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The effects of Bilirubin and Bilirubin-di-taurate on ischemia reperfusion injury in a rat model of kidney transplantation

Dissertation

Zum Erwerb des Doktorgrades der Medizin an der Medizinischen Fakultät der Ludwig Maximilian Universität zu München.

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Die Neugier steht immer an erster Stelle eines Problems, das gelöst werden will.

Galileo Galilei (15.02.1564 - 08.01.1642)
Italienischer Physiker und Astronom

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2. Introduction

2.1 History of transplantation

2.1.1 Transplantation and mythology

From time immemorial transplantation of organs or tissues has exerted a strong fascination on humanity.

The earliest evidence of orthotopic transplantation has been bequeathed from the Bronze Age where a circular disk of bone was removed from the calvarium to presumably relieve intracranial pressure and later replaced as an autograft. (1)

One of the oldest reports originates from hinduistic mythology of the 12 century before Christ and describes the creation of Ganesha, the god of wisdom and remover of obstacles, by Shiva, combining an elephant head with an anthropomorphic body.

In Greek mythology the Chimera was a monstrous fire-breathing creature composed of the parts of multiple animals consisting of a lioness body with a tail terminated in a snake's head and a goat head arising on the back at the centre of the spine. Homer's description in his Iliad is the earliest surviving literary reference. (2) (Fig. 1.2)

The modern term chimerism, describing the coexistence of donor and host cells arose from this chimera.

Figure 1.1: Ganesha (3)

Figure 1.2: Bronze figure of a chimera

In 430 b.c. the Chinese priest and physician Pien Ch'iao was born, who was reputed for his excellent diagnostic skills, excellence in pulse taking and acupuncture therapy was the main character of the first tale describing surgical transplantation procedures. He exchanged two hearts of two soldiers afflicted with an unbalanced equilibrium of energies. To achieve acceptance of the heart he administered powerful herbs after the transplantation. (4)

The two brothers and Christian physicians Saint Cosmas and Saint Damian are considered the patron saints of transplantation for their reported miraculous painless amputation of the ulcerated leg of a Christian Roman deacon and the subsequent transplantation of a healthy leg of a dead Ethiopian Moor. (Fig.1.2) (5)

Figure 1.2: Saint Cosmos and Damian performing a miraculous transplantation (6)

2.1.2 Advances in surgery

The first more reasonable account of early transplantation dates from the second century BC in which the Indian surgeon Sushruta preformed autografted skin transplantations to perform nose reconstructions. Encouraged by these attemps the Italian surgeon Gasparo Tagliacozzi performed rhinoplasty using tissue flap autografts. However he failed in performing the same procedure with allografts and attributed it to the „force and power of individuality". (7)

The beginning of the 20th century would mark several milestones for the advances in succesfull transplantation techniques.

In 1902 the Austrian surgeon Emmerich Ullmann performed the first succesfull kindey transplantation in dogs. The kindeys were transplanted into the neck using

magnesium tubes for vessel anastomosis and the ureter was led through the skin to document urine production. (8)

Alexis Carrel achieved another milestone by performing the fist successful transplantation of arteries and veins. In later studies, he successfully transplanted a dog's kidney to its neck. Thanks to those new techniques a fundament was laid for later transplantations and awarded him in 1912 the Nobel Prize. (9).

In 1933 the Ukrainian surgeon Yu Yu Voronoy performed the first human kidney transplantation from a deceased donor. The recipient, a young woman suffering from acute renal failure due to mercuric chloride poisoning in a suicide attempt survived for only four days. Unfortunately the transplanted organ never functioned and autopsy showed necrosis of the graft. Between 1933 and 1949 Voronoy performed 5 additional transplantations. None of his attempts was successful (10).

In 1948 the French surgeon Küss suggested, after performing several kidney transplantations in dogs, a heterotopic placement of the organs into the iliac fossa. (11).

In 1954 Joseph E. Murray at the Peter Bent Brigham hospital in Boston was the first to successfully transplant a human kidney between two HLA-identical twins. In 1962 the same team around Murray transplanted the first human cadaveric kidney. Unfortunately the new organ only functioned for 9 months. (12).

Figure 1.3: First succesfull kidney transplantation (13)   **Figure 1.4:** Joseph E. Murray (14)

Thomas E. Starzl was the first to successfully transplant a human liver in Denver/Colorado in 1963. Unfortunately the fist three patients died after 2-3 weeks.

In the same year James D. Hardy performed the first successful single lung transplantation at the University of Missisippi. His patient, a 58-year-old man with carcinoma of the left main-stem bronchus died 18 days after the transplantation was performed due to renal failure. (15)

In 1966 Dr. R. Lillehei at the University of Minneapolis performed the first pancreas transplantation.

On the night of 2/3 December 1967, Christian Barnard performed the world's first successful human orthtotopic heart transplantation at the University of Cape Town. His patient, a 53-year-old man suffered from severe coronary insufficiency. Two weeks after surgery the patients condition began to deteriorate after initial postoperative recovery and developed radiographic infiltrates in the lungs. On the 18th day post-operatively the patient died from severe pneumonia and septicaemia. (16)

Figure 1.5: Barnard and his patient Louis Washhansky (17)

Until that time only cadavers were used for transplantation (currently termed non-heart-beating donors) and many patients died after transplantation. In 1968 a Harvard committee, chaired by the anaesthesiologist Beecher, introduced the definition of brain death, which dramatically increased the number of available organs. (18)

In 1989 the first multi-organ transplantation in adults (pancreas, liver, small bowel) was performed by Prof. Margreiter at the University of Innsbruck (19).

Nine years later the French surgeon Dubernard performed the first successful hand transplantation in Lyon. (20)

2.1.3   Advances in immunosupression

Since the discovery of the human circulatory system by William Harvey around 1650 many blood transfusions where made between animals. Jean Baptiste Denise performed the first documented successful blood transfusion between an animal (lamb) and a 15-year-old boy who suffered from fever in 1667 (21). The first successful documented human-to-human blood transfusion was performed by the British obstetrician James Blundell. He transfused blood from a man to his wife to

replace the amount of blood she had lost during childbirth. (22) After those early attempts it was Karl Landsteiner who discovered the different blood groups. He was awarded the Noble prize in 1930 for his work. (23)

A milestone in understanding the immune system was achieved by the French Prof. Dausset in 1958 with the discovery of the major histocompatibility complex (MHC). Those molecules are displayed on cell surfaces and are responsible for lymphocyte recognition and antigen presentation. It is very rare if not even impossible that two individuals share the same set of MHC molecules. Thanks to this discovery it became clear that successful long-term transplantation could only be achieved by inhibition of the immune system of the recipient.

Peter Medawar a British zoologist who was awarded the Nobel Prize in 1960 for his work and the discovery of acquired tolerance. Medawar was performing skin grafts for fire victims during World War II. He transplanted skin grafts from the patients self (autografts) and grafts from another individual (allograft). After macroscopic and histological observation of the transplanted grafts he observed a good outcome after performing autograft transplantation and signs of rejection by infiltration of lymphocytes when allograft transplantation was performed. With his work he demonstrated that rejection was a cell mediated immunological problem rather than due to surgical problems. (24)

Murray treated his 12 first allogeneic kidney transplantation recipients with total body irradiation and subsequent bone marrow transplantation. Only one of his patients became a long-term survivor.

A real breakthrough in the era of immunosupression was achieved by Schwartz and Dameshek by demonstrating that 6-mercaptopuirne (6-MP) suppressed antibody production and prolonged skin allograft survival. After the replacement of 6-MP by it's derivate Azathioprine and the combination with corticosteroids, this immunosuppressant regime became the standard therapy for the following 20 years. (25).

In 1978 Cyclosporine (CsA) was introduced to the field of solid organ transplantation. Jean-Francois Borel isolated this substance from the fungus Tolypocladium inflatum and was able to demonstrate immunosuppressive effects in animals. However severe side effects as hepatotoxicity and nephrotoxicity where observed. (26)

In 1983 Tacrolimus was isolated in 1983 from a soil bacterium and showed similar characteristics as CsA. In 2000 Sirolimus (SRL) was introduced into clinical practice. Kahan could demonstrate that it acts early in the cell cycle (G1-phase) by inhibition of the regulatory kinase mammalian target of rapamycine (mTOR). SRL likely to be more effective when used in combination with CsA. Due to its mTOR inhibition it acts

strongly anti-proliferative, thereby showing anti-tumour effects, in particular in the formation of malignancy after transplantation. Moreover, on of the major advantages is the reduced nephrotoxicity as compared to CsA.

Mycophenolate mofetil (MMF) was introduced 1997 into clinical practice. It is the most effective and selective inhibitor of the de novo purine synthesis and replaced Azathioprine in many centres.

Nowadays in most centres a combination maintenance therapy in addition to a induction therapy is used.

2.1.4 Present

Thanks to the advances in basic research and the implementation of new surgical techniques solid organ transplantation can be seen as clinical routine. However chronic rejection, acute rejection episodes, ischemia reperfusion injury and the in part severe side effects of immunosuppressant drugs need to be considered.

Moreover the goal of organ transplantation to represent a long-term treatment after end stage organ failure has not yet been achieved as the following graph demonstrates (27).
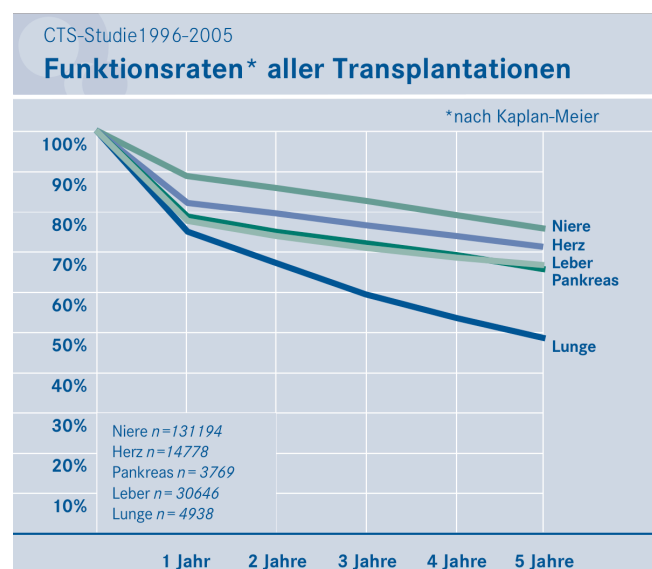

Fig.1.7: 5 year graft survival in more than 400 transplantation centres in over 45 countries. (27)

2.2 Allograft rejection

In solid organ transplantation three forms of rejections can be distinguished: Hyperacute, acute and chronic rejection.

