Thiamine, Fasting and the Kidney

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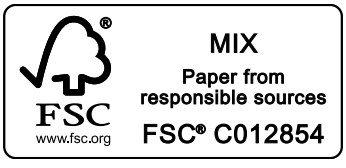

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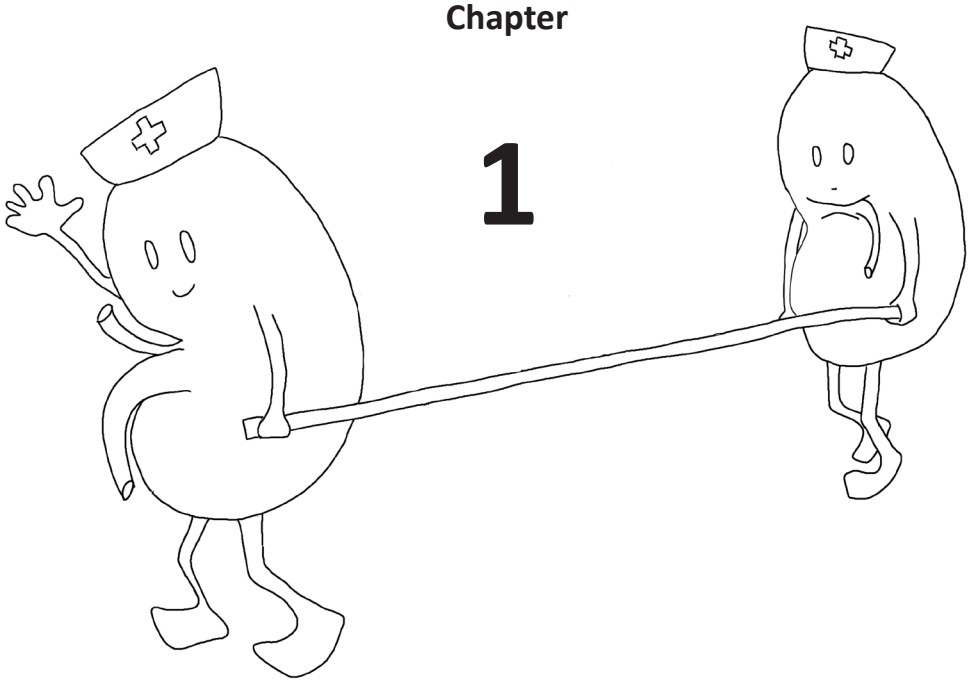
1

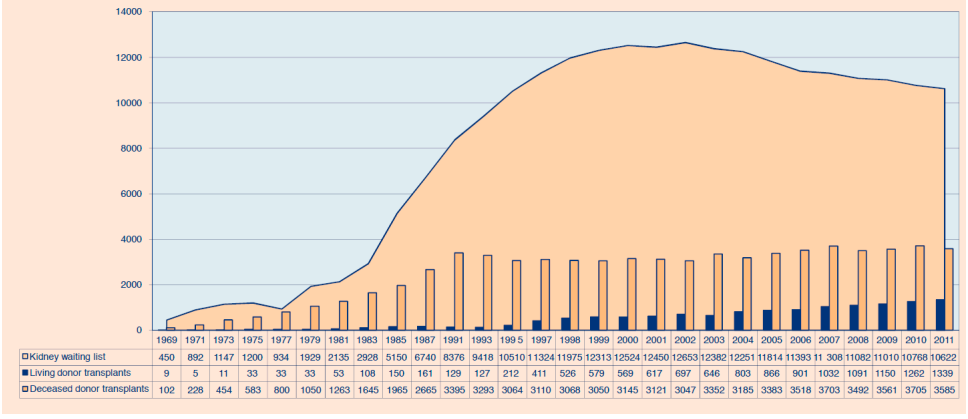


Introduction and Aim

Introduction

Kidney transplantation

Kidney transplantation is a life saving therapy for patients with renal failure. Patients who receive a new kidney live longer and have better quality of life compared to those on organ replacement therapy(1,2). Moreover, kidney transplantation is more cost-effective than organ replacement therapy(3-5). In the Eurotransplant zone the median age of deceased donors increased dramatically and linearly over the past twenty years(6). After 1998, the number of patients on the waiting list stabilized and steadily declined after 2004 (Figure 1). Median waiting time to transplantation has also decreased after 2008; although it was still more than four years. For every 10 transplanted patients, one patient will die while waiting for a kidney transplantation, indicating the need for donor organs.



	1969	1971	1973	1975	1977	1979	1981	1983	1985	1987	1991	1993	199 5	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011
Kidney waiting list	450	892	1147	1200	934	1929	2135	2928	5150	6740	8376	9418	10510	11324	11975	12313	12524	12450	12653	12382	12251	11814	11393	11 308	11082	11010	10768	10622
Living donor transplants	9	5	11	33	33	33	53	108	150	161	129	127	212	411	526	579	569	617	697	646	803	866	901	1032	1091	1150	1262	1339
Deceased donor transplants	102	228	454	583	800	1050	1263	1645	1965	2665	3395	3293	3064	3110	3068	3050	3145	3121	3047	3352	3185	3383	3518	3703	3492	3561	3705	3585

Figure 1. Dynamics of the Eurotransplant kidney transplant waiting list and transplants between 1969 and 2011

The worldwide increasing demand for donor organs has resulted in a gradual shift towards acceptance of suboptimal donor organs from deceased donors. These donors are referred to as expanded criteria donors, defined as any donor aged ≥ 60 years or a donor aged 50 to 59 years with two of the following features: pre-existing history of systemic hypertension, terminal serum creatinine level > 130 µmol/L, or death resulting from a cerebrovascular accident. The criteria for the definition of expanded criteria donors was based on the presence of variables that increased the risk for graft failure by 70% compared with a standard criteria donor kidney(7). Inherent to this definition is the worse outcome after transplantation when receiving an expanded criteria donor kidney than when receiving a standard criteria donor kidney. However, despite poorer outcomes, mortality is decreased when receiving a kidney from an expanded criteria donor compared to maintenance on dialysis therapy(1,8,9).

Approximately 70% of transplanted kidneys exhibit immediate function after transplantation, thereby ending further dependence of transplanted patients on renal replacement therapy(10). However, delayed graft function is an important problem. Around 30% of the grafts do not have immediate function and have to continue dialysis in the first week after transplantation. The end of the spectrum is primary non-function, which occurs in 2-15% of cases. On the long-term delayed graft function is an independent risk factor of graft loss and significantly shortens the half life of the kidney(11,12). Risk factors of delayed graft function include the use of kidneys from non-heart beating donors, necessity for inotropic support of the circulation of the donor, donor age > 55 years and co-morbidity of the donor caused by diabetes and hypertension(10). Also, if the donor has to stay more than 5 days on the intensive care unit before donation the risk for delayed graft function is increased(13,14).

Ischaemia-reperfusion injury

Ischaemia-reperfusion injury is inevitable to transplantation. Ischaemia starts during brain dead and/or decreased cardiac output or harvest of the organ in case of a living kidney donation. Long-term hypothermic kidney storage prior to transplantation adds to ischaemic tissue damage(15). Reperfusion injury develops in thehours or days after the initial insult. Repair and regeneration processes occur together with cellular apoptosis, autophagy and necrosis(16). Interventions in the process of ischaemia-reperfusion injury can already be started before organ recovery by donor pre-treatment and during preservation(17-19).

After ischaemia, a switch to the anaerobic glucose metabolism pathway occurs within minutes. Anaerobic metabolism generates only a minimal amount of high-energy posphates, which is definitely insufficient to meet the demands of aerobic tissues(20). Low-energy phosphates are permeable to the cellular membrane and thereby escape to the extracellular compartment. This results in deprivation of substrate for the synthesis of high-energy phosphates even when there is restitution of the blood flow. Free radicals are formed during global ischaemia, when small amounts of oxygen are available.

After reperfusion injury, repair and regeneration processes occur together with cellular apoptosis, autophagy and necrosis. As apoptosis needs energy, it occurs mostly upon reperfusion. The rapid burst of free radicals shortly following reperfusion is a well-documented phenomenon(21). Mitochondria are the gatekeeper to cellular injury during reperfusion, because they are the source of free radical production as well as a source of radical-scavenging potential.

Thiamine

Thiamine is a B-vitamin (vitamin B1) naturally present in whole grains and vegetables. It is highly soluble in water and has a slight thiazole odour with a bitter taste. Thiamine is not synthesized in mammalians and hence it is an essential micronutrient(22). Thiamine is phosphorylated to thiamine monophosphate, thiamine pyrophosphate and thiamine

triphosphate. Thiamine pyrophosphate is the 'active' thiamine co-enzyme for at least three enzymes involved in glucose metabolism. These are transketolase, pyruvate dehydrogenase, and α-ketoglutarate dehydrogenase(23-25). These enzymes play a role in both the regeneration of reduced glutathione (from oxidized glutathione) as substrate for anti-oxidant enzymes and the regeneration of adenosine triphosphate (ATP) for maintenance of energy-requiring metabolic processes(26-30). Thereby thiamine is crucial for optimal regeneration of ATP and reduced glutathione in cells. Both processes are crucial in ischaemia-reperfusion injury, especially during reperfusion when the tissue demands of ATP and reduced glutathione are particularly high, in order to counterbalance events as acute cell swelling and production of oxygen free radicals(26,27). Existence of tissue thiamine deficiency during reperfusion may therefore be an important determinant of the occurrence of acute tubules necrosis and concomitant delayed graft function in kidney transplantation.

Subclinical thiamine deficiency is also thought to play a role in diabetic nephropathy. It is shown that plasma thiamine is lower to that of controls in both type 1 and type 2 diabetes(31). In experimental diabetes thiamine and benfotiamine, a thiamine monophosphate pro-drug, supplementation reversed increased diuresis and glucosuria without changes in glycemic status and also corrected dislipidemia(32). Moreover in diabetes accumulation of triosephosphate is a potential trigger for biochemical dysfunction leading to the development of diabetic complications, such as diabetic nephropathy, neuropathy and retinopathy(33). This may be prevented by disposal of excessive triosephosphate via the reductive pentosephosphate pathway. In mild thiamine deficiency, as in diabetes, this pathway is impaired by decreased expression and activity of transketolase. In experimental diabetes correction of thiamine deficiency restored disposal of triosephosphates. This prevented multiple mechanisms of biochemical dysfunction: activation of protein kinase C, activation of the hexosamine pathway, increased glycation and oxidative stress(34). Thereby thiamine supplementation could prevent diabetic complications.

This thesis primarily focuses on thiamine deficiency and supplementation in ischeamia-reperfusion injury and diabetic nephropathy. As we unexpectedly found a protective effect rather than a detrimental effect of thiamine deficiency when thiamine deficiency was complicated with weight loss, we surmised whether it might have been the weight loss that exerted protective effects.

Fasting

Nutrition around surgery is a recurrent issue in experimental and clinical research related to the safety of anaesthesia on the one hand and the metabolic response to surgical trauma on the other hand. Studley was the first in 1936 to report a negative correlation between (excessive) preoperative weight loss and surgical outcome following major abdominal and thoraric surgery(35). The appreciation that a large number of hospitalized patients suffer from undernutrition has further fuelled the attention paid to preoperative and postoperative

feeding. Although the literature is replete with studies showing the adverse effects of fasted state for surgical patients, older studies as well as recently published studies indicate that different types of dietary restriction in well-nourished patients may in fact be beneficial as a way of protecting against acute organ stress(36). Dietary restriction can be performed by means of different regimens such as fasting (no food intake), alternate day fasting and caloric restriction (reduced daily caloric intake).

Many studies have demonstrated that substantial reduction of food intake to 30-60% below ad libitum intake levels can increase lifespan in a wide variety of species(37,38). Moreover caloric restriction has been shown to decrease the incidence and age of onset of many age-related diseases, increase resistance to toxicity and stress and maintain function at more youthful-like states compared to controls eating at libitum or near at libitum levels. Also studies in nonhuman primates have indicated that many physiological responses in monkeys parallel those observed in rodents on caloric restriction(39). In addition, primate studies relating several of those responses to markers of disease, such as blood lipids and hormones, also indicate a reduced incidence of chronic disease, such as heart disease and diabetes. Recently, it has been shown that caloric restriction in nonhuman primates decreases the percentage of animals suffering from age-related disease, as well decreases age-related mortality. Perhaps the most compelling feature of the animals is their appearance at old age (see Figure 2)(39).

The effect of caloric restriction points towards the hormesis hypothesis, which states that caloric restriction imposes a low level of stress on the organism that in turn activates stress responses providing protection against a variety of aging processes. The concept of hormesis is derived from toxicology where the term implies that small doses of a toxin might have long-term beneficial consequences as a means of conditioning the organism toward enhanced stress responses. Such a concept would be in tune with an evolutionary perspective provided by the ‘soma theory of aging’, which proposes that when faced with low energy availability an organism must shift its energy investment away from growth and reproductive processes to energy investment in somatic maintenance and repair. This hypothesis would predict that it is adaptive for the organism under caloric restriction to use available energy to enhance its protection against stress. It is well established that caloric restriction induces stress, manifested as higher levels of circulating glucocorticoids(40).

Also in various experimental models of kidney disease and injury has caloric restriction shown to be protective. Caloric restriction prevents glomerular enlargement, podocyte hypertrophy, proteinuria and glomerulosclerosis in aging rats(41). Moreover caloric restriction attenuated the increased susceptibility of aged rats to renal ischaemic injury *in vitro* and *in vivo*(42,43). The length of time on a restricted diet required for the onset of increased stress resistance is not well characterized in any organism. In mice a three day fasting period before renal ischaemia-reperfusion injury led to increased protection compared to one day fasting period(43).

The effects of caloric restriction could partly be explained by the fact that caloric restriction has been shown to reduce lipid peroxidation and protein oxidation, down regulation of the inflammatory response and upregulation of several metabolic pathways as fatty acid synthesis, mitochondrial fatty acid β-oxidation, glycolysis and gluconeogenesis(44).
In humans there is very little data on caloric restriction. But in overweight patients with chronic proteinuric nephropathies a low-calorie normoprotein diet associated with moderate weight loss reduced proteinuria(45).

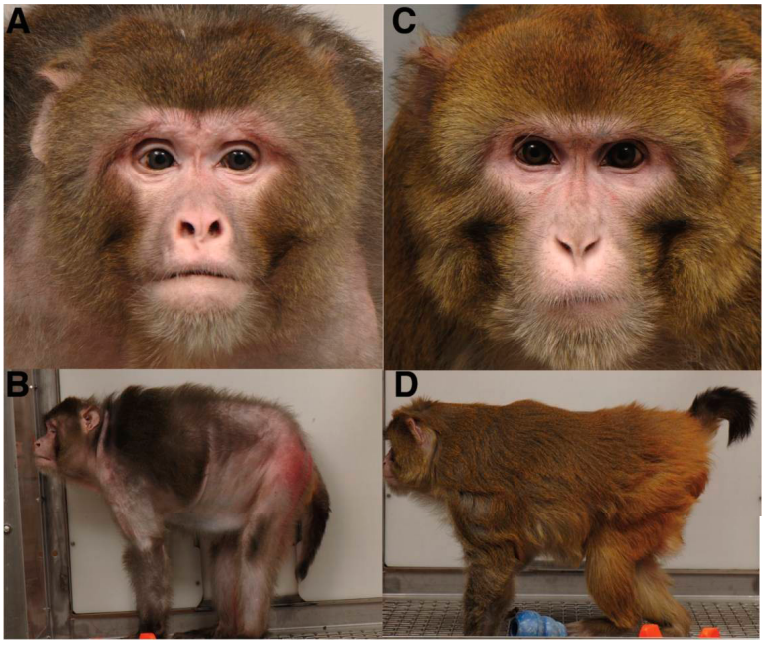


Figure 2. Colman, R.J. et al. Science, 2009: 325, 201-204
Animal appearance in old age
A and B: Control animal at 27.6 years of age (average life span).
C and D: Age-matched animal on caloric restriction.

Non-esterified fatty acids and malondialdehyde

During a period of fasting metabolism will switch from using glucose as a primary energy source to using fat as a primary energy source. Fat reserves are released in the blood. Thereby fatty acid levels in blood will rise during starvation. However non-esterified fatty acids are deemed to be detrimental in kidney injury, especially in proteinuric models. Albuminuria is a well characterized and valued risk factor for progression of kidney injury in different types of diseases. Albumin carries various substances, including fatty acids. Even in non-proteinuric rats albumin is filtered in the kidney and is followed by rapid endocytosis into proximal tubule cells(46). Thereby in proteinuric and non-proteinuric patients proximal tubule cells will be exposed to substances that are carried by albumin such as fatty acids.

It has been argued that not albumin rather than the fatty acids bound to albumin are toxic to proximal tubule cells and thereby underlie deterioration of the renal interstitium. This is supported by *in vitro* as well as *in vivo* data(47-55). The model used in the *in vivo* studies is a protein overload model in which albumin is injected in rats intra-peritoneal. However in Axolotls, an amphibian animal in which the kidney has open and closed nephrons towards the peritoneal cavity, but also in rats it was showed that when comparing delipidated albumin with delipidated albumin-reloaded with fatty acids no significant renal effect was seen of the fatty acids(56). Axolotls were used because concern was raised whether fatty acids, which have a very short half life in the circulation, will reach the kidney after passing through the circulation(57-59). It was suggested that the delipidation procedure itself may have been responsible for observations of less renal injury after injection of delipidated albumin versus regular albumin in the previous mentioned studies.
When fatty acids, especially polyunsaturated fatty acids, are oxidized malondialdehyde is formed(60,61). Malondialdehyde is long known as an indicator of reactive oxygen species formation in ischaemia-reperfusion damage. Reactive oxygen species causes lipid peroxidation of cell and organelle membranes, thereby forming malondialdehyde. By means of this lipid peroxidation the structural integrity and capacity for cell transport and energy production is disrupted. High plasma malondialdehyde levels are increased in conditions associated with renal injury in focal segmental glomerulosclerosis, diabetic nephropathy and renal replacement therapy(62-65). After kidney transplantation malondialdehyde levels decrease. High malondialdehyde levels in the donor are correlated with delayed graft failure and acute rejection after kidney transplantation(66). Despite malondialdehyde levels decreasing after kidney transplantation they are still elevated in renal transplant recipients more than a year after kidney transplantation compared to levels in healthy controls(67-69). Thereby this is suggesting ongoing damage of reactive oxygen species. However, levels of non-esterified fatty acids are also correlated to malondialdehyde levels. Thereby malondialdehyde may not be merely an indicator of reactive oxygen species, but also be correlated to the nutritional status of the patient. Moreover it has been shown that high intake of polyunsaturated fatty acids and also exercise will increase malondialdehyde levels(70-74). Thereby high malondialdehyde levels could be a measurement of a healthy lifestyle as well.

Ketogenic diet

It is interesting that to mimic the metabolism of fasting in the 1920s a ketogenic diet was designed(75). The ketogenic diet was at that time introduced as a treatment for epilepsy. With the development of anti-epileptic drugs use of the ketogenic diet fell into disfavour. However, the ketogenic diet is still used as treatment for refractory epilepsia(76). The ketogenic diet constitutes of a very low proportion of carbohydrate and a high proportion of fat. Patient intolerance of the ketogenic diet as it is a high fat diet is a major contributor to therapeutic

failure. As a consequence of the high fat intake, the ketogenic diet has been reported to have high blood cholesterol, increased incidence of nephrolithiasis, and dilated cardiomyopathy as side effects(77,78). The ketogenic diet raised ketonebodies, especially β-hydroxybutyrate, as effective as fasting. B-hydroxybutyrate has been shown to increase metabolic efficiency, by increasing the energy of the redox span between site I and site II of the electron transport system. This results in increased energy release by the electron. Additionally ketone bodies can bypass a blockade of pyruvate dehydrogenase thereby providing an alternative source of mitochondrial acetyl-CoA(79,80). Another aspect of ketone bodies is their ability to oxidize co-enzyme Q. Since the half-reduced semiquinone of co-enzyme Q is a major source of mitochondrial free radicals, ketone bodies can decrease production of mitochondrial free radicals(79-81). Also ketone bodies reduce the mitochondrial nicotinamide adenine dinucleotide (NAD+) to NADH, thereby favouring reduction of glutathione and this results in reducing free radical production(82). The effect of the ketogenic diet on refractory epilepsia was even more pronounced when it was combined with caloric restriction.

Aim

The principle aim of this thesis was to investigate the role of thiamine deficiency in ischaemia-reperfusion injury. The alleged importance of thiamine deficiency in ischaemia-reperfusion injury and its role in delayed graft function are described in **chapter 2**. In **chapter 3** the development of thiamine deficiency in different tissues is studied. The effects of experimental thiamine deficiency on ischaemia-reperfusion injury are studied in **chapter 4**. In **chapter 5** we describe the results of a double-blind, randomized, placebo-controlled clinical trial on supplementation of benfotiamine, a thiamine derivate with a high bioavailability, in diabetic nephropathy. Driven by the unexpected protective effects of thiamine deficiency in **chapter 4** which we surmised to be associated with weight loss, we investigated the effects of fasting on renal disease in the second part of this thesis. In **chapter 6** we therefore studied the effects of fasting on renal ischaemia-reperfusion injury. The association of non-esterified fatty acids and malondialdehyde on graft failure in a stable renal transplant recipient cohort were investigated in **chapter 7** and **chapter 8** respectively. On the basis of these studies the effect of caloric restriction, as a prolonged equivalent of fasting, was studied in a proteinuric rat model in **chapter 9**. In that chapter we also tested the hypothesis that the effect of caloric restriction is mediated by ketogenesis.

In **chapter 10** in English and in **chapter 11** in Dutch the results of the studies are summarized, the implications of these findings and future approaches to conduct further studies are discussed.

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Chapter

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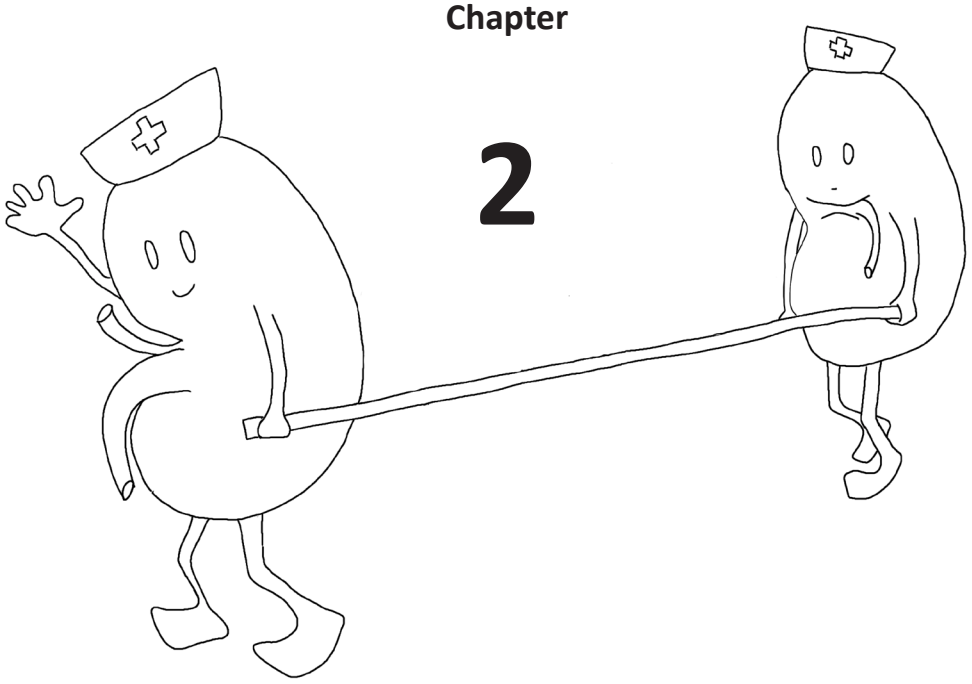

Tissue Thiamine Deficiency as Potential Cause of Delayed Graft Function after Kidney Transplantation

Thiamine Supplementation of Kidney Donors may Improve Transplantation Outcome

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Abstract

Delayed graft function is an important medical problem after renal transplantation. It occurs in approximately 30% of cases, and is not only associated with more prolonged and complicated hospitalisation, but also with earlier graft loss on the long-term.

Delayed graft function is the consequence of acute tubular necrosis caused by ischaemia-reperfusion injury, with insufficiently opposed toxic effects of reactive oxygen species and insufficient ATP regeneration. An optimal tissue thiamine status is pivotal for scavenging of reactive oxygen species and regeneration of ATP.

There are several reasons to suppose that tissue thiamine availability is suboptimal in donor kidneys prior to reperfusion in transplantation. These reasons include a high prevalence of untreated thiamine deficiency at admission of donors to intensive care units, quick exhaustion of body thiamine stores during periods of non-feeding or inappropriate feeding during hospital stays of donors, and loss of the water-soluble vitamin into water-based organ preservation solutions.

We therefore hypothesize that a suboptimal tissue thiamine status is a cause of delayed graft function after renal transplantation, and that it can be prevented with thiamine supplementation.

Introduction

Renal transplantation is the preferred treatment for most patients with end stage renal disease, both in terms of quality of life and survival(1,2). Approximately 70% of transplanted kidneys exhibit immediate function, thereby ending further dependence of transplanted patients on renal replacement therapy by dialysis(3-9). Another 30%, however, exhibit delayed graft function, which is a form of acute renal failure that results in posttransplantation oliguria. Delayed graft function is usually defined as the necessity for continuation of dialysis in the first week after transplantation or beyond(6,10). In approximately 50% of the patients with delayed graft function, dialysis can be discontinued before the 10th day after transplantation, in approximately 33% between day 10 and 20 after transplantation, and after more than 20 days in 10–15% of cases(8).

Delayed graft function after kidney transplantation is not just a matter of dialysis until the organ starts functioning. The end of the spectrum of delayed graft function, primary non-function, is a medical disaster, which occurs in 2–15% of cases(8). In these cases, the whole transplantation procedure was not only in vain, but a re-transplantation is generally more difficult because of sensitization of the immune system and scar tissue. This threat of never functioning of the graft and necessity for continuation of dialysis associated with delayed graft function is usually not in keeping with expectations of patients and their social environment. This can be very burdening in a psychosocial sense. More direct medical consequences of delayed graft function are complicated post-transplant management, increased duration of hospitalisation, increased costs after transplantation and increased allograft immunogenicity with a higher risk of acute rejection episodes(8,9). It is, however, not only these early consequences, which make occurrence of delayed graft function a highly unwanted event. Delayed graft function also adversely affects long-term outcome after renal transplantation. It is acknowledged as an independent predictor of graft loss, with a 2.9 times higher risk of late graft loss for delayed than for immediate function(10). Its importance for long-term graft outcome is further corroborated by reports about half-lives of 11.5 and 12.9 years for kidneys with immediate function, compared with 7.2 and 8.0 years for those with delayed function, respectively(10,11).

Risk factors for DGF are multiple, and include kidneys from non-heart beating donors, necessity for inotropic support of the circulation of the donor, donor age > 55 years and co-morbidity of the donor caused by diabetes and hypertension(8). Also pre-donation intensive care unit stays > 5 days are a risk factor for delayed graft function(12,13).

We hypothesize that suboptimal donor tissue thiamine availability at the moment of reperfusion of transplanted kidneys is an important – and preventable – cause of delayed graft function. Reasons are set out below.

Why would thiamine availability be suboptimal in a donor kidney before transplantation?

Thiamine is a water-soluble B-vitamin (vitamin B1). The normal steady-state human whole body thiamine store is estimated at 30 mg(14). It is difficult to maintain these stores without continuous supplementation with thiamine from food or other resources(15). In fasting obese subjects, metabolism facilitated by thiamine becomes already compromised within a few days of absence of any intake(15). Such findings of subclinical thiamine deficiency are very common in patients at admission to intensive care units(16). This is quite imaginable because thiamine intake is often already marginal in the general population(17), and thiamine availability may be further compromised by increased requirements in conditions with increased metabolic rates, such as inter-current illnesses(16). It has been demonstrated that presence of biochemical thiamine deficiency at admission to an intensive care unit is associated with an almost 50% increase in mortality(16). Nevertheless, thiamine supplementation is not routinely given in intensive care units. It is furthermore unusual to feed potential donors during their stay at intensive care units, and if feeding is applied is it often inappropriate(18,19).

Intensive care patients – admitted with a subclinical biochemical thiamine deficiency and some worsening of this state during the days prior to donation – are the typical kidney donors. Low thiamine availability may in part explain why intensive care unit stays > 5 days of donors have been recognised as a risk factor for the occurrence of delayed graft function. Thiamine deficiency is also more prevalent in elderly people of in the general population than in younger ones(20). This is also consistent with the notion that old age of donors is a risk factor for delayed graft function.

A further cause of loss of thiamine from donor tissue may be the preservation of the organ prior to transplantation. Preservation solutions for cold storage and machine perfusion are water-based and do not contain thiamine. Thiamine is water-soluble, and may therefore diffuse from the organ into the solution during cold flushing prior to storage and during storage itself. It has indeed been demonstrated that substantial amounts of thiamine are lost by diffusion into the preservation fluid during cold storage of kidneys prior to transplantation(21). It is not known whether this is also true for machine perfusion, but losses may be even greater, because preservation solution volumes used for machine perfusion are larger than for cold storage, and equilibration of thiamine concentration with the whole volume of the solution may be facilitated by continuous recirculation of the solution through the organ by active pumping. Greater unopposed losses of thiamine during preservation by machine perfusion versus cold storage may be one reason why high expectations of machine perfusion as a superior method of storage have not been redeemed(22-24).

It is, in summary, highly likely that thiamine availability of donor kidneys is suboptimal at the time of implantation.

Why would suboptimal tissue thiamine availability pose a transplanted kidney at increased risk for delayed graft function?

It is intrinsic to transplantation that donor kidneys are exposed to a period of ischaemia prior to implantation(8). Ischaemia starves tissue of oxygen and nutrients and causes accumulation of metabolic waste products. The main biochemical changes are inhibition of oxidative metabolism, depletion of ATP and an increase in anaerobic glycolysis. The depletion of ATP shuts down Na^+-K^+-ATPase activity, resulting in increasing intracellular concentrations of sodium and swelling of cells(25). The anaerobic glycolysis results in accumulation of lactic acid, which subsequently translates into lowering intracellular pH, lysosomal instability and activation of lytic enzymes(8). Binding of transition metals to their carrier proteins is also inhibited, which leads to an increase in intracellular concentrations of free iron, which is a strong catalyst for reactions that generate oxygen radicals(26).

Transplantation cannot be performed without reinstitution of blood flow. This reperfusion activates a sequence of events that plays a pivotal part in the development of delayed graft function(8). Adequate recovery of ATP regeneration is an obvious prerequisite for prevention of cell death and concomitant organ dysfunction(25,27,28). Reperfusion produces reoxygenation, a return to aerobic metabolism including oxidative phosphorylation, and production of ATP. However, reactive oxgen species (ROS) are also generated in huge amounts in ischaemic tissues after reperfusion(26). Mitochondria are the most important source(27). Normally, mitochondria produce a small, but steady amount of superoxide as by-product of respiration. This superoxide is then converted by mitochondrial superoxide dismutase to hydrogen peroxide, and this in turn is reduced to water by glutathione peroxidase, using glutathione (GSH) as a substrate, which is converted to oxidized glutathione (GSSG). These naturally occurring antioxidant enzymes in the kidney counteract the cellular effects of oxygen free radicals under normal conditions(26,27). During reperfusion of ischaemic tissue, however, the protective ability of these scavengers can be overwhelmed by rapid generation of reactive oxygen species, which initiates lipid peroxidation of cell membranes, disruption of the cytoskeleton, loss of polarity of renal tubular epithelial cells, and cell death(25,29). All together, these phenomena result in the acute tubular necrosis (ATN) which is the anatomical substrate of delayed graft function.

As a co-enzyme of three enzymes involved in glucose metabolism (transketolase in the pentosephospate shunt, and pyruvate dehydrogenase and α-ketoglutarate dehydrogenase in the citric acid cycle), thiamine is crucial for the optimal regeneration of ATP and GSH in cells (Figure 1)(30-34). Thiamine deficiency compromises metabolic fluxes through the pentose phosphate shunt and the citric acid cycle, whereby it constrains regeneration of ATP form ADP and regeneration of reduced glutathione (GSH) from its oxidized from (GSSH) (30,31). Tissue demands of ATP and GSH, and requirements for regeneration of ATP from ADP and GSH from GSSH are particularly high at the moment of reperfusion, in order to

counterbalance such events as acute cell swelling and production of oxygen free radicals, respectively(25-27). Existence of tissue thiamine deficiency at the moment of reperfusion may therefore be an important determinant of the occurrence of ATN and concomitant delayed graft function in kidney transplantation.

Antioxidants such as ascorbic acid have been shown to be beneficial in ameliorating ischaemia-reperfusion injury, but not potent enough to really antagonise DGF(35). An intrinsic constraint of antioxidants like ascorbic acid is that only one oxygen free radical molecule can be scavenged by one antioxidant molecule. The promise of an indirect antioxidant like thiamine is that it facilitates regeneration of GSH (and ATP), whereby one molecule of thiamine can scavenge many oxygen free radicals(30).

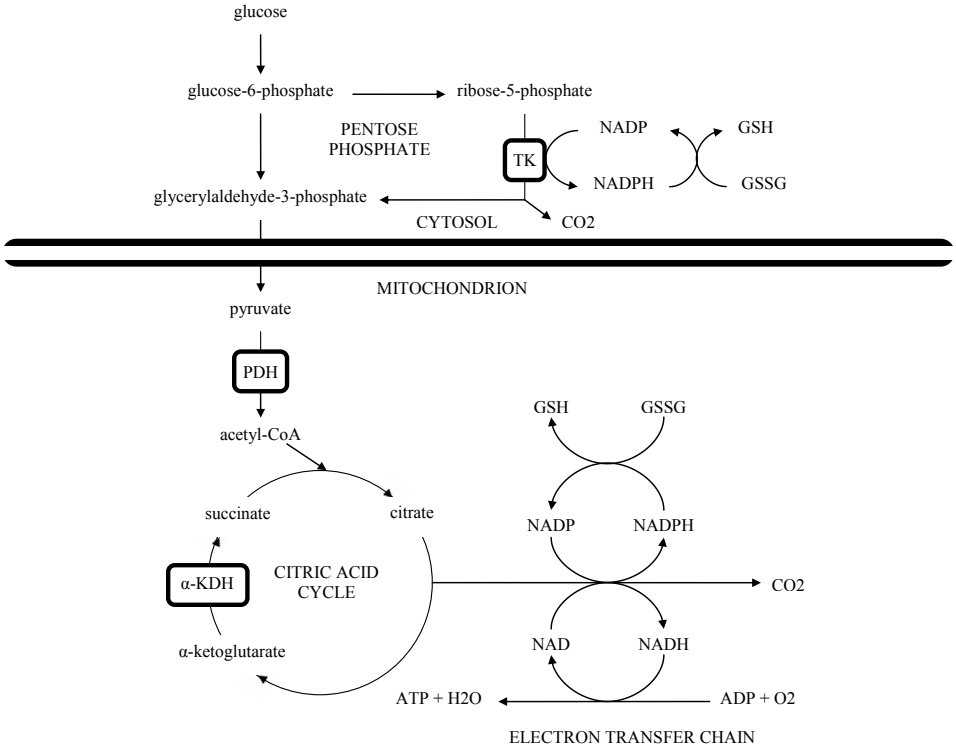

Figure 1. The critical role of thiamine availability in glucose metabolism and its linkage with ATP and GSH generation

If thiamine availability is suboptimal, the flux of glucose metabolites along the cytosolic enzyme TK (transketolase) and the mitochondrial enzymes PDH (pyruvate dehydrogenase) and α-KDH (α-ketoglutarate dehydrogenase) will become impaired. This will translate into a suboptimal capacity for regeneration of reduced glutathione (GSH) from oxidized glutathione (GSSG) and a suboptimal capacity for regeneration of ATP from ADP.

