under the mc6 Waldner fume hood (Waldner Laboreinrichtungen GmbH, Co. KG, Wangen im Allgäu, Germany). 1 mL of TRIzol™ Reagent (ThermoFischer scientific, Waltham, MA, USA) was added to each Eppendorf tube (Eppendorf, Hamburg, Germany) containing a heart and was homogenized using a homogenizer (Thomas Scientific, Swedesboro, NJ, USA). Upon complete homogenization, 0.2 mL of Chloroform (Sigma Aldrich, Taufkirchen, Germany) was added to the samples to start the lysis process. Eppendorf tubes were closed tightly and samples were incubated for 3 min on ice. Samples were centrifuged for 15 min at 14000 x g at 4°C in an Eppendorf 5415R centrifuge. This centrifugation step separated the mixture into a lower red phenol-chloroform phase, an interphase and a colorless upper aqueous phase. The aqueous phase containing RNA was transferred to a new Eppendorf tube. 0.5 mL of isopropanol (AppliChem, Darmstadt, Germany) was added to this aqueous phase and was incubated to 10 min on ice, followed by a centrifugation step for 10 min at 12000 x g at 4°C. This yielded a small RNA pellet at the bottom of the Eppendorf tube. Supernatant was discarded and the RNA pellet was resuspended in 1 mL of ethanol (AppliChem, Darmstadt, Germany). Here the samples were vortexed shortly followed by centrifugation for 5 min at 7500 x g at 4°C. Supernatant was discarded and the RNA pellet was air dried under the fume hood for 10-15 min.

In order to solubilize the RNA, it was resuspended in 30 µL of RNase free water, DEPC (AppliChem, Darmstadt, Germany). In the end, RNA samples were incubated in water bath at 55°C for 15 min.

A Nanodrop 2000 UV-Vis Spectrophotometer (VWR International GmbH, ThermoFischer Scientific, Wilmington, USA) was used to determine the total RNA yield. This nanodrop

provided absorbance at 260 nm to determine total nucleic acid content while absorbance at 260/280 nm ratio determined sample purity.

2.3.5.2 cDNA synthesis

cDNA was synthesized using the RNA values obtained from the Nanodrop. These RNA samples were diluted to 2000 ng/μL with RNA free DEPC water in a 0.2 mL PCR clean Eppendorf tube (Eppendorf, Hamburg, Germany). A high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) was used to synthesize cDNA. cDNA reverse transcription kit manufacturing protocol was followed to prepare the master mix exactly with exception of using a volume of 5.8 μL per reaction instead of 10 μL. Last but not the least, T-Gradient Biometra thermocycler (Biometra, Göttingen, Germany) was used to reverse transcription of the RNA under a controlled program (10 min at 25°C, 120 mins at 37°C, 5 sec at 85°C and hold at 4°C). After completion of the reverse transcription, all the samples were diluted with 230 μL of DEPC water.

2.3.5.3 qPCR.

Expression of 7 target genes was assessed to characterize the ischemia reperfusion injury: TNF-α, IL-6, IL-1β, HIF-1α, ICAM-1, IL-10 and NDRG-3 (Applied Biosystems, Foster City, CA, USA). Taqman Gene Expression kit (Applied Biosystems, Foster City, CA, USA) was used. DEPC water, respective probes and Taqman gene expression kit master mix was added to a 1.5 mL PCR clean Eppendorf tube (Eppendorf, Hamburg, Germany) as instructed by the protocol of manufacturer. 6.65 μL of cDNA and 27 μL of the prepared reagent mixtures were added to a 0.2 mL PCR clean Eppendorf tube. Content was mixed and transferred to a Hard-Shell® 384-Well 480 PCR plate (Bio-Rad Laboratories GmbH, Munich, Germany) in 10 μL triplicates. In the end, quantitative expression of assessed genes was measured using a ViiA™ 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). At completion of the rtPCR programme, Ct values were transported to Excel for further analysis.

2.3.6 Histology

21 days after the last ischemia reperfusion injury, hemodynamic parameters were measured (as described in section 2.3.3) and hearts were excised for histological analysis.

2.3.6.1 Preparation of hearts

Animals were euthanized and heart samples were excised. Hearts were washed in PBS solution and were fixated in 4% zinc formalin (Merck KGaA, Darmstadt, Germany) at 4°C for about 18 hours. Then the hearts were taken out of zinc formalin and were washed in distilled water for 2 hours. In the end, hearts were dehydrated in 70% alcohol (Otto Fischer GmbH & Co, KG, Saarbrücken, Germany) for one day at 8°C.

2.3.6.2 Paraffin embedding

Hearts were taken out of 70% alcohol and were transferred to specially designed plastic cassettes labelled specifically for the respective heart. In order to completely dehydrate the hearts, following steps were performed.

Table Nr. 3: Tissue dehydration steps.

Cassette Nr.	Solution	Time	Agitation
1	70% Isopropanol	3 h	2
2	80% Isopropanol	1 h	2
3	80% Isopropanol	1 h	2
4	90% Isopropanol	1 h	2
5	90% Isopropanol	1 h	2
6	96% Isopropanol	2 h	2
7	100% Isopropanol	2 h	2
8	100% Isopropanol	2 h	2

At this stage, all but tiny residue of tightly bound (molecular) water should have been removed from the heart samples. Xylene was used as clearing agent to infiltrate the heart samples with the wax.

Table Nr. 4: “Clearing” of heart samples.

Cassette Nr.	Solution	Time	Agitation
9	Xylene	1 h	1
10	Xylene	1 h	1

The heart samples are now ready for infiltration with wax. The paraffin embedding station (Medax GmbH & Co. KG, Neumünster, Germany; Fig. 24) was turned on well in time to calibrate. Paraffin was melted and a disposable plastic vessel was filled with this melted paraffin in a way that there was a small solid layer of paraffin at the bottom of the plastic vessel and the rest of the vessel was filled with melted paraffin. Then the heart was picked up using forceps and was placed in the plastic vessel containg the paraffin with the aorta on the top and apex at the bottom. In the end, paraffin was allowed to solidify and stored at room temperature.

Fig. 24: Tissue embedding station.

2.3.6.3 Sectioning of heart samples using microtome

The Microme HM 355 (Microm GmbH, Walldorf, Germany (Fig. 25) was used to cut the embedded hearts in sections. The microtome was switched on and the water bath temperature was set to 42C°. The plastic embedment vessel was cut down using a standard sharp knife. The knife was heated enough to melt the paraffin a little using a standard burner. Once the knife was hot, a little paraffin was melted and few drops of melting paraffin were dropped on a specially customized piece of wood to fix the paraffin embedded heart onto this. Then this setup was allowed to cool down until the paraffin embedded heart was fixed onto the wood. This block was then mounted on the microtome. In the step the paraffin block was trimmed in relatively bigger sections (10 μm) until the desired levels of heart sections were reached. Now the thickness of the heart sections was changed to 5 μm. The sections of the sliced heart slipped down into the water bath where they were allowed to stretch for about 10 seconds minimum to eliminate all the folds and wrinkles. After stretching a microscope glass slide was dipped into the water and moved under the heart sections in a position that allowed attaching the sections with one edge to the slide. Finally, the slide was pulled out of the water bath slowly to allow the sections to settle on the glass slide. Special care was given to avoid any air bubble under the heart sections. Then these glass slides were put into a pre-heated oven at 62C°for about 6 hours for fixation. In the end, these slides were stored in slide boxes.

Fig. 25: A microtome, installed with water bath and heat sensor.

2.3.6.4 Sirius Red staining

A standard procedure to visualize the cardiac fibrosis is Sirius red staining of heart sections.
Sirius Red (0.1%) solution was prepared by mixing 0.1 g Direct Red 80 (Aldrich Chemical Company, WI, USA) to 100 ml of saturated picric acid (Fluka GmbH, USA, Art.Nr. 80456), the mixture was allowed to settle for 5 to 10 min and then filtered. Staining process was started by dewaxing and rehydrating the heart sections. Then the sections were stained in Sirius Red (0.1%) solution for 15 min. In the next step, the sections were immersed in deionized water 5 times followed by 5 times immersing in 70% alcohol, 5 times in 90% alcohol and finally 10 times in 100% alcohol to complete dehydration. Lastly, these sections were dipped in xylene (Sigma Aldrich, Taufkirchen, Germany) 15 times and were mounted in a resinous medium i.e., Entellan (Sigma Aldrich, Taufkirchen, Germany).

The stained heart sections were then photographed in 4-fold magnification (2 ms exposure time and 3-4 light intensity. Multiple pictures of each heart section were taken. Analysis software AnalySIS® (Olympus Soft Imaging System GmbH, Münster, Germany) was used to merge the pictures into one picture. After this, the image was converted into a HSI (Hue Saturation Intensity). By image optimization, contrasts were reinforced until the collagen appeared deep red and intact myocardium deep yellow (Fig. 26). Photoshop (Adobe Photoshop CS2 Vers.: 9:02; 1990-2005) was used to remove the irrelevant collagen (such as pericardium and vessel walls) and the right ventricle. In the end, the percentage of collagenous infarct tissue was calculated in the left ventricle using the program MakroPrisca (AnalySIS ® 3.0 Version 2.8; Olympus Soft Imaging System GmbH, Münster, Germany). The received values were transferred to GraphPad Prism 5 (GraphPad Software, San Diego, USA) for further statistical analysis.

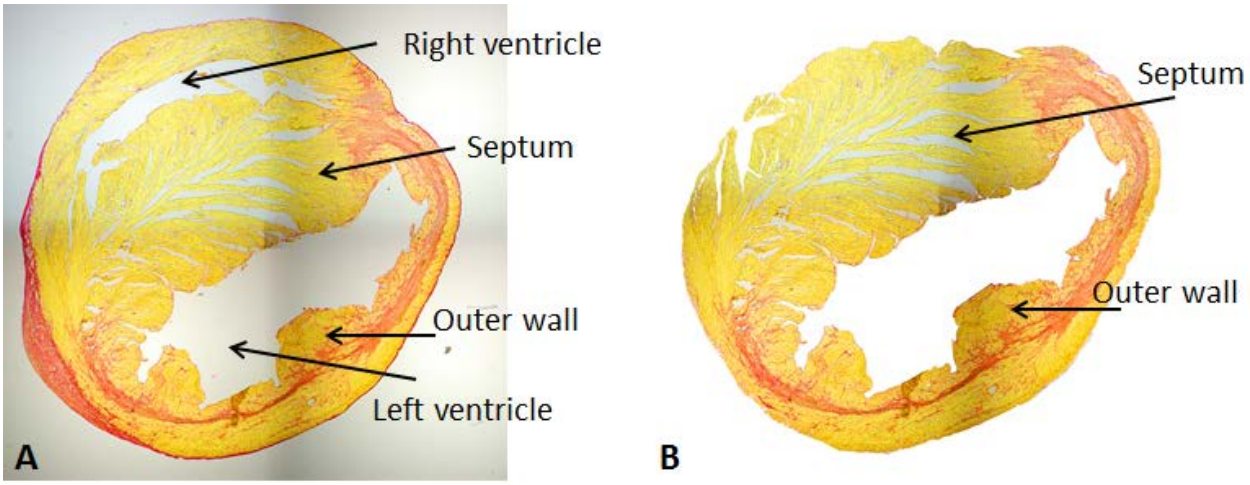

Fig. 26: A typical example of Sirius red stained section (A) before and (B) after processing.

2.4 Statistical analysis

The statistic evaluation was accomplished with the help of the software Prism version 5.0 and 6.0. It enabled us to rapidly determine the statistical significance by one-way ANOVA with Bonferroni's and Holm-Sidak's multiple comparisons as post-test. All data were expressed as mean (Mean) and standard error of the mean (SEM). The statistical differences with an error probability of $p < 0.05$ were considered significant. <0.05= *, <0.01= ** and 0.005= ***.

3 Results

3.1 Animals

3.1.1 Number of animals used

A total of 245 C57BL/6 wild type animals of 10 ± 2.1 week old were examined during the whole study. These mice were divided into different experimental groups for different experiments.

3.1.2 Mortality rate

In this study, the mortality rate after thread implantation around LAD until the end of the experiments depending on the group varied in different experimental groups (Fig. 27). The mortality rate during the thread implantation around the LAD was mainly attributed to in/extubation of the trachea and by injuring the heart tissue with the catheter. Wound healing and sepsis during the one full week of recovery period between initial operations (thread implantation around LAD) and the ischemia induction played a huge role towards the mortality rate. A lot of mice mortality rate was attributed to arrhythmia during and after the LAD occlusion.

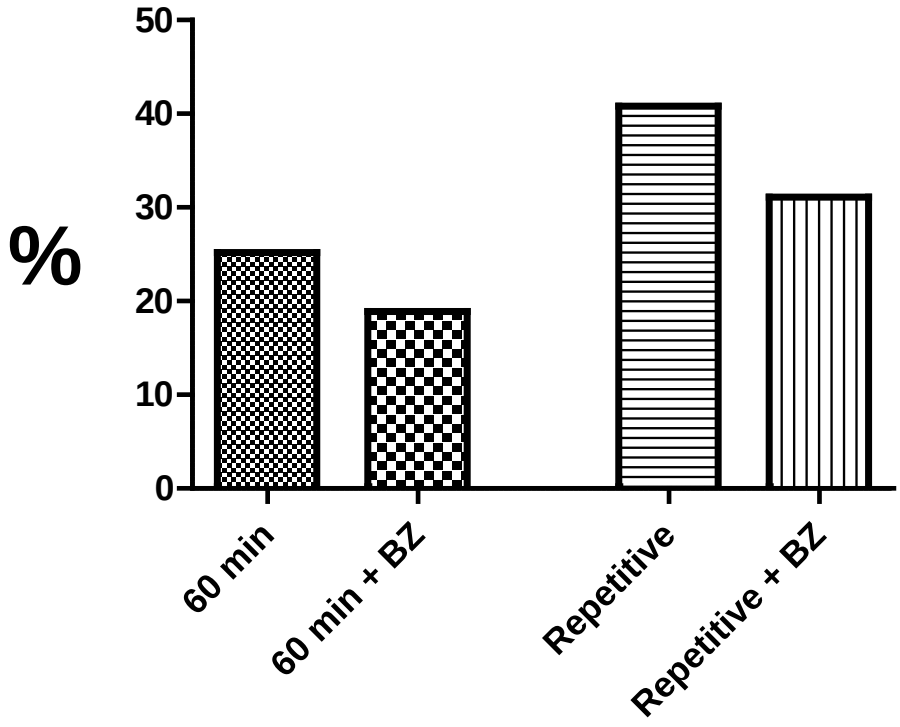

Fig. 27: Relative mortality in the different groups. 25.2% of the animals died in 60 min group (n= 52), in the 60 min + BZ mortality was reduced to 18.9% (n=43); the highest mortality of 40.8% was observed in the repetitive group (n=57) while a mortality rate of 31.1% was observed in the repetitive + BZ group (n=41).

3.1.3 Body weight loss

Our finding that bortezomib treatment reduced the mortality rate was also supported by the finding that the animals that were administrated with bortezomib during the course of the experiment reduced less weight in comparison to their counterparts. The weight loss may be taken as a sign for physical stress. The bortezomib treatment significantly reduced the weight loss effect (Fig. 28) in comparison to its counterpart experimental groups.

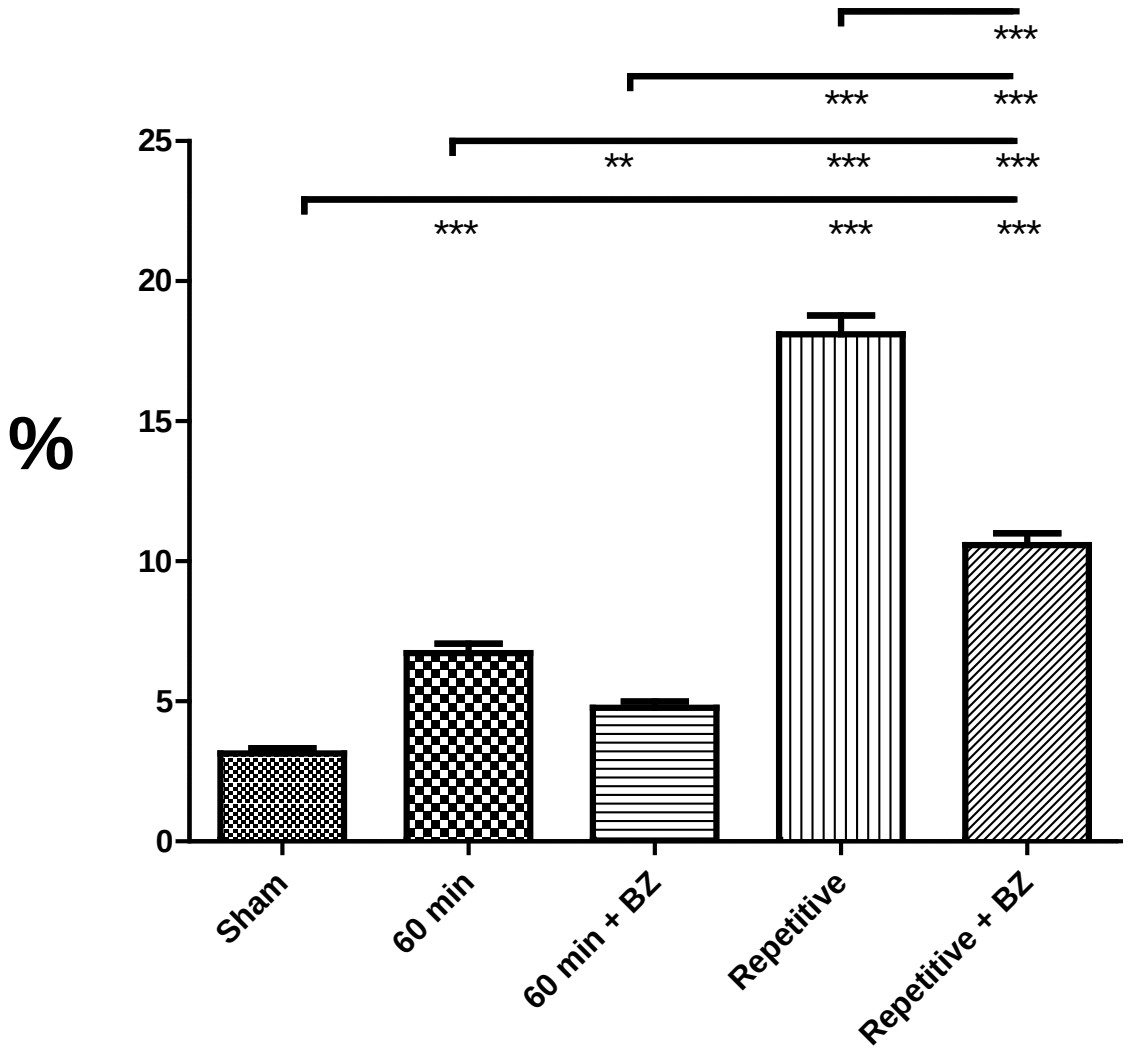

Fig. 28: Percentage of the body weight loss during the course of the whole experiment. The mice in the sham group lost 3.1 ± 0.7% (n=40) while the mice in the 60 min group lost 6.7 ± 1.1% of their body weight (n= 52), addition of BZ reduced this to 4.7 ± 0.9% (n=50). Repetitive short ischemias increased weight loss to 18.1 ± 3.7% (n=55), addition of BZ reduced the weight loss to 10.1 ± 2.6% also in the repetitive group (n=52).

Table Nr. 5: Average body weight of the mice in each experimental group (in grams).

Body weight (BW)	Sham	60 min	Repetitive	60 min + BZ	Repetitive + BZ
BW at start	23.1 ± 2.1	21.8 ± 1.5	24.7 ± 1.2	24.0± 1.3	23.8 ± 1.3
BW at end	22.2 ± 1.8	20.3 ± 1.3	23.5± 1.9	19.7± 1.6	21.7± 1.8
%age decrease	3.5%	6.7%	4.7%	18.1%	10.1%

3.1.3 Effects of LAD occlusion on ECG recording

The successful induction of ischemia by the occlusion of LAD was made sure by keeping a constant eye on the ECG monitoring unit. Before the occlusion of the LAD, ECG looks normal as in the non-operated mouse consisting of P- wave, QRS complex and T- wave (Fig. 29-A). The ECG pattern of the mouse heart looked different than that of humans because of the faster action potential and higher heart rate. After the beginning of the LAD occlusion, ST segment was elevated confirming the induction of myocardial ischemia (Fig. 29-B) followed by the reperfusion phase where ST peaks were lowered again (Fig. 29-C) that led to the negative ST complex after 24 hours of ischemia as a sign of expired ischemia (Fig. 29-D).

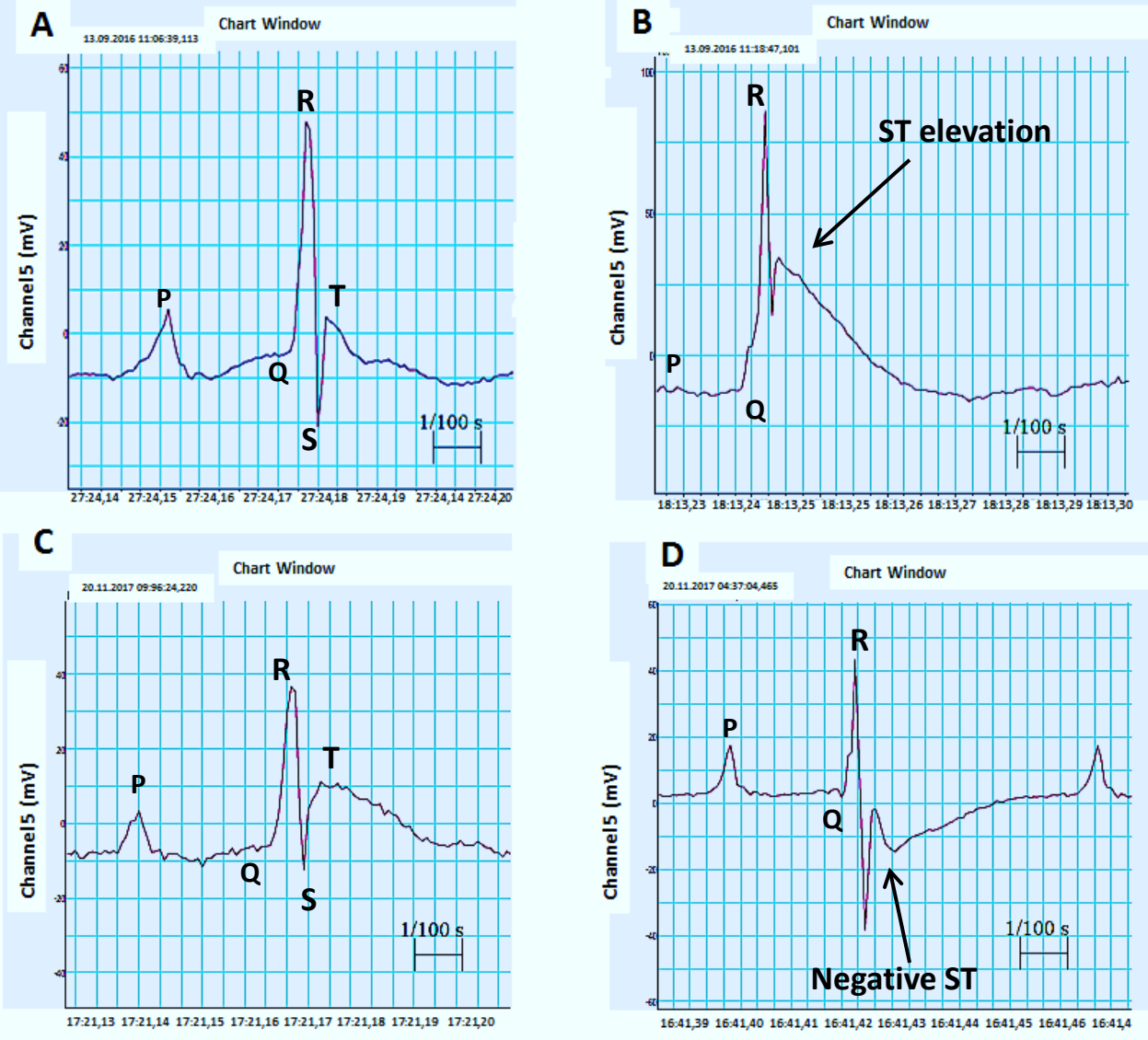

Fig. 29: Different situations of mice ECG according to Einthoven at different times during I/ R experimental protocol. (A) Normal ECG. (B) ST elevation showing the induction of ischemia. (C) Reperfusion phase after the ischemic phase. (D) Negative ST segments after 24 hours of ischemia (1 h).

