Dissertation

Relaxin in organ preservation: a large animal study

submitted by

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Statutory Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgment has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Guidelines of the Medical University of Graz on Good Scientific Practice“.

Augustinas Bausys,
Date 2022-03-15

Disclosure

Part of this theses has been published previously:

All co-authors listed below actively contributed to the results of the thesis and the publications resulting from the thesis project:

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Some parts of the thesis have been quoted verbatim from the previously published, theses project related, articles listed above. The permission to reproduce text, figures, tables, and other content published in *International Journal of Molecular Sciences* from the respective copyright holder MDPI.

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Table of Contents

Abbreviations and Definitions

4-HNE: 4-Hydroxynonenal

BAX: BCL2 Associated X Protein

BCL2L1: BCL2 Like 1

big ET: Big Endothelin

CASP8: Caspase 8

CP: Carbonylprotein

DCD: grafts and organs after circulatory death

DGF: delayed graft function

ECD: extended criteria donor

ESRD: end-stage renal disease

GPX3: Glutathione Peroxidase 3

GSH: reduced glutathione

GSS: Glutathione Synthetase

HMP: hypothermic machine perfusion

HSP70.2: Heat Shock Protein 70.2

I/R: ischemia-reperfusion

IPC: Ischemic preconditioning

IPoC: Ischemic postconditioning

IRI: ischemia/reperfusion injury

KTx: kidney transplantation

MDA: Malondialdehyde

MLKL: Mixed-lineage kinase domain-like protein

MP: Machine perfusion

MPO: Myeloperoxidase

MSC: mesenchymal stem cells

NAC: N-acetylcysteine

NFKB: Nuclear Factor of Kappa Light Polypeptide Gene Enhancer In B-cells

NMP: normothermic machine perfusion

OXSR1: Oxidative Stress Responsive Kinase 1

PPARA: Peroxisome Proliferators Activated Receptor Alpha

RIPKI1: Receptor Interacting Serine/Threonine Kinase 1

ROS: reactive oxygen species

SCS: static cold storage

SOD2: Superoxide Dismutase 2

TOS: Total oxidant status

UW: University of Wisconsin

Abstract in German

Im Zuge der Nierentransplantaiton ist ein Ischämie-/ Reperfusionsschaden unvermeidlich und kann zu verzögerter Transplantatfunktion, Transplantatversagen, erhöhter postoperativer Morbidität und Mortalität führen. Relaxin, ein insulinähnliches Peptidhormon, schützt in Nagetiermodellen vor Ischämie-/ Reperfusionsschäden der Niere. Klinische Studien sind, aufgrund bisher fehlender Daten aus Großtierversuchen, noch nicht durchführbar. Diese Studie wurde entworfen, um die Wirkung von Relaxin (supplementiert in Custodiol®) im Setting der statischen kalten Lagerung in einem klinisch relevanten Großtiermodell der Nierentransplantation zu untersuchen.

In dieser verblindeten, randomisierten, Placebo kontrollierten experimentellen Studie wurden 19 Spenderschweinen nach Perfusion mit Custodiol®, supplementiert mit Relaxin (5 oder 20 nmol/l) oder Placebo, beide Nieren entnommen. Die Organe wurden anschließend für 24 Stunden (rechte Niere) bzw. 48 Stunden (linke Niere) in eiskaltem Custodiol®, supplementiert mit Relaxin oder Placebo, gelagert. Nierenproben wurden für eine PCR-Analyse entnommen, um oxidativen Stress und Apoptose / Nekroptose-assoziierte Gencluster zu untersuchen. Des Weiteren wurde mittels Immunhistochemie, Apoptose und durch oxidativen Stress induzierte Zellschädigungen dokumentiert. Anschließend wurden bei 2 separaten Empfängertieren, nach jeweils einseitiger Nephrektomie rechts eine Nierentransplantation durchgeführt und die Empfänger 28 Tage lang beobachtet. Blutbiochemie, Nierenfunktionsparameter so wie Marker für oxidativen Stress, Lipidperoxidation und Endothelzellschädigung (Carbonylprotein; Malondialdehyd; 4-Hydroxynonenal; Myeloperoxidase; Major Endothelin; und Gesamtoxidationsmittelstatus) wurden bestimmt.

Die Studie zeigte, dass Relaxin die SOD2- und NFκB-Genexpression auf 1.35 fache ($p=0.042$) bzw. 1.25 fache ($p=0.019$) erhöhte. Gleichzeitig wurde die RIPK1-Expression auf 0.82 fache ($p=0.016$) der jeweiligen Kontrollgruppen reduziert. Darüber hinaus senkte Relaxin die Expression von RIPK1 und MLKL signifikant und reduzierte die Anzahl der Caspase-3- und MPO-positiven Zellen in Nierentransplantaten nach der statischen kalten Lagerung.

Dies ist die erste Großtierstudie, die eine signifikante Verringerung der ischämischen Schädigung nach statischer kalter Lagerung von Custodiol®, supplementiert mit Relaxin, unter

Verwendung von sowohl antioxidativen als auch anti-apoptose-/antinekroptose-Mechanismen zeigt. Um Relaxin als neues Supplement zur Bekämpfung des Ischämie-/ Reperfusionsschadens im Rahmen der statischen kalten Lagerung im humanen Setting erfolgreich implementieren zu können, sind weiterführende klinische Studien jedoch unbedingt erforderlich.

Abstract in English

Renal ischemia-reperfusion injury is unavoidable in the course of kidney transplantation and can lead to increased postoperative morbidity/mortality, delayed graft function and graft failure. Relaxin, an insulin-related peptide hormone, protects against renal ischemia-reperfusion injury in rodent models. Although it has yet to be tested in clinical settings given lack of large animal studies. Thus, this study was designed to investigate the effect of Relaxin supplemented to Custodiol® for static cold storage in a large animal kidney transplantation model.

This study was designed as blinded, randomized, and placebo-controlled experimental study. Kidneys from 19 donor pigs were retrieved after perfusion with Custodiol® supplemented with Relaxin (5 or 20 nmol/l) or placebo. Right and left kidneys underwent static cold storage for 24 or 48 hours, respectively. Kidney samples were harvested for PCR analysis to investigate oxidative stress. Apoptosis/necroptosis-related gene panels and immunohistochemistry were performed to document apoptosis and oxidative stress-induced cell damage. Subsequently, kidneys were transplanted after unilateral right nephrectomy, and recipients were followed up for 28 days. Blood biochemistry parameters representing kidney function, including indicators of oxidative stress, lipid peroxidation, and endothelial cell damage (Carbonyl protein; Malondialdehyde; 4-hydroxynonenal; Myeloperoxidase; Big Endothelin; and Total Oxidant Status) were determined.

The results of the study showed that perfusion with Relaxin upregulated SOD2 and NFKB gene expression to 1.35-fold ($p=0.042$) and 1.25-fold ($p=0.019$) of controls, respectively. At the same time, RIPK1 expression was downregulated to 0.82-fold ($p=0.016$) of corresponding controls. Furthermore, after static cold storage, Relaxin significantly downregulated RIPK1 and MLKL expression and decreased the number of Caspase 3- and MPO-positive cells in kidney grafts. This is the first large animal study demonstrating that ischemic injury was significantly diminished after static cold storage in Custodiol® supplemented with Relaxin via both antioxidant and anti-apoptotic/anti-necroptotic mechanisms. Clinical trials are warranted to implement synthetic human Relaxin as a novel additive to preservation solutions to combat ischemia-reperfusion injury in kidney transplantation.

1. Introduction

1.1.Kidney transplantation: a historical perspective

Currently, kidney transplantation (KTx) may be considered the excellent choice for renal replacement therapy in end-stage renal disease (ESRD). It demonstrates longer survival and superior quality of life when compared to conventional hemodialysis (1,2). According to the data of the Global Observatory on Donation and Transplantation produced by the WHO-ONT collaboration, more than 80 000 kidney transplants were performed worldwide in 2020. Such success has been the product of tremendous advances in many fields of medicine, which could not have been possible without endless experimental and clinical research (3).

The first successful experimental KTx was performed by the Austrian surgeon Emerich Ullman in 1902 when he autotransplanted a dog kidney (4). Several decades later, in 1933, the Ukrainian surgeon Yurii Voronoy achieved the first human KTx, using 6 h anoxic cadaver kidneys implanted in a young woman suffering from acute renal failure because of mercury poisoning (5). Unfortunately, the patient died 2 days after due to ABO incompatibility (5). Later, in 1945, a group of surgeons - Landsteiner, Hufnagel, and Hume from the Peter Bent Brigham Hospital in Boston, transplanted a human cadaver kidney to the brachial artery and cephalic vein of a young woman suffering acute renal failure. The woman's native kidneys recovered within several hours, and the allograft was successfully excised afterwards (6). After five years, the first intra-abdominal human renal transplant was done in Chicago by a team led by Lawler (7). The donor's kidney was retrieved from a dead donor with cirrhosis and implanted into a patient suffering polycystic kidney disease (7). Surprisingly, the kidney functioned for at least 53 days. After ten months, it was found to be shrunken, discolored, and rejected, and thus was removed. At those times, there were no methods to avoid graft rejection and the chance of success was minimal. However, the lack of dialysis or alternative treatments for renal failure justified further investigation of KTx in otherwise doomed patients (8). In 1951 several teams from France performed nine KTx, where most of the donors were guillotined criminals (8). Through these operations, the standard technique for kidney transplantation was established. Kuss created the method of retroperitoneal kidney placement in the pelvis with re-vascularization via the iliac

vessels and ureter anastomosis to the bladder. This technique is still the foundation of current transplantations (8). The first eight recipients died within days or weeks without meaningful transplant function. However, the ninth transplant was unique. It was the first using a living donor, who was the patient's mother. Different than previous, this kidney promptly functioned for about 3 weeks after KTx (8). At the same time, the KTx program in the previously mentioned Peter Bent Brigham Hospital resulted in 9 KTx between 1951 and 1953. Four kidneys functioned for a short period post-transplant and one of them for 5.5 months before rejection (8). Such results may sound morbid in current times, however, in the 1950's it represented an opportunity for further research.

The first long-term success after KTx was achieved in Boston on December 23, 1954, when Joseph Murray avoided rejection by using the patient's identical twin as the donor. The kidney functioned immediately in the operating room and continued to have good kidney function until cardiac death 8 years later (9). Despite the immediate enthusiasm and worldwide acknowledgment, the latter findings of Murray's team were much more important. After a significant amount of evidence proved that the immune system is responsible for graft failure, Murray's team used total body irradiation with or without donor's bone marrow transplantation as a method for recipient conditioning in 1958 (10). Several months later, Jean Hamburger and colleagues used the same irradiation treatment for successful KTx in Paris, at first for dizygotic twin cases and then for non-twin recipients (11). Despite some success, the initial results with such protocol were poor since less than 10 % of allograft recipients survived at least 3 months (12). The next advancement in KTx occurred when Tom Starzl utilized drug-induced immunosuppression to improve KTx outcomes. He created a „cocktail" consisting of prednisone and azathioprine instead of irradiation (8). These findings provided a strong background for further research and development of KTx strategies. The combination of advanced surgical and anesthesiological techniques, immunosuppression medicine, and improved coordination of the transplant process have cultivated our transplant practices today.

1.2.Kidney transplantation: current issues and future perspectives

Nowadays, the short-term outcomes of KTx are excellent with 1-year graft survival rates exceeding 95 % and even higher rates of patients' survival. On the other hand, long-term outcomes did not improve over the last decade (13). Long-term survival is better in transplanted patients compared to those on dialysis. Although, a large discrepancy still exists between the transplant population and the general community (14). Depending on the age of the KTx patient, the risk of death may be up to 10-fold higher than the standard population, or in other words - equivalent to a patient 30 years older (14). Furthermore, despite major improvements in graft loss rates in the short-term, graft survival beyond 10 years has not advanced equally (14). Chronic allograft dysfunction and graft loss have multiple etiologies, which commonly overlap. These include nephrotoxicity of calcineurin inhibitors used to prevent rejection, damage resulting from chronic antibody-mediated rejection, and initial ischemia mediated organ injury at time of transplant (14). Despite the above-mentioned challenges of KTx, it is still the first-line option for patients with ESRD. Organ shortage is the greatest limiting factor facing the field of transplantation today (15,16). With the rise in ESRD patients, the lack of organs widens the gap between demand and supply. As the waiting time for transplantation increases, more patients are dying while still listed (15,17,18). Moreover, the current COVID-19 pandemic had an additional negative impact on transplant activity in all countries, most of all kidney transplants (19). To resolve this shortage of donor kidneys, the use of extended criteria donor (ECD) grafts and organs after circulatory death (DCD) is increasing. However, these organs are known to be very susceptible to ischemia/reperfusion injury (IRI) (15,20,21). Thus, the impact of IRI is an increasing problem, and prevention strategies to better protect grafts are urgently required.

1.3.*Ischemia-reperfusion injury in the kidney transplantation process*

IRI is inevitable during the KTx process, and the injury occurs through several phases of the procedure. The warm ischemia phase starts immediately after blood flow to the donor kidney stops and lasts until cold perfusion begins. The severity of renal injury at this phase is related to the time of warm ischemia and strongly depends on the type of donor. The shortest time of warm ischemia, usually only several minutes, occurs in living donors and standard donation after brain death donors. However, this phase may be much longer in the case of DCD, where relative

ischemia between the withdrawal of life-sustaining measures to cold organ flush may last up to 2 hours. Prolonged warm ischemia time damages the kidney graft and leads to impaired outcomes of KTx. This includes higher rates of delayed graft function (DGF) and increased probability for rejection (21,22). The cold ischemia phase starts immediately after the warm ischemia phase ends and lasts until reperfusion after organ implantation. Prolonged cold ischemia time is among the major risk factors for DGF and graft survival (23,24). The reperfusion stage take place after the revascularization of the graft. This is the final and biologically most severe stage of injury. It results in derangement of cellular reparative systems and increase in inflammatory responses (25).

IRI may cause cellular damage via different pathways, but the main mechanisms include oxidative stress-mediated injury, increase in intracellular calcium, mitochondrial damage, leak of iron ions, and inflammatory immune response (21,26). Ischemia give rise to a series of intracellular changes such as a shift to anaerobic metabolism, accumulation of bioproducts which cause acidosis, an increase of intracellular hyperosmolarity, and depletion of adenosine triphosphate (ATP) stores (21,25). Consequently, lysosome membranes are destabilized, and thus leaking enzymes causing a breakdown of the cell structure. Also, Na^+/K^+ ATPase activity is inhibited, therefore intracellular Na^+ concentration dramatically increases. Failure of energy-dependent transporters cause an upsurge in intracellular Ca^{2+} levels with the accumulation of Na^+ ions. Osmotic imbalance is responsible for the increased water influx and cellular edema, which damage the integrity of organelles such as the endoplasmic reticulum and mitochondria. Injury of endoplasmic reticulum results in the accumulation of misfolded proteins. Glucose deprivation and redox state imbalance provoke an unfolded protein response leading to apoptosis. The calcium overload promotes the activation of calcium-dependent proteases and the generation of reactive oxygen species (ROS) within mitochondria. This causes permeability transition pores to open allowing cytochrome C to leak and activate the caspases pathway. In that way apoptosis may be initiated. As the signals for oxidative phosphorylation are lost and ATP is depleted, the process becomes irreversible and cellular necrosis occurs. A relatively small quantity of cells are lost through these ischemia-mediated processes. Tissue damage in the reperfusion phase is much more significant (21,25,26).

Reperfusion phase damage represents impaired cellular reparative systems and inflammatory reactions in response to ischemic injury. Following oxygen supply restoration and extracellular pH normalization, there is a steep increase of ROS production that causes direct injury to cellular membranes, DNA, and inflammatory responses. The cells which were subjected to previous ischemia-related injury are especially sensitive to oxidative stress, because of their reduced antioxidant systems capacity. Additionally, after cells die from injurious stimuli, the normal intracellular products are released. They trigger the innate immune system to release cytotoxic cells into injured tissue. In addition, the complement system is activated, and the production of chemokines and cytokines increase. Simultaneously, endothelial cells of micro-vessels upregulate the expression of adhesion molecules and promote the production of vasoconstrictive substances. Reperfusion injury promotes a prothrombotic state and vascular permeability. Together with this inflammatory response and reduced perfusion from microvasculature failure, significant damage occurs. (21,25,26). IRI remains a main cause for poor donor graft function or failure, increased postoperative morbidity, and mortality after KTx. Thus, it is among the main targets of research to improve organ quality and prevent transplantation-related complications.

1.4. Current strategies to prevent IRI in kidney transplantation

Currently, different strategies to ameliorate IRI in KTx have been investigated including interventions in donor and recipient organs before, during, or after transplantation. Figure 1 shows an overview of these therapies.

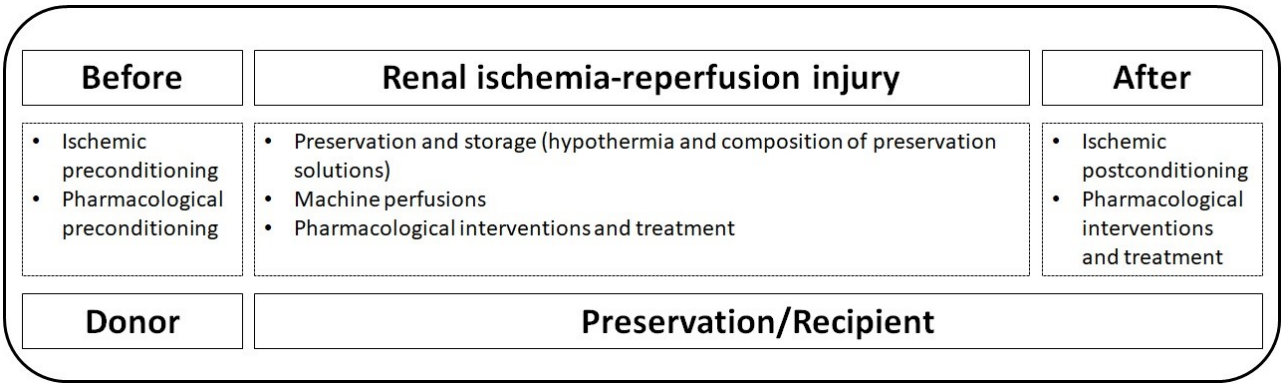

Figure 1. Available strategies to prevent ischemia-reperfusion injury at different stages of kidney transplantation.

