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The Inhibition of Prolyl Endopeptidase as a Potential Therapeutic Target for the Treatment of Ischemic-Reperfusion

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Abstract

Ischemia/reperfusion injury (IRI) is a complex pathophysiological process characterized by disturbances in blood circulation caused by various factors, such as traumatic shock, surgery, organ transplantation and thrombus. Severe metabolic dysregulation and destruction of tissue structure are observed after restoration of blood flow to ischemic tissue. The heart and kidneys, being highly perfused organs, are very susceptible to ischemic and subsequent reperfusion injury. The incidence of myocardial and renal IRI has a high morbidity and mortality rate. Several studies have shown that among the mechanisms involved in the pathophysiology of IRI are inflammation, apoptosis and angiogenesis.

Recently, the involvement of prolyl endopepetidase (PREP) in inflammatory response and angiogenesis has been demonstrated. Therefore, the aim of this PhD project is to investigate the effects of a selective PREP inhibitor, KYP-2047, in the regulation of inflammation, apoptosis and angiogenesis, in *in vivo* models of myocardial ischemia reperfusion (MI/R) and kidney ischemia/reperfusion (KI/R).

Myocardial I/R was induced by coronary artery occlusion (15 minutes) followed by reperfusion, whereas kidney I/R was induced by renal artery occlusion (30 minutes), followed by reperfusion. The mice were pre-treated intraperitoneally with KYP-2047 at doses of 2.5 and 5 mg/kg in cardiac ischemia and treated at doses of 0.5, 1, and 5 mg/kg in renal ischemia. Hearts and kidneys were removed and processed for histological, immunohistochemical and biomolecular analysis.

Inhibition of PREP, through KYP-2047, reduced histological damage, neutrophil accumulation and inflammation in both models. In addition, KYP-2047 was able to modulate angiogenesis and reduce apoptosis in renal and cardiac context. This protective

effect of KYP-2047 could be related to the modulation of NF-κB and MAPK pathways in MI/R injury and to the activation of PP2A in KI7R injury.

Therefore, these results demonstrated the protective effect of KYP-2047, highlighting the role of PREP inhibition in cardiac and renal ischemia, suggesting PREP as a therapeutic target for MI/R and KI/R injury.

Introduction

Ischemia is a vascular disease characterized by a reduction in blood flow to a specific organ or tissue, resulting in a lack of oxygen and nutrients in the affected organ or tissue [1]. In some cases, restoring blood flow after a prolonged period of ischemia can cause so-called reperfusion injury, which causes irreversible cellular and microvascular damage that overlaps the initial ischemic insult, exacerbating tissue damage, initiating an inflammatory cascade with excessive production of reactive oxygen species (ROS) [2]. The tissue damage caused by ischemia and that attributable to reperfusion are so interconnected that they are called "ischemia-reperfusion" (IR) damage [3]. The molecular mechanisms underlying I/R damage are complex and remain poorly understood, therefore, the understanding of these mechanisms is fundamental for the development of therapeutic strategies [4]. In the heart, I/R injury contributes to adverse cardiovascular conditions, such as coronary artery disease (CAD), which is one of the most common cardiovascular diseases, with a high mortality rate [5]. In the kidney, I/R injury contributes to pathological conditions, called acute kidney injury (AKI), which is a clinical syndrome with rapid kidney dysfunction and high mortality rates [6].

The pathophysiology of I/R damage is complex and involves several pathways, such as the activation of inflammatory mediators (e.g. TNF-α and some interleukins) and the release of neutrophils [7]. During I/R injury, alterations in angiogenic processes and stimulation of apoptosis also occur, which significantly contributes to cardiac and renal dysfunction [8, 9].

Recently, the involvement of new molecules in the inflammatory and angiogenic processes of some diseases has been discovered and among these, a key role is played by prolyl endopeptidase (PREP), a proteolytic enzyme belonging to the serine protease family and involved in the release of pro-angiogenic and pro-inflammatory factors [10].

PREP is expressed in all tissues, but localized only in specific cell types, especially in the heart, kidneys and brain [11]. Therefore, considering the role of PREP in inflammatory and angiogenic processes, PREP inhibitors may represent a new therapeutic strategy [12]. In particular, KYP-2047 is the most selective inhibitor of PREP, having an excellent ability to reach PREP even intracellularly [13]. Therefore, on the basis of these evidences, the aim of this PhD project was to evaluate the effects of PREP inhibition by KYP-2047 in *in vivo* mouse models of myocardial I/R and kidney I/R.

Chapter 1: Ischemia Reperfusion Injury

1.1 Ischemia Reperfusion Injury: focus on Heart and Kidney

Ischemia is a vascular disease characterized by the reduction of blood flow, causing a severe deficit of oxygen and nutrients in the affected organ and/or tissue; in case of prolonged oxygen deficiency, an irreversible process is generated that leads to cell death (or necrosis) of the organs and/or tissues involved [3]. In some cases, the restoration of blood flow after a prolonged period of ischemia can cause what is known as reperfusion injury, which causes irreversible cellular and microvascular damage that overlaps with the original ischemic insult. The tissue damage caused by ischemia and that attributable to reperfusion are so interconnected that they are called "ischemia-reperfusion" (IR) damage [14].

Under ischemic conditions, reduced oxygen supply blocks the mitochondrial respiratory chain, resulting in accumulation of respiratory chain intermediates and depletion of adenosine triphosphate (ATP), thus leading to stimulation of anaerobic glycolysis [15]. A reduction in ATP and pH levels then occurs, causing an imbalance in electrolyte exchange, with increased intracellular concentrations of Ca^{2+}, Na^{+} and K^{+} ions [16]. The accumulation of Ca^{2+} in the cytosol activates various enzymes such as phospholipases and proteases involved in inflammation [17], while the accumulation of Na^{+} and other osmotically active particles such as lactate and inorganic phosphate, leads to intracellular edema that further affects cytoplasmic organelles and cell membrane integrity, which can eventually lead to cell death [18]. These factors induce mitochondrial dysfunction and activation of pro-inflammatory cytokines, releasing mediators involved in the inflammatory cascade [19].

With the next phase of reperfusion, through restoration of blood flow, there is an excessive production of oxygen-derived free radicals (ROS) and nitrogen-derived free radicals (RNS), which exceeds the antioxidant capacity of the cell, leading it to major oxidative

stress. ROS cause endothelial damage, with activation and aggregation of macrophages and platelets and accumulation of neutrophils in the ischemic area [20]. They can also activate transcription factors, including hypoxia-inducible factor 1 (HIF-1) [21] and nuclear factor-kappa B (NF-κB) [22], leading to the synthesis of pro-inflammatory cytokines (IL-6, TNFα, IL-1, IL-8) and adhesion molecules [23]. Activated inflammatory cells in turn release free radicals, pro-inflammatory and chemotactic mediators at the damaged site, supporting and amplifying tissue changes and cell death by necrosis and apoptosis [24].

Ischemia-reperfusion injury is thus a two-stage pathological condition characterized by an initial disruption of blood flow, resulting in oxygen deprivation, followed by subsequent restoration of perfusion and oxygen and nutrient supply [25]. Although the mechanisms of ischemia-reperfusion are complex and still not understood, changes in vascular permeability and mucosal barrier integrity are deeply related [26, 27].

Situations in which IR damage can occur are, for example, myocardial infarction and cerebral stroke [28]. Reperfusion injury is a primary concern in surgery and especially in organ transplantation surgery [29]. Organs most susceptible to ischemia include heart, kidneys and brain, as they require constant blood flow to carry out their vital functions [30]. In each affected organ, ischemia can manifest itself with different symptoms and lead to serious clinical complications, as organs respond in different ways [3]. The brain, for example, may suffer a cerebral infarction (ischemic stroke) after only a few minutes of ischemia, while skeletal muscle tissue may endure longer before suffering irreversible damage [31, 32].

1.2 Myocardial ischemia

1.2.1 Definition

Cardiac ischemia, also known as myocardial ischemia, is defined as an imbalance between myocardial oxygen supply and demand, meaning it occurs when blood flow to the coronary arteries, which supply oxygen to the heart muscle, is reduced or blocked (Figure 1) [33]. Unlike other tissues, the myocardium (muscle tissue of the heart) is highly dependent on a constant supply of oxygen to maintain its contractile function and to ensure proper blood flow throughout the body; consequently, even a brief reduction in oxygen supply can severely impair cardiac function [34].

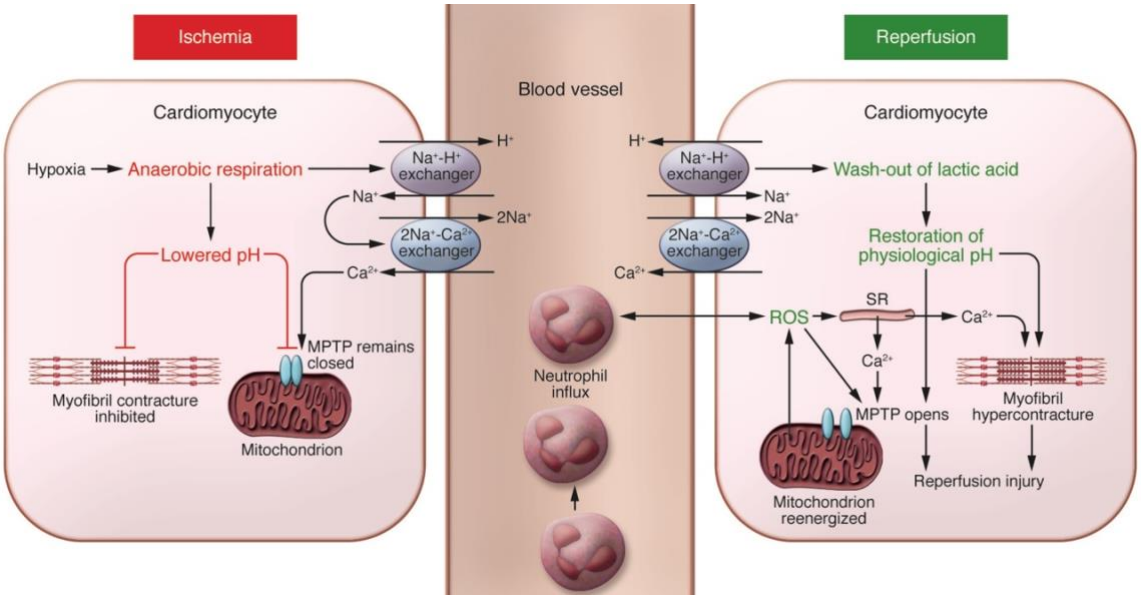

Figure 1. Schematic illustrating of acute myocardial ischemia-reperfusion injury [35].

1.2.2 Epidemiology and risk factors

Myocardial ischemia, associated with coronary artery disease (CAD), which is one of the most common cardiovascular diseases globally, is a major cause of morbidity and mortality worldwide [36].

Myocardial ischemia has a higher incidence in Western countries (North America, Europe) due to the high prevalence of risk factors such as obesity, smoking, and sedentary lifestyles. In contrast, in countries such as sub-Saharan Africa the incidence is lower but increasing due to urbanization and adoption of Western lifestyles [37-39].

Cardiac ischemia is a condition that increases in a correlated manner with advancing age; in fact, it appears to be more common in adults. Studies have shown that the risk of developing cardiac ischemia increases significantly after age 45 in men and age 55 in women [40]. This age-related risk is due to the accumulation of atherosclerotic plaques over time and the onset of other cardiovascular risk factors such as hypertension and diabetes [41].

Men are generally more likely to develop myocardial ischemia at a younger age than women, but after menopause, the risk in women increases and may approach or even exceed that of men, probably due to the loss of the protective effects of estrogen on the cardiovascular system [42].

The pathogenesis of cardiac ischemia is strongly influenced by several risk factors that accelerate the process of atherosclerosis or impair the function of the coronary arteries [43].

Non-modifiable risk factors:

- Age: the risk of myocardial ischemia increases with age.

- Gender: men are at greater risk early in life, while postmenopausal women increase their risk.
- Family history: a positive family history for cardiovascular disease increases risk.

Modifiable risk factors:

- Hypertension.
- Hyperlipidemia: high levels of LDL cholesterol and low levels of HDL cholesterol contribute significantly to atherosclerosis.
- Smoking.
- Diabetes mellitus.
- Obesity: increasing globally, especially in urban areas, contributes to a higher burden of ischemic heart disease.
- Sedentary lifestyle: lack of physical activity is a growing problem in both developed and developing countries.

1.2.3 Etiopathogenesis

The main mechanism behind cardiac ischemia is coronary artery disease, which is characterized by the formation of atherosclerotic plaques in the coronary arteries [43]. In fact, the endothelial wall of the coronary arteries can be damaged by factors such as hypertension, smoking, and hypercholesterolemia, and this damage makes the endothelium more permeable to lipids, especially low-density lipoprotein (LDL), which infiltrate the arterial walls. Oxidized LDL activates a local inflammatory response, calling up monocytes that turn into macrophages, which in turn phagocytize oxidized LDL [44]. As inflammation progresses, smooth muscle cells migrate from the tunica media to the tunica intima of the arterial wall, where they proliferate and secrete extracellular matrix,

contributing to the formation of fibrous plaque (*atheroma*), consisting of a central lipid core, inflammatory cells, fibrous tissue, and smooth muscle cells [45]. As the size of the plaque increases, the lumen of the coronary arteries progressively narrows, reducing the blood supply to the heart, especially under conditions of increased energy requirements, such as during exercise or stress [46]. This causes episodes of ischemia, which manifest clinically as stable angina pectoris.

In addition, the fibrous covering of the atherosclerotic plaque can rupture, exposing the highly thrombogenic lipid core to the bloodstream, this triggers platelet aggregation and thrombus formation. If the thrombus completely occludes the coronary artery, blood flow is stopped, leading to acute myocardial infarction (AMI) (Figure 2) [47].

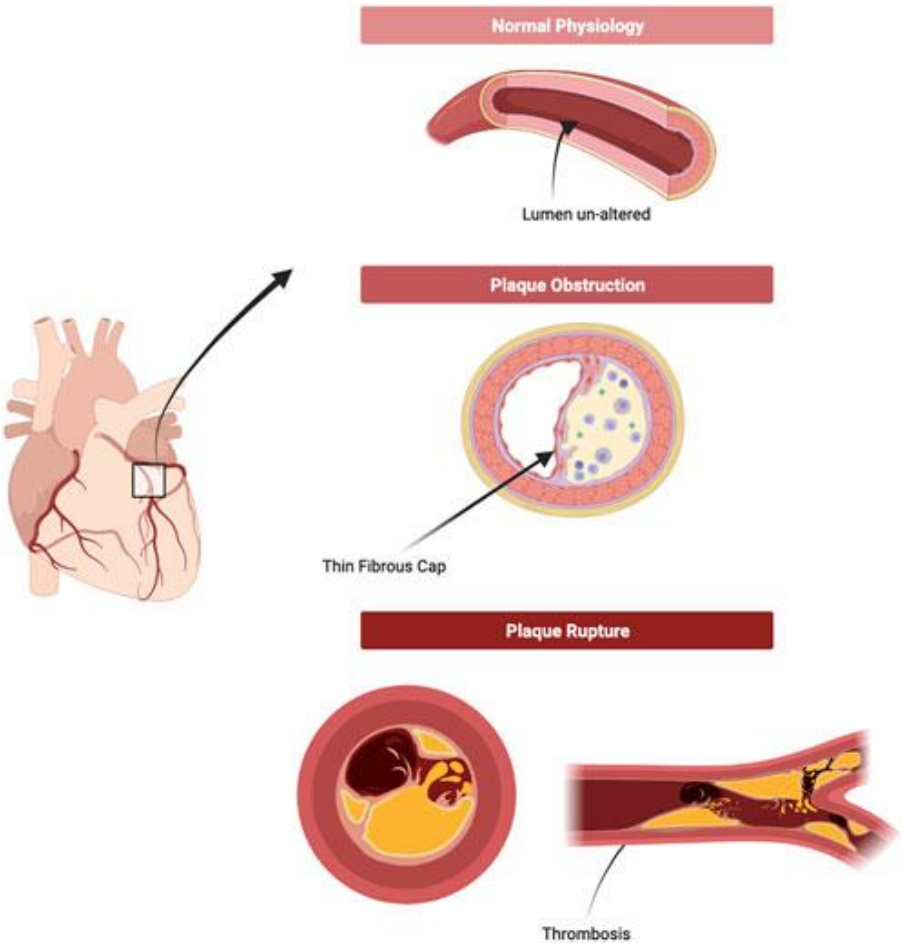

Figure 2. Plaque formation and rupture [48]

In some cases, cardiac ischemia may be caused or aggravated by *coronary vasospasm*, a temporary narrowing of the coronary arteries due to contraction of vascular smooth muscle. This phenomenon affects mostly medium- and small-caliber arteries, can occur both in the presence and absence of atherosclerotic plaques, and can drastically reduce the blood supply to the myocardium.

