

Cumulative dissertation

**UTERUS PRESERVATION: REDUCTION OF ISCHEMIA-REPERFUSION  
INJURY AND STATIC COLD STORAGE DAMAGE USING RELAXIN AND  
ERYTHROPOIETIN IN EXPERIMENTAL RAT UTERUS MODEL**

submitted by

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## **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 organizations that have contributed to the research for this thesis. Due acknowledgement 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”.

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I confirm that all co-authors have agreed to include their published data in my dissertation. All manuscripts were published in peer-reviewed journals by MDPI AG, Basel, Switzerland under Creative Commons Attribution (CC BY 4.0) “Open Access” license, which allows for unlimited distribution and reuse as long as appropriate credit is given to the original source and any changes made compared to the original are indicated. The parts of published work are being reproduced identically in this dissertation.

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## ABBREVIATIONS

ATP	Adenosine triphosphate
AUFI	Absolute uterine factor infertility
BAX	B-cell lymphoma protein 2 associated X
BCL-2	B-cell lymphoma protein – 2
CI	Cold ischemia
CXCL-2	Chemokine (C-X-C motif) ligand – 2
eNOS	Endothelial nitric oxide synthase
EPO	Erythropoietin
EPO-R	Erythropoietin receptor
GR	Glucocorticoid receptor
GSH	Glutathione
HES1	Hairy and enhancer of split 1
HIF1a	Hypoxia-inducible factor – 1 alpha
HTK	Histidine-tryptophan-ketoglutarate
ICAM-1	Intracellular adhesion molecule – 1
IL-10	Interleukin – 10
iNOS	Inducible nitric oxide synthase
IRI	Ischemia – reperfusion injury
IVF	In vitro fertilization
MCP1	Monocyte chemoattractant protein – 1
MDA	Malondialdehyde
MMF	Mycophenolate mofetil
MPO	Myeloperoxidase
MRKH	Mayer-Rokitansky-Küster-Hauser
NFKB	nuclear factor kappa B
NFKB1	nuclear factor NF-kappa-B 1
NICD	Notch intracellular domain
NO	Nitric oxide
qPCR	Quantitative polymerase chain reaction
RLX	Relaxin
ROS	Reactive oxygen species
RXFP1	Relaxin-like family peptide receptor 1
SCS	Static cold storage

SOD	Superoxide dismutase
SOD2	Superoxide dismutase – 2
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
UTx	Uterus transplantation
UW	University of Wisconsin

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## ZUSAMMENFASSUNG

Uterus-Transplantation (UTx) ist die einzige Behandlung für Frauen mit uterusbedingter Infertilität, die es den betroffenen Frauen erlaubt, sowohl genetisch Mutter zu werden als auch die Schwangerschaft und Geburt selbst zu erleben. Jedoch wird UTx häufig negativ durch die statisch gekühlte Aufbewahrung (SCS) und ischämische Reperfusionsschäden (IRI) beeinflusst. Relaxin (RLX) und Erythropoetin (EPO) sind Substanzen, bei denen gezeigt werden konnte, dass sie in verschiedenen Modellen zu SCS und IRI entzündungshemmend, anti-apoptotisch und anti-fibrotisch wirken.

Diese kumulative Arbeit soll die schützenden Wirkungen von RLX bzw. EPO bei Ratten in Modellen zu SCS und IRI unterstreichen und die morphologischen und biologischen Veränderungen im Uterusgewebe der Ratten untersuchen. Zusätzlich haben wir Gen-Panels untersucht, die im Zusammenhang mit oxidativem Stress und Apoptose stehen, sowie die Wirkmechanismen der experimentellen Substanzen.

Zuerst wurde Uterus SCS untersucht. Dazu wurden Uterushörner aus 15 Sprague Dawley Ratten entnommen und zufällig in drei Gruppen (n = 10 je Gruppe) aufgeteilt und bei 4°C in einer HTK-N-Lösung aufbewahrt, und zwar mit oder ohne Zusätze: 10 IU/ml EPO oder 20 nmol/l RLX. Gewebeproben wurden nach 8 und 24 Stunden nach Konservierung entnommen. Danach wurde Uterus-IRI simuliert. Dazu wurden 80 Ratten zufällig in 8 Gruppen (zu je 10) aufgeteilt und entweder mit Saline, 5 µg/kg humanem Relaxin-2, 4000 IU/kg rekombiniertem humanem Erythropoetin oder Kombinationen daraus vorbehandelt. Ischämie wurde erreicht, indem die Aorta und die Arterien in den Ovarien 60 min lang gedrosselt wurden, gefolgt von 120 min Reperfusion und Probenentnahme.

In beiden Teilen wurden Histologie, biochemische und immunhistochemische Marker des Uterusgewebes untersucht. Zusätzlich wurden bei den Geweben, die IRI ausgesetzt waren, Gen-Panels untersucht. Die Morphologie des Uterusgewebes, die Spiegel von MDA und SO sowie die Zahl von TUNEL-positiven Zellen zeigten schwere Schäden in HTK-N-Lösung nach 24 Stunden Lagerung SCS ohne Zusätze. Diese Schäden wurden deutlich abgemildert, wenn RLX der Konservierungslösung hinzugefügt wurde. EPO zeigte hingegen keine positiven Auswirkungen. Eine Vorbehandlung mit RLX führte im Vergleich mit den Kontrollgruppen während IRI zum Erhalt der normalen Morphologie des Gewebes, reduzierte den MDA-Spiegel und die Zahl der TUNEL-positiven Zellen, beibehaltener SOD-Aktivität und gesteigerten Genexpressionen NICD und HES1. Eine Vorbehandlung mit EPO reduzierte die MDA-Spiegel. Im Ergebnis zeigt unsere Studie, dass RLX sich als Zusatz zu einer HTK-N-Lösung bei Lagerung von Uterus per SCS positiv auf den Gewebeerhalt auswirkt, indem Entzündungen

und Apoptose gehemmt und die Morphologie des Gewebes erhalten bleibt. Vorbehandlung mit RLX, EPO oder einer Kombination aus EPO und RLX kann während IRI die Schäden am Uterusgewebe deutlich abmildern. Die Kombination aus RLX und EPO war so effektiv wie RLX allein, mit der Ausnahme, dass die Kombination eine positive Wirkung hinsichtlich der Expression entzündungshemmender Gene war.

## ABSTRACT

Uterus transplantation (UTx) is the only treatment method for women with absolute uterine factor infertility and it enables women to be both genetical mothers and to experience pregnancy and childbearing. However, UTx is negatively affected by uterus static cold storage (SCS) and ischemia-reperfusion injury (IRI). Relaxin (RLX) and erythropoietin (EPO) are the substances that have already shown anti-inflammatory, anti-apoptotic and anti-fibrotic effects in several SCS and IRI models.

This cumulative thesis aims to highlight the protective effect of RLX or/and EPO in rat uterus SCS and IRI models and to examine morphological and biochemical changes in rat uterine tissue. Additionally, we aimed to investigate gene panels, associated with oxidative stress, apoptosis and possible acting mechanisms of the experimental substances.

Firstly, uterus SCS was investigated. Therefore, uterus horns retrieved from 15 Sprague Dawley rats were randomly assigned into three groups (n = 10/group) and kept at 4°C in HTK-N solution without or with different additives: 10 IU/mL EPO or 20 nM RLX. Tissue samples were taken after 8 and 24 h of preservation.

Secondly, uterus IRI was simulated. Therefore, eighty rats were randomly assigned into eight groups (n = 10/group) and pretreated with either saline, 5 µg/kg human relaxin-2, 4000 IU/kg recombinant human erythropoietin or their combination. Ischemia was achieved by clamping the aorta and ovarian arteries for 60 min, following 120 min of reperfusion and tissue sampling. For both parts uterine tissue histology, biochemical and immunohistochemical markers were analyzed. Additionally, gene panels were examined for tissues exposed to IRI. Uterine tissue morphology, MDA, SOD levels and the TUNEL-positive cell number showed severe damage in HTK-N solution without additives after 24 h of preservation during SCS. This damage was significantly attenuated by adding RLX to the preservation solution whereas EPO showed no favorable effect. Pretreatment with RLX preserved normal tissue morphology, reduced MDA levels, MPO- and TUNEL-positive cell count, preserved SOD activity and upregulated NICD and HES1 gene expression when compared to the control group during IRI. Pretreatment with EPO reduced MDA levels.

In conclusion, our study shows that RLX as an additive to HTK-N solution can serve as an effective uterine tissue preservative in a SCS setting by inhibiting inflammation, apoptosis and preserving tissue morphology. Pretreatment with RLX, EPO or a combination of both EPO and RLX during IRI significantly alleviates uterine tissue damage. The combination of RLX with EPO was as effective as RLX alone, except the combination yielded a beneficial effect on anti-inflammatory gene expression.

# INTRODUCTION

## Absolute uterine factor infertility (AUF)

Absolute uterine factor infertility is a form of infertility resulting from the absence or an abnormality of the uterus that prevents conception and maintenance of a pregnancy. AUF affects 1 in 500 women of reproductive age or about 1.5 million women worldwide (1).

There are two major groups of causes responsible for AUF – congenital or acquired. Congenital causes refer to Müllerian malformations which occur during fetal formation and can result in agenesis or a non-functional uterus. Uterus agenesis, also known as Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome, is a rare condition with an estimated prevalence of 1 in 4500 females (2). This congenital disorder is characterized by the aplasia of the uterus and upper third of the vagina with normal external genitalia and typical reproductive endocrine function (2).

Other Müllerian malformations are more frequent, however, only a few types of these malformations cause AUF (3). T-shaped or hypoplastic uterus cover less than 5 percent of all Müllerian malformations, but have a huge impact on the infertility rate as only up to 10 percent of women conceive and give birth even after using assisted reproduction technologies (4).

Acquired AUF is mainly caused by hysterectomies during the reproductive period. There are approximately 600,000 hysterectomies performed in the United States annually, and more than 40 percent of these surgeries are done for women younger than 44 years (5). Hysterectomies are usually performed due to benign conditions (large myomas, endometriosis, abnormal bleeding), malignancy, or life-threatening obstetric complications (abnormal placentation, postpartum hemorrhage, or sepsis) (6). Intrauterine adhesions are defined as scar tissue within the uterine cavity and typically form in response to infection or surgery (7). Although this condition can be treated by doing a hysteroscopy, 30 percent of women are unable to conceive and give birth even after successful surgery (8).

Women having AUF and seeking motherhood nowadays have only two established options – either to adopt a child or to use a gestational carrier. However, surrogacy is limited to only a few countries worldwide and raises many ethical, religious, and cultural issues. Moreover, none of these choices enable women to be both genetical mothers and to experience pregnancy and childbearing. Uterus transplantation (UTx) is the only treatment method for women with AUF and is now being offered as a standard procedure in the United States (9).

## History of UTx

### **Animal studies**

Successful kidney transplantation in 1954 was one of the most remarkable achievements in medicine during the last 70 years (10). In the following years, other life-saving organs were transplanted giving a chance to live for patients having end-stage diseases. UTx is a life-enhancing rather than life-saving operation and it gained interest later in the 1960s.

Most important animal studies in the field of UTx are reported in a Table 1. The first experiments on UTx were performed on dogs and published by Eraslan et al. in 1966 (11). These experiments aimed to address tubal factor infertility, as it was considered to be the largest group of female infertility. Eraslan and colleagues did an autologous combined uterus and ovaries transplantation and achieved multiple pregnancies. Later, more experiments on dogs were performed, unfortunately, lack of effective immunosuppressive therapy limited the survival of the transplants (12,13). In vitro fertilization (IVF) was the main breakthrough in fertility treatment in the previous century (14). Steptoe and Edwards reported the first human IVF baby born in 1978 after the reimplantation of an embryo for a woman with occluded tubes. This new treatment method was ideal for women having tubal factor infertility, therefore UTx remained relevant exclusively for patients with an absence or a non-functioning uterus.

Further research in various animal models was conducted around the 2000s to address different aspects related to UTx, such as surgery, immunosuppression, ischemia-reperfusion injury (IRI), graft rejection, and fertility (15–18). El-Akouri et al. published a few experiments in a syngeneic mouse UTx model reporting pregnancies and healthy offspring (15,16). No graft rejection was observed after UTx as animals were genetically identical and there was no need for immunosuppression. A few years later experiments in the allogeneic mouse model identified graft rejection which was reduced by cyclosporine monotherapy (18,19). In separate experiments, graft rejection rates were even lower using tacrolimus than cyclosporine A for immunosuppression (20,21). Other studies in rat models aimed to evaluate surgery related aspects therefore did a syngeneic orthotopic UTx (22). The authors concluded that the pregnancy rate after natural mating was comparable to a non-transplanted group. Díaz-García and colleagues were the first to demonstrate that perinatal outcomes of the offspring after allogeneic UTx were the same compared to controls (23). Moreover, authors later found that an extended warm ischemia period of up to 4 hours had a significant negative effect on the viability of uterine graft (24).

There were a few attempts to transplant the uterus in a rabbit model. Confino et al. performed transplantations during which he fixated the uterus to the broad ligament for revascularization, thereafter few animals presented with preserved endometrial and myometrial structure (25). Later studies conducted by Saso et al. aimed to demonstrate the feasibility of the aorto-caval vascular patch technique and to achieve pregnancies following natural mating after allogeneic UTx (26). The technique was successful, but no conception was achieved because of the blocked cornua. Saso et al. later concluded that a low long-term survival rate could be determined by challenging vascular anastomosis in a rabbit model (27).

The sheep model is considered to be conducive for UTx experiments because sheep are comparable to humans regarding size and anatomy of pelvic vasculature (1). Dahm-Kähler et al. in 2008 demonstrated the feasibility of the sheep autotransplantation and the usage of sheep model in further UTx studies (28). Wranning et al. studied ewes uterus samples after static cold storage (SCS) and concluded that short-time cold storage did not induce any reperfusion damage (29). Moreover, they determined that the Perfadex preservation solution was superior to Ringer's acetate in terms of decreased oxidative stress and inflammation. In subsequent experiments, the same research group auto-transplanted the uterus with oviduct and ovary. After mating three months later, 60 percent of transplanted animals achieved pregnancies and delivered lambs (30). It was the first successful auto-UTx in an animal model comparable in size to a human. Ramirez et al. performed an allogeneic sheep UTx with immunosuppression of cyclosporine and demonstrated a fully developed lamb which was delivered via cesarean section (31).

The pig is another animal model, which is suitable for UTx research due to its similarity to human anatomy. In 2006, Wranning et al. aimed to establish a method for pig uterus auto-transplantation and evaluate reperfusion results after 1-2 hours of cold ischemia (CI) (17). The authors performed 19 UTx, of which seven were well-flushed and suitable for further cold storage. After reperfusion, analysis of transplanted organs revealed that two uteri had no severe IRI based on blood gases, metabolite parameters and histology. Although acceptable reperfusion was achieved, authors concluded that UTx research on the pig model is difficult due to the large total uterus size compared to the small available vessels for anastomosis. Sieunarine et al. reported the development of thrombosis in the anastomosis sites, especially in small re-anastomosed vessels (32). In 2009, Avison et al. performed UTx in mini-pigs and transplanted uteri in the lower abdominal cavity of the recipient (33). Intravenous tacrolimus immunosuppression was administered for the first 12 posttransplant days, followed by oral cyclosporine for maintenance. A survival rate of 50 percent was reported during 12 months of

follow-up. Authors reported acute graft rejection in several animals, which were effectively reversed with increased doses of immunosuppressant drugs.

The beginning of UTx experiments in non-human primates was another huge step further in UTx research. Primates such as baboons have a relatively large uterus and their reproductive physiology closely resembles humans, hence these animals are widely used in the field of reproductive medicine (34). Enskog et al. in 2010 performed ten autologous UTx in baboons, unfortunately, only 20 percent of the transplanted animals resumed menstrual cycle showing restored uterine and ovarian function (35). The method was altered by changing the anastomosis type and procurement of graft vessels (36). It led to three times higher long-term function compared to previous experiments conducted by the same group. However, no pregnancies were achieved despite mating for  $\geq 5$  menstrual cycles. Post-mortem analysis revealed tubal blockage and adhesions. In 2013, Johannesson et al. performed allogeneic UTx in a baboon from a living donor and examined different immunosuppression protocols including anti-thymocyte globulin and triple immunosuppressive scheme consisting of tacrolimus, corticosteroids and mycophenolate mofetil (MMF) (37). The study concluded that living donor UTx in a non-human primate model is safe for a donor as all living donors survived full follow-up time. Graft rejection was observed in all recipients within the first weeks after the transplantation, however, authors were not able to conclude whether graft injury was induced by rejection or reduced blood flow after the surgery.

Another research team in Japan performed autologous UTx in a small cynomolgus macaque model which resulted in the first pregnancy after UTx in a non-human primate species (38,39). A follow-up study was conducted by the same authors on allogeneic UTx in a cynomolgus macaque model (40). A restoration of menstruation was observed in an animal receiving a triple immunosuppression scheme of tacrolimus, methylprednisolone and MMF.

Although a few of the abovementioned animal models were not suitable for UTx due to challenging surgery peculiarities, they still had a significant impact on further UTx studies.

**Table 1.** Animal studies on UTx

Author, year	Animal model	Transplantation technique	Main findings, advantages, disadvantages
Eraslan et al., 1966 (41)	Dog	Replantation of uterus and ovaries <i>en bloc</i>	First to report successful uterine-ovarian replantation

			<p>Functional integrity of uterus and ovaries in two examined dogs.</p> <p>Two successful deliveries (nine and three healthy puppies).</p> <p>One pregnancy at the time of report.</p>
El-Akouri et al., 2002 (15)	Mice	Syngeneic heterotopic uterine transplantation	<p>First successful syngeneic uterus transplantation.</p> <p>Pregnancies and deliveries of same size and weight offspring as in native controls.</p>
El-Akouri, 2003 (16)	Mice	Syngeneic heterotopic uterine transplantation	<p>Pregnancy rates are comparable to those of native controls.</p>
El-Akouri et al., 2006 (19)	Mice	Allogeneic uterine transplantation	<p>First murine model to study rejection patterns in uterine transplantation</p> <p>Important model for further immunosuppressant studies</p>
Wranning et al., 2007 (18)	Mice	Allogeneic uterine transplantation	<p>Immunosuppression with Cyclosporine A delays rejection of grafted uterus.</p>
Akhi et al., 2013 (20)	Rat	Allogeneic orthotopic transplantation	<p>Immunosuppression with Tacrolimus reduces allotransplanted uterus rejection and normalizes IP-10 and IL-1a, prevents infiltration with T-lymphocytes.</p>
Groth et al., 2012 (21)	Rat	Allogeneic orthotopic transplantation	<p>Cyclosporine A reduces rejection of uterine transplant, normalizes</p>

			proinflammatory cytokine IL-1a and protein galectin-1 mRNA levels.
Wranning et al., 2011 (22)	Rat	Syngeneic orthotopic transplantation	Successful pregnancies after uterine transplantation.  Similar pregnancy rate, offspring count, and growth trajectory of pups when compared to controls
Diaz-Garcia et al., 2013 (24)	Rat	Syngeneic orthotopic transplantation	A prolonged warm ischemia time has negative effect on uterus survival after UTx.
Diaz-Garcia et al., 2014 (23)	Rat	Allogeneic orthotopic transplantation	Pregnancy rate and perinatal outcomes after allogeneic UTx are similar to controls.
Saso et al., 2014 (26)	Rabbit	Allogeneic uterine cross transplantation	Feasibility of macrovascular patch technique for uterine allotransplantation.  No conception was achieved.
Saso et al., 2014 (27)	Rabbit	Allogeneic uterine cross transplantation	Low long-term survival rate is probably associated with usage of a rabbit model.
Dahm-Kähler et al., 2008 (28)	Sheep	Orthotopic auto-transplantation	Feasibility of sheep orthotopic UTx with unique vasculature.  Model suitable for further experiments examining uterine viability and pregnancies.

Wranning et al., 2008 (29)	Sheep	One horn auto-transplantation	Short-time SCS of sheep uterus has no serious harm during reperfusion.  Perfadex reduced oxidative stress and inflammation when compared to Ringer's acetate.
Wranning et al., 2010 (30)	Sheep	Orthotopic auto-transplantation	Fertility and successful pregnancies after auto-transplantation.
Ramirez et al., 2008 (42)	Sheep	Orthotopic allotransplantation	First reported ewe uterine allotransplantation.
Wranning et al., 2006 (17)	Pig	Auto-transplantation	First report of uterus auto-transplantation in a pig model.  Large size of the uterus and small vessels limits this model for further use in UTx studies.
Avison et al., 2009	Mini-pig	Heterotopic allogeneic transplantation	Successful UTx in minipigs using a heterotopic model.
Oliveira et al., 2017 (43)	Pig	Orthotopic allogeneic transplantation	Feasibility of this model.  Rejection of 5 of 6 grafts on 7 <sup>th</sup> postoperative day.
Zhang et al., 2018 (44)	Pig	Orthotopic allogeneic transplantation	Allogeneic UTx is safe and feasible.  Long-term survival and resumed menstruation in study animals.
Enskog et al., 2010 (35)	Baboon	Autotransplantation	Feasibility of UTx by vascular anastomosis in a baboon model.