1.4.1. Strategies to prevent IRI by interventions in the organ donor

Donor preconditioning could be defined as treatment or intervention in donors before organ retrieval to improve organ quality. Different interventions for preconditioning of kidney donors have been investigated in a clinical and experimental setting:

- Ischemic preconditioning (IPC): This is a concept of increased organ tolerance to IRI after preconditioning it with brief ischemia and reperfusion periods. The benefit of IPC was first described by Murray et al. in 1986, showing reduced ischemic insult in the canine myocardial infarction model (27). The effect size of IPC was much larger compared to pharmacological interventions and this phenomenon was reproducible in different experimental models (28,29), including the renal IRI model (30). However, results are inconsistent, as some experimental studies failed to show that IPC attenuates renal dysfunction or morphological damage in large animal models (31,32). Moreover, IPC is an invasive procedure, requiring direct clamping of renal pedicle vessels. Therefore, IPC did not gain much clinical attention.

  Przyklenk and colleagues showed that IPC could be used remotely, in such that periods of ischemia can be applied to one vascular bed while the other remains protected (33). In clinical practice, remote IPC can be induced using blood-pressure cuffs in legs or forearms. Such non-invasive approach has revived interest in the IPC strategy (34). An experimental IRI model showed promising result, demonstrating that remote IPC diminished kidney injury and dysfunction (35–39). Similar findings were also seen in a clinical study showing remote IPC reduces the rate of acute kidney injury in high-risk patients after cardiac surgery (40). After such evidence, IPC was also studied in KTx. However, a large-scale RCT failed to show the benefit of remote IPC for deceased donor KTx (41,42).

- Pharmacological preconditioning: Various drugs have been proposed for donor preconditioning before organ procurement to attenuate IRI. Substances with antioxidative or immunosuppressive features are among the most commonly used in pre-clinical and clinical studies. Immunosuppressive drugs play a crucial role in modern transplantation medicine to prevent immunological response mediated graft rejection. Hence these drugs may also have some properties against IRI (43). An experimental study on rat KTx documented that preconditioning donor animals with tacrolimus with or without rapamycin significantly reduced acute tubular necrosis, level of apoptosis, and pro-inflammatory cytokines in grafts at early stage post-transplantation (44). The increasing amount of evidence suggests that the cytoprotective effect of tacrolimus may be achieved through different mechanisms including promotion of microcirculation and intracellular ATP levels, suppression of ROS elaboration, potential antioxidative activities, and immunomodulatory effects (45). Another large class of drugs investigated for preconditioning is antioxidants. N-acetylcysteine (NAC) is one of the most studied substances among these. It acts as a reduced glutathione (GSH) precursor, which is a direct antioxidant and a substrate of several antioxidant enzymes. Moreover, NAC may act as a direct antioxidant for some oxidant species such as NO_2 and HOX or its antioxidant activity can be mediated by its ability to break thiolated proteins (43,46). In an experimental setting, NAC application before ischemia and immediately before reperfusion prevents nephrotoxicity caused by I/R (47). Furthermore, NAC was shown to have a protective role in pre-clinical intestinal and heart transplantation models (48,49). Despite promising results in experimental studies, a large-scale RCT showed that donor pre-treatment with NAC did not improve outcomes in patients undergoing KTx (50).
- Dietary preconditioning: Short-term dietary restriction or fasting have a beneficial effect against kidney injury and dysfunction in mice IRI models (51). Also, it is safe and feasible in living human kidney donors (52). On the other hand, the pilot study which confirmed feasibility failed to show the efficacy in human KTx (52). Pre-clinical and clinical research is necessary to elucidate the effect of such dietary preconditioning.

1.4.2. Strategies to prevent IRI by interventions in the recipient

- Ischemic postconditioning (IPoC): The concept has many similarities with the previously described IPC technique. In this case, the "conditioning" intervention is performed in the recipient before the start of reperfusion, which is a more controllable time point (53). IPoC showed its potential in a series of experimental studies (54–59). Mechanisms for kidney protection include those observed in IPC, in addition to some unique benefits (53). IPoC attenuates ROS formation, promotes the production of ROS scavengers, alleviates mitochondria injury, prevents complement activation, and reduces the inflammatory response (53). Similar to IPC, the remote approach for IPoC is available as shown in experimental studies (36,60). However, the clinical data for the efficacy of direct or remote IPoC is inconsistent. A small-scale RCT demonstrated that remote IPoC may enhance the early recovery of renal function after KTx (61), but another pilot study failed to show any beneficial effect on postoperative outcomes (62). Furthermore, a recent meta-analysis showed that various types of conditioning do not improve DGF after KTx (63).
- Pharmacological interventions and treatment: Many pharmacological agents including spingosine 1 phosphate analogs, adenosine A2A receptor agonists and other adenosine analogs, statins, fibrates, inducible nitric oxide synthase inhibitors, antioxidants, and antioxidant enzymes, thrombomodulin and activated protein C, erythropoietin and its derivatives showed efficacy in pre-clinical models against acute kidney injury and IRI (29,64). However, most of these differently acting substances aiming against IRI are not used in routine clinical practice. Currently, statins and erythropoietin are among the most studied in clinical trials (29). Some retrospective studies have suggested that statins have renoprotective effects against contrast-induced acute kidney injury (65,66), but these findings were not confirmed in a high-quality prospective RCT (29,67). Statins were investigated in a transplant setting as well, but a recent meta-analysis showed no positive effect on transplant rejection rates, graft function, or patient survival after KTx (68). Similarly, erythropoietin was shown to prevent kidney IRI in the experimental setting, but such findings were not replicated in clinical studies (29). Moreover, treatment with high-dose human recombinant erythropoietin resulted in significantly increased systolic blood

pressure. Thus, not only the efficacy but also the safety of such treatment was questioned (69).

Cellular therapy by mesenchymal stem cells (MSC) has been suggested as an innovative approach for kidney IRI. Experimental small animal studies demonstrated that MSC minimized kidney IRI by antioxidative and anti-inflammatory mechanisms (70,71). However, these findings were not confirmed in large animal models (72). MSC was tested in very small pilot clinical studies, which suggested that they are feasible and potentially beneficial in KTx. They may help to reduce the dose of conventional immunosuppression (29,73), but its role against IRI needs to be elucidated in future research.

1.4.3. Strategies to prevent IRI by interventions during the preservation phase

- Machine perfusion (MP): Organ preservation techniques are always necessary in cadaveric kidneys to minimize IRI. MP techniques were originally discovered in the 1930s but lost attraction in the 1950s when SCS became the dominating technique due to reduced costs, easier logistics, and non-inferior clinical outcomes. However, MP has regained a role in KTx in the last two decades (74). During MP, the kidney is connected to a special machine that pumps preservation solutions through the kidney. It maintains hemodynamic simulation and eliminates metabolic waste (29). MP can be classified into hypothermic machine perfusion (HMP) and normothermic machine perfusion (NMP) based on the temperature at which the organ is preserved. HMP is currently the most used technique, in which the kidney is perfused with colloid cell-free solution at 1–8° C (74). Similar to SCS, HMP aims to prolong the life and preserve the quality of the harvested kidney by hypothermia, which reduces the metabolism and oxygen demands at a cellular level. Additionally, dynamic circulation in HMP continuously provides nutrients and eliminates metabolic waste from the kidney. Maintenance of hemodynamic stimulus promotes endothelial cells to sustain physiologic morphology and function, thus improving vascular reactivity (74,75). HMP is increasingly being used for the preservation of kidneys, especially from ECDs after its theoretical efficacy was proved in

a large-scale RCT (34,76). Nevertheless, even if MP is beneficial for graft preservation, it can still be improved by optimizing the quality of the preservation solutions.

NMP is another available and emerging technique for organ storage at KTx. It maintains the procured organ at temperature and pressure close to physiological. The perfusate used in NMP is a nutrient supplemented oxygen carrier, such as red blood cell-based solutions, artificial hemoglobin solutions, or acellular solutions. They ensure optimal oxygen delivery and support metabolism. Potential advantages of NMP include avoidance of cold ischemia, restoration of ATP levels, and the ability to evaluate renal function by measuring urine output. However, there is no consensus on the most optimal protocols for NMP. Variations have been used including a period of NMP after hypothermic preservation or a prolonged period of NMP. Most of the studies investigating NMP are experimental and only limited data exists on its application in clinical KTx (74,77). The results of currently ongoing and future clinical trials are needed to clear up the benefits of NMP over current hypothermic techniques before its wider introduction.

– Preservation solutions: Cold storage remains the gold standard in kidney preservation (29). Therefore, optimization of conditions during cold ischemia is fundamental. Different organ preservation solutions have been developed to prevent against IRI and warrant immediate and appropriate organ function after transplantation (78). The time of ex vivo graft preservation is a very attractive timepoint for novel therapeutic interventions because this step is a long and unavoidable part of KTx. Also, its practical application is relatively simple. Thus, many different pharmacologically active agents have been proposed as potential additives to preservation solutions (79). Different molecules targeting inhibition of inflammation, coagulation disruption, oxidative stress attenuation, prevention of apoptosis, and modulation of the hypoxia-inducible factor signaling pathway have been shown to be effective in experimental studies (79). Also, during ex-vivo preservation gene therapy can be applied without the risk of ectopic transgene expression. Such therapy may aim to reduce inflammation, apoptosis, and oxidative stress in grafts. For example, preclinical studies have shown that delivery of antisense oligodeoxynucleotides targeting intercellular adhesion molecule-1 or Midkine can attenuate renal IRI. Adenovirus-mediated antisense-ERK2 therapy decreased chronic

allograft nephropathy. siRNA-mediated p53 silencing reduced ischemic kidney injury and donor kidney treatment in situ with an adenovirus expressing HO-1 improved recipients' survival (79).

Despite the efficacy of various pharmacological additives in experimental models, translation to clinical practice is slow. There is a need for large-animal studies, which could provide patient-like data and facilitate implementation (79).

1.5. Relaxin – potential substance against IRI

Relaxin (RLX) is a peptide hormone discovered in 1926 by Frederick Hisaw. The researcher noticed that injection of serum from pregnant guinea pigs caused the pubic symphysis of virgin guinea pigs to "relax" (80). RLX is a two-chain polypeptide that is a member of the protein hormone family that also includes insulin and insulin-like growth factors (81). The peptide encoded by the RLN2 gene is the main circulating form of RLX (82). Primarily RLX is produced by the corpus luteum during pregnancy and non-pregnancy in females, with the highest plasma levels during pregnancy (81). In males, RLX is synthesized in the prostate. The heart atria are an additional source of RLX in both genders (81). RLX has long been considered a pregnancy hormone, responsible for typical changes in connective tissue composition, implantation process regulation, myometrial activity, and labor (83). However, in recent years novel actions of RLX have been discovered including antifibrotic, antioxidant, anti-inflammatory, hemodynamic, and cytoprotective actions (81,84). These newly discovered properties of RLX attracted the attention of researchers investigating new drugs against IRI. Thus, RLX was tested in a series of pre-clinical studies in the field of liver, lung, heart, and kidney transplantation.

1.6. Current evidence for the beneficial effect of Relaxin in solid organ transplantation

Boehnert and colleagues extensively investigated RLX as a supplement for UW or HTK preservation solutions through rat liver I/R (85–87). RLX at concentrations of 32 ng/mL or 64

ng/mL significantly decreased MPO and MDA levels in perfusate and liver tissue, and improved oxygen supply in grafts (85–87). A liver transplantation model in mice showed that 5 μg/kg RLX administered intravenously immediately before reperfusion improved recipients' survival, decreased the level of apoptosis and necrosis, and attenuated neutrophil/macrophage accumulation in grafts (88,89). The mechanistic insights of these studies showed that RLX effect against IRI in the liver is achieved by acting on its primary receptor (RXFP1) and on hepatocyte glucocorticoid receptors. Furthermore, it also works on the Notch1 signaling pathway, which controls cell proliferation, differentiation, fate, and death (84,88,89).

Alexiou et al. showed a benefit of RLX as an additive to Krebs–Henseleit preservation solution against lung IRI in isolated lung perfusion models (90,91). RLX significantly ameliorated I/R-induced endothelial cell injury, inflammation, and oxidative stress as shown by decreased levels of endothelin-1, neutrophil elastase, myeloperoxidase, and malondialdehyde. The mechanisms of action against IRI in lungs included early and moderate induction of inducible nitric oxide synthase and a protective effect via balanced extracellular signal-regulated kinase-1/2 and phosphatidylinositol-3 kinase stimulation (84,90,91).

RLX has a great variety of effects on the cardiovascular system including improved arterial distensibility, renal blood flow, and increased cardiac output (92,93). These properties made RLX a promising substance for acute heart failure treatment, thus it was tested in a large-scale placebo-controlled RCT (RELAX-AHF) in humans (93). The results of the RCT demonstrated that treatment with RLX was associated with dyspneic relief and improvement in other clinical outcomes such as a reduction of 180-day mortality. It was also well tolerated and safe. However, it had no effect on the rate of readmissions. (93). Given these encouraging results, the subsequent RELAX-AHF-2 clinical study was initiated to confirm the reduced 180-day mortality in patients hospitalized for acute heart failure (94).

However, the trial failed to show that RLX can reduce cardiovascular disease-related 180 days mortality rate (94). These studies did not aim to investigate RLX impact on IRI, but rather demonstrated the feasibility in applying the substance in a clinical setting. RLX effects on IRI in the heart has been investigated in pre-clinical studies including mice, rats, guinea pigs, and porcine models (95–99). The myocardial infarction model in wild-type and endothelial nitric oxide synthase knockout mice demonstrated that pre-treatment with 10 μg/kg RLX administered

subcutaneously before ischemia and before reperfusion improved animal survival, reduced the size of the infarct zone, preserved left ventricle function, and reduced apoptosis in the heart (95). RLX administration before an ischemic event or before reperfusion was similarly effective. Genetic deletion of eNOS abolished the effect of RLX, therefore proving that RLX attenuates myocardial IRI and the subsequent caspase-1 activation via eNOS-dependent mechanisms (95). Similar to this study, many authors accentuate that RLX works by inducing NO synthase (84). However, NO has a dual role in myocardial IRI (97). The study by Masini et al. demonstrated that RLX prevents IRI in rats' hearts by its ability to promote endogenous NO production. The same effect was achieved by administering L-N^{w}-(1-iminoethyl)lysine (L-NIL) - a selective inhibitor of inducible NO synthase. Opposite to RLX, this agent inhibits the production of NO caused by reperfusion in ischemic tissues. The authors hypothesized that such phenomena can be explained by another feature of L-NIL, in which NO is converted to peroxynitrite, a molecule that has significant oxidative capacity (97). The same study group showed that when RLX was used as a supplement to perfusion solution at the dose of 30 ng/mL it was effective against IRI (98). RLX improved coronary flow, NO generation, and decreased malondialdehyde production and calcium overload. Also, it inhibited mast cell granule exocytosis, prevented histamine release, and preserved the structure and function of the myocardium (98). All of these effects were shown to be blunted by the NO synthase inhibitor (98). The only available large-animal study on RLX against heart IRI is a porcine model of myocardial infarction (96). This study investigated three doses of RLX (1.25, 2.5, and 5 μg/kg) administered as a continuous infusion through a right atrial catheter at the time of reperfusion (96). Like previous small animal studies, it also showed that RLX reduced levels of myocardial injury markers (serum myoglobin, CK-MB, troponin T), decreased cardiomyocyte apoptosis, diminished cardiomyocyte contractile dysfunction, and preserved ventricular performance (96). The effect of RLX was dose-dependent and 5 μg/kg of RLX resulted in almost no ultrastructural changes in the myocardium following IRI (96).

Despite kidneys being the most transplantable solid organ worldwide, no experimental KTx studies have investigated RLX. Only two studies have looked at it against kidney IRI in non-transplant rat models (84). These small animal studies investigated RLX as an intravenously administered drug before ischemia or before and after reperfusion. Both studies showed consistent results, in which RLX was effective against renal IRI (100,101). RLX improved renal

function after reperfusion, ameliorated histological injury, and had anti-apoptotic and anti-inflammatory effects (100,101).

All above-mentioned studies were consistent in demonstrating RLX protective effects against IRI in the liver, lung, heart, and kidney. However, the majority of data comes from non-transplant, small animal, IRI models. Currently, there is a lack of evidence from clinically relevant large animal organ transplantation models, which prevents the use of RLX in clinical trials (20). Therefore, this experimental study was designed to evaluate the impact of RLX supplemented to Custudiol® on a panel of genes, specifically those involved in oxidative stress and apoptosis. Further, the study investigated apoptosis and inflammation in grafts by immunohistochemistry, also the RLX impact on serum markers of oxidative stress, lipid peroxidation, and endothelial cell injury in a clinically relevant porcine KTx model.

2. Materials & Methods

The description of some parts of the Material & Methods section may be similar to those published previously by *Bausys et al.; 2021* (20).

2.1.Study approval

Republic of Austria federal ministry of education, science, and research approval was obtained before this study was started (BMWFW-66.010/0104-WF/V/3b/2016). Austrian national laws and 3Rs, principles of laboratory animal care were followed for all animal experiments.

2.2.Animals

Fifty-seven (19 triplets) domestic pigs (sus scrofa domesticus) were used for the study. Each triplet consisted of the same generation siblings - one male pig (donor) and two female pigs (recipients) weighing between 35 and 50 kg. Study animals were kept in the Medical University of Graz animal facility under standard conditions having access to water and standard porcine

food (PorkoCidKorn F, Garant, Graz, Austria) ad libitum. All experimental animals were acclimatized for 2 weeks before the start of the experiments. A week before the experiments donor and recipients' blood cross-matching was checked and KTx was performed only if no signs of hemolysis or macro- and microagglutination has been observed (20).

