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THE EFFECTS OF SELECTIVE COX-2 INHIBITORS ON LOCAL AND REMOTE INTESTINAL ISCHEMIA/REPERFUSION INJURY IN RATS

PhD thesis

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List of Abbreviations

Bax	-	Bcl-2 associated X-protein
BCA	-	Bicinchoninic acid assay
Bcl-2	-	B cell lymphoma-2
BSA	-	bovine serum albumin
CAT	-	catalase
CPB	-	cardiopulmonary bypass
CEL	-	celecoxib
C_{max}	-	peak plasma concentration
COX-1	-	cyclooxygenase-1
COX-2	-	cyclooxygenase-2
ECM	-	extracellular matrix
ER	-	endoplasmic reticulum
GI-	-	gastrointestinal
HO-1	-	heme oxygenase-1
H_2O_2	-	hydrogen peroxide
IFN-γ	-	interferon-γ
IL -	-	interleukin
IL-1β	-	interleukin-1β
IL-6	-	interleukin-6
IL-10	-	interleukin-10
I/R	-	ischemia/reperfusion
ISC	-	ischemia
LAD	-	left anterior descending artery

LPA2R - lysophosphatidic acid receptor type 2

MAP - mean arterial blood pressure

MI - myocardial infarction

MMP - matrix metalloprotease

MMP-2 - matrix metalloprotease-2

MMP-9 - matrix metalloprotease-9

MOF - multiple organ failure

MPO - myeloperoxidase

mPTP - mitochondrial permeability transition pore

NF-κB - nuclear factor-κB

NO - nitrogen oxide

NSAID - non-steroidal anti-inflammatory drug

PBS - phosphate-buffered saline

PG - prostaglandin

PGD_2 - prostaglandin D_2

PGE_2 - prostaglandin E_2

PGI_2 - prostacyclin

PI3K - phosphoinositide 3-kinase

PPAR-γ - peroxisome proliferator-activated receptor gamma

PTX3 - pentraxin 3

RBC - red blood cell

REP - reperfusion

ROF - rofecoxib

ROS - reactive oxygen species

RONS - reactive oxygen and nitrogen species

SMA - superior mesenteric artery

SOD - superoxide dismutase

TIMP - tissue inhibitors of metalloproteinase

TLR4 - Toll-like receptor 4

TNF-α - tumor necrosis factor-α

TXA_2 - thromboxane A_2

VEH - vehicle

6-keto $PGF_{1\alpha}$ - 6-keto-prostaglandin $F_{1\alpha}$

1 Introduction

1.1 Pathomechanism of ischemia/reperfusion injury

The phenomenon of ischemia/reperfusion (I/R) injury is of pivotal importance in medicine and has been extensively studied over the last 40 years (1). I/R injury results in both functional and structural impairment of the affected tissue, with reperfusion injury serving as the primary driver of tissue damage (1). I/R injury across all tissues follows a similar pathomechanism. The interruption of the blood supply leads to ischemic injury, which rapidly damages metabolically active tissues. Paradoxically, the restoration of blood flow to ischemic tissue initiates a cascade of events that may cause additional cell injury, known as reperfusion injury (Figure 1) (2). Upon restoration of blood supply, the molecular and biochemical changes occurring during ischemia predispose tissues to free radical-mediated damage. The initial site of abnormality during ischemia has been emphasized in the cellular mitochondria, which is particularly vital for producing adenosine triphosphate for organ recovery (3).

The ischemic period results in the reduction of oxidative phosphorylation in the mitochondria and triggers the production of ATP anaerobically, leading to lactic acid accumulation during anaerobic glycolysis (4). The limited cellular ATP inactivates ATP-dependent Ca^{2+} reuptake by the endoplasmic reticulum (ER), leading to the lethal elevation of the cytoplasmic Ca^{2+} levels. For the reduction of intracellularly high Ca^{2+} levels, the mitochondrial Ca^{2+} uptake is also responsible, which leads to the opening of mitochondrial permeability transition pore (mPTP) in the inner mitochondrial membrane (4), resulting in ROS formation and further impairment of ATP supply (2, 5, 6). Excessive reactive oxygen species (ROS) production contributes to the increased expression of several antioxidant enzymes, such as catalase (CAT) and superoxide dismutase (SOD), which act as antioxidants to neutralize ROS (7).

Moreover, heme oxygenase-1 (HO-1) expression is rapidly upregulated during I/R injury due to the increase of ROS, which plays a pivotal role in maintaining antioxidant and oxidant homeostasis by exerting antioxidant functions (6). It also contributes to preserving microcirculation, has anti-apoptotic effects, and exhibits anti-inflammatory properties (8, 9).

Tissue damage triggers the production of cytokines and chemokines, which, along with ROS, promote the infiltration of leukocytes, especially neutrophil granulocytes. These cells further enhance the expression of these proteins, such as myeloperoxidase (MPO), interleukin-(IL-) 1β, IL-6, IL-10, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) (2, 10, 11).

The neutrophil infiltration is a critical component in the pathogenesis of I/R injury (4, 10, 12). Firstly, MPO, an enzyme produced by neutrophils, plays an essential role in further tissue damage in the I/R patomechanism by generating reactive substances deleterious to tissue integrity (13). On the other hand, neutrophils exacerbate tissue degradation through the release of toxic proteases, such as cathepsin G and matrix metalloproteinases (MMPs), which further compromise the structural integrity of the affected tissues (13).

MMPs are zinc-dependent neutral endopeptidases capable of degrading nearly all extracellular matrix (ECM) components. They play crucial roles in both physiological and pathological processes, including tissue remodeling, chronic degenerative disorders, cancer progression, and diabetes (14, 15). Under normal conditions, MMPs regulate angiogenesis, tissue repair, and ECM remodeling, influencing cellular functions such as survival, inflammation, and intracellular signaling (14). They also control the release and activation of cytokines, chemokines, growth factors, and other bioactive molecules (16). MMPs are categorized into membrane-type MMPs, collagenases, gelatinases, stromelysins, and matrilysins (17). Gelatinases, specifically MMP-2 and MMP-9, are involved in cellular processes such as angiogenesis and neurogenesis, and they contribute to basal lamina disruption, resulting in cell death (17). These enzymes are synthesized as inactive proforms and are activated through proteolysis, a process known as zymogen activation (14).

Tissue damage during I/R can lead to cell death. I/R-induced cell death was long believed to occur solely through necrosis, an unregulated and irreversible process. However, it has been recognized that several cells subjected to I/R die in a programmed manner via apoptosis (18). Furthermore, the level of apoptosis could reflect the severity of tissue injury (19).

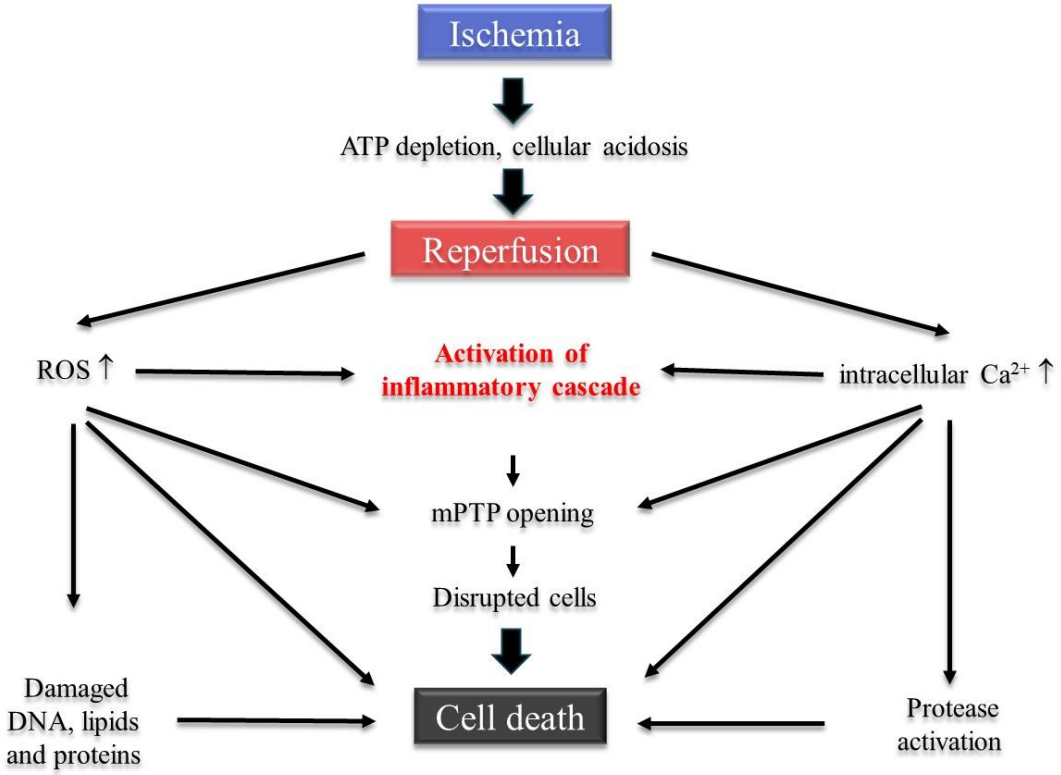


Figure 1. Pathomechanism of I/R injury.

Ischemia reduces oxidative phosphorylation in the mitochondria, triggering anaerobic ATP production and lactic acid accumulation. Limited ATP disrupts Ca^{2+} reuptake in the ER, leading to cytoplasmic Ca^{2+} overload during reperfusion. Mitochondrial Ca^{2+} uptake activates the mitochondrial permeability transition pore (mPTP), causing reactive oxygen species (ROS) formation and further ATP depletion. Tissue damage induces the release of cytokines and chemokines, promoting leukocyte infiltration—especially neutrophils—which amplify inflammation and contribute to cell death. Adapted from Kalogeris et al, 2016 (2).

Organs exhibit varying susceptibility to I/R injury, which can be tolerated differently depending on the magnitude and duration of the circulation interruption (Figure 2) (20). Additionally, it is influenced by the type of organ affected (2, 20). The lungs are particularly sensitive to I/R injury, probably due to their extensive capillary surface area, which provides a large surface area for immune cell infiltration (21, 22). The mucosal layer of the small intestine is particularly susceptible to ischemic insult, the underlying mechanisms of which will be discussed below (2).

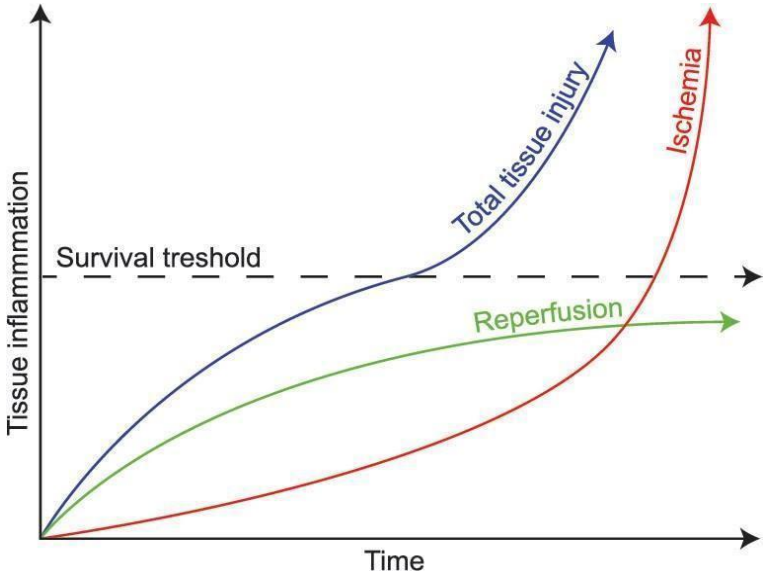


Figure 2. Association of tissue damage with duration of ischemia and reperfusion.
Total tissue injury depends on the period of ischemia and the reperfusion component. Adapted from Bulkley, 1987 (20).

1.2 Remote intestinal I/R injury

I/R injury is a complex pathological process that not only affects the initially ischemic organ but can also lead to damage in distant organs (23). During reperfusion, ROS, inflammatory proteins, cytokines, and chemokines are released from the affected organ into the circulation. These circulating mediators may then reach distant organs. Upon arrival, they can activate molecular pathways that initiate inflammatory responses similar to those observed in the originally injured tissue (12). Immune cells released into the circulation and locally activated can further exacerbate damage to distant organs (12).

It is important to emphasize the impact of I/R injury on remote organs and its critical association with patient survival. I/R injury of the kidney has been implicated in damage to various distant organs, including the lungs and liver, through the induction of systemic inflammation and hemodynamic alterations (21, 22). During liver surgery, I/R-induced hepatic injury has been observed to contribute to dysfunction in remote organs (24). Hepatic I/R injury can lead to severe pulmonary complications that significantly affect postoperative mortality, particularly following liver transplantation (24). Lung injury, driven by systemic inflammation, oxidative stress, and endotoxin translocation, contributes to acute respiratory distress syndrome, prolonging the need for ventilatory support and increasing the risk of death (25). Hepatic I/R

injury substantially raises the risk of acute kidney injury, which occurs in up to 95% of liver transplant cases, leading to elevated postoperative complications and mortality (26).

The intestinal mucosa is highly susceptible to the effects of I/R injury (27, 28). First of all, the intestinal mucosa has a high need for oxygen due to its high metabolic rate, which is strongly damaged by the abolishment of blood supply (2). The increased sensitivity of enterocytes located at the tips of the villi is due to their position at the terminal end of the central arteriole's distribution and the relative insufficiency of collateral blood flow, which together result in lower partial oxygen pressure in distal enterocytes compared to those residing in the cripts (28, 29). The resulting mucosal damage increases permeability and facilitates bacterial translocation *in vivo* and humans (23, 30). This, along with the production of large amounts of proinflammatory compounds and free radicals, may lead to damage in distant organs, potentially resulting in systemic inflammation and MOF in humans, resulting in increased morbidity and mortality (23, 31, 32).

Therefore, the impact of I/R injury on the small intestine, as a distant organ, has been investigated regarding I/R-induced damage in various organs. The effect of lower limb I/R on the small intestine has been studied in several animal models, where it was found that histopathological changes in the gut, along with increased permeability, were observed as early as two hours after limb reperfusion (33-35). Park *et al.* found that renal I/R injury contributed not only to liver injury but also to severe small intestinal damage, characterized by increased vascular damage and leukocyte infiltration (36). Moreover, experimental models confirm that liver I/R disrupts intestinal integrity, increasing permeability and the translocation of endotoxins, while clinical studies highlight its role in overall survival (37).

Among the organs susceptible to I/R injury, the heart is particularly vulnerable (38). Recanalization of the occluded vessel is vital for survival; however, the restoration of perfusion induces I/R injury in the myocardium, thereby exacerbating tissue damage (38). Myocardial ischemia (or infarction, MI) has a high global prevalence and remains a leading cause of mortality worldwide. Therefore, it seems to be a logical approach to investigate the impact of myocardial I/R injury on remote organ damage. Nevertheless, limited literature data are available regarding the effects of myocardial I/R on the small intestine, even though disruption of the intestinal mucosal barrier may contribute to increased mortality by promoting systemic injury.

Cardiopulmonary bypass (CPB) is a life-saving procedure used during open-heart surgery to temporarily take over the function of the heart and lungs. It makes it possible to perform operations on the heart without blood flow. After finishing the operation, circulation of the heart

is re-established, which is equivalent to cardiac ischemia/reperfusion. CPB-induced abdominal complications are relatively rare (0,5-1,1%), although 59-67% belong to acute mesenteric ischemia (AMI), which has a high mortality rate due to the late diagnosis and treatment (39-42). Furthermore, acute heart failure, where the revascularisation of coronary arteries means myocardial I/R injury, can lead to hypovolemia, which diminishes blood flow to abdominal organs, potentially resulting in intestinal damage (43). Investigating intestinal impairment during cardiac I/R injury is vital for understanding the pathomechanism and developing strategies to reduce damage. In an animal model of MI, intestinal barrier impairment was noted 17 days after reperfusion (44). Similarly, increased intestinal permeability was observed in patients with ST-segment elevation MI undergoing primary percutaneous coronary intervention, persisting for up to 7 days. Plasma levels of gut-derived bacterial products correlated with systemic inflammation and predicted adverse cardiovascular events, suggesting a critical role for intestinal damage in post-MI outcomes (45).

Early detection of remote intestinal injury after cardiac I/R may provide prognostic insights and guide interventions aimed at preserving intestinal integrity, potentially reducing systemic inflammation and improving outcomes. Therefore, the first experiment examined the early effects of myocardial I/R on the small intestine.

1.3 Mesenteric I/R injury

Mesenteric I/R injury is a critical condition characterized by the restoration of blood supply to the intestines following a period of ischemia, resulting in significant tissue damage. Acute mesenteric I/R injury can be subdivided into three categories (42):

- Occlusive arterial mesenteric ischemia: This is the most prevalent form, accounting for approximately 68.6% of cases, commonly caused by embolism or thrombosis.
- Non-occlusive mesenteric ischemia: Constituting roughly 15.1% of cases, this type is frequently associated with low blood flow conditions.
- Mesenteric venous thrombosis: Represents about 11.5% of cases.
- Other forms of acute mesenteric ischemia: less than 5%, which was not detailed due to the lack of information from the examined studies.

The prevalence of acute mesenteric ischemia approximates 0.1%, with the risk increasing with advancing age (46). Despite its low prevalence, the high mortality rate, which ranges from 24-52%, is contingent on its etiology, as well as the patient's age and overall health status (42). Early diagnosis remains challenging due to the atypical presentation of symptoms.

Non-occlusive mesenteric ischemia is typically induced by low splanchic blood flow, induced by hypovolemia, septic shock, cardiac insufficiency, or even intestinal transplantation or chronic atherosclerosis, leading progressively to stenosis (23, 43). Restoring mesenteric blood flow is essential for the survival of ischemic intestinal tissue. However, the reperfusion phase often leads to a worsening of injury, involving molecular mechanisms similar to those seen in reperfusion injury of other organs (23, 47). These include oxidative stress, inflammation, and microvascular dysfunction (23).

Due to the high mortality rate associated with intestinal I/R (42), various therapeutic approaches are currently under investigation. The primary treatment modalities for intestinal I/R include interventional radiological revascularization and surgical intervention (48-50). However, no pharmacological agents are presently available to mitigate the tissue damage induced by reperfusion. Moreover, in surgical procedures involving substantial hemodynamic alterations, such as cardiopulmonary bypass or organ transplantation, the administration of a pharmacological agent capable of reducing potential reperfusion injury would be highly beneficial. Consequently, our research has focused on elucidating novel therapeutic strategies to address this critical need.

1.4 Cyclooxygenase enzymes and non-steroidal anti-inflammatory drugs

Cyclooxygenase (COX) enzyme has two isoforms (51). The COX-1 isoform is constitutively expressed in various tissues and is involved in maintaining homeostatic functions (51). While the COX-2 isoform is minimally expressed or absent in most healthy tissues, but can be significantly upregulated during inflammatory conditions, including I/R injury (51-56). COX enzymes catalyze the conversion of arachidonic acid into prostaglandins (PGs), particularly PGE_2 and PGD_2, which are essential for maintaining normal physiological functions (51).

COX-1-derived PGs are crucial for maintaining the integrity of the gastric mucosa. They stimulate mucus and bicarbonate secretion, creating a protective barrier against gastric acid, preventing ulcer formation, and maintaining gastric mucosa and microcirculation (52). In the kidneys, COX-1-derived PGs play a vital role in regulating renal hemodynamics and the glomerular filtration rate. PGE_2 and prostacyclin (PGI_2) promote vasodilation of afferent arterioles, counterbalancing the effects of vasoconstrictive agents such as angiotensin II, thereby ensuring adequate blood flow to the kidneys, supporting proper filtration processes, and increasing natrium and water excretion (57, 58). Although COX-1 was initially believed to be

the sole physiologically expressed COX isoform in the kidneys, recent studies have demonstrated that COX-2 is also constitutively expressed in specific renal locations and is highly regulated in response to changes in intravascular volume (59, 60).