3.2 Triphenyl tetrazolium chloride staining

After following the respective experimental protocol, a set of mice underwent triphenyl tetrazolium chloride staining (TTC staining). Hearts were explanted, histologically processed and

stained with TTC. This made possible to quantify the myocardial ischemic injury, the infarct size and the area at risk. Area at risk and infarcted portions could be seen in the stained pictures. Hearts of the four experimental groups (60 min, 60 min+ BZ, repetitive and repetitive + BZ) were compared. Figure 30 shows the effects of ischemia in different experimental groups. Infarct sizes were measured using these digitized TTC staining pictures. It clearly shows the bigger infarct size in 60 min group where almost the whole area at risk is infarcted in comparison to other three experimental groups. In each case, infarct size is expressed as the percentage inside the area at risk. 60 min groups showed a significantly bigger infarct size (47.2%) than the counterpart group; 60 min + BZ (23.12%) and both repetitive (16.7%) and repetitive + BZ (13.61%) groups.

The groups those were administrated with bortezomib showed significantly smaller infarcts than their respective counterpart non-administrated groups (60 min + BZ; 23.12% vs 60 min; 47.2% and repetitive + BZ; 13.61% vs repetitive; 16.7%). It was also noticed that the infarct size was significantly reduced in repetitive + BZ group (13.61%) compared to the 60 min + BZ group (23.12%) (Fig. 30-B)

A


60 min
60 min + BZ
Repetitive
Repetitive + BZ

B

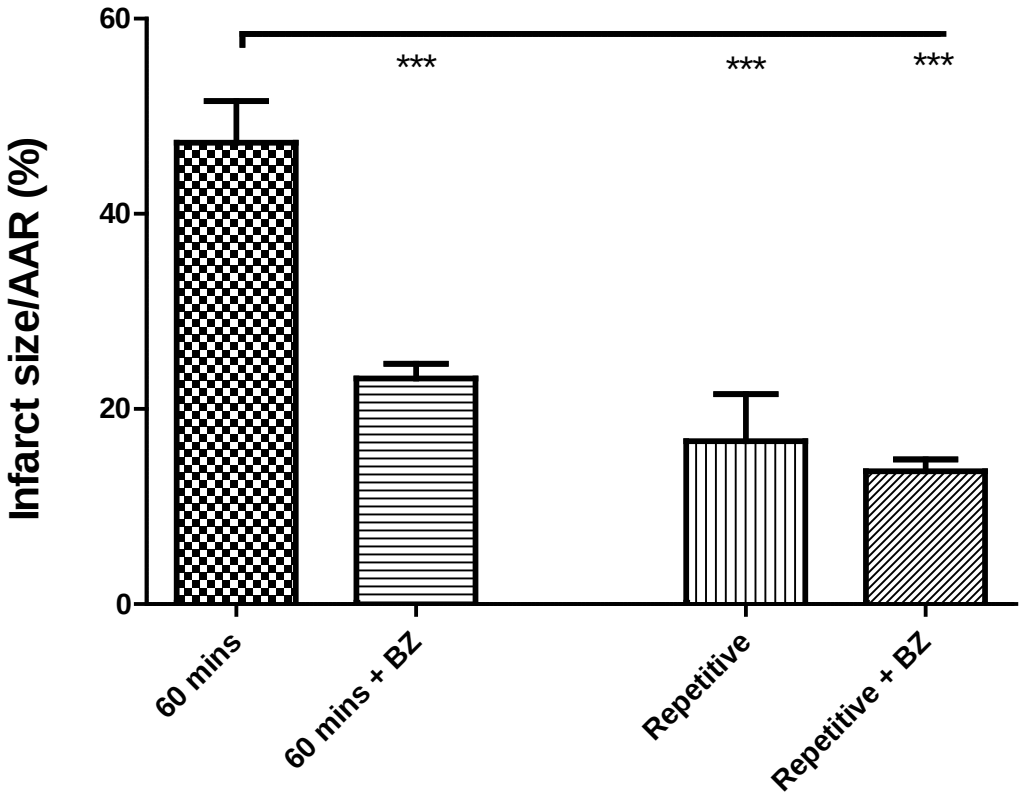
Infarct size/AAR (%)
60
40
20
0
***
***
***
60 mins
60 mins + BZ
Repetitive
Repetitive + BZ

Fig. 30: Infarct size/AAR in different experimental groups after 24 hours of last ischemia. The 60 min group produced the largest infarct size/AAR that was 47.2 ± 2.1% (n=8) while infarct size/AAR was found to be only 23.12 ± 1.7% in 60 min + BZ group (n=8). The repetitive group exhibited 16.7 ± 1.3% (n=9) while infarct size/AAR was found to be 13.61 ± 1.25% in repetitive + BZ group (n=8).

3.3. Hemodynamic parameters after 24 hours

Hemodynamic parameters were recorded 24 hours after the last ischemia. In chapter 3.3.1 the measured results together with the calculated significances are presented in the figures and the respective values are given in the legends. The text paragraphs focused on the significant differences between the groups and the relative changes.

3.3.1 Ejection fraction

Hemodynamic measurement revealed that the ejection fraction was significantly reduced in the 60 min group in comparison to all other experimental groups (Fig. 31). Compared to the sham group, 60 min of ischemia reduced the ejection fraction by 47 ± 4.1%. Repetitive short ischemias in advance as well as BZ treatment protected against the injury caused by the 60 min I/R thus reduction in ejection fraction was only 4.5 ± 3.4% in the repetitive group, 3.3 ± 3.6% in the 60 + BZ group, and 17.9 ± 4.7% in the repetitive + BZ group. Thus the group with the largest IR/AAR ejected the least ventricular volume.

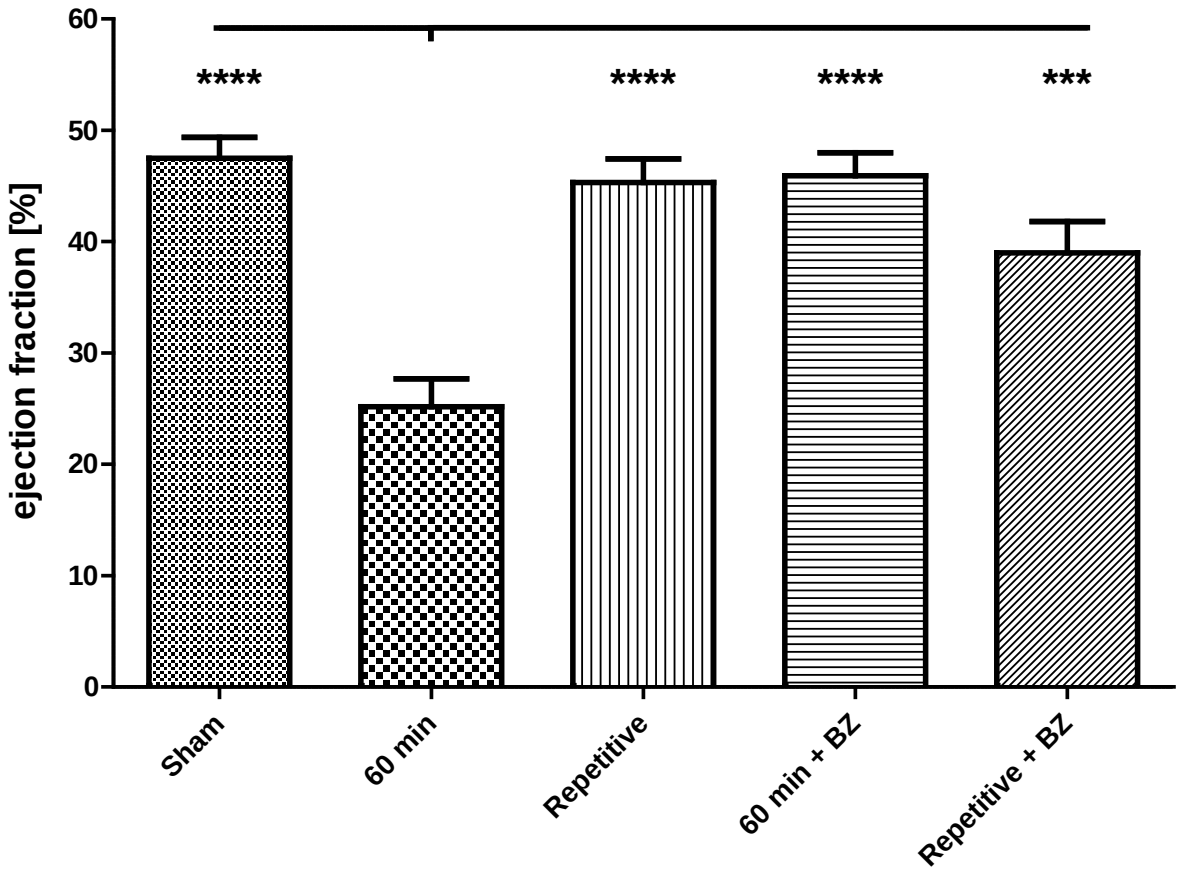

Fig. 31: Ejection fraction (%) in different experimental groups after 24 hours of last ischemia. The mice in the sham group ejected 47.4 ± 1.8% of the left ventricular volume (n=10). 24 h after a 60 min ischemia the mice ejected 25.1 ± 2.5% which was significantly less (n=11). Preceding short repetitive ischemias led to an ejection fraction of 45.3 ± 2.1% (n=7), BZ treatment to 46.6 ± 2.08% (n=9) and a combination of both (Repetitive + BZ) to 39.1 ± 2.8% (n=14).

3.3.2 End-systolic pressure

Fig. 32 showed that the end-systolic pressure was significantly reduced in the 60 min group and in the 60 min + BZ group. In comparison to the sham group, 60 min of I/R reduced the end-systolic pressure by 19.8 ± 4.1% in the 60 min group and 15.6 ± 3.0% in the 60 min + BZ group. These results are in line with our TTC results (Fig. 30) where these two groups exhibited the largest infarct size, hence leading to reduced end-systolic pressure and the reduced/compromised

cardiac function. Repetitive short ischemias, prior to 60 min long ischemia seemed to reduce the injury caused by the following 60 min long ischemia, because I/R reduced end-systolic pressure only by 4.1 ± 2.8% in the repetitive group and 9.8 ± 3.2% in the repetitive + BZ group.

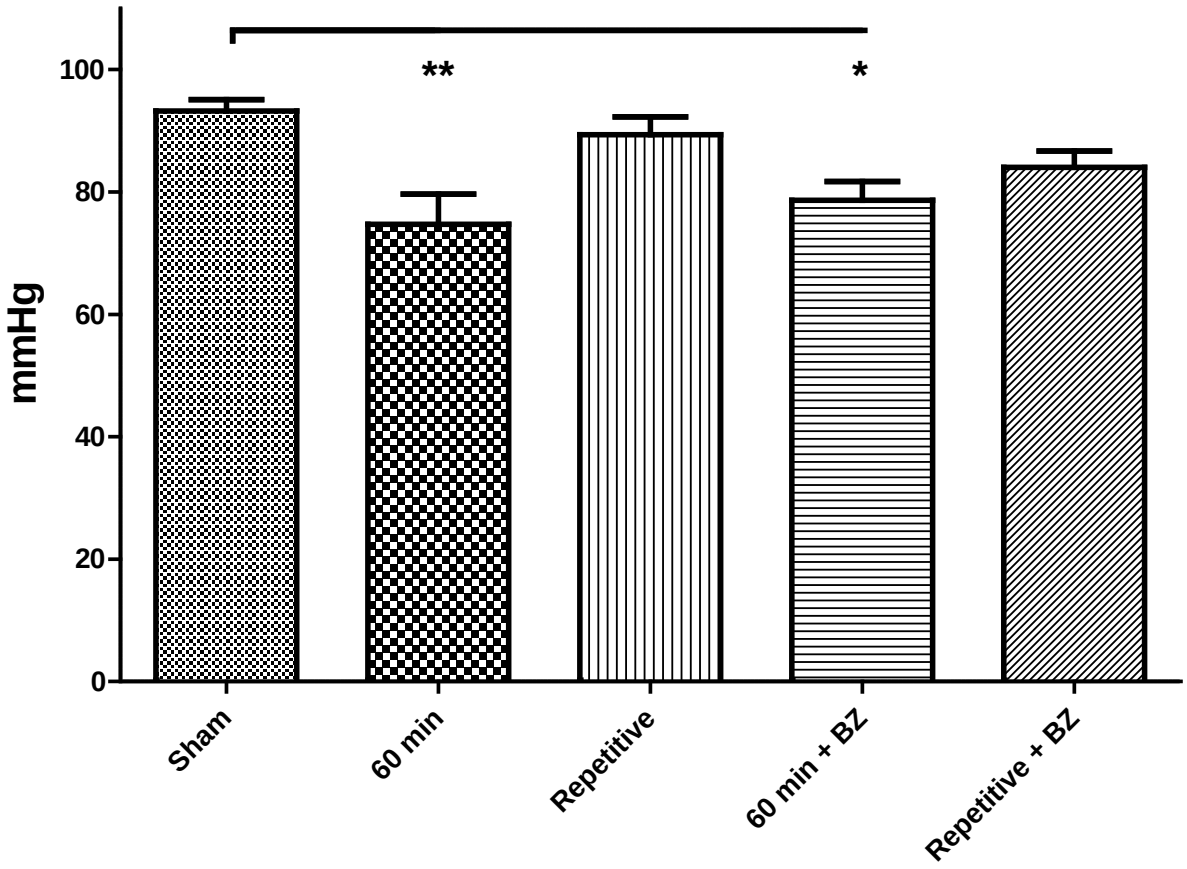

Fig. 32: End-systolic pressure (mmHg) in different experimental groups after 24 hours of last ischemia. End-systolic pressure was noted to be 93.2 ± 1.8 mmHg in the sham group (n=10). 24 h after a 60 min ischemia the end-systolic pressure was found to be 74.7 ± 4.9 mmHg which was significantly less (n=11). Preceding the short repetitive ischemia led to an end-systolic pressure of 89.3 ± 2.9 mmHg (n=7), BZ treatment to 78.6 ± 3.0 mmHg (n=8) and a combination of repetitive and BZ treatment to 84.0 ± 2.6 mmHg (n=14).

3.3.3 End-systolic volume

End-systolic volume is the left ventricular blood volume at the end of the contraction (systole) and the beginning of the ventricular filling (diastole). Fig. 33 shows that the end-systolic volume was significantly increased after the 60 min ischemia by 112.4 ± 7.7% as compared to the sham group. Pre-conditioning the heart with repetitive short ischemias in the repetitive group, bortezomib treatment (60 min + BZ group) and the combination of both repetitive and bortezomib treatment (repetitive + BZ group) seemed to reduce the injury caused by the following 60 min long ischemia, because the volume was only insignificantly increased in these groups when compared to the sham group. The repetitive group exhibited an increase of 51.2 ± 6.3%, 60 min + BZ group showed an increase of 24.6 ± 4.9% and the repetitive + BZ group exhibited 38.2 ± 6.2% increase in comparison to the sham group. Furthermore the volume was significantly smaller in these groups than in the 60 min group.

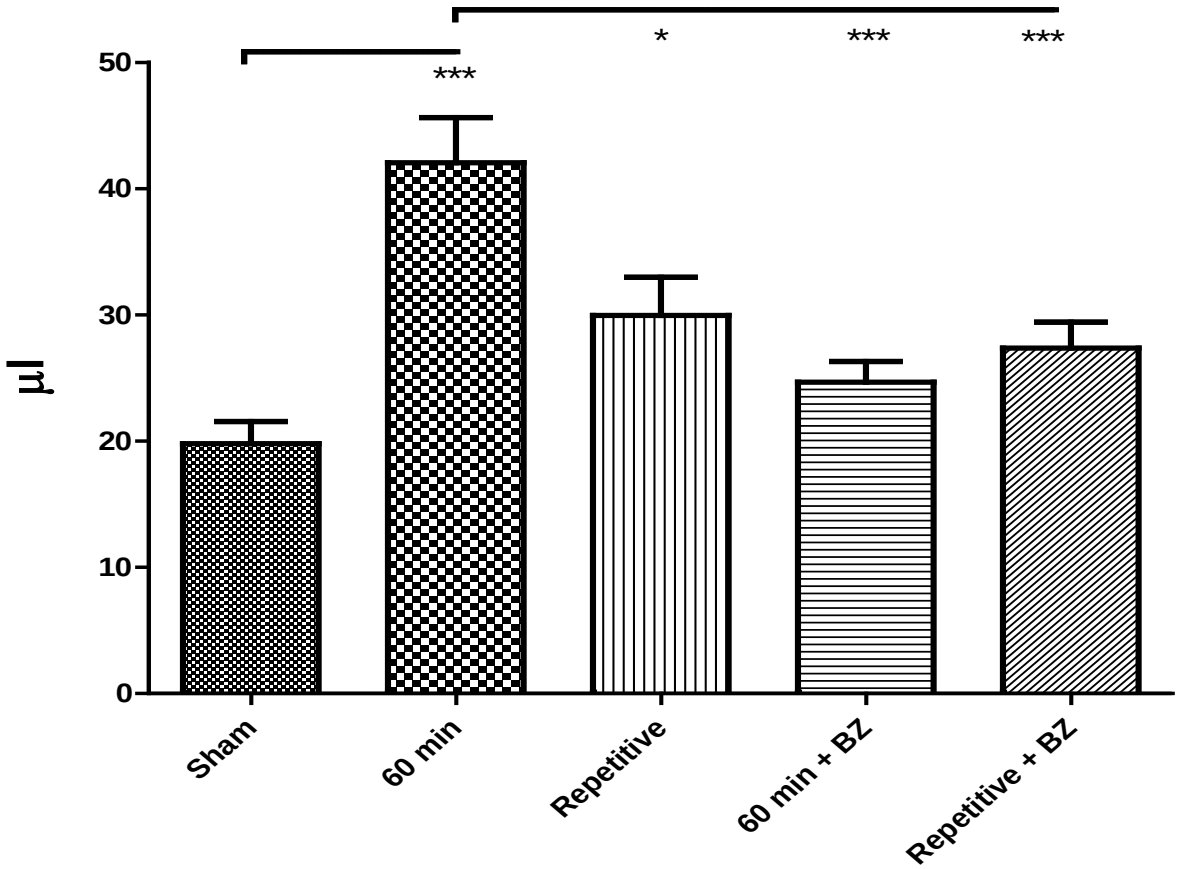

Fig. 33: End-systolic volume (µl) in different experimental groups after 24 hours of last ischemia. End-systolic volume was noted to be 19.7 ± 1.7 µl in the sham group (n=10). 24 h after a 60 min ischemia the end-systolic volume was found to be 42.0 ± 3.5 µl which was significantly higher (n=11). Preceding the short repetitive ischemia led to an end-systolic volume of 29.9 ± 3.0 µl (n=7), BZ treatment to 24.6 ± 1.6 µl (n=9) and a combination of repetitive and BZ treatment to 27.3 ± 2.0 µl (n=14). All these were significantly smaller than in 60 min group.

3.3.4 End-diastolic pressure

In the case of end-diastolic pressure (Fig. 34), we did not find any significant difference between the experimental groups. The 60 min group exhibited a little higher end-diastolic pressure in comparison to the sham group (28 ± 9.7%) which could be taken as a sign for a disturbed cardiac function but this increased end-diastolic pressure was not found to be significant to the sham and other experimental groups. Moreover, the repetitive group exhibited 3.9 ± 5.4% reduction in the end-diastolic pressure in comparison to the sham group. 2.9 ± 4.7% increase was noted in the 60 min + BZ group and 15.0 ± 6.8% in the repetitive + BZ group in comparison to the sham group.

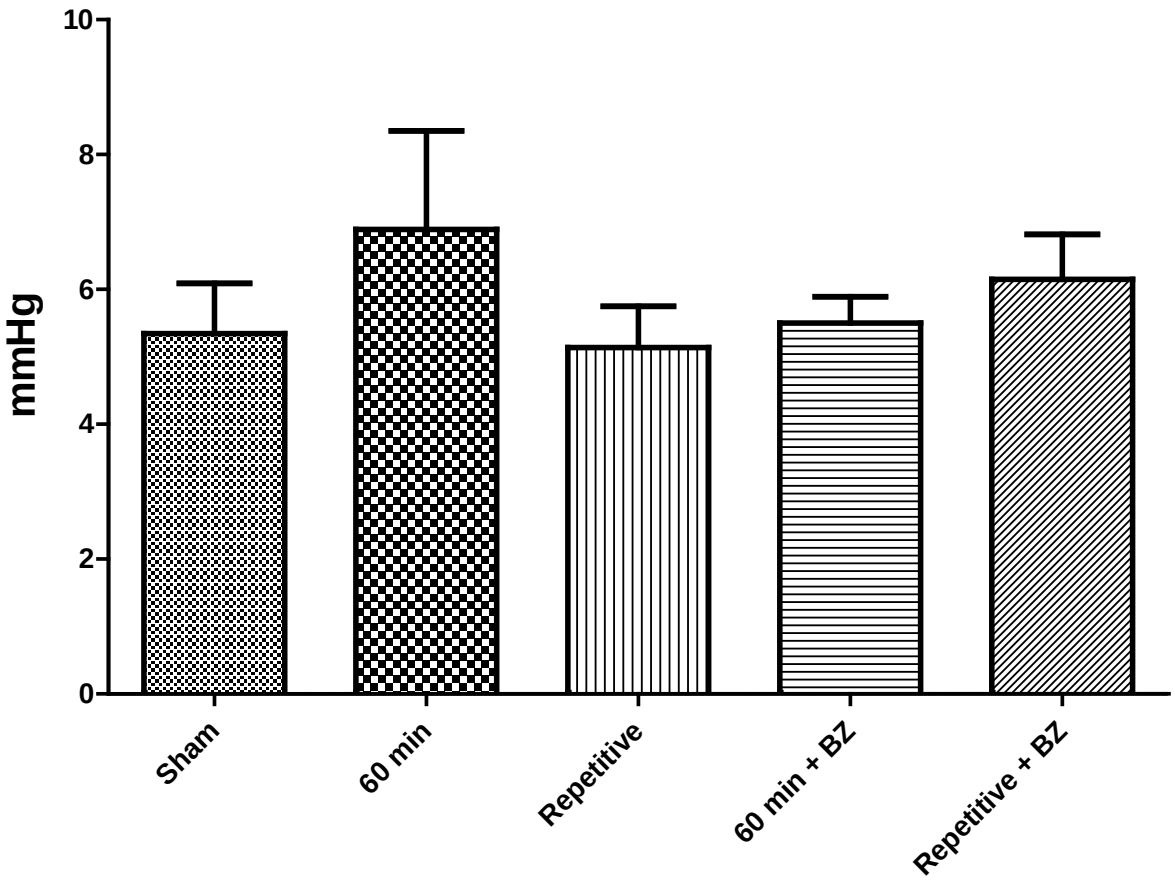

Fig. 34: End-diastolic pressure (mmHg) after 24 hours of last ischemia. End-diastolic pressure was noted to be 5.3 ± 0.7 mmHg in sham group (n=10). 24 h after a 60 min ischemia the end-diastolic pressure was found to be 6.8 ± 1.4 mmHg (n=11). Preceding short repetitive ischemia led to an end-diastolic pressure of 5.1 ± 0.6 mmHg (n=7), BZ treatment to 5.5 ± 0.4 mmHg (n=9) and a combination of repetitive and BZ treatment to 6.14 ± 0.6 mmHg (n=14).