Evidence for a beneficial effect of thiamine in ischaemia-reperfusion injury

Thiamine has been shown to have a protective effect on hypoxia-induced cell death in cultured neonatal cardiomyocytes(36). After 24 h of hypoxia, the death rate of cultured neonatal cardiomyocytes was approximately 41.5% in the absence of addition of thiamine to the culture-medium, whereas the death rate dose dependently decreased to 20.6% with addition of 20 μM of thiamine. In a study in dogs, it was shown that after ligation of the left anterior descending coronary artery the amount of damaged tissue forming the border zone of myocardial infarction was reduced from 7.9% to 3.5% (P<0.02) by treatment with intra- aortic balloon pumping (IABP) in combination with treatment with high dose thiamine versus no treatment(37). It was not investigated whether this effect was due to the treatment with IABP, thiamine or both. One might think that the effect has to be attributed to the treatement with IABP, but later studies corroborate a role of thiamine(38,39). In these studies, it was shown that thiamine pyrophosphate supplementation alone had beneficial effects to ischaemic canine myocardium. It was, however, suggested that this was due to systemic hemodynamic effects of thiamine rather than effects of thiamine on metabolism(38).

The most compelling evidence for a beneficial effect of thiamine supplementation in prevention of ischaemia-reperfusion injury comes from a study with transient middle cerebral artery occlusion and reperfusion in rats(40). In rats with a normal baseline thiamine status, both acute (60 mg/kg of thiamine 30 min before application of ischaemia) and chronic (seven days of pretreatment with 2% of thiamine in the drinking water) supplementation resulted in a significant reduction in infarct size after 30 min of transient cerebral artery occlusion.

Conclusions

In conclusion, many donor kidneys may suffer from a suboptimal thiamine status before reperfusion. Thiamine is necessary for optimal cellular regeneration capacity of antioxidant GSH and ATP, which are both required for antagonism of ischaemia-reperfusion injury in tissues. Therefore, we hypothesize that suboptimal tissue thiamine availability during ischaemia-reperfusion of donor kidneys is an important determinant of DGF due to ATN after renal transplantation, and that supplementation of the donor will result in improved outcome.

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Chapter

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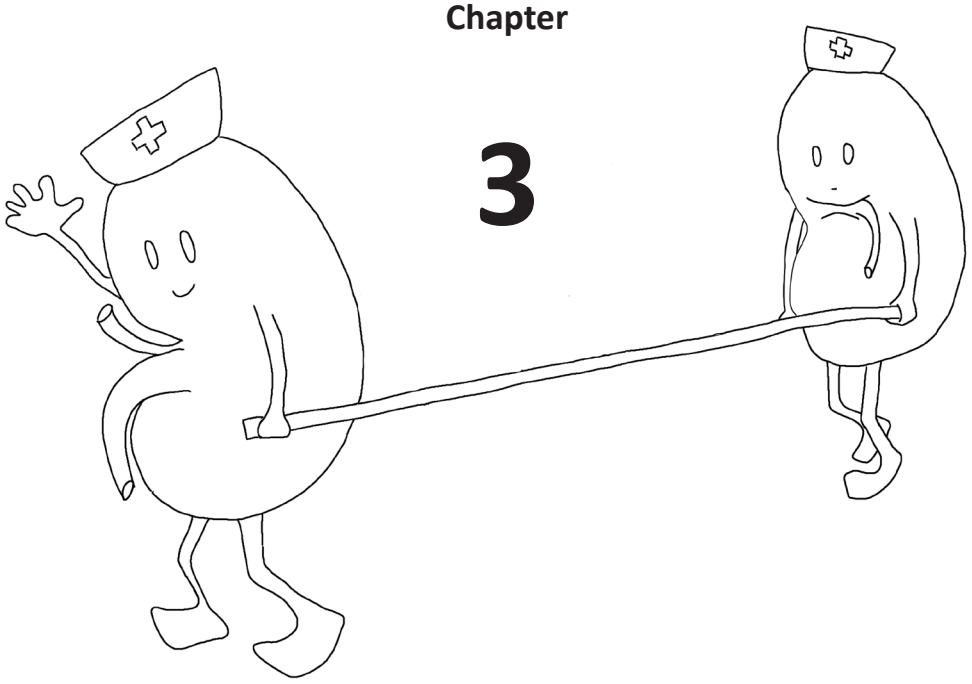

Are Brain and Heart Tissue prone to the Development of Thiamine Deficiency?

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Abstract

Thiamine deficiency is a continuing problem leading to beriberi and Wernicke encephalopathy. The symptoms of thiamine deficiency develop in the heart, brain and neuronal tissue. Yet, it is unclear how rapid thiamine deficiency develops and which organs are prone to development of thiamine deficiency. We investigated these issues in a thiamine deficient animal model.

Twenty-four male Lewis rats were fed a thiamine deficient diet, which contained 0.04% of normal thiamine intake. Six control rats were fed 200 µg of thiamine per day. Every week a group of six rats on the thiamine-deficient diet was sacrificed and blood, urine and tissue were stored. Blood and tissue transketolase activity, thiamine and thiamine metabolites were measured and PCR of thiamine transporter-1 (ThTr-1) was performed.

Transketolase activity was significantly reduced in red blood cells, liver, lung, kidney and spleen tissue after two weeks of thiamine deficient diet. In brain tissue, transketolase activity was not reduced after up to four weeks of thiamine deficient diet. The amount of thiamine pyrophosphate was also significantly conserved in brain and heart tissue (decrease of 31% and 28% respectively), compared to other tissues (decrease of ~70%) after four weeks of thiamine deficient diet. There was no difference between tissues in ThTr-1 expression after four weeks of thiamine deficient diet.

Despite the fact that the heart and the brain are predilection sites for complications from thiamine deficiency, these tissues are protected against thiamine deficiency. Other organs could be suffering from thiamine deficiency without resulting in clinical signs of classic thiamine deficiency in beriberi and Wernicke encephalopathy.

Introduction

Thiamine deficiency is well-known as a risk-factor for brain damage as a consequence of untreated Wernicke encephalopathy in chronic alcoholics(1-5). Chronic alcoholism hampers maintenance of adequate body thiamine stores by inhibiting uptake of thiamine from food in the gut and by promoting release in urine(1,2,6). Timely recognition and treatment of Wernicke encephalopathy with thiamine supplementation can acutely cure the delirant state and can prevent translation to permanent brain damage manifesting itself as Korsakoff syndrome(7,8). These are, however, the typical clinical entities, representing severe cases of thiamine deficiency. It is estimated that unrecognized milder atypical cases of Wernicke encephalopathy which translate into atypical cases of Korsakoff syndrome occur more often(8).

Post-mortem studies have demonstrated that many elderly people in nursing homes exhibit brain damage typical of having been inflicted by thiamine deficiency without a prior history of chronic alcoholism(6,9). However, thiamine deficiency is also under diagnosed in alcoholics(10-12). Many current guidelines recommend prophylactic adminstration of thiamine in chronic alcoholics in order to prevent the occurrence of Wernicke encephalopathy and subsequent brain damage. In response to these guidelines, many countries now add thiamine preventively to alcoholic beverages(6,13-16). However, such measures do not affect high risks groups, such as elderly people with low thiamine intake coupled with easy exhaustion of body stores, and therefore such populations remain at risk for thiamine deficiency(5,17,18).

Another predilection place for complications of thiamine deficiency is the heart, resulting in heart failure. Although the role of thiamine deficiency in heart failure has been reported in chronic alcholics (i.e., cardiac beriberi), thiamine deficiency may also be a contributing factor to heart failure in many cases in the general population(19-21).

Despite the fact that the heart and the brain seem predilection sites for complications from thiamine deficiency, it is not known whether these tissues are more susceptible to development of thiamine deficiency than others. In this study, we followed the course of biochemical and functional thiamine status in different tissues during the development of thiamine deficiency. The goal of our experimental design was to determine the organ specific vulnerability to thiamine deficiency and to chart the time course of the development of this deficiency.

Materials and Methods

Animals

The principles of laboratory animal care (NIH publication no. 85-23, revised 1996) were followed. All experimental procedures were approved by the Committee for Animal

Experiments of the University of Groningen. Nine to ten weeks old inbred male Lewis rats weighing 275 gram with a range of 25 gram (Harlan, Horst, The Netherlands) were used.

Thiamine deficient diet

The diet was obtained from Arie Blok (Woerden, The Netherlands). The diet contained 0.16 µg/kg thiamine derived from casein, which is 20% of the diet. This resulted in an intake of 0.04% thiamine relative to a normal diet.

Both the control (n=6) and the thiamine deficient groups (n=24) were fed the thiamine deficient diet. Control rats were supplemented with 200 µg thiamine in a 2.5% sucrose-solution on voluntary and oral basis. The rats in the thiamine deficient groups were fed 2.5% sucrose-solution without thiamine.

Experimental design

Thirthy male Lewis rats were individually housed allowing for daily determination of weight and food intake. In the first 14 days all rats were fed a thiamine deficient diet with supplementation of thiamine, thereafter rats were randomly assigned to the experimental groups (n=6 per group). The control group was sacrificed at time of randomization. The experimental groups were sacrificed after 1, 2, 3 or 4 weeks after randomization, receiving respectively 1, 2, 3 or 4 weeks of unsupplemented thiamine deficient diet.

Before sacrificing the animals, urine was collected for 24 hours and volume was assessed. Urine samples were snapfrozen and stored at -80 °C. After rats were anaesthesized with isoflurane, 250-IU heparin was perfused through the penile vein. This was followed by cannulation of the aorta and a 5 mL blood sample was taken. Plasma and red blood cells were separated and stored. After a full body flush of 40 mL 0.9% NaCl at 4 °C, in order to prevent red blood cells disturbing the transketolase and thiamine and thiamine metabolite measurements in the tissues, the organs were removed, snap frozen and stored at -80 °C.

Transketolase activity

Transketolase (TK) activity was measured by using Chamberlain's kinetic method(22). The reagents were purchased at Sigma Aldrich (United Kingdom).

Tissue homogenates (100-150 mg tissue homogenised in 500 µL 10 mmol/L Na_2HPO_4) and red blood cell membranes (sedimented by centrifugation, 20000 g, 30 min) were prepared for the measurements. The absorbance at 340 nm was monitored in intervals of 20 minutes for 2 h and the rate of linear decrease in the absorbance was used to deduce the rate of oxidation of NADH, which is limited by the TK activity.

When the substrate ribose-5-phosphate (R-5-P) is taken away from the assay cocktail, the reaction should run to a minimum. This was the case for all tissues, except for heart tissue, in which the reaction proceeded even in the absence of R-5-P (data not shown). Therefore it was considered that TK activity could not be measured in heart tissue.

Thiamine and phosphorylated metabolites

Thiamine pyrophosphate (TPP), thiamine monophosphate (TMP) and thiamine (THM) were determined by HPLC with fluorimetric detection after pre-column derivatization of thiamine and its phosphate esters to their respective thiochrome counterparts, as described previously(23). Thiamine excretion was calculated as concentration of thiamine in urine times the 24-h urine volume and is expressed in mmol/24 h. Total thiamine metabolite content was calculated as the sum of TPP, TMP and THM concentration in plasma and tissue, and is expressed as µmol/L in plasma and pmol/mg protein in tissue.

Real-time PCR of thiamine transporter-1 (ThTr-1)

Tissue preparation for real-time PCR was done as previously described(24). The expression of ThTr-1 was normalized relative to the mean cycle threshold (CT) value of the β-actin gene. Results were finally expressed as 2-ΔCT, which is an index of the relative amount of mRNA expressed in each tissue. The standard deviation of the triplicate CT values was used if the coefficient of variation was less than 3%.

Statistical analyses

Analyses were performed with PASW version 18.0.3 (IBM SPSS Inc., Chicago, IL). Data are presented as means ± SD. For analysis of change in transketolase activity and total thiamine content with duration of thiamine deficient diet, groups of rats sacrificed before and at different times after start of the thiamine deficient diet were compared by means of ANOVA with post-hoc analysis according to Tukey. For analysis of differences in transketolase activity, thiamine metabolites and ThTr-1 expression between tissues after four weeks of thiamine deficient diet, percentages change from baseline were compared by means of ANOVA with post-hoc analyses according to Tukey. A *P*-value of 0.05 was considered statistically significant.

Results

Body weight and food intake

We measured body weight and food intake daily. In the first two stabilization weeks on the thiamine deficient diet with supplementation of 200 µg of thiamine to all groups, there was no significant difference in growth between the groups (*P*=0.68 and *P*=0.62 for the respective weeks) (Figure 1). In the subsequent weeks of feeding a thiamine deficient diet, growth in thiamine deficient rats was steadily retarding, with growth rates of 22.3 ± 4.3 g in the first week of thiamine deficient diet, 16.1 ± 3.1 g in the second week of thiamine deficient diet, 11.2 ± 4.9 g in the third week of thiamine deficient diet and -0.3 ± 14.9 g in the fourth week of thiamine deficient diet.

There was also no significant difference in food intake between the groups in the two weeks before start of the experiment, with intakes of 21.2 ± 2.5 g/day in the first week and 20.6 ± 2.3 g/day in the second week in control group vs. 22.1 ± 3.3 g/day in the first week and 21.0 ± 2.4 g/day in the second week in thiamine deficient group (resp. *P*=0.49 and *P*=0.71). Thereafter, the food intake declined, with intake of 20.2 ± 2.2 g per day after one week of thiamine deficient diet, 19.2 ± 2.2 g after two weeks of thiamine deficient diet, 20.2 ± 1.6 g after three weeks of thiamine deficient diet and 17.4 ± 3.1 g after four weeks of thiamine deficient diet.

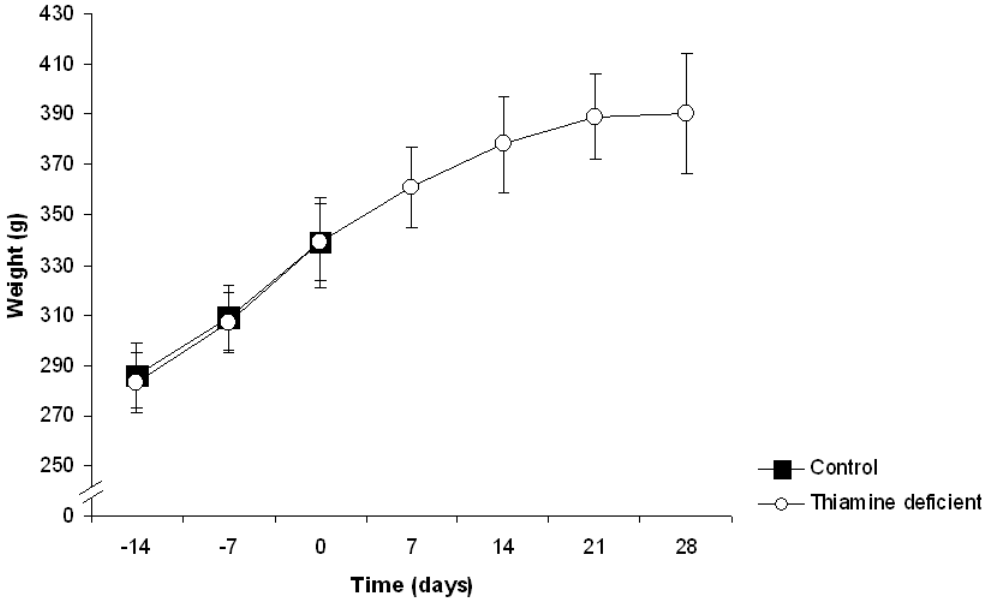

Figure 1. Weight of control vs. thiamine deficient animals.

Transketolase activity, thiamine and phosphorylated metabolites and ThTr-1 expression

The TK activity decreased significantly after two weeks of thiamine deficient diet and on, in all tissues and red blood cells (RBCs), except brain tissue (Figure 2). In RBCs, liver and lung tissue there was a significant further decrease in TK activity at four weeks of thiamine deficient diet as compared to two weeks of thiamine deficient diet, which was not the case in kidney and spleen tissue.

After one week of thiamine deficient diet thiamine excretion fell sharply from 195 ± 85 mmol/24 h to 8 ± 2 mmol/24 h, and remained low during the further weeks (Figure 3). Total thiamine content in plasma was significantly lower after two weeks of thiamine deficient diet compared to baseline and was not significantly lower when comparing four weeks of thiamine deficient diet with two weeks of thiamine deficient diet. This was also the case for heart, kidney and spleen tissue. Total thiamine metabolite content in liver and lung tissue was significantly lower after two weeks of thiamine deficient diet but was also significantly lower when comparing four weeks of thiamine deficient diet to two weeks of thiamine deficient diet. In contrast to other tissues, total thiamine metabolite content in brain tissue was not significantly lower after two weeks of thiamine deficient diet. However, after three weeks of thiamine deficient diet total thiamine metabolite content was significantly lower when compared to baseline.

In Table 1 percentages of transketolase activity, TTP, TMP, THM and total thiamine metabolite content between different tissues after four weeks of thiamine deficient diet were compared. Transketolase activity in brain tissue was significantly higher than in all other tissues. Transketolase activity in spleen tissue was significantly lower than in all other tissues. In addition, there was preservation of TPP in brain and heart tissue after 28 days of thiamine deficient diet. There was a decrease of TPP in most tissues approximately 70% after four weeks of thiamine deficient diet, but in the heart this was only 27.8%. The TPP levels were relatively well maintained in brain tissue, with a decrease of only 31.3% after four weeks of thiamine deficient diet. Percentage of TPP in brain and heart tissue was significantly higher after 28 days of thiamine deficient diet compared to all other tissues. This preservation of TPP was also shown in the TK activity of the brain, which had not decreased during thiamine deficiency. TMP and THM contents in brain were also well preserved, however in heart tissue there was overall no difference in preservation of these contents compared to other tissues. Thiamine deficiency caused no difference in the expression of ThTr-1 between the different tissues.

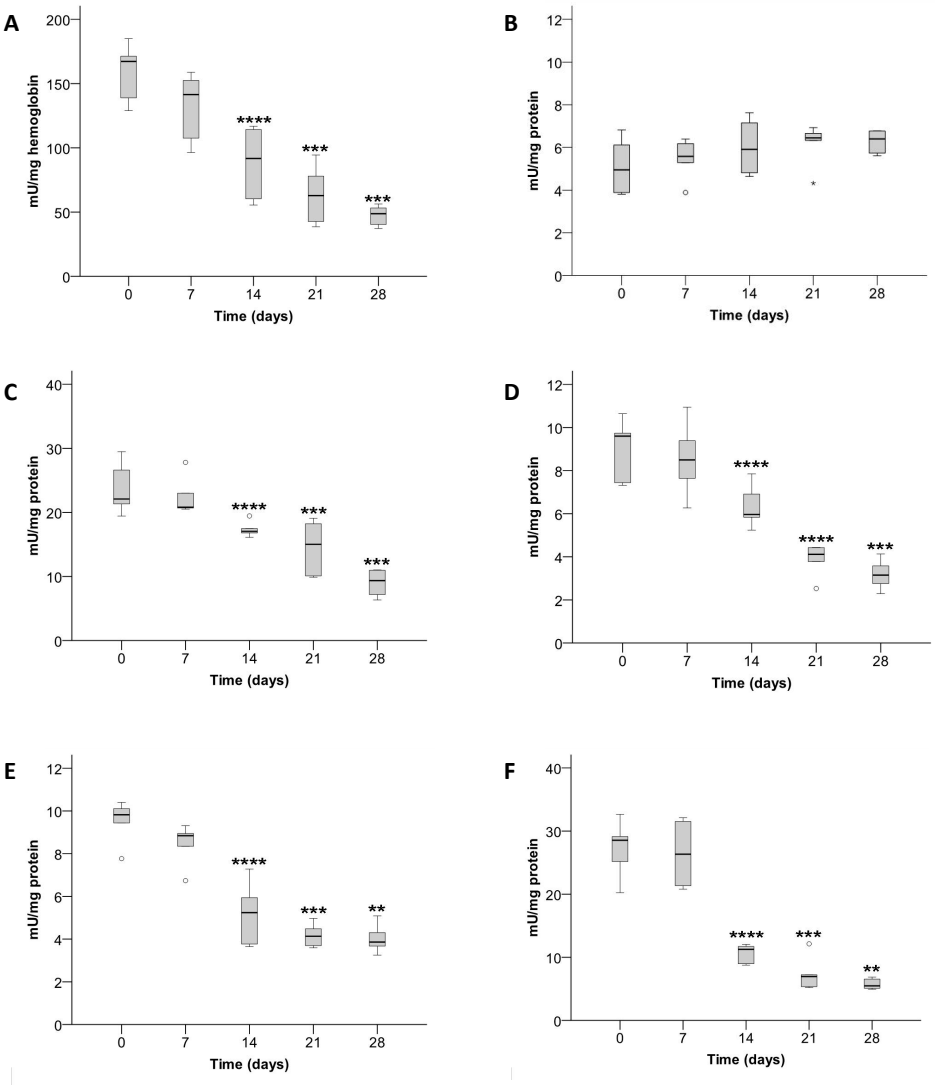

Figure 2: Boxplots of transketolase (TK) activity expressed as mU/mg hemoglobin or mU/mg protein
A: TK activity of red blood cells[a]; B: TK activity of brain tissue; C: TK activity of liver tissue[a]; D: TK activity of lung tissue[a]; E: TK activity of kidney tissue[a]; F: TK activity of spleen tissue[a].
[a]: p-value Anova <0.05.
****: Post-hoc Tukey *P*-value <0.05 compared to the value one week earlier.
***: Post-hoc Tukey *P*-value <0.05 compared to the value two weeks earlier.
**: Post-hoc Tukey *P*-value <0.05 compared to the value three weeks earlier.
*: Post-hoc Tukey *P*-value <0.05 compared to the value four weeks earlier.

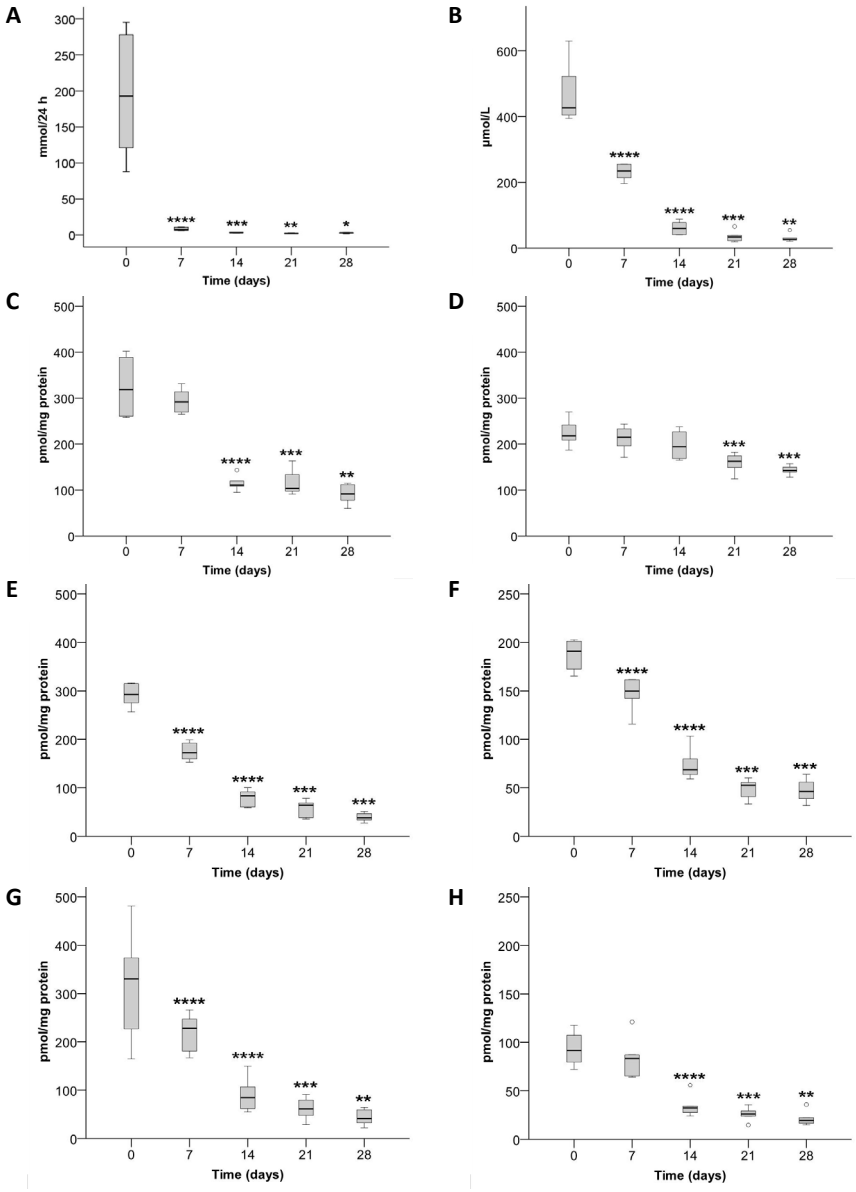

Figure 3: Amount of total thiamine metabolites in urine, plasma and different tissues, expressed as mmol/24 h, µmol/L or pmol/mg protein

A: thiamine excretion in urine[a]; B: total thiamine metabolite content in plasma[a]; C: total thiamine metabolite content in heart tissue[a]; D: total thiamine metabolite content in brain tissue[a]; E: total thiamine metabolite content in liver tissue[a]; F: total thiamine metabolite content in lung tissue[a]; G: total thiamine metabolite content in kidney tissue[a]; H: total thiamine metabolite content in spleen tissue[a].

[a]: p-value Anova <0.05.

****: Post-hoc Tukey *P*-value <0.05 compared to the value one week earlier.

***: Post-hoc Tukey *P*-value <0.05 compared to the value two weeks earlier.

**: Post-hoc Tukey *P*-value <0.05 compared to the value three weeks earlier.

*: Post-hoc Tukey *P*-value <0.05 compared to the value four weeks earlier.

Table 1. Percentage of transketolase activity, thiamine pyrophosphate, thiamine monophosphate, thiamine and total thiamine metabolite contents in tissues

	Brain	Heart	Kidney	Liver	Lung	Spleen	Anova-*P*
Transketolase activity (mU/mg)							
Baseline (absolute value)	5.1 ± 1.2	NA	9.5 ± 1.0	23.5 ± 3.8	9.1 ± 1.4	27.4 ± 4.2	
Four weeks from baseline (%)	123.4 ± 10.6[a]	NA	42.1 ± 6.6	38.5 ± 8.2	35.1 ± 7.3	21.0 ± 2.9[a]	<0.001
Thiamine pyrophosphate (pmol/mg)							
Baseline (absolute value)	46.5 ± 11.3	78.6 ± 13.2	127.1 ± 42.4	116.3 ± 17.7	73.5 ± 9.8	59.4 ± 14.3	
Four weeks from baseline (%)	68.7 ± 14.2[b]	72.2 ± 15.2[c]	24.1 ± 8.5	24.8 ± 5.3	36.1 ± 11.3	28.8 ± 8.7	<0.001
Thiamine monophosphate (pmol/mg)							
Baseline (absolute value)	131.6 ± 12.8	197.5 ± 47.1	38.9 ± 13.0	37.7 ± 8.1	65.0 ± 4.3	6.2 ± 2.0	
Four weeks from baseline (%)	60.8 ± 2.7[a]	14.3 ± 4.1	9.9 ± 2.5	6.6 ± 2.2	17.2 ± 4.9	24.0 ± 30.8	<0.001
Thiamine (pmol/mg)							
Baseline (absolute value)	131.6 ± 12.8	197.5 ± 47.1	38.9 ± 13.0	37.7 ± 8.1	65.0 ± 4.3	6.2 ± 2.0	
Four weeks from baseline (%)	68.4 ± 8.5[a]	12.3 ± 4.9	5.9 ± 3.2	5.7 ± 1.5	19.3 ± 4.5[b]	10.4 ± 4.2	<0.001
Total thiamine metabolite content (pmol/mg)							
Baseline (absolute value)	223.6 ± 28.8	324.2 ± 61.4	317.8 ± 111.4	291.2 ± 23.1	187.3 ± 16.3	93.4 ± 16.9	
Four weeks from baseline (%)	64.0 ± 4.4[a]	28.1 ± 6.4[d]	13.7 ± 5.2	13.5 ± 3.0	25.2 ± 6.2[d]	23.0 ± 8.2[d]	<0.001
ThTr-1 expression (fold induction)							
Baseline (absolute value)	0.80 ± 0.05	1.19 ± 0.29	1.01 ± 0.18	1.24 ± 0.31	1.04 ± 0.08	1.09 ± 0.20	
Four weeks from baseline (%)	101 ± 5	102 ± 19	112 ± 21	111 ± 36	122 ± 20	118 ± 40	0.73

NA: not assessed. [a]: $P<0.05$ for difference from all other tissues. [b]: $P<0.05$ for difference from all other tissues except from heart tissue. [c]: $P<0.05$ for difference from all other tissues except from brain tissue. *d*: $P<0.05$ for difference from brain, kidney and liver tissue.

Discussion

Brain and heart are predilection sites for complications from thiamine deficiency. However, the brain especially seems to be protected from loss of thiamine and thiamine metabolites. The heart appeared also protected from loss of TPP. We found no increase in expression of the thiamine transporter ThTr-1.

Previously thiamine deficiency has been studied in several animal models. Often these models consisted of administration of the antimetabolites oxythiamine and pyrithiamine, but deprivation of thiamine from the diet has also been used. Although earlier reports suggested that the deficiency state induced by pyrithiamine resembled that caused by dietary deprivation of thiamine, more recent studies have revealed substantial difference between the two approaches(25,26). Dietary deprivation studies found relatively preserved brain transketolase activities until neurological symptoms developed(25). After onset of symptoms decreases of up to 85% were found, in particular in the lateral vestibular nucleus. TK activity decreases both in vulnerable and spared regions(25,27,28). In another dietary thiamine deprivation study, a close relation between the decreases in brain transketolase activity and decrease in transketolase mRNA was found(28). A previous study on the course of tissue thiamine derivate concentrations during dietary deprivation of thiamine found relative preservation of contents of brain tissue compared to other tissues, but did not specifically investigate the course of concentrations of the heart and did not include data on transketolase activity or thiamine transporter expression(29). Another study that documented tissue thiamine contents found reductions of TPP of 50-67% in liver, brain and kidneys after 8 days of dietary thiamine deprivation(30). This study did also not report on tissue transketolase expression or thiamine transporter expression.

We found brain and heart tissue to be relatively protected against development of thiamine-deficiency. Apparently, these tissues have a mechanism or mechanisms by which they can better preserve thiamine concentrations than other tissues.

Unexpectedly, we found no upregulation of the thiamine transporter ThTr-1. It remains to be determined whether other thiamine transporters or thiamine conserving mechanisms are upregulated. Thiamine is transported across the membrane by ThTr-1 and ThTr-2, both a product of the same SLC19 gene family(31). They are ubiquitously expressed and both capable of thiamine transport. There is a significant structural similarity between the carriers. ThTr-1 and ThTr-2 share a similarity in the amino acid sequence of 70%. In vitro it was shown that thiamine deficiency upregulates both ThTr-1 and ThTr-2(32). The transcellular movement of thiamine and thiamine homeostasis has not yet completely been resolved. Therefore it could be that other thiamine transporters or thiamine conserving mechanisms are upregulated.

A limitation of the study design is not including a control group which was terminated at the end of the study. Thereby a direct comparison between control and thiamine deficiency could not been made. The period of thiamine deficient diet was not long enough to see the maximal effect of thiamine deficiency on brain tissue, but was long enough to compare development of thiamine deficiency between different tissues.

TPP is considered to be an essential coenzyme in several biochemical pathways in the brain. However, these pathways are common to every cell except erythrocytes, because the citric acid cycle is present in all mitochondria-containing cells. Therefore decreasing amounts of thiamine pyrophosphate could also lead to deficiencies in biochemical pathways in other organs.

The implication of these findings is that if recognizable signs of thiamine deficiency show in brain and heart tissues, other organs are also affected with thiamine deficiency. This could explain the findings in diabetes and kidney disease, in which (benfo)thiamine supplementation has been shown to reduce complications of these diseases(23,33-35).

Thiamine deficiency is now almost exclusively linked to alcohol abuse(5,17). Nevertheless, the elderly and people with comorbidity could be at risk for thiamine deficiency(13). Furthermore, patients with congestive heart failure and type 2 diabetes are more prone to thiamine deficiency(19,21,23). In diabetic patients, especially those with micro-albuminuria, it has been shown that urinary excretion of thiamine is increased(36). In heart failure it has been shown that thiamine supplementation will increase mean left ventricular ejection fraction(37,38). Thiamine deficiency has also been described in various disease states as hyperemesis gravidarum, critically ill patients admitted to the intensive care unit, refeeding syndrome, Alzheimer's disease, and after bariatric surgery(39,40).

There are unusual findings reported in Wernicke encephalopathy in non-alcoholic patients(41,42). This could be due to the fact that other tissues are thiamine deficient as well. This could explain why severe dysphagia could be the presenting symptom of Wernicke-Korsakoff syndrome in a non-alcoholic man(42). Food intake was also reduced in this study. Dysphagia is common in the elderly, and this could be both a symptom of thiamine deficiency and a cause of thiamine deficiency. From animal studies it is known that thiamine deficiency is complicated by loss of food intake and weight loss(43). A patient with alcohol abuse may be able to develop a more severe state of thiamine deficiency than in a patient without alcohol abuse(44).