COX-1 plays a pivotal role in platelet function through the production of thromboxane A_2 (TXA_2), which promotes platelet aggregation and vasoconstriction (61, 62). In contrast, COX-2-derived PGI_2 facilitates vasodilation by enhancing nitric oxide (NO) production and inhibiting platelet aggregation (61, 62). The balance between COX-1 and COX-2 activities is essential for maintaining vascular physiological functions and regulating hemostasis (61, 62).

Agents capable of inhibiting COX enzymes were first identified by Vane in 1971 and are known as non-steroidal anti-inflammatory drugs (NSAIDs) (63). These anti-inflammatory compounds block the biosynthesis of PGs, which are involved in various physiological and pathophysiological processes (63). COX-2-derived PGs are responsible for initiating inflammation, vasodilation, edema, hyperalgesia, and fever (64). NSAIDs are among the most widely used medications to alleviate or prevent these symptoms (Figure 3) (65).

Based on the isoenzyme inhibited by the drug, NSAIDs can be categorized into three types:

1. non-selective COX inhibitors
2. selective COX-2 inhibitors
3. selective COX-1 inhibitors

NSAIDs are effective for the short-term management of acute pain, inflammation, and fever. In contrast, long-term use is appropriate for the treatment of chronic conditions such as arthritis and musculoskeletal disorders, which are increasingly prevalent in aging populations (65). Over many years, it has been discovered that long-term administration of non-selective COX inhibitors is associated with several side effects, of which GI side effects are of particular importance. Firstly, NSAIDs have topical irritative effects on the gastric mucosa (66). Secondly, depletion of PGs, produced by COX-1, increases the risk of developing GI ulcers and bleeding (67). Namely, PGs have gastroprotective effects, helping in gastric mucosal regeneration, maintenance of gastric microcirculation, and maintaining balance between healthy acid and bicarbonate production (66).

Consequently, significant efforts have been dedicated to developing anti-inflammatory agents that selectively inhibit COX-2, which are anticipated to minimize adverse effects. Numerous studies have demonstrated that selective COX-2 inhibitors, known as coxibs, resulted in reduced gastric injury compared to non-selective NSAIDs (68-70).

Despite the initial optimism surrounding the gastroprotective properties of COX-2 inhibitors, the introduction of these agents to the market from 2000 to 2008 corresponded with

an increased incidence of cardiovascular events among users (62, 71). Specifically, the administration of selective COX-2 inhibitors resulted in an altered TXA_2/PGI_2 ratio: prothrombotic TXA_2 levels remain unaltered due to its production by COX-1, while PGI_2 levels, which inhibit platelet aggregation, are reduced, thereby inducing a prothrombotic state (61). Consequently, both valdecoxib and rofecoxib, associated with a high frequency of adverse cardiovascular effects, were withdrawn from the market (68, 72-74). However, celecoxib, parecoxib, and etoricoxib continued to be utilized in clinical practice (74, 75).

Parecoxib is used primarily for the short-term management of acute postoperative pain in parenteral form, particularly in situations where oral administration is not feasible due to nausea, vomiting, or inability to swallow post-surgery (76). The treatment with parecoxib should not exceed three days due to the potentially increased cardiovascular risk of cardiovascular events (76).

Etoricoxib is approved for the symptomatic relief of pain and inflammation in various conditions, including osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, and chronic lower back pain (77). Etoricoxib is not recommended for long-term use due to its associated cardiovascular adverse effects, despite being primarily indicated for chronic conditions characterized by persistent pain. For these conditions, intermittent treatment with the lowest effective dose is advised (77).

Celecoxib is used for treating chronic conditions involving joint, muscle, and bone pain, similar to the indications for etoricoxib (78). According to literature data, celecoxib, compared to other coxibs, is associated with a lower cardiovascular risk, likely due to its lower COX-2 selectivity (78, 79).

Even though rofecoxib has been withdrawn from the market due to its high cardiovascular risk (68, 72), it remains a valuable compound in preclinical studies due to its high COX-2 selectivity. This property makes rofecoxib particularly useful for examining the pathophysiological roles of COX-2 inhibition, such as in cancer prevention research, thereby aiding the development of future therapeutic strategies (80). Thereby, several studies have aimed to explore the potential therapeutic value of selective COX-2 inhibitors in I/R injury of various organs.

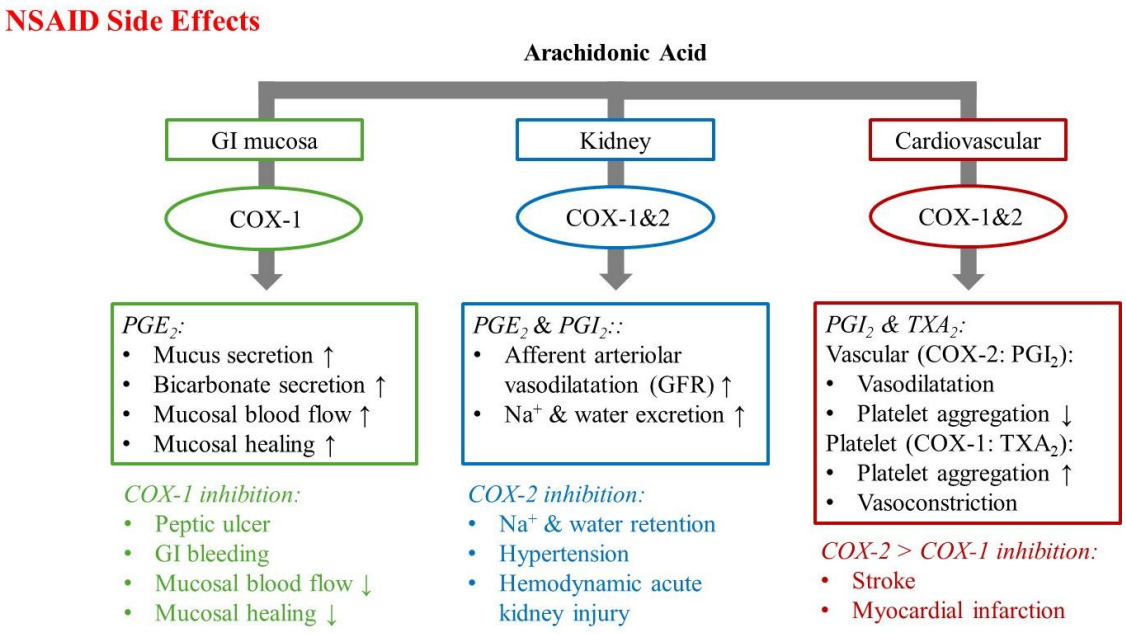


Figure 3. The major side effects of NSAIDs and their association with the inhibition of the different COX isoforms.

Cyclooxygenase (COX) -1-derived prostaglandins (PGs) are essential for maintaining gastric mucosal integrity, regulating renal hemodynamics, and supporting platelet function, promoting protective mechanisms in the stomach and kidneys. The dynamic interplay between COX-1 and COX-2 activities is essential for the proper functioning of the vascular system and the regulation of hemostasis, underscoring their physiological and pathophysiological significance in various biological processes (81).

Abbreviations: GI: gastrointestinal, PGE_2: prostaglandin E_2, PGI_2: prostacyclin, TXA_2: thromboxane A_2

1.5 Effects of selective COX-2 inhibitors on remote I/R injury

Rapid upregulation of COX-2 in response to I/R can be observed in numerous organs, including the heart (53, 82, 83), kidney (84, 85), liver (86), and intestines (55, 87). There is evidence suggesting that COX-2 inhibitors may also attenuate remote organ injury following limb or renal ischemia-reperfusion (88-90). However, existing literature presents conflicting evidence regarding the role of COX-2 enzyme expression in local I/R injury in different organs, with studies indicating both protective and detrimental effects (53, 55, 83, 91-93). Namely, COX-2 can contribute to tissue damage by inducing cytokine and chemokine production and promoting immune cell migration (55, 56, 94). COX-2 also plays a crucial role in tissue protection. COX-2-derived PGs can enhance mucosal resilience to injury and facilitate repair processes (54, 95). Additionally, PGs may help reduce epithelial apoptosis during I/R-induced intestinal damage (93, 96). The subsequent section will elucidate the impact of selective COX-2 inhibitors on I/R-induced damage in various organs.

In numerous studies examining the effects of COX-2 inhibition on cardiac I/R injury, selective COX-2 inhibitors have been found to confer protective effects (83, 92, 97-100). Saito *et al.* identified that the generation and release of PGs play a deleterious role in cardiac I/R injury by upregulating the expression of inflammatory mediators (83, 97). Experimental data indicate that selective COX-2 inhibitors can mitigate cellular apoptosis, suggesting they may reduce the extent of cell death in cardiac I/R injury (92, 98, 99). Namely, the increased production of COX-2-derived prostanoids may represent an adaptive response that confers cellular protection against I/R injury (53, 91). In contrast, other studies have demonstrated that selective COX-2 inhibitors exacerbated cardiac I/R injury (83, 92, 97, 98, 100).

Ozturk *et al.* demonstrated that celecoxib mitigated I/R injury in the liver by modulating oxidative stress and reducing antioxidant levels (86). Conversely, in multiple transgenic mouse models with hepatocyte-specific COX-2 expression, a protective effect of COX-2 against liver I/R damage was observed (101, 102). Motino *et al.* reported that elevated COX-2 expression led to decreased plasma levels of proinflammatory cytokines, attenuated apoptosis during liver I/R injury, and diminished oxidative stress (101). Furthermore, the protective role of COX-2 was linked to an upregulation of PGE_2 expression (101). Specifically, COX-2 alleviated I/R injury at the mitochondrial level by preventing proteolytic cleavage and maintaining mitochondrial respiratory function (102).

Selective COX-2 inhibitors have complex effects on renal I/R injury, as the inhibition of both COX isoenzymes can induce vasoconstriction and impair water excretion (59, 60).

Thus, selective COX-2 inhibition may produce both beneficial and adverse outcomes. On one hand, inhibiting COX-2 can attenuate the production of pro-inflammatory PGs, subsequently reducing inflammation and tissue damage during renal I/R injury (103). Furthermore, studies have demonstrated that COX-2 enzyme inhibition can mitigate oxidative stress, suppress neutrophil infiltration and activation, and decrease cell death in renal I/R injury (85). Conversely, administration of the selective COX-2 inhibitor parecoxib has been shown to exacerbate renal dysfunction after I/R injury, and COX-2 knockout mice exhibited more severe I/R-induced damage compared to their wild-type counterparts (104).

The aforementioned evidence illustrates that the selective inhibition of COX-2 can exert either protective or harmful effects on I/R injury across different organ systems. Furthermore, within an organ subjected to I/R injury, the impact of COX-2 inhibition can be multifaceted and contradictory. In addition, the therapeutic efficacy of selective COX-2 inhibition in attenuating remote organ injury secondary to I/R events in non-target organs remains inconclusive. The conflicting data suggest that the effects of COX-2 inhibitors on I/R injury are highly organ-specific and complex.

1.6 Role of selective COX-2 inhibitors in intestinal I/R injury

The early upregulation of COX-2 gene expression in instances of intestinal I/R injury is indicative of its involvement as a therapeutic target (55, 87). However, COX-2 likely has a dual role in intestinal I/R injury, similar to that in other organs.

Accordingly, the effect of selective COX-2 inhibitors on intestinal I/R injury is inconclusive. According to the results of most available studies, non-selective (56, 105) and selective COX-2 inhibitors (55, 94, 106-109) provide varying degrees of protection against I/R-associated small intestinal inflammation and tissue damage. However, while the number of publications on this topic remains limited, more specific findings have emerged.

More specifically, while the selective COX-2 inhibitors FK3311 and NS-398 were both shown to mitigate intestinal I/R injury (55, 106, 107), the protective effect of NS-398 was found to be influenced by the sex of the animals used (110). Conversely, celecoxib and firocoxib demonstrated only moderate protection in rat models (94, 108), whereas parecoxib exhibited variable results depending on the study (56, 109).

In contrast, there is substantial evidence suggesting that PGs produced by COX-2 may also possess mucoprotective properties in the context of intestinal I/R injury (87, 93). For example, research indicates that PGs synthesized by both COX-1 and COX-2 play a crucial role

in the recovery of the mucosal barrier in ischemia-compromised porcine ileum (87), and the deletion of the COX-2 gene is associated with more severe injury and heightened epithelial apoptosis following intestinal I/R in murine models (93). These studies suggest that selective COX-2 inhibitors could exacerbate intestinal I/R injury, similar to their damaging effects in gastric I/R injury. Literature data demonstrate that COX-2-derived PGs are vital for preserving gastric mucosal integrity during I/R episodes (54), and selective COX-2 inhibitors exacerbated mucosal damage and hindered the healing of gastric lesions (54, 95, 108, 109).

2 Objectives

2.1. As outlined in the Introduction, the intestine is highly susceptible to IR injuries occurring in distal organs, but little is known about the intestinal consequences of cardiac IR injury. Furthermore, existing literature on the impact of selective COX-2 inhibitors on local and remote IR injuries is conflicting. Therefore, the aim of my first study was to assess the effect of the highly selective COX-2 inhibitor rofecoxib on remote intestinal injury caused by cardiac IR. More specifically, I sought to answer the following questions:

2.1.1. Can rofecoxib treatment reduce early mucosal damage to the intestine caused by cardiac IR injury?

2.1.2. Does rofecoxib influence inflammation and oxidative stress, which are hallmarks of remote intestinal injury after cardiac I/R injury?

2.1.3. Are MMPs involved in the effects of rofecoxib on cardiac I/R-induced intestinal damage?

2.1.4. Does altered intestinal blood circulation contribute to cardiac I/R-induced intestinal damage?

2.2. Because the role of COX-2 in mesenteric I/R injury is unclear, my second study aimed to evaluate and compare the effect of two selective COX-2 inhibitors, celecoxib and rofecoxib, on mesenteric I/R-induced intestinal damage. My questions were as follows:

2.2.1. Can celecoxib and rofecoxib treatment reduce mesenteric I/R-induced intestinal inflammation?

2.2.2. Do celecoxib and rofecoxib influence mesenteric I/R-provoked intestinal mucosal damage?

2.2.3. Do the non-COX-dependent effects of celecoxib and rofecoxib explain the difference in their impact on intestinal injury following mesenteric I/R?

2.2.4. Can the different COX selectivity of celecoxib and rofecoxib explain the difference in their impact on intestinal mucosal damage caused by mesenteric I/R?

3 Methods

3.1 Animals

Experiments were carried out on male Wistar rats (Semmelweis University, Budapest, Hungary, and Toxicoop Ltd., Budapest, Hungary). Animals were housed in a temperature-controlled (22 ± 2°C) and humidity-controlled room at a 12 h light/dark cycle. Food and water were available ad libitum.

3.2 Ethical Considerations

All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments. All procedures conformed to the Directive 2010/63/EU on European Convention for protecting animals used for scientific purposes. The experiments were approved by the National Scientific Ethical Committee on Animal Experimentation and permitted by the government (Food Chain Safety and Animal Health Directorate of the Government Office for Pest County (PEI/001/1493-4/2015, PE/EA/1784-7/201 and 7PE/EA/1118-6/2020)).

3.3 Study design

3.3.1 Animal models of cardiac I/R-induced remote intestinal injury

Male Wistar rats were used to evaluate the effects of long-term rofecoxib treatment on the remote intestinal effects of myocardial I/R injury. The experimental protocol consists of two studies, whose implementation is discussed in separate subsections for the sake of clarity (111, 112).

3.3.1.1 Evaluating the impact of COX-2 inhibition on the remote intestinal effects of cardiac I/R injury

Male Wistar rats, weighing between 180-280 g, were allocated into groups of 7-11 animals each. They were administered intragastrically with either a vehicle (1% hydroxyethylcellulose) or rofecoxib (5 mg/kg) [4-(4’ -methylsulfonylphenyl)-3-phenyl-2-(5H)-furanone] (MedChem Express, Sollentuna, Sweden) in a volume of 0.33 ml/100 g once daily for 28 days. The dosage of rofecoxib was selected based on previous animal studies (110), pharmacokinetic similarities between rats and humans (113), and extrapolation from the maximum recommended daily dose (50 mg) used in clinical practice for a 60-kg individual, as per Reagan-Shaw *et al.* (114). Additionally, the efficacy and COX-2 selectivity of this rofecoxib

dose were confirmed, as it inhibited COX-2-derived PGE_2 synthesis by nearly 100% without affecting gastrointestinal PGE_2 levels produced by COX-1 (112). Noteworthy, chronic rofecoxib treatment did not increase the mortality of animals, and did not reduce the cardiac level of 6-keto prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$, the stable metabolite of PGI_2), which has been associated with rofecoxib-induced cardiotoxicity (vehicle: 40.6 ± 6.3 pg/mg tissue, rofecoxib: 49.4 ± 7.3 pg/mg tissue, n=7/group, p=0.38) (62).

On the 29th day, 24 hours after the final rofecoxib administration, all rats were anesthetized with pentobarbital (60 mg/kg intraperitoneally) and underwent thoracotomy. The rats were ventilated using a rodent ventilator (Ugo-Basile, Gemonio, Italy) with a tidal volume of 6.2 ml/kg at a rate of 69 ± 3 breaths/min according to body weight. Blood pressure was continuously monitored via the carotid artery (AD Instruments, Bella Vista, Australia), and body temperature was maintained at 37°C using a heating pad. The right carotid artery was cannulated for mean arterial blood pressure (MAP) measurement (AD Instruments, Bella Vista, Australia) and fluid supplementation with saline containing 10 IU/kg heparin. The completion of these procedures marked the start of the experiment (0 min). Forty minutes later, two groups of rats treated with either vehicle or rofecoxib underwent a sham operation, where the left anterior descending coronary artery (LAD) was isolated but not occluded (groups 1 and 3, VEH SHAM and ROF SHAM). In the other two groups (groups 2 and 4, VEH I/R and ROF I/R), cardiac I/R injury was induced by occluding the LAD for 30 minutes, followed by 120 minutes of reperfusion. Additionally, all animals received intraperitoneal injections of 100 IU/kg heparin three times during the surgical procedure, at 35, 65, and 185 minutes. The study design and experimental protocol are illustrated in Figure 4.

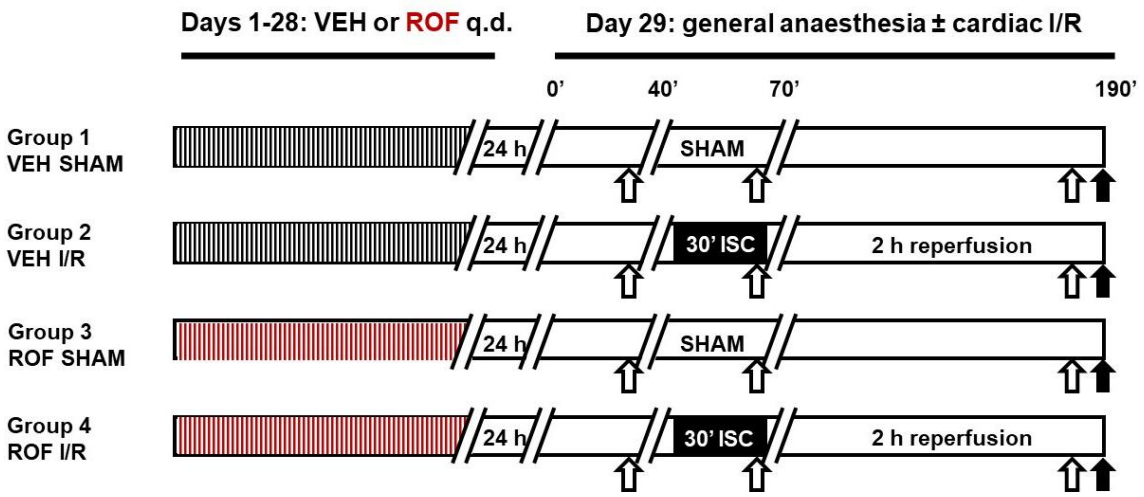


Figure 4. Protocol of cardiac I/R-induced remote intestinal injury.

