Modeling of isolated kidney perfusion systems in transplantation ischemic tolerance

PhD thesis

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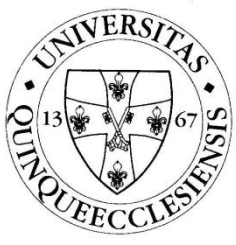

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1. Introduction

Kidney transplantation is considered the best treatment for patients with end stage renal disease. Ischemia-reperfusion injury (IRI) is an evitable event after deceased donor transplantation and influences short term and long-term graft outcome. After kidney transplantation, the main consequences are DGF (delayed graft function), acute and chronic graft rejection and chronic graft dysfunction. The better understanding of IRI mechanisms will help to find further improvements in donated organ survival. Almost 30 % of DGF following kidney transplantation is owed to IRI. Caspases, the classical effector enzymes of apoptosis, are able to induce inflammation following IRI, which is one of the most important non-specific and non-immunologic factor affecting not only DGF but also late allograft dysfunction. Transplantation of an ischemic organ can lead to distant organs' dysfunction. Several mediators are released into systemic circulation during IRI and these can be disadvantageous at distant tissues and organs (native kidneys, liver, lungs and heart). The released mediators can improve permeability of capillaries and more cytokines will secreted, like TNF-alpha, IL-1 and IL-6, which cause severe cell response and it can easily end in cell necrosis or apoptosis.

Transplanted kidneys are damaged organs and drugs can be used either alone or in combination to modify the effects of ischemia and reperfusion besides perfusion solutions and perfusion methods. Further investigations are required to eliminate the effects of IRI during kidney transplantation.

1.1. Endoplasmic reticulum stress in general

Organelle autoregulation is a main characteristic of eukaryotic cells. The endoplasmic reticulum (ER), Golgi apparatus, mitochondria, lysosomes, peroxisomes, and nucleus also have this feature. This is the basis of proper cell function and it is strictly regulated to keep cellular homeostasis on a stable level. ER is a cell structure which is responsible for secretory and membrane protein synthesis and folding. Solely correctly folded proteins are transported to the Golgi apparatus; forasmuch unfolded or not correctly folded proteins are degraded by ER-associated degradation (ERAD). Several physiologic stresses (increased secretory load), pathological stresses (presence of mutated proteins, which cannot fold in the ER) can lead to an inequality between the demand

of protein folding and the ER's capacity for protein folding, causing ER stress (ERS). The unfolded protein response (UPR) is the collective term of signal transduction pathways that are involved in ERS sensing and responding. The most phylogenetically conserved UPR signaling pathway is IRE1. IRE1α is presented in every cell of mammals, whereas IRE1β is expressed by intestinal epithelial cells. The method is mediated through the splicing of *Xbp-1* mRNA and the spliced *Xbp-1* encodes a transcription factor of the basic-leucine zipper (B-ZIP) family whose genetic targets code for proteins and their function is to enhance ER protein-folding capacity and degradation of misfolded ER proteins, thus protecting the cell by reducing the ERS stimulus.

IRE1 signaling can also promote cell death after ERS by activating caspases. TRAF2, the adaptor molecule, interacts with murine procaspase-12 and ERS disrupts this interaction, possibly by causing the IRE1 kinase domain to bind TRAF2 which leads to the conversion of procaspase-12 into the active enzyme. The role of caspases-12 in cell death promotion after ERS is controversial, although Nakagawa et al. published a study, where murine *Caspase-12*$^{-/-}$ cells are largely resistant to cell death induced by ERS, and another group observed no significant resistance to ER stress when they generated *Caspase-12*$^{-/-}$ cells independently.

1.2. Pioglitazone

The peroxisome proliferator-activated receptor-γ (PPARγ) belongs to the family of nuclear receptors which control the expression of a huge number of genes and act like sensors of hormones, vitamins, endogenous metabolites and xenobiotic compounds. The group of thiazolidinediones (TZDs) is the most widely studied PPARγ ligands. Activation of PPARγ results in insulin sensitization by opposing the effect of TNFα in adipocytes and enhances glucose metabolism. The PPARγ agonist Pioglitazone (Pio) is used as an antidiabetic drug in the treatment of type 2 diabetes as efficient insulin sensitizer. Recent studies show that Pio protects kidneys, myocardium and brain against IRI. The potential role of Pio and other PPARγ agonists as nephroprotective agents is demonstrated in non-diabetic models of renal injury (such as IRI and induced renal toxicity by drug or chemical). Renoprotective properties of Pio exhibits via facilitation of endothelium-dependent vasodilatation through amending abnormalities in NO production, developing the antioxidant profile and control of the expression of inflammatory mediators and apoptotic factors.

2. Aims

According to our idea, there are three methods, which can model the transplantation's process. In the first part, we test Pio in one concentration - which is mostly recommended by literature - and we administer it in various stages of ischemia-reperfusion in IRI model. In the second part, we focus on Pio in several doses and the possible toxic effect, and the selection of the most effective dose are our goals. In the third part, we make from the above mentioned two methods a mixture, and the purpose is to test the drug in different concentrations in a perfusion-reperfusion model, which is the closest to the clinical application.

1. We aim to investigate the effect of PPAR-γ agonist Pioglitazone in ischemia-reperfusion injury. The parameters are superoxide-dismutase activity, TNF-α, IL-1β, IL-6 and catalase activity. We would like to prove that Pio can reduce IRI by decreasing the level of inflammatory cytokines and it help to maintain the balance between ROS and antioxidants. First, we would like to examine the histological changes by hematoxylin-eosin staining. In our first experimental method, it is a crucial question if there is any difference in the timing of treatment. The aim is to show that Pio has positive effect on injured kidneys.
2. We suppose that Pio has different effect on kidneys and liver. The aim is to ascertain this deviation regarding *in situ* whole body perfusion. Another aim to this method is to investigate the extent of endoplasmic reticulum stress in case of liver and kidneys. Our expectation is that Pio decrease the ER stress. To measure this, we would like to perform Western-blot analysis.
3. We would like to compare the *in situ* perfusion to the *in situ* perfusion-reperfusion model. To demonstrate this difference, we use the same kits and experimental conditions, and we would like to measure the catalase activity, SOD activity and the level of inflammatory cytokines, furthermore, the histological parameters to evaluate the visible changes in kidneys. Our aim is to answer the question, either *in situ* perfusion or *in situ* perfusion-reperfusion result the better outcome in the case of kidneys.