Hyperacute rejection occurs immediately, within minutes of reperfusion and is based on pre-existing circulating anti-donor-MHC antibodies of the recipient. Due to an immediate binding of those antibodies to MHC-antigens on the surface of endothelial cells of the grafted organ, platelets are activated as well as the complement system. This reaction leads to subsequent loss of the transplanted organ. In order to prevent a severe systemic inflammatory response the organ needs to be directly removed. To minimize the occurrence of this reaction, recipients on waiting lists are screened for the presence of circulating antibodies by performing panel reactive antibody tests (PRA's).

Acute rejection episodes are cell mediated. They occur when a mismatch between the MHC system between the recipient and the donor are present. The risk of acute rejection episodes is highest in the first three months after transplantation. Thanks to new immunosuppressant drugs acute rejection episodes can be treated if recognized. However the occurrence of acute rejection episodes are a main risk factor for the development of chronic rejection. Acute rejection is a cell mediated inflammatory response. Mostly T-cells accumulate around the vessels of the newly transplanted organ and leads through activation and production of inflammatory cytokines to necrosis of the graft.

Chronic rejection describes the slow dysfunction of the transplanted organ. It is associated with progressive sclerosis of the vessels of the allograft leading to an unstoppable loss of the allograft. Due to this pathomechanism chronic rejection is now termed chronic allograft vasculopathy (CAV). The clinical presentation and the pathomechanism of CAV depends on the transplanted organ:

Chronic rejection in kidney allografts is characterized by vascular neointimal proliferation and glomerular sclerosis. This leads to a decreased glomerular filtration rate, an increase in plasma creatinine levels and resulting proteinuria and arterial hypertension (28).

In heart allografts chronic rejection presents itself with myocardial infarction, arrhythmias and sudden death due to arterio- and arteriosclerosis of the coronary arteries (29).

An serum elevation of liver enzymes and bilirubin due to obstructive cholangiopathy and arteriopathy with the representation of foam cells and a change and obliteration of the portal vein branches are typical characteristics of chronic rejection in liver transplants (30).

Bronchiolitis obliterans (BOS) is the clinical term used for the occurrence of chronic rejection in lung allografts. It is characterized by organized inflammation centred on the respiratory and terminal bronchioles (31).

2.3   Heme catabolism

Heme is synthesized in the mitochondria and cytosol of erythroid cells (85%) and hepatocytes.

In hepatocytes the synthesized heme is incorporated into the P450 cytochromes, which is important for detoxification. In erythroid cells all of the synthesized heme is used for incorporation into hemoglobin.

Red blood cells represent the largest repository for heme in the human body. Those cells have an average life span of 120 days. Thus about 6g/day of heme are accumulated. The first step of heme catabolism is the oxygenation, in which the heme ring is being opened by the endoplasmatic reticulum enzyme, heme oxygenase.

Three isoforms of heme oxygenase have been characterized so far.

HO-1 is expressed at low levels, except in the spleen where HO-1 levels are constantly high. Under stress conditions such as radiation, exposure to H_2O_2, heavy metals, cytokines such as IL-6 or TNF-α and lipopolysaccharide (LPS) it can rapidly be upregulated.

HO-2 is expressed in most cell types under homeostatic conditions.

A third heme oxygenase (HO-3) is not catalytically active, but is thought to work in oxygen sensing.

The oxidation of heme results in the formation of biliverdin, ferric iron (Fe^{3+}) and carbon monoxide. This is the only known reaction of the human body where CO is produced. In the next step a second bridging methylene is reduced by biliverdin reductase resulting in the production of bilirubin (Fig. 1.8)

Fig. 1.8: Heme degradation resulting in the formation of free iron, CO, biliverdin and bilirubin

Bilirubin formed in this reaction is not water-soluble and is bound to albumin and transported to the liver where it dissociates from albumin and is up taken by the hepatocytes. In hepatocytes bilirubin-UDP-glucuronyltransferase (bilirubin-UGT) adds 2 equivalents of glucuronic acid to bilirubin, which produces a more water-soluble form. This form is now actively secreted into the bile. In the intestine bilirubin is acted on by bacteria to produce the final porphyrin products, urobilinogen and urobilin, which are found in the faeces. (Fig. 1.9)

Fig. 1.9: Bilirubin catabolism

2.3.1 Inherited disorders of bilirubin catabolism:

As mentioned above, biliary excretion is the main route of disposal of bilirubin. Decreased excretion of bilirubin leads to the well known clinical presentation of jaundice. There are several inherited syndromes that lead to non-haemolytic hyperbilirubinemia.

2.3.1.1   Inherited forms of unconjugated hyperbilirubinemia

Three form of inherited unconjugated hyperbilirubinemia can be distinguished into the mildest form, **Gilberts syndrome** (GS) and two more severe clinical presentations, **Crigler-Najar Syndrome** (CN-syndrome) I and II.

GS is the most common herediditary form of hyperbilirubinaemia and can be found in 5% of the population. The main clinical symptom is harmless jaundice caused by reduced activity of the enzyme glucur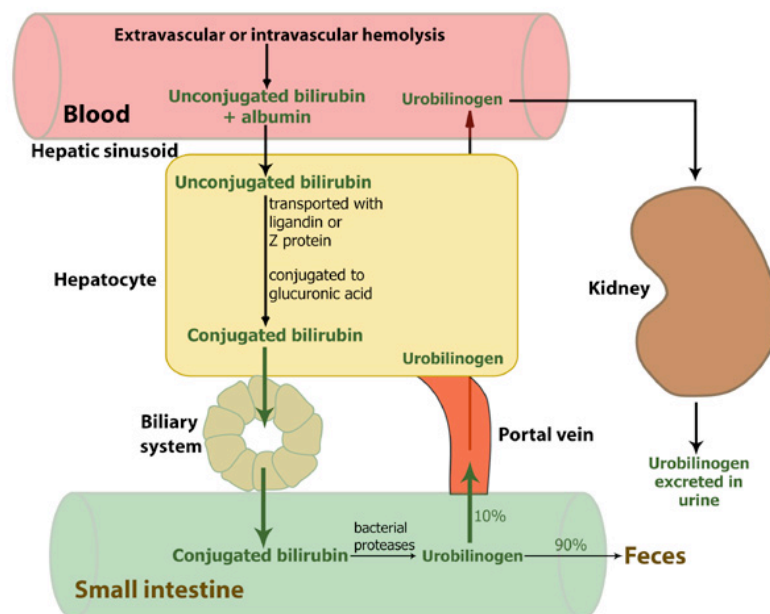onyltransferase. GS is generally considered to be an autosomal recessive disorder. Patients suffering from GS tend to have serum bilirubin levels from 1-6 mg/dl. Vitek et al published the first study on coronary heart disease (CAD) risk and individuals suffering from GS and found a significant decrease in the incidence of CAD in those patients. Vitek explained this occurrence with the increased serum antioxidant capacity of those patients (32).

In 1952 Crigler and Najar described the first seven patients suffering from severe non-haemolytic jaundice (33). Six of their patients died in early childhood with kernicterus and since this first report the lethal inherited disorder is called Crigler-Najar (CN) syndrome. In 1969 Arias et al. could distinguish between two forms of CN syndrome. Patients suffering from CN-syndrome type I did not respond to Phenobarbital injection (enzyme induction) and had no traces of bilirubin glucuronides in the bile. Patients suffering from this type of CN-syndrome normally died in early childhood. In contrast patients suffering from CN-syndrome Type II responded to Phenobarbital treatment and bilirubin glucuronides could be found in the bile. Those patients normally survived into adulthood. The combination of less severe hyperbilirubinaemia and the presence of bilirubin glucuronides in the bile indicate that patients suffering from CN-syndrome II have some residual bilirubin

glucuronidating activity whereas patients suffering from CN-syndrome I lack this activity completely (34).

2.3.1.2   Inherited forms of conjugated hyperbilirubinemia

First described in 1954 by the two pathologists from Washington Dubin and Johnson, this inherited form of hyperbilirubinemia is characterized by conjugated hyperbilirubinaemia with normal liver enzymes, a deposition of pigments into the liver, and a unique pattern of urinary excretion of heme metabolites (coproporphyrins). **Dubin-Johnson-syndrome** (DJS) is normally following a autosomal recessive inheritance. The pathomechanism of DJS is a mutation in an apical canalicular membrane protein responsible for the excretion of conjugated bilirubin called the multidrug resistance protein 2 (MRCP2). Patients suffering from DJS are normally asymptomatic. Worsening of jaundice due to pregnancy and the intake of oral contraceptives is a well-known feature of this syndrome (35).

Rotor syndrome represents another similar clinical presentation of conjugated hyperbilirubinemia. This syndrome follows an autosomal recessive inheritance with unknown aetiology. Patients suffering from Rotor syndrome show a non-itching jaundice with occasionally abdominal pain and fever. In contrast to patients suffering from DJS the liver pigmentation is missing (36).

