NOVEL ASPECTS OF CARDIAC ISCHEMIA/REPERFUSION INJURY IN TRANSLATIONAL DRUG DEVELOPMENT

PhD thesis

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Budapest

2021

1. Introduction

1.1. Major reasons for attrition in drug development

Drug development is a time consuming, complex, and expensive process that consists of preclinical and clinical phases. Attrition in drug development in later phases, such as phase 3 and phase 4, may lead to sunk costs, increased patient risks and waste of time of scientists. The leading causes of attrition in drug development programs are related to unexpected cardiotoxicity and failure of translation of preclinical efficacy.

Some cardiotoxic effects may manifest only in the presence of cardiac diseases, e.g., in myocardial ischemia/reperfusion (I/R) conditions and/or in the presence of cardiovascular comorbidities and comedications. Since they remain undetected during preclinical and early clinical toxicology and safety studies, we termed this phenomenon "hidden cardiotoxicity". Hidden cardiotoxicity fails to be detected, since the current International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines require the assessment of drug safety only in healthy animals and healthy human volunteers.

High attrition rates in drug development and frequent clinical safety issues suggest that more sensitive methods that, e.g., model disease states are required for toxicity studies, including *in vivo*, *ex vivo* and *in vitro* models of myocardial I/R and/or comorbidities.

Another important aspect of attrition in drug development is related to failure of translating preclinical efficacy to clinical efficacy. To date, reperfusion therapy is the only available treatment option to reduce the risk of post-myocardial infarction heart failure (post-MI HF). A plethora of cardioprotective therapies effective even in large animal models have been described. Since failure rate of translation of efficacy to clinical phases is still considerable, there is a need for bridging the translational gap between preclinical and clinical trials. Potential reasons of the failed translation of the preclinical data might be the use of suboptimal post-MI HF animal models with low clinical relevance.

Clinically relevant pig models with long-term follow up and serial cardiac magnetic resonance imaging (CMRI) of post-MI HF are prerequisites for final proof-of-concept studies before entering into clinical trials in drug and medical device development.

1.2. Hidden cardiotoxicity of rofecoxib and need to detect it in preclinical safety assessment of drugs

Rofecoxib, a nonsteroidal anti-inflammatory drug (NSAID) from the group of selective cyclooxygenase-2 (COX-2) inhibitors, was withdrawn from the market in 2004 due to an increased risk of cardiovascular adverse effects observed in the VIGOR and APPROVe trials. A cumulative meta-analysis involving 42 174 patients concluded that rofecoxib should have been withdrawn several years earlier. Later, in another meta-analysis by Zhang et al, that included 116 094 participants, it was shown that the use of rofecoxib was associated with increased risk of arrhythmias. Other details and mechanisms of cardiotoxicity of rofecoxib were revealed including inhibition of protection against I/R injury, prevention of production of epi-lipoxins, increase in blood pressure and inhibition of vascular remodeling. Since none of the aforementioned cardiotoxicities had been revealed in safety assessment of rofecoxib, according to our definition, rofecoxib had hidden cardiotoxic properties. In addition, according to the scientific statement of the American Heart Association the use of COX-2 inhibitors should be avoided in HF patients, since they may exacerbate underlying myocardial dysfunction.

From details above we can conclude that early detection of hidden cardiotoxicity of rofecoxib could have reduce a number of serious adverse events that appeared only in phase IV trials and even after withdrawal.

Since the hidden cardiotoxicity of an unknown number of drugs, including rofecoxib, remained unrevealed during preclinical safety assessment, there is a need to develop novel methods to predict cardiotoxicities of drugs using *in vivo, ex vivo* and *in vitro* disease models such as I/R injury and to assess effect of drugs on intrinsic cardioprotection, e.g., elicited by ischemic preconditioning (IPC).

