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

PhD thesis

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1 Introduction

1.1 Pathomechanism of ischemia/reperfusion injury

Ischemia/reperfusion (I/R) injury is a significant pathological process characterized by tissue damage following the restoration of blood flow after ischemia. Initially, ischemia disrupts mitochondrial oxidative phosphorylation, leading to anaerobic glycolysis, lactic acid accumulation, and ATP depletion. Reperfusion paradoxically worsens injury by generating reactive oxygen species (ROS), leading to oxidative stress, calcium overload, and mitochondrial dysfunction through the opening of mitochondrial permeability transition pores (mPTPs).

These biochemical events activate antioxidant responses (e.g., catalase (CAT), superoxide dismutase (SOD), heme oxygenase-1 (HO-1)) and initiate inflammation. Cytokines and chemokines (e.g., tumor necrosis factor-α (TNF-α), interleukin (IL) -1β, IL-6) promote neutrophil infiltration, amplifying tissue injury through enzymes such as myeloperoxidase (MPO) and matrix metalloproteinases (MMPs). MMPs degrade extracellular matrix (ECM) components, contributing to inflammation and tissue remodeling. I/R injury leads to both necrosis and apoptosis, the latter being increasingly recognized as a key marker of injury severity.

1.2 Remote organ damage in I/R injury

I/R injury affects not only the target organ but also distant tissues via systemic inflammation and circulating mediators.

The small intestine is particularly sensitive to I/R due to its high metabolic rate and the anatomical vulnerability of villus tip enterocytes. Ischemic damage increases mucosal permeability and promotes bacterial translocation, contributing to systemic inflammation and multiple organ failure. Studies show that I/R in organs such as the heart or kidneys can induce histological and functional damage in the intestine, highlighting its role in systemic pathophysiology.

In cardiac conditions like myocardial infarction or during cardiopulmonary bypass (CPB), remote intestinal injury has been observed. Increased gut permeability correlates with systemic inflammation and worsened clinical outcomes, suggesting that intestinal barrier dysfunction contributes to post-cardiac I/R complications.

1.3 Mesenteric I/R injury

Mesenteric I/R injury results from the restoration of blood flow to previously ischemic intestinal tissue, and reperfusion exacerbates injury via oxidative stress, leukocyte activation, and endothelial dysfunction. Mesenteric ischemia is associated with high mortality, which can arise from arterial occlusion, non-occlusive ischemia (e.g., due to shock or heart failure), or venous thrombosis. It can occur in different surgical settings, such as organ transplantation or CPB. Despite low prevalence, early diagnosis remains difficult, and treatment is primarily surgical.

There are no approved pharmacological agents currently that prevent mesenteric I/R injury. However, pharmacological interventions would be beneficial in reducing reperfusion-related harm.

1.4 Cyclooxygenase enzymes and COX-2 inhibitors

Cyclooxygenase (COX) enzymes convert arachidonic acid to prostaglandins (PGs). COX-1 is constitutively expressed and involved in maintaining gastric, renal, and vascular homeostasis. COX-2 is typically inducible and is upregulated during inflammation, including I/R injury.

Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit COX enzymes and are categorized as non-selective and selective COX-2 inhibitors. While selective COX-2 inhibitors (coxibs) reduce inflammation with fewer gastrointestinal side effects, concerns emerged about cardiovascular risks due to altered prostanoid

balance—specifically reduced prostacyclin (PGI_2) and increased thromboxane A_2 (TXA_2).

Selective COX-2 inhibitors such as celecoxib, etoricoxib, and parecoxib remain in use, though with caution. Rofecoxib, despite market withdrawal, is still used in experimental models due to its high COX-2 selectivity.

1.5 COX-2 inhibition in I/R injury

COX-2 expression increases in various organs during I/R injury, including the heart, kidneys, liver, and intestines. However, studies report conflicting effects of selective COX-2 inhibition. In cardiac models, COX-2 inhibitors can reduce apoptosis and inflammation, while others suggest that COX-2-derived PGs may be protective for cardiomyocytes. Similarly, in hepatic I/R injury, both beneficial and detrimental effects of COX-2 inhibition have been reported. Renal I/R studies reveal complex outcomes, with COX-2 inhibitors both attenuating inflammation and impairing renal function.