2.3.2 Beneficial effects of Bilirubin/Biliverdin:

As mentioned above, heme oxygenase 1 (HO-1) is induced in response to cellular stress and is responsible for converting the prooxidant heme molecule into equimolar quantities of biliverdin (BV), carbon monoxide (CO), and iron. BV is then converted to bilirubin (BR) by the enzyme biliverdin reductase. Recent experimental groups could demonstrate that induction of the HO system is an important endogenous mechanism for cytoprotection and that the downstream products of heme degradation, CO, BR, and BV, may mediate these powerful beneficial effects. These molecules, which were once considered to be toxic metabolic waste products, have recently been shown to have dose-dependent vasodilatory, antioxidant, and anti-inflammatory properties that are particularly desirable for tissue protection during organ transplantation. In fact, recent work has demonstrated that administration of

exogenous CO, BR, or BV may offer a simple, inexpensive method to substitute for the cytoprotective effects of HO-1 in a variety of clinically applicable models.

2.3.2.1 Bilirubin can prevent from atherosclerosis

One of the major risk factors for the development of atherosclerosis has been suggested to be the oxygenation of low-density lipoprotein molecules (LDL) by free radicals, particularly reactive oxygen species (ROS) (37). Those oxidatively modified LDL's are than taken up by intimal macrophages leading to the formation of foam cells (38).

Many research groups attempted to prevent the oxygenation of LDL with known antioxidants as Vitamins A,C,E and ß-carotine (a form of vitamin A) (38).

As mentioned above, Vitek et al. could demonstrate in a clinical trial that the occurrence of ischemic heart disease in patients with Gilberts syndrome was significantly decreased if compared to patients with normal bilirubin levels. The authors accounted this observation to the elevated serum antioxidant capacity and thereby the protection of LDL oxygenation (32).

In the following years many publications supported Viteks finding showing an inverse effect of high serum bilirubin levels and the occurrence of atherosclerosis (39-44).

Based on these findings Oellinger et al. hypothesized that bilirubin had beneficial effects on intimal hyperplasia in a model of carotid balloon injury. In their experimental design he performed carotid injury in hyperbilirubinemic Gunn rats and showed a significant decrease in intimal hyperplasia after injury if compared to Wistar rats. Comparable results could be achieved by one-hour local pre-treatment with biliverdin in wild type Wistar rats (45). The underlying mechanism by which BV/BR exerts its beneficial effect could be demonstrated in an *in vitro* experimental setting. In a proliferation assay of vascular smooth muscle cells they could demonstrate that administration of either BV or BR led to an arrest in cell cycle leading to a stop in proliferation (45).

2.3.2.2 Bilirubin can modulate the immune system

Transplantation of solid organs has become clinical routine as successful treatment of end stage organ failure. However, the life long immunosupression and the hereby caused increased morbidity due to a higher rate of infections and the occurrence of cancer need to be taken in account (46). In addition therapeutic strategies to prevent or decrease the damage caused by ischemia reperfusion injury or chronic rejection are missing (47). For this reason less toxic strategies improving quality of life and survival of transplanted recipients need to be developed.

Sima et al. was the first to describe immunosuppressive effects of bilirubin in 1980. In his experiments he could demonstrate a direct effect of bilirubin on lymphocytes and granculocytes *in vitro* as well a decrease in antibody forming cells in the spleens of mice being immunized and treated with bilirubin (48).

Due to findings that HO-1 induction could counteract both acute and chronic rejections of transplanted solid organs arose hope that similar effects could be achieved through exogenous administration of its downstream products biliverdin and bilirubin (49-51).

Yamashita et al. could demonstrate the immunmodulating effects of biliverdin in a murine model of heart transplantation. His group administered BV at a dose of 35mg/Kg once before transplantation and then daily for 13 days after transplantation and could achieve a significant prolonged survival of B6AF1 allografts in DBA/2 recipients to a median survival of 20.5 days compared to 11.5 days in untreated mice. When the injection rate of BV was increased to three doses per day the graft survival could be prolonged to over 200 days. Those long-term survivors where then challenged with a second heart allograft from FVB "third party" mice. Those hearts where promptly rejected whereas second DBA/2 allografts were accepted without any further immunosuppressive treatment. BV treatment induced donor-specific tolerance through a significant decrease of immunocompetent cell infiltration into the graft (52). The acceptance of the heart could be explained by the suppression of alloreactive T-cell responses.

2.3.2.3 Bilirubin ameliorates ischemia reperfusion injury

In recent publications it has been documented that local HO-1 over expression in the transplanted graft ameliorates IRI. Same protective effects were demonstrated for its downstream products bilirubin and biliverdin (53-55).

The effect of bilirubin on renal IRI is so far controversially discussed in recent publications.

Kirkby et al. clamped in their model of renal IRI the pedicles of both kidneys for 30 minutes followed by a 6h reperfusion period. Bilirubin was administered intravenously at a dose of 1.5mg/Kg 1h before reperfusion and continuously during reperfusion respectively at a dose of 20mg/Kg 1h before and during ischemia (bolus administration). After 6h of reperfusion animals where sacrificed and tissue samples as well as serum samples where collected for further analyses. This experimental design did not show any significant protective effects of bilirubin administration on renal IRI. Only serum creatinine levels in the 20mg/Kg treatment group showed a significant decrease if compared to the vehicle control. However, neither the intravenous bolus nor continuous infusion of bilirubin led to a significant protection to the renal medulla (56).

Protective effects of bilirubin administration to prevent the damage caused by renal IRI could be demonstrated in an isolated perfused rat kidney model. The kidneys were flushed with BR or control perfusate before 20 min of warm ischemia. Subsequently a 2h perfusion period was performed. BR treatment resulted in an improved vascular resistance, urinary output, glomerular filtration rate, tubular function and mitochondrial integrity (57).

Clark et al. could demonstrate protective effects of bilirubin treatment on cardiac ischemia reperfusion injury. In their experimental setting hearts of male Lewis rats were exposed to 30 min of ischemia followed by 60 min ex-vivo reperfusion period in a Langendorff heart setting. Administration of bilirubin to the perfusion solution led to a significant increase in cardiac recovery, reduced infarct size and preserved mitochondrial integrity (58)

2.3.2.4 Bilirubin and cancer

Since oxidative stress is considered to be a major contributing factor to carcinogenesis and bilirubin is a potent antioxidant, it has been proposed that bilirubin may have protective effects on the occurrence of malignancy (59,60).

A prospective study from Belgium examined the association between serum total bilirubin levels and cancer mortality in a representative sample of the Belgian population consisting of 5460 men and 4843 women. This group could observe significant decreased cancer mortality in male individuals with high normal BR serum concentrations. This association did not reach significance in the female group (61).

Keshavan et al. could demonstrate *in vitro* that the administration of unconjugated bilirubin to HCT15 colon adenocarcinoma cells led to a significant reduction in viable carcinoma cells via induction of apoptosis through activation of caspase-9 (62).

2.4 Harmful effects of bilirubin

2.4.1 Kernicterus:

Kernicterus represents the irreversible damage of the brain, especially the basal ganglia, caused by unconjugated bilirubin (not bound to albumin) in newborn. This symptom may be due to several pathological processes.

Rhesus incompatibility between mother and fetus can lead to severe hemolysis of fetal red blood cells leading to an elevated serum level of unconjugated bilirubin. Since the blood brain barrier of newborn is not fully developed, the unconjugated bilirubin can easily infiltrate the brain and interfere with normal neuronal development. Another cause for the occurrence of kernicterus is the above described syndrome of Crigler-Najar Type I.

Depending on the level of exposure the effects of kernicterus to the newborns brain range from unnoticeable to severe brain damage.

2.5   Ischemia reperfusion injury

Cardiovascular diseases that are initiated by local or systemic ischemia remain the main cause of death in the United States and Europe (62).

Ischemia is a consequence of interruption of blood supply during solid organ transplantation. Subsequently damage to metabolically active tissue occurs as a consequence of hypoxia and the lack of nutrients. If blood flow is restored to the ischemic organ (reperfusion) a series of events occur leading to an additional cell injury.

During IRI a series of interactions between the vascular endothelium and the innate immune system occurs leading to an inflammatory burst (63).

Organ ischemia induces a decrease in cellular oxidative phosphorylation resulting in a failure to resynthezise energy rich phosphated such as ATP and phosphocreatinine. This again leads to the alteration of ATP dependent membrane ionic pump function, favouring the entry of calcium, sodium and water into the cell.

Within the endothelium, ischemia leads to the expression of certain proinflammatory cytokines, while repressing other protective gene products (constitutive nitric oxyde synthethase, thrombomodulin).

All those processes during ischemia lead to the increased tissue vulnerability and further injury during reperfusion.

2.5.1 Role of reactive oxygen species during ischemia reperfusion injury:

Reperfusion of ischemic tissue leads to an increased formation of reactive oxygen species (ROS) such as superoxide anions (O_2^-), hydroxyl radicals (OH^-) and hydrogen peroxide (H_2O_2). During normal cell metabolism low levels of reactive oxygen species (ROS) are formed continuously and play important roles in cellular homeostasis, mitosis, differentiation, and signaling (64). ROS formed under physiological conditions are inactivated by endogenous scavenging enzymes, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase.

During ischemia ATP is degraded to hypoxanthine. Under physiological circumstances hypoxanthine is oxidized by xanthine dehydrogenase to xanthine. During ischemia however, hypoxanthine dehydrogenase is converted to xanthine

oxidase. Xanthine oxidase needs oxygene to be able to catalyze hypoxanthine to xanthine. For this reason an excess of hypoxanthine is built up during ischemia in the cell. During reperfuison, oxygen is re-introduced into the cell and the conversion of hypoxanthine by xanthine oxidase results in the formation of high levels of toxic ROS (65).

Since the endogenous scavenging enzymes are not efficient enough to neutralize the formed ROS, direct damage of the cellular membranes are caused by liped peroxidation (66). In addition ROS increases leukocyte activation, chemotaxis and leukocyte endothelial adherence after IRI.

2.5.2 Role of the complement system during ischemia reperfusion injury:

During ischemia reperfuison the complent system is activated and the formation of several pro-inflammatory cytokines is increased. Most important are the anaphylatoxins $C3_a$ and $C5_a$ and the complement components iC3b and C5b-9.