1.2.4 Classification

Clinical manifestations of myocardial ischemia include:

- ***Transient myocardial ischemia*:** blood spontaneously flows back to the myocardium in a short period of time (usually 15 minutes), without permanent damage to cardiac muscle tissue [49]. This reversible condition can lead to angina pectoris, a pain in the retrosternal region and surrounding areas such as the arms and neck. Angina is defined as stable when chest pain arises predictably following physical exertion and the patient finds relief at rest; unstable when the manifestation is unpredictable, the pain occurs even when the subject is at rest and is a more dangerous form that can quickly lead to heart attack [50, 51].
- ***Acute myocardial ischemia:*** oxygen supply suddenly decreases in a severe and prolonged manner, such that the myocardium goes into necrosis. Cardiac cells, in fact, can withstand 20 to 360 minutes of oxygen and/or nutrient deprivation, after which it dies and, in that case, can degenerate into myocardial infarction [52].
- ***Chronic cardiac ischemia:*** ischemia is due to persistent obstruction over time, causing reduced blood flow even under resting conditions [53].
- ***Silent cardiac ischemia*:** a completely asymptomatic condition. Symptoms may occur during diagnostic examinations after exertion especially in individuals who have

already suffered a heart attack or have undergone transplants and revascularization surgeries [54].

1.2.5 Diagnosis

The diagnosis of cardiac ischemia is made by a combination of clinical evaluation, laboratory tests, imaging studies, and functional tests to confirm the presence of ischemia and assess its severity [55, 56].

1.2.5.1 Clinical Evaluation

The first step for a correct diagnosis of myocardial ischemia is the patient's clinical history and a thorough examination.

The classic symptom of cardiac ischemia is angina pectoris, often described by patients as a feeling of pressure or compression in the chest; such pain may radiate to the arms, neck, jaw, or back. Other symptoms, which arise depending on the type and severity of ischemia, may be dyspnea, hyperhidrosis, asthenia, nausea, and dizziness [57].

The doctor should also, assess the patient's risk factors for coronary artery disease, such as hypertension, diabetes, smoking, sedentary lifestyle, etc.

Physical examination may reveal signs of other cardiovascular diseases or complications, such as abnormal heart sounds, jugular venous distension, or signs of heart failure [58].

1.2.5.2 Electrocardiogram (ECG)

The ECG allows the electrical activity of the heart to be recorded and is one of the most important and widely used tools in the diagnosis of myocardial ischemia [59].

An ECG can show:

- ST-segment changes

- T-wave inversions
- Arrhythmias

1.2.5.3 Blood Tests

Some blood markers are useful in the diagnosis of myocardial ischemia, particularly if myocardial infarction is suspected [60]:

- **Cardiac troponins (troponin I and troponin T):** elevated levels are a strong indicator of myocardial damage, as they are proteins released into the bloodstream when there is damage to the heart muscle [61].
- **Creatine kinase-MB (CK-MB):** another enzyme that increases in the blood when heart muscle is damaged but is less specific than troponin [62].
- **B-type natriuretic peptide (BNP) or NT-proBNP:** prognostic markers useful in cases of patients with congestive heart failure (CHF) that may accompany ischemia [63].

1.2.5.4 Stress Testing

Stress tests are used to assess the performance of the heart under high workload conditions and detect ischemia that may not be present at rest, as in the case of silent cardiac ischemia [64].

- **Stress test:** the patient walks on a treadmill or pedals on an exercise bike while cardiac activity (ECG) is monitored. If there is myocardial ischemia, symptoms such as chest pain, ECG changes (such as ST-segment depression) or arrhythmias may appear during the test.

- **Pharmacological stress test:** if a patient cannot exercise, drugs such as dobutamine or adenosine, which stimulate the effects of exercise on the heart, are administered having a good safety profile [65].
- **Exercise Stress Echocardiography (ESE):** combines a stress test with an echocardiogram to visualize the movement of the heart during stress; abnormalities in wall movement may indicate the presence of ischemia [66].
- **Nuclear stress test:** involves the injection of a radioactive tracer (such as thallium or technetium) and the use of a gamma chamber to assess blood flow to the heart both at rest and during stress. Reduced tracer uptake in some areas suggests ischemia [67].

1.2.5.5 Coronary Angiography and Computed Tomography Coronary Angiography (CTCA)

In coronary angiography (or cardiac catheterization), a catheter is inserted into a blood vessel and then guided to the coronary arteries, into which a contrast dye is injected so that the arteries can be visualized by X-ray. This test allows the location and severity of any obstruction in the coronary arteries to be shown and is useful in guiding therapeutic decisions, such as angioplasty or coronary artery bypass surgery.

CTCA is a noninvasive alternative to traditional coronary angiography. It uses computed tomography to create detailed images of the coronary arteries and detect atherosclerotic plaques or obstructions [67].

1.2.5.6 Echocardiogram

An echocardiogram (ultrasound of the heart) is often used to assess heart function and structure [68]. It can detect:

- Wall motion abnormalities: areas of the heart that are not contracting properly due to ischemia.
- Ejection fraction: a measure of how well the heart is pumping blood, which can be reduced in cases of significant ischemia or heart damage.
- Valvular heart disease or other structural issues: can contribute to ischemia and complicate treatment.

1.2.5.7 Cardiac magnetic resonance imaging (CMR)

A cardiac magnetic resonance imaging scan provides highly detailed images of the heart's structure and function. It can identify ischemic areas (areas with reduced blood flow) and also can differentiate between reversible and irreversible ischemic damage (such as scarring from a previous infarction) [69].

1.2.5.8 Holter Monitor

For patients with intermittent symptoms, a Holter monitor can be used. It continuously records the heart's electrical activity over 24-48 hours or longer, detecting episodes of ischemia-related arrhythmias or silent ischemia (ischemia without symptoms) [70].

1.2.5.9 Positron Emission Tomography (PET) Scan

A PET scan can evaluate myocardial perfusion and metabolism, offering high sensitivity for detecting ischemia, especially in cases where other tests may be inconclusive [71].

1.2.5.10 Intravascular Ultrasound (IVUS) and Fractional Flow Reserve (FFR)

The Intravascular ultrasound provides detailed images of the inside of the coronary arteries, helping to assess the severity and composition of atherosclerotic plaques. Instead,

the Fractional flow reserve measures the pressure differences across a coronary artery stenosis to assess whether it is causing significant ischemia [72].

1.2.6 Therapeutic approaches

Treatment approaches for myocardial ischemia serve to restore adequate blood flow to the heart muscle, relieve symptoms, prevent progression to myocardial infarction, and reduce mortality. Treatment may vary according to the type and severity of ischemia, but also according to the patient's overall health. In fact, for one patient it may be enough just to change lifestyle, for another it will require medication, etc.

1.2.6.1 Lifestyle changes

Lifestyle changes are critical for both prevention and management of myocardial ischemia [73].

- **Diet:** a healthy diet rich in fruits, vegetables, whole grains, lean protein and healthy fats helps control blood pressure, cholesterol levels and body weight.
- **Physical activity:** regular aerobic exercise (walking, biking, or swimming) helps improve cardiovascular fitness, control weight, and reduce risk factors such as hypertension and hyperlipidemia.
- **Quitting smoking:** one of the most effective ways to reduce the risk of myocardial ischemia and coronary artery disease.
- **Weight management:** maintaining a healthy weight reduces the risk of developing hypertension, diabetes and high cholesterol, which contribute to ischemia.
- **Stress reduction:** meditation and relaxation exercises can help reduce the negative impact of chronic stress on heart health.

1.2.6.2 Pharmacological Therapy

In pharmacological therapy, drugs are used to relieve symptoms, prevent further ischemic episodes, and reduce the risk of complications such as myocardial infarction or heart failure. Specifically, the most used drugs are nitrates, beta-blockers, calcium channel blockers, antithrombotic and statins [74].

Nitrates, such as Nitroglycerin, are used for acute symptoms relief and long-term management. They increase the distribution of nitric oxide and dilate blood vessels, improving blood flow to the heart and reducing chest pain [75].

Beta-blockers (BB) or Beta-adrenergic antagonists, such as Metoprolol and Atenolol, reduce heart rate and contractility by blocking the β1 adrenergic receptor, thereby decreasing the heart's oxygen demand. They are commonly prescribed after a heart attack to prevent further ischemia.

Calcium channel blockers (CAs), such as Amlodipine and Diltiazem, block the L-type calcium receptor, relaxing coronary arteries, improving blood flow to the heart and reducing cardiac workload by lowering blood pressure.

Anti-thrombotic, such as Aspirin and Heparin, reduce the risk of blood clot formation.

Statins (Atorvastatin, Simvastatin) reduce LDL cholesterol levels and intervene in the progression of atherosclerosis by stabilizing atherosclerotic plaques.

1.2.6.3 Revascularization Procedures

In cases where medication and lifestyle changes are insufficient, revascularization procedures are used to restore blood flow to the heart muscle.

Percutaneous transluminal coronary angioplasty (PTCA) is a minimally invasive technique that involves the use of a balloon catheter that is inflated at lesions to dilate the arteries and the subsequent placement of a stent (a small wire mesh tube) to keep the

artery open. The stents may or may not be coated with useful drugs to prevent narrowing of the artery [76].

Coronary Artery Bypass Grafting (CABG) is a surgical procedure that allows arteries or veins taken from the patient, to be used to restore adequate blood flow, thereby reducing the risk of myocardial infarction and death. It involves taking a healthy artery or vein from another part of the body and then grafting it to bypass the blocked coronary artery, thereby improving blood flow to the heart. It is a recommended procedure in patients with severe obstruction, left main coronary artery disease, or in cases where PTCA is not feasible [77].

1.2.6.4 Experimental Therapies

New therapeutic treatments are currently being developed, such as the use of stem cells to regenerate damaged heart tissue or gene therapy to promote the growth of new blood vessels in the heart muscle so as to improve blood flow [78].

1.3 Renal Ischemia

1.3.1 Definition

Ischemic renal disease, or ischemic nephropathy, is a pathological condition characterized by a reduction or blockage of blood flow to the kidneys, resulting in tissue damage [79]. This impairs the kidneys' ability to effectively filter blood, leading to a buildup of toxins and potentially fatal electrolyte imbalances. The common outcome of ischemic nephropathy is renal fibrosis followed by atrophy and chronic renal failure [80]. Ischemic nephropathy is characterized also, by a reduction of kidney size [81] and a significant decrease in glomerular filtration rate (GFR) (Figure 3) [82].

Renal I/R injury is a common cause of acute kidney injury, a disorder characterized by damage to tubular epithelial cells and associated with renal dysfunction, prolonged hospitalization, possible development of chronic kidney disease, and high rates of morbidity and mortality in patients [83].

Renal I/R injury can often occur after a kidney transplant, major vascular surgery, or sepsis [84, 85]. I/R renal injury represents an incurable perioperative complication; Therefore, there is an urgent need to develop new strategies to preserve renal function after renal ischemia [86]. In addition, since the kidneys are vital organs for the maintenance of body homeostasis, timely diagnosis and treatment of renal ischemia are essential to prevent serious complications.

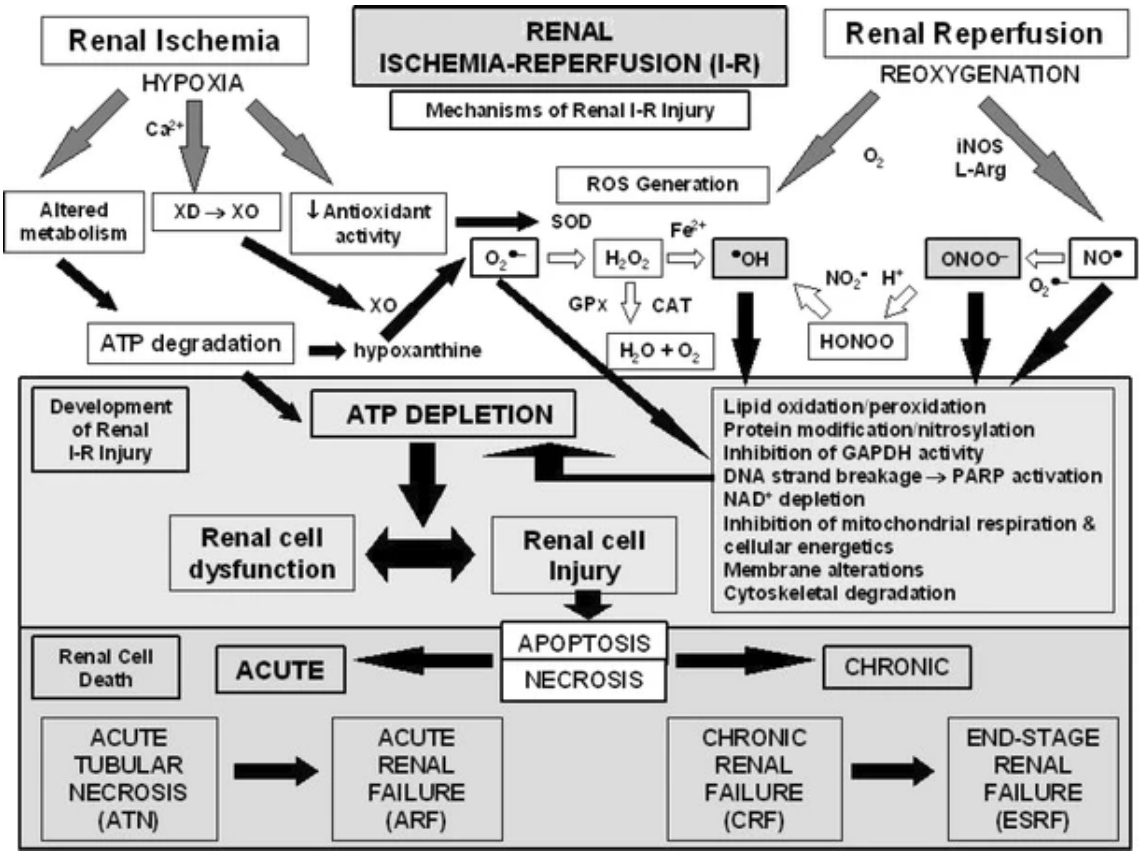

Figure 3. Mechanism of renal ischemia-reperfusion injury [87]

Renal ischemia can be divided according to duration and severity into acute and chronic, and each has a distinct pathogenetic mechanism.

1. **Acute renal ischemia:**

It occurs when blood flow to the kidneys is abruptly reduced or stopped, as happens in cases of thrombosis, embolism, or hypovolemic shock. Rapid intervention is crucial to avoid permanent damage to kidney tissues, such as the development of acute tubular necrosis (ATN) [88] and ischemic renal failure (ARF) [89].

2. **Chronic renal ischemia:**

A form commonly associated with renal artery stenosis (RAS) or vascular disease [90]. There is a gradual narrowing of the renal arteries that leads to a reduction in blood flow and oxygen supply to the kidney, causing progressive damage to tubular and glomerular cells. It involves progressive and irreversible damage such as interstitial fibrosis, renal atrophy, chronic renal failure (CRF) leading to end-stage renal failure (ESRF) [87]. In addition, chronic ischemia is associated with increased intraglomerular pressure, which causes further damage and worsens kidney function [91].

1.3.2 Epidemiology and risk factors

Renal ischemia, especially in the acute form, is associated with AKI, a condition that affects a large proportion of hospitalized patients. In fact, a study has shown that AKI of ischemic origin affects 5-7% of hospitalized patients [92]. AKI is estimated to affect about 500 individuals out of 100,000 [93]. The ischemic nephropathy represents the cause of 5% to 22% of advanced renal disease in patients older than 50 years [90]. Renal ischemia is responsible for 7% of end-stage renal disease and in the United States it is among the main causes of renal failure leading to dialysis [94].

The pathogenesis of renal ischemia is influenced by several risk factors [95], such as:

- **Age:** the risk of renal ischemia increases with age, increasing the prevalence of cardiovascular disease such as atherosclerosis, which is the principal cause of ischemia. In fact, a prevalence of 18% in patients aged 64 to 75 years and 42% in patients over 75 years of age is found [82]. After age 50, or in general older patients, are particularly susceptible to chronic ischemia.
- **Gender**: the risk is higher in women than men.
- **Family history:** a positive family history for cardiovascular disease increases risk.
- **Smoking.**
- **Hypertension**: chronic hypertension damages the renal arteries promoting the development of ischemia.
- **Hyperlipidemia:** high levels of LDL cholesterol and low levels of HDL cholesterol contribute significantly to atherosclerosis.
- **Diabetes mellitus**: represents an important risk factor as it promotes atherosclerosis in both large and small vessels (microangiopathy).
- **Cardiovascular disease:** patients with cardiovascular disease such as angina or congestive heart failure present a higher risk.
- **Nephrotoxic drugs:** the prolonged use of drugs such as NSAID (non-steroidal anti-inflammatory drugs) or ACE-inhibitors can impair renal blood flow in particular in patients with bilateral stenosis or pre-existing renal failure.