These data suggest that COX-2 inhibitors may have organ-specific and context-dependent effects, influenced by dosage, timing, and the balance between inflammatory and protective PGs.

1.6 Selective COX-2 inhibitors in intestinal I/R injury

In intestinal I/R injury, COX-2 expression is upregulated early, making it a potential therapeutic target. Several studies demonstrate that selective COX-2 inhibitors can reduce histological damage and inflammation during intestinal I/R. However, COX-2-derived PGs also have mucoprotective functions—supporting mucosal healing, reducing apoptosis, and maintaining epithelial barrier integrity. Some studies show that COX-2 gene deletion worsens injury in intestinal I/R, mirroring findings in gastric models.

Thus, while selective COX-2 inhibitors may confer protective effects, they could also impair mucosal recovery, depending on the

timing and context of administration. This dual role emphasizes the complexity of COX-2 targeting in intestinal I/R injury and necessitates further investigation.

2 Objectives

2.1. Study I – Cardiac I/R-induced remote intestinal injury

The first objective was to examine whether rofecoxib, a highly selective COX-2 inhibitor, could alleviate intestinal injury induced by myocardial I/R. This study aimed to answer the following questions:

2.1.1 Can rofecoxib reduce early mucosal injury in the small intestine caused by cardiac I/R?

2.1.2 Does rofecoxib modulate inflammation and oxidative stress in remote intestinal tissue?

2.1.3 Are matrix MMPs involved in the intestinal effects of rofecoxib?

2.1.4 Does cardiac I/R impair intestinal blood flow? Can the possible reduction in intestinal microcirculation contribute to intestinal injury?

2.2. Study II – Mesenteric I/R-induced local intestinal injury

The second objective was to evaluate and compare the effects of two selective COX-2 inhibitors—celecoxib and rofecoxib—on intestinal injury induced by mesenteric I/R. Key research questions included:

2.2.1 Do celecoxib and rofecoxib reduce inflammation associated with mesenteric I/R?

2.2.2 Can these agents prevent or attenuate intestinal mucosal injury following local I/R?

2.2.3 Do non-COX-related properties of celecoxib and rofecoxib contribute to differences in their effects on mesenteric I/R?

2.2.4 Can the difference in COX-2 selectivity between celecoxib and rofecoxib explain the observed variation in intestinal protection?

3 Methods

3.1 Animals

Male Wistar rats were housed under controlled conditions (22 ± 2°C, 12 h light/dark cycle) with free access to food and water.

3.2 Ethical considerations

Experiments complied with Directive 2010/63/EU and were approved by the relevant ethical committees to minimize animal suffering.

3.3 Study design

Two main animal models were used:

1. *Cardiac I/R-induced remote intestinal injury*: Rats were treated daily once with vehicle or rofecoxib (5 mg/kg) for 28 days. On day 29, myocardial I/R injury was induced by 30 min LAD occlusion followed by 120 min reperfusion. At the end of reperfusion, intestinal samples were collected for further analysis.
2. *Small intestinal microcirculation after cardiac I/R*: In a separate rat cohort, jejunal microcirculation was measured by laser Doppler imaging during reperfusion after cardiac I/R.

3.4 Local small intestinal I/R injury model

Rats were treated with celecoxib (10 or 100 mg/kg), rofecoxib, or vehicle for 8 days. On day 8, superior mesenteric artery (SMA) occlusion for 30 min followed by 120 min reperfusion was performed. At the end of reperfusion, intestinal samples were collected for further analysis.

3.5 Macroscopic and histological evaluation

Macroscopic damage was graded on a 0–4 hyperemia scale. Jejunal tissues were formalin-fixed, paraffin-embedded, sectioned (4–5 μm), and hematoxylin and eosin-stained. For mesenteric I/R, the Swiss-roll technique was used to assess longer segments. Images were acquired via Olympus BX51 microscope with DP50 camera. Histological injury was blindly scored by two pathologists using the Mantyh scale (cardiac I/R) and Park/Chiu scale (mesenteric I/R).