3.3.5 End-diastolic volume

End-diastolic volume was measured in all the experimental groups (Fig. 35). The figure below shows that the end-diastolic volume was significantly increased in the 60 min group due to I/R by 53.7 ± 6.0% and in the repetitive group by 49.5 ± 5.5% in comparison to the sham group. Bortezomib administration reduced the I/R dependent rise in the end-diastolic volume to a

nonsignificant increase of 24.3 ± 3.9% in the 60 min + BZ group and 22.1 ± 4.8% in the repetitive + BZ group.

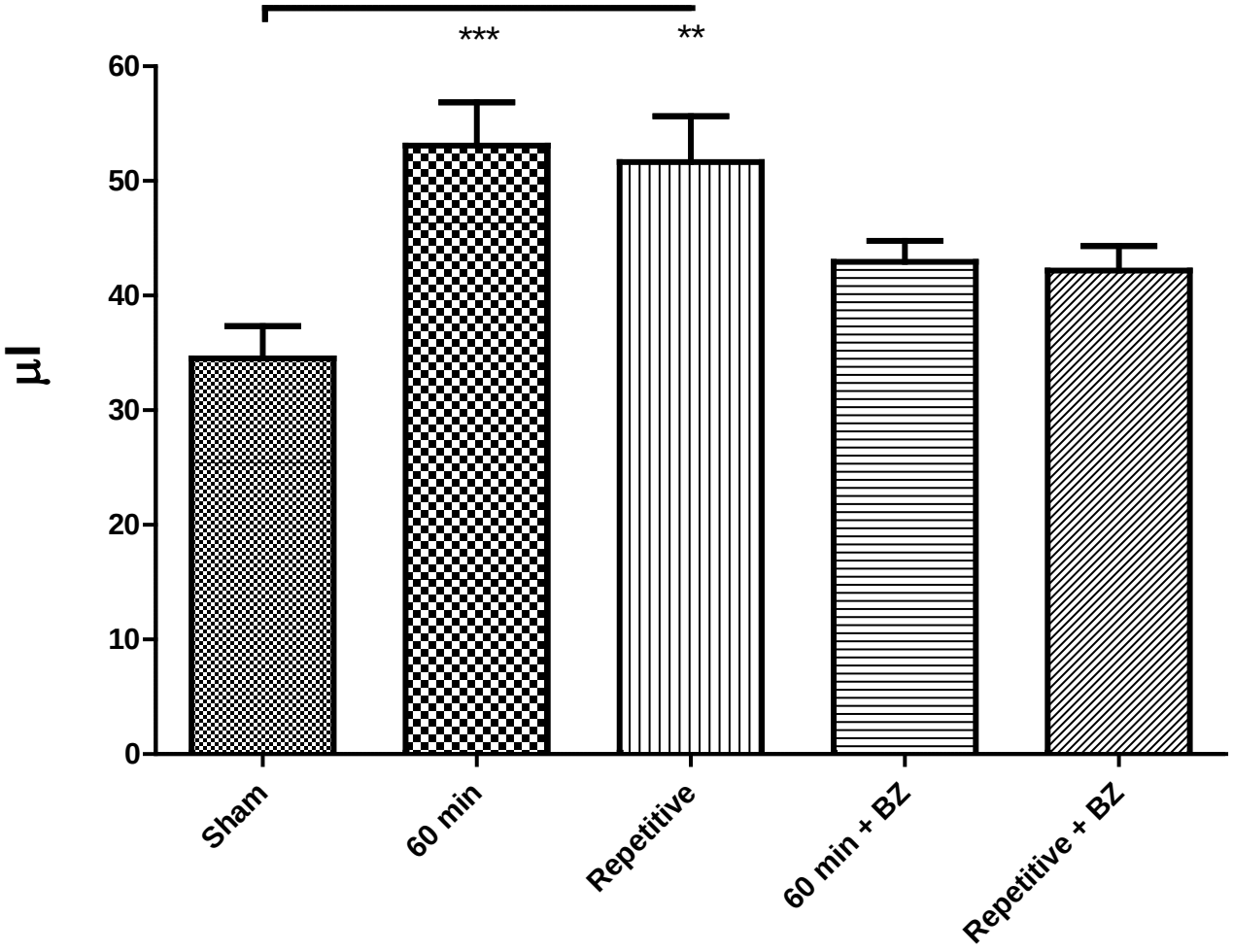

Fig. 35: End-diastolic volume (µl) in different experimental groups after 24 hours of last ischemia. End-diastolic volume was noted to be 34.5 ± 2.8 µl in the sham group (n=10). 24 h after a 60 min ischemia the end-diastolic volume was found to be 53.0 ± 3.7 µl which was significantly higher (n=11). Preceding the short repetitive ischemia led to a significantly higher end-diastolic volume of 51.6 ± 3.9 µl (n=7), BZ treatment led to 42.9 ± 1.8 µl (n=9) and a combination of repetitive and BZ treatment to 42.1 ± 2.1 µl (n=14).

3.3.6 dP/dt_{max}

dP/dt_{max} is the maximum rate of rise in left ventricular pressure during systole. It is taken as a measure of the global left ventricular contractility. The faster contractile force is developed, the higher is the rate of rise in left ventricular pressure. dP/dt_{max} (Fig. 36) was measured in all the experimental groups. dP/dt_{max} was significantly reduced in 60 min group in comparison to WT sham group by 30.1 ± 5.1%. Repetitive short ischemia pre-treatment increased the dP/dt_{max}value in the repetitive group by 13.4 ± 3.74% in comparison to the sham group. In case of bortezomib treatment the dP/dt_{max}value was suppressed by only 3.6 ± 4.7% in the 60 min + BZ group thus preventing the influence of I/R on $dP/dt_{max.}$ Treating the mice with both bortezomib and repetitive short ischemias was less protective as I/R reduced dP/dt_{max} in this group by 20.5 ± 4.5% compared to the sham group. Furthermore dP/dt_{max} was significantly smaller in this group than in the repetitive group.

These results are again in agreement with our TTC findings (Fig. 30) where the 60 mins group exhibited the largest infarct size, hence reduced dP/dt_{max} and compromised cardiac function. However, the repetitive + BZ group exhibited the smallest infarct but small dP/dt_{max}.

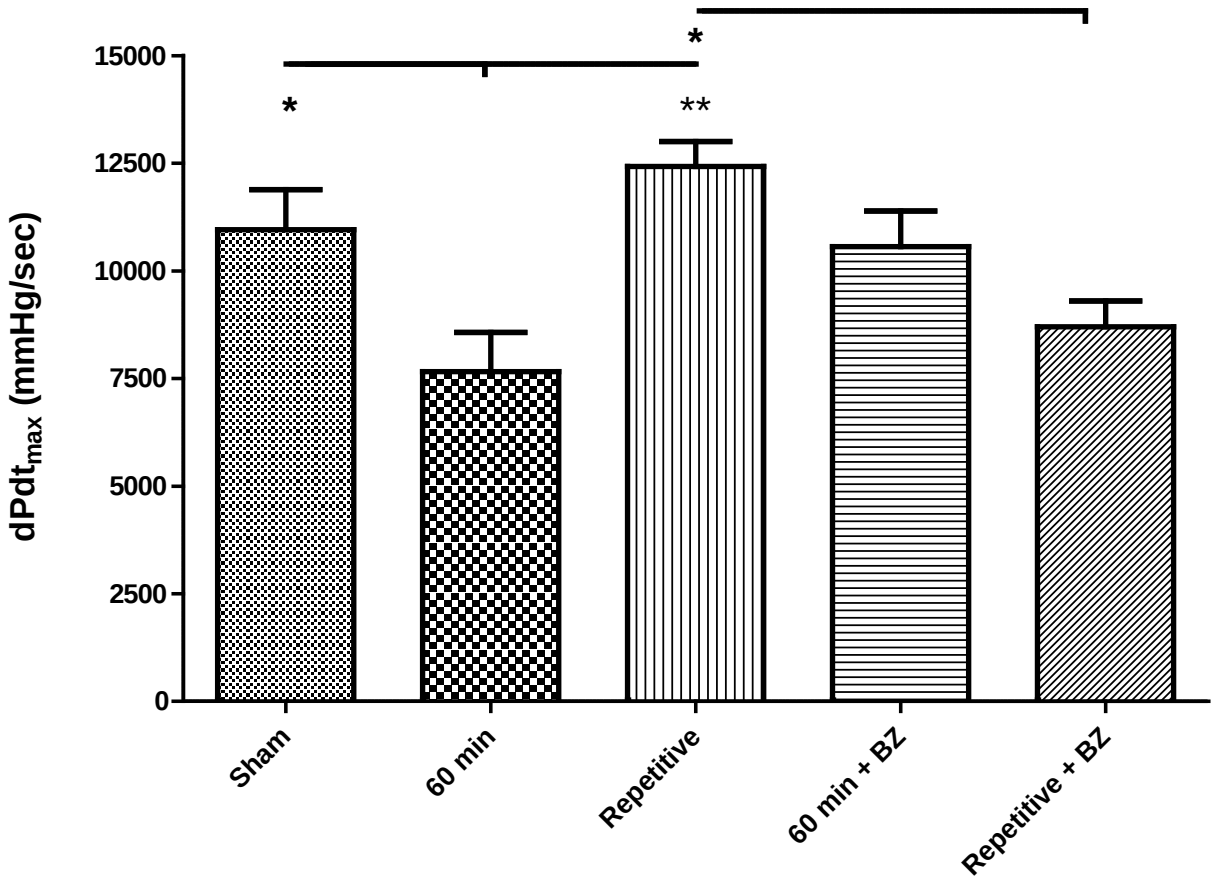

Fig. 36: dP/dt_{max} (mmHg/sec) in different experimental groups after 24 hours of last ischemia. The sham group exhibited a dP/dt_{max}value of 10957.3 ± 931.8 mmHg/sec (n=10). 24 h after a 60 min long ischemia reduced the dP/dt_{max}value to 7655.9 ± 913.4 mmHg/sec in the 60 min group (n=11). Preceding short repetitive ischemia led to a dP/dt_{max}value of 12426 ± 579 mmHg/sec in the repetitive group (n=7). dP/dt_{max} value was found to be 10559.6 ± 827 mmHg/sec in the 60 min + BZ group (n=9) and 8701.01 ± 597 mmHg/sec in the repetitive + BZ group (n=14).

3.3.7 dP/dt_{min}

Likewise $dP/dt_{max,}$ dP/dt_{min} is also highly dependent on contractility. It represents the peak of the first derivative of the falling left ventricular pressure vs time during relaxation period of the heart. dP/dt_{min} can be taken as a measure of the stiffness of the left ventricle.

The dP/dt_{min} was found to be significantly reduced in the 60 min group in comparison to the sham group by 30.20 ± 4.8%. Repetitive short ischemia treatment compensated the deleterious effects of the following long lasting 60 min ischemia as dP/dt_{min} was recorded just 3.4 ± 4.3% less than the sham group. In the presence of bortezomib, I/R suppressed the dP/dt_{min}value by 16.5 ± 4.2% in the 60 min + BZ group and by 18.47 ± 4.1% in the repetitive + BZ group in comparison to the sham group (Fig. 37). However, the reduction of the dP/dt_{min} in the 60 min group reached significance only vs the sham group and the repetitive group. These results are again in line with our TTC results where 60 min group (Fig. 30) exhibited largest infarct size.

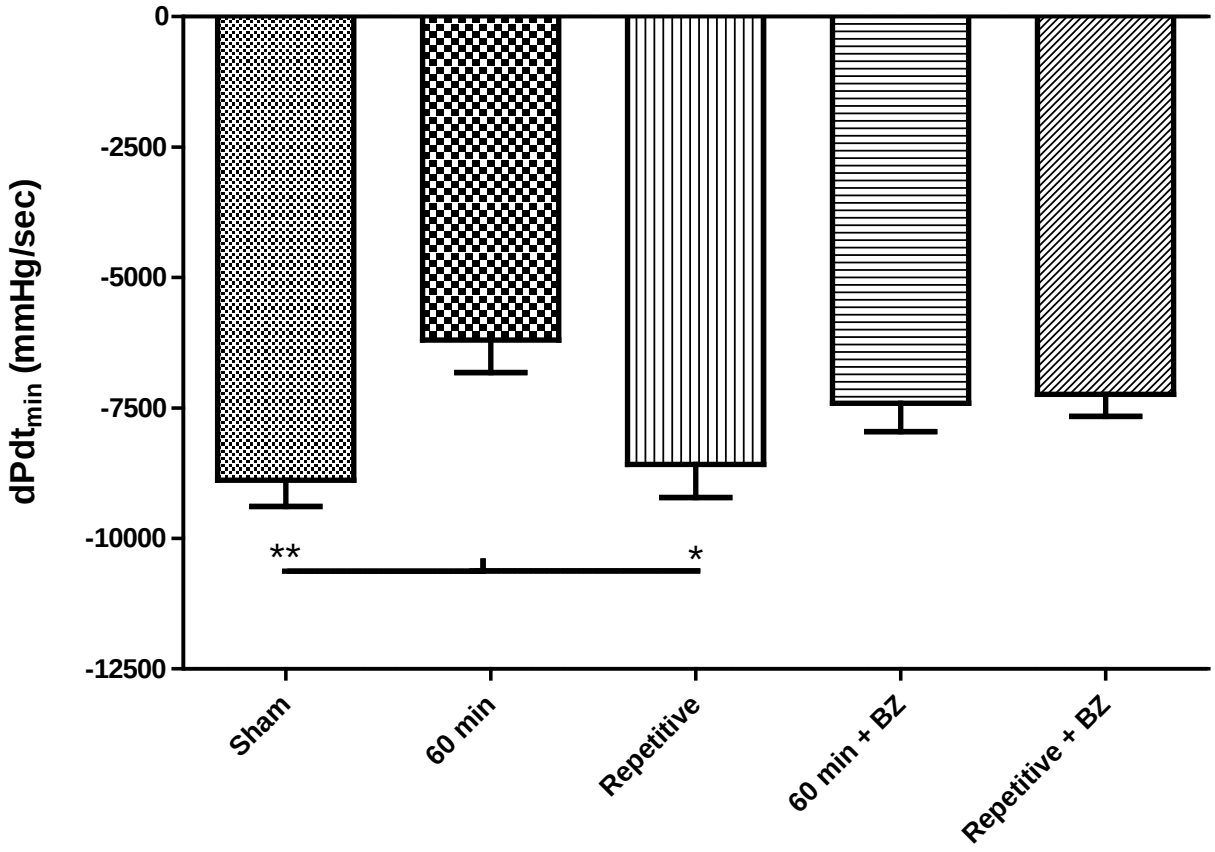

Fig. 37: dP/dt_{min} (mmHg/sec) in different experimental groups after 24 hours of last ischemia. The sham group exhibited a dP/dt_{min}value of -8882.1 ± 508 mmHg/sec (n=10). 60 min long ischemia reduced the dP/dt_{min}value to -6199.1 ± 620 mmHg/sec (n=11). Preceding short repetitive ischemia led to a dP/dt_{min}value of -8579.1 ± 639 mmHg/sec (n=7). dP/dt_{min} value was

found to be -7412.7 ± 539 mmHg/sec in the 60 min + BZ group (n=9) and -7241.5 ± 418 mmHg/sec in the repetitive + BZ group (n=14).

3.3.8 Heart rate

Heart rate was monitored in all the experimental groups but no significant difference was noted between any of the experimental groups (Fig. 38). The repetitive + BZ group exhibited the maximum difference in comparison the sham group and that was found to be 7.7 ± 3.5%. The 60 min group, the repetitive group and the 60 min + BZ group showed 1.13 ± 3.14%, 1.8 ± 2.8% and 0.1 ± 2.0% decrease respectively in the heart rate in comparison to the sham group.

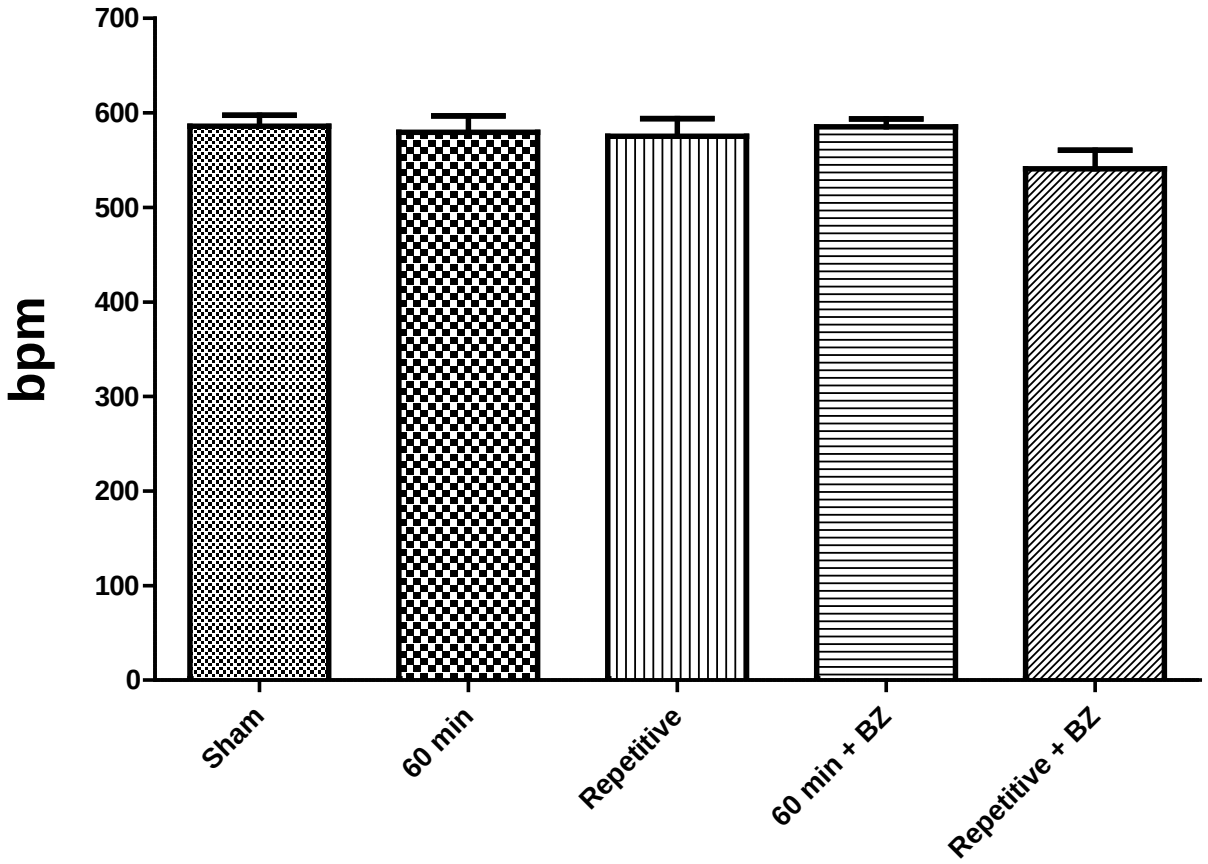

Fig. 38: Heart rate (bpm) in different experimental groups after 24 hours of last ischemia. The sham group exhibited the heart rate of 586 ± 11.4 bpm (n=10). 24 h after a 60 min long ischemia reduced the heart rate value to 579.3 ± 17.6 bpm (n=11). Pre-conditioning the heart with short

repetitive ischemias led to a heart rate of 575.1 ± 18.7 bpm (n=7). The heart rate was found to be 585.1 ± 8.4 bpm in the 60 min + BZ group (n=9) and 540.7 ± 19.6 bpm in the repetitive + BZ group (n=14).

3.3.9 Cardiac output

Cardiac output specifies the amount of the blood pumped into the body within 1 min. This can be used to calculate the total peripheral resistance, if the blood pressure is known. Fig. 39 shows the cardiac output in the different experimental groups. I/R injury reduced the cardiac output by 22.6 ± 5.2% in the 60 min group in comparison to the sham group. Pre-conditioning the mice with repetitive ischemias prior to the following 60 min long ischemia increased the cardiac output insignificantly by 34.0 ± 3.9% in the repetitive group in comparison to the sham group. Also in the 60 min + BZ group cardiac output after I/R was found to be increased by 17.3 ± 4.4% when compared with the sham group. In the repetitive + BZ group I/R reduced the cardiac output insignificantly by 8.7 ± 5.8% in comparison to the sham group.

In the repetitive and the 60 min + BZ groups, the cardiac output was significantly higher than in the 60 min group and the repetitive group was significantly above the repetitive + BZ group. Although I/R did not reduce the cardiac output in any of the experimental groups significantly, the 60 min group developed the lowest performance again.

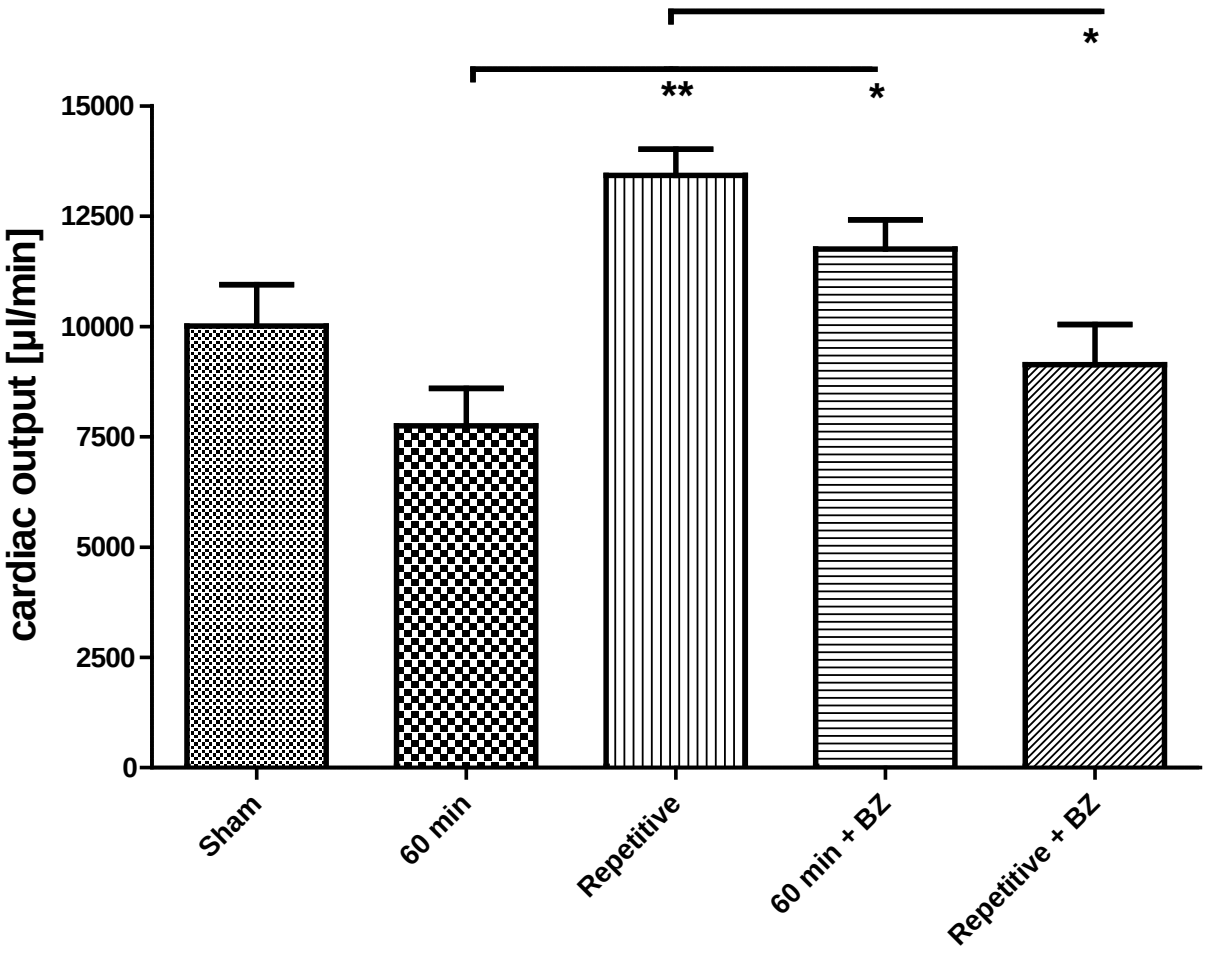

Fig. 39: Cardiac output (µl/min) in different experimental groups after 24 hours of last ischemia. The cardiac output was found to be 10011.6 ± 934 µl/min (n=10) in the sham group. 24 h after a 60 min long ischemia reduced the cardiac output to 7749.7 ± 846 µl/min (n=11). The repetitive group exhibited the cardiac output of 13423.1 ± 546 µl/min (n=7). The cardiac output was found to be 11752.5 ± 662 µl/min in the 60 min + BZ group (n=9) and 9134.02 ± 910 µl/min in the repetitive + BZ group (n=14).