It is therefore important to investigate the prevalence of thiamine deficiency and the effectiveness of thiamine supplementation in other disease states than classical Wernicke-Korsakoff syndrome.

Future investigations could focus on risk-populations of thiamine deficiency other than patients with alcohol abuse. Supplementation of thiamine could be helpful in disease states other than the classical beri-beri or Wernicke encephalopathy. Resolving how brain and heart tissue are relatively protected against development of thiamine deficiency may help

in unraveling how bodily thiamine homeostasis is regulated during circumstances of relative shortness and how it can be that deficiency states are particularly expressed in these tissues, particularly in circumstances of chronic alcoholism.

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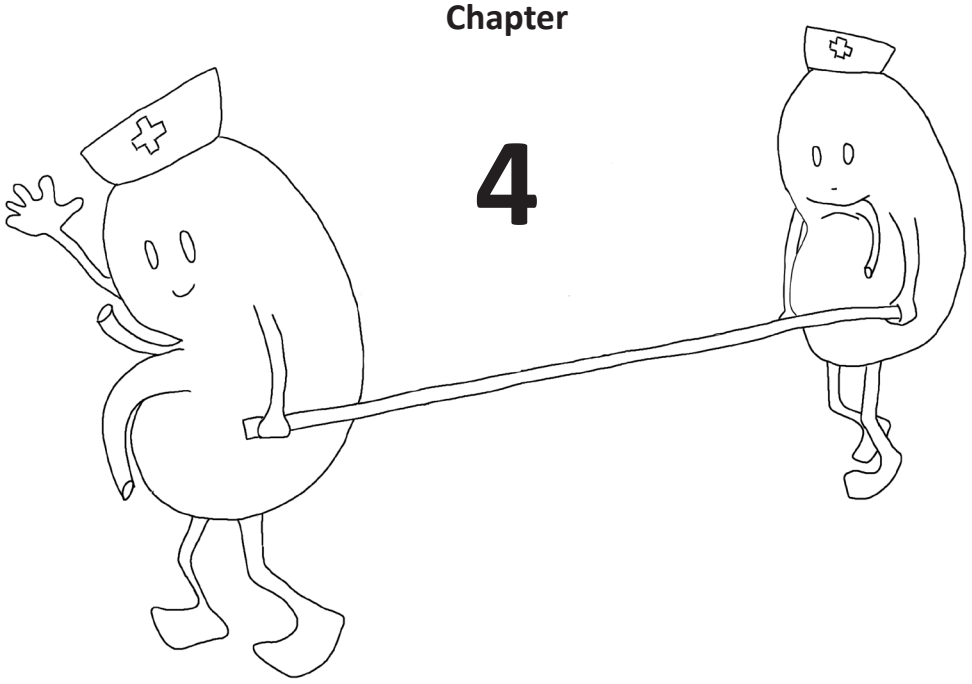
Chapter

4



Severe Thiamine Deficiency Complicated by Weight Loss Protects against Renal Ischaemia-Reperfusion Injury in Rats

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Abstract

Ischaemia and subsequent reperfusion (I/R) injury is a common cause of acute renal failure after renal transplantation. Thiamine is a water-soluble B-vitamin, which easily becomes deficient with intercurrent illnesses, excessive alcohol intake and malnutrition. Thiamine is a cofactor for enzymes that play a role in maintenance of cellular anti-oxidant capacity and ATP. We hypothesized that thiamine deficiency (TD) leads to increased susceptibility for I/R injury in kidneys.

Adult male Lewis rats were fed a thiamine free diet, with (controls, CON) or without (thiamine deficient, TD) supplementation of 400 µg thiamine/day. After two weeks and four weeks period of diet left kidneys were subjected to ischaemia and reperfusion, combined with contra-lateral nephrectomy. Plasma creatinine was assessed after one day and four days, followed by sacrifice. Renal tissue was processed for assessment of thiamine content, transketolase activity (TKA), and immunohistochemial staining for macrophages (ED1) and Kidney injury molecule-1 (Kim-1) and PCR expression of Kim-1 and Monocyte chemotactic protein-1 (MCP-1).

Mean (SEM) weight at time of I/R was not significant different after two weeks of diet (340 ± 3.6 g in CON vs. 333 ± 5.2 g in TD (*P*=0.26)), but was significantly higher in CON after four weeks of diet (356 ± 7.4 g vs. 320 ± 5.3 g (*P*=0.002)). Transketolase activity (TK) was in both experiments significantly higher in CON compared to TD. At the moment of ischaemia-reperfusion procedure there was no significant difference in plasma creatinine. After two weeks of thiamine deficient diet there was no significant difference after one day of ischaemia-reperfusion (42.6 ± 2.0 vs. 43.2 ± 3.9 µmol/L, *P*=0.89), however after four weeks of thiamine deficient diet plasma creatinine was significantly higher in CON compared to TD (71.7 ± 8.4 vs. 161.8 ± 31.9 µmol/L, *P*=0.02).

In conclusion, systemic TD associated with weight loss protects against renal I/R injury rather than increasing susceptibility. This is most likely described to a protective effect of generalized catabolism.

Introduction

Delayed graft function, usually defined as the necessity for continuation of dialysis in the first week after transplantation or beyond, is an important complication after renal transplantation, which occurs in approximately 30% of cases(1-8). Delayed graft function is not just a matter of dialysis until the organ starts functioning: although dialysis can be discontinued before the 20th day after transplantation in approximately 80% of the patients with delayed graft function, 10-15% remain dialysis-dependent for even longer periods of time, and at the end of the spectrum there are 2-15% of cases in which the transplanted kidney will never start functioning (primary non-function)(6).

Medical consequences of delayed graft function are not only complicated post-transplant management, increased duration of hospitalisation, increased costs after transplantation and increased allograft immunogenicity with a higher risk of acute rejection episodes, but also adverse effects on long-term outcome(6,7). Delayed graft function is an acknowledged independent risk factor for late graft failure, with a 2.9 times higher risk for delayed than for immediate function(7). Its importance for long-term graft outcome is further corroborated by reports about half-lives of 11.5 and 12.9 years for kidneys with immediate function, compared with 7.2 and 8.0 years for those with delayed function, respectively(7,9).

Results from ischaemia-reperfusion studies of the heart in dogs, and of the heart and cerebrum in rats, suggest that thiamine is protective against tissue injury mediated by hypoxia and reperfusion(10-12). Kidneys (and other organs harvested for transplantation) may be deficient in thiamine at the moment of reperfusion, because thiamine is a water-soluble B-vitamin (vitamin B1) of which is difficult to maintain whole body stores without continuous supplementation with thiamine from food or other resources(13). In fasting obese subjects it has been documented that metabolism facilitated by thiamine is already compromised within a few days of absence of any intake(13). Subclinical thiamine deficiency has been documented to be particularly common in patients at admission to intensive care units(14). Thiamine deficiency may be common in donors because intensive care patients are the typical kidney donors. Thiamine deficiency may become even more pronounced because it is unusual to feed potential donors during their stay at intensive care units, and if feeding is applied is it often inappropriate(15,16).

We hypothesize that tissue thiamine deficiency of transplanted kidneys is an important – and potentially treatable – risk factor for occurrence of delayed graft failure in transplanted kidneys(17). We aimed to investigate the effect of severe tissue thiamine deficiency on ischaemia-reperfusion injury in rat kidneys.

Materials and methods

Experimental design

Male inbred Lewis rats (± 270 g) (Harlan, Zeist, The Netherlands) were fed a thiamine-deficient diet (Arie Blok, Woerden, The Netherlands). The diet only contained trace amounts of thiamine (0.16 μg/kg, equalling approximately 0.04% of thiamine in regular chow) derived from casein, which constitutes 20% of the thiamine-deficient diet. Control animals were orally supplemented with 400 μg thiamine per day in a 2.5% sucrose-solution. Thiamine-deficient groups were provided with the same volume of the 2.5% sucrose-solution without thiamine. Rats were individually housed allowing for daily determination of body weight and food intake. After respectively two and four weeks of thiamine deficient diet ischaemia-reperfusion procedures were performed. Briefly, anesthesia was induced by 5% isoflurane, and maintained on 3% isoflurane. The rats were placed on a homothermic table to maintain core body temperature at 37 °C. The left kidney was subjected to a period of warm ischaemia, followed by reperfusion. Nephrectomy of the contralateral right kidney was performed during ischaemia of the left kidney. Blood was withdrawn before inducing warm ischaemia, after one day of reperfusion and after four days of reperfusion.

Sacrificing the rats was started with induction of deep anaesthesia with isoflurane, 250-IU heparin was perfused through the penile vein. This was followed by cannulation of the aorta and a 5 mL blood sample was taken. After a full body flush of 40 mL 0.9% NaCl at 4 °C, in order to obtain optimal tissue for morphology, and to prevent red blood cells disturbing transketolase activity measurement. Kidney tissue samples were snapfrozen and stored at -80 °C and in 4% formalin. Plasma and red blood cells were also stored at -80 °C.

All experimental procedures were approved by the Committee for Animal Experiments of the University of Groningen and performed according to the principles of laboratory animal care (NIH publication no. 85-23, revised 1985).

Experiment 1

Thiamine deficient (TD) group (n=12) were given thiamine deficient diet and sucrose-solution for four weeks, control rats (CON) were given thiamine deficient diet and oral thiamine supplementation. After four weeks ischaemia-reperfusion procedure was performed, with the left kidney subjected to 45 minutes of warm ischaemia, followed by one or four days reperfusion before termination.

Experiment 2

Thiamine deficient (TD) group (n=12) were given thiamine deficient diet and sucrose-solution for two weeks, control rats (CON) were given thiamine deficient diet and oral thiamine supplementation. After two weeks the ischaemia-reperfusion procedure was performed, with the left kidney subjected to 30 minutes of warm ischaemia, followed by one day of

reperfusion before termination.

Measurements

Thiamine and transketolase activity in renal tissue

Before measurements, 100-150 mg of renal tissue was homogenised in 500 µL 10 mM Na_2HPO_4 and centrifuged at 20,000 g for 30 min. Supernatant was used for assays. Tissue transketolase activity was measured according to the kinetic method of Chamberlain et al(18). Thiamine pyrophosphate (TPP), thiamine monophosphate (TMP) and thiamine (THM) were determined by HPLC with fluorimetric detection after pre-column derivatization of thiamine and its phosphate esters to their respective thiochrome counterparts, as described previously(19). Reagents were purchased from Sigma Aldrich (Gillingham, United Kingdom).

Immunohistochemistry of renal tissue

Parrafin sections (4 µm) were dewaxed and subjected to heat-induced antigen retrieval by overnight incubation at 80 °C in 0.1 mol/L Tris-HCl buffer (pH 9). Kidney injury molecule-1 (Kim-1) was stained using a rabbit polyclonal antibody (Kim-1 peptide 9, V. Bailly), monocytes/macrophages were detected using a mouse monoclonal antibody (ED-1; Serotec, Kidlington, UK). After washing, primary antibodies were detected using the appropriate horseradish peroxidase-conjugated secondary and tertiary antibody (DakoCytomation, Glostrup, Denmark). Peroxidase activity was developed by the addition of 3,3'-diaminobenzidine tetrahydrochloride. Sections were counterstained with hematoxylin eosin/periodic acid schiff. Expression was quantified by counting positive cells in the renal interstitium in case of ED-1 and computerized morphometry was used to measure Kim-1.

RNA isolation and real-time PCR

Tissue preparation for real-time PCR is previously described(20). The expression of Kim-1 and Monocyte chemotactic protein-1 (MCP-1) were determined. For each gene the expression was normalized relative to the mean cycle threshold (CT) value of the β-actin gene. Results were finally expressed as 2-ΔCT, which is an index of the relative amount of mRNA expressed in each tissue. The standard deviation of the triplicates of the CT values was accepted, if the coefficient of variation was less than 3%.

Other biochemical measurements

Plasma creatinine concentration was measured by Roche enzymatic method. Tissue protein concentration was measured in homogenised tissue samples according to Bradford.

Statistical analysis

Data was analysed using PASW version 18.0.3 (IBM SPSS Inc., Chicago, IL), and expressed as the average ± standard error of the mean (SEM). Statistical significance of difference was assessed by Student's T-tests (for independent and paired samples). PCR results are presented as fold induction times 100. Differences and correlations were considered significant if the *P*-value<0.05.

Results

Experiment 1

Thiamine deficiency

Induction of thiamine deficiency by feeding a thiamine-deficient diet resulted in decrease in food intake and weight loss. The course of body weight is shown in Figure 1A. In the third week, growth of CON was significantly higher than in TD (20.16 ± 1.3 g vs. 11.3 ± 2.5 g, *P*=0.006). In the fourth week, CON gained weight, whereas TD lost weight (13.5 ± 6.8 g vs. -17.3 ± 2.8 g, *P*<0.001), resulting in a significant difference in body weight before ischaemia-reperfusion (356 ± 7.4 g vs. 320 ± 5.3 g, *P*=0.002). Decrease in growth preceded decrease in food intake: food intake in CON and TD was similar at day 14 (21.2 ± 0.5 g vs. 20.0 ± 0.5 g, *P*=0.12) and day 21 of the experiment (18.9 ± 0.5 g vs. 18.1 ± 1.6 g, *P*=0.63). At the day of ischaemia-reperfusion, food intake was significantly lower in thiamine-deficient rats than in control rats (13.6 ± 2.3 g vs. 20.2 ± 0.8 g resp., *P*=0.006).

The contralateral kidney was used to assess renal biochemical and functional thiamine status at the moment of ischaemia-reperfusion (Table 1). Concentrations of the three forms of thiamine in renal tissue (thiamine pyrophosphate (TPP), thiamine monophosphate (TMP) and unphosphorylated thiamine (THM)) were all three significantly higher in CON than in TD (all *P*<0.001). This translated into a significantly higher functional activity of the thiamine-dependent enzyme transketolase in CON (*P*<0.001).

Plasma creatinine concentrations

There was no difference in baseline plasma creatinine concentrations prior to ischaemia-reperfusion between CON and TD (16.9 ± 0.8 µmol/L vs. 16.7 ± 0.7 µmol/L, *P*=0.88). At the first day after ischaemia-reperfusion, plasma creatinine concentrations were significantly higher in CON than in TD (161.8 ± 31.9 vs. 71.7 ± 8.4 µmol/L, *P*=0.02). At four days after ischaemia-reperfusion, plasma creatinine concentrations were still slightly higher in CON, but this difference was not significant (68.2 ± 19.1 vs. 40.5 ± 3.5 µmol/L, *P*=0.22).

Markers of damage and inflammation

TBARS was borderline significant in spot-urine one day after ischaemia-reperfusion between CON and TD (0.73 ± 0.12 vs. 0.43 ± 0.04, *P*=0.06). Four days after ischaemia-reperfusion

there was no difference between CON and TD (1.03 ± 0.09 vs. 0.83 ± 0.22, *P*=0.39). Immunohistochemistry for Kim-1 showed no significant between CON and TD at baseline ($0.10x10^{-3}$ ± $0.05x10^{-3}$ %area vs. $5.3x10^{-3}$ ± $3.8x10^{-3}$ %area, *P*=0.26). At the first day after ischaemia-reperfusion Kim-1 was significantly higher in CON than in TD (2.5 ± 0.4 %area vs. 1.4 ± 0.2 %area, *P*=0.04). Four days after ischaemia-reperfusion there was no significance difference for Kim-1 between CON and TD (2.1 ± 0.8 %area vs. 1.0 ± 0.5 %area, *P*=0.37).
Immunohistochemistry for ED-1 showed no significant different between CON and TD at baseline (3.3 ± 0.2 vs. 3.9 ± 0.2 cells per view, *P*=0.10). One day after ischaemia-reperfusion ED-1 counts were borderline significantly higher in CON compared to TD (18.0 ± 2.6 vs. 10.7 ± 2.1 cells per view, *P*=0.06). There was no significant difference after four days between CON and TD (33.4 ± 14.3 vs. 11.1 ± 2.6 cells per view, *P*=0.29).
PCR results for Kim-1 and MCP-1 are shown in Table 2. There were no significant differences between CON and TD.

Experiment 2

Thiamine deficiency

Two weeks of thiamine deficient diet did not result in a decrease in food intake and weight loss. The course of body weight is shown in Figure 1B. At day 14 weight was 340 ± 3.6 g in CON and 333 ± 5.2 g in TD (*P*=0.26). Growth was not different between CON and TD. Growth in week two was 27.2 ± 0.9 g in CON and 25.4 ± 2.1 g in TD (*P*=0.47). Food intake was also not different between CON and TD. Food intake in week two was 21.1 ± 1.3 g in CON and 20.5 ± 1.8 g in TD (*P*=0.80) .
The contralateral kidney was used to assess renal functional thiamine status at the moment of ischaemia-reperfusion. Transketolase activity was higher in CON than in TD (91 ± 1.4 vs. 59 ± 1.2 mU/mg protein, *P*<0.001)

Plasma creatinine concentrations

There was no difference in baseline plasma creatinine concentration prior to ischaemia-reperfusion between CON and TD (16.8 ± 0.5 vs. 16.4 ± 0.5 µmol/L, *P*=0.51) and 1 day after ischaemia-reperfusion injury between CON and TD (42.6 ± 2.0 vs. 43.2 ± 3.9 µmol/L, *P*=0.89).

Markers of damage and inflammation

TBARS in spot urine one day after ischaemia-reperfusion was not significant different between CON and TD (1.45 ± 0.08 vs. 1.64 ± 0.11, *P*=0.17). Before ischaemia-reperfusion Kim-1 in paraffin coupes was not significantly different between thiamine-deficient and control rats ($2.0x10^{-3}$ ± $0.4x10^{-3}$ %area vs. $9.4x10^{-3}$ ± $7.4x10^{-3}$ %area, P=0.34). There was also no difference one day after reperfusion between CON and TD (0.4 ± 0.1 %area vs. 0.4 ± 0.1 %area, P=0.85).

PCR results of Kim-1 and MCP-1 are shown in Table 2. At time of ischaemia-reperfusion the expression of Kim-1 was significantly increased in CON compared to TD (47.0 ± 0.4 vs. 7.7 ± 1.2, *P*=0.042). After ischaemia-reperfusion there were no significant differences between expression of Kim-1 and MCP-1 in CON or TD.

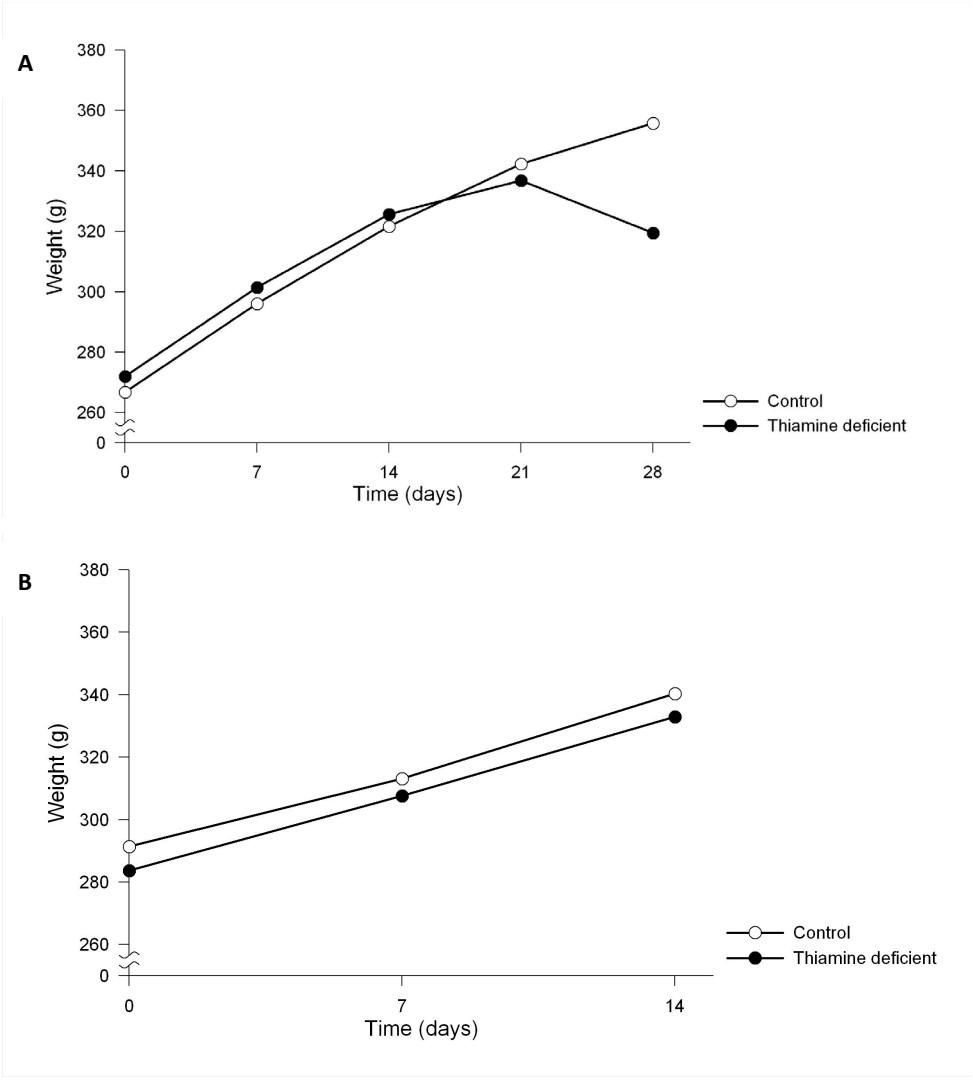

Figure 1. Body weight. A: Experiment 1; B: Experiment 2

Table 1. Experiment 1: Transketolase activity and thiamine and thiamine metabolites

	Control	Thiamine deficient	*P*-value
TK activity	13.9 (0.7)	7.7 (0.5)	<0.001
TPP	81.2 (4.0)	15.7 (2.3)	<0.001
TMP	26.2 (1.5)	0.4 (0.1)	<0.001
THM	90.2 (4.6)	2.5 (0.3)	<0.001

TK activity is expressed as mU/mg protein. TPP, TMP and THM are expressed as pmol/mg protein.

Table 2. PCR results of Kim-1 and MCP-1

		Control	TD	*P*-value
Experiment 1				
Kim-1	baseline	0.5 ± 0.2	0.1 ± 0.01	0.18
	1 day after I/R	41.7 ± 8.8	37.7 ± 7.0	0.74
	4 days after I/R	9.8 ± 1.9	8.6 ± 2.4	0.72
MCP-1	baseline	0.6 ± 0.1	0.4 ± 0.2	0.30
	1 day after I/R	1.5 ± 0.1	1.4 ± 0.09	0.29
	4 days after I/R	0.58 ± 0.04	0.55 ± 0.09	0.68
Experiment 2				
Kim-1	baseline	47 ± 0.4	7.7 ± 1.2	0.04
	1 day after I/R	1228 ± 521	575 ± 103	0.21
MCP-1	baseline	4.3 ± 0.7	3.7 ± 0.8	0.59
	1 day after I/R	9.3 ± 2.4	8.0 ± 1.2	0.64

PCR results are presented as fold induction times 100.

Discussion

In this study we found that a four weeks period of thiamine deficient diet, which was associated with weight loss, led to protection against renal ischaemia-reperfusion injury. This was not true after a two week period of thiamine deficient diet. In contrast to our hypothesis, thiamine deficiency appeared to protect against reperfusion injury, rather than to increase it. The protective effect was manifested by significantly lower plasma creatinine concentrations one day after ischaemia-reperfusion in thiamine-deficient rats compared to control rats and lower expression of Kim-1 in immunohistochemical staining of kidney tissue.

Prior to ischaemia-reperfusion, we found growth retardation in the third week of feeding the thiamine-deficient diet, followed by weight loss in the fourth week. Growth retardation preceded a decline in food intake, suggesting less efficient energy metabolism to underlie weight loss. Da Cunha et al. also showed that in thiamine deficient rats, preceding the decrease in food intake, weight gain is reduced and after four weeks bodyweight will dramatically decrease(21). In experiment 1, the ischaemia-reperfusion procedure was performed after four weeks of thiamine deficient diet, when bodyweight was reducing slightly as well as a moderate reduction in food intake. In experiment 2, ischaemia-reperfusion procedure was performed after two weeks of thiamine deficient diet, when there was no difference in bodyweight and food intake.

Renal tissue thiamine deficiency was proven biochemically by lower concentrations of thiamine and phosphorylated metabolites and functionally by lower TK. Moreover it is shown by Klooster et al. that after two weeks of thiamine deficiency there is no further decrease in TK in renal tissue. So after two weeks thiamine deficiency reaches biochemically the maximum decrease in TK, without decrease in bodyweight and food intake(22).

Few other studies have to date investigated the effect of thiamine supplementation on ischaemia-reperfusion injury. One study investigated the effect of thiamine supplementation in prevention of cerebral ischaemia-reperfusion injury in rats(11). In rats with a normal baseline thiamine status, both acute and chronic supplementation resulted in a significant reduction in infarct size after 30 min of transient cerebral artery occlusion. In another study, it was shown that after ligation of the left anterior descending coronary artery the amount of damaged tissue forming the border zone of myocardial infarction was reduced by treatment with intra-aortic balloon pumping (IABP) in combination with treatment with high dose thiamine versus no treatment in dogs(23). It was not investigated whether this effect could be contributed to the treatment with IABP or to thiamine. One might think that the effect has to be attributed to the treatment with IABP, but later studies corroborate a role of thiamine(10,24). In these studies, it was shown that thiamine pyrophosphate supplementation alone had beneficial effects to ischaemic canine myocardium. It was, however, suggested that this was due to systemic hemodynamic effects of thiamine rather

than on metabolism(10). However, results of an in vitro study of the effect of thiamine on hypoxia-induced cell death in cultured neonatal cardiomyocytes, suggest that metabolic effects play a role as well(12).

An obvious difference between our studies and these previous studies is that we investigated the effect of thiamine deficiency rather than supplementation. Moreover, we investigated the effect on ischaemia-reperfusion injury in kidneys rather than brain or heart. Apart from differences between tissues, a potential explanation for the discrepancy between our study, in which we found a protective effect of thiamine deficiency, and the other studies, in which a protective effect was found for thiamine supplementation, is that induction of thiamine deficiency in our study was accompanied by weight loss and anorexia. Interestingly, it is known that fasting protects against ischaemia-reperfusion injury in hearts, liver and kidney(25-27). Currently, dietary restriction protocols prior to renal transplantation are conducted(28).

Another potential explanation for our finding of a protective effect of thiamine deficiency against ischaemia-reperfusion could lie in that systemic thiamine deficiency impaired influx of inflammatory cells into the renal interstitium after ischaemia-reperfusion. It has indeed been demonstrated that thiamine deficiency impairs function and migration of neutrophils(29,30). It has also been suggested that a prolonged catabolic state resulting in growth retardation leads lower numbers of circulating white blood cell and impaired influx of neutrophils after induction of pneumonia(31,32). After four weeks period of thiamine deficient diet we consistently found significantly lower numbers of neutrophils, macrophages and expression of Kim-1 in thiamine-deficient rats than in controls before ischaemia-reperfusion, but no consistent differences between thiamine-deficient rats and controls after ischaemia-reperfusion injury. Apparently, either thiamine deficiency itself or the catabolic state that was induced by thiamine deficiency led to lower expression of inflammatory markers and damage markers in renal tissue.

Based on these considerations and our observation of a protective effect of thiamine deficiency complicated by anorexia and weight loss, we hypothesize that a protective effect of fasting on ischaemia-reperfusion injury could explain the effect seen in the thiamine deficient group after four weeks of thiamine deficient diet.

In conclusion, we have demonstrated a protective effect of severe thiamine deficiency complicated by weight loss and anorexia against ischaemia-reperfusion injury in kidneys. Our study points towards a potential protective role of weight loss and fasting in preventing ischaemia-reperfusion injury in kidneys.

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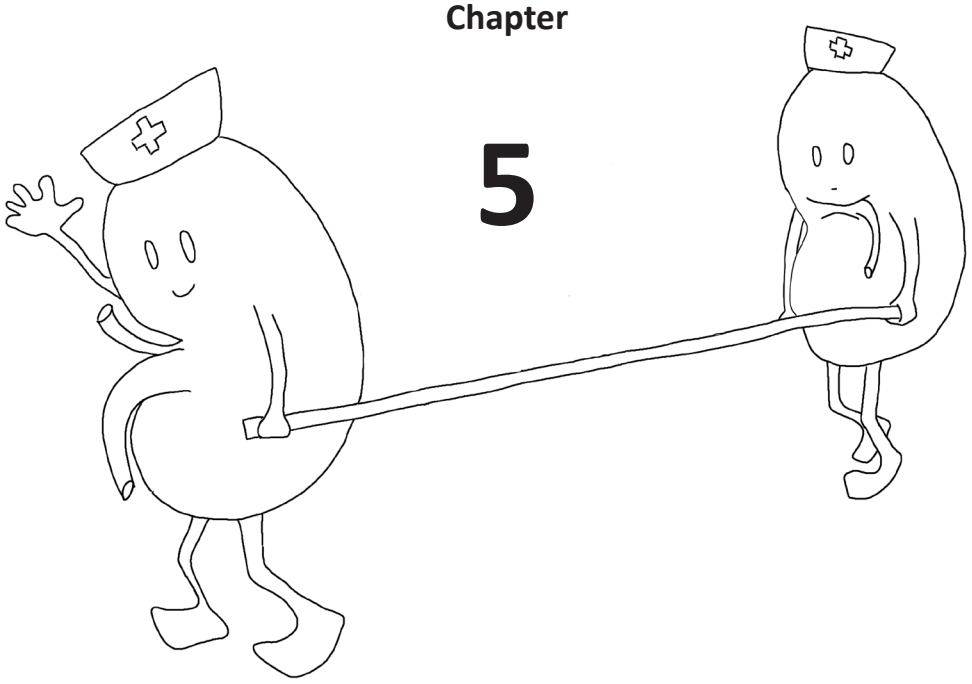
Chapter

5



A Double-Blind, Randomized, Placebo-Controlled Clinical Trial on Benfotiamine Treatment in Patients with Diabetic Nephropathy

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Abstract

Benfotiamine, a lipid-soluble thiamine derivative, has been suggested as an agent that can prevent occurrence and deterioration of diabetic complications, including diabetic nephropathy. We aimed to investigate the effect of benfotiamine on urinary excretion of albumin (UAE) and the tubular damage marker kidney injury molecule 1 (KIM-1) in patients with type 2 diabetes and nephropathy.

In this double-blind, placebo-controlled trial, patients with type 2 diabetes and high-normal to micro-albuminuria (UAE 15-300 mg/24h) despite use of angiotensin-converting enzyme inhibitors (ACE-Is) or angiotensin-receptor blockers (ARBs), were randomly assigned to receive 12-week treatment with benfotiamine (900mg/day) or placebo. Thiamine status was assessed by whole blood thiamine concentrations, erythrocyte transketolase activity, and thiamine pyrophosphate effect. Primary outcome measures were 24h-UAE and 24h urinary KIM-1 excretion.

In 39 patients assigned to benfotiamine and 43 patients assigned to placebo, median [interquartile range] baseline 24h-UAE was 90 [38; 267] vs 97 [48; 177] mg/24h, respectively, and 24h-KIM-1 was 1.67 [0.9; 2.4] vs 1.56 [1.1; 1.9] µg/24h respectively. Benfotiamine treatment resulted in significant improvement in all three domains of thiamine status (P<0.001). After 12 weeks of treatment with benfotiamine, there were no significant reductions in 24h-UAE and 24h-KIM-1 compared to placebo (ΔUAE: -9 [-53; 34] vs -7 [-56; 65] mg/24h respectively, P=0.36; ΔKIM-1: -0.014 [-0.23; 0.56] vs -0.043 [-0.36; 0.19] µg/24h respectively, P=0.09).

In patients with type 2 diabetes and nephropathy, high-dose benfotiamine treatment for 12 weeks as add-on therapy to ACE-Is or ARBs did not reduce urinary excretion of albumin or KIM-1 despite improving thiamine status.

Introduction

The incidence of diabetes related complications, like diabetic nephropathy (DN), increases, also in the perspective of the worldwide increase in prevalence of type 2 diabetes mellitus(1). Diabetes has become the leading cause of end-stage renal disease (ESRD), with in some countries more than 40% of all new cases of ESRD occurring in patients with diabetes(2).
ESRD caused by diabetes can be explained by different pathophysiological mechanisms, including induction of glomerular endothelial damage, which in turn leads to albuminuria(3). Albuminuria as such also plays an etiological role inducing tubulointerstitial inflammation and fibrosis with increasing albuminuria(4,5).
Improving glycaemic control has shown to reduce the risk of the development of microalbuminuria(6,7). Once established, reduction of albuminuria, in particular by using angiotensin-converting enzyme inhibitors (ACE-Is) and angiotensin-receptor blockers (ARBs), is the cornerstone in preventing or retarding the occurrence of ESRD(8,9). Despite this successful therapy, there are still people with diabetes progressing to ESRD. Therefore, there is a great need for further adjunctive treatments which can help to prevent ESRD.
Recently, patients with type 1 and type 2 diabetes were found to have low plasma thiamine concentrations, due to increased thiamine loss with urine(10). Additionally, thiamine and benfotiamine have recently been proposed as agents that can prevent occurrence and deterioration of diabetic complications(11,12). Benfotiamine is a lipophilic thiamine derivative with higher bioavailability compared to thiamine(13). In animal experimental studies, benfotiamine has a beneficial effect on microvascular complications(11,14).
We aimed to investigate whether additional treatment with benfotiamine in patients with type 2 diabetes and increased urinary albumin excretion rate on ACE-Is or ARBs results in reduction in urinary excretion of albumin or tubulointerstitial damage markers.