2.3. Experimental design

The study was designed as a blinded, randomized, placebo-controlled trial (Figure 2). Animals were randomized to RLX or Placebo groups. Custodiol® solution (HTK, Dr. Franz Köhler Chemie GmbH, Bensheim, Germany) supplemented with 5 or 20 nM of Relaxin (Relaxera, Bensheim, Germany) or placebo (5mg/ml Mannitol with PBS; Relaxera, Bensheim, Germany) were used for organ flushing and preservation, respectively. After randomization, both kidneys were perfused with RLX- or placebo-supplemented preservation solution and retrieved from donor pigs. Immediately after, each kidney was packed in 1000 ml of the respective study solution and placed on crushed ice for SCS. The right and left kidneys were implanted into randomly assigned sibling recipients after 24 or 48 hours of SCS, respectively. RLX was not administered for reperfusion. Following KTx, animals were followed up for 28 days or until premature euthanasia. Afterward, all experimental animals were euthanized for organ sampling (20). Table 1 shows the numbers of kidneys in subgroups of the experiment.

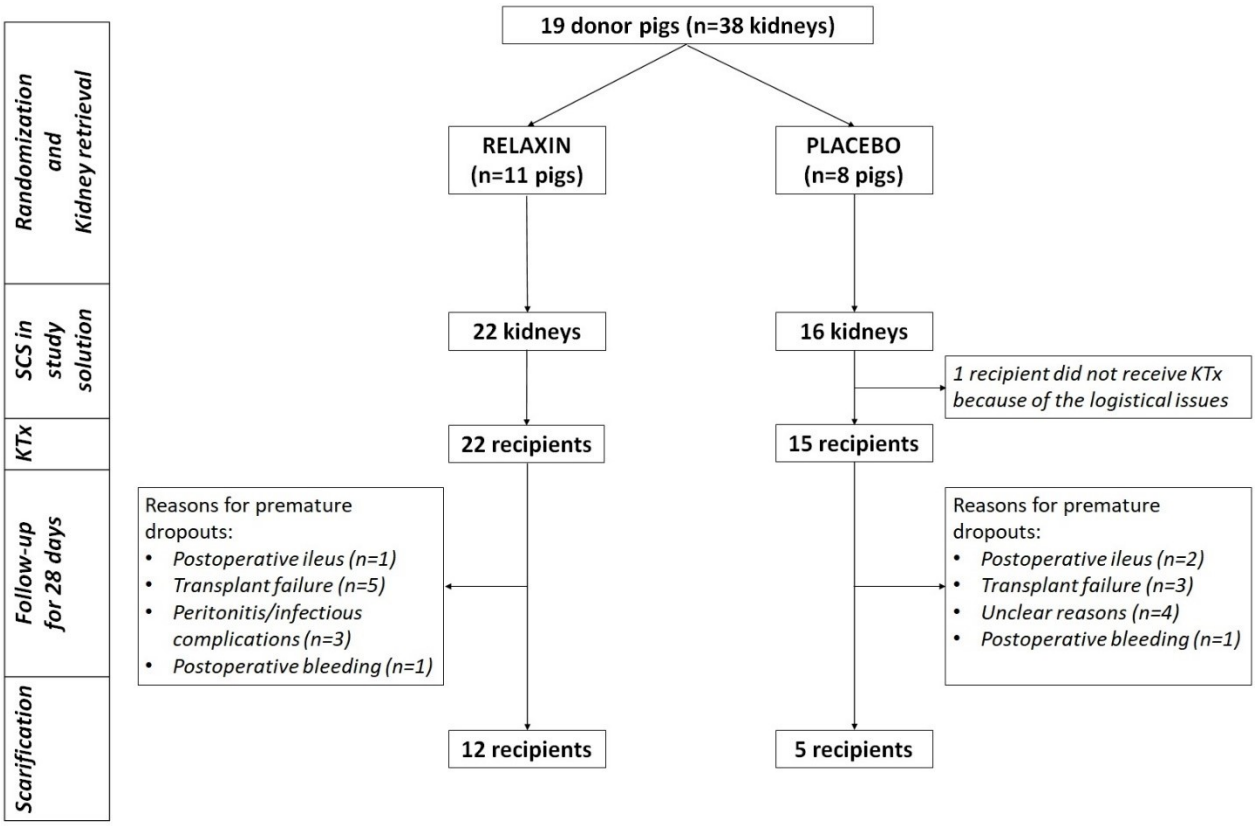

Figure 2. Flowchart study design. Adapted from Bausys et al., 2021 (20) with permissions of MDPI.

SCS: static cold storage; KTx: kidney transplantation.

Subgroups of the experiment	Number of animals, n
Placebo 24 h SCS	n=8
Placebo 48 h SCS	n=8
Relaxin 5nM 24 h SCS	n=5
Relaxin 5nM 48 h SCS	n=5
Relaxin 20nM 24 h SCS	n=6
Relaxin 20nM 48 h SCS	n=6

Table 1. The numbers of kidneys in different subgroups. SCS: static cold storage.

2.4.Kidney perfusion and retrieval

A midline laparotomy was performed under general anesthesia and the aorta was prepared for catheterization 3 cm down to the renal artery. After heparin (200 IU/kg; i/v) injection the distal part of the aorta was ligated, and the perfusion catheter was placed. The proximal part of the aorta at the level of the aortic hiatus was cross-clamped and gravity perfusion with 4000 ml of Custodiol®±RLX started. At the same time cooling with crushed ice and cold saline solution was performed. After these steps were completed, kidneys were retrieved (20).

2.5.Kidney implantation

Animals were premedicated with midazolam (0.5-1 mg/kg; i/m), ketamine (10-15 mg/kg; i/m) and azaperone (2mg/kg; i/m). Anesthesia was induced with Propofol (i/v) and maintained by sevoflurane (1-2 %) and remifentanil (20-100 µg/kg/h). A single dose of Amoxicillin (500 mg; i/v) was used for antibiotic prophylaxis before skin incision. Ranitidine (50 mg; i/v) and Carprofen (4mg/kg; i/v) were administered at the time of anesthesia for analgesia and peptic ulcer prevention. Anticoagulation protocols included heparin (200 IU/kg; i/v) injection before the start of perfusion for donors and double therapy with heparin (200 IU/kg; i/v) and aspirin (500 mg; i/v) before revascularization of the donor organ for recipients. Implantation started with midline laparotomy under general anesthesia and right nephrectomy to remove the native kidney. Afterward, KTx was performed. First, end-to-side renal vein anastomosis with vena cava inferior was done using a continuous 6/0 Prolene (Ethicon, Somerville, New Jersey, USA) suture. Second, end-to-side arterial anastomosis between the renal artery and aorta was constructed using continuous 5/0 Prolene (Ethicon, Somerville, New Jersey, USA) sutures (Figure 3). Vessels were unclamped after heparinization (200 IU/kg; i/v) and renal ischemic time ended. Uretrocystostomy was performed using continuous 3/0 polydioxanone (Ethicon, Somerville, New Jersey, USA) suture on a double J stent. After precise hemostasis, the abdominal wall was closed (20).

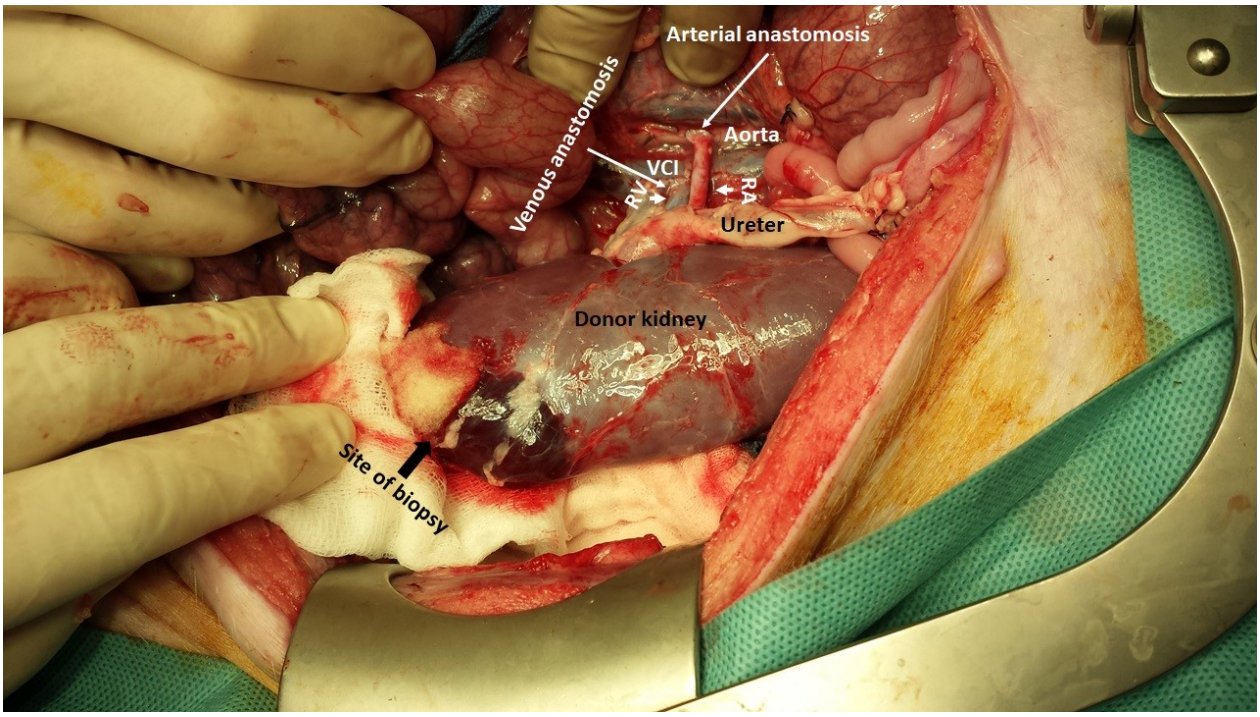

Figure 3. Kidney transplantation in pigs: view after the creation of renal vein and renal artery anastomosis.

RV: renal vein; RA: renal artery; VCI: vena cava inferior

2.6. Immunosuppression and postoperative care

The immunosuppression protocol started intraoperatively by injecting prednisolone (250 mg; i/v) and basiliximab (20 mg; i/v). Later, immunosuppression was maintained with daily administered tacrolimus (30 mg; p/o), basiliximab (20 mg; i/v) on a postoperative day (POD) 4, and prednisolone once a week. Tacrolimus concentration was monitored weekly, and the dose was adjusted if necessary. Trough levels were reached within 2 days and ranged between 6 – 8 ng/dl.

Postoperative care protocols consisted of:

I. Pain management: fentanyl (100 µg/h; transdermally) for the first 7 days after KTx, later carprofen (4 mg/kg; s/c) and buprenorphine (5-10 µg/kg) when necessary.

II. Antibiotic therapy: benzylpenicillin/dihydrostreptomycin (100/100 mg; i/m) on the POD 1, later once a week.

III. Anticoagulation therapy: daily aspirin (100 mg; p/o) and weekly depo-heparin (25 000 IU; i/v).

IV. Peptic ulcer prevention: daily Pantoprazole (40 mg; p/o)

To evaluate the perfusion and survival of the graft abdominal ultrasound was performed on POD 1 and later once a week (Figure 4). Graft failure was defined as graft thrombosis on follow-up ultrasound or if the graft was not viable at the time of explantation (20).

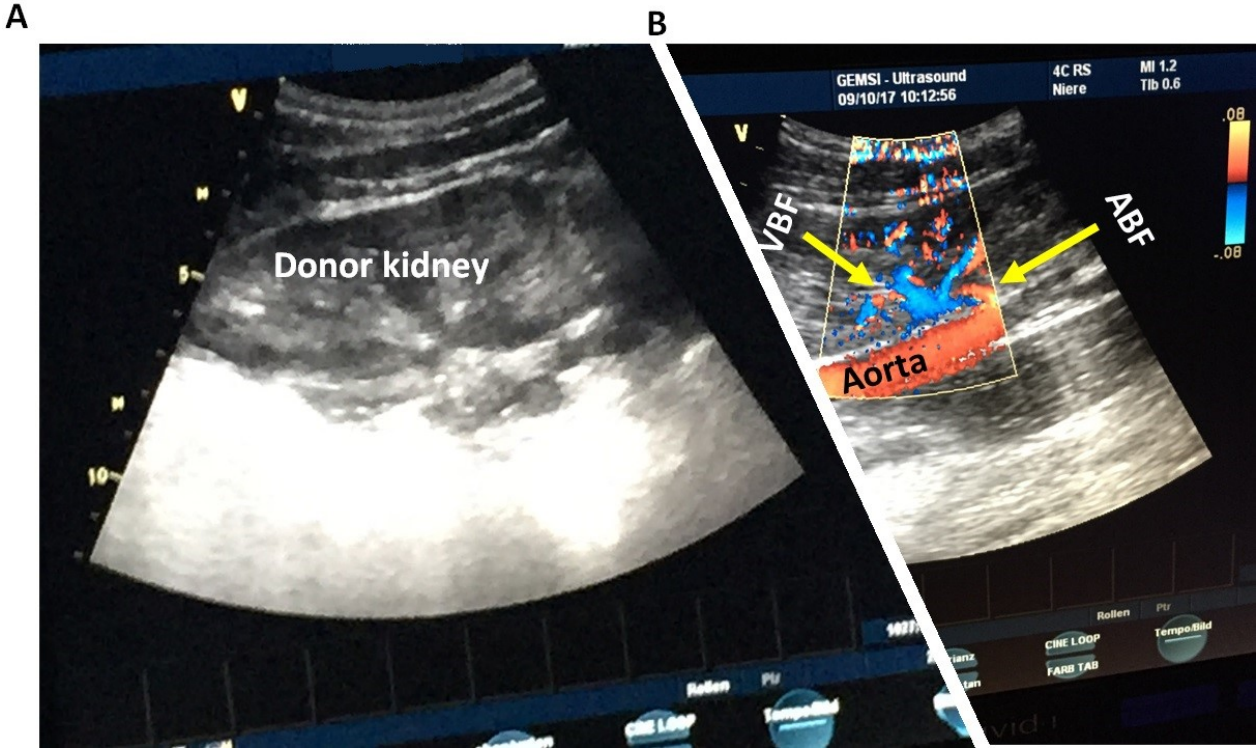

Figure 4. Representative pictures of abdominal ultrasound to examine graft status after kidney transplantation.

Donor's kidney was visualized on abdominal ultrasound (A) and Doppler ultrasonography was performed to visualize arterial, venous blood flow, and kidney perfusion (B).

ABF: arterial blood flow; VBF: venous blood flow.

2.7. Tissue and blood sampling

Tissues (kidney, ureter, renal artery, and renal vein) were sampled after perfusion, after SCS, and at the end of the experiment. Blood samples were collected at baseline, just before KTx, and later on, POD: 1, 7, 14, 21, and 28 (20).

2.8. Whole blood and serum measurements

Blood biochemistry, electrolytes, and blood gases were measured with the i-STAT system (Abbot, Chicago, USA) (20). Oxidative status representing blood parameters: carbonyl protein (CP), malondialdehyde (MDA), and 4-hydroxynonenal (4-HNE) were measured in the commercial laboratory (Institute fur Laborwissenschaften Dr. Greilberger GmbH, Laßnitzhöhe, Austria). Myeloperoxidase (MPO), Big Endothelin (big ET), and Total Oxidant Status (TOS) were measured in an industrial laboratory by the Immunodiagnostik company (Bensheim, Germany).

2.9. Immunohistochemistry

After sampling, kidney tissue was placed in 4 % neutral buffered formalin and transferred to 80 % ethanol after 24 hours. For immunohistochemistry, 3µm paraffin-embedded sections were prepared according to standard protocols. To evaluate the occurrence of apoptosis, an anti-caspase 3 antibody (Abcam, Cambridge, UK; dilution 1:200, mouse monoclonal) was used in combination with the UltraVision LP Detection System HRP Polymer (Thermo Fisher Scientific, Waltham, MA, USA) and DAB chromogen (Dako, Via Real Carpinteria, CA, USA). A similar technique was used to evaluate inflammation and oxidative stress by myeloperoxidase (MPO) antibody (Dako, Via Real Carpinteria, CA, USA; dilution 1:800, Rabbit Polyclonal). After staining, all slides were scanned, and images were viewed using the Aperio ImageScope ver.12.3.2.8013 software (Leica Biosystems Imaging, Wetzlar, Germany). For the semi-

quantification of activated Caspase 3 and MPO positive cells, three independent investigators reviewed at least five randomly assigned areas of the slide and quantitatively graded the staining (negative, slightly positive, positive, strongly positive). The results were transferred to a score from 0 to 3 and the mean of the observations was used for statistical analysis (20).

2.10. qPCR

Kidney tissue samples were snap-frozen and stored in liquid nitrogen until nucleic acid extraction. Tissue (50-100mg) was homogenized in 1 mL TRIzol reagent in combination with a MagNA Lyser (Roche Diagnostics GmbH, Mannheim, Germany). Isolation of RNA was done according to the protocol provided by the manufacturer. Quality and quantity of RNA were determined by Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Two micrograms of RNA were used for reverse transcription (High-Capacity cDNA RT Kit; Thermo Fisher Scientific, Waltham, MA, USA) according to the protocol provided by the manufacturer in a final volume of 20 µL.

Real-time PCR amplification and melting analysis were performed using a BioRad CFX96 TouchTM System (Bio-Rad Laboratories Ges.m.b.H., Vienna, Austria). cDNA corresponding to an equivalent of 5 ng RNA was added to a reaction mix containing Promega GoTaq® qPCR Master Mix (Promega, Madison WI, USA) containing 1 µM of each primer in a final reaction volume of 10 µL. The PCR reaction mixture was subjected to an initial denaturation at 95 °C for 10 seconds, followed by 45 cycles of denaturation at 95 °C for 10 seconds, annealing at 58 °C for 20 seconds, and elongation at 72 °C for 30 seconds followed by a melting curve (60 to 95 °C). For detailed information on primers used see Table 2.