Due to the activation of the complement, several endothelial leukocyte adhesion molecules are expressed like vascular cell adhesion molecule 1 (VCAM1), intracellular adhesion molecule 1 (ICAM-1), E-selectin and P-selectin (67). In addition to increased leukocyte-endothelial interaction, the activation of the complement system alters vascular homeostasis by inhibiting endothelium dependent relaxation (67).

2.5.3 Role of leukocytes during ischemia reperfusion injury:

As mentioned above, during IRI molecules are expressed on the surface of endothelial cells to facilitate the interaction between leukocytes and endothelial cells. In a first step leukocytes interact with endothelial P-selectin, which interacts with the leukocyte counter receptor, P-selecitn glycoprotein 1. This initial binding between endothelial cells and leukocytes is described as "leukocyte rolling". The followed step is the firm adhesion of the leukocyte to the endothelium via binding of the leukocyte to endothelial expressed ICAM-1. Leukocyte transmigration into the interstitial compartment is facilitated by expression of platelet-endothelial cell adhesion molecule 1 (PECAM-1). This leukocyte recruitment and the hereby-caused release of ROS, proteases and elastases leads to and increased microvascular permeability, edema, thrombosis and cell death (67).
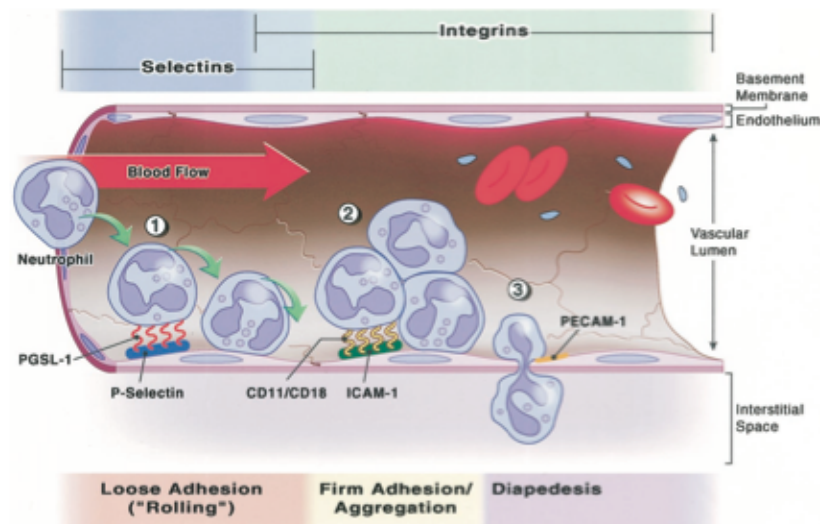
Fig.2.0: Leukocyte-endothelial cell interaction during ischemia reperfusion injury (67).

3. Aim of the study

Nowadays organ transplantation can be seen as an established therapeutic option for end stage organ failure. The transplantation procedure consists out of cold preservation and warm revascularization resulting in ischemia reperfusion injury which severity affects the outcome of transplantation.

The severity of I/R injury can be seen as a prognostic factor regarding primary non-function of the transplanted graft and the development of chronic graft failure leading to late graft loss.

The main pathomechanism of I/R injury consists of oxidative stress during cold preservation and an excess formation of reactive oxygen species (ROS) during reperfusion.

Aim of this study was to test the potent antioxidant bilirubin in a kidney ischemia reperfusion model of the rat and to examine its effects on IRI. For this purpose we treated rats receiving kidney allografts after 18h of cold ischemia with bilirubin-dt/ biliverdin/dt in doses assigned previously in an ischemia reperfusion clamp model of the rat. To evaluate the potential protective effects of bilirubin on IRI serum creatinine levels and estimated glomerular filtration rate were assigned.

4. Material and Methods

4.1   Reagents

Bilirubin	Frontier Scientifc
Bilirubin-ditaurate	Frontier Scientifc
NaCl 0,15 M	Fluka
PBS	Invitrogen

4.2   Animal surgery equipment

Bipolar diathermia	Martin
Heparin	Heparin Immuno®
Ketasol	Graeub
Liquid Nitrogen	Linde
Operating microscope	Zeiss
Ringer's lactate solution	Braun AG
Saline solution (NaCl 0.9%)	Fresenius
Standard microsurgical instruments	S&T AG
Suture material	Ethicon / USS DA
Xylasol	Graeub
Chemical analyzer Cobas Plus	Roche
Metobolic cage	Harvard Apparatus
Isoflurane	Benson Medical

4.3   Animal housing:

Animals were housed in single rat cages with free access to water and food during the conduction of the experiments according to regulation of the Institutional Animal Care and Use Committee.

4.4 Anesthesia:

Rats were anesthetized using a isoflurane vaporizer.

4.5 Kidney clamp model in the rat

Male Lewis rats were anesthetized (80 mg/kg, i.p. pentobarbital) and prepared for surgery in a sterile surgical field. A long abdominal incision is made from the xyphoid to the symphysis pubis. The bowel is covered with moist gauze and retracted to the left. The right kidney is being explanted after ligation of the ureter and the renal artery and vein. The left renal artery is exposed and dissected so that a vascular clamp can be easily applied.

4.6 Orthotopic kidney transplantation in the rat

4.5.1 Donor Operation

Each animal was anesthetized (80 mg/kg, i.p. pentobarbital) and prepared for surgery in a sterile surgical field. A long abdominal incision is made from the xyphoid to the symphysis pubis. The bowel is covered with moist gauze and retracted to the right. The right ureter is ligated at its entrance to the bladder and transected. The left ureter is mobilized from its surrounding tissue and the bladder is ligated at its button. The left kidney is completely mobilized from its retroperitoneal attachments. 1% xylocaine is applied around the renal pedicle to avoid vasospasms. The infrarenal inferior vena cava and the aorta are mobilized and tied proximally above the renal pedicle and distally just proximal to the bifurcation. A 25-guage needle is inserted into the infrarenal aorta, and the left kidney is perfused with approximately 3mls of cold heparinized, isotonic saline and subsequently with UW solution. The perfusion of the left kidney will cause the organ to become pale. The solution should become clear once all circulating blood has been removed. After perfusion the kidney with the complete bladder is removed and the organ is placed in UW solution at 4°C for 18h of cold ischemia until transplantation is performed.

4.6.2 Recipient operation

Each animal will be anesthetized (80 mg/kg, i.p. pentobarbital) and prepared for surgery in a sterile surgical field. Body temperature will be kept constant at 37°C during the whole operation using an electric heating pad. A long abdominal incision is made and the bowel is again covered and retracted to the right. The left abdominal wall is retracted to the left with a small hook. The left ureter is identified and transected. The renal pedicel of the left recipient kidney is mobilized, tied, and transected, and the left recipient kidney is completely removed. After mobilization and ligation of the lumbar branches, the aorta and inferior vena cava (IVC) are cross-clamped infrarenal and close to the bifurcation, respectively. The arterial reconstruction is performed by end-to-side anastomosis between the aortic stump of the donor and recipient aorta, using a 10-0 uninterrupted nylon suture, as the venous reconstruction is done by end to-side anastomosis between the recipient renal vein and the IVC of the donor. After completion of the vascular anastomosis, the distal and proximal vascular clamps are removed. Bladder anastomosis is performed using the bladder patch of the donor bladder and the recipient bladder using 7-0 Vicryl uninterrupted suture. After nephrectomy of the contralateral kidney, the bowel is replaced in its original position. The abdomen is irrigated to remove any debris and closed in two layers (inner layer absorbable: outer layer non-absorbable), using 4-0 sutures. To rehydrate the animals, 3ml of Saline is injected i.v. and 7ml of Saline is given s.c. Postoperatively, animals are placed on a heating pad while they recovered from the anesthesia and have free access to food immediately after transplantation.

For the collection of urine and calculation of the estimated glomerular filtration rate (eGFR) rats are palced in metabolic cages for 24h. After 24h of reperfusion animals are sacrificed under isoflurane anaesthesia and blood samples as well as tissue samples of the transplanted kidney are harvested and snap frozen for further analyzes.
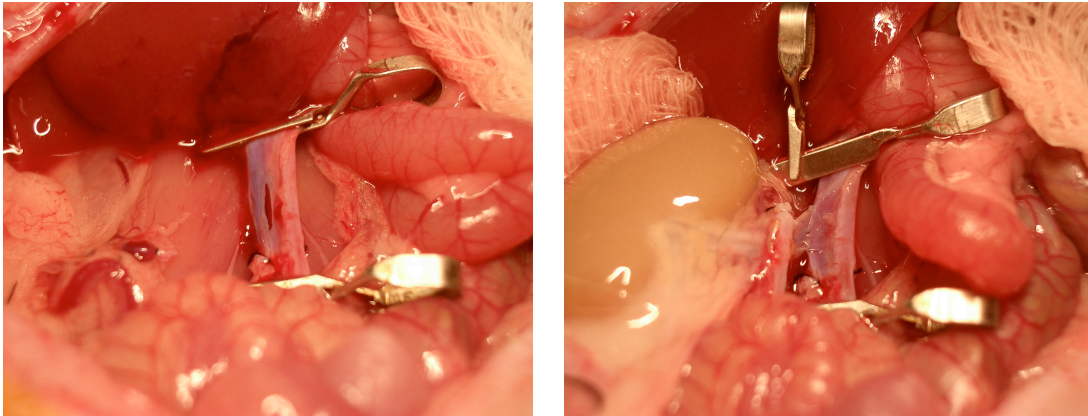
Fig.2.0: Preparation and clamping of the inferior vena cava and the aorta (left image). Venal anastomosis between the inferior vena cava of the recipient and the renal vein of the donor (right image).
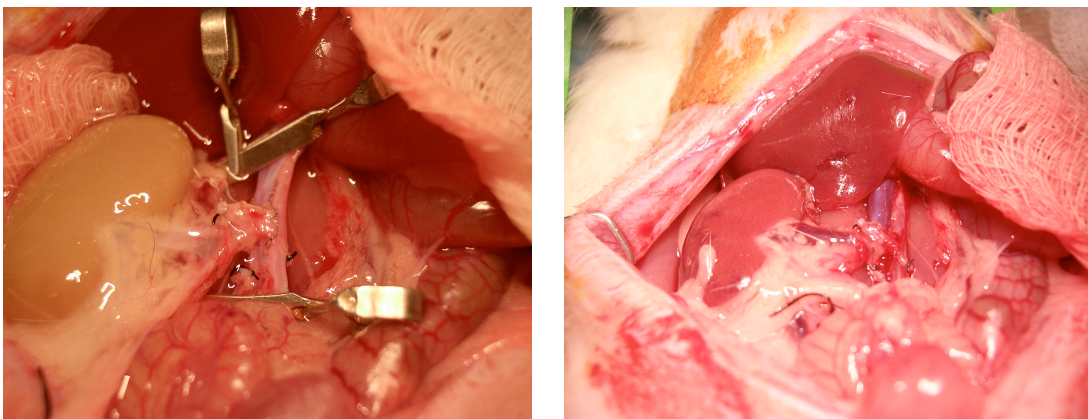