1.3. Need for improvement of translation efficacy of cardioprotective and post-myocardial infarction heart failure therapies

In addition to toxicity reasons, lack of efficacy is the another leading causes of attrition in drug development programs. Since failure rate of translation of efficacy to clinical phases is still considerable, there is a need for bridging the translational gap between preclinical and clinical trials. The way how to improve translation is a focus of great interest. According to ESC working group on cellular biology, improvement of the preclinical assessment of novel cardioprotective and post-MI HF therapies is needed. Potential reasons for

failed translation in terms of efficacy of cardioprotective therapies and therapies of post-MI HF are related to use of inadequate models, endpoints, study designs and protocols. Guideline on the relevance of rigor and reproducibility in preclinical studies on cardioprotection recommends CMRI as a clinically relevant model for measurement of ventricular function in pigs.
Based on aforementioned observations, it can be concluded that for higher translational efficacy the use of large animal model, e.g., pigs, and clinically relevant protocols and study designs are needed.
Clinically relevant pig models of post-MI HF are prerequisites for final proof-of-concept studies before entering clinical trials in most of the cardiovascular drug and medical device development projects. Therefore, a comprehensive characterization of the closed-chest post-MI HF models in Göttingen minipigs and Landrace pigs with the assessment of ventricular functions and anatomy using CMRI during long-term follow up may be useful for choosing the most fitting large animal models to study post-MI HF and to develop novel therapies.

2. Objectives

2.1. Establishing a preclinical model with I/R to detect hidden cardiotoxicity of rofecoxib

We aimed to investigate that hidden cardiotoxicity of rofecoxib could have been detected in early preclinical phases in pathological conditions using cellular- (*in vitro*), and small animal models (*ex vivo* and *in vivo*) of acute I/R injury and to test the effect of rofecoxib on intrinsic cardioprotection elicited by IPC.

2.2. Characterization of post-MI HF pig model that resembles human pathology

We also aimed to characterize post-MI HF in Göttingen minipigs in comparison to Landrace pigs to show whether any of these models reflect post-MI HF parameters comparable to humans.

3. Methods

3.1. Methods to reveal hidden cardiotoxicity of rofecoxib

With aim to investigate hidden cardiotoxicity of rofecoxib we used *in vivo, ex vivo* and *in vitro* models of acute I/R injury. Hidden cardiotoxicity of a drug is revealed if the drug inhibits cell survival signaling (induced by IPC) or activates deleterious cell signaling induced by I/R injury.

For *in vivo* experiments (Figure 1), male Wistar rats were treated with 5.12 mg kg−1 rofecoxib or with its vehicle, 1% hydroxyethylcellulose by oral gavage once daily for 4 weeks. Rofecoxib- and vehicle-treated animals were then subjected to I/R with or without IPC. I/R was induced by occlusion of the left anterior descending coronary artery (LAD) and IPC was elicited by 3 cycles of brief LAD occlusion and reperfusion before I/R. Animals received reperfusion. Mortality, arrhythmia analysis and infarct size (IS) were the endpoints.

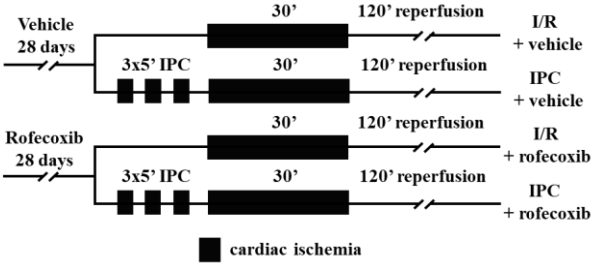

Figure 1. In vivo ischemia/reperfusion (I/R) injury study protocol. *IPC: ischemic preconditioning.*

For *ex vivo* experiments (Figure 2) left ventricular papillary muscles from male Wistar rats were used. Left ventricular papillary muscle preparations were mounted in a tissue chamber and were stimulated. Transmembrane potentials were recorded while using the conventional microelectrode technique. Papillary muscles were subjected to normoxic conditions (in normoxic solution) or to simulated I/R (sI/R) conditions (in ischemic solution) with or without IPC. Solutions contained either vehicle, 1 or 10 μM rofecoxib. Action potential parameters were quantified.