3. Materials and methods

3.1. Ischemia-reperfusion injury model

3.1.1. Animal model

Thirty male Wistar rats of the same age, weighting between 250-300 g, were used for this study. The rats were housed in standardized cages, under standard conditions (temperature was 25±2 ℃, in air filtered room), with 12/12-hour light and dark cycle and were fed with standard rat chow and water *ad libitum*. The study protocol was approved by the National Scientific Ethical Committee on Animal Experimentation. (Number: BA02/2000-38/2019.)

3.1.2. Experimental protocol

The animals were divided into three groups (10 rats in each group). The first group was the control group, operation and ischemic condition was induced but they got the solvent of the drug (PBS+10 % DMSO) intraperitoneal (i.p.) one hour prior to the ischemic period. The second group was treated with Pioglitazone (20 mg/kg) one hour prior to the ischemic period, ordered from Sigma-Aldrich, St. Louis, Missouri, USA. In these two groups, after 60 minutes of ischemia, reperfusion was started for 90 minutes. The third group was first anesthetized, then ischemia was induced and prior to the start point of reperfusion, we administered 20 mg/kg Pioglitazone i.p. and after it the reperfusion was induced. To standardize the study, all procedures were performed at similar time points in all groups. The drug was freshly solved into PBS + 10 % DMSO solution before the administration. *(Fig. 1.)*

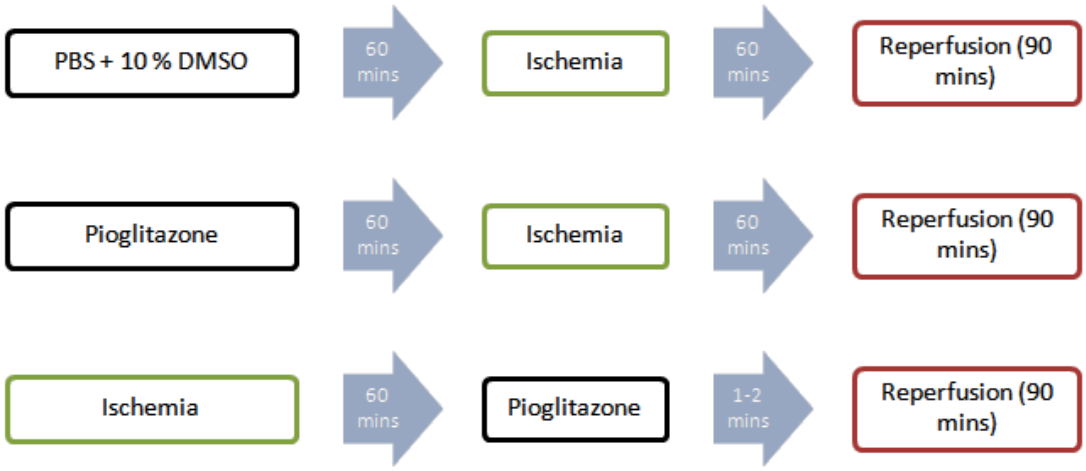

Figure 1: Investigation groups: 1: control, 2: IR preconditioning with Pio, 3: IR postconditioning with Pio (IR – ischemia-reperfusion; Pio – Pioglitazone)

3.1.3. Surgical procedure

The rats were preoperatively anesthetized with an intraperitoneal (i.p.) application of a mixture consisting of ketamine (2.7 ml/kg) and diazepam (2.7 ml/kg). The ratio was 1:1. The skin of the abdomen was depilated using an animal depilatory agent. During the operation, the animals were placed on a heated pad and ECG monitoring was also used. After median laparotomy, we administered heparin into mesenterial vein, then the left renal pedicle was clamped by the microaneurysm clamp. The clamp was removed 60 minutes after clamping and the rat was monitored alive for 90 minutes during reperfusion period. After 90 minutes, the animals were sacrificed, and kidney and blood samples were collected. The blood samples were centrifuged, and plasma was collected and stored at -80 °C. Kidney samples were stored immediately at -80 °C within individual containers. This method is widely used.

3.2. *In situ* whole body perfusion model

3.2.1. Animal model

Sixty male Wistar rats of the same age, weighting between 250-300 g, were used for this study. The rats were housed in standardized cages, under standard conditions (temperature was 25±2 ℃, in air filtered room), with 12/12-hour light and dark cycle and were fed with standard rat chow and water *ad libitum*. The study protocol was approved by the National Scientific Ethical Committee on Animal Experimentation. (Number: BA02/2000-38/2019.)

3.2.2. Experimental protocol

Animals were divided into six groups, ten rats in each group. The first group was the control (sham operated). The second group was the KH control (KH – Krebs-Henseleit buffer), the third group was the KH + Pio 10 mg/kg, the fourth group was the KH + Pio 20 mg/kg, the fifth group was the KH + Pio 30 mg/kg and the sixth group was the KH + Pio 40 mg/kg. To standardize the study, all procedures were performed at similar time points in all groups. The drug was freshly solved into PBS + 10 % DMSO solution before the administration. The perfusion solution's temperature was maintained on 20 ℃ during the whole study. (*Table 1*)

Table 1: The groups with the treatments

Groups (n=10 in each groups)	Treatments
1. group	control (sham operated)
2. group	perfused with KH
3. group	perfused with KH + 10 mg/kg Pio
4. group	perfused with KH + 20 mg/kg Pio
5. group	perfused with KH + 30 mg/kg Pio
6. group	perfused with KH + 40 mg/kg Pio

3.2.3. Surgical procedure

The rats have been preoperatively anesthetized with an intraperitoneal (i.p.) application of a mixture consisting of ketamine (2.7 ml/kg) and diazepam (2.7 ml/kg). The ratio was 1:1. The skin of the abdomen was depilated using an animal depilatory agent. During the operation, the animals

were placed on a heated pad and ECG monitoring was also used. After middle laparotomy, the infrarenal abdominal aorta and inferior vena cava were dissected and heparin (400 IU/kg) was administered into mesenterial vein. After a few minutes, the inferior vena cava was catheterized (22 gauge) and the in situ whole body perfusion was initiated. In each experimental group, 200 ml perfusion solution was used. The perfusion equipment was set to 150 ml/h, and for one animal the perfusion lasted 80 minutes in all experimental groups. At the same time with catheterizing the vein, on the aorta we made a small incision, and it was the outflow of the perfusate which was removed from the abdominal cavity by suction. (*Fig.2*) The sham group was handled as a treated group without perfusion, in which the blood circulation was intact. During the experiment, the animals were sacrificed by bleeding out. At the end of the perfusion protocol, kidneys and liver were taken out and were placed immediately at -80 °C within individual containers.