Fig.2.1: Arterial anastomosis end-to-side between the recipient aorta and the donor aorta (left image). Result after 5 minutes of reperfusion (right image).

4.7   Treatment

Before kidney transplantation was performed a series of kidney ischemia reperfusion studies was accomplished to ascertain the right warm ischemic time and the right dosing regimen of bilirubin/bilirubin-ditaurate (bilirubin-dt) respectively biliverdin.

In the clamping model a treatment regimen of 2 doses of biliverdin given intravenously at a dose of 17.5 mg/Kg before clamping and directly after reperfusion showed to be the most effective.

For the kidney transplantation model rats where treated with 3 doses of 10mg/Kg bilirubin-dt applied intravenously 1h before, 15min before and 10min after reperfusion. (Fig. 2.2) After 24 h of reperfusion rats where sacrificed and tissue samples as well as blood samples where taken for further analyzes.
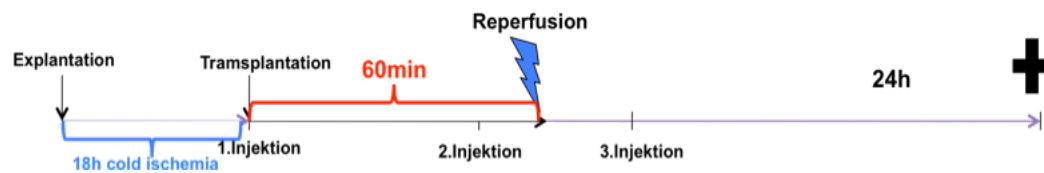
Fig.2.2: Experimental set-up for the kidney transplantation model

4.8   Haematoxylin-Eosin Staining

Haematoxylin-eosin staining was performed using the following protocol:

Slides were taken out of the freezer and air dried to remove moisture. Subsequently the slides were placed into a conical tube containing 0,1% Mayers-Haemotoxylin for 10 minutes.

In a Copling jar slides were washed with cool running ddH20 for 5 minutes.

slides were placed into a clonical tube containing 0,5% eosin for 5 minutes.

Slides were dipped in distilled H_2O until the eosin stops streaking. To dehydrate the slides they were placed into 50% ethanol for 10 minutes, then in 70% ethanol for another 10 minutes. Specimens were equilibrated in 95% ethanol for 1 minute and in 100 % ethanol for another 30 seconds.

In a last step, slides were dipped in xylene for several times. A drop of Permount (xylene based) was placed on the slides using a glass rod and a coverslip was mounted.

4.9   Statistical analyzes

Statistical analyses were performed using Student's t-test and the data are displayed with prism version 4 for Mac. $p < 0,05$ was defined as significant and $p < 0,01$ as highly significant. All data are presented as mean values ± standard deviation. The relative densities of protein bands were analyzed by measuring optical density using the Image J® software.

4.10 Experimental groups

Group I: Clamping of the left renal pedicle (artery and vein) for 30 min. (n=8)

Group II: Clamping of the left renal pedicle for 45 min. (n=8)

Group III: Clamping of the right renal artery for 45 min. (n=8)

Group IV: Clamping of the right renal artery for 60 min. (n=8)

Group V: Clamping of the right renal artery for 45min and treatment with a total of 3 doses of biliverdin at a dose of 3mg/Kg given i.v. (n=8)

Group VI: Clamping of the right renal artery for 45min and treatment with a total of 3 doses of biliverdin at a dose of 10mg/Kg given i.v. (n=8)

Group VII: Clamping of the right renal artery for 45min and treatment with a total of 2 doses of biliverdin at a dose of 17.5mg/Kg given i.v. (n=8)

Group VIII: Kidney transplantation after 18h of cold ischemia (n=8)

Group IX: Kidney transplantation after 18h of cold ischemia and treatment with 3 doses of bilirubin-dt at a dose of 10mg/Kg given i.v. (n=8)

Group X: Kidney transplantation after 18h of cold ischemia and treatment with 3 doses of bilirubin-dt at a dose of 3mg/Kg given i.v. (n=8)

Group XI: Kidney transplantation after 18h of cold ischemia and treatment with 3 doses of bilirubin-dt at a dose of 30mg/Kg given i.v. (n=8)

Group XII: Kidney transplantation after 18h of cold ischemia and treatment with a bilirubin-dt flush at a dose of 125 μM. (n=3)

Group XIII: Kidney transplantation after 18h of cold ischemia and treatment with 3 doses of bilverdin-dt at a dose of 10mg/Kg given i.v. (n=3)

5. Results

5.1 *Clamping of the renal pedicle and its effect on serum creatinine levels*

The first experiments we conducted were used to find the perfect model in which a series of dose studies could be performed. For this purpose renal pedicles respectively renal arteries where clamped for different times and serum creatine levels where controlled at different time points.

Clamping of the renal pedicle led to a significant increase in serum creatinine levels after 30 minutes respectively 45 minutes of warm ischemia. In rats where warm ischemic time was calculated with 30 minutes renal function recovered after 72h compared to those where clamping was performed for 45 minutes. (Fig 4.1)
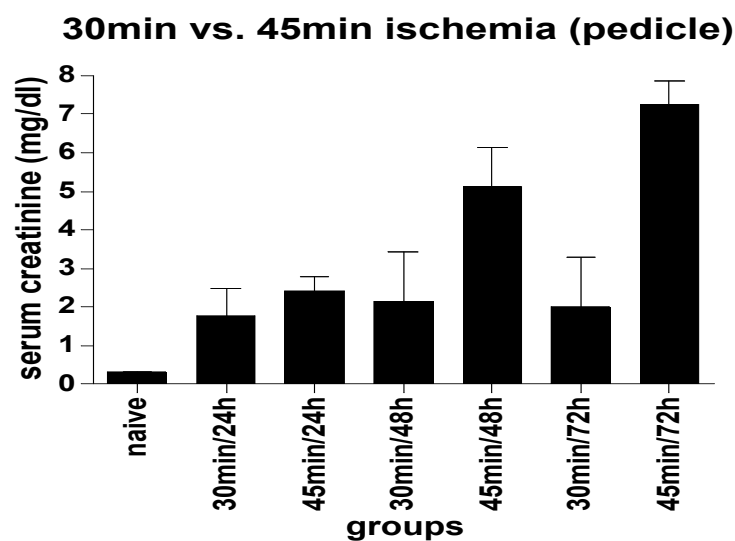

Fig. 4.1: Serum creatinine levels of rats (n=8) in which the left renal pedicle (artery and vein) has been clamped for 30 and 45 minutes respectively. The right kidney has been removed before clamping. In the 30 minutes group, standard deviations were high with some rats showing no increase of creatinine at all. In the 45 minutes group kidneys did not recover from ischemia reperfusion injury until day 3, when animals were sacrificed.

5.2 Clamping of the renal artery alone vs. clamping of the renal pedicle

Since clamping of the renal pedicle for 45 minutes led to death of the operated animals another series of experiments was performed in which only the renal artery was clamped for 45 minutes. Fig. 4.2 shows the time course of serum creatinine levels of clamping the renal artery alone respectively the renal pedicle. All rats, that underwent clamping of the renal artery recovered after a maximum increase in serum creatinine up to (1.4±0.4) at 24h. In contrast to rats in which the whole renal pedicle has been clamped for the same time, all animals recovered from ischemia reperfusion injury.

pedicle vs. artery allone
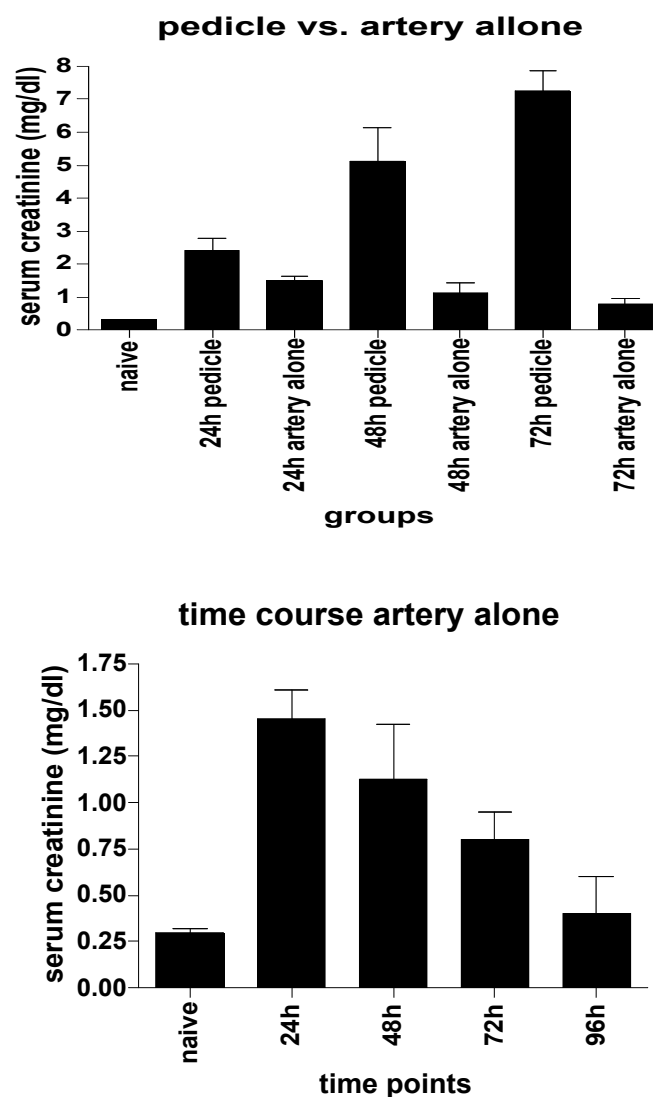
time course artery alone

Fig. 4.2 a+b: Serum creatinine levels of rats (n=8) in which the left renal pedicle (artery and vein) or the artery alone has been clamped for 45 minutes. The right kidney has been removed before clamping.