Gene expression was determined using the Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories Ges.m.b.H., Vienna, Austria) via the Cq regression method embedded in the program. All PCR reactions were done in duplicates. Relative gene expression was calculated using multiple reference genes (ACTB & GAPDH) by the Vandesompele method (102–104). This method was used for accurate normalization of real-time quantitative PCR data by geometric averaging of multiple internal control genes (20). The equation used for calculations was:

$$Relative\ gene\ expression = \frac{(E_{GOI})^{\Delta CtGOI}}{GeoMean[(E_{REF})^{\Delta CtREF}]}$$

E: primer efficacy; GOI: gene of interest; REF: reference genes; GeoMean: geometric mean; ΔCt=Calibrator Ct – Sample Ct;

Accession Number	Forward (5'->3')	Reverse (5'->3')	Product Length
ACTB (Actin Beta)			
XM_021086047.1 XM_003124280.5	CTCCAGAGCGCAAGTACTCC	ACTCCTGCTTGCTGATCCAC	90bp
GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase)			
NM_001206359.1	CCGTGTGTTCCGTGCATTG	GCCAAATCCGTTCACTCCGA	71bp
GSS (Glutathione Synthetase) (102)			
NM_001244625.1	AAGAAGCTGCCAAGATCCTC	ATTCTCTATGGCACGCTGGT	155bp
GPX3 (Glutathione Peroxidase 3) (102)			
NM_001115155.1	GAGACAACTCGGAGATTCTG	GGAACGTGTAGAACTTCTGC	126bp
OXSR1 (Oxidative Stress Responsive Kinase 1) (102)			
NM_214342.1	CCGAAGTTATGGAACAGGTC	GATCATTCTGCAGTGTCAGC	147bp
SOD2 (Superoxide Dismutase 2) (102)			
NM_214127	CCTACGTGAACAACCTGAAC	GATACAGCGGTCAACTTCTC	247bp
HSP70.2 (Heat Shock Protein 70.2) (102)			
NM_213766.1	AGGTGCAGGTGAGCTACAAG	CTGCGAGTCGTTGAAGTAGG	158bp

PPARA (Peroxisome Proliferators Activated Receptor Alpha) (102)			
NM_001044526.1	TGAAGTTCAATGCGCTGGAG	TTGAGCACATGCACGATACC	139bp
BCL2L1 (BCL2 Like 1) (102)			
NM_214285.1	TGAGTCGGATCGCAACTTGG	ATCGGTTGAAGCGTTCCTGG	150bp
NFKB1 (Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-cells) (102)			
NM_001048232.1	GAGGTGCATCTGACGTATTC	CACATCTCCTGTCACTGCAT	138bp
BAX (BCL2 Associated X Protein) (102)			
XM_003127290	GCTGACGGCAACTTCAACTG	CCGATCTCGAAGGAAGTCCA	141bp
MLKL (Mixed-lineage kinase domain-like protein)			
XM_003481791.4	TTGGAAAACACCACGAGGGA	CCCTTCTTGGGTTTGTGTGC	77bp
RIPK1 (Receptor Interacting Serine/Threonine Kinase 1)			
XM_005665536	CACTCGGAGAAATCAAGGCAG	CTGCGCCCTGATGGTTACAAAA	86bp
CASP8 (Caspase 8)			
NM_001031779.2	CCAGGATTTGCCTCCGGTTA	TCACTGTCCAAATGTTCCCCA	99bp

***Table 2*.** Genes used for quantification and primer information. Adapted from Bausys et al., 2021 (20) with permissions of MDPI.

2.11. Statistical analysis

Statistical analysis was performed using SPSS v.25.0 (SPSS Inc., Chicago, Illinois, USA). Data are presented as median and quartiles (Q1, Q3) unless stated differently. Differences between groups were analyzed using non-parametric tests - Mann Whitney U test or Kruskal-Wallis test. For related sample analysis, the Wilcoxon-Signed rank test was used. Graft survival

was defined by the Kaplan-Meier method and compared by log-rank test. All statistical tests were 2-sided. P-values < 0.05 were considered statistically significant. To evaluate the effect of 24 or 48 hours of SCS and treatment with 5 vs 20 nM of RLX, a subgroup analysis was performed.

3. Results

The description of some parts of the Results section may be similar to those published previously by *Bausys et al.; 2021* (20).

3.1. Apoptosis/necroptosis gene expression after kidney perfusion and static cold storage

In total, 19 donor animals were randomized to RLX (n=11) or placebo (n=8) groups. Thirty-eight kidneys (RLX n=22; Placebo=16) were successfully retrieved after perfusion with Custodiol®±RLX and placed for SCS in the respective study solution. Perfusion with RLX supplemented solution upregulated SOD2 (Figure 5) and NFKB (Figure 6) genes expression to 1.35-fold (p=0.042) and 1.25-fold (p=0.019) of corresponding controls, while downregulated MLKL expression to 0.82-fold (p=0.021) of controls in the placebo group. Moreover, after SCS, RLX downregulated RIPK1 and MLKL expression to 0.82-fold (p=0.016) and 0.81-fold (p=0.010) of corresponding controls in the placebo group (Figure 6) (20).

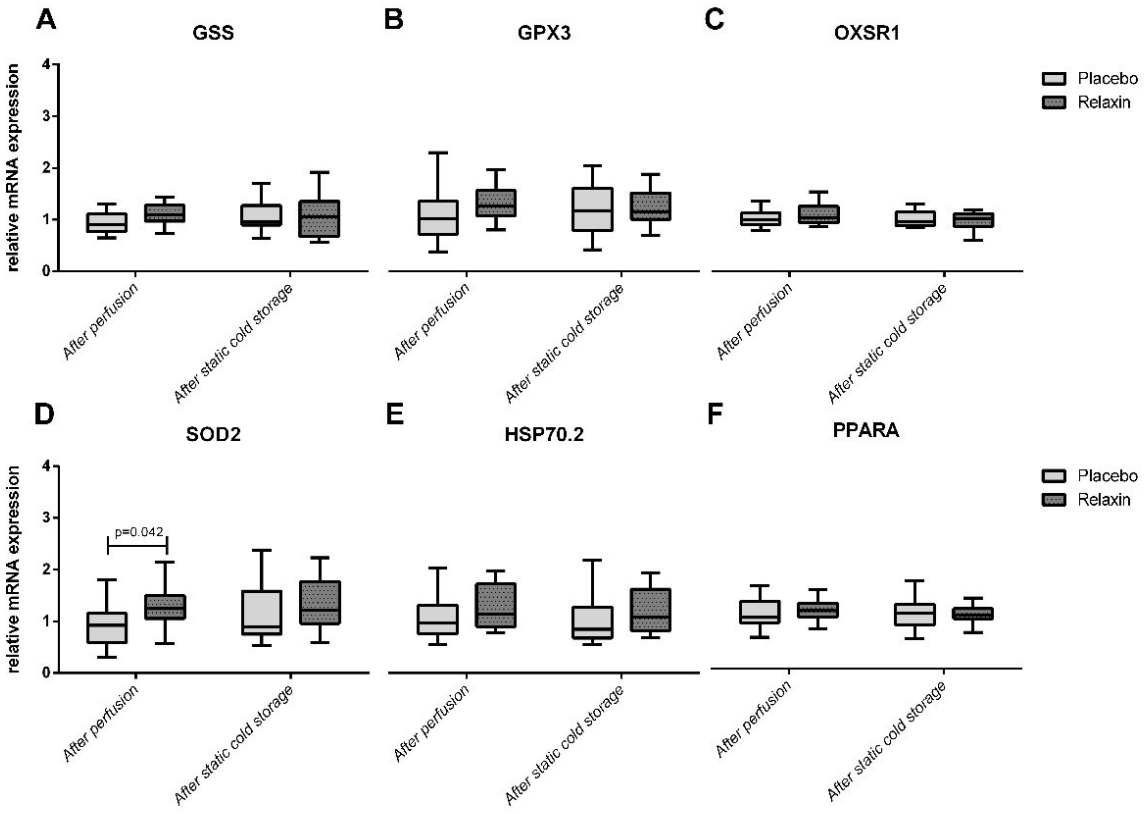

Figure 5. Oxidative stress-related genes expression in kidney tissue after perfusion and static cold storage in Relaxin and Placebo groups. Adapted from Bausys et al., 2021 (20) with permissions of MDPI.

GSS: Glutathione Synthetase; GPX3: Glutathione Peroxidase 3; OXSR1: Oxidative Stress Responsive Kinase 1; SOD2: Superoxide Dismutase 2; HSP70.2: Heat Shock Protein 70.2; PPARA: Peroxisome Proliferators Activated Receptor Alpha.

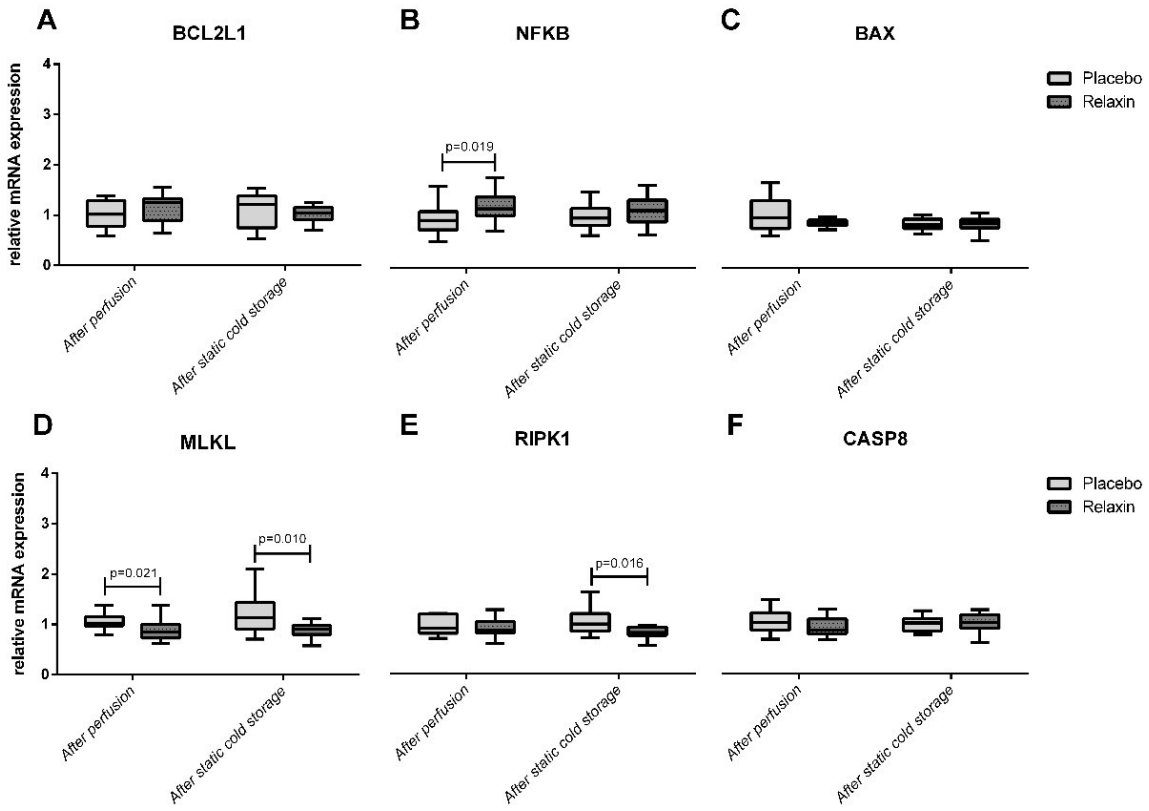

Figure 6. Apoptosis and necroptosis-related genes expression in kidney tissue after perfusion and static cold storage in Relaxin and Placebo groups. Adapted from Bausys et al., 2021 (20) with permissions of MDPI.

BCL2L1: BCL2 Like 1; NFKB: Nuclear Factor of Kappa Light Polypeptide Gene Enhancer In B-cells; BAX: BCL2 Associated X Protein; MLKL: Mixed-lineage kinase domain-like protein; RIPKI1: Receptor Interacting Serine/Threonine Kinase 1: CASP8: Caspase 8.

3.2. Oxidative stress and kidney injury markers after KTx

Oxidative stress (MDA; TOS; CP; MPO), lipid peroxidation (4-HNE), and endothelial cell damage (big ET) plasma markers were similar between the RLX and Placebo groups throughout the course of the experiment (Table 3).

	RLX	Placebo	p value

MDA [µM]; Median (Q1; Q3)	Baseline	0.62 (0.49; 1.31)	0.59 (0.37; 0.96)	0.363
	POD1	0.81 (0.45; 0.96)	0.79 (0.49; 0.90)	0.803
	POD7	0.82 (0.53; 1.18)	0.78 (0.54; 1.45)	0.808
	POD14	0.88 (0.78; 1.03)	0.88 (0.60; 1.38)	0.876
	POD21	0.83 (0.63; 1.07)	0.58 (0.41; 0.92)	0.165
	POD28	0.98 (0.79; 1.27)	1.03 (0.85; 1.16)	0.999
TOS [µM]; Median (Q1; Q3)	Baseline	1593 (799; 2389)	1218 (631; 2915)	0.897
	POD1	1339 (945; 3376)	2107 (1526; 3218)	0.487
	POD7	3302 (1804; 4603)	3730 (941; 5618)	0.733
	POD14	2690 (1040; 3667)	2092 (1675; 4631)	0.639
	POD21	1989 (1645; 4885)	3870 (754; 4588)	0.679
	POD28	2600 (1155; 4266)	2667 (304; 5622)	0.733
CP [pmol/mg]; Median (Q1; Q3)	Baseline	3215 (2646; 4074)	4234 (3348; 4846)	0.068
	POD1	3525 (2659; 4099)	3045 (2274; 4494)	0.559
	POD7	3516 (2597; 4168)	3769 (2186; 4942)	0.999
	POD14	3383 (2471; 4152)	3370 (2666; 3919)	0.876
	POD21	3702 (2502; 4060)	3829 (3123; 4915)	0.513
	POD28	3929 (2090; 4483)	4283 (2428; 4850)	0.733
4-HNE [µM]; Median (Q1; Q3)	Baseline	0.32 (0.21; 0.59)	0.47 (0.18; 0.69)	0.517
	POD1	0.21 (0.11; 0.66)	0.34 (0.13; 0.53)	0.999
	POD7	0.50 (0.23; 0.94)	0.33 (0.05; 0.72)	0.256
	POD14	0.09 (0.05; 0.32)	0.40 (0.12; 0.50)	0.149
	POD21	0.14 (0.08; 0.83)	0.28 (0.14; 0.55)	0.679
	POD28	0.10 (0.07; 0.36)	0.17 (0.06; 0.28)	0.999
Big ET [pmol/l]; Median (Q1; Q3)	Baseline	0.26 (0.14; 0.59)	0.26 (0.10; 1.01)	0.936
	POD1	0.29 (0.18; 0.47)	0.27 (0.08; 1.14)	0.749
	POD7	0.24 (0.16; 0.32)	0.22 (0.10; 0.42)	0.710
	POD14	0.23 (0.15; 1.20)	0.18 (0.02; 0.31)	0.713
	POD21	0.15 (0.08; 0.23)	0.17 (0.00; 0.21)	0.999

	POD28	0.14 (0.13; 0.24)	0.15 (0.04; 0.18)	0.964
MPO [ng/ml]; Median (Q1; Q3)	Baseline	29 (22; 40)	27 (23; 37)	0.642
	POD1	26 (22; 42)	28 (24; 48)	0.674
	POD7	35 (31; 59)	28 (25; 34)	0.087
	POD14	35 (27; 66)	24 (20; 29)	0.016
	POD21	35 (27; 98)	41 (29; 53)	0.999
	POD28	33 (26; 55)	35 (23; 43)	0.616

Table 3. Oxidative stress (MDA; TOS; CP, MPO), lipid peroxidation (4-HNE), and endothelial cell damage (big ET) markers in the Relaxin and Placebo groups after kidney transplantation. MDA: Malondialdehyde; TOS: Total oxidant status; CP: Carbonylprotein; 4-HNE: 4-Hydroxynonenal; big ET: Big Endothelin; MPO: Myeloperoxidase; POD: post-operative day

3.3. Blood biochemistry and blood gases after KTx

Sodium, potassium, chloride, hemoglobin, lactate, and pH were not different in the RLX and Placebo groups throughout the course of the experiment (Table 4).

		RLX	Placebo	p value
Sodium [mM]; Median (Q1; Q3)	Baseline	140 (139; 141)	140 (138; 141)	0.699
	POD1	139 (137; 141)	140 (137; 142)	0.553
	POD7	138 (133; 140)	139 (138; 140)	0.357
	POD14	139 (138; 140)	137 (135; 140)	0.301
	POD21	140 (138; 141)	138 (136; 139)	0.022
	POD28	137 (137; 138)	138 (137; 138)	0.385
Potassium [mM]; Median (Q1; Q3)	Baseline	3.5 (3.3; 3.7)	3.5 (3.3.; 3.7)	0.601
	POD1	3.5 (3.4; 3.7)	3.6 (3.5; 3.7)	0.445
	POD7	3.8 (3.3; 4.3)	3.7 (3.6; 4.0)	0.745
	POD14	4 (3.8; 4.2)	3.6 (3.4; 3.8)	0.016
	POD21	3.8 (3.4; 4.0)	3.7 (3.6; 4.2)	0.562

	POD28	3.8 (3.4; 4.0)	4.2 (3.9; 4.7)	0.067
Chloride [mM]; Median (Q1; Q3)	Baseline	101 (100; 102)	101 (99; 102)	0.960
	POD1	96 (94; 97)	95 (92; 99)	0.497
	POD7	94 (87; 98)	97 (96; 99)	0.110
	POD14	99 (96; 100)	95 (90; 98)	0.031
	POD21	98 (96; 99)	96 (94; 98)	0.147
	POD28	98 (96; 99)	97 (95; 101)	0.999
Haemoglobin [g/dl]; Median (Q1; Q3)	Baseline	8.5 (7.8; 8.9)	8.5 (8.2; 9.2)	0.511
	POD1	8.5 (7.7; 9.5)	8.1 (7.5; 9.5)	0.724
	POD7	7.8 (7.1; 8.5)	8.5 (7.8; 9.5)	0.089
	POD14	8.2 (7.5; 9.8)	9.0 (7.7.; 9.9)	0.492
	POD21	8.5 (7.8; 10.3)	9.1 (8.0; 10.0)	0.456
	POD28	8.5 (7.8; 10.3)	8.8 (7.9; 10.8)	0.682
Lactate [mM]; Median (Q1; Q3)	Baseline	2.2 (1.6; 3.0)	2.3 (1.8; 3.0)	0.827
	POD1	2.7 (1.9; 4.3)	2.4 (1.5; 3.3)	0.306
	POD7	2.6 (2.1; 5.2)	1.8 (1.0; 6.0)	0.198
	POD14	3.0 (2.2; 3.8)	3.4 (1.4; 6.2)	0.999
	POD21	3.0 (2.2; 4.3)	2.7 (2.3; 4.5)	0.958
	POD28	3.0 (2.3; 4.1)	2.5 (2.2; 5.3)	0.733
pH; Median (Q1; Q3)	Baseline	7.43 (7.39; 7.45)	7.45 (7.40; 7.48)	0.257
	POD1	7.44 (7.39; 7.49)	7.43 (7.37; 7.51)	0.646
	POD7	7.42 (7.38; 7.46)	7.45 (7.38; 7.53)	0.297
	POD14	7.38 (7.36; 7.44)	7.38 (7.33; 7.39)	0.462
	POD21	7.37 (7.32; 7.42)	7.42 (7.33; 7.45)	0.328
	POD28	7.48 (7.43; 7.51)	7.49 (7.40; 7.54)	0.820

Table 4. Sodium, potassium, chloride, hemoglobin, lactate, and pH levels in the Relaxin and Placebo groups after kidney transplantation.