Johannesson et al., 2012 (36)	Baboon	Autotransplantation with end-to-side or end-to-end anastomoses	Modified technique is a safe procedure demonstrating resumed long-term function of the uterus.
Johannesson et al., 2013 (37)	Baboon	Allogeneic orthotopic transplantation	All recipients presented with rejection within first weeks after UTx.  Authors suggest optimization of immunosuppression protocols to avoid rejection.
Kisu et al., 2012 (38)	Macaque	Autotransplantation	Feasibility of uterine autotransplantation in cynomolgus monkeys.
Mihara et al., 2012 (39)	Macaque	Autotransplantation	First natural pregnancy and in a non-human primate after UTx.
Kisu et al., 2014 (40)	Macaque	Orthotopic allotransplantation	Recovery of uterine function after UTx.

## Human studies

Main human studies in UTx are summarized in Table 2. The first human UTx was performed in 2000 in Saudi Arabia (45). Uterus was transplanted from a living donor to a 26-year-old recipient, who experienced a hysterectomy due to massive peripartum bleeding. The surgery went without major donor or recipient complications. However, on the 99<sup>th</sup> postoperative day, patient presented with heaviness and foul-smelling vaginal discharge. Speculum examination and doppler ultrasound confirmed vascular thrombosis urging the immediate hysterectomy. Postoperative analysis showed no signs of graft rejection. First human UTx encouraged other research teams to develop further programs on this topic.

Ozkan et al. performed the world's second UTx but utilized a uterus from a deceased donor (46). A donor was a brain-dead 22-year-old nulliparous woman, who suffered cerebral trauma in a car accident. During a two hour-long procedure, uterus was the first procured organ and

was transplanted to a 21-year-old recipient with MRKH syndrome. The immunosuppression scheme of prednisolone, azathioprine and tacrolimus was maintained and the first IVF attempts started 18 months after the transplantation. However, only two early miscarriages were noted after several IVF cycles (47). Although delivery of a near-term baby was not achieved, this study was a big step further showing successful implantation after uterus allotransplantation in humans.

The Swedish team led by professor Mats Brännström began a clinical trial on living donor UTx in 2013 (48). Nine recipients were included in this trial, of which eight women had MRKH syndrome and one experienced radical hysterectomy with pelvic lymphonodectomy due to cervical cancer five years before the transplantation. The majority of donors were related to recipients as they were mothers, sister and maternal aunt. The remaining two donors were a mother-in-law and a family friend. The retrieval of the uterus lasted from 10 to 13 hours including the retrieval of uterine arteries with internal iliac arteries as well as vein supply from deep uterine veins together with internal iliac vein segments. The duration for uterus implantation was 4-5 hours. The first report of this trial noted viable organs with restored menstruation in seven out of nine recipients after 6 months of the follow-up period. Two grafts were rejected and had to be removed due to thrombotic uterine vessels bilaterally and persistent uterine infection. All women with viable grafts became pregnant after either one or repeated embryo transfer procedures, however, one patient experienced repeated miscarriages (49,50). The remaining recipients delivered healthy babies.

In 2015 Wei et al. transplanted the uterus which was procured during robotic-assisted laparoscopy, removed through the vagina and implanted using traditional laparotomy (51). Menstruation resumed two months after the UTx and the delivery of a healthy baby was achieved after the fifth embryo transfer (52).

A clinical trial including the biggest number of deceased donors at that time was reported by Chmel et al. (53). In total nine UTx were performed, of which four grafts were procured from deceased donors, the remaining five living donors were related to the recipients (four mothers and maternal aunt). In most cases, ovarian veins were chosen as the only venous outflow. Two thrombotic events were reported resulting in a hysterectomy on the 5<sup>th</sup> and 15<sup>th</sup> postoperative days. Two years later, the authors published another article reporting two childbirths after successful deceased and living donor UTx (54). Another three-case interventional study was performed in Tübingen, Germany (55). It was the first study reporting

an aborted attempt to transplant the uterus due to resistance of graft uterine arteries and an inability to perfuse the graft.

Johannesson et al. recently reported twelve livebirths after UTx performed by an American team in Dallas (56). In this prospective study 20 women received uterine grafts from 18 living and 2 deceased donors. Authors calculated a live-birth rate per technically successful UTx which is 79 percent. Fronek et al. published interim results of their UTx study performed in Czech Republic (57). In total 10 UTx were performed procuring grafts from five living and five deceased donors. Three livebirths were reported, one of them was achieved after uterus donation from nulliparous donor.

**Table 2.** Human studies on UTx

Author, year of publication, country	Number of cases	Recipients	Donors	Main findings, outcomes
Fageeh et al., 2002, Saudi Arabia (45)	1	Postpartum hysterectomy	Living	No donor or recipient related complications.  Thrombosis and hysterectomy on 99th postoperative day.
Ermar Akar et al., 2013, Turkey (47)	1	MRKH	Brain-dead	Resumed estrous cycle.  Two spontaneous abortions.
Brännström et al., 2014, Sweden (48)	9	8 MRKH and 1 hysterectomy for cervical cancer	Living	Seven grafts survived 3 months period.  Two graft failures.  In total 8 healthy babies.
Wei et al., 2017, China (51)	1	MRKH	Living	First successful robotic-assisted uterus procurement.  Resumed menstruation.

				Healthy baby after fifth IVF cycle.
Flyckt et al., 2016, United States (58)	1	MRKH	Deceased	First deceased donor UTx.
Ejzenberg et al., 2018, Brazil (59)	1	Unknown	Deceased	First livebirth after UTx from a deceased donor.
Testa et al., 2017, United States (60)	5	MRKH	Living	Three grafts failures on 6 <sup>th</sup> , 12 <sup>th</sup> and 16 <sup>th</sup> postoperative days.  Resumed menstruation and two healthy babies.
Brännström et al., 2017, Serbia (61)	1	Unknown	Living	Graft survival at 3 months
Brucker et al., 2017, Germany	2	MRKH	Living	Two successful UTxs.  One aborted attempt due to resistance of grafts uterine vessels.
Brännström et al., 2018, Sweden (62)	1	Unknown	Living	Successful donor surgery using robotic approach.
Chmel et al. 2019, Czech Republic (53)	9	MRKH	Four deceased and five living	Largest group of deceased donors.  Two grafts removals on 7 <sup>th</sup> and 15 <sup>th</sup> postoperative days.

				Seven successful grafts after at least 6 months of follow-up period.
Johannesson et al., 2021, United States (56)	20	18 MRKH and 2 after hysterectomies for leiomyomas	18 living and 2 deceased donors	Six graft failures. 14 successful transplants. 11 successful pregnancies resulting in 12 livebirths.
Fronek et al., 2021, Czech Republic (57)	10	MRKH	5 living and 5 deceased donors	Two graft removals due to thrombosis. Three livebirths.

MRKH – Mayer Rokitansky Kuster-Hauser syndrome, IVF – in vitro fertilization

## Transplantation from a living donor

Based on Organ Procurement and Transplantation Network data, in 2021 one third of all solid organs in the United States were transplanted from living donors and the kidney remains the most commonly donated organ of this type. Several organs can be transplanted from a living donor such as kidney, liver, lung, small intestine and pancreas. All of them are considered life-saving transplants. UTx however, is rather a life-enhancing than a life-saving procedure. In addition, the uterus is the first ephemeral organ in transplantation as it is removed if no further pregnancies are expected.

UTx from living donors has a few advantages when compared to deceased donors (Table 3). A detailed evaluation of a possible donor and her uterus before the operation is one of the major advantages of living donors. A meticulous gynecological and obstetrical history as well as preoperative examination including extensive imaging can be obtained. Gynecological ultrasound examination, magnetic resonance imaging and computed tomography with angiograms can be performed to better understand the anatomy of uterine vessels and increase the viability of a transplanted organ (63). Moreover, the selection of genetically related donors (usually mothers) is beneficial, because of the lower probability of a human leukocyte antigen mismatch (48). Some authors also highlight that nowadays the demand for solid organs does not meet the supply, which causes longer waiting lists, especially for vital organs

(64). Although the uterus is a non-vital organ, a donation from a living donor could increase the donor pool. Up to date, the majority of all successful UTx cases were achieved after living donor donation showing the efficiency of this method.

On the other hand, living donor donation is associated with various surgery-related complications which can be completely avoided when utilizing organs from deceased donors (65). During uterus retrieval, a radical hysterectomy is performed with a more complex and broad dissection of uterus vessels and ureters compared to a hysterectomy due to benign causes (66). The risk of Clavien-Dindo grade III or IV surgical complications reaches 20 percent in previously reported UTx from living donors (53,66,67). More than 10 percent of living donor cases required additional surgical intervention due to ureter injuries, vaginal cuff dehiscence or evacuation of feces under general anesthesia (45,68,53,48,67,60). Other donors experienced less severe complications such as wound infection, anemia, bladder hypotonia and urinary tract infection (69). Implementation of modified surgical techniques has already reduced the total operation time for organ retrieval and has a positive effect on reducing surgery-related complications (69).

## Transplantation from a deceased donor

UTx from a deceased donor has one undisputed advantage that donor suffers no surgical risk during surgery (Table 3). It also eliminates psychological harm to the donor (69). In addition, longer vascular grafts and pedicles for both arteries and veins can be obtained during organ procurement operation from a deceased donor (53). It minimizes the risk of thrombosis as wider vascular anastomoses are associated with quicker restoration of blood flow to the graft (1). Also, more extensive resection of the bladder peritoneum is beneficial for uterus placement in the recipient (53). Dissection in the deep pelvis does not urge to preserve pelvic nerves and important anatomical structures thus reducing the total surgery time. However, transplantation from a deceased donor has a few notable disadvantages.

First of all, evaluation of a potential graft before the transplantation is important for a successful surgery and graft survival. However, limited time for evaluation could cause issues especially in donors who are close to or after menopause. These donors have narrower uterine vessels, hence they need to be carefully evaluated before the organ retrieval (70). Angiography before the explantation is a preferred method, but it is not possible in all cases, therefore angiography on the back-table could be applied as an alternative (60). Secondly, the usage of nulliparous donors is not recommended, as their uterine vessels are narrower and there is no definite test

for the feasibility to achieve pregnancy in those donors (71). Some authors argue that the total duration of organ retrieval operation can be even longer in deceased donors because it is hard to identify small vessels after perfusing them with preservation solutions (72). Despite the abovementioned limitations, there have already been published successful UTx cases from deceased donors proving its feasibility.

Priore et al. investigated whether the human uterus can be retrieved from a multi-organ, brain-dead, heart-beating donor (73). He revealed that the human uterus can tolerate CI for up to 12 hours and show no signs of tissue changes. This finding was important as the uterus, as a not life-supportive organ, is not prioritized and is usually retrieved lastly during the organ procurement procedure thus experiencing relatively long ischemic time.

**Table 3.** Advantages and disadvantages of living and deceased donors

	Living donor	Deceased donor
Advantages	<ul style="list-style-type: none"> <li>• Meticulous evaluation of donor before the transplantation.</li> <li>• Availability of genetically related donors to avoid human leukocyte antigen mismatch.</li> <li>• UTx from living donors could increase donor pool.</li> </ul>	<ul style="list-style-type: none"> <li>• No surgical risk for donor.</li> <li>• Elimination of psychological harm to donor.</li> <li>• Availability of longer vascular pedicles which reduces risk of thrombosis.</li> <li>• Reduced surgical time.</li> </ul>
Disadvantages	<ul style="list-style-type: none"> <li>• Surgery related donor complications</li> </ul>	<ul style="list-style-type: none"> <li>• Limited time for donor evaluation.</li> <li>• Not recommended to procure grafts from nulliparous women.</li> </ul>

## Organ preservation

### Warm and cold ischemia

During organ transplantation, metabolic changes can impair graft survival and result in worse patient outcomes (74). Ischemia is one of the most challenging conditions that occurs in an organ during the transplantation and is divided into “cold” and “warm” periods. According to international standards, CI is described as a period during which the organ is stored *ex vivo* in hypothermic conditions. It begins with the flushing of the graft with cold preservation solution and lasts until the graft is placed into the recipient’s abdomen. Warm ischemia is divided into two phases. The first one occurs after the cessation of blood flow to the graft in the donor and extends till the organ is perfused with the preservative solution. The second phase starts with placing the organ into the abdomen and is counted till vascular clamps are released after surgical anastomosis (75). Total warm ischemia time includes both of the warm ischemia phases.

### Static cold storage

Nowadays, SCS remains the main preservation method in solid organ transplantation. It aims to keep a procured organ under hypothermic conditions to reduce its metabolic requirements

(76). Since the first attempts to preserve the grafts in the 1960s, SCS became the golden standard for organ preservation (77,78). In SCS, a procured organ is flushed with preservation solution at 0-4°C, submerged into the preservation solution and kept at the same temperature until transplantation (79). The underlying mechanism of SCS is hypothermia which slows down metabolic activity by 10 times (80). Despite the reduced cell metabolism, prolonged CI leads to adenosine triphosphate (ATP) depletion and causes anaerobic respiration with lactic acid formation (81). Cellular acidosis interrupts transmembrane ion pumps (Na/K<sup>+</sup> and Ca<sup>2+</sup>) causing an influx of ions and water into the cell and resulting in cell swelling. The accumulation of intracellular Ca<sup>2+</sup> releases cytochrome C and other mitochondrial enzymes into the cytosol and initiates cell death by apoptosis (82). The pro-oxidant environment contributes to the formation of reactive oxygen species (ROS) at early reperfusion. The knowledge of these mechanisms of cell death in the SCS setting enables the development of preservation solutions which could alleviate processes leading to cell injury.

### **Preservation solutions**

Cold preservation solutions are designed to minimize processes during SCS or to provide substrates for regeneration (83). The composition of organ preservation solutions varies and includes substances that counteract electrolyte and water movement across cell membrane preventing it from swelling. In addition, different buffers can help to control pH changes as well as antioxidants can prevent free radical molecules (81). Amino acids can also be included in the composition of preservation solutions as precursors for ATP production.

Collins solution was the first preservation solution introduced into the market (77). It was used in kidney transplantation and could preserve organs for up to 24 hours enabling organ sharing between distant locations. Collins solution was low in sodium and high in potassium simulating the intracellular ion balance. The osmotic balance between the solution and the organ was preserved by using high concentrations of glucose as it suppressed water and electrolyte shifts through the membrane and therefore reduced tissue edema. A phosphate buffer was also included in the solution to minimize intracellular acidosis. Although the Collins solution of this composition was exploited for more than ten years, an improved version of Collins C2 solution was introduced with a higher concentration of glucose to protect against tissue edema (84). The formation of magnesium phosphate precipitates was another issue that led to a modified Euro-Collins solution without magnesium sulfate. In 1976 at The Eurotransplant Meeting, it was acknowledged as a standard perfusate as it showed its superiority over other solutions in experimental data (85).

University of Wisconsin (UW) solution was created in 1987 by Belzer and Southard to extend liver and pancreas tolerance to preservation which at that time lasted only 4-8 hours using Collins solution (86). This developed solution brought new components aiming to alleviate cold storage and ischemia-reperfusion caused tissue damage. First of all, new impermeants such as lactobionic acid and raffinose replaced glucose as these substances cannot cross the membrane due to their charge and size and therefore are considered to prevent cell swelling (83). In addition, hydroxyethyl starch was added to increase oncotic pressure, however, the solution itself became more viscous. A few ingredients were also introduced to address oxidative stress (allopurinol and glutathione) and to generate ATP from its substrate (adenosine) (86). The development of the UW solution mirrored its wide use in clinical practice.

Institute George Lopez-1 solution is another preservation solution that has a similar composition as the UW solution except its ionic balance is near the extracellular proportion of  $\text{Na}^+/\text{K}^+$  (87). In addition, polyethylene glycol was substituted for hydroxyethyl starch which led to significantly reduced lipid peroxidation as demonstrated in the experimental kidney and isolated hepatocytes perfusion model (88,89).

Celsior solution was initially created for cardioplegia, but later it showed its effect during cold organ preservation of various solid organs such as the pancreas, liver, lungs and kidneys (90–94). It contains high molecular mass impermeants such as mannitol and lactobionic acid and reduced antioxidant glutathione and glutamic acid concentrations (95). Intracellular calcium overload and acidosis are prevented by adding histidine as a buffer. Celsior solution has a lower viscosity, which may cause better flushing of hepatic vasculature and biliary tree during liver procurement (92).

Celsior solution was not the only which was initially developed for cardioplegia. In the 1970s German physiologist, Hans Jürgen Bretschneider designed a cardioplegic solution which is nowadays known under the names of Custodiol or histidine-tryptophan-ketoglutarate (HTK) solution (96). It mimics intracellular sodium concentration with an elevated extracellular potassium concentration. This electrolyte composition decreases cardiac electric activity and provokes cardiac arrest during diastole (97). A macromolecule of mannitol was chosen as an impermeant to prevent cell swelling caused by hypothermia. The solution contains histidine/histidine hydrochloride buffer to preserve physiological pH which is usually affected by hypothermia and anaerobic metabolism. Histidine and ketoglutarate were supplemented as substrates for energy supply and amino acid tryptophan serves as an antioxidant and cellular membrane protective substance. Despite the fact that HTK solution was developed for cardioplegia, in 1990 it was tested in a clinical liver transplantation setting and proved its

feasibility and successful employment in this field (98). HTK solution was approved by the Food and Drug Administration in the United States in 2002 for liver preservation (99). However, after a few years of HTK solution application in clinical practice, some authors raised concerns regarding HTK solution toxicity most likely associated with histidine (86). This substance has been chosen as an effective buffer in several preservation solutions, unfortunately, after using it in higher concentrations as it is in HTK solution, it can have a detrimental effect (100). Rauen et al. in their study aimed to analyze the mechanism of histidine toxicity and possible ways to prevent it (101). Authors revealed that histidine provokes iron-dependent ROS formation further causing cell injury and death. Interestingly, histidine derivatives examined in this study showed no toxic effect, as well as the addition of membrane-permeable iron chelators to the preservation solution prevented ROS formation (102,103). These findings encouraged to modify the original HTK solution and develop a novel HTK-N solution (104). In this improved solution histidine was partially replaced by its derivate N-acetyl-histidine and chloride was substituted with lactobionate and aspartate. In addition, arginine, glycine and alanine were supplemented. Changes in composition urged to evaluate HTK-N superiority over its original precursor, therefore many studies have been conducted aimed to answer this question. More than 20 animal studies have already been published analyzing HTK-N effect in heart, liver, lung, kidney and small bowel transplantation setting (105–110). The majority of the conducted studies revealed HTK-N superiority over HTK solution and reported significantly lower apoptotic index, reduced oxidative stress, preserved better liver and heart microcirculation and prolonged organ survival. The capabilities of HTK and HTK-N solutions were also analyzed in the rat uterus cold preservation model (111). The authors concluded that samples preserved in HTK-N solution presented with less tissue edema, lower myeloperoxidase (MPO) infiltration and higher superoxide dismutase (SOD) activity. There is still no consensus on the best preservation solution for uterus cold preservation, however, solutions that are suitable for the kidney can be used for the uterus too (72). The majority of the research teams use the HTK solution, while others utilize UW or Euro-Collins solution (45,46,48,55). Higher viscosity solutions are not recommended for uterus preservation, because it may be challenging to flush the uterus graft due to high resistance in the uterine vessels (72).

### **Ischemia-reperfusion injury**

IRI is one of the most important events affecting graft survival during solid organ transplantation. As all grafts are subjected to ischemia, a complex process of ischemia-reperfusion is inevitable after organ implantation to the recipient (112). This process is described as the paradoxical deterioration of cell dysfunction after the blood flow is restored to

hypoxia affected tissues (113). As the definition suggests organ injury during IRI occurs in two phases. First, ischemia is caused by a cessation of blood flow to the organ, which experiences cellular damage caused by hypoxia and lack of nutrients during organ storage. The second phase is a reperfusion, which further aggravates the cell injury through ROS production, calcium overload and mitochondrial dysfunction.

