Beneficial Effects of Preoperative Dietary Restriction

Tessa van Ginhoven

COLOFON

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Part one

General introduction

Chapter 1

Introduction

People have always been searching for methods to stay young and live longer. For example, in the European middle ages alchemists were looking for the "potion of life". This elixir, also known as the elixir of immortality and sometimes equated with the philosopher's stone, is a legendary potion, or drink, that grants the drinker eternal life or eternal youth. So far no elixir has been found. The same holds for the fountain of youth, a legendary spring that reputedly restores the youth of anyone who drinks of its waters. Despite all these efforts it was not until the beginning of the twentieth century that a non-invasive way to prolong life-, and healthspan was found: dietary restriction.

DIETARY RESTRICTION

Dietary restriction (DR), a reduction in daily energy intake without causing malnutrition, is able to extend lifespan. This was first reported by C.M. McCay in 1935[1]. He divided his rats into three groups based on their nutritional regimen: ad libitum (unrestricted) diet, 30% restriction of caloric intake starting from the time of weaning and starting 2 weeks after weaning. Rats on 30% DR lived twice as long when compared to the ad libitum fed group. Hereafter, the life-extending action was found to occur in both genders of many different rat and mouse strains, as well as in non-mammalian species such as fish, flies, and water fleas[2-7]. Recently, it was reported that DR is also able to increase the lifespan of non-human primates[8], suggesting that DR could also work in humans. Although studies investigating the effect of DR in humans on lifespan are lacking there are reports which imply that DR might work in humans. Epidemiological data showed that people living on the Island of Okinawa (Japan), which were not exposed to the westernisation of their diet and adhered to a relatively calorie-restricted diet, had a higher centenarian rate compared to the people on the "mainland" of Japan[9]. Furthermore, during the first mission in Biosphere 2 (a 12700 m^2 artificial, materially-closed ecological system) the eight humans were capable of producing only 83% of the calorie intake of a normal human diet, leading tot calorie restriction. Medical markers indicated that their health during this fase of the the experiment was excellent and it was concluded that non-obese humans on a low-calorie, nutrient-dense diet show physiologic, hematologic, hormonal, and biochemical changes resembling those of rodents and monkeys on such diets[10]. In addition, the CALERIE trial (a randomized controlled trial were participants were randomized to 6 months of a control diet or 25% calorie restriction) reports a reduced risk for cardiovascular events in healthy non-obese individuals[11], and improved insulin sensitivity in non-obese humans adhering to a DR diet[12].

THE EFFECT OF DIETARY RESTRICTION ON AGING AND OXIDATIVE STRESS

Aging can be defined as the process of growing old or maturing. One of the most supported theories about why we age is the free radical theory of Harman[13]. He postulated that aging and the degenerative diseases associated with it are due to the deleterious effects of free radicals on cell constituents and connective tissues. Although it has been shown that DR delays the aging process and extends lifespan, the mechanisms underlying this effect have not been elucidated. Rubner et al.[14] were the first to suggest that metabolic rate (i.e. energy metabolism per unit of body mass) is negatively correlated with the length of life. With the subsequent discovery that energy metabolism generates reactive oxygen species that cause molecular oxidative damage, a potential biochemical mechanism emerged that linked DR to oxidative damage and aging. DR is indeed able to attenuate the age-associated increase in lipid peroxidation[15], the accumulation of oxidized proteins[16] and the accumulation of oxidative damage to DNA[17]. Furthermore, long-term DR lowers steady-state levels of oxidative stress, decreases mitochondrial electron and proton leak in mammalian cells and attenuates damage resulting from intracellular oxidative stress[18-21]. The concept that DR, as a relatively non-invasive method, is able to reduce and/or protect against oxidative stress is interesting from a clinical perspective. DR can be performed by means of different regimens such as calorie restriction (CR; reduced daily calorie intake), fasting (no food intake), and alternate day fasting (ADF). In addition, DR can be divided into long-term (months-years) or short-term (days-weeks) interventions. Long-term interventions are however not amendable in the clinical setting. Therefore, this thesis focuses on short-term DR.

OXIDATIVE STRESS IN THE CLINICAL SITUATION

Ischemia and reperfusion injury

Chapter 2 offers a review of the literature on the protective effects of short-term DR against clinically relevant forms of oxidative stress such as ischemia-reperfusion (I/R) injury. Ischemia is a deprivation of sufficient blood supply, accompanied by the lack of oxygen, which is an essential source for cellular energy metabolism, resulting in damage or dysfunction of tissue. Reperfusion injury refers to the additional damage caused when blood supply is restored after a period of ischemia. Many complex mechanisms are involved in the injury caused by I/R, including an inflammatory response and oxidative damage through the induction of oxidative stress. I/R injury is unavoidable in case of liver transplantation and is commonly induced during major liver resections when vascular occlusion techniques, such as the Pringle maneuver, are used to control bloodloss[22]. Renal transplantation is considered the treatment of choice for people

with end-stage renal disease. I/R injury negatively influences the outcome after kidney transplantation[23,24]. Delayed graft function is primarily a consequence of I/R injury and contributes to the loss of kidney grafts[25]. The development of a protective non-invasive strategy against I/R injury is therefore warranted to improve clinical outcome after kidney transplantation as well as liver surgery and transplantation. In previous studies we have demonstrated that dietary restriction protects against I/R injury[26]. Both 3 days of fasting and 2 weeks of reduced (30%) caloric intake prior to renal I/R resulted in significant protection against I/R injury in mice. In **chapter 3** we aimed to extend these observations and elucidate the mechanisms of protection by short-term fasting against hepatic I/R injury.

In **chapters 4 and 5** we aimed to identify the factor(s) responsible for the beneficial effect of short-term DR on I/R injury. During short-term stress responses, activation of the hypothalamic-pituitary-adrenal axis stimulates the release of glucocorticoids from the adrenal gland. Glucocorticoids are important mediators in these stress response pathways[27] and essential in limiting and resolving inflammation[28]. I/R injury induces inflammation, which is responsible for many of its detrimental consequences[29]. Fasting acts as an acute stressor and increases levels of corticosterone in rodents[30]. We therefore tested the hypothesis that the protection against I/R injury imposed by fasting is mediated by increased systemic levels of corticosterone in **chapter 4**. Fasting also leads to an increase in serum levels of ghrelin[31]. The complete structure of ghrelin has been identified as an [*O*-*n*-octanoyl-Ser 3]-peptide. The *n*-octanoyl moiety is essential for the activity of ghrelin[32]. Acylated ghrelin is the endogenous ligand for the growth hormone secretagogue receptor[31]. Interestingly, ghrelin has been shown to reduce ischemia-related problems after skin flap transfer[33] and to protect against renal I/R injury[34]. The influence of ghrelin levels on renal I/R injury was examined in **chapter 5**.

Postoperative inflammation

Surgical procedures induce hematogenic tumor cell dissemination into the blood-stream as reflected by increased circulating tumor cells (CTC) present during surgical procedures[35]. The importance of CTC is underlined by an increased hepatic metastasis rate in CTC-positive patients, when compared to CTC-negative patients[36]. Almost every surgical trauma causes oxidative stress[37,38] and provokes an acute phase reaction. This response is characterized by increased levels of pro-inflammatory cytokines such as interleukin-6 and tumor necrosis facto alpha (TNF-α), increased levels of C-reactive protein and leukocytosis[39,40]. Surgery also decreases the number of circulating B- and T-cells, which may lead to a temporary impairment of cellular immunity[41,42]. These changes in the immune system are thought to play an important role in enhancing the metastatic potential of pre-existing or intraoperatively spilled CTC. The inflammatory reaction with elevated levels of local and systemic proinflammatory cytokines results

in the up-regulation of adhesion molecules, such as E-selectin which may promote outgrowth of metastases by facilitating tumor cell adhesion to the endothelium of several organs such as the liver. Secondly, the induction of a pronounced immunosuppressive period after major surgery may impair the innate effector cell function of Kupffer cells and natural killer cells, which have an important role in the eradication of tumor cells retained in the liver vasculature. Impairment of their activity may result in an increased risk of the development of hepatic metastases[35,43,44].

The perioperative period may provide a window of opportunity in which the adhesion and outgrowth of CTC in the liver can be reduced, leading to less metastatic lesions and possibly lower patient morbidity and mortality rates. DR is associated with extended longevity[1] and reduced cancer incidence[8,45-48]. Short-term preoperative DR for one week reduces angiogenesis and growth in a mouse brain tumor model[49]. In addition, we reported that short-term DR prior to both renal and hepatic I/R injury reduces the expression of pro-inflammatory cytokines and adhesion molecules[50]. In **chapter 6** we used a murine model to determine the effect of short-term preoperative DR on tumor cell adhesion and hepatic tumor load after inoculation with tumor cells.

FEASIBILITY OF PREOPERATIVE DIETARY RESTRICTION IN THE CLINICAL SETTING

The experimental studies show that the beneficial effects of DR are induced rapidly and can be tapped for clinically relevant benefits such as protection against I/R injury. The maximum protection against renal I/R injury in the mouse was induced by both three days of preoperative water-only fasting and two weeks of preoperative reduced (30%) caloric intake. Preoperative DR may therefore be a non-invasive way to reduce I/R injury following organ transplantation[25]. Unfortunately, preoperative fasting is currently considered an unwanted necessity as it reduces patient well-being and induces peripheral insulin resistance[51,52]. The concept of reducing I/R injury by slightly longer periods of preoperative fasting or preoperative dietary restriction is novel and so far no clinical studies have been conducted. It is known that humans in general have difficulties to adhere to prescribed diets and it is unknown if people are able to adhere to a diet in preparation for surgery, which causes distress itself. We therefore describe in **chapter 7** a pilot study where we investigated whether a relatively mild preoperative DR regimen was feasible in the clinical setting and explored the effects of preoperative DR in live kidney donors on both the donor and recipient. We studied live kidney donors, who are a healthy, relatively homogenous patient group undergoing a standardised operation, rendering them a suitable study population and designed a DR regimen involving three preoperative days of 30% DR and one day of fasting. To assess the effect of preoperative

DR on renal transplant function, we measured graft function on the first postoperative day by means of renographs and during the first month by serum creatinine levels in the recipient. In **chapter 8** we focussed on the effect of preoperative DR on the postoperative acute phase response. We hypothesized that short-term DR reduces the postoperative acute phase response in humans. This theory was based on the results of our experimental studies where short-term DR reduces the inflammatory response after surgically induced acute oxidative stress[50], and on others who found reduced serum cytokine levels after surgery[53,54]. Furthermore, TNF-α levels are lower and well-being is improved in asthma patients after short-term DR[55], supporting the use of DR in the clinical situation. In **chapter 9** the results of the studies performed in this thesis are summarized and discussed.

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Chapter 2

The use of preoperative nutritional interventions to protect against hepatic ischemia-reperfusion injury

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ABSTRACT

Preoperative fasting was introduced in the 19th century to reduce the risk of aspiration pneumonia while under general anesthesia. During the last decades, the value of preoperative fasting has been questioned and more liberal guidelines have been proposed, such as the use of preoperative carbohydrate-rich drinks. Here we review both old and new evidence supporting the view that slightly longer periods than overnight fasting are beneficial for an entirely different purpose: protection against certain types of stress, such as ischemia-reperfusion injury. We provide a framework to explain these benefits as well as future applications and alternatives to induce the protection afforded by nutritional interventions.

INTRODUCTION

Perioperative nutrition is a recurrent issue in experimental as well as clinical research related to the safety of anesthesia on the one hand, and the metabolic response to surgical trauma on the other hand. Hiram Studley (1936) was the first to report a negative correlation between (excessive) preoperative weight loss and surgical outcome following major abdominal or thoracic surgery[1]. Although it is difficult to draw firm conclusions from this study, since there was no control group that failed to lose weight or actually gained weight prior to surgery, in the clinical setting malnutrition has been proved to indeed be a risk factor for surgical complications[2-4].

The appreciation that a large portion of hospitalized patients suffers from undernutrition has further fuelled the attention for pre- and postoperative feeding. Pre- and postoperative nutritional support may not only reduce the complications of surgery, as shown in a randomized clinical trial by Beattie et al.[5], but may also speed up postoperative recovery[6-8].

As early as in 1858 John Snow wrote that the best time for an operation is before breakfast (thus after a night of fasting) arguing that "the possibility of vomiting constitutes an unpleasantness and inconvenience which is desirable to avoid"[9]. Anesthesia relaxes the gag reflex, and increases the chance for either pulmonary aspiration or aspiration pneumonia. To reduce the risk of pulmonary aspiration, patients in the end 18th and early 19th century were mainly allowed only a cup of tea up to a few hours before surgery[9]. With little evidence to prove its usefulness, this practice has evolved to become the accepted "nil by mouth from midnight" regimen, which has been widely used in the 20th century. During the last decades, the value of preoperative fasting has been questioned, and it was recently shown that the intake of clear fluids up to two hours before surgery did not increase the gastric residual volume or risk of aspiration as compared to overnight fasting[10]. Nowadays, a six hour fast from solid foods and a two hour fast from clear liquids prior to surgery is accepted as safe for healthy individuals[11].

Along with the increasing understanding that patients require an optimal nutritional status before surgery, it was shown that surgery induces resistance to the actions of insulin, which may be ameliorated by the preoperative administration of carbohydrates. Randomized studies, where glucose was administered either as an infusion or as a carbohydrate-rich drink taken two to three hours before surgery, were found to reduce the postoperative insulin resistance and increase the patients subjective well-being before and after surgery[12-17].

Although the literature is replete with studies showing the adverse effects of the fasted state for surgical patients[18-20], there are a number of older studies as well as emerging new data in the field indicating that different types of DR (dietary restriction, defined as reduced food intake without malnutrition) in well-nourished patients

may in fact be beneficial as a way of protecting against acute organ stress. DR can be performed by means of different regimens such as CR (calorie restriction, reduced daily calorie intake), fasting (no food intake) and ADF (alternate day fasting), which are associated in experimental literature with lifespan extension and increased stress resistance in a wide range of organisms[21-24]. In this review we provide an overview of the studies lending support to the beneficial effects of DR in the context of increased resistance to surgical stress in the liver. More generally we will provide a perspective that attempts to explain how various types of dietary restriction, including CR, fasting and ADF, upregulate endogenous cell resistance mechanisms and how these benefits may be further explored in liver transplantation and surgery.

FASTING PROTECTS AGAINST HEPATIC ISCHEMIA-REPERFUSION INJURY

Ischemia-reperfusion injury is unavoidable during liver transplantation and is commonly induced during liver resections when vascular occlusion techniques, such as the Pringle maneuver, are used. The effects of fasting on ischemia-reperfusion injury were investigated in the nineties, since it was suggested that donor nutritional status may affect the outcome after liver transplantation and that starvation of donors, due to prolonged stay in the intensive care unit, may adversely affect the transplanted liver[25].

Aiming to investigate the effects of duration of donor fasting on the outcome after orthotopic liver transplantation (OLT) in a rat model, Sumimoto et al.[26] found to their surprise that livers from fasted donors were more viable than livers from fed donors. After 45 minutes of warm ischemia 50% of the recipients survived when the liver was obtained from a fed donor, whereas 80% survived when the liver was obtained from a three-day-fasted donor. Increasing the warm ischemia time to 60 minutes resulted in 100% mortality in the fed donor group. In contrast, if the donor was fasted for three days, 89% of the transplanted animals survived for seven days. Livers that were cold-stored for 30 hours were 50% viable, and fasting for one to three days did not affect this outcome. However, if the donor was fasted for four days, 100% survival was obtained. After 44 hours cold preservation, only 29% of the recipients survived for seven days. If the donor was fasted for four days, survival increased to 83%. In addition, liver function, bile production, and serum transaminases were better in livers from the fasted donors than from the surviving fed rats.

The glycogen content of the liver has been studied as a possible factor determining the outcome after OLT. As glycogen provides energy to maintain cellular function, glycogen was expected to reduce ischemic preservation injury. The results of these experiments are somewhat paradoxical, since both fasting (glycogen depletion), but also feeding plus oral glucose supplementation (glycogen restoration) are beneficial

for the survival after transplantation of cold stored liver grafts. Glucose supplementation in rats, prior to harvesting the liver, could result in newly synthesized glycogen, which could lead to reduced hepatocellular damage[27]. Since this study also found that fasting, and fasting plus oral glucose supplementation had similar beneficial effects, an alternative explanation is that glucose supplemented rats obtain their calories from glucose and consume less chow, and thus are restricted in calorie intake from food. However, Sumimoto et al.[28] reported that, in a rat model, four days of donor fasting resulted in the highest survival rate of the recipient, while the fasted group with glucose supplementation had high glycogen levels, but the worst survival rates.

Cold storage induces microvascular injury to the sinusoidal lining cells and this relates directly to graft viability. Six hours after transplantation the livers from fed rats showed significantly more apoptosis of the sinusoidal lining cells compared to the livers from the fasted group and electron micrographs showed sinusoidal spaces filled with more cell debris as well as inflammatory cells and Kupffer cells in the fed group[29]. The decrease of apoptotic sinusoidal lining cells observed after fasting may be related to the protection of the liver graft from reperfusion injury afforded by fasting, and partially explain the survival benefit of fasted livers.

In 1995, Sankary et al.[30] showed that recipient survival after OLT in a rat model after twelve hours of cold ischemia was significantly higher using donors that were fasted for 48 hours compared to ad libitum fed donors. In addition, they showed that fasting was associated with significantly lower tumor necrosis factor alpha serum levels after transplantation, which suggested a lower Kupffer cell activation.

When cells are exposed to stress, the expression of heat shock proteins (HSP's) is transcriptionally upregulated[31]. HSP's are cytoprotective molecular chaperones that aid in protein folding, refolding and degradation. In 1998, Takahashi et al.[32] showed that HSP-60 and HSP-70 were upregulated in livers from fasted rats after 72 hours of cold storage. These fasted livers were significantly more viable than normal fed livers after cold storage. Recent data suggest that HSP-70 is hepato-protective during hepatic ischemia-reperfusion injury, which suggests that upregulation of HSP's is (partially) responsible for the protective effect of fasting[33].