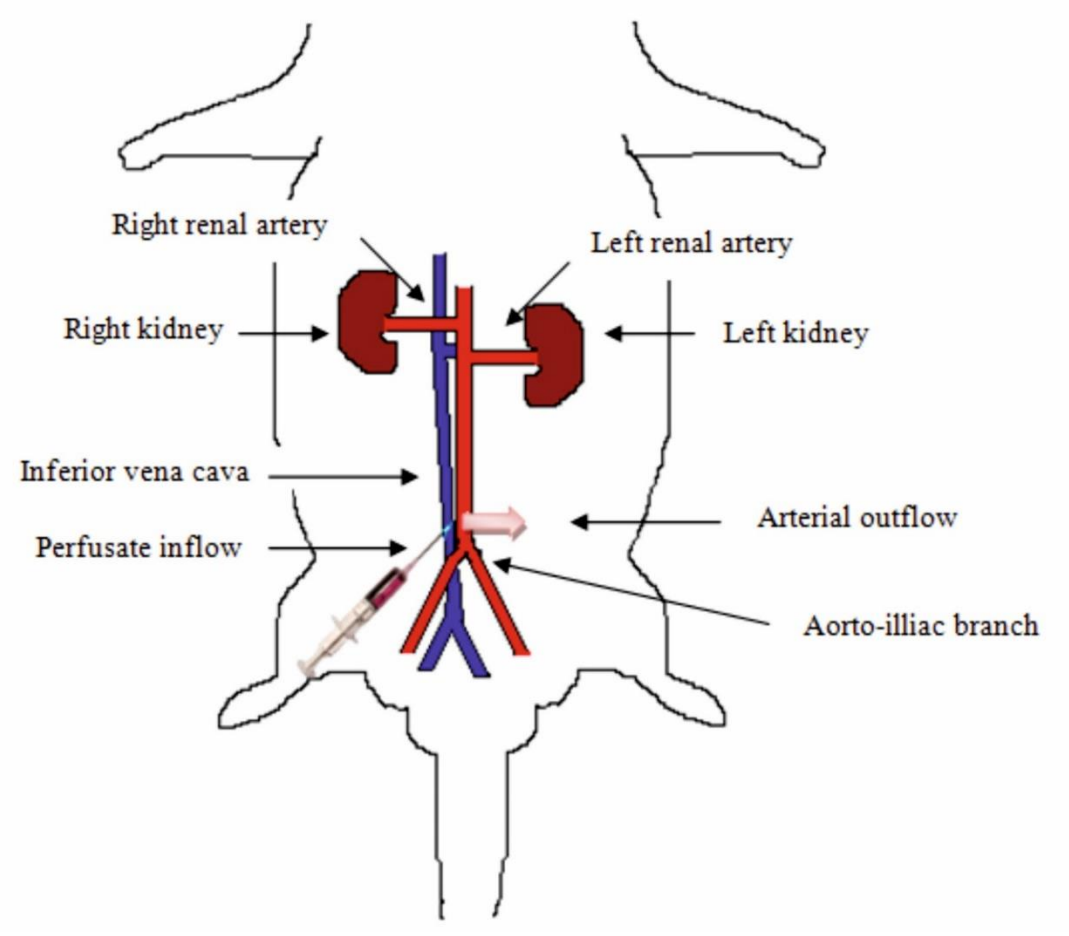

Figure 2: Schematic representation of an animal regarding *in situ* perfusion model

3.3. *In situ* perfusion-reperfusion model

3.3.1. Animal model

Sixty male Wistar rats of the same age, weighting between 250-300 g, were used for this study. The rats were housed in standardized cages, under standard conditions (temperature was 25±2 ℃, in air filtered room), with 12/12-hour light and dark cycle and were fed with standard rat chow and water *ad libitum*. The study protocol was approved by the National Scientific Ethical Committee on Animal Experimentation. (Number: BA02/2000-38/2019.)

3.3.2. Experimental protocol

Animals were divided into ten groups, six rats in each group. To standardize the study, all procedures were performed at similar time points in all groups. The drug was freshly solved into PBS + 10% DMSO solution before the administration. The perfusion solution's temperature was maintained on 20 ℃ during the whole study. (*Table 2*)

Table 2: The experimental groups, six rats in each group

Control groups	Treated groups
KH control	KH perfused
KH + 10 mg/kg Pio_control	KH + 10 mg/kg Pio perfused
KH + 20 mg/kg Pio_control	KH + 20 mg/kg Pio perfused
KH + 30 mg/kg Pio_control	KH + 30 mg/kg Pio perfused
KH + 40 mg/kg Pio_control	KH + 40 mg/kg Pio perfused

3.3.3. Surgical procedure

The rats have preoperatively anesthetized with an intraperitoneal (i.p.) application of a mixture consisting of ketamine (2.7 ml/kg) and diazepam (2.7 ml/kg). The ratio was 1:1. The skin of the abdomen was depilated using an animal depilatory agent. During the operation, the animals were placed on a heated pad and ECG monitoring was also used. After middle laparotomy, the abdominal and suprarenal aorta section and vena cava were dissected, and heparin was administered into mesenterial vein. Then the suprarenal aorta was clamped by a microsurgical

vascular clamp and the abdominal aorta was catheterized (22 gauge). The perfusion machine was connected to the catheter and the flow was maintained in each group on 110 ml/h. The perfusate volume was 55 ml. After 30 minutes the perfusion was finished, and the catheter was removed and the whole was clamped. (*Fig. 3*) The reperfusion phase lasted 60 minutes. Thereafter the animals were sacrificed by bleeding out. The blood was centrifuged, and plasma and kidney samples were placed immediately at -80 °C within individual containers.