POD: post-operative day

3.4. Immunohistochemistry for Caspase 3 and MPO after kidney perfusion and static cold storage

The number of Caspase 3 and MPO positive cells was similar between RLX or Placebo groups after perfusion (Table 5). However, SCS increased the number of apoptotic cells. RLX additive to Custodiol® prevented such an increase in the kidney, renal vein, and ureter tissue (Table 5). Similarly, RLX prevented an increase of MPO positive cells in kidneys after SCS as well (20). Figures 7 and 8 show representative staining for Caspase 3 and MPO.

		Caspase 3			**Myeloperoxidase**		
		RLX	Placebo	p value	RLX	Placebo	p value
Kidney	After perfusion	0 (0; 0)	0 (0; 0)	*0.999*	0 (0; 0)	0 (0; 0)	*0.999*
	After SCS	0 (0; 0.5)	1 (0; 1.6)	*0.005*	0 (0; 0.5)	1 (0; 1.3)	*0.017*
Renal artery	After perfusion	0 (0; 0)	0 (0; 0)	*0.999*	0 (0; 0)	0 (0; 0)	*0.999*
	After SCS	0 (0; 0)	0 (0; 0.2)	*0.650*	0 (0; 0)	0 (0; 0)	*0.660*
Renal vein	After perfusion	0 (0; 0)	0 (0; 0)	*0.811*	0 (0; 0)	0 (0; 0)	*0.999*
	After SCS	0 (0; 0)	0.8 (0; 1)	*0.027*	0 (0; 0)	0 (0; 1)	*0.066*
Ureter	After perfusion	0 (0; 0)	0 (0; 0)	*0.999*	0 (0; 0)	0 (0; 0)	*0.999*
	After SCS	0 (0; 0)	0.6 (0; 1)	*0.019*	0 (0; 0)	0 (0; 0)	*0.839*

Table 5. Immunohistochemical evaluation for activated Caspase-3 positivity as well as Myeloperoxidase positivity in kidneys after perfusion and static cold storage in Placebo and Relaxin groups. Adapted from Bausys et al., 2021 with permissions of MDPI.

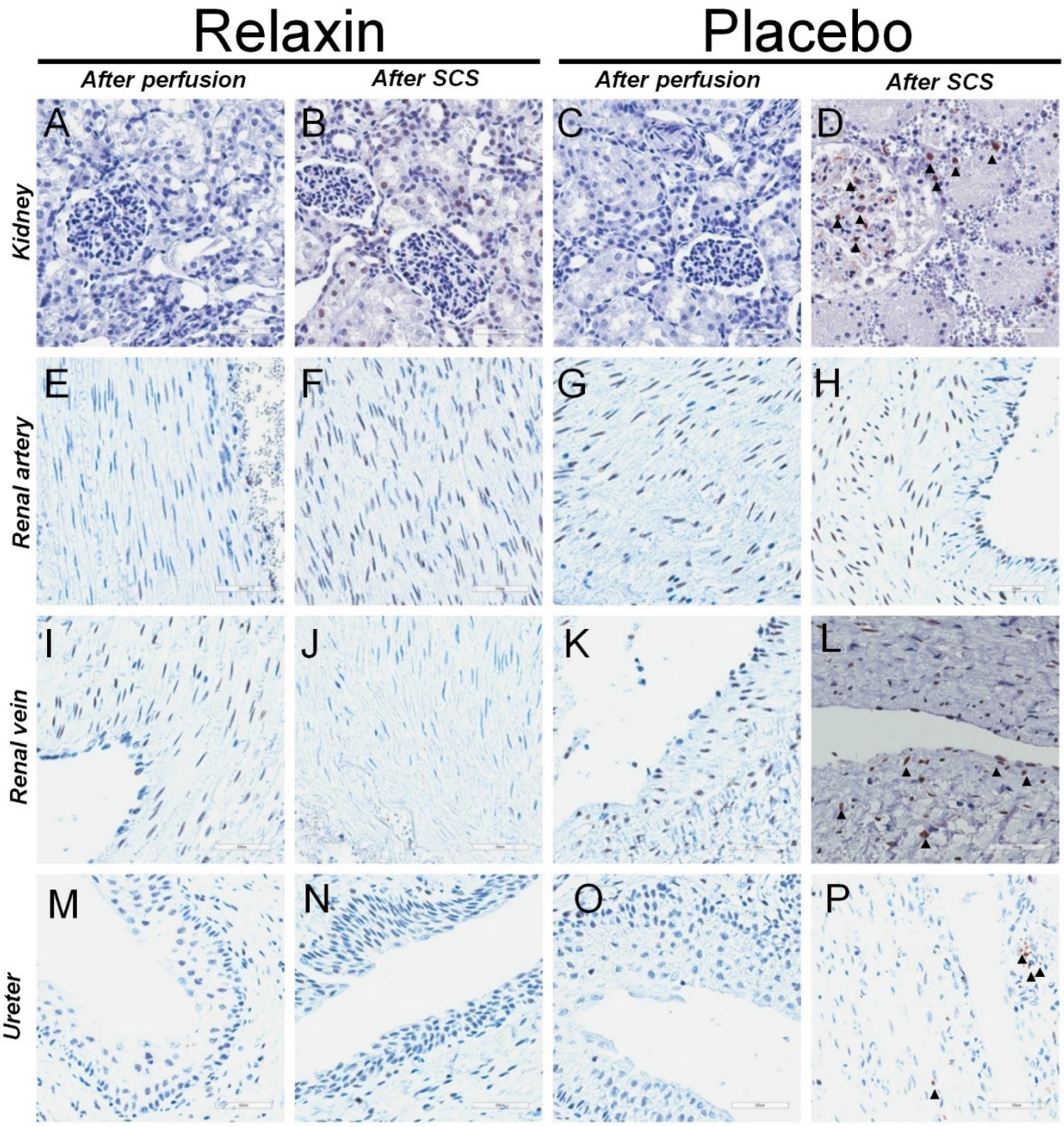

Figure 7. Pictures depicting typical staining for activated Caspase 3 in the kidney, renal artery, renal vein, and ureter tissues after perfusion and static cold storage in RLX or control. Representative staining for kidney tissue at 100× magnification for Caspase 3 after perfusion (A) and static cold storage (B) with RLX and after perfusion (C) and static cold storage (D) with Custodiol®. Caspase 3 positive cells were more common after SCS in a Placebo group. Representative staining for renal artery tissue against Caspase 3 after perfusion (E) and static cold

storage (F) with Custodiol® supplemented with Relaxin or after perfusion (G) and static cold storage (H) with Placebo. Representative staining for renal vein tissue against Caspase 3 after perfusion (I) and static cold storage (J) with Relaxin or after perfusion (K) and static cold storage (L) with Placebo. Caspase 3 positive cells were more common after SCS in a Placebo group. Representative staining for ureter tissue against Caspase 3 after perfusion (M) and static cold storage (N) with Relaxin or after perfusion (O) and static cold storage (P) with Placebo. Caspase 3 positive cells were more common after SCS in a Placebo group. All scale bars represent 50 μm. Adapted from Bausys et al., 2021 (20) with permissions of MDPI.

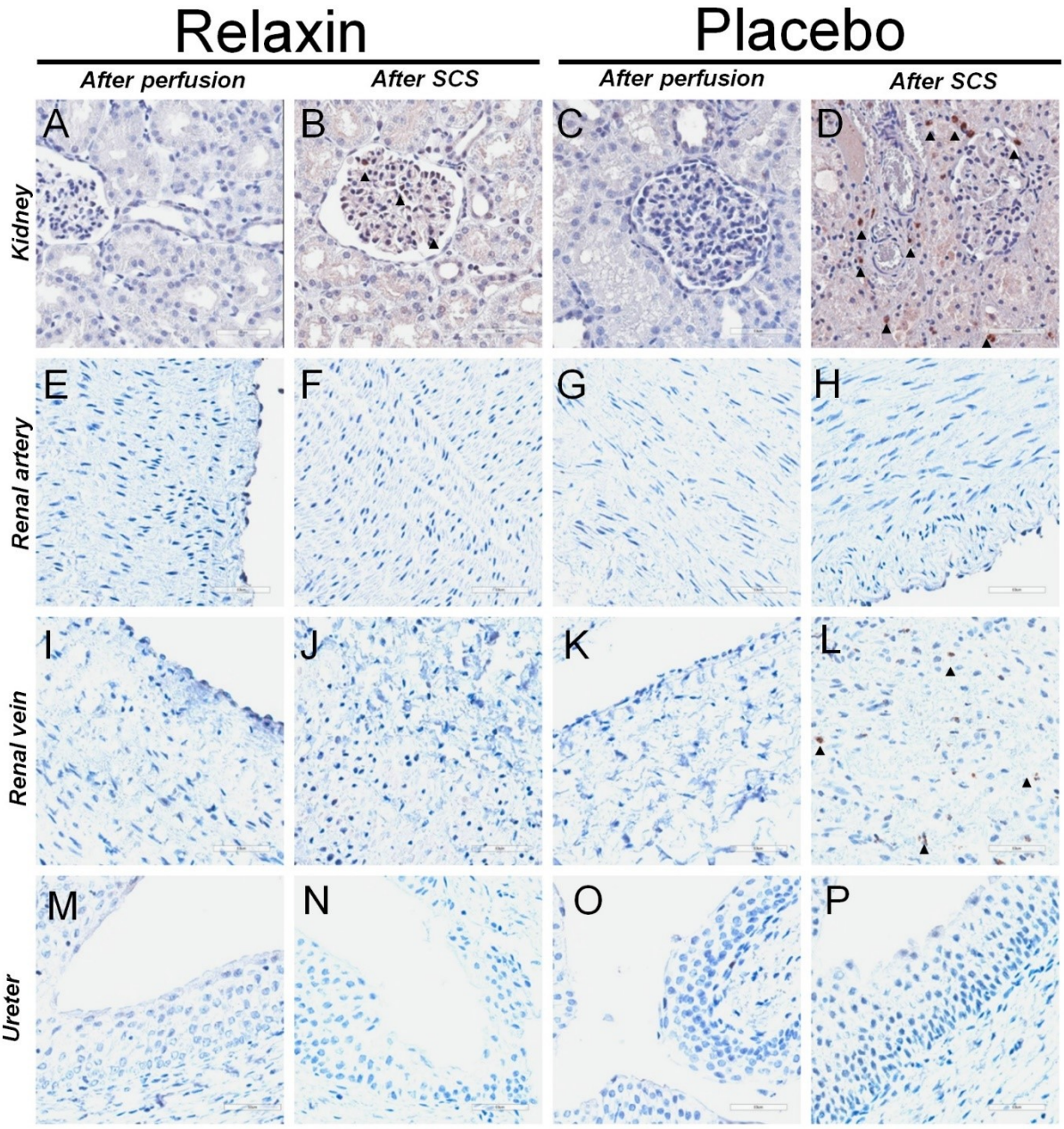

Figure 8. Representative pictures of staining for myeloperoxidase positive cells in the kidney, renal artery, renal vein, and ureter tissues after perfusion and static cold storage in Relaxin or Placebo. Representative staining for kidney tissue at 100× magnification against myeloperoxidase positive cells after perfusion (A) and static cold storage (B) with Relaxin or after perfusion (C) and static cold storage (D) with Placebo. Myeloperoxidase-positive cells were more common after SCS in a Placebo group. Representative staining for renal artery tissue

against myeloperoxidase positive cells after perfusion (E) and static cold storage (F) with Relaxin or after perfusion (G) and static cold storage (H) with Placebo. Representative staining for renal vein tissue against myeloperoxidase positive cells after perfusion (I) and static cold storage (J) with Relaxin or after perfusion (K) and static cold storage (L) with Placebo. Representative staining for ureter tissue against myeloperoxidase positive cells after perfusion (M) and static cold storage (N) with Relaxin or after perfusion (O) and static cold storage (P) with Placebo. All scale bars represent 50 μm. Adapted from Bausys et al., 2021 (20) with permissions of MDPI.

3.5. Kidney transplantation and graft function

Following SCS, all kidneys were transplanted, except one organ from the Placebo group, which was missed because of logistical reasons. Recipients' 28-days survival rate was 54.5 % (12 of 22) and 33.3 % (5 of 15) in the RLX and Placebo groups, respectively (p=0.315). Remaining pigs dropped out prematurely because of transplant failure (RLX: n=5; 22.7 % vs. Placebo: n=3; 20.0 %, p=0.843) or transplant non-related postoperative complications (RLX: n=5; 22.7 % vs. Placebo: n=7; 46.6 %, p=0.126). No differences were observed regarding graft survival between the study groups (Figure 9). Kidney function representing parameters - creatinine and blood urea nitrogen (BUN) peaked on POD 1 but to a similar level in both groups, as it remained through the course of the experiment (Figure 10) (20).

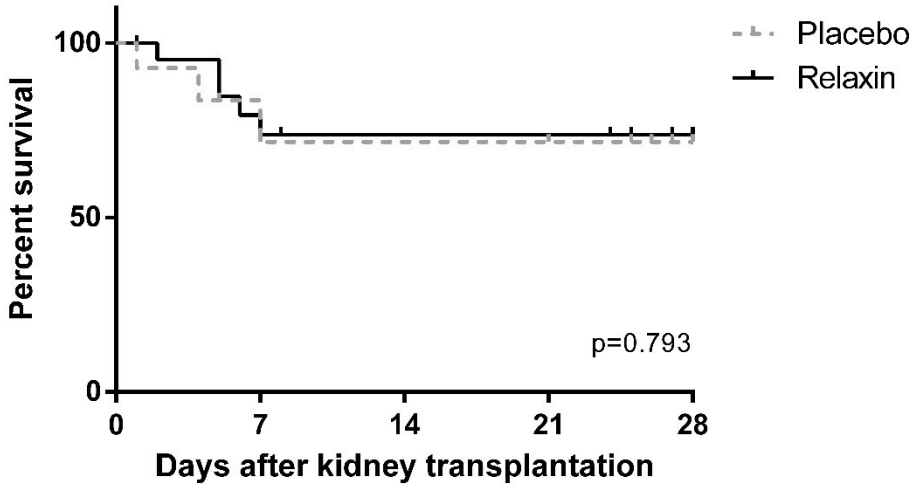

Figure 9. Graft survival after kidney transplantation in Placebo and Relaxin groups. Adapted from Bausys et al., 2021 (20) with permissions of MDPI.

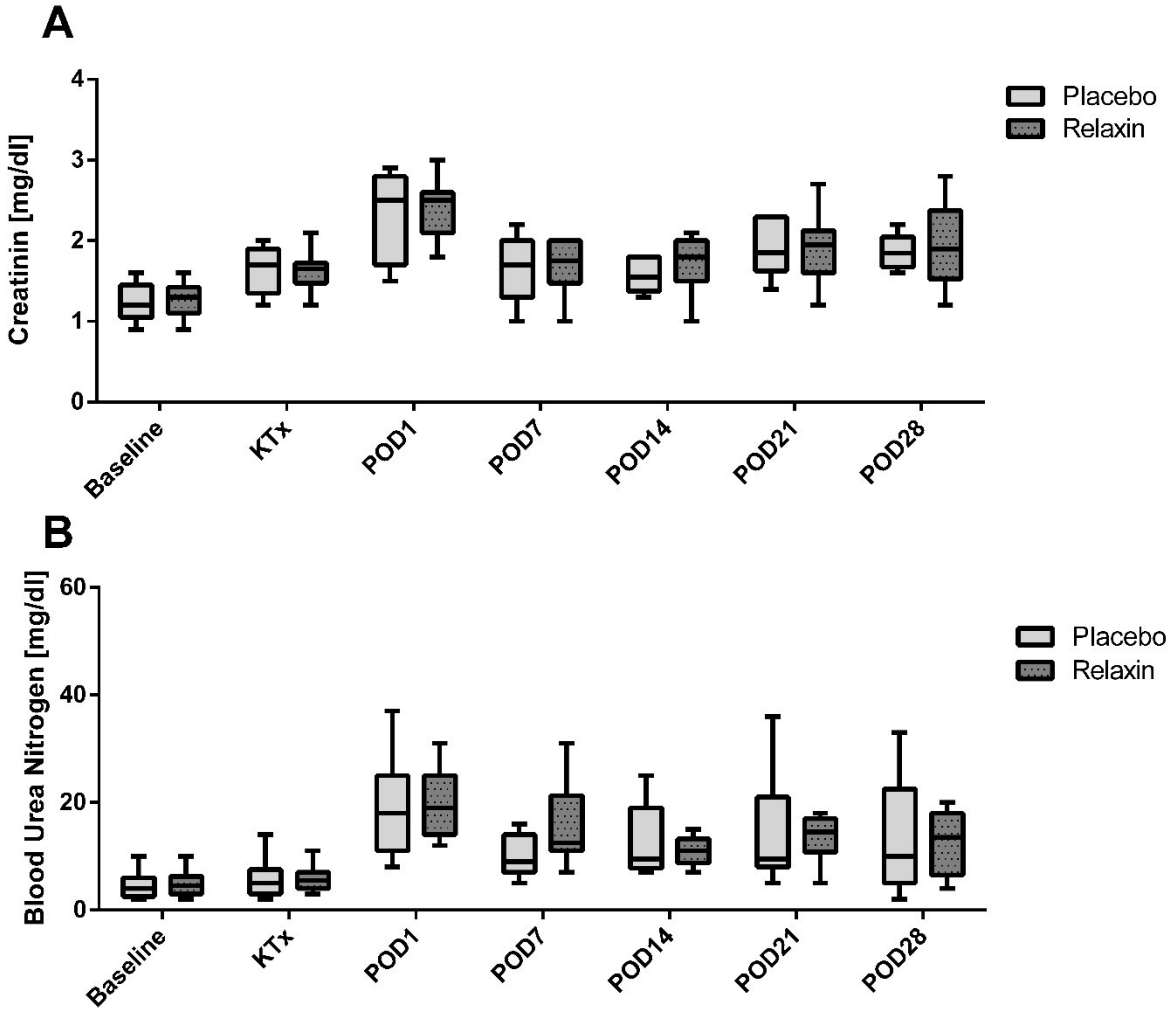

Figure 10. Plasma creatinine (A) and blood urea nitrogen (B) levels after kidney transplantation in Placebo and Relaxin groups. POD: postoperative day.