1.3.3 Diagnosis

The renal ischemia is difficult to estimate, it is often asymptomatic and in addition, there are no precise diagnostic criteria of ischemic nephropathy [96]. The diagnosis of renal

ischemia required a combination of clinical evaluation, laboratory and imaging tests, and functional tests to confirm the presence of ischemia and assess its severity [97].

- **Urine tests**: the presence of proteinuria or granulated casts is indicative of tubular damage. In addition, microscopic urinalysis may reveal signs of tubular necrosis.
- **Blood tests**: increased serum creatinine is a sign of reduced kidney function. Electrolytes can also be altered, with potassium and phosphate levels often elevated due to reduced renal filtration [98].
- **Duplex Doppler ultrasound (DDS):** used to evaluate the blood flow of the kidney or kidney system and can detect signs of arterial stenosis [99].
- **Magnetic resonance angiography (MRA):** the procedure involves the injection of a bolus of a gadolinium chelate and the subsequent three-dimensional volumetric data collection sensitized to the T1 shortening effects of gadolinium. It is used to assess the stenosis or occlusion of the renal arteries, accurately identifying the degree of vascular compromise [100].
- **Computed Tomography (CT) and Magnetic Resonance Imaging (MRI):** used to visualize the anatomy of the kidneys and identify vascular obstructions, can provide detailed images of renal blood flow and parenchyma [82].

1.3.4 Therapeutic approaches

Treatments approaches for renal ischemia serve to restore adequate blood flow to relieve symptoms and prevent development of acute or chronic renal dysfunction. Lifestyle changes are critical for both prevention and management of ischemia, for example quitting smoke is very useful. It is essential to maintain adequate hydration to improve

renal perfusion. In addition, a balanced diet rich in fruits, vegetables, whole grains, lean protein and healthy fats helps control blood pressure, cholesterol levels and, if necessary, restricting sodium and protein intake can help prevent overload of work on the kidneys [81].

1.3.4.1 Pharmacological Therapy

In pharmacological therapy, drugs are used to relieve symptoms, prevent further ischemic episodes, and reduce the risk of complication [101]. Specifically, the most used drugs are:

- **Vasodilators:** reduce intra-renal pressure and help prevent further damage to kidney tissues. ACE inhibitors (such as Enalapril and Lisinopril) and angiotensin receptor blockers (ARBs, such as Losartan and Valsartan) are used to lower blood pressure and improve blood flow to the kidneys.
- **Anti-thrombotic:** prevent the formation of new clots and facilitate the dissolution of existing ones. The most used are Aspirin and Heparin.

1.3.4.2 Revascularization Procedures

Revascularization procedures are indicated in patients with a decrease in renal artery diameter greater than 75%, progressive deterioration of renal function with renovascular disease and renal failure caused by aortic, unilateral, or bilateral renal artery thrombosis [102]. Revascularization procedures are also used to restore blood flow to the heart muscle, when medication and lifestyle changes are insufficient.

Percutaneous transluminal renal angioplasty (PTRA) is a minimally invasive technique used to dilate the narrowed renal artery using a balloon in case of hemodynamic relevant renal artery stenosis. Often, a stent is inserted to keep the artery open and ensure adequate blood flow [103].

Chapter 2: KYP-2047

2. KYP-2047

KYP- 2047 (4-phenylbutanoyl-L-prolyl-2(S)-cyanopyrrolidine) (Figure 4) is a prolyl endopeptidase (PREP) inhibitor, synthesized at the University of Kuopio, Finland as described above [104]. Structurally, is based on the structure of another inhibitor, SUAM-1221, but differs from it by for an additional electrophilic nitrile group in the 2S-position of the pyrrolidine ring. KYP-2047 has a MW 339.4 g/mol and possess a K_i of 0.023 nM.

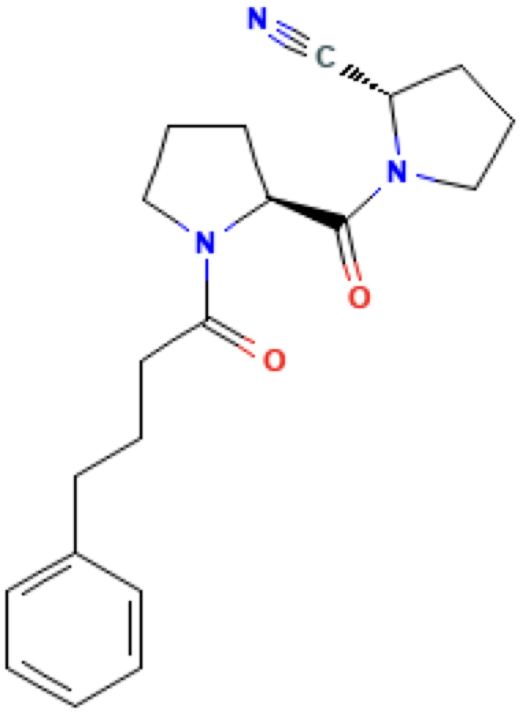

Figure 4. Chemical structure of KYP-2047

[PubChem, https://pubchem.ncbi.nlm.nih.gov/compound/kyp-2047]

2.1 Prolyl endopeptidase (PREP)

PREP, also known as prolyl oligopeptidase (POP) or post-proline cleavage enzyme, is an enzyme encoded in humans by the PREP gene. It is an intracellular enzyme (molecular mass 80kDa) belonging to the serine protease family, specifically the S9 subfamily that differs from the others, trypsin and subtilisin, in their specificity to recognize the peptide substrate and in their different catalytic triad [105]. PREP hydrolyzes the peptide bond at the C-terminal end of a proline residue. In addition to its enzymatic activity, PREP can cleave short proline-containing peptides (less than 3 kDa) that are involved in the activation of the inflammatory response, the development of neurodegenerative diseases, and the outcome of autoimmune diseases [106].

PREP is expressed in different organs and tissues (such as brain, heart, liver, kidney, lung, intestine) [107, 108] and is involved in the hydrolysis of bioactive peptides containing proline, such as angiotensin, arginine-vasopressin, substance P and neurotensin [109, 110]. It has also been shown to be involved in several other physiological and pathological functions such as inflammation [111].

Being involved in the release of pro-angiogenic and anti-fibrogenic molecules [112, 113], prolyl endopeptidase inhibitors may represent a novel approach to the treatment of many inflammatory diseases. The chemical structures of PREP inhibitors can be divided into P1 (usually contains a proline like moiety), P2 (may contains aliphatic chain or a ring) and P3 (usually contains an aromatic ring) [114] (Figure 5).

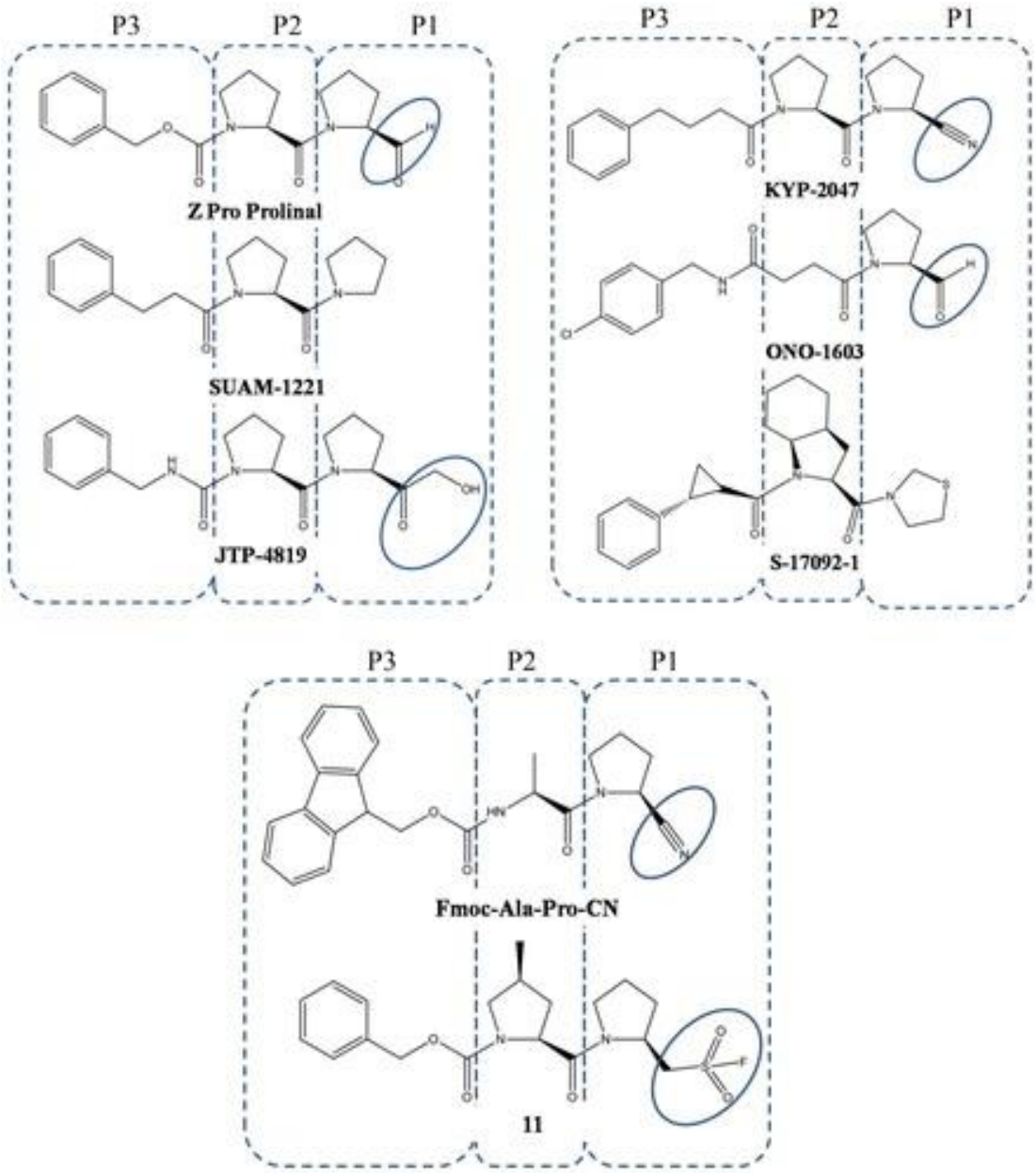

Figure 5. Structure of PREP inhibitors [114]

Specifically, the inhibitor KYP-2047 possesses a good ability to reach and inhibit prolyl endopeptidase at the intracellular level [115], showing the ability to inhibit this enzyme more effectively than other inhibitors [116]. Klimaviciusa et al. demonstrated greater potency of KYP-2047 than ZPP (Z-pro-prolinal) inhibitor in primary cortical neurons of rats and neuroblastoma cells [117]; indeed, it inhibits about 85% of prolyl-endopeptidase activity, showing a half-life of about 4-8 hours.

2.2 Mechanism of action of KYP-2047

KYP-2047 works by blocking the enzyme activity of PREP, inducing conformational changes. In fact, KYP-2047 is able to bind to Ser554 in the active site of the PREP enzyme, stabilizing the inactive "closed" conformation of the enzyme and therefore, preventing its proteolytic activity [118] (Figure 6).

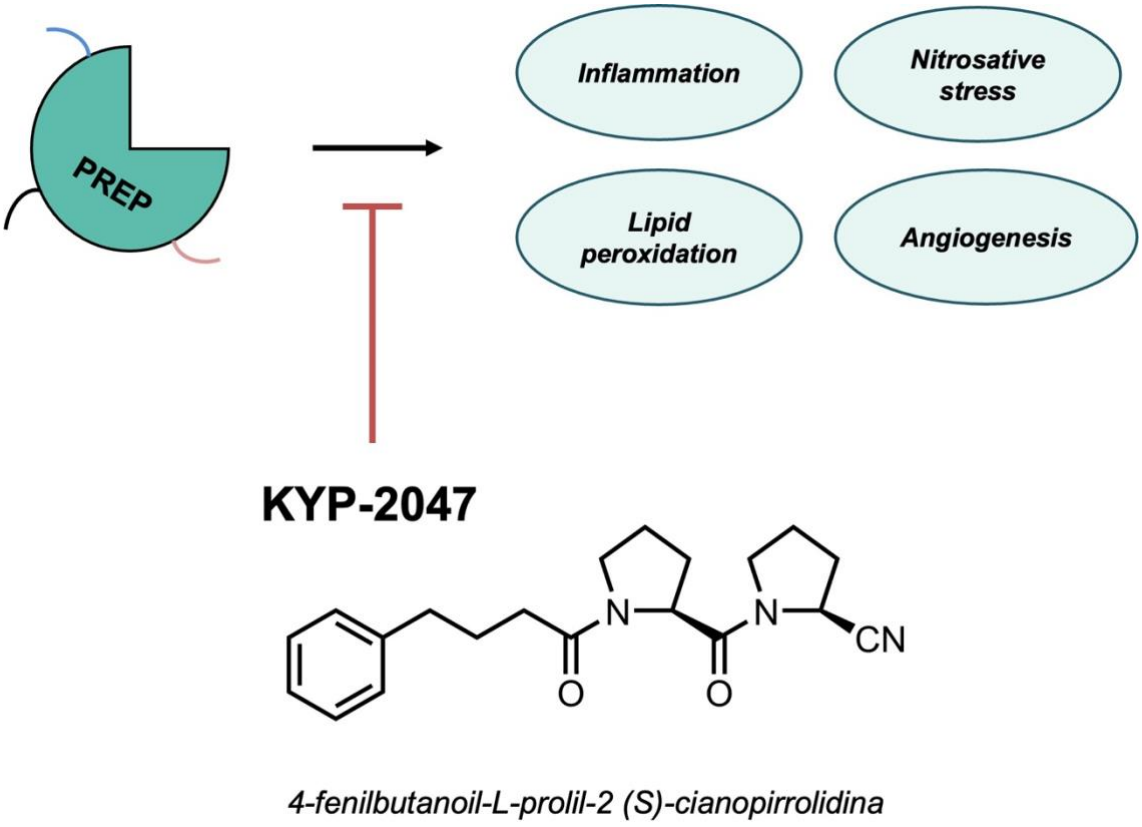

Figure 6. Mechanism of inhibition of PREP by KYP-2047 [Created with BioRender]

KYP-2047 has been shown to penetrate the blood-brain barrier, distribute equally between the cortex, hippocampus and striatum, and penetrate the brain parenchyma and exhibits, likewise, high lipophilicity, allowing it greater penetration through biological membranes *in vivo*. In addition to exerting a neuroprotective role, KYP-2047 has been shown to have

significant effects on inflammatory and angiogenic processes, thus making it a possible therapeutic strategy for different diseases.

2.3 Role of KYP-2047 in Inflammation

Recent studies have demonstrated an involvement of PREP in important disease processes, such as α-synuclein aggregation and neutrophil-linked inflammation [115]. The inflammation is a response of the body to damage related to chemical, physical or microorganism insults, to inactivate or destroy the pathogen involved, remove irritants and lay the foundations for tissue repair [119]. As demonstrated by several studies PREP inhibition by KYP-2047 reduces the production of pro-inflammatory cytokines, such as TNF-α IL-6 [120-122]. This anti-inflammatory effect could be crucial in the management of diseases in which chronic inflammation plays a key role.

2.4 Role of KYP-2047 in Angiogenesis

Some studies demonstrated the ability of KYP-2047 to modulate angiogenesis, a process of forming of new blood vessels, often associated with tumor progression [123, 124]. It is a complex process, consisting of a cascade of events regulated by pro- and anti-angiogenic factors, such as the vascular endothelial growth factor (VEGF) or the transforming growth factor β (TGFβ). Angiogenesis plays a fundamental role in embryonic development and tissue growth; in adults, however, angiogenesis is found mainly in pathological states such as tumor growth and states of chronic inflammation (rheumatoid arthritis [125], pulmonary fibrosis [126], etc).

Chapter 3: Aim of the thesis

3. Aim of the thesis

Based on the important role of PREP in inflammatory and angiogenic processes, PREP inhibitors, such as KYP-2047, may represent a new therapeutic strategy for ischemia/reperfusion injury. Therefore, this PhD project aims to evaluate the protective effect of KYP-2047 to counteract the inflammatory and angiogenic process involved in the pathophysiology of I/R. In particular, the evaluation was carried out on the preventive effects of KYP-2047 in a mouse model of myocardial I/R and on the protective effect of KYP-2047 in a mouse model of renal I/R as post-treatment, emphasizing the ability of KYP-2047 to modulate angiogenesis, inflammation, fibrosis, apoptosis and nitrosative stress.

Chapter 4: Materials and Methods

4. Materials and methods

4.1 Materials

KYP-2047 (Sigma, CAS No.: SML020) was purchased by Sigma-Aldrich (Milan, Italy). All other chemicals used in this study were of the highest commercial grade available. All the stock solutions were prepared in non-pyrogenic saline (0.9% NaCl, B. Braun Melsungen AG, Berlin, Germany).