Using a similar model as above, Uchida et al.[34], showed in 2000 that four days of fasting significantly increased the levels of hemoxygenase in the liver after 24 hours of cold storage. As hemeoxygenase-1 is an inducible stress protein which confers cytoprotection against oxidative stress in vitro and in vivo, and upregulation of heme-oxygenase-1 has been shown to confer protection against hepatic cold preservation injury, it is likely also involved in the protective effect of fasting[35]. In contrast, fasting for 36 hours in male rats reduced the levels of catalase and copper, zinc-superoxide dismutase (anti oxidant enzymes) while the activity of glutathione peroxidase remained the same[36] (Table 1).

Table 1: Studies reporting beneficial effects of donor fasting prior to orthotopic liver transplantation.

First author	Year	Animal	Model	Intervention	Outcome compared to ad libitum fed control group
Sumimoto, R	1993	Rat, Brown Norway	OLT[1]: - warm ischemia (45/60 min[1]) - cold ischemia (30/44 hours)	24, 48, 72, and 96 hours water only fasting	- Fasting induced higher recipient survival rates after cold and warm ischemia and lower ALT[1] and AST[1] serum levels
Sun, X	2001	Rat, Wistar	OLT: - cold ischemia (24 hours)	96 hours water only fasting	- Fasting induced higher 14 days survival rates in the recipient (0% vs. 90%) - Fasting reduced the number of apoptotic SLC[1] compared to the ad libitum fed donors - Fasting reduced the LDH[1] serum levels six hours after transplantation
Sankary, H	1995	Rat, Lewis	OLT: - cold ischemia (8, 12 hours)	48 hours water only fasting	- Fasting resulted in significantly higher recipient survival rates after 12 hours ischemia (0% vs. 83%) - Fasting resulted in a significant decrease of peripheral blood TNF[1]- α levels after reperfusion
Takahashi, Y	1998	Rat, Brown Norway	OLT: - cold ischemia (48, 60, 72, 96 hours)	24, 48, 72, and 96 hours water only fasting	- 96 h fasting resulted in significantly higher recipient survival rates after 48 and 72 hours ischemia. - HSP[1]-60 and HSP-70 showed an increased expression after four days of fasting
Uchida, Y	2000	Rat, Lewis	OLT: - cold ischemia (24 hours	48 hours water only fasting	- Fasting induced higher 7 days survival rates in the recipient (0% vs. 87.5%) - Fasting induced expression of HO[1]-1 in Kupffer cells - Tissue GSH content was less reduced in livers from fasted donors
Sumimoto, R	1996	Rat, Brown Norway	OLT: - cold ischemia (30-44 hours)	96 hours water only fasting: with and without oral glucose water suppletion	- Fasting induced higher recipient survival rates after cold ischemia, while glucose suppletion lowered survival

[1] Orthotopic liver transplantation (OLT), minutes (min), Aspartate amino transferase (AST), Alanine amino transferase (ALT), Sinusoidal lining cells (SLC), Lactate dehydrogenase (LDH), Tumor necrosis factor (TNF), Heat shock protein (HSP), Heme-oxygenase (HO)

The effect of donor fasting was also studied in a large animal model. Donor pigs were divided into three groups: group one was fasted for seven days and received intravenous administration of saline; group two was fed ad libitum, and group three

was fasted for seven days, but given 20% glucose intravenously. The mean survival time after OLT in the last group (group three) was 37.2 days, significantly longer than 5.8 +/- 0.7, and 9.8 +/- 2.0 days in groups one and two, respectively[37]. In another study five days of fasting resulted in deteriorated adenosine triphosphate synthesis and less sinusoidal lining cell viability when compared to one day of fasting. The survival in the one day fasted group was 75% (1/4, death due to technical error) in contrast to 25% in the five day fasted group[38]. Both studies indicate that extended periods of fasting in pigs do not protect against ischemia-reperfusion injury in an OLT model.

Several studies used isolated perfusion models of the liver to examine the effect of nutritional interventions on ischemia-reperfusion injury after cold and/or warm storage. Results of these studies differ from those of the orthotopic transplant models. Using isolated liver perfusion, fasted livers release more transaminases in the perfusate than livers of fed animals[39-42]. These studies suggest that isolated liver perfusion is not a suitable model to reveal the beneficial effects of fasting observed in in-vivo studies.

The previous results were obtained using animals with healthy livers. Fatty livers are known to be more sensitive to the deleterious effects of ischemia-reperfusion injury, and livers with more than 60% steatosis are currently regarded as unsuitable for transplantation[43]. Since the incidence of obesity and the concomitant incidence of steatosis is rapidly increasing[44,45] this leads to the loss of potential donors. In 1999 Caraceni et al.[46] used fed and fasted rats with normal or fatty livers, induced by a choline deficient diet, who underwent one hour of warm hepatic ischemia followed by reperfusion. Whereas survival was similar in fasted and fed rats with normal livers (90% vs. 100%), 18 hours of water only fasting dramatically reduced the survival in rats with fatty livers (14% vs. 64%). The duration of the fasting period could be a determining factor.

In rats with steatosis, induced by feeding a choline deficient diet for 28 days, two or four days of fasting had no effect on the severity of steatosis, but afforded a time dependent increase in the protection against warm and cold preservation injury and ischemia-reperfusion injury[47].

In summary, fasting for one to four days protects livers from cold preservation injury and results in higher survival rates after OLT in animal studies. Proposed mechanisms include an upregulation of cytoprotective molecules such as HSP's and heme oxygenase-1. The depletion or augmentation of donor glycogen stores did not affect the outcome. The beneficial effects of preoperative fasting were not found when isolated liver perfusion models were used.

A FRAMEWORK TO UNDERSTAND THE BENEFICIAL EFFECT OF FASTING

Why did the former studies, showing beneficial effects of fasting, with their enormous potential to influence preoperative nutritional care, have so little measurable impact in the past decade and a half since they were first published? First, these studies were published at a time when a shift in preoperative nutritional care was underway, namely replacing strict preoperative overnight fasting guidelines with more liberal ones, and later with liquid carbohydrate-rich beverages specifically to avoid the catabolic state associated with fasting and to improve subjective perioperative well-being[12-17]. Second, a number of prior and subsequent studies demonstrated detrimental effects of fasting and/or malnutrition in various different experimental systems (e.g. isolated liver perfusion), leaving the overall picture cloudy and controversial[14,48,49]. Finally, these studies lacked a mechanistic framework in which to understand the results and make predictions on what would and wouldn't work and why, which is essential in order to have an impact on clinical practice. In this section we aim to provide a framework to understand these results.

The effects of long-term DR regimens have been widely studied and provide mechanistic insights with which the effect of DR on acute stress resistance may be explained. DR is the most robust, non-invasive intervention that increases lifespan and reduces the rate of aging[50]. This life-extending action has been found to occur in both genders of many different rat and mouse strains, as well as in hamsters and non-mammalian species such as fish, flies, and water fleas[50-55]. Long-term DR lowers steady-state levels of oxidative stress, decreases mitochondrial electron and proton leak in mammalian cells, attenuates damage resulting from intracellular oxidative stress[23,56-58], reduces the susceptibility to chronic diseases, and retards age-associated functional decline[22,59]. DR also augments antioxidant defence systems, and increases stress resistance to both oxidative and non-oxidative challenges in models of extended longevity. Hormesis is a common biological phenomenon in which exposure to a low intensity stressor induces a general adaptive response that has net beneficial effects on the cellular and/or organismal level, including protection against subsequent, higher dose exposures as well as to different types of stress[60-62]. DR has been proposed to act as a mild stressor that extends longevity through hormetic mechanisms[63,64]. Interestingly, ischemic preconditioning, a procedure used to protect against ischemic insult that entails brief period(s) of ischemia prior to a longer ischemia time, is also thought to function via hormesis[65].

DR may be performed by various regimens, namely: calorie restriction, fasting and alternate day fasting. CR (calorie restriction) refers to an intervention in which the total daily calories provided to an animal or organism is limited to a certain percentage of the animals' normal daily intake. ADF (alternate day fasting) regimens involve a "feast day" on which food is consumed ad libitum that alternates with a "fast day" on which

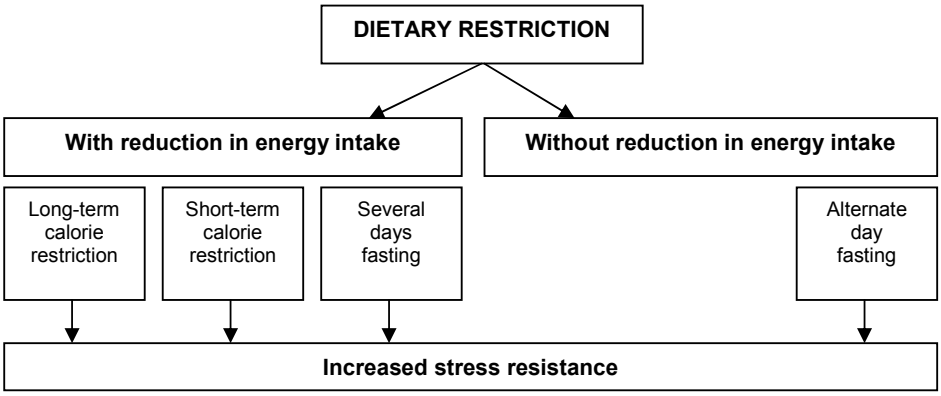

Figure 1: Overview of various forms of dietary restriction which are capable of inducing increased stress resistance. Calorie restriction refers to an intervention in which the total daily calories provided to an animal or organism is limited to a certain percentage of the animals ad libitum daily intake. Alternate day fasting regimens involve a "feast day" on which food is consumed ad libitum that alternates with a "fast day" on which food is withheld.

food is withheld. A key difference in the ADF approach is that overall calorie intake needs not to be limited. The alternating days of fasting are sufficient to act as a low dose stressor inducing a hormetic response[66], which can also extend lifespan and protect multiple organ systems against diseases in rodents[67,68]. All regimens can be applied for longer (lifetime–years) or shorter (months-days) time periods (Figure 1). Although long-term regimens induce many beneficial effects, four weeks of CR is able to induce many of the genomic expression changes seen after long-term CR. Short-term CR induced all of the changes seen after long-term CR on xenobiotic metabolism and stress response/chaperone protein gene expression. It also reproduced 67% of the effects of long-term CR on inflammatory response gene expression. These results suggest that the effects seen after long-term CR are induced rapidly, and that short- and long-term CR may act via a common protective mechanism[69].

Although the mechanisms responsible for the upregulation of defence systems during both long-term and short-term DR are not well understood, we are now able to see a mechanistic framework to explain the early studies on nutritional interventions in liver donors. The data and insights discussed below reveal new areas of applications that may therapeutically benefit from the changes triggered by the low-grade stress induced by DR as predicted by the hormesis hypothesis.

PROTECTION BY SHORT-TERM DIETARY RESTRICTION EXTENDS TO OTHER ORGANS, AND IS NOT LIMITED TO ISCHEMIA-REPERFUSION INJURY

Studies have been reported in which DR is used in the context of enhanced stress resistance to prevent or reduce injury in clinically relevant situations, such as ischemia-reperfusion injury. As described below, different organ systems such as brain, heart, liver, and retina were shown to enjoy protection by various forms of clinically applicable DR regimens.

Broad protection against ischemia-reperfusion injury

DR has recently been shown to facilitate the functional recovery of ischemically damaged neurons in the brain. The performance of DR rats in spatial tasks after an ischemic insult, such as the radial arm maze, was significantly better than that of ad libitum fed rats[70]. Furthermore, DR prior to cerebral ischemia-reperfusion resulted in a highly significant decrease in infarct volume when compared with the ad libitum fed group. Immunoblot analysis showed that levels of HSP-70 were greatly increased in neuronal tissue of DR mice compared with ad libitum fed controls[71]. In the heart, DR attenuated the postischemic inflammatory response of rats subjected to fifteen minutes of partial cardiac ischemia-reperfusion injury compared to ad libitum fed animals. This was shown by a reduced activation of nuclear factor kappa beta and faster return to baseline of antioxidant enzymes[72]. Similar benefits were found in the retina of rats subjected to DR. DR was neuroprotective in the retina following ischemia, and this was associated with increased levels of HSP-70[73].

Unfortunately, the onset of heart attack and cerebral ischemia is unpredictable, and thus not readily amenable to planned nutritional interventions. However, Plunet et al.[74] showed that DR may also be effective when applied after the injury. After surgical induction of cervical spinal cord injury, rats that were on DR showed a 50% reduction in lesion volume and improved regeneration and behavioral recovery.

DR protects the liver against various toxic insults

DR for three weeks protects rats against a lethal dose of the hepatotoxic compound TA (thioacetamide). DR rats showed 70% survival compared with 10% in ad libitum fed rats. Paradoxically, DR and ad libitum fed animals showed similar hepatocellular injury, and the survival benefit was due to stimulation of tissue repair in the DR group resulting in arrest of progressive injury and enhanced regeneration[75]. Expression of hepatocyte growth factor was consistently higher in the livers of DR rats after the administration of TA. Epidermal growth factor receptor expression was higher in DR rats before TA administration and remained higher until 48 hours after TA intoxication. DR induced a 2-fold increase in hepatic inducible nitric oxide synthase activity, which is consistent

with early cell division in DR rats after TA challenge. These data suggest that the augmented liver tissue repair after TA-induced hepatotoxicity in DR rats is due to faster and higher expression of growth stimulatory cytokines and growth factors[75]. Protection from acetaminophen hepatotoxicity has been found in mice that had been exposed to DR for eight months, as shown by negligible increases of serum alanine amino transferase and lactate dehydrogenase in the DR group and high levels of alanine amino transferase and lactate dehydrogenase in the ad libitum controls[76].

DISCUSSION AND FUTURE PERSPECTIVES

Although animal studies suggest potential uses for DR in the clinic, there are several drawbacks that need attention. Randomized clinical trials have shown that preoperative carbohydrate-rich drinks contribute to better insulin sensitivity and increased patient well-being. However, clinical studies on the effects of preoperative DR are currently lacking. Recently, a study was published in which human subjects adhered to a DR diet for three months, which led to a significant increase in verbal memory scores compared to the ad libitum group[77]. In addition, the CALERIE trial reports a reduced risk for cardiovascular events in healthy non-obese individuals[78], and improved insulin sensitivity in non-obese humans adhering to a DR diet[79]. Furthermore, overweight patients with asthma subjected to DR revealed improved well-being and reduced levels of circulating tumor necrosis factor alpha, brain-derived neurotrophic factor, and ceramides[80]. These studies indicate that DR in humans is feasible and capable to exert beneficial effects. However, more clinical studies are needed to develop DR regimens (length, reduction, and timing) for different pathological conditions.

Secondly, animal studies have shown that DR protects organs against various forms of stress. It is not known whether surgical patients benefit more from preoperative feeding or from the beneficial effects of DR on an organ specific level. Recently it was shown that two days of fasting is able to confer protection against the adverse side effects of a high dose of the chemotherapeutic agent etoposide in mice[81,82]. Etoposide displays a generalized toxicity profile ranging from myelosuppression to liver and neurological damage. This suggests that DR acts on an organismal rather than on a single organ-specific level.

Thirdly, protein restriction without a reduction in calories has been shown to increase maximum longevity in rats and mice as well[83]. Although the magnitude of these increases is around 30–40% of that of DR, neither carbohydrate[84] nor lipid restriction[85,86] exerted these effects. Restriction of proteins could therefore be another way to induce the effects seen after DR. These data also show that the beneficial effects of preoperative carbohydrate-rich drinks and DR may not be mutually exclusive.

Finally, the use of DR mimetics may be a way to overcome the problems associated with DR in surgical patients. A DR mimetic can be loosely defined as any pharmacological intervention that produces beneficial effects of DR without causing or requiring a significant reduction in calorie intake. One compound that has received considerable attention as DR mimetic is resveratrol, a naturally-occurring polyphenol found in red wine. Resveratrol induces genomic changes which resemble many of the genetic alterations induced by DR[87] and, at doses that can be readily achieved in humans, mimics aspects of DR, including an increased resistance to oxidative stress[88,89]. In addition, resveratrol treatment decreases liver injury induced by ischemia-reperfusion injury by significantly increasing glutathione reductase, Cu/Zn-superoxide dismutase, and catalase activities[90].

CONCLUSIONS

Together these results support an emerging view that the increased resistance to stress, that is associated with longevity in animals on long-term DR, may be tapped for short-term benefits. These range from neuroprotection and resistance to the adverse effects of chemotherapy, to protection against preservation- and ischemia-reperfusion injury in organ allografts, cardiothoracic surgery, and liver resection. Although these data are robust and convincing, more research is needed to identify the appropriate diet for each condition. The notion that protein restriction, and not DR per se can induce similar effects, may offer new avenues to combine preoperative nutrition (carbohydrate rich beverages) with restriction of proteins and thereby protect the target organ without compromising patient well-being. Furthermore, new drugs are able to mimic the protective effects of DR and must be studied more extensively in relation to ischemia-reperfusion injury of the liver.

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Part two

Experimental studies

Chapter 3

Short-term fasting protects mice against hepatic ischemia-reperfusion injury and does not affect liver regeneration

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ABSTRACT

Background: We have shown that brief periods of fasting induce functional changes similar to long-term dietary restriction in mice, including protection from ischemia-reperfusion (I/R) injury. Here, we investigated the mechanisms of protection induced by fasting, and determined its effect on liver regeneration following partial hepatectomy.
Methods: Partial hepatic ischemia (75 minutes) was induced in ad libitum fed and one, two and three days fasted mice. In following experiments we performed a 35% hepatectomy in ad libitum fed and 3 day fasted mice.
Results: Preoperative fasting for two and three days significantly decreased hepatocellular I/R injury. Hepatic gene expression of heme oxygenase-1 (HO-1), superoxide dismutase-2 (SOD2), glutathione peroxidase-1 (Gpx1), and glutathione reductase (GSR) was significantly upregulated in three day fasted mice prior to I/R injury and six hours hereafter. Furthermore, after reperfusion p-selectin and interleukin-6 (IL-6) were significantly down-regulated, and superoxide radical generation and neutrophil influx were significantly attenuated in the fasted mice. Preoperative fasting did not affect hepatocyte proliferation 48 hours and liver weight 5 days following a partial hepatectomy. Hepatic gene expression of tumor necrosis factor-alpha (TNF-α), IL-6, and transforming growth factor-beta 1 (TGF-β1) were significantly up-regulated in three days fasted mice at baseline and following resection.
Conclusions: Upregulation of the stress response gene HO-1 and the mitochondrial antioxidant enzymes SOD2, Gpx1, and GSR at baseline, and a better response following reperfusion, likely underlie the protection induced by short-term fasting against hepatic I/R injury. Because fasting does not affect liver regeneration after partial hepatectomy, it may be a promising new strategy to protect the liver against I/R injury in the clinical situation.