3.3.10 Cardiac index

In cardiac hemodynamics, the cardiac index is a very important parameter which is related to the cardiac output and correlates the performance of the heart according to the weight of the organism. Cardiac index was also found to be significantly reduced in the 60 min group

compared to the 60 min + BZ group (Fig. 40). I/R decreased the cardiac index in the 60 min group only slightly as compared to the sham group (4.8 ± 5.6%). Repetitive short ischemia treatment prior to the following 60 min long ischemia significantly increased the cardiac index by 62.1 ± 4.7% in comparison to the sham group even after I/R. Also 60 min + BZ group exhibited an increase in cardiac index by 33.9 ± 5.6% while the repetitive + BZ group showed a cardiac index (2.1 ± 6.6%) in the same range as the sham group. However, due to the high cardiac index in the repetitive group those of the sham, the 60 min and the repetitive + BZ groups were significantly smaller.

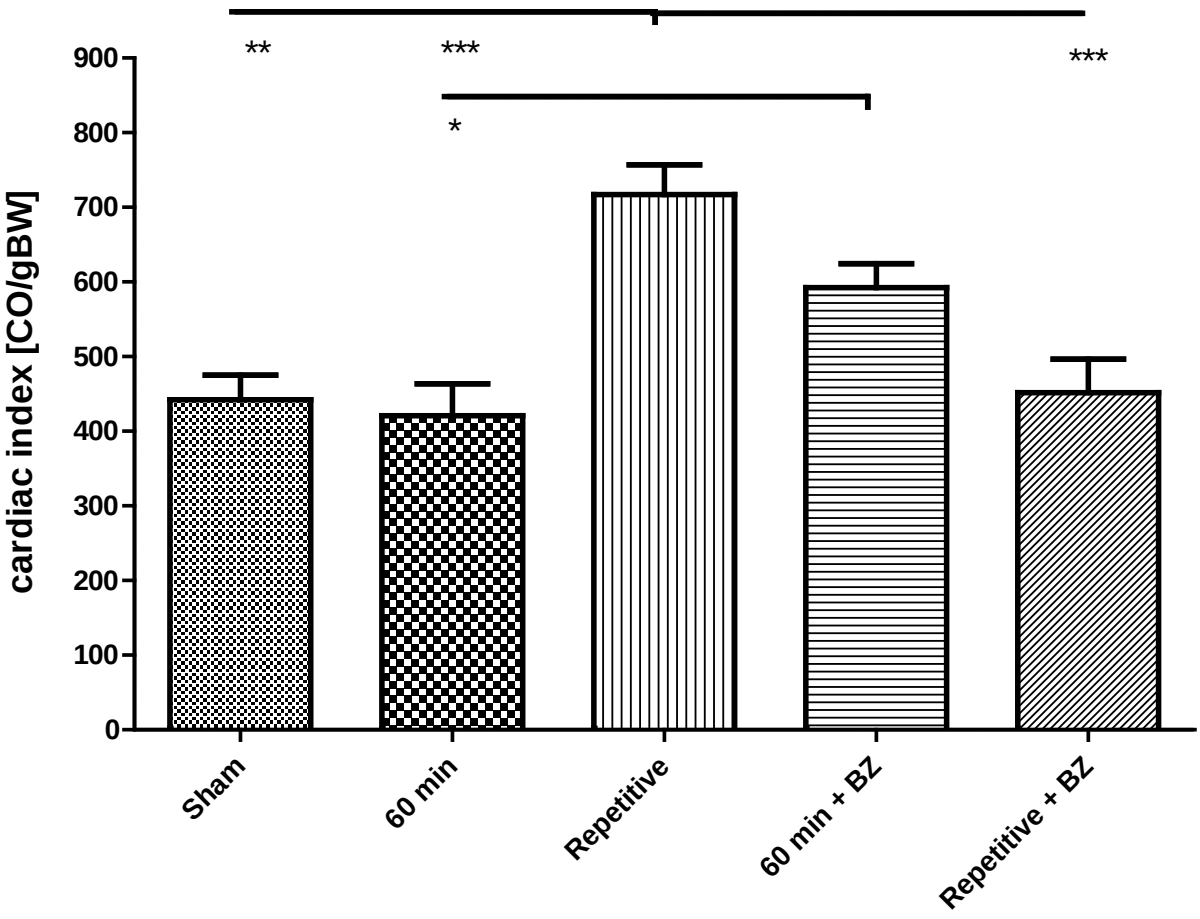

Fig: 40. Cardiac index (CO/gBW) in different experimental groups after 24 hours of the last ischemia. The sham group showed a cardiac index of 442.3 ± 32.7 CO/gBW (n=10). 24 h after a 60 min long ischemia reduced the cardiac index to 420.7 ± 42.7 CO/gBW (n=11). The repetitive group showed a cardiac index of 717.0 ± 39.8 CO/gBW (n=7). The cardiac index was found to

be 592.2 ± 32 CO/gBW in the 60 min + BZ group (n=9) and 451.7 ± 44.7 CO/gBW in the repetitive + BZ group (n=14).

3.4 Inflammation

As a result of an ischemic insult, an inflammation was expected. Therefore different indicators of the inflammation were investigated after 24 h of reperfusion.

3.4.1 Tumor necrosis factor α

Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine that is involved in systemic inflammation. As result of the proteasome inhibition and repetitive ischemia treatment, a subsequent decrease in TNF-α was expected.

Unexpectedly, the TNF-α value was found to be lower in the 60 min group than in the sham group (35.6 ± 4.3%); however, this reduction did not reach the level of significance. The repetitive ischemia treatment also reduced the TNF-α value by an insignificant 19.1 ± 5.9%. The experimental groups which were treated with bortezomib exhibited increases in the TNF-α value but these were also insignificant. The TNF-α value was found to be increased by 51 ± 9.9% while the repetitive + BZ group showed augmentation in the TNF-α value by 1 ± 0.7% in comparison to the sham group (Fig. 41).

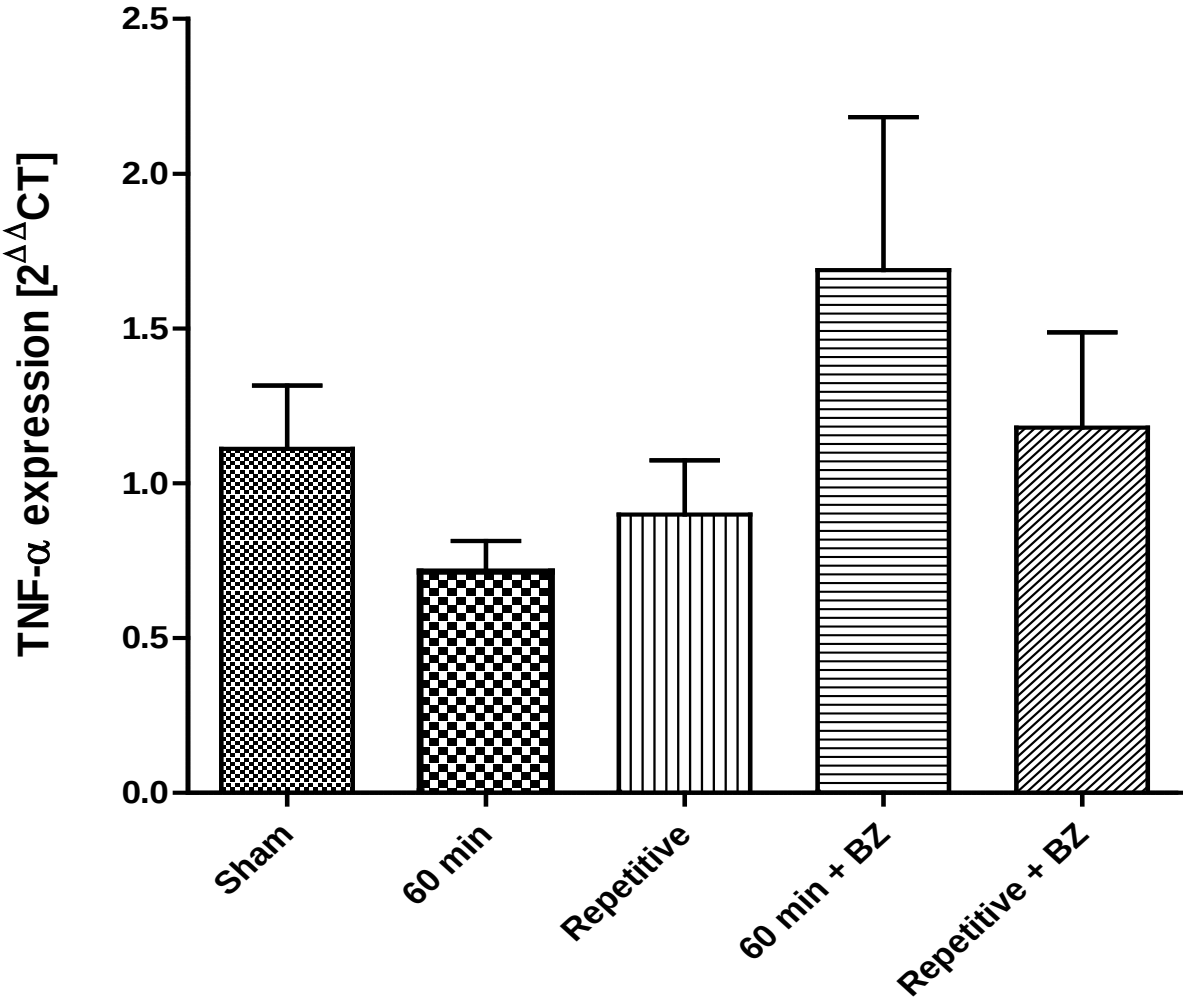

Fig 41: mRNA expression of TNF-α after 24 hours of the last ischemia. The sham group exhibited the TNF-α value of about 1.114 ± 0.20 (n=5). 24 hours after the 60 min I/R, the TNF-α value was reduced to 0.71 ± 0.09 (n=5) while the short repetitive ischemia treatment reduced it to 0.8 ± 0.17 (n=5). Administering the bortezomib to these two groups slightly increased the TNF-α value to 1.68 ± 0.49 in 60 min + BZ group (n=5) and 1.18 ± 0.30 in the repetitive + BZ group (n=5)

3.4.2 Interleukin-6

Expression of another pro-inflammatory cytokine, interleukin-6 (IL-6) was investigated too (Fig. 42). It was found that the expression of IL-6 was significantly increased in 60 min + BZ group by 21079 ± 145.1% as compared to the sham group. Expression of the IL-6 was also significantly higher than in the repetitive group. IL-6 expression was also increased in the 60 min group (4007 ± 86.2%), the repetitive group (2080 ± 54.5%) and the repetitive + BZ group (3622 ± 56.4%) in comparison to the sham group but these increases were not found to be significant.

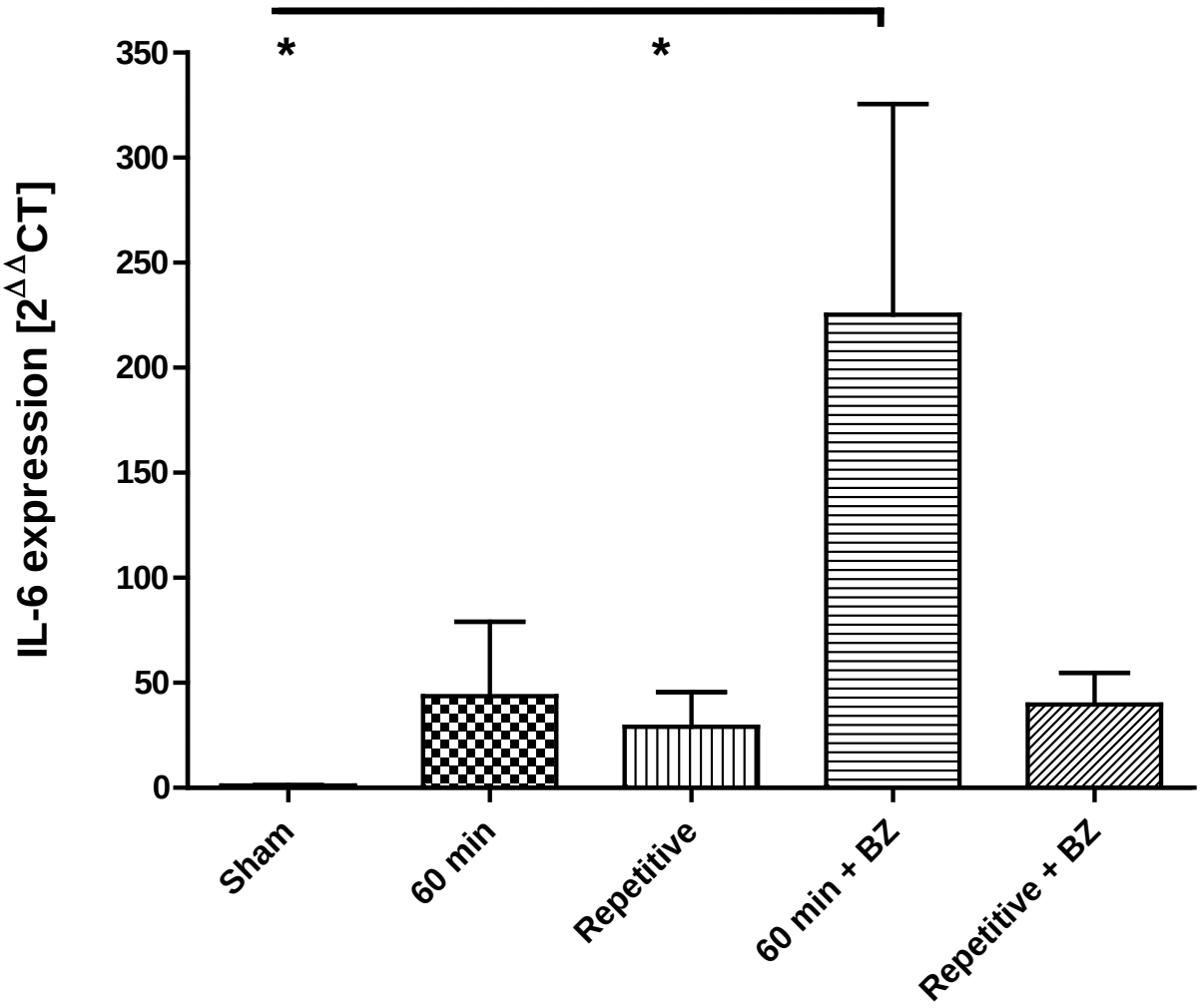

Fig. 42: mRNA expression levels of IL-6 after 24 hours of the last ischemia. The sham group exhibited an IL-6 value of 1.06 ± 0.16 (n=5). 60 min ischemia and preceding short repetitive ischemias increased the IL-6 value to 43.7 ± 35.3 (n=5) and 28.9 ± 16.6 (n=5) respectively. 60

min + BZ group exhibited a significantly augmented IL-6 value of 225.3 ± 100.2 (n=5) while the repetitive + BZ group showed the slight increase in IL-6 to 39.5 ± 15.1 (n=5).

3.4.3 Interleukin- 1β

The pro-inflammatory cytokine interleukin-1β (IL-1β) expression was also investigated in all experimental groups (Fig. 43). Significantly higher levels of IL-1β expression were found in 60 min + BZ group in comparison to the sham group (8500 ± 87.8%) the 60 min group (597 ± 32.1%) and the repetitive + BZ group (978.6 ± 23.9%). Although the IL-1β expression in the 60 min + BZ group was also far above the repetitive group, it did not reach the level of significance. IL-1β expression in the repetitive group was insignificantly elevated by (1732.9 ± 52.7%) compared to the sham mice.

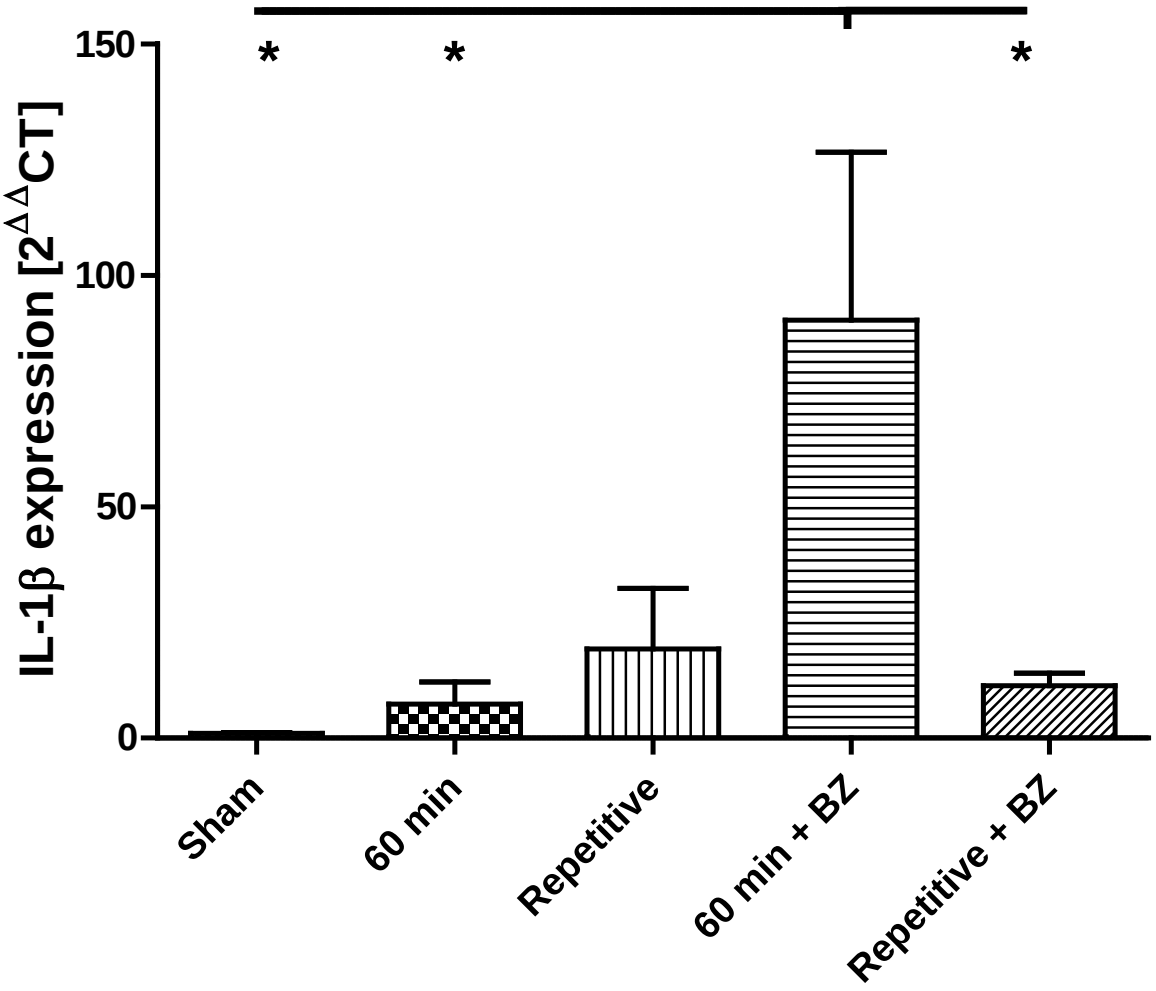

Fig. 43: Expression of IL-1β in different experimental groups after 24 hours of the last ischemia. The sham groups exhibited the IL-1β value of 1.05 ± 0.14 (n=5). 60 min ischemia and preceding short repetitive ischemia increased the IL-1β value to 7.3 ± 4.8 (n=5) and 19.2 ± 13.0 (n=5) respectively. 60 min + BZ group exhibited a significantly augmented IL-1β value of 90.32 ± 36.2 (n=5) while the repetitive + BZ group showed an increase in IL-1β to 36.3 ± 2.6 (n=5).

3.4.4 Hypoxia-inducible factor1-α

Since the proteasome system is involved in the regulation of hypoxia-inducible factor (HIF) stability and activity, we analyzed the expression of HIF-1α (Fig. 54). The 60 min + BZ group and the repetitive + BZ group showed significant augmentation in the level of HIF-1α expression

in comparison to the sham group (124.69 ± 7.0% and 133.1 ± 7.1% respectively). The 60 min group (33.2 ± 6.1%) and the repetitive group (80.0 ± 7.0%) also showed an increased HIF-1α level of expression but this increase did not reach the level of significance.

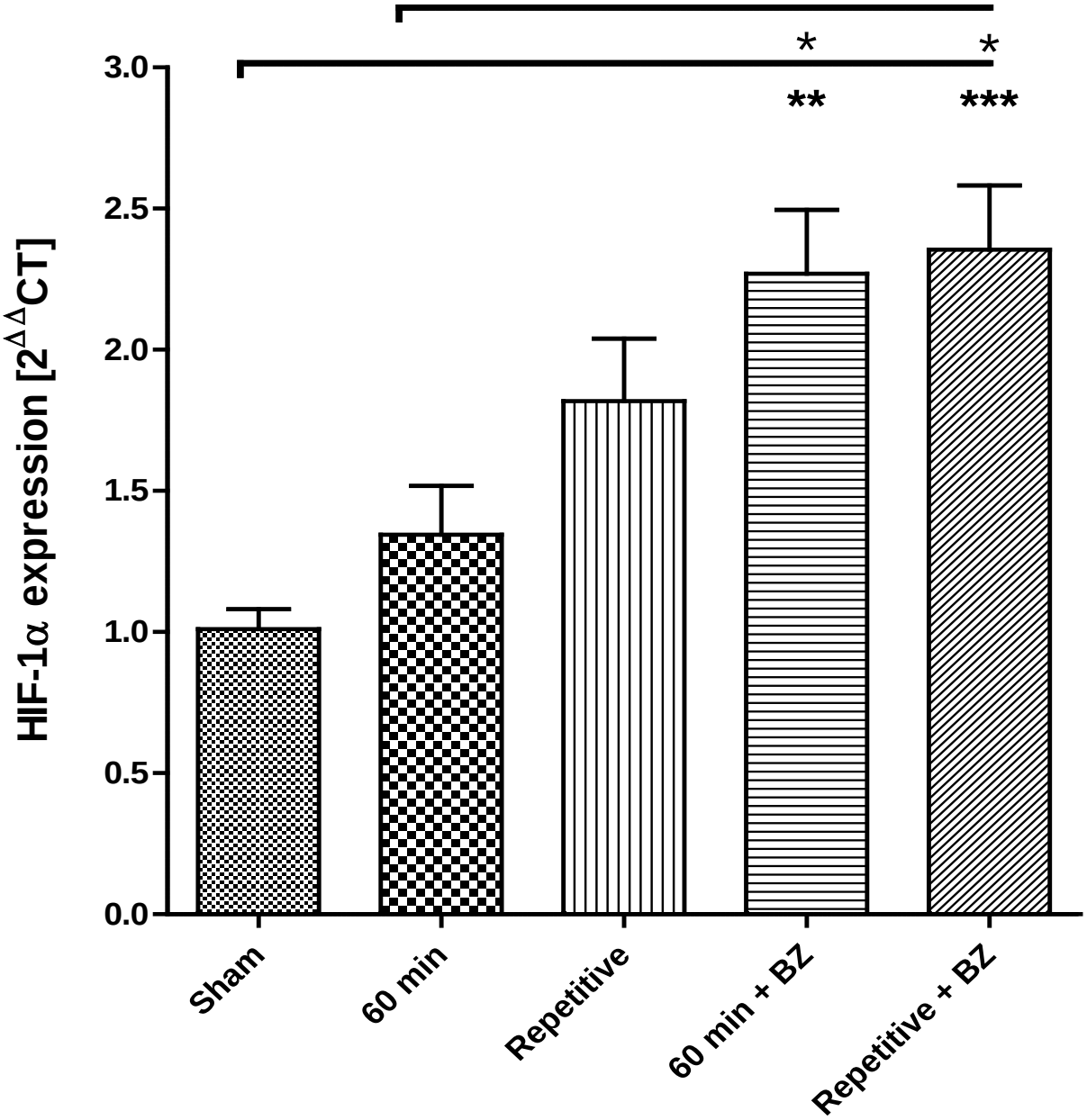

Fig. 44: mRNA levels of hypoxia-inducible factor in different experimental groups after 24 hours of the last ischemia. The sham group exhibited the HIF-1α expression of 1.00 ± 0.007 (n=5). 60 min long ischemia increased the HIF-1α expression level to 1.3 ± 0.17 (n=5) while treatment

with short repetitive ischemia increased the HIF-1α expression even more to 1.8 ± 0.2 (n=5). Administering these two groups with bortezomib significantly increased the HIF-1α expression level to 2.2 ± 0.2 (n=5) in 60 min + BZ group and 2.35 ± 0.22 (n=5) in the repetitive + BZ group.