Materials and methods

Patients

Participants were recruited from the outpatient department population of the Isala Clinics in Zwolle, The Netherlands. Inclusion criteria were diagnosis of type 2 diabetes according to American Diabetes Association criteria, age between 40 and 75 years, active DN as indicated by urinary albumin excretion (UAE) in the high-normal or microalbuminuric range (UAE 15-300 mg/24-hour urine, or spot urine albumin/creatinine 1.25-25 mg/mmol in males and 1.75-35 mg/mmol in females) in at least two out of three samples within 2-6 weeks in advance of inclusion in the trial despite treatment with ACE-Is and/or ARBs in an unchanged dose for at least 3 months, glycated hemoglobin (HbA_{1c}) < 8.5% and estimated glomerular filtration rate calculated using the Modification of Diet in Renal Disease formula (eGFR-MDRD) > 30 ml/min(15,16). Exclusion criteria were participation in another study ≤

one month before joining this study, renal impairment by causes other than diabetes, liver enzymes (AST and ALT) ≥ three times upper limit of normal values (normal values: AST < 40 IU/L, ALT < 45 IU/L), hyper-/hypothyroidism, a blood pressure > 160/90 mmHg, severe cardiac function disturbances or heart rhythm disturbances, neoplasm, severe general diseases or mental disorders, drug abuse, pregnancy or lactation, active menses during the past year, hypersensitivity to benfotiamine or other constituents of the study medication, use of vitamin B-containing supplements during the last 3 months and use of nonsteroidal anti-inflammatory drugs more than 3 times per week. Additionally, it was required that no changes had been made in prescription of cholesterol lowering medication, blood pressure lowering drugs and oral hypoglycaemic agents during 3 months prior the study. In total 2711 patients registered in our local Diabetes Electronic Management System (DEMS) were screened for eligibility(17). Those who fulfilled inclusion criteria were informed about the study by sending information per mail. Patients who accepted were included after written informed consent was obtained. This trial was conducted in accordance with the Helsinki Declaration, approved by the medical ethics committee of the Isala Clinics, and registered in the clinical trial registry (ClinicalTrial.gov) under number: NCT00565318.

Procedures

Patients were randomised to benfotiamine 300 mg t.i.d. (daily dose 900mg) or placebo for 12 weeks. Benfotiamine and placebo tablets were prepared by Wörwag pharma (Böblingen, Germany) and packed in numbered boxes, unrecognized from each other, according to a computer-generated randomisation list which was prepared by an independent statistician. Independent pharmacists dispensed the medication box with the lowest available number to each patient. Neither the researchers nor the patients knew into which group they had been allocated.

During a run-in phase, patients were instructed how to collect 24-hour urine and asked not to change their usual diet or daily activity during the study, particularly during the week preceding their clinical visits. Patients were instructed to take one tablet after the three main meals, every day. In case of suspected side effects, patients were asked to contact the study physician who instructed them to stop the study medication until disappearance of complaints for a maximum of one week. When complaints disappeared, study medication was resumed once again. All participants were evaluated at baseline, after 6 weeks, and after 12 weeks of treatment. Patients were asked to deliver a 24-hour urine collection to the laboratory on each visit. At the laboratory, additional morning spot-urine sample and blood samples were taken. On the last visit, tablets were counted to assess compliance. Non-compliance was considered if less than 80% of the study medication had been taken. At the end of the study, after data collection and laboratory analyses had been completed, the randomisation list was provided to the researchers for unblinding.

Laboratory analyses

Thiamine concentration was measured in whole blood by HPLC, reference range 90-200 nmol/L, lower limit of detection 10 nmol/L, upper limit of detection 300 nmol/L(18). Erythrocyte transketolase (TK)-activity (expressed in mU/mgHb) and thiamine pyrophosphate (TPP) effect (expressed as %) were measured according to the kinetic method of Chamberlain et al. in washed erythrocyte samples after being haemolysed by mixing with Aqua Purificata(19). Reagents were purchased from Sigma Aldrich® (Gillingham, United Kingdom). Thiamine deficiency was considered present if TPP effect >15%.(20) All these results were left unrevealed until all patients had completed the study. Urinary albumin was measured by immunonephelometry (Behring Nephelometer, Mannheim, Germany) with a threshold of 1.8-2.3 mg/L and intra- and inter-assay coefficients of variation of less than 2.2 and 2.6%, respectively. Urinary kidney injury molecule-1 (KIM-1) was measured by ELISA, lowest limit of detection: 0.12 ng/mL, intra- and inter-assay coefficients of variation: 7.9% and 14.4%, respectively(21). The other tubular markers, neutrophil gelatinase-associated lipocalin (Ngal, R&D Systems, Abingdon, UK) and α1-microglobulin (α1-m, Fitzgerald, Concord MA, USA; ICL Inc, Newberg, OR, USA), were measured using routine ELISA and competitive EIA assays, respectively. HbA_{1c} was measured with Primus Ultra2 system using high-performance liquid chromatography. Other laboratory measurements were performed according to standard hospital procedures. Creatinine clearance was calculated from 24-hour urinary creatinine excretion and plasma creatinine.

Statistical analyses

Normally distributed variables are presented as means ± standard deviations (SD) and variables with a skewed distribution as medians and interquartile ranges (IQR). Q-Q plot was used to assess whether variables were distributed normally or skewed. χ2 test was used to compare non-continuous variables. Changes were analyzed by ANOVA for repeated measurements. *P*-values for change over time are presented. Additionally, changes in outcome measures from baseline to 6 weeks (Δ 6 weeks) and from baseline to 12 weeks (Δ 12 weeks) were computed. Positive changes indicate increase over time and negative changes indicate decrease over time. Comparisons of changes in primary and secondary parameters between groups were performed by an unpaired Student's T-test (in case of normal distribution) or Mann-Whitney-U test (in case of skewed distribution). Multivariate regression analysis was used to adjust for baseline differences between groups.

To test our hypothesis that benfotiamine reduces 24-hour urinary excretion of albumin and KIM-1 (primary outcome measures), 38 evaluable patients per group were required to detect an effect of size 0.65 (power 80%, α = 0.05, one-sided test). To compensate for possible drop-out, we planned to enroll 43 patients per group. Secondary outcome parameters were ratio of albumin over creatinine in 24-hour urine and spot morning urine samples, 24-hour urinary excretion of tubulointerstitial damage markers (α1-m and Ngal), and ratios of these

markers and KIM-1 over creatinine concentration in 24h urine collections. A *P*-value of 0.05 or less was considered statistically significant. One-sided *P*-values were calculated for primary outcome measures and two-sided *P*-values were calculated for the other outcome measures. Statistical analyses were done by using a commercially available program (SPSS for Windows, version 16.0., Chicago, IL, USA).
Intention-to-treat analysis and per-protocol analysis were planned. In case of drop-out, data was not replaced and these patients had then to be excluded from analysis. Non-compliance and change in concomitant medications (including ACE-I or ARB) were considered as deviations from study protocol that lead to exclusion from per-protocol analysis.

Results

Patient flow and baseline characteristics at randomization
Participants were recruited from February 2008 till February 2009. A CONSORT diagram of the study is shown in Figure 1.
43 patients were randomized to benfotiamine and 43 to placebo. In the benfotiamine group, 2 patients did not complete the study because of newly diagnosed malignancy (lung cancer and stomach cancer, both were considered not causally related to study medication) and 2 others withdrew informed consent (one complained of dizziness and the other complained of urticaria and dry mouth). Because of this missing follow-up data, 39 patients out of 43 were analyzed in this group. In the placebo group, all patients finished the study and were analyzed. Baseline characteristics of the two groups are shown in Table 1.

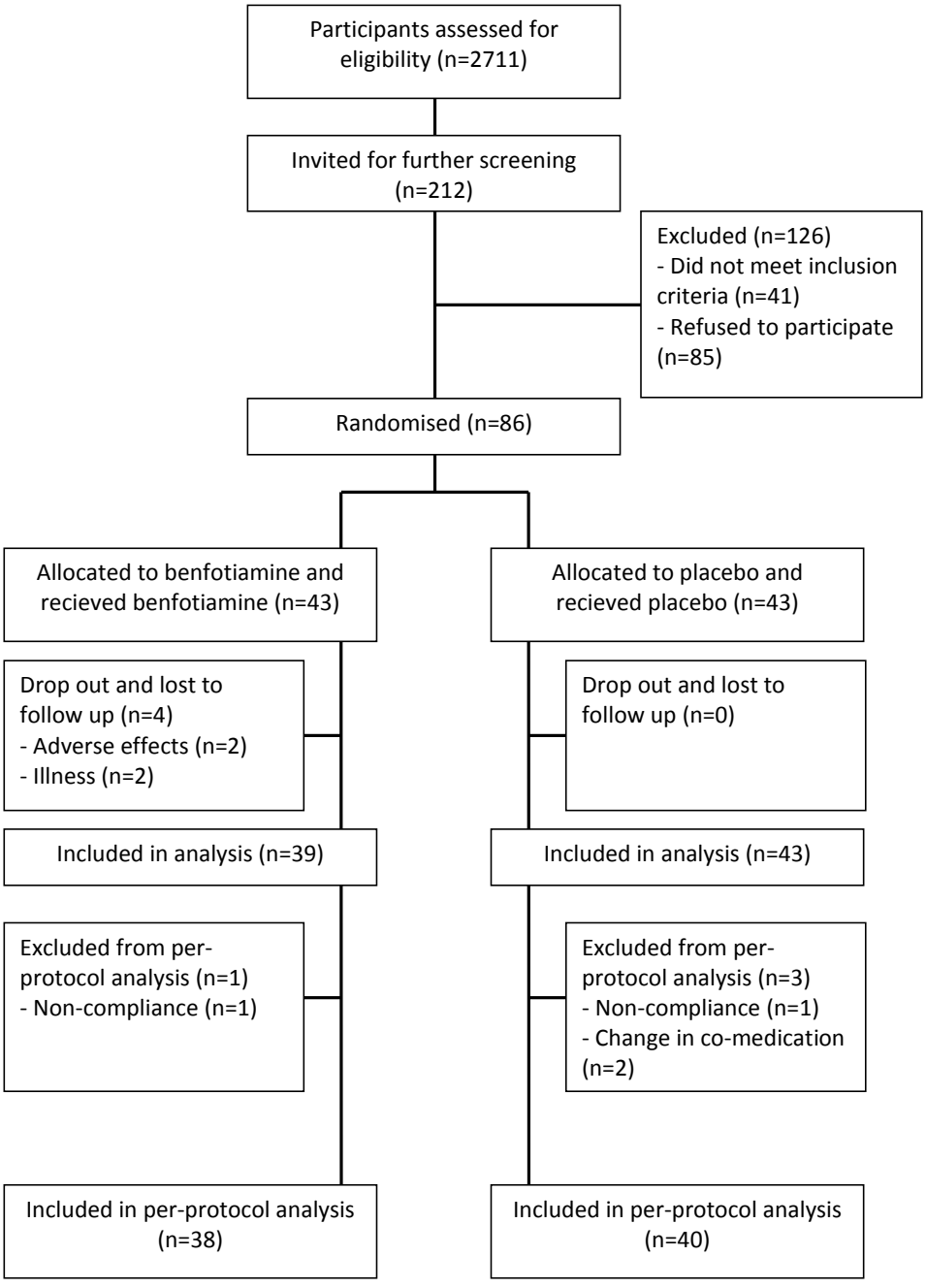

Figure 1: CONSORT flow diagram of the study

Table 1: Baseline characteristics of study population at baseline according to randomisation group

	Benfotiamine	Placebo	*P*-value
General			
n	39	43	
Age (years)	65.3 ± 5.9	64.6 ± 6.1	0.63
Males, n (%)	30 (76.9)	33 (76.7)	0.98
BMI (kg/m^2)	32.1 ± 5.1	31.9 ± 5.9	0.93
Duration of diabetes (years)	12 [9; 18]	10 [7; 18]	0.41
Systolic blood pressure (mmHg)	140 ± 16	137 ± 20	0.48
Diastolic blood pressure (mmHg)	76 ± 8	76 ± 10	0.91
Smoking, n (%)	10 (26)	10 (24)	0.72
Insulin treatment, n (%)	31 (79)	29 (67)	0.22
Oral hypoglycemic agents, n (%)	19 (49)	29 (67)	0.05
HbA_{1c} (%)	7.3 ± 0.9	7.4 ± 0.9	0.55
LDL-cholesterol (mmol/L)	1.9 ± 0.7	1.8 ± 0.9	0.37
HDL-cholesterol (mmol/L)	1.2 ± 0.3	1.1 ± 0.3	0.18
Triglycerides (mmol/L)	1.8 [1.4; 2.6]	2.1 [1.4; 3.4]	0.11
Serum creatinine (µmol/L)	84 ± 19	87 ± 23	0.51
Creatinine clearance (mL/min)	135 ± 51	130 ± 58	0.69
Cystatin C (mg/L)	1.01 ± 0.21	1.03 ± 0.23	0.66
Thiamine status			
Thiamine (nmol/L)	126 ± 23	120 ± 23	0.39
Transketolase activity (mU/mgHb)	0.41 ± 0.10	0.38 ± 0.11	0.69
TPP effect (%)	6.2 [1.0; 11.6]	9.1 [4.6; 15.5]	0.15
TPP effect > 15%, n (%)	6 (15)	10 (23)	0.37
Primary outcome parameters			
UAE (mg/24 hours)	90 [38; 267]	97 [48; 177]	0.70
KIM-1 (µg/24 hours)	1.67 [0.9; 2.4]	1.56 [1.1; 1.9]	0.73
Secondary outcome parameters			
Spot urine UACR (mg/mmol)	10.3 [3.7; 23.4]	7.6 [4.3; 13.3]	0.60
24h UACR (mg/mmol)	9.3 [2.4; 16.8]	6.2 [3.4; 10.5]	0.47
KIM-1/creatinine (ng/mmol)	103 [63; 158]	99 [79; 141]	0.96
Urinary α1-m (mg/24 hours)	9.4 [4.3; 24.4]	8.2 [4.3; 20.3]	0.96
Urinary α1-m/creatinine (mg/mmol)	0.57 [0.28; 1.38]	0.64 [0.30; 1.35]	0.78
Urinary Ngal (mg/24 hours)	131.5 [66.8; 226.8]	122.2 [53.5; 224.2]	0.73
Urinary Ngal/creatinine (mg/mmol)	6.68 [4.25; 13.91]	7.68 [4.22; 18.86]	0.93

Data are n (%), mean ± standard deviation, or median [interquartile range]. HbA_{1c}, glycated hemoglobin; TPP, thiamine pyrophosphate; UAE, urinary albumin excretion; KIM-1, kidney injury molecule-1; UACR, urinary albumin-creatinine ratio; α1-m, α1-microglobuline; Ngal, neutrophil gelatinase associated lipocalin.

Table 2: Summary of absolute changes in primary outcome, secondary outcome, and clinical characteristics after 6 and 12 weeks of intervention (Benfotiamine vs Placebo)

	Benfotiamine	Placebo	*P*-value
Primary outcome parameters			
UAE (mg/24h)			
Δ 6 weeks	-3 [-57; 51]	12 [-61; 40]	0.37
Δ 12 weeks	-9 [-53; 34]	-7 [-56; 65]	0.36
Urinary KIM-1 excretion (µg/24h)			
Δ 6 weeks	0.084 [-0.16; 0.36]	-0.100 [-0.35; 0.17]	0.02
Δ 12 weeks	-0.014 [-0.23; 0.56]	-0.043 [-0.36; 0.19]	0.09
Secondary outcome parameters			
24h UACR (mg/mmol)			
Δ 6 weeks	-0.2 [-7.0; 3.4]	-0.1 [-2.6; 1.6]	0.74
Δ 12 weeks	-0.1 [-4.3; 2.1]	-0.1 [-3.3; 4.6]	0.40
Spot urine UACR (mg/mmol)			
Δ 6 weeks	-0.7 [-4.9; 1.7]	-1.4 [-4.6; 2.2]	0.94
Δ 12 weeks	-0.4 [-8.2; 2.4]	1.1 [-3.4; 3.5]	0.21
Urinary KIM-1/creatinine (ng/mmol)			
Δ 6 weeks	-4 [-22; 40]	11 [-41; 9]	0.14
Δ 12 weeks	2 [-26; 27]	4 [-37; 14]	0.55
Urinary α1-m excretion (mg/24h)			
Δ 6 weeks	0.4 [-3.9; 7.1]	0.8 [-7.5; 5.9]	0.94
Δ 12 weeks	0.4 [-5.2; 5.3]	-0.2 [-7.8; 5.6]	0.63
Urinary α1-m/creatinine (mg/mmol)			
Δ 6 weeks	0.05 [-0.42; 0.52]	-0.01 [-0.65; 0.37]	0.60
Δ 12 weeks	0.05 [-0.17; 0.32]	-0.02 [-0.70; 0.39]	0.54
Urinary Ngal excretion (mg/24h)			
Δ 6 weeks	-0.8 [-19; 23]	-0.9 [-18; 17]	0.97
Δ 12 weeks	3.8 [-17; 31]	-4.2 [-19; 2]	0.08
Urinary Ngal/creatinine (mg/mmol)			
Δ 6 weeks	0.54 [-3.45; 3.61]	-0.73 [-4.24; 1.22]	0.33
Δ 12 weeks	-1.23 [-3.64; 1.40]	0.37 [-3.08; 2.39]	0.23
Clinical characteristics			
Systolic blood pressure (mmHg)			
Δ 6 weeks	-1 [-9; 9]	6 [-9; 16]	0.25
Δ 12 weeks	0 [-6; 11]	4 [-7; 14]	0.98
Diastolic blood pressure (mmHg)			
Δ 6 weeks	1 [-5; 6]	-1 [-6; 5]	0.59
Δ 12 weeks	1 [-5; 6]	0 [-5; 6]	0.95

HbA_{1c} (%)			
Δ 6 weeks	-0.1 [-0.4; 0.1]	-0.1 [-0.4; 0.1]	0.78
Δ 12 weeks	-0.1 [-0.4; 0.3]	0.1 [-0.3; 0.1]	0.92
Serum creatinine (μmol/L)			
Δ 6 weeks	6 [0; 9]	2 [-4; 5]	0.08
Δ 12 weeks	4 [0; 8]	0 [-3; 4]	<0.001
Creatinine Clearance (mL/min)			
Δ 6 weeks	0 [-21; 21]	5 [-24 ; 40]	0.35
Δ 12 weeks	3 [-29; 34]	4 [-34; 24]	0.92
Cystatine C (mg/L)			
Δ 6 weeks	-0.05 [-0.30; 0.14]	0.13 [-0.20; 0.36]	0.10
Δ 12 weeks	0.04 [-0.22; 0.34]	0.05 [-0.28; 0.46]	0.72
LDL-cholesterol (mmol/L)			
Δ 6 weeks	-0.05 [-0.30; 0.14]	0.13 [-0.20; 0.36]	0.10
Δ 12 weeks	0.04 [-0.22; 0.34]	0.05 [-0.28; 0.46]	0.72
HDL-cholesterol (mmol/L)			
Δ 6 weeks	-0.06 [-0.12; 0.06]	0.02 [-0.05; 0.10]	0.02
Δ 12 weeks	-0.01 [-0.07; 0.13]	0.02 [-0.07; 0.10]	0.66
Triglycerides (mmol/L)			
Δ 6 weeks	0.17 [-0.16; 0.67]	-0.13 [-0.66; 0.20]	0.01
Δ 12 weeks	-0.13 [-0.52; 0.60]	-0.28 [-0.55; 0.11]	0.18

Data are median [interquartile range]; Δ 6 weeks/12 weeks, change from baseline to 6 weeks/12weeks; UAE, urinary albumin excretion; KIM-1, kidney injury molecule-1; UACR, urinary albumin-creatinine ratio; α1-m, α1-microglobuline; Ngal, neutrophil gelatinase associated lipocalin; HbA_{1c}, glycated hemoglobin; eGFR-MDRD, estimated glomerular filtration rate calculated using Modification of Diet in Renal Disease formula.

Thiamine status

Effects of thiamine status are shown in Figure 2. In patients receiving benfotiamine, whole blood thiamine concentrations increased, reaching the upper limit of detection (300 nmol/L) in all patients at 12 weeks. Erythrocyte TK-activity also significantly increased after 12 weeks of treatment in the benfotiamine group compared to placebo (median [IQR] change after 12 week 0.13 [0.05; 0.18] versus 0.04 [-0.03; 0.06] mU/mgHb in benfotiamine and placebo, respectively, P<0.001). Concomitantly, there was a significant decrease in TPP-effect in the benfotiamine group (median [IQR] change after 12 week -9.9 [-14.1; -3.6] % versus -1.4 [-9.9; 3.6] % in benfotiamine and placebo, respectively, P= 0.002). At 12 weeks, no patients in the benfotiamine group and 2 patients (5%) in the placebo group had thiamine deficiency defined as TPP effect > 15%.

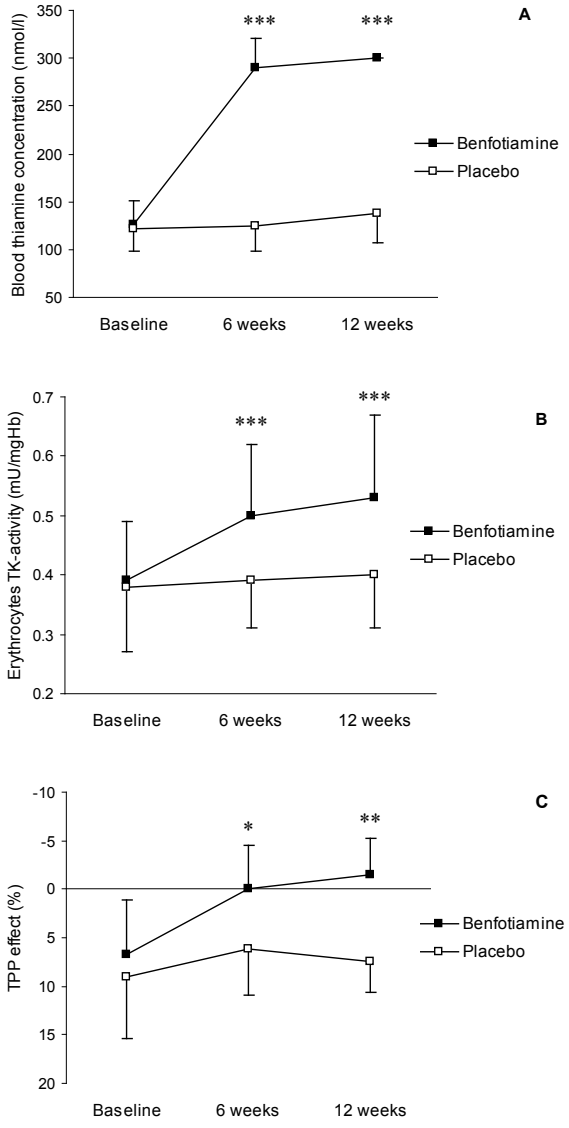

Figure 2: Effects of intervention on thiamine status parameters after 6 and 12 weeks according to group (benfotiamine vs placebo)
A. mean values and standard deviations of blood thiamine concentration.
B. mean values and standard deviations of erythrocyte transketolase activity.
C. median values and interquartile ranges of TPP effect.

TK, transketolase; TPP, thiamine pyrophosphate. *P<0.05 **P<0.01; ***P<0.001, compared with changes from baseline in placebo group.

Table 3: Baseline characteristics and changes in thiamine status parameters, primary outcome measures, secondary outcome measures, and clinical characteristics over time

	Benfotiamine (n = 39)		
	Baseline	6 weeks	12 weeks
Baseline characteristics			
Males, n (%)	30		
Age (years)	65.3 ± 5.9		
BMI (kg/m^2)	32.1 ± 5.1		
Duration of diabetes (years)	12 [9-18]		
Insulin treatment, n (%)	31 (79)		
Oral hypoglycaemic agents, n (%)	19 (49)		
Plasma thiamine (nmol/L)	31.8 ± 7.7		
Thiamine status			
Thiamine (nmol/L)	126 ± 23	290 ± 31	300 ± 0
TK-activity (mU/mgHb)	0.41 ± 0.10	0.51 ± 0.12	0.53 ± 0.15
Primary outcome parameters			
UAE (mg/24h)	90 [38-267]	75 [49-280]	72 [38-199]
U-KIM-1 (µg/24h)	1.67 [0.95-2.47]	1.51 [0.86-2.59]	1.68 [1.06-2.40]
Secondary outcome parameters			
24h UACR (mg/mmol)	10.3 [3.7-23.4]	6.1 [3.0-17.7]	4.9 [2.5-18.4]
Spot urine UACR (mg/mmol)	9.3 [2.4-16.8]	5.8 [3.7-17.9]	7.1 [3.6-17.8]
U-KIM-1/creatinine (ng/mmol)	103 [63-158]	95 [66-170]	96 [77-148]
U-α1m (mg/24h)	9.4 [4.3-24.4]	11.9 [4.4-20.2]	11.2 [4.1-18.8]
U-α1m/creatinine (mg/mmol)	0.6 [0.3-1.4]	0.7 [0.3-1.3]	0.6 [0.3-1.2]
U-Ngal (mg/24h)	131 [67-227]	118 [77-229]	115 [73-284]
U-Ngal/creatinine (mg/mmol)	6.7 [4.3-13.9]	6.2 [3.4-15.9]	5.1 [3.2-12.9]
Clinical characteristics			
SBP (mmHg)	140 ± 16	139 ± 14	143 ± 17
DBP (mmHg)	76 ± 8	77 ± 10	76 ± 9
HbA_{1C} (%)	7.3 ± 0.9	7.1 ± 0.9	7.3 ± 1.0
Plasma creatinine (µmol/l)	84 ± 19	89 ± 19	88 ± 20
Creatinine Clearance (mL/min)	135 ± 51	129 ± 53	133 ± 45
Cystatin C (mg/L)	1.01 ± 0.21	1.06 ± 0.22	1.09 ± 0.23
LDL-cholesterol (mmol/L)	1.9 ± 0.7	1.9 ± 0.8	2.1 ± 0.8
HDL-cholesterol (mmol/L)	1.2 ± 0.3	1.1 ± 0.3	1.2 ± 0.3
Triglycerides (mmol/L)	1.8 [1.4-2.6]	1.9 [1.4-2.8]	1.7 [1.2-2.6]

Data are mean ± standard deviation or median [interquartile range]. BMI, body mass index; TK, transketolase; UAE, urinary albumin excretion; U-KIM-1, urinary excretion of kidney injury molecule-1; UACR, urinary albumin-excretion ratio; U- α1m, urinary excretion of α1-microglobulin; U-Ngal, urinary excretion of neutrophil gelatinase-associated lipocalin; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA_{1c}, glycated hemoglobin.

Placebo (n = 43)			*P*-value
Baseline	6 weeks	12 weeks	
33			0.98
64.6 ± 6.1			0.63
31.9 ± 5.9			0.93
10 [7-18]			0.41
29 (67)			0.22
29 (67)			0.05
31.6 ± 9.8			0.92
122 ± 23	124 ± 25	138 ± 30	<0.001
0.38 ± 0.11	0.39 ± 0.08	0.41 ± 0.10	<0.001
97 [48-177]	99 [43-200]	96 [45-200]	0.36
1.56 [1.06-1.83]	1.56 [1.06-1.83]	1.39 [1.02-2.01]	0.12
7.6 [4.3-13.3]	7.4 [2.8-11.0]	7.1 [4.0-12.5]	0.37
6.2 [3.4-10.5]	8.2 [3.9-14.2]	8.1 [4.6-15.9]	0.58
99 [79-141]	89 [58-130]	81 [66-150]	0.37
8.2 [4.3-20.3]	9.0 [5.7-21.1]	10.2 [2.5-19.7]	0.33
0.6 [0.3-1.3]	0.6 [0.3-1.4]	0.7 [0.2-1.1]	0.47
122 [53-224]	112 [52-218]	99 [52-222]	0.17
7.7 [4.2-18.9]	6.4 [3.2-15.1]	8.5 [3.3-13.1]	0.18
137 ± 20	140 ± 20	140 ± 17	0.60
76 ± 10	76 ± 9	76 ± 10	0.68
7.4 ± 0.9	7.2 ± 0.9	7.2 ± 0.9	0.33
87 ± 23	89 ± 25	87 ± 21	0.04
130 ± 58	139 ± 58	131 ± 64	0.57
1.03 ± 0.23	1.10 ± 0.26	1.11 ± 0.23	0.53
1.8 ± 0.9	1.8 ± 0.9	1.9 ± 0.9	0.55
1.1 ± 0.3	1.1 ± 0.3	1.1 ± 0.3	0.25
2.1 [1.4-3.4]	2.2 [1.4-2.9]	2.0 [1.2-2.9]	0.06

Comparison of baseline characteristics was performed by unpaired Student's T-test (for normally distributed variables) or Mann-Whitney-U test (for non-normally distributed variables). X2-test was used to compare non-continuos variables. Changes in thiamine status parameters, primary outcome measures, secondary outcome measures, and clinical characteristics over time were analyzed by ANOVA for repeated measures, with log-transformation of variables with skewed distribution prior to analysis.

Primary and secondary outcome parameters

Changes in primary outcome parameters, secondary outcome parameters and clinical characteristics are shown in Table 2 and Table 3. Significant differences in primary or secondary outcome parameters were neither found after 6 nor after 12 weeks of treatment between the benfotiamine group and the placebo group. Change in UAE between baseline and 12 weeks was -18 mg/24h in the benfotiamine and -1 mg/24h in the placebo group. For individual differences, respective changes were -9 [-53;34] mg/24h and -7 [-56;65] mg/24h. Adjustment for differences in baseline use of oral hypoglycemic agents, and prevalence of TPP>15% in multivariate regression analyses did not reveal any relevant different results (data not shown).

With respect to clinical characteristics, there was a trend towards increase in plasma creatinine in the benfotiamine group compared to placebo, which reached significance after 12 weeks of treatment, but this was not accompanied by changes in creatinine clearance or cystatin C. In addition, there was a significant increase in serum triglycerides (TG) and a significant decrease in HDL-cholesterol in the benfotiamine group compared to placebo group at 6 weeks, which were not significant any more at 12 weeks.

Side effects

During the study, no serious adverse effects occurred. In the benfotiamine group one patient contacted the study physician because of nausea and heartburn. Attempt to stop the medication for one week and to retry resulted in symptoms to reappear. This patient continued the study with a reduced dose of 1 tablet/day (300mg) and was therefore categorised as non-compliant. In the placebo group, one patient was non-compliant, as concluded by more than 50 tablets (>20% of the total amount) not being taken at the end of the study. Besides, two protocol deviations occurred: ACE-I was stopped and antibiotic treatment was initiated for prostatitis in one patient and another patient suffered from ACE-I-induced angioedema and was then switched to ARB. As a consequence, 38 in the benfotiamine group and 40 in the placebo group were available for the per-protocol analysis. The results of these per-protocol analyses (data not shown) were not materially different from the presented analyses.

Discussion

In this double-blind placebo-controlled trial, we found that 12 weeks of treatment with high-dose benfotiamine did not result in a decrease in urinary excretion of albumin or tubulointerstitial damage markers, such as KIM-1, Ngal, and α1-m. On the other hand, high-dose benfotiamine did result in improvement in thiamine status, as reflected by whole blood thiamine concentrations, erythrocyte TK-activity and TPP effect.

Our findings contrast with earlier findings from studies with low dose (7mg/kg) and high dose

(70 mg/kg) of thiamine and benfotiamine treatment in animal models with streptozotocin-induced diabetes, in which 24 weeks of treatment protected against a further increase in urinary albumin excretion already after 6 weeks of treatment(11, 14). Although no clear evidence of dose-response relationship regarding albuminuria was found in these studies, only high-dose benfotiamine suppressed oxidative stress. The relatively high dose of benfotiamine (900 mg/day) in our study is still less than 70mg/kg and might be insufficient to achieve all therapeutic goals in humans.

Our results also contrast with results of a randomised, double-blind placebo-controlled pilot study of 12 weeks of high dose (300 mg/day) oral thiamine supplementation in 40 Pakistani patients with type 2 diabetes(22). In this study, a median decrease of 17.7 mg/24h (33%) in UAE within the thiamine treated group was observed after 12 weeks of treatment.

We investigated the effect of benfotiamine instead of thiamine in addition to existing ACE-I or ARB treatment, while in the study by Rabbani et al, more than 50% of the patients was not on such treatment(22). Nevertheless, in a separate analysis of that study it was reported that presence or absence of ACE-I/ARB treatment made no difference in outcome(23). Another point is that we investigated Caucasian patients, where only about 20% had thiamine deficiency at baseline by the “thiamine effect” criterion, while Rabbani et al. investigated Pakistani patients with low plasma thiamine concentration. Thus, a difference in basal thiamine status, background diet or genetic susceptibility for the effects of benfotiamine/thiamine supplementation might also play a role.

In their study in rats, Babaie-Jadidi et al. suggested that benfotiamine has a renal hemodynamic effect, antagonizing renal hyperfiltration, similar to ACE-I and ARB treatment(11,24). Consistent with this putative mechanism, we found a small but significant increase in plasma creatinine, but this was not paralleled by changes in cystatin C or creatinine clearance. Yet, measured glomerular filtration rate would have been necessary in order to elucidate renal effects of benfotiamine.

Finding no effect on urinary albumin excretion and tubulointerstitial damage markers in patients with type 2 diabetes and DN, it is important to realise that thiamine and benfotiamine are supposed to antagonise the detrimental effects of hyperglycaemia. In line with this, it has been shown that these agents interfere with at least three biochemical pathways by which hyperglycaemia otherwise exerts its detrimental effects, including formation of advanced glycation end-products(14). Still, changes may take much time. For example, it took 4-5 years of lowering of HbA_{1c} in patients with type 1 diabetes in the Diabetes Control and Complications Trial before a difference could be discerned between subjects with strict metabolic control and standard therapy regarding urinary albumin excretion rate(6). Likewise, a similar delay in separation of curves was reported in patients with type 2 diabetes in the United Kingdom Prospective Diabetes Study(25). Therefore, given the biology of DN and the long duration of detrimental effects of hyperglycaemia to be reversed, it is more likely that our study was too short to demonstrate an effect of an agent interfering with

glucotoxicity. A larger trial with longer follow-up of at least 4 years is necessary to investigate possible preventive effects of benfotiamine in DN.

Few adverse events were observed in the benfotiamine group, but none of these was serious. Two premature terminations of the study were caused by newly diagnosed malignancies; a lung cancer and a stomach cancer. These two cases were assessed by the treating physician as not causally related to the study medication and not unusual considering the demographic background of the study population.

One limitation of our study is that it was not powered to detect a small effect of benfotiamine on urinary albumin excretion as an add-on therapy besides existing ACE-I or ARB treatment. Our study was explorative and used a one-sided hypothesis based on the likelihood of improvement in outcome measures. However, after 12 weeks of treatment, the median change in UAE was -9 mg/24h in the benfotiamine group versus -7 mg/24h in the placebo group. It is questionable whether such a difference would be considered clinically relevant and a much larger study would have been necessary to find this difference statistically significant.

In conclusion, 12-week treatment with benfotiamine in patients with type 2 diabetes and mild diabetic nephropathy did not reduce urinary excretion of albumin or tubulointerstitial damage markers. Long-term intervention studies are likely to be necessary to discern whether benfotiamine treatment has an effect on development and course of diabetic nephropathy.