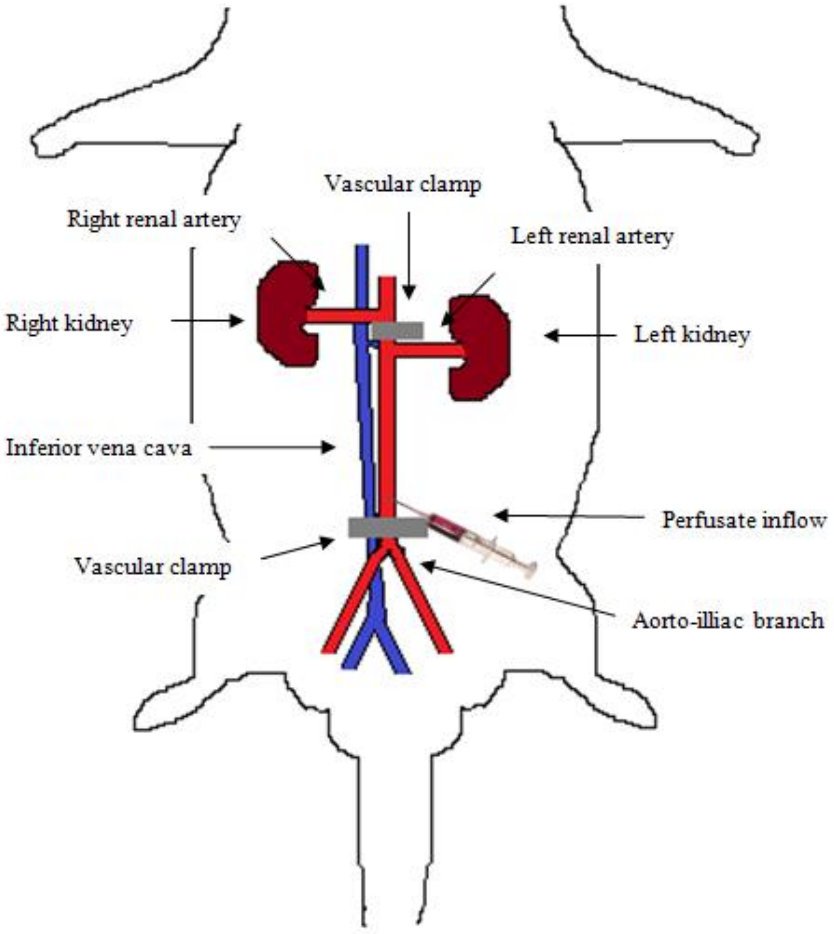

Figure 3: Schematic representation of an animal in *in situ* perfusion-reperfusion model

3.4. Analytical methods

3.4.1. Biochemical analysis

We measured the level and activity of IL-1β, IL-6, TNF-α, SOD and catalase from the kidney samples to verify the results. SOD and catalase activity was measured (inflammatory cytokines were not analyzed, due to lack of reperfusion) from kidney and liver samples regarding *in situ* perfusion model. IL-1β, IL-6 and TNF-α are the main indicators of inflammatory response and their level in blood and tissue samples were studied by the Rat IL-1 beta ELISA Kit, Rat IL-6 Elisa Kit, Rat TNF alpha ELISA Kit (Abcam, Cambridge, UK) following the manufacturer's protocol. To measure the level of oxidative stress, we used the Superoxide Dismutase Activity Assay Kit and Catalase Activity Assay Kit (Abcam, Cambridge, UK) following the manufacturer's protocol.

3.4.2. Histopathological analysis

Hematoxylin eosin staining protocol

Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, cut in 3 micrometers thick sections with a rotational microtome (Microm HM 325, Thermo Scientific Ltd.) and mounted on coated glass microscope slides. After deparaffinization and rehydration, samples were stained with hematoxylin, bluing was performed with tap water, and tissues were stained with eosin, dehydrated in alcohol, cleared in xylene and mounted with permanent mounting medium.

3.4.3. Western blot protocol

Kidney and liver tissue samples were frozen in liquid nitrogen, then manually pulverized in mortar and dissolved in ice-cold lysis buffer (containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM Na_3VO_4, 100 mM NaF, 5 μM $ZnCl_2$, 10% glycerol, and 1% Triton X-100 plus 10 μg/ml of the protease inhibitor aprotinin). Lysates were subjected to centrifugation at 40 000 x g at 4 °C for 30 minutes, and then the protein concentration of the supernatants was determined using Protein Assay Dye Reagent Concentration (Bio-Rad Laboratories, Inc., Hercules, California, USA) and light absorption measurement at 595 nm. Samples containing 30 μg of denatured total protein have been prepared and loaded onto 10% polyacrylamide gels. Proteins separated based on size have been electro-blotted for half an hour onto PVDF membranes using the Trans-Blot Turbo semi-dry system (Bio-Rad Laboratories, Inc., Hercules, California, USA),

then blocked in 3% BSA dissolved in Tris-buffered saline containing 0.2% Tween 20. Probing of the membranes with the primary antibodies (caspase12 and XBP1 [Sigma-Aldrich, St. Louis, Missouri, USA]) diluted 1:1000 in the blocking solution followed at 4 °C overnight. Binding of the antibodies to the membrane was detected by a secondary anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) diluted 1:10,000. The enhanced chemiluminescent signal was visualized using a G:box gel documentation system (Syngene, India). All membranes were then stripped from the antibodies and detected again as above for possible loading differences using a primary antibody against GAPDH (Cell Signaling Technology, Danvers, Massachusetts, USA) at a dilution rate of 1:3000. ImageJ software was used to analyze the blots, and for the statistical evaluation, one-way analysis of variance (ANOVA) with Bonferroni correction was used.