5.3   45 min clamping of the renal artery vs. 60 min clamping of the renal artery

To further evaluate if longer clamping of the renal artery would lead to more significant increase in creatinine levels, leading to a more reliable model, we conducted another series of experiments in which the renal artery was clamped for 60 minutes. However the injury set by 60 minutes of warm ischemia was so severe that kidney function did not recover. (Fig. 4.3)
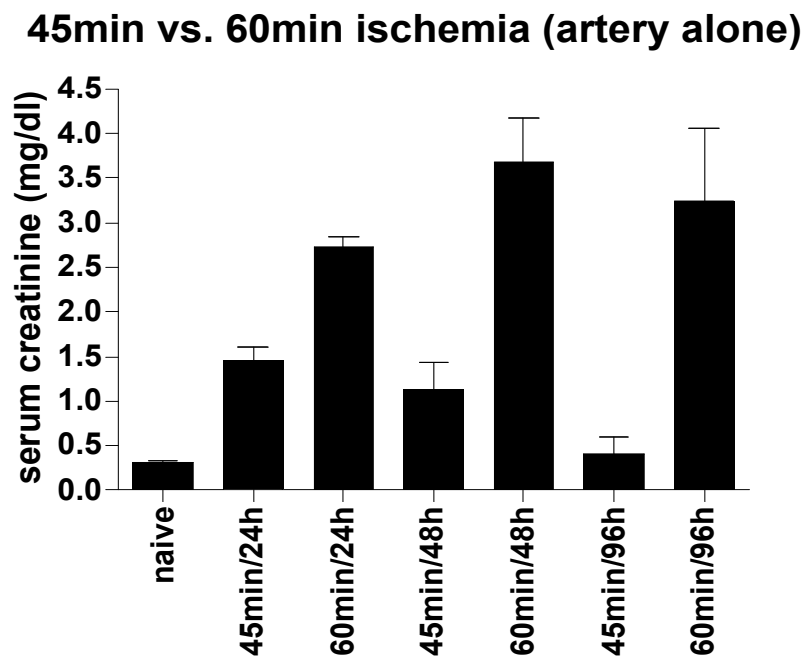

Fig. 4.3: Serum creatinine levels of rats (n=8) in which the left renal artery has been clamped for 45 or 60 minutes respectively. The right kidney has been removed before clamping.

5.4 Effects of biliverdin on kidneys after 45minutes of warm ischemia and 24h reperfusion

Since the injury caused by clamping of the artery for 45 minutes showed to bet the most reproducible model in which kidney function could recover, we decided to work with this model to conduct our dose studies to test the effect of biliverdin and bilirubin-dt on ischemia reperfusion injury. Clamping of the renal artery for 45 minutes led to a significant increase of creatinine levels and BV treatment significantly decreased creatinine serum levels in a dose dependent manner.
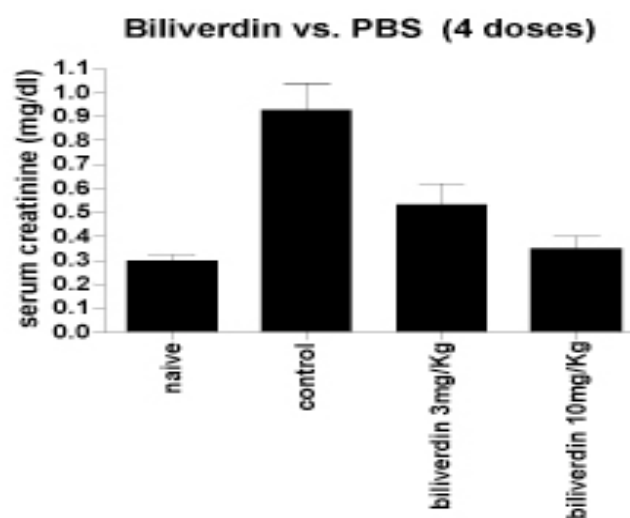

Fig. 4.4: Serum creatinine levels of rats (n=8) in which the left renal artery has been clamped for 45 minutes. The right kidney has been removed before clamping. Rats have been treated with biliverdin i.v. 15 minutes before clamping, 15 minutes before reperfusion, immediately thereafter and 10 hours after reperfusion.

5.5 Hematoxylin Eosin staining of kidney samples after 45min clamping of the renal artery

After 45 minutes of warm ischemia and 24h of reperfusion, kidneys where harvested and formalin fixed. Hematoxylin-Eosin staining of paraffin embedded kidneys was performed. Picture1 shows a kidney after 45min clamping of the artery alone and reperfusion over 24h. Picture 2 shows a representative picture of a kidney after biliverdin i.v. treatment at a dose of 17.5 mg/Kg 1h before clamping and shortly thereafter. (Magnification 20x).
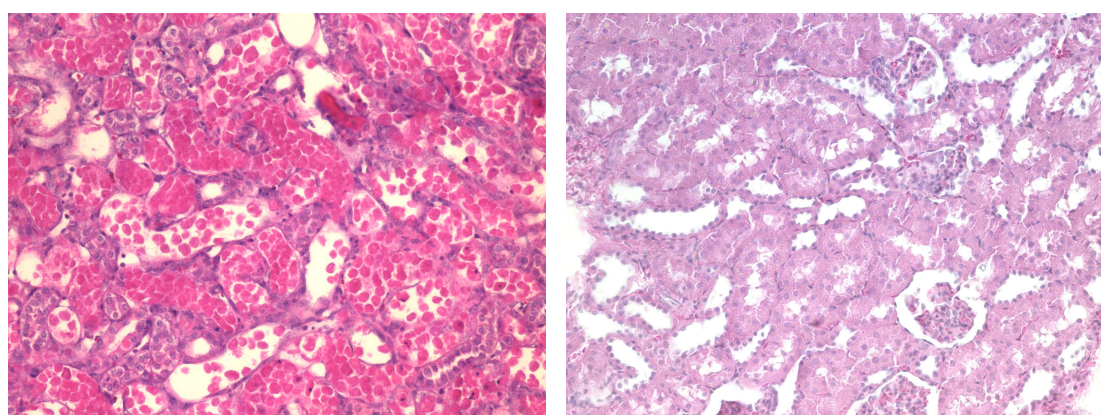

Fig. 4.5: Representative images of kidneys after 45 minutes of warm ischemia and 24h of reperfusion. The left image shows the vehicle control the right image the BV treated kidney.

5.6 Effects of bilirubin-ditaurine treatment after kidney transplantation after 18h of cold ischemia

To be able to test BR-dt treatment in a more clinical relevant model we decided to test the potential protective effect of BR-dt on IRI in a rat kidney transplantation model.

For this purpose orthotopic kidney transplantation was performed in male Lewis rats after 18h of cold ischemia and animals where treated with BR-dt at different doses. BR-dt treatment of transplanted rats resulted in a significant decrease of serum creatinine levels if compared to vehicle treated controls after 24 and 48h at a dose of 10mg/Kg administered in three intravenous injections (1h before, 15min before and 10 min after reperfusion). In addition the estimated glomerular filtration rate was significantly increased in the BR-dt treated group if compared to the vehicle treated control (Fig 4.6)

Fig.4.6: Serum creatinine levels after 24h of rats (n=6) in which renal transplantation was performed after 18h cold and 1h warm ischemia. Rats have been treated with 3 doses of 10mg/Kg Br-dt 1h before, 15min before and 10 min after reperfusion. The right kidney has been removed after reperfusion.