INTRODUCTION

Temporary occlusion of hepatic in-flow (Pringle maneuver) and hepatic in- and out-flow (total hepatic vascular occlusion) are routinely used techniques during extended liver surgeries such as hepatic resection, and liver transplantation[1-2]. These prolonged interruptions of hepatic blood flow result in ischemia-reperfusion (I/R) injury. Hepatic I/R injury is characterized by progressive hepatocellular injury and hepatocyte loss. It is considered to be a risk factor for potentially lethal primary-, or delayed non-functioning of the liver[3-4], as well as a risk factor for distant organ damage such as kidney, heart and lung[5-6]. To reduce the negative consequences of hepatic I/R injury during liver surgery and liver transplantation, development of a protective strategy against I/R is warranted.

Dietary restriction (DR), defined as reduced food intake without causing malnutrition, has been reported to extend lifespan in several organisms, including non-human primates[7]. DR is associated not only with extended longevity, but also with prolonged health span[8-9], and improved resistance against various stressors[10-12]. We have recently shown that the beneficial effects of DR can be induced rapidly. Both three days of fasting and one month of 30% DR were able to confer protection against hepatic I/R injury[13].

Although previous studies have shown similar beneficial effects of fasting against hepatic I/R injury in liver transplant models[14], the mechanisms responsible for this protective effect remained unknown and no connection with DR was made. As these results ran against their gut feeling and studies in an isolated rat liver perfusion models later opposed these findings[15], the issue remained mired in controversy to this day.

In the present study, we aimed to elucidate the mechanisms of protection induced by short-term fasting against hepatic I/R injury. Because in the clinic postoperative outcome is determined by the severity of I/R injury and the regenerative capacity of the liver, we also investigated the effects of short-term fasting on liver regeneration after partial hepatectomy. Here, we show that upregulation of the mitochondrial antioxidant enzymes SOD2, Gpx1, and GSR and the stress response gene HO-1 likely underlie the protection against hepatic I/R injury by fasting, and that short-term fasting does not affect liver regeneration.

MATERIALS AND METHODS

Animals

C57BL/6 male mice (~25 grams) were obtained from Harlan, Horst, the Netherlands. All mice had free access to water and chow (Special Diet Services, Witham, United Kingdom), unless mentioned otherwise. The one, two or thee day water-only fasting

regimen was applied by transferring mice to clean cages without food at 17:00 hours (n = 3-4 animals per cage). No mortality occurs during these fasting regimens. The experimental protocol was approved by the Animal Experiments Committee under the Dutch National Experiments on Animals Act and complied with the 1986 directive 86/609/EC of the Council of Europe.

Surgical procedures

All operations were performed between 9:00 and 13:00 hours. Mice were anesthetized by isoflurane/N_2/O_2 inhalation, and placed on a heating plate to maintain body temperature. Partial (~70%) hepatic I/R injury was induced by occluding the blood flow to the left lateral and median liver lobes with an atraumatic microvascular clamp for 75 minutes. After clamp removal, restoration of blood flow in the ischemic liver lobes was observed. For partial (~35%) hepatectomy (PH) the left liver lobe was resected. All mice received 0.5 mL of phosphate buffered saline subcutaneously postoperatively after which they were placed under a heating lamp to recover from anesthesia. After surgery all animals had free access to food and water.

Hepatocellular injury

Mice were anesthetized, and blood was drawn by retro-orbital puncture before surgery (baseline; t = 0 hours) and at six and twenty-four hours post-reperfusion. Sera were analyzed for alanine aminotransferase (sALAT) at the central clinical chemical laboratory of the Erasmus University Medical Center. The percentage of necrosis (0, 0-25, 25-50, 50-75, 75-100 or 100%) was scored by two independent observers blinded to the treatment on 3 μm thick H&E stained liver sections at a magnification of 100x in five microscopic fields per section.

Immunohistochemistry

Frozen (5 μm) and paraffin embedded (3 μm) liver sections were stained with monoclonal antibodies against neutrophils or proliferating cell nuclear antigen (PCNA), and visualized based on alkaline phosphatase- and HRP-conjugated secondary antibodies, respectively. In five microscopic fields per section the number of neutrophils or PCNA positive cells was counted by two independent observers blinded to the treatment at magnifications of 200-400x.

Superoxide radical production

Superoxide radical production in the liver was measured as described earlier[16], using 10 μM dihydroethidium. At least 300 nuclei per section were counted in 2-3 consecutive sections by an independent observer blinded to the treatment.

Liver weight/total body weight ratio

Wet liver weights and total body weights of fed and three day fasted mice were determined at baseline and five days after PH. This was also measured from three day fasted mice who were refed for five days without undergoing any surgery. Liver weight/total body weight (LW/TBW) ratio was calculated as follows: wet liver weight divided by total body weight.

Quantitative RT-PCR

Total RNA was extracted from frozen liver tissue using Trizol reagent (Invitrogen, Breda, the Netherlands), purified by a DNase treatment (RQ1 RNase-Free DNase; Promega Benelux B.V., Leiden, the Netherlands), and reverse transcribed to cDNA using random hexamer primers, and Superscript II RT (both from Invitrogen, Breda, the Netherlands) according to manufactures instructions. Quantitative real-time PCR was performed using a MyiQ Single-color Real-Time PCR Detection System with SYBR Green incorporation (both from Bio-Rad Laboratories B.V., Veenendaal, the Netherlands). Primer sequences are available upon request. Relative expression was calculated using the equation $2^{-(\Delta Ct\ sample - \Delta Ct\ control)}$. Each sample was tested at least in duplicate.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Differences in groups were analyzed by Mann-Whitney U tests using SPSS (version 15). Differences were considered significant at P values less than 0.05.

RESULTS

Short-term fasting protects against hepatic I/R injury

At baseline there were no significant differences in sALAT levels between the fed and fasted mice. However, six hours after reperfusion sALAT levels were significantly lower in two (P=0.004) and three (P=0.004) day fasted mice when compared to the ad libitum fed mice (Figure 1A). At twenty-four hours post-reperfusion sALAT levels remained significantly lower in two (P=0.03) and three (P=0.02) days fasted mice when compared to the control group. Twenty-four hours after I/R ijnury histological examination of livers from preoperative fed mice shows large areas of necrosis (69% ± 6 of the examined area). This was significantly lower in two (25% ± 0, P=0.01) and three (28% ± 6, P=0.001) day fasted mice (Figure 1B).

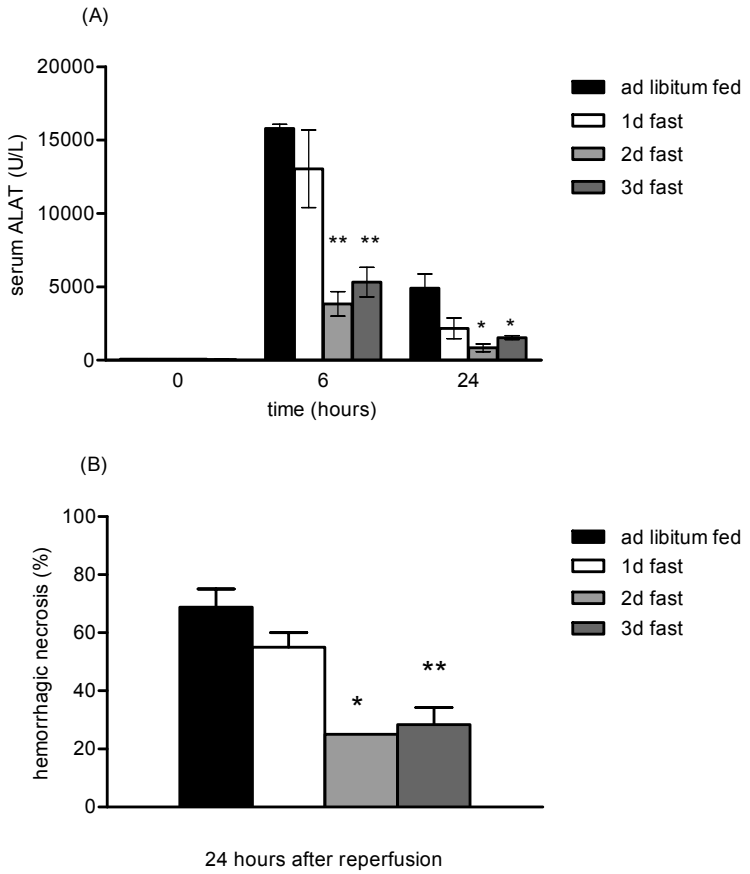

Figure 1: Hepatocellular injury. (A) Following reperfusion, serum alanine aminotransferases (sALAT) concentrations were significantly lower in two and three day preoperative fasted mice (n = 5-8 per group per time point). (B) Twenty-four hours post-reperfusion, livers from preoperative fasted mice contained significantly less hemorrhagic necrosis (n = 3-11 per group). Data are expressed as the mean±SEM. **P*<0.05; ***P*<0.01 vs. preoperative fed mice.

Short-term fasting reduces the inflammatory response after hepatic I/R

Development of hepatic I/R injury is a biphasic inflammatory process. In the first phase reactive oxygen species are generated, while the second phase is characterized by the release of pro-inflammatory cytokines such as interleukin-6 (IL-6), the expression of adhesion molecules such as p-selectin, and infiltration of neutrophils[17]. We investigated the effect of three days of preoperative fasting on the inflammatory response after hepatic I/R. Six hours post-reperfusion significantly less superoxide radicals were produced in livers from preoperative fasted animals compared to the control group (644 ± 41 vs. 1453 ± 235, P=0.02) (Figure 2A). Hepatic mRNA expression levels for IL-6 were low and comparable in both groups at baseline (2.7±1.3 vs. 1.1±0.2 in fed livers, P=NS). In contrast, p-selectin expression at baseline was significantly higher in

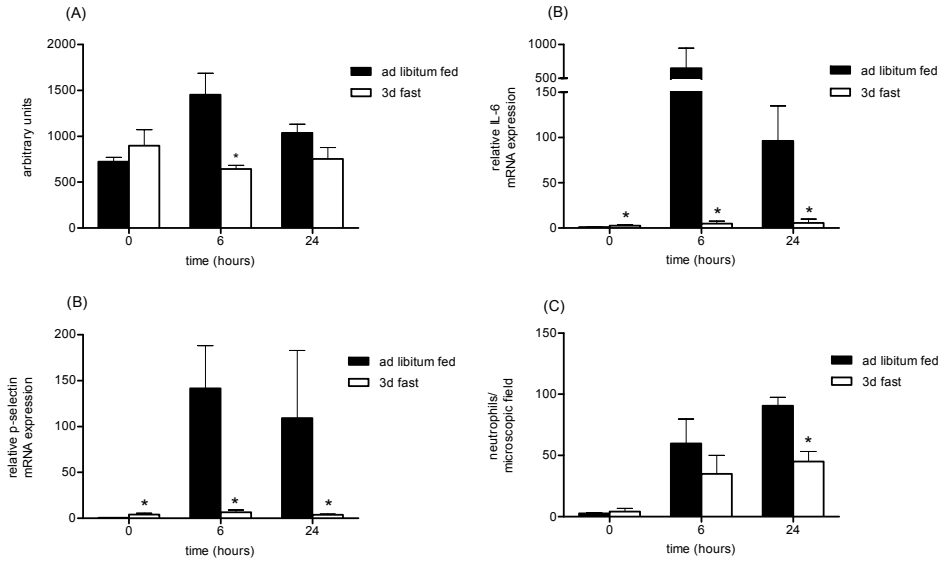

Figure 2: Inflammatory response. (A) Six hours post-reperfusion significantly less superoxide radical production was detected in livers from preoperative fasted mice (n = 4-8 per group per time point). (B) Following reperfusion, hepatic mRNA expression levels of p-selectin and interleukin-6 (IL-6) were significantly reduced in livers from preoperative fasted mice. Data was normalized for beta-2-microglobulin and expressed relative to preoperative fed mice at t = 0h (n = 3-5 per group per time point). (C) Lower numbers of neutrophils were detected in livers from preoperative fasted mice post-reperfusion (n = 3-6 per group per time point). Data are expressed as the mean±SEM. *$P<0.05$ vs. preoperative fed mice.

livers from fasted animals (4.2±1.4 vs. 0.7±0.1 in fed livers, P=0.03). Six hours after I/R injury both inflammatory markers were significantly lower in livers from preoperative fasted animals (p-selectin P=0.03; IL-6 P=0.02) when compared to the fed group. At twenty-four hours post-reperfusion this difference remained significant for p-selectin (P=0.03), but not for IL-6 (Figure 2B). Significantly less neutrophils were present in livers from preoperative fasted animals (P=0.02) at twenty-four hours post-reperfusion (Figure 2C).

Fasting up-regulates mitochondrial antioxidant enzymes and the stress response gene HO-1

In response to hepatic I/R, heat shock proteins (Hsp), and antioxidants are produced which mitigate hepatocellular injury[18-19]. We investigated the effect of three days of fasting on Hsp70 and heme oxygenase-1 (HO-1) gene expression, and on the mitochondrial antioxidant enzyme activities of superoxide dismutase 2 (SOD2), glutathione peroxidase 1 (Gpx1), glutathione reductase (GSR), and superoxide dismutase 1 (SOD1). At baseline, HO-1 expression was eight times higher in livers from fasted mice (8.8±2.2 vs. 1.1±0.2 in fed mice, P=0.01). Six hours after reperfusion, HO-1 expression signifi-

cantly increased in livers from both groups versus their baseline values (preoperative fasted: 23.3 fold increase, P=0.01; preoperative fed: 15.0 fold increase, P=0.02), with significantly higher levels in livers from preoperative fasted animals when compared with preoperative fed animals (203.8±51.9 vs. 16.5±2.1 in fed, P=0.02) (Figure 3A).

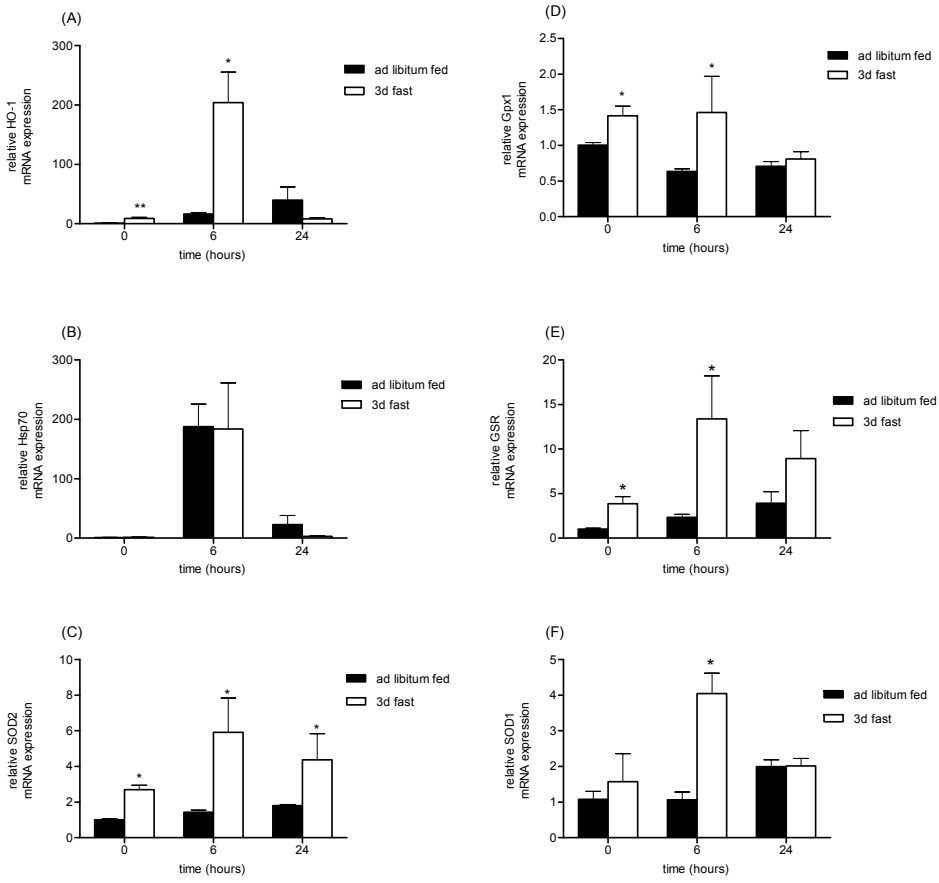

Figure 3: Hepatic gene expression of the stress response genes HO-1, and Hsp70, and the mitochondrial antioxidant enzymes SOD2, Gpx1, GSR, and SOD1. (A) Heme oxygenase-1 (HO-1) levels were significantly up-regulated at baseline, with a maximum expression six hours post-reperfusion in livers from preoperative fasted mice. (B) Heat shock protein 70 (Hsp70) levels peaked six hours post-reperfusion in both groups before returning to baseline values. (C) Superoxide dismutase 2 (SOD2) expression was significantly up-regulated in livers from preoperative fasted mice at baseline, and peaked to significantly higher levels six hours after reperfusion. (D) A significant increase in glutathione peroxidase 1 (Gpx1) expression levels was found in preoperative fasted mice at baseline, which remained significantly elevated six hours post-reperfusion. (E) At baseline, and six hours post-reperfusion, glutathione reductase (GSR) expression was significantly up-regulated in livers from preoperative fasted mice. (F) Six hours after reperfusion superoxide dismutase 1 (SOD1) peaked in livers from preoperative fasted mice. Data was normalized for beta-2-microglobulin and expressed relative to preoperative fed mice at t = 0h (n = 3-5 per group per time point). Data are expressed as the mean±SEM. $^{**}P<0.01$, $^{*}P<0.05$ vs. preoperative fed mice.