Male Wistar rats were treated with vehicle (VEH, 1% hydroxyethylcellulose) or rofecoxib (ROF, 5 mg/kg) for 28 days once daily (q.d.). On the 29th day, rats were subjected to sham operation (groups 1 and 3, VEH SHAM and ROF SHAM) or cardiac ischemia (ISC) followed by reperfusion (groups 2 and 4, VEH ischemia/reperfusion [I/R] and ROF I/R). Number of animals surviving the whole protocol: 5-8/group. White arrow: intraperitoneal injection of 100 IU/kg heparin; black arrow: termination of animals and tissue sampling.

At the end of the reperfusion phase, the rats were euthanized, and plasma samples were collected. The small intestines were excised, and the mucosa was flushed with cold saline and photographed for subsequent macroscopic analysis. Full-thickness sections of the distal jejunum were snap-frozen in liquid nitrogen and stored at -80°C for further biochemical assays. Additional segments of intestinal tissue were fixed in 10% formalin for histological examination.

Intestinal tissue and plasma samples were obtained exclusively from animals that survived the entire experimental protocol. While all animals in the VEH SHAM and ROF SHAM groups (n=8/group) survived, one of the seven animals in the VEH-treated I/R group succumbed to ventricular fibrillation, resulting in a 14% mortality rate. In contrast, treatment with rofecoxib following cardiac I/R was associated with an increased mortality rate, with four out of nine animals (44%) dying due to arrhythmias (111).

3.3.1.2 Study II. Evaluating the effect of cardiac I/R injury on small intestinal microcirculation

This study was conducted on a separate cohort of rats with body weights matching those of the vehicle-treated animals in Study I on the day of surgery (320-440 g). The animals

underwent the same anesthesia and surgical procedures as previously described, including a median laparotomy to gently expose the distal jejunum. Small intestinal microcirculation was assessed using a PeriScan PIM II laser Doppler perfusion imager (Perimed, Stockholm, Sweden) positioned 12 cm above the jejunal surface. Each scan covered an area of 31 x 31 sampling points within 59 seconds, followed by a 1-second pause. Consequently, imaging was performed every minute. To minimize fluid and heat loss and prevent tissue desiccation during the reperfusion period, the opened abdomen was intermittently covered with saline-moistened gauze for 5-minute intervals from the 95th to the 180th minute (i.e., from 95 to 100 minutes, from 105 to 110 minutes, and so forth) (115). Perfusion values during these intervals were not recorded. Perfusion patterns were analyzed using LDPIwin 2.6 software (Perimed, Stockholm, Sweden), and values were expressed as a percentage of the basal (pre-occlusion) values recorded at 10 minutes.

3.4 Animal model of local small intestinal I/R injury

Two *in vivo* experiments were conducted involving 48 rats, each weighing between 250-350 g. The rats were randomly assigned to three groups and treated once daily for 8 days with either celecoxib (10 and 100 mg/kg) [2,3,5,6-tetradeuterio-4-[5-(4-methyl phenyl)-3-(trifluoromethyl)pyrazol-1-yl]benzenesulfonamide)] (Merck Millipore, Burlington MA, USA) or its vehicle (1% hydroxyethylcellulose) via gavage. The 10 mg/kg dose of celecoxib was reported to be selective for COX-2 over COX-1 (116), and our preliminary studies confirmed the high efficacy and COX-2 selectivity of celecoxib at this dosage.

The higher dose was selected based on studies indicating that celecoxib can also elicit COX-independent effects at this concentration (117, 118). On the 8th day, 2 hours after the final drug administration, the animals in each group were further divided into two subgroups, with 8 rats in each, and anesthetized by intraperitoneal injection of pentobarbital (60 mg/kg). Following an upper median laparotomy, the animals were subjected to either a sham operation, where the SMA was isolated but not occluded, or to I/R injury by occluding the SMA for 30 minutes followed by 120 minutes of reperfusion (Figure 5).

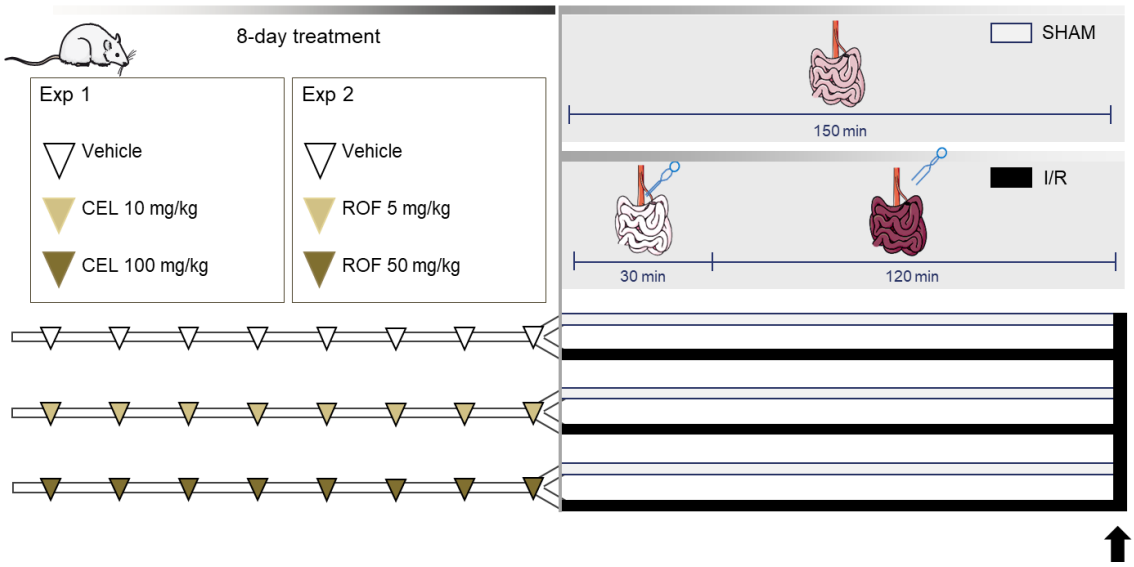


Figure 5. Experimental protocol of mesenteric I/R injury.

Male Wistar rats were treated with vehicle (1% hydroxyethylcellulose), celecoxib (CEL, 10 and 100 mg/kg), or rofecoxib (ROF, 5 and 50 mg/kg) for 8 days once daily. On the 8th day, 2 h after the last drug administration, rats were subjected to either sham operation or mesenteric ischemia/reperfusion (I/R). n=7-8/group. Black arrow: termination of animals and tissue sampling.

During surgery, the animals' body temperature was maintained at 37°C using a heating pad, and the depth of anesthesia was monitored by periodically assessing pedal reflexes. At the end of the reperfusion period, the rats were euthanized, and their small intestines were excised. The mucosa of the small intestine was flushed with cold saline, and three samples, each 1-2 cm in length, were taken from the same region of the distal jejunum, 13-15 cm from the ileocecal junction. The first two specimens were snap-frozen in liquid nitrogen and stored at -80°C for subsequent measurement of protein and gene expressions. The third specimen was fixed in 10% formalin for histological analysis.

In the second experiment, essentially the same protocol was followed, except that the rats were treated with rofecoxib instead of celecoxib (Figure 5). The doses of rofecoxib were selected based on previous studies conducted by other groups and our research (112, 116).

This I/R injury protocol was chosen based on the results of our preliminary experiments aimed at inducing sufficient intestinal injury and inflammation without significant mortality (Figure 6) (119).

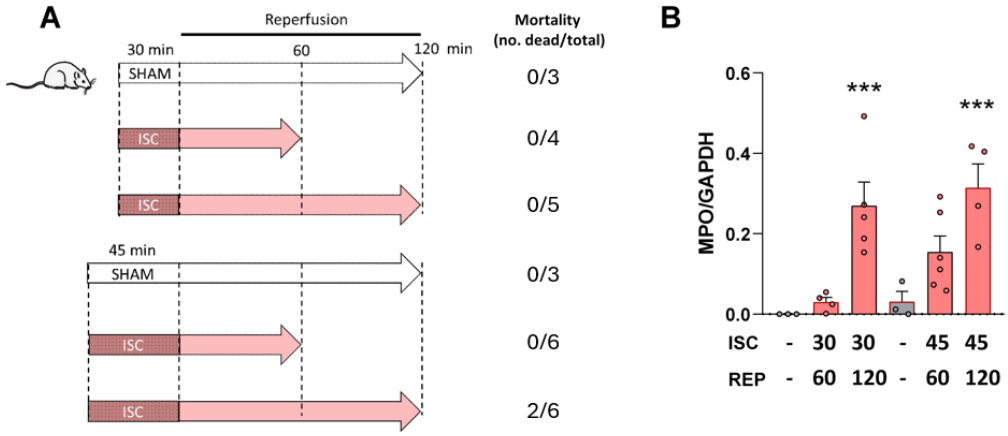


Figure 6. Analysis of mortality and jejunal MPO level in I/R models with different ischemic and reperfusion time periods

Panel A: *Experimental protocol and mortality rate of different time intervals of ischemia and reperfusion compared to respective SHAM-groups (n=3-6/group).* ***Panel B:*** *Intestinal inflammation was measured according to jejunal MPO level via Western blot. Circles represent the data of each rat, bars indicate the mean + SEM. For statistical analysis, one-way ANOVA was used, followed by Fisher's LSD post hoc test. p<0.001 vs respective SHAM (Laszlo et al, 2024).*

3.5 Macroscopic evaluation of intestinal damage

In the remote I/R injury model, high-resolution photographs of the entire small intestinal mucosa were meticulously analyzed and scored in a blinded manner as follows: 0, no visible morphological alteration; 1, small (1-2 mm) hyperemic area at one site; 2, small (1-2 mm) hyperemic areas at two or more sites; 3, extensive (>2 mm) hyperemic area at one site; 4, extensive (>2 mm) hyperemic areas at two or more sites (112).

3.6 Histological analysis

Samples from the distal jejunum were excised, fixed in 10% formalin, embedded in paraffin, sectioned at 4-5 μm, and stained with hematoxylin and eosin. In the local intestinal I/R model, the Swiss-roll technique was employed to obtain histological samples, facilitating the examination of a longer segment of the small intestine. Digital micrographs were captured using an Olympus BX51 microscope and an Olympus DP50 camera. In both experiments, histological injury was assessed in a blinded manner by two histopathologists. Intestinal samples from cardiac I/R-induced intestinal injury were evaluated using a modified version of

the scoring system described by Mantyh *et al.* (Table 1) (120). The total histological score, ranging from 0 to 9, was calculated based on the sum of partial scores.

Table 1. Criteria for quantitative estimation of the small intestinal injury after cardiac I/R.

	0	**1**	**2**	**3**
Epithelial damage	none	destruction of villus tips	destruction of up to one half of villus	complete villus destruction
Congestion and edema	none	minimal increase in crypt spacing, rare RBC-containing vessels	moderate increase in crypt spacing, up to one-half of vessels contain RBCs	widely spaced crypts, numerous RBC-containing vessels in the lamina propria
Cellular infiltration	none	mild cellular infiltration	moderate cellular infiltration	numerous leukocytes throughout the lamina propria

RBC–red blood cell

The intestinal samples from the intestinal I/R injury model were assessed according to the Chiu/Park classification (Table 2) (121, 122). Histological injury was graded on an eight-point scale, ranging from 0 (normal mucosa) to 8 (transmural infarction). Representative pictures were captured by Eclipse E200 microscope and scanned by a Panoramic 1000 Digital Slide Scanner.

Table 2. Criteria for quantitative estimation of the mucosal damage after mesenteric I/R according to Park/Chiu grading system (121, 122).

Grade	Park-Chiu
0	Normal mucosa
1	Subepithelial space at villus tips
2	Extension of the subepithelial space with moderate lifting
3	Massive lifting down the sides of the villi, some denuded tips
4	Denuded villi, dilated capillaries
5	Disintegration of lamina propria
6	Crypt layer injury
7	Transmucosal infarction
8	Transmural infarction

3.7 Cytokine measurements

In the myocardial I/R-induced injury, the jejunal levels of various cytokines were quantified using either Luminex xMAP technology or ELISA. Excised and snap-frozen jejunal tissues were pulverized and homogenized following the manufacturer's instructions. The ELISA kit (Invitrogen, Camarillo, CA, USA) was used to measure the protein levels of TNF-α, while the Milliplex Multiplex assay (Merck Millipore, Burlington, MA, USA) was used to determine the levels of IL-1β and IL-10 using a customized Milliplex Rat Cytokine/Chemokine Magnetic Bead Panel. The total protein concentration of the samples was determined using a bicinchoninic acid assay kit (Thermo Scientific Pierce Protein Research Products, Rockford, IL, USA) with bovine serum albumin (BSA) as a standard, and cytokine amounts were expressed in pg/mg of total protein.

3.8 Western Blot

Distal jejunal tissues were homogenized using a TissueLyser (Qiagen, Venlo, Netherlands) in a lysis buffer supplemented with a protease inhibitor cocktail (cOmplete ULTRA Tablets, Roche, Basel, Switzerland) and PMSF (Sigma, St. Louis, MO, USA). The homogenates were centrifuged twice at 1500×g for 15 minutes at 4 °C, and the supernatants

were collected. Protein concentration was measured using the bicinchoninic acid assay (BCA, Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of protein (20 µg) were mixed with Pierce Lane Marker reducing sample buffer (Thermo Fisher Scientific, Waltham, MA, USA), loaded, and separated on a 4-20% precast Tris-glycine SDS polyacrylamide gel (Bio-Rad, Hercules, CA, USA). Proteins were then transferred electrophoretically onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA) at 200 mA overnight.

Membranes were blocked with either 5% nonfat dry milk (Bio-Rad, Hercules, CA, USA) or 5% bovine serum albumin (BSA, a9647, Merck Millipore, Burlington, MA, USA) in Tris-buffered saline containing 0.05% Tween-20 (0.05% TBS-T; Sigma, St. Louis, MO, USA) at room temperature for 2 hours. They were then incubated overnight at 4 °C with primary antibodies against COX-2 (#12282, 1:500, Cell Signaling Technology, Danvers, MA, USA), COX-1 (4841, 1:500, Cell Signaling Technology, Danvers, MA, USA), myeloperoxidase (MPO, AF3667, 1:1000, R&D Systems, Minneapolis, MN, USA), pentraxin 3 (PTX3, ab125007, 1:1000, Abcam, Cambridge, UK), claudin-1 (ab15098, 1:1000, Abcam, Cambridge, UK), occludin (ABT146-25UG, 1:1000, Merck Millipore, Burlington, MA, USA), phospho-Akt (#9271, 1:1000, Cell Signaling Technology, Danvers, MA, USA), and Akt (#9272, 1:1000, Cell Signaling Technology, Danvers, MA, USA). This was followed by a 2-hour incubation at room temperature with an appropriate HRP-linked secondary antibody. GAPDH (D16H11, 1:1000, Cell Signaling Technology, Danvers, MA, USA) and Akt (for phospho-Akt) were used as loading controls. Membranes were trimmed before antibody treatment if the bands of interest were far apart. Each experiment was repeated at least twice.

Signals were detected using a chemiluminescence kit (Bio-Rad, Hercules, CA, USA) and visualized with a Chemidoc XRS+ system (Bio-Rad, Hercules, CA, USA). Relative protein levels were quantified by densitometric analysis using Image Lab Software version 6.1.0 (Bio-Rad, Hercules, CA, USA). The intensity of each band was quantified and normalized to the intensity of the respective loading control (GAPDH or Akt).

3.9 qRT-PCR

Total RNA was extracted from 10 to 30 mg of small intestine tissue using the QIAzol extraction method (Qiagen, Hilden, Germany). The RNA concentration was measured with a Nanophotometer (Implen GmbH, Munich, Germany). Reverse transcription was performed on 1 µg of total RNA using the Sensifast cDNA synthesis kit (Bioline, London, UK) following the manufacturer's protocol. Target genes were amplified using the LightCycler® 480 II instrument

(Roche, Germany) with the SensiFAST SYBR Green master mix (Bioline, UK). Expression levels were calculated using the $2^{-\Delta\Delta CT}$ method, with Rpl13a serving as the reference gene. The sequences of the primers used for determination were as follows: heme oxygenase-1 (HO-1) forward AAG AGG CTA AGA CCG CCT TC, HO-1 reverse GCA TAA ATT CCC ACT GCC AC (Accession number: NM_012580.2), peroxisome proliferator-activated receptor-γ (PPAR-γ) forward CCC ACC AAC TTC GGA ATC AG, PPAR-γ reverse GGA ATG GGA GTG GTC ATC CA (Accession number: NM_013124), interleukin 1β (IL-1β) forward TGG CAA CTG TCC CTG AAC TC, IL-1β reverse GGG CTT GGA AGC AAT CCT TAA TC (Accession number: NM_031512.2), interleukin-10 (IL-10) forward GAA CCA CCC GGC ATC TAC TG, IL-10 reverse AGG AGT TGC TCC CGT TAG C (Accession number: NM_012854.2), B cell lymphoma-2 (Bcl-2) forward TGA GTA CCT GAA CCG GCA TC, Bcl-2 reverse TAT AGT TCC ACA AAG GCA TCC CAG (Accession number: NM_009741.5), Bcl-2 associated X-protein (Bax) forward AGT GTC TCC GGC GAA TTG G, Bax reverse CAC GTC AGC AAT CAT CCT CTG C (Accession number: NM_007524.4) and Rpl13a forward GGA TCC CTC CAC CCT ATG ACA, Rpl13a reverse CTG GTA CTT CCA CCC GAC CTC (Accession number: NM_173340.2). At least two repetitions were performed for each experiment.

3.10 COX enzyme activity assay

The total COX enzyme activity of homogenized intestinal samples (10 µl) was measured by a fluorescent COX-activity assay kit (700200, Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Sample homogenization was performed as described in Section 2.4. The fluorescence ($\lambda_{excitation}$= 535 nm and $\lambda_{emission}$ = 590 nm) was recorded at 5 min by a Varioskan™ LUX Multimode Microplate Reader (Thermo Fisher Scientific, Waltham, MA, USA). All samples were measured in duplicates. To assess the contribution of COX-1 and COX-2 isoforms to total COX enzyme activity, the highly selective COX-1 inhibitor SC-560 was added to separate sample aliquots (final well concentration: 3.47 µM). Enzyme activities were expressed as percentage of the mean activity of the vehicle-treated sham-operated groups.

3.11 Quantification of 6-keto $PGF_{1\alpha}$

The quantification of 6-keto $PGF_{1\alpha}$ levels in jejunal tissue was performed using an ELISA, following the protocol provided by the manufacturer (Cayman Chemical, Ann Arbor, MI, USA). In brief, the tissues were homogenized in precooled acetone containing 10 µM indomethacin and subsequently centrifuged at 10,000 g for 10 minutes at 4 °C. Acetone was then evaporated from the supernatants using a vacuum centrifuge, and the resulting residues were reconstituted in assay buffer for the determination of 6-keto $PGF_{1\alpha}$.

3.12 SOD and CAT assays

The enzymatic activities of SOD and CAT in the jejunum were quantified using assay kits following the manufacturer's protocols (Cayman Chemical, Ann Arbor, MI, USA). The SOD assay employs a tetrazolium salt to detect superoxide radicals generated by xanthine oxidase and hypoxanthine, measuring the activity of all three SOD isoforms (Cu/Zn, Mn, and Fe-SOD). One unit of SOD is defined as the amount of enzyme required to achieve 50% dismutation of the superoxide radical. The total protein concentration of the supernatants was determined, and SOD activity was expressed in units per milligram of protein.