3.4.5 Intercellular Adhesion Molecule 1

Intercellular Adhesion Molecule 1 (ICAM-1) expression was also investigated because of its high dependency on NF-κB and TNF-α and its function as vasodilative factor (Fig. 45). It was found that the expression level of ICAM-1 was significantly increased in the 60 min group (20.77 ± 7.7%), the repetitive group (90.9 ± 10.6%) and the 60 min + BZ group (132.5 ± 10.6%) in comparison to the sham group. ICAM-1 also showed an increased tendency in repetitive group + BZ but this augmentation was not found to be significant (57.8 ± 3.6%).

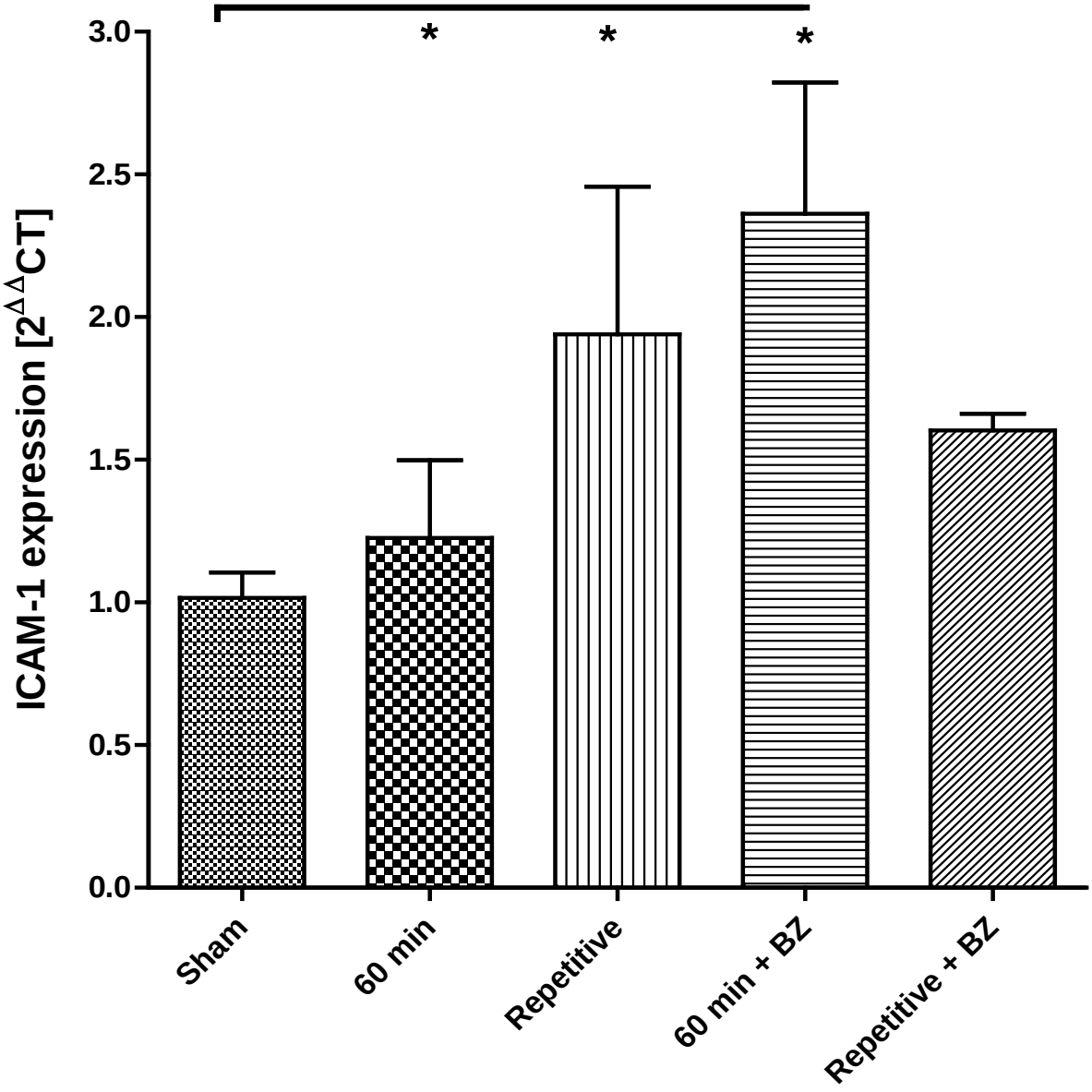

Fig. 45: Expression of ICAM-1 in different experimental groups after 24 hours of the last ischemia. The sham group exhibited the ICAM-1 expression value of 1.01 ± 0.08 (n=5) while the 60 min group and the repetitive ischemia group were found to have an ICAM-1 expression value of 1.2 ± 0.27 (n=5) and 1.93 ± 0.51 (n=5) respectively. On bortezomib treatment, the 60 min + BZ group exhibited the ICAM-1 expression value of 2.3 ± 0.46 (n=5) while the repetitive + BZ group was found to have an ICAM-1 expression value of 1.60 ± 0.05 (n=5).

3.4.6 Interleukin 10

Like IL-1, the anti-inflammatory cytokine interleukin 10 (IL-10) is also highly dependent on the NF-κB and TNF-α (Fig. 46). The pattern of the expression of IL-10 also followed the same trend as did in case of the IL-1β (Fig. 43) and IL-6 (Fig. 42). The IL-10 expression was found to be significantly increased in the 60 min + BZ group in comparison to the sham (1192.2 ± 27.4%) and all other groups. The 60 min ischemia treatment increased the IL-10 value by 282.0 ± 14.4% when compared to the sham group. Preceding short repetitive ischemias in repetitive group and administering the bortezomib in repetitive + BZ group also augmented the IL-10 expression by 207.1 ± 12.6% and 337.3 ± 15% respectively in comparison to the sham group.

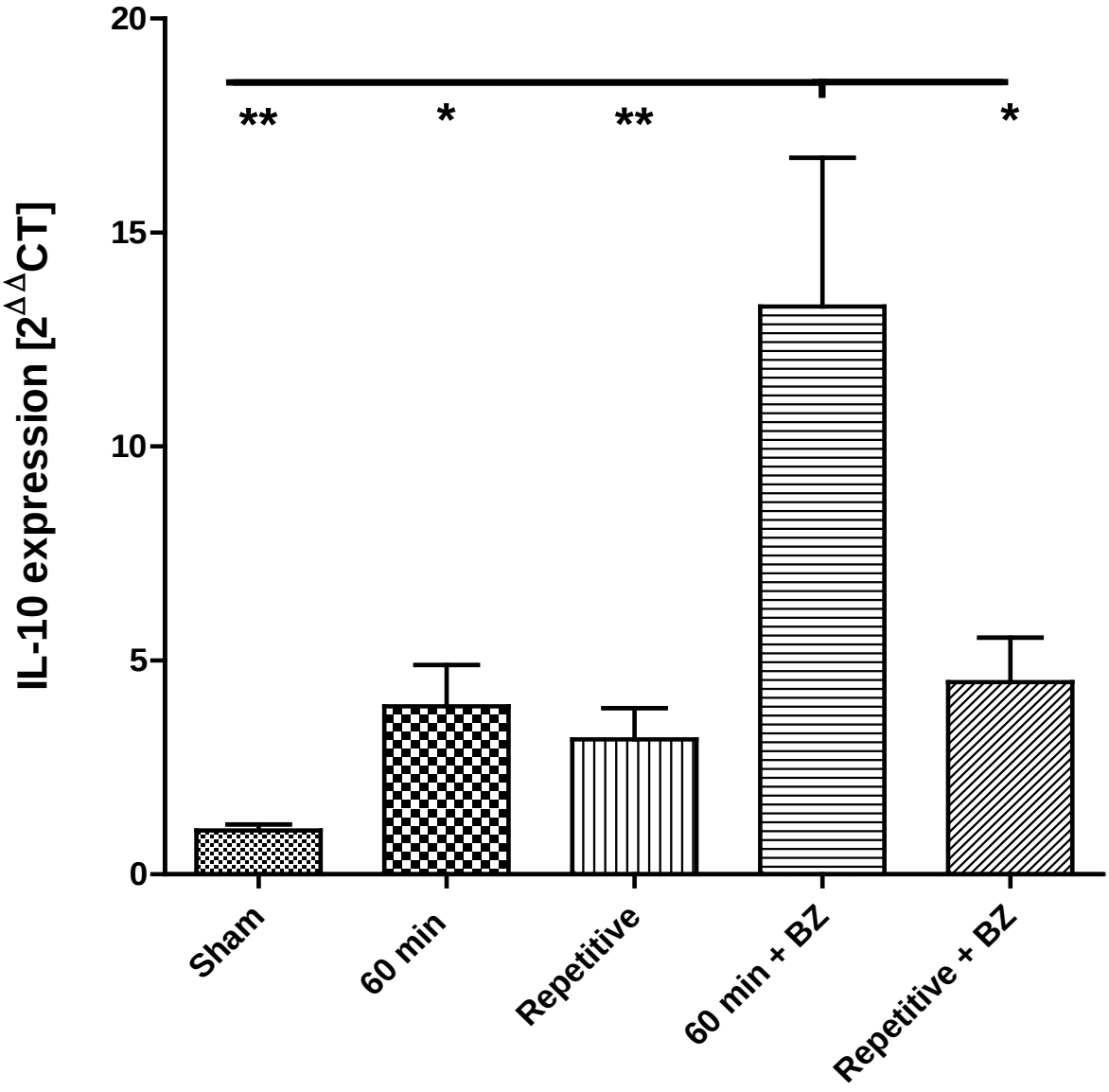

Fig. 46: mRNA expression levels of interleukin-10 in different experimental groups after 24 hours of last ischemia. The sham group exhibited the IL-10 value of 1.0 ± 0.13 (n=5) while the 60 min group and the repetitive group were found to exhibit the IL-10 value, with a slight increase, of 3.9 ± 0.9 (n=5) and 3.1 ± 0.7 (n=5) respectively. Moreover, the 60 min + BZ group and the repetitive + BZ group showed to have IL-10 expression value of 13.2 ± 3.4 (n=5) and 4.49 ± 1.0 (n=5) respectively.

3.5 Recordings 21 days after I/R

3.5.1 Sirius Red staining:

After 21 days of the last ischemia, hearts were excised, fixed, sections were prepared and these were stained with Sirius red (figs. 47 and 48).

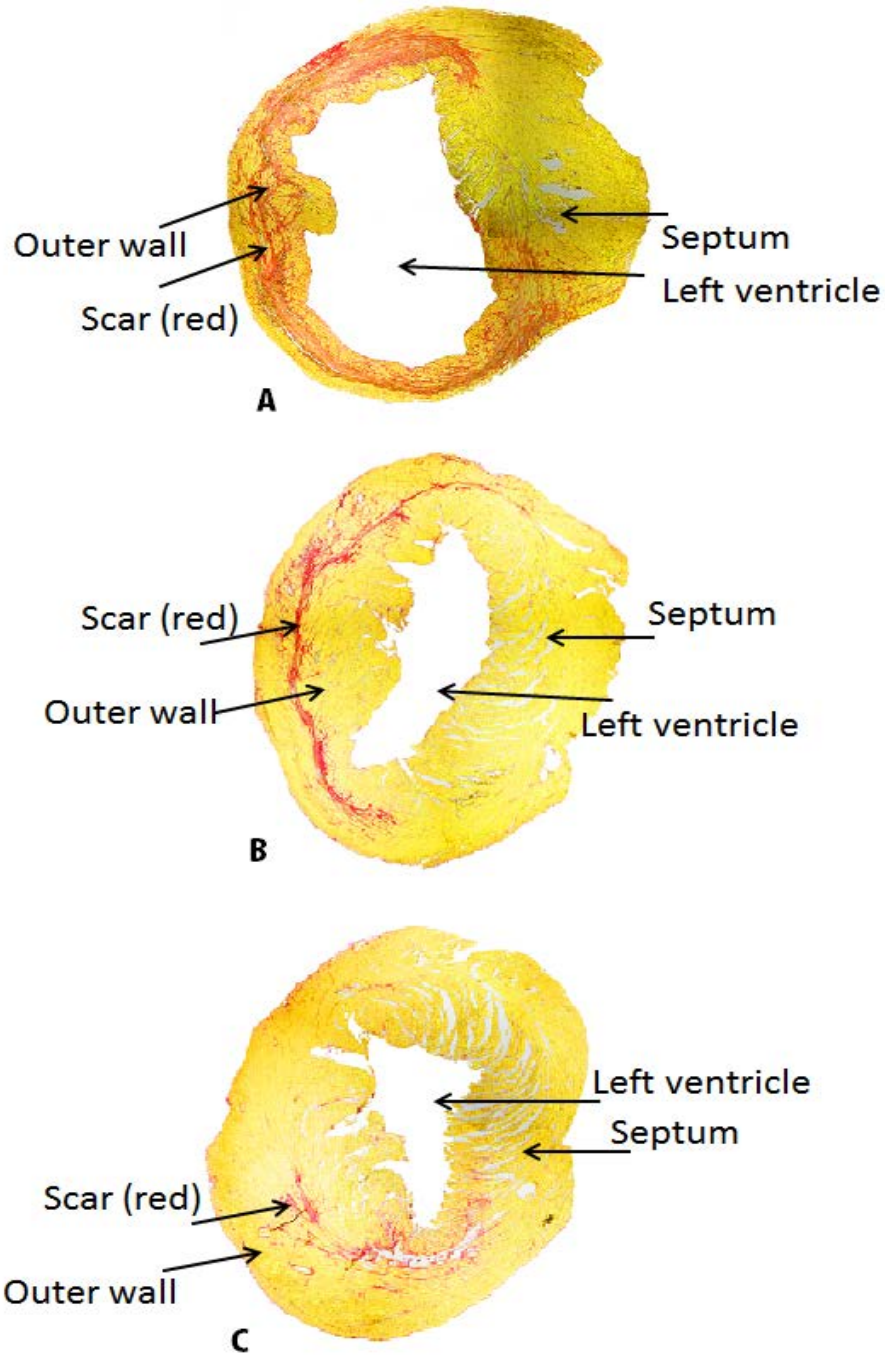

Fig. 47: Sirius red stained heart sections of (A) the 60 min + 21 d group, (B) the 60 min + BZ + 21 d group and (C) the repetitive + 21 d group showing their scar sizes.

The 60 min + 21 d group and the 60 min + BZ + 21 d group exhibited a huge increase in percentage scar size in comparison to the sham group (541.3% and 440% increase respectively). On the other hand, the repetitive + 21 d group showed only an increase of 4.95% in comparison to the sham group.

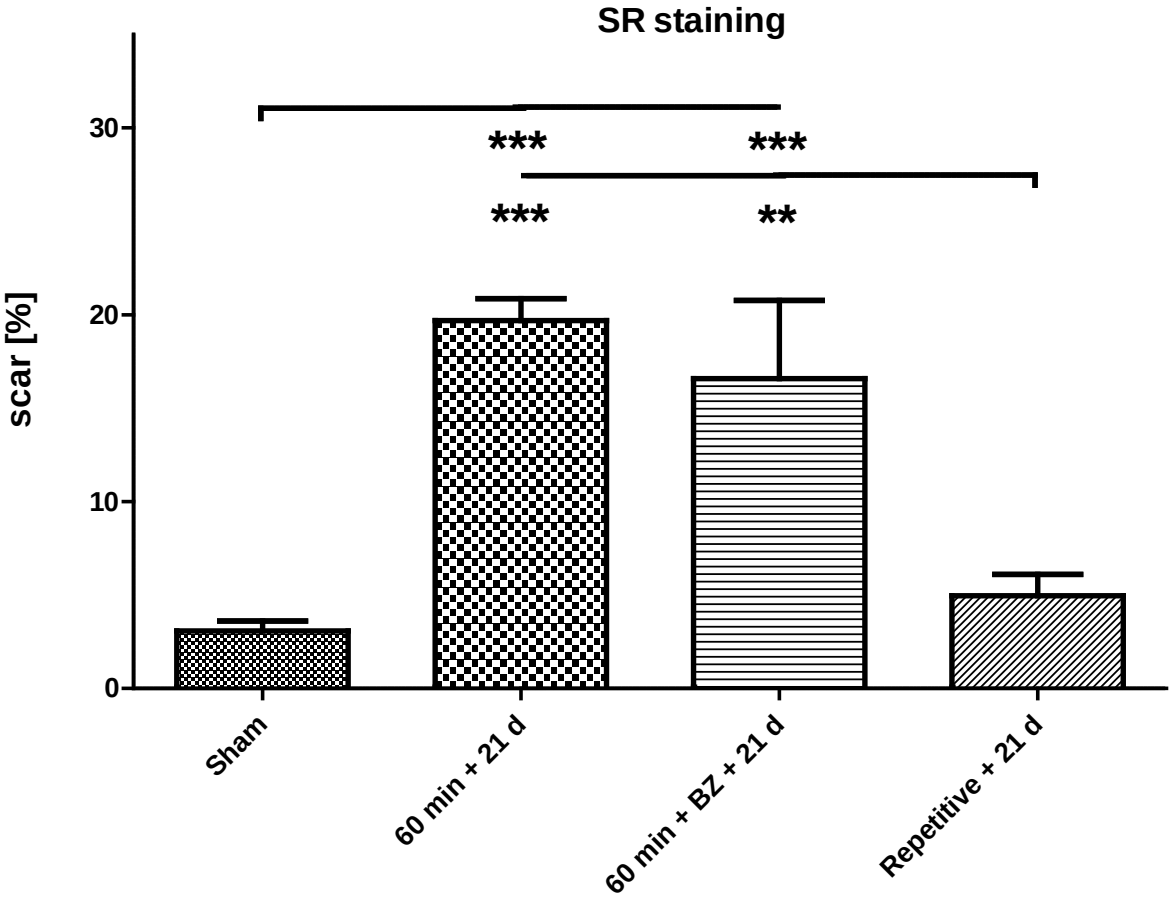

Fig. 48: Percentage scar in different experimental groups after 21 days of the last ischemia. The sham group and the 60 min + 21 d group exhibited 3.07 ± 0.7% (n=8) and 19.69 ± 4.45% (n=9) scar respectively while 60 min + BZ + 21 d and the repetitive + 21 d group exhibited 16.58 ± 3.87% (n=9) and 4.95 ± 1.15% (n=8) scar in the left ventricle.

3.5.2 Hemodynamic parameters after 21 days

Hemodynamic parameters were recorded 21 days after the last ischemia. In chapter 3.5.2 the measured results together with the calculated significances are presented in the figures and the respective values are given in the legends. The text paragraphs focused on the significant differences between the groups and the relative changes.

3.5.2.1 Ejection fraction

The hemodynamic investigation showed that the ejection fraction is significantly reduced in all the experimental groups in comparison to sham group after 21 days of the last ischemia (Fig. 49). 60 min of ischemia treatment reduced the ejection fraction significantly by 67.04 ± 3.1% in comparison to the sham group. The experimental groups treated with bortezomib as well as repetitive short ischemias also exhibited a significant reduction in ejection fraction by 62.6 ± 2.7% and 32.5 ± 3.6% respectively in comparison to the sham group.

It’s also to be noticed that the level of ejection fraction is significantly higher in repetitive group in comparison to 60 min group and 60 min + BZ group.

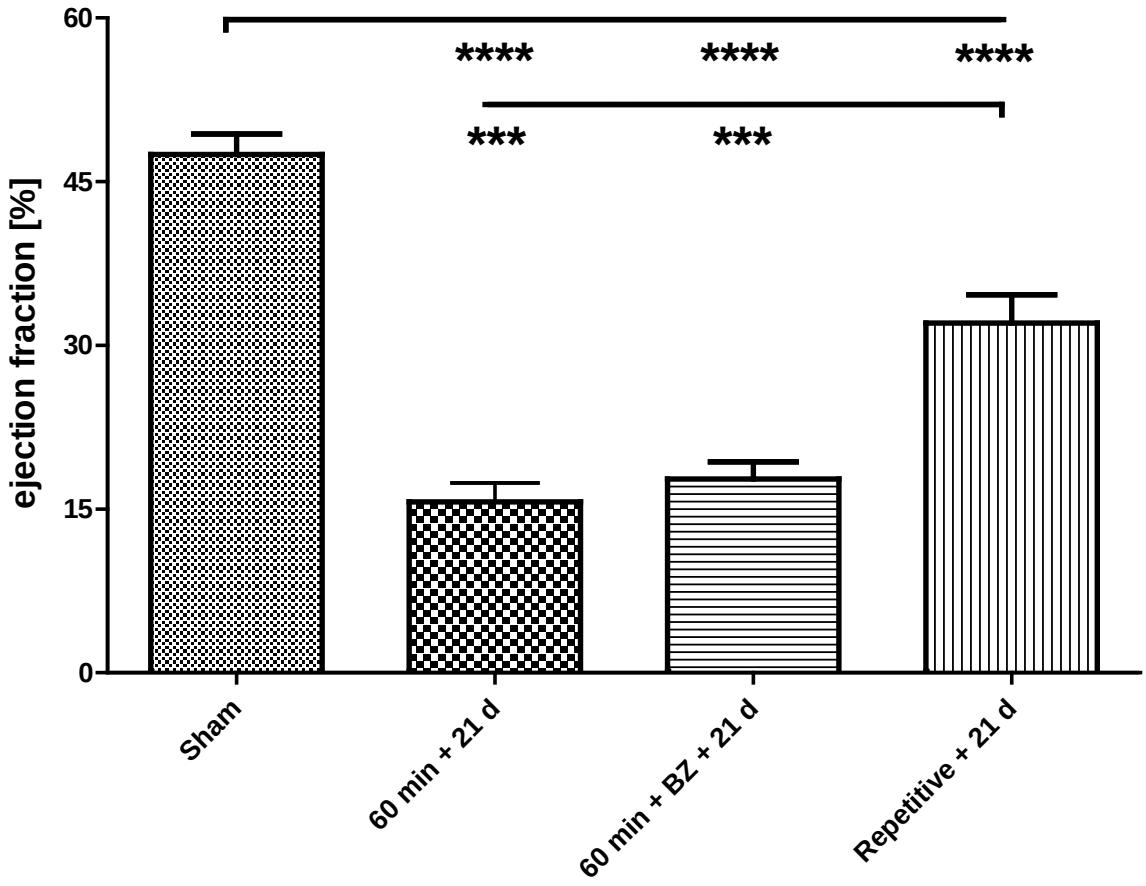

Fig. 49: Ejection fraction (%) in different experimental groups after 21 days of the last ischemia. The mice in the sham group ejected 47.4 ± 1.8% of the left ventricular volume (n=10). 21 d after a 60 min ischemia the mice ejected 15.6 ± 1.7% which was significantly less (n=7). The bortezomib treatment led to an ejection fraction of 17.7 ± 1.5% (n=5), while preceding short repetitive ischemias led to an ejection fraction of 32.0 ± 2.6% (n=6).