Twenty-four hours post-reperfusion HO-1 expression remained elevated in livers from preoperative fed mice, while it returned to baseline in preoperative fasted animals. No significant difference in Hsp70 expression was found at baseline, or at six hours post-reperfusion, when Hsp70 expression peaked in both groups before returning to baseline values at twenty-four hours post-reperfusion (Figure 3B). In livers from three day fasted animals expression levels of SOD2, Gpx1, and GSR were significantly upregulated at baseline (SOD2: 2.7±0.2 vs. 1.0±0.1, P=0.01; Gpx1: 1.4±0.1 vs. 1.0±0.0, P=0.03; GSR: 3.9±0.8 vs. 1.0±0.1 in fed livers, P=0.01) (Figure 3C-E). In the preoperative fasted group, SOD2 expression increased 4.2 times (5.9±1.9 vs. 1.4±0.1 in preoperative fed livers, P=0.03), Gpx1 2.5 times (1.5±0.5 vs. 0.6±0.0 in preoperative fed livers, P=0.03), GSR 5.0 times (13.4±4.8 vs. 2.7±0.0 in preoperative fed livers, P=0.03), and SOD1 3.6 times (4.0±0.6 vs. 1.1±0.2 in preoperative fed livers, P=0.02) at six hours post-reperfusion (Figure 3F). Twenty-four hours post-reperfusion SOD2 (4.4±1.5), Gpx1 (0.8±0.1), GSR (8.9±3.1), and SOD1 (2.0±0.2) expression levels decreased in livers from preoperative fasted animals compared with six hours post-reperfusion. In livers from preoperative fed animals no significant changes in expression levels were observed as compared with preoperative fed animals at six hours post-reperfusion.

Fasting does not affect liver regeneration after partial hepatectomy

An important determinant of postoperative liver function is the capacity of the liver to regenerate. To investigate liver regeneration we determined LW/TBW ratios. At baseline, liver weight (*P*=0.05), total body weight (*P*=0.05) and LW/TBW ratios (*P*=0.05) were significantly lower in three day fasted mice when compared to fed mice (Figure 4A, 4B). Preoperative fed mice did not eat much during the first days after PH, while preoperative fasted mice started eating rapidly. In the fed group, liver weight on post-resection day five was significantly lower (*P*=0.03) when compared to their baseline value. In contrast, livers from preoperative fasted mice were significantly heavier five days after PH when compared to baseline (*P*=0.03). Liver weight of fasted mice after five days of refeeding, without any intervention, was also significantly (*P*=0.03) higher when compared to baseline (Figure 4C). LW/TBW ratios on post-resection day five of preoperative fasted animals were significantly higher than preoperative fed animals (0.06±0.00 vs. 0.04±0.00, *P*=0.01) (Figure 4D). However, after correction for the increase in liver weight due to the "fasting and refeeding effect" no difference in liver weight, expressed as a percentage of the liver without PH, was observed (Figure 4E). We next investigated hepatocyte proliferation by PCNA staining (Figure 4F). At baseline, a significant reduction in the number of PCNA positive cells was found in livers from three day fasted animals (0±0 vs. 4±2 in fed animals, *P*=0.01). In a time course following PH, PCNA positive cells peaked at forty-eight hours without a significant difference between both groups (35±14 vs. 27±5 in preoperative fed animals).

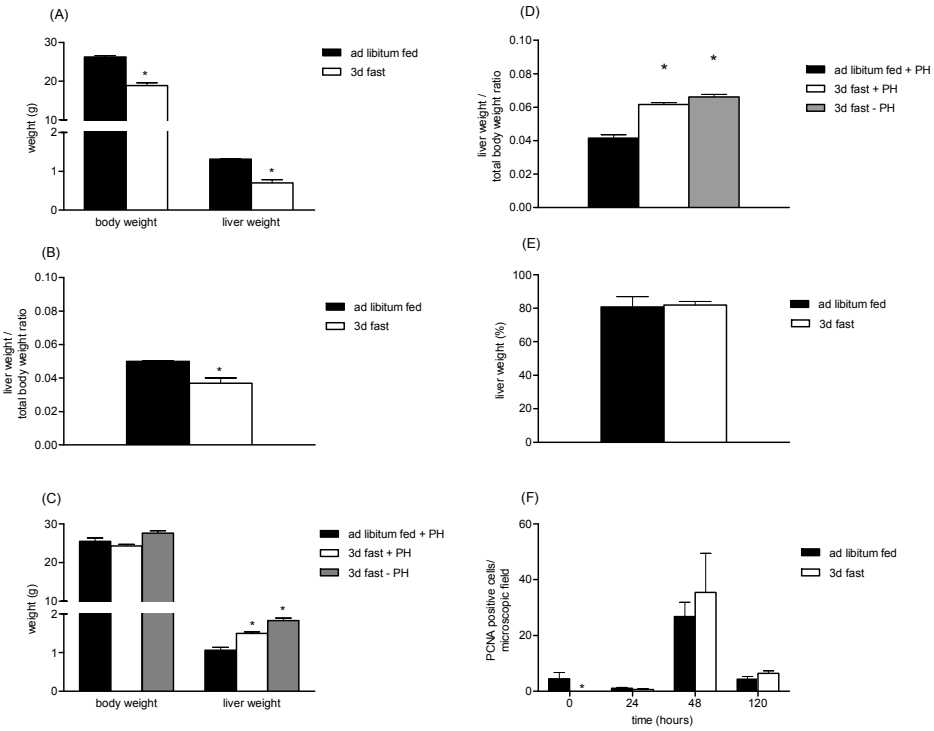

Figure 4: Liver regeneration. (A) At baseline, liver and total body weight were significantly decreased in three day fasted mice (n = 3 per group). (B) Liver weight/total body weight ratio was significantly decreased in three day fasted mice at baseline (n = 3 per group). (C) Bodyweight of the mice five days after PH, and the control group (Fasted – 5 days refeeding). Livers from preoperative fasted mice with and without partial hepatectomy (PH) were significantly heavier on postoperative day five (n = 4-5 per group). (D) On post-resection day five, liver weight/total body weight ratios were significantly increased in preoperative fasted mice with and without PH (n = 4-5 per group). (E) Relative liver weight on postoperative day five was expressed as a percentage of the weight at baseline (ad libitum fed group) or as a percentage of the liver weight after three days of fasting and five days of refeeding without liver resection (fasted group), to compensate for the increase in liver weight by fasting and refeeding. No significant change in relative liver weight was observed between both groups (n = 3-5 per group). (F) Hepatocyte proliferation assessed by proliferating cell nuclear antigen (PCNA) staining peaked at forty-eight hours in both groups (n = 4-5 per group). Data are expressed as the mean±SEM. *P<0.05 vs. preoperative fed mice.

Fasting differentially affected cytokine and growth factor expression after PH

Because liver regeneration is initiated by the expression of genes involved in hepatic growth and proliferation, such as tumor necrosis factor-alpha (TNF-α)[20] and IL-6[21], we investigated the effects of preoperative fasting on these markers using qRT-PCR. TNF-α expression was significantly increased in livers from preoperative fasted animals at twenty-four hours post-resection (8.6±2.1 vs. 0.4±0.1 at baseline, P=0.03) and returned towards baseline values at forty-eight hours post-resection (Figure 5A). TNF-α expression in livers from preoperative fed animals remained unaffected. Before PH,

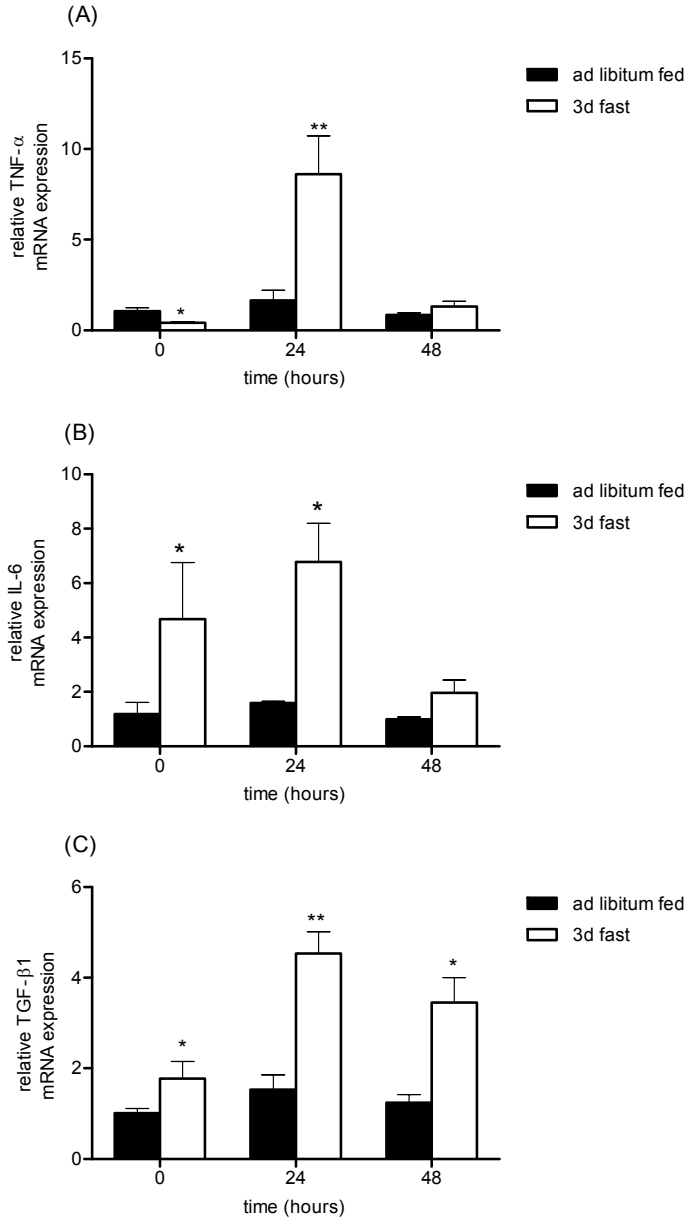

Figure 5: Growth factor kinetics after PH. (A) Hepatic mRNA expression levels of tumor necrosis factor-alpha (TNF-α) peaked twenty-four hours post-resection in livers from preoperative fasted mice (n = 4-5 per group per time point). (B) Interleukin-6 (IL-6) levels were already significantly up-regulated at baseline and remained so until twenty-four hours after partial hepatectomy (PH). (C) In livers from pre-operative fasted mice transforming growth factor-beta1 (TGF-β1) expression was up-regulated at baseline, and peaked at twenty-four hours post-resection. Data was normalized for beta-2-microglobulin and expressed relative to preoperative fed mice at t = 0h (n = 4-5 per group per time point). Data are expressed as the mean±SEM. *P<0.05; **P <0.01 vs. preoperative fed mice.

IL-6 expression was significantly up-regulated in livers from preoperative fasted animals (4.7±2.0 vs. 1.2±0.4 in fed animals, *P*=0.04) and remained significantly higher until twenty-four hours post-resection. In livers from preoperative fed mice IL-6 expression remained unaffected (Figure 5B). TGF-β1 is one of the factors involved in the termination response of liver regeneration[22]. Before PH, expression levels were significantly up-regulated in fasted livers (1.8±0.4 vs. 1.0±0.1 in fed livers, *P*=0.02) (Figure 5C). After PH, significantly higher expression levels were found in livers from preoperative fasted animals (24h: 3.0 times higher, *P*=0.009; 48h: 2.8 times higher vs. preoperative fed animals, *P*=0.03).

DISCUSSION

Long-term DR is associated with extended longevity and improved stress resistance in multiple experimental models. We recently showed that the beneficial effects of DR can be induced rapidly. Short-term DR as well as brief periods of preoperative fasting induced many of the transcriptional changes observed after long-term DR, and both protected against I/R injury[13]. The objective of the present study was to elucidate the mechanisms of protection induced by fasting. In addition, we investigated the effect of preoperative fasting on liver regeneration after partial hepatectomy. Both two- and three days of preoperative fasting offered significant protection against hepatic I/R injury. Moreover, we showed that the beneficial effects of three days of preoperative fasting were likely achieved by higher expression levels of genes encoding for mitochondrial matrix residing antioxidant defense enzymes SOD2, Gpx1, and GSR, and the stress response gene HO-1 at baseline, and a more expeditious and pronounced response post-reperfusion.

Consistent with previous studies[23-25], we found that hepatic I/R resulted in increased superoxide radical formation, up-regulation of p-selectin and IL-6 mRNA expression levels, and increased neutrophil infiltration. Although at baseline these markers were slightly elevated in livers from preoperative fasted mice, after I/R injury they were significantly lower.

HO-1 overexpression at baseline and after reperfusion is a critical factor in protection against hepatic I/R injury. Up-regulation of HO-1 expression after hepatic I/R has been shown to reduce graft injury in human liver transplant patients[26]. In addition, pharmacologically induced baseline HO-1 overexpression decreased I/R mediated hepatocellular injury in several animal models. For example, pretreatment with the HO-1 inducer cobalt protoporphyrin reduced hepatocellular injury after reperfusion in rat and mouse models[27-29]. In contrast, inhibition of HO-1 expression in the liver at baseline and post-reperfusion, results in higher sALAT levels, more hepatocellular ne-

crosis and apoptosis, higher neutrophil numbers and an increase in pro-inflammatory cytokine synthesis[28]. Our finding that three days of preoperative fasting induced HO-1 and strongly increased its expression after reperfusion suggests that HO-1 plays an important role in the beneficial effects of preoperative fasting.

Mitochondria are considered a major intracellular source of reactive oxygen species generation during hepatic I/R injury[30]. To minimize oxidative stress, these organelles contain a variety of antioxidant enzymes. Overexpression of these enzymes protects I/R injury prone organs such as the liver and the heart[31-32]. We demonstrate that three days of preoperative fasting significantly increases the baseline expression of these mitochondrial antioxidants SOD2, Gpx1 and GSR, and following I/R injury. These data suggest that protection against hepatic I/R injury by short-term fasting is in part mediated by increased resistance against mitochondrial oxidative stress.

Because in the clinic I/R injury occurs in situations when the liver needs to regenerate to maintain function, we investigated the effects of short-term preoperative fasting on liver regeneration. As hepatic ischemia impairs liver regeneration following partial hepatectomy[33], we chose to study a partial hepatectomy *per se* without concomitant hepatic ischemia. Although the combination is often encountered in the clinical setting.

In accordance with previous observations[34-35] we found a decrease in liver weight after three days of fasting. Furthermore, if animals are refed after a period of fasting the liver increases in weight to weights more than their normal value[36-38]. Therefore, it is not surprising that livers from preoperative fasted mice gained more weight than that those from preoperative fed mice after a PH. However, if we correct the liver weight for this "fasting-refeeding effect" the liver weight was similar in preoperative fed and fasted animals five days after PH. If we take into account the unaffected PCNA rate and the LW/TBW ration, our data suggest that preoperative fasting does not affect liver regeneration following hepatectomy. However, we must take into account that our model consists of a 30% PH, this induces a relatively minor regenerative response. Future experiments will be conducted in a 70% PH model.

In the scarcity of nutrients during the fasting period, cellular signaling shifts towards a survival mediated response, and represses proliferation[39]. We found that three days of fasting up-regulates baseline expression of the regeneration termination cytokine TGF-β1, and down-regulates the hepatic regeneration cytokine TNF-α. In contrast, IL-6 expression was increased in fasted livers at baseline. IL-6 has a role in both liver regeneration and inflammation[40]. Studies have shown that glycogen depletion results in hepatic injury[41-42]. The slight increase in IL-6, and p-selectin expression at baseline after three days of fasting, points towards the induction of a low grade inflammatory response by fasting. It is possible that this low grade inflammatory response contributes to the induction of cytoprotective and antioxidant genes, and preconditions the liver to a stronger response following injury.

Following partial hepatectomy, TNF-α, IL-6, and TGF-β1 showed a stronger and more expeditious response in livers of fasted mice. This suggests that to compensate for the hepatic injury caused by fasting at baseline, higher TNF-α and IL-6 levels facilitates an enhanced regenerative response. This is followed by an increase in TGF-β1 expression to terminate this enhanced regeneration response. The net result is a proliferative response that is similar to that in livers from preoperative fed animals.

In conclusion, this study shows that preoperative fasting ameliorates hepatic I/R injury via up-regulation of baseline levels of the mitochondrial antioxidant enzymes and the stress response gene HO-1, and a more expeditious and pronounced response of these genes following I/R injury. The baseline up-regulation of these genes, as well as TNF-α and TGF-β1 suggest that preoperative fasting acts as a low-level stressor, preconditioning the liver for other types of stress such as partial hepatectomy and hepatic I/R injury. Because preoperative fasting does not significantly affect liver regeneration in our model it could be a promising new non-invasive strategy to protect the liver against the detrimental effects of hepatic I/R injury during liver transplantation and major liver resection.

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Chapter 4

Preoperative fasting induces protection against renal ischemia-reperfusion injury by a corticosterone-independent mechanism

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ABSTRACT

Background: Three days of fasting protects mice against lethal renal ischemia-reperfusion (I/R) injury. We hypothesize that the protection imposed by fasting is mediated by increased levels of corticosterone, induced by the stress of food deprivation.

Methods: C57BL/6 mice were fasted for one, two or three days after which serum corticosterone levels were determined. Mice underwent a bilateral adrenalectomy (ADX) or sham procedure and ten days later they were fasted prior to renal I/R injury. Furthermore, another group of mice was given a corticosterone receptor antagonist or a vehiculum while fasting prior to I/R injury. Bilateral renal I/R injury was induced by clamping the artery and vein of the left and right kidney simultaneously for 37 minutes. Survival and kidney function were determined.