The initial trigger of ischemia is the cessation of blood flow to the organ, which further results in the deficiency of ATP, glucose and other substances necessary for cell metabolism (114). Depletion of ATP molecules in mitochondria switches cell metabolism to anaerobic and impairs the functioning of Na<sup>+</sup>/K<sup>+</sup> ion pumps causing increased N<sup>+</sup>, Ca<sup>2+</sup> and H<sup>+</sup> concentrations in cells (115,116). An increased concentration of intracellular ions and higher osmolarity causes a surge of water resulting in cell swelling (117). In addition, intracellular calcium overload promotes the translation of xanthine dehydrogenase to xanthine oxidase, which is crucial in the formation of ROS (118). If reperfusion does not occur, calcium overload also leads to the deterioration of vital cellular enzymes such as phospholipases, lysozymes, and proteases and further results in cell death (117). Once the oxygen supply is restored oxygen catalyzes the degradation of hypoxanthine to uric acid and the liberation of a superoxide anion, which is finally converted to the hydroxyl radical. An increase in hydroxyl radical production leads to an extensive peroxidation of all cellular lipid structures and ultimately causes the disruption of membrane permeability and cell death (113). Moreover, upon reperfusion blood flow normalizes extracellular pH by washing out accumulated H<sup>+</sup> ions from the intravascular space and creates osmotic imbalance resulting in water influx which finally causes permanent membrane damage (119). It has been established that cell death during IRI happens via apoptosis and necrosis, however, necrosis contributes to a bigger infarct size than apoptosis (120,121). Parallel abovementioned pathophysiological processes happen in all organs undergoing ischemia-reperfusion.

Research in organ transplantation is rapidly evolving, aiming to elucidate the optimal preservation solution for particular organs. One of the key methods is to use various substances helping to alleviate tissue injury during transplantation.

## Relaxin

Relaxin (RLX) is a peptide hormone, primarily discovered by the reproductive endocrinologist Dr. Frederick Hisaw and his colleagues in 1926 (122). Hisaw demonstrated that the injection of serum from a pregnant guinea pig or rabbit to a virgin guinea pig causes the relaxation of a

pubic ligament within 8 hours. After it was successfully isolated from pig corpus luteum and placenta, this hormone was named after “Relaxin” (123). In subsequent experiments, new data regarding RLX structure and its biological action accumulated.

RLX belongs to the relaxin peptide family, which comprises seven peptides that are structurally similar to insulin (RLX 1,2,3 and INSL 3, 4, 5, 6) (124). All peptides contain A- and B-chains linked by two disulfide bonds and an additional disulfide bond in chain A. The most prevalent isoform in humans is RLX-2 which is equivalent to RLX-1 in other mammalian species and is usually referred to as RLX (125). Some authors refer to recombinant human relaxin – 2 as Serelaxin (126–129). RLX binds to a specific G-protein coupled relaxin-like family peptide receptor – 1 (RXFP1), which can be found in various human organs and their systems such as reproductive organs, brain, kidney, heart, lung, bone marrow, skin (124,130). The function of RLX slightly differs between organs.

RLX effect on the reproductive system is very important, especially for pregnancy and parturition (130). It is essential for the remodeling and expansion of the vagina, cervix and uterus during pregnancy (131). Moreover, RLX seems to stimulate myometrium cells to increase their elastase activity which is an indicator of tissue remodeling and growth (132). Moreover, long-term RLX infusion to women stimulates endometrial growth which is mediated by activating vascular endothelial growth factor and its ability to promote neoangiogenesis (133).

RLX has shown its effect to cause cervical changes during pregnancy. Increased levels of RLX provoke higher levels of matrix metalloproteinases in cervical cells and therefore cause the degradation of collagen which leads to a softened cervix (134). In addition, animal studies also revealed that RLX increased pig and rat cervix weight twice by inhibiting stromal cell apoptosis and increasing their proliferation (51,135,136). RLX concentration in women also correlates with cervical length and diameter till mid-pregnancy when RLX concentration is at its peak (137).

Studies indicate that RLX has an effect on other organ systems as it is produced by various cell types and has autocrine and paracrine actions (131). As mentioned before, RLX is involved in tissue remodeling by regulating cervical stroma cells collagen turnover. Interestingly, RLX production in various tissues is necessary for regular collagen turnover as RLX knock-out mice presented with severe fibrosis in the heart, lungs or kidney (138–140). Furthermore, RLX is considered to be partly responsible for cardiovascular adaptations during pregnancy such as increased blood volume, cardiac output and decreased systemic vascular resistance (130). Its acting mechanism is mediated via the induction of nitric oxide synthase which produces a

vasodilator nitric oxide (NO) causing a decrease in systemic vascular resistance (141). RLX effect is also apparent in the setting of human acute heart failure by inhibiting two main mediators of vasoconstriction - angiotensin II and endothelin, thus reducing systemic vascular resistance and improving blood flow in kidneys (142). These findings of RLX action are important and may be beneficial in clinical practice.

Due to the abovementioned effects and RLX ability to inhibit fibrosis and apoptosis in animal and human tissues, this hormone was also introduced in organ transplantation research (143). To date, there are only preclinical animal studies aiming to examine RLX potency to reduce ischemia reperfusion injury and thus expand the organ donor pool or to improve graft condition (125,126,144–147) (Table 4). These promising results in already published papers validate RLX abilities in various solid organs, however, it was never tested on the uterus.

**Table 4.** RLX effects on various animal models

Author	Organ	Animal model	Effect of RLX
Kageyama et al. (126,144)	Liver	Mouse	<ol style="list-style-type: none"> <li>1. Enhanced overall survival.</li> <li>2. Reduced cell apoptosis.</li> <li>3. Reduced sinusoidal congestion, swelling/vacuolization.</li> <li>4. Works by binding to GR receptors present in hepatocytes.</li> <li>5. Stimulates the signaling pathway of Notch 1.</li> </ol>
Boehnert et al. (148–150)	Liver	Rat	<ol style="list-style-type: none"> <li>1. Reduced reperfusion injury indicated by decreased levels of MDA and MPO.</li> </ol>
Yoshida et al. (146)	Kidney	Rat	<ol style="list-style-type: none"> <li>1. Enhanced kidney function.</li> <li>2. Reduced TNF-alpha increase after ischemia-reperfusion (IR).</li> <li>3. Improved tissue appearance after IR.</li> <li>4. Decreased apoptosis.</li> <li>5. Decreased expression of TNFR1 mRNA in the kidney</li> </ol>

Collino et al. (125)	Kidney	Rat	<ol style="list-style-type: none"> <li>1. Elevated clearance of creatinine.</li> <li>2. Reduced injury to renal cells following ischemia-reperfusion (IR).</li> <li>3. Prevented the decrease in MnSOD and CuZnSOD caused by IR.</li> <li>4. Lowered the activity of MPO.</li> <li>5. Decreased expression of ICAM-1.</li> <li>6. Prevented I/R induced increase of IL-1b, IL-18, and TNF-alpha</li> </ol>
Bausys et al. (151)	Kidney	Pig	<ol style="list-style-type: none"> <li>1. Reduced Caspase-3 and myeloperoxidase positive cell counts.</li> <li>2. Upregulated SOD and NFkB genes, downregulated MLKL and RIPK1 expression.</li> </ol>
Alexiou et al. (147,152)	Lung	Rat	<ol style="list-style-type: none"> <li>1. Decreased biochemical and structural indicators of pulmonary injury.</li> <li>2. Lowered activity of NE and MPO.</li> <li>3. Reduced production of MDA.</li> <li>4. Reduced production of ET-1 by endothelial cells.</li> <li>5. Provides protection against IR-induced lung injury through early and moderate induction of iNOS, which depends on balanced stimulation of ERK-1/2 and PI3K.</li> </ol>
Valle Raleigh et al. (129)	Heart	Mouse	<ol style="list-style-type: none"> <li>1. Enhanced survival following ischemia-reperfusion injury (IRI).</li> <li>2. Decreased the area of myocardial infarction.</li> <li>3. Maintained left ventricle fractional shortening and end-systolic diameter.</li> </ol>

			4. Reduced caspase-1 activity in the heart following IRI.
Perna et al. (153)	Heart	Pig	<ol style="list-style-type: none"> <li>1. Lowered levels of MPO and MDA, decreased tissue calcium overload.</li> <li>2. Reduced apoptosis in cardiomyocytes.</li> <li>3. Decreased ultrastructural cardiomyocyte damage.</li> <li>4. Improved heart contractability.</li> </ol>
Masini et al. (154–156)	Heart	Rat	<ol style="list-style-type: none"> <li>1. Lowered levels of MPO and MDA, decreased tissue calcium overload.</li> </ol>

UW—University of Wisconsin; SOD—superoxide dismutase; GR—glucocorticoid receptor; ET-1 endothelin 1, MDA—malondialdehyde; MPO—myeloperoxidase; NE—neutrophil elastase; IRI—ischemia-reperfusion injury, iNOS – inducible nitric oxide synthase.

## Erythropoietin

Erythropoietin (EPO) is a glycoprotein hemopoietic hormone that is synthesized in response to hypoxic conditions and is the main cytokine responsible for erythropoiesis (157). It was first isolated in 1977 from the urine of patients having aplastic anemia (158). During fetal development, liver is the initial site of EPO production, after birth EPO is synthesized in kidney interstitial cells (159).

EPO is essential for erythropoiesis – it regulates the survival of progenitor cells, stimulates the proliferation and maturation of normoblasts to mature erythrocytes (160,161). The development of recombinant human EPO in 1989 was a striking achievement for the treatment of chronic kidney disease as it significantly improved patients' quality of life (162,163).

The effect of EPO is realized via the EPO receptor (EPO-R), which belongs to the cytokine receptor family (164). EPO receptors are expressed not only in erythroid cells but also in the cardiovascular system, brain, endocrine, digestive, respiratory, male and female reproductive systems (161). In recent years, studies revealed that EPO is produced in tissues exposed to hypoxia, inflammation or trauma and can even have a direct effect on immune cells (165). EPO protective effect against IRI has already been demonstrated in kidney, liver, brain, heart, lung and small intestine in animal models (166–172) (Table 5.). Recent studies suggest that the EPO effect is mediated by its antiapoptotic, anti-inflammatory and antioxidative properties

(173). Favorable results of EPO usage in IRI and its well tolerance propose its introduction into further organ transplantation research.

**Table 5.** Protective effects of EPO in various organs

Author	Organ	Animal model	Effect of EPO
Imamura et al. (166)	Kidney	Rat	<ol style="list-style-type: none"> <li>1. Induced hypoxia inducible factor – 1alpha</li> <li>2. Induced vascular endothelial growth factor messenger RNA.</li> <li>3. Reduced apoptotic cell number compared to controls.</li> </ol>
Guneli et al. (167)	Intestine	Rat	<ol style="list-style-type: none"> <li>1. Decreased number of positive cells.</li> <li>2. Improved histological tissue appearance.</li> <li>3. Increased catalase activity.</li> <li>4. Decreased myeloperoxidase activity and malondialdehyde concentration.</li> </ol>
Yilmaz et al. (168)	Liver	Rat	<ol style="list-style-type: none"> <li>1. Preserved liver tissue morphology.</li> <li>2. Reduced blood serum alanine aminotransferase, tumor necrosis factor, interleukin-2 levels.</li> <li>3. Reduced liver tissue malondialdehyde.</li> </ol>
Lipšic et al. (169)	Heart	Rat	<ol style="list-style-type: none"> <li>1. Reduced infarct area in a heart, accompanied by better left ventricle function.</li> <li>2. Reduced tissue apoptosis.</li> </ol>
Wu et al. (171)	Lung	Rat	<ol style="list-style-type: none"> <li>1. Attenuated lung edema and neutrophil infiltration.</li> <li>2. Decreased lung microvascular permeability.</li> <li>3. Ameliorated pulmonary oxygenation function.</li> </ol>

Kumral et al. (172)	Brain	Mouse	<ol style="list-style-type: none"><li>1. Reduced apoptosis in brain</li><li>2. Attenuated lipid peroxidation.</li><li>3. Preserved antioxidant levels.</li></ol>
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## Hypothesis and aims

We hypothesized that RLX and EPO or the combination of these two substances could alleviate uterine tissue injury caused by SCS and IRI.

The study was divided into two parts:

- Part A – Static cold storage
- Part B – Ischemia-reperfusion injury

The specific aims of this study were:

Part A:

1. To analyze RLX and/or EPO effects on uterine tissue morphological changes by calculating the histological score.
2. To evaluate apoptotic cell count in uterine tissue.
3. To calculate tissue oxidative stress marker SOD activity and lipid peroxidation marker malondialdehyde (MDA) concentrations in uterine tissue.

Part B:

1. To analyze RLX and/or EPO effect on uterine tissue morphological score.
2. To measure tissue biochemical markers involved in oxidative stress (SOD activity) and lipid peroxidation (MDA concentration).
3. To calculate MPO-positive cells in uterine tissue.
4. To calculate apoptotic cell number.
5. To analyze the expression of various genes related to oxidative stress and apoptosis. Also, to investigate if the RLX effect could be mediated via the Notch1 signaling pathway.

## DISCUSSION

Some results were discussed in a similar manner to my first author publications (174,175).

Within my dissertation, I aimed to investigate the effects of RLX and EPO during the UTx scenario, which consisted of experiments focusing on uterus SCS and experiments emphasizing on IRI.

UTx is the only treatment method for women suffering from AUI, which explains its relevance in clinical research worldwide. One of the most important events during UTx is the organ preservation procedure determining organ condition and its further survival. RLX and EPO are two independent substances helping to alleviate tissue injury during SCS or ischemia-reperfusion in various solid organ transplantation models. However, RLX and EPO were never meticulously investigated as preservatives in the uterus.

From the SCS part of the study we determined that the use of RLX as an additive in the preservation solution attenuated uterine damage. This was demonstrated by preserved uterine tissue histology score, tissue SOD activity, reduced apoptotic cell count and tissue MDA levels. Unfortunately, the use of EPO did not result in any favorable effects. In the next part, representing IRI, we showed that pretreatment with RLX maintained normal tissue histology, decreased the levels of MDA, MPO and TUNEL-positive cells, sustained SOD activity, and increased the expression of NICD and HES1 genes in comparison to the control group. Additionally, pretreatment with EPO was not that effective and only reduced the levels of MDA.

Uterus preservation during SCS was the first part of my research during which we implemented uterine tissue storage in HTK-N solution without or with RLX/EPO. Uterus SCS is an unavoidable step during UTx, aiming to reduce metabolic requirements under hypothermic conditions. Slowed-down metabolism delays the onset of anaerobic respiration, ATP depletion and slows further cascade resulting in irreversible tissue damage.

### Uterine tolerance to various CI times

Uterine tolerance to different CI times was examined in several animal studies. However, there were only a few studies examining separate SCS methods and not performing a full UTx procedure or sampling before implantation. El-Akouri et al. demonstrated that the uterus is a robust organ and cold ischemia up to 24 hours in either UW or 0.9% NaCl solution did not affect tissue morphological or microscopical appearance (16). In contrast, Zitkute et al.

demonstrated that histological changes, such as tissue edema, signs of necrosis, as well as higher MPO expression and lower SOD activity may be detected in uterine tissue even after 8 hours of preservation in Custodiol solution (111). In comparison, these changes were significantly less evident in uterine tissue samples kept in Custodiol-N solution. Morphological and biochemical changes were even more pronounced in samples after 24 hours of preservation. Tricard et al. performed autologous UTx in ewes and concluded that prolonged cold ischemia up to 24 hours in Celsior solution had no effect on tissue apoptosis and tissue contractility (176). In our study, we used Custodiol-N solution as a control and supplemented it with RLX or EPO. We did not observe any significant differences regarding total histological score, apoptosis and biochemical markers between all groups after 8 hours of preservation. The impact of prolonged ischemia up to 48 hours was also investigated by a Swedish professor Brännström followed by 48 hours of *ex vivo* normothermic reperfusion (177). He concluded that biopsies taken after 48 hours of CI prior to reperfusion indicated no morphological changes. However, reperfusion injury significantly affected uterine tissue causing severe edema, degraded glandular structures, which were more prominent in specimens reperfused after 48 hours of CI. Priore et al. studied the possibility to retrieve the human uterus from a multi-organ, brain-dead, heart-beating donor (155). He demonstrated that the human uterus could withstand CI for up to 12 hours without exhibiting any tissue alterations. This discovery was significant since the uterus, as not a vital organ is frequently removed last during organ procurement procedures and hence experiences considerably prolonged ischemia times.

## Preservation solutions and additives to increase uterus tolerance to CI

As there is no consensus regarding the best preservation solution for uterus storage during UTx, different research groups worldwide use distinct solutions or aim to add supplements possibly helping to alleviate tissue damage. The most intensely studied solutions for uterus preservation are Perfadex, IGF1, HTK (Custodiol), Celsior, and University of Wisconsin solution (176,59,178–180,50,181,37,177). Barun et al. supplemented the HTK solution with prostacyclin analog Iloprost and investigated its effect after 4 and 24 hours of cold preservation in a rat model (182). The authors concluded that supplementation with Iloprost preserved tissue histopathologic appearances after 24 hours of CI when compared to samples kept in a plain HTK solution. Moreover, two oxidative stress markers (nitric oxide (NO) and MDA) were used to assess the level of tissue damage caused by SCS. No significant differences were observed between the groups in terms of NO and MDA concentrations. Ugurlu et al. conducted a similar study and supplemented the HTK solution with acetyl L-carnitine which was used for uterus preservation (183). Results of this study indicated that acetyl L-carnitine preserved

normal histology of all three uterine layers and caused significantly lower vascular endothelial growth factor receptor – 2 immunoreactivity in the myometrium, luminal and glandular epithelium when compared to samples preserved for 24 hours in a plain HTK solution. However, tissue histology was evaluated by only describing the sample's appearance without using a definite scoring system, therefore statistical analysis to compare morphological changes between the groups was not possible.

## Preservation solution supplementation with RLX

Neither RLX nor EPO were investigated as supplements to the preservative solutions used in the UTX setting. There are only a few studies examining the effect of RLX on cell damage in liver or kidney preservation models (145,151). Boehnert et al. used a standard solution similar to the UW solution and supplemented it with 64 ng/ml of RLX (145). The animal number in this study was too small to draw reliable conclusions. However, the authors reported a tendency of RLX to reduce tissue MDA and MPO levels. Bausys et al. performed pig kidney transplantation and used RLX as an additive to Custodiol solution for kidney perfusion and SCS (151). In this study, RLX reduced the number of MPO- and Caspase-3-positive cells number. Moreover, gene expression analysis revealed that RLX upregulated SOD2 and nuclear factor kappa B - 1 (NFKB1) expression while downregulating receptor-interacting serine/threonine-protein kinase – 1 and mixed lineage kinase domain like pseudokinase.

## Preservation solution supplementation with EPO

There is also a limited number of studies aiming to reduce tissue damage by supplementing preservation solution with EPO. Eipel et al. conducted a study and kept mice either steatotic or lean livers in HTK solution supplemented with EPO at 4°C temperature for 24 hours. Results revealed that EPO significantly reduced transaminase release in steatotic livers. Moreover, EPO prevented steatotic liver denudation of endothelial lining, but worsen it in lean livers. These findings suggested implementing EPO for the preservation of steatotic livers to increase success after transplantation. Watson et al. analyzed EPO as a cardioprotective agent when supplemented to Celsior solution for the hypothermic IRI model (184). Isolated rat hearts were kept in a Celsior solution for either 6 or 10 hours in hypothermic conditions, following 45 minutes of reperfusion and determination of myocardial functional recovery. Results revealed that the application of EPO in a dose-dependent manner significantly improved myocardial function evaluated after 6 hours of preservation. When preservation time was extended to 10

hours, EPO favorable effect was only visible when EPO was used in a combination with other cardioprotective substances zonisipride and glyceryl trinitrate.

Our study results are in line with findings reported by Boehnert et al. and similar to those reported by Bausys et al. In our study, we observed a significant decrease in tissue MDA and apoptotic cell number after supplementing HTK-N solution with RLX while the effect of EPO was not significant. However, it showed a tendency to reduce tissue apoptotic cell number, MDA and to preserve SOD activity.

## Pathophysiological IRI effect on uterine tissue

The phenomenon of IRI and its impact on uterine tissue was the second part of my thesis. IRI is a complex mechanism that is unavoidable in every solid organ transplantation. The beginning of this process is a cessation of blood flow to the organ, which causes the depletion of the energy-carrying molecule ATP and switches aerobic metabolism to anaerobic (114). The deficiency of ATP impairs the normal functioning of ion pumps and leads to increasing calcium, sodium and hydrogen ion concentrations intracellularly. Higher concentrations of these ions in the cells cause an influx of water resulting in cell swelling (117). Calcium overload is one of the most important events which further generates ROS by promoting the formation of xanthine oxidase (118). Moreover, prolonged calcium overload accelerates cell death by degrading various cellular enzymes such as nucleases, lysozymes, phospholipases and proteases contributing to faster necrosis and apoptosis (117). Cell damage during reperfusion is even more severe as restoration of oxygen catalyzes the formation of hydroxyl radicals. This ROS molecule initiates lipid peroxidation and disrupts cellular membrane permeability leading to cell death. Additionally, ROS are highly active with all major biological molecules and have deleterious effects. Although mitochondria are one of the main producers of all ROS, these organelles are very susceptible to lipid peroxidation as chemical reactions occur on membranes. Once the membranes are disrupted, the production of ATP is decreased.