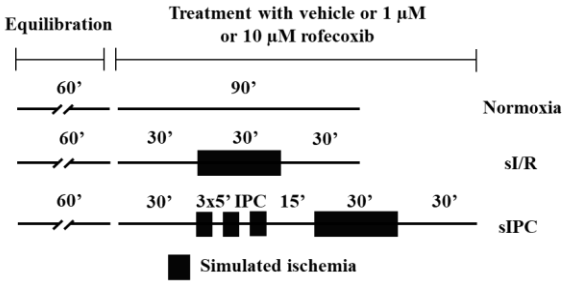

Figure 2. Ex vivo simulated ischemia/reperfusion (sI/R) injury study protocol. *sIPC: simulated ischemic preconditioning.*

For *in vitro* experiments (Figure 3) cells were isolated from hearts of adult male Wistar rats. Cells were subjected to normoxic (in normoxic solution) or to sI/R conditions (in ischemic solution). Solutions contained either vehicle or rofecoxib in increasing doses (0.1, 0.3, 1, 3, and 10 µM). Calcein staining was performed to assess cell viability.

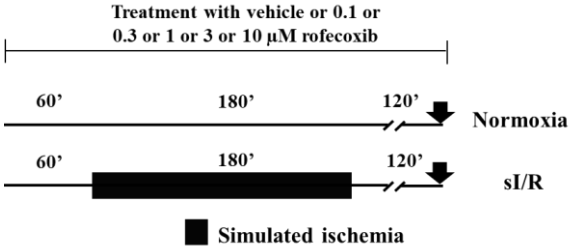

Figure 3. In vitro simulated ischemia/reperfusion (sI/R) injury study protocol.

3.2. Methods for characterization of post-MI HF pig model that resembles human pathology

With aim to characterize post-MI HF in Göttingen minipigs in comparison to Landrace pigs, myocardial infarction (MI) was induced by intraluminal balloon occlusion of the LAD for 120 min in Göttingen minipigs and for 90 min in Landrace pigs, followed by reperfusion. Body weights were measured, and CMRI was performed to assess cardiac morphology and function at baseline in both breeds and at 3 and 6 months in Göttingen minipigs and at 2 months in Landrace pigs, respectively.

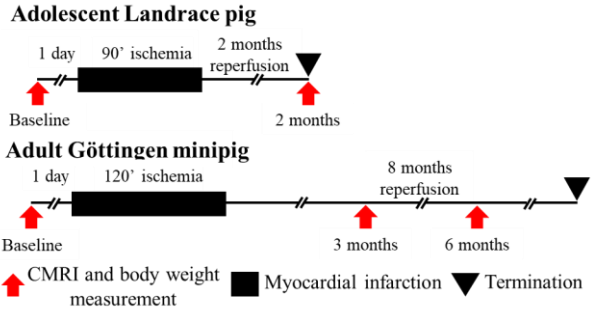

Figure 4. Experimental protocol for post-myocardial infarction-induced heart failure in Landrace pigs and Göttingen minipigs. *CMRI: cardiac magnetic resonance imaging.*

4. Results

4.1. Chronic rofecoxib treatment increased acute mortality during cardiac ischemia/reperfusion

Rofecoxib treatment increased the mortality rate (Figure 5) as compared to the pooled data of other groups.

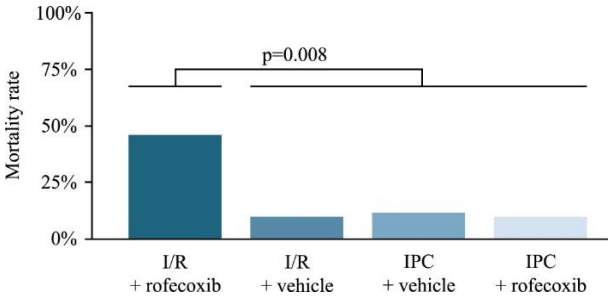

Figure 5. *Rofecoxib treatment increased the mortality rate in the ischemia/reperfusion (I/R) group in vivo when compared to the pooled data of other groups (OR = 7.73, CI 95% = 1.70–34.97, p < 0.008). IPC: ischemic preconditioning.*

4.2. Chronic rofecoxib treatment increased arrhythmia score in cardiac ischemia/reperfusion

The peak arrhythmia scores were achieved in the I/R+vehicle groups after 10 min of ischemia. Arrhythmia scores in 5-min intervals declined gradually

starting from the 50th min in the I/R+vehicle group but remained elevated in the I/R + rofecoxib group (Figure 6).