4.2 Animals

The study was conducted on adult male CD1 mice (25–30 g, 6-8 weeks of age, Envigo, Milan, Italy). The animals were housed in a controlled environment and placed in steel cages in a room maintained at 22 ± 1 °C with a 12-h light/12-h dark cycle. Mice were supplied with water and standard rodent food. Animal study was approved by the University of Messina Review Board following Italian regulations on the use of animals (DM116192) and Directive legislation (EU) (2010/63/EU).

4.3 Myocardial Ischemia/Reperfusion (MI/R)

A mouse model of MI was performed by occlusion of the left anterior descending coronary artery (LAD) as previously described [127]. Specifically, mice were anesthetized with a mixture of ketamine and xylazine and then, placed on a heating pad to maintain core body temperature at 37 °C during surgery. Briefly, animals underwent thoracotomy at the fifth left intercostal space, the pericardium was opened, and a 6-0 silk thread was placed around the LAD approximately 1–2 mm below its origin. LAD was kept occluded for 15 minutes and ligation was subsequently released so as to allow

reperfusion of the previously ischemic myocardium. The duration of reperfusion was predetermined at 2 hrs. Following the surgical procedure, the mice were left to recover under a heat lamp and under observation for 6 hours. Timings were chosen based on the literature to maximize the reproducibility of functional myocardial damage while minimizing mortality in these animals [127]. KYP-2047 was administrated to mice at doses of 2.5 and 5 mg/kg 24 hours before the surgical procedures. The dose and route of administration of KYP-2047 were chosen based on previous studies [121]. Hearts were collected for analysis, determining the size of ischemic injury.

4.3.1 Experimental Group

Mice were randomly divided into the following groups:

- *Sham:* mice were subjected to surgical procedures, except for MI/R and were kept under anesthesia for the duration of the experiment. (N=8).
- *Sham + KYP-2047 2.5 mg/kg group:* mice were subjected to surgical procedures, except for MI/R and were kept under anesthesia for the duration of the experiment. KYP-2047 (2.5 mg/kg 0.001% DMSO i.p) was administered 24hours before the surgical procedures (N=8).
- *Sham + KYP-2047 5 mg/kg group*: mice were subjected to surgical procedures, except for MI/R and were kept under anesthesia for the duration of the experiment. KYP-2047 (5 mg/kg 0.001% DMSO i.p) was administered 24hours before the surgical procedures (N=8).
- *MI/R*: mice were subjected to coronary artery occlusion (15 min), followed by reperfusion (2h) (N=8):

- *MI/R + KYP-2047 2.5 mg/kg group*: mice were subjected to coronary artery occlusion (15 min), followed by reperfusion (2h). KYP-2047 (2.5 mg/kg 0.001% DMSO i.p) was administered 24hours before the surgical procedures (N=8);
- *MI/R + KYP-2047 5 mg/kg group*: mice were subjected to coronary artery occlusion (15 min), followed by reperfusion (2h). KYP-2047 (5 mg/kg 0.001% DMSO i.p) was administered 24hours before the surgical procedures (N=8).

Results for the KYP-2047-treated Sham groups were not shown because neither toxicity nor improvement were seen compared to the Sham group.

4.3.2 Histological evaluation

Heart tissues were collected and processed for histological analysis, as previously described [128]. Briefly, the whole heart was removed and was fixed in formaldehyde buffered solution (10% phosphate buffered saline) for 24h at room temperature, dehydrated by graded ethanol and included in paraffin. Subsequently heart tissue sections (7 µm) were deparaffinized with xylene, stained with hematoxylin/eosin, and studied with light microscope (Zeiss Milan, Italy). Every piece was viewed at a magnification of 2,5x and 20x (200 µm and 50µm scale bar). For the quantitative estimation of I/R lesions, histological studies were performed by two blinded investigators and the score scale-point was chosen based on previous studies [127].

4.3.3 Masson's trichrome

The degree of fibrosis collagen accumulation in the heart tissues was assessed using Masson's trichrome stain, following the manufacturer's instructions (Bio-Optica, Milan, Italy), as previously described [121]. Images were shown at 2,5x and 20x magnification (respectively, 200µm and 50µm scale bar), using a Nikon Eclipse Ci-L microscope.

4.3.4 Toluidine Blue Staining

Heart tissues were stained with toluidine blue (Bio-Optica, Milan, Italy) to assess the quantity of mast cells and their degranulation, as previously described [129]. Briefly, sections were deparaffinized in xylene and dehydrated using ethanol graded sequences and then stained for 5 min. Sections were placed in absolute alcohol, cleared in xylene, and fixed using Eukitt (Bio-Optica, Milan, Italy). Images were shown at 40× magnification (20 μm scale bar). The number of metachromatic stained mast cells was obtained by counting in five high-power fields (40×) per section using a Nikon Eclipse Ci-L microscope.

4.3.5 Immunohistochemistry

Immunohistochemical analysis was performed as previously described [130]. The heart sections (7 μm) were incubated overnight with the primary antibody at room temperature. The used antibodies were anti-mast cell tryptase (1:100, Santa Cruz Biotechnology, sc-59587), anti-nitrotyirosine (1:100, Merck-Millipore, 06-284), anti-VEGF (1:100, Santa Cruz Biotechnology, sc-7269) and anti-CD34 (1:100, Santa Cruz Biotechnology, sc-74499). The following day the sections were washed with PBS and incubated with a secondary antibody for 1h at room temperature. A chromogenic substrate (brown DAB) detected the reaction, and the images were taken using a microscope and AxioVision software. Sections were then observed with a Nikon Eclipse Ci-L microscope. The percentage area of immunoreactivity (determined by the number of positive pixels) was expressed as percentage (%) of total tissue area (red staining) at 20x magnification (50μm scale bar).

4.3.6 Terminal deoxynucleotidyl transferase-mediated UTP end labeling (TUNEL) assay

The TUNEL staining was performed using a cell death detection kit following the manufacturer's instructions (Roche), as previously described [131]. The sections, after being deparaffinized and hydrated, were permeabilized with 0.1 M citrate buffer and then incubated in the TUNEL reaction at 37° C for 60 minutes in the dark. For TUNEL staining, 2,5x and 20x magnification (respectively, 200μm and 50μm scale bar) were showed.

4.3.7 Western blot

The heart tissues were collected and then processed for western blot analysis as previously described [132]. Lysates were used for the detection of inhibitor kappa B-alpha (IκB-α), interleukine-18 (IL-18), p-p38 MAPK and p-ERK in cytosolic fraction. At the same way, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) was quantified in nuclear fraction. Membranes were incubated overnight at 4° C with the following primary antibodies: anti-IκB-α (1:500; Santa Cruz Biotechnology sc-1643), anti-IL-18 (1:500; Santa Cruz Biotechnology sc-7954, Dallas, TX, USA), anti- p-p38 MAPK (Thr180/Tyr182) (1:500; Cell Signaling, 9211S), anti-p-ERK (1:500; Santa Cruz Biotechnology sc-7383), anti ERK 1/2 (1:500, ; Santa Cruz Biotechnology sc-514302) and anti-NF-κB (1:500; Santa Cruz Biotechnology sc-8008). Then, membranes were incubated with peroxidase-conjugated bovine anti-mouse secondary antibody (1:1000, Jackson ImmunoResearch, USA) for 1 h at room temperature. Signals were detected through an enhanced chemiluminescence (ECL) detection system reagent and standardized to β-actin (1:500; Santa Cruz Biotechnology sc-47778) or lamin A (1:500; Santa Cruz Biotechnology sc-518013) levels. The relative expression of the protein bands

was quantified by densitometry with Bio-Rad ChemiDoc™ XRS software (Bio-Rad, Milan, Italy).

4.3.8 Statistical analysis

Data are expressed as mean ± standard deviation (SD) of N observations, in which N represents the number of animals. Data are representative of at least three independent experiments. The results were examined by one-way ANOVA analysis of variance followed by a Bonferroni post hoc test for multiple comparisons. Only a p-value less than 0.05 was considered significant.

4.4 Kidney Ischemia/Reperfusion (KI/R)

A mouse model of KI/R was performed as previously described [133]. Specifically, mice were anesthetized with a mixture of ketamine and xylazine and then, placed on a heating pad to maintain core body temperature at 37 °C during surgery. Briefly, animals were subjected to a midline laparotomy and then underwent to a bilateral renal ischemia. KI/R was induced by occluding renal arteries and veins for 30 minutes. Renal clamps were subsequently removed, and the kidney were observed for the next 5 minutes to confirm complete reperfusion. Timings were chosen based on the literature to maximize the reproducibility of functional renal damage while minimizing mortality in these animals. KYP-2047 was administrated to animal at doses of 0,5, 1 and 5 mg/kg 5 minutes before the reperfusion. Following the surgical procedure, the mice were left to recover under a heat lamp and under observation for 6 hours. The dose and route of administration of KYP-2047 were chosen based on previous studies [134]. Kidneys were collected for analysis.

4.4.1 Experimental Group

Mice were randomly divided into the following groups:

- *Sham:* mice were subjected to surgical procedures, except for KI/R (N=8);
- *KI/R*: mice were subjected to renal ischemia (30 min), followed by reperfusion (6 hrs) plus administration of saline (N=10);
- *KI/R + KYP-2047 0.5 mg/kg group*: mice were subjected to mice were subjected to renal ischemia (30 min), followed by reperfusion (6 hrs) and administration of KYP-2047 (0.5 mg/kg 0.001% DMSO i.p) 5 minutes before the reperfusion (N=10);
- *KI/R + KYP-2047 1 mg/kg group*: mice were subjected to mice were subjected to renal ischemia (30 min), followed by reperfusion (6 hrs) and administration of KYP-2047 (1 mg/kg 0.001% DMSO i.p) 5 minutes before the reperfusion (N=10);
- *KI/R + KYP-2047 5 mg/kg group*: mice were subjected to mice were subjected to renal ischemia (30 min), followed by reperfusion (6 hrs) and administration of KYP-2047 (5 mg/kg 0.001% DMSO i.p) 5 minutes before the reperfusion (N=10).

4.4.2. Histological evaluation

Kidney tissues were collected and processed for histological analysis, as previously described [128]. Briefly, renal samples were fixed in formaldehyde-buffered solution (10% phosphate-buffered saline) at room temperature for 24h, dehydrated with ethanol, and included in paraffin. Subsequently, renal tissue sections (7 μm) were deparaffinized with xylene, stained with hematoxylin/eosin and studied with light microscope (Zeiss Milan, Italy). Every piece was viewed at a magnification of 10x and 40x (respectively, 100 μm and 20 μm scale bar). For the quantitative estimation of I/R lesions, histological studies were performed by two blinded investigators and the score scale-point was chosen based on previous studies [135].

4.4.3. PAS Staining

Glycogen stores were assessed using PAS staining, as previously described [136]. Renal sections were scored from 0, no staining of glycogen granules, to 3 in case of more intense staining. The images are shown at 20x magnification (50 μm scale bar).

4.4.4 Assessment of Renal Function

Blood urea nitrogen (BUN) and serum creatinine levels were assessed from blood samples as previous described [137].

4.4.5 Blue Toluidine Staining

Renal tissues were stained with toluidine blue (Bio-Optica, Milan, Italy) to assess the quantity of mast cells and their degranulation, as previously described [129]. Briefly, sections were deparaffinized in xylene and dehydrated using ethanol graded sequences and then stained for 5 min. Sections were placed in absolute alcohol, cleared in xylene, and fixed using Eukitt (Bio-Optica, Milan, Italy). Images were shown at 40× magnification (20 μm scale bar). The number of metachromatic stained mast cells was obtained by counting in five high-power fields (40×) per section by using a Nikon Eclipse Ci-L microscope.

4.4.6 Masson's Trichrome Staining

The degree of fibrosis collagen accumulation in the kidney tissues was assessed using Masson's trichrome stain, following the manufacturer's instructions (Bio-Optica, Milan, Italy), as previously described [121]. Images were shown at 20x magnification (50μm scale bar), using a Nikon Eclipse Ci-L microscope.

4.4.7. ELISA Kit for Pro-Collagen 1 (Colla1) Evaluation

The collagen content was evaluated using the mouse Pro-Collagen I alpha 1 ELISA Kit (Abcam, #ab210579), as previous described [138]. Briefly, samples were homogenized in 300 µL lysis buffer (750 µL, Pierce #87787, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with a protease inhibitor cocktail (Sigma-Aldrich, Rehovot, Israel). Samples were subsequently centrifuged at 14,000× *g* for 10 min at 4 °C and supernatants were collected, aliquoted, and subjected to analysis.

4.4.8 Western Blot Analysis

The kidney tissues were collected and then processed for western blot analysis as previously described [132]. Lysates were used for the detection of IκB-α, inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2), NF-κB-inducing kinase (NIK), B-cell lymphoma 2 (Bcl-2), Bcl-2 associated agonist of cell death (BAD), tumor necrosis factor (TNF-α), interleukine-6 (IL-6), protein phosphatase 2 (PP2A), and p-PP2A in cytosolic fraction. At the same way, NF-κB was quantified in nuclear fraction. Membranes were incubated overnight at 4° C with the following primary antibodies: IκB-α (1:500; Santa Cruz Biotechnology sc-1643), anti-COX-2 (1:500; Cayman 160106), iNOS (1:500, Abcam ab3523), anti-Bcl-2 polyclonal antibody (1:500 Santa Cruz Biotechnology sc-7382), anti-Bad (1:500, Santa Cruz Biotechnology sc-8044), anti TNF-α (1:500, Santa Cruz Biotechnology sc-52746), anti-IL-6 (1:500, Santa Cruz Biotechnology sc-57315) PP2A (1:500; Merck 05–421), p-PP2A (1:500; Thermo Fisher Scientific PA5-36874) and anti-NF-κB (1:500; Santa Cruz Biotechnology sc-8008). Then, membranes were incubated with peroxidase-conjugated bovine anti-mouse secondary antibody (1:1000, Jackson ImmunoResearch, USA) for 1 h at room temperature. Signals were detected through an enhanced chemiluminescence (ECL) detection system reagent

and standardized to β-actin or lamin levels. The relative expression of the protein bands was quantified by densitometry with Bio-Rad ChemiDoc™ XRS software (Bio-Rad, Milan, Italy).

4.4.9. TUNEL Staining

The TUNEL staining was performed using a cell death detection kit following the manufacturer's instructions (Roche), as previously described [131]. The kidney sections, after being deparaffinized and hydrated, were permeabilized with 0.1 M citrate buffer and then incubated in the TUNEL reaction at 37° C in the dark for 60 minutes. For TUNEL staining 40x magnification (20μm scale bar) were showed.

4.4.10 Immunohistochemical Localization of TGF-β and VEGF

Immunohistochemical analysis was performed as previously described [130]. The kidney sections (7 μm) were incubated overnight with the primary antibody at room temperature. The used antibodies were anti-TGFβ (1:100, Santa Cruz Biotechnology, sc-130348) and anti-VEGF (1:100, Santa Cruz Biotechnology, sc-7269). The following day the sections were washed with PBS and incubated with a secondary antibody for 1h at room temperature. A chromogenic substrate (brown DAB) detected the reaction, and the images were taken using a microscope and AxioVision software. Sections were observed using a Nikon Eclipse Ci-L microscope. The percentage area of immunoreactivity (determined by the number of positive pixels) was expressed as percentage (%) of total tissue area (red staining) and images were shown at 20x magnification (50μm scale bar).

4.4.11 Real-Time PCR

Total RNA of kidney was isolated following the manufacturer's instructions (RNeasy Mini Kit; Qiagen). RNA concentration was determined by a NanoDrop™ 1000 (Thermo Scientific, Waltham, MA, USA) and 4μg of total RNA was reverse transcribed into cDNA, following the manufacturer's instructions (PrimeScript RT Master Mix, Takara, Japan). Real-time PCR amplifications were performed with the use of the ABI 7500 system (Applied Biosystems, Waltham, MA, USA), as previous described [139]. The analysis was performed to evaluate the expression of TNF-α and IL-6 mRNA in the kidney samples.

PCR primers for all analyzed genes were:

TNF-α gene: GTGATCGGTCCCAACAAGGATGGTGGTTTGCTACGACGTG

IL-6 gene: AAGTCCGGAGAGGAGACTTCAGCCATTGCACAACTCTTTTCTCATT

4.4.12 Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM) of N observations, in which N represents the number of animals. Data are representative of at least three independent experiments. Results were examined by one-way ANOVA analysis of variance followed by a Bonferroni post hoc test for multiple comparisons. Only a p-value less than 0.05 was considered significant.