The CAT assay quantifies the peroxidative function of CAT to determine enzyme activity, based on its reaction with methanol in the presence of an optimal concentration of hydrogen peroxide (H_2O_2). The produced formaldehyde was quantified spectrophotometrically using 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole as the chromogen. One unit of CAT is defined as the amount of enzyme that produces 1 nmol of formaldehyde per minute at 25°C, with CAT activity expressed in nmol/min/mg tissue.

3.13 Measuring MMP-2 and MMP-9 activities by gelatin zymography

The activity of MMP-2 and MMP-9 was evaluated using gelatin zymography on plasma samples collected after 120 minutes of reperfusion. Gelatinolytic activities of MMPs were analyzed as previously described (123). Briefly, 8% polyacrylamide gels were copolymerized with gelatin (2 mg/ml, type A from porcine skin), and 50 µg of protein per lane was loaded. An internal standard (American Type Culture Collection, Manassas, Virginia) was included in each gel to normalize activities between gels. Following electrophoresis (90 V, 90 min), gels were washed with zymogram renaturation buffer (Novex, Carlsbad, CA, USA) for 40 minutes.

Samples were then incubated for 20 hours at 37°C in zymogram development buffer (Novex, Carlsbad, CA, USA).

In a separate experiment, one plasma sample from each group was loaded into the gel in four replicates. After renaturation, the gel was divided into four sections, each incubated in a development buffer containing vehicle or rofecoxib at concentrations of 0.1, 1, or 10 μM, respectively, to determine whether rofecoxib directly inhibits MMP activity. The 1 μM concentration of rofecoxib was selected based on the peak plasma concentration (C_{max}) observed after a single 5 mg/kg oral dose in rats (123, 124).

Gels were subsequently stained with 0.05% Coomassie brilliant blue in a methanol-acetic acid-water mixture [2.5:1:6.5 (v/v)] and destained in an aqueous solution of 4% methanol and 8% acetic acid (v/v) to remove nonspecific Coomassie binding. For positive controls, a gelatinase zymography standard containing human MMP-2 and MMP-9 (Chemicon Europe Ltd., Southampton, UK) was used. For negative controls, lanes containing tissue samples were excised after gel renaturation and incubated for 20 hours at 37°C in development buffer with the calcium chelator EGTA (10 mM). Gelatinolytic activities were visualized as clear bands against a dark blue background. Gels were scanned using a transilluminator, and band intensities were quantified with Quantity One software (BioRad, Hercules, CA, USA), normalized to the internal standard, and expressed in arbitrary units.

3.14 Statistical analysis

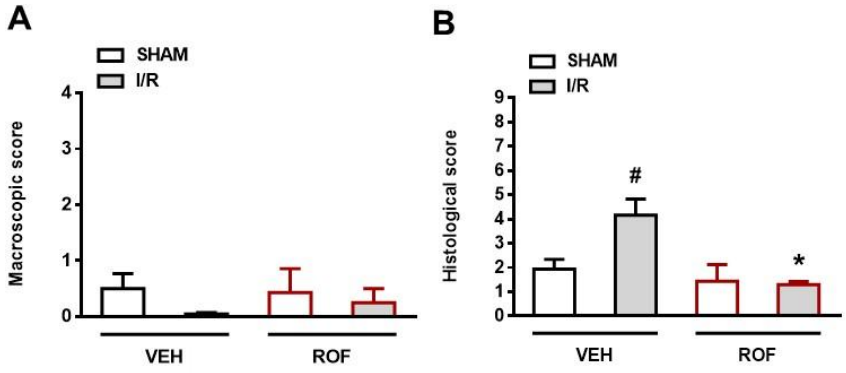
Data are expressed as mean + SEM. Statistical analysis of the data was performed with one-way or two-way ANOVA, followed by Fisher's LSD post hoc test and uncorrected Dunn's post hoc test (in case of histological score of mesenteric I/R injury). In case of nonparametric values, statistical data analysis was performed with Mann-Whitney (pairwise comparison) or Kruskal-Wallis tests, followed by Dunn's post hoc test. Two-way repeated measures ANOVA was used to compare the time course of blood pressure and jejunal blood flow changes, followed by Holm-Sidak post hoc test. Correlations between MMP-values and histological scores were calculated by Spearman test. Outliers detected by Grubb's test were excluded from the analyses. In the *in vitro* assays, all samples were measured at least in duplicates, and most measurements were repeated two times. In all cases, a probability of $p<0.05$ was considered statistically significant.

4 Results

4.1 The effects of rofecoxib on cardiac I/R-evoked intestinal injury

4.1.1 Rofecoxib treatment ameliorated the mild histological intestinal damage following cardiac I/R injury

Ischemia induced by a 30-minute occlusion of the LAD, followed by a 2-hour reperfusion period, did not produce any observable macroscopic changes in the small intestines of either vehicle- or rofecoxib-administered rats. (Figure 7A). Conversely, histopathological examination revealed early substantial modifications in the jejunal mucosa of vehicle-administered rats subjected to myocardial I/R injury compared to sham-operated controls (Figure 7B). This was predominantly characterized by subepithelial edema with dilated vessels containing numerous erythrocytes and was noted in the majority of specimens (5 out of 6 rats). Additionally, in half of the I/R-treated animals, an increased infiltration of leukocytes (including lymphocytes, macrophages, and granulocytes) in the lamina propria, including extravasated ones, was detected, indicating augmented vascular permeability (Figure 7C). The epithelial integrity and villous architecture were largely preserved, although early signs of epithelial disruption at the apices of some villi were observed in 5 out of 6 specimens (Figure 7C). Overall, the histological score was significantly elevated in the I/R group relative to the sham-operated group. However, such morphological alterations were absent in the I/R group treated with rofecoxib, suggesting that COX-2 inhibition mitigated remote intestinal damage post-cardiac I/R (Figures 7B and 7C). Histopathological evaluation was conducted according to the criteria outlined in Table 1.



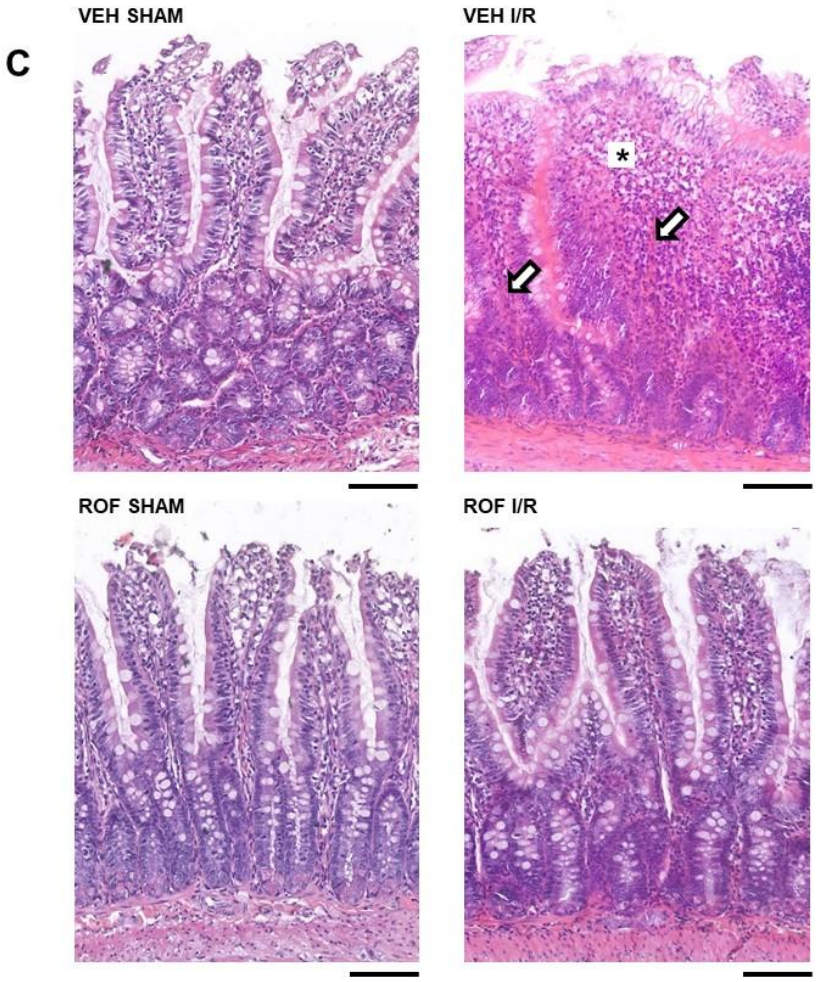


Figure 7. Panels A, B: *The effects of 4-week treatment with vehicle (VEH) and rofecoxib (ROF, 5 mg/kg) on macroscopic (**A**) and histological scores (**B**) of jejunum. Data are expressed as mean + SEM. For pairwise comparison of respective treatment groups, Mann-Whitney test was used, n=5-8/group (*p<0.05 vs. respective VEH, #p<0.05 vs. respective SHAM).* ***Panel C*** *demonstrates representative histological micrographs (scale bar: 100 µM, hematoxylin and eosin staining). Cardiac ischemia/reperfusion (I/R) injury induced histological alterations in vehicle-treated (VEH) rats. White arrows demonstrate dilated capillaries with numerous red blood cells in the lumen, whereas increased cellularity of the lamina propria with increased number of granulocytes, including extravasated ones, is marked by asterisk.*

4.1.2 Rofecoxib treatment inhibited the development of mild intestinal inflammation following cardiac I/R injury and increased the SOD activity in the I/R group compared to the respective vehicle-treated group

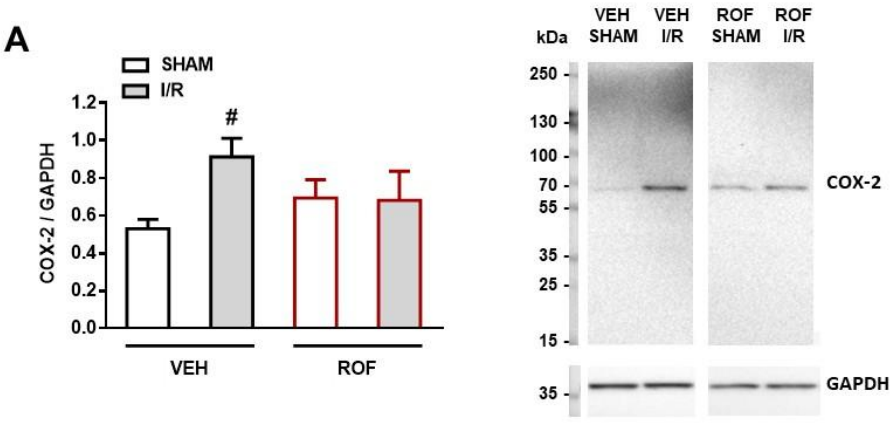
Given that histological analysis indicated mild intestinal inflammation in the vehicle-treated I/R group, our subsequent objective was to support this by quantifying various inflammatory mediators implicated in I/R injury. Initially, the intestinal protein expression of COX-2 was evaluated by Western blot, as its upregulation is a prompt reaction to I/R. In

contrast to sham-operated animals with negligible COX-2 expression, vehicle-treated rats after cardiac I/R exhibited markedly elevated COX-2 protein levels in the jejunum. Conversely, in rofecoxib-treated animals, cardiac I/R did not augment intestinal COX-2 expression relative to the respective sham group (Figure 8A). The protein levels of the constitutive isoform COX-1, unlike COX-2, remained consistent across all groups regardless of treatment (Figure 8B).

The expression of COX-2 can be stimulated by various pro-inflammatory cytokines, such as TNF-α (11). TNF-α plays a crucial role in orchestrating the inflammatory response to I/R injury in most tissues (2). However, no differences in intestinal TNF-α protein levels were observed between the groups using ELISA (Figure 8C).

The tissue concentrations of the anti-inflammatory cytokine IL-10 were also quantified by ELISA, since it is known to be produced during acute inflammation by various cell types in parallel with pro-inflammatory cytokines to modulate immune responses (125). As depicted in Figure 8D, there were only moderate variations in IL-10 levels, though these somewhat reflected the alterations in histological scores and COX-2 concentrations. Specifically, the highest IL-10 protein levels were observed in the vehicle-treated I/R group, whereas significantly lower levels were found in rofecoxib-treated animals subjected to I/R.

Likewise, the highest concentration of 6-keto $PGF_{1\alpha}$ (the stable metabolite of PGI_2), predominantly synthesized by COX-2 during inflammation, was observed in the vehicle-treated I/R group (Figure 8E) (126), although the differences between the groups did not achieve statistical significance.



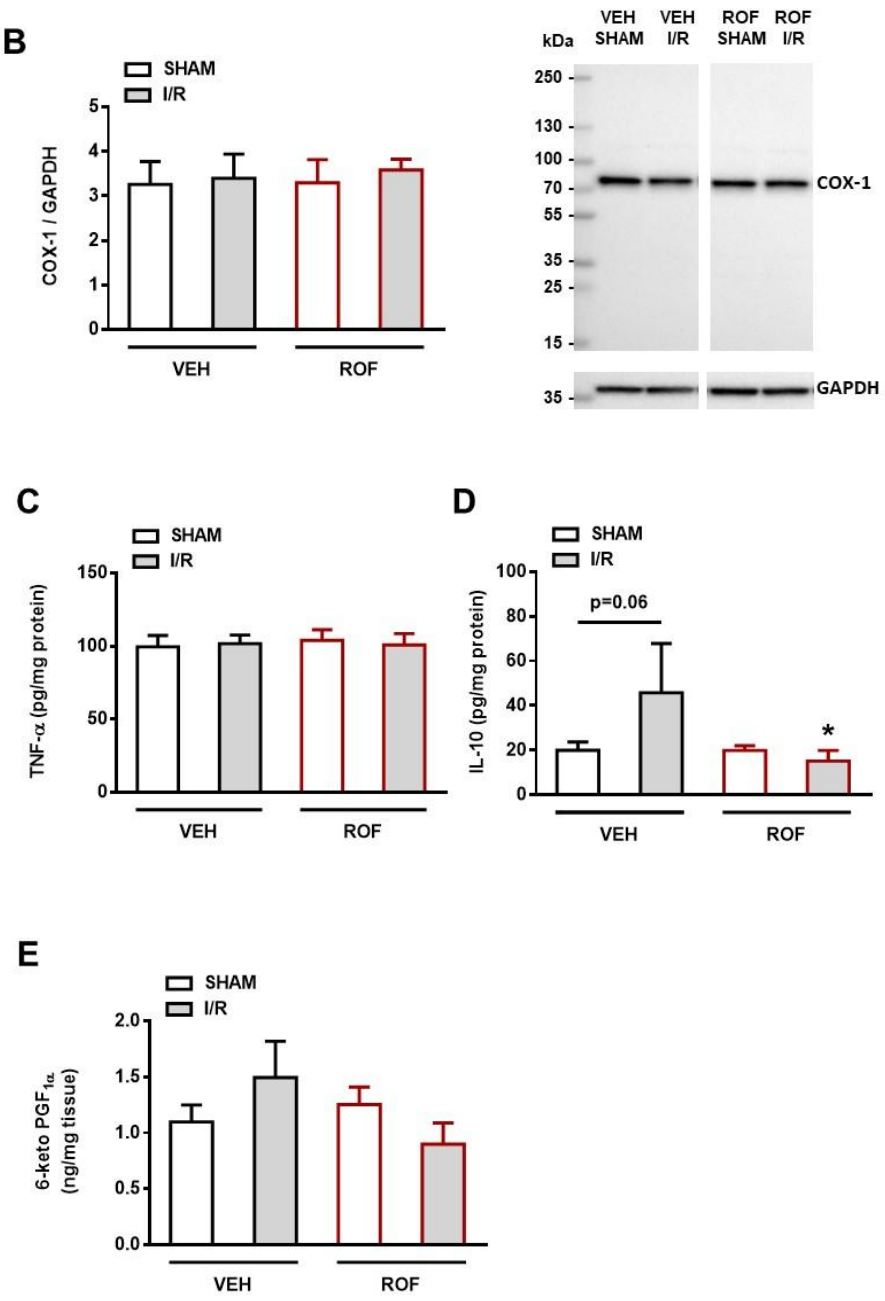


Figure 8. *Jejunal protein levels of cyclooxygenase-2 (COX-2, **A**), COX-1 (**B**), tumor necrosis factor-α (TNF-α, **C**), interleukin-10 (IL-10) (**D**) and 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$, **E**) in rats treated with vehicle (VEH) or rofecoxib (ROF, 5 mg/kg) for 4 weeks and subjected to sham operation or cardiac ischemia/reperfusion (I/R) injury. Results are expressed as mean + SEM. For statistical analysis, two-way ANOVA was used, followed by Fisher's LSD post hoc test, n=5-8/group (*p<0.05 vs. respective VEH, #p<0.05 vs. respective SHAM).*

Finally, given that oxidative stress and reactive oxygen and nitrogen species (RONS) are well-known contributors to the pathogenesis of I/R injury, it was aimed to assess the tissue activities of SOD and CAT, two primary antioxidant enzymes involved in neutralizing toxic

oxygen metabolites and alleviating cellular damage (7, 127, 128). While CAT activities were consistent across all groups, total SOD activity showed a tendency to increase in the vehicle-treated I/R group and significantly rose in the rofecoxib-treated I/R group (Figures 9A and 9B).

Superoxide anion, along with nitric oxide, is one of the primary RONS generated during I/R (27), and SOD catalyzes its dismutation into H_2O_2 and molecular oxygen. Therefore, the elevated SOD activity in rofecoxib-treated animals may reflect an augmented defensive response to heightened intracellular superoxide levels (7).

Collectively, these findings suggest that 2 hours of reperfusion following cardiac ischemia induced only mild responses in the small intestine, which were mitigated by rofecoxib treatment.

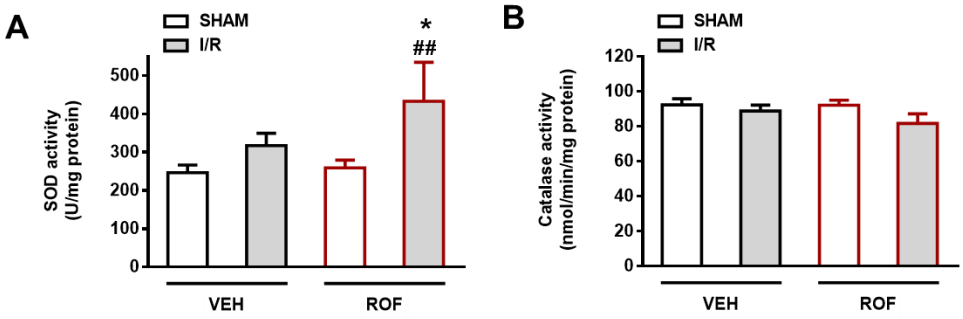


Figure 9. *Jejunal activities of the antioxidant superoxide dismutase (SOD, **A**) and catalase (CAT, **B**) in rats treated with vehicle (VEH) or rofecoxib (ROF, 5 mg/kg) for 4 weeks and subjected to sham operation or cardiac ischemia/reperfusion (I/R) injury. Results are expressed as mean + SEM. For statistical analysis, two-way ANOVA was used, followed by Fisher's LSD post hoc test, n=5-8/group (*p<0.05 vs. respective VEH, ##p<0.01 vs. respective SHAM).*

4.1.3 The activity of MMP-2 but not MMP-9 in the plasma correlated with the intestinal histological score, but rofecoxib did not inhibit their activity *in vitro*

The crucial involvement of specific types of MMPs, particularly MMP-2 and MMP-9, in I/R injury has been evidenced in various organs, including the heart (129, 130) and gastrointestinal tract (131). Additionally, according to the literature data, they contribute to remote I/R injury as well (132). In the next step, it was aimed to determine activities of MMP-2 and MMP-9, the predominant MMP types in the myocardium, using plasma samples analyzed via gelatin zymography. Gelatin zymography revealed two distinct bands for both MMP-2 (72 and 75 kDa) and MMP-9 (86 and 92 kDa) (Figure 10), representing different zymogen and active forms (123). Analysis of band intensities demonstrated that cardiac I/R elevated the

plasma activity of both MMP-2 isoforms in the vehicle-treated group, which was mitigated by rofecoxib treatment (Figures 11A and 11B). Moreover, plasma MMP-2 activities exhibited a relatively weak but statistically significant positive correlation with the histological scores of intestines (Figures 11C and 11D).