Results: Fasting significantly increased corticosterone levels. Only 8% of the ADX mice which were fasted prior to I/R injury survived, whereas all sham-ADX operated mice survived I/R injury after fasting. After ADX and fasting, 70% of the mice subjected to sham-I/R succumbed to the surgical procedure. After fasting with concomitant blockade of the glucocorticoid receptor all animals survived renal I/R.

Conclusions: Three days of fasting protects against I/R injury and increases serum corticosterone levels. ADX renders mice incapable of withstanding subsequent abdominal surgery. Glucocorticoid receptor blockade does not interfere with the protective effects of fasting. Thus, the protection against renal I/R injury induced by preoperative fasting is mediated by corticosterone-independent mechanisms.

INTRODUCTION

Renal transplantation is considered the treatment of choice for people with end-stage renal disease. One of the factors negatively influencing the outcome after kidney transplantation is ischemia-reperfusion (I/R) injury[1,2]. Delayed graft function is primarily a consequence of I/R injury and contributes to the loss of kidney grafts[3]. We have previously shown that dietary restriction protects against I/R injury[4]. Both 3 days of fasting and 2 weeks of reduced (30%) caloric intake prior to renal I/R resulted in protection against I/R injury in mice. Dietary restriction increased baseline levels of cytoprotective and antioxidant genes and resulted in a more expeditious and pronounced response of these genes to I/R injury[4,5]. The mechanism by which dietary restriction induces this protection remains elusive.

During short-term stress responses, activation of the hypothalamic-pituitary-adrenal axis stimulates the release of glucocorticoids from the adrenal gland. Glucocorticoids are one of the main mediators in these stress response pathways[6] and are essential in limiting and resolving inflammation[7]. I/R injury induces inflammation, which is responsible for its detrimental consequences[8]. Prolonged fasting acts as an acute stressor and increases levels of corticosterone in rodents[9]. We hypothesized that the protection against I/R injury imposed by fasting is mediated by increased systemic levels of corticosterone. We quantified serum corticosterone levels after three days of fasting and subjected mice to a bilateral adrenalectomy (ADX) and treatment with the glucocorticoid receptor antagonist Mifepristone during fasting. The effect of glucocorticoid receptor blockade on the increased expression of cytoprotective and antioxidant genes induced by fasting was determined to investigate the relationship between fasting, corticosterone, and the expression profile of these genes.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice with an average weight of 25 g were purchased from Harlan (Horst, The Netherlands). All mice were maintained under standard conditions with a 12 hour light/dark cycle and were allowed food and water *ad libitum*. The experimental protocol was approved by the Animal Experiments Committee under the Dutch National Experiments on Animals Act and complied with the 1986 directive 86/609/EC of the Council of Europe.

Fasting protocol

Mice in the fed group were allowed unrestricted access to food. Mice in the fasting groups were transferred to a clean cage at 5:00 pm and withheld food for 3 days. All animals were given continuous access to water or 0.9% NaCl (discussed next).

Bilateral adrenalectomy

Mice were anaesthetized by isoflurane inhalation (5% isoflurane initially and then 2% with oxygen for maintenance). Body temperature was maintained by placing the animals on heating pads until recovery from anesthesia. A small incision (0.5 cm) was made in the left and right flanks after which the adrenal glands were identified. Diathermy coagulation was performed to remove the adrenal glands from the surrounding tissue. Wounds were closed in two layers using 5/0 Safil (B.Braun Medical B.V., Oss, The Netherlands) sutures. Sham animals underwent the same procedure without removal of the adrenal glands. After surgery, 0.5 mL phosphate-buffered saline (PBS) was administered subcutaneously for maintenance of the fluid balance. Postoperatively, all animals were given access to 0.9% NaCl to ensure adequate salt balance. The experiments were resumed following a recovery period of 10 days. Corticosterone levels were determined as described below to confirm complete removal of the glands.

Bilateral renal I/R injury

All surgical procedures were conducted between 9.00 and 12.00 hours. Mice were anaesthetized by isoflurane inhalation (5% isoflurane initially and then 2% isoflurane with a 1:1 air:oxygen mixture for maintenance of anaesthesia). Body temperature was maintained by placing the animals on heating pads until recovery from anesthesia. Following a midline abdominal incision, the renal artery and vein of both the left and right kidney were occluded simultaneously, by using atraumatic microvascular clamps, for 37 minutes. In a previous study we showed that this ischemic time induces a mortality rate of 40%[4]. After macroscopic confirmation of ischemia (purple color), the incision was covered with PBS-soaked gauze and the animal was covered with an aluminum foil blanket to maintain body temperature. Following release of the vascular clamp, restoration of blood-flow was confirmed by the kidney returning to normal color. The abdominal wound was closed in two layers using 5/0 Safil sutures. Directly after closing the abdomen 0.5 mL of PBS at body temperature was injected subcutaneously for maintenance of fluid balance.

Glucocorticoid receptor blockage

Mifepristone is a potent glucocorticoid type II receptor antagonist that also blocks the progesterone receptor, albeit to a much lesser extent. Mifepristone (RU-38486, Sigma-Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich)

to a final concentration of 500 mg/mL. This stock solution was diluted 850- or 85-fold with PBS before intraperitoneal injection yielding a final DMSO concentration of 0.12% or 1.16%, respectively, to minimize the effect of DMSO on I/R injury[10]. As these treatments differ in final DMSO concentration, we used two vehicle solutions containing either 0.12% or 1.16% DMSO to correct for this difference.

Serum measurements

Blood samples were obtained under anesthesia by retro-orbital venous plexus puncture (during the experiments) or heart puncture (at the end of the experiment). Serum urea levels were determined using a QuantiChrom assay kit, DIUR-500 (Gentaur, Brussels, Belgium). Serum corticosterone was determined using a corticosterone ELISA kit (Sigma Aldrich) according to the manufacturer's protocol. Corticosterone serum levels were determined from blood samples obtained between 9:00 and 10:00 am.

Influence of fasting and ADX on corticosterone levels

Animals were fed ad libitum or fasted for one, two, or three days (n = 6/group), after which they were scarified by exsanguination under anesthesia. Furthermore, blood samples were obtained from ADX and sham(ADX)-operated mice (n = 6/group) after a 3-day fast. Determination of corticosterone levels was performed to confirm complete removal of the adrenal glands (Figure 1A).

Survival following renal I/R injury after ADX and subsequent fasting

ADX mice (n = 13) or sham (ADX)-operated mice (n = 8) underwent three days of fasting and subsequent renal I/R. Animals were observed twice a day for one week to monitor survival. In addition, survival was assessed in fasted ADX mice that had been subjected to a sham I/R procedure (n = 8) (Figure 1B)

Survival following renal I/R injury after mifepristone treatment

To asses the effect of glucocorticoid receptor blockade on renal I/R injury after a three day fast several experiments were performed. First, either the vehicle (PBS containing 0.12% DMSO, n = 6) or mifepristone (10 mg/kg, n = 6) was injected intraperitoneally (i.p.) 30 minutes prior to I/R after 3 days of fasting. Next, vehicle (n=6) or mifepristone (n=6) were administered once daily at 17.00 during the 3-day fast before renal I/R was applied. Finally, either vehicle (PBS containing 1.16% DMSO) (n = 8) or mifepristone in a ten times higher dose (100 mg/kg, n = 8) was injected daily i.p. during the 3-day fast before renal I/R was applied. To investigate the effects of mifepristone on renal I/R without preoperative fasting, vehicle (n = 6) or mifepristone (100 mg/kg, n = 6) was administered daily i.p. to ad libitum fed control mice (no ADX) during three days preceding I/R injury (Figure 1C). Mifepristone was administered in dosages that have

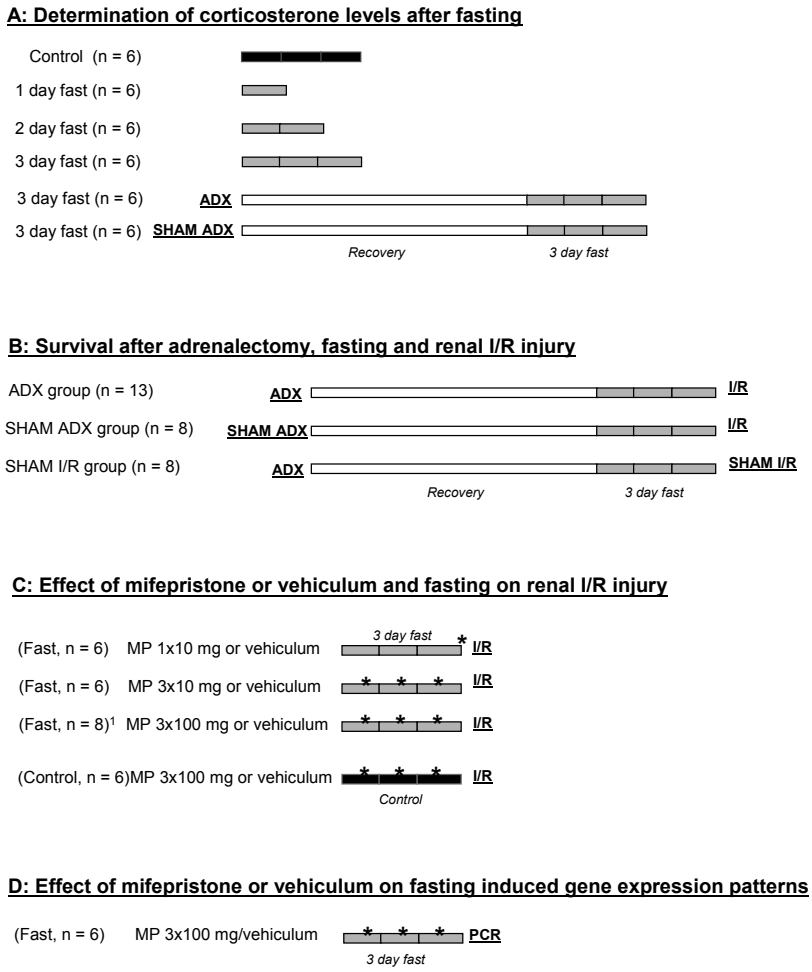

Figure 1: (A) Corticosterone levels were determined in control animals and in animals after 1, 2, or 3 days of fasting. Furthermore, corticosterone levels were determined in animals after ADX or sham-ADX and subsequent fasting. (B) Animals were subjected to either a bilateral adrenalectomy or a sham procedure. After a recovery period of ten days, animals in all groups were fasted for 3 days followed by either bilateral renal ischemia and reperfusion injury or a sham procedure. Survival was monitored following this second operation. (C) All animals were subjected to three days of fasting while mifepristone or the vehiculum was administered, except for the last group which was fed ad libitum. The asterisk (*) indicates administration of mifepristone or the vehiculum. Next, all groups were subjected to renal I/R injury and survival was monitored.[1] Renal function was measured in this group. MP = Mifepristone. (D) During the 3-day fast, either vehicle or mifepristone was injected daily i.p. The effect of mifepristone treatment on the expression of anti-oxidant genes in the liver was assessed after the 3-day fast.

been reported to effectively block all glucocorticoid receptors[11,12]. Serum corticosterone levels increase after administration of mifepristone[11] due to feedback inhibition of the pituitary gland. Therefore, increased corticosterone levels were used to indirectly assess blockade of the glucocorticoid receptors by mifepristone.

Quantitative real-time PCR

During the 3-day fast, either vehicle (PBS containing 1.16% DMSO, n = 6) or mifepristone 100 mg/kg i.p. (n = 6) was injected daily i.p. (Figure 1D). Since the most robust upregulation of cytoprotective and antioxidant genes upon fasting was observed in the liver; we investigated the effect of mifepristone treatment on the expression of these genes in the liver. Livers were harvested and snap frozen in liquid nitrogen after the 3-day fast. For gene expression analysis, total RNA was extracted from frozen liver tissue using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To prevent contamination by genomic DNA, the isolated RNA was purified by a DNase treatment (RQ1 Rnase-Free Dnase; Promega, Madison, WI, USA). Two µg of total RNA was reverse transcribed to cDNA using random hexamer primers (Invitrogen), and Superscript II RT (Invitrogen) according to manufacturer's instructions. Quantitative real-time PCR was performed using a MyiQ Single-color Real-Time PCR Detection System (Bio-Rad Laboratories, Herculus, CA, USA) in combination with SYBR Green as DNA probe (Bio-Rad Laboratories). The following primers were used: B2m, forward 5'-TCACTGACCGGCCTGTATGC-3,' reverse 5'-GAGGCGGGTGGAACTGTGTT-3,' Hsp32/HO-1, forward 5'-GAAGGCTTTAAGCTGGTGATGG-3,' reverse 5'-CTTCGGTGCAGCTCCTCAGG-3,' SOD2, forward 5'-TCTGGCGGGAGATGTTACAA-3,' reverse 5'-GGGCTCAGGTTTGTCCAGAAAAT-3,' GSR, forward 5'-CCGCCTGAACACCATCTAT-3,' reverse 5'-TTCCCATTGACTTCCACCG-3,'. Relative mRNA expressions were calculated using the equation $2^{-(\Delta Ct,\ sample\ -\ \Delta Ct,\ control)}$. Each sample was assayed in duplicate.

Statistical analysis

Categorical data are presented as number (percentage) and continuous variables as mean ± SEM (normal distribution, assessed visually and by means of Shapiro-Wilks test) or median ± interquartile distance (no normal distribution). Means between two groups were compared using either the non-parametric Mann-Whitney U test or the t-test for parametric data. Survival curves were compared using a log-rank (Mantel-Cox) test. P-values of <0.05 were considered significant. All analyses were performed using Statistical Package for the Social Sciences 15.0 (SPSS, Chicago, IL).

RESULTS

Fasting induces increased levels of corticosterone

Baseline corticosterone levels were 298±40 nmol/L. One, 2, and 3 days of fasting significantly increased corticosterone levels compared with baseline to 1135±163 nmol/L ($p = 0.0022$), 1253±234 nmol/L ($p = 0.0022$), and 1287±167 nmol/L ($p = 0.0022$),

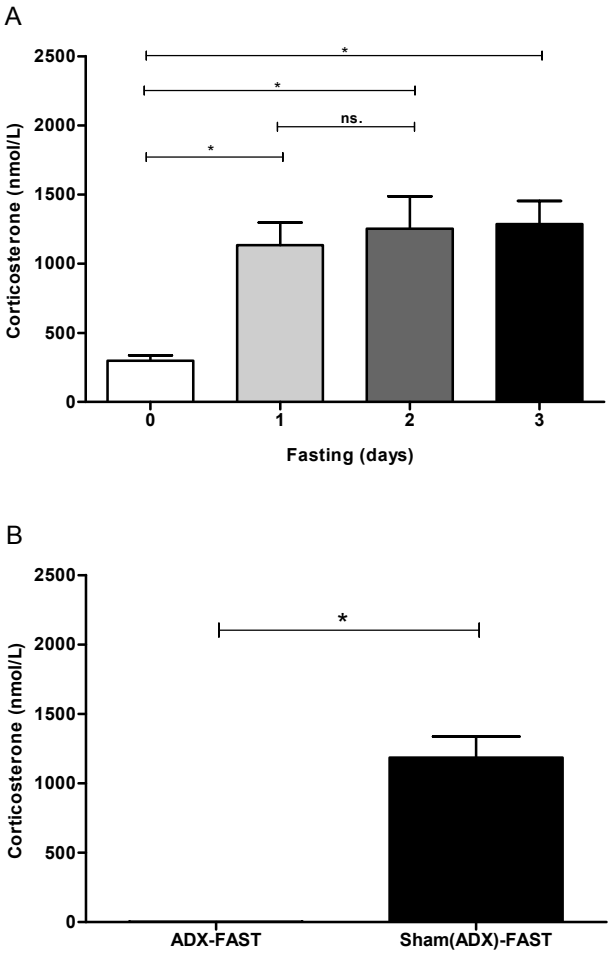

Figure 2: (A) Animals were fasted for 0, 1, 2, or 3 days after which the serum corticosterone levels were determined. Data are presented as mean±SEM. An asterisk (*) designates a statistically significant difference between the indicated groups (p = 0.0022 for all comparisons). Ns. = not statistically different. (B) ADX-FAST animals underwent an adrenalectomy 10 days prior to fasting and subsequent I/R. Sham (ADX)-FAST animals served as a control group. Animals in this group underwent a sham adrenalectomy 10 days prior to fasting. Data are presented as mean±SEM. An asterisk (*) designates a statistically significant difference between the indicated groups (p = 0.0022).

respectively (Figure 2A). ADX in combination with 3 days of fasting led to significantly reduced corticosterone values of 2.6±0.3 nmol/L, when compared to the sham(ADX)-operated group, who had corticosterone levels of 1186±150 nmol/L (p=0.0022) after a 3-day fasting period (Figure 2B).

ADX abolishes the protective effect of fasting on renal I/R injury

Mice recovered rapidly from the ADX as reflected by their return to preoperative weight on postoperative day 2. When ADX mice were subjected to a 3-day fast followed by renal I/R injury only 8% of the animals survived (Figure 3A). In contrast, survival of sham(ADX)-operated mice after fasting and subsequent I/R was 100% ($p < 0.0001$). To determine whether the high mortality rate was due to I/R injury or the absence of adrenal glands, the survival of ADX mice subjected to a sham I/R procedure after 3 days of fasting was assessed (Figure 3B). The 7-day survival in this group was 30%, similar to the ADX mice that had undergone renal I/R ($p = 0.501$), indicating that mice are not able to withstand abdominal surgery after bilateral adrenalectomy.