Chapter 5: Results

5.1 Results of MI/R

5.1.1 Preventive effects of KYP-2047 on histological assessment

The histological examination showed the presence of tissue damage, necrosis and neutrophilic infiltration in the heart undergoing ischemia-reperfusion (Fig. 1B, B1, see histological score in Fig. 1E) compared to the sham group, in which the structure of the myocardial tissue assumed the typical normal architecture (Fig. 1A, A1, see histological score in Fig. 1E). In fact, myocardial tissue in the I/R group showed significant infiltration of inflammatory cells and disordered arrangement of myocardial fibers [140]. On the contrary, the I/R+KYP-2047 group showed reduced necrosis and a more orderly arrangement of cardiomyocytes; moreover, the degree and severity of cell necrosis were found to be significantly lower after pretreatment with KYP-2047 at the highest dose of 5 mg/kg (Fig. 1C, C1 and 1D, D1, see histological score in Fig. 1E).

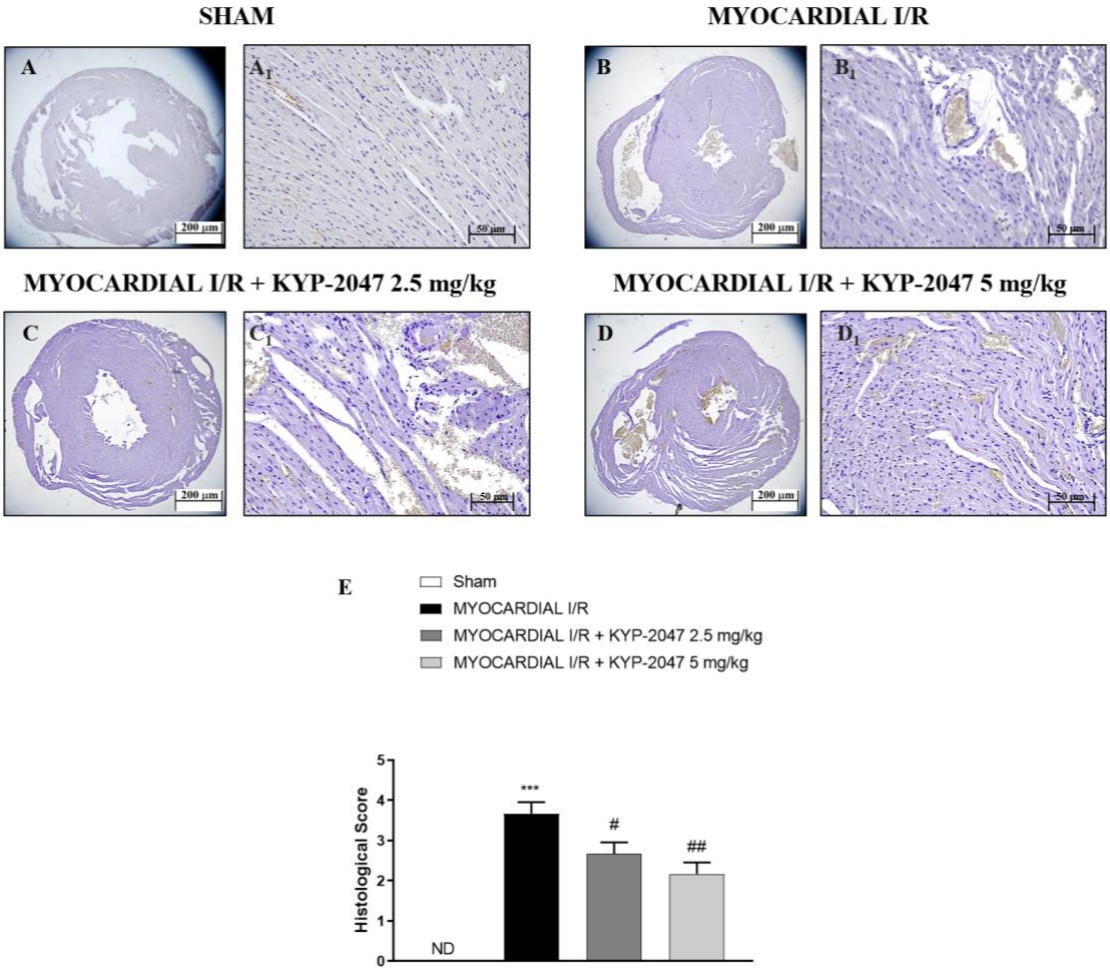

Figure 1. Effect of KYP-2047 on histological assessment. Sham (A, A1), MI/R (B, B1), MI/R + KYP-2047 2.5 mg/kg (C, C1), MI/R + KYP-2047 5 mg/kg (D, D1); see histological score (E). Images were shown at 2,5× and 20× magnification (respectively 200µm and 50µm scale bar). Data are expressed as mean ± SD of N = 8 mice/group. $^{***}p$ <0.001 vs sham; $^{\#}p$ <0.05 vs MI/R. $^{\#\#}p$ <0.01 vs MI/R.

5.1.2 Role of KYP-2047 treatment to reduce fibrosis

Masson's trichrome staining was used to evaluate the heart tissue of mice, specifically, red staining indicates healthy tissue, while blue staining indicates the fibrotic scar area. Masson's trichrome showed an increase in the level of collagen deposition in MI/R group (Fig. 2B, B1) compared to the sham group (Fig. 2A, A1) due to the formation of a

thrombus in the tissue [141]. The treatment with KYP-2047 at both doses of 2.5 and 5 mg/kg was not found to be able to reduce the collagen deposition (respectively, Fig. 2C, C1 and Fig. 2D, -D1).

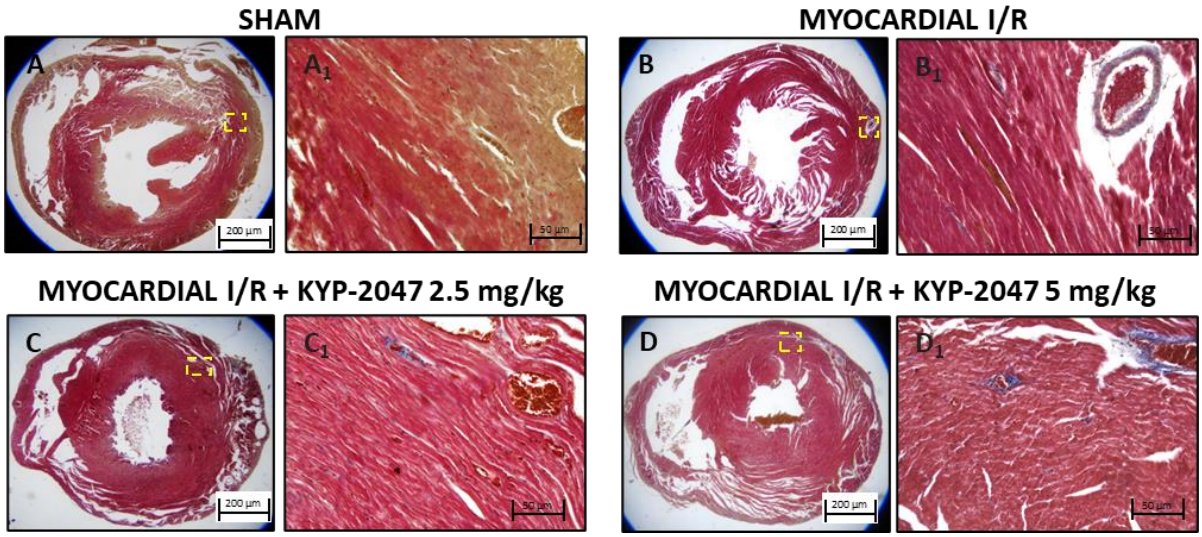

Figure 2. Effect of KYP-2047 on collagen content. Sham (A, A1), MI/R (B, B1), MI/R + KYP-2047 2.5 mg/kg (C, C1), MI/R + KYP-2047 5 mg/kg (D, D1). Images were shown at 2,5x (A-D) and 20x (A1–D1) magnification (respectively 200µm and 50µm scale bar).

5.1.3 Preventive effect of KYP-2047 on myocardial injury by mast cells staining and tryptase evaluation

Blu toluidine was performed to evaluate the presence of mast cells in myocardial tissue [142]. Data showed an increase of mast cells (MCs) infiltration in the heart tissues of MI/R group (Fig. 3B, see mast cells density Fig. 3E), compared to sham group (Fig. 3A, see mast cells density Fig. 3E). Whereas treatment with KYP-2047 at both doses of 2.5 mg/kg and 5 mg/kg reduced MC infiltration in heart tissues (respectively, Fig. 3C and Fig. 3D, see mast cells density Fig. 3E). Tryptase can be used as a marker of mast cell activity [143], so its expression was evaluated by immunohistochemistry. Data showed an increase of tryptase positive staining in MI/R group (Fig. 3G, see percentage of total

tissue area Fig. 3L) compared to sham group (Fig. 3F, see percentage of total tissue area Fig. 3L). On the other hand, tryptase positive staining increased following the treatments with KYP-2047 (Fig. 3H-I, see percentage of total tissue area Fig. 3L).

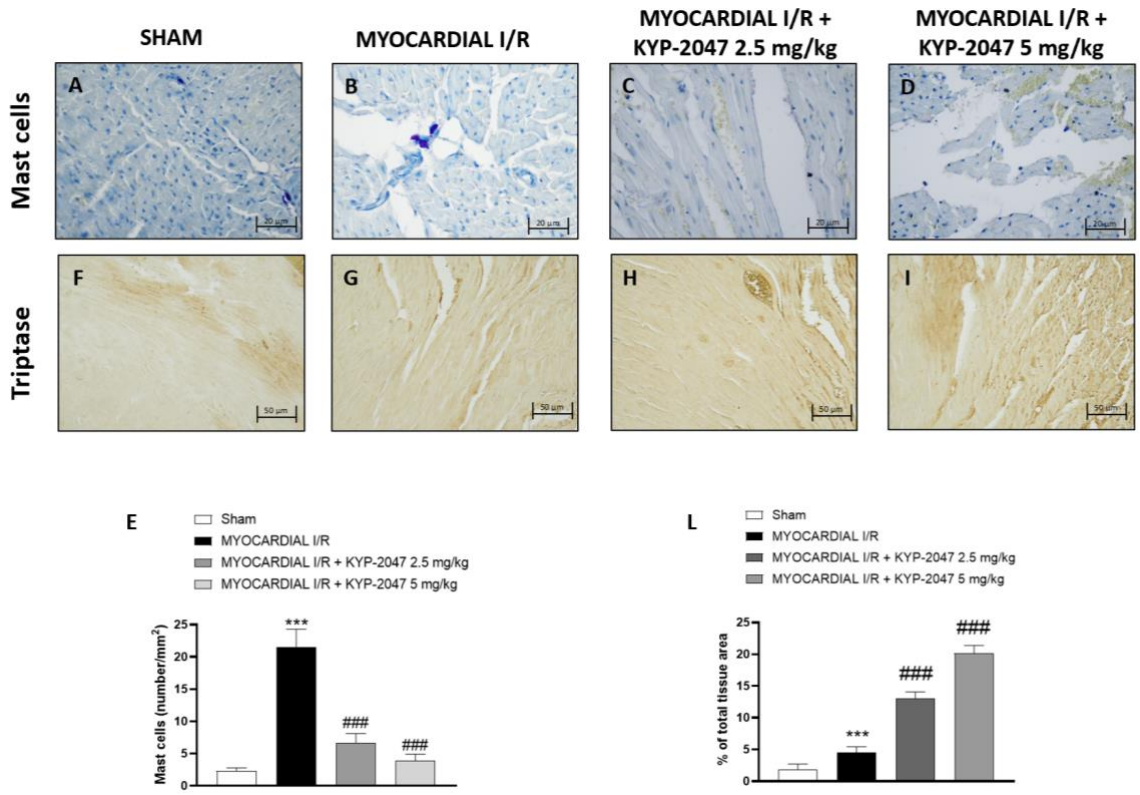

Figure 3. Effect of KYP-2047 on myocardial injury. Blue toluidine staining for mast cells degranulation in Sham group (A), MI/R group (B), MI/R + KYP-2047 2.5 mg/kg group (C), MI/R + KYP-2047 5 mg/kg group (D). Mast cells graph (number/mm^2) (E). Tryptase evaluation by immunohistochemical. Sham group (F), MI/R group (G), MI/R + KYP-2047 2.5 mg/kg group (H), MI/R + KYP-2047 5 mg/kg group (I). See percentage of total tissue area (L). Images were shown at 40× (A-D) and 20× (F-I) magnification (respectively 20µm and 50µm scale bar). Data are expressed as mean ± SD of N = 8 mice/group. $^{***}p$ <0.001 vs sham; $^{###}p$ <0.001 vs MI/R.

5.1.4 Effect of KYP2047 on angiogenesis

Vascular endothelial growth factor (VEGF) plays an important role in myocardial angiogenesis and its expression is regulated by hypoxia and cytokines [144]. Therefore, to evaluate the effect of KYP-2047 on angiogenesis, VEGF was evaluated by immunohistochemistry. Data showed an increased positive staining for VEGF in MI/R group (Fig 4B, see percentage of total tissue area Fig. 4E), compared to sham group (Fig. 4A, see percentage of total tissue area Fig. 4E). Furthermore, the expression of CD34, a marker of angiogenesis [145], was evaluated and data showed an increase of positive cells for CD34 in MI/R group (Fig 4G, see percentage of total tissue area Fig. 4L), compared to the sham group (Fig 4F, see percentage of total tissue area Fig. 4L), index of the ability to stimulate angiogenesis after injury. Data also, suggest that KYP-2047 treatments, at both doses of 2.5 mg/kg and 5 mg/kg was able to reduce both VEGF and C34 positive cells, as shown respectively in Fig. 4C-D (see percentage of total tissue area Fig. 4E) and Fig. 4H-I (see percentage of total tissue area Fig. 4L).

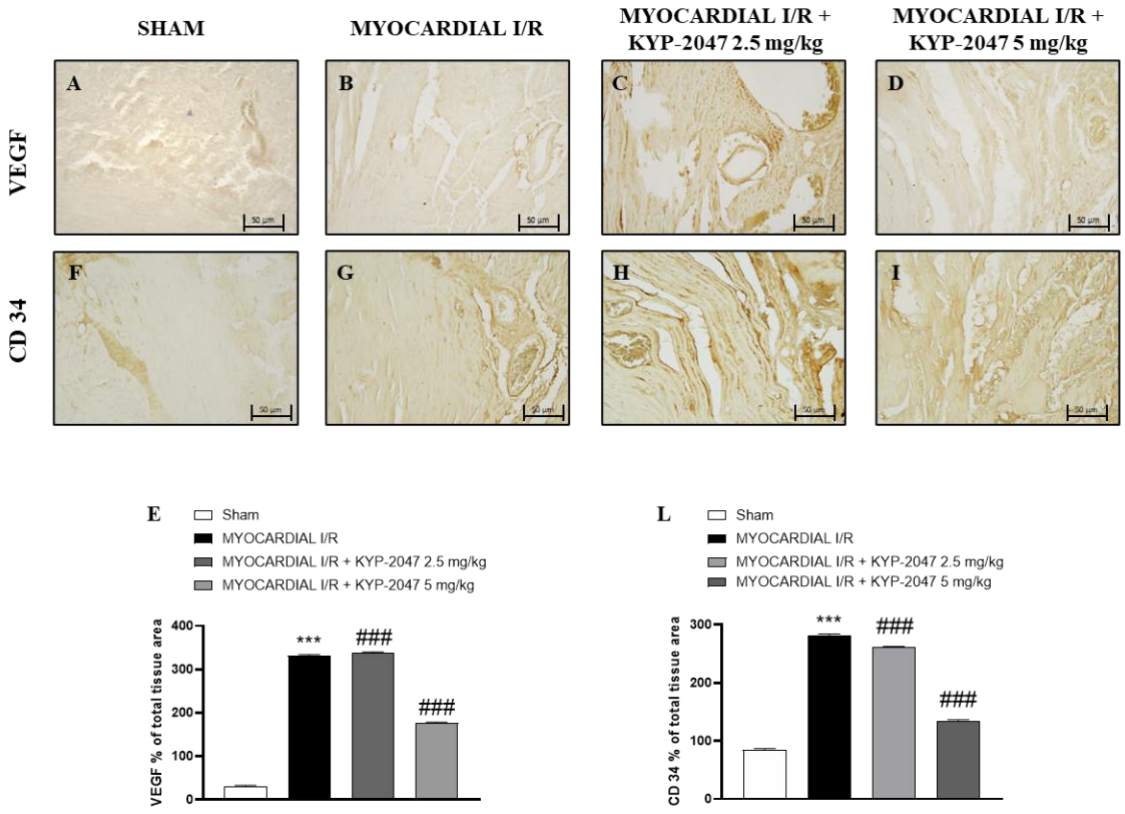

Figure 4. **Effect of KYP-2047 on angiogenesis**. Immunohistochemistry of VEGF. Sham group (A), MI/R group (B), MI/R + KYP-2047 2.5 mg/kg group (C), MI/R + KYP-2047 5 mg/kg group (D), see percentage of total tissue area (E). Immunohistochemistry of CD34. Sham group (F), MI/R group (G), MI/R + KYP-2047 2.5 mg/kg group (H), Myocardial I/R + KYP-2047 5 mg/kg group (I). See percentage of total tissue area (L). Images were shown at 20× magnification (50μm scale bar). Data are expressed as mean ± SD of N = 8 mice/group. ***p <0.001 vs sham; ###p <0.001 vs MI/R.