Oxidative stress occurs due to an imbalance between the production of ROS and tissue antioxidant capacities typically happening in the tissues affected by ischemia-reperfusion injury (185). It is considered to be one of the major mechanisms affecting the outcome of organ transplantation (186). Antioxidants are substances that inhibit oxidation and therefore neutralize ROS (187). They play an important role in the human body by protecting tissues against oxidative stress. One of the most important antioxidants in the human body is glutathione (GSH) which forms a cellular redox buffer and is crucial for protecting cell integrity.

Aslan et al. measured GSH, SOD and MDA concentrations after a combined uterus and ovaries IRI and demonstrated that GSH and SOD concentrations were significantly lower after 90 min of reperfusion (188). These findings proved that classical markers can also be used in the uterus IRI model when evaluating the extent of oxidative stress.

## Substances applied to alleviate uterine tissue damage during IRI

Various chemical substances are being investigated in uterine tissue which could alleviate cell damage caused by IRI. Some of these tested substances have other purposes in the human body such as immunoregulation or pain management, however, studies indicate that they can also be helpful in tissue preservation. Sahin et al. investigated the effect of the immunosuppressant drug Tacrolimus on uterine IRI consisting of 30 minutes of ischemia and 60 minutes of reperfusion (189). The same dose of tacrolimus was administered intravenously either 30 minutes before ischemia or at the time of reperfusion. The authors revealed that Tacrolimus inhibited lipid peroxidation and increased tissue antioxidant capacities. Moreover, post-ischemic administration of tacrolimus showed a significantly better effect when compared to pre-ischemic use. Atalay et al. in 2015 concluded a similar study examining the protective effect of a short-acting opioid analgesic remifentanil which previously showed its potential to suppress the inflammatory response in a septic mice model and to reduce liver IRI (190). In this model, rats were continuously infused with remifentanil during 2-hour of ischemia followed by 1 hour of reperfusion. Authors concluded that remifentanil reduced tissue degeneration, infiltration with leukocytes and lipid peroxidation, as well as preserved the antioxidant capacities of SOD and catalase. Another immunosuppressive agent mycophenolate mofetil (MMF) was under investigation by a Turkish team led by Sahin Ersoy (191). Rats were given MMF by gavage 5 days prior to the experiment. By clamping the aorta and both ovarian arteries rat uteruses were subjected to 30 minutes of ischemia and 4 hours of reperfusion. Pretreatment with MMF showed a favorable effect as samples in the MMF group presented with significantly less cellular and tissue damage, a lower apoptosis rate when compared to the control group. Moreover, the use of MMF significantly reduced uterine tissue MDA, MPO, 8-hydroxy-2'-deoxyguanosine and serum ischemia modified albumin levels, as well as preserved SOD activity. The authors concluded that the MMF effect was mainly fulfilled by its anti-oxidative and anti-inflammatory action. This study also stood out from others due to a relatively long reperfusion period, which lasted 4 hours. In 2017 Aslan et al. performed a study in the uterus and ovary IRI model and investigated the protective effect of kisspeptin and oxytocin, which are both reproductive hormones acting as potential antioxidants (188). Experimental agents were administered 30 minutes prior to 1.5-hour-long ischemia and same length reperfusion

periods. Results indicated that injection of oxytocin, kisspeptin or a combination of these two substances significantly preserved ovarian and uterine histological score, had an anti-oxidant effect based on decreased MDA levels, increased SOD activity and GSH level when compared to a control group. Another hormone melatonin was also investigated along with the amino-acid glycine in a rat uterus IRI model (192). The substances were supplemented to the animals via gavage for 5 days and 2 hours before ischemia on the experimental day. Ischemia and reperfusion lasted for 1 hour each followed by uterus procurement for biochemical, immunohistochemical and histological evaluation. The total histological score was significantly lower after pretreatment of animals with melatonin, glycine or a combination of these two substances when compared to a control group. Furthermore, a combination of melatonin and glycine significantly reduced tissue edema. Melatonin used alone only showed a tendency to reduce tissue edema and the number of inflammatory cells. Glycine tended to reduce edema and perimeter thickening, however, these results lacked significance. Moreover, melatonin, glycine or a combination of these two agents reduced tissue MPO expression and preserved SOD activity when compared to a control group.

In recent years, natural substances were also investigated in the uterus IRI model in order to reduce tissue damage. Crocetin is a carotenoid naturally found in saffron and *gardenia jasminoides* fruits (193). In a study done by Peng et al. animals were pretreated with ascending doses of crocetin for 5 days and subjected to 1 hour of ischemia and 3 hours of reperfusion. Results revealed that the administration of crocetin effectively reduces inflammation, apoptosis and oxidative stress when compared to a control group. In addition, these results manifested in a dose-dependent manner. The outcomes improved with increasing doses applied. Moreover, authors suggest that the effect of crocetin was probably mediated by the nuclear factor erythroid 2-related factor-2, and heme oxygenase-1 pathway. Resveratrol is another natural compound found in the skin of grapes, peanuts, berries and recently is being prescribed as a dietary supplement (194). In various studies it demonstrated the ability either to neutralize ROS or to upregulate genes related to cellular defense systems (195). A study examining the resveratrol effect on uterine IRI demonstrated favorable results (196). The experimental agent significantly reduced MDA concentration, tissue MPO activity and increased SOD and catalase activity when compared to a control group. Concentrations of the abovementioned parameters were comparable to those in the sham-operated group. By measuring various cytokines, the authors concluded that the effect of resveratrol was achieved by suppressing pro-inflammatory cytokines (interleukin – 6, tumor necrosis factor –  $\alpha$ ) and increasing levels of anti-inflammatory cytokines (interleukin – 10 (IL-10), interleukin – 37). Another important finding of this study was that resveratrol activates the PI3K-AKT signaling pathway which inhibits oxidative stress (197).

In our study, we used EPO, which was previously investigated in a uterus IRI model (198). In a study published by Tsompos et al. four animal groups were subjected to 45 minutes of ischemia and either to 60 or 120 minutes of reperfusion. Two groups were used as control, and another two groups received intravenous EPO injections at the start of reperfusion. The EPO dose of 10 mg per kilogram of body weight described in this study is technically almost unachievable and raises some concerns as volume per animal exceeds the recommended amount even using EPO at the highest concentration available from the supplier mentioned in that paper. However, the authors did not reply to our concerns with an explanation of how they applied such a high dose of EPO. Moreover, uterine tissue changes were evaluated superficially, as the authors referred to histological parameters used for ovarian IRI, not including important tissue areas such as endometrium or endometrial glands. As the authors only examined tissue histology and found no favorable effect of EPO, we decided to modify the protocol. We chose to investigate tissue biochemical markers, tissue infiltration with MPO, apoptosis and expression of various genes.

Our results from the rat IRI model suggest that EPO tended to reduce uterine tissue histological score. However, due to the wide confidence interval it did not reach statistical significance. When used alone EPO reduced tissue infiltration with MPO, which proves its anti-inflammatory effect. Similar findings were reported in a study done by Karaca et al. in a rat ovary IRI model (199). Administration of EPO 30 minutes before ischemia reduced tissue MPO activity when compared to control.

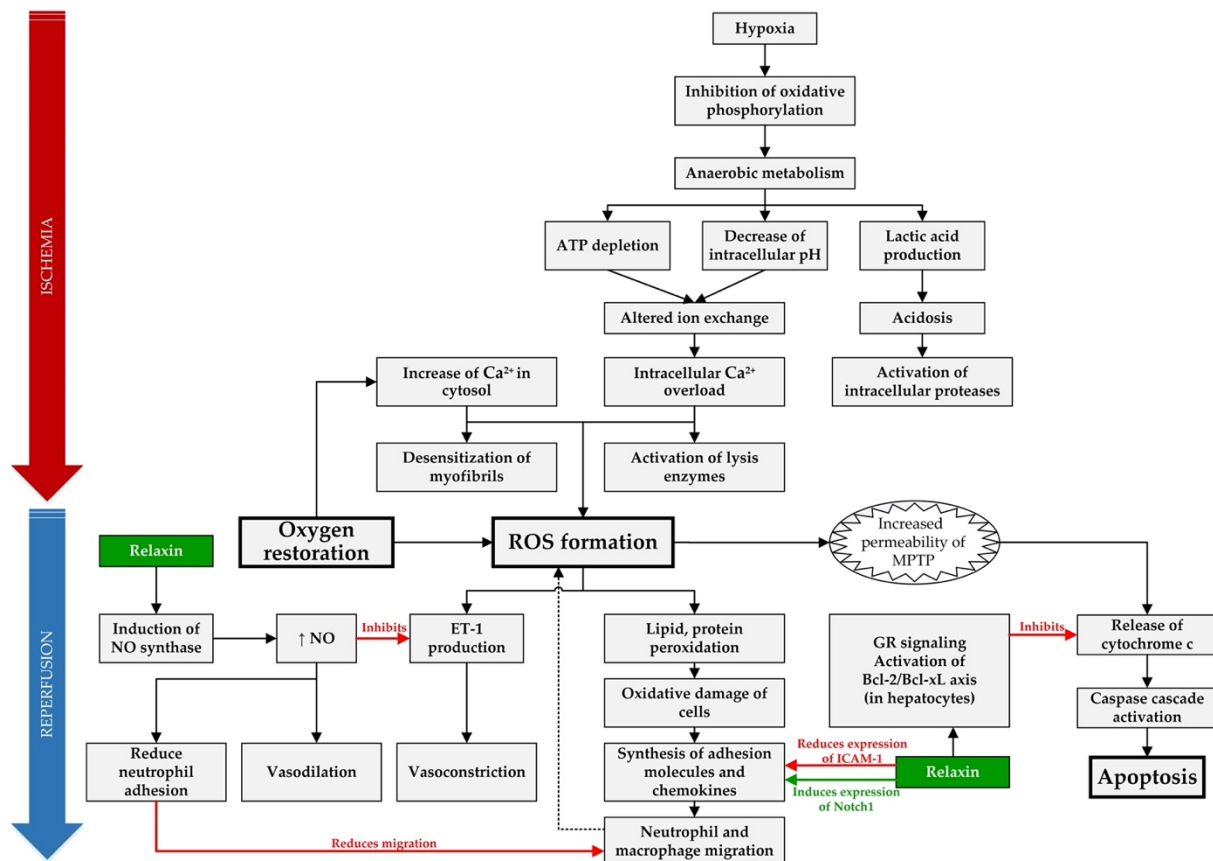
## RLX protective effect in solid organ IRI setting

This is the first time when RLX was applied as a protective agent in a rat uterus IRI model. Results suggest that RLX has anti-oxidative, anti-inflammatory and anti-apoptotic properties and thus can significantly preserve the uterus. Comparable effects were already described in different organ IRI models (143). Yoshida et al. demonstrated that RLX can also significantly alleviate IRI-induced histological damage and reduce apoptotic cell count (200). In another kidney IRI model, Collino et al. analyzed the RLX effect after 60 minutes of ischemia and 6 hours of reperfusion (201). The authors concluded that administration of RLX reduced tissue MPO activity, preserved IRI-induced SOD activity and attenuated renal cell damage. Alexiou et al. performed a few studies examining the RLX effect in a rat lung IRI model (152,202). Lung samples after 60 minutes of ischemia and the same length of reperfusion presented with significantly lower biochemical and morphological markers as well as lower MDA production and reduced activity of neutrophil elastase when compared to controls (202). Furthermore, in

subsequent experiments, Alexiou et al. extended ischemia and reperfusion times for up to 90 minutes each and analyzed a possible acting mechanism of RLX (152). There are several studies in a heart IRI model aiming to evaluate RLX properties in heart tissue (129,153,154). Despite differences between already published study designs, the RLX effect is undeniable. Administration of RLX reduced myocardial infarct size and improved heart contractile function (129,153). Analysis of biochemical markers showed similar results as reported in other organs – reduced tissue MPO activity, MDA concentration, lower apoptosis rate and Caspase-3 activity in cardiomyocytes. RLX efficacy in IRI animal models leads the way to clinical studies. RELAX-AHF was a phase III randomized, placebo-controlled clinical trial which aimed to investigate if patients with acute heart failure administering Serelaxin (SER, a recombinant human relaxin-2) would have greater dyspnoea relief when compared to standard of care or placebo (127). A cohort with 1161 patients showed that SER treatment for acute heart failure was related to the improvement of patients' symptoms and significantly reduced cardiovascular mortality through day 180. However, the subsequent clinical trial of RELAX-AHF-2 did not confirm the SER effect, as it was not able to reduce the risk of death from cardiovascular causes compared to the placebo (128).

## Acting mechanism of RLX

The effect of RLX in IRI models was mediated via several pathways compiled in our previously published review (Figure 1) (143). First of all, RLX reduces the expression of intracellular adhesion molecule 1 (ICAM-1) which serves as an endothelial ligand for neutrophil receptors and activates specific kinases resulting in increased cytokine and ROS production during oxidative stress (201,203). A few studies also suggest that in oxidative stress RLX acts via macrophages by inducing the Notch1 signaling pathway which is associated with cell fate, differentiation, proliferation and regeneration (126,204,205). Collino et al. reported that the renoprotective effect of RLX is related to the up-regulation of iNOS and activation of eNOS, both having an important role in the NO pathway (201). Increased formation of NO during IRI is advantageous, as NO causes local vasodilation, inhibits leucocyte and platelet adhesion and stimulates angiogenesis (206). Moreover, RLX realizes its anti-apoptotic effect via binding to glucocorticoid receptors (GR) and inhibiting cytochrome c release from mitochondria (144).



**Figure 1.** RLX acting pathways during ischemia-reperfusion. Reprinted from: Jakubauskiene L, Jakubauskas M, Leber B, Strupas K, Stiegler P, Schemmer P. Relaxin Positively Influences Ischemia—Reperfusion Injury in Solid Organ Transplantation: A Comprehensive Review.

## EPO and RLX mediated gene expression

Within my thesis, we aimed to analyze gene panels associated with inflammation, apoptosis or genes, which could potentially explain RLX or EPO acting mechanism. First of all, results from our analysis revealed that the eNOS gene was upregulated upon administering the combination of EPO and RLX. Previously published data suggest, that both of the tested substances can upregulate this gene, however, in our study, this result was only achieved by using a combination of EPO and RLX. The main role of NO is the regulation of vascular tone (207). Physiological response to NO varies from therapeutic to toxic (208). During IRI, NO reacts with superoxide and produces peroxynitrite which causes vascular dysfunction mainly by decoupling eNOS and ceasing NO production in vascular endothelium (208,209). Interestingly, an additional supply of NO and thiols can deactivate peroxynitrite and restore impaired regulation of vascular tone (210). Our findings are in line with reports by Bani et al.,

that RLX upregulates eNOS in the myometrium, endometrial stroma cells, glands and surface epithelium and does not affect iNOS expression in mouse uterus (211).

Hypoxia-inducible factor – 1 alpha (HIF1a) and NFkB1 were hypoxia associated genes that we included in our analysis based on results in IRI models (212,151). HIF1a is upregulated in ischemic conditions and initiates downstream expression of genes involved in cell survival, angiogenesis, and oxygen-independent ATP production (213). Few studies report that RLX activates ERK-1/2 pathways which further targets NFkB1 and stimulates the expression of iNOS (152). In our study, we could not confirm these findings as none of the abovementioned genes were significantly changed in ischemic conditions. On the other hand, Bausys et al. perfused porcine kidneys with Custodiol solution supplemented with RLX and demonstrated that RLX can upregulate NFkB1 and therefore alleviate subsequent kidney injury (151,214).

B-cell lymphoma protein 2 associated X (BAX) and B-cell lymphoma protein – 2 (BCL-2) are two cytoplasmic proteins known for their ability to inhibit or promote apoptosis, respectively (215). Kagayama et al. presented a murine liver transplantation model with a prolonged cold storage period for up to 18 hours in which RLX alleviated IRI and improved survival rate by increasing expression of hepatocyte GR and BAX/BCL-2 (144). RLX action via GR was proven only in hepatocytes, however, GR are also expressed in the uterus, therefore RLX action could hypothetically be mediated through them (216). Shawky et al. demonstrated that EPO preconditioning (24 hours or 30 minutes before liver ischemia) significantly increased the expression of the anti-apoptotic gene BAX and preserved hepatic structure when compared to controls (217). Unfortunately, our BAX, BCL-2 gene analysis revealed no significant changes in gene expression.

Monocyte chemoattractant protein - 1 (MCP1) is a chemokine that is upregulated during IRI and promotes the recruitment of monocytes/macrophages to ischemia-reperfusion affected sites (218,219). Macrophages produce chemokines which are responsible for leukocyte migration to inflamed tissues (220). Chemokine (C-X-C motif) ligand 2 (CXCL-2) expression is increased early during reperfusion, when neutrophil accumulation is not yet detectable, proposing that the primary neutrophil recruitment to the ischemic site is driven by CXCL2 (221). Kageyama et al. demonstrated that RLX suppresses MCP1 and CXCL2 expression in macrophage cultures as well as in stressed transplanted livers, suggesting that RLX mediates hepatoprotection via the regulation of macrophages (126). This RLX-induced tissue protection was not observed in the uterus IRI model in our study, as both of the abovementioned genes did not differ from the controls.

IL-10 is an anti-inflammatory cytokine that is produced by various types of cells and plays an important role in limiting host immune response (222). Studies in IRI models suggest that it alleviates tissue damage by inhibiting apoptosis and promoting the production of pro-inflammatory cytokines (223). Gene analysis in our study revealed that pretreatment with a combination of EPO and RLX significantly increased the expression of IL10 in uterine tissue when compared to the control group, interestingly both substances when administered alone did not yield any result. Kageyama et al. in the already mentioned study demonstrated that IL-10 was also significantly upregulated in a murine liver transplantation model after RLX was administered during reperfusion (126).

RLX effect through the Notch1 signaling pathway has not been fully studied, however, research in liver transplantation models provides the first evidence that RLX action is possibly mediated via Notch intracellular domain (NICD) and hairy and enhancer of split 1 (HES1) target genes (126). Notch1 is a transmembrane receptor that is involved in the cell-fate specification, differentiation, communication between adjacent cells and apoptosis (205). Studies indicate that RLX may act via Notch1 signaling in human umbilical vein endothelial cells, cardiac fibroblast, cardiomyocytes and mice liver transplants (126,204,224,225). After ligand binding to Notch1, NICD is released and translocates towards the nucleus (226). NICD inhibits NFkB1 and therefore suppresses tissue inflammation (227). In addition, activation of the HES1 pathway leads to blockage of caspase-3, which is responsible for apoptosis. Kageyama et al. reported that RLX attenuates IRI in mouse liver transplantation by inducing Notch1 and increasing the expression of NICD and HES1 (126). We observed similar patterns in our study's gene analysis. Pretreatment with RLX alone or in combination with EPO significantly upregulated the expression of Notch1 downstream genes, suggesting that the RLX effect may be mediated via Notch1 signaling in uterine tissue.

## Strengths and weaknesses

To our knowledge, this was the first experimental study analyzing RLX and EPO protective effects both in uterus SCS and IRI models. UTx is a complex procedure during which the uterus is subjected to warm and cold ischemia periods, thus covering both ischemia stages is crucial in further improving graft quality.

On the other hand, this study has a few limitations, which have to be addressed. First of all, this is an animal study, which results were not further confirmed in other species. Although we covered both cold and warm ischemia periods, this model still lacks representation of

conditions occurring during full organ transplantation. In addition, during experiments, we did not synchronize rats according to their diestrus cycle, which could have an impact on the outcome. However, to overcome this issue we randomly divided rats into experimental groups which we believe resulted in similar study groups. A similar approach was chosen in other rat uterus IRI studies (188,191,192).

## Conclusions

In conclusion, our study documents the efficacy of RLX in uterine tissue SCS and IRI models. The protective effect of RLX was mediated via anti-apoptotic, anti-inflammatory and lipid-peroxidation limiting properties. Primary data in gene expression suggest RLX acting mechanism via the Notch1 signaling pathway. Additionally, our research shows EPO anti-inflammatory effect in the uterus IRI model. The combination of substances did not yield any additional benefit except for the upregulation of anti-inflammatory genes. These findings should be fully elucidated in the upcoming studies before introducing experimental substances to clinical practice.

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Review

# Relaxin Positively Influences Ischemia—Reperfusion Injury in Solid Organ Transplantation: A Comprehensive Review

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**Abstract:** In recent decades, solid organ transplantation (SOT) has increased the survival and quality of life for patients with end-stage organ failure by providing a potentially long-term treatment option. Although the availability of organs for transplantation has increased throughout the years, the demand greatly outweighs the supply. One possible solution for this problem is to extend the potential donor pool by using extended criteria donors. However, organs from such donors are more prone to ischemia reperfusion injury (IRI) resulting in higher rates of delayed graft function, acute and chronic graft rejection and worse overall SOT outcomes. This can be overcome by further investigating donor preconditioning strategies, graft perfusion and storage and by finding novel therapeutic agents that could reduce IRI. Relaxin (RLX) is a peptide hormone with antifibrotic, antioxidant, anti-inflammatory and cytoprotective properties. The main research until now focused on heart failure; however, several preclinical studies showed its potentials for reducing IRI in SOT. The aim of this comprehensive review is to overview currently available literature on the possible role of RLX in reducing IRI and its positive impact on SOT.