3.6 Cytokine measurement

Jejunal cytokine levels (TNF-α, IL-1β, IL-10) were quantified by ELISA or multiplex assays and normalized to total protein.

3.7 Western blot

Western blotting was performed to assess protein expression in tissue samples. After SDS-PAGE and membrane transfer, membranes were incubated with primary and HRP-conjugated secondary antibodies, followed by chemiluminescent detection. Targets included COX-1, COX-2, MPO, PTX3, claudin-1, occludin, phospho-Akt, and total Akt. Protein levels were normalized to GAPDH or total Akt for phospho-Akt.

3.8 qRT-PCR

Total RNA was extracted from tissue samples and reverse-transcribed into cDNA. Quantitative real-time PCR was performed using gene-specific primers and fluorescent probes to monitor amplification. Expression levels of HO-1, PPAR-γ, IL-1β, IL-10, Bcl-2, and Bax were quantified relative to the reference gene Rpl13a.

3.9 COX enzyme activity assay

Total COX activity in intestinal homogenates was measured fluorometrically, with selective inhibition to distinguish COX-1 and COX-2 contributions.

3.10 6-keto $PGF_{1\alpha}$ quantification

Jejunal levels of 6-keto $PGF_{1\alpha}$ were measured by ELISA after tissue homogenization and sample preparation.

3.11 SOD and CAT assays

SOD and CAT activities in jejunal samples were measured with commercial kits. SOD activity, based on superoxide radical dismutation, was expressed as U/mg protein. CAT activity, measured via formaldehyde production, was reported in nmol/min/mg tissue.

3.12 Measuring MMP-2 and MMP-9 activity by gelatin zymography

Plasma MMP-2 and MMP-9 activities were assessed by gelatin zymography. Samples were run on gelatin gels, renatured, and incubated to reveal enzymatic digestion as clear bands. Band intensity was quantified against an internal standard. Rofecoxib's inhibitory effects on plasma MMP activity were also tested at different concentrations.

3.13 Statistical analysis

Data were presented as mean + SEM. Various ANOVA tests (one-way, two-way, repeated measures), nonparametric tests (Mann-Whitney, Kruskal-Wallis), and correlation analyses (Spearman) were applied based on data type. Post hoc tests included Fisher's LSD, Dunn's, and Holm-Sidak. All in vitro measurements were done at least in duplicates and repeated when possible. Outliers were removed via Grubb's test, and significance was set at $p<0.05$.

4 Results

4.1 4.1 Effects of rofecoxib on cardiac I/R-induced intestinal injury

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

A 30-minute LAD occlusion followed by 2-hour reperfusion did not induce visible macroscopic changes in the small intestines of either vehicle- or rofecoxib-treated rats. However, histological analysis revealed mild but significant jejunal mucosal alterations in vehicle-treated I/R rats compared to sham controls, mainly subepithelial edema, vascular dilation, and leukocyte infiltration, with largely preserved epithelial integrity. These histological changes were absent in rofecoxib-treated I/R rats, indicating COX-2 inhibition reduced remote intestinal injury.

4.1.2 Rofecoxib reduced intestinal inflammation and increased SOD activity post-cardiac I/R

Vehicle-treated I/R rats showed elevated intestinal COX-2 protein levels, which were prevented by rofecoxib. COX-1 levels remained unchanged across groups. Intestinal TNF-α protein levels did not differ between groups, while IL-10 levels were moderately elevated in vehicle-treated I/R rats but reduced by rofecoxib. 6-keto PGF1α levels tended to be higher in vehicle I/R rats, but without statistical significance. Total SOD activity increased significantly with rofecoxib in the I/R group, suggesting enhanced antioxidant defense, whereas CAT activity remained stable. These results indicate that mild intestinal inflammation and oxidative stress induced by cardiac I/R were alleviated by rofecoxib.