3.6.Subgroup analysis

3.6.1. 24. vs. 48 hours of SCS in Placebo and RLX groups

Oxidative stress-related genes (Figure 11) and apoptosis, necroptosis-related gene expression (Figure 12), oxidative stress (MDA; TOS; CP; MPO), lipid peroxidation (4-HNE), and endothelial cell damage (big ET) markers (Table 6), immunohistochemistry (Table 7), graft function representing parameters (Figure 13) and graft survival (Figure 14) were similar between grafts stored for 24 and 48 hours within the RLX and placebo groups (20).

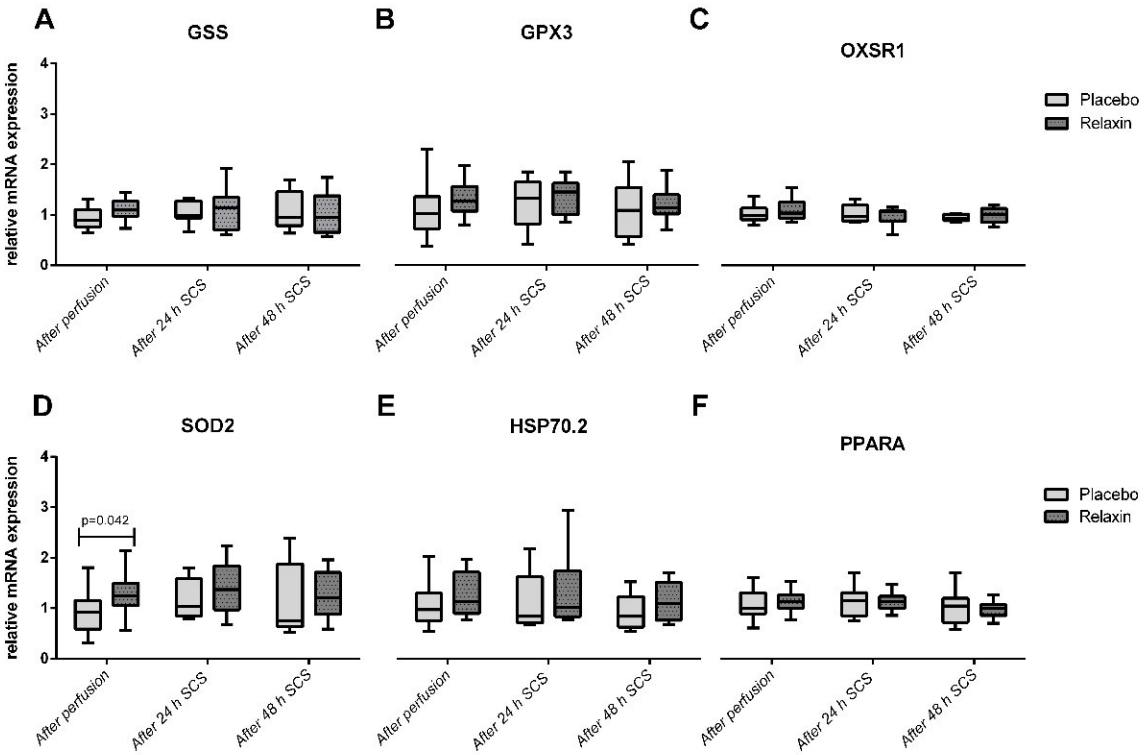

Figure 11. Oxidative stress-related genes expression in kidney tissue after perfusion and static cold storage in Relaxin and Placebo groups for 24 and 48 hours. Adapted from Bausys et al., 2021 (20) with permissions of MDPI.

GSS: Glutathione Synthetase; GPX3: Glutathione Peroxidase 3; OXSR1: Oxidative Stress Responsive Kinase 1; SOD2: Superoxide Dismutase 2; HSP70.2: Heat Shock Protein 70.2; PPARA: Peroxisome Proliferators Activated Receptor Alpha.

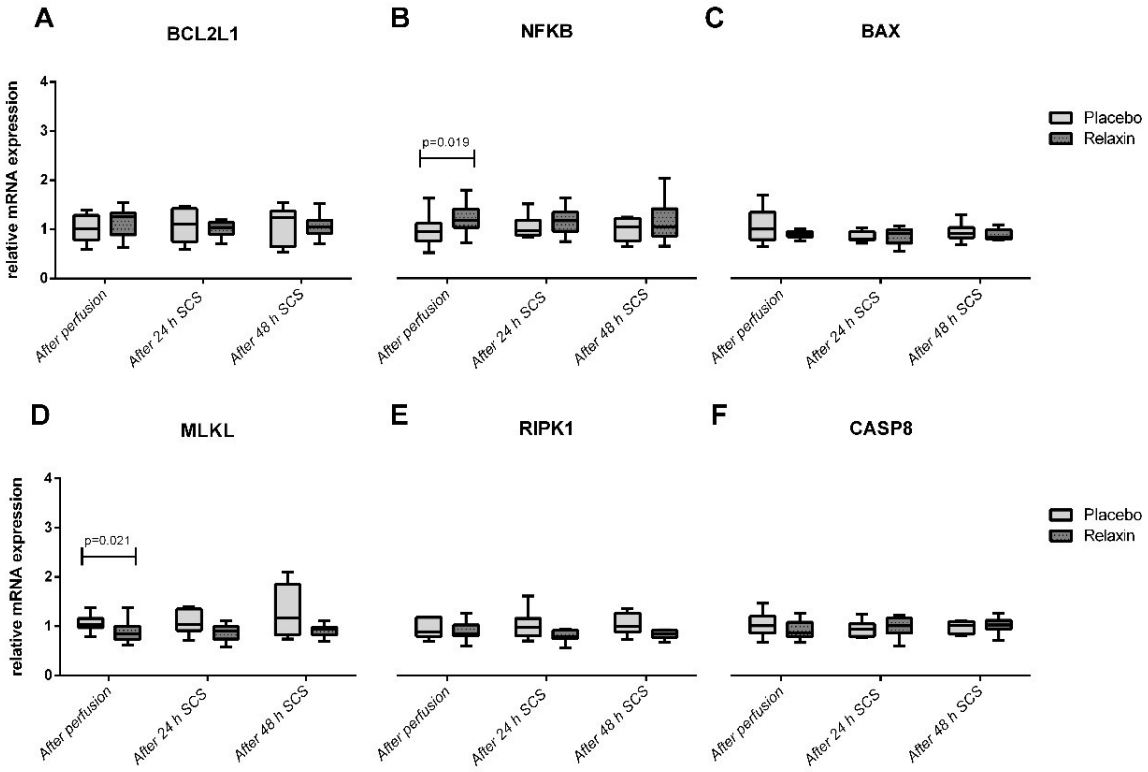

Figure 12. Apoptosis and necroptosis-related genes expression in kidney tissue after perfusion and static cold storage in Relaxin and Placebo groups for 24 and 48 hours. Adapted from Bausys et al., 2021 (20) with permissions of MDPI.

BCL2L1: BCL2 Like 1; NFKB: Nuclear Factor of Kappa Light Polypeptide Gene Enhancer In B-cells; BAX: BCL2 Associated X Protein; MLKL: Mixed-lineage kinase domain-like protein; RIPKI1: Receptor Interacting Serine/Threonine Kinase 1: CASP8: Caspase 8.

		Placebo 24 h SCS	Placebo 48 h SCS	RLX 24 h SCS	RLX 48 h SCS	p value
MDA [µM]; Median (Q1; Q3)	Baseline	0.47 (0.27; 0.88)	0.67 (0.34; 1.47)	0.70 (0.49; 1.28)	0.61 (0.49; 1.39)	0.614
	POD1	0.53 (0.41; 1.17)	0.82 (0.70; 0.93)	0.78 (0.45; 0.96)	0.83 (0.47; 1.00)	0.830
	POD7	1.21 (0.53; 1.21)	0.78 (0.58; 1.19)	0.57 (0.45; 0.86)	1.02 (0.64; 1.47)	0.504
	POD14	1.02 (0.49; 1.02)	0.88 (0.71; 0.88)	0.91 (0.84; 0.91)	0.83 (0.42; 0.99)	0.767
	POD21	0.86 (0.49; 0.86)	0.58 (0.33; 0.58)	0.88 (0.80; 1.30)	0.65 (0.32; 1.10)	0.163
	POD28	1.13 (1.08; 1.13)	0.90 (0.81; 0.90)	0.92 (0.56; 1.13)	1.21 (0.82; 1.52)	0.523
TOS [µM]; Median (Q1; Q3)	Baseline	1054 (602; 2935)	1381 (653; 2617)	1826 (637; 2141)	1405 (814; 3878)	0.976
	POD1	2107 (1312; 3218)	2281 (1411; 3464)	1016 (613; 2512)	2249 (1245; 4211)	0.276
	POD7	2960 (1120; 2960)	4123 (969; 5682)	2199 (1497; 3953)	4173 (1461; 5551)	0.858
	POD14	1769 (1445; 1769)	4109 (1904; 4109)	1414 (1040; 1414)	2967 (1405; 5040)	0.516
	POD21	3201 (1347; 3201)	3870 (160; 3870)	1807 (1060; 4992)	2172 (1611; 5210)	0.947
	POD28	2667 (748; 2667)	3062 (156; 3062)	1260 (832; 5936)	3055 (1910; 3669)	0.975
CP [pmol/mg];	Baseline	4138 (3051; 5311)	4330 (3255; 5084)	3841 (3051; 4266)	2766 (1881; 3564)	0.143

Median (Q1; Q3)	POD1	2287 (1927; 4012)	3699 (2650; 5285)	3525 (2149; 4079)	3536 (2659; 4633)	0.464
	POD7	3361 (1981; 3361)	3769 (2494; 5239)	3530 (2482; 3530)	3435 (2581; 4213)	0.981
	POD14	3723 (3144; 3723)	3370 (2189; 3370)	3143 (233; 3143)	3767 (2699; 5162)	0.557
	POD21	4547 (3562; 4537)	3829 (2684; 3829)	3694 (2005; 4084)	3710 (3013; 4494)	0.843
	POD28	4782 (4654; 4782)	2926 (1931; 2926)	2645 (1899; 4195)	3958 (3019; 5018)	0.183
4-HNE [µM]; Median (Q1; Q3)	Baseline	0.42 (0.09; 0.67)	0.53 (0.25; 1.35)	0.33 (0.28; 0.54)	0.32 (0.10; 1.34)	0.792
	POD1	0.20 (0.08; 0.53)	0.35 (0.16; 0.73)	0.18 (0.11; 0.66)	0.22 (0.11; 2.17)	0.944
	POD7	0.36 (0.02; 0.36)	0.33 (0.07; 0.70)	0.50 (0.34; 2.06)	0.42 (0.19; 1.54)	0.655
	POD14	0.28 (0.17; 0.28)	0.48 (0.08; 0.48)	0.06 (0.02; 0.06)	0.24 (0.07; 0.46)	0.233
	POD21	0.55 (0.35; 0.55)	0.17 (0.11; 0.17)	0.09 (0.06; 0.14)	0.76 (0.38; 1.80)	0.048
	POD28	0.17 (0.16; 0.17)	0.17 (0.03; 0.17)	0.08 (0.03; 0.23)	0.26 (0.09; 0.40)	0.472
Big ET [pmol/l]; Median (Q1; Q3)	Baseline	0.28 (0.07; 0.58)	0.22 (0.12; 1.26)	0.24 (0.15; 0.56)	0.28 (0.13; 0.68)	0.997
	POD1	0.27 (0.13; 1.42)	0.30 (0.03; 1.39)	0.28 (0.17; 0.34)	0.33 (0.26; 0.60)	0.891
	POD7	0.23 (0.10; 0.23)	0.16 (0.05; 0.37)	0.21 (0.15; 0.28)	0.25 (0.11; 0.34)	0.857

	POD14	0.30 (0.03; 0.30)	0.13 (0.01; 0.13)	0.26 (0.14; 2.72)	0.22 (0.10; 2.08)	0.617
	POD21	0.17 (0.01; 0.17)	0.17 (0.01; 0.17)	0.15 (0.10; 0.22)	0.15 (0.04; 0.23)	0.979
	POD28	0.18 (0.04; 0.18)	0.15 (0.03; 0.15)	0.14 (0.10; 1.28)	0.13 (0.09; 0.87)	0.981
MPO [ng/ml]; Median (Q1; Q3)	Baseline	25.0 (19.4; 34.4)	30.2 (25.0; 45.3)	31.0 (29.3; 53.6)	23.1 (21.3; 35.2)	0.444
	POD1	24.3 (21.2; 48.3)	33.4 (27.6; 58.5)	27.8 (22.2; 63.7)	25.0 (22.0; 38.8)	0.236
	POD7	25.2 (22.5; 25.2)	32.1 (26.2; 56.6)	49.5 (32.7; 80.2)	34.2 (31.9; 46.9)	0.110
	POD14	22.2 (16.7; 22.2)	25.1 (24.8; 25.1)	66.0 (39.5; 84.0)	28.5 (25.6; 35.8)	0.017
	POD21	30.2 (27.6; 30.2)	51.0 (50.4; 51.0)	51.1 (28.8; 118.6)	31.4 (26.4; 78.4)	0.281
	POD28	24.9 (19.0; 24.9)	37.2 (33.2; 37.2)	37.5 (24.5; 37.5)	31.5 (26.6; 69.9)	0.571

Table 6. Oxidative stress (MDA; TOS; CP, MPO), lipid peroxidation (4-HNE), and endothelial cell damage (big ET) markers in the Relaxin and Placebo subgroups stored for 24 and 48 hours after kidney transplantation.

MDA: Malondialdehyde; TOS: Total oxidant status; CP: Carbonylprotein; 4-HNE: 4-Hydroxynonenal; big ET: Big Endothelin; MPO: Myeloperoxidase

		Caspase 3			Myeloperoxidase		
		RLX	Placebo	p value	RLX	Placebo	p value
Kidney	After perfusion	0 (0; 0)	0 (0; 0)	*0.999*	0 (0; 0)	0 (0; 0)	*0.999*
	After 24 hours SCS	0 (0; 1)	1 (0.2; 1.5)	*0.068*	0 (0; 0)	0.5 (0; 1.25)	*0.177*
	After 48 hours SCS	0 (0; 0)	1.1 (0; 1.9)	*0.051*	0 (0; 1)	1 (0.25; 1.5)	*0.062*
Renal artery	After perfusion	0 (0; 0)	0 (0; 0)	*0.999*	0 (0; 0)	0 (0; 0)	*0.999*
	After 24 hours SCS	0 (0; 0)	0 (0; 0.5)	*0.768*	0 (0; 0)	0 (0; 0)	*0.999*
	After 48 hours SCS	0 (0; 0)	0 (0; 0.6)	*0.768*	0 (0; 0)	0 (0; 0.5)	*0.594*
Renal vein	After perfusion	0 (0; 0)	0 (0; 0)	*0.811*	0 (0; 0)	0 (0; 0)	*0.999*
	After 24 hours SCS	0 (0; 0.1)	0 (0; 1)	*0.513*	0 (0; 0)	0 (0; 1.1)	*0.284*
	After 48 hours SCS	0 (0; 0)	1 (0.3; 1)	*0.019*	0 (0; 0)	0.5 (0; 1)	*0.199*
Ureter	After perfusion	0 (0; 0)	0 (0; 0)	*0.999*	0 (0; 0)	0 (0; 0)	*0.999*
	After 24 hours SCS	0 (0; 0)	0 (0; 1)	*0.240*	0 (0; 0)	0 (0; 0)	*0.768*

	After 48 hours SCS	0 (0; 0)	0.8 (0.1; 2)	*0.036*	0 (0; 0)	0 (0; 0.5)	*0.606*

Table 7. Immunohistochemical evaluation for activated Caspase-3 positivity as well as Myeloperoxidase positivity in kidneys after perfusion and static cold storage for 24 and 48 hours in Placebo and Relaxin groups.

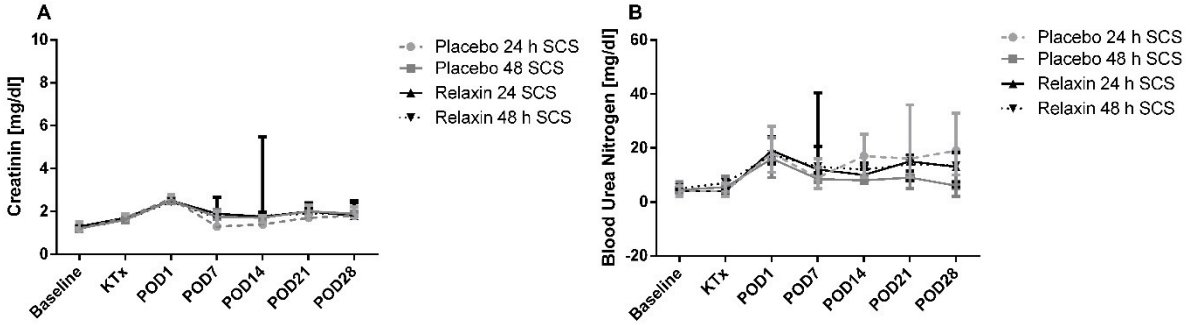

Figure 13. Plasma creatinine (A) and blood urea nitrogen (B) levels after kidney transplantation in Placebo and Relaxin subgroups were stored for 24 and 48 hours. Adapted from Bausys et al., 2021 (20) with permissions of MDPI.