5.1.5 Effect of KYP 2047 on nitrosative stress and DNA damage

Nitrosative stress results associated with cardiovascular disease [146] and protein tyrosine nitration increases after myocardial ischemia-reperfusion injury [147]. Therefore, nitro tyrosine content was evaluated by immunohistochemistry and data showed an increased nitrotyrosine content (Fig. 5B, see percentage of total tissue area Fig. 5E) compared with

sham group (Fig. 5A, see percentage of total tissue area Fig. 5E). On the contrary, the pre-treatment with KYP 2047 reduced the nitrotyrosine positive staining (Fig. 5C-D, see percentage of total tissue area Fig. 5E).

Furthermore, western blot analysis showed a basal expression of IκBα in sham group, while in MI/R group the expression was decreased significantly, due to the increased IκBα cytosolic degradation (Fig. 5F, F2). At the same time, nuclear NF-κB levels increased significantly in MI/R group compared to sham group (Fig. 5G). The treatment with KYP 2047, at both doses, significantly restored IκBα and NF-κB expression (respectively Fig 5F, F2 and 5G). The inhibition of nuclear translocation of NF-κB by IκBα phosphorylation blockade could attenuate I/R injury [148]. Considering IL-18 and NF-κB-responsive proinflammatory cytokine, its expression was evaluated by western blot analysis and data showed increased IL-18 levels in MI/R group compared to sham group; treatment with KYP-2047 at both doses significantly reduced IL-18 levels (Fig 5F, F1).

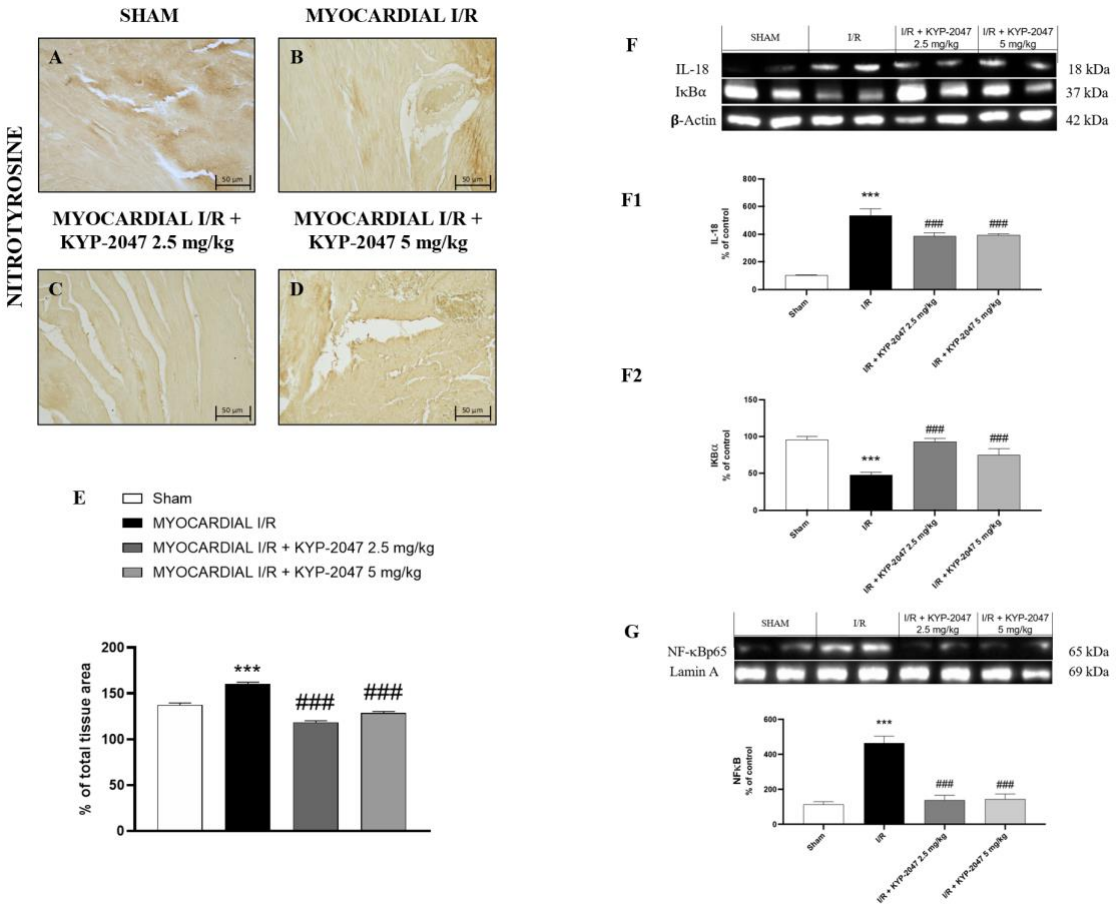

Figure 5. Effect of KYP-2047 on nitrosative stress and DNA damage. Immunohistochemistry for nitrotyrosine. Sham group (A), MI/R group (B), MI/R + KYP-2047 2.5 mg/kg group (C), MI/R + KYP-2047 5 mg/kg group (D), see percentage of total tissue area (E). Images were shown at 20× magnification (50μm scale bar). Western blot and relative densitometric analysis of IL-18 (F, F1) IκB-α (F, F2), and NF-κB (G). Data are expressed as mean ± SD of N = 8 mice/group. $^{***}p$ <0.001 vs sham; $^{###}p$ <0.001 vs MI/R.

5.1.6 Effect of KYP 2047 on apoptosis

Apoptosis represents a pathologic feature in myocardial ischemia [149], for these reason TUNEL assay was performed to evaluate the effect of KYP-2047 on apoptosis. Data showed an intense upregulation in apoptotic cells in MI/R group (Fig. 6B,B1, see positive

cells Fig. 6E), as compared to sham group (Fig. 6A,A1, see positive cells Fig. 6E), whereas KYP-2047, at both doses of 2.5 mg/kg and 5 mg/kg, showed a reduction in apoptosis process (respectively Fig. 6C, C1 and Fig. 6D, D1, see positive cells Fig. 6E).

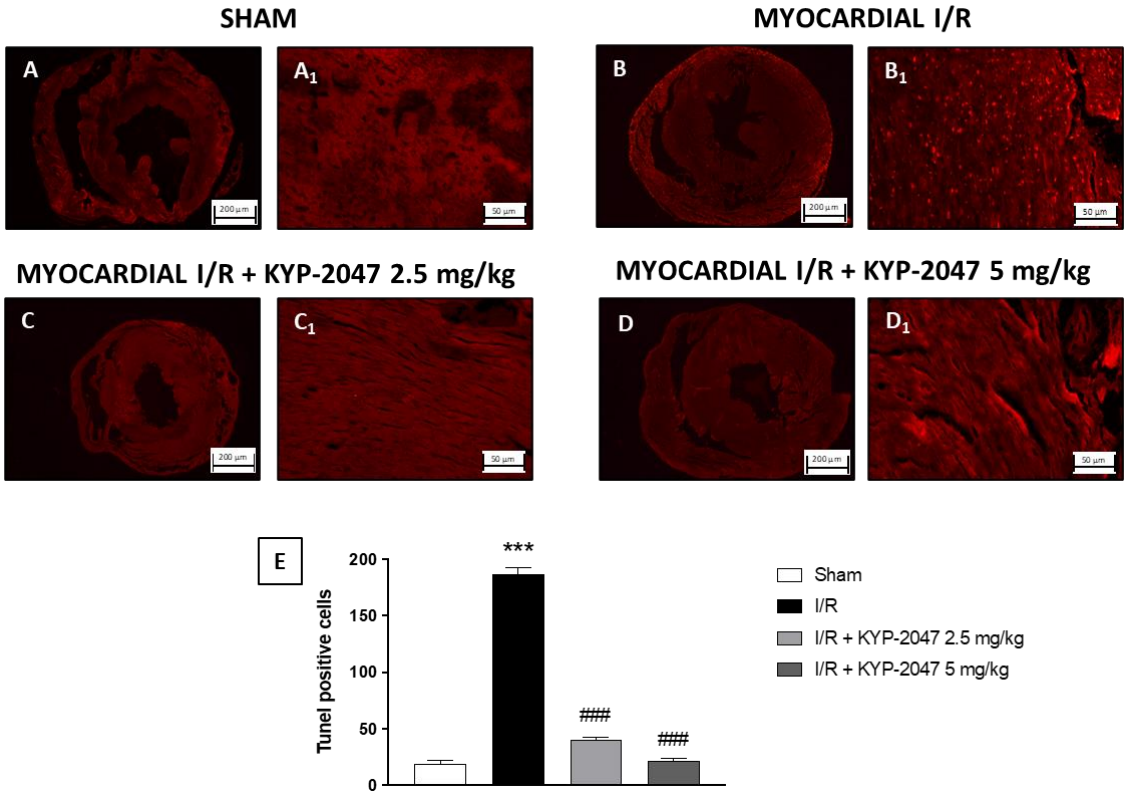

Figure 6. Effect of KYP 2047 on apoptosis. Sham (A, A1), MI/R (B, B1), MI/R + KYP-2047 2.5 mg/kg (C, C1), MI/R + KYP-2047 5 mg/kg (D, D1). See TUNEL positive cells (E). Images were shown at 2,5x (A-D) and 20x (A1–D1) magnification (respectively 200µm and 50µm scale bar). Data are expressed as mean ± SD of N = 8 mice/group. $^{***}p$ <0.001 vs sham; $^{###}p$ <0.001 vs MI/R.

5.1.7 Role of MAPK in MI/R

Myocardial ischemia activates numerous families of protein kinases, including MAP kinases, ERK 1/2, JNK 1/2 and p38 MAPKα/β [150]. The activity of p38 is regulated by

phosphorylation at the end of the cascade composed of MAPK kinases (MKK) and MKK kinases (MEKK) [151]. Western blot analysis showed that pre-treatment with KYP-2047 was able to reduce p-38-MAPK expression compared to I/R damage (Fig. 7A). ERK 1/2 belongs to the family of serine-threonine kinases known as mitogen-activated protein kinases (MAPKs). 30-31 Western blot analysis showed that p-ERK levels appeared to be reduced in the I/R group compared to sham group, while pre-treatment with KYP-2047 increased its expression (Fig. 7B), suggesting its cardioprotective role, however in the literature the role of ERK1/2 in the I/R injury is controversial [152].

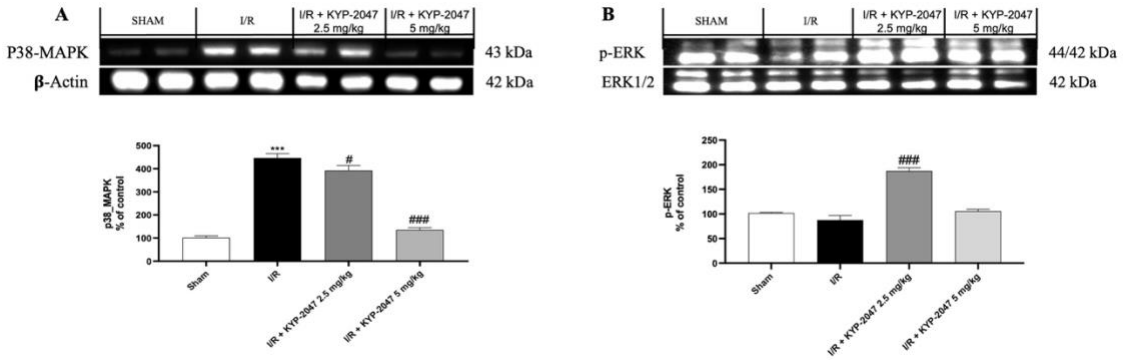

Figure 7. Role of MAPK in MI/R. Western blot and relative densitometric analysis of p38-MAPK (A) and p-ERK (B). Data are expressed as mean ± SD of N = 8 mice/group. ***p<0.001 vs sham; $^{###}p$ <0.001 vs MI/R.

5.2 Results of renal ischemia

5.2.1 The effect of KYP-207 on histological damage

AKI is characterized histologically by major reductions in glomerular filtration rate, tubular cell necrosis, glomerular injury and signs of tubular obstruction [153]. H&E staining showed a significant histological alteration in KI/R group (Fig. 1B, B1, see

tubular injury score Fig. 1F), see tubular injury score 1F) compared to sham (Fig. 1A, A1, see tubular injury score Fig. 1F). Treatments with KYP-2047 at doses of 1 and 5 mg/kg restored kidney dysfunction observed during the reperfusion (respectively Fig. 1D, D1 and E, E1, see tubular injury score Fig. 1F). On the contrary, the treatment with KYP-2047, at the lowest dose of 0.5 mg/kg, did not significantly improve the histological tubular alteration (Fig. 1C, C1, see tubular injury score Fig. 1F) and for this reason, the study was continued only with the higher doses that appeared protective.

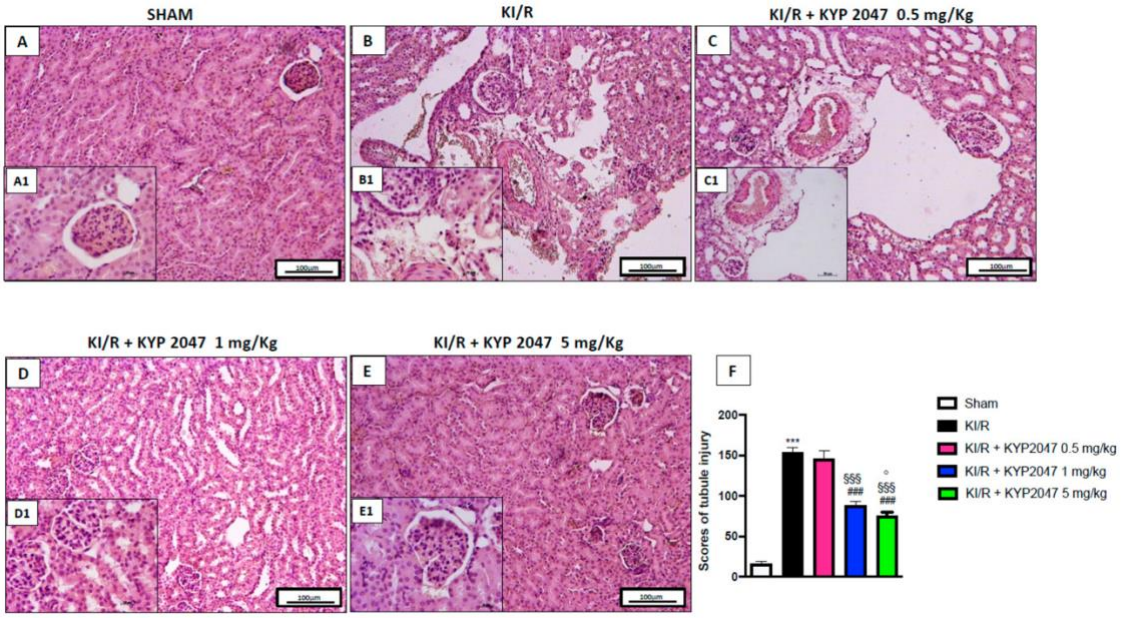

Figure 1. Role of KYP-2047 treatment on histological damage induced by KI/R. H&E staining. Sham (A, A1), KI/R (B, B1), KI/R + KYP-2047 0.5 mg/kg (C, C1), KI/R + KYP-2047 1 mg/kg (D, D1), KI/R + KYP-2047 5 mg/kg (E, E1), tubular injury score F. Images were shown at 10× (A-E) and 40x (A1-E1) magnification (respectively 100µm and 20µm scale bar). Data represent the means of at least three independent experiments. One-way ANOVA followed by Bonferroni post-hoc. *** $p < 0.001$ vs Sham; ### $p <$

0.001 vs KI/R; §§§ $p < 0.001$ vs KI/R+ KYP2047 0.5 mg/kg; ° $p < 0.05$ vs KI/R+ KYP-2047 1 mg/kg.

5.2.2 The effects of KYP2047 on KI/R dysfunction and renal markers

KI/R causes a loss of brush border, degeneration of tubular epithelial cells from the basement membrane and necrosis of tubular cell. PAS staining was performed to emphasize renal injury consisting of tubular atrophy, blebbing tubular structures and irregular tubular cytoplasm [154]. Results showed the results a significant tubular atrophy in KI/R group (Fig. 2B, see PAS-positive area Fig. 2E) compared to sham group (Fig. 2A, see PAS-positive area Fig. 2E). KYP-2047, at both doses of 1 and 5 mg/kg, significantly lowered the percentage (%) of PAS-positive area (respectively Fig. 2C and D, see PAS-positive area Fig. 2E).