4.1.3 Plasma MMP-2 activity correlated with intestinal injury and was reduced by rofecoxib

Cardiac I/R increased plasma MMP-2 activity, which correlated positively with intestinal histological scores and was attenuated by rofecoxib. In contrast, MMP-9 activity showed no significant changes or correlation. In vitro assays confirmed rofecoxib did not directly inhibit MMP-2 or MMP-9 activity, suggesting its effect involves reduced MMP-2 expression rather than direct enzymatic inhibition.

4.1.4 Cardiac I/R caused mild, transient jejunal hypoperfusion

Laser Doppler imaging showed that cardiac I/R induced a moderate (~15%) but transient reduction in jejunal blood flow. Jejunal microcirculation normalized within 15 minutes after reperfusion, despite the fact that systemic blood pressure did not normalize and continued to decline during reperfusion. Thus, mild intestinal histological damage after cardiac I/R is unlikely due to sustained intestinal ischemia.

4.2 Effects of chronic COX-2 inhibitors on mesenteric I/R injury

4.2.1 Celecoxib, but Not Rofecoxib, Reduced Intestinal Inflammation After Mesenteric I/R

Mesenteric I/R caused macroscopic intestinal injury and elevated neutrophil marker MPO and pentraxin 3 (PTX3) levels. Celecoxib partially mitigated the increase in these inflammatory markers at high doses, whereas rofecoxib failed to reduce MPO or PTX3. Celecoxib also suppressed COX-2 protein and IL-1β mRNA upregulation induced by mesenteric I/R, effects not observed with rofecoxib.

4.2.2 Effects of celecoxib and rofecoxib on mesenteric I/R-induced mucosal damage

4.2.2.1 Neither celecoxib nor rofecoxib reduced histological injury

Histological examination revealed that mesenteric I/R induced mucosal damage, including epithelial disruption and villous injury. Neither celecoxib nor rofecoxib treatment at tested doses alleviated these histological changes, despite celecoxib's anti-inflammatory effects.

4.2.2.2 High-dose Celecoxib prevented disruption of tight junction proteins

Mesenteric I/R decreased jejunal claudin-1 and occludin protein levels, indicative of tight junction disruption. This effect was prevented by high-dose celecoxib but not by rofecoxib, suggesting differential impacts on mucosal barrier integrity.

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

Celecoxib and rofecoxib had different effects on intestinal inflammation and barrier proteins, suggesting mechanisms beyond COX-2 inhibition. Celecoxib increased the Bax/Bcl-2 ratio—a marker of apoptosis—following I/R injury, indicating a proapoptotic effect, whereas rofecoxib did not. Moreover, rofecoxib reduced the elevation in Akt phosphorylation observed following ischemia/reperfusion injury, in contrast to celecoxib.

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

To determine whether the distinct effects of high-dose celecoxib and rofecoxib on intestinal I/R inflammation were related to their

differential COX selectivity, total COX activity was measured in jejunal tissues, with and without the COX-1-selective inhibitor SC-560.

Mesenteric I/R significantly increased intestinal COX activity in vehicle-treated rats. This I/R-induced rise was prevented by both celecoxib and rofecoxib. Notably, celecoxib also reduced COX activity in sham-operated animals in contrast to rofecoxib. SC-560 also reduced COX activity in sham-operated rats. Its effect was mimicked by celecoxib, but not by rofecoxib, suggesting that high-dose celecoxib inhibited COX-1, whereas rofecoxib maintained COX-2 selectivity.

5 Discussion

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

This study shows that chronic rofecoxib treatment reduces small intestinal histological injury following cardiac I/R. Although rofecoxib was withdrawn from clinical use due to cardiovascular risks reported in the VIGOR and APPROVe trials, it remains a useful research tool for exploring the remote effects of COX-2 inhibition. The current findings confirm its cardioprotective effects and reveal, for the first time, significant intestinal histopathological changes as early as two hours after myocardial reperfusion. Prior studies suggested intestinal injury develops days to weeks post-MI, but this study indicates a much earlier onset. Observed intestinal damage includes subepithelial edema, increased vascular permeability, and leukocyte infiltration. These findings highlight the importance of early detection and intervention to mitigate remote intestinal injury after cardiac I/R.