3.4.4. Statistical analysis

For statistical evaluation, one-way analysis of variance (ANOVA) was used, followed by adequate post hoc tests (Dunnett's, Sidak) for multiple comparisons. All Western blots were performed independently in triplicates. Regarding statistical evaluation, one-way ANOVA was used, followed by post-hoc analysis of Bonferroni. All data are represented as the mean ± SD. The difference was considered statistically significant when the p-value was less than 0.05 and classified by asterisks as follows: $p<0.05$ (*); $p<0.001$ (**); $p<0.0001$ (***). The statistical analysis was calculated through GraphPad Prism software for Windows (version 5.03).

4. Results and discussion

4.1. Ischemia-reperfusion injury model

4.1.1. Results

First, to answer the question, that Pio in which setting could reduce more the oxidative stress, we performed SOD and catalase activity assays. All the control groups depict significant difference compared to the ischemic groups. However, we cannot detect any crucial deviation between the two treated groups.

The expression of IL1-beta, IL6 and TNF-alpha was detected by ELISA. Control samples depict statistically significant difference compared to treated groups, and the most effective time point of the treatment is in case of IL1-beta the Pio treatment before the initiation of reperfusion period

(pre-Pio reperfusion ischemia). However, the Pio treatment before the induction of ischemic period effectively reduced the IL6 and TNF-alpha expressions compared to the control ischemic group. However, there were not any significant difference between the two treated groups, pre-Pio 1h ischemic and pre-Pio reperfusion ischemic.

The histopathological analysis did not depict any apoptotic or oncotic sign in control groups. The basic tissue structure was kept. However, in control ischemic group, the swelled tubules and Bowman's capsules and eosinophilia represent the damage in kidney tissue. The difference between the treated groups is in the swelled endothelial tubules. In pre-Pio 1h ischemic group, arrowhead shows the swelling, but not the same extent as in the previous group, the pre-Pio 1h ischemic. The pre-Pio reperfusion ischemic correlates to the control's tissue structure. The histopathological findings correlate to the ELISA and Western blot results.

Caspase 12 expression was measured performing Western blot. Control samples' bands are weaker and the untreated, but ischemic (control ischemic) group's pixel density is significantly higher compared to control and pre-Pio 1h ischemic group. However, the Pio administration before reperfusion initiation (pre-Pio reperfusion ischemic group) could reduce the Caspase 12 expression, not in a statistically significant extent.

Treated groups decreased the XBP1s expression compared to control ischemic group. Control ischemic and the treated groups differed significantly to their controls. In case of XBP1u, control groups show significant deviation from non-treated but ischemic (control ischemic) and treated groups. The most effective time point was the Pio administration one-hour before the initiation of ischemia compared to control ischemic group in decreasing XBP1u expression. However, the pattern of the bands depicts as in previous results, any significantly between control ischemic and pre-Pio reperfusion ischemic groups were not ascertained by statistical analysis.

4.1.2. Discussion

IRI can cause severe problems in microcirculation and it may lead to patient's higher morbidity and prolonged hospitalization. Cellular dysfunction, interstitial oedema, inflammatory cytokine expression can lead to cell death. The length of the ischemic period has a great importance on the caused damage. In case of our IRI model, the 45 mins ischemic and 90 mins reperfusion periods were used, since in international literature it is recommended. A longer ischemic period (over 60

minutes) causes irreversible injury and significant cell death, which has adverse consequences on the organ function and can lead to renal failure.

Based on international literature, gender and/or sexual steroids may play a role in the recovery from ischemic injury in non-renal organs, and postischemic organ dysfunction is influenced by gender and sexual steroids. Müller et al. published an article about this issue and according to the published papers' conclusions, male Wistar rats were chosen in all experiments presented in this thesis. They concluded that female rats have relative protection against postischemic renal failure, furthermore, in intact males the effects of androgens upon ischemic kidney damage seemed to be mediated by endothelin-induced vascular changes. Robert et al. examined the gender difference and sex hormone production in rodent renal ischemia reperfusion injury and repair. Overall, after ischemia, renal function recovery and tissue injury was gender-dependent and the differences were associated with a modulation of sex hormone production and a modification of tissue remodeling and proliferative cell processes.

IRI method has a wide variety in application. Huge number of drugs were tested whether they antioxidant, anti-inflammatory or kidney protective agents are or not. Our aim was to characterize the effect of Pio administration in a single dose but different time point. Therefore, SOD and catalase activity, inflammatory cytokines (IL1-beta, IL6, TNF-alpha) were measured, histopathological changes and endoplasmic reticulum stress markers (Caspase 12, XBP1s and XBP1u) were analyzed. For further experiments, first the conclusion was made and the Pio treatment one-hour before initiation of ischemic period was preferred, since this experimental group represented our expectations and served as answer to hypotheses. The pre-Pio 1h ischemic group could decrease not only the oxidative stress markers and inflammatory cytokines, but also the ERS markers. Thus, we hypothesize that Pio has a preventive effect in IRI against oxidative stress, inflammation and ERS, and the consequent cell death.

IRI method has been used since 1980's and several drugs and agents were applied to determine their effects and applicability. Our department has numerous publications and contribution with other researchers among IRI induction and pre- and post-conditional methods.

According to international literature, Pio's anti-inflammatory, antioxidant, and kidney-protective effects were proven. However, the beneficial function in ERS regarding IRI rat kidney model was

not examined yet. Therefore, to compare the two treated groups' influence on above mentioned markers, as a pilot study, we considered it important to compare their effects, the results of which served as a basis for further experiments such as in situ perfusion model and in situ perfusion-reperfusion model.

Previously as above was mentioned, Pio's protective effect against oxidative stress and inflammation were examined and proven, however, ERS markers were not examined in IRI setting with Pio yet. However, NRK-52E cells were exposed by hypoxia/reoxygenation injury and the cytoprotective effect of Pio was declared.