In contrast, although plasma MMP-9 activities showed similar trends, they neither differed significantly between the groups (Figures 11E and 11F) nor correlated with the histological scores (Figures 11G and 11H).

These findings suggest that increased MMP-2 activity, but not MMP-9, in the circulation is associated with early intestinal injury following cardiac I/R, and the protective effect of rofecoxib could be linked to reduced MMP-2 activity.

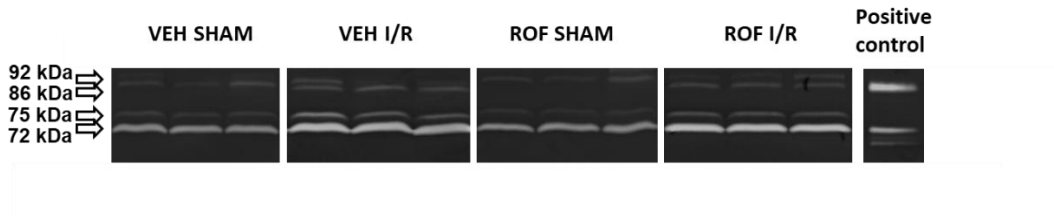
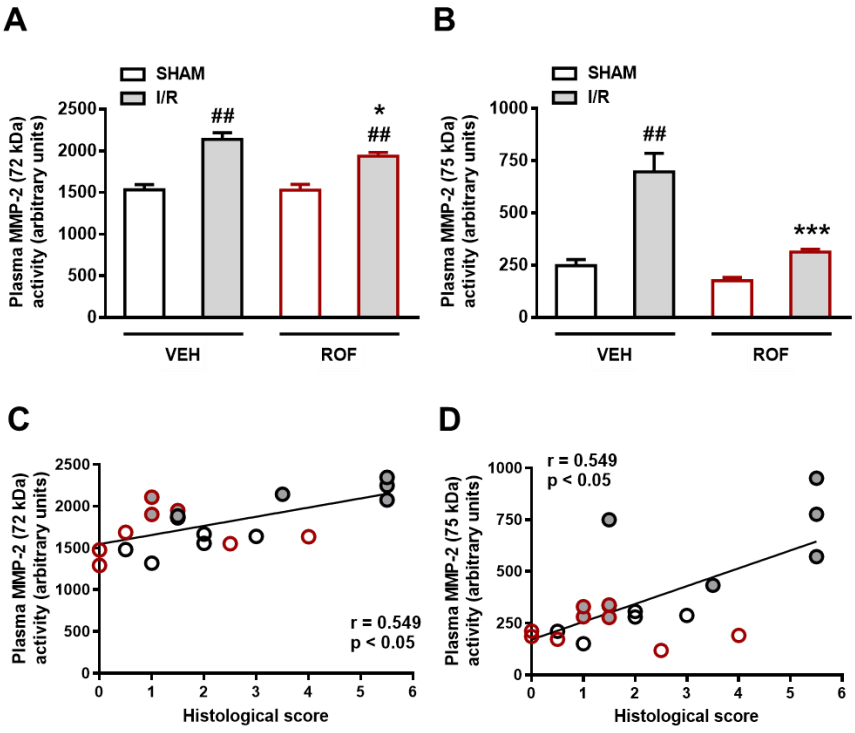


Figure 10. *Representative zymograms of plasma matrix metalloproteinase-2 (MMP-2) (72 and 75 kDa) and MMP-9 activities (86 and 92 kDa) in vehicle- (VEH, 1% hydroxyethylcellulose) and rofecoxib-treated (ROF, 5 mg/kg) rats subjected to sham operation or cardiac ischemia/reperfusion (I/R).*



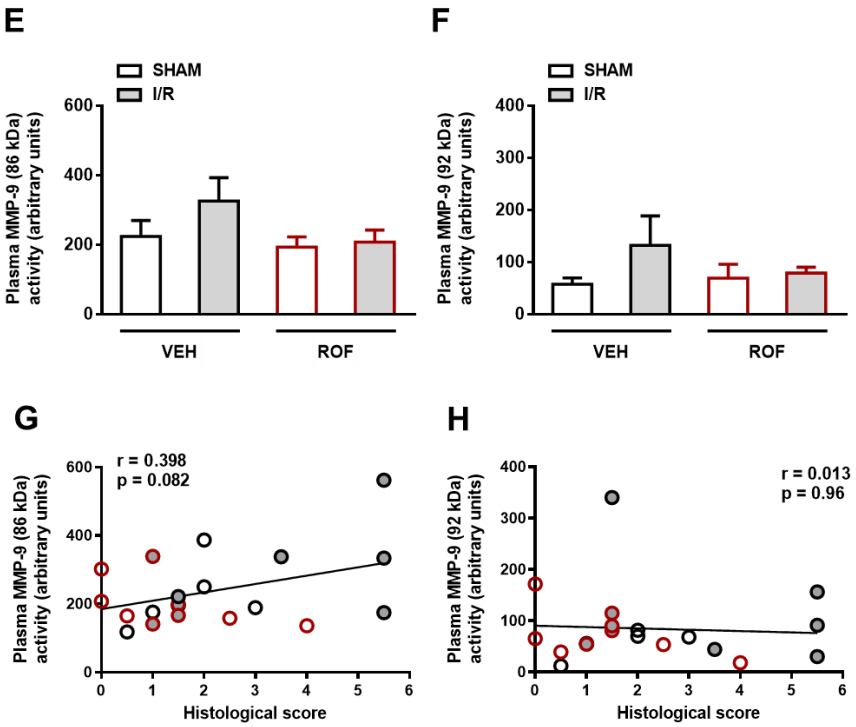


Figure 11. Panels A, B, E and F*: Gelatinolytic activities of matrix metalloproteinase-2 (MMP-2, 72 kDa, **A**; 75 kDa, **B**) and of MMP-9 (86k Da, **E**; 92 kDa, **F**) in plasma samples of animals treated with vehicle (VEH) or rofecoxib (ROF, 5 mg/kg) for 4 weeks and subjected to sham operation or cardiac ischemia-reperfusion (I/R) injury. Results are expressed as mean + SEM. For statistical analysis, two-way ANOVA was used, followed by Fisher's LSD post hoc test, n=4-5/group (*p<0.05 and ***p<0.001 vs. respective VEH, ##p<0.01 vs. respective SHAM).* ***Panels C, D, G and H****: Correlations between MMP-2 (72 kDa, **C**; 75 kDa, **D**) and of MMP-9 (86 kDa, **G**; 92 kDa, **H**) and histological scores of rats in the VEH SHAM (empty black circles), VEH I/R (grey filled black circles), ROF SHAM (empty red circles) and ROF I/R (grey filled red circles) groups. Correlations were calculated by Spearman test.*

To determine whether the reduced MMP-2 activity observed in the rofecoxib-treated I/R group results from direct inhibition of MMP-2 activity by rofecoxib or from diminished protein expression, the gelatinolytic activities of plasma samples from each group were assessed using gels incubated with varying concentrations of rofecoxib.

As shown in Figure 12, none of the tested rofecoxib concentrations inhibited the activity of either MMP isoform *in vitro*, suggesting that the decreased plasma MMP-2 activity in rofecoxib-treated I/R rats may be due to reduced enzyme synthesis at the level of the heart.

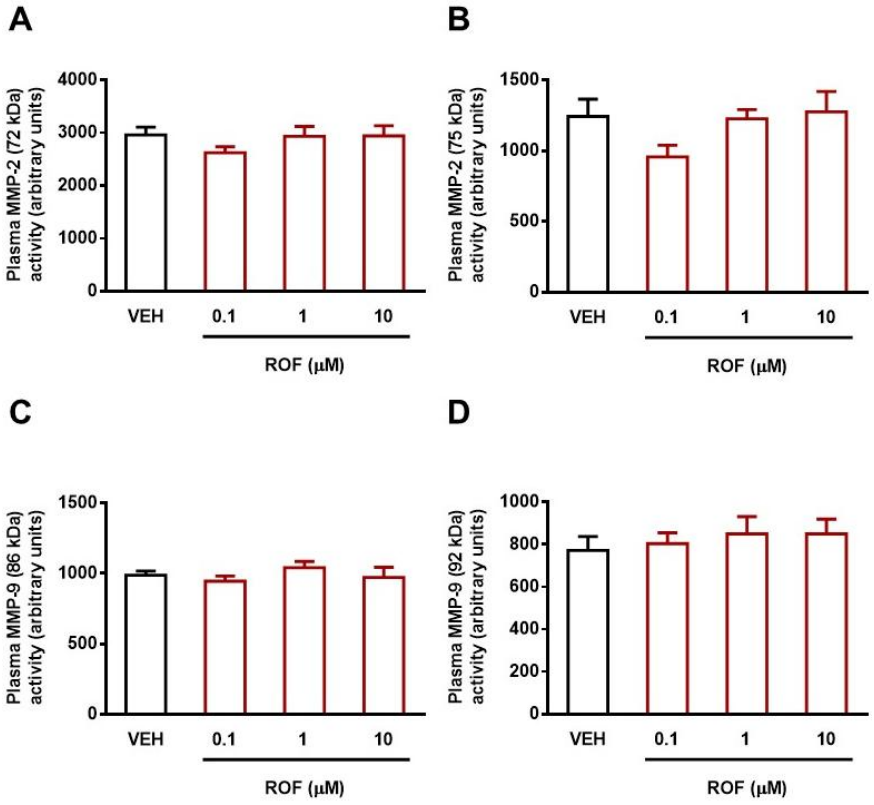


Figure 12. *The effects of different concentrations of rofecoxib (ROF, 0.1, 1, and 10 μM) in vitro on the gelatinolytic activities of matrix metalloproteinase-2 (MMP-2 72 kDa,* ***A****; 75 kDa,* ***B****) and MMP-9 (86 kDa,* ***C****; 92 kDa,* ***D****). Data are expressed as mean + SEM. For statistical analysis, one-way ANOVA was used, followed by Fisher's LSD post hoc test, n=5/group.*

4.1.4 Cardiac I/R provoked mild, transient jejunal hypoperfusion, normalized after the abolishment of occlusion

We investigated whether the mild mucosal damage observed in the small intestine following cardiac I/R could be attributed to decreased small intestinal perfusion. Therefore, in a separate study, we assessed jejunal microcirculation using laser Doppler perfusion imaging in weight-matched rats while simultaneously measuring systemic blood pressure. In sham-operated animals, both MAP and jejunal blood flow remained stable throughout the observation period (Figure 13). A 30-minute occlusion of LAD induced a prompt reduction in blood pressure, partly due to transient arrhythmias, which was accompanied by impaired jejunal microcirculation. However, this impairment was moderate (averaging a 15.5% reduction, peaking at 31.6%) and transient, with microcirculation normalizing within 15 minutes. Meanwhile, the systemic blood pressure of the animals failed to recover completely and remained lower until the end of the experiment.

Our findings demonstrate that cardiac I/R caused only mild and temporary impairment of intestinal microcirculation, indicating that the histological damage in the intestine following I/R was unlikely due to intestinal ischemia.

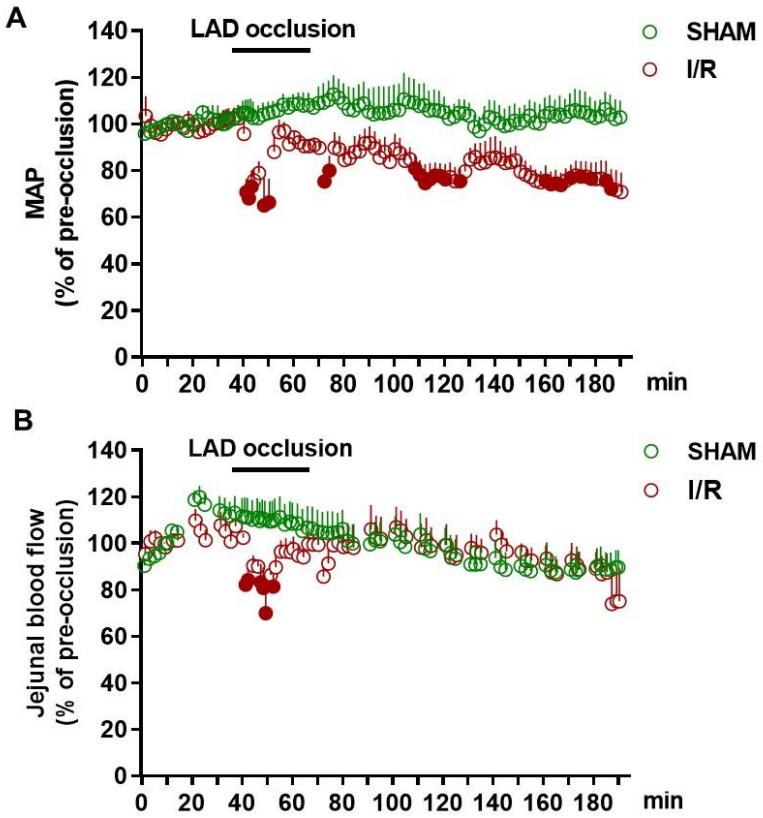


Figure 13. *The effects of sham operation and occlusion of left anterior descending (LAD) coronary artery for 30 min followed by 120 min of reperfusion on the mean arterial pressure (MAP,* ***A****) and jejunal blood flow (****B****). Results are expressed as percentages of the baseline values registered at 10 min (i.e., 30 min before LAD occlusion). Filled circles represent p<0.05 vs. respective SHAM values. Circles and error bars represent mean + SEM. For statistical analysis, two-way repeated measures ANOVA was used, followed by Holm-Sidak post hoc test, n=6-9/group.*

4.2 Effects of chronic administration of selective COX-2 inhibitors, rofecoxib and celecoxib on mesenteric I/R injury

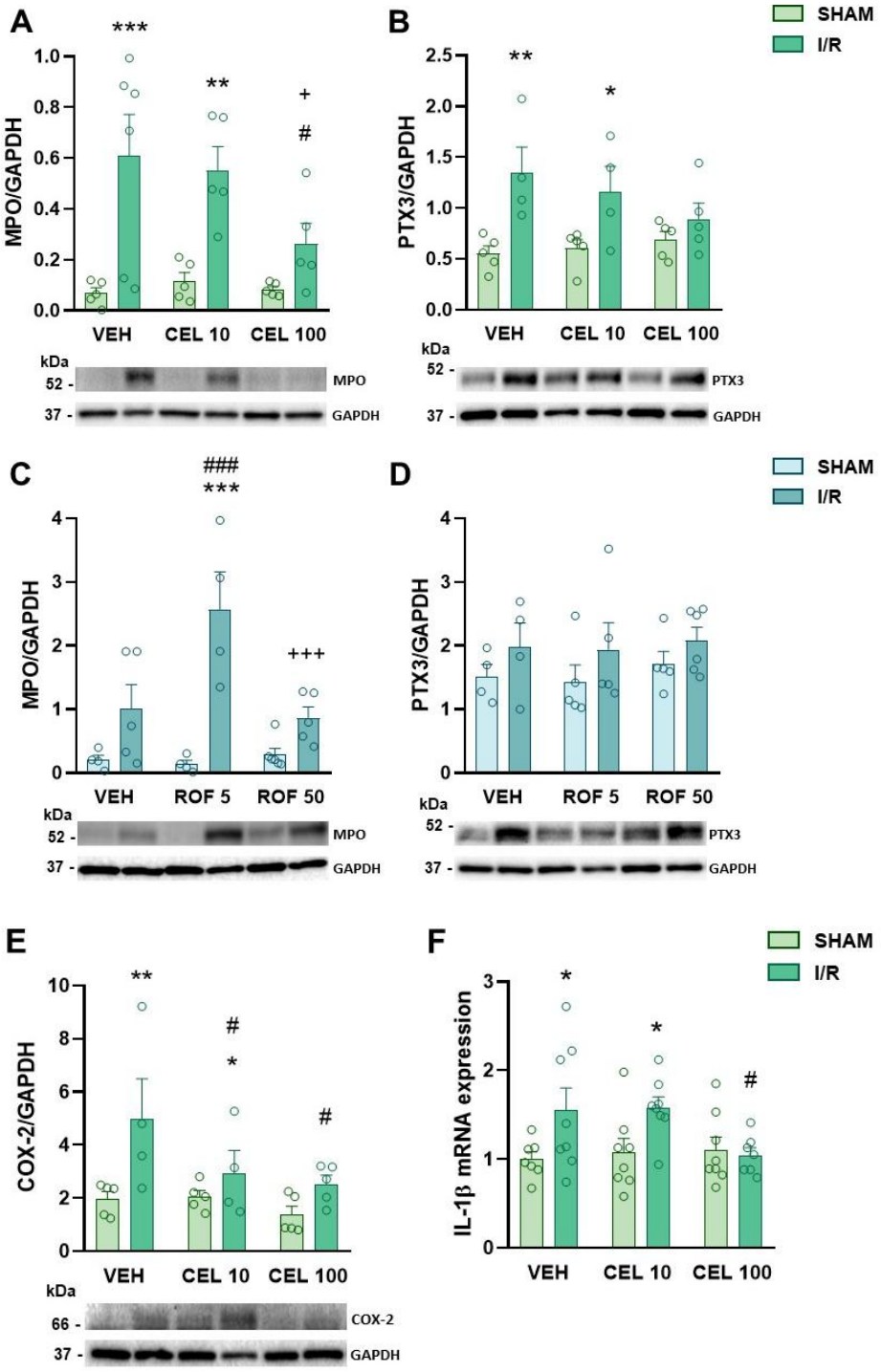
4.2.1 Celecoxib, but not rofecoxib, reduced the severity of intestinal inflammation induced by mesenteric I/R

There were no observable macroscopic alterations in the small intestines of the sham-operated rats. In contrast, the intestines of rats subjected to mesenteric I/R were characterized by lividity, edema, and hemorrhages. Our primary objective was to evaluate the severity of intestinal inflammation induced by mesenteric I/R in both control (vehicle-treated) and COX-2 inhibitor-treated animals. As the recruitment and activation of neutrophils are crucial components of I/R-induced inflammation and mucosal injury (10), the tissue levels of the neutrophil marker MPO were quantified via Western blot analysis. Intestinal I/R was associated with an upregulation of MPO, which was partially mitigated by celecoxib treatment. However, a significant reduction in MPO was only achieved in animals receiving the higher dose of celecoxib. Conversely, rofecoxib failed to mitigate the I/R-induced increase in MPO levels; notably, at a lower dose, it further augmented this effect (Figures 14A and 14C).

Subsequently, the protein levels of pentraxin 3 (PTX3) were measured, a long pentraxin family member released by various cell types in response to proinflammatory cytokines and other inflammatory signals. PTX3 plays a pivotal role in regulating I/R-induced intestinal inflammation (133). Among the treatments tested, only the highest dose of celecoxib prevented the I/R-induced increase in PTX3 (Figures 14B and 14D).

Treatment with celecoxib, but not with rofecoxib, also attenuated the intestinal upregulation of COX-2 protein in animals subjected to mesenteric I/R (Figures 14E and 4G) as well as the increase of IL-1β mRNA levels (Figures 14F and 14H), as quantified by qRT-PCR, a well-established inducer of COX-2 expression (134).