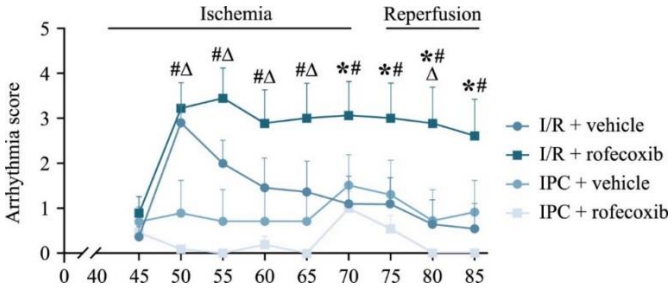

Figure 6. *Arrhythmia scores in 5-min intervals declined gradually starting from the 50th min in the I/R+vehicle (ischemia/reperfusion) group but remained elevated in the I/R + rofecoxib group (*p < 0.05 I/R + vehicle vs. I/R + rofecoxib). IPC (ischemic preconditioning) prevented initial increase of arrhythmia score (#p < 0.05 IPC + rofecoxib vs. I/R + rofecoxib, Δp < 0.05 IPC + vehicle vs. I/R + rofecoxib).*

4.3. Rofecoxib decreased infarct size and did not interfere with cardioprotection by ischemic preconditioning

We measured IS to explore the effect of rofecoxib on I/R injury and cardioprotection by IPC. Rofecoxib reduced IS (I/R + rofecoxib) as compared to the vehicle-treated (I/R + vehicle) group (Figure 7). IS was significantly smaller in the IPC+vehicle group as compared to I/R+vehicle. Chronic rofecoxib treatment did not affect IS-limiting effect of IPC in IPC+rofecoxib when compared to the IPC+vehicle group.

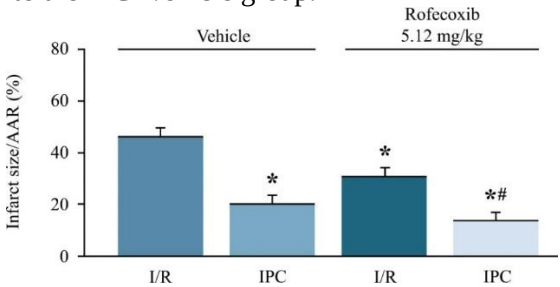

Figure 7. *Chronic rofecoxib treatment reduced infarct size in animals subjected to cardiac ischemia/reperfusion (I/R) and did not interfere with cardioprotection by ischemic preconditioning (IPC). (*p < 0.05 vs. I/R + vehicle, #p < 0.05 vs. I/R + rofecoxib). AAR: area at risk.*

4.4. Rofecoxib increased the action potential duration in rat isolated papillary muscles at the end of simulated ischemia/reperfusion and this effect was not observed ischemic preconditioning group

In a collaboration with the Department of Pharmacology and Pharmacotherapy from University of Szeged, *ex vivo* simulated ischemia/reperfusion (sI/R) and simulated ischemic preconditioning (sIPC) experiments were performed on isolated rat left ventricular papillary muscles in order to analyze the effect of rofecoxib on cardiac action potential parameters. Rofecoxib treatment did not change action potential duration at 75% of repolarization (APD75) (Figure 8 A and B) in normoxic conditions. As expected, the 30 min simulated ischemia significantly shortened APD75 (Figure 8 A) in all groups that were subjected to ischemia when compared to the respective normoxic groups. However, in the presence of sI/R rofecoxib increased APD75 (Figure 8 B) upon reperfusion following the 30 min simulated ischemia. In the sIPC group, these effects of rofecoxib on APD were not seen during reperfusion (Figure 8 B).