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

Histological changes in the jejunum after cardiac I/R were associated with increased COX-2 expression, reflecting an early cellular stress response. Although selective COX-2 inhibition shows protective effects in some I/R models, others report worsened outcomes. Oxidative stress, marked by increased RONS, commonly induces antioxidant enzymes like CAT and SOD, but their activity varies across organs and experimental conditions. In this study, rofecoxib enhanced SOD activity under I/R. According to some literature data, similar findings were observed with celecoxib or parecoxib in I/R injury of other organs, indicating a potential antioxidant benefit of COX-2 inhibitors. These findings suggest that COX-2 inhibitors can exert a positive modulatory effect on tissue antioxidant capacity, probably via the inhibition of PG synthesis. Despite histological jejunal injury, cytokine and oxidative stress markers showed minimal changes, suggesting only mild remote intestinal injury in this cardiac I/R model. These data imply that rofecoxib's protective effect was likely mediated remotely, possibly at the level of the heart, rather than direct intestinal COX-2 inhibition.

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

MMP-2 and MMP-9 degrade extracellular matrix components and are activated by oxidative stress and cytokines during I/R, contributing to increased vascular permeability and neutrophil infiltration. They play key roles in cardiovascular diseases and tissue remodeling after cardiac injury, with MMP-2 affecting cardiomyocyte function and MMP-9 produced mainly by inflammatory cells. While their role in remote intestinal injury is not well understood, this study shows that plasma activity of MMP-2—but not MMP-9—correlates with early

intestinal damage after cardiac I/R. Rofecoxib prevented the rise in cardiac I/R-induced plasma MMP-2 activity. Rofecoxib might result in reduced intestinal injury by inhibiting MMP-2 release rather than its enzymatic function due to the lack of direct inhibition of MMP-2 in the plasma. This aligns with evidence that COX-2 inhibition reduces MMP-2 expression, linking rofecoxib's protective effect to its cardioprotective action. Tissue inhibitors of metalloproteinases (TIMPs) regulate MMP activity and extracellular matrix turnover. Selective MMP-2 inhibitors have shown promise in mitigating intestinal injury after cardiac I/R.

5.1.4 Impaired intestinal microcirculation is unlikely to play a role in the development of remote intestinal injury following cardiac I/R

To explore if impaired intestinal microcirculation contributes to remote intestinal injury after cardiac I/R, jejunal blood flow was measured using laser Doppler imaging. The results showed only a moderate (30%) and temporary reduction in jejunal perfusion. Previous studies indicate the small intestine can withstand ischemia up to 2 hours without major damage if blood flow stays above 50% of baseline. This level of perfusion maintains adequate oxygen consumption. Therefore, impaired microcirculation is unlikely to be the main cause of remote intestinal injury in this model.

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

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

This study compared the effects of two selective COX-2 inhibitors, celecoxib and rofecoxib, on intestinal inflammation caused by mesenteric I/R in rats. Celecoxib at a low dose (10 mg/kg) had minimal impact, while a higher dose (100 mg/kg) significantly reduced inflammation. Rofecoxib, despite being more selective for

COX-2, failed to alleviate intestinal inflammation at any tested doses (5 and 50 mg/kg). These results suggest that selective COX-2 inhibition alone may not effectively reduce I/R-induced intestinal inflammation. The differing protective effects may involve additional mechanisms beyond COX-2 inhibition.

5.2.2 Celecoxib and rofecoxib do not reduce mesenteric I/R-induced histological injury; however, high-dose celecoxib preserves tight junction proteins

Celecoxib at 10 mg/kg showed limited protection against I/R-induced loss of intestinal tight junction proteins and did not reduce histological damage, consistent with previous studies. Although a higher dose of celecoxib prevented the loss of tight junction proteins, it still failed to reduce mucosal injury. Rofecoxib, despite greater COX-2 selectivity, was ineffective in preventing both histological damage and tight junction disruption, even at high doses. These results suggest that selective COX-2 inhibition alone is insufficient to protect the small intestine from I/R injury. This may be due to the dual role of COX-derived prostaglandins, which have both proinflammatory and mucosal protective effects. Inhibiting COX-2 reduces inflammation but may also impair mucosal blood flow and delay tissue healing.