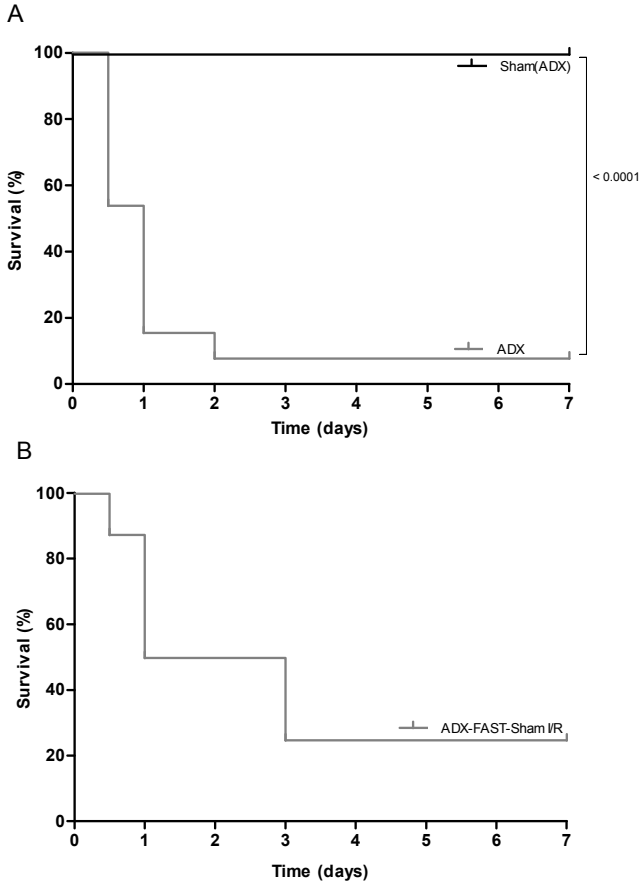

Figure 3: (A) Survival of adrenalectomized (ADX) mice vs. sham(ADX) mice after a 3-day fast and subsequent renal I/R injury. Survival in the sham-operated group is 100% vs. 8% in the adrenalectomized group ($p < 0.0001$). (B) Survival of adrenalectomized mice after a 3-day fast and subsequent sham I/R injury. Survival in the sham-operated group is 30%. This is not statistically different from the survival of the ADX group in figure 3A ($p = 0.5010$).

Glucocorticoid receptor blockade does not affect the benefits of fasting on renal I/R injury

To asses the effect of glucocorticoid receptor blockade on renal I/R injury after and during a three day fast several experiments were performed. In the first experiment mice received either 10 mg/kg mifepristone or vehicle after 3 days of fasting and 30 minutes prior to renal I/R injury. In both groups survival was 100%. Subsequently, we increased the frequency of mifepristone administration to once daily during the 3-day fast preceding I/R injury. All animals survived the experiment. When a tenfold higher mifepristone dosage (100 mg/kg) was given, again all animals in the control and mifepristone groups survived I/R after the 3-day fast. Following the high dose of

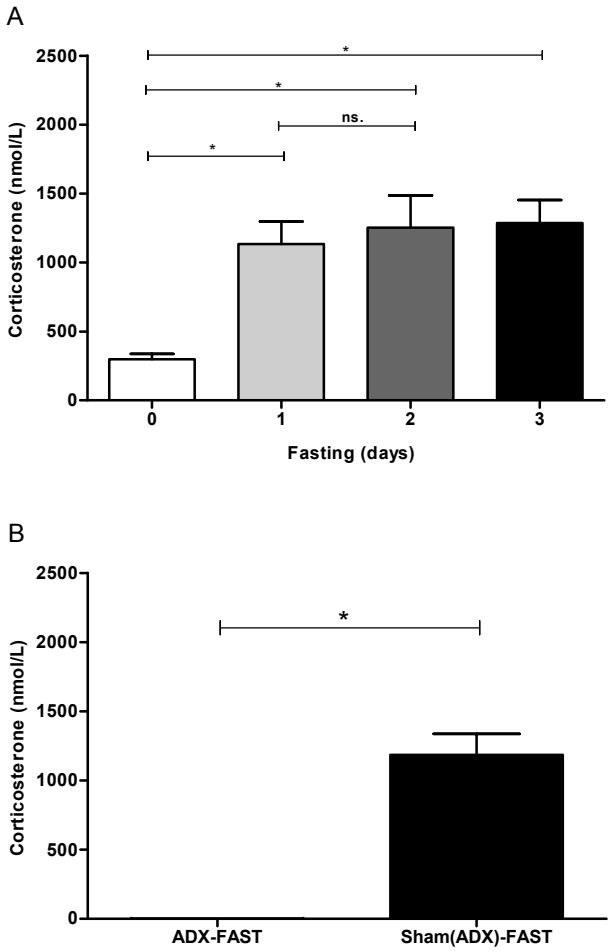

Figure 4: Kidney function after renal I/R injury as indicated by serum urea values. Mifepristone treatment was given once daily (100mg/kg) during the 3-day fast preceding I/R. The control group received a vehicle. There were no statistically significant differences between both groups.

mifepristone, kidney function assessed by serum urea concentrations before and 24 and 48 hours after I/R, showed no differences between the two groups (Figure 4). To rule out that mifepristone or the vehicle interfered with the renal I/R injury model, the three day treatment as described above was applied to ad libitum fed control mice (no ADX, only I/R injury). The survival of mifepristone and vehicle-treated mice (Figure 5A)

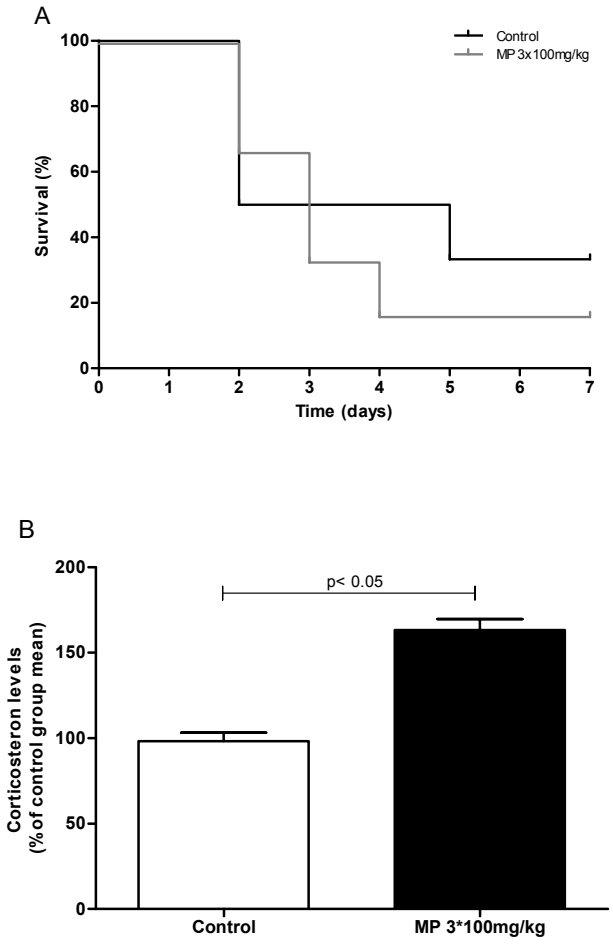

Figure 5: (A) Survival of mifepristone-treated and vehicle-treated control animals after renal I/R injury. Mifepristone (100mg/kg) was administered once daily, starting 3 days prior to I/R. Vehicle (PBS containing 1.16% DMSO) was administered to the control group. There was no significant difference in survival. Survival was similar to that observed in control mice without treatment[4] (data not shown).
(B) Corticosterone levels of mifepristone-treated and control animals after a three day fast. Mifepristone (MP) (100mg/kg) was administered once daily during the three day fast (n = 4). Vehicle (PBS containing 1.16% DMSO) was administered to the control group, during the three day fast (n = 4). After the three day fast corticosterone levels were measured and expressed as a percentage of the control group. Corticosterone levels were significantly (p=0.0268) higher in the mifepristone treated group, when compared to the control group. This indicates blockade of the glucocorticoid receptor.

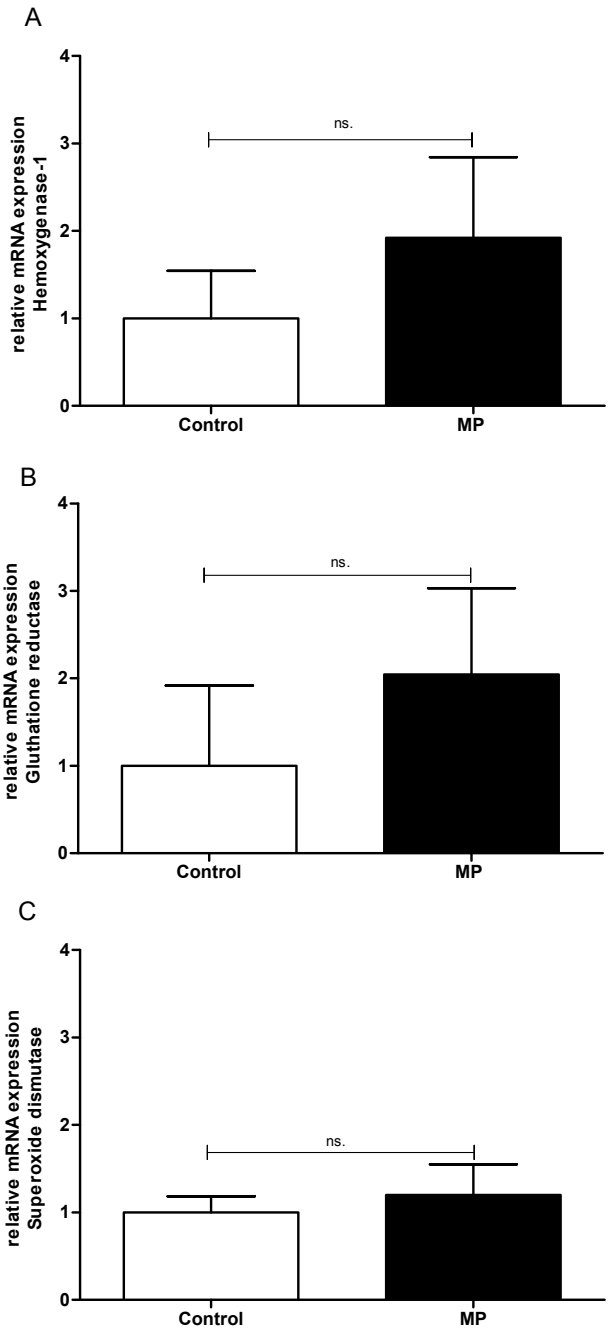

Figure 6: Hepatic mRNA expression levels of hemoxygenase-1(A), glutathione reductase (B), and superoxide dismutase-2 (C). Mifepristone treatment was given once daily (100mg/kg) during a 3-day fast after which the livers were harvested (n=6). The control group (n=6) received a vehicle. There were no statistically significant differences in mRNA expression between both groups.

was similar to the survival of untreated (no mifepristone or vehicle), ad libitum fed mice[4]. To confirm effective glucocorticoid receptor blockade by mifepristone serum levels of corticosterone were measured. Corticosterone levels were significantly increased ($p<0.05$) in mifepristone-treated animals, confirming adequate blockade of the glucocorticoid receptors during fasting and subsequent I/R (Figure 5B).

The effect of mifepristone on fasting-induced upregulation of cytoprotective genes.

We have previously shown that 3 days of fasting led to significantly higher baseline expression levels of antioxidant defense genes in the liver[4]. Here, we determined mRNA expression levels of hepatic tissue after 3 days of fasting with or without mifepristone treatment (three days, 100mg/kg/day). No significant differences were observed in mRNA expression levels of hemoxygenase-1, glutathione reductase, and superoxide dismutase, suggesting that corticosterone receptor inhibitor treatment did not interfere with the induction of cytoprotective and antioxidant genes by fasting (Figure 6).

DISCUSSION

Renal ischemia and reperfusion injury (I/R) negatively influences the outcome of kidney transplantation. Strategies to reduce I/R injury are important to improve patient survival as well as graft function and survival, as I/R is one of the main factors contributing to graft loss[3]. We have recently reported that fasting is able to protect both kidney and liver against I/R injury[4]. Current experiments were designed to investigate whether the protection afforded by fasting against I/R injury is mediated by increased levels of corticosterone. Fasting led to significantly higher levels of corticosterone when compared to ad libitum feeding[9]. Bilateral ADX was performed to investigate the effect of corticosterone on renal I/R injury. After ADX, mice exhibited higher mortality rates after I/R compared with control mice. However, survival after laparotomy in ADX mice without I/R injury resulted in similar mortality rates. These experiments did not address our hypothesis that the protection afforded by fasting is due to increased corticosterone levels.

Mifepristone, a glucocorticoid receptor antagonist, blocks the downstream signaling of the glucocorticoid receptor. The use of mifepristone therefore enables controlled studies on the effects of corticosterone on renal I/R injury without bilateral ADX. Glucocorticoid receptor blockade 30 minutes prior to I/R injury did not abolish the protective effects of fasting on renal I/R injury. This suggests that either glucocorticoid receptor blockade does not interfere with the protective effects of fasting or that fasting induces its protection during the three days fast. The latter is supported by elevated levels of cor-

ticosterone already after one day of fasting. Therefore, mifepristone was administered daily during the 3-day fast. However, this regime did not affect the protective effect of fasting on renal I/R injury. Finally, a higher dosage of mifepristone was used based on previous studies[13]. Again, this regime did not abolish the protection afforded by fasting on renal I/R injury. Survival rates and kidney function were similar in both the treatment and the control group. We therefore conclude that fasting-induced protection against renal I/R injury is mediated by corticosterone/glucocorticoid receptor-independent pathways. This is partially in line with earlier reports indicating that mice subjected to social stress[13] or high physiological titers of endogenous glucocorticoids[14] exhibited exacerbated ischemic injury. In contrast, a study in rats concluded that bilateral ADX prevents renal I/R injury[15]. However, this protection is probably induced by the depletion of mineralocorticoid hormones only, as these rats were supplemented with dexamethason, a potent exogenous glucocorticoid agonist. Administration of exogenous glucocorticoids is known to protect against cerebral[16], cardiac[17,18], and renal I/R injury[19]. In addition, clinical studies have shown that donor pre-treatment with steroids significantly decreased tissue (liver) and serum expression of proinflammatory cytokines[20] after I/R injury. A recent prospective randomized study investigated the effects of donor pretreatment with methylprednisolone on organ function and outcome after liver transplantation. The use of steroids significantly reduced I/R injury and inflammation and improved graft function[21]. We did not administer exogenous glucocorticoids in our model because they are already known to improve I/R injury and because our hypothesis predicted the involvement of endogenous steroids.

If increased levels of endogenous corticosteroids do not mediate the protective effects of fasting, the question remains which mechanisms do contribute to the induced protection. In previous experiments we have shown that three days of fasting lead to significantly higher expression levels of cytoprotective and antioxidant defense genes in the kidney and liver[4,22]. The strongest response to fasting was observed in the liver; therefore we investigated the effect of mifepristone treatment on the expression of these cytoprotective and anti-oxidant genes in the liver. The present study demonstrated that mifepristone treatment did not interfere with the upregulation of antioxidant defense systems. It would be interesting to investigate whether exact mimicking of corticosterone induction by fasting, by corticosteroid administration, would be able to increase the expression of cytoprotective genes as well. However, as it is difficult, if not impossible, to duplicate the physiological response to fasting, we have not performed these additional experiments. Together, these data support a hypothesis that the up-regulated expression of these genes was instrumental in the protection afforded by fasting against I/R injury, but that these changes are independent of corticosterone. Future experiments are warranted to investigate the relation between these fasting induced changes in gene expression patterns and I/R injury.

In conclusion, our data demonstrate that fasting increases serum corticosterone levels. However, the protective effect of fasting on I/R injury is induced independently of corticosterone levels and glucocorticoid receptor availability. The upregulation of antioxidant genes is independent of the availability of the glucocorticoid receptor. The latter may represent an important clue to elucidate the mechanisms by which fasting affords protection against I/R injury.

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Chapter 5

Preoperative fasting induced protection against renal ischemia-reperfusion injury is independent of ghrelin in mice

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ABSTRACT

Introduction: One of the factors negatively influencing the outcome after kidney transplantation is ischemia-reperfusion (I/R) injury. Preoperative fasting is able to confer protection against I/R injury. We hypothesized that the protection imposed by preoperative fasting is mediated by increased levels of acylated ghrelin.

Methods: Male C57BL/6 mice, 10-12 weeks old, were fasted for one, two or three days after which acylated ghrelin levels were determined. Ad libitum fed mice were injected with acylated ghrelin or PBS prior to renal I/R injury. Furthermore, mice were fasted for three days during which they were injected with a growth hormone secretagogue receptor antagonist, to block the effects of ghrelin, or a vehiculum. Bilateral renal I/R injury was induced by clamping the artery and vein of the left and right kidney simultaneously for 37 minutes. Kidney function was assessed by means of serum urea values determined at 24 and 48 hours after reperfusion.

Results: Fasting significantly increased acylated ghrelin serum levels. Ghrelin suppletion in ad libitum fed animals or ghrelin receptor blockade in fasted animals did not affect renal function after I/R injury.

Conclusion: Our data suggest that the increased levels of acylated ghrelin induced by fasting do not mediate its protection against renal I/R injury.

INTRODUCTION

Renal transplantation is considered the treatment of choice for people with end-stage renal disease. One of the factors negatively influencing the outcome after kidney transplantation is ischemia-reperfusion (I/R) injury[1,2]. Delayed graft function is primarily a consequence of I/R injury and contributes to the loss of kidney graft[3]. Currently there are no therapies to prevent or treat ischemic injury.