In conclusion, histopathological findings correlate to the ELISA and Western blot results since Pio was not depicted any toxic effects in the administered dose and decreased the inflammation by diminishing the expression of the examined cytokines and reduced the oxidative stress. ERS markers depicted that Pio has a protective impact against the organelle's stress upon IRE1 pathway. However, further experiments are needed to determine the most effective quantity of Pio regarding IRI and our other models.

4.2. *In situ* whole body perfusion model

4.2.1. Results

4.2.1.1. Rat kidney results

SOD and catalase activity ELISA's were performed to specify the most effective dosage of administered Pio regarding in situ perfusion rat model. A dose dependent manner can be observed. Except the 10 mg/kg Pio group, a significant difference was not only between the KH (perfused without Pio) and treated groups (20, 30, 40 mg/kg Pio), but also in 10 mg/kg Pio and 30, 40 mg/kg Pio groups, furthermore, 10 and 20 mg/kg Pio groups depict significantly higher SOD and catalase activity compared to 40 mg/kg Pio group.

In the hematoxylin-eosin-stained histopathological analyses demonstrates, that the control group's main tissue structure was kept, and we cannot detect any signs of apoptosis or swelling the tubules or Bowman's capsules. However, in the Krebs-Henseleit-treated group (KH), the swelling of tubules and Bowman's capsule can be seen. In a dosage dependent manner, we can observe a positive effect of the drug, since in the KH+40 mg/kg Pio group, the tissue structure correlates to the control's sample.

To conclude the ERS decreasing effect of Pio, we performed Caspase-12 and XBP1 Western blots. The positive effect of Pio can be detected in a dose-dependent manner. Western blot analysis was performed to examine the expressions regarding Caspase 12 and XBP1, both from liver and kidney samples, and explore the effect of the different doses regarding Pio. Expression of Caspase 12 was lower, dependent on the dosage of the drug regarding kidney samples. Pio was efficient in all the four administered doses. The Western blot analysis exhibited the significant differences in the case of XBP1 expression in kidney samples. The pattern of the pixel density appears similar in the case of XBP1s and XBP1u, however, the XBP1u had weaker bands and the expression of these proteins was lower. Nevertheless, the 40 mg/kg dose of Pio was significantly effective in reducing the expression of both XBP1s and XBP1u.

4.2.1.2. Rat liver results

Rat liver samples were also analyzed, and the effect of Pio regarding SOD and catalase activity was measured. The above-mentioned dose-dependent manner can be observed. Control group depict significantly lower SOD and catalase activity compared to all other experimental groups. KH + 30 and 40 mg/kg Pio dosages significantly decreased the oxidative stress markers' activity compared to KH group. KH + 10 mg/kg Pio group similarly as KH group, had significantly higher SOD and catalase activity compared to KH + 40 mg/kg Pio group.

Sections of rat livers from different treated groups were stained with hematoxylin and eosin. The control group depicted a normal appearance. In the non-treated ischemic group (KH), we can detect pronounced nodular fibrosis of the parenchyma with hypertrophic hepatocytes and a swelling of the liver cells. In KH + 20 Pio and KH + 30 Pio groups, the basic tissue structure was mainly kept. We cannot detect any signs of oncosis or apoptosis among the treated groups. However, the changes are not significant, but viewing all the samples from the liver, the tendency of the protective effect of the administered drug can be very well detected.

The expression of Caspase 12 regarding liver samples was significantly lower at 10 and 20 mg/kg Pio concentrations compared to the KH group.

In regard to the liver samples, the pattern of the bands appeared similar, however, the significantly effective dosages of Pio were the 30 and the 40 mg/kg analyzing XBP1s and XBP1u.

4.2.2. Discussion

The use of a proper drug to decrease the damage caused by IRI plays a crucial role in transplantation and surgery. Approximately 30% of DGF following kidney transplantation is linked to IRI. In our experiment, we chose a drug, Pio, which is used as an insulin sensitizer agent in treatment of type 2 diabetes and according to published literature, it has anti-inflammatory and antioxidant effects, and can decrease the amount of cell damage and apoptosis. The initial ERS response is a defensive mechanism to detect unfolded and misfolded proteins and maintain ER homeostasis. Prolonged perturbation of ER triggers the activation of an adaptive signaling pathway referred to as UPR. Long-term UPR activation can lead to apoptotic programmed cell death. In the present study, we demonstrated the use of Pio as an antioxidant agent can suppress the expression of ERS markers. Activation of IRE1 induces altered communication between ER and mitochondria, leading to dysfunction in mitochondria, metabolic imbalance, and cell death.

According to the results of SOD and catalase activity both from kidney and liver samples, a dose-dependent manner can be observed. Therefore, Pio in our novel in situ perfusion setting kept its antioxidant activity and protected kidneys and livers from cell death.

In the control group, we observed an enhanced expression of XBP1 and Caspase 12 and in a dose-dependent manner, Pio decreased the level of these ERS markers. These findings implicate Pio as a therapeutic target for the protection of ERS and ultimately cell death. The ratio of the XBP1s/XBP1u presents the activity of IRE1 pathways, however, different gels and membranes were used to detect XBP1s and XBP1u. Therefore, the calculation of the ratio is not necessarily reliable.

In our experimental model, we used the *in situ* whole body perfusion system to perfuse the kidneys and liver with Krebs-Henseleit solution modified with different dosages of Pio. Dosages used were 10, 20, 30 and 40 mg/kg Pio. The literature stated these concentrations can be protective against cell damage and apoptosis, and a higher dose level is more effective. This *in situ* perfusion model is suitable for mimicking the kidney or liver transplantation, when clinicians perfuse the organ with organ preservative solution, which helps to keep the organ functional and does not modify the cell structure. This process is inevitably necessary to decrease DGF and the

consequently repeated transplantation. According to our results, we conclude Pio is suitable for reducing the cell damage and decreasing potential ERS and apoptosis.

Concentrations used were based on published literature. *Singh et al.* used in their experiment, 20 and 40 mg/kg Pio orally 1 h prior to IRI induction. According to their results, the 40 mg/kg dosage decreased the serum urine acid, blood urea nitrogen, serum nitrogen and microproteinuria concentrations. Furthermore, Pio in higher concentrations lowers the myeloperoxidase activity regarding tissue. They highlight the renoprotective effect of Pio in diabetic and non-diabetic models in reference to a kidney injury.