5.2.3 Rofecoxib reduces mesenteric I/R-induced Akt phosphorylation; celecoxib increases intestinal apoptosis

COX inhibitors can affect non-COX molecular targets, influencing their therapeutic and adverse effects. Celecoxib, in contrast to rofecoxib, upregulates HO-1, a cytoprotective enzyme, and activates the Akt signaling pathway. Both coxibs increase PPAR-γ expression. All of the mentioned off-target effects can be linked to protection against intestinal I/R injury. However, in this study, celecoxib did not alter HO-1, PPAR-γ gene expression, or Akt phosphorylation, whereas rofecoxib reduced I/R-induced Akt activation, which may

explain its limited anti-inflammatory effects. The PI3K/Akt pathway is crucial for cell survival and inflammation regulation, suggesting rofecoxib's inability to sustain Akt activation could limit its efficacy. Furthermore, celecoxib increased the pro-apoptotic Bax/Bcl-2 ratio after I/R, indicating its COX-2-independent pro-apoptotic effect, which was previously described in the literature. This suggests celecoxib's limited protective effect may be partly due to its promotion of apoptosis, which counterbalanced its anti-inflammatory action.

5.2.4 Selective COX-2 inhibition alone is insufficient to protect the intestinal mucosa from mesenteric I/R injury

In rats undergoing mesenteric I/R, a high dose (100 mg/kg) of celecoxib significantly reduced inflammation and prevented tight junction protein loss, compared to any tested doses of rofecoxib. Although 100 mg/kg celecoxib was described to be selective for COX-2, our results suggest it partially inhibited COX-1 activity. This partial COX-1 inhibition possibly contributed to its enhanced anti-inflammatory effect. This observation aligns with the current understanding that effective prevention of mucosal injury requires the simultaneous inhibition of both COX-1 and COX-2. Supporting this, COX-1-preferential drugs like flunixin reduce intestinal I/R inflammation, showing COX-1-derived prostanoids also drive inflammation. These findings suggest that inhibiting both COX isoforms may be necessary to effectively counteract severe intestinal I/R inflammation. Thus, celecoxib's dual inhibition at high doses may explain its superior protective effects in this model.

6 Conclusions

6.1. In the initial experiment, the findings are summarised as follows:

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

6.1.2. Remote intestinal damage was associated with mild mucosal inflammation, which was attenuated by rofecoxib treatment, indicating that prolonged administration of rofecoxib effectively mitigates remote intestinal injury following cardiac I/R.

6.1.3. The observed intestinal changes correlated with increased plasma MMP-2 activity, but not MMP-9, indicating MMP-2 as a potential biomarker for early detection and assessment of intestinal damage induced by cardiac I/R.

6.1.4. The brief and moderate decrease in small bowel circulation caused by cardiac I/R likely did not significantly contribute to the observed intestinal injury.

6.2. 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 was included in the experimental design to compare its effects on mesenteric I/R injury:

6.2.1. Celecoxib was more effective than rofecoxib in mitigating I/R-induced small intestinal inflammation in rats.

6.2.2. Despite celecoxib's anti-inflammatory effect, it failed to prevent the mucosal histomorphological alterations. Rofecoxib was also ineffective in preventing the I/R injury.

6.2.3. Celecoxib's pro-apoptotic effects may limit its tissue protection despite anti-inflammatory action, while rofecoxib's reduction of Akt phosphorylation during I/R may explain its reduced anti-inflammatory efficacy.

6.2.4. Celecoxib showed anti-inflammatory effects only at the higher dose, where COX-2 selectivity was lost, suggesting that COX-1 may also contribute.

7 Bibliography of the candidate's publications

7.1 Peer-reviewed publications related to the dissertation

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