3.5.2.2 End-systolic pressure

Fig. 50 shows that the end-systolic pressure in all the experimental groups after 21 days of last ischemia was not altered significantly. All the groups exhibited almost the same end-systolic pressure as in the sham group. In comparison to the sham group, 60 min of ischemia reduced the end-systolic pressure by only non-significant 3.04 ± 4.8%. The experimental groups treated with bortezomib as well as repetitive short ischemias also exhibited an insignificant reduction in end-

systolic pressure by only 9.6 ± 2.3% and 0.99 ± 3.3% respectively in comparison to the sham group.

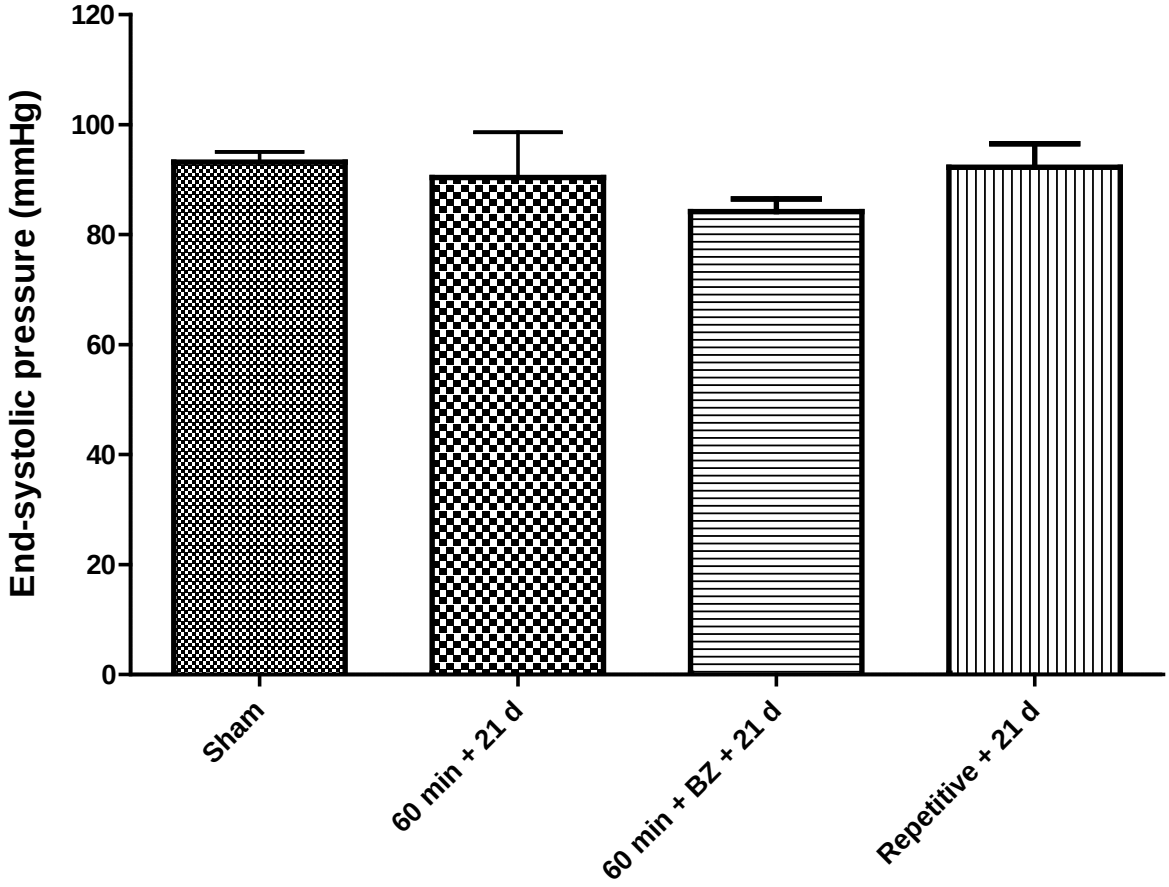

Fig. 50: End-systolic pressure (mmHg) in different experimental groups after 21 days of the last ischemia. The end-systolic pressure was noted to be 93.2 ± 1.8 mmHg in the sham group (n=10). 21 d after 60 min long ischemia the end-systolic pressure was found to be 90.3 ± 8.2 mmHg (n=7). The bortezomib treatment led to an end-systolic pressure of 84.1 ± 2.3 mmHg (n=5), while preceding short repetitive ischemias led to an end-systolic pressure of 92.2 ± 4.2 mmHg (n=6).

3.5.2.3 End-systolic volume

Fig. 51 demonstrates the end-systolic volume in different experimental groups. The end-systolic volume was found to be significantly increased in all of the experimental groups in comparison

to the sham group. The 60 min long ischemia significantly increased the end-systolic volume by 175 ± 8.5%, bortezomib led to a significant increase by 309 ± 7.9% and preceding short repetitive ischemia prior to the following 60 min long ischemia led to an end-systolic volume increase by 152.0 ± 4.7% in comparison to the sham group. End-systolic volume of the 60 min + BZ group was even significantly above those of the 60 min and the repetitive group.

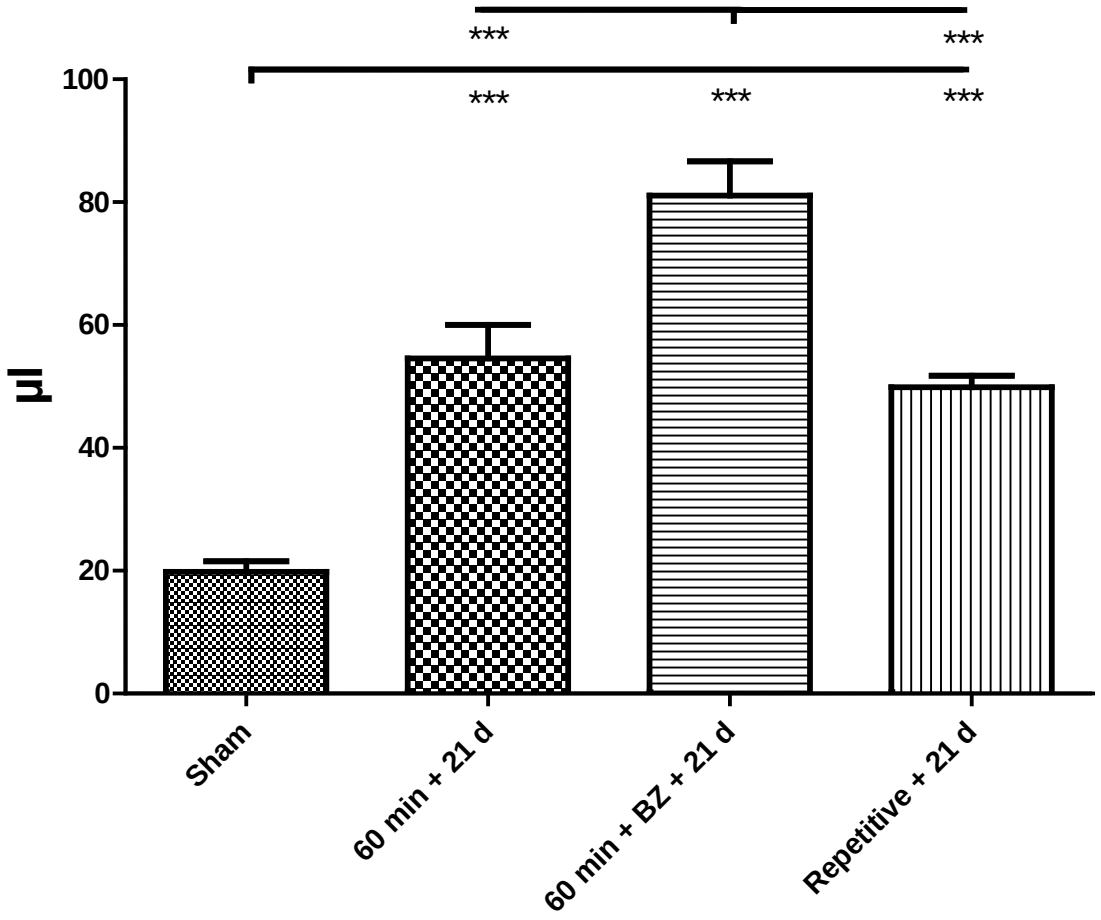

Fig. 51: End-systolic volume (µl) in different experimental groups after 21 d of the last ischemia. The end-systolic volume was noted to be 19.7 ± 1.7 µl in the sham group (n=10). 21 d after 60 min long ischemia the end-systolic volume was found to be 54.5 ± 5.4 µl (n=7). The bortezomib treatment led to an end-systolic volume of 81.0 ± 5.5 µl (n=5), while preceding short repetitive ischemias led to an end-systolic volume of 49.8 ± 1.8 µl (n=6).

3.5.2.4 End-diastolic pressure

Like the end-systolic pressure after 24 h, the end-diastolic pressure after 21 days of the last ischemia was not significantly different either between all of the experimental groups (Fig. 52). End-diastolic pressure in all the experimental groups was found to be a little higher than in the sham group but this increase was not significant. 60 min long ischemia led to non-significant increase in the end-diastolic pressure by 27.6 ± 6.8% in comparison to the sham group. The bortezomib treatment non significantly increased the end-diastolic pressure by 55.4 ± 8.2% while short repetitive ischemia before the long lasting 60 min ischemia led to a non-significant increase by 17.2 ± 5.5% in comparison to the sham group.

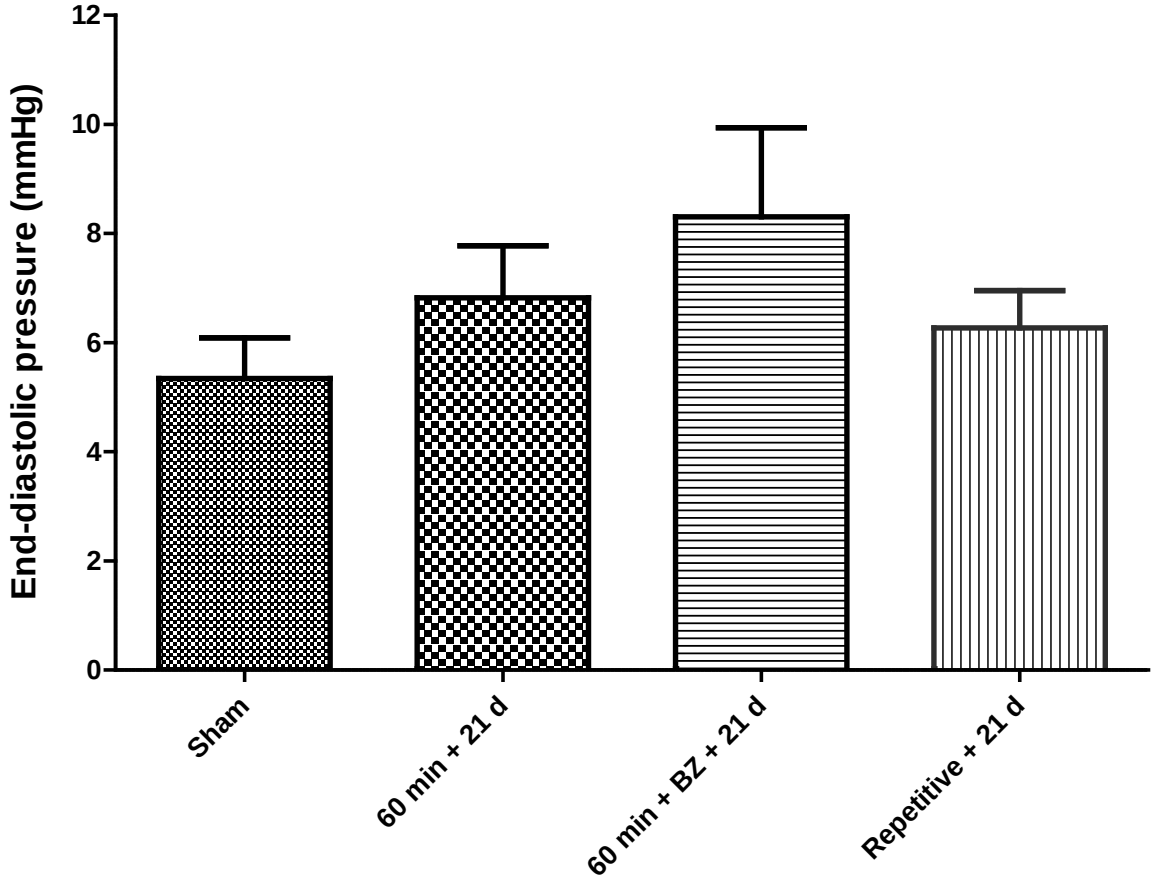

Fig. 52: End-diastolic pressure (mmHg) in different experimental groups after 21 days of the last ischemia. The end-diastolic pressure was noted to be 5.3 ± 0.7 mmHg in the sham group (n=10). 21 d after 60 min long ischemia the end-diastolic pressure was found to be 6.8 ± 0.9 mmHg

(n=7). The bortezomib treatment led to an end-diastolic pressure of 8.3 ± 1.6 mmHg (n=5), while preceding short repetitive ischemias led to an end-diastolic pressure of 6.2 ± 0.6 mmHg (n=6).

3.5.2.5 End-diastolic volume

The end-diastolic volume was measured in all the experimental groups (Fig. 53). The statistical analysis showed that the end-diastolic volume is significantly increased in all the experimental groups in comparison to the sham group. The 60 min long ischemia significantly increased the end-diastolic volume by 77.9 ± 6.2%, bortezomib treatment led to a significant increase by 172 ± 6.5% and preceding short repetitive ischemia prior to the following 60 min long ischemia led to an end-diastolic volume increase by 100.8 ± 4.1% in comparison to the sham group. Again performance of the 60 min + BZ group was significantly worse than that of the other experimental groups.

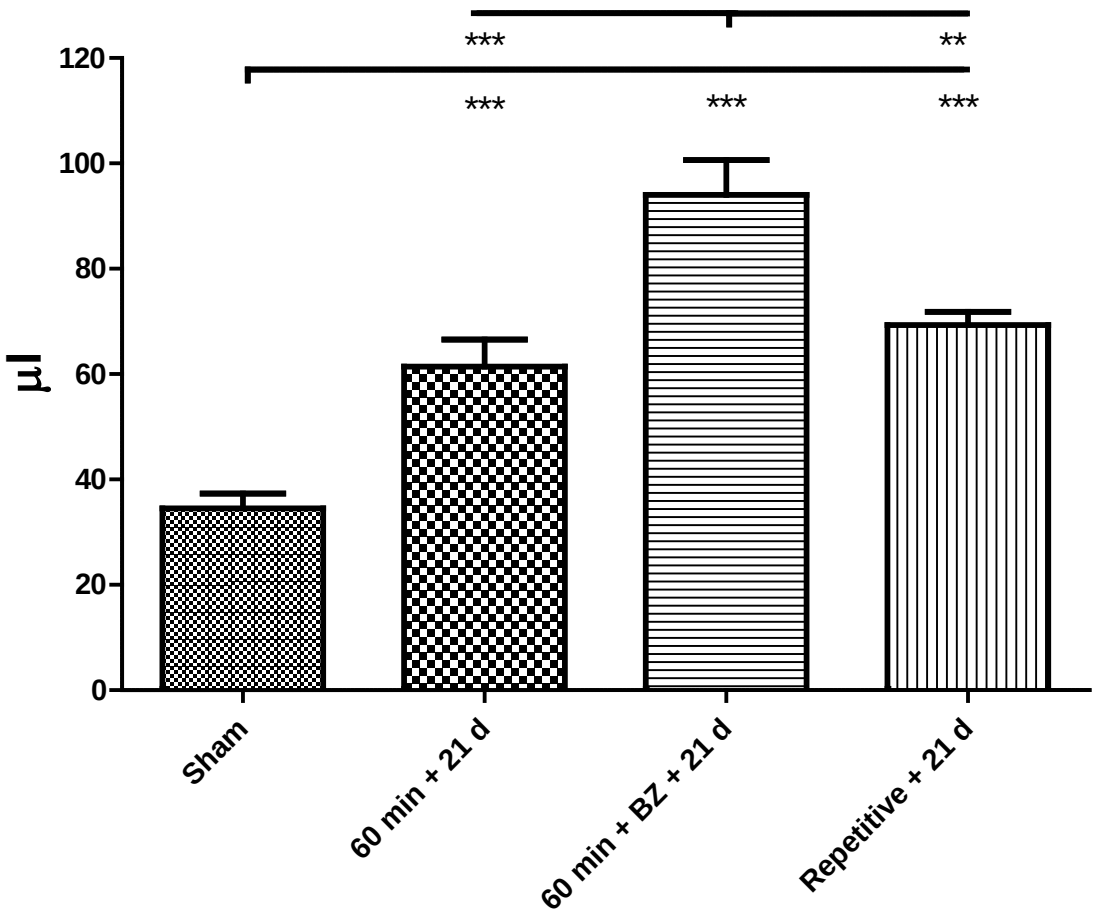

Fig. 53: End-diastolic volume (µl) in different experimental groups after 21 d of the last ischemia. The end-systolic volume was noted to be 34.5 ± 2.8 µl in the sham group (n=10). 21 d after 60 min long ischemia the end-systolic volume was found to be 61.4 ± 5.0 µl (n=7). The bortezomib treatment led to an end-systolic volume of 94.0 ± 6.5 µl (n=5), while preceding short repetitive ischemias led to an end-systolic pressure of 69.3 ± 2.4 µl (n=6).

3.5.2.6 dP/dt_{max}

Likewise dP/dt_{max} after 24 hours of the last ischemia (Fig. 54), dP/dt_{max} after 21 days of last ischemia also found to be significantly reduced in the 60 min group as well as 60 min + BZ group as compared to the sham group. 60 min of ischemia significantly reduced the dP/dt_{max} value by 23.7 ± 5.5% in comparison to the sham group. Treating the mice with bortezomib also significantly reduced the dP/dt_{max} value by 32.5 ± 4.3% in comparison to the sham group.

Treating the mice with preceding short repetitive ischemia increased the dP/dt_{max} value by 8.0 ± 4.1%.

These results are in support of our TTC results (Fig. 30) where the 60 min group exhibited the largest infarct, hence reduced contractility leading to reduced dP/dt_{max} and compromised cardiac function. It is also to be noted here that the dP/dt_{max} value has significantly increased in the repetitive group as compared to the 60 min group and the 60 min + BZ group (Fig. 54)

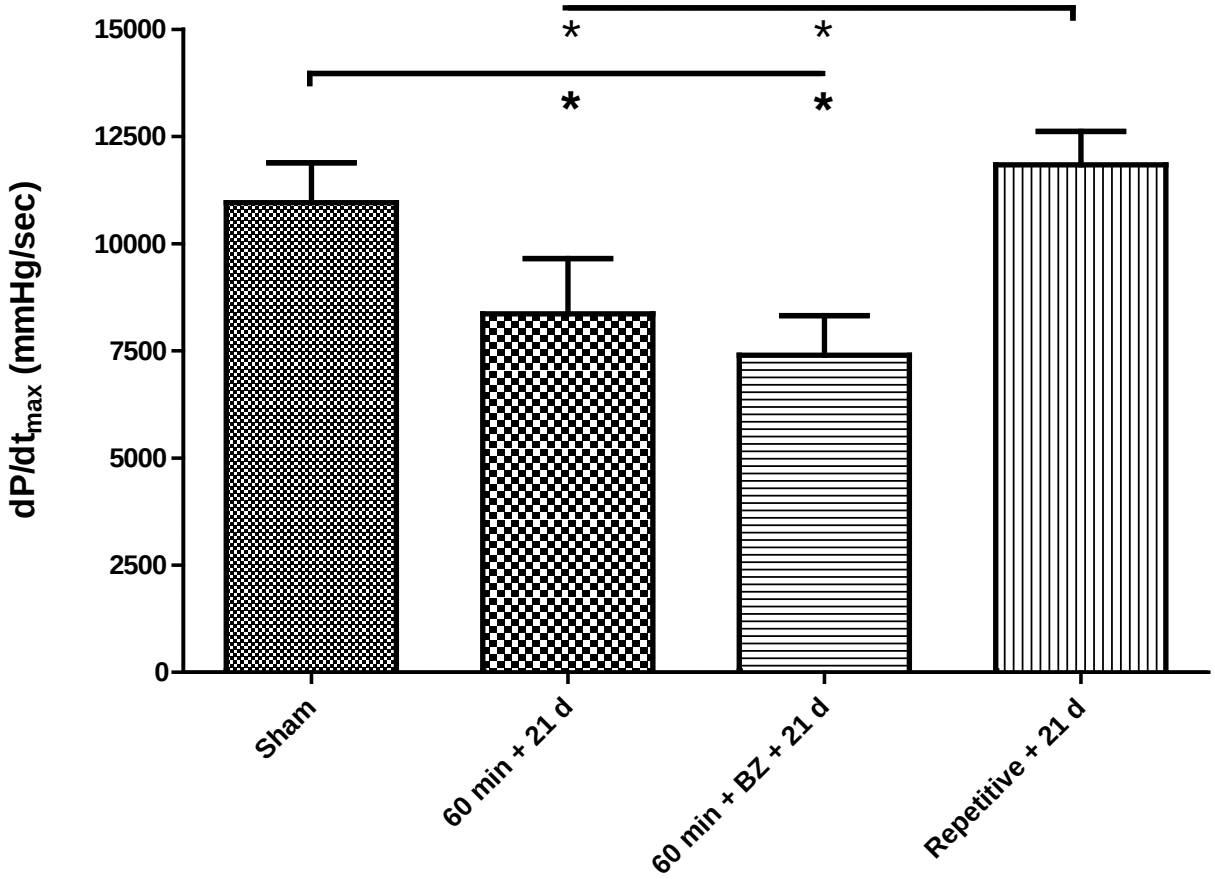

Fig. 54: dP/dt_{max} (mmHg/sec) in different experimental groups after 21 d of the last the ischemia. The dP/dt_{max} value was noted to be 10957.3 ± 931.8 mmHg/sec in the sham group (n=10). 60 mins ischemia significantly reduced the dP/dt_{max} value to 8360.2 ± 1289.5 mmHg/sec (n=7) while bortezomib significantly reduced it to 7393.8 ± 926.9 mmHg/sec (n=5). Preceding short repetitive ischemias increased the dP/dt_{max} value to 11839.5 ± 780.1 mmHg/sec (n=6).

3.5.2.7 dP/dt_{min}

dP/dt_{min} values were also investigated 21 days after the last ischemic injury in all the experimental groups. It was found that the dP/dt_{min} value decreased significantly in 60 min group and the 60 min + BZ group in comparison to the sham group (Fig. 55). dP/dt_{min} was reduced in 60 min group by 33.9 ± 3.9% and by 26.7 ± 3.5% in 60 min + BZ group in comparison to the sham group. Repetitive short ischemia increased the dP/dt_{min} value by a mere margin of 11.5 ± 4.4% when compared to the sham group.

These results are in line with dP/dt_{min} results after 24 hours of the last ischemia (Fig. 55). It could also be seen here that dP/dt_{min} values in the repetitive group are significantly higher than in the 60 min group and the 60 min + BZ group. This result is again in support of dP/dt_{min} values after 24 hours of the last ischemia (Fig. 55). Overall, dP/dt_{min} values after 21 days of the last ischemia can be explained by the TTC results (Fig. 30) where 60 min group was found to have the biggest infarct size, hence, reduced contractility.