To set the perfusion time and perfusate volume, we used a perfusion fixation protocol and modified it since we used the vena cava as inflow and abdominal aorta section as outflow. The protocol recommended, passing through the abdominal aorta of the rat, we need 100 ml/h flow rate, thus, in our experiment, we initiated perfusion from 100 ml/h and then slowly increased the flow rate until the kidney and the liver became opalescent/white. According to the protocol, 250-300 ml of perfusate solution is optimal for one rat.

The group of thiazolidinediones (TZDs) is the most widely studied PPARγ ligands. Activation of PPARγ results in insulin sensitization by opposing the effect of TNFα in adipocytes and enhances glucose metabolism. Recent studies show Pio protects kidneys, myocardium, and the brain against IRI. The potential role of Pio and other PPARγ agonists are nephroprotective agents and is demonstrated in non-diabetic models of renal injury (such as IRI and induced renal toxicity by a drug or chemical). Renoprotective properties of Pio exhibits via facilitation of endothelium-dependent vasodilatation through amending abnormalities in NO production, developing the antioxidant profile and control of the expression of inflammatory mediators and apoptotic factors. Additionally, with regards to the insulin sensitizing effect of Pio, it can reduce inflammation and consequences of oxidative stress in IRI model. Experiments under hypoxic conditions on NRK-52 cells proved Pio increased the rate of cell survival and decreased the injury caused by hypoxia/reperfusion. According to TUNEL assay, they ascertain Pio can reduce the rate of apoptotised cells in treated groups compared with the untreated, hypoxia/reperfusion control group. Furthermore, Pio has endothelial protective functionality which can be used beside methotrexate (MTX) therapy.

Previously, the effect of Pio was studied in IRI rat models, yet, in an *in situ* perfusion set, we cannot find any results. To the best of our knowledge, this is the first study in which Pio effects were thoroughly examined regarding an *in situ* perfusion model in reference to ERS and histological changes. We conclude Pio is most effective in acute use and in higher concentrations.

4.3. *In situ* perfusion-reperfusion model

4.3.1. Results

A novel in-situ perfusion-reperfusion model was performed to test the effect of Pio in different dosages. As in the case of the other experimental models of this PhD thesis, the oxidative stress markers' activity and inflammatory cytokines' expression were measured and analyzed comparing the controls and perfused groups. KH + 40 Pio perf. group was the most effective and had the lowest SOD and catalase activity.

Inflammatory cytokines' expressions were measured by ELISA, and the groups were compared to each other. We focused on the difference between the perfused but not treated and the treated groups' statistical relation. According to the results, all the three graphs depict a dose dependent manner of Pio, since the highest concentration diminished to the greatest extent the expression of IL1-beta, IL6 and TNF-alpha.

In control histological sections any oncosis or apoptosis cannot be detected, the basic tissue structure is kept. However, in KH perf. group, swelled Bowman's capsules and endothelial tubules and eosinophilia can be observed. According to the sections, the perfusion with KH + 20 and 30 mg/kg Pio depict less histological abnormality and their tissue structure correlate to the control groups'.

The protein expression of Caspase 12 was measured by Western blot. According to previous results, the KH + 40 Pio perf group diminished the expression of Caspase 12, however, significant difference can be observed between KH perf and KH 20, 30 and 40 Pio perf. groups, and between KH + 10 Pio perf. and KH 30 and 40 Pio perf. groups.

Protein expression of XBP1s (56 kDa) compared to KH perf. group, the KH + 30 and 40 mg/kg Pio perf. groups significantly diminished the expression of XBP1s. According to the pixel density of XBP1u's (29 kDa) protein expression, all treated groups, except KH + 10 Pio perf., could

decrease the XBP1s' protein expression, and the KH + 40 Pio perf. performed it to the greatest extent compared to KH perf. group.

4.3.2. Discussion

The importance of diminishing IRI is unquestionable, considered as a relevant factor in determining high morbidity and mortality in several diseases such as myocardial infarction, ischemic stroke, AKI, and trauma. Especially, in organ transplantation as well as in major surgery IRI cause severe complication and influences the clinical outcome. As above was mentioned, ischemic period results in reduces metabolic supply with respect to the demand within an ischemic organ. Due to severe hypoxia microvascular dysfunction develops. Furthermore, in reperfusion phase, instead of restoring the normal conditions, further damages cause the activation of several mechanisms (innate and adaptive immune response and cell death program).

In modeling the human transplantation protocol, these findings are important, since a preservation solution can be perfused, and it could maintain the cell and tissue structure under reperfusion period. By this method, the incidence of DGF could be diminished and the survival rate could be increased.

For more than 5 decades, the cold storage (a preservation solution is perfused into the organ and the organ is stored at hypothermic conditions) in organ preservation was successfully used and decreased the organ failure since the method reduced the metabolic requirements of organs and attenuated ischemic injury. Several preservation solutions are available in the clinic and the effectivity is getting better. University of Wisconsin Solution (UW)/Viaspan and Histidine-tryptophan-ketoglutarate (HTK)/Custodiol solution are the most common preservation solutions and the perspectives on abdominal organ preservation are similar. However, HTK shows conflicting results with respect to pancreatic cellular edema, several studies were noted its advantageous effect in liver transplant against biliary complications than UW. In case of kidney, HTK is associated with higher graft loss and increased DGF in marginal deceased donors. UW serves as a reference standard for use during multiorgan recoveries but has its own competitors, especially HTK solution.

Mitochondria plays important roles in oxidative stress and crosstalk in ERS, inflammasome and autophagy in type 2 diabetes. ER is an organelle with essential functions, including protein

synthesis and processing. After the initiation of UPR, ERS is activated through three major signaling pathways: IRE1, ATF6 and PERK pathways. According to our knowledge, ERS is involved in several types of kidney diseases, such as renal fibrosis, diabetic nephropathy, AKI, CKD.