**Keywords:** liver transplantation; kidney transplantation; heart transplantation; lung transplantation; relaxin; serelaxin; review

## 1. Introduction

In recent decades, solid organ transplantation (SOT) has increased the survival and quality of life for patients with end-stage organ failure by providing a potentially long-term treatment option [1–3]. Nowadays, SOT is not only performed as a lifesaving procedure but also purely for quality of life enhancement measures as the examples of uterus, penis, face or limb transplantation show [4–7]. This evolution of SOT is possible due to technical and pharmacological advances and standardization of procedures. Although the availability of organs for transplantation has increased throughout the years, the demand greatly outweighs the supply. Kidney, liver, heart and lung remain the most demanded organs, which account for more than 98% of all SOT. According to the Organ Procurement and Transplantation Network data, in 2018, in the United States, there were 36,529 SOTs. Unfortunately, this number is inadequate as the waiting list currently has around 113,000 of possible recipients. The same year over 6000 patients died waiting for a transplant. In 2018, in the Eurotransplant member states, 7350 SOTs were performed; however, there were 1896 patients who died or became unfit while waiting for SOT. Several authors suggest organ shortage to be the biggest challenge in transplant medicine [8,9]. One possible solution for this problem is to extend the potential donor pool by using

extended criteria donors (ECD) [10]. As these donors are older and have more comorbidities, organs are more prone to ischemia reperfusion injury (IRI) resulting in higher rates of delayed graft function, acute and chronic graft rejection and worse overall SOT outcomes [11–15]. There is a huge scientific effort to find ways in reducing IRI in order to increase the number of suitable organs procured from ECD. This can be achieved by further investigating donor preconditioning strategies, graft perfusion and storage and by finding novel therapeutic agents that could reduce IRI [16–20]. Relaxin (RLX) is a peptide hormone, which was first isolated from the ovaries and named by Dr. Frederick Hisaw in 1926 after experiments with guinea pigs showed RLX ability to relax the pubic ligament [21]. RLX belongs to the insulin family of structurally related molecules. The majority of circulating RLX in primates is encoded by the RLN2 gene and has a high affinity to cognate receptor relaxin family peptide receptor 1 (RXFP1) [22,23]. These receptors are scattered throughout the body and are found not only in the reproductive organs but also in kidney, lung, heart, liver, brain, arteries and blood cells [24]. Although for a long time RLX was considered strictly as a pregnancy hormone, several researchers demonstrated its antifibrotic, antioxidant, anti-inflammatory and cytoprotective actions and the role in hemodynamic changes [25–28]. These newly discovered effects prompted to investigate RLX as a pharmacotherapeutic agent in preclinical and even clinical settings. The main research until now focused on the heart failure; however, several preclinical studies showed its potential for reducing IRI in SOT (Table 1).

The aim of this comprehensive review is to overview currently available literature on the possible role of RLX in reducing IRI and its positive impact on SOT.

## 2. Materials and Methods

The literature search was performed in PubMed, Web of Science, EMBASE and [ClinicalTrials.gov](https://www.clinicaltrials.gov) online databases. The following combination of Medical Subject Headings (MeSH) and keywords with the employment of “AND” or “OR” Boolean operators were used:

“Organ Transplantation” OR “Transplantation” OR “Organ transplant” OR “liver transplantation” OR “uterus transplantation” OR “kidney transplantation” OR “heart transplantation” OR “lung transplantation” OR “pancreas transplantation” OR “Organ Preservation Solutions” OR “Perfusion” OR “Perfusion machine” OR “Reperfusion Injury” OR “Ischemia Reperfusion Injury” OR “Cold Storage” OR “Cold ischemia” OR “hypoxia-reoxygenation” OR “reperfusion” OR “ischemia” OR “ischaemia” OR “ischemic reperfusion” OR “ischaemic reperfusion” AND “Relaxin” OR “Serelaxin” OR “Recombinant human relaxin”.

Database specific search strategies are provided as a Supplementary Materials. The search was restricted to English language only without a time limitation. Most recent search was performed on 20 October 2019.

At least two researchers reviewed the abstracts for the inclusion. After relevant abstracts were identified, full-text articles were retrieved and re-reviewed. Reference lists from selected studies were examined, and relevant articles were also included. A flowchart of the literature search according to the PRISMA guideline is provided as a Supplementary Materials [29].

**Table 1.** Relaxin application in preclinical trials.

Author	Organ	Experimental Animal	Intervention	RLX Application	Effect of RLX
Kageyama et al. [30,31]	Liver	Mice	Liver transplantation after 18 h of cold storage in UW solution without RLX.	rhRLX dose of 5 µg/kg intravenously at the onset of reperfusion.	<ol style="list-style-type: none"> <li>1. Improved overall survival.</li> <li>2. Decreased number of apoptotic cells.</li> <li>3. Attenuated sinusoidal congestion, edema/vacuolization, and hepatocellular necrosis.</li> <li>4. Acts through hepatocyte GR receptors.</li> <li>5. Induces Notch 1 signaling pathway.</li> </ol>
Boehnert et al. [32,33]	Liver	Rats	Isolated healthy liver perfusion model after 5 h of cold or warm ischemia in HTK solution with or without RLX.	64 ng/mL of rhRLX was used in the preservation solution.	<ol style="list-style-type: none"> <li>1. Protective effect against reperfusion injury indicated by reduced levels of MDA and MPO.</li> </ol>
Boehnert et al. [34]	Liver	Rats	Isolated healthy liver perfusion model after 3.5 h of cold or warm ischemia in UW solution with or without RLX.	32 ng/mL of rhRLX was used in the reperfusion solution and 64 ng/mL of RLX in the preservation solution.	<ol style="list-style-type: none"> <li>1. Protective effect against reperfusion injury indicated by reduced levels of MDA and MPO.</li> </ol>
Yoshida et al. [35]	Kidney	Rats	Kidney IRI model with 45 m of ischemia and 24 h of reperfusion.	500 ng/h of porcine RLX 2 h before onset of reperfusion using an osmotic minipump under the skin.	<ol style="list-style-type: none"> <li>1. Improved renal function.</li> <li>2. Attenuated post-IR increase in TNF-<math>\alpha</math>.</li> <li>3. Ameliorated histological damage after IR.</li> <li>4. Reduced the number of apoptotic cells.</li> <li>5. Reduced the expression of TNFR1 mRNA in the kidney.</li> </ol>
Collino et al. [36]	Kidney	Rats	Kidney IRI model with 60 m of ischemia and 6 h of reperfusion.	5 µg/kg of rhRLX intravenously at the onset of reperfusion and again 3 h after reperfusion.	<ol style="list-style-type: none"> <li>1. Increased creatinine clearance.</li> <li>2. Attenuated renal cell damage after IR.</li> <li>3. Abolished IR-induced reduction in MnSOD and CuZnSOD.</li> <li>4. Reduced the activity of MPO.</li> <li>5. Reduced expression of ICAM-1.</li> <li>6. Prevented I/R-induced rise in IL-1<math>\beta</math>, IL-18 and TNF-<math>\alpha</math>, levels.</li> </ol>

Table 1. Cont.

Author	Organ	Experimental Animal	Intervention	RLX Application	Effect of RLX
Alexiou et al. [37]	Lung	Rats	Isolated healthy lung perfusion model using Krebs–Henseleit solution. Lungs exposed to 60 m of ischemia and 60 m of reperfusion.	5 nmol/L of rhRLX in the perfusion solution.	<ol style="list-style-type: none"> <li>1. Reduced biochemical and morphological markers of pulmonary injury.</li> <li>2. Reduced the activity of NE and MPO.</li> <li>3. Decreased the production of MDA.</li> <li>4. Inhibited production of ET-1 from endothelial cells.</li> </ol>
Alexiou et al. [38]	Lung	Rats	Isolated healthy lung perfusion model using Krebs–Henseleit solution. Lungs exposed to 90 m of ischemia and 90 m of reperfusion.	5 nmol/L of rhRLX in the perfusion solution.	<ol style="list-style-type: none"> <li>1. Protective effect on IR-induced lung injury via early and moderate iNOS induction, dependent on balanced ERK-1/2 and PI3K stimulation.</li> </ol>
Valle Raleigh et al. [39]	Heart	Mice (C57BL and eNOS knockout).	Heart IRI model with 30 m of ischemia and 24 h of reperfusion.	10 µg/kg of rhRLX subcutaneously 60 m before IRI or 5 m before the onset of reperfusion.	<ol style="list-style-type: none"> <li>1. Improved survival after IRI.</li> <li>2. Reduced myocardial infarct size.</li> <li>3. Preserved left ventricle fractional shortening and end-systolic diameter.</li> <li>4. Attenuated caspase-1 activity in the heart after IRI.</li> </ol>
Perna et al. [40]	Heart	Pigs	Heart IRI model with 30 m of ischemia and 3 h of reperfusion.	1.25; 2.5; or 5 µg/kg of rhRLX given upon the onset of reperfusion by a continuous infusion through the right atrial catheter.	<ol style="list-style-type: none"> <li>1. 5 µg/kg of RLX afforded the best myocardial protection.</li> <li>2. Reduced MPO and MDA, tissue calcium overload.</li> <li>3. Reduced apoptosis in cardiomyocytes.</li> <li>4. Decreased ultrastructural signs of cardiomyocyte damage.</li> <li>5. Improved the contractile performance of the heart.</li> </ol>
Masini et al. [41]	Heart	Rats	Heart IRI model with 30 m of ischemia and 60 m of reperfusion	100 ng of porcine RLX dissolved in 500 µL of saline injected intravenously 30 m before ischemia.	<ol style="list-style-type: none"> <li>1. Reduced MPO and MDA, tissue calcium overload in the left ventricle.</li> </ol>

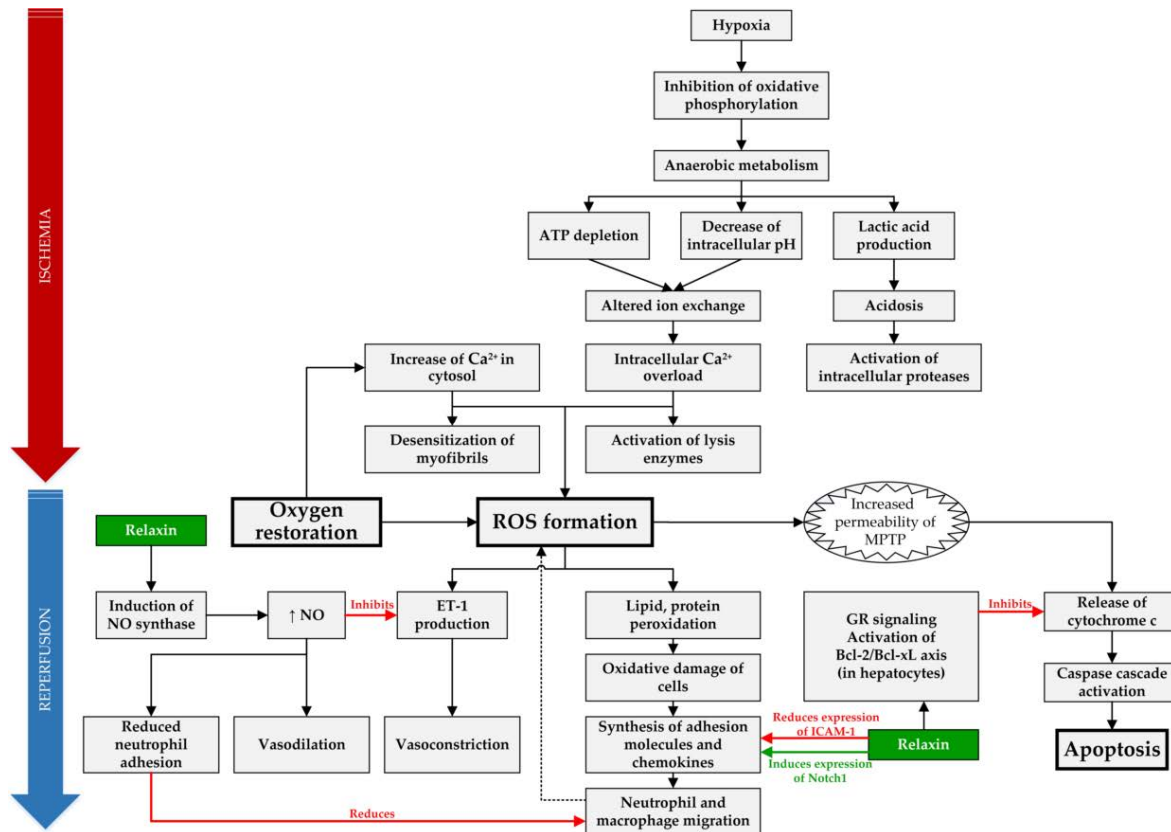
Table 1. Cont.

Author	Organ	Experimental Animal	Intervention	RLX Application	Effect of RLX
Masini et al. [42]	Heart	Guinea pigs	Isolated healthy heart perfusion model using a modified Tyrode solution supplemented by a IRI model with 20 m of ischemia and 20 m of reperfusion.	30 ng/mL of porcine RLX in the perfusion solution	<ol style="list-style-type: none"> <li>1. Reduced ischemia-reperfusion induced increase of MDA.</li> <li>2. Reduced the calcium content.</li> <li>3. Retained unchanged histamine amounts compared to control.</li> <li>4. Reduced the light transmittance across mast cells showing decreased content of secretory granules.</li> <li>5. Reduced histological changes caused by IRI.</li> <li>6. Retained the heart contractility unchanged as compared to basal mean value.</li> </ol>
Masini et al. [43]	Heart	Guinea pigs	Isolated healthy heart perfusion model using a modified Tyrode solution supplemented by a IRI model with 20 m of ischemia and 20 m of reperfusion. IRI model with 30 m of ischemia and 60 m of reperfusion was used.	30 ng/mL of porcine RLX in the perfusion solution In the second IRI model 100 ng of porcine RLX injected intravenously 30 m before ischemia.	<ol style="list-style-type: none"> <li>1. Increased the amount of NO<sub>2</sub> in the perfusates.</li> <li>2. Increased coronary flow through stimulation of NO production.</li> <li>3. Reduced leukocytes infiltration and lipid peroxidation.</li> <li>4. Decreased IR-induced release of histamine and LDH.</li> </ol>

rhRLX—recombinant human relaxin; UW—University of Wisconsin; SOD—superoxide dismutase; GR—glucocorticoid receptor; MDA—malondialdehyde; MPO—myeloperoxidase; NE—neutrophil elastase; IRI—ischemia-reperfusion injury.

### 3. Comprehensive Review

A total of 14 articles examined the possible role of RLX in SOT and are summarized in Table 1, and the molecular mechanisms, which can be targeted and positively influenced by RLX during IRI, are compiled in Figure 1.



**Figure 1.** Overview of targets of RLX in IRI. In IRI setting, RLX has anti-inflammatory effect by reducing expression of intracellular adhesion molecule 1 (ICAM-1), inducing expression of Notch1 in macrophages and by reducing neutrophil adhesion through increased synthesis of nitric oxide (NO) [30,36]. Furthermore, it acts as an anti-apoptotic agent in hepatocytes by binding to the glucocorticoid receptors (GR) and inhibiting release of cytochrome c from mitochondria [31]. Lastly, RLX causes vasodilation through increased NO production, which acts as a vasodilator and reduces vasoconstriction by inhibiting endothelin 1 (ET-1) production [38]. (ROS—reactive oxygen species, MPTP—mitochondrial permeability transition pore).

#### 3.1. Liver Transplantation (LTx)

Numbers of patients waiting for LTx are increasing continuously resulting in a constant enlargement of the gap between organ need and organ availability. Therefore, new techniques to expand the donor pool are coming to clinical practice. Among others, living donor LTx, splitting grafts, the use of machine perfusion or donors who are older or after cardiac death aim to increase the available donor organs [44]. As the transplantations from ECD is rising, and it is known that these organs are more prone to IRI, research in reducing IRI is mandatory [11]. A total of 5 publications analyzing RLX potency in liver were identified. 3 publications by Boehnert et al. evaluated the role of RLX in a perfused rat liver model [32–34]. In the first experimental model, University of Wisconsin (UW) solution was used for preservation and the reperfusion was performed with a hemoglobin free solution, additional doses of 32 ng/mL and 64 ng/mL of RLX were added, respectively. Liver ischemia was induced for 3.5 h applying either 20 °C (warm) or 4 °C (cold) preservation conditions [34]. In the second experimental model, liver ischemia was induced for 5 h, and instead of UW, the HTK solution

was used with 64 ng/mL of RLX in the preservation solution [32,33]. The authors concluded that RLX had a protective effect against IRI, which was indicated by reduced levels of malondialdehyde (MDA) and myeloperoxidase (MPO) and by the increased scattered light intensity (indicates increased oxygen supply).

The only SOT model examining the role of RLX in the liver was an orthotopic LTx model in mice [30,31]. The liver was stored in 4 °C UW solution for 18 h before implantation, and 5 µg/kg RLX was injected intravenously at the time of reperfusion. RLX significantly improved overall survival rate, decreased the number of apoptotic cells, attenuated sinusoidal congestion, edema/vacuolization and hepatocellular necrosis. However, the main finding of this study was that RLX acts not through its primary cognate receptor RXFP1, but through hepatocyte glucocorticoid receptors. Moreover, Kageyama et al. conducted an additional study using the same protocol and discovered that RLX in liver acts by inducing the macrophage Notch 1 signaling pathway, which is involved in cell fate decision, proliferation, differentiation and regeneration [30,45].

Overall, RLX shows promising results in LTx models, therefore it may be a promising substance to be tested in clinical LTx studies.

### 3.2. Kidney Transplantation (KTx)

According to Global Observatory on Donation and Transplantation (GODT) data, a total of 75,664 KTx were performed worldwide in 2018, retaining it the most transplantable solid organ. Although KTx from living donors are performed quite often, the majority of kidneys are transplanted from deceased donors, which are known to be more prone to IRI [46]. It has been shown that IRI in kidneys is one of the major causes of delayed graft function, chronic graft dysfunction and graft rejection [47].

There are no experimental KTx models using RLX as a protective agent; however, two publications analyzing the role of RLX in reducing kidney IRI in rats [35,36]. In a study by Yoshida et al. an osmotic minipump was used to administer 500 ng/h of RLX for 24 h starting 2 h before ischemia. Another experiment was described in the same publication in which the RLX was infused via the same osmotic minipumps just at the time of reperfusion. The authors found that RLX improves renal function after reperfusion; it also ameliorates the histological damage to the kidney and has an anti-apoptotic effect. Interestingly, staining of RXFP1 showed that damaged cells did not express these receptors, suggesting that pretreatment with RLX should be more effective. However, this idea contradicts the result, which showed increased kidney function when RLX was administered at the time of reperfusion compared to the pretreatment group. Another study by Collino et al. used a similar IR model in rats. Rats were subjected to 1 h of ischemia followed by 6 h of reperfusion. In the experimental group, RLX was administered at the dose of 5 µg/kg intravenously at the beginning of reperfusion and again 3 h after reperfusion. The findings were identical to the previously mentioned study and showed that RLX improved renal function after IR and also ameliorated the histologically verified damage to the kidney. Additionally, RLX reduced local leukocyte recruitment and oxidative stress. None of the above mentioned studies investigated the exact signaling mechanisms of RLX in renal cells; however, they provide data suggesting the involvement of the nitric oxide pathway [35,36].

Although there are some methodological differences between these two studies concerning RLX administration time or duration of reperfusion, both studies concluded that RLX may have beneficial effect in reducing kidney IRI.

### 3.3. Lung Transplantation (LuTx)

First successful LuTx was performed by James Hardy in 1963 [48]. Since then, more than 69,000 procedures have been performed worldwide with the median survival reaching 6.7 years [49]. Although the survival almost doubled in the last 30 years, efforts are necessary to further improve outcome as the rate of primary graft dysfunction after LuTx can reach 30% [50]. This is mostly due to

the IRI endured during transplantation [51]. Some authors agree that methods to reduce IRI are deeply needed to improve both short- and long-term outcomes after LuTx [52].

Currently, there are no studies investigating the effect of RLX in experimental LuTx models. Overall, there are only two publications about the potential role of RLX in protecting lungs from IRI by Alexiou et al. [37,38]. In the first study, an isolated lung perfusion model was described using Krebs–Henseleit solution containing 5 nmol/L of RLX. Lungs were exposed to 60 m of ischemia and 60 m of reperfusion [37]. The results of this study showed marked reduction of biochemical and morphological markers of pulmonary injury and reduced activity of neutrophil elastase (NE) and MPO. In addition, the production of MDA also was decreased, as well as production of endothelin-1 (ET-1) from endothelial cells was inhibited by adding RLX to the perfusate. The second study was similar, except the ischemia and reperfusion times were prolonged to 90 m [38]. This study was mainly aimed to determine pathways through which RLX acts in the lungs. By adding various inhibitors to the perfusate, authors discovered RLX protective effect via early and moderate inducible nitric oxide synthase (iNOS) induction, dependent on balanced extracellular signal-regulated kinase-1/2 (ERK-1/2) and phosphatidylinositol-3 kinase (PI3K) stimulation. This experiment also showed that RLX does not affect IRI in lungs if it is applied only during ischemia or only during the reperfusion cycles. Although there is evidence that RLX can reduce IRI in an isolated rat lung model, there are no studies using RLX in an experimental LuTx model. Further investigation is needed in order to better understand the true effect of RLX in LuTx.