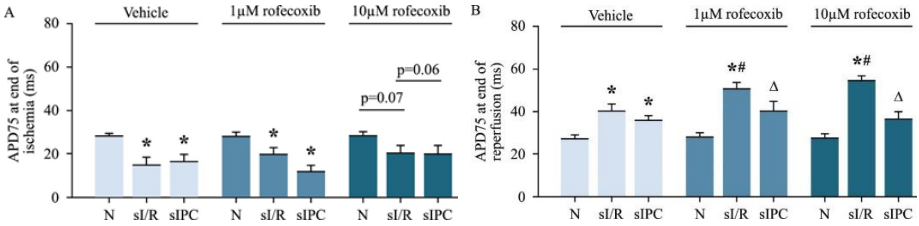

Figure 8. *(A) action potential duration at 75% of repolarization (APD75) decreased by the end of 30 min simulated ischemia in the simulated ischemia/reperfusion groups (sI/R) and simulated ischemic preconditioning groups (sIPC). (B) Rofecoxib increased the APD75 in adult rat isolated papillary muscles at the end of reperfusion and this effect was reversed by sIPC (*p<0.05 vs. corresponding normoxia group, #p<0.05 vs. sI/R +vehicle, Δp<0.05 vs. corresponding sI/R group).*

4.5. Rofecoxib treatment increased viability of isolated adult rat cardiac myocytes in normoxia and in simulated ischemia/reperfusion injury

In vitro sI/R experiments were performed in order to analyze the effect of rofecoxib on viability of isolated cardiac myocytes. sI/R caused significant cell death (Figure 9) as compared to normoxic control, which was reversed by rofecoxib treatment at 0.1, 0.3, 1, and 3 μM concentration, respectively, thereby supporting the *in vivo* data showing the IS reduction by rofecoxib (Figure 9).

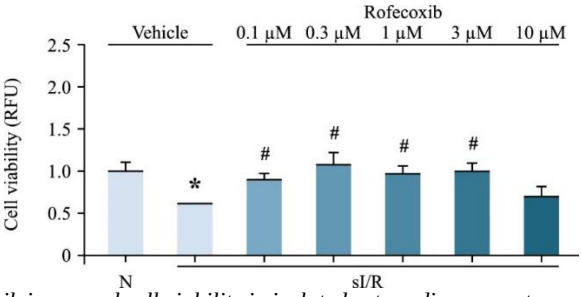

Figure 9. *Rofecoxib increased cell viability in isolated rat cardiac myocytes exposed to simulated ischemia/reperfusion (sI/R). Normoxia (N)+vehicle group was set to 1 relative fluorescence (RFU) arbitrary unit and all of the data were normalized to the averaged sI/R group (*p < 0.05 vs. Normoxia+vehicle, #p < 0.05 vs. sI/R+vehicle).*

4.6. Myocardial scar sizes were comparable between the two pig breeds

To measure the extent of cardiac scar as a consequence of AMI, CMRI was performed. Scar sizes and BARI scores (Bypass Angioplasty Revascularization Investigation Myocardial Jeopardy Index) were comparable between the two breeds measured at the 2nd month of follow-up in Landrace pigs, and at the 3rd and 6th month in Göttingen minipigs (Figure 10 A and B).

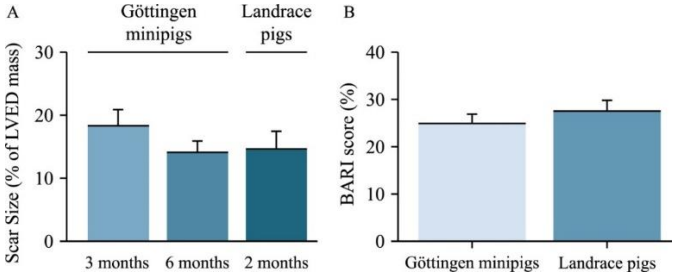

Figure 10. *(A) Left ventricular scar sizes in Göttingen minipigs and Landrace pigs measured by cardiac magnetic resonance imaging. Scar size is shown as a ratio of mass of infarction to the mass of left ventricle at end of diastole (LVED). (B) BARI (Bypass Angioplasty Revascularization Investigation Myocardial Jeopardy Index) scores in Göttingen minipigs and Landrace pigs measured before coronary occlusion.*