Collectively, of the two pharmacological agents tested, only celecoxib diminished the severity of intestinal inflammation provoked by mesenteric I/R. However, even the higher dose of celecoxib resulted in only a partial reduction of the inflammatory mediators measured. There were no signs of inflammation in any of the sham-operated rats, suggesting that 8-day administrations of celecoxib and rofecoxib had no significant impact on intestinal mucosal integrity.


A
B
C
D
E
F
SHAM
I/R
MPO/GAPDH
PTX3/GAPDH
COX-2/GAPDH
IL-1β mRNA expression
VEH
CEL 10
CEL 100
ROF 5
ROF 50
kDa
MPO
PTX3
COX-2
GAPDH
52 -
37 -
66 -

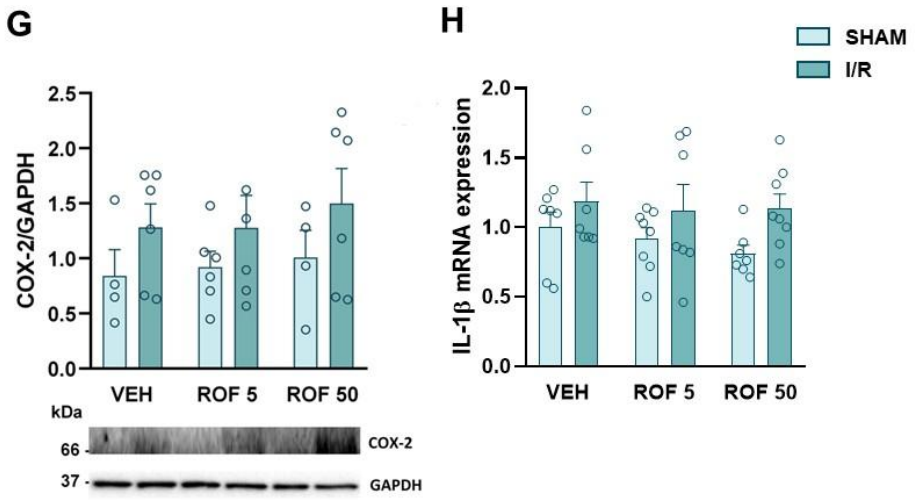


Figure 14. *Small intestinal levels of myeloperoxidase (MPO,* ***A****,* ***C****), pentraxin 3 (PTX3,* ***B****,* ***D****), cyclooxygenase-2 proteins (COX-2,* ***E****,* ***G****), and mRNA levels of interleukin-1β (IL-1β,* ***F****,* ***H****) in rats treated with vehicle (VEH), celecoxib (CEL, 10 and 100 mg/kg) or rofecoxib (ROF, 5 and 50 mg/kg) for 8 days and then subjected to sham operation or mesenteric ischemia/reperfusion (I/R). Circles represent the data of each rat, bars indicate the mean + SEM. For statistical analysis two-way ANOVA was used, followed by Fisher's LSD post hoc test, n=4-8/group (*p<0.05, **p<0.01, ***p<0.001 vs respective SHAM, $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs VEH I/R, $^{+}$p<0.05, $^{+++}$p<0.001 vs CEL 10 I/R).*

4.2.2 The effects of celecoxib and rofecoxib on the intestinal mucosal integrity in mesenteric I/R injury

4.2.2.1 Neither celecoxib nor rofecoxib mitigated the histological injury caused by mesenteric I/R.

In the next step, it was examined whether treatment with celecoxib and rofecoxib could mitigate mucosal injury induced by intestinal I/R. No significant mucosal damage was observed in any of the sham-operated rats, although, in some animals treated with the higher dose of celecoxib, we observed some mild changes, such as slightly dilated villi and epithelial detachment (subepithelial Gruenhagen's space) (Figures 15 and 16). Mesenteric I/R elicited various morphological alterations, ranging from Gruenhagen's space or destruction of the epithelium to more severe damage to the villi and occasionally even the crypts. Interestingly, celecoxib treatment did not influence I/R-induced histological injury at the tested doses, despite its effect in reducing mucosal inflammation (Figure 15). Similarly, neither dose of rofecoxib impacted the I/R-induced histological changes in the mucosa (Figure 16).

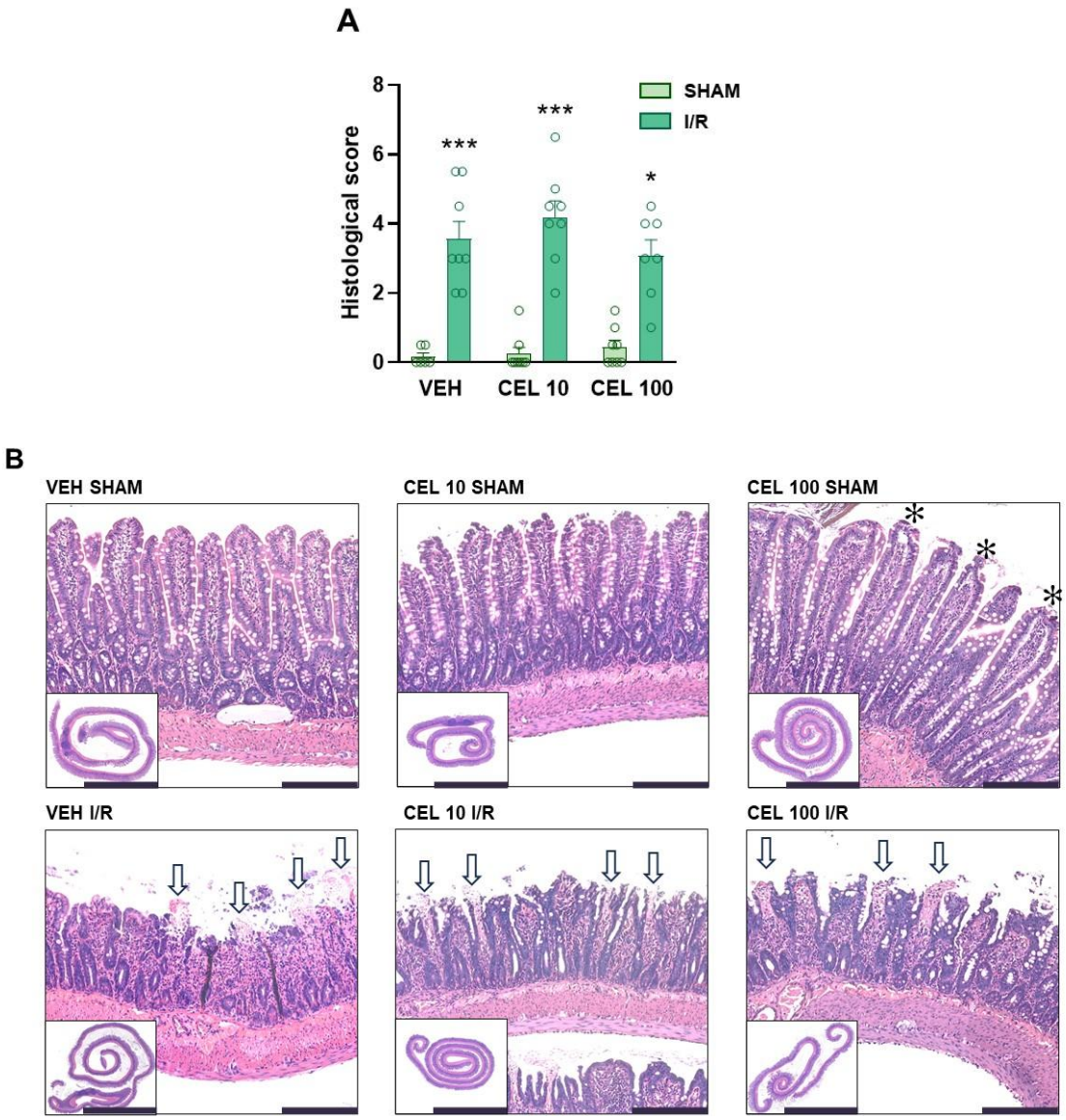


Figure 15. *The effect of vehicle (VEH) and celecoxib (CEL, 10 and 100 mg/kg) on the histomorphology of the small intestinal mucosa in sham-operated and mesenteric ischemia/reperfusion (I/R)-exposed rats.* ***Panel A:*** *Histological scores. Circles represent the data of each rat, bars indicate the mean + SEM. For statistical analysis, Kruskal-Wallis test was performed, followed by uncorrected Dunn's test, n=7-8/group (*p<0.05, ***p<0.001 vs respective SHAM).* ***Panel B:*** *Representative histological images of the small intestines of VEH- and CEL-treated rats. Hematoxylin and eosin staining, low magnification scale bar (lower left images): 5 mm, high magnification scale bar: 200 µm. White arrows mark denuded villi with exposed lamina propria and capillaries, asterisks show moderate lifting of the epithelial layer from the lamina propria (Gruenhagen's space).*

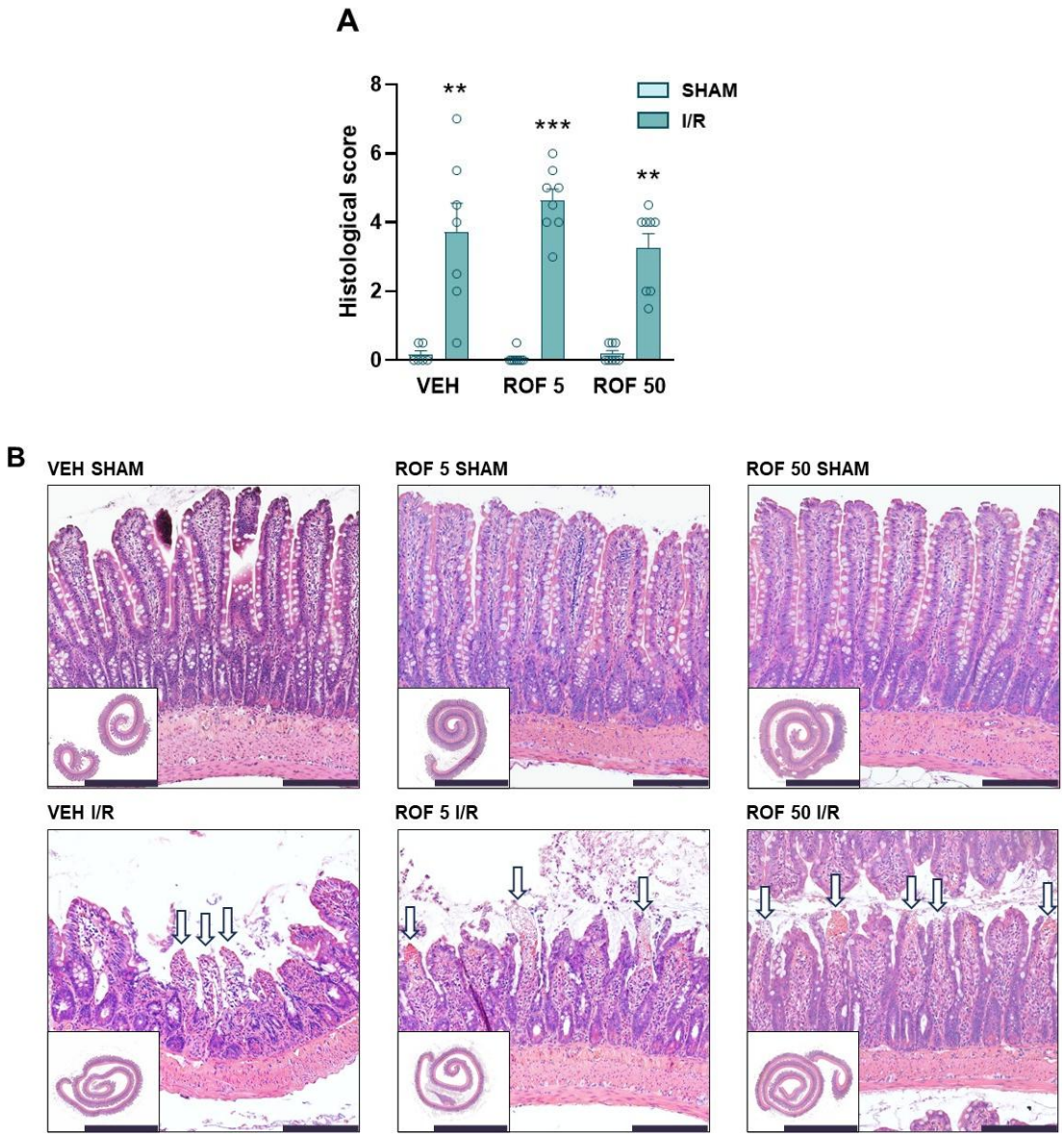


Figure 16. *The effect of vehicle (VEH) and rofecoxib (ROF, 5 and 50 mg/kg) on the histomorphology of the small intestinal mucosa in sham-operated and ischemia/reperfusion (I/R) -exposed rats.* ***Panel A:*** *Histological scores. Circles represent the data of each rat, bars indicate the mean + SEM. For statistical analysis, Kruskal-Wallis test was performed, followed by uncorrected Dunn's test, n=7-8/group (**p<0.01, ***p<0.001 vs respective SHAM).* ***Panel B:*** *Representative histological images of the small intestines of VEH- and ROF-treated rats. Hematoxylin and eosin staining, low magnification scale bar (lower left images): 5 mm, high magnification scale bar: 200 µm. White arrows mark denuded villi with exposed lamina propria and capillaries.*

4.2.2.2 *High-dose celecoxib, but not rofecoxib, prevented the disruption of tight junction proteins*

According to most literature data, I/R-induced mucosal damage is associated with the disruption of tight junction proteins, such as claudin-1 and occludin (135). Consequently, we evaluated their expression using Western blot analysis. The jejunal level of claudin-1 was significantly decreased in response to I/R in both cohorts, and this effect was abrogated by the higher dose of celecoxib, but not by rofecoxib (Figures 17A and 17C). The measurement of occludin provided essentially similar results; the reduction in occludin expression elicited by I/R was mitigated by celecoxib, whereas it was not abolished by rofecoxib (Figures 17B and 17D).

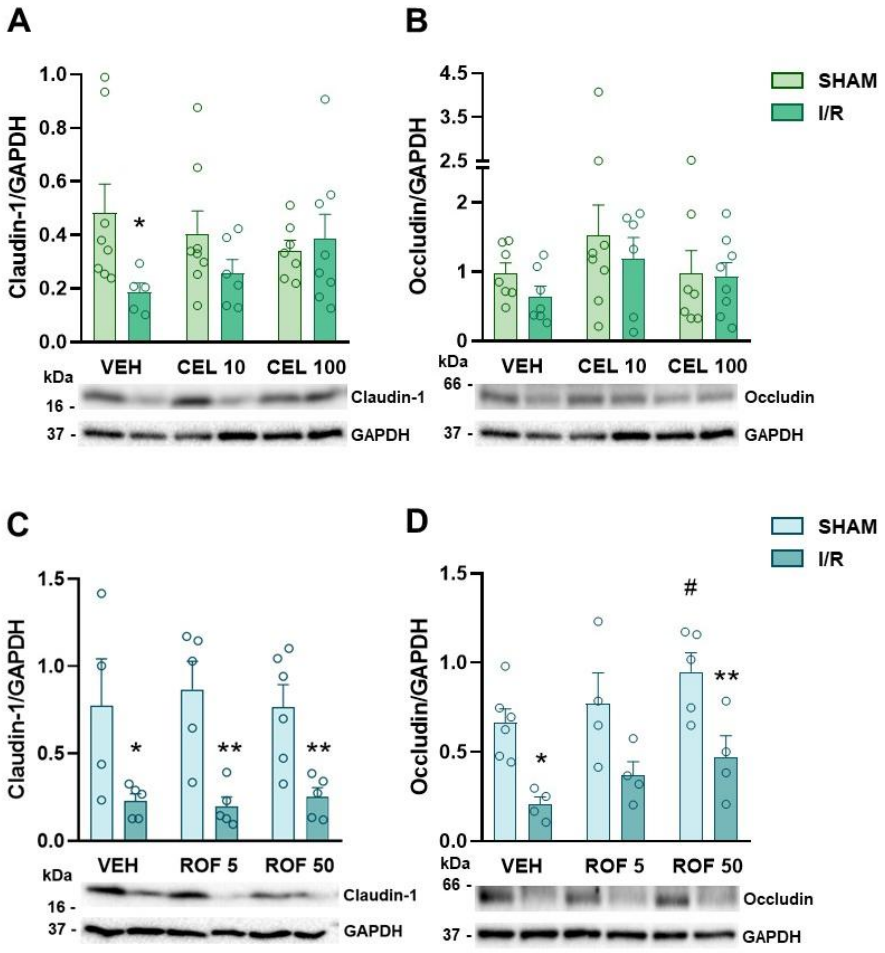


Figure 17. *Small intestinal expressions of claudin-1 (**A**, **C**) and occludin proteins (**B**, **D**) in rats treated with vehicle (VEH), celecoxib (CEL, 10 and 100 mg/kg) or rofecoxib (ROF, 5 and 50 mg/kg) for 8 days and then subjected to sham operation or mesenteric ischemia/reperfusion (I/R). Representative bands of claudin-1 (**A**, **C**) and occludin (**B**, **D**) are derived from the same animals; therefore, images of GAPDH proteins are identical. Circles represent the data of each rat, bars*

*indicate the mean + SEM. For statistical analysis, two-way ANOVA was used, followed by Fisher's LSD post hoc test, n=4-8/group (*p<0.05, **p<0.01 vs respective SHAM, $^{\#}$p<0.05 vs VEH SHAM).*

4.2.3 Celecoxib increased I/R-induced intestinal apoptosis, whereas rofecoxib reduced the I/R-induced phosphorylation of Akt

Given that celecoxib and rofecoxib exhibited differential effects on intestinal inflammation and tight junction proteins despite both nearly completely inhibiting COX-2 activity at the administered doses (112, 136), it was hypothesized that the observed difference might be independent of COX inhibition, and we evaluated the levels of some known off-targets of COX-2 inhibitors.