Emerging data suggest that the beneficial effects of long-term dietary restriction (defined as a reduction in energy intake without malnutrition) can be tapped for clinically relevant benefits such as protection against hepatic (I/R) injury[4] and the toxic side effects of chemotherapy[5]. We have previously shown that dietary restriction protects against renal I/R injury[6]. Three days of fasting prior to renal I/R injury protected against I/R injury in mice. Preoperative fasting increased baseline levels of cytoprotective and antioxidant genes and resulted in a more expeditious and pronounced response of these genes to I/R injury[6,7]. The mechanism by which dietary restriction induces this protection remains elusive. The mechanism by which dietary restriction induces this protection remains elusive. Although animal studies suggest that dietary restriction protects against I/R injury, translation of these results to the clinical setting poses a challenge. Patients may not respond to dietary restriction similarly as healthy animals or humans. Therefore, future research should focus on dietary restriction mimetics or agents that may impinge on (some) of the protective pathways induced by dietary restriction. To pharmaceutically mimic the effects of dietary restriction, its mechanisms need to be elucidated. In this study we sought to determine the role of ghrelin in the protection induced by fasting against renal I/R injury in mice. Fasting leads to several hormonal changes, among which is an increase in serum levels of ghrelin[8]. The complete structure of ghrelin has been identified as an [*O*-*n*-octanoyl-Ser 3]-peptide. The *n*-octanoyl moiety is essential for the activity of ghrelin[9]. Acylated ghrelin, the endogenous ligand for the growth hormone secretagogue receptor (GHSR) has recently been implicated in the control of food intake and energy balance[8]. Interestingly, survival rate after gut I/R injury increased significantly if ghrelin was administered just prior to reperfusion. In addition, ghrelin treatment reduced serum levels of TNF-α and IL-6, and reduced neutrophil infiltration in distant organs[10]. This suggests that acylated ghrelin is capable of reducing the inflammatory response to I/R injury. In addition, ghrelin has been shown to reduce ischemia related problems after skin flap transfer[11] and to protect against renal I/R injury[12]. We hypothesized that the protection against I/R injury imposed by fasting is mediated by increased systemic levels of acylated ghrelin. We quantified serum acylated ghrelin levels after one, two and three days of fasting. Furthermore, ad libitum fed mice were injected with acylated ghrelin prior to renal I/R injury and fasting mice were treated with the competitive GHSR antagonist [D-lys-3]-GHRP-6[13] prior to renal I/R injury.

METHODS AND MATERIALS

Animals

Male C57BL/6 mice with an average weight of 25 grams were purchased from Harlan (Horst, The Netherlands). All mice were maintained under standard conditions with a 12 h light/dark cycle. Mice in the fed group were allowed unrestricted access to food. Mice in the fasting groups were transferred to a clean cage at 5:00 pm and withheld food for 1, 2, or 3 days. The experimental protocol was approved by the Animal Experiments Committee under the Dutch National Experiments on Animals Act and complied with the 1986 directive 86/609/EC of the Council of Europe.

Serum measurements

Blood was obtained under anesthesia (isoflurane inhalation,5% isoflurane initially and then 2% isoflurane with a 1:1 air:oxygen mixture for maintenance of anaesthesia) by retro-orbital venous plexus puncture (during the experiments) or heart puncture (at the end of the experiment). Urea levels were determined in the serum of the animals using a QuantiChrom assay kit, DIUR-500 (Gentaur, Brussels, Belgium). Acylated ghrelin levels were determined in plasma using an acylated ghrelin ELISA kit (BioVendor, Modrice, Czech Republic) according to the manufacturer's protocol. Acylated ghrelin levels were determined from plasma samples obtained between 9:00 and 10:00. After cardiac puncture, 400 µL of blood was transferred directly into 1 ml EDTA containing tubes (MiniCollect, Greiner Bio-one) pre-filled with p-hydroxymercuribenzoic acid, leading to a final concentration of 1 mM. Samples were directly centrifuged (3.500 rpm; 10 min; 4°C) after which 100 µL of the supernatant was transferred to a separate tube containing 10 µL 1NHCl. After centrifugation (3,500 rpm; 5 min; 4°C) the supernatant was transferred to another vial and stored at -20°C until assayed.

Bilateral renal I/R injury

Renal I/R procedures were conducted between 9.00 and 12.00 hours. Mice were anesthetized with isoflurane inhalation (5% isoflurane initially and then 2% isoflurane with a 1:1 air:oxygen mixture for maintenance of anaesthesia). Body temperature was maintained by placing the animals on heating pads until recovery from anesthesia. Following a midline abdominal incision, the renal artery and vein of both the left and right kidney were occluded simultaneously, by using atraumatic microvascular clamps, for 37 minutes. Previously we showed that this ischemic time induces a mortality rate of 40%[6]. After macroscopic confirmation of ischemia (purple color), the incision was covered with PBS-soaked gauze and the animal was covered with an aluminum foil blanket to maintain body temperature. Following release of the vascular clamp, restoration of blood-flow was confirmed by the kidney returning to normal color. The

abdominal wound was closed in two layers using 5/0 Safil sutures. Postoperatively mice received a single subcutaneous injection of 0.5 ml of PBS.

Influence of fasting on acylated ghrelin levels

Animals were fed ad libitum (n = 10) or fasted for one (n = 6), two (n = 6), or three days (n = 14), after which they were killed by exsanguination under anesthesia. Acylated ghrelin levels were measured as described above.

Influence of ghrelin administration on kidney function after renal I/R injury

Acylated ghrelin (Polypeptide Group, Strasbourg, France) (100 µg/kg, n = 6) or vehicle (phosphate buffered saline, 100uL, n = 6) was injected subcutaneously two times a day with 12 hour intervals during the 3-days before renal I/R. Renal function was determined by measuring serum urea levels at 24 and 48 hours after I/R. The acylated ghrelin dosage was based on previous studies showing protection against renal I/R injury[12].

Influence of GHSR blockage on kidney function after renal I/R injury

GHSR antagonist (200nmol/100µL PBS, [D-Lys-3]-GHRP-6, Bachem, Weil am Rhein, Germany, n = 6) or vehicle (phosphate buffered saline, 100µL, n = 6) was administered intraperitoneally twice daily with a 12 hour interval during the 3-day fast before renal I/R. Renal function was determined by measuring serum urea levels 24 and 48 hours after I/R. The GHSR antagonist dosage is based on previous studies showing that antagonism of the ghrelin receptor reduces food intake[13].

Statistical analysis

Continuous variables are presented as means ± SEM (normal distribution, assessed visually and by means of Shapiro-Wilks test). One-way ANOVA was used to asses whether fasting significantly altered acylated ghrelin levels. Two-way ANOVA was used to asses if time or treatment significantly influenced renal function after I/R injury. Thereafter, means between the intervention groups and the control group were compared using the t-test for parametric data. P-values of <0.05 were considered significant. All analyses were performed using Statistical Package for the Social Sciences 15.0 (SPSS, Chicago, IL).

RESULTS

Influence of fasting on acylated ghrelin levels

To investigate if fasting influences plasma levels of acylated ghrelin, we measured these levels after fasting. Baseline acylated ghrelin levels were 95.51±10.80 pg/ml. One, two, and three days of fasting significantly increased acylated ghrelin levels compared with

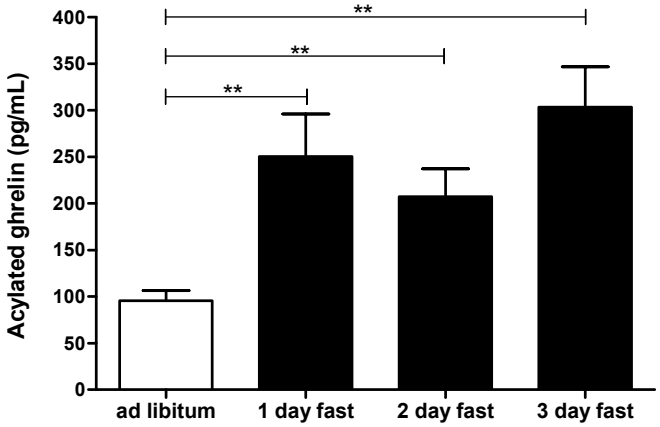

Figure 1: Effect of fasting on plasma ghrelin levels. Mice were fasted for one, two, or three days. Control mice were allowed unrestricted access to food. One, two, and three days of fasting was associated with significantly increased acelyted ghrelin levels as compared with ad libitum fed mice. The bars and error bars represent mean ± SEM. One-way ANOVA showed a significant difference between the means ($p = 0.0016$) A T-test was used to compare the fasted groups with the control group. (**, $p < 0.01$ compared to ad libitum fed control mice. Ad libitum $n = 10$, one day fasting $n = 6$, two days fasting $n = 6$, three days fasting $n = 14$.)

ad libitum fed mice (250.6±45.57 pg/ml ($p = 0.0013$), 207.4±29.78 pg/ml ($p = 0.0075$), and 303.3±43.30 pg/ml ($p = 0.0014$), respectively) (Figure 1).

Influence of ghrelin administration to ad libitum fed mice on renal function after I/R injury

We next determined whether administering acylated ghrelin to ad libitum fed mice would protect against renal I/R injury. There was no significant difference in serum urea levels between groups at 24 hours after reperfusion (Ghrelin vs. PBS; 40.0±1.1 mmol/ml vs 40.2 ±1.0 mmol/ml, p = ns). In both groups, serum urea values were increased 48 hours after reperfusion, when compared to 24 hours after reperfusion. Again no statistically significant differences were observed between both groups (Ghrelin vs. PBS; 157.5 ± 2.4 mmol/ml vs 151.8 ± 2.4 mmol/ml, p = ns) (Figure 2).

Influence of GHSR antagonist administration to fasting mice on renal function after I/R injury

As shown previously, three days of fasting protects against renal ischemia and reperfusion injury. Serum urea values at 48 hours after reperfusion were significantly lower in fasted mice vs. fed mice (57.4±20.0 mmol/ml vs. 157.5±2.4 mmol/ml, p = 0.008).

Next, we determined whether administration of a GHSR antagonist to fasting mice would abolish the protective effect of fasting on renal I/R injury. There was no significant difference between groups regarding renal function 24 hours after reperfusion (serum

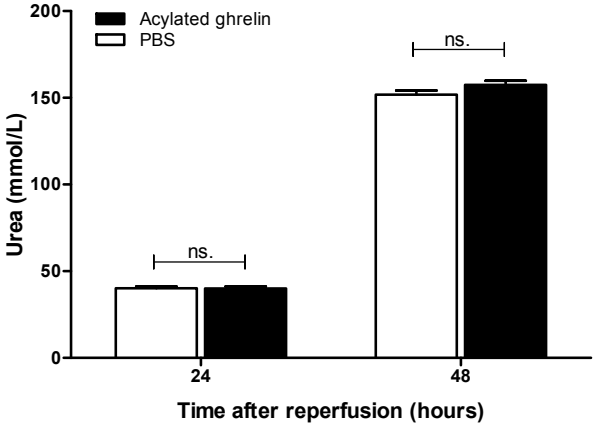

Figure 2: Effect of acylated ghrelin treatment on renal I/R injury. Acylated ghrelin was administered subcutaneously twice daily during a 3-days period before renal I/R. Control mice were treated with PBS at the same time-points. Serum urea levels were determined at 24 and 48 hours after reperfusion. Acylated ghrelin administration did not affect serum urea levels when compared with PBS-treated administration. The bars and error bars represent mean ± SEM. Statistical comparisons were made using a two-way ANOVA. (ns., not significantly different, *n* = 6 mice/ group.)

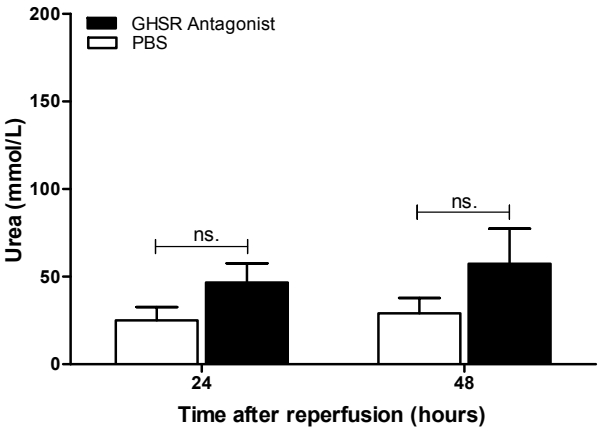

Figure 3: Effect of GHSR antagonist on renal I/R injury. GHSR antagonist was administered intraperitoneally twice daily during 3-days before renal I/R. Control mice were treated with PBS at the same time-points. Serum urea levels were determined at 24 and 48 hours after reperfusion. GHSR antagonist treatment did not affect serum urea levels compared with PBS-treated control mice. The bars and error bars represent mean ± SEM. Statistical comparisons were made using a two-way ANOVA. (ns., not significantly different, *n* = 6 mice/ group.)

urea levels in GHSR antagonist treated vs. PBS treated controls; 46.6±11.1 mmol/ml vs 25.1±7.5 mmol/ml, p = ns). Serum urea levels at 48 hours after reperfusion were comparable to those at 24 hours after reperfusion. Again no statistically significant differences were observed between both groups (GHSR antagonist vs. PBS; 57.4±20.0 mmol/ml vs 29.1±8.7 mmol/ml, p = ns) at 48 hours after reperfusion (Figure 3).

DISCUSSION

Renal ischemia and reperfusion injury (I/R) negatively influences the outcome of kidney transplantation. Strategies to reduce I/R injury are important to improve patient survival as well as graft outcome, as I/R is one of the main factors contributing to graft loss[3]. Recently we observed that preoperative fasting induces protection against both renal and hepatic I/R injury[6]. In the present study we hypothesized that the protection afforded by fasting is mediated by increased levels of acylated ghrelin.

We found that fasting leads to significantly increased serum levels of acylated ghrelin, which is in accordance with the literature[14]. These levels were increased from day one, and remained elevated during the three day fast. To mimic increased acylated ghrelin levels during fasting, we administered acylated ghrelin twice daily during three preoperative days in ad libitum fed mice. However, no effect on renal function after I/R injury was detected. It has been shown previously by Takeda et al., that treatment with acylated ghrelin improves renal function after I/R injury[12]. We administered acylated ghrelin before the induction of renal I/R, whereas Takeda et al. gave ghrelin both before and after I/R, which may explain the observed difference in efficacy.

Acylated ghrelin is the endogenous ligand for the growthhormone secretagogue receptor (GHSR)[8]. A GHSR antagonist [D-lys-3]-GHRP-6[13] was used to block the receptor mediated effects of elevated ghrelin levels during fasting. Despite using a dosing protocol shown to be effective in suppressing the biological activity of ghrelin, no difference in postoperative renal function was observed between treated and control animals. Furthermore, the protective effect of fasting on kidney function after I/R injury was not affected by GHSR antagonist administration.

Limitations of our study are that, we do not know whether our acylated ghrelin suppletion schedule exactly mimics the effect of fasting on acylated ghrelin levels. Although the dosage we used is based on existing literature, we did not determine whether the GHSR antagonist blocked the ghrelin receptor adequately. Furthermore, we show that there is no effect on renal function by means of serum urea values, but this does not exclude the possibility that other parameters of renal damage, such as histology, indicate differences between the groups. However, in previous studies[6] we found that there is a strong correlation between urea values, histological damage, and mortality due to ischemia induced acute kidney injury. Although we cannot exclude a protective effect of ghrelin administration on renal I/R injury, our data suggest that the increased levels of acylated ghrelin induced by fasting do not mediate protection against renal I/R injury. We therefore reject the hypothesis that the protection against renal I/R injury afforded by fasting is induced by increased levels of acylated ghrelin.

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Chapter 6

Preoperative dietary restriction reduces hepatic tumorload by reduced E-selectin mediated adhesion in mice

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ABSTRACT

Background: Inflammatory responses facilitate metastasis by increasing expression of adhesion molecules. Dietary restriction (30% reduction in daily calorie intake) reduces the expression of adhesion molecules and protects against surgically induced inflammation. DR might therefore beneficially interfere with surgery induced inflammation and subsequent adhesion of circulating tumorcells.

Methods: BALB/c mice were subjected to two weeks dietary restriction prior to inoculation with tumor cells. Intrasplenic injection of $5.0x10^4$ c26-colon carcinoma cells was followed by splenectomy. Hepatic tumor load was scored after ten days as a percentage (tumor surface/total liver surface) on H&E stained sections. Liver mRNA expression of adhesion molecules was determined and the effect of serum from dietary restriction mice on in vitro tumor growth and adhesion capacity was assessed.

Results: Preoperative dietary restriction significantly reduced mRNA expression levels of E-selectin ($p=0.0087$) and hepatic tumor load($p=0.036$). Dietary restriction serum did not affect in vitro cell growth, but reduced in vitro adhesion of c26 cells to endothelial cells ($p=0.0043$).

Conclusions: Preoperative dietary restriction reduces hepatic tumor load after injection with tumorcells. Reduced adhesion to endothelial cells and reduced mRNA expression of E-selectin suggest that dietary restriction reduces tumor load by lowering the adhesion of circulating tumor cells to hepatic vascular endothelium.

INTRODUCTION

Colorectal cancer is the third most common cancer worldwide, with a cumulative lifetime risk of approximately 5% in the United States. Each year approximately 150.000 patients present with colorectal cancer and over 55.000 deaths are attributed to this disease[1]. In addition, in Europe almost 200.000 new cases of colorectal cancer occur every year[2] and this incidence is estimated to increase with 45% in the next two decades[3]. Surgical resection of the primary tumor remains the treatment of choice. Unfortunately, 30% to 50% of all patients undergoing curative resections subsequently develop either a local recurrence or distant metastases, predominantly in the liver, resulting in increased mortality[4]. Most recurrences are observed within two years after an operation. It is hypothesized that viable circulating tumor cells (CTC) play an important role in the pathogenesis of distant metastases. CTC were first detected in colorectal cancer patients more than 50 years ago[5] and are mainly detected in portal blood[6]. Recently, a meta-analysis showed a significantly increased hepatic metastases rate of 21% in CTC positive patients compared with 8% in negative patients which emphasizes the influence of CTC on hepatic metastasis formation[7]. Surgery increases the number of CTC due to tumor handling. In addition it enhances the metastatic potential of pre-existing or intra-operatively spilled CTC due to several factors. First, surgery inevitably leads to tissue trauma which evokes an inflammatory reaction with elevated levels of local and systemic proinflammatory cytokines. These cytokines subsequently result in the up-regulation of adhesion molecules, such as E-selectin, on liver endothelial cells, which may promote metastases outgrowth by facilitating tumor cell adhesion. Secondly, the induction of a pronounced immunosuppressive period after major surgery may impair the innate effector cell function of Kupffer cells and natural killer cells. These cells have an important role in eradication of tumor cells retained in the liver vasculature. Impairment of their activity may result in an increased risk of hepatic metastases development[8-10].