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Chapter

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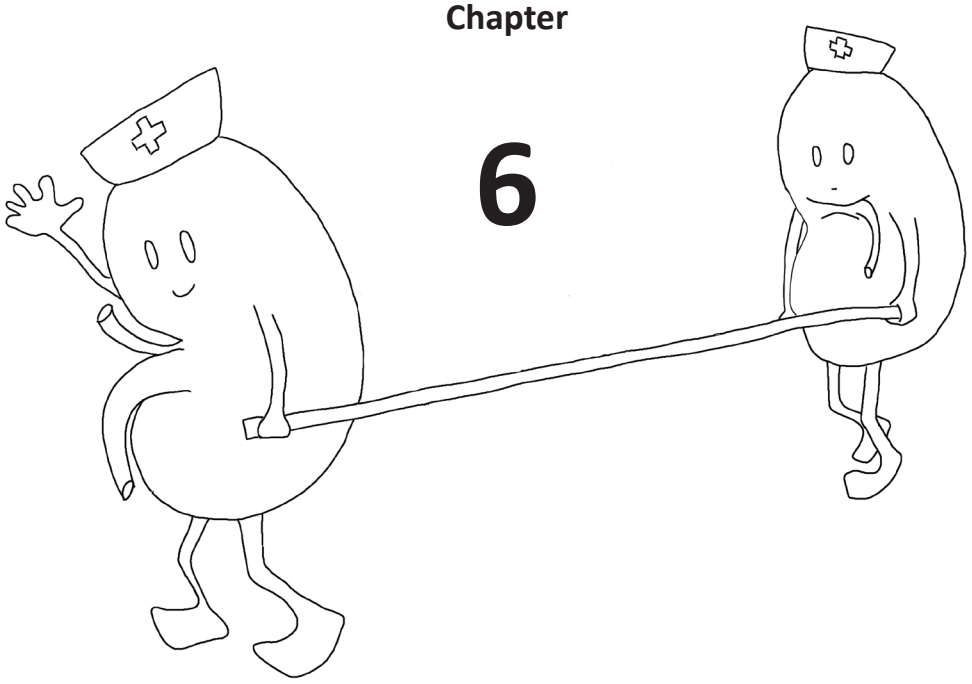

The Effect of Fasting on Renal Ischaemia-Reperfusion Injury

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Abstract

Donor nutritional status may affect end-organ damage after surgical intervention. Indeed in liver and heart tissue there was a profound protective effect of fasting on ischaemia-reperfusion injury. However, data of fasting on ischaemia-reperfusion injury in the kidney is scarce. We therefore aimed to investigate the effect of short-term perioperative fasting on renal ischaemia-reperfusion injury in rats.

Adult male Lewis rats were fed ad libitum or fasted for 48 hours prior to ischaemia-reperfusion procedure with water ad libitum. The left kidney was subjected to 45 minutes of warm ischaemia, combined with nephrectomy of the right kidney. After ischaemia-reperfusion procedure only water was allowed for both groups. Animals were sacrificed 24 hours after ischaemia-reperfusion injury and plasma and kidney tissue were retrieved.

Mean (± SEM) weight at time of ischaemia-reperfusion was significantly higher in ad libitum fed than in fasted animals (332 ± 3.2 vs. 286 ± 2.5 g, $P<0.001$). At sacrifice there was no difference between ad libitum fed and fasted animals in plasma creatinine, ureum, AST or LDH nor was there any difference between ad libitum fed and fasted animals in renal gene expression for Kim-1, MCP-1, α-SMA, BCL-2 or BAX (all $P>0.05$).

In conclusion, dietary food restriction may be a powerful method to reduce ischaemia-reperfusion injury in various tissues. However, in this study we did not show an effect of 48 hours fasting on renal ischaemia-reperfusion injury on biochemical markers or gene-expression levels. It remains to be elucidated whether longer period(s) of fasting or caloric restriction will be protective in renal ischaemia-reperfusion injury in rats.

Introduction

Perioperative nutrition is a recurrent issue in experimental and clinical research related to the safety of anaesthesia and the metabolic response to surgical trauma. The effects of fasting on ischaemia-reperfusion injury in liver and heart have been investigated previously since it was hypothesized that a poor nutritional status of the donor may adversely affect transplant outcome. It has been shown that malnutrition has proven to be a risk factor of surgical complications(1). The attention was drawn to a large number of hospitalized patients suffering from undernutrition, fueling the hypothesis that preoperative and postoperative feeding would be beneficial. In a randomized clinical trial by Beattie et al. preoperative and postoperative nutritional support reduced complications from surgery(2). Later it was shown that preoperative and postoperative nutrition also speeds up postoperative recovery(3-5). In contrast to these findings, in animal studies a protective effect of fasting on ischaemia-reperfusion injury in liver and heart was found(6-10). Recently these beneficial effects have also shown to be substantiated in renal ischaemia-reperfusion injury in mice(11). However, data of fasting on ischaemia-reperfusion injury in the kidney is scarce.

Dietary restriction can be performed by means of different regimens such as fasting, alternate day fasting and caloric restriction (reduced daily caloric intake). Caloric restriction results in increased lifespan in a wide variety of species such as yeast, worms, flies and mice(12). The mechanism of caloric restriction is related to a decrease in the incidence and onset of age-related diseases and the increase in resistance to toxicity and stress(13,14). The effects of long-term dietary restriction regimens have been widely studied and provide mechanistic insights on the effect of fasting on acute stress resistance. Long-term dietary restriction lowers steady-state levels of oxidative stress, decreases mitochondrial electron and proton leak in mammalian cells, and attenuates damage resulting from intracellular oxidative stress(15-17). Dietary restriction also augments antioxidant defence systems and increases stress resistance to both oxidative and non-oxidative challenges in models of extended longevity. Dietary restriction has been proposed to act as a mild stressor that extends longevity through hormetic mechanisms(18,19). Interestingly, ischaemic preconditioning, a procedure used to protect against ischaemic insult that entails brief periods of ischaemia prior to a longer ischaemia time, is also thought to function via hormesis(20).

Ischaemia-reperfusion injury is inevitable in the process of transplantation. During ischaemia a lack of blood flow results in a state of tissue oxygen and nutrient deprivation characterized by ATP depletion, loss of ion gradients across membranes and build up of toxic by products. Restoration of blood flow causes further damage by a burst of reactive oxygen species and subsequently by inflammatory mediators in response to tissue damage.

We hypothesized that fasting prior to renal ischaemia-reperfusion injury in rats will attenuate ischaemia-reperfusion injury. We therefore aimed to investigate the effect of perioperative fasting on ischaemia-reperfusion injury in rat kidneys.

Materials and methods

Experimental design

Twelve male inbred Lewis rats (± 320 g) (Harlan, Zeist, The Netherlands) were kept under standard laboratory conditions (temperature 20-24 °C, relative humidity 50-60%, 12 h light/12 h dark). Rats were individually housed allowing for daily determination of body weight. Control group (C) (n=6) was allowed ad libitum intake of food and water, the fasting group (F) (n=6) was allowed only water ad libitum 48 hours before the ischaemia-reperfusion procedure was performed. Briefly, anesthesia was induced by 5% isoflurane, and the rats were subsequently maintained on 3% isoflurane. The rats were placed on a homothermic table to maintain core body temperature at 37 °C. The left kidney was subjected to 45 minutes of warm ischaemia, followed by reperfusion. Nephrectomy of the contralateral right kidney was performed during ischaemia of the left kidney. During the first 24 hours after reperfusion only water was allowed for C and F. At sacrifice the rats was were anesthetized with isoflurane and 250-IU heparin was perfused through the penile vein. This was followed by cannulation of the aorta after which a 5 mL blood sample was taken. A full body flush with 40 mL 0.9% NaCl at 4 °C, was performed in order to obtain optimal tissue for morphology. Midcoronal kidney samples were snap frozen and stored at -80 °C or processed in 4% formalin for paraffin embedding. Plasma was stored at -80 °C.

All experimental procedures were approved by the Committee for Animal Experiments of the University of Groningen and performed according to the principles of laboratory animal care (NIH publication no. 85-23, revised 1985).

Biochemical measurements

Plasma creatinine concentration was measured by Roche enzymatic method. Plasma ureum, plasma aspartate transaminase (AST) and plasma lactate dehydrogenase (LDH) were measured by routine analysis on Roche Modular.

RNA isolation and real-time PCR

Tissue preparation for real-time PCR was performed as described previously(21). The expression of a marker for tubules injury Kidney injury molecule-1 (Kim-1), a marker of macrophages Monocyte chemotactic protein-1 (MCP-1), a pro-fibrotic marker α-Smooth muscle actin (α-SMA), and the apoptosis markers B-cell lymphoma-2 (BCL-2) and BCL-2-assiociated protein X (BAX) were determined. For each gene the expression was normalized relative to the mean cycle threshold (CT) value of the β-actin gene. Results were finally expressed as 2-ΔCT, which is an index of the relative amount of mRNA expressed in each tissue. The standard deviation of the triplicates of the CT values was accepted, if the coefficient of variation was less than 3%.

Immunohistochemistry

Immunohistochemistry was performed on 3 µm kidney sections stained for necrosis with periodic acid-Schiff (PAS). Area of necrosis was expressed as percentage of cortex of the kidney.

Statistical analysis

Data was analyzed using PASW version 18.0.3 (IBM SPSS Inc., Chicago, IL), and expressed as the average ± standard error of the mean (SEM). Statistical significance of difference was assessed by Student's T-tests. Differences were considered significant if the *P*-value<0.05.

Results

Bodyweight

Fasting resulted in significant reduction of bodyweight (Figure 1). Before the start of the fasting period there was no difference in bodyweight between C and F (327 ± 3.0 vs. 320 ± 3.4 g, *P*=0.20). At time of ischaemia-reperfusion weight in C was significantly higher than in F (332 ± 3.2 vs. 286 ± 2.5 g, *P*<0.001). In the 24 hours after ischaemia-reperfusion procedure C lost weight (*P*<0.001) whereas F maintained their weight although there were still fasted (*P*=0.45). However, at time of sacrifice weight in C was still significantly higher than in F (316 ± 4.0 vs. 284 ± 4.4 g, *P*<0.001).

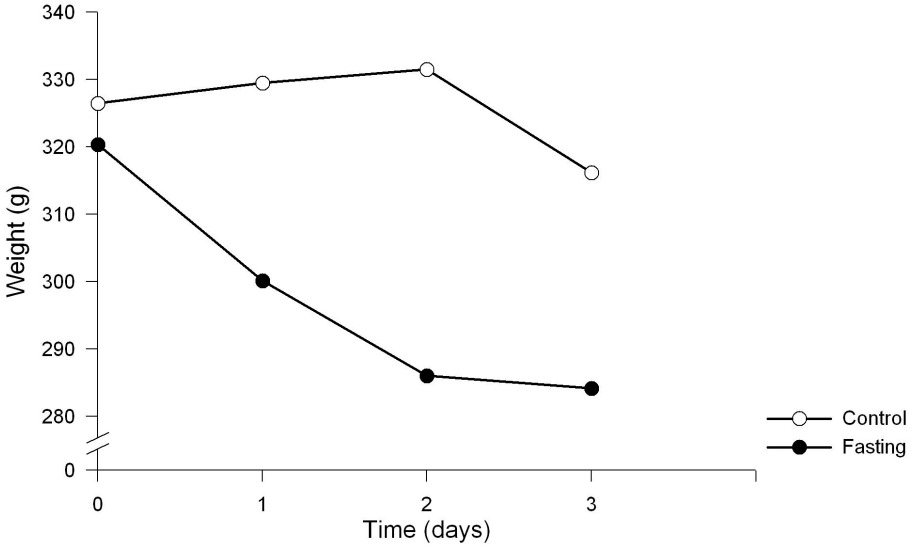

Figure 1: Body weight

Biochemical measurements

After 24 hours after reperfusion there was no difference between C and F in plasma creatinine, plasma ureum, plasma AST or plasma LDH (see Table 1).

RT-PCR results

At baseline there was no significant difference between C and F in the expression of Kim-1, MCP-1, α-SMA, BCL-2 and BAX (all $P>0.05$). After 24 hours after reperfusion there was also no difference in the expression of the genes analyzed. Expression of the genes when comparing C to F was respectively for Kim-1, 27 ± 4.7 vs. 30 ± 2.1, $P=0.50$, MCP-1 0.15 ± 0.03 vs. 0.14 ± 0.01, $P=0.76$, α-SMA, 0.78 ± 0.18 vs. 0.55 ± 0.10, $P=0.31$, BCL-2, 0.55 ± 0.12 vs. 0.50 ± 0.07, $P=0.70$, and BAX, 1.21 ± 0.20 vs. 1.01 ± 0.18, $P=0.45$.

Immunohistochemistry

After 24 hours after reperfusion there was no difference in percentage of necrosis of the cortex. Percentage of necrosis of the cortex was 15.5 ± 2.4% in C, and 15.5 ± 2.3% in F, $P=1.00$. Histology of the cortex of the kidney after 24 hours after reperfusion is shown in Figure 3. Necrotic strings of tubules with loss of nuclei are shown between normal tubules.

Table 1. Biochemical measurements in plasma 24 hours after ischaemia-reperfusion injury

	Control	48 h Fasted	*P*-value
Kreatinine	128 ± 17	172 ± 18	0.12
Ureum	26.8 ± 1.9	26.4 ± 1.9	0.89
AST	177 ± 9.0	154 ± 13	0.33
LDH	143 ± 20	120 ± 11	0.17

Kreatinine is expressed as µM, ureum is expressed as mM, AST and LDH are expressed as U/L.

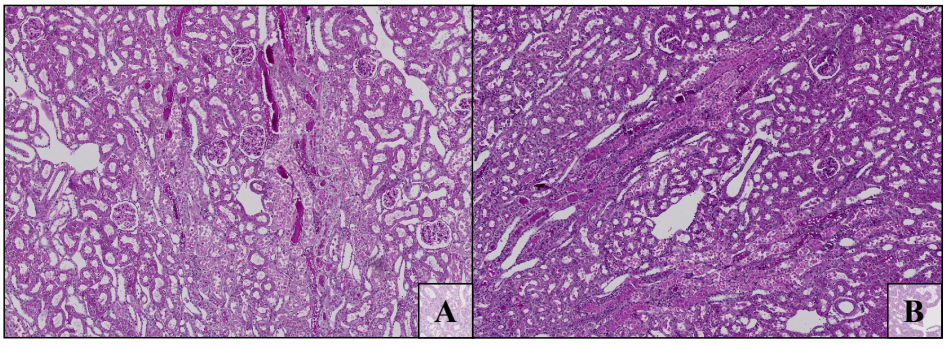

Figure 3. PAS staining. A: Control. B: 48 h Fasted

Discussion

It is well known that excessive preoperative weight loss is associated with negative surgical outcome after major abdominal and thoracic surgery. Studley et al. first reported on this phenomenon with great limitations to the study(22). In this study there was no control group that did not loose weight or actually gained weight prior to surgery. Thereafter, other studies reported on nutritional status in surgical patients and did show that malnutrition is a risk factor for surgical complications(23-25).

Nowadays there is increasing evidence that pre-operative dietary restriction is protective in ischaemia-reperfusion injury by up-regulating endogenous cell resistance mechanisms(11,26-29). Hormesis is a common biological phenomenon in which exposure to a low intensity stressor induces a general adaptive response that has net beneficial effect. It has been proposed that dietary restriction acts through hormetic mechanisms, just as ischemic preconditioning(18-20). Dietary restriction (mostly studied as caloric restriction in longevity models) has shown to exert profound tissue level changes in metabolism with a generalized shift from carbohydrate to fat metabolism. Four pathways have been implicated in this effect: these are the insulin like growth factor/insulin signalling pathway, the sirtuin pathway, the adenosine monophosphate activated protein kinase pathway and the target of rapamycin pathway(30). These different pathways may interact and may all play important roles mediating different aspects of the respons. In different models of ischaemia-reperfusion injury and solid organ tranplantation long term dietary restriction has shown to increase stress resistance through upregulating of heat-shock proteins, hemeoxygenase-1 and nuclear factor kappa beta(31-34). Its also attenuates damage from intracellular oxidative stress and lowers the levels of oxidative stress; as well antioxidant levels are faster returning to baseline(15,16,31,35). In liver tissue it has been shown that there is stimulation of tissue repair after DR due to faster and higher expression of growth stimulatory cytokines and growth factors(36,37).

In our study we found no effect of 48-hours fasting period prior to renal ischaemia-reperfusion injury in rats. A 48-hours fasting period led to weight reduction of approximately 10%. As to be expected from a surgical procedure weight in control rats decreased after ischaemia-reperfusion injury. However, in rats fasted for 48-hours weight was maintained after ischaemia-reperfusion injury, despite they continued fasting. After 24 hours of reperfusion there was no significant difference between C and F in biochemical measurements, including plasma creatinine and plasma ureum, or in gene expression.

The time frame of restriction or fasting prior to ischaemia-reperfusion injury has still to be elucidated. In mice it has been shown that three days of fasting was even more protective in renal ischaemia-reperfusion injury than one day(11). However, due to concerns about animal welfare and lack of clinical applicability, when applying an even longer period of fasting prior to ischaemia-reperfusion injury, we decided not to extend the period of fasting

prior to ischaemia-reperfusion injury. The functional protection afforded by fasting prior to ischaemia-reperfusion injury is rapidly lost within hours of refeeding(11). Therefore we applied also a fasting period in C and F in the 24-hours after ischaemia reperfusion injury.
The fact that a 48-hours fasting period failed to show significant differences after ischaemia-reperfusion injury in rats, whereas a 24-hours fasting period in mice showed protective effects, could imply that in humans even longer period of fasting is necessary. Thereby this method would not be applicable in clinical setting and concerns about malnutrition will rise. However, caloric restriction will be a better option in clinical setting. Recently, it has been shown that is possible for living kidney donors to adhere to a combined 24 hours of fasting with three days of 30% caloric restriction(38). In this pilot study there were no beneficial effects on post-operative graft function. Therefore in humans even longer and more extensive dietary regimens may be needed. This would also provide more possibilities for errors, non-compliance and increased work load of the dieticians involved.
In conclusion, dietary food restriction may be a powerful method to reduce ischaemic damage in various organs. In the time frame and the species studied in the present paper we did not observe these beneficial effects in the kidney. Future studies aiming at longer periods of fasting or caloric restriction and a longer follow up may further shed light on the present findings.

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Chapter

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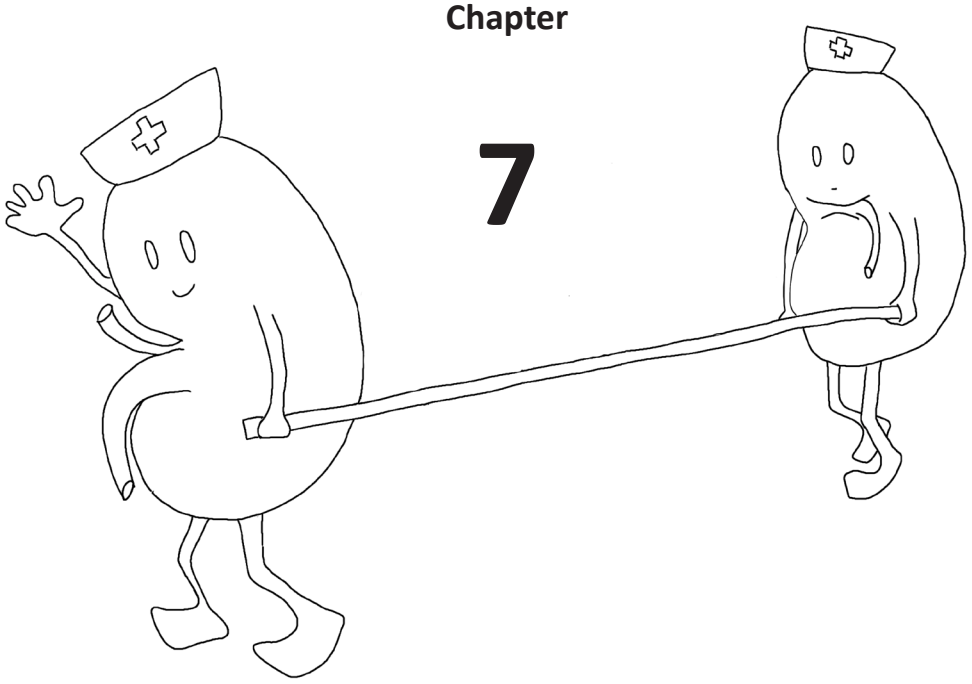

Non-Esterified Fatty Acids and Development of Graft Failure in Renal Transplant Recipients

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Abstract

Chronic transplant dysfunction is the most common cause of graft failure on the long term. Proteinuria is one of the cardinal clinical signs of chronic transplant dysfunction. Albumin-bound fatty acids (FA) have been hypothesized to be instrumental in the etiology of renal damage induced by proteinuria. We therefore questioned wheter high circulating FA could be assiociated with an increased risk for future development of graft failure in renal transplant recipients (RTR). To this end, we prospectively investigated the association of fasting concentrations of circulating non-esterified fatty acids (NEFA) with development of graft failure in RTR.

Baseline measurements were performed between 2001-2003 in outpatient RTR with a functioning graft >1 year. Follow-up was recorded until May 19, 2009. Graft failure was defined as return to dialysis or retransplantation.

We included 461 RTR at a median (interquartile range [IQR]) of 6.1 (3.3-11.3) years post-transplant. Median (IQR) fasting concentrations of NEFA were 373 (270-521) µmol/L. Median (IQR) follow-up for graft failure beyond baseline was 7.1 (6.1-7.5) years. Graft failure occurred in 23(15%), 14(9%), and 9(6%) of RTR across increasing gender-specific tertiles of NEFA (P=0.04). In a gender-adjusted Cox-regression analysis, log-transformed NEFA level was inversely associated with development of graft failure (hazard ratio, 0.61; 95% confidence interval, 0.47-0.81; P<0.001).

In this prospective cohort study in RTR, we found an inverse association between fasting NEFA concentrations and risk for development of graft failure. This association suggests a renoprotective rather than a tubulo-toxic effect of NEFA. Further studies on the role of different types of NEFA in the progression of renal disease are warranted.

Introduction

Chronic transplant dysfunction is the most common cause of graft failure in the long term and clinically characterized by a slow but steady decline in allograft function, with proteinuria as one of its cardinal signs(1-3). Interstitial fibrosis accompanied with tubular atrophy is the most important histopathological entity associated with chronic transplant dysfunction(4-6).

Ultrafiltrated proteins are thought to play an important role in the pathogenesis of chronic tubulointerstitial injury, the major cause for changes in renal structure and decline of function(7, 8). Albumin is the major determinant of ultrafiltrate and nephrotic urine. Albumin isolated from plasma carries various substances, including fatty acids (FA)(9). Recently it has been discovered that even in nonproteinuric rats renal albumin filtration is approximately 50 times greater than previously measured and is followed by rapid endocytosis into proximal tubule cells. Thereby even in nonproteinuric patients proximal tubule cells will be exposed to substances that are carried by albumin, such as FA(10,11).

It has been argued that the FA bound to albumin rather than the albumin per se are toxic to proximal tubular cells and thereby underlie the deterioration of the renal interstitium associated with proteinuria. In vitro it has been shown that albumin-bound FA are efficiently taken up by proximal tubular epithelial cells, where they induce adverse effects, such as altered cellular growth,(12) disturbed metabolism,(12,13) increased apoptosis,(14) production of extracellular matrix proteins,(15) reactive oxygen species (ROS)(15, 16) and the release of lipid metabolites(15,17,18). All these actions can contribute to the development and progression of interstitial damage. The concept of albumin-bound FA nephrotoxicity is supported by in vivo evidence. In rodents, it is found that FA bound to albumin aggravate albumin-induced nephropathologic effects, including cortical apoptosis,(19) tubulointerstitial inflammation(19-21) and glomerular injury(21).

To the best of our knowledge, the contribution of albumin-bound FA to nephrotoxicity has not been tested in humans. The development of graft failure after renal transplantation provides an interesting concept for testing nephrotoxic effects of FA in humans.

We therefore aimed to prospectively investigate whether fasting concentrations of circulating non-esterified fatty acids (NEFA) are associated with the development of graft failure in renal transplant recipients (RTR).

Materials and Methods

The current prospective study was a predefined part of a larger study and incorporated in the Groningen Renal Transplant Outpatient Program, details of which have been published previously(22-24). Between August 2001 and July 2003, all adult RTR who had a functioning graft for more than 1 year were eligible to participate. Patients with known or apparent

systemic illnesses (e.g., malignancies or opportunistic infections) were considered ineligible. Of 847 eligible RTR, a total of 606 (72%) signed written informed consent. Mortality and graft failure were recorded for all RTR until May 19, 2009. Graft failure was censored for death and defined as return to dialysis or retransplantation. For the current study, 461 patients were included at a median of 6.1 years post transplant. Subjects with a combined kidney-pancreas transplantation were excluded. For patients who died with a functioning graft (n= 88 in our study population), duration of follow-up was calculated until the date of death. For patients with graft failure (n= 46), duration of follow-up was calculated using the date of the start of dialysis or retransplantation.

The institutional review board approved the study protocol (METc01/039). Funding sources had a role neither in the collection and analyses of data nor in publication of the manuscript. Literature indicates that, if samples are not collected on ice or are retrieved as serum or heparin plasma, levels of NEFA can become falsely high due to release of fatty acids from triacylglycerols and cholesterol esters present in plasma lipids, presumably as a consequence of activity of circulating lipases(25-27). We therefore took special precautions to collect and prepare samples before the assessment of NEFA. Blood for separation of EDTA plasma was drawn in ice-chilled tubes after an 8- to 12-h overnight fasting period. It was kept on ice until centrifugation at 4°C within 1 h after collection and then stored at -80 °C for a maximum of one month until assessment of NEFA concentrations. If samples could not be processed within 1 h after collection or if assays could not be performed within 1 month after frozen storage, NEFA concentrations were not assessed. NEFA concentrations were measured by means of an enzymatic colorimetric commercial assay (WAKO Diagnostics, Richmond). This assay measures the total amount of NEFA present in plasma, of which the largest part is bound to albumin(28,29). Serum creatinine levels were determined using a modified version of the Jaffé method (MEGA AU 510; Merck Diagnostica, Darmstadt, Germany). Creatinine clearance was calculated from 24-h urinary creatinine excretion and serum creatinine. Total urinary protein concentration was analyzed using the Biuret reaction (MEGA AU 510; Merck Diagnostica, Darmstadt, Germany). Both class I and class II anti-HLA antibodies were assessed by enzyme-linked immunosormbent assay (LATM20x5; One Lambda, Canoga Park, CA).

Statistical analyses

Analyses were performed with PASW version 18.0.3 (IBM SPSS Inc., Chicago, IL). Parametric variables are given as mean ± SD. Nonparametric variables are given as median (IQR). A priori, we decided to divide subjects in tertiles based on fasting NEFA concentrations stratified for gender; differences between the groups were tested for statistical significance with the Mann Withney U test in case of a non-parametric variable; the chi-square test was used in case of a categorical variable. Kaplan-Meier survival analysis with log-rank testing was performed for prospective analysis of graft loss. We then proceeded with univariate and

multivariate Cox-regression analyses of log-transformed NEFA concentrations. A two-sided *P*-value of <0.05 was considered to be statistically significant.

Results

Baseline measurements were obtained from 461 RTR at median, with an interquartile range (IQR) of 6.1 (3.3-11.3) years after renal transplantion. Median (IQR) fasting concentrations of NEFA for men were 333 (249-463) µmol/L. Minimum and maximum values were 95 and 1001 µmol/L, respectively. Median (IQR) fasting concentrations of NEFA for women were 438 (302-564) µmol/L. Minimum and maximum values were 73 and 1139 µmol/L, respectively. Cutoff points for tertiles were 277 and 418 µmol/L for men and 350 and 521 µmol/L for women. Distributions of NEFA concentrations are shown in Figure 1. In Figure 2 a graph of NEFA concentrations and urinary protein excretion is depicted.

Recipient-related baseline characteristics according to the gender-stratified tertiles of NEFA concentrations are shown in Table 1. High-density lipoprotein cholesterol increased significantly with rising NEFA concentrations. Triglycerides were significant different between the tertiles, but there was no dose-effect relation. There was no difference between the tertiles for anti-human leukocyte antigen (HLA) antibodies, plasma albumin, insulin concentrations and weight-related measures.

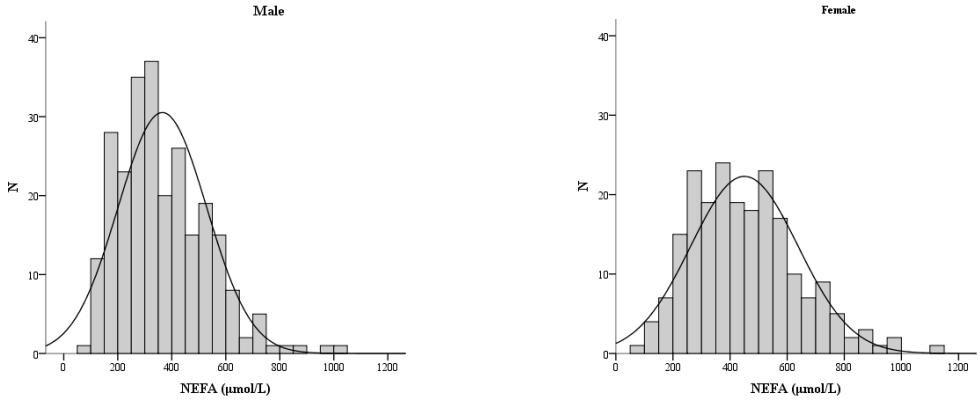

Figure 1. Histogram of NEFA concentrations

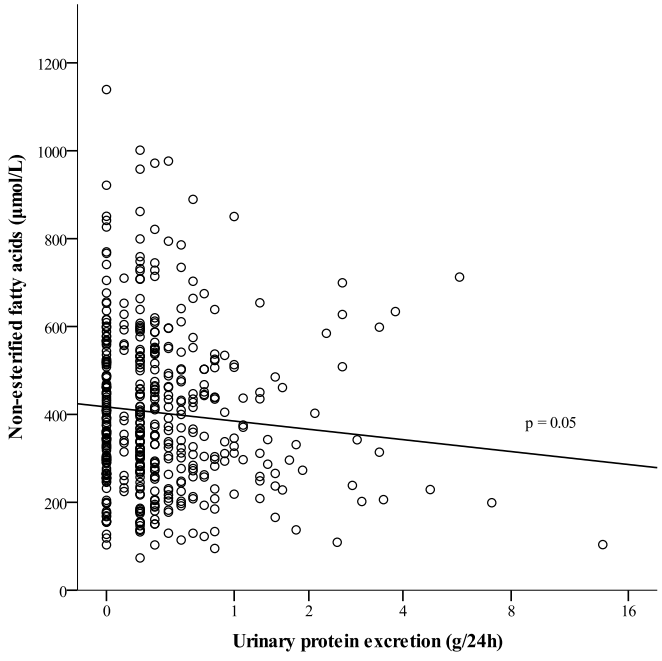

Figure 2. Graph of non-esterified fatty acid concentrations and urinary protein excretion

Table 1. Recipient-related baseline characteristics

	Gender stratified tertiles of NEFA			
	1st	2nd	3rd	*P*
N	154	154	153	
Recipient demographics				
Time until baseline (years)	7.1 [3.6-12.2]	6.0 [3.4-10.9]	5.9 [2.7-10.9]	0.37
Dialysis prior Tx (months)	26 [12-48]	28 [14-51]	28 [16-47]	0.55
Age (years)	51.7 ± 12.2	51.7 ± 11.5	52.3 ± 12.4	0.88
Male gender, n (%)	84 (55)	84 (55)	83 (54)	1.00
Anti-HLA antibodies				
Negative[a], n (%)	122 (79)	119 (77)	125 (82)	0.92
Borderline, n (%)	6 (4)	6 (4)	5 (3)	
Positive[b], n (%)	26 (17)	29 (19)	23 (15)	
Body composition measurements				
Weight (kg)	76.3 ± 12.4	77.1 ± 13.4	76.1 ± 13.1	0.79
Body mass index (kg/m^2)	25.6 ± 3.7	26.0 ± 4.1	26.3 ± 4.8	0.33
Waist circumference (cm)	96.3 ± 12.5	97.4 ± 13.7	97.4 ± 14.1	0.72
Hip circumference (cm)	98.7 ± 8.8	99.3 ± 8.5	99.5 ± 9.3	0.72
Waist hip ratio	0.98 ± 0.11	0.98 ± 0.11	0.98 ± 0.10	0.95
Current smoking, n (%)	40 (26)	31 (20)	31 (20)	0.37
Blood pressure				
MAP (mm Hg)	142 ± 17	139 ± 15	141 ± 16	0.24
ACE-I or ARB, n (%)	63 (41)	47 (31)	53 (35)	0.16
β-blocker, n (%)	104 (68)	95 (62)	83 (54)	0.06
Glycemia				
Fasting plasma glucose (mmol/L)	4.8 ± 1.1	4.9 ± 1.8	4.8 ± 1.1	0.64
Diabetic status, n (%)	25 (16)	22 (14)	26 (17)	0.80
Fasting insulin (µU/mL)	12.2 ± 6.9	12.9 ± 7.1	13.0 ± 8.5	0.60
Fasting pro-insulin (pmol/L)	18.8 [12.6-28.2]	17.4 [11.2-27.8]	16.2 [10.5-23.8]	0.07
HOMA-index	2.7 ± 1.8	2.9 ± 2.1	2.9 ± 2.3	0.68
HbA_{1C} (%)	6.7 ± 1.1	6.6 ± 1.1	6.5 ± 1.0	0.49
Lipids				
Plasma albumin (g/L)	41 ± 3	41 ± 3	40 ± 4	0.97
Cholesterol (mmol/L)	5.5 [4.8-6.2]	5.7 [4.9-6.2]	5.7 [5.1-6.2]	0.21
HDL cholesterol (mmol/L)	1.0 [0.8-1.3]	1.0 [0.8-1.2]	1.1 [0.9-1.4]	0.006
Triglycerids (mmol/L)	1.8 [1.3-2.4]	2.0 [1.6-2.7]	1.9 [1.4-2.8]	0.03
LDL cholesterol (mmol/L)	3.6 [3.0-4.2]	3.6 [3.0-4.2]	3.5 [3.0-4.1]	0.78
Use of statin at inclusion, n (%)	76 (49)	77 (50)	77 (50)	0.99

Parametric variables are expressed as mean ± SD, nonparametric variables as median (interquartile range). Tx, transplantation; [a] Both class I and class II HLA antibodies negative, [b] Class I or class II HLA antibodies positive; MAP, mean arterial pressure; ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin-receptor blockers; HOMA, homeostatic model assessment; HBA1C, glycated haemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Transplant-related characteristics are shown in Table 2. Serum creatinine decreased significantly with increasing NEFA concentrations. There was a difference in the prescription of proliferation inhibitors, but this was not a dose-dependent relation. Follow-up for graft failure beyond baseline was 7.1 (6.1-7.5) years. During this follow-up, occurrence of graft failure resulting in return to dialysis or retransplantation decreased significantly with increasing NEFA concentrations at baseline: 23 (15%), 14 (9%), and 9 (6%) for the consecutive tertiles, respectively (*P*=0.04). A corresponding Kaplan-Meier curve for the gender-stratified tertiles of NEFA concentrations is shown in Figure 3.