POD: postoperative day.

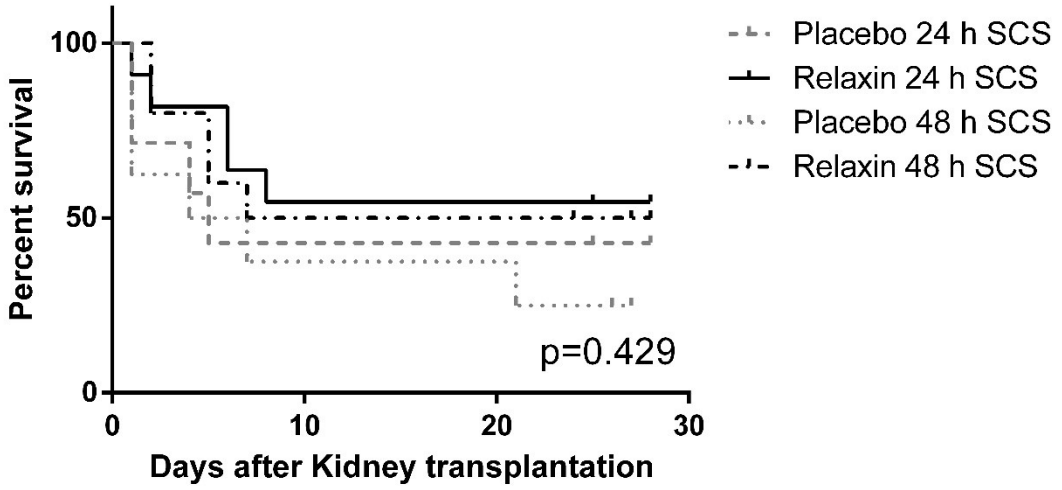

Figure 14. Graft survival after kidney transplantation in Placebo and Relaxin subgroups stored for 24 and 48 hours. Adapted from Bausys et al., 2021 (20) with permissions of MDPI.

3.6.2. High vs. low concentration of RLX

Oxidative stress-related genes (Figure 15) and apoptosis, necroptosis-related gene expression (Figure 16), oxidative stress (MDA; TOS; CP; MPO), lipid peroxidation (4-HNE), and endothelial cell damage (big ET) markers (Table 8), immunohistochemistry (Table 9), graft function representing parameters (Figure 17) and graft survival (Figure 18) were similar across animals treated with 5 versus 20 nM RLX.

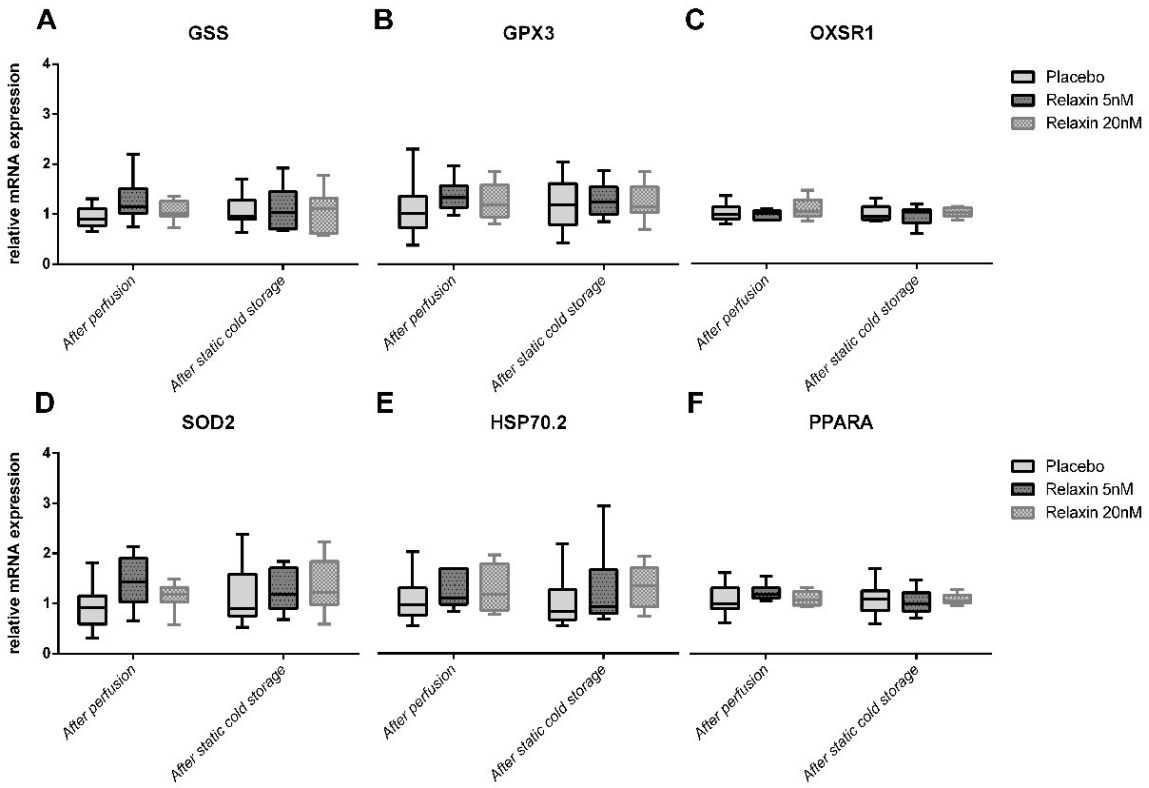

Figure 15. Oxidative stress-related genes expression in kidney tissue after perfusion and static cold storage in Placebo, Relaxin 5nM, and Relaxin 20nM subgroups. Adapted from Bausys et al., 2021 (20) with permissions of MDPI.GSS: Glutathione Synthetase; GPX3: Glutathione Peroxidase 3; OXSR1: Oxidative Stress Responsive Kinase 1; SOD2: Superoxide Dismutase 2; HSP70.2: Heat Shock Protein 70.2; PPARA: Peroxisome Proliferators Activated Receptor Alpha

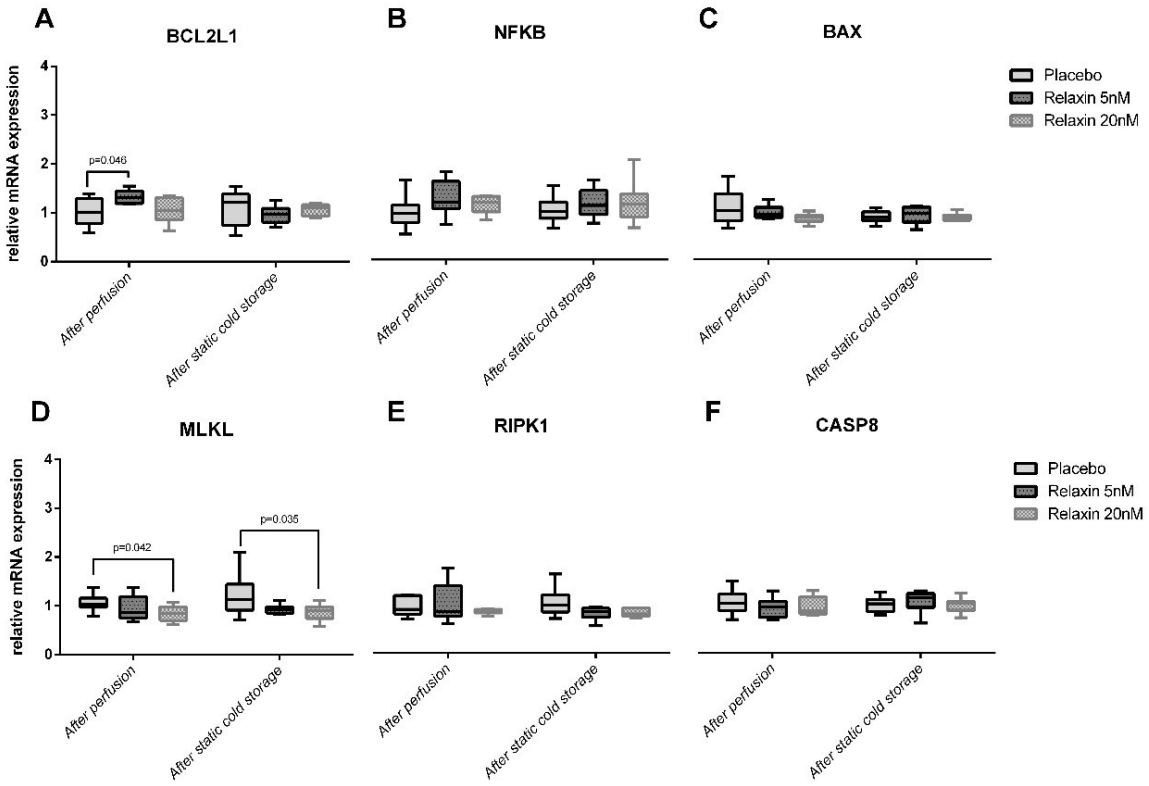

Figure 16. Apoptosis and necroptosis-related genes expression in kidney tissue after perfusion and static cold storage in Placebo, Relaxin 5nM, and Relaxin 20nM subgroups. Adapted from Bausys et al., 2021 (20) with permissions of MDPI.

BCL2L1: BCL2 Like 1; NFKB: Nuclear Factor of Kappa Light Polypeptide Gene Enhancer In B-cells; BAX: BCL2 Associated X Protein; MLKL: Mixed-lineage kinase domain-like protein; RIPKI1: Receptor Interacting Serine/Threonine Kinase 1: CASP8: Caspase 8.

		RLX 5nM	RLX 20 nM	Placebo	p value
MDA [µM]; Median (Q1; Q3)	Baseline	0.94 (0.49; 1.46)	0.56 (0.49; 0.89)	0.59 (0.37; 0.96)	0.492
	POD1	0.93 (0.77; 1.05)	0.51 (0.32; 0.83)	0.79 (0.49; 0.90)	0.048

	POD7	0.88 (0.79; 1.28)	0.53 (0.42; 1.00)	0.78 (0.54; 1.45)	0.141
	POD14	0.83 (0.42; 0.90)	1.03 (0.84; 1.03)	0.88 (0.60; 1.38)	0.447
	POD21	0.84 (0.46; 1.46)	0.80 (0.67; 0.93)	0.58 (0.41; 0.92)	0.339
	POD28	1.12 (0.88)	0.85 (0.44; 1.13)	1.03 (0.85; 1.16)	0.404
TOS [µM]; Median (Q1; Q3)	Baseline	1121 (799; 3130)	1841 (787; 2389)	1218 (631; 2915)	0.962
	POD1	1339 (1066; 3877)	1679 (897; 2755)	2107 (1526; 3218)	0.711
	POD7	3109 (1616; 4421)	3302 (1393; 6357)	3730 (941; 5618)	0.871
	POD14	2967 (1453; 5040)	1414 (977; 1414)	2092 (1675; 4631)	0.486
	POD21	3309 (1237; 5398)	1803 (1709; 4035)	3870 (754; 4588)	0.775
	POD28	2816 (1463; 4266)	2157 (944; 5530)	2667 (304; 5622)	0.913
CP [pmol/mg]; Median (Q1; Q3)	Baseline	3338 (2099; 4074)	3215 (2646; 4338)	4234 (3348; 4846)	0.182
	POD1	3525 (2781; 4408)	3493 (2640; 4079)	3045 (2274; 4494)	0.786
	POD7	2975 (2470; 4523)	3530 (3103; 4201)	3769 (2186; 4942)	0.835
	POD14	3311 (2366; 5162)	3383 (3143; 3383)	3370 (2666; 3919)	0.970

	POD21	3856 (2123; 4339)	3702 (2839; 3954)	3829 (2123; 4915)	0.761
	POD28	3929 (2066; 4318)	3536 (2122; 4595)	4283 (2428; 4850)	0.898
4-HNE [µM]; Median (Q1; Q3)	Baseline	0.46 (0.21; 0.61)	0.31 (0.13; 0.45)	0.47 (0.18; 0.69)	0.610
	POD1	0.32 (0.12; 2.77)	0.15 (0.11; 0.50)	0.34 (0.13; 0.53)	0.554
	POD7	0.59 (0.19; 1.54)	0.50 (0.23; 2.00)	0.33 (0.05; 0.72)	0.482
	POD14	0.18 (0.02; 0.46)	0.09 (0.06; 0.09)	0.40 (0.12; 0.50)	0.291
	POD21	0.43 (0.09; 1.21)	0.07 (0.06; 0.80)	0.28 (0.14; 0.55)	0.318
	POD28	0.17 (0.06; 0.40)	0.10 (0.06; 0.29)	0.17 (0.06; 0.28)	0.953
Big ET [pmol/l]; Median (Q1; Q3)	Baseline	0.20 (0.13; 0.62)	0.28 (0.15; 0.59)	0.26 (0.10; 1.01)	0.878
	POD1	0.30 (0.12; 0.67)	0.29 (0.26; 0.39)	0.27 (0.08; 1.14)	0.764
	POD7	0.22 (0.09; 0.28)	0.25 (0.19; 1.67)	0.22 (0.10; 0.42)	0.537
	POD14	0.19 (0.04; 0.23)	0.28 (0.18; 4.24)	0.18 (0.02; 0.31)	0.264
	POD21	0.14 (0.06; 0.18)	0.21 (0.09; 0.24)	0.17 (0.01; 0.21)	0.473
	POD28	0.14 (0.09; 0.21)	0.13 (0.10; 3.18)	0.15 (0.04; 0.18)	0.990

MPO [ng/ml]; Median (Q1; Q3)	Baseline	31.0 (23.0; 61.3)	28.3 (21.5; 34.8)	27.9 (23.2; 37.1)	0.398
	POD1	26.0 (23.1; 65.3)	27.0 (22.0; 38.6)	28.0 (24.2; 48.9)	0.817
	POD7	33.7 (30.5; 59.4)	40.7 (34.1; 61.1)	28.9 (25.2; 34.9)	0.200
	POD14	30.6 (24.8; 84.1)	44.9 (28.1; 66.3)	24.2 (20.8; 29.3)	0.047
	POD21	60.4 (27.0; 130.7)	35.0 (22.4; 47.9)	41.7 (29.5; 53.0)	0.769
	POD28	52.4 (32.9; 108.0)	27.3 (24.5; 34.9)	35.2 (23.5; 43.0)	0.082

Table 8. Oxidative stress (MDA; TOS; CP, MPO), lipid peroxidation (4-HNE), and endothelial cell damage (big ET) markers in the Placebo, Relaxin 5nM, and Relaxin 20nM subgroups.

MDA: Malondialdehyde; TOS: Total oxidant status; CP: Carbonylprotein; 4-HNE: 4-Hydroxynonenal; big ET: Big Endothelin; MPO: Myeloperoxidase

		Caspase 3				**Myeloperoxidase**			
		RLX 5nM	RLX 20nM	Placebo	p value	RLX 5nM	RLX 20nM	Placebo	p value
Kidney	After perfusion	0 (0; 0)	0 (0; 0)	0 (0; 0)	*0.999*	0 (0; 0)	0 (0; 0)	0 (0; 0)	*0.999*
	After SCS	0.5 (0; 1)	0 (0; 0)	1 (0; 1.6)	*0.001*	0.1 (0; 1)	0 (0; 0)	1 (0; 1.3)	*0.009*
Renal artery	After perfusion	0 (0; 0)	0 (0; 0)	0 (0; 0)	*0.999*	0 (0; 0)	0 (0; 0)	0 (0; 0)	*0.999*
	After SCS	0 (0; 0)	0 (0; 0)	0 (0; 0.2)	*0.732*	0 (0; 0)	0 (0; 0)	0 (0; 0)	*0.329*

Renal vein	After perfusion	0 (0; 0.3)	0 (0; 0)	0 (0; 0)	*0.449*	0 (0; 0)	0 (0; 0)	0 (0; 0)	*0.999*
	After SCS	0 (0; 0.3)	0 (0; 0)	0.8 (0; 1)	*0.013*	0 (0; 0)	0 (0; 0)	0 (0; 1)	*0.014*
Ureter	After perfusion	0 (0; 0)	0 (0; 0)	0 (0; 0)	*0.999*	0 (0; 0)	0 (0; 0)	0 (0; 0)	*0.999*
	After SCS	0 (0; 0)	0 (0; 0)	0.6 (0; 1)	*0.002*	0 (0; 0)	0 (0; 0)	0 (0; 0)	*0.626*

Table 9. Immunohistochemical evaluation for activated Caspase-3 positivity as well as Myeloperoxidase positivity in kidneys after perfusion and static cold storage in Placebo, Relaxin 5nM, and Relaxin 20 nM subgroups. Adapted from Bausys et al., 2021 (20) with permissions of MDPI.

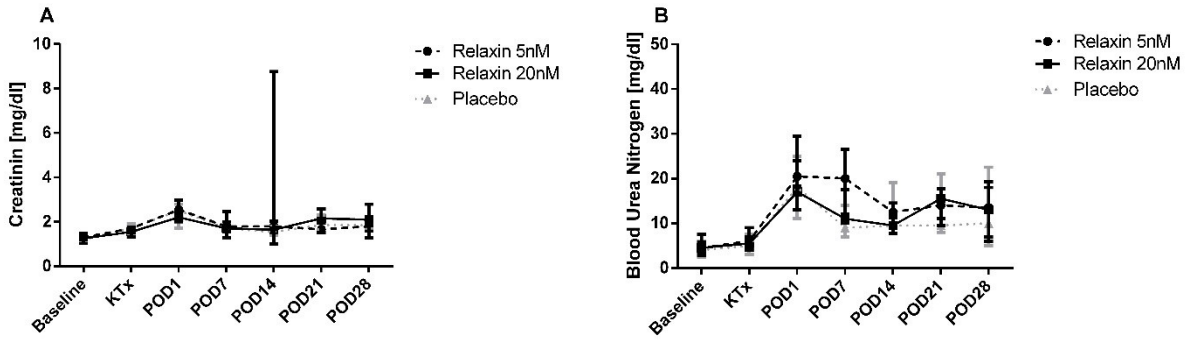

Figure 17. Plasma creatinine (A) and blood urea nitrogen (B) levels after kidney transplantation in Placebo, Relaxin 5nM, and Relaxin 20nM subgroups. Adapted from Bausys et al., 2021 (20) with permissions of MDPI.