4.7. Increase in left ventricular mass was more pronounced in Landrace pigs during follow-up

The cardiac growth rate was measured by CMRI. Left ventricular end-diastolic (LVED) mass in Göttingen minipigs increased only moderately at 6 months (Figure 11 A). In contrast, in Landrace pigs, LVED mass increased by almost 100% at 2 months (Figure 11 B).

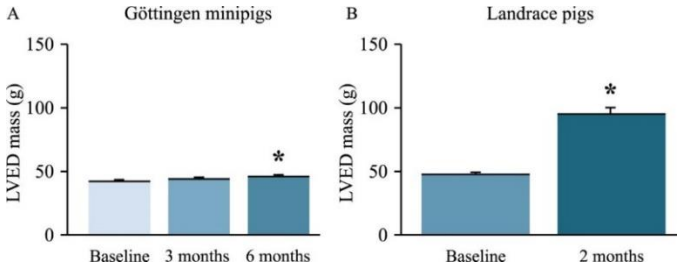

Figure 11. *(A) Left ventricular end diastolic (LVED) mass (g) of Göttingen minipigs and (B) Landrace pigs measured by cardiac magnetic resonance imaging (*p<0.05 vs. corresponding baseline).*

4.8. Left ventricular ejection fraction decreased only in Göttingen minipigs

LVEF, as the most widely used parameter of left ventricular systolic function, was measured by CMRI. MI resulted in a significant decrease in LVEF in minipigs at 3 months and 6 months (Figure 12 A). In Landrace pigs LVEF did not change after 2 months (Figure 12 B).

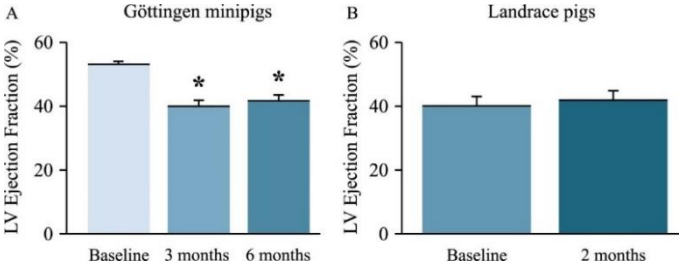

Figure 12. *(A) Left ventricular (LV) ejection fraction (%) of Göttingen minipigs and (B) Landrace pigs measured by cardiac magnetic resonance imaging (*p<0.05 vs. corresponding baseline).*

4.9. Left atrial volume indexed to body surface area increased only in Göttingen minipigs, but both the breeds developed pulmonary edema following myocardial infarction

In order to further examine signs of HF, we performed measurement of the left atrial volume indexed to body surface area (LAVi). LAVi increased by 34% in Göttingen minipigs after 6 months (Figure 13 A) and did not change significantly in Landrace pigs after 2 months (Figure 13 B). Moreover, the presence or absence of pulmonary edema was assessed by CMRI on the localizer images. Pulmonary edema was observed in both breeds as a result of cardiac decompensation.

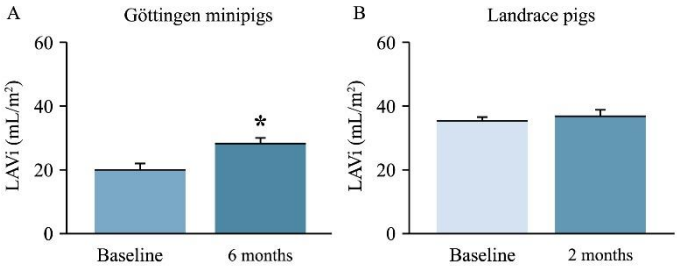

Figure 13. *(A) Left atrial volume indexed to body surface area (LAVi) in mL/m2 in Göttingen minipigs and (B) Landrace pigs measured by cardiac magnetic resonance imaging (*p<0.05 vs. corresponding baseline).*