We subsequently investigated whether NEFA concentrations are independently associated with graft failure (Table 3). In a prospective Cox-regression analysis, gender-adjusted log-transformed NEFA levels were inversely associated with development of graft failure (hazard ratio [HR], 0.61; 95% confidence interval [CI], 0.47-0.81; *P*<0.001). After adjustment for plasma albumin, donor age, creatinine clearance and urinary protein excretion the HR was 0.73 (95% CI, 0.56-0.95); *P*=0.02. This model was the basis for further adjustments. Further additional adjustment for diabetes-related measurements, including diabetic status, glucose concentration and HbA_{1C} did not materially change the association (HR, 0.73; 95% CI, 0.56-0.95; *P*=0.02 [model 3]). Also, additional adjustment for the use of ciclosporine or tacrolimus and the trough levels of ciclosporine or tacrolimus did not materially change the results (HR, 0.74; 95% CI, 0.57-0.97; *P*=0.03 [model 4]). Similarly, additional adjustment for the use of azathioprine or mycophenolate and for anti-HLA antibodies and type of rejection did not materially change the association (HR, 0.73; 95% CI, 0.56-0.96; *P*=0.02 [model 5] and HR, 0.74; 95% CI, 0.57-0.97; *P*=0.03 [model 6], respectively).

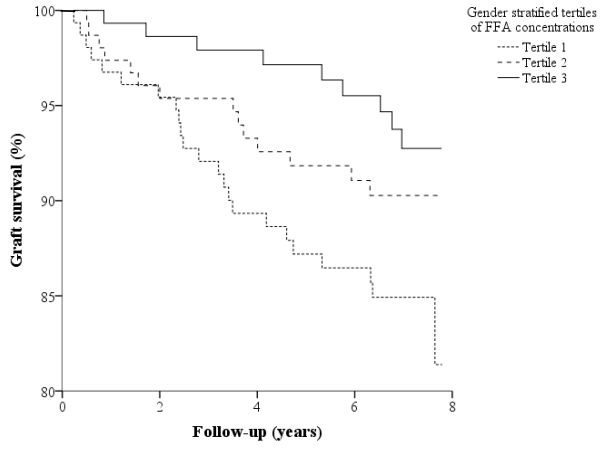

Figure 3. Kaplan-Meier survival curve of gender-stratified tertiles of FFA concentrations for graft failure, *P*=0.04 according to log-rank test

Table 2. Transplanted kidney-related baseline characteristics

	Gender stratified tertiles of NEFA			
	1st	2nd	3rd	*P*
N	154	154	153	
Donor characteristics				
Age (years)	38 ± 16	38 ± 15	37 ± 15	0.75
Male gender, n (%)	84 (55)	86 (56)	84 (55)	0.98
Deceased donor transplant, n (%)	137 (89)	129 (84)	137 (90)	0.24
Transplantation procedure				
Warm ischaemia time (min)	37 [30-45]	35 [30-45]	35 [30-45]	0.91
Cold ischaemia time (h)	23 [17-28]	22 [14-27]	22 [16-28]	0.35
Oliguric time > 1 h, n (%)	33 (21)	27 (18)	29 (19)	0.68
Rejection				
Rejection therapy, n (%)	67 (44)	65 (42)	58 (38)	0.58
Cellular rejection, n (%)	53 (34)	42 (27)	46 (30)	0.39
Steroid resistent rejection, n (%)	11(7)	18 (12)	9 (6)	0.15
Vascular rejection, n (%)	3 (2)	5 (3)	1 (1)	0.26
Renal allograft funtion				
Serum creatinine (µmol/L)	137 [115-177]	139 [116-165]	130 [107-152]	0.03
Creatinine clearance (ml/min)	60 ± 24	62 ± 21	62 ± 21	0.49
Proteinuria (>0.5 g/24h), n (%)	45 (29)	43 (28)	39 (25)	0.78
Urinary protein excretion (g/24 h)	0.2 [0.0-0.5]	0.2 [0.0-0.5]	0.2 [0.0-0.5]	0.62
Immunosuppression				
Prednisolone dose (mg/day)	10 [7.5-10]	10 [7.5-10]	10 [7.5-10]	0.38
Ciclosporine , n (%)	90 (58)	102 (66)	108 (71)	0.08
Tacrolimus, n (%)	22 (14)	18 (12)	19 (12)	0.78
Azathioprine, n (%)	64 (42)	60 (39)	36 (24)	0.002
Mycophenolate, n (%)	49 (32)	63 (41)	66 (43)	0.10
Ciclosporine trough level (µg/L)	108 [78-140]	95 [70-122]	107 [84-133]	0.06
Tacrolimus trough level (µg/L)	7.9 [5.8-9.8]	8.7 [8.1-11.3]	8.7 [5.2-10.7]	0.32

Parametric variables are expressed as mean ± SD, whereas nonparametric variables are given as median (interquartile range), unless otherwise noted. Statistical analyses were performed with the Mann-Withney U test in case of a nonparametric variable. The χ^2 test was used in case of a categorical variable.

Table 3. Univariate and multivariate Cox regression analyses of the association of NEFA with graft failure in renal transplant recipients

Model	HR (95% CI) for log-transformed NEFA	*P*-value
1	0.61 [0.47-0.81]	<0.001
2	0.73 [0.56-0.95]	0.02
3	0.73 [0.56-0.95]	0.02
4	0.74 [0.57-0.97]	0.03
5	0.73 [0.56-0.96]	0.02
6	0.74 [0.57-0.97]	0.03

Model 1: crude model. Model 2: model 1 + adjustment for plasma albumin, donor age, creatinine clearance, and urinary protein excretion. Model 3: model 2 + adjustment for diabetic status, glucose concentration, HbA_{1c}. Model 4: model 2 + adjustment for use and trough level of ciclosporine, use and trough level of tacrolimus. Model 5: model 2 + adjustment for use of azathioprine and mycophenolate. Model 6: model 2 + adjustment for Anti-HLA antibodies and type of rejection.

Discussion

In this study, we show that high fasting concentrations of NEFA are not associated with an increased risk for development of graft failure in RTR. Rather, high concentrations of NEFA appeared to be associated with a decreased risk of graft failure. This relation was independent of plasma albumin, donor age, creatinine clearance, proteinuria, diabetes related measurements, use of ciclosporine and tacrolimus, trough levels of ciclosporine and tacrolimus, use of azathioprine or mycophenolate, anti-HLA antibodies and type of rejection. In obesity, NEFA are elevated and cause insulin resistance in all major insulin target organs including skeletal muscle and liver. Increased levels of NEFA have been shown to closely relate to cardiovascular risk markers and mortality in coronary heart disease patients(30,31). In RTR, NEFA have been shown to be associated with obesity, insulin resistance and atherosclerosis(32). In this study, however, there was no significant difference in diabetic status, insulin concentrations, pro-insulin concentrations, homeostatic model assessment (HOMA) and HbA_{1c} levels between the tertiles. The levels of NEFA in our cohort were not as high as the mean ± standard deviation values of 600 ± 350 μmol/L reported in the former study in RTR and were only marginally higher than the 300 ± 200 μmol/L that was reported for the control group in the mentioned study on NEFA in RTR(32). Other studies did not find a difference in levels of NEFA between patients with chronic kidney disease (CKD) versus controls(33-35). In these respective studies, levels of NEFA were 278 ± 116 μmol/L in patients with CKD stage 2 to 3 versus 327 ± 43 μmol/L in normotensive controls,(33) 960 ± 370 μmol/L in nondialyzed uremic patients versus 760 ± 297 μmol/L in healthy controls(35) and 480 ± 306 μmol/L in haemodialysis subjects versus 530 ± 108 μmol/L in healthy controls matched for age, sex and body mass index(34). Differences between studies may depend on population characteristics, bus also on differences between assays and precautions taken during sample collection and preparation.

To the best of our knowledge, this is the first prospective study in renal transplant patients that relates fasting concentrations of NEFA to graft failure. Most in vivo studies have shown a detrimental effect of NEFA on the kidney. However, there is a large confounding factor in many of these studies, because they compare delipidated FA-free albumin with untreated FA-containing albumin FA(19,20,36-38). In this way, it is the delipidation procedure that is compared rather than the FA present on albumin. The delipidation procedure may remove other substances besides FA, or modify chemical activity or structure of albumin(39).

However, *in vitro* studies that compared the effect of delipidated albumin with that of delipidated albumin reloaded with FA, thereby preventing confounding by comparison of the delipidation procedure, showed altered cellular growth,(12) disturbed metabolism,(12,13) increased apoptosis,(14) increased fibronectin production(15) and increased ROS production,(15,16) which may all be considered detrimental.

In vivo studies of protein overload in the rat and axolotl compared delipidated albumin with delipidated albumin loaded with oleic acid(39). In the study in rats, no significant renal effect was seen of addition of oleic acid. Because FA bound to albumin have a very short half life in the circulation it was surmised that absence of a significant effect could be due to the fact that albumin-bound FA administered in the peritoneal cavity did not reach the kidney after passing through the circulation(9,40,41). Axolotls have a subset of nephrons that drain on the peritoneal cavity, so that intraperitoneal injection of albumin selectively target these nephrons, without necessity of first passing the circulation to reach the kidney. In a substudy with these axolotls no significant effect of albumin loaded with oleic acid was also seen. In the study in rats as well as in the study in axolotls, there was a trend toward a protective rather than nephrotoxic effect.

In line with these studies with oleic acid in rats and axolotls, recent studies found protective effects of monounsaturated NEFA (such as oleic acid) and polyunsaturated FA, but not of saturated NEFA in vitro(42,43). Thereby, these studies suggest that type of NEFA is important in determining either a protective or a detrimental effect of albumin-bound FA on the kidney during proteinuria. NEFA can be classified according to chain length and absence or presence of double bonds(44). In our study, in which we, for the first time, show the effect of fasting concentrations of NEFA on graft failure in RTR, we have not measured different types of NEFA. It would have been interesting to investigate whether the NEFA profile in the circulation matters to the outcome of graft failure.

This study has some limitations. First, this study is a single-center study, and the findings need to be confirmed in other central or multicenter studies. Furthermore, the study population almost entirely consisted of Caucasians; the applicability of our results to more racially diverse renaltransplantion population may be limited. We have no data collected in control groups to compare the concentrations of NEFA in this population compared with, for example, a population with chronic kidney disease or healthy individuals. Another point is that baseline samples for our study were collected from 2001 to 2003, so results could be

different if a current cohort would be sampled. Another limitation is that we have no repeated measurements of NEFA. Most epidemiological studies use a single baseline measurement to predict outcomes, which adversely affects predictive properties of variables associated with outcomes. If intraindividual variability of predictive parameters is taken into account, this results in much stronger relations with outcomes. Many immunosuppressants are albumin-bound, while almost all NEFA that are present in the circulatlion are also albumin-bound, although this binding is very loose and noncovalent(28,29). Therefore, it could be possible that NEFA have an effect on the bioavailability of immunosuppressant. We have no information on that issue in our cohort. Further research into a potential effect of albumin-bound NEFA on the biological availability of immunosuppressants could be of interest. An important strength of this study is that there was no loss to follow-up.

Monounsaturated FA and polyunsaturated have been shown to have different effects. It has, for instance, been found that an anti-inflammatory effect of monounsaturated FA specifically protected against inflammation induced by saturated NEFA, whereas polyunsaturated FA had an anti-inflammatory effect not only against saturated FA but also against inflammation induced by tumor necrosis factor-α and lipopolysaccharide(43). Previous studies already have shown that polyunsaturated FA have an anti-inflammatory effect by different mechanisms, including nuclear factor-κB suppression,(45,46) and alteration of lipid rafts particularly relating to the function of toll-like receptors(47-49). Therefore, it may be hypothesized that the protective role of monounsaturated FA on tubulointerstitial injury is limited to lipotoxicity, whereas polyunsaturated FA have a broad protective effect on tubulointerstitial injury.

In conclusion, RTR with high concentrations of plasma NEFA have a decreased risk of graft failure. A new evaluation of the role of NEFA in proteinuric conditions is warranted.

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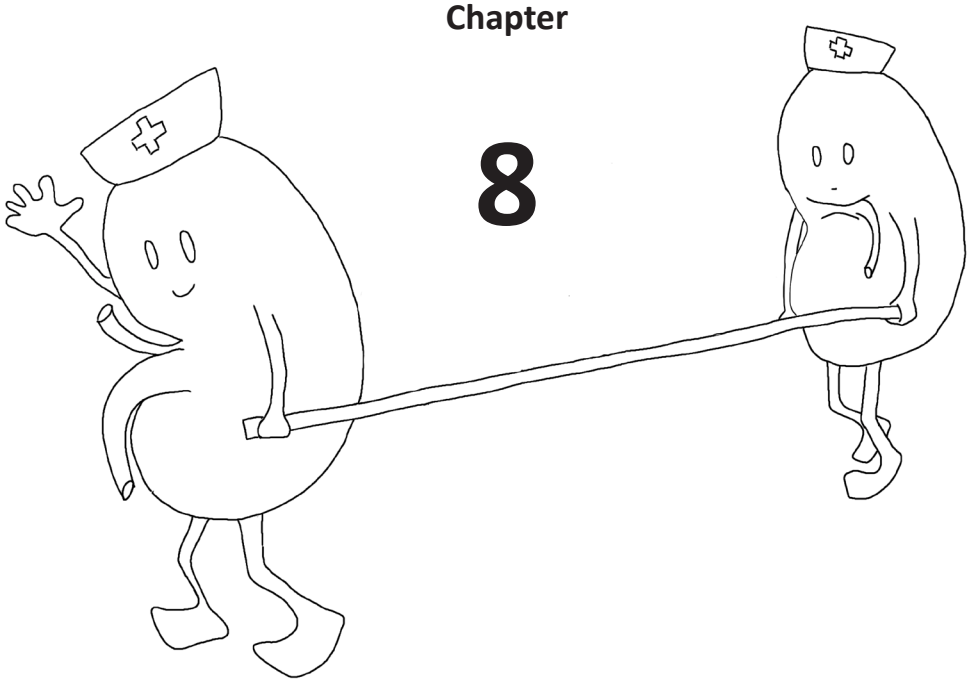
Chapter

8



Malondialdehyde and development of graft failure in renal transplant recipients

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In submission

Abstract

Malondialdehyde (MDA) is considered a marker of oxidative stress. Since oxidative stress is implicated in the pathophysiology of chronic transplant dysfunction, we aimed to investigate the association between plasma MDA levels and the risk to develop graft failure in renal transplant recipients (RTR).

Baseline measurements were performed between 2001-2003 in outpatient RTR with a functioning graft >1 year. MDA was measured using the thiobarbituric acid method. Follow up was recorded until May 19, 2009. Graft failure was defined as return to dialysis or re-transplantation.

We included 596 RTR at a median, interquartile range (IQR) of 6.0 (2.8-11.6) years post-transplant. Follow-up for graft failure beyond baseline was 6.9 (6.0-7.4) years. Fasting levels of MDA were inversely associated with the development for graft failure in a prospective univariate Cox-regression analysis (HR=0.60 [95%CI 0.49-0.74], $P<0.001$). This association remained significant after adjustment for plasma albumin, serum creatinine and proteinuria (HR=0.76 [0.62-0.94], $P=0.01$).

MDA levels are inversely associated with risk for development of graft failure. Our results suggest that MDA levels are not merely a measurement of oxidative stress. Intake of unsaturated fatty acids and exercise might contribute to this finding. This is a new area of investigation in renal transplant recipients.

Introduction

Malondialdehyde (MDA) is a by-product formed from the reaction of free radicals with unstaturated fatty acids in lipids and therefore considered a marker of oxidative stress(1,2). It is long known that reactive oxygen species (ROS) are formed in increased amounts during ischaemia and reperfusion of the kidney and that this contributes to renal injury on the short term. ROS cause lipid peroxidation of cell and organelle membranes, thereby disrupting the structural integrity and capacity for cell transport and energy production. In line with this, it has been found that high MDA levels in the donor correlate with delayed graft failure in RTR(3).

Plasma MDA levels are increased in patients with end stage renal disease, attributed to dialysis treatment. Consequently, MDA levels decrease after kidney transplantation.(4-7) Nevertheless, even more than a year after renal transplantation MDA levels are elevated in RTR compared to healthy controls(4,8,9).

Leading causes of late allograft loss are patient mortality and development of chronic transplant dysfunction. Chronic transplant dysfunction is characterized clinically by a slow decline in transplant function over time, albeit onset and progression may vary among patients. Increased oxidative stress is also thought to play a role in chronic transplant dysfunction and development of late graft failure(10).

There are many cross-sectional studies on MDA levels in RTR, early after transplantation as well as more than a year after transplantation(4,6-9). However, none of these studies prospectively investigated whether MDA levels are associated with the development of graft failure in RTR. We aimed to prospectively investigate whether MDA levels are associated with the development of graft failure in RTR.

Materials and methods

The current prospective study was part of a larger study and incorporated in the Groningen Renal Transplant Outpatient Program, details of which have been published previously(11). Between August 2001 and July 2003, all adult renal allograft recipients who had a functioning graft for more than 1 year were eligible to participate. Patients with known or apparent systemic illnesses (e.g., malignancies or opportunistic infections) were considered ineligible. A total of 606 out of 847 (72%) eligible renal transplant recipients signed written informed consent. There was no difference between participating and not-participating RTR with regard to age, gender, BMI, serum creatine, creatinine clearance, and proteinuria(12). Physical activity was recorded as desribed previously(13). Graft failure was recorded for all renal transplant recipients until May 19, 2009. Graft failure was censored for death and defined as return to dialysis or retransplantation. For the current study, 596 patients were included with measured fasting concentrations of malondialdehyde. For patients with

graft failure (n=54), duration of follow-up was calculated using date of start of dialysis or retransplantation.

The institutional review board approved the study protocol (METc01/039). The study was performed according to the Helsinki Declaration of 1975, as revised in 2000. Funding sources had neither a role in the collection and analyses of data, nor in publication of the manuscript.

Blood was drawn after an 8- to 12-h overnight fasting period. Analytical methods have been described earlier(12). MDA was determined as follows: after binding to thiobarbituric acid, the subsequently formed TBARS were extracted in a butanol layer, measured with fluorescence spectrophotometer at 485/590 nm (Beun de Ronde FL600, Abcoude, The Netherlands).

Statistical analyses

Analyses were performed with PASW version 18.0.3 (IBM SPSS Inc., Chicago, IL). Parametric variables are given as means ± SD. Nonparametric variables are given as median (interquartile range). Subjects were divided in tertiles based on MDA levels, differences between the groups were tested with the Mann Withney U test in case of a non-parametric variable; the chi-square test was used in case of a categorical variable. Kaplan-Meier survival analysis with log-rank testing was performed for prospective analysis of graft loss. We then proceeded with univariate and multivariate Cox-regression analyses of MDA. We performed a pre-defined multivariate analysis with variables known from literature that determine graft loss. A two-sided *P*-value of <0.05 was considered to be statistically significant.

Results

Baseline measurements were obtained from 596 RTR, who participated at a median, interquartile range (IQR) of 6.0 (2.8-11.6) years post-transplantation. Follow-up for graft failure beyond baseline was 6.9 (6.0-7.4) years. Fasting levels of MDA were 5.39 (4.30-6.46) µmol/L. Minimum and maximum values were 1.98 and 15.91 µmol/L, respectively. Cutoff points for the tertiles were 4.69 and 6.04 µmol/L.

RTR-related characteristics according to tertiles of MDA levels are shown in Table 1. Patients were older and follow-up was shorter with higher plasma levels of MDA. Furthermore, waist circumference was greater with higher plasma levels of MDA. In line also the waist-hip-ratio was significantly greater with higher plasma levels of MDA. There was no difference between the tertiles for weight and BMI. Angiotensin-converting enzyme(ACE)-inhibitors and angiotensin receptor blockers (ARBs) were used less frequently by RTR with high MDA levels. However, there was no difference in prescription of β-blockers or in mean-arterial pressure. Current smoking was significantly different between the tertiles, although there was no dose-effect relation. There was no significantly difference in physical activity levels

between the tertiles.

Transplant-related characteristics are shown in Table 2. Creatinine clearance was significantly higher with greater plasma levels of MDA, there was no difference between the tertiles for proteinura. There was a significant difference between the tertiles in prescription of calcineurin inhibitors, although there was no dose-effect relation.

Occurrence of graft failure, defined as return to dialysis or retransplantation decreased significantly with higher plasma MDA levels (P<0.001). In the lowest tertile 31 (16%) RTR had graft failure, in the second tertile 19 (10%) and in the third tertile 4 (2%). A corresponding Kaplan-Meier curve for the tertiles of plasma MDA levels is shown in Figure 1.

We subsequently investigated whether plasma MDA levels are an independent predictor of graft failure (Table 3). In a prospective Cox-regression analysis MDA levels were significant associated with development of graft failure (HR=0.60 [0.49-0.74], P<0.001). After adjustment for plasma albumin, serum creatinine and urinary protein excretion the hazard ratio was 0.77 [0.63-0.95], P=0.01. This model was the basis of further adjustments with variables that were independently associated with plasma MDA levels and variables known from literature that determine graft failure. Further additional adjustment for recipient age, time between baseline measurement and transplantation and recipient gender did not materially change the association (HR=0.77 [0.62-0.96], P=0.02 (model 3). After additional adjustment for weight, waist circumference and hip circumference and after additional adjustment for C-reactive protein the association remained significant (resp. HR=0.78 [0.64-0.96], P=0.02 (model 4) and HR=0.79 [0.64-0.97], P=0.02 (model 5)). After additional adjustment for mean arterial pressure and use of an ACE-inhibitors or ARB, additional adjustment for high-density lipoprotein cholesterol, triglycerides and low-density lipoprotein cholesterol and additional adjustment for prednisolone dose, use of calcineurin inhibitors and use of proliferation inhibitors the association did not materially change (resp. HR=0.78 [0.64-0.96], P=0.02 (model 6), HR=0.76 [0.61-0.93], P=0.01 (model 7) and HR=0.75 [0.61-0.93], P=0.01 (model 8)).

Table 1. Recipient-related baseline characteristics

	Tertiles of malondialdehyde (µmol/L)			
	1.98-4.68	4.69-6.04	6.05-15.91	*P*
N	199	199	198	
Recipient demographics				
Dialysis prior Tx (months)	25 [12-45]	27 [14-49]	30 [14-53]	0.43
Age (years)	49.9 ± 12.5	51.6 ± 11.2	53.1 ± 12.3	0.03
Time until baseline (years)	7.9 [4.1-12.7]	5.6 [2.5-11.1]	5.6 [2.6-8.8]	0.002
Male gender, n (%)	98 (49)	115 (58)	112 (57)	0.18
Anti-HLA antibodies				
Negative [a], n (%)	153	161	167	0.32
Borderline, n (%)	8	6	8	
Positive [b], n (%)	38	32	23	
Body composition measurements				
Weight (kg)	75.3 ± 12.7	77.8 ± 13.8	77.7 ± 14.0	0.11
Body mass index (kg/m$^{2)}$	25.9 ± 4.0	25.9 ± 4.4	26.5 ± 4.5	0.30
Waist circumference (cm)	94.7 ± 12.8	97.2 ± 14.0	99.6 ± 14.0	0.001
Hip circumference (cm)	98.6 ± 8.9	99.5 ± 9.6	100.5 ± 8.2	0.10
Waist hip ratio	0.96 ± 0.10	0.98 ± 0.11	0.99 ± 0.11	0.02
Smoking				
Current smoking, n (%)	55 (28)	34 (17)	42 (21)	0.04
Ex smoking, n (%)	84 (42)	85 (43)	83 (42)	0.98
Physical activity (MET-min/day)	95 [16-294]	107 [30-282]	127 [34-295]	0.92
CRP (mg/L)	1.9 [0.8-4.5]	1.8 [0.8-4.4]	2.7 [0.9-5.9]	0.08
Plasma albumin (g/L)	40 ± 3	41 ± 4	41 ± 4	0.01
Blood pressure				
MAP (mmHg)	140 ± 17	141 ± 15	142 ± 15	0.63
ACE-I or ARB use, n (%)	85 (43)	67 (34)	54 (27)	0.005
B-blocker use, n (%)	130 (65)	119 (60)	119 (60)	0.44
Glycemia				
Fasting plasma glucose (mmol/L)	4.8 ± 1.2	4.8 ± 1.2	4.8 ± 1.6	0.95
Diabetes, n (%)	34 (18)	25 (13)	30 (15)	0.45
Fasting insulin (mU/L)	13.3 ± 7.8	13.2 ± 7.5	13.2 ± 8.9	0.99
Fasting pro-insulin (pmol/L)	16.9 [9.8-25.9]	17.2 [11.2-26.7]	17.1 [10.9-26.8]	0.76
HOMA-index	2.3 [1.6-3.4]	2.3 [1.7-3.7]	2.1 [1.6-3.5]	0.66
HbA_{1C} (%)	6.4 ± 1.0	6.6 ± 1.0	6.6 ± 1.1	0.25
Lipids				
Total cholesterol (mmol/L)	5.5 [4.9-6.2]	5.6 [4.8-6.2]	5.7 [5.1-6.3]	0.53
HDL cholesterol (mmol/L)	1.1 [0.9-1.3]	1.0 [0.8-1.3]	1.1 [0.9-1.3]	0.54
Triglycerides (mmol/L)	1.8 [1.4-2.4]	2.0 [1.4-2.7]	2.0 [1.5-2.9]	0.08
LDL cholesterol (mmol/L)	3.5 [3.0-4.1]	3.5 [2.9-4.0]	3.5 [3.0-4.2]	0.16
Statin use, n (%)	106 (53)	92 (46)	99 (50)	0.37

Parametric variables are expressed as mean ± SD, whereas nonparametric variables are given as median (interquartile range), unless otherwise noted. Statistical analyses were performed with the Mann-Withney U test in case of a nonparametric variable. The χ^2 test was used in case of a categorical variable. Tx, transplantation; HLA; Human Leukocyte Antibody; [a] Both class I and class II HLA antibodies negative, [b] Class I or class II HLA antibodies positive; MET, metabolic equivalent of task; CRP, C-reactive protein; MAP, mean arterial pressure; ACE-I, angiotensin-converting enzyme inhibitor, ARB, angiotensin receptor blocker; HOMA, homeostatic model assessment; HbA_{1C}, glycated haemoglobin, HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Table 2. Transplanted kidney-related baseline characteristics

	Malondyaldehyde (µmol/L)			
	1.98-4.68	4.69-6.04	6.05-15.91	*P*
N	199	199	198	
Donor characteristics				
Donor age (years)	37 ± 16	38 ± 15	35 ± 15	0.29
Donor male gender, n (%)	116 (58)	105 (53)	104 (53)	0.48
Deceased donor transplant, n (%)	168 (84)	172 (86)	177 (89)	0.34
Transplantation procedure				
Warm ischeamia time (minutes)	37 [30-35]	35 [30-45]	35 [30-44]	0.26
Cold ischeamia time (hours)	21 [15-27]	22 [15-27]	22 [15-27]	0.93
Oliguric time > 1 hour, n (%)	40 (20)	42 (21)	37 (19)	0.85
Rejection therapy, n (%)	106 (53)	104 (52)	115 (58)	0.46
Renal allograft funtion				
Serum creatinine (µmol/L)	140 [111-176]	139 [116-168]	127 [105-149]	0.002
Creatinine clearance (mL/min)	59 ± 23	61 ± 22	65 ± 23	0.02
Proteinuria (>0.5 g/24h), n (%)	61 (31)	59 (30)	46 (23)	0.21
Immunosuppression				
Prednisolone dose (mg/day)	10 [7.5-10]	10 [8.75-10]	10 [7.5-10]	0.05
Calcineurin inhibitors, n (%)	35 (18)	17 (9)	20 (11)	0.01
Proliferation inhibitors, n (%)	47 (24)	58 (29)	52 (26)	0.46

Parametric variables are expressed as mean ± SD, whereas nonparametric variables are given as median (interquartile range), unless otherwise noted. Statistical analyses were performed with the Mann-Withney U test in case of a nonparametric variable. The χ2 test was used in case of a categorical variable. Calcineurin inhibitors, ciclosporine and tacrolimus; proliferation inhibitors; azathioprine and mycophenolate.

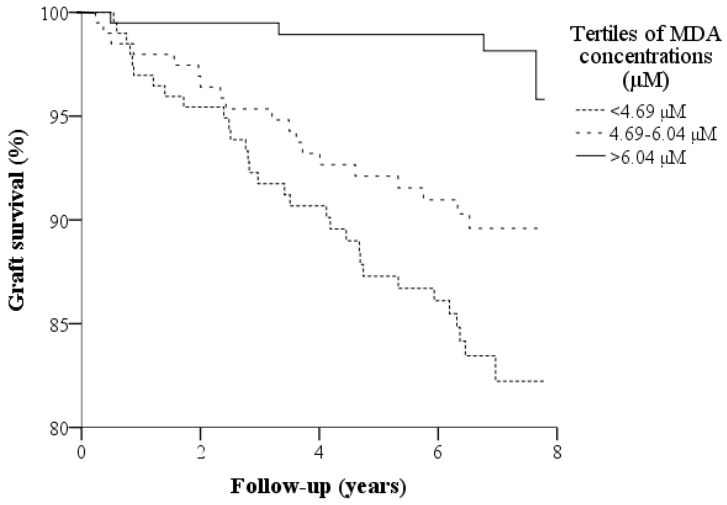

Figure 1. Kaplan-Meier survival curve of MDA concentrations (μM) at baseline for graft failure. P<0.001 according to log-rank test

Table 3. Univariate and multivariate Cox regression analyses of determinants of graft failure in renal transplant recipients

Model	HR (95% CI) for malondialdehyde	*P*-value
1	0.60 [0.49-0.74]	<0.001
2	0.77 [0.63-0.95]	0.01
3	0.77 [0.62-0.96]	0.02
4	0.78 [0.64-0.96]	0.02
5	0.79 [0.64-0.97]	0.02
6	0.78 [0.64-0.96]	0.02
7	0.76 [0.61-0.93]	0.01
8	0.75 [0.61-0.93]	0.01

Model 1: crude model. Model 2: model 1 + adjustment for plasma albumin, plasma creatinine and proteinuria. Model 3: model 2 + adjustment for recipient age, time between transplantation and baseline and gender. Model 4: model 2 + adjustment for weight, waist circumference and hip circumference. Model 5: model 2 + adjustment for CRP. Model 6: model 2 + adjustment for MAP and use of ACE-I or ARB. Model 7: model 2 + adjustment for HDL cholesterol, triglycerides and LDL cholesterol. Model 8: model 2 + adjustment for prednisolone dose, calcineurin inhibitors and proliferation inhibitors.

Discussion

In the present study, we found a longitudinal, prospective association of high levels of MDA with a decreased risk for development of graft failure in RTR. This relation was independent of recipient and transplant related characteristics.

To the best of our knowledge this is the first prospective study in RTR that relates levels of MDA to graft failure. Earlier cross-sectional studies reported that plasma MDA levels are increased in conditions associated with renal injury, like focal segmental glomerulosclerosis, diabetic nephropathy, dialysis and in renal transplant recipients(14-17).

The main source of MDA is the peroxidation of polyunsaturated fatty acids with two or more methylene-interrupted double bonds(18). MDA is the principal and most studied product of polyunsaturated fatty acid peroxidation. Because of analytical limitations, methods currently available for the direct measurement of ROS are of limited applicability. Instead, it is more common to measure not the ROS themselves but the damage that they cause. The justification is that the damage caused by ROS matters rather than the total amount of ROS produced(1,19). A potential drawback for the use of MDA for measurement of oxidative stress is that it may be present in ingested food and can be absorbed through the gastrointestinal tract(18). Salmon intake will directly raise MDA levels(20). Also, exercise may increase MDA levels(21-23). In acute phase after renal transplantation, diet and exercise might have less contribution than the ischaemic insult of the renal transplantation itself. In the chronic state, more than a year after transplantation, the contribution of diet and exercise at the MDA levels might be greater than the ischaemic insult at time of transplantation. Thereby, MDA might not be a measurement for oxidative stress alone, especially not in chronic disease states. In our study there was no relation between physical activity and MDA levels. Unfortunately, we were not able to determine whether there could be a relation between fish intake and MDA levels, since we collected no data on that. However, intake of fish oil has been shown to give a rise in glomerular filtration rate and creatinine clearance while reducing the mean arterial pressure(24). It is thought that this effect is by reducing nephrotoxic effects of cyclosporine A, which induces hemodynamic imbalance. Thereby fish oil intake may reduce graft failure.

Patients on hemodialysis have also been reported to have high MDA levels(5). Some studies showed that dialysis treatment is the main source of increased oxidative injury compared to the renal disease itself. Consequently, MDA levels have been reported to decrease after renal transplantation(4,7). But even long after renal transplantation MDA levels are still increased compared to healthy individuals(8,9). We reported levels of MDA of 5.39 (4.30-6.46) µmol/L at a median of 6.0 years after renal transplantation. These MDA levels are higher compared to other studies. Moreno et al. also included RTR at at least 1 year after renal transplantation and reported levels of 2.6 µmol/L in RTR(9). This was significantly higher than levels in healthy subjects with a mean of 1.4 µmol/L. Kim et al. included RTR after 2.7 ± 0.6 years after renal transplantation, and reported levels of MDA of 2.3 ± 0.1

µmol/L in RTR and 1.9 ± 0.1 µmol/L in healthy subjects(8). All studies, including ours used the same method to determine MDA. A possible explanation is that in the study of Kim et al., RTR were much younger (approximately 35 years of age), compared to RTR in our study (approximately 50 years of age). In line with this, we found that age of the recipient was independently associated with plasma levels of MDA. More importantly we determined plasma MDA levels after an overnight fasting period of 8- to 12-hours. Because lipolysis is activated in the fasting state, MDA levels could be higher after a period of overnight fasting. The other studies did not mention an overnight fasting period before blood withdrawal.

In our study, we found RTR with high MDA are less frequently on ACE-inhibitors or ARBs than with low MDA. Previously, it was shown in an interventional study that treatment with ACE-inhibitor or ARB causes a decrease in MDA levels in RTR(25). Our results are in line with this study. The mechanisms underlying these pharmacological effects of ACE-inhibitors are not fully understood. Potentiation of bradykinin and of free radical scavenger action by the ACE-inhibitors has been postulated(26). A possible mechanism by which ARB improve endothelial function is by reducing NADH-/NADPH-oxidase-mediated superoxide anion formation that is stimulated by angiotensin II(27).