POD: postoperative day.

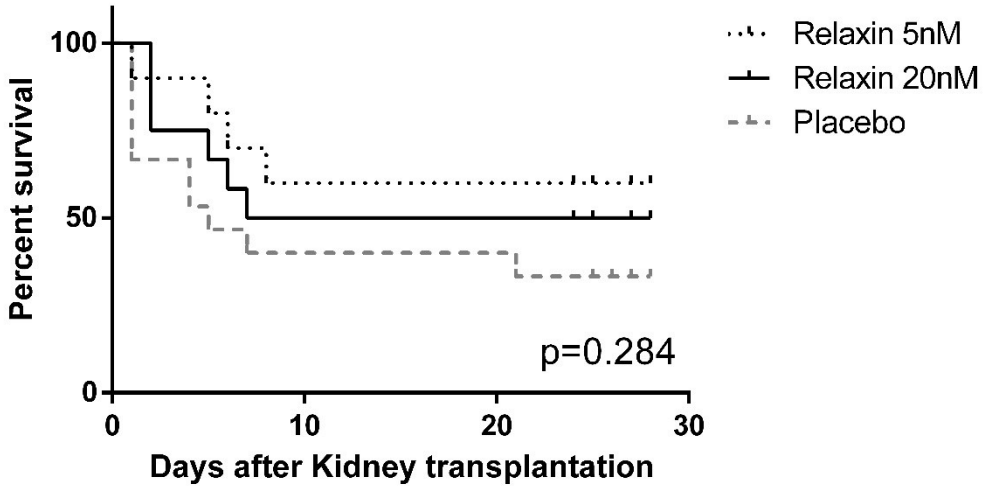

Figure 18. Graft survival after kidney transplantation in Placebo, Relaxin 5nM, and Relaxin 20nM subgroups. Adapted from Bausys et al., 2021 (20) with permissions of MDPI.

4. Discussion

This is the first experimental study that investigated RLX as additive to conventional Custodiol® to prevent IRI in a large animal experimental KTx model. Results demonstrate that RLX impacts genes involved in oxidative stress and apoptosis/necroptosis which in turn improve graft conditions. Addition of RLX reduced inflammation and cell death after SCS as well as demonstrated beneficial effects against IRI-related pathways. Perfusion with RLX supplemented Custodiol® upregulated the expression of antioxidant (SOD2) and anti-apoptotic (NFkB) genes in kidney grafts. Further, RLX downregulated pro-apoptotic/necroptotic (RIPK; MLKL) genes after SCS. Also, it improved graft condition by decreasing the number of Caspase 3 and MPO positive cells after SCS (20).

Renal IRI, which is an unavoidable part of KTx, is a major cause of oxidative stress. The phenomenon is caused by a disrupted balance between production and accumulation of ROS, leading to cellular damage and graft failure (103,105–107). ROS are molecules involved in many

physiological processes, however, can also spontaneously react with lipids, proteins, or nucleic acids and thus harm cells (107–109). Therefore, under standard physiological conditions, levels of ROS are precisely controlled (109). Endogenous antioxidative systems become a self-defense mechanism. Multiple genes participate in scavenging ROS or detoxifying enzymes that can prevent ROS-mediated ischemic insult thus protecting oxidative stress-induced injury (102,110). Mitochondria are the main source of ROS but are also most vulnerable to it. Oxidative stress in these organelles causes deterioration of electron transport, disruption of oxidative phosphorylation, and increased membrane permeability consequently causing mitochondrial dysfunction (111). Mitochondria localized manganese superoxide dismutase (SOD2) is one of the key enzymes to protect cells and particularly mitochondria, against oxidative stress. It catalyzes the dismutation of two superoxide radicals generating hydrogen peroxide and oxygen (112). The SOD2 protein dysfunction in IRI is well documented, thus it is a therapeutic target against renal IRI (113).

The simplest strategy, administration of exogenous superoxide dismutase, is limited effectiveness because of insufficient bioavailability (102,114). In contrast, the alternative strategy - upregulation of SOD genes diminish ROS-induced cellular damage and promotes the apoptosis-suppressive effects (115,116). As demonstrated in our study, perfusion by Custodiol® supplemented with RLX upregulates SOD2 expression in the kidney. Thus RLX may improve graft resistance to oxidative stress-mediated injury (20). Overexpression of SOD2 has a major role in protection against IRI because it decreases steady-state O_2 levels thereby preventing the formation of other biologically active ROS, such as ONOO-. Also, it reduces membrane lipid peroxidation and tyrosine nitration following ischemia (117). Furthermore, SOD2 overexpression prevents the decrease in mitochondrial membrane potential, decline in ATP levels, and increase in calcium release, caspase-3 activation, and TNF-α dependent or independent mechanisms leading to apoptotic cell death. This includes apoptosis elicited by ROS (117). While RLX upregulates SOD2, it had no impact on other oxidative stress-related genes investigated in the present study. GSS increases intracellular levels of glutathione which protects cells from unstable oxygen-containing molecules, such as hydrogen peroxide and peroxynitrite (102). GPX3 encodes a protein that catalyzes the reduction of hydrogen peroxide, also organic hydro- and soluble lipid hydroperoxides (118). OXSR1 encoded product protein is involved in regulating the actin

cytoskeleton and reactions to osmotic stress. Further, it participates in processes controlling whether cells proliferate or die by apoptosis (102). HSP70.2, a member of the HSP 70 family, is included in cell protection mechanisms by facilitating refolding of denatured proteins and preventing proteins aggregation induced by lethal stimulus (102,119). PPARA inhibits activated T-cells to produce TNF-α, gamma-interferon and interleukin-2. Thus it may be involved in acute graft rejection (20,102,120,121).

Besides above mentioned SOD2 roles against oxidative stress, increased activity of it has been shown to prevent apoptotic cell death via the receptor-mediated pathway (122). As RLX upregulates SOD2 expression, we further investigated pro-apoptotic BAX (123) and anti-apoptotic genes including BCL2L1 (124) and NFKB (102) which are known to impact the inflammatory response in renal injury (20,125). In this study, RLX had no impact on BCL2L1 and BAX gene expression but upregulated NFKB expression in kidney tissue after perfusion (20). The transcription factor NFKB has been recognized as a pivotal mediator of gene expression induced by pathogens or proinflammatory cytokines (126–128). Reduced NFKB activity is associated with increased cell sensitivity to DNA-damaging insults (126). The exact genes contributing to the anti-apoptotic effect by NFKB in case of cellular injury remain unknown, but zinc finger protein A20 and SOD2 genes are suspected (126). Contrary, some studies suggest that NFKB may have an opposite pro-apoptotic role (129). Cytotoxic insult on neuronal cells by glutamine is accompanied by the upregulation of NFKB which seems to lead to cell death. However, blocking its activation with pharmacological substances prevents cells from neurotoxicity (126,130). Other studies which support NFKB's pro-apoptotic action includes human embryonic, mammary carcinoma, and mouse neuroblastoma cell lines (126,131,132). Therefore, it is clear that the NFKB's role to attenuate or promote apoptosis may ultimately depend on the nature of the apoptosis-inducing stimulus and the type of cells (126). In certain cells, NFKB achieves the opposite functions by mediating distinct genes through cells specific transcription factors (126). In the case of renal IRI, lipopolysaccharides induced pre-activation of NFKB mitigates the kidney damage by preventing apoptosis as shown in a previous experimental study (133,134). Despite a few exceptions mentioned above where NFKB may contribute to cell death, in the majority of cases, the expression of its target genes promotes cellular survival (135). Thus it is not surprising, that ROS produced during I/R trigger a NFKB response and that target

genes of NFKB promote survival (135). Among the main ROS-induced signaling pathways, the crosstalk between NFKB and JNK is paramount. This interaction prevents sustained JNK activation, therefore decreasing cell death through apoptotic and necroptotic pathways (135–137). Furthermore, NFKB activity impacts ROS by increasing production of antioxidative proteins. Among the well-known targets is SOD2 (135,138). The present study demonstrated that RLX antioxidant properties are mediated by the NFKB-SOD2 pathway, as both of these genes were upregulated after SCS in RLX supplemented Custodiol® (20). Moreover, SCS in RLX supplemented Custodiol® downregulated the expression of genes (RIPK1 and MLKL) involved in the mediation of programmed cell death via apoptotic or necroptotic pathways (139–141). Necroptosis is a regulated inflammatory mode of cell death mimicking features of apoptosis and necrosis and also includes specific necrosome formation. The crucial factors of this pathway are protein RIPK3 and its substrate MLKL (142,143). The proposed mechanisms of necroptosis promotion include the phosphorylation of MLKL by RIPK3 which leads to indispensable changes in the "latch" of this pseudokinase. This step is necessary for the formation of specific oligomers that are capable of directly disrupting the plasma membrane by migrating to it and binding to phosphatidylinositol lipids (144). The presence of necroptosis in ischemic kidney injury was first identified by Linkermann et al. who demonstrated that necrostatin-1, a chemical inhibitor of RIPK1, decreased renal dysfunction and tissue damage in a mouse model of IRI (145). Further, the role of necroptosis in the pathogenesis of ischemic kidney injury was also confirmed in two in vitro studies on human and rat renal tubular cells (146,147). Similarly, this was also shown in renal IRI genetic models, where the ischemic injury was prevented in Rip3-knockout mice (148,149). Necrostatin-1 was not effective in Rip3-knockout mice, indicating that RIPK3 participates in renal ischemia-induced necroptosis (148,149). In the field of kidney transplantation, RIPK3-mediated necroptosis is known to promote graft inflammatory injury leading to impaired allograft survival (150), while blocking necroptosis through RIPK1 alleviates the renal IRI (145). The non-necroptotic role of RIPK in kidney injury has been demonstrated as well (151). The kidney fibrosis model in RIPK3 knockout mice showed RIPK3 as a key regulator of fibrogenesis in vivo (151). Moreover, RIPK3-regulated fibrogenesis is independent of MLKL. It activates AKT-dependent regulation of the metabolic enzyme - ATP citrate lyase in fibroblasts (151). Therefore, inhibition of the RIPK pathway is a potentially effective therapeutic target in

transplantation (20,150,152). Likewise, MLKL is among potential therapeutic targets against IRI as well (153). As mentioned previously, our experiments demonstrated RLX as an effective substance against necroptosis because it downregulates both of these genes (RIPK and MLKL) after SCS.

Furthermore, immunohistochemical staining for oxidative stress and apoptosis was performed to demonstrate the role of examined genes and to confirm that the cascades described above contributed to kidney graft quality. The present study used antibodies against MPO as markers of inflammation and oxidative stress (154). MPO is an enzyme produced by activated neutrophils and infiltrates the kidney in IRI (154,155). Ischemia not only stimulates the migration of neutrophils but also activates leukocytes within the kidney vasculature (156). The neutrophils' role in the pathogenesis of IRI was shown in several previous experimental models. For example, seeing a reduced insult in mice lacking adhesion molecules or blocking immune cells adhesion by pharmacological substances (155). Physiologically, MPO is the central enzyme contributing to lipoprotein peroxidation, which is necessary for the formation of reactive nitrogen species that have anti-pathogenic activity against bacteria, viruses, and parasites. MPO can be considered a biomarker of oxidative stress during IRI because it stimulates nitric oxide consumption, induces endothelial dysfunction, and causes the generation of numerous oxidative reactants (154). These toxic oxygen derivatives are capable to damage various cells and tissues (20,156). Moreover, some previous in vitro studies suggest that MPO mediates caspase-3 activation leading to apoptotic cell death (157). Our experiments demonstrated an increased number of MPO-positive cells in grafts after SCS in a conventional preservation solution, but the most important this increase was diminished by supplementing Curtodiol® with RLX.

Another marker investigated in the present study was activated Caspase 3. This immunohistochemical staining was used for the evaluation of apoptotic cells because Caspase 3 is the final effector in apoptotic death and is a widely described reliable marker of apoptosis (20,102). The results of the study showed an increased number of Caspase 3 positive cells after SCS in conventional preservation solution. On the contrary, RLX prevented such an increase. A positive RLX effect was observed not only in kidney tissue but also in other parts of the graft including the renal vein and ureter tissues (20). Such results of immunohistochemistry confirmed the findings of the gene expression analysis. Together these results clearly indicate that

RLX as a supplement to Custodiol® is effective to improve the preservation of kidney grafts. RLX not only upregulated anti-apoptotic genes and downregulated pro-apoptotic/necroptotic genes, but these changes also resulted in the reduced number of Caspase 3 and MPO positive cells in kidney grafts (20). Our findings are consistent with previous reports from small animal models that demonstrated an organ-protective effect of RLX against ischemic injury by preventing leucocyte recruitment and oxidative stress (100,158). Because of these known features, RLX has already been proposed as an effective additive to preservation solutions for liver and lung transplantation (85,90,100). There is evidence that the kidney may have the greatest uptake of exogenous RLX, although to date only two studies investigated RLX against kidney IRI (100,101). Yoshida et al. showed that RLX downregulated renal TNF receptor 1 mRNA expression, reduced plasma TNF-α levels, decreased apoptotic kidney cells number, and improved kidney function when administered at a dose of 500 ng/h just before the onset of reperfusion in rat kidney IRI model (101). A similar positive effect of RLX against kidney IRI was demonstrated by Collino et al. (100). Five µg/kg of RLX intravenously at the onset of reperfusion and 3 h after reperfusion attenuated renal damage and improved organ function by reducing local lipid peroxidation and free radical-induced DNA damage, also by increasing antioxidant enzymes – SOD expression/activity (100). Furthermore, RLX prevented neutrophil activation, as suggested by decreased MPO activity. It also diminished I/R induced increase of various cytokines levels, including IL-1b, IL-18, and TNF-alfa (100). However, to date, there was no experimental study investigating RLX in a KTx model. Our study was the first to show its renoprotective effect in a clinically-relevant large animal KTx model.

Despite RLX prevented ischemic injury in grafts, it had no impact on renal function (Creatinine; BUN), oxidative stress (MDA; TOS; CP, MPO), lipid peroxidation (4-HNE), and endothelial cell damage (big ET) markers after KTx. These unexpected results may be associated with the design and limitations of the present experiment. In this study, RLX was used as a supplement to a conventional preservation solution, rather than systemic treatment. Thus, only a minimal amount of peptide reached systemic blood flow from the graft vascular bed. Such concentrations are minimal when compared to those achieved in studies where systemic treatment with RLX at doses of 5 µg/kg was used for recipients at the time of reperfusion (20,89,100,159). Also, in current experiments donors and recipients were healthy, and young

animals. They do not represent the real-world clinical situation where both – donors and recipients may be elderly patients suffering from various comorbidities. Also, our recipients underwent only uni-lateral nephrectomy before KTx, which does not mimic terminal renal failure. The remaining native kidney may have concealed the study treatment impact on kidney function representing parameters (BUN and Creatinine) (20). Another limitation of our study which could have masked the impact of RLX on post-transplant graft function was a relatively high rate of premature dropouts (20). This was not unexpected, because the long-term follow-up of pigs after KTx may be extremely challenging. It is known from previous experimental KTx models, that a postoperative mortality rate of 25 % could be expected. Moreover, about one-third of animals do survive 10-60 days after surgery because of various complications (20,86,160,161). Despite the challenges of large animal models (162), they are crucial in the field of transplantation to investigate the novel substances' safety and therapeutic efficacy. The similarity in size, embryology, physiology, and pathogeneses of diseases makes large animal models an ideal tool for human disease research (163). In comparison, the small-animals studies are excellent for defining the fundamental biological mechanisms, but they are not easily translatable to humans. (164,165). Young animals used in murine models have an immature immune system, which does not correctly reflect most clinical situations. Furthermore, in rodents, the expression of major histocompatibility complex (MHC) antigens is restricted to antigen-presenting cells. Rodents have a reverse activity cycle compared to humans or large animals; thus, the chronobiology is different. Moreover, different pharmacokinetic parameters between rodents and large animals/humans may lead to incorrect extrapolation (164). Therefore, in the transplant field, any novel treatment efficacy and toxicity should be investigated in the large animal experiment before they can proceed to clinical studies (165). Among large animal models suitable for transplant research non-human primates, pigs and dogs are available (164). Pigs are ethically more accepted than non-human primates or dogs, thus this model is becoming more popular (164,165). Also, European authorities' regulations do not require colony breeding and allow domestic pigs from farms to be used in laboratory research. This regulation reduces the costs and increases the availability of experimental animals. Pigs have other advantages over other large animals as well. These include easy breeding, human-like anatomy, physiology, and immunology, relatively easy adaptation to experimental conditions, low rate of diseases, and the

ability for pig genetic engineering (164). Thus, pig models are critical to developing surgical procedures and novel preservation solutions in transplantation (163). Also, the main differences between the groups shown in this study represent data from qPCR experiments. Despite, that differences between RLX and placebo groups were significant, it should be considered that mRNA levels do not necessarily correlate with protein levels because of the potential impact of post-translational regulations. The present study lacks experiments showing protein levels; thus, future studies will have to elucidate the unclarities and show if RLX impact on various gene expression correlates to corresponding protein levels.

Despite some limitations, this is the first study that demonstrated the benefit of RLX against IRI in a large animal KTx model. Our results confirm former findings arising from small-animal models that have shown RLX as an effective substance against IRI in the liver, lung, heart, and kidney (85–87,89,95,97,100,166,167). Demonstrating similar effects in large animal transplantation models was necessary to build the bridge from bench to bedside. Now that our study confirmed encouraging effect of RLX against IRI in pig KTx, the subsequent investigation in first human clinical trials may be justified.

In conclusion, this large animal KTx study revealed that RLX added to Custodiol® upregulates antioxidant and anti-apoptotic genes while downregulates pro-apoptotic/necroptotic genes. The effect of RLX most likely includes apoptotic pathways and oxidative stress-related mechanisms as demonstrated by decreased numbers of Caspase 3 and MPO positive cells in kidney grafts. Clinical studies are needed to implement RLX as a novel additive to preservation solutions diminishing IRI in human transplantation.

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