#### 3.4. Heart Transplantation (HTx)

World's first HTx was performed by Christiaan Barnard in 1967. Although the results after the first transplantations were poor, the development of modern immunosuppression therapy led to the median survival of around 15 years nowadays [53]. HTx remains crucial in medicine as it is the gold standard treatment in end-stage heart failure [54]. However, the demand of donors exceeds the available supply, requiring new strategies to overcome organ shortage [55]. Extension of the donor pool can be achieved by using ECD organs including organs with prolonged ischemia time or donation after circulatory death. Such grafts are more susceptible to IRI which translates to higher primary graft dysfunction and worse overall outcome after SOT [56]. Novel therapeutic agents are in great demand to further push the development of HTx.

The effect of RLX on the heart is widely studied showing that RLX is increasing coronary flow or remodeling post-infarction cardiac tissue in acute or chronic heart failure [57]. Although in a recent randomized clinical trial RLX showed no beneficial effect when treating acute heart failure, Dschietzig argues that there is a need to further investigate its effects as the trial had flaws [58,59]. In our review, we only included studies that would investigate the benefit of RLX in HTx but no studies that would directly examine the effect of RLX in HTx could be identified. However 5 experimental animal studies that showed the beneficial RLX effect in reducing IRI in heart [39–43] were identified.

Valle Raleigh et al. conducted a study in wild-type and endothelial nitric oxide synthase (eNOS) knockout mice model using RLX as possible agent reducing IRI after myocardial infarction [39]. Mice were pretreated with RLX (10 µg/kg) subcutaneously 1 h before ischemia or 5 m before reperfusion. The heart ischemia was induced for 30 m by occlusion of the left descending coronary artery, followed by reperfusion for 24 h. The results of this study showed that in both RLX treated groups, the overall survival rate was improved compared to controls. In addition, RLX also reduced myocardial infarct size, preserved left ventricle fractional shortening and end-systolic diameter. Administered at reperfusion, RLX reduced activity of caspase-1 in the heart of wild-type mice. These results were not observed in eNOS knockout mice, confirming a previously reported finding that RLX attenuates IR-induced heart injury via NO-dependent pathways. Interestingly, the administration time of RLX did not influence the effect, as the results in both pretreatment and reperfusion groups did not differ. Another study was performed by Perna et al. using a swine model in which myocardial infarction was induced by ligating the left anterior descending coronary artery and causing ischemia for 30 m with a followed reperfusion

of 3 h [40]. In this study, RLX was administered at three different doses of 1.25, 2.5, and 5 µg/kg as a continuous infusion through the right atrial catheter for 20 m, starting at the time of reperfusion. The positive RLX effect was dose dependent, and the dose of 5 µg/kg showed the best myocardial protection. The administration of RLX lowered IRI markers, such as MPO and MDA levels, and the tissue calcium overload. RLX reduced apoptosis in cardiomyocytes and decreased the ultrastructural signs of cardiomyocyte damage. In the 5 µg/kg of RLX group almost no ultrastructural changes after IRI were observed.

Most of the articles emphasize that RLX acts by inducing NO synthase, and interestingly, Masini et al. prove that NO synthase can have dual roles in the myocardium [41]. In their study, RLX or a selective inhibitor of inducible NO synthase were used 30 m before ischemia in an experimental rat model. Unexpectedly, the results revealed that IRI can be reduced not only by increasing NO with the help of RLX but also by reduction applying a selective NO synthase inhibitor. It seems that the protection against IRI can be achieved by controlling the amount of NO in the tissue.

Masini et al. conducted another study in a perfused guinea pig heart model in which ischemia and reperfusion lasted for 20 m each, and RLX was added to the perfusate at the dose of 30 ng/mL [42]. RLX showed its effect by reducing MDA, calcium and histamine content compared to controls. Moreover, it reduced histological changes caused by IRI and the light transmittance across mast cells showing decreased content of secretory granules. One of the most important findings of this study was that RLX retained unchanged heart contractility function as compared to baseline values. In their other similar study, it was shown that RLX reduces myocardial IRI by stimulating the NO production and increasing coronary flow [43].

Overall, RLX in all studies showed a protective effect in non-transplant IRI animal models. There are concerns how these studies would be reproduced with human subjects, as the results from a recent randomized clinical trial examining the effect of RLX in acute heart failure were disappointing [58].

### 3.5. Possible Limitations of the Studies

Several possible limitations arise from these studies. Firstly, only one study examines the role of RLX in a transplantation setting, while all the others analyze the effect of RLX in reducing IRI in a non-transplant setting, such as isolated organ models or cold organ storage. Secondly, all included studies investigate RLX effect only on healthy organs. Up to now, no studies on extended criteria donor (ECD) organs, comprising among others organs after prolonged ischemia, from donors after circulatory death (DCD) and organs affected by hepatic steatosis were performed. As Lee et al. showed, RLX could be beneficial in attenuating hepatic steatosis and fibrosis in mice with non-alcoholic fatty liver disease [60]. Theoretically, this finding could be used for improving ECD organ quality prior to transplantation; however, this still needs to be elucidated and experimental and clinical studies are needed to prove the concept. Lastly, there are some methodological differences between the studies, which should also be taken into account. For instance, RLX was administered at different concentrations and routes; the ischemia and reperfusion times varied. Surprisingly, despite these differences, all included studies showed promising results.

## 4. Conclusions

Although RLX in a SOT setting is not widely studied, our review of currently available literature demonstrates a positive RLX effect in this field. Results from the studies show that it attenuates histological damage, inhibits apoptosis and reduces biochemical markers associated with IRI. Most importantly, RLX preserves the overall function of the organ and improves the overall survival. All these findings support a rationale to conduct first clinical studies to apply RLX as a potential substance in order to maintain organ integrity and ameliorate transplantation success after IRI.

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## Abbreviations

SOT	Solid organ transplantation
IRI	Ischemia reperfusion injury
RLX	Relaxin
ECD	Extended criteria donors
RXFP1	Relaxin family peptide receptor 1
MeSH	Medical Subject Headings
rhRLX	Recombinant human relaxin
UW	University of Wisconsin
SOD	Superoxide dismutase
GR	Glucocorticoid receptor
MDA	Malondialdehyde
MPO	Myeloperoxidase
NE	Neutrophil elastase
GODT	Global Observatory on Donation and Transplantation
LTx	Liver transplantation
KTx	Kidney transplantation
HTx	Heart transplantation
LuTx	Lung transplantation
ERK1/2	Extracellular signal-regulated kinase-1/2
PI3K	Phosphatidylinositol-3 kinase
iNOS	Inducible nitric oxide synthase
eNOS	Endothelial nitric oxide synthase
ROS	Reactive oxygen species
MPTP	Mitochondrial permeability transition pore
ET-1	Endothelin-1

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## Article

# Relaxin and Erythropoietin Significantly Reduce Uterine Tissue Damage during Experimental Ischemia–Reperfusion Injury

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**Abstract:** Successful uterus transplantation, a potential treatment method for women suffering from absolute uterine infertility, is negatively affected by ischemia–reperfusion injury (IRI). The aim of this study is to investigate the protective effect of relaxin (RLX) or/and erythropoietin (EPO) on experimental uterus IRI. Eighty rats, randomly assigned into eight groups ( $n = 10/\text{group}$ ), were pretreated with either saline, 5  $\mu\text{g}/\text{kg}$  human relaxin-2, 4000 IU/kg recombinant human erythropoietin or their combination. Ischemia was achieved by clamping the aorta and ovarian arteries for 60 min, following 120 min of reperfusion and tissue sampling. For sham animals, clamping was omitted during surgery. There were no differences in tissue histological score, malondialdehyde (MDA) and superoxide dismutase (SOD) levels, myeloperoxidase (MPO) and TUNEL-positive cell count between all sham-operated rats. Pretreatment with RLX preserved normal tissue morphology, reduced MDA levels, MPO and TUNEL-positive cell count, preserved SOD activity and upregulated NICD and HES1 gene expression when compared to the control group. Pretreatment with EPO reduced MDA levels. In conclusion, pretreatment with RLX, EPO or a combination of both EPO and RLX significantly alleviates uterine tissue damage caused by IRI.

**Keywords:** transplantation; uterus; ischemia–reperfusion injury; relaxin; erythropoietin



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## 1. Introduction

Approximately 1.5 million women worldwide suffer from absolute uterine factor infertility and therefore have no chance to be both genetic and gestational mothers to their children [1]. Uterus transplantation (UTx) nowadays is considered to be the only treatment method for women who do not have a uterus due to congenital (uterus agenesis in Mayer–Rokitansky–Küster–Hauser syndrome) or acquired (hysterectomies due to large myomas, peripartum bleeding or sepsis and malignancies) causes [2,3]. Moreover, ethical, legal and technical issues of UTx are being discussed to implement this method for transgender individuals [4].

The first successful human UTx was performed in 2014 by a Swedish team led by Brännström [2]. Since then, the research interest in UTx has been rapidly growing as an increased number of surgical teams have already realized more than 40 successful UTx, including only a few utilizing uterine grafts from deceased donors, worldwide [5–7]. Organ procurement from a living donor enables the extensive evaluation of potential grafts before transplantation, including function, anatomy and the presence of vascular abnormalities [8]. On the other hand, living donors have a high risk of surgery-related complications, such as

urinary tract and bowel injuries, bleeding and vaginal cuff dehiscence [1,2,9]. Considering the potential risks for living donors, research on the use of brain-dead donor organs is of growing interest. During organ donation, the uterus is retrieved last and therefore experiences longer warm ischemia times, resulting in reduced survival of the transplanted uterus [10].

Ischemia–reperfusion injury (IRI) plays a significant role during solid organ transplantation and can cause severe tissue alterations, leading to graft dysfunction and rejection [10]. The effect of IRI is mediated by several mechanisms, including overproduction of reactive oxygen species (ROS), neutrophil infiltration, endothelial dysfunction resulting in apoptosis and necrosis of cells in transplanted tissue [11]. Knowledge of the basic mechanisms underlying IRI allows the introduction of pharmacological adjuvants that could increase transplanted tissue tolerance to IRI [12].

Relaxin (RLX) is an insulin-related peptide hormone that has already demonstrated its antifibrotic, anti-inflammatory, antioxidative and cytoprotective effects during solid organ transplantation [13]. However, it has never been applied as a protective agent against IRI in a uterus model.

Erythropoietin (EPO) is an endogenous hormone that is already being used in clinical practice for treating anemia. Studies of hepatic and renal IRI models revealed that EPO can significantly alleviate IRI by reducing lipid peroxidation and serum tumor necrosis factor- $\alpha$  levels, increasing superoxide dismutase (SOD) activity and preserving tissue histology [14,15].

The aim of this study is to investigate the protective effect of RLX or/and EPO pretreatment on uterine tissue IRI in a rat model.

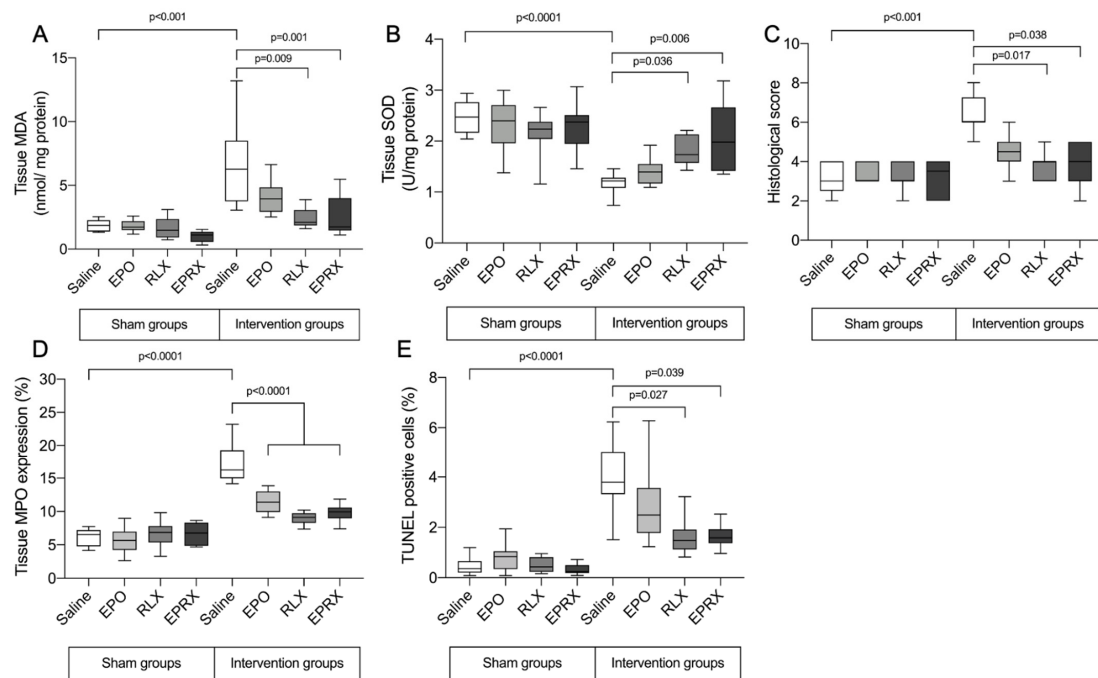
## 2. Results

### 2.1. Tissue Biochemical Analysis

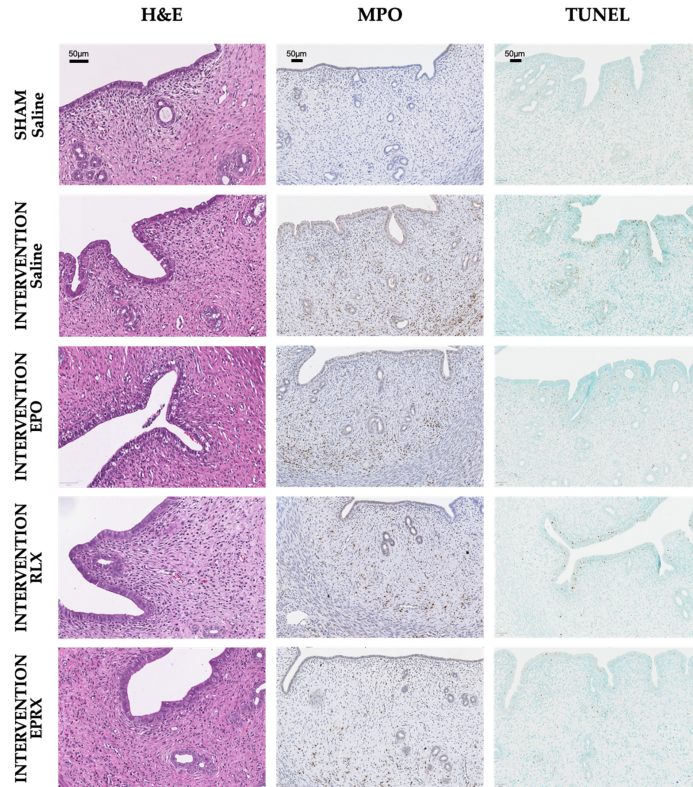
Malondialdehyde (MDA) and SOD concentrations were similar in all sham-operated groups (Figure 1). Uterine IRI led to significantly higher tissue MDA levels 2 h after reperfusion between the groups. The increase in tissue MDA levels was attenuated in groups pretreated with RLX and RLX with EPO compared to the saline-pretreated group. Additionally, SOD activity was significantly decreased in the saline-pretreated group (Figure 1). However, SOD activity remained higher in groups pretreated with RLX or RLX in combination with EPO compared to the control group. Pretreatment with EPO alone showed only a tendency to return MDA and SOD activity to normal.

### 2.2. Morphology, Neutrophil Infiltration and TUNEL Assay

Histological and immunohistochemical analysis revealed no significant differences between all sham-operated rats (Figures 1 and 2). Samples of sham-operated rats presented with normal uterine tissue morphology—normally organized endometrial glands, only a mild infiltration with polymorphonuclear cells, with no vasoconstriction or hemorrhage. Contrarily, signs of severe uterine tissue architecture disruption were observed in all intervention groups; the highest histological score was evident in the intervention group pretreated with saline (6.0 (IQR 6.0; 7.3)). Pretreatment with RLX or RLX with EPO attenuated tissue damage, as samples in these groups presented with significantly lower histological scores (6.0 (IQR 6.0; 7.3) vs. 4.0 (IQR 3.0; 4.0) and 6.0 (IQR 6.0; 7.3) vs. 4.0 (IQR 3.0; 5.0),  $p = 0.017$  and  $p = 0.038$ , respectively).



**Figure 1.** Uterine tissue lesion markers. MDA—malondialdehyde, SOD—superoxide dismutase, MPO—myeloperoxidase, TUNEL—terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling EPO—erythropoietin, RLX—relaxin, EPRX—erythropoietin and relaxin. Data presented as median (Q1; Q3). (A–C,E) Kruskal–Wallis test; (D) ANOVA test.



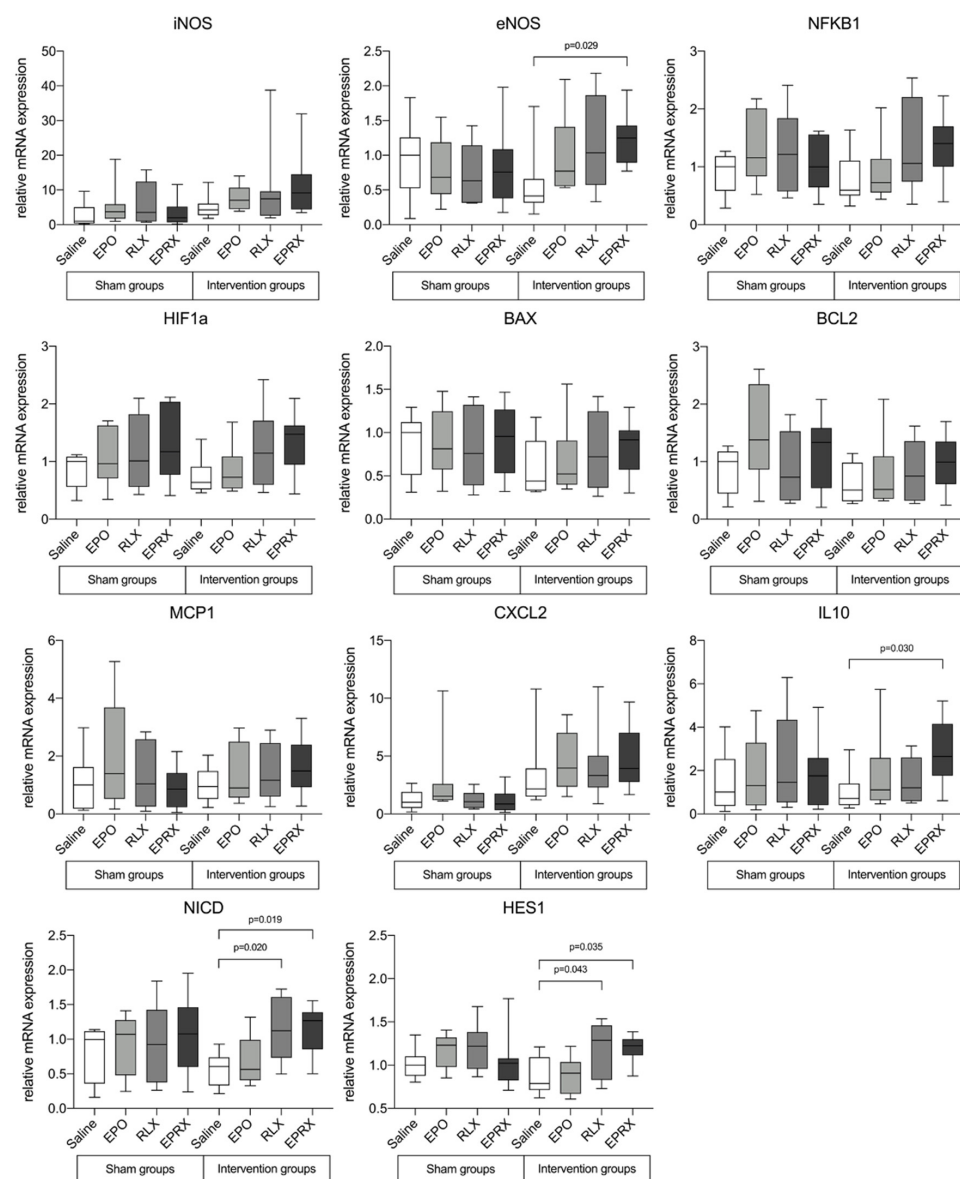
**Figure 2.** Micrographs of histological changes, MPO expression and TUNEL-positive cells in uterine tissue between sham + saline and all intervention groups. H&E—hematoxylin and eosin, MPO—myeloperoxidase, TUNEL—terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling EPO—erythropoietin, RLX—relaxin, EPRX—erythropoietin and relaxin.