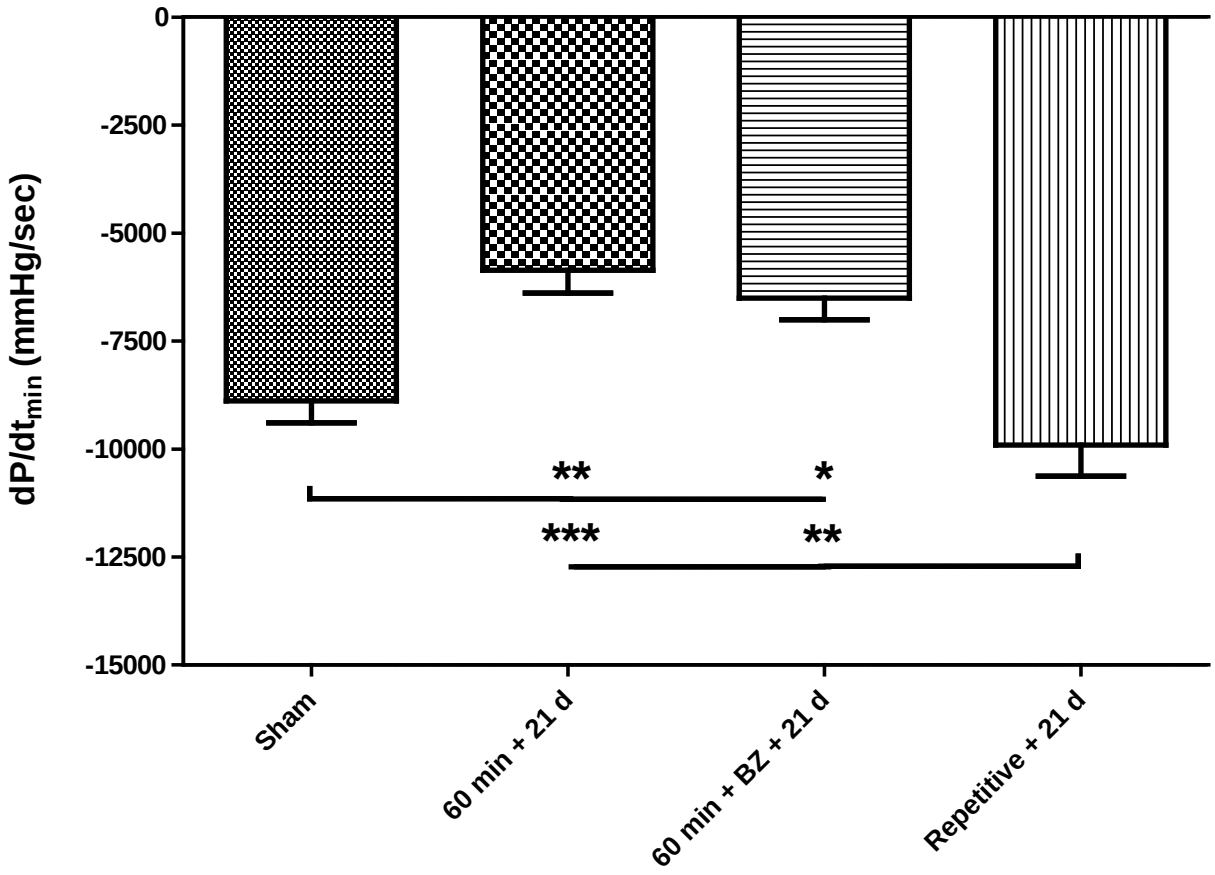

Fig. 55: dP/dt_{min} (mmHg/sec) in different experimental groups after 21 d of the last the ischemia. The dP/dt_{max} value was noted to be -8882.1 ± 508.4 mmHg/sec in the sham group (n=10). 60 mins ischemia significantly reduced the dP/dt_{max} value to -5862.5 ± 524.1 mmHg/sec (n=7) while bortezomib significantly reduced it to -6502.8 ± 503.4 mmHg/sec (n=5). Preceding short repetitive ischemias increased the dP/dt_{max} value to -9905.8 ± 721.8 mmHg/sec (n=6).

3.5.2.8 Heart rate

Heart rate was monitored in all the experimental groups but no significant difference was found between any of the experimental groups (Fig. 56). 60 min of ischemia treatment and repetitive ischemias slightly but insignificantly increased the heart rate by 3.6 ± 3.0% and 5.2 ± 2.9%

respectively in comparison to the sham group. Additionally, bortezomib treatment reduced the heart rate a little but again insignificantly by 3.3 ± 2.1% in comparison to the sham group.

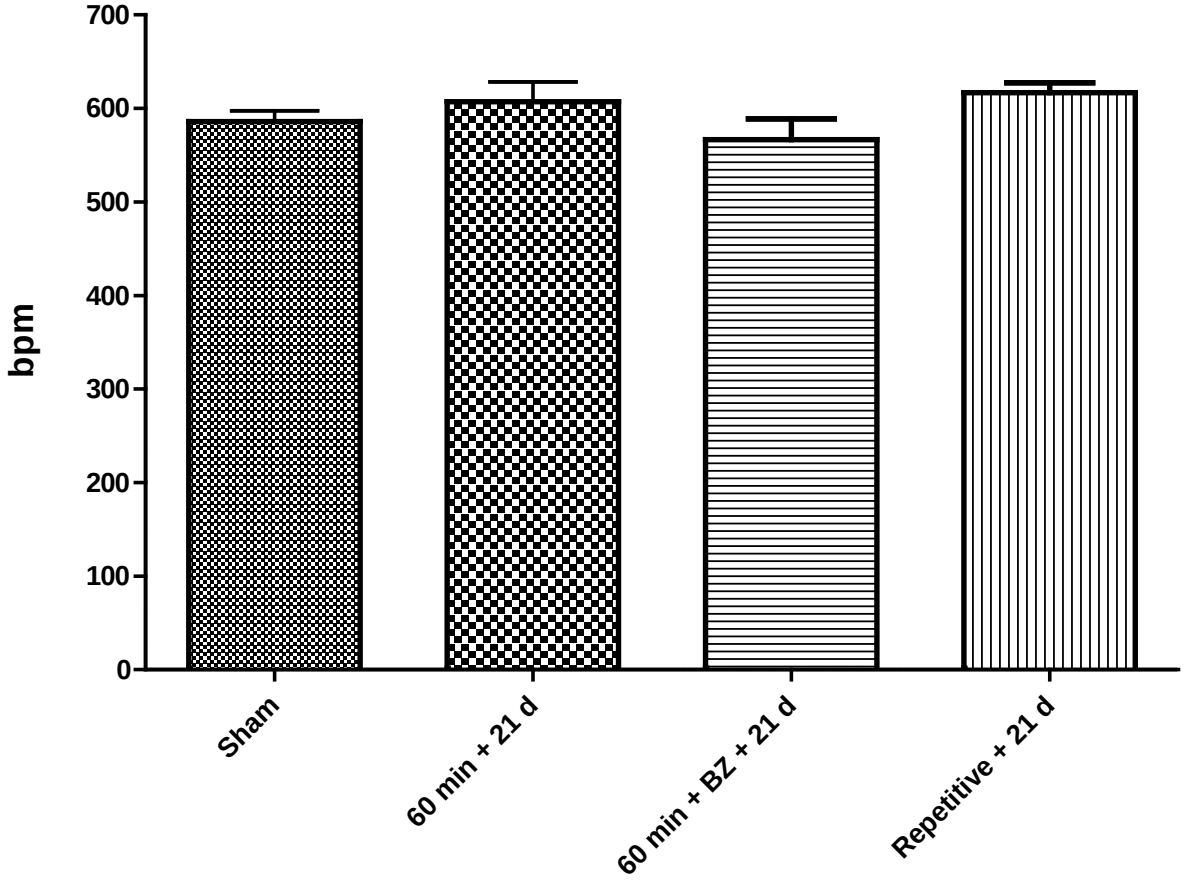

Fig. 56: Heart rate (bpm) in different experimental groups after 21 d of the last ischemia. The sham group exhibited the heart rate of 586 ± 11.2 bpm (n=10). 21 d after a 60 min long ischemia increased the heart rate to 607.1 ± 21.2 bpm (n=7). The group of animals who were administered with doses of bortezomib exhibited a heart rate of 566.6 ± 22.3 bpm (n=5) while pre-conditioning the hearts with repetitive short ischemias led to a heart rate of 616.6 ± 10.8 bpm (n=6).

3.5.2.9 Cardiac output

Cardiac output after 21 days of the last ischemia exhibited the same pattern as after 24 hour of the last ischemia. 60 min group found to have the significantly reduced cardiac output compared

to/ vs. the sham group and the repetitive group (Fig. 57). 60 min + BZ group exhibited almost the unchanged cardiac output as in the sham. 60 min ischemia treatment reduced the cardiac output significantly by 42.6 ± 4.1% while short repetitive ischemias increased the cardiac output values significantly by 40.0 ± 5.9% in comparison to the sham group. The bortezomib treatment prevented the I/R dependent reduction of cardiac output when compared to the sham group. The recorded difference between the two groups was found to be 4.1 ± 4.7%.

This data is in support of our earlier found TTC staining results (Fig. 30) where 60 min group showed the largest infarct size that must have led to compromised contractility, hence reduced cardiac output.

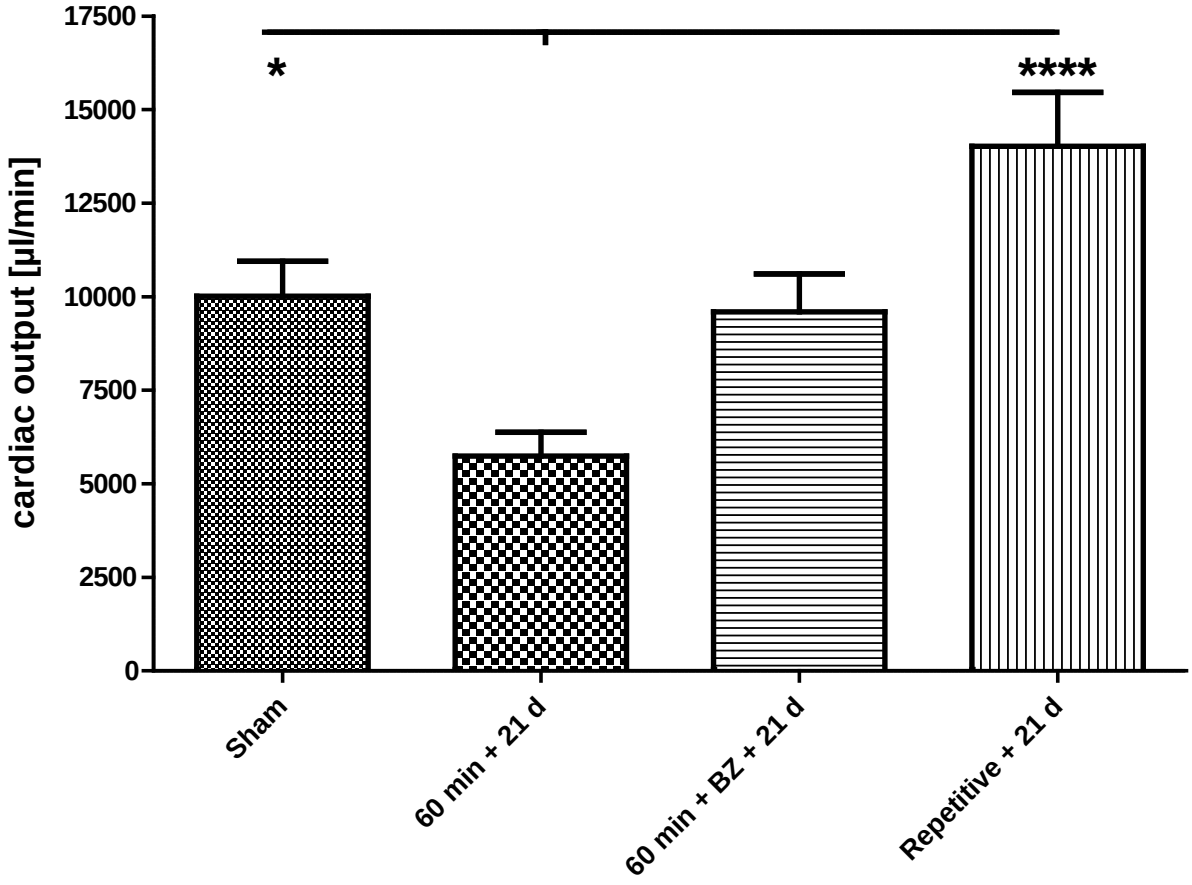

Fig. 57: Cardiac output (µl/min) in different experimental groups after 21 d of the last ischemia. The sham group exhibited the cardiac output value 10011.6 ± 934.7 µl/min (n=10). 21 d after the 60 min long ischemia reduced the cardiac output to 5737.3 ± 641.7 µl/min (n=7) while

bortezomib treatment reduced it slightly to 9593.2 ± 1017.3 µl/min (n=5). Short repetitive ischemias prior to 60 min long ischemia increased the cardiac output significantly to 14021.8 ± 1435.7 µl/min (n=6).

3.5.2.10 Cardiac index

Like cardiac output (Fig. 57), cardiac index was also found to be significantly reduced in the 60 min group by 39.7 ± 4.2% in comparison to the sham group. Repetitive short periods of ischemia prior to the following 60 min ischemia significantly altered the cardiac index to a mammoth increase of 32.9 ± 5.3% in comparison to the sham group. The bortezomib treatment prevented the influence of I/R on the cardiac index as no significant difference to the sham group could be detected (increase by 9.8 ± 4.8%).

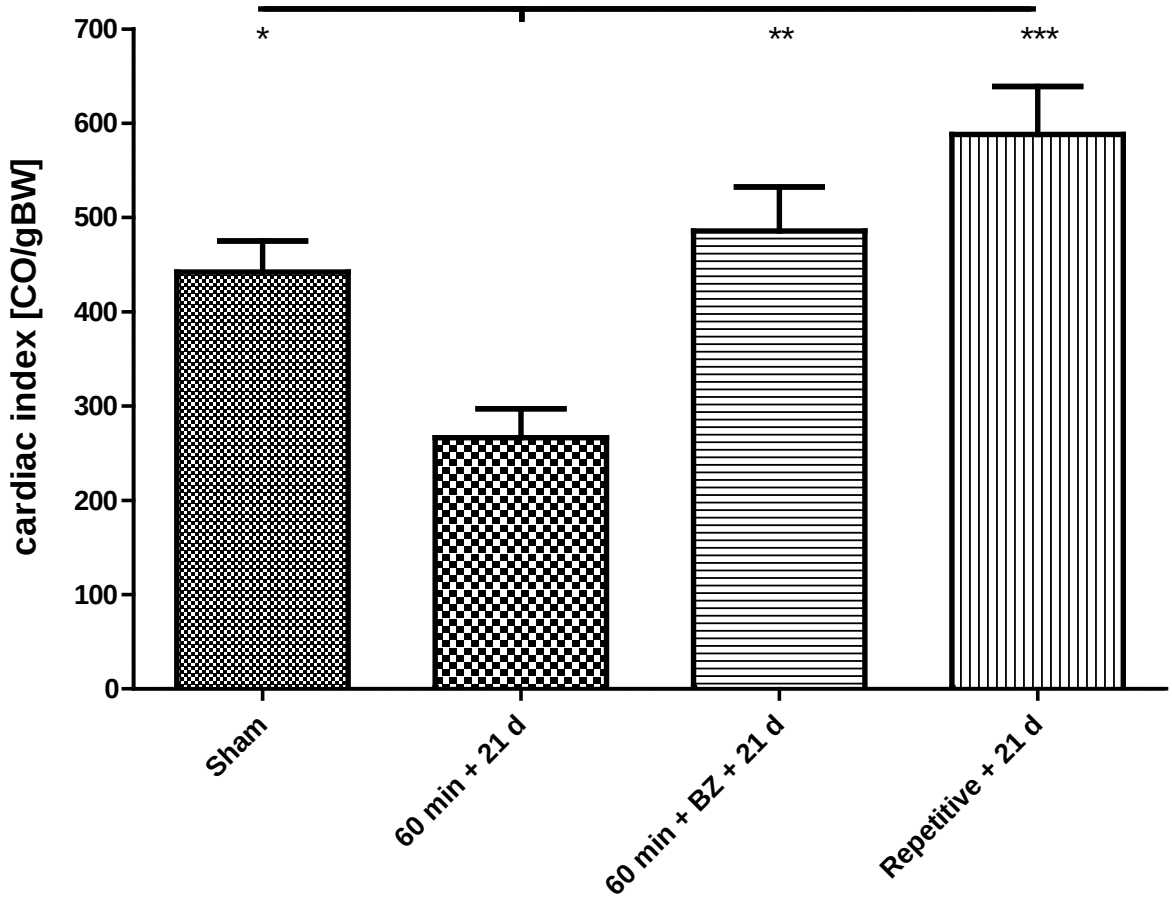

Fig. 58: Cardiac index (CO/gBW) in different experimental groups after the 21 d of the last ischemia. The sham group exhibited the cardiac output value 442.3 ± 32.7 CO/gBW (n=10). 21 d after the 60 min long ischemia reduced the cardiac output to 266.6 ± 30.7 CO/gBW (n=7) while bortezomib treatment led to a cardiac index of 485.7 ± 46.8 CO/gBW (n=5). Short repetitive ischemias prior to 60 min long ischemia increased the cardiac output significantly to 588.0 ± 50.9 CO/gBW (n=6).

4 Discussion

In the presented project, we compared the role of preconditioning by repetitive short ischemias and NF-κB inhibition. The UPS system has gained a great attention during the last decade reinforcing the idea of a potent therapeutical drug in myocardial diseases (Hedhli and Depre 2010; Yu and Kem 2010). Beneficial effects of bortezomib have been observed in acute myocardial infarction which was attributed to the blockade of the NF-κB (Huang et al., 2008; Marfella et al., 2009; Yu et al., 2005). Based on these findings, we investigated the effects of proteasome inhibition during myocardial ischemia reperfusion injury. We analyzed the role of both NF-κB and HIF in these processes as well as hemodynamic parameters.

In the present study, we reported for the first time that inhibition of the UPS by bortezomib successfully decreased cardiac injury in mice in the situation of 60 min ischemia as well as previous repetitive interruptions in LAD blood flow. Bortezomib treatment reduced the infarct size and improved ventricular function analyzed by Millar catheter. These results were accompanied by an attenuated body weight loss and an improved contractility. mRNA and histological data implicated that these findings can be in part attributed to increased HIF-1α expression.

4.1 Methodology

4.1.1 Mouse as experimental model

In this study, mice were used as a research model. This is justified as both human and mice are mammals, have high resemblance in anatomy, physiology and genetics (Oliver et al., 2007), as well as a reproducibility of the results. In terms of ventricular structure, the mouse heart is comparable to that of humans (Doevendans et al., 1998). Being mammals, the physiology and gene function is similar to great extent. Both possess about 95% of genetic similarity (Kim et al., 2010). Using mice models allows the researcher to apply “knock out, knock in and knockdown”, techniques used to simulate various diseases which are affecting humans too (Braun and Willnow, 1996; Cutler et al., 2007; Oliver et al 2007). Other than this, short and accelerated

lifespan of mice allows the scientists to save a lot of money, time and space required to perform the experiments.

Using mice as research tool has got some disadvantages too. The coronary anatomy differs between the two mammal species. Small mammals, in comparison to the large mammals, have high energy demand. The cardiac output and the cardiac index varies between mouse and human (Barbee et al., 1992). Mice have higher heart rates thus the ECG pattern and the underlying action potential are different to humans. Also the potassium channels are different between the two species (Nerbonne et al., 2001). Nevertheless, mice have become the most popular animal model in cardiac physiology in the last two decades.

4.1.2 Age of the mice

A mouse gets to mature adult age when the animal is 3-6 months old. It is known that the I/R injury is a difficult process for the animals to go through. Furthermore, during growing up the heart undergoes structural and functional changes that affect its function under stress, its natural cardioprotection and remodeling process (Lakatta, 2015). Therefore, adult mature mice almost 3 months old were used in this study to rule out "survival of the fittest" to the maximum, and also because the mice of this age group were used mostly in the published data. In addition, mice of this age were investigated in earlier studies of our fellow research groups (Babiker et al., 2006; Baumgarten et al., 2006; Ehrentraut et al., 2011; Meyer et al., 2008; Velten et al., 2012; Weisheit et al., 2014).

4.1.3 Closed chest model for ischemia reperfusion injury

The technique of "closed chest model" is in use since 2000 to study the effects of the ischemia reperfusion injury in mice (Nossuli et al., 2000). The often used "open chest model" means that the infarction is performed with the chest open. This has a huge disadvantage as it triggers greater inflammatory reactions and post-operative trauma (Kim et al., 2007; Stapel et al., 2006). In our investigation, we also used the closed chest model, but our model was much less traumatic

than the conventional closed chest model. Kim et al., 2012 has introduced this procedure to ligate the LAD. This technique consists of the following steps, the ribs of the mouse were visualized, and the thorax was entered with the help of a forceps by making a hole through the intercostal space and pleura (fig. 17). Neither any rib nor the sternum was cut during this process, making it much more convenient for the experimental animal triggering less inflammatory reactions and minimal trauma.

4.1.4 Mortality rate

Inducing repetitive ischemia injury to murine heart is a sensitive experimental procedure which demands elaborated technique, expertise and steady hands. Still the mortality rate could differ from one experiment to the other, depending on the nature of the experiment. There is a wide spectrum of mortality rates available in literature, ranging from 15% to 60 mortality rate of 15% was reported by the Liu et al, (2017) while Stapel et al, (2006) found a mortality rate of about 20%. Other than this, mortality rate of 30 % (Kassiri et al., 2005) has also been published. A very high mortality rate of 60% has detected after 60 min ischemia in mice (Kandalam et al., 2010). In our study, the mortality rate was found to be 25% in the 60 min group comparable to Stapel et al. (2006) and 40% in the repetitive groups (fig. 27), although the duration of ischemia was also 60 min. The mortality rate in the repetitive groups can be explained by the high number of anesthesia, intubations and I/R.

Furthermore, high mortality rates may in part be dependent on the surgeon i.e. little experience and skillfulness may lead to bleeding of the heart or vessels, respiratory failure, heart failure or pneumothorax for instance.

4.1.5 Anesthetics

The easy controllable volatile anesthetics such as halothane and isoflurane possess cardioprotective properties in the form of a pre-conditioning influence which could easily distort results of the experiments. Experiments on dogs and rabbits showed that only 15 to 30 mins of

isoflurane treatments just before the occlusion produced a pre-conditioning influence and hence led to a reduction in the infarct size (Cason et al., 1997). In addition to the pre-conditioning effect isoflurane was found to develop post-conditioning effects to nearly the same extent (Kersten et al., 1997).

To avoid these influences of the volatile anesthesia on the infarct size, animals received a special recipe of injectable anesthetics, for ischemia reperfusion insult consisting of atropin, xylacin, ketanest-S and 0.9% NaCl (table Nr: 2) (Kim et al., 2012; Kim et al., 2014). Isoflurane was used only for the initial operation which was performed seven days before the ischemia and the final recording of hemodynamic parameters and sacrificing.

4.2 Infarction

4.2.1 60 min group

There have been some earlier projects, successfully completed in our working group, on the topic of myocardial I/R injury e.g. the experimental animals were treated with 60 min of ischemia followed by 24 h of reperfusion injury. These animals produced about 50% of infarct/AAR (Stapel et al., 2006). 45% infarct/AAR was found by a US group in wild type animals while 66% infarct/AAR was found in CD-1 deficient mice after 60 min of ischemia followed by 24 h of reperfusion injury (Eckle et al., 2006). 46% of infarct/AAR in wild type mice and 49% infarct/AAR in C3H/HeJ mice have also been reported in the literature (Kim et al., 2007). In our investigation, 47% infarct/AAR was found after 60 min of ischemia followed by 24 h of reperfusion injury in C57/BL6 mice (fig. 30-B), this is in good agreement with earlier studies.

4.2.2 Repetitive group

In this study, it has been shown that repetitive short periods of ischemia reperfusion treatment reduced the infarct sizes significantly in C57BL/6 mice. The mice treated with 15 min of repetitive ischemia for 6 consecutive days followed by 60 min of long ischemia exhibited 64.6%

smaller infarct size than those who were given just 60 min long ischemia only once. Lavine et al. (2013) have found 52% reduction in infarct size in C57/B6 mice after repetitive ischemia. The reduction in infarct size by repetitive short periods of ischemia has been shown first by Murry et al., 1986 in dogs. Where 4 cycles of 5 min of ischemia followed by 5 min of reperfusion before a sustained occlusion of coronary artery for 40 mins limited the infarct size to 25% of that seen in the control group. Another study showed the reduced infarct size by 69% after 5 mins of ischemia and 5 mins of reperfusion before 60 mins of long ischemia in circumflex coronary artery in dogs (Przyklenk et al., 1993). Ischemic pre-conditioning manifests not only in reduced infarct size, but also in improved recovery of contractile function during reperfusion, reduced incidence of arrhythmias in most species but not in pigs, and improved endothelial function (Schulz et al., 2001; Skyschally et al., 2008; Yellon et al., 2003).