5. Conclusions

5.1. Hidden cardiotoxicity of rofecoxib can be revealed by experimental I/R models

We demonstrated for the first time, in the literature that rofecoxib increased acute mortality due to its proarrhythmic effect as indicated by increased APD75 during I/R. We also showed that rofecoxib did not interfere with the cardioprotective effect of IPC and that IPC was able to protect against rofecoxib-induced hidden cardiotoxicity.

These findings highlight the value of testing drug candidates for hidden cardiotoxicity in disease models such as I/R injury.

5.2. Göttingen minipig model is superior to the Landrace pig to follow-up the development of post-MI HF

We have found that despite the equal scar sizes and BARI scores in the two breeds, left ventricular dysfunction characterized by decreased LVEF was observed only in Göttingen minipigs. In addition, this was the first characterization of post-MI HF in Göttingen minipigs in comparison to commonly used Landrace pigs, showing that the Göttingen minipig model reflects post-MI HF parameters comparable to humans.

These findings highlight that Göttingen minipig model is superior to the Landrace pig to follow-up the development of post-MI HF.

6. Bibliography of the candidate's publications

6.1. Own publications involved in the current thesis

1. Brenner GB, Makkos A, Nagy CT, Onódi Z, Sayour NV, Gergely TG, Kiss B, Görbe A, Sághy É, Zádori ZS, Lázár B, Baranyai T, Varga RS, Husti Z, Varró A, Tóthfalusi L, Schulz R, Baczkó I, Giricz Z, Ferdinandy P. (2020) Hidden Cardiotoxicity of Rofecoxib Can be Revealed in Experimental Models of Ischemia/Reperfusion. Cells, 9.

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Coronary Occlusion/Reperfusion Model in Göttingen Minipigs and Landrace Pigs. Journal of Visualized Experiments, doi:10.3791/61901.

6.2. Own publications not involved in the current thesis

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7. Acknowledgements

I would like to express my sincere gratitude to the people, without whom I would not have been able to complete my PhD studies.
First and foremost, I would like to thank my supervisor Dr. Zoltán Giricz for his support, guidance and patience. I am also very thankful to Prof. Dr. Péter Ferdinandy, director of the Department of Pharmacology and Pharmacotherapy, Semmelweis University.
I would like to express my appreciation for my Colleagues in the Cardiovascular and Metabolic and the MTA-SE System Pharmacology Research Group of the Department of Pharmacology and Pharmacotherapy, Semmelweis University.
I would like to thank the research students especially Dr. Sayour Nabil and Dr. Tamás Gergely for their collaborative effort during previous years.
I would like to express my thanks to all the members of the Pharmahungary Group.
Furthermore, I would like to thank all the Colleagues in the Department of Pharmacology and Pharmacotherapy University of Szeged, Moritz Kaposi Somogy County Teaching Hospital, Faculty of Agricultural and Environmental Sciences in Kaposvar, Heart and Vascular Center of Semmelweis University, Quark Pharmaceuticals, Heart Institute of Medical School of University of Pécs, I cannot forget to thank my lovely family, especially my Mom, Brother, Wife, Daughter and friends for all the support during these very intense years of PhD studies.
The present studies were supported by the National Research, Development and Innovation Officece of Hungary (NKFIA; NVKP-16-1-2016-0017 National Heart Program), the Higher Education Institutional Excellence Programme of the Ministry of Human Capacities in Hungary (within the framework of the Therapeutic development thematic programme of the Semmelweis University), Quark Pharmaceuticals Inc, MTA-SE System Pharmacology Research Group (Department of Pharmacology and Pharmacotherapy, Semmelweis University, Budapest, Hungary).
GB.B. was supported by EFOP-3.6.3-VEKOP-16-2017-00009 and Richter Gedeon Inc. scholarship.