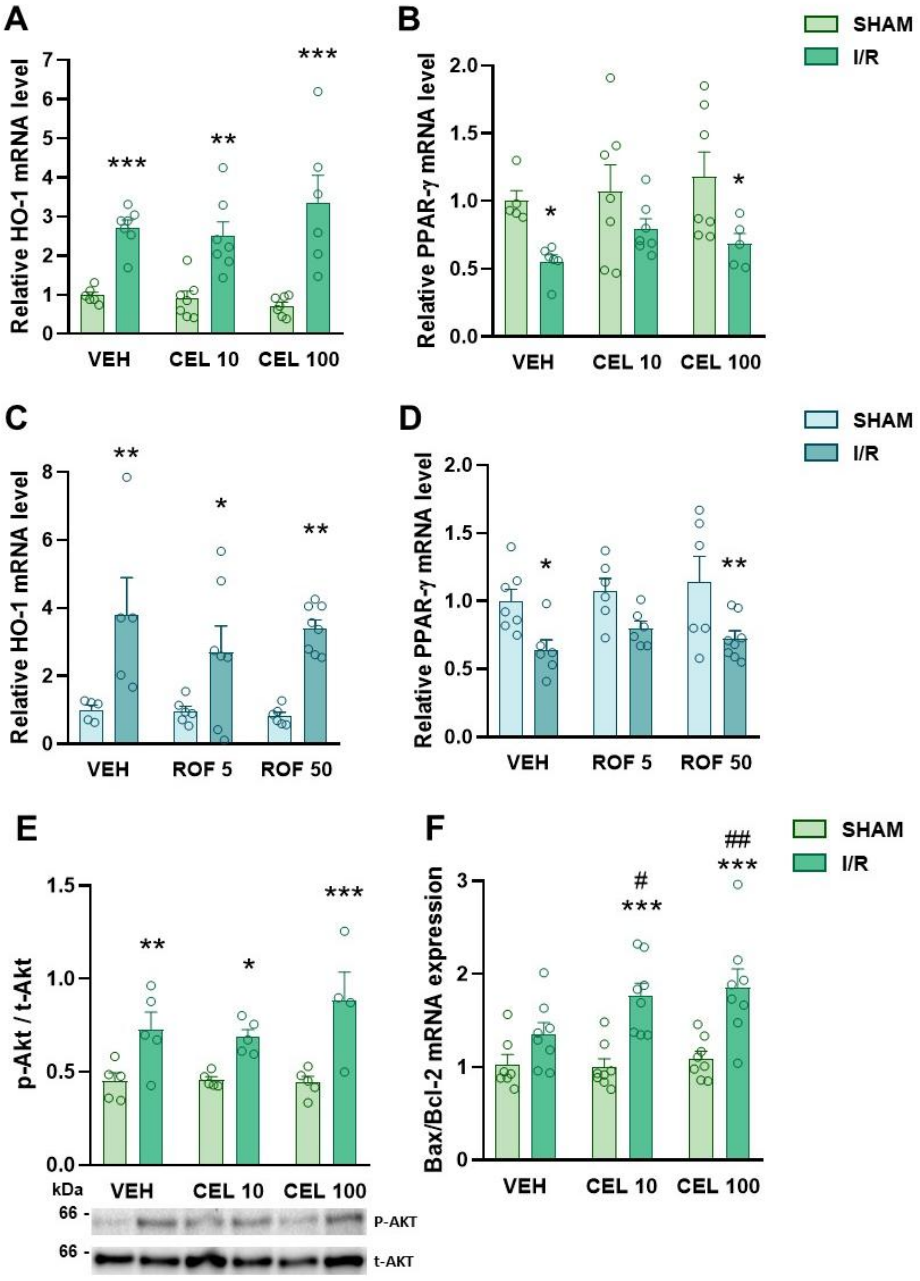
Initially, the expression of the stress-inducible gene HO-1 was quantified. This gene has previously been shown to be upregulated by celecoxib *in vitro*, but not by rofecoxib (137, 138). HO-1 is known to be induced during intestinal I/R, where it contributes to tissue protection (9). Although our findings confirmed that I/R increases HO-1 expression, no differences were observed between the HO-1 levels of vehicle- and drug-treated animals, either in the sham-operated or I/R-injured groups (Figures 18A and 18C).

Next, the expression of peroxisome proliferator-activated receptor gamma (PPAR-γ), a nuclear receptor, was assessed, which is able to mediate anti-inflammatory effects in intestinal I/R injury and activated by several COX inhibitors (139), including celecoxib and rofecoxib (140, 141). Mesenteric I/R significantly reduced intestinal PPAR-γ expression, but neither the basal nor the I/R-induced expression levels of PPAR-γ were significantly affected by celecoxib or rofecoxib (Figures 18B and 18D).

Since celecoxib can activate the phosphoinositide 3-kinase (PI3K)/Akt pathway (137), which has been shown to reduce inflammation and barrier damage in intestinal I/R injury (142), the phosphorylation of Akt was also determined. Contrary to expectations, celecoxib treatment had no effect on phospho-Akt levels in either the sham-operated or I/R-injured groups, while rofecoxib prevented the I/R-induced elevation of phospho-Akt in a dose-dependent manner (Figures 18E and 18G).

Finally, it is known that apoptosis is a major form of epithelial cell death induced by I/R injury (18). Celecoxib has also been shown to increase epithelial apoptosis (143). Therefore, we investigated whether the inability of celecoxib to mitigate I/R-induced mucosal injury, despite its anti-inflammatory effect, might be related to increased apoptosis. Consequently, the gene expression of Bax and Bcl-2 was measured and the Bax/Bcl-2 ratio, an important marker of apoptosis, was calculated (144). The Bax/Bcl-2 ratio exhibited a modest, non-significant increase in response to I/R, which was enhanced by celecoxib but unaffected by rofecoxib (Figures 18F and 18H).

Collectively, celecoxib augmented I/R-induced apoptosis, whereas rofecoxib reduced the I/R-induced elevation of phospho-Akt.



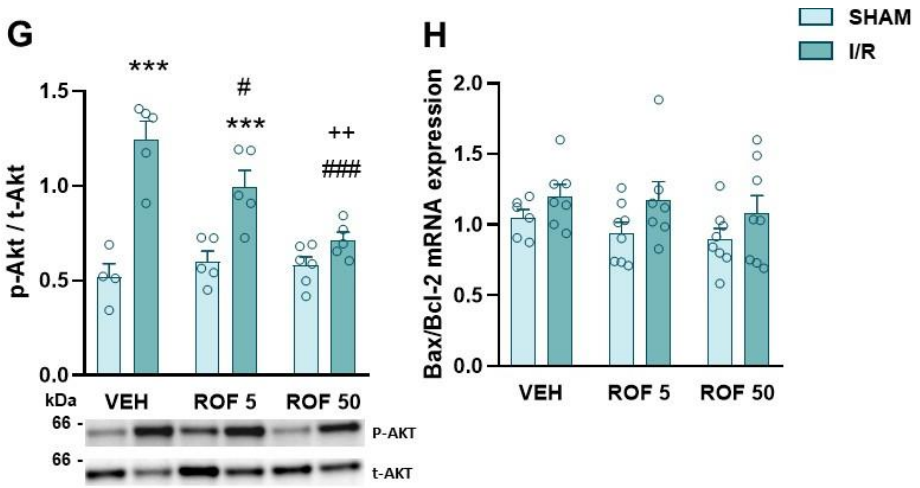


Figure 18. *Gene expression of heme oxygenase-1 (HO-1,* ***A****,* ***C****) and peroxisome proliferator-activated receptor-γ (PPAR-γ,* ***B****,* ***D****), and the ratio of phosphorylated to total Akt protein (p-Akt/Akt,* ***E****,* ***G****) and Bax to Bcl-2 mRNA (****F****,* ***H****) in the small intestine of rats treated with vehicle (VEH), celecoxib (CEL, 10 and 100 mg/kg) or rofecoxib (ROF, 5 and 50 mg/kg) for 8 days and then subjected to sham operation or mesenteric ischemia/reperfusion (I/R). Circles represent the data of each rat, bars indicate the mean + SEM. For statistical analysis two-way ANOVA was used, followed by Fisher's LSD post hoc test, n=4-8/group (*p<0.05, **p<0.01, ***p<0.001 vs respective SHAM, $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs VEH I/R, $^{++}p<0.01$ vs ROF 5 I/R).*

4.2.4 High-dose celecoxib, but not rofecoxib, reduced the activity of COX-1 in the small intestine

Finally, considering that both COX-2 and COX-1 can mediate prostanoid release during inflammatory processes (64), it was investigated whether the differential effects of high-dose celecoxib and rofecoxib on intestinal I/R inflammation could be attributed to their distinct influence on COX-1 activity. To this end, the total COX activities were quantified in the small intestines of celecoxib- and rofecoxib-treated animals using an assay kit, and the contribution of COX-1 to total COX activity was evaluated by measuring the COX activities of the same samples both in the presence and absence of SC-560, a highly selective COX-1 inhibitor.

Mesenteric I/R significantly augmented intestinal total COX activity in vehicle-treated rats (VEH SHAM vs VEH I/R, p=0.009) (Figure 19A). In contrast, COX activity in celecoxib- and rofecoxib-treated I/R-exposed animals remained comparable to that of the vehicle-treated sham-operated group (VEH SHAM vs CEL I/R and ROF I/R, p=0.85 and p=0.21, respectively), indicating that both celecoxib and rofecoxib treatments could abrogate the I/R-induced elevation of COX activity. However, celecoxib, but not rofecoxib, also reduced total COX

activity in sham-operated animals (VEH SHAM vs CEL SHAM, p=0.003) (Figure 19B). Additionally, giving the COX-1 inhibitor SC-560 into the samples decreased total COX activity in both vehicle- and rofecoxib-treated sham-operated rats (VEH SHAM vs VEH SHAM + SC-560, p=0.002, ROF SHAM vs ROF SHAM + SC-560, p=0.006), but it did not further reduce the COX activity in celecoxib-treated sham-operated animals.

These findings imply that high-dose celecoxib attenuated COX-1 activity in the small intestine, whereas rofecoxib maintained selectivity for COX-2 at its elevated dose.

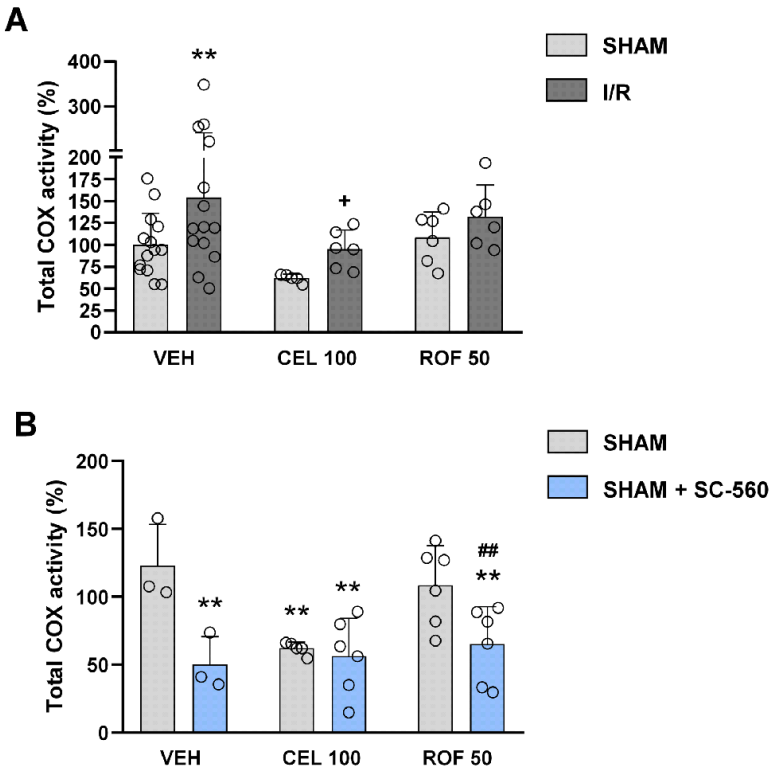


***Figure 19. Panel A**: Total cyclooxygenase (COX) activity in the small intestine of rats treated with vehicle (VEH), celecoxib (CEL, 100 mg/kg), or rofecoxib (ROF, 50 mg/kg) for 8 days and then subjected to sham operation or mesenteric I/R. **Panel B**: COX activity of some samples was also measured in the presence of SC-560, a highly selective COX-1 inhibitor, to assess the contribution of COX-1 and COX-2 to total COX activity. Circles represent the data of each rat, bars indicate the mean + SEM. For statistical analysis, two-way ANOVA was used, followed by Fisher's LSD post hoc test, n=3-14/group (**$p<0.05$ vs VEH SHAM, $^{+}p<0.05$ vs VEH I/R, $^{\#\#}p<0.01$ vs ROF 50 SHAM).*

5 Discussion

The small intestinal mucosa is highly susceptible to I/R injury (27, 28). Despite its crucial physiological role in nutrient absorption and the potentially life-threatening consequences of extensive damage, relatively little research has been conducted on protective strategies against I/R-induced injury in the small intestine. Furthermore, no known pharmacological agents are currently capable of effectively preserving small intestinal tissue.

Moreover, significant damage to the small intestine facilitates the translocation of aggressive luminal factors and bacteria into the circulation, potentially leading to systemic tissue injury and increasing the risk of sepsis and MOF (23, 30-32). In light of these concerns, our research aimed to assess whether COX-2 inhibitors provide protective effects in local and remote intestinal I/R models.

COX-2 is rapidly upregulated during inflammatory processes and plays a key role in producing pro-inflammatory cytokines and chemokines (55, 64, 87). Therefore, we hypothesized that inhibiting COX-2 activity could mitigate both local and remote intestinal damage by reducing the inflammatory cascade associated with I/R injury.

5.1 Chronic rofecoxib treatment reduced cardiac I/R-induced intestinal injury

5.1.1 Chronic rofecoxib treatment alleviated the cardiac I/R-induced mild histological injury in the intestine

A key finding of the present study is that chronic administration of rofecoxib can alleviate histological damage in the small intestine following cardiac I/R. It is crucial to emphasize that rofecoxib reduced cardiac I/R injury in these rats (111), in line with previous studies, which indicated that different COX-2 inhibitors provide cardioprotection after both permanent and transient myocardial ischemia (92, 97-100). However, it is worth noting that the infarct size-reducing effect of rofecoxib has limited clinical relevance (111), as this compound was withdrawn from the market due to serious adverse cardiovascular effects observed in the VIGOR and APPROVe trials (68, 72). Nevertheless, it has proven to be a valuable test compound for analyzing the remote intestinal effects of selective COX-2 inhibition on myocardial protection after cardiac I/R.

Importantly, this study provides the first evidence of significant histopathological changes in the rat small intestine as early as 2 hours following reperfusion of the ischemic myocardium. Recent investigations have reported that secondary intestinal damage can also

occur in response to both transient and permanent coronary artery occlusion in rodent models, as well as in MI patients, as well as in MI patients (44, 45, 145). Furthermore, intestinal injury may increase the risk of adverse cardiovascular outcomes post-MI, highlighting the critical need for early detection and preventive interventions to reduce remote intestinal damage following cardiac I/R (45). In these studies, based on the time points of analysis and the identification of morphological and functional changes, intestinal injury was expected to develop over days to weeks (44, 45). However, our findings reveal that structural mucosal histological changes in the intestine occur two hours after reperfusion of the ischemic myocardium, indicating a much more rapid onset, similar to I/R-induced injury in the limbs or the kidney (34-36). At this early stage, remote intestinal histopathological changes are primarily characterized by mild subepithelial edema, increased vascular permeability, and cellular infiltration of leukocytes.

5.1.2 Rofecoxib treatment reduced cardiac I/R-induced intestinal inflammation and also increased the level of the antioxidant SOD

The histological alterations were associated with a significant increase in COX-2 protein expression in the jejunum, indicating an early cellular response to I/R across various organ systems (53, 54, 84), including the small intestine (55, 56). Furthermore, COX-2 upregulation has been observed in tissues distant from the primary site of ischemic injury (90, 146). However, the role of selective COX-2 inhibition on ischemia reperfusion injury is controversial. In some models, selective COX-2 inhibition has been shown to exert a protective effect (55, 56, 88-90, 92, 98, 99), whereas in other experiments it has demonstrated a deleterious effect (53, 54, 93, 95).

The local overproduction of RONS has been extensively documented in I/R injury (2, 5), increasing the production of antioxidant enzymes such as CAT and SOD also in the intestine (128). However, activity of antioxidant enzymes in I/R models has demonstrated contradictory results, varying according to species, tissue type, and the specific experimental design. For instance, CAT and SOD activities are frequently reported to decrease immediately following reperfusion in cerebral and renal I/R models (147-149). In contrast, elevated CAT and SOD activities have been observed during a 4-hour reperfusion period following 4 hours of ischemia in limb tissues (150), and after 30 minutes of ischemia followed by 60 minutes of reperfusion in cardiac tissue (151).

During I/R injury, increased expression of COX-2 may play a role in enhancing antioxidant defenses by modulating the expression or activity of antioxidant enzymes (53, 91). However, the inhibition or genetic deletion of COX-2 may abrogate these protective effects,

resulting in aggravated tissue injury and diminished antioxidant response (53, 91). Our results show a significant increase in SOD activity in the rofecoxib-treated I/R group, indicating that rofecoxib may enhance SOD enzyme activity under conditions of oxidative stress. Celecoxib has been demonstrated to enhance SOD activity following renal and hepatic I/R injury (89, 152-154). In an intestinal I/R model, Li and Zheng reported that I/R exposure led to increased mucosal concentrations of malondialdehyde and NO (94). Whereas the treatment with the selective COX-2 inhibitor parecoxib resulted in a dose-dependent attenuation of intestinal RONS levels, along with a concomitant elevation in SOD enzymatic activity (90). Collectively, these findings suggest that COX-2 inhibitors can exert a positive modulatory effect on tissue antioxidant capacity, probably via the inhibition of PG synthesis (155). To the best of my knowledge, there is no published evidence whether rofecoxib directly upregulates SOD expression or increases the SOD activity through off-target mechanisms.

In the remote intestinal I/R injury model, we noted an early, mild upregulation of intestinal COX-2 following cardiac I/R injury. Despite the histopathological evidence of tissue injury, jejunal cytokine levels and oxidative stress markers exhibited either no significant changes or only minor fluctuations after cardiac I/R. These findings suggest that the cardiac I/R model utilized in this study resulted in only mild intestinal injury, which was insufficient to trigger substantial cytokine release or oxidative damage, unlike other remote intestinal I/R models with comparable reperfusion durations (1–5 hours) (36). Notably, a 6-minute episode of cardiac arrest followed by cardiopulmonary resuscitation in rats caused histopathological injury in the jejunum within 6 hours, while a significant increase in tissue cytokine levels was detected only after 24 hours (156). These data show that the severity and onset of intestinal inflammation may largely depend on the I/R model employed, and histological analysis could reveal early morphological changes preceding significant cytokine elevation. In conclusion, these results suggest that the protective effect of rofecoxib was likely not due to inhibition of COX-2 at the intestinal level but was initiated at a remote site, most probably in the heart, which thereafter prevented the development of intense inflammation and higher upregulation of COX-2 in the gut.

5.1.3 MMP-2 likely plays a crucial role in the development of cardiac I/R-induced remote intestinal damage, and its plasma level is influenced by rofecoxib.

Various circulating mediators have been implicated in the pathophysiological mechanisms underlying remote organ dysfunction following I/R. MMP-2 and MMP-9, which are responsible for the degradation of EMC, are primarily involved in breaking down collagen

type IV, a major component of the basement membrane (15, 16). They can be activated by oxidative stress and cytokine signaling during I/R, leading to increased vascular permeability, enhanced neutrophil infiltration, and the release of pro-inflammatory cytokines, which exacerbate tissue damage (157, 158).

MMPs are crucial in cardiovascular diseases, contributing to conditions such as aneurysm formation, coronary artery disease, atherosclerosis, MI, and heart failure (159-161). MMP-2 and MMP-9 particularly facilitate ECM degradation, supporting both tissue repair and remodeling following cardiac injury (107, 130, 161). MMP-2 plays a vital role in cardiomyocyte contractility, proliferation, apoptosis, and transcriptional regulation in different organs (162, 163). MMP-9 is primarily synthesized by inflammatory cells such as macrophages and neutrophil granulocytes (130). MMP-9 influences early-stage arterial remodeling in the heart, increases arterial pressure, and contributes to cardiac failure (164). Examination of MMP-2 and -9 may be useful in the investigation of remote I/R damage, as they are involved in the pathogenesis of remote pulmonary injury following limb I/R (132, 165).

However, their role in remote intestinal injury remains underexplored. Our study provides the first evidence that MMP-2, but not MMP-9, contributes to early-stage remote intestinal damage following cardiac injury. A significant correlation was observed between the gelatinolytic activity of plasma MMP-2 and intestinal histopathological scores, while no such correlation was found for MMP-9. This suggests that only specific MMP isoforms are involved in the pathogenesis of intestinal injury after cardiac I/R, at least within the present experimental conditions.

Rofecoxib prevented the elevation of plasma MMP-2 activity in response to cardiac I/R, and the decrease in plasma MMP-2 activity correlated with a reduction in intestinal histological scores. The low plasma MMP-2 activity observed in rofecoxib-treated I/R animals likely results from the inhibition of MMP-2 release or synthesis rather than direct inhibition of MMP-2 enzymatic activity since rofecoxib did not inhibit MMP-2 gelatinolytic activity *in vitro*. These findings are consistent with previous studies showing that COX-2 and PGE_2 upregulate MMP-2 expression in tumor cells and atherosclerotic plaques (166, 167). Moreover, pharmacological inhibition of COX-2 has been shown to reduce MMP-2 activity by suppressing its gene transcription (166, 167). Therefore, the protective effect of rofecoxib in mitigating remote intestinal injury following cardiac I/R may be due to its cardioprotective properties, potentially linked to reduced MMP-2 release from the heart.