Traditional markers of AKI, such as creatinine (Cr) and blood urea nitrogen (BUN) can be evaluated for a possible altered balance [155]. Data showed that levels of Cr and BUN were increased in KI/R group compared to sham group, while KYP-2047, at both doses of 1 and 5 mg/kg, significantly reduced both levels (respectively Fig. 2F and G).

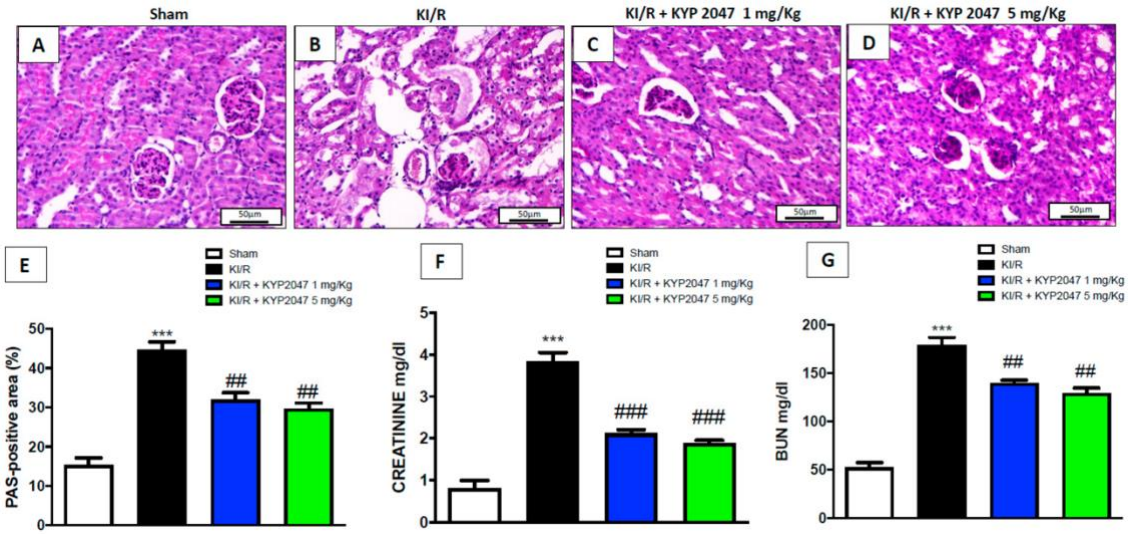

Figure 2. The Effects of KYP-2047 on KI/R dysfunction and renal markers. PAS staining of Sham (A), KI/R (B), KI/R + KYP-2047 1 mg/kg (C), KI/R + KYP-2047 5 mg/kg (D), % PAS-positive area (F). Images were shown at 20× magnification (50μm scale bar). Serum evaluation of creatinine (Cr) and blood urea nitrogen (BUN) expressed as mg/dl (respectively F and G). Data represent the means of at least three independent experiments. One-way ANOVA followed by Bonferroni post-hoc. *** $p < 0.001$ vs Sham; ### $p < 0.001$ and ## $p < 0.01$ vs KI/R.

5.2.3 The effects of KYP2047 on fibrosis

KI/R is characterized by a constantly declining of GFR, associated to a progressive renal fibrosis [156]. The proliferation of fibroblasts promotes the production and secretion of pro-collagen I, a fundamental precursor of collagen [157]. Masson's trichrome staining was performed to evaluate fibrosis grade and in addition, an ELISA kit for the pro-collagen I content was used to confirm the results. Data showed a significant increase in collagen content in KI/R group (Fig. 3B see fibrosis score Fig. 3E) compared to sham group (Fig. 3A see fibrosis score Fig. 3E). Treatments with KYP-2047, at both doses of 1 and 5 mg/kg, significantly reduced fibrosis state and the amount of collagen (respectively, Fig. 3C and D, see fibrosis score Fig. 3E). ELISA kit confirmed the results, showing an important increase of pro-collagen I quantity in KI/R group compared to sham group and a significant decrease after treatments with KYP-2047 at both doses of 1 and 5 mg/kg (Fig. 3F).

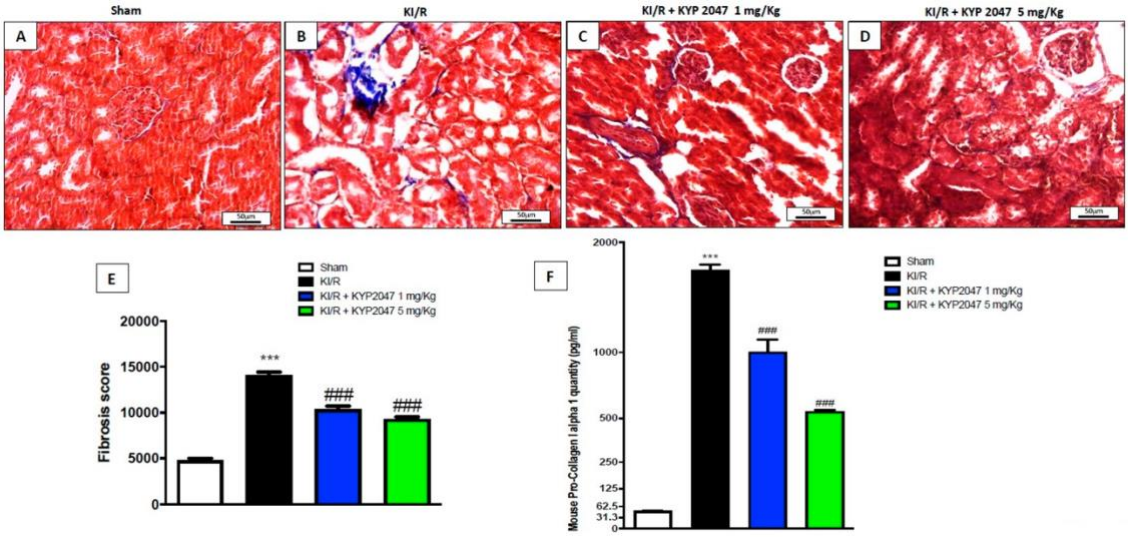

Figure 3. Effect of KYP-2047 on fibrosis. Masson's trichrome staining of Sham group (A), KI/R group (B), KI/R + KYP-2047 1 mg/kg group (C), KI/R + KYP-2047 5 mg/kg group (D), see fibrosis score (E). Images were shown at 20× magnification (50μm scale bar). Pro-collagen quantity expressed as pg/mL (F). Data represent the means of at least three independent experiments. One-way ANOVA followed by Bonferroni post-hoc. *** $p < 0.001$ vs Sham; ### $p < 0.001$ vs KI/R.

5.2.4 Effect of KYP-2047 on inflammatory state

Several kidney diseases caused by ischemia are characterized by an inflammatory state due to the damage released by damaged necrotic cells [158]. It has been shown that in renal damage, the inflammatory state is mediated by crosstalk between the canonical and non-canonical pathways of NF-κB [159]. The canonical NF-κB pathway, mediated by the kinase complex containing IKKα, IKKβ and IKKγ, which phosphorylates IκB bound to NF-κB dimers, leading to the degradation of IκB. In addition, the NIK kinase, which induces NF-κB, plays a role in phosphorylation through the activation of the IKKα [160]. Western blot analysis highlighted the anti-inflammatory activity of KYP-2047 through

the reduction of NF-κB and NIK expressions, compared to KI/R group (respectively Fig.4 B and C). Instead, treatments with KYP-2047 significantly prevented IκBα cytosolic degradation (Fig. 4A). Ischemia is associated with the induction of the pro-inflammatory enzymes, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) [161]. As expected, showed an important increase of COX-2 and iNOS expression in KI/R group, compared to sham group, while the treatment with KYP-2047 significantly reduced the inflammatory enzyme protein levels at both doses of 1 and 5 mg/kg (respectively Fig. 4D and E).

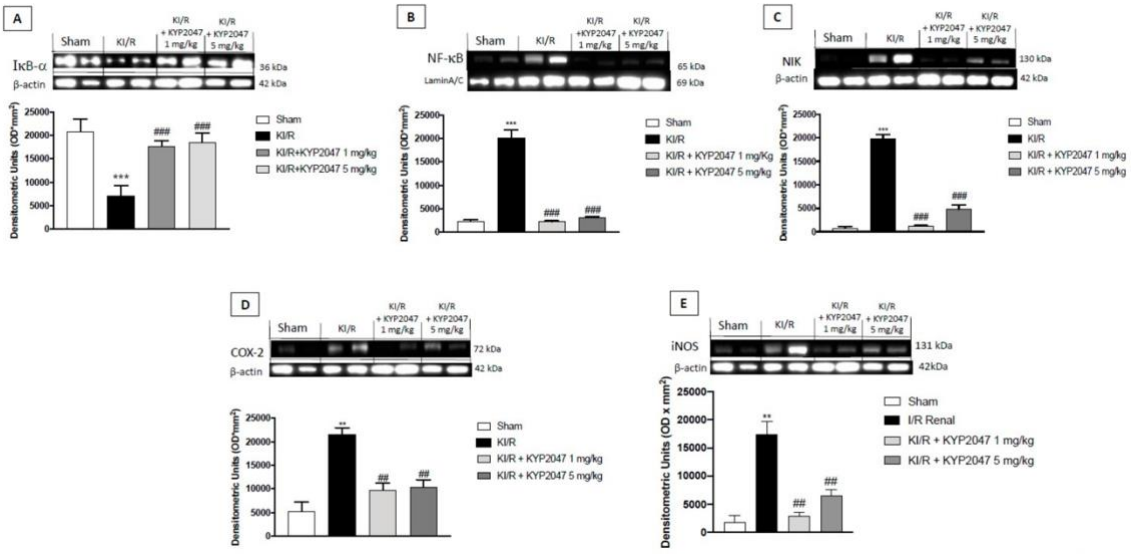

Figure 4. Effect of KYP-2047 on inflammatory state. Western blot and relative densitometric analysis of IκB-α (A) NF-κB (B), NIK (C), COX-2 (D) and iNOS (F). Data represent the means of at least three independent experiments. One-way ANOVA followed by Bonferroni post-hoc. *** $p < 0.001$, ** $p < 0.01$ vs Sham; ### $p < 0.001$, ## $p < 0.01$ vs KI/R.

5.2.5 The Effects of KYP-2047 on Inflammatory Mediators

Ischemia-reperfusion injury leads to an acute inflammatory state characterized by the deleterious role of mast cells and in case of persistent inflammation increases the risk of developing chronic kidney disease [162].

Blu toluidine staining showed a significant increase of mast cells degranulation in KI/R group (Fig. 5B, see mast cell density Fig. 5E) compared to sham group (Fig. 5A, see mast cell density Fig. 5E). KYP-2047, at both doses of 1 and 5 mg/kg, significantly reduced mast cell activation (respectively Fig. 5C and D, see mast cell density Fig. 5E).

Pro-inflammatory cytokines, such as tumor necrosis factor α (TNF- α) and IL-6 result increase after ischemia-reperfusion injury [163]. The Real Time-PCR showed a significant increase in renal TNF-α and IL-6 mRNA expression in KI/R group compared to sham group, while treatment with KYP-2047 considerably decreased their expression (respectively Fig. 5F and G). Western blot analysis confirmed the modulation of pro-inflammatory cytokines observed with RT-PCR; indeed, KYP-2047 significantly decreased TNF-α and Il-6 protein levels compared to KI/R group (respectively, Fig. 5H and I).

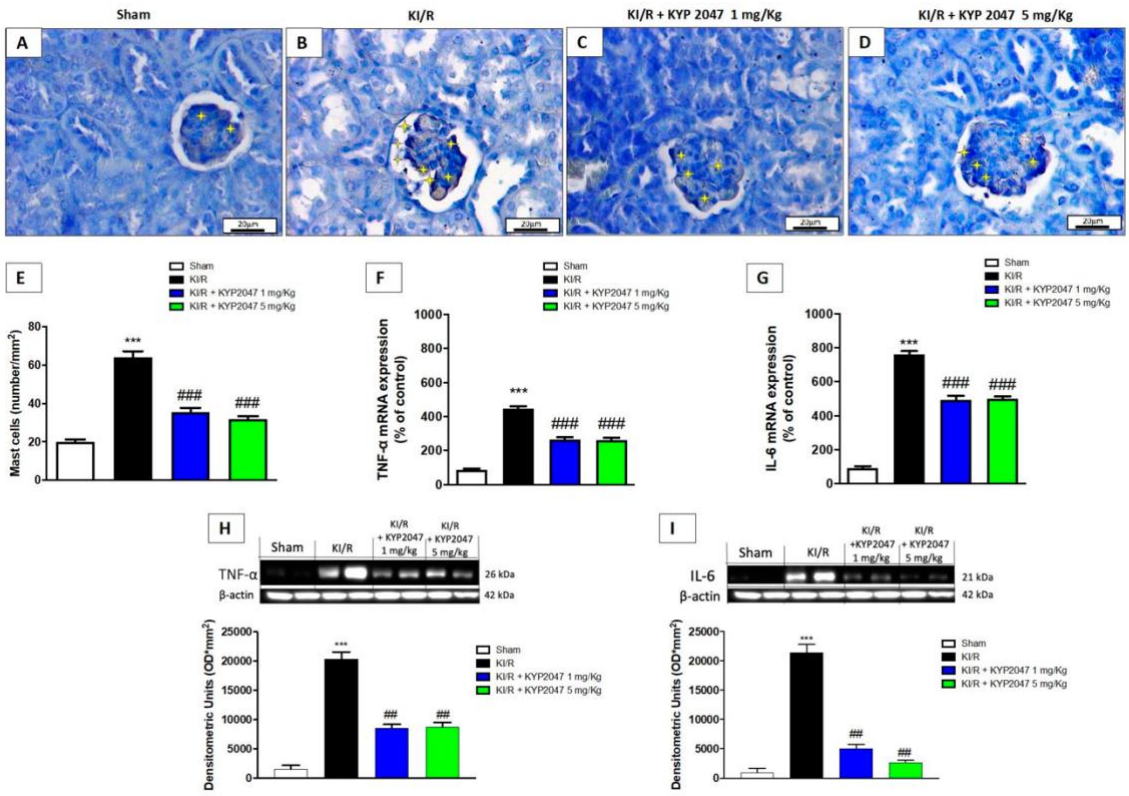

Figure 5. The Effects of KYP-2047 on Inflammatory Mediators. Mast cells degranulation by toluidine blue staining: Sham group (A), KI/R group (B), KI/R + KYP-2047 1 mg/kg group (C), KI/R + KYP-2047 5 mg/kg group (D), mast cell graph (number/mm^2) (E). Images were shown at 40x magnification (20µm scale bar) and the yellow stars represented the mast cells. RT-PCR for the evaluation of mast cell–derived TNF-α (F) and IL-6 (G). Western blot and relative densitometric analysis of TNF-α (H) and IL-6 (I). Data represent the means of at least three independent experiments. One-way ANOVA followed by Bonferroni post-hoc. *** $p < 0.001$ vs Sham, ## $p < 0.01$ and ### $p < 0.001$ vs KI/R.

5.2.6 Effects of KYP-2047 on angiogenesis

Early stages of I/R injury appear to be associated with an antiangiogenic response, while the consequent hypoxia may activate angiogenic factors, such as TGF-β and VEGF; in

particular, angiogenesis seems to be involved in the restoration of ischemic damage [9]. Immunohistochemical analysis demonstrated a positive staining for both TGF-β and VEGF markers in KI/R group (respectively, Fig. 6B and 6G, see percentage (%) of total tissue area Fig. 6E and 6J), compared to sham group (respectively, Fig. 6A and 6F, see percentage (%) of total tissue area Fig. 6E and 6J). Treatment with KYP-2047, at both doses of 1 and 5 mg/kg, significantly reduced the positive staining for TGF-β (respectively, Fig. 6C-D, see percentage (%) of total tissue area Fig. 6E). Instead, a minor significant difference in VEGF reduction was observed with KYP-2047 at the higher dose (Fig. 6I, see percentage (%) of total tissue area Fig. 6J), compared to the effect of the lowest dose of 1 mg/kg (Fig. 6I, see percentage (%) of total tissue area Figure 6J).