Myeloperoxidase (MPO) expression was the highest in the intervention + saline group (Figures 1 and 2). The MPO-positive cell count was significantly lower in all intervention groups, after EPO, RLX or EPO with RLX application.

The intervention + saline group presented with the highest terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cell number, which was significantly lower in the intervention + RLX and intervention + EPO and RLX groups (Figures 1 and 2). EPO alone did not have any influence in lowering apoptotic cell numbers in the respective group compared to the intervention + saline group.

### 2.3. Gene Expression after Ischemia–Reperfusion Injury

Real-time quantitative polymerase chain reaction (qPCR) analysis revealed changes in several inflammatory and apoptosis genes between experimental groups in rat uterine tissue. No changes in the expression of iNOS, NFKB1, HIF1, BAX, BCL2, MCP1 and CXCL2 genes were detected after reperfusion in rat uterus tissue (Figure 3).



**Figure 3.** Quantitative RT-PCR of mRNA expression of iNOS, eNOS, NFKB1, HIF1A, BAX, BCL2, MCP1, CXCL2, IL10, NICD and HES1. Data were normalized to GAPDH gene expression. EPO—erythropoietin, RLX—relaxin, EPRX—erythropoietin with relaxin. Data are shown as median (Q1, Q3). The Kruskal–Wallis test was used in all cases.

Pretreatment with RLX combined with EPO significantly upregulated the expression of eNOS, IL10, NICD and HES1 1.25- ( $p = 0.029$ ), 2.65- ( $p = 0.030$ ), 1.27- ( $p = 0.019$ ) and 1.22-fold ( $p = 0.035$ ), respectively. Furthermore, RLX alone resulted in significantly upregulated NICD and HES1 gene expression (1.12-fold,  $p = 0.020$  and 1.29-fold,  $p = 0.043$ , respectively). Unfortunately, EPO alone had no significant impact on gene expression.

### 3. Discussion

Our study demonstrated that pretreatment with RLX or/and EPO significantly alleviates uterine tissue damage caused in an experimental IRI model. This is reflected by reduced lipid peroxidation, neutrophil infiltration, apoptosis, increased anti-inflammatory response and differential regulation of expression of genes involved in inflammation and apoptosis. In addition, pretreatment with RLX or RLX + EPO preserved uterine tissue morphology during IRI, which is important for further graft function.

IRI is a major factor limiting successful outcomes in solid organ transplantation [16]. It is defined as a multifactorial inflammatory condition involving hypoxia, metabolic stress, leukocyte extravasation, cellular death pathways and activation of the immune response [11].

Understanding these underlying mechanisms enables us to apply potential therapeutic approaches aimed at alleviating the deleterious effects of IRI. Currently, only several studies report the use of such agents to reduce IRI in the uterus. Sahin et al. report that the use of the calcineurin inhibitor Tacrolimus reduces oxidative stress in the uterus during IRI and significantly suppresses histopathological changes [17]. In a similar study, Sahin Ersoy et al. investigated another immunosuppressant, mycophenolate mofetil (MMF) [18]. This study determined that oxidative stress markers, such as 8-hydroxy-2'-deoxyguanosine (8-OHdG), MDA, MPO and ischemia-modified albumin, and cellular apoptosis were significantly attenuated in rats treated with MMF. Moreover, the use of MMF reduced IRI-induced uterus morphological changes. Comparable findings were reported by Aslan et al., who used oxytocin and kisspeptin premedication to significantly lower histological alterations and oxidative stress markers [19]. In essence, the same results were achieved by Atalay et al. by continuously administering remifentanyl during ischemia–reperfusion [20]. Recently, the use of melatonin and glycine was shown to significantly reduce tissue MPO expression and increased SOD activity [21].

EPO is a glycoprotein hormone that stimulates erythropoiesis and is synthesized in the kidneys and fetal liver. In our study, it significantly decreased the number of MPO-positive cells in the uterus; however, a contradictive study by Tsompos et al. concluded that the use of EPO did not have any protective effects during uterus IRI [22]. This study mostly relied only on histological changes and no other IRI readouts were taken into account. Furthermore, their reported EPO dosage draws some suspicion, as it is extremely high and almost technically unachievable.

RLX is a peptide hormone that is being widely studied in transplantation research as a protective agent against IRI. It acts through various target molecules and pathways, some of them being organ-specific. RLX acts on leukocytes as it induces the expression of Notch1 in macrophages and reduces the expression of intracellular adhesion molecule 1, as well as neutrophil adhesion through the increased synthesis of nitric oxide [23,24]. The hepatoprotective mechanism of RLX is unique as it acts via glucocorticoid receptors and inhibits the release of cytochrome c from mitochondria, thus suppressing apoptosis [25]. Furthermore, RLX increases organ perfusion as it reduces vasoconstriction by inhibiting endothelin 1 production and increases vasodilation through the amplified production of nitric oxide [26]. With the qPCR analysis in our study, we confirmed that RLX acts through the Notch1 signaling and upregulates the expression of NICD and HES1. NICD further blocks NFkB, which is responsible for tissue inflammation and activates the HES1 pathway, blocking Caspase-3 and reducing cellular apoptosis [27].

qPCR analysis revealed another interesting result showing that the RLX and EPO combination upregulated eNOS gene expression. Inducible NOS expression was not

affected by any of the experimental substances. Pretreatment with RLX alone showed only a tendency to upregulate eNOS gene expression, but this unfortunately did not reach significance. We could only speculate that the results were not significant due to the small sample size. These findings are partly in line with previously reported results of a study published by Bani et al. [28]. The synergistic effect of the RLX and EPO combination managed to significantly upregulate the expression of IL-10, which plays a central role in limiting the immune response [29]. Kageyama et al. reported that RLX alone managed to increase the expression of the IL-10 gene in liver tissue; however, there are no studies examining the effects of RLX and EPO combination on reducing IRI [23].

#### 4. Materials and Methods

##### 4.1. Animals

Eighty adult female (12-week-old) Sprague Dawley rats weighing 250–330 g were used in this experiment. All animals were obtained from Janvier Labs (Le Genest-Saint-Isle, France) and kept in the Division for Biomedical Research at the Medical University, Graz. An acclimatization period of 7 days before the experiments was applied for the animals under controlled environmental conditions on a 12 h light/dark cycle with room temperature set at  $22 \pm 1$  °C and with access to fresh water and standard rat chow ad libitum. All experimental procedures and protocols were performed according to the 3R guidelines and were approved by the Austrian Ministry for Science, Research and Economy on the 17 September 2019 (Approval number: BMWF-66.010/0154-V/3b/2019).

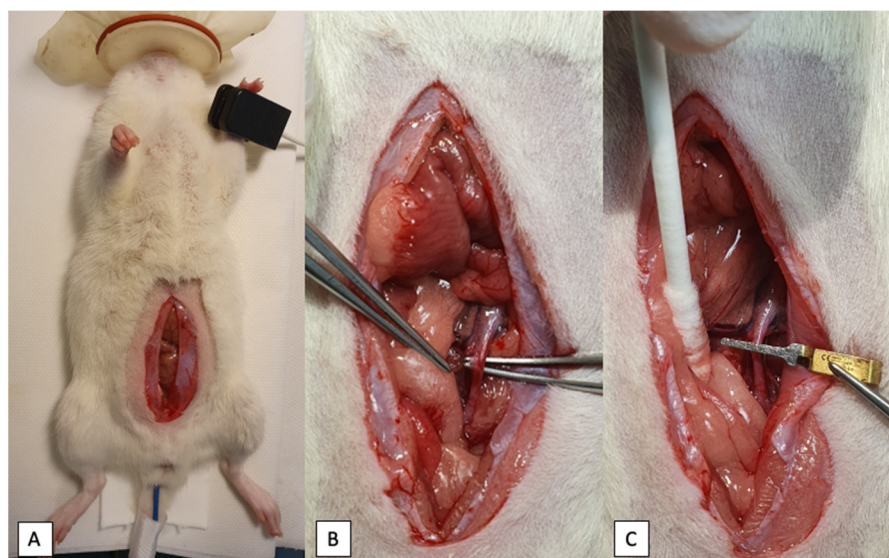
##### 4.2. Experimental Design

Before the experiments, rats were randomly assigned into eight groups ( $n = 10$ /group) and underwent either sham or experimental surgery. Each group was pretreated either with saline or 4000 IU/kg recombinant human erythropoietin (Erypo; Janssen-Cilag GmbH, Germany) or 5 µg/kg human relaxin-2 (Immundiagnostik AG, Bensheim, Germany), or the combination of RLX and EPO.

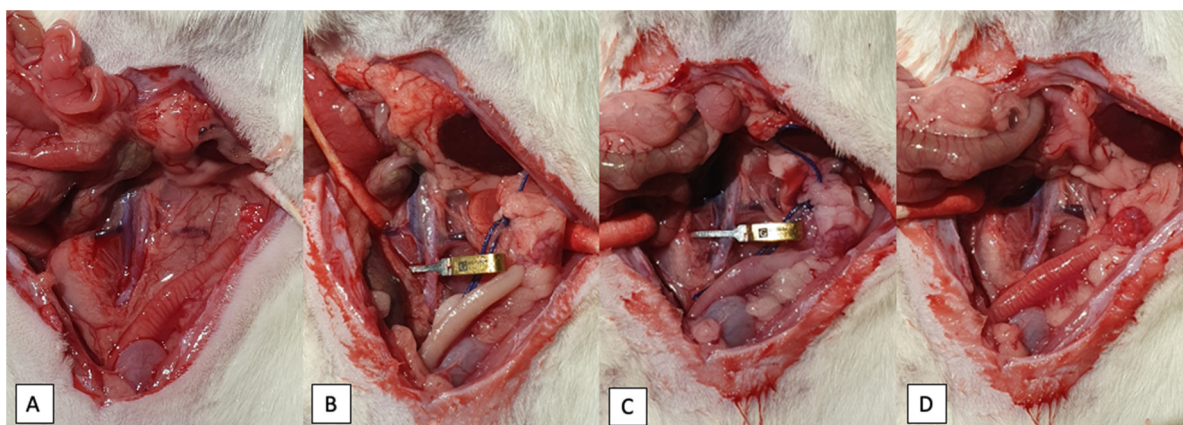
On the day of the experiment, animals were anesthetized in an anesthesia box using 2% 2 L/min isoflurane (Piramal-Isoflurane, Piramal Critical Care Deutschland GmbH, Hallbergmoos, Germany). After anesthesia induction, a mixture of anesthetic agents was injected intramuscularly, consisting of 0.15 g/kg medetomidine (Domitor, Orion Pharma, Vienna, Austria), 2 mg/kg midazolam (Midazolam, Accord Healthcare GmbH, Germany) and 5 µg/kg fentanyl (Fentanyl-Hamel, Hameln, Germany). During the experiment, rats were kept on a heating pad to maintain their body temperature at 37 °C, which was measured using a rectal probe. Heart rate and blood oxygen saturation were monitored constantly using a pulse oximeter device (Model 9847V; Nonin Medical BV, Amsterdam, The Netherlands), placed on one of the forelimbs.

After the anesthesia had been achieved, a single injection of saline, EPO (4000 IU/kg), RLX (5 µg/kg) or EPO (4000 IU/kg) with RLX (5 µg/kg) resuspended in 200 µL of sterile saline was administered through the lateral tail vein using a 26 G needle (B.Braun, Melsungen, Germany). After the operation field was shaved and disinfected, a 3–4 cm midline laparotomy was performed and uterus, ovaries and relevant vessels were identified (Figure 4). Exactly 30 min after the injection of the respective substances, ischemia was induced by clamping the distal abdominal aorta approximately 1 cm above the bifurcation with a 20–25-g pressure microvascular bulldog clamp (Geister Medizintechnik GmbH, Tuttlingen, Germany). Ovarian arteries with surrounding fatty tissue were occluded bilaterally using surgical polyglactin 2 (Vicryl 2, Ethicon, Johnson & Johnson International) ligature. Sham-operated rats underwent the same dissection procedure except for the exclusion of the abovementioned vessels. After clamping the vessels, the cessation of blood flow was ascertained by observing a pale uterus color (Figure 5B). The abdominal wall was sutured continuously with polyglactin 4-0 suture and the animal was placed in a sideways position to ensure proper lung ventilation and oxygen supply. After 60 min of ischemia, reperfusion was initiated by releasing the clamp and sutures from the abdominal aorta and

ovarian arteries bilaterally. By inspecting the uterus color, the restoration of blood flow was confirmed (Figure 5D). Reperfusion was maintained for 120 min. After this period, uterus and ovaries were procured and animals were euthanized by exsanguination. One uterus horn was immediately flash-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for biochemical analysis, and the other uterus horn was fixed in 4% formalin solution for further histological evaluation.



**Figure 4.** Surgical procedure for IR injury model. (A)—positioning of an animal prior to experiment, a 3–4 cm midline laparotomy. (B)—identification of the vessels, dissection of the aorta. (C)—occlusion of the aorta with microvascular bulldog clamp.



**Figure 5.** Blood flow changes in uterus during the experiment. (A)—before ischemia; (B)—start of ischemia; (C)—before reperfusion; (D)—start of reperfusion.

#### 4.3. Tissue Morphological Evaluation

Uterine tissue was fixed in 4% formalin and embedded in paraffin after routine histological procedures were performed. Tissue sections of  $2\ \mu\text{m}$  were stained with hematoxylin and eosin and afterward evaluated by a blinded pathologist using an already published scoring system for uterus IRI [10] (Table 1). Uterus tissue score was calculated according to seven parameters seen under light microscopy.

**Table 1.** Light microscopy morphological scoring system for evaluation of the uterus IRI model.

Score	0	1	2
Inflammatory cells	Absent	Moderate amount of cells	Severe infiltration of cells
Vasoconstriction	Absent	Moderate < 20% small vessels	Severe > 20% small vessels
Hemorrhage	Absent	Subendometrial	Myometrial plus endometrial
Necrosis	Absent	<20% *	>20% *
Edema	Absent	<50% *	>50% *
Thrombosis	Absent	<50% of the vessels	>50% of the vessels
Endometrial loss of cells	Absent	<20% *	>20% *

\* Percentages are calculated as (surface of the affected area/surface of the whole section) × 100.

#### 4.4. Biochemical Analysis

Malondialdehyde (MDA) as a lipid peroxidation marker and antioxidant SOD activity were chosen for biochemical analysis. Frozen tissue samples were homogenized with ceramic beads in a phosphate-buffered solution or lysis buffer (catalog number: ab118970, Abcam, Cambridge, UK) using the MagNA Lyser instrument (Roche Life Science, Mannheim, Germany) and centrifuged at 10,000 rpm for 10 min at 4 °C. The clear supernatant was collected and the following kits were used: Pierce™ BCA Protein Assay Kit (catalog number: 23225, Thermo Fisher Scientific, Waltham, MA, USA), Lipid Peroxidation (MDA) Assay Kit (catalog number: ab118970, Abcam), Superoxide Dismutase Colorimetric Activity Kit (catalog number: EIASODC, Invitrogen). All kits were utilized according to the manufacturer's manual. Tissue MDA and SOD levels were measured and expressed per milligram (mg) of protein.

#### 4.5. Immunohistochemical (IHC) Staining

Uterus tissue sections were stained with anti-MPO antibodies to evaluate tissue infiltration with neutrophils. After deparaffinization and antigen retrieval in a pressure cooker, 3% H<sub>2</sub>O<sub>2</sub> solution in methanol was used to block the activity of endogenous peroxidase. Primary anti-MPO antibodies (catalog number: A0398, Dako, Glostrup, Denmark) were diluted 1:800 in Dako REAL Antibody Diluent (Dako, Glostrup, Denmark) and applied for 60 min at room temperature. The UltraVision LP Detection System: HRP Polymer (Thermo Fisher Scientific, Waltham, MA, USA) and DAB Chromogen (Dako, Via Real Carpinteria, CA, USA) were used to visualize the target antigen. Sections were counterstained with hematoxylin, scanned and evaluated. Anti-MPO positive cells were quantified as the percentage of positive cells among all cells using QuPath software (open-source software for Quantitative Pathology, version 0.2.0).

#### 4.6. TUNEL Assay

Apoptotic cells were assessed using the TUNEL Assay Kit—HRP-DAB (catalog number: Ab206386, Abcam). The method was applied according to the manufacturer's manual. Stained sections were scanned and positive cells were counted using QuPath software (open-source software for Quantitative Pathology, version 0.2.0).

#### 4.7. Real-Time qPCR

Uterus tissue samples were snap-frozen and stored in liquid nitrogen until nucleic acid extraction. The tissue sample (weighing 50–100 mg) was homogenized in 1 mL TRIzol reagent (Invitrogen, Carlsbad, CA, USA) using a MagNA Lyser instrument (Roche Life Science, Mannheim, Germany) at 6500 rpm for 30 s. Nucleic acid was extracted according to the manufacturer's protocol. RNA was quantified spectrophotometrically using the 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  $\mu$ L. Real-time PCR amplification and melting curve analysis were performed using a BioRad CFX96 Touch™ System (Bio-Rad Laboratories GmbH, Vienna, Austria). The amount of 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  $\mu$ M of each primer in a final reaction volume of 10  $\mu$ L. The PCR reaction mixture was subjected to an initial denaturation at 95 °C for 10 s, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 58 °C for 20 s and elongation at 72 °C for 30 s, followed by a melting curve (60 to 95 °C). For detailed information on primers used, see Table 2. Gene expression was determined using Bio-Rad CFX Manager 3.1 (BioRad Laboratories GmbH, Vienna, Austria), using the Cq regression method embedded in the program. All PCR reactions were performed in duplicate, and relative gene expression was calculated by the  $2^{-\Delta\Delta C_T}$  method, using GAPDH as a housekeeping gene.

**Table 2.** Genes used for quantification and primer information.

Acc. Number	Forward Primer (5′–3′)	Reverse Primer (5′–3′)	Product Length
<i>GAPDH—Glyceraldehyde-3-Phosphate Dehydrogenase</i>			
NM_017008.4	AGTGCCAGCCTCGTCTCATA	GGTAACCAGGCGTCCGATAC	77 bp
<i>eNOS—Endothelial Nitric Oxide Synthase</i>			
NM_021838.2	ATTGGCATGAGGGACCTGTG	CCGGGTGTCTAGATCCATGC	81 bp
<i>iNOS—Inducible Nitric Oxide Synthase</i>			
NM_012611.3	TTGGTGAGGGGACTGGACTTT	CCGTGGGGCTTGTAGTTGA	86 bp
<i>HIF1<math>\alpha</math>—Hypoxia Inducible Factor 1 Subunit Alpha</i>			
NM_024359.1	CGGCGAGAACGAGAAGAAAA	ACTCTTTGCTTCGCCGAGAT	89 bp
<i>BAX—BCL2-Associated X Protein</i>			
NM_017059.2	GACACCTGAGCTGACCTTGG	AGTTCATCGCCAATTGCCT	87 bp
<i>BCL2—B-Cell Lymphoma 2</i>			
NM_016993.1	GACTGAGTACCTGAACCGGC	GCATGCTGGGGCCATATAGT	89 bp
<i>MCP1—Monocyte Chemoattractant Protein-1</i>			
NM_031530.1	CTCCTCCACCACTATGCAGG	GATGCTACAGGCAGCAACTG	71 bp
<i>NFKB1—Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-Cells</i>			
NM_001276711.1	GGACAACCTATGAGGTCTCTGGG	TTCAATGGCCTCTGTGTAGCC	82 bp
<i>NICD—Notch Intracellular Domain</i>			
NM_001105721.1	CGGGACATCACGGATCACAT	ATTCATCCAAAAGCCGCACG	88 bp
<i>HES1—Hairy And Enhancer Of Split 1</i>			
NM_024360.3	ATGACAGTGAAGCACCTCCG	GGTACTTCCCAACACGCTC	85 bp
<i>IL10—Interleukin 10</i>			
NM_012854.2	ACCCGGCATCTACTGGACT	GTTTTCCAAGGAGTTGCTCCC	89 bp
<i>CXCL2—C-X-C Motif Chemokine Ligand 2</i>			
NM_053647.1	CTGACTACACCACCTCCACAC	GCCTTCAAAGCCCTCTGACT	87 bp

#### 4.8. Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 23 program (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA). Distribution of variables was investigated using the Shapiro–Wilk test. Normally distributed data were analyzed by one-way ANOVA test. If any differences were observed, pairwise post-hoc Tukey’s test was used. Non-normally distributed data were evaluated by a Kruskal–Wallis test. When overall significant differ-

ences were observed, pairwise significance was tested using Dunn's post-hoc test. Data are reported as median and quartiles (Q1; Q3). Differences were considered statistically significant when  $p < 0.05$ .