Given that circulating MMP-2 during myocardial I/R may significantly contribute to remote intestinal injury, MMP-2 inhibitors could serve as potential therapeutic targets. MMP

inhibitors, such as doxycycline and o-phenanthroline, can mitigate myocardial damage during I/R injury (129, 168-170). This aligns with our finding that cardiac-derived MMP-2, but not MMP-9 release, may contribute to small intestinal damage.

Additionally, there are endogenous MMP inhibitors known as tissue inhibitors of metalloproteinases (TIMPs) (171). TIMPs regulate the pericellular proteolysis of ECM and cell surface proteins by inhibiting MMP activity and are an important component of ECM turnover regulation (172). Elevated levels of TIMPs are believed to result in ECM accumulation, leading to fibrosis, whereas the loss of TIMP responses results in enhanced matrix proteolysis (173). TIMP-2 and MMP-2 exhibit a dual regulatory interaction involving both activation and inhibition, with structural and functional nuances critical to ECM dynamics (174). TIMP-2 facilitates the activation of pro-MMP-2 by forming a trimolecular complex with MMP-14 (MT1-MMP) (174). While at higher concentrations, TIMP-2 binds to the catalytic site of active MMP-2, blocking its proteolytic activity (174). A broad-spectrum MMP inhibitor, such as ilomastat, was evaluated in cardiac I/R models, resulting in infarct size reduction (175, 176), but its application is limited due to off-target effects in clinical use. Certain synthetic MMP-2 inhibitors, such as MMPI-1154, have been tested in acute MI, where they reduced the infarct area by 50% (175).

5.1.4 The impairment of intestinal microcirculation is unlikely to contribute to remote intestinal damage after cardiac I/R.

To investigate whether compromised intestinal microcirculation plays a role in the pathogenesis of remote intestinal injury induced by cardiac I/R, we assessed jejunal perfusion using laser Doppler imaging. Our findings indicate that the impairment of local microcirculation is unlikely to be a primary driver of remote intestinal injury. Namely, cardiac I/R resulted in only a moderate (30%) and transient decline in jejunal perfusion in our experiment. Importantly, previous studies suggest that the small intestine can tolerate ischemic episodes lasting up to 2 hours without sustaining significant injury (177). This protection is maintained as long as blood flow remains above 50% of baseline levels (177). Under these conditions, oxygen consumption is preserved (177).

5.2 Celecoxib and rofecoxib exert different impact on mesenteric I/R injury

In the first study, we identified that the administration of COX-2 inhibitor rofecoxib attenuated mucosal injury in the small intestine elicited by myocardial I/R insult. Due to these

observations, the subsequent phase of our research was designed to evaluate whether pharmacological inhibition of COX-2 confers a protective effect in the local mesenteric I/R-induced tissue damage. Rofecoxib was selected due to its previously established efficacy in mitigating cardiac I/R-associated remote intestinal injury. Celecoxib was chosen due to its clinical availability and its high pKa-value (pKa: 11,1) (112). Non-acidic character promotes avoiding the direct topical, irritative effects of coxibs (112).

According to the available literature, the role of COX-2 in mesenteric ischemia-reperfusion remains inconclusive. Based on the majority of available, though limited, studies, both non-selective and selective COX-2 inhibitors appear to confer varying degrees of protection against I/R–induced inflammation and tissue injury in the small intestine (55, 56, 94, 105, 106, 178-180). Although COX-2 knockout mice exhibited more severe tissue injury following mesenteric I/R (93). Moreover, variations in COX-2 inhibitors may contribute to the observed differences in outcomes. Selective COX-2 inhibitors like FK3311 and NS-398 were shown to reduce intestinal injury caused by I/R (55, 105, 180), although the protective effect of NS-398 was also found to vary depending on sex (107). On the other hand, celecoxib and firocoxib showed only limited protection in rats (56, 106), and the results with parecoxib have been mixed across different studies (94, 178).

These discrepancies may arise from variations in research methodologies, such as differences in the experimental animal models employed or the I/R protocols utilized. Alternatively, they might be attributed to the distinct pharmacological characteristics of the COX-2 inhibitors. Consistent with this, our present data indicate that celecoxib, but not rofecoxib, alleviated I/R-induced intestinal inflammation, reinforcing the hypothesis that COX-2 inhibitors can exhibit divergent efficacy in mitigating intestinal I/R injury.

5.2.1 Celecoxib and rofecoxib demonstrated different effects on mesenteric I/R-induced intestinal inflammation

This study highlights the contrasting effects of two selective COX-2 inhibitors, celecoxib and rofecoxib, on small intestinal inflammation induced by mesenteric I/R in rats. Celecoxib, administered at a dose (10 mg/kg) sufficient to nearly completely and selectively inhibit COX-2 activity (116), demonstrated only a marginal effect in alleviating I/R-induced intestinal inflammation. In contrast, a higher dose of celecoxib (100 mg/kg) significantly reduced tissue inflammation in rats exposed to mesenteric I/R. Conversely, rofecoxib, which has a greater selectivity for COX-2 than celecoxib (181), was ineffective in alleviation of

inflammation. These findings imply that selective inhibition of COX-2 alone may not be able to alleviate I/R-induced small intestinal inflammation, and the variability in the protective effects of certain COX-2 inhibitors may be influenced by additional mechanisms.

5.2.2 Celecoxib and rofecoxib did not reduce the histological damage associated with mesenteric I/R, although high-dose celecoxib effectively preserved the integrity of tight junction proteins

Celecoxib, administered at a dose of 10 mg/kg, showed only limited effectiveness in preventing the I/R-induced loss of intestinal tight junction proteins and had no significant impact on the extent of histological damage. These findings align partially with previous studies, which demonstrated that celecoxib at the same dosage provided only partial protection against intestinal injury in a comparable experimental protocol using female rats (56). However, while high-dose celecoxib prevented the mesenteric I/R-provoked loss of tight junction proteins, it still failed to mitigate mucosal injury.

In addition, rofecoxib, which is more selective for COX-2 than celecoxib (181), was ineffective in mitigating histological injury, even at high doses. Additionally, rofecoxib did not prevent the mesenteric I/R-induced disruption of tight junction proteins. Taken together, these results imply that selective COX-2 inhibition alone does not suffice to protect the small intestine from I/R-induced histological damage. A possible explanation for this may involve the complex role of COX-derived PGs in the gut, which have both proinflammatory and mucoprotective functions (64, 66). Inhibition of COX-2 and its PG products can mitigate inflammation by reducing vasodilation, vascular permeability, and immune cell activation. However, the decreased PG levels resulting from COX-2 inhibition may also impair mucosal circulation, further delaying the healing of already compromised tissues (64, 66).

5.2.3 Rofecoxib reduced the I/R-induced phosphorylation of Akt, whereas celecoxib increased I/R-induced intestinal apoptosis

Previous studies have revealed several non-COX molecular targets that can be influenced by COX inhibitors, potentially affecting both their therapeutic outcomes and adverse effects (182, 183). Notably, different COX inhibitors exhibit distinct abilities to modulate these signaling pathways. For example, celecoxib, unlike rofecoxib, has been found to upregulate the expression of HO-1, an enzyme that plays a cytoprotective and anti-inflammatory role, by reducing ROS production and activating Akt (117, 137, 138). In contrast, both celecoxib and rofecoxib have been shown to increase the expression of PPAR-γ (140, 184). Activation of HO-

1, Akt, and PPAR-γ has been shown to confer protection against intestinal I/R injury (9, 139, 142). Additionally, PPAR-γ has been implicated in modulating the protective effects of the selective COX-2 inhibitor NS-398 against I/R injury (105). Based on these findings, we hypothesized that the differential effects observed between celecoxib and rofecoxib may, at least in part, reflect the contribution of COX-independent molecular pathways. However, our analysis revealed no significant alterations in HO-1 or PPAR-γ gene expression, nor Akt phosphorylation, in the intestinal tissue of celecoxib-treated animals. Notably, while rofecoxib exhibited no impact on these molecular markers under sham conditions, it significantly attenuated the I/R-induced activation of Akt. The PI3K/Akt signaling cascade plays a pivotal role in regulating cell survival and inflammatory responses (185). Therefore, it is plausible that the limited anti-inflammatory efficacy of rofecoxib may stem from its inability to sustain Akt activation following I/R insult. Nevertheless, this interpretation requires further experimental validation.

The dual role of PGs may explain the phenomenon that celecoxib had anti-inflammatory effects but did not protect against mesenteric I/R-induced mucosal damage. PGs have been shown to protect against I/R-induced intestinal damage by reducing epithelial apoptosis (93, 96), whereas a reduction in COX-2 expression in mice lacking Toll-like receptor 4 (TLR4), MyD88, or lysophosphatidic acid type 2 receptor (LPA2R) was associated with diminished inflammation but enhanced mucosal damage in various gut injury models (93, 186, 187). The potential involvement of increased apoptosis in limiting celecoxib's protective effect is further supported by the significantly elevated Bax to Bcl-2 ratio in celecoxib-treated I/R-exposed rats. Notably, rofecoxib had no effect on this ratio, consistent with previous studies reporting that celecoxib induces a more pronounced apoptotic response than rofecoxib (143, 188). This also suggests that the pro-apoptotic impact of celecoxib in this model is independent of COX-2 inhibition.

5.2.4 Selective COX-2 inhibition is not sufficient to protect intestinal mucosa from mesenteric I/R injury

In rats exposed to mesenteric I/R, the 100 mg/kg dose of celecoxib significantly reduced inflammation and prevented the loss of tight junction proteins, in contrast to all doses of rofecoxib. Previous studies examining whole blood TXA_2 synthesis or PGE_2 levels in the dorsal skin or small intestine as indicators of COX-1 activity suggested that 100 mg/kg dose of celecoxib retains selectivity for COX-2 (116, 189, 190). However, our observations indicate

that high-dose celecoxib may have partially inhibited intestinal COX-1 activity. Specifically, total COX activity in the small intestine of celecoxib-treated sham-operated rats was lower compared to vehicle-treated rats and more similar to the activity observed in samples treated with the COX-1 inhibitor SC-560. The histological analysis revealed mild morphological alterations in the mucosa of sham-operated rats treated with high-dose celecoxib (Figures 15A and 15B), suggesting a partial inhibitory effect on COX-1. This observation is consistent with the understanding that mucosal injury typically requires concurrent inhibition of both COX-1 and COX-2 (191, 192). Thus, the enhanced efficacy of high-dose celecoxib in our model may be explained by COX-1 inhibition. Evidence suggests that COX-1-preferential drugs, such as flunixin and flurbiprofen, can also reduce I/R-induced intestinal inflammation (56, 179), supporting the conception that COX-1-derived prostanoids contribute to inflammatory processes (64). Additionally, drugs with lower selectivity for COX-2, such as piroxicam and meloxicam, were found to be more effective than parecoxib in reducing intestinal I/R damage (178). These findings suggest that inhibiting both COX isoforms may be crucial for effectively alleviating severe inflammation induced by intestinal I/R.

6 Limitations

Some limitations should be considered in the study of remote intestinal injury following cardiac I/R. Rofecoxib was chosen due to its physicochemical properties, specifically, its relatively high pKa value (8.6), in contrast to more acidic COX-2 inhibitors, such as etoricoxib (pKa: 4.6) and parecoxib (pKa: 4.9) (112, 193). This characteristic of rofecoxib may reduce the risk of direct, topical GI mucosal damage often associated with acidic NSAIDs. However, it has been withdrawn from the market, as the results have less translational value.

Furthermore, the association between MMP-2 and remote intestinal damage is based on correlation analysis, and further studies are required to determine whether MMP-2 activity plays a direct role in intestinal injury. Moreover, since heart samples from infarcted animals were used for other studies, we do not have data on cardiac COX-2 and MMP-2 expression.

Both studies were conducted on male Wistar rats, despite known sex-based differences in I/R injury outcomes (194-196). While COX-2 inhibition has shown protective effects in both sexes (56, 98), the results cannot be directly extrapolated to females, and further research is necessary to evaluate sex-specific responses. Lastly, the I/R models utilized - 30 minutes of ischemia followed by 120 minutes of reperfusion - are commonly used in experimental protocols but may not completely replicate clinical cardiac or mesenteric I/R conditions.

7 Conclusions

7.1. In the initial experiment, the findings are summarized as follows:

7.1.1. Early histopathological alterations were observed in the rat small intestine as early as two hours after cardiac I/R injury.

7.1.2. Remote intestinal damage was accompanied by mild mucosal inflammation, which was mitigated by rofecoxib administration. Furthermore, rofecoxib treatment enhanced the activity of SOD in rats subjected to I/R injury. These findings suggest that prolonged rofecoxib administration effectively ameliorates remote intestinal injury resulting from cardiac I/R.

7.1.3. The observed intestinal changes were correlated with an increase in circulating MMP-2 activity, but not MMP-9, indicating that plasma MMP-2 activity may serve as a potential biomarker for the early detection and assessment of intestinal damage induced by cardiac I/R.

7.1.4. The transient and moderate (15-30%) reduction in small bowel circulation is unlikely to have contributed significantly to the observed intestinal injury.

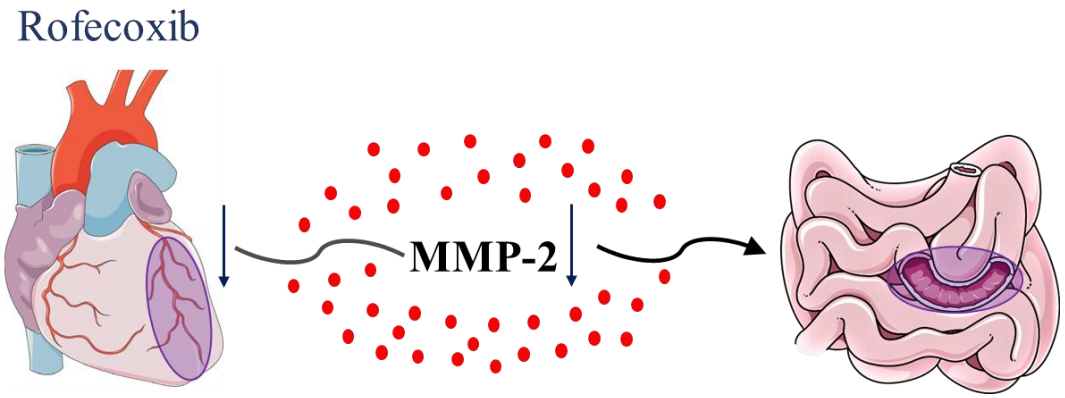


Figure 20. Rofecoxib attenuates cardiac I/R-induced remote intestinal injury by reducing MMP-2 activity.

Rofecoxib mitigates cardiac ischemia/reperfusion (I/R)-induced remote intestinal injury, likely by reducing matrix-metalloprotease-2 (MMP-2) activity at the cardiac level, potentially through decreased synthesis and/or release of MMP-2 as a result of a smaller myocardial infarct size. This figure contains artworks produced by Servier Medical Art (http://smart.servier.com).

7.2. Since rofecoxib was shown to protect against I/R damage to the small intestine in the previous model, it was used in the local intestinal I/R experiment. Celecoxib, a clinically approved selective COX-2 inhibitor, was included in the second model. This second

investigation evaluated the effects of two selective COX-2 inhibitors on mesenteric I/R-induced injury, with the following key findings:

7.2.1. Celecoxib was more effective compared to rofecoxib in mitigating I/R-induced small intestinal inflammation in rats.

7.2.2. Although celecoxib effectively inhibited intestinal inflammation, even at high doses, it failed to prevent the histomorphological alterations observed in the I/R-affected mucosa.

7.2.3. The pro-apoptotic properties of celecoxib may contribute to its inability to protect against tissue damage, despite its anti-inflammatory effects. Additionally, rofecoxib reduced Akt phosphorylation during I/R, which may account for its limited anti-inflammatory efficacy.

7.2.4. Celecoxib exhibited anti-inflammatory effects only at the higher dose, at which it lost selectivity for COX-2, suggesting that COX-2 alone is not responsible for I/R-induced intestinal damage. It indicates that COX-1 may also contribute to the pathological process.

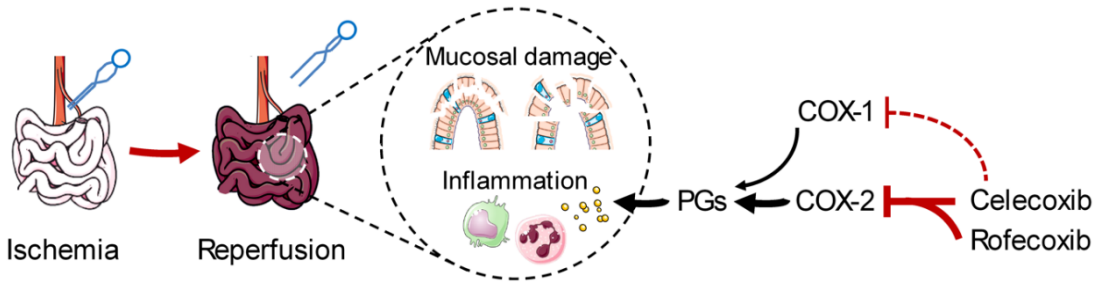


Figure 21. High-dose celecoxib, but not rofecoxib, alleviated mesenteric I/R-induced intestinal inflammation.

120 minutes of reperfusion following 30 minutes of upper mesenteric artery (SMA) occlusion induced jejunal inflammation and histopathological injury. Treatment with the selective cyclooxigenase-2 (COX-2) inhibitors celecoxib and rofecoxib did not mitigate the histopathological injury. However, administration of high-dose celecoxib attenuated small intestinal inflammation, via inhibition of COX-1 activity, potentially due to the reduction of proinflammatory and pro-apoptotic effects mediated by prostaglandins (PGs). Figure 21 contains artworks produced by Servier Medical Art (http://smart.servier.com).

8 Summary

Myocardial I/R can induce remote organ injury, including that of the small intestine, which may contribute to its overall morbidity and mortality. Therefore, we investigated whether cardiac I/R elicits early remote alterations in the intestinal tissue and whether prolonged administration of rofecoxib - a selective COX-2 inhibitor - can attenuate these effects. Notably, histopathological examination revealed mild mucosal inflammation in the small intestine as early as two hours following cardiac I/R, which was independent of cardiac I/R-induced altered mesenteric perfusion. The intestinal involvement and the protective effect of rofecoxib were correlated with elevated plasma activity of MMP-2 but not MMP-9. Rofecoxib appears to exert protective effects on remote intestinal tissues, potentially through cardioprotection via infarct size reduction. These findings suggest that plasma MMP-2 may serve as an early biomarker for cardiac I/R-induced intestinal injury.

As rofecoxib was shown to be protective in remote I/R-induced small intestinal injury in the previous model, it was chosen as a test compound in the study of mesenteric I/R, which still has a high mortality rate in clinical practice. We also applied celecoxib as a clinically used selective COX-2 inhibitor in the local intestinal I/R injury model.

Celecoxib was more effective compared to rofecoxib in alleviating I/R-induced small intestinal inflammation in rats. Celecoxib demonstrated anti-inflammatory effects only at higher doses, at which it lost its selectivity for COX-2. This suggests that COX-2 inhibition alone may not be sufficient to prevent I/R-induced intestinal damage, and that COX-1 may also play a role in the pathological process. However, neither celecoxib, even at high doses, nor rofecoxib was able to prevent the histological damage induced by mesenteric I/R. The pro-apoptotic properties of celecoxib may explain its lack of protective effect against tissue injury, despite its anti-inflammatory actions. Moreover, rofecoxib was found to reduce Akt phosphorylation during I/R, which may account for its limited anti-inflammatory efficacy.

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