This is the first study, where kidney perfusion was performed *in situ* using subnormothermic (20 ℃) KH solution modified with Pio in different dosages. *In situ* perfusion-reperfusion model is a combination of IRI and our novel *in situ* perfusion rat model. Our aim was to determine the optimal dosage of Pio, which were the same as in the second chapter of the thesis. The hypothesis was, that does the reperfusion phase modify the effect of Pio and how. According to the results, the highest dosage of the drug (KH + 40 mg/kg Pio perf. group) was an effective decreasing factor of oxidative stress, inflammatory cytokines and ERS markers. Histopathological findings correlate to the ELISA and Western blot results, however, on HE stained kidney sections the 20 and 30 mg/kg Pio perf. groups depicted less tissue damage.

5. Conclusion

Pio is a clinically applicable and non-toxic agent, which can increase the ischemic tolerance of tissues in decreasing the endoplasmic reticulum stress and consequent apoptosis. The results support the finding in which Pio has an ERS decreasing effect in higher dosage levels, both in the case of kidney and liver perfusion. Furthermore, we could not detect any necrotic effect regarding Pio, which may indicate for a short period of time, an acute administered higher dosage of this PPARγ agonist Pioglitazone can help in maintaining the basic organ structure in the case of both the kidney and liver. Further experiments are needed to examine other PPARγ agonists referenced this methodological set and prove their potential role in ischemia and organ preservation by in situ perfusion. In our preliminary study, we aimed to confirm the protective effect of Pio in our novel in situ perfused rat model. Other ERS pathways should be examined in this experimental set to get a broader knowledge of ERS reducing activity of Pio. We can conclude that the highest dosage has anti-inflammatory, anti-oxidative and ERS inhibitory effects, furthermore, histopathological results prove Pio's effectiveness.

Our third aim was, to compare the second and the third experimental set of this study, however, the administered dosages of Pio was similar, but the periods of perfusion not. Therefore, we should carefully conclude any correlation between the two methods.

Due to enzyme polymorphism, application on humans differs from the applied protocols, dosages, and time periods of Pio. Therefore, the modeling of a real-time rat kidney transplantation is planned, where our experiments will be merged and the time periods, administered dosages of Pio and the surgical procedure will be tested and improved.

6. Novel findings

1. In our **first study**, we investigated and proved the protective effect of Pio in inflammation, oxidative stress and ERS which was dedicated to the group of 1h before the initiation of ischemic period. In the traditional IRI experimental setting ERS markers were analyzed for the first time and established a basis to the other experimental sets.
2. Based on the **second chapter**, there was a difference between kidney and liver samples regarding Pio metabolism, since in case of kidney tissue, higher dosage was most preventive, however, the liver sections represented in middle dosages (20 and 30 mg/kg Pio) the similar improvement. According to our knowledge and compared to the international literature, a novel in situ perfusion system was examined and raises the question of the use of Pio in clinical transplantation protocol.
3. In the **third part** of the study, the results were correlated to the other two performed protocols', and the 40 mg/kg Pio dosage depicted a protective effect against IRI compared to the KH-perfused group. On the basis of our knowledge, this novel experimental setting is suitable for preclinical drug testing and improving of our tool against IRI.
4. Overall, we can conclude, that Pio is applicable in higher dosages and has an advantageous effect on kidney and liver samples in IRI, *in situ* perfusion and *in situ* perfusion-reperfusion models.

7. List of publication and presentation related to the thesis

Cumulative impact factor: **1.741**

Publication

Telek V, Erlitz L, Caleb I, Nagy T, Vecsernyés M, Balogh B, Sétáló G Jr, Hardi P, Jancsó G, Takács I. Effect of Pioglitazone on endoplasmic reticulum stress regarding in situ perfusion rat model. Clinical Hemorheology and Microcirculation. 2021. DOI: 10.3233/CH-211163. **IF 1.741 (Q2) /under publication/**

Presentations

Telek V, Erlitz L, Caleb I, Nagy T, Vecsernyés M, Balogh B, Sétáló G Jr, Hardi P, Jancsó G, Takács I. A novel treatment on endoplasmic reticulum stress regarding in situ perfused rat model. DOSZ Science and Innovation Conference 29-30th of January 2021

Telek V, Erlitz L, Caleb I, Nagy T, Vecsernyés M, Balogh B, Sétáló G Jr, Hardi P, Jancsó G, Takács I. Could Pioglitazone decrease endoplasmic reticulum stress regarding a novel in situ perfusion rat model? Magyar Haemorheologiai Társaság XXVII. Kongresszusa 23rd of April 2021.

7.1. Other publication and presentations

Cumulative impact factor: **1.901**

Publication

Borocz K, Csizmadia Z, Markovics A, Meszaros V, Farkas K, **Telek V**, Varga V, Maloba GO, Bodo K, Najbauer J, Berki T, Nemeth P. Development of a robust and standardized immunoserological assay for detection od anti-measles IgG antibodies in human sera. Journal of Immunological Methods. 2019. DOI: 10.1016/j.jim.2018.07.009 **IF 1.901 (Q2)**

Presentations

Telek V, Rapp J, Bognár A, Nagy G, Minier T, Czirják L, Berki T, Simon D. Analysis of PI3K pathway in B cells in early diffuse cutaneous systemic sclerosis. XVI. János Szentágothai Multidisciplinary Conference and Student Competition. 14-15th of February 2019. Pécs

Telek V, Rapp J, Balogh P, Minier T, Czirják L, Berki T, Simon D. PI3K jelátviteli útvonal aktivációjának vizsgálata autoreaktív B-sejtekben. 49. Membrán-Transzport Konferencia, 14-17th May 2019. Sümeg,

Telek V, Rapp J, Bognár A, Nagy G, Minier T, Czirják L, Berki T, Simon D. Innate immune activation of B cells in early diffuse cutaneous systemic sclerosis. Magyar Immunológiai Társaság 47. Vándorgyűlése 17-19th of October 2018 Bükfürdő

Telek V, Rapp J, Nagy G, Minier T, Czirják L, Berki T, Simon D. Alterations of PI3K pathway associated molecules in B cells in early diffuse cutaneous systemic sclerosis. 1st Medical Conference for PhD Students and Experts of Clinical Sciences. 27th of October 2018 Pécs

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