## 5. Conclusions

In conclusion, pretreatment with RLX, EPO or a combination of both EPO with RLX significantly alleviates uterine tissue damage caused by IRI. Combination of RLX with EPO was as effective as RLX alone, except that the combination of these substances yields an additional benefit for anti-inflammatory gene expression. Further studies are warranted before introducing these novel agents into clinical practice.

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**Data Availability Statement:** All data relevant to the study are included in the article.

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## Article

# Experimental Static Cold Storage of the Rat Uterus: Protective Effects of Relaxin- or Erythropoietin-Supplemented HTK-N Solutions

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**Abstract:** Uterus transplantation (UTx) is the only treatment method for women with absolute uterine infertility. Currently, the number of grafts retrieved from deceased donors is increasing; hence, prolonged cold ischemia time is inevitable. Thus, this study was designed to assess the effect of the novel relaxin (RLN)- or erythropoietin (EPO)-supplemented Custodiol-N (HTK-N) solutions in an experimental uterus static cold storage (SCS) model. A total of 15 Sprague Dawley rats were used. Uterus horns were randomly assigned into three groups ( $n = 10$ /group). SCS was performed by keeping samples at 4 °C in HTK-N solution without or with different additives: 10 IU/mL EPO or 20 nM RLN. Tissue samples were taken after 8 and 24 h of preservation. Uterine tissue histology, and biochemical and immunohistochemical markers were analyzed. No significant differences in SCS-induced tissue damage were observed between groups after 8 h of preservation. Uterine tissue histology, MDA, SOD levels and the TUNEL-positive cell number showed severe damage in HTK-N without additives after 24 h of preservation. This damage was significantly attenuated by adding RLN to the preservation solution. EPO showed no favorable effect. Our study shows that RLN as an additive to an HTK-N solution can serve as an effective uterine tissue preservative in the uterus SCS setting.

**Keywords:** relaxin; erythropoietin; cold storage; animal model; uterus



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## 1. Introduction

Uterus transplantation (UTx) is gaining interest as being the only treatment method for women with absolute uterine infertility. It affects 1 in 500 women of reproductive age and results either from congenital or acquired causes [1]. Uterine agenesis in Mayer–Rokitansky–Küster–Hauser syndrome is one of the congenital causes, which is relatively rare with a prevalence of 1 in 4500 women, while hysterectomy is the second most common female surgery mainly performed due to large myomas, peripartum bleeding, postpartum sepsis or malignancies [2]. UTx can serve as an alternative to adoption or surrogacy and enable a woman to have a childbirth experience [3].

The first UTx was performed in Saudi Arabia in 2000 [4]. Unfortunately, the transplanted graft had to be removed due to thrombosis three months after UTx. In 2014, a Swedish team led by Brännström successfully performed UTx, which resulted in the first live birth [2]. Since then, more than 40 UTxs have been successfully performed including transplantations from deceased donors [1,5]. There is an interest in improving protocols for UTx from deceased donors as this eliminates the risk of donor morbidity and mortality [6]. Furthermore, although the surgical procedure for performing UTx from a living or deceased

donor is the same, longer graft vascular pedicles can be dissected from deceased donors, which further increases the probability of the success of the transplant [6,7]. For these reasons, research into the factors influencing the success of UTx from deceased donors is needed.

One of the primary factors influencing the ability to perform UTx from deceased donors is the time during which the ex situ uterus is stored. Static cold storage (SCS) has been the standard method for solid organ preservation since the 1960s [8]. The use of this method relies on reduced temperature, which causes the decrease in cell metabolic activity to the minimum required for survival and ultimately prolongs graft viability [9,10]. The susceptibility to SCS varies by organ and is shortest for the heart (4–6 h), whereas the kidney and liver can successfully survive cold ischemia for up to 44 h [11]. Previously published studies report uterine tolerance to cold ischemia for at least 6 h with maintained contractile function and histological characteristics [12]. Custodiol-N is a novel preservative solution developed after base Custodiol® solution was supplemented with glycine, alanine, iron chelators and sucrose instead of mannitol [13]. Custodiol-N showed its superiority to Custodiol® in a uterus SCS model likely by reducing tissue edema and inhibiting oxidative stress [14].

In recent years, a concerted scientific effort has been dedicated to finding ways to reduce tissue damage following SCS [15,16]. In particular, studies addressing whether the additional substances could be employed to minimize tissue damage during SCS.

Relaxin (RLN) is a peptide hormone, which belongs to the insulin family of structurally-related molecules [17]. The RLN receptor (RXFP1) is expressed on many organs, such as the kidney, heart, brain, arteries, lungs and even blood cells [18]. It plays an important role during the early stages of pregnancy and is highly expressed in reproductive tissues such as the ovaries, uterus (both endometrium and smooth muscles) and placenta. This hormone has already been studied in cell culture and animal models and demonstrated its antiinflammatory, antifibrotic, cytoprotective and antioxidant properties as well as a role in hemodynamic changes [19–22]. Given its central role, it is also under investigation in transplantation research [23].

Erythropoietin (EPO) is an endogenous hormone, produced by interstitial fibroblasts in the kidney or fetal liver under hypoxic conditions [24]. It is used for anemia treatment in people who need dialysis or have cancer. EPO receptors were found outside the hemopoietic system, such as in the uterus' epithelial and stromal cells [25,26]. EPO showed its antioxidant effect by reducing lipid peroxidation and increasing superoxide dismutase (SOD) activity [27]. Moreover, preservation solutions supplemented with EPO reduced liver damage during SCS [28].

This study was designed to investigate the protective effect of RLN or EPO in a rat uterus SCS model and to examine morphological and biochemical changes in rat uterine tissue over different SCS time periods.

## 2. Materials and Methods

### 2.1. Animals

A total of 15 female (12 weeks old, weighing 250–330 g) Sprague Dawley rats (Janvier Labs, Le Genest-Saint-Isle, France) were used for this study. All rats were housed in the Division for Biomedical Research at the Medical University, Graz, and kept under controlled environment with a 12-h light/dark cycle,  $22 \pm 1$  °C temperature and unlimited access to fresh water and standard rat chow. Before conducting the experiments, rats were acclimatized to the new surroundings for 7 days. The study was approved by the Austrian Ministry for Science, Research and Economy on the 17th of September 2019 (approval number: BMBWF-66.010/0154-V/3b/2019) and conducted according to the 3R guidelines.

### 2.2. Experimental Design

A total of 15 rats were used in an experimental SCS model. The initial anesthesia was achieved with 2% 2 L/min isoflurane (Piramal-Isoflurane, Piramal Critical Care Deutsch-

land GmbH, Hallbergmoos, Germany). After the induction, anesthetic agent mixture consisting of 0.15 g/kg medetomidine (Domitor, Orion Pharma, Vienna, Austria), 2 mg/kg midazolam (Midazolam, Accord Healthcare GmbH, Munich, Germany) and 5 µg/kg fentanyl (Fentanyl-Hamel, Hameln, Germany) was injected intramuscularly. After midline laparotomy, the uterus was procured, and each uterus horn was randomly assigned into one of three groups ( $n = 10$ /group). Uterus SCS was performed by using Custodiol-N (HTK-N) solution in different compositions: Group HTKN (HTK-N + no additives), Group EPO (HTK-N + 10 IU/mL recombinant human erythropoietin (Erypo; Janssen-Cilag GmbH, Germany)) and Group RLN (HTK-N + 20 nM synthetic human relaxin-2 (Relaxera, Bensheim, Germany)). The concentrations for experimental substances were chosen according to previously published studies [28,29]. Uterine horns were gently flushed with the respective experimental preservation solution, immediately packaged into the same solution and placed on ice at 4 °C. Tissue samples were taken after 8 and 24 h of preservation. These timepoints were chosen based on our research group published data [14]. One part of the sample was flash-frozen in liquid nitrogen and stored at −80 °C for biochemical analysis and the other part was fixed in 4% formalin for histological analysis.

### 2.3. Tissue Morphological Evaluation

Uterine tissue was fixed in 4% formalin and embedded in paraffin and sections of 2 µm were stained with hematoxylin and eosin (H&E). Slides were evaluated by a blinded pathologist using an adapted scoring system for uterus SCS damage and analyzing the whole section at 100× magnification (Table 1) [14].

**Table 1.** Light microscopy morphological evaluation scoring system for uterus SCS model.

	Score			
	0	1	2	3
Edema	Absent	<15%	15–30%	>30%
Necrosis	Absent	<15%	15–30%	>30%
Smooth muscle contraction	Absent	Present		
Impaired integrity of surface epithelium	Absent	Present		
Disruption of epithelial cells	Absent	Present		
Endometrial loss of cells	Absent	Present		

Note Percentages are calculated as (surface of the affected area/surface of the whole section) × 100.

### 2.4. Biochemical Analyses

Two biochemical markers were chosen to evaluate the tissue oxidative stress. Malondialdehyde (MDA) is the final product of polyunsaturated fatty acids' peroxidation in the cells and SOD acts as an agent against reactive oxygen species [30,31].

Frozen tissue samples were homogenized with ceramic beads in a phosphate-buffered solution (PBS) or Lysis buffer using the MagNA Lyser instrument (Roche Life Science, Mannheim, Germany). After homogenization, samples were centrifuged at 10,000 rpm for 10 min at 4 °C. The following assay kits, according to the manufacturer's manuals, were performed using the collected supernatant: Pierce™ BCA Protein Assay Kit (catalog number: 23225, Thermo Fisher Scientific, Waltham, MA, USA), Lipid peroxidation (MDA) assay kit (catalog number: ab118970, Abcam, Cambridge, United Kingdom), Superoxide Dismutase Colorimetric Activity Kit (catalog number: ELASODC, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Tissue MDA and SOD levels were measured and expressed per milligram (mg) of tissue protein.

### 2.5. Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL) Assay

Cell apoptosis was evaluated using TUNEL Assay Kit—HRP-DAB (catalog number: Ab206386, Abcam, Cambridge, United Kingdom). The staining was performed according to the manufacturer's manual.

Stained sections were scanned and positive cells in whole section were counted using QuPath software (open source software for Quantitative Pathology, version 0.2.0) [32]. The number of positive cells was expressed as the percentage of stained cells of total nuclei.

### 2.6. Statistical Analysis

Statistical analysis was performed using SPSS 23.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA). Variable distribution was determined using the Shapiro–Wilk test. As most of the continuous variables were non-normally distributed, the Kruskal–Wallis test and Dunn's post hoc test were used for analysis. Data are reported as median and quartiles (Q1; Q3). Statistical significance was considered when  $p < 0.05$ .

## 3. Results

### 3.1. Tissue Biochemical Analysis

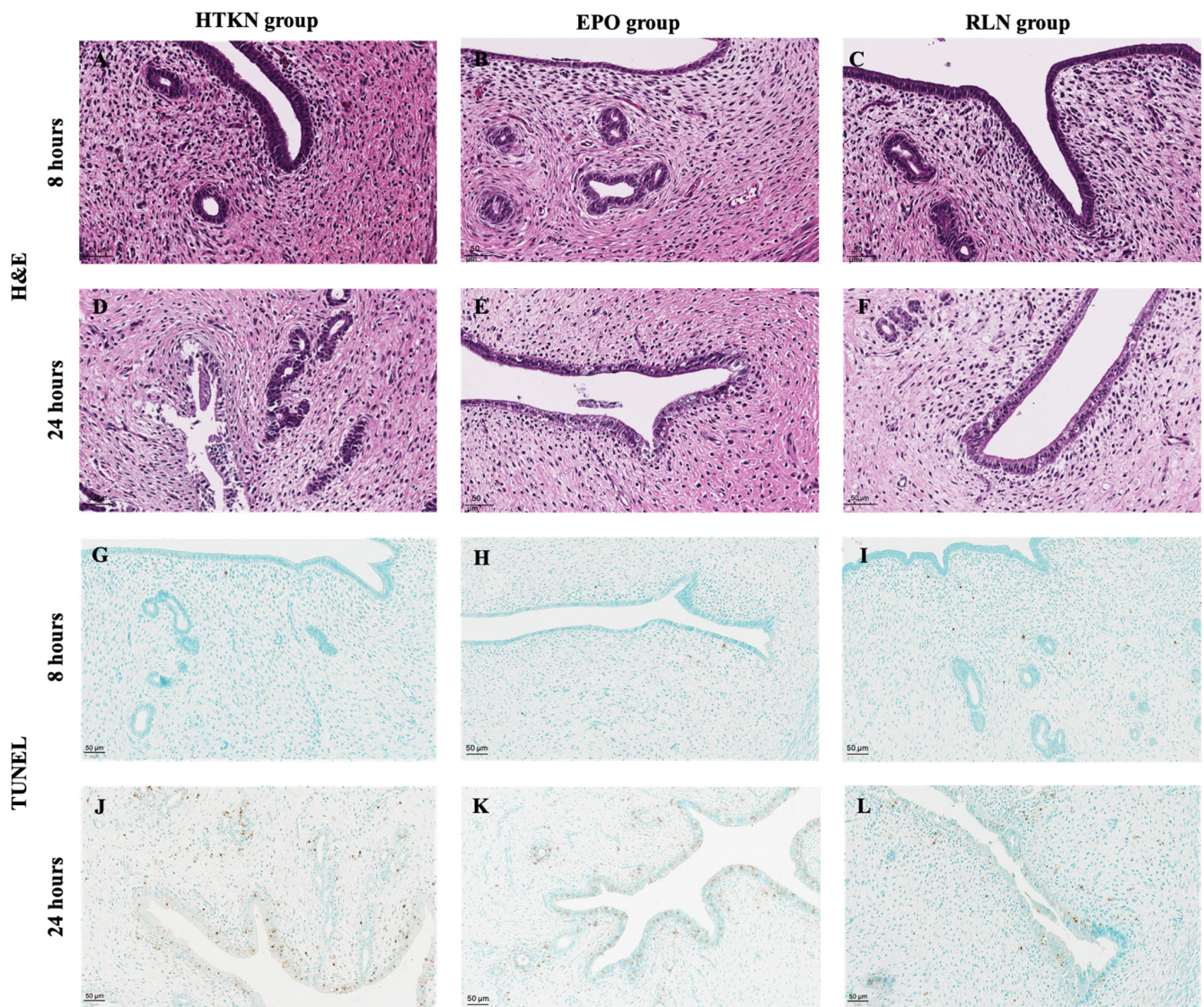
After 8 h of organ preservation, no significant differences in tissue MDA concentration and SOD activity were observed between the groups (Figure 1A,B). However, after 24 h of preservation, MDA levels increased in all groups compared to 8 h and differences between the groups after 24 h became significantly different. MDA concentration was highest in the HTKN group with no additives. MDA concentration in samples of the RLN group was significantly lower compared to samples in the HTKN group. Adding RLN to the preservative solution inhibited lipid peroxidation as samples after 24 h presented with similar MDA levels when compared to the 8 h group. EPO did not have a significant effect on MDA concentration after 24 h of SCS.

Moreover, differences in SOD activity between the groups became evident only after 24 h of SCS. The lowest SOD activity was observed in the HTK-N without additives group. In samples kept in the HTK-N solution with RLN, SOD activity remained significantly higher and was even similar compared to samples measured 8 h after preservation. No significant differences were observed in samples kept in the HTK-N solution supplemented with EPO.

### 3.2. Histology and Immunohistochemistry

The histological examination of uterine tissue showed no differences between groups after 8 h of preservation (Figures 1C and 2). Samples of all groups presented with mild tissue edema and minor changes in the epithelium (Figure 2). In contrast, preservation of 24 h led to significant uterine tissue injury. Although no tissue necrosis was observed across any group, samples exhibited signs of severe edema, smooth muscle contraction and changes in the endometrium. According to our adapted scoring system, samples kept in HTK-N with no additives or with EPO had a higher tissue injury score compared to samples kept in the HTK-N solution supplemented with RLN.





**Figure 2.** Micrographs of histological changes (A–F) and TUNEL-positive cells (G–L) in uterine tissue after 8 h and 24 h of preservation. (A–C) Samples after 8 h of preservation presented with mild tissue edema and minor changes in the epithelium. (D,E) Preservation of 24 h in HTK-N without additives or with EPO caused signs of tissue severe edema, smooth muscle contraction and changes in the endometrium. (F) Preservation of 24 h in HTK-N solution with RLN, samples presented with tissue edema, vacuolization of the endometrium and preserved integrity of surface epithelium. HTK-N—Custodiol-N, EPO—erythropoietin, RLN—relaxin, H&E—hematoxylin and eosin, TUNEL—terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

#### 4. Discussion

Here we investigated whether RLN or EPO could alleviate the negative impact of SCS on uterus tissue in a rat uterus SCS model. The results of this study revealed that RLN could serve as an effective additive to an HTK-N preservation solution by reducing lipid peroxidation, oxidative stress and apoptosis and preserving uterine tissue morphology.

Many animal studies have already been conducted to assess uterus myometrial tissue tolerance to cold ischemia. From recent studies it is known that the murine uterus can tolerate cold ischemia for up to 24 h and still obtain a pregnancy [33]. Our study demonstrates that cold ischemia lasting up to 24 h has a negative impact on the uterine tissue resulting in biochemical and histological changes, but the damage can be diminished by adding RLN to the preservation solution. These findings are important as the uterus is not a vital organ

and therefore is procured lastly from a brain-dead donor, resulting in an extended ischemic time [7].

SCS induces several pathophysiological mechanisms, which ultimately result in tissue damage. It impairs the activity of Na<sup>+</sup>/K<sup>+</sup> ATPase, which causes cell swelling, depletes ATP and shifts cell metabolism to an anaerobic state; furthermore, one of the most important damage pathways is the formation of excess reactive oxygen species [10]. Therefore, in this study we decided to use the particular uterine tissue lesion indicators (lipid peroxidation (MDA) and antioxidative (SOD) markers, impairment of tissue morphology, and apoptotic cell number) as their changes are associated with posttransplant outcomes [34,35].

Various preservation solutions, as well as different additives, have already been used in the experimental and clinical practice of UTx. The most studied preservation solutions are Celsior<sup>®</sup>, HTK (Custodiol<sup>®</sup>), University of Wisconsin solution, Perfadex<sup>®</sup> and IGF-1<sup>®</sup> [1,36–43]. Some studies revealed that adjuvants such as L-carnitine and Iloprost to an HTK solution significantly attenuate uterine tissue alteration after 24 h of cold ischemia when compared to preservation in a plain HTK solution [15,16]. Currently, there is no consensus concerning the best storage fluid for uterus SCS. However, in a previous study, our group found that Custodiol-N is superior to Custodiol in a uterus SCS model most likely by inhibiting oxidative stress and reducing tissue edema [14]. Therefore, we investigated the possibility to further improve HTK-N solution in the setting of uterine SCS.

RLN is one of the protective agents that is already being widely studied in preclinical kidney, lung, heart and liver transplantation research [23,29,44–53]; however, to our knowledge, this has not yet been applied in a uterine transplant. RLN acts through different target molecules and pathways of which some are organ related [54]. It reduces expression of intracellular adhesion molecule 1, induces expression of Notch1 in macrophages and reduces the adhesion of neutrophils through the increased synthesis of nitric oxide [23,45]. In hepatocytes, it acts via glucocorticoid receptors and inhibits the release of cytochrome c from mitochondria showing antiapoptotic properties [51]. Moreover, RLN reduces vasoconstriction via the inhibition of endothelin 1 production and increases vasodilation through the increased production of nitric oxide [47].

Our study results revealed that RLN added to an HTK-N solution reduces MDA concentration and preserves SOD activity in rat uterine horns after SCS. Moreover, this translated into the histological findings such as reduced morphological damage and the inhibition of cellular apoptosis. Our findings are in line with the reported results from a similar study by Boehnhart et al. in which the effects of RLN were tested on liver tissue during SCS [55]. This study reported that RLN managed to sustain lower MDA and myeloperoxidase levels in hepatocytes.

The second preservative additive used in our study was EPO. Several of its protective properties were demonstrated in a variety of studies [27,56,57]. We chose this substance as, currently, there are no studies analyzing its properties on uterus SCS. Unfortunately, the addition of EPO to the HTK-N solution at our tested dose did not yield any additional protective properties to the uterine tissue in our study.

There are several limitations of our study that should be addressed. First of all, this is an animal study conducted in an experimental rat model. Even though these experimental results are promising, it remains unclear if they will fully generalize to human tissues and organs. Furthermore, our study design mainly focused on the uterine tissue's morphologic and biochemical changes, and we did not examine the possible functional changes, which may be crucial for a successful uterus transplantation. Lastly, the human uterus is much thicker than the rat uterus, making it even more prone to ischemia. This limits the translational value of the study.

## 5. Conclusions

In conclusion, our study shows that RLN as an additive to an HTK-N solution can serve as an effective uterine tissue preservative in a uterus SCS setting. Further studies are warranted for introducing its usage in clinical practice.

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