5.7   Effects of bilirubin-dt treatment after kidney transplantation after 18h of cold ischemia

Treatment of transplanted animals with BR-dt at a dose of 3mg/Kg respectively 30mg/Kg did not show beneficial effects against IRI, as demonstrated in serum creatinine levels, if compared to the vehicle treated control.
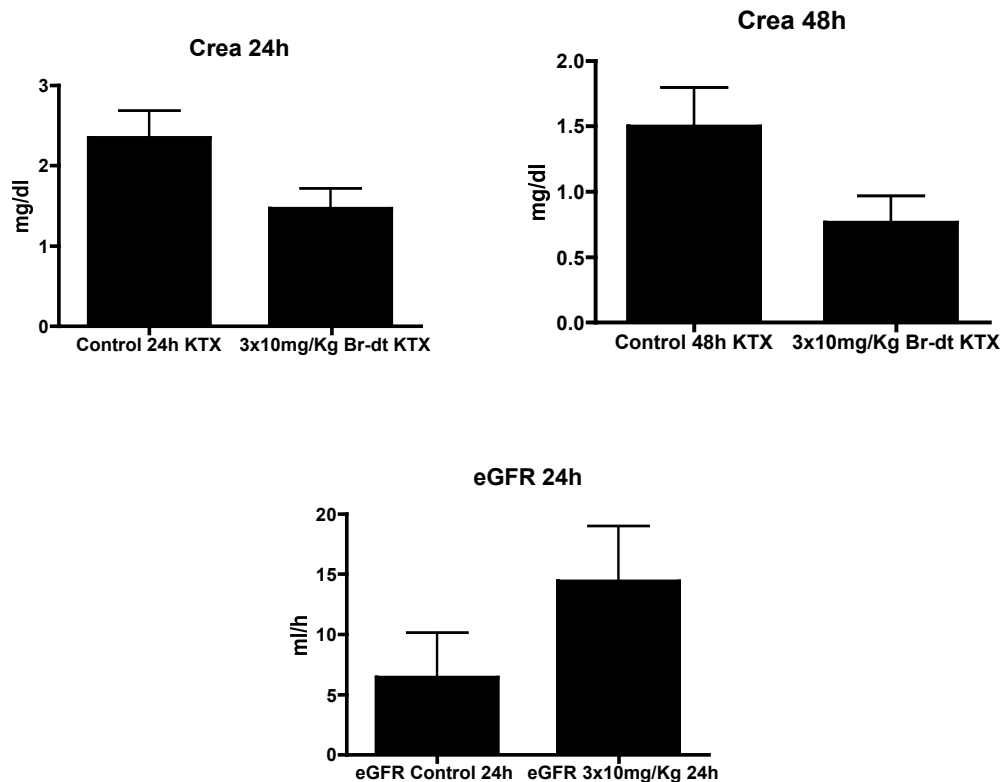
Fig.4.7: Serum creatinine levels after 24h of rats (n=6) in which renal transplantation was performed after 18h cold and 1h warm ischemia. Rats have been treated with 3 doses of 3mg/Kg Br-dt 1h before, 15min before and 10 min after reperfusion. The right kidney has been removed after reperfusion.

5.8   Effects of bilirubin-dt flush treatment after kidney transplantation after 18h of cold ischemia

If kidneys where flushed with 125μM BR-dt prior to transplantation no beneficial effect on IRI could be achieved if compared to the vehicle treated control.
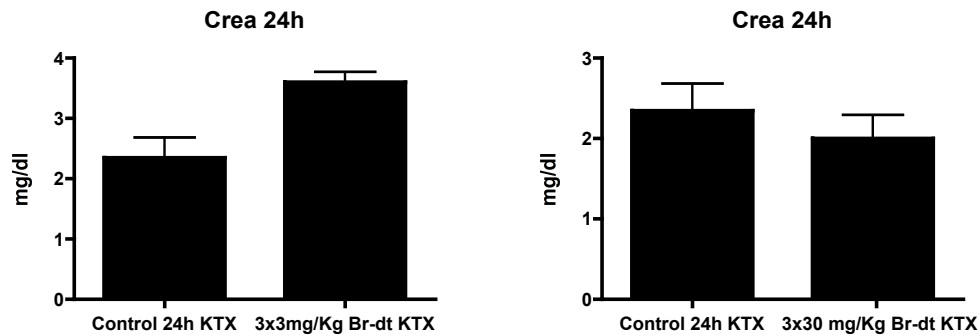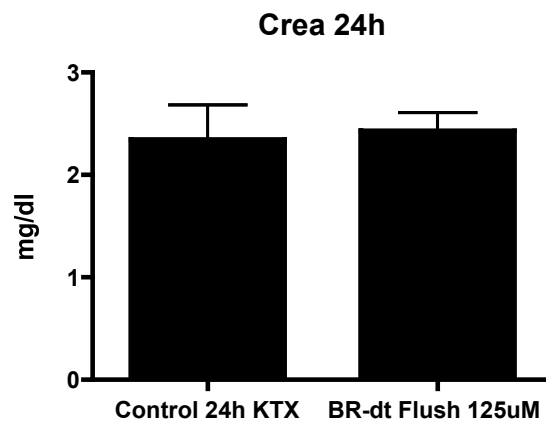

Fig. 4.8: Serum creatinine levels after 24h of rats (n=3) in which renal transplantation was performed after 18h cold and 1h warm ischemia. Kidneys have been flushed with 125uM BR-dt before transplantation. The right kidney has been removed after reperfusion.

6. Discussion

6.1. Discussion of the rationale

Transplantation of solid allogeniec organs requires life long intake of immunosuppressive drugs to prevent the occurrence of acute rejection episodes. Most centres are using immunosuppressive protocols in which a combination of calcineurin inhibitors and antiproliferative agents are successful combined with an induction therapy (e.g. anti-thymocyte globulin, IL2-antagonists). The potent immunosuppressive effect of those drugs leads however to severe side effects as a higher incidence of tumours and viral, fungal and bacterial infections.

Unfortunately the introduction of more potent immunosuppressive agents has not let to the expected improvement of long term outcome after solid organ transplantation. (Fig. 1.7).

Ischemia reperfusion injury occurs during the re-establishment of blood flow in the transplanted organ leading to an abrupt delivery of O_2. Since the graft was exposed to ischemia during the time of transplantation, the mitochondrial electron transport chain cannot instantly use O_2, leading to an accumulation of O_2, which is now available for oxidative enzymes, consequently leading to the formation of reactive oxygen species (ROS). These ROS can lead to DNA damage, oxidative modification of proteins and lipid peroxidation, which show cytotoxic effects.

The severity of ischemia reperfusion is held responsible for delayed graft function (DGF) of the transplanted organ as well as the long-term outcome after kidney transplantation.

Aim of our study was to investigate the potential protective effects of BR in a rodent model of kidney transplantation.

6.2. Discussion of the model and methods

In our experimental setup we decided to work with two different animal models of ischemia reperfusion injury. The kidney clamp model was chosen because of its easy technical feasibility and its high reproductivity. With this model a dose study was

performed in order to find the right protective dose regimen to be tested in the clinical more relevant kidney transplantation model.

6.2.1 Warm ischemia reperfusion model of the rat:

In the first experimental setting clamping of the whole renal pedicle was performed. This experimental setting however led to a severe damage of the kidney and animals didn't recover from surgery after 76h due to acute renal failure.

If however only clamping of the renal artery was performed, the increase in serum creatinine levels was not as severe and animals could recover from surgery. We think that the higher damage in the pedicle group can be explained by the occurrence of thrombosis in the clamped kidney, since venous outflow was clamped during the 45 minutes of warm ischemia.

If clamping of the renal pedicle was performed the kidney showed a dark blue colour after 45minutes of warm ischemia and the reperfusion period took much longer if compared to the artery alone group.

For this reason we decided to work with the ischemia reperfusion injury model of the rat, in which only the artery is clamped.

In our clamping data, 3 administrations of biliverdin in a dose of 10mg/Kg led to significant decrease of serum creatinine levels after 45 minutes of warm ischemia and 24h of reperfusion.

Encouraged by these findings we decided to work with this dose regimen to test the potential beneficial effects of bilirubin on ischemia reperfusion injury in a more clinical relevant model of kidney transplantation in the rat.

6.2.2 Kidney Transplantation Model:

Kidney transplantation is a well-established model in transplantation research and experimental microsurgery (73,74).

Fisher and Lee first described the heterotopic rat kidney transplantation model in 1965 (75) and the orthotopic technique was published in 1968 by Daniller et al. (76).

Kidneys were explanted 18h prior to transplantation and preserved in 4°C cold UW-solution. After this period of 18h cold ischemia kidneys were transplanted orthotopically into the recipient rat and the warm ischemic time was kept constantly at 60 minutes. Serum concentrations of creatinine and BUN were taken at different time points.

Serum creatinine levels, serum BUN levels were significantly decreased if compared to the untreated control if animals were treated with 3 injections of 10mg/kg Biliruibn-dt.

Lower (3x3mg/Kg) or higher (3x20mg/Kg) doses of Bilirubin-dt administration could not achieve protection against the caused ischemia reperfusion injury.

Encouraged by earlier findings in our laboratory that bilirubin rinse of transplanted hearts showed beneficial effects against ischemia reperfusion injury (unpublished data) we tested this treatment regimen in our kidney transplantation model. As demonstrated in Fig. 4.8 bilirubin-dt rinse of the transplanted kidney prior to transplantation at a dose of 125µM did not protect the organ from ischemia reperfusion injury.

*6.*3 Bilirubin and HO-1:

The heme oxygenase system is the rate-limiting step in the conversion of heme into free iron, carbon monoxide and biliverdin.

HO-1 is activated by cellular stress such as inflammation, ischemia, radiation or hypoxia (68) and is known to play a crucial role in maintaining oxidant/antioxidant homeostasis during cell injury (69).

In 1987, Stocker et al. was the first to introduce the idea that BR is one of the most important endogenous antioxidant in the serum (70). In subsequent conducted studies it could be demonstrated that induction of HO-1 leads to BR-mediated reduction of oxidative stress in animal models of renal ischemia (71) and cardiac ischemia (72).
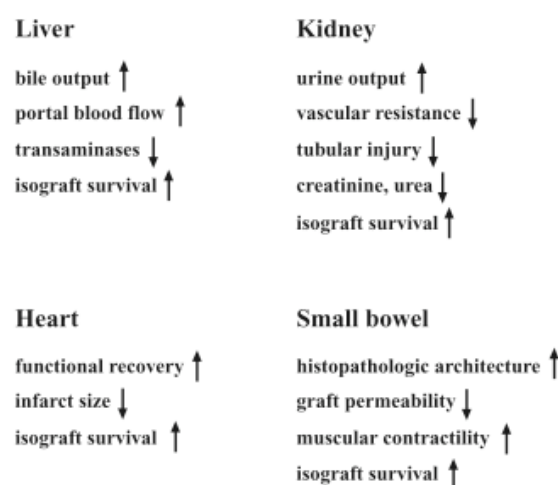

Fig. 4.9: Effects of bilirubin and biliverdin in animal models of IRI

The chemical induction of HO-1 to improve kidney function after ischemia reperfusion injury has been the target of many ongoing and already published data. Those could demonstrate that pharmacological induction of HO-1 led to a better functional and structural outcome after ischemia reperfusion injury to the kidney. In contrast inhibition of HO-1 led to more severe injury after reperfusion (77).