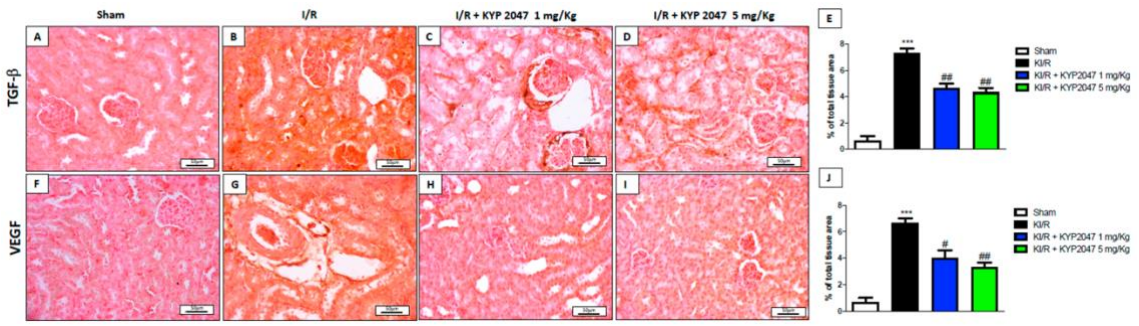

Figure 6. Effects of KYP-2047 on angiogenesis. Immunohistochemical evaluation of TGF-β: Sham group (A), KI/R group (B), KI/R + KYP-2047 1 mg/kg group (C), KI/R + KYP-2047 5 mg/kg group (D), % total tissue area (E). Immunohistochemical evaluation of VEGF: Sham group (A), KI/R group (B), KI/R + KYP-2047 1 mg/kg group (C), KI/R + KYP-2047 5 mg/kg group (D), % total tissue area (E). Images were shown at 20× magnification (50μm scale bar). Data represent the means of at least three independent experiments. One-way ANOVA followed by Bonferroni post-hoc. *** $p < 0.001$ vs Sham; ## $p < 0.01$ # $p < 0.05$ vs KI/R.

5.2.7 Effect of KYP-2047 on apoptosis

One of the mechanisms associated to KI/R is the tubular cell death by apoptosis occurs predominantly as a result of reperfusion [164]. TUNEL staining showed an increase in apoptotic cells in KI/R group (Fig. 7B, see graph of percentage (%) apoptosis Fig. 7E), compared to sham group (Fig. 7A, see graph of percentage (%) apoptosis Fig. 7E); while, the treatment with KYP2047, at both doses of 1 and 5 mg/kg, reduced the apoptotic process (respectively, Fig. 7C and D, see graph of percentage (%) apoptosis Fig. 7E). Western blot analysis confirmed this result, demonstrated a modulation in the expression of apoptotic markers, like BAD and Bcl-2 [165]. Data showed an increase of the expression of the pro-apoptotic marker Bad in KI/R group, compared to sham group, and a restore of its expression after treatment with KYP-2047 at both doses of 1 and 5 mg/kg (Fig. 7F). On the contrary, the expression of the anti-apoptotic Bcl-2 was decreased in KI/R group compared to sham group, while KYP-2047 was able to restore its expression (Fig. 7G).

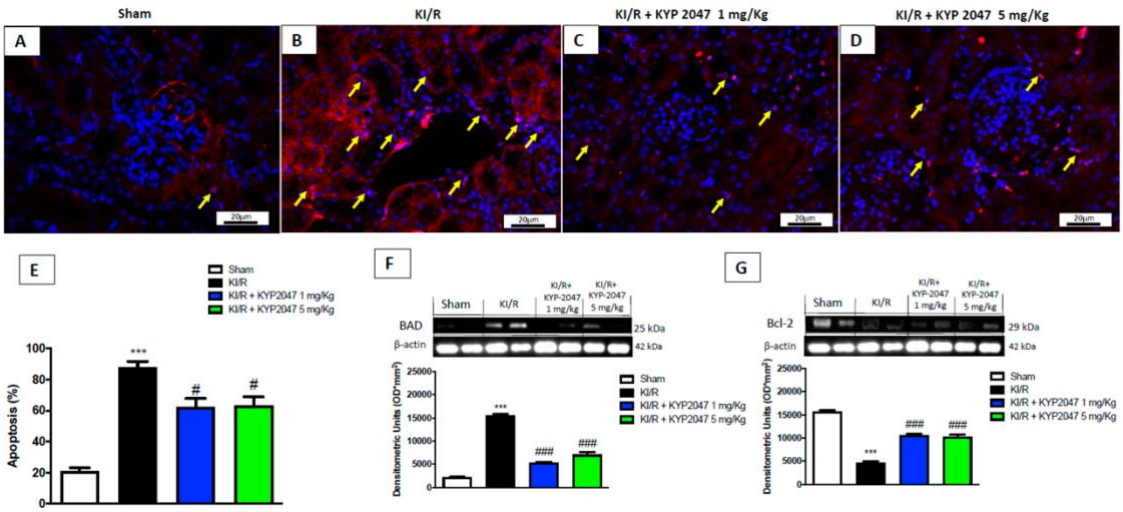

Figure 7. Effects of KYP-2047 on apoptosis. TUNEL staining: Sham group (A), KI/R group (B), KI/R + KYP-2047 1 mg/kg group (C), KI/R + KYP-2047 5 mg/kg group (D),

apoptosis (%) score (E). Images were shown at 40x magnification (20μm scale bar) and the yellow arrows represented the cells in apoptosis. Western blot and relative densitometric analysis of BAD (F) and Bcl-2 (G). Data represent the means of at least three independent experiments. One-way ANOVA followed by Bonferroni post-hoc. *** $p < 0.001$ vs Sham; ### $p < 0.001$ and # $p < 0.05$ vs KI/R.

5.2.8 Effect of KYP-2047 on the modulation of PP2A activity

Several studies demonstrated that the physiological role of PREP is to regulate PP2A and that the inhibition of PREP increases PP2A activity [166, 167]. Furthermore, PP2A acts an important role in protection against kidney inflammation [168]. To investigate whether PP2A is activated (i.e., dephosphorylated) by KYP-2047 during KI/R, the activated form of PP2A, and the inactivated form (pPP2A) were evaluated by western blot analysis. Data showed a significant increase of PP2A expression (Fig. 8A) and a significant decrease of pPP2A expression (Fig. 8B) after the treatment with KYP-2047 at both doses of 1 and 5 mg/kg. Furthermore, data showed that KYP-2047 significantly decreased the ratio of pPP2A to total PP2A compared to KI/R group (Fig. 8C).

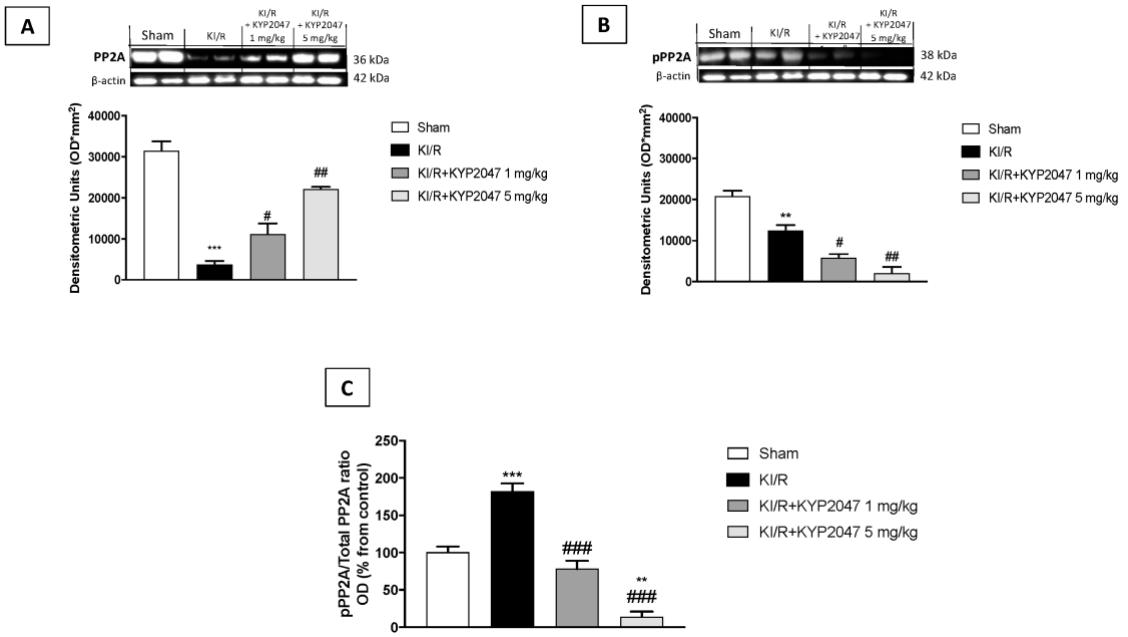

Figure 8. Effect of KYP-2047 on the modulation of PP2A activity. Western blot and relative densitometric analysis of PP2A (A), pPP2A (B) and pPP2A/PP2A ratio expressed as percentage of control (C). Data represent the means of at least three independent experiments. One-way ANOVA followed by Bonferroni post-hoc. ** $p < 0.01$, *** $p < 0.001$ vs Sham; ### $p < 0.001$, ## $p < 0.01$, and # $p < 0.05$ vs KI/R.

Chapter 6: Discussion

6. Discussion

Ischemia is a vascular disease characterized by a reduction in blood flow resulting in a lack of oxygen and nutrients in the affected organ or tissue [169]. The subsequent reperfusion phase, characterized by the restoration of blood flow, causes irreversible cellular and microvascular damage which, in addition to the initial ischemic damage, exacerbates tissue damage, initiating an inflammatory cascade with excessive production of reactive oxygen species [170]. The molecular mechanisms underlying I/R injury are complex and poorly understood, therefore, understanding these mechanisms is crucial for the development of new and effective therapeutic strategies [171].

In the heart, I/R damage is characterized by the death of cardiomyocytes due to excessive ROS production, resulting in damage to myocardial tissue [172]. MI/R can contribute to the development of adverse cardiovascular conditions, such as coronary artery disease, one of the most common cardiovascular diseases with a high mortality rate [173].

In the kidney, I/R damage involves several pathways, such as the release of neutrophils, the activation of inflammatory mediators, alterations in angiogenic processes, and stimulation of apoptosis, which contributes significantly to renal dysfunction [81]. KI/R can contribute to pathological conditions, such as acute kidney injury, a clinical syndrome with rapid kidney dysfunction and high rates of morbidity and mortality [174].

Several studies have demonstrated the involvement of PREP in the processes of inflammation and angiogenesis, obtaining pharmaceutical interest for various diseases [121, 175]. The aim of this PhD project is to investigate the effects of the most selective PREP inhibitor, KYP-2047, in the regulation of inflammation, apoptosis and angiogenesis, in *in vivo* models of myocardial ischemia/reperfusion and kidney ischemia/reperfusion. In particular, this project evaluated the preventive effects of KYP-

2047 in a mouse model of MI/R and the protective effect with KYP-2047 in a mouse model of KI/R as post-treatment.

Unfortunately, there is no effective treatment for reperfusion injury and efforts to find an efficient preventive approach are still ongoing. In the past 3 decades, there have been many successful animal studies on how to prevent reperfusion injury; nonetheless, translation to the clinical setting has been not always satisfactory. In this study, pretreatment with KYP-2047 in the context of MI/R significantly reduced the severity and degree of cell necrosis, improving the architectural state of the myocardial tissue which is more compact and better organized than damaged myocardial tissue rich in neutrophilic infiltration [176]. Similarly, treatment with KYP-2047 restored renal function and reduced histological alteration after KI/R, such as endothelial injury, local edema and arteriolar vasoconstriction. AKI is in fact characterized by rapid renal dysfunction [177] as confirmed by the results that showed an increase in serum creatinine and urea levels, diagnostic markers of AKI, following KI/R damage and these levels were then restored by treatment with KYP-2047.

At the basis of the disease process of I/R injury there are several signaling pathways in addition to an obvious cellular lesion, such as fibrosis [27]. Masson's trichrome and pro-collagen type I deposition have shown a greater presence of fibrotic tissue and increased collagen content following I/R damage, both in myocardial and renal tissue. Pretreatment with KYP-2047 did not exert any protective effect on myocardial tissue, on the contrary, treatment with KYP-2047 reduced renal fibrosis, bringing collagen production back to a physiological level, highlighting a possible involvement of PREP in the fibrotic process.

Several studies demonstrated the involvement of MCs in the pathophysiology of I/R injury, in particular in the heart and kidney [178-180], highlighting how MC depletion leads to an improvement in cardiac or renal function. In fact, MCs exert a deleterious

effect by acting already in the early stages of I/R injury, through the release of chymase and tryptase and degranulation which, causes the release of inflammatory cytokines such as IL-6, TNF-α [181, 182]. The inhibition of PREP, by KYP-2047, significantly reduced the degranulation of MCs both in the cardiac and renal tissue and in KI/R it also reduced the production of the pro-inflammatory cytokines; however, pre-treatment with KYP-2047 was unable to reduce tryptase release into myocardial tissue.

In response to ischemic damage, start a process of neoangiogenesis, a formation of new blood vessels to re-establish microcirculation and preserve tissue perfusion, which is essential for organ function [183, 184]. Among the various angiogenic factors, VEGF plays a key role in promoting the growth of blood vessels [185], as confirmed by immunohistochemistry which showed an increase in VEGF levels after MI/R and KI/R injury. KYP-2047 reduced VEGF levels in both cardiac and renal tissue and was also able to decrease the levels of two other factors involved in angiogenesis [186, 187]: CD34 in myocardial tissue and TGFβ-1 in renal tissue. As a result, treatment with KYP-2047 may be able to reduce cascade events associated with high expression of these mediators.

Another consequence of I/R damage is nitrosative and oxidative stress and high levels of ROS and RNS can damage DNA, leading to cell death [188-191]. One of the main features of nitrosative stress is tyrosine nitration, which causes cell membrane damage, DNA strand breaks, and activation of cascade signal responses that lead to cell death [192]. Interestingly, recent work revealed a possible mechanism by which comorbidities such as prediabetes, hyperglycemia, and metabolic syndrome may abrogate cardioprotective interventions due to increased NADPH oxidase-induced ROS and increased iNOS-induced NO [193]. Therefore, decreasing nitration through inhibition of iNOS or NOX activity may help to restore the cardiac intrinsic pathways of protection. In this study, in MI/R, pre-treatment with KYP-2047 reduced nitrotyrosine levels, which

plays a role in the tyrosine nitration process, demonstrating the preventive effect of KYP-2047 against nitrosative stress and therefore consequent cell death, suggesting a translating preclinical cardio-protection

Necrosis induced by oxidative stress is promoted by NF-κB which also regulates the expression of several genes involved in the inflammatory response during I/R damage [194]; therefore, its inhibition could exert a protective effect [195]. In this thesis, the ability of KYP-2047 to counteract the inflammatory process in both MI/R and KI/R was demonstrated through the modulation of the canonical NF-κB pathway mediated by IκBα. In KI/R, the ability of KYP-2047 to modulate the non-canonical NF-κB pathway through a reduction of NIK has also been demonstrated [196]. The anti-inflammatory action of KYP-2047 was confirmed by the reduction of inflammatory mediators, such as IL-18 in MI/R and the two inflammatory enzymes, iNOS and COX2 in KI/R [197, 198].

NF-κB in addition to releasing pro-inflammatory factors, also promotes apoptosis [199] and, although programmed cell death is necessary, its dysregulation can cause kidney and cardiac atrophy and dysfunction [200, 201]. These results confirmed a high presence of apoptotic cells in MI/R and KI/R injury and also demonstrated the ability of KYP-2047 to reduce the apoptotic process as showed in TUNEL assay, decreasing markers, such as BAD and Bcl2.

The apoptotic process is associated with the activation of p38 MAPK, a pro-apoptotic mediator in cardiac myocytes, and with the activation of phosphorylated substrates of ERK1/2 [202, 203]. Pretreatment with KYP-2047 inhibited the activation of p38 MAPK and activated ERK1/2, exerting a protective effect towards cardiac cells. Therefore, modulation of the MAPK pathway could represent a possible mechanism of action of KYP-2047 in exerting a preventive protective effect in MI/R.

Several studies have shown that PREP regulates the action of PP2A and that the inhibition of PREP increases PP2A activity [106]. PP2A has been shown to play a key role in protecting against kidney inflammation [204, 205]. This thesis demonstrates the involvement of PP2A, highlighting the ability of KYP-2047 to modulate PP2A activity through PREP inhibition. Modulation of PP2A could represent a possible mechanism of action of the anti-inflammatory effect of KYP-2047 in KI/R.

Chapter 7: Conclusions

7. Conclusions

These results demonstrated the protective effects of KYP-2047 in I/R injury. In particular, the protective effects of the pretreatment in MI/R injury could be related to the modulation of NF-κB and MAPK pathways; although this research primarily sought to explain the mechanism by which PREP is inhibited in IRI, it paves the way for future pharmacological-based approaches to MI/R prevention.

Instead, in KI/R context, the protective effects of the treatment could be associated to the activation of PP2A, mediated by PREP inhibition. Additionally, this study, focused on KYP-2047, as a novel compound for which the on-target IR mechanisms are clearly defined to prevent the IR-induced nitrosative stress.

In conclusion, the results of this PhD project suggest that PREP inhibition by KYP-2047 may represent a therapeutic strategy for I/R damage.

Chapter 8: References

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