This protective effect of the repetitive ischemia treatments has been attributed to reduced ATP depletion and/or reduced catabolite accumulation during the sustained occlusion (Murry et al., 1986). The repetitive ischemia governed cardioprotection may partially depend on Protein Kinase C (PKC) activation and VEGF over-expression, angiogenesis and increased capillary density after 3 cycles of 3 min ischemia and 5 mins of reperfusion in the Sprage Dwarley rats (Kawata et al., 2001). Lavine et al. (2017) have named microvasculature as a potential reason of improved cardiac function and 52% reduced infarct size in 2-4 months old C57/B6 mice after repetitive ischemia for 3 times 15 min ischemia every other day. This is a protocol which is relatively near to ours. Pre-conditioning the rat brain with 3 times 5 mins of ischemia and 15 min reperfusion followed by 48 hours of reperfusion significantly downregulates the expressions of NF-κB in adult male Sprague-Dawley rats (Tu et al., 2015). The possible reasons for this reduction in infarct size in our study are discussed later.

4.2.3 60 min + bortezomib and repetitive + bortezomib groups

It has been found in this study that the experimental groups which were treated with BZ exhibited reduced infarct size compared to their counterpart experimental groups. Here we are reporting for the first time that the administering mice with bortezomib reduced the infarct size by 51.01% in 60 min + BZ group and by 71.1% in repetitive + BZ group in comparison to the 60

min group. Furthermore, treating the mice with bortezomib in repetitive + BZ group reduced the infarct/AAR size by 18.5% in comparison to the repetitive group. This is in agreement with previously published results of Huang et al. (2008), Marfella et al. (2009) and Yu et al. (2005), although there were differences in the animal model, the I/R duration, the dosage of the BZ and its time of application (pre- or post-infarction). The earlier studies attributed the BZ dependent cardio protection to inhibition of NF-κB (Marfella et al., 2009) and the upregulation of the G-protein coupled receptor kinase 2 (Huang et al., 2008; Yu et al., 2005). As G-protein coupled receptor kinase 2 is responsible for the β-adrenergic receptor (β-AR) sensitivity, the authors proposed that the blockade of its degradation by bortezomib protects against malignant ventricular tachyarrthymias (Huang et al., 2008; Yu et al., 2005). Though these were findings in animal models of acute infarction they are in line with our results in a chronic setting of repetitive ischemia-reperfusion similar to clinical situations of angina pectoris.

Bortezomib, apart from myocardium, has been reported to be responsible for its protective role in many other parts of the body too. Chen et al. (2013) showed that bortezomib reduced retinal functional impairment after 45 mins of ischemia. They also found that administering bortezomib reduced the inflammatory mediators such as NF-κB. This is in support of hypotheses where we proposed that the bortezomib treatment plays an important role in reduction of the infarct size by blocking the NF-κB pathway. Protective role of bortezomib has been well documented in steatotic liver (Triveedhi et al., 2014), non steatotic liver (Zaouali et al., 2013), hemorrhagic shocks in rats (Bach et al., 2013), renal ischemia reperfusion injury (Huber et al., 2009), peripheral nerve reperfusion injury (Park et al., 2009), diabetic myocardium (Marfella et al., 2009), attenuates skeletal muscle reperfusion injury by blocking the pathway of NF-κB activation (Park et al., 2007) and in cancer therapy (Zavrski et al., 2007).

4.3 I/R injury induced changes in hemodynamics

It is known from the literature that the ischemic injury affects the cardiac functions. Cardiac function in our experimental model was analyzed by a Millar pressure volume catheter 24 h and 21 days after I/R.

4.3.1 Hemodynamic parameters after 24 h

The 60 min group developed significantly reduced end-systolic pressure and an increased end-systolic as well as end-diastolic volume. Increase in end-diastolic pressure did not reach the level of significance. Most other parameters of cardiac function were calculated on the base of these four measured values. Consequently, ejection fraction calculated from end-systolic and end-diastolic volumes was also reduced as well as dP/dt_{max} and dP/dt_{min}. The significantly impaired cardiac function 24 h after I/R was comparable to some other published data (Goltz et al., 2015; Kim et al., 2006; Kim et al., 2014; Li et al., 2013; Markowski et al., 201; Remme, 2000).

All pre-conditioned groups exhibited less impaired cardiac function than the 60 min group. Interestingly, short periods of ischemia reperfusion, bortezomib treatment and combination of both in advance had significantly reduced the malignant influence of I/R on end-systolic volume, as well as the ejection fraction and cardiac output. Among the pre-conditioned groups the repetitive I/R treatment resulted in the best hemodynamic function 24 h after I/R as this group was significantly better than the 60 min group in most parameters. A calculation of the average heart beat volume reveals 21.1 µL in the repetitive group, which was the highest in all groups and nearly double of that in the 60 min group (11.0 µL).

The repetitive group exhibited also somewhat better cardiac function than the 60 min + BZ group, however, the level of significance was not reached in any of the recorded parameters. The performance of the 60 min + BZ group did not differ significantly from the sham group with the exception of the systolic pressure, which was reduced in the 60 min + BZ group, i.e. the mice in the 60 min + BZ group did not suffer very much from I/R injury. A comparison of the 60 min + BZ group with the 60 min group revealed significantly better function in 60 min + BZ group as its end-systolic volume was significantly smaller and its ejection fraction as well as its cardiac output and cardiac index were significantly higher than the in the 60 min group.

The repetitive + BZ exhibited significantly increased cardiac function vs the 60 min group in ejection fraction and end-systolic volume only. Furthermore, in comparison to the repetitive group the dP/dt_{max}, the cardiac output and the cardiac index were significantly reduced in the repetitive + BZ group. Taken together concerning the cardiac function 24 h after I/R the protection against I/R injury was least distinct in the repetitive + BZ group and best in the

repetitive group. Due to the relatively weak performance of the repetitive + BZ group, this was excluded in long term experiments.

This well preserved cardiac function, in the repetitive group is accordance with earlier published data. Ji et al. (2013) found a preserved ejection fraction and dP/dt_{max} after 2 cycles of 5 min ischemia/ 5 min reperfusion prior to the 30 min long ischemia in rats. Better ejection fraction has also been reported in C57/Bl6 mice after 15 min x 3 times ischemia followed by 24 h of reperfusion (Lavine et al., 2013).

4.4 Investigation of inflammatory mediators 24 h after I/R

Two basically different treatments were applied in order to reduce the ischemic injury in the heart. One was preconditioning with repetitive short periods of ischemia. The second was the application of the bortezomib, an inhibitor of ubiquitin proteasome system. Both methods were also applied in combination. The hypothesis behind the repetitive periods of short ischemia was that the tissue becomes used to ischemia by application of short nondestructive periods of ischemia. These ischemic periods excite a well-controlled inflammatory process in the tissue. At the end of the pre-conditioning process, the long lasting ischemia with its succeeding strong inflammation may be managed better by the myocardium, thus resulting in smaller ischemic injury. Bortezomib is applied with the aim to reduce the post-ischemic inflammation. In the present study, bortezomib was applied in a pre-conditioning as well as post-conditioning substance. The idea was to help the tissue by reducing the post-ischemic inflammation and thereby to dampen the post-ischemic insult. Regarding the infarct size as well as the hemodynamic performance after 24 h of reperfusion, both repetitive pre-conditioning as well as the bortezomib treatment proved to be successful. According to the theory, one would expect smaller levels of the inflammatory mediators after the pre- and post-conditioning treatments; therefore the expression of inflammatory mediators was investigated in this study.

As HIF-1α is a protein, which induces post-hypoxic processes as a transcription factor, one would expect elevated levels of this protein after ischemia. Indeed, in all groups, which were exposed to I/R, increased HIF-1α mRNA was detected reaching the levels of significance only in

the 60 min + BZ and the repetitive + BZ groups. Our study is the first one to show report that HIF-1α was upregulated by a combination of BZ and repetitive I/R treatment. This may be attributed to the observation that bortezomib by itself also influences the HIF-1α level. Concerning pro-inflammatory mediators like IL-6, IL-1β, TNF- α and ICAM- 1, the results were somewhat surprising. IL-6 and IL-1β as were expressed at the significantly highest levels in the 60 min + BZ group, clearly above all groups. All the other groups except the 60 min + BZ exhibited moderate and insignificant increases in the pro-inflammatory cytokines.

ICAM-1 was expressed at clearly elevated levels in all groups which were exposed to I/R, but the level of significance was reached only in the 60 min, the 60 min + BZ and the repetitive groups not in the repetitive + BZ group. ICAM-1 is expressed constitutively on endothelial cells as well as on leukocytes. Its expression is upregulated by the pro-inflammatory cytokines IL-1β and TNF-α (Yang et al., 2005). It facilitates transmigration leukocytes through the endothelium. Up-regulated ICAM-1 expression may be taken as a sign for a preceding by increased expression of IL-1β and TNF-α. The time-point of measuring the expression of TNF-α, IL-1 β and IL-6 may have been too late to detect the peak expression of these early cytokines in the 60 min and the repetitive group.

The high expression of pro-inflammatory mediators in the 60 min + BZ group was accompanied by a significant up-regulation of the anti-inflammatory cytokine IL-10. As bortezomib was applied to dampen the inflammatory response, one would expect the lowest expression of the inflammatory reactions in the bortezomib groups but the reverse happened. Furthermore, the infarct size, as detected by the TTC staining, was biggest in the 60 min group and significantly smaller in all the other groups. Also the cardiac performance was better in the three pre-conditioned groups compared to the 60 min group thus correlating nicely to the infarct sizes. However, 24 h after the reperfusion, inflammatory mediators were highest in the 60 min + BZ group with small infarct as well as second best hemodynamic performance at the same time.

A possible explanation for the high expression of pro- and anti-inflammatory mediators could be that bortezomib did not act anti-inflammatory in our hands. In our study bortezomib yielded the expected result as a significant cardioprotection concerning infarct size and cardiac function was reached in the 60 min + BZ group. Furthermore, the mortality and the weight loss was reduced in

both bortezomib groups compared to their counterparts (60 min and repetitive group). Thus there was an effect of bortezomib.

In a preceding study we demonstrated in murine macrophages (RAW 264.7) that bortezomib is able to prevent the LPS dependent NF-κB translocation into the nucleus (Pesch, 2011). In an additionally performed study with experiments to characterize the myocardium after the period of short repetitive I/R cycles, before the 60 min ischemia the effect of bortezomib on the myocardium was tested (Gölz et al., 2011). After the repetitive six short I/R cycles of 15 min NF-κB was found to be increased in the nuclei of the cardiomyocytes in the ischemic zone (Fig. 59). Application of bortezomib could dampen this. This may be taken as a sign for the expected downregulation of NF-κB translocation to the nucleus in cardiomyocytes by bortezomib. Thus the up-regulation of the cytokines in the 60 min + BZ group did not seem to be depending on ineffective bortezomib.

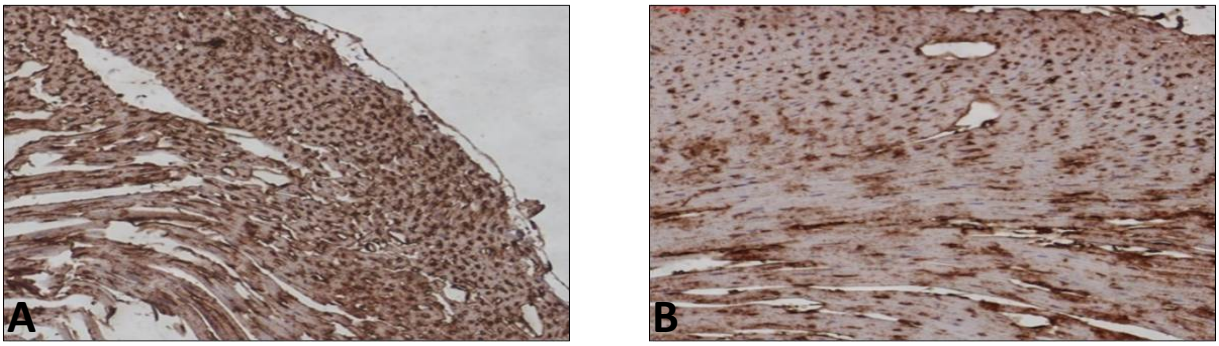

Fig. 59: NF-κB 24 h after 6 short repetitive ischemias visualized by an antibody in the nuclei of cardiomyocytes in the murine heart with (A) and without (B) bortezomib treatment. The brown colour is much less intense after bortezomib treatment indicating less NF-κB in the nuclei (Gölz et al., 2011).

But the question why we detected increased pro-inflammatory mediators in the 60 min + BZ group is still open. This group had a pre-conditioning with bortezomib which should reduce the inflammatory mediators in the myocardium prior to I/R. In a preceding unpublished study our group monitored the development of inflammatory mediators during the pre-conditioning timespan. At the end the level of IL-1β and TNF-α were not significantly different between the sham and the 60 min + BZ groups. This may be taken as a weak indicator for an anti-

inflammatory influence of bortezomib prior to I/R. The last bortezomib injection was applied at the end of the 60 min ischemia this may help to reduce the infarct size measured by TTC 24 h later. But the influence of bortezomib ceases and a strong delayed inflammation may develop in a rebound reaction. This inflammation was detected in our PCR recordings 24 h after reperfusion. Possibly a further injection of bortezomib might have prevented this reaction.

4.5 Morphology and cardiac function 21 days after I/R

4.5.1 Scar size

As mentioned before in the long-term observation we included only three experimental groups and the sham group. Evaluation of the scar size revealed that the scars of the animals in the in the 60 min and in the 60 min + BZ groups were significantly larger than in the sham and in the repetitive group, whereas the scar size did not differ between the sham and the repetitive group. The scar size did not correlate/reflect to the infarct size/AAR, as the 60 min + BZ exhibited a significantly smaller (by 49%) infarct size/AAR relation than the 60 min group. Obviously, the remodeling process differed due to the presence of bortezomib, as the relatively small infarct size/AAR relation in the 60 min + BZ group did not result in a smaller scar than in the 60 min group.

However, according to Clarke et al. (2016) it is not only the size of the scar, which is important for the recovery of the cardiac function, also the position of the scar is very important. The position of the scar was in the results discussed here not crucial, as the ligature of the left anterior descendent artery was always performed at the same place. Furthermore, collagen crosslinking and orientation have been reported to influence post-infarct cardiac pumping function. Bortezomib has been shown to influence remodeling in different ways. Hypertrophic remodeling neonatal rat cardiomyocytes as well as in hypertensive rats was significantly reduced by proteasome inhibition (Meiners et al., 2008). However, in murine neonatal cardiomyocytes as well as in mouse hearts *in vivo* proteasome inhibition caused hypertrophic growth (Tang et al., 2010). After aortic constriction the proteasome inhibitor MG262 facilitated ventricular dilatation and functional decompensation via activation of the calcineurin-NFAT (nuclear factor of

activated T cells) pathway. Thus applied over a longer time proteasome inhibition may as well act in a deleterious way. Therefore, cardiac function was recorded in the four groups investigated for the scar sizes.

4.5.2 Hemodynamic parameters after 21d

Evaluation of cardiac function revealed significantly reduced ejection fraction as well as decreased dP/dt_{max} and dP/dt_{min} in the 60 min group in comparison to the sham group. This attenuation of ejection fraction was evident in significantly increased end-systolic and end-diastolic volumes. The calculated cardiac output too was significantly diminished in this group in comparison to the sham group. All these results were in line with another study where dP/dt_{max} and dP/dt_{min} were found to be significantly diminished 21 days after I/R (Goltz et al., 2015). It is to be noted here that the end-systolic and end-diastolic pressures were recuperated in 60 min group and in all other experimental groups to an extent that no significant difference was noted in comparison to the sham group. Taken together, the hemodynamic parameters in 60 min group are supported by the finding that this group exhibited the largest scar after 21 days.

Unlike in 24 h measurements, 60 min + BZ group exhibited significantly reduced ejection fraction in comparison to the sham group. This attenuation in ejection fraction was supported by severely increased end-systolic and end-diastolic volumes as well as significantly reduced dP/dt_{max} and dP/dt_{min}. However, the cardiac output was higher than the sham group but did not reach the level of significance and was less impaired than in the 60 min group. Again, the end-systolic and end-diastolic pressures were recuperated after 21 d. The end-systolic and end-diastolic volumes in the 60 min + BZ group were significantly higher not only versus the sham group but versus the 60 min group too. Taken together, literature supports our results as swine after 60 min of ischemia and 21 days of reperfusion exhibited smaller left ventricular infarct but attained in impaired cardiac function after preconditioning the hearts with PS-159 proteasome inhibitor (Sihag et al., 2013).

After 21 days of reperfusion, the repetitive group exhibited the most well preserved cardiac function in comparison to the sham group. Ejection fraction, end-systolic and end-diastolic

pressure, end-systolic and end-diastolic volume as well as dP/dt_{max}, dP/dt_{min} and cardiac output were found to be better preserved in the repetitive group than any other experimental group.

Thus three weeks after the 24 h hemodynamic recordings the picture had changed completely. The 60 min group as well as 60 min + BZ group showed impaired cardiac performance and significantly increased scars compared to the repetitive group. The increased inflammation after 24 h of reperfusion in the 60 min + BZ group may explain the decline in the cardiac performance in the 60 min + BZ group in this follow-up experiment.

5 Conclusion

The first hypothesis, that repetitive ischemia reperfusion cycles simulating Angina pectoris will raise the HIF-levels and reduce the sensitivity of the myocardium to inflammation, was proven to be correct. 24 h after I/R HIF mRNA was increased though not significantly above sham level. However, HIF was also not different from the significantly increased levels in the bortezomib groups. Pro- and anti-inflammatory cytokines did not differ to the 60 min group.

The second hypothesis that bortezomib treatment will raise HIF-levels was proven. However, the predicted decreased inflammation could not be shown. In contrast, the investigation performed 24 h after I/R revealed increased levels of inflammatory mediators. This was interpreted as a rebound reaction.

The third hypothesis, that ischemic injury could be reduced and cardiac function could be preserved better by the three treatments, was proven, however to different degrees. All three treatments reduced infarct size/AAR significantly, but the cardiac function was best in the repetitive group.

The fourth hypothesis that remodeling after I/R may be facilitated and better recovery of long term cardiac function will be supported by the treatments could be shown only for the repetitive group.

Taken together, the pre-conditioning with repetitive ischemias was successful improving cardiac performance even in the long term. However, this is not a treatment, which can be applied to people after ischemia reperfusion. The treatment with bortezomib helped the mice to survive, reduced infarct size and attenuated the influence of the I/R injury on cardiac performance. However, these positive effects did not lead to an improved remodeling as the scar was the same as in the 60 min group. Also the cardiac performance was less well preserved than in the repetitive group. A possible explanation for the disappointing long term results may be that the inflammation was increased in a rebound reaction. Thus a different timing of the bortezomib treatment may help prolong the short term positive influences on I/R injury. Possibly a post-conditioning treatment with bortezomib repeated daily for a few days may help to prevent the deteriorating influences of a too strong inflammation. Another positive result of this study is that

HIF-1α was increased in all groups. Perhaps a specific way to increase HIF-1α may help to reduce the I/R injury.

6 Abstract

Myocardial infarction (MI) is a state when the flow of blood stops in a coronary artery or a succeeding vessel resulting in hypoxia leading to damage in the downstream cardiac tissue. Ischemia/reperfusion (I/R) injury leads to significant tissue damage and initiation of inflammatory reactions. The roles of nuclear factor (NF)-κB and hypoxia inducible factor (HIF) in myocardium are still under discussion. It has been reported, that reduction of NF-κB activity by attenuation of the ubiquitin proteasome system (UPS) exerts bbeneficial effects on I/R injury in the myocardium. Furthermore, preceding short periods of ischemia before I/R injury like in Angina pectoris are known to protect the heart. Based on these findings, we hypothesized that repetitive short cycles of I/R will raise the cardiac HIF-levels and attenuate NF-κB levels, thus reduce inflammation. It was also hypothesized that pre- and post-conditioning with bortezomib (BZ), a blocker of UPS, will decrease ischemic injury, help to preserve cardiac function and facilitate remodeling after I/R.

9-12 weeks old female C57BL/6 mice underwent LAD constriction or sham surgery. Mice were divided into 5 different groups. Sham group; 60 min group treated with 15 min sham ischemia daily for 6 days prior to 1 x 60 min ischemia; repetitive group treated with 15 min ischemia daily for 6 days prior to 1 x 60 min ischemia, repetitive + BZ group received the same protocol complemented with 3 doses of BZ; 60 min ischemia + BZ group received the same treatment as the 60 min group complemented with 3 doses of BZ. 24 h post I/R injury changes were monitored in all groups. 21 d post ischemia success of remodeling was investigated in the sham, the 60 min, the 60 min + BZ and the repetitive groups.

24 h after I/R, 60 min group exhibited the biggest infarct size while all other groups developed significantly smaller infarct size. The 60 min group exhibited significantly reduced end-systolic pressure and ejection fraction as well as reduced dP/dt_{max} and dP/dt_{min} accompanied by an increased end-systolic as well as end-diastolic volume. All pre-conditioned groups exhibited less impaired cardiac function than the 60 min group. Interestingly, short periods of I/R, BZ treatment and combination of both in advance significantly reduced the malignant influence of I/R on the cardiac performance. Among the pre-conditioned groups the repetitive I/R treatment resulted in the best hemodynamic function 24 h after I/R.

The investigation of inflammatory mediators 24 h after I/R revealed that all I/R groups exhibited increased levels of HIF-1α being highest in both BZ groups. IL-6 and IL-1β as well as ICAM-1 were expressed at the significantly highest levels in the 60 min + BZ group.

Evaluation of the scar size after 21 d after I/R revealed that the scars of the animals in the 60 min and in the 60 min + BZ groups were significantly larger than in the sham and in the repetitive group, whereas the scar size did not differ significantly between later groups. Evaluation of the cardiac function revealed totally different results than after 24 h post I/R. Significantly reduced ejection fraction as well as decreased dP/dt_{max} and dP/dt_{min} was found in the 60 min group in comparison to the sham group. It is to be noted here that the end-systolic and end-diastolic pressures were compensated in 60 min group and in all other experimental groups. Surprisingly the 60 min + BZ group developed only slightly better cardiac function than the 60 min group. The repetitive group exhibited the most well preserved cardiac function in comparison to the I/R groups. Ejection fraction, end-systolic and end-diastolic pressure, and volume as well as dP/dt_{max}, dP/dt_{min} and cardiac output were found to be better preserved in the repetitive group than in any other experimental group.

Taken together, the pre-conditioning with repetitive ischemia was successful in improving cardiac performance even in the long term. This study is the first one to report the upregulation of HIF-1α as result of BZ and repetitive I/R treatment. All experimental groups which were exposed to I/R exhibited increased HIF levels. In general, we did not gain the desired attenuation in inflammation by the BZ treatment. Also the BZ treatment was not found to be as successful as the repetitive treatment in long term. However, repetitive treatment cannot be applied in clinical setting. Thus, possibly another specific way to increase HIF level may help or a new protocol of BZ treatment has to be established in order to reduce I/R injury.

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7 Appendix

7.1 List of figures

7.2 List of tables

8 Declaration

I hereby declare that this dissertation has been written only by the undersigned and without any assistance from third parties. Furthermore, I confirm that no sources have been used in the preparation of this dissertation other than those indicated in the dissertation itself. This dissertation was not submitted in any form for another degree at any university or other institution of tertiary education.

Bonn, _____ 2019

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Muhammad Ajmal Ayub

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10 Publications

Eichhorn L, Dolscheid-Pommerich R, Erdfelder F, **Ayub MA**, Schmitz T, Werner N, Jansen F (2017) Sustained apnea induces endothelial activation. Clin Cardiol 40: 704-709.

Eichhorn L, Weisheit CK, Gestrich C, Peukert K, Duerr GD, **Ayub MA**, Erdfelder F, Stöckigt F (2018) A Closed-chest Model to Induce Transverse Aortic Constriction in Mice. J Vis Exp 134. doi: 10.3791/57397.