The protective actions of HO-1 induction during ischemia reperfusion injury are realized via its downstream products CO, Biliverdin and Free iron. Those molecules effectuate their beneficial actions through inhibiton of apotosis, the scavenging of free radicals and the maintenance of medullary blood flow. Moreover leukocyte recruitment and platelet aggregation are inhibited (Fig 5.0).
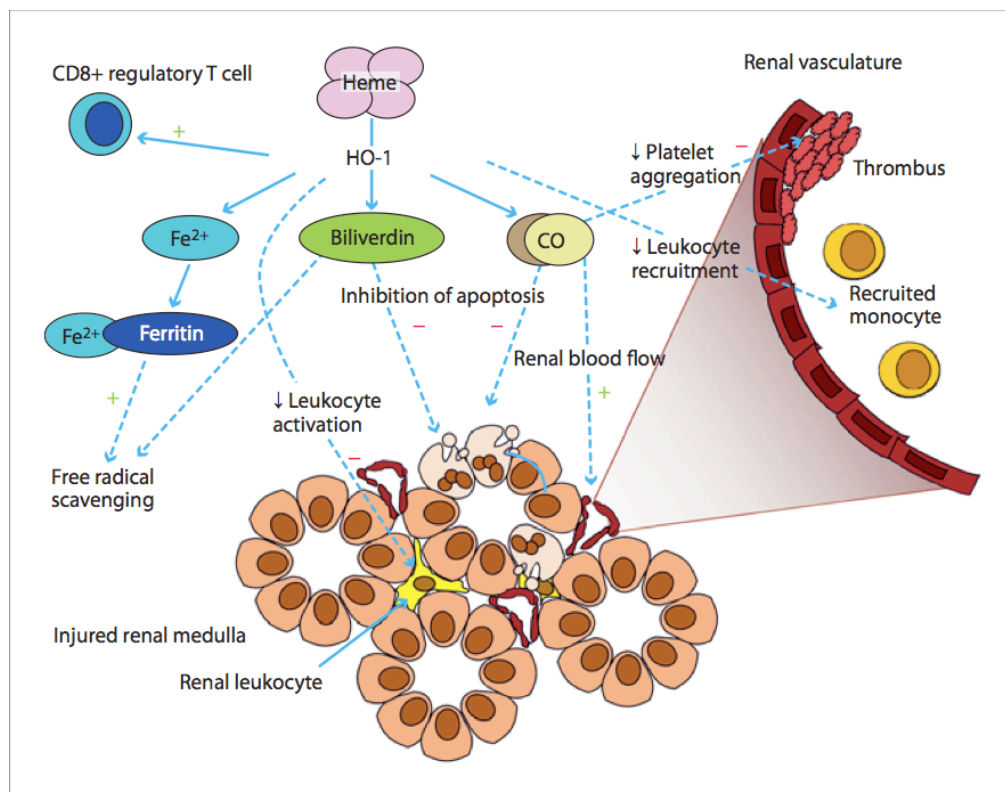

Fig. 5.0: Putative actions of HO-1 during ischemia reperfusion injury. (78)

6.4. Exogenous administration of Bilirubin leads to improved graft function after 18h of cold ischemia .

Aim of our study was to examine if exogenous administered Biliverdin/Bilirubin led to similar protective effects on transplanted kidneys that have been exposed to ischemia reperfusion injury.

Kirkby et al. tested the effect of intravenous administered bilirubin on ischemia reperfusion injury in the rat. His group clamped both renal pedicles for 30min and analyzed the potential protective effect of bilirubin administration after 6h of reperfusion. They could not observe a beneficial effect if bilirubin was administered at a dose of 5mg/Kg respectively 20mg/Kg 1h before and during ischemia (51). One explanation for these negative results could be the tested model. As mentioned above, clamping of the renal pedicle for 45minutes led to a severe injury of the tested kidney and the animals did not recover from surgery, suggesting, that the caused damage was to severe.

In our model of warm ischemia reperfuison injury, if only the artery alone was clamped, exogenous bilirubin administration via i.p. injection led to significant decrease in serum creatinine levels after 24h of reperfusion showing an intact cell integrity of the treated kidney if compared to the control group. (Fig 4.4, Fig 4.5).

In summary we could show that exogenous, systemic administration of bilirubin-dt could achieve an improved kidney function after 18h of cold ischemia and isogenic kidney transplantation.

One explanation for the observed protective effect of bilirubin administration could be its potent antioxidant effect. However more research needs to be conducted to understand and explain the underlying mechanism by which bilirubin exerts its protective effects.

Moreover, bilirubin needs to be tested in an allogenic kidney transplantation model in order to test the potential protective effects of bilirubin in a more clinical relevant model.

7. Abstract:

Background:

Heme oxygenase-1 (HO-1) is the rate-limiting enzyme in the conversion of heme into biliverdin, carbon monoxide (CO) and free iron. Biliverdin is then subsequently reduced to bilirubin by the enzyme bilverdin reductase. In the past decades a lot of effort was conducted to investigate the beneficial effects of HO-1 and its end products biliverdin/bilirubin and CO.

Due to intensive research, solid organ transplantation can nowadays be seen as clinical routine. However ischemia reperfusion injury (IR), acute rejection episodes and the occurrence of chronic rejection remain main problems.
The severity of IRI can be seen as a prognostic factor for early graft function, immunogenecity of grafts as well as for long term graft survival.
The goal of our experiments was to investigate the potential beneficial effects of bilirubin and biliverdin on ischemia reperfusion in a kidney transplantation model of the rat.

Methods:

Two different sets of experiments were performed:
First, kidneys of Lewis rats were exposed to 60 minutes of warm ischemia by clamping the renal artery followed by a 24h reperfusion period. This model was used to find the optimal dosing regimen of bilirubin/biliverdin before the more clinical relevant model of kidney transplantation in the rat was performed. We found that three doses of 10mg/Kg bilirubin were the most effective dose regimen to protect kidneys from ischemia reperfusion injury.
In the second set of experiments, kidney transplantation was performed in Lewis rats. Kidneys were harvested and stored in 4°C cold UW-solution for 18h. Subsequently the kidneys were transplanted isotopically into the recipient rat. Time of warm ischemia was kept in all experiments constantly at 60 minutes. After 24h of reperfusion tissue samples and serum were harvested for further analyses.

Results:

Systemic treatment of bilirubin led to a significant amelioration of organ function after ischemia reperfusion injury as assessed by measuring serum creatinine levels and BUN levels after 24h of reperfusion. In addition treated animals showed increased eGFR and a better cell integrity as histomorphological analyses could demonstrate.

Conclusion:

Systemic treatment with bilirubin and bilverdin has beneficial effects on graft function after ischemia rerperfusion injury.

Hintergrund:

Heme oxygenase-1 (HO-1) ist das geschwindigkeits- bestimmende Enzym bei der Umwandlung von Häme zu Biliverdin, Kohlenmonoxid und freiem Eisen. Biliverdin wird dann in einem folgendem Schritt durch das Enzym Biliverdin-Reduktase in Bilirubin umgewandelt. In den letzten Jahrzehnten wurde intensive Forschung betrieben um die protekitven Eigenschaften von HO-1 und seiner Endprodukte Biliverdin/Bilirubin und Kohlenmonoxid zu untersuchen.

In der heutigen Zeit kann man die Transplantation von soliden Organen dank großer Forstschritte auf dem Gebiet der Immunsupression und durch die Verbesserung chirurgischer Techniken als klinische Routine ansehen. Trotzdem bleiben weiterhin große Probleme bestehen, wie akute Abstoßungsreaktionen sowie das Auftreten der chronischen Abstoßung und des Ischämie Reperfusionsschadens.
Die Schwere des Ischämie Reperfusionsschadens dient als prognostischer Faktor für die frühzeitige Organfunktion als auch für das Langzeitüberleben des Transplantats.
Ziel dieser Arbeit war es, den potentiell protektiven Effekt von Bilirubin und Biliverdin in einem Ischämie-Reperfusionsmodell zu testen.

Methoden:

Nieren von Lewis Ratten wurden 60 Minuten warmer Ischämie ausgesetzt, indem die Nierenarterie geklemmt wurde. Dieses Modell diente der Erforschung der optimalen Dosis von Bilirubin und Biliverdin bevor das aufwendigere und klinisch relevantere Modell der Nieren Transplantation in der Ratte durchgeführt wurde. Eine Dosis von insgesamt 30mg/Kg administriert in 3 Einzeldosen a 10mg/Kg stellte sich als die effektivste Therapie dar.
Für das Nierentransplantationsmodel wurden Nieren von Lewis Ratten explantiert und für 18h in 4°C kalter UW-Lösung präserviert. Hiernach wurden die Nieren in unten beschriebener Technik in die Empfängerratte transplantiert. Nach 24h Reperfusion wurden Gewebeproben sowie Serum für die Durchführung weiterer Untersuchungen entnommen.

Resultate:

Die systemische Behandlung mit Bilirubin führte zu einer signifikanten Besserung der Organfunktion nach Ischämie Reperfusion wie wir durch die Bestimmung der Serumkreatinin- und Serumharnstoff-Werte demonstrieren konnten. Außerdem führte die Behandlung mit Bilirubin zu einer signifikant gesteigerten eGFR sowie einer Erhaltenen Zellintegrität, wie histologisch gezeigt werden konnte.

Schlussfolgerung:

Systemische Behandlung mit Bilirubin und Bilivedin zeigt protektive Effekte auf die Organfunktion nach Ischämie-Reperfusionsschaden.

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