This study has some limitations. First, the present study is a single center study and the findings need to be confirmed in other centra and/or multicenter studies. Futhermore, the study population almost entirely consisted of Caucasian ethnicity, the applicability of our results to more racially diverse renal transplant population may be limited. Another point is that baseline samples for our study were collected from 2001 to 2003, so results could be different if a current cohort would be sampled. However, to allow for evaluation of effect on graft failure and mortality, follow-up beyond a certain baseline is required, necessitating analyses to be performed in RTR that have been investigated in the past. Another limitation is that we have no repeated measurements of MDA. However, most epidemiological studies use a single baseline measurement to predict outcomes, which adversely affects predictive properties of variables associated with outcomes. If intra-individual variability of predictive parameters is taken into account, this results in much stronger relations with outcomes(28,29). An important strength of this study is that there was no loss to follow-up.

In conclusion high plasma MDA levels are associated with a decreased risk for development of graft failure in renal transplant recipients. This is a new area of investigation in renal transplant recipients.

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Chapter

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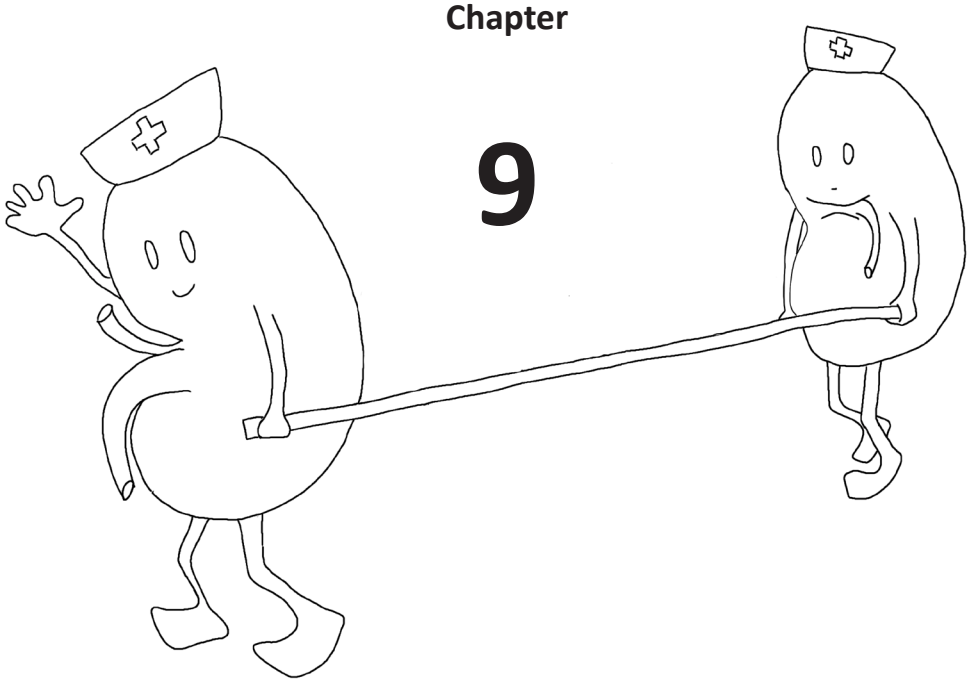

Effects of caloric restriction and ketogenesis on established proteinuria in Münich-Wistar-Frömter rats

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In preparation

Abstract

Caloric restriction induced at young age delays onset of age-related proteinuria, possibly due to stimulation of ketogenesis. It is unknown whether caloric restriction and/or ketogenesis reduce established proteinuria. We studied the effect of caloric restriction and a ketogenic diet in a model of structural and functional kidney damage, with main outcome on proteinuria.

Münich-Wistar-Frömter (MWF) rats (n=56) and Wistar rats (n=14) were nephrectomised at 22 wks of age. At 26-wks of age, MWF rats were divided in 4 groups: normal diet ad libitum, normal diet caloric restricted (to 60% of normal diet ad libitum), ketogenic diet ad libitum and ketogenic diet caloric restricted (to 60% of ketogenic diet ad libitum). After a diet period of 22 weeks rats were sacrificed.

At the end of the study ad libitum fed groups had higher proteinuria, lower creatinine clearance, higher mean arterial pressure, more glomerular sclerosis, and more interstitial fibrosis compared to corresponding caloric restricted groups. There was no effect of the ketogenic diet.

In conclusion, caloric restriction with 60% caloric intake of corresponding control group reverses established proteinuria in MWF rats. It also prevented the decrease of creatinine clearance, increase in blood pressure and glomerular sclerosis and interstitial damage. These beneficial effects could not be attributed to stimulation of ketogenesis.

Introduction

Experimental and clinical data have highlighted proteinuria as a powerful predictor of progression of kidney disease and proteinuria reduction as an important strategy to retard or prevent loss of kidney function(1,2). It has also been shown that treatment modalities that lower proteinuria independently of lowering blood pressure could prevent decrease in kidney function(3). Therefore lowering proteinuria has become a specific target in renoprotective treatment.

Caloric restriction induced at young age has shown to protect against age related development of proteinuria(4,5). A study of Yu et al. showed that death of ad libitum fed rats was associated with severe renal lesions while rats with caloric restriction induced at young age lived longer and showed almost none of such lesions at time of death(6). Indicating that caloric restriction could not only postpone renal lesions but also prevents its occurrence. The effect of caloric restriction induced at middle age or after establishment of age-related changes, such as proteinuria, has been less frequently explored, but is more relevant with respect to human health. Caloric restriction induced in aldult rats showed to reduce proteinuria, but it was not stated whether proteinuria was established at the start of the experiment(7,8). Although several hypotheses have been proposed to explain the effect of caloric restriction, the precise mechanisms are still unknown. Beneficial effects of caloric restriction could be attributed to different mechanisms including attenuation of oxidative damage, inflammation, reduction of plasma glucose, and reduction of blood pressure(9-12). One characteristic of caloric restriction is that ketone bodies are increased(13). Ketone bodies have been attributed many beneficial effects, with decrease of apoptosis, reduction of free radical formation and increased viability in tissue culture models of Alzheimer's and Parkinson's disease(14). Effects of ketogenic diet (also called low-carbohydrate diet) are also researched in the field of weight loss and diabetes, as a means to loose weight, reduce diabetic medication and lowering of blood pressure(15,16). In fact, recently it has been shown that the ketogenic diet could reduce proteinuria in experimental diabetic nephropathy(17).

We aimed to investigate (1) whether caloric restriction induced could prevent further development of proteinuria and renal damage after proteinuria was already esthablished and (2) whether this effect was mediated by ketogenesis. To this purpose we used Münich-Wistar-Frömter rats as model for spontaneous proteinuria with mild hypertension(18-21). This rat strain is characterized by a glomerular hypertension with hyperfiltration, as a consequence over time this rat strain develops extensive glomerulosclerosis and interstitial damage. It is an excellent animal model for chronic kidney damage.

Material and methods

Animals

Male Wistar rats (n=14) aged 16 weeks were obtained from Harlan (The Netherlands) and MWF rats (n=56) aged 16 weeks were obtained from Harlan (USA). The principles of laboratory animal care (NIH publication no. 85-23, revised 1985) were followed. The protocol was approved by the Committee for Animal Experiments of the University of Groningen (Permit number: 4690F). All efforts were made to minimize suffering.

Experimental design

Rats were fed custom made diets from Harlan (ref. number TD.09675 (normal diet) and TD.09676 (ketogenic diet) (Table 1). All animals were fed the normal diet ad libitum for eight weeks prior to randomisation. During this period blood pressure measurement performed by pneumatic tail cuff method was trained. At 22 weeks of age rats were uni-nephrectomised. By uni-nephrectomization the time frame of the development of proteinuria and structural and functional kidney damage was increased. Baseline measurements were taken after uni-nephrectomization. Proteinuria and blood pressure were assessed twice at 24 and 25 weeks of age, and the mean of these values were calculated as baseline measurement. Blood withdrawal for baseline measurement was performed at 25 weeks of age. Rats were randomly assigned to different treatment groups. Wistar rats served as a control group and were fed normal diet ad libitum (Wistar ND-AL). MWF rats were divided in normal diet fed ad libitum (MWF ND-AL), normal diet fed 60% of caloric intake of MWF ND-AL (MWF ND-CR), ketogenic diet fed ad libitum (MWF KD-AL) and ketogenic diet fed 60% of caloric intake of MWF KD-AL (MWF KD-CR). We excluded two animals that died from complications of nephrectomy and five animals that had symptoms of large Granular Lymphocytic Leukemia. Large Granular Lymphocytic Leukemia is reported in Fischer 344 rats as the one of the most common causes of death(22). It has not been reported in this extend in other strains. The cases of large Granular Lymphocytic Leukemia were distributed throughout the experimental groups, thereby it is unlikely to be related to caloric restriction or the ketogenic diet. Münich-Wistar-Frömter rats are not often studied; thereby the spontaneous cancer incidence in this strain is not well characterized. Treatment period begun at 26 weeks of age and duration of treatment was 22 weeks. Every four weeks blood pressure measurement was performed and urine and plasma samples were collected. Group size of rats that completed the study were Wistar ND-AL, n=13; MWF ND-AL, n=13; MWF ND-CR, n=13; MWF KD-AL, n=11 and MWF KD-CR, n=13. At the end of the experiment rats were anaesthesized with isoflurane, 50-IU heparin was perfused through the penile vein. This was followed by cannulation of the aorta and a 5 mL blood sample was taken. After a full body flush of 40 mL 0.9% NaCl at 4˚C, kidneys were removed and processed in 4% formalin for paraffin embedding.

Table 1. Composition of diets

TD.09675 Control diet		TD.09676 Ketogenic diet	
Kcal/g	3.7	Kcal/g	6.4
Formula	g/Kg	Formula	g/Kg
Casein	130.0	Casein	220.0
DL-Methionine	2.0	DL-Methionine	3.0
Corn Starch	514.6	Vegetable Shortening	533.98
Maltodextrin	100.0	Corn Oil	86.2
Sucrose	100.0	Cellulose	87.97
Vegetable Shortening	30.0	Mineral Mix (79055)	22.2
Corn Oil	30.0	CaP, dibasic	20.1
Cellulose	52.0	Calcium Carbonate	8.5
Mineral Mix (79055)	13.37	Magnesium Oxide	0.42
CaP, dibasic	12.1	Vitamin Mix (40060)	14.5
Calcium Carbonate	5.1	Choline Bitartrate	3.0
Magnesium Oxide	0.25	TBHQ, antioxidant	0.13
Vitamin Mix (40060)	8.7		
Choline Bitartrate	1.8		
TBHQ, antioxidant	0.08		
	% Kcal		% Kcal
Protein	12.6	Protein	12.1
Carbohydrate	72.4	Carbohydrate	0.6
Fat	15.0	Fat	87.5

Biochemical measurements and immunohistochemistry

Plasma and urinary creatinine, urinary ureum and urinary protein were measured along with routine analysis. Plasma β-hydroxybutyrate (BHB) was measured using BHB FS reagens from DiaSys (Holzheim, Germany). Creatinine clearance was calculated as the product of urinary creatinine concentration and 24 h urine volume divided by plasma creatinine, and was corrected for kidney weight. Creatinine clearance at baseline was corrected for the weight of the nephrectomised kidney, creatinine clearance at the end of the study was corrected for the weight of the kidney recovered at termination.

Immunohistochemistry was performed on 3 µm kidney sections stained for focal glomerulosclerosis with periodic acid-Schiff (PAS) and pro-fibrosis was stained using monoclonal mouse anti-smooth muscle actin (α-SMA) antibody (α-SMA clone 1A4, Sigma, St. Louis, MO). Scoring of focal glomerulosclerosis was performed as described previously, and expressed per glomerulus(23). Scoring of α-SMA was expressed percentage of positive stained area in cortex and outer medulla, including the vessels.

Statistics

Analyses were performed with PASW version 18.0.3 (IBM SPSS Inc., Chicago, IL). Parametric variables were given as mean ± standard deviation, non-parametric variables were given as median, interquartile range. In case of parametric variables differences between groups were tested with ANOVA and post-hoc Tukey. To test whether there was difference within a group over time, a paired sample T-test was used. In case of non-parametric variables differences between groups were tested with Kruskall-Wallis and post-hoc Mann-Whitney U. Correlations in MWF rats were controlled for the fact that there were different experimental groups. A *P*-value of 0.05 was considered statistical significant.

Results

Course of body weight

The course of body weight during the study is shown in Figure 1. Weight of Wistar ND-AL was significantly higher than weight of MWF ND-AL at baseline (453 ± 34 g vs. 364 ± 33 g, *P*<0.001). There was no difference in weight between MWF groups at baseline (*P*=0.44). At the end of the study weight of Wistar ND-AL was higher than in the different MWF groups (all *P*<0.001). Weight in MWF fed ad libitum was higher than in corresponding caloric restricted groups (both *P*<0.001). There was no significant difference between the two MWF ad libitum groups (*P*=0.13), or between the two MWF caloric restricted groups (*P*=0.68).

At baseline kidney weight was higher in Wistar ND-AL than in MWF ND-AL (1.7 ± 0.2 g vs. 1.2 ± 0.2 g, *P*<0.001), of which the latter was not different from the other MWF groups (MWF ND-CR 1.2 ± 0.1 g, MWF KD-AL 1.2 ± 0.1 g, MWF KD-CR 1.2 ± 0.1 g; *P*=0.21). Kidney weight at the end of the study was higher in Wistar ND-AL (2.4 ± 0.3 g) than in MWF ND-AL (1.8 ± 0.3 g). Kidney weight at termination was lower in MWF ND-AL compared to MWF KD-AL (2.2 ± 0.1 g) (*P*<0.001). Between MWF fed caloric restricted there was no significant difference in kidney weight at termination (MWF ND-CR 1.2 ± 0.1 g, vs. MWF KD-CR 1.3 ± 0.1 g, *P*=0.29). Kidney weight in MWF fed ad libitum was higher than in MWF fed caloric restricted (resp. both *P*<0.001).

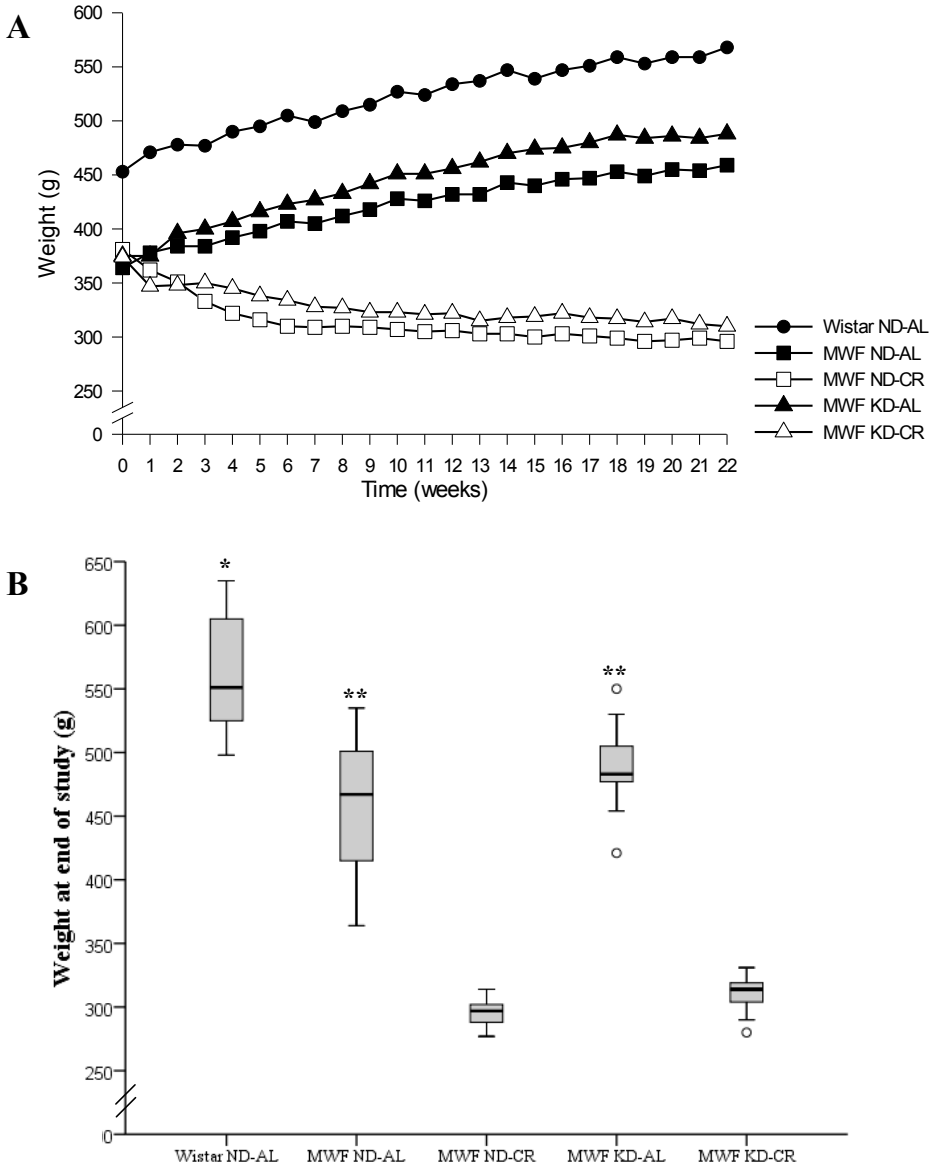

Figure 1. Body weight

A: Course of bodyweight during the experiment. B: Body weight at end of study, 48 weeks of age, after 22 weeks on experimental diet; * Wistar vs. MWF ND-AL, *P*<0.001; ** AL vs. corresponding CR, both *P*<0.001.

Focal glomerulosclerosis and fibrosis

PAS staining of the different MWF groups is shown in Figure 2. Focal glomerulosclerosis in MWF ad libitum groups, with scores of 1.14 ± 0.31 for normal diet and 1.41 ± 0.15 for ketogenic diet, was higher than in corresponding MWF caloric restricted groups, with scores of 0.33 ± 0.19 for normal diet and for 0.48 ± 0.37 ketogenic diet (both $P<0.001$). There was no difference in focal glomerulosclerosis between MWF fed ad libitum ($P=0.26$) and not between MWF fed caloric restricted ($P=0.68$). Focal glomerulosclerosis in Wistar ND-AL (0.14 ± 0.15) was significantly lower compared to MWF fed ad libitum (resp. both $P<0.001$). However, there was no difference in focal glomerulosclerosis in Wistar ND-AL compared to MWF fed caloric restricted (resp. $P=0.60$ normal diet compared and $P=0.07$ ketogenic diet compared).

Also fibrosis as measured by α-SMA in MWF ad libitum groups, with positive area of 6.6 ± 1.3% for normal diet and 7.1 ± 1.8% for ketogenic diet, was higher than in MWF caloric restricted groups, with positive area of 4.3 ± 1.4% for normal diet (resp. $P=0.006$) and 4.7 ± 2.1% for ketogenic diet (resp. $P=0.008$). There was no difference in fibrosis between MWF fed ad libitum($P=0.92$) and not between MWF fed caloric restricted ($P=0.92$). Fibrosis in Wistar ND-AL (4.0 ± 1.5%) was significantly lower compared to MWF ND-AL and MWF KD-AL (resp. $P=0.002$ and $P<0.001$), but was not significantly different compared to MWF ND-CR and MWF KD-CR (resp. $P=1.00$ and $P=0.84$).

As above described and also shown in Figure 2 caloric restriction prevented the occurrence of focal glomerulosclerosis and fibrosis during the experiment. Another feature is the dilatation of tubules in MWF fed ad libitum, this is probably caused by Tamm-Horsfall protein-casts.

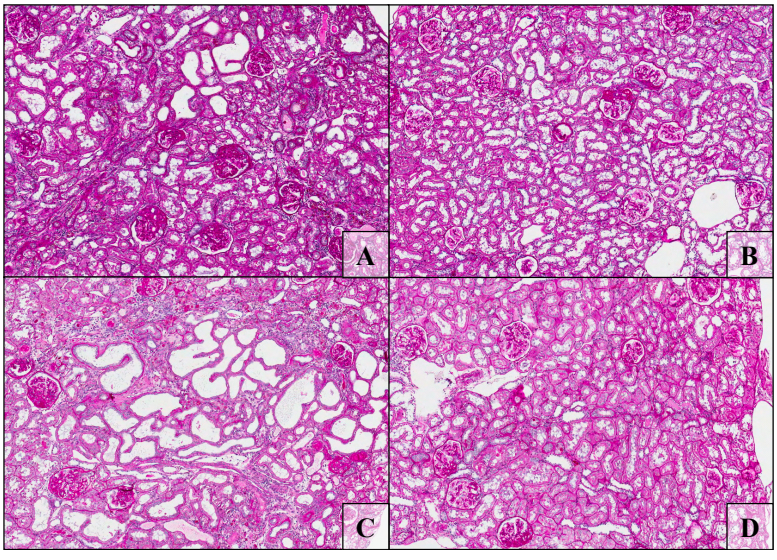

Figure 2. PAS staining. A: MWF ND-AL. B: MWF ND-CR. C: MWF KD-AL. D: MWF KD-CR

Kidney function

Kidney function was assessed by plasma creatinine and creatinine clearance. Results are shown in Table 2. At baseline there was no significant difference in plasma creatinine or creatinine clearance between Wistar ND-AL and MWF ND-AL, or between the different MWF groups. At the end of the study plasma creatinine was higher in MWF fed ad libitum, compared to corresponding MWF fed caloric restricted (*P*=0.01 normal diet compared and *P*=0.004 ketogenic diet compared). Creatinine clearance at the end of the study in Wistar ND-AL was significantly higher compared to MWF fed ad libitum (resp. both *P*<0.001). But there was no significant difference in creatinine clearance between Wistar ND-AL compared to MWF caloric restricted (resp. vs. MWF ND-CR, *P*=0.99 and vs. MWF KD-CR, *P*=0.17). Creatinine clearance was significantly lower in ad libitum fed groups compared to corresponding caloric restriction groups (*P*<0.001 normal diet compared and *P*=0.001 ketogenic diet compared). Caloric restriction protected creatinine clearance from decreasing during the experiment.

Table 2. Kidney function, proteinuria and blood pressure

	Wistar ND-AL	MWF ND-AL	MWF ND-CR	MWF KD-AL	MWF KD-CR	*P**	*P***
Plasma creatinine (µmol/L)							
Baseline†	38 ± 4	43 ± 6	43 ± 6	42 ± 5	42 ± 4	0.08	0.94
End of study‡	42 ± 4	94 ± 34	56 ± 15 [a]	89 ± 47	45 ± 14 [a]	<0.001	<0.001
% Δ	12 ± 14	121 ± 76	32 ± 34 [a]	106 ± 95	8 ± 31 [a]	<0.001	<0.001
Creatinine clearance (mL/min/g)							
Baseline†	1.5 ± 0.3	1.4 ± 0.4	1.3 ± 0.2	1.4 ± 0.4	1.4 ± 0.3	0.93	0.86
End of study‡	1.1 ± 0.3	0.5 ± 0.2	1.0 ± 0.2 [a]	0.5 ± 0.3	0.9 ± 0.2 [a]	<0.001	<0.001
% Δ	-23 ± 27	-61 ± 16	-22 ± 25 [a]	-67 ± 17	-39 ± 18 [a]	<0.001	<0.001
Proteinuria (mg/24 h)							
Baseline†	10 ± 12	58 ± 21	55 ± 19	67 ± 25	60 ± 30	<0.001	0.64
End of study‡	57 ± 71	112 ± 21	28 ± 8 [a]	131 ± 48	35 ± 21 [a]	0.008	<0.001
% Δ	525 ± 428	123 ± 101	-46 ± 14 [a]	131 ± 136	-43 ± 19 [a]	<0.001	<0.001
Mean arterial pressure (mmHg)							
Baseline†	115 ± 14	125 ± 9	134 ± 7	132 ± 8	133 ± 10	0.12	0.09
End of study‡	121 ± 10	165 ± 29	129 ± 9 [a]	177 ± 17	130 ± 13 [a]	<0.001	<0.001
% Δ	6 ± 11	29 ± 23	-9 ± 8 [a]	35 ± 19	-2 ± 14 [a]	0.004	<0.001

* P-value Wistar ND-AL vs. MWF ND-AL; **Anova P-value between MWF groups; † 26 weeks of age, on normal diet; ‡ 48 weeks of age, after 22 weeks on experimental diet; [a] MWF AL vs. corresponding MWF CR, *P*<0.05; [b] MWF ND-AL vs. MWF KD-AL, *P*<0.05; [c] MWF ND-CR vs. MWF KD-CR, *P*<0.05.

In Figure 3 the correlation of creatinine clearance at the end of the study with glomerulosclerosis and fibrosis is shown. As expected creatinine clearance was strongly negatively correlated with glomerulosclerosis and fibrosis (resp. R=-0.58, *P*<0.001 with glomerulosclerosis and R=-0.43, *P*=0.003 with fibrosis). This indicates that kidney function correlate with structural lesions of the kidney.

Proteinuria

Levels of proteinuria are shown in Table 2. At the end of the study proteinuria was lower in Wistar ND-AL than in MWF ND-AL (*P*<0.001) There was no difference in proteinuria between different MWF groups (*P*=0.64). At the end of the study proteinuria in MWF fed ad libitum were increased when compared to corresponding caloric restricted groups (both *P*<0.001). There was no significant difference in proteinuria between the two MWF ad libitum groups (*P*=0.36) or between the two MWF caloric restricted groups (*P*=0.90). More importantly proteinuria at the end of the study was significantly reduced in caloric restricted animals when comparing to levels at baseline (MWF ND-CR, *P*<0.001; MWF KD-CR, *P*<0.001). In ad libitum fed animals proteinuria increased during the experiment (MWF ND-AL, *P*<0.001; MWF KD-AL, *P*=0.008).

In Figure 3 we show the correlation of proteinuria at the end of the study with glomerulosclerosis and fibrosis. Also proteinuria strongly correlated with glomerulosclerosis (R=0.65, *P*<0.001), but not with fibrosis (R=0.17, *P*=0.25). Proteinuria was not correlated with creatinine clearance (R=-0.11, *P*=0.48).

Blood pressure

At baseline mean arterial pressure was lower in Wistar ND-AL than in MWF ND-AL (*P*<0.001). There were no differences between different MWF groups (*P*=0.09). At the end of the study mean arterial pressure was significantly higher in MWF fed ad libitum compared to corresponding caloric restricted groups (both *P*<0.001). Mean arterial pressure at the end of the study was not significantly different between the two MWF ad libitum groups (*P*=0.39), or between the two MWF caloric restricted groups (*P*=0.66). In MWF ND-CR mean arterial pressure at the end of the study was even significantly lower compared to mean arterial pressure at baseline (*P*=0.002). In MWF KD-CR mean arterial pressure at the end of the study was not significantly different from mean arterial pressure at baseline (*P*=0.54).

In Figure 3 the correlation of mean arterial pressure at the end of the study with glomerulosclerosis and fibrosis is shown. Mean arterial pressure at the end of the study was not correlated with glomerulosclerosis and fibrosis (resp. R=0.21, *P*=0.16, with glomerulosclerosis and R=0.19, *P*=0.20, with fibrosis). Mean arterial pressure was not correlated with creatinine clearance either (R=-0.28, *P*=0.06).

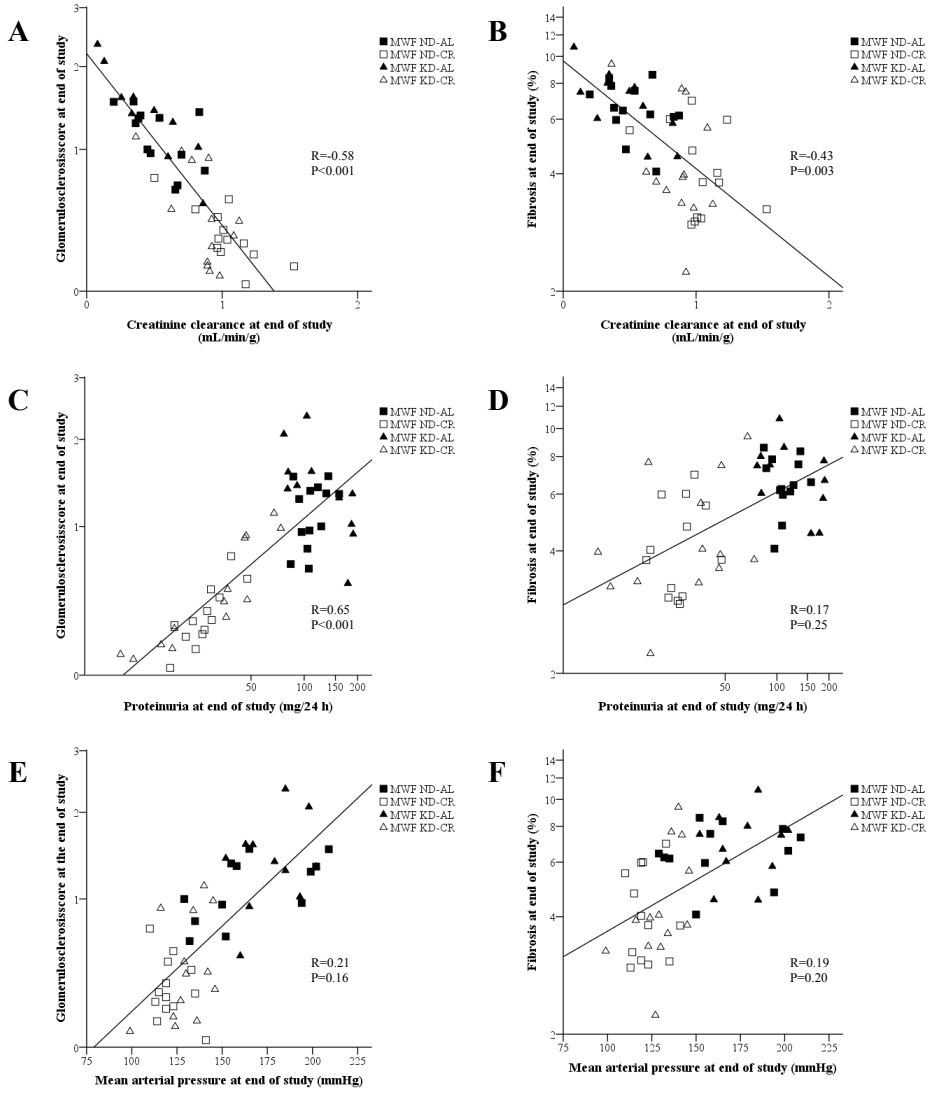

Figure 3. Correlation of creatinine clearance and proteinuria with glomerulosclerosis and fibrosis

A: Correlation of creatinine clearance with glomerulossclerosis. B: Correlation of creatinine clearance with fibrosis. C: Correlation of proteinuria with glomerulosclerosis. D: Correlation of proteinuria with fibrosis. E: Correlation of mean arterial pressure with glomerulosclerosis. F: Correlation of mean arterial pressure with fibrosis.

BHB

At the end of the study BHB concentrations in Wistar ND-AL (0.60 ± 0.54 mmol/L) were not different from MWF ND-AL (0.71 ± 0.58 mmol/L) (*P*=1.00). There was no difference in BHB concentrations between MWF fed ad libitum (resp. MWF KD-AL 0.99 ± 0.33 mmol/L, *P*=0.83). However BHB concentrations in MWF fed ad libitum were lower than the corresponding MWF fed caloric restricted (resp. MWF ND-CR 1.80 ± 0.93 mmol/L, *P*=0.006 normal diet compared and MWF KD-CR 3.73 ± 1.09 mmol/L, *P*=0.006 ketogenic diet compared). In MWF fed caloric restricted ketogenic diet significantly raised BHB concentrations (*P*<0.001). However there was no difference in kidney function between these groups, concluding that the ketogenic diet had no effect on preserving kidney function.

Biochemistry

Glucose, cholesterol and triglycerides levels are shown in Table 3. There were no differences in glucose, cholesterol or triglyceride levels at baseline. At the end of the study glucose, cholesterol and triglyceride levels were all significantly higher in MWF fed ad libitum when compared to corresponding MWF fed caloric restricted. When comparing MWF fed ad libitum, triglyceride levels were lower in MWF ND-AL compared to MWF KD-AL (*P*=0.009). And when comparing MWF fed caloric restricted, cholesterol levels were higher in MWF ND-CR when compared to MWF KD-CR (*P*<0.001).

Table 3. Biochemistry

	Wistar ND-AL	MWF ND-AL	MWF ND-CR	MWF KD-AL	MWF KD-CR	*P**	*P***
Glucose (mmol/L)							
Baseline†	10.6 ± 2.2	11.0 ± 1.9	11.1 ± 2.3	10.5 ± 1.5	11.0 ± 1.9	0.99	0.88
End of study‡	13.7 ± 3.5	12.8 ± 2.1 [a]	8.6 ± 3.8	13.8 ± 1.7 [a]	8.6 ± 1.8	0.92	<0.001
% Δ	34 ± 43	19 ± 26 [a]	-19 ± 43	34 ± 26 [a]	-21 ± 18	0.80	<0.001
Cholesterol (mmol/L)							
Baseline†	2.3 ± 0.5	2.6 ± 0.4	2.6 ± 0.3	2.9 ± 0.5	2.7 ± 0.5	0.37	0.16
End of study‡	3.6 ± 1.5	4.0 ± 0.4 [a]	3.0 ± 0.3 [c]	4.3 ± 0.3 [a]	2.2 ± 0.6	0.72	<0.001
% Δ	50 ± 36	45 ± 32 [a]	19 ± 16 [c]	51 ± 36 [a]	-15 ± 21	1.00	<0.001
Triglycerides (mmol/L)							
Baseline†	1.3 ± 0.4	1.7 ± 0.9	1.5 ± 0.7	1.8 ± 0.9	1.6 ± 1.0	0.75	0.90
End of study‡	1.8 ± 1.2	1.6 ± 0.6 [a,b]	0.7 ± 0.3	2.4 ± 0.9 [a]	0.5 ± 0.2	0.98	<0.001
% Δ	45 ± 112	27 ± 109 [a]	-51 ± 28	59 ± 84 [a]	-62 ± 23	0.98	<0.001

* *P*-value Wistar ND-AL vs. MWF ND-AL; ** Anova P-value between MWF groups; † 26 weeks of age, on normal diet; ‡ 48 weeks of age, after 22 weeks on experimental diet; [a] MWF AL vs. corresponding MWF CR, *P*<0.05; [b] MWF ND-AL vs. MWF KD-AL, *P*<0.05; [c] MWF ND-CR vs. MWF KD-CR, *P*<0.05.

Discussion

In this study we found that caloric restriction reverses established proteinuria in MWF rats. Caloric restriction also prevented the decrease of creatinine clearance, the increase in blood pressure and glomerulosclerosis and fibrosis. Ketogenic diet fed ad libitum had no effect on measurements of renal damage. Ketogenic diet fed caloric restricted did not perform better than normal diet fed caloric restricted. Therefore we conclude that the beneficial effects of caloric restriction cannot be attributed to stimulation of ketogenesis.

Caloric restriction has extensively shown to reduce proteinuria and age related glomerular nephropathy(4,5). Usually caloric restriction is already induced at young age. We induced caloric restriction when MWF rats already showed increased proteinuria. In our study caloric restriction was even capable to lower proteinuria beyond baseline levels, at levels similar to the Wistar control group. McKiernan et al. studied the effect of caloric restriction induced in 18 months old Fischer x Brown Norway rats and showed significant reduction of age-related changes in the kidney(8). However, it was stated in the abstract that in this study caloric restriction was induced before the onset of "significant age-related changes".

Caloric restriction has been studied in MWF rats by Macconi et al(24). This study showed that caloric restriction prevented development of proteinuria in MWF rats. However, also in this study reversibility has not been shown. Dietary treatment period was 2 months, and caloric intake in the caloric restriction group was on average 50% of the normal diet group. There was no difference in systolic blood pressure after two months of dietary treatment. In our experiment blood pressure was considerably lower in rats fed caloric restricted when compared to rats fed ad libitum. This difference could be explained by the difference in duration of dietary treatment period.

In our experiment blood pressure was higher in ad libitum fed animals compared to caloric restricted animals. In MWF rats it has been shown that ACE-inhibition normalizes blood pressure and thereby markedly decreases proteinuria(25). The effect of caloric restriction is probably at least partly explained by lowering the blood pressure. However, blood pressure was not correlated with glomerulosclerosis, fibrosis or creatinine clearance. Beside this caloric restriction or weight reduction could be because of blood pressure lowering qualities, an important treatment modality in patients with nephropathy and hypertension.

In our study we did not show an effect of the ketogenic diet. Recently Poplawalski et al. showed a protective effect of a ketogenic diet in diabetic nephropathy(17). They conclude that the protective effect of the ketogenic diet could be partially explained by reduction of glucose metabolism. Whereas caloric restriction decreased plasma glucose, we did not show an effect of the ketogenic diet on plasma glucose. This difference could be due to difference in protein concentrations in the ketogenic diet used. Poplawalski et al. used a ketogenic diet with 8% protein, 5% carbohydrate and 87% fat, whereas we used a ketogenic diet with 12.1% protein, 0.6% carbohydrate and 87.5% fat. The group of Poplawalski showed earlier

that an Atkins-type diet with low-carbohydrate and standard protein intake of 20% would fail to loose weight, but also do not significantly increase blood ketone levels and have a modest effect to reduce plasma glucose(26). It was hypothesized that the plasma glucose was derived largely from gluconeogenesis from the amino acids in the diet. Interestingly when protein was decreased to 8% mice started to loose weight, however they consumed the same number of calories as mice on the high protein diet. The diet we used contained an intermediate amount of protein, and was designed to match protein intake compared to normal diet. In our study we did not see a difference between calorie intake, protein intake and weight between normal diet and ketogenic diet fed ad libitum.

Another difference between our experiment and that of Poplawalski et al., is that we balanced the control and ketogenic diet to protein intake. In the experiment of Poplawalski et al. the protective effect of the ketogenic diet could be possibly partly be attributed to protein restriction(17). In our study there was no difference in protein intake between the ad libitum groups and between the caloric restricted groups. So if we could have shown an effect of the ketogenic diet, than this was independently of protein restriction. However, in our study caloric restriction was not controlled for protein intake. It is not certain that the effects we see of caloric restriction are not at least partly due to protein restriction. However literature comparing the effect of caloric restriction and protein restriction, show that caloric restriction is more important than protein restriction(27).

In this study kidney weight in ad libitum groups were increased when compared to caloric restricted groups. However, creatinine clearance per gram of kidney weight was lower in ad libitum fed rats than in caloric restricted rats. Immunohistochemistry revealed dilation of tubules in ad libitum fed groups, and not in caloric restricted groups. Dilatation of the tubules is probably caused by Tamm-Horsfall protein casts, which were present in ad libitum fed animals. Tubular casts and tubular dilatation could possible explain the increased kidney weight of ad libitum fed rats as compared to the decreased kidney function. Also in other studies it is shown that proteinuria results in the formation of casts with concomitant deterioration of kidney function(28,29).

It is worthwhile to mention that the ketogenic diet is designed for and clinically used in refractory epilepsia(30). Possible mechanism of the ketogenic diet in refractory epilepsia is also thought to be by lowering plasma glucose, as well as in diabetic nephropathy. Therefore this indicates that the ketogenic diet or other pharmacological interventions mimicking the ketogenic diet will be beneficial in states with impaired glucose metabolism. In our experiment we used a model of spontaneous nephropathy without the involvement of diabetes or impaired glucose metabolism. In this model a reduction of glucose metabolism is unlikely to prevent or reverse nephropathy. However, caloric restriction may have a wider range of indications in which it would be beneficial. The mechanism by which caloric restriction acts is probably independently of ketogenesis in non-diabetic states.

It is also possible that the effect of caloric restriction was induced by intermittent fasting,

rather than by caloric restriction. Ad libitum fed animals were allowed to eat for 24 hours per day, but caloric restriction animals eat their portion in about half an hour and thereafter they had no access to food. It has been shown that mice fasting for 4 consecutive days, every 2 weeks, do not decrease in body mass, but that the development of high grade proteinuria was delayed(31). Therefore it could be that the stress response of intermittent fasting is more involved in the protective effect against nephropathy than the caloric restriction itself. In conclusion, the beneficial effects of caloric restriction on established proteinuria in MWF rats, are not mediated by ketogenesis. Other concomitant effects of caloric restriction as reduced protein intake or intermittent fasting cannot be excluded as factors in the protective effects of caloric restriction in this experiment. Further research to elucidate the positive effects of caloric restriction is warranted.

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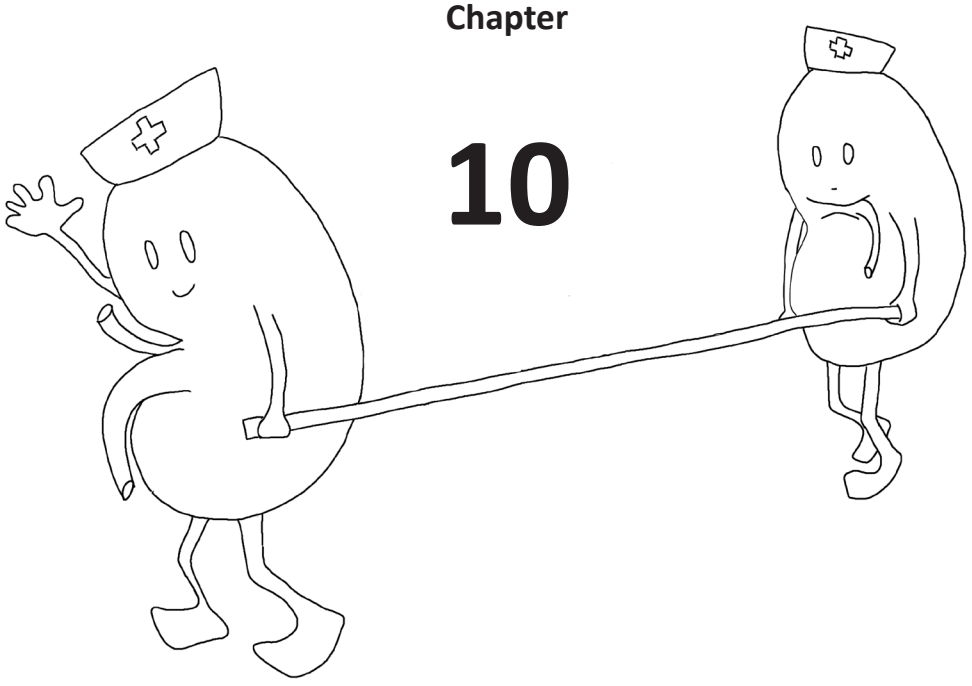
Chapter

10



Summary and Future Perspectives

Summary

Ischemia-reperfusion injury is inevitable to transplantation. During ischemia adenosine triphosphate (ATP) is depleted coincided with the formation of free radicals. Thiamine pyrophosphate is the 'active' thiamine co-enzyme for at least three enzymes involved in glucose metabolism. These enzymes play a role in both the regeneration of reduced glutathione (GSH) as substrate for anti-oxidant enzymes and the regeneration of ATP for maintenance of energy-requiring metabolic processes. Thereby thiamine is crucial for optimal amounts of GSH and for regeneration of ATP in cells.

In **chapter 2** the hypothesis why thiamine deficiency could be detrimental in kidney transplantation is described. Thiamine is a B-vitamin (vitamin B1) and an essential micronutrient for mammalians. It is difficult to maintain thiamine stores without continuous supplementation with thiamine from food or other resources. Subclinical thiamine deficiency is very common in patients at admission to intensive care units. Intensive care patients are the typical kidney donors. A further cause of thiamine deficiency may be related to the preservation procedure of the organ prior to transplantation. Thiamine is water-soluble, and may diffuse from the organ into the preservation solution during cold flushing and during storage. Thiamine is required for optimal regeneration of GSH and ATP in cells. In the reperfusion phase tissue demands of GSH and ATP are high in order to counterbalance events as production of reactive oxygen species and acute cell swelling. Several studies have shown that thiamine supplementation was beneficial in ischaemia-reperfusion models of different organs. This leads to the hypothesis that thiamine deficiency is an important determinant of the occurrence of acute tubule necrosis and delayed graft function in kidney transplantation.

In **chapter 3** we describe the development of thiamine deficiency in different tissues. This study showed that brain and heart tissue are relatively protected by thiamine deficiency. However clinical signs of thiamine deficiency appear first in these tissues. This indicates that when signs of thiamine deficiency are clinically present such as Wernicke's encephalopathy and wet beriberi also other tissues then brain and heart are affected. Subclinical thiamine deficiency of certain tissues could thereby well exist. Moreover this study gives a rationale for the period of thiamine deficient diet as used in **chapter 4**. It showed that kidney tissue is already deficient after two weeks of feeding a thiamine deficient diet. This deficient state is prior to weight loss of the animals and before they decrease their dietary food intake.

In **chapter 4** the influence of thiamine deficiency on ischaemia-reperfusion injury in the kidney was tested. In contrast to our hypothesis, we could not show that thiamine deficiency aggravated ischaemia-reperfusion injury. Moreover when thiamine deficiency

was complicated by a decreased food intake and weight loss this prevented ischaemia-reperfusion injury. However there was no effect of thiamine deficiency when it was not complicated by decreased food intake and weight loss. Thereby we surmised that the weight loss and reduced food may explain the unexpected protective effect.

In **chapter 5** we describe a double-blind, randomized, placebo-controlled clinical trial, in which we tested the hypothesis whether benfotiamine could decrease albuminuria in diabetic nephropathy. In this study there was no effect of benfotiamine treatment on albuminuria and other markers of kidney damage. However thiamine status improved in the study period, indicating proper adherence to the study treatment. In studies that showed an effect of thiamine supplementation on albuminuria in diabetic nephropathy there were less patients on angiotensin-converting-enzyme inhibitor and angiotensin receptor blocker treatment. This indicates that patients who are optimally treated for diabetic nephropathy do not benefit from (benfo)thiamine supplementation as much as suboptimal treated patients do.

In **chapter 6** we did not show an effect of peri-operative fasting on ischaemia-reperfusion injury. However, this could be due to timeframe and experimental design. The duration of fasting was 48 h prior to ischaemia-reperfusion procedure, and it could be that to induce a protective effect in rats longer period of fasting is necessary. However, longer period of fasting prior to ischaemia-reperfusion procedure would not be applicable in the clinical setting. Instead of fasting also other forms of dietary restriction could be used, such as caloric restriction. As fasting is known to play a role in ischaemia-reperfusion injury and kidney disease we proceeded to study the correlation of non-esterified fatty acids and malondialdehyde in renal transplant recipients. In **chapter 7** we describe the protective effects of non-esterified fatty acids on graft failure in stable renal transplant recipients. In experimental studies it has been described that non-esterified fatty acids carried by albumin are detrimental for kidney function. However the effect of non-esterified fatty acids had not been measured in stable renal transplant recipients. In this chapter we showed that non-esterified fatty acids protect against graft loss. Recent *in vitro* studies suggest that type of non-esterified fatty acids is important in protective or detrimental effects. Malondialdehyde has, as described in **chapter 8**, been shown to be correlated with lower change of graft failure in stable renal transplant recipients. Malondialdehyde is historically seen as a marker of oxidative stress. However malondialdehyde excretion will also rise when salmon is consumed or after exercise. Thereby it could be rather a marker of lifestyle than of oxidative stress in stable renal transplant recipients.

In **chapter 9** we tested the hypothesis whether caloric restriction in combination with ketogenic diet protects against nephropathy in a proteinuric rat model. We introduced

caloric restriction with or without the combination of a ketogenic diet in a proteinuric rat model when proteinuria had already developed. Thereby we attempted to mimick the clinical situation in which a patient is treated when proteinuria is already established. We showed that caloric restriction lowers proteinuria and decreases renal damage. This was irrespective of ketogenesis since a ketogenic diet did not shown any changes. This suggests that other mechanisms rather than ketogenesis underlie the beneficial effects of caloric restriction. One mechanism could be the effect of caloric restriction on lowering the blood pressure.

Future perspectives

Our initial hypothesis was that thiamine deficiency aggravates ischaemia-reperfusion injury of the kidney. Since we noted no effect of thiamine deficiency we assume that there is no further base to test thiamine supplementation in human kidney transplantation. However, we found in those studies that decreased food intake and weight loss was protective for ischaemia-reperfusion injury. These findings were confirmed by other groups in the literature. Currently feasibility studies to apply caloric restriction in kidney donors are undertaken.

We found no effect of benfotiamine in diabetic nephropathy in patients on established angiotensin-converting-enzyme inhibitor or angiotensin receptor blocker treatment. However a study in Pakistani patients showed that thiamine supplementation lowered albuminuria. In that study less than 50% of patients used angiotensin-converting-enzyme inhibitors or angiotensin receptor blockers, were in our study all patients used at least one of these medications. Also baseline albuminuria was two times lower than in our study. This could indicate that (benfo)thiamine supplementation is a therapeutic option to slow down progression of albuminuria in early stages of diabetic nephropathy or in developing countries were treatment with angiotensin-converting-enzyme inhibitors or angiotensin receptor blockers is less common or deemed too expensive.

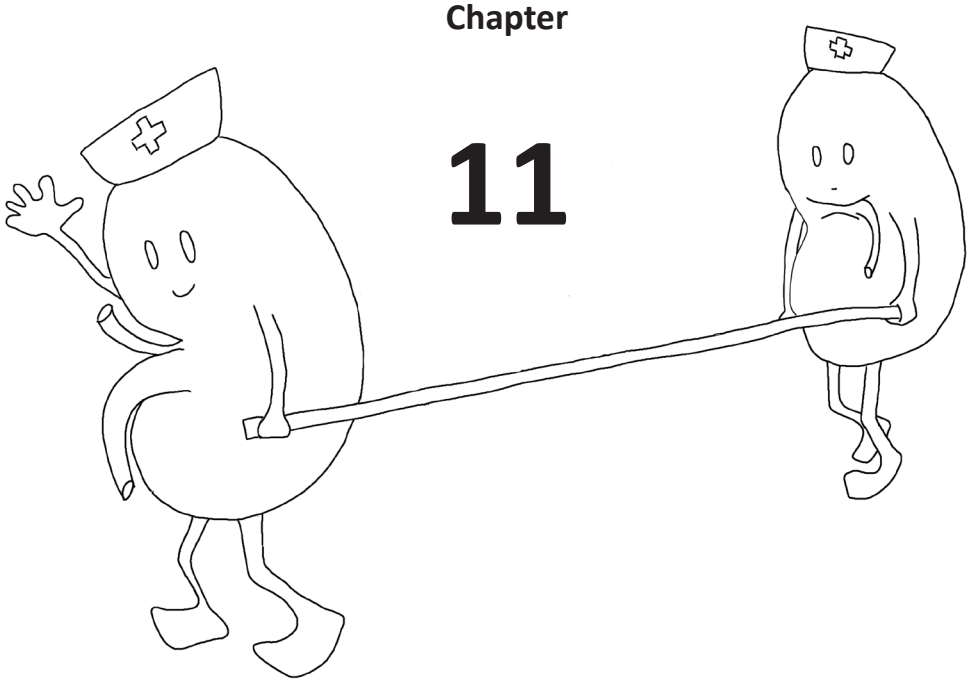
High levels of fasting non-esterified fatty acids and malondialdehyde are protective against graft failure in stable renal transplant recipients. Both markers are historically described as being detrimental for the kidney. However recent *in vitro* studies showed that different types of non-esterified fatty acids exert different effects. Further studies should reveal whether intake of polyunsaturated fatty acids is a protective factor in graft failure in stable renal transplant recipients. Malondialdehyde is historically seen as a marker of oxidative stress, but after consumption of salmon and after exercise malondialdehyde levels will also increase. Thereby malondialdehyde could be a measure of healthy lifestyle in stable renal transplant recipients, rather than a marker of oxidative stress. If increased consumption of salmon will increase malondialdehyde levels on long term and will prevent graft failure

has to be further studied. Currently a national study is undertaken to evaluate effects of a three month exercise programme in renal transplant recipients. It would be interesting to test whether malondialdehyde is increased by this exercise programme and whether it is associated with lower graft failure.

We also showed that caloric restriction reverses proteinuria in established experimental renal disease. Further studies have to reveal whether protein intake is in play, irrespective of caloric restriction. Since caloric restriction was very effective in reducing proteinuria and preventing structural and function kidney damage, it is most likely that the effect is mediated by modulation of the blood pressure. Another possibility is that the effect is due to intermitting fasting rather than to caloric restriction. It has been shown in mice that intermitting fasting with no decrease in body mass delayed the development of proteinuria. The blood pressure lowering capacities of caloric restriction as well as the intermittent fasting component should be further investigated. The capability of caloric restriction to reverse proteinuria and lowering the blood pressure is also of interest in patients with nephropathy and/or hypertension.

Chapter

11



Samenvatting en Toekomstperspectief

Nederlandse samenvatting

Ischemie-reperfusie schade treedt op tijdens niertransplantatie en is niet te voorkomen. Gedurende ischemie wordt adenosine trifosfaat (ATP) verbruikt en tegelijkertijd worden vrije radicalen gevormd. Hierdoor treedt schade op. Thiamine pyrofosfaat is de 'actieve' thiamine cofactor voor tenminste drie enzymen die een rol spelen in het glucose metabolisme. Deze enzymen zijn betrokken bij zowel de regeneratie van gereduceerd glutathion (GSH), een belangrijk substraat voor antioxidant enzymen, als bij de regeneratie van ATP, een energiebron benodigd voor het behoud van essentiële metabole processen. Hierdoor is thiamine cruciaal voor optimale hoeveelheden GSH en ATP.

In **hoofdstuk 2** wordt de hypothese waarom thiamine deficiëntie ongunstig zou zijn tijdens niertransplantatie beschreven. Thiamine is een B-vitamine (vitamine B1) en een essentieel sporenelement voor zoogdieren, inclusief mensen. Het is moeilijk om thiamine voorraden op peil te houden zonder continue inname van thiamine via voedsel of andere bronnen. Subklinische thiamine deficiëntie is een veel voorkomend probleem bij patiënten die worden opgenomen op een intensive care afdeling. Veel nierdonoren zijn patiënten die (gaan) overlijden op een intensive care afdeling. Een voortgaande verergering van thiamine deficiëntie kan veroorzaakt worden door de preservatie procedure van een orgaan voorafgaand aan een transplantatie. Thiamine is wateroplosbaar, en kan diffunderen vanuit het orgaan naar de preservatie vloeistof gedurende het doorspoelen van het orgaan en de opslag. Thiamine is nodig voor een optimale regeneratie van GSH en ATP in cellen. In de reperfusie fase is de vraag naar GSH en ATP verhoogd met als doel tegenwicht te geven aan gebeurtenissen zoals de productie van reactieve zuurstofradicalen en het optreden van celoedeem. Verscheidene studies hebben aangetoond dat thiamine suppletie een positief effect heeft in ischemie-reperfusie modellen van verschillende organen. Dit leidde tot de hypothese dat thiamine deficiëntie een belangrijke factor is in het ontstaan van acute tubulus necrose en het verlate functioneren van het niertransplantaat na een niertransplantatie.

In **hoofdstuk 3** beschrijven wij de ontwikkeling van thiamine deficiëntie in verschillende weefsels. Deze studie laat zien dat hersenen- en hartweefsel relatief beschermd zijn tegen thiamine deficiëntie. Echter klinische signalen van thiamine deficiëntie ontstaan het eerste vanuit deze organen. Dit betekent dat als signalen van thiamine deficiëntie zich klinisch presenteren, zoals Wernicke encefalopathie en beriberi, andere weefsels dan de hersenen en het hart al langer zijn aangedaan. Subklinische thiamine deficiëntie van bepaalde weefsels kan dus voorkomen. Deze studie liet zien dat nierweefsel al thiamine deficiënt was na een thiamine deficiënt dieet gedurende twee weken, daarom hebben wij in **hoofdstuk 4** gekozen voor een dieetperiode van twee en vier weken. Het tijdspunt na twee weken thiamine deficiënt dieet lag voor het moment dat de dieren als gevolg van thiamine

deficiëntie gewicht verloren en minder voedsel opnamen, wat wel optrad na vier weken thiamine deficiënt dieet.

In **hoofdstuk 4** werd het effect van thiamine deficiëntie op ischemie-reperfusie schade in de nier getest. In tegenstelling tot onze hypothese konden we niet aantonen dat thiamine deficiëntie de ischemie-reperfusie schade verergerde. Bovendien wanneer thiamine deficiëntie werd gecompliceerd door een verlaagde voedselinname en gewichtsverlies voorkwam dit de ischemie-reperfusie schade. Echter wanneer thiamine deficiëntie niet werd gecompliceerd door een verminderde voedselopname en gewichtsverlies was er geen effect. Hierdoor kwamen we tot de conclusie dat het gewichtsverlies en de verminderde voedselopname het onverwachte beschermende effect zouden kunnen verklaren.

In **hoofdstuk 5** beschrijven wij een dubbelblind, gerandomiseerd, placebo-gecontroleerd onderzoek waarin wij de hypothese testten of benfotiamine albuminurie in diabetische nefropathie zou kunnen verlagen. In deze studie werd geen effect van benfotiamine behandeling op albuminurie of andere markers van nierschade gezien. De thiamine status verbeterde echter gedurende de studie periode, dit gaf aan dat de patiënten therapietrouw waren. In studies die een effect lieten zien van thiamine suppletie op albuminurie in patiënten met diabetische nefropathie, werden minder patiënten behandeld met renine-angiotensine-aldosteronsysteem remmers. Dit geeft aan dat patiënten die optimaal worden behandeld voor diabetische nefropathie niet in die mate profiteren van (benfo)thiamine suppletie als suboptimaal behandelde patiënten.

In **hoofdstuk 6** konden we geen effect van peri-operatief vasten op ischemie-reperfusie schade aantonen. Dit kan mogelijk worden verklaard door de tijdsspanne en het experimentele ontwerp. De duur van de periode van vasten was 48 uur voorafgaand aan de ischemie-reperfusie procedure, en mogelijkerwijs zou het voor ratten noodzakelijk zijn een langere periode van vasten te ondergaan voordat er een beschermend effect op ischemie-reperfusie schade optreedt. Een langere periode van vasten voorafgaand aan de ischemie-reperfusie procedure zal echter niet toepasbaar zijn in de klinische setting. In plaats van vasten zouden mogelijk ook andere vormen van verminderde voedselopname kunnen worden gebruikt, zoals calorische restrictie.

Omdat vasten een rol speelt in ischemie-reperfusie schade en in nierziekten, hebben wij de rol van vrije vetzuren en malondialdehyde in niertransplantatiepatiënten onderzocht.

In **hoofdstuk 7** hebben wij de beschermende effecten van vrije vetzuren op transplantaatfalen in niertransplantatiepatiënten beschreven. In experimentele studies is aangegeven dat vrije vetzuren welke worden gebonden door albumine, de nierfunctie verslechteren. Het effect

van vrije vetzuren werd echter niet eerder in niertransplantatiepatiënten bepaald. In dit hoofdstuk hebben wij laten zien dat een hoge concentratie vrije vetzuren beschermend zijn tegen nierfalen in niertransplantatiepatiënten. Recente *in vitro* experimenten geven de suggestie dat verschillende typen vrije vetzuren een verschillend effect hebben, die overwegend positief dan wel negatief kunnen zijn.

Zoals wij hebben beschreven in **hoofdstuk 8** is ook malondialdehyde gecorreleerd met een lagere kans op transplantaatfalen in niertransplantatiepatiënten. Malondialdehyde wordt gezien als een marker van oxidatieve stress. Malondialdehyde excretie stijgt echter ook na het eten van zalm of na fysieke inspanning. Hierdoor zou malondialdehyde eerder een maat kunnen zijn van een gezonde levensstijl dan van oxidatieve stress in niertransplantatiepatiënten.

In **hoofdstuk 9** hebben wij de hypothese onderzocht of calorische restrictie in combinatie met een ketogeen dieet beschermt tegen nefropathie in een proteïnurisch experimenteel diermodel. Calorische restrictie werd gegeven met of zonder de combinatie van een ketogeen dieet in een experimenteel diermodel na de ontwikkeling van proteïnurie. Hierdoor probeerden we de klinische situatie na te bootsen waarin een patiënt wordt behandeld nadat de aanwezigheid van proteïnurie werd geconstateerd. Deze studie laat zien dat calorische restrictie de proteïnurie verlaagd en dat het een positief effect had op nierschade. Dit was onafhankelijk van ketogenese, omdat het ketogene dieet niet leidde tot een andere uitkomst dan het controle dieet. Dit wekt de suggestie dat andere mechanismen dan ketogenese ten grondslag liggen aan de effecten van calorische restrictie. Een van die mechanismen zou het effect van calorische restrictie op verlaging van de bloeddruk kunnen zijn.

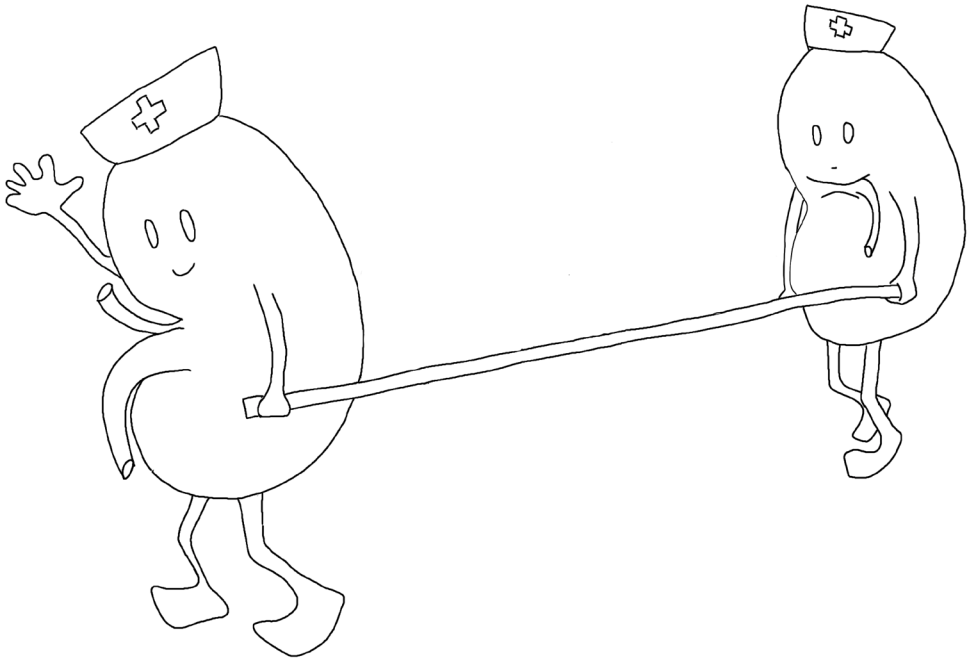
Toekomstperspectief

Onze aanvankelijke hypothese was dat thiamine deficiëntie ischemie-reperfusie schade in de nier zou verergeren. Omdat wij geen effect van thiamine deficiëntie op ischemie-reperfusie schade konden aantonen veronderstellen wij dat er geen verdere basis is om thiamine suppletie rond de niertransplantatie te onderzoeken. Wij vonden echter wel dat een verminderde voedselinname en gewichtsverlies een beschermend effect heeft tegen ischemie-reperfusie schade. Deze bevindingen werden bevestigd door andere onderzoeksgroepen. Momenteel worden er haalbaarheidsonderzoeken naar het toepassen van calorische restrictie in nierdonoren ondernomen.
Wij vonden geen effect van benfotiamine op diabetische nefropatie in patiënten behandeld met een renine-angiotensine-aldosteronsysteem remmer. Een onderzoek in Pakistaanse patiënten liet echter zien dat thiamine supplementatie de albuminurie verlaagde. In deze

studie werd minder dan 50% van de patiënten behandeld met een renine-angiotensine-aldosteronsysteem remmer. In onze studie werden alle patiënten behandeld met minstens één medicijn uit deze groep. De albuminurie concentratie was tweemaal zo laag in onze studie in vergelijking tot de Pakistaanse studie. Dit zou kunnen betekenen dat (benfo) thiamine suppletie een therapeutische optie is om de progressie van albuminurie te vertragen tijdens het beginstadium van diabetische nefropathie of in ontwikkelingslanden waar behandeling met een renine-angiotensine-aldosteronsysteem remmer minder voorkomend of te kostbaar is.

Hoge concentraties van vrije vetzuren en malondialdehyde zijn verlagen de kans op transplantaatfalen bij niertransplantatiepatiënten. Beide markers worden in de literatuur beschreven als schadelijk voor de nier. In recente in vitro studies werd echter aangetoond dat verschillende typen vrije vetzuren verschillende effecten hebben. Toekomstige experimenten zullen moeten uitwijzen of de inname van meervoudig onverzadigde vetzuren het risico op transplantaatfalen bij niertransplantatiepatiënten verlaagt. Malondialdehyde wordt enkel gezien als een marker van oxidatieve stress, maar men weet nu dat na inname van zalm of na lichamelijke inspanning de concentraties malondialdehyde eveneens verhoogd kunnen zijn. Hierdoor zou malondialdehyde een maat voor een gezonde levensstijl kunnen zijn. Of een verhoogde inname van zalm of lichamelijke inspanning malondialdehyde concentraties op de langere termijn zal verhogen en of dit de kans op nierfalen zal verlagen moet nog worden onderzocht. Momenteel wordt er een nationaal onderzoek uitgevoerd naar de effecten van een drie maanden durend trainingsprogramma in niertransplantatiepatiënten. Het zal interessant zijn om te onderzoeken of malondialdehyde verhoogd is bij deelnemers aan dit trainingsprogramma en of dit gerelateerd kan worden met een lagere kans op transplantaatfalen.

Wij hebben ook laten zien dat calorische restrictie de proteïnurie verlaagt tot onder het startniveau in een experimenteel diermodel van nierschade. Verdere studies zullen moeten uitwijzen of dit wordt veroorzaakt door verminderde inname van eiwitten, onafhankelijk van calorische restrictie. Omdat calorische restrictie zeer effectief was in het verminderen van proteïnurie en zowel structurele als functionele nierschade voorkwam, is het meest waarschijnlijk dat dit effect deels veroorzaakt wordt door veranderingen in de bloeddruk. Een andere mogelijkheid is dat het effect wordt veroorzaakt door intermitterend vasten in plaats van alleen calorische restrictie. Bij experimenten met muizen is aangetoond dat intermitterend vasten, zonder afname in lichaamsgewicht, de ontwikkeling van proteïnurie vertraagt. Het bloeddruk verlagende effect van calorische restrictie als wel de component van intermitterend vasten in calorische restrictie zal verder moeten worden onderzocht. De eigenschappen van calorische restrictie om de proteïnurie en bloeddruk te verlagen zijn mogelijk gunstig voor patiënten met nefropathie en/of hypertensie.



Dankwoord
en
Curriculum Vitae

Dankwoord

Promoveren doe je niet alleen, want er staan twee geweldige paranimfen aan mijn zijde. Maar voor het voorbereidende werk, de experimenten, vele discussies en het schrijven van de artikelen zijn vele malen meer personen bij betrokken dan dat de lezer uit het proefschrift zelf zou kunnen opmaken.
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Anouk en Fré, jullie zijn twee geweldige kinderen. Jullie maken mijn leven compleet.

Curriculum Vitae

De auteur van dit proefschrift, Astrid Klooster, werd geboren op 2 december 1985 te Winschoten. Zij groeide op in Vriescheloo, gelegen nabij de Duitse grens in Oost-Groningen. Aansluitend aan het behalen van het Gymnasium diploma aan het Dollard College te Winschoten, startte zij in 2004 met de studie Geneeskunde aan de Rijksuniversiteit Groningen. Haar eerste JSM proefproject werd uitgevoerd in 2006 getiteld 'De buikomvang bij vrouwen als voorspeller van het ontstaan van type 2 diabetes mellitus' onder leiding van Dr. Stephan J.L. Bakker. Daarna volgde het JSM proefproject en de wetenschappelijke stage naar thiamine tekort en ischemie-reperfusie schade van de nier. Hier werd de basis voor haar MD/PhD-traject gelegd. In 2009 begon zij haar MD/PhD-traject, waarin coschappen en onderzoek elkaar afwisselden. Na het doorlopen van de senior co-schappen in het Scheper Ziekenhuis te Emmen en de semi-arts stage op de afdeling nefrologie van het Universitair Medisch Centrum Groningen onder leiding van Prof. dr. Willem J. van Son werd zij in augustus 2012 bevorderd tot arts. In april 2013 is zij gestart als AIOS pathologie in het Universitair Medisch Centrum Groningen.

Astrid is in 2007 getrouwd met Pieter van der Burg en zij wonen in Oostwold, waar ze een vleeskuikens- en akkerbouwbedrijf hebben. Zij hebben twee kinderen, Anouk en Fré.