Several interventions have demonstrated their beneficial effects on perioperative tumor metastasis i.e. the "no touch technique"[11,12], the use of immunosuppressive drugs to blunt the proinflammatory cytokine response[13], and blockade of alpha2 integrins on tumor cells to reduce adhesion on endothelial cells[8]. The perioperative period may provide a window of opportunity in which the adhesion and outgrowth of circulating tumor cells in the liver can be reduced, leading to less metastatic lesions and possibly lower patient morbidity and mortality rates.

We investigated the effect of preoperative dietary restriction (DR) on perioperative adhesion and outgrowth of CTC. DR, reduced food intake without causing malnutrition, is associated with extended longevity[14] and reduced cancer incidence[15-19]. Short-term preoperative DR for one week reduces angiogenesis and growth in an mouse

brain tumor model[20]. Recently, we reported that short-term DR prior to both renal and hepatic ischemia and reperfusion injury reduces the expression of pro-inflammatory cytokines and adhesion molecules[21]. Here, we used a murine model to determine the effect of short-term pre-operative DR on tumor cell adhesion and hepatic tumor load after inoculation with tumor cells. We demonstrate that pre-operative DR reduces hepatic tumor load. Furthermore, we demonstrate that a two week DR regimen reduces the hepatic expression of the endothelial cell specific adhesion molecule, E-selectin. In vitro, serum from DR mice was able to reduce the adhesion of tumor cells to endothelial cells. Our results indicate that DR might be a valuable addition to the multimodality treatment of patients with colorectal malignancies.

MATERIALS AND METHODS

Animals

Male BALB/c mice (+/- 25 gram) were purchased from Charles River (The Netherlands, Maastricht). Mice were housed separately under standard laboratory conditions and allowed to acclimatize for one week. The experimental protocol was approved by the Animal Experiments Committee under the Dutch National Experiments on Animals Act and complied with the 1986 directive 86/609/EC of the Council of Europe.

Dietary restriction

After one week of acclimatization, food intake was measured daily during one week. Thereafter, mice were randomized to either the control or the experimental group. Control mice were fed standard rodent chow (SDS, Hope Farms, Woerden, The Netherlands) ad libitum (= AL group). Experimental mice received only 70% of the daily caloric intake by means of standard rodent chow leading dietary restriction (= DR group). Mice were subjected to two weeks of DR prior to the surgical intervention. Postoperatively, all mice were allowed ad libitum access to food.

Cell culture

The murine colon carcinoma cell line C26 (kindly provided by Dr. R. Schiffelers, Utrecht University, The Netherlands) was cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma Aldrich, St. Louis, MO, USA) supplemented with 10 percent fetal calf serum, penicillin (100 units/ml) and streptomycin (100 units/ml) in a five percent carbon dioxide environment. Near confluent cultures were harvested by brief trypsinization (0.05 trypsin in 0.02 per cent ethylenediamine tetra-acetic acid (EDTA)). For the surgical procedure, cells were harvested and after centrifugation, single-cell suspensions were prepared in phosphate buffered saline (PBS) to a final concentration

of 5.0×10^4 cells/100 µL or 10.0×10^4 cells/100 µL. Cell viability was determined by trypan blue staining, and was always at least 98 percent.

Induction of circulating tumor cells

For induction of hepatic tumor growth mice were anaesthetized with isoflurane inhalation. Surgical procedures were performed under aseptic conditions. Body temperature was maintained by placing the mice on heating pads. Following a left lateral flank incision, the spleen was localized and C26 colorectal carcinoma cells were injected into the splenic parenchyma (total volume 100µL, n = 6 per group). This experiment was divided into two sub-experiments. In sub-experiment 1: 5.0×10^4 cells were injected, in sub-experiment 2: 10.0×10^4 cells were injected intra-splenically After 10 minutes, the spleen was removed to prevent intrasplenic tumor growth. Single tumor cells reach the liver through the portal vein, where a subset grows out to form intrahepatic micrometastases. Metastases were allowed to develop for 10 days. Morphological assessment of tumor growth was performed on right lower liver lobes harvested ten days after tumor induction.

Determination of hepatic tumor load

Intrahepatic tumor load was scored as the percentage of hepatic tissue that has been replaced by tumor cells (hepatic tumor percentage, HTP). Digital images were captured from two non-sequential hematoxylin-eosin–stained sections of the right lower liver lobe using virtual microscopy (Hamamatsu NanoZoomer). Using specific software (NanoZoomer Digital Pathology, NDP) the HTP ratio was determined by two independent observers blinded to treatment (Figure 1). We obtained less than 5% intra- and interobserver variability. The mean HTP per slide was used to compare the AL-group and the CR-group.

Serum collection for in vitro assays

Blood was obtained from mice after two weeks DR or AL access to food by means of cardiac puncture under general anesthesia. Serum was stored at -80ºC until further analysis, without pooling of the serum.

In vitro growth curves

C26 colon carcinoma cells were cultured in DMEM containing 10% foetal calfs serum and 200 U/mL penicillin/streptomycin. Cells were suspended in serum free DMEM containing in a concentration of 5.25×10^4 cells per mL. Subsequently, 10% mouse serum obtained from individual AL or DR mice was added to reach a final concentration of 5.0×10^4 cells. These cells were plated in triplicate on 5 different 96-wells plates. Plates were analyzed 6, 24, 48, 72 and 96 hours after seeding. Cell proliferation

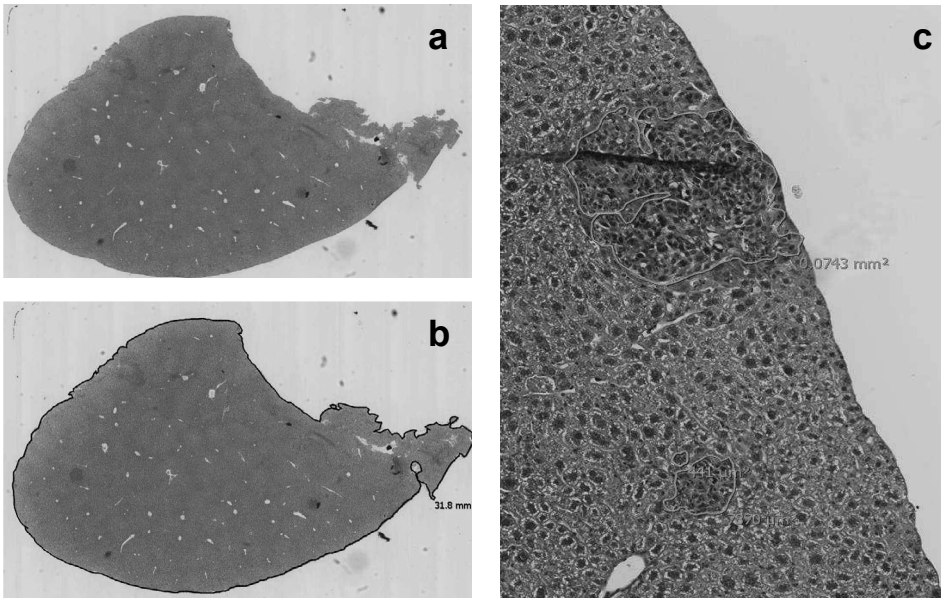

Figure 1: Histological analysis of tumor load in liver tissue. Hepatic tumor growth was induced in BALB/C mice by intrasplenic injection of C26 tumorcells. Histopathologic analyses were performed on hematoxylin and eosin-stained liver sections. Digital images of stained sections were captured using a virtual microscopy system. Intrahepatic tumor load was determined as the percentage of hepatic tissue that has been replaced by tumor cells. (A) Microscopic appearance of a liver section (magnification 2x). (B) Manual indication of the total liver section surface using the computerized system. Surface indicated in black. (C) Microscopic appearance of C26 tumor areas present in liver tissue using the computerized system, tumor indicated (magnification 20x).

was determined by a colorimetric assay using tetrazolium salt (XTT, Sigma- Aldrich). XTT was dissolved in DMEM until a final concentration of 1.0 mg/ml was obtained. N-methyl dibenzopyrazine methyl sulfate (PMS, Sigma-Aldrich) was added to the XTT solution to achieve a final concentration of 7.6 µg/ml. Subsequently, 50 µL of the XTT-solution was added to the experimental wells followed by one hour incubation at 37ºC. Absorbance of the samples was measured spectrophotometrically (ELISA plate reader, Victor, Perkin Elmer) and cell content was expressed as the optical density at wavelength of 490 nanometer.

In vitro adhesion assay

Human umbilical vascular endothelial cells (HUVEC) (kindly provided by dr. A. Seynhave, Erasmus University Rotterdam, The Netherlands) at passage 2 were maintained in EGM-2-MV Bullet kit medium (Sigma Aldrich). Confluent monolayers were passaged by 0.025% trypsin/0.01% EDTA and cells were used up to passage six. C26 colon carcinoma cells were cultured as described earlier. To quantify C26 tumor cell adhesion to HUVEC, a standardized cell adhesion assay was used as described previously[22].

Briefly, endothelial monolayers were established in 96-well microtiter plates (Perkin Elmer, Groningen, The Netherlands). Confluent HUVEC were trypsinized and 2×10^4 endothelial cells were plated in each well followed by incubation at 37°C, 95% relative humidity, 5% CO_2. Medium was daily replaced by fresh medium until HUVEC reached confluence in 3 to 4 days, confirmed by light microscopy.

To quantify tumor cell adhesion, trypsinized tumor cells (1×10^6 cells/ml) were labeled with calcein-AM (Molecular Probes, The Netherlands, Leiden) and 3×10^4 c26 cells were added to the HUVEC monolayer in the presence of 10% mouse serum, obtained from individual AL or CR mice. Assays were performed in triplo. Thereafter plates were centrifuged for 1 minute at 80 × g in a Heraeus centrifuge and incubated at 37°C for 1 hour. After this, wells were washed twice with medium to remove non-adherent tumor cells. The remaining fluorescence per well was measured on a Perkin Elmer plate reader using 485 nm excitation and 530 nm emission filters.

Real time quantitative PCR

RNA was isolated from liver tissue obtained after two weeks of 30% DR or ad libitum access to food. For gene expression analysis, total RNA was extracted from frozen liver tissue using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To prevent contamination by genomic DNA the isolated RNA was purified by a DNase treatment (RQ1 Rnase-Free Dnase; Promega, Madison, WI, USA). Two microgram of total RNA was reverse transcribed to cDNA using random hexamer primers (Invitrogen), and Superscript II RT (Invitrogen) according to manufacturers instructions.

E-selectin mRNA expression level was determined by real-time quantitative PCR (RT-PCR) using an AppliedBiosystems 7700 PCR machine (Foster City, CA, USA) and quantified by normalization against ABL as previously[23]. Each sample was tested in duplicate. All values were normalized to the mean relative expression calculated for the AL group, which was assigned a value of 1.

Statistical analysis

Categorical data are presented as number (percentage) and continuous variables as mean ± SEM (normal distribution, assessed visually and by means of Shapiro-Wilks test) or median ± interquartile distance (no normal distribution). Means between two groups were compared using either the non-parametric Mann-Whitney U test or the t-test for parametric data. Mixed models are used to analyse repetitive measurements. P-values of <0.05 were considered significant. All analyses were performed using Statistical Package for the Social Sciences 15.0 (SPSS, Chicago, IL).

RESULTS

Dietary restriction

In both groups mean daily food intake was 4.0 gram (95% CI (3.9-4.1)). Dietary restriction was performed by reducing the intake to 70% of the ad libitum intake, which is 2.8 gram per day/mouse. During dietary restriction, the intake of the control group

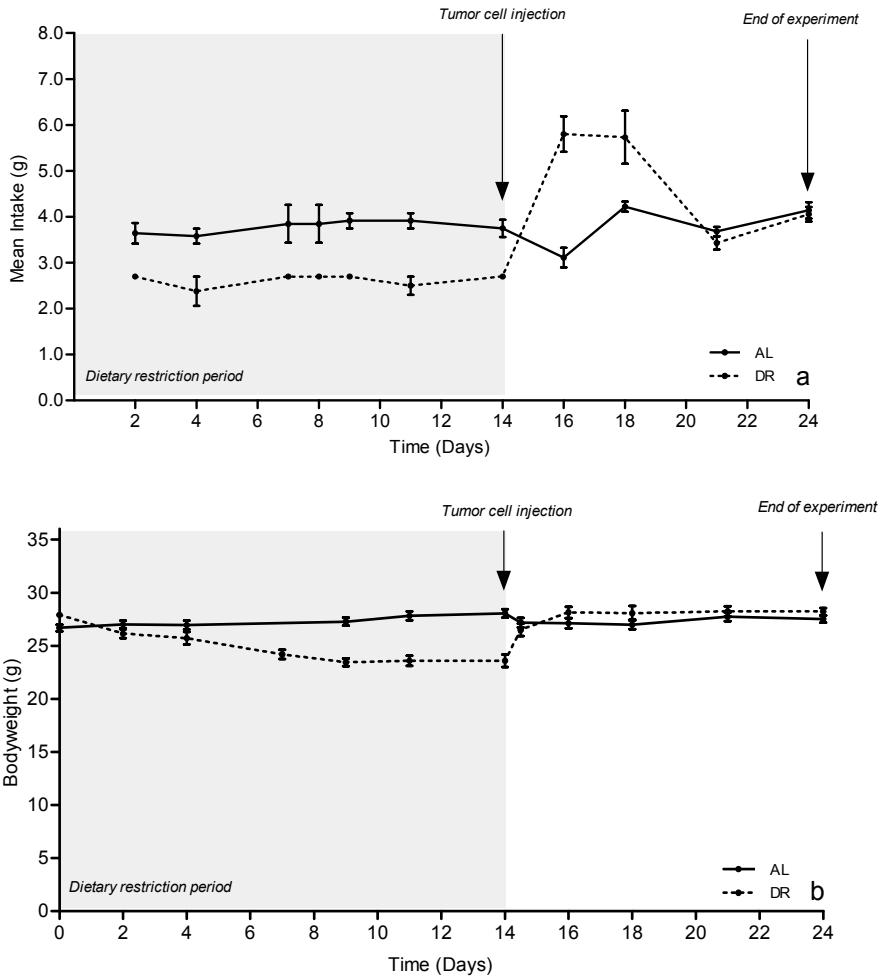

Figure 2: Food intake and bodyweight during the experimental period. Mice were fed *ad libitum* or dietary restricted to 70% of the daily caloric intake during 14 days prior to surgery. After intrasplenic injection all mice were allowed to eat *ad libitum*. (A) Daily food intake was monitored from the onset of diet till the mice were sacrificed. In mice randomized to dietary restriction a 70% caloric intake was achieved during the dietary period. After surgery caloric intake showed a rapid increase in mice subjected to dietary restriction (B) Mean body weight was monitored during the experiment. All mice regained their pre-dietary weight within two days after surgery.

remained constant. Weight loss, an objective measurement of decreased caloric intake, was 15.6 ± 3.3% in the intervention group during DR, while the control group gained 5.0 ± 1.3% bodyweight during the same period (Figure 2A). After intra-splenic tumor injection, all mice were allowed to eat ad libitum, resulting in "catch up" intake of the DR group (Figure 2B).

Hepatic tumor load

We examined whether preoperative dietary restriction affected hepatic tumor load after inoculation with CTC. Therefore 5.0 x 10^4 tumor cells were injected intrasplenically. In

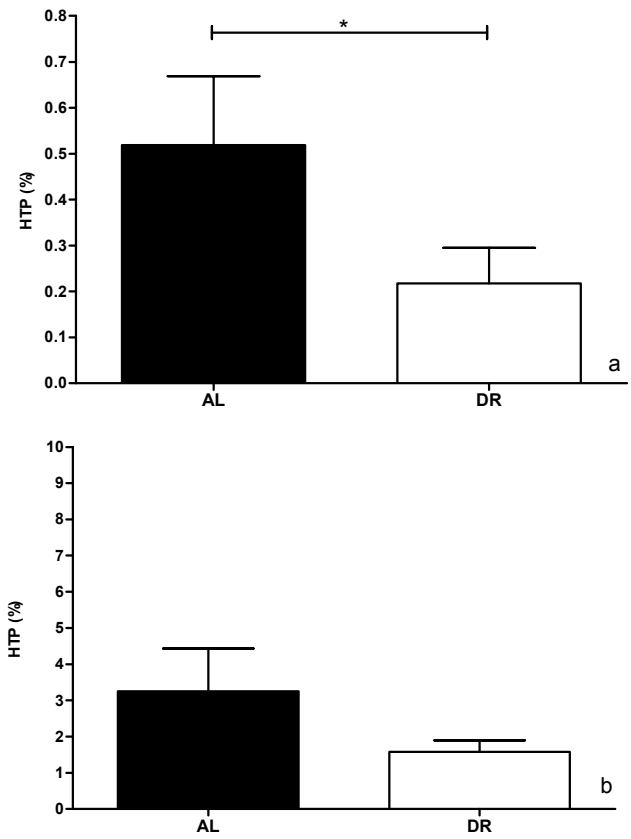

Figure 3: Effect of caloric restriction on hepatic tumorgrowth. Hepatic tumorgrowth was induced in BALB/c mice by intrasplenic injection of tumorcells followed by splenectomy. Mice were fed ad libitum or dietary restricted to 70% of the normal daily caloric intake during 14 days prior to surgery. Intrahepatic tumor load was determined in liver sections obtained ten days after surgery. (A) Caloric restriction was associated with reduced intrahepatic tumor load (HTP) after intrasplenic injection of 5.0 x 10^4 tumor cells. (B) Caloric restriction did not reduce intrahepatic tumor load (HTP) after intrasplenic injection of 1.0 x 10^5 tumor cells. (n = 2 sections per mice, 5 mice / group, *, p=0.036). Data are presented as median ± interquartile distance.

mice who underwent DR prior to tumor inoculation hepatic tumor load was significantly reduced to 0.11% as compared to 0.62% in the control group (p=0.036) (Figure 3A). Next we performed the experiments by injection of a larger tumor volume (1.0 x 10^5) to increase the number of CTC. In both control and DR mice higher tumor loads were found (Figure 3B). Although hepatic tumor load in DR mice was consistently lower this did not reach statistically significance (p=0.41). Collectively these data suggest that preoperative DR is able to reduce hepatic tumor load after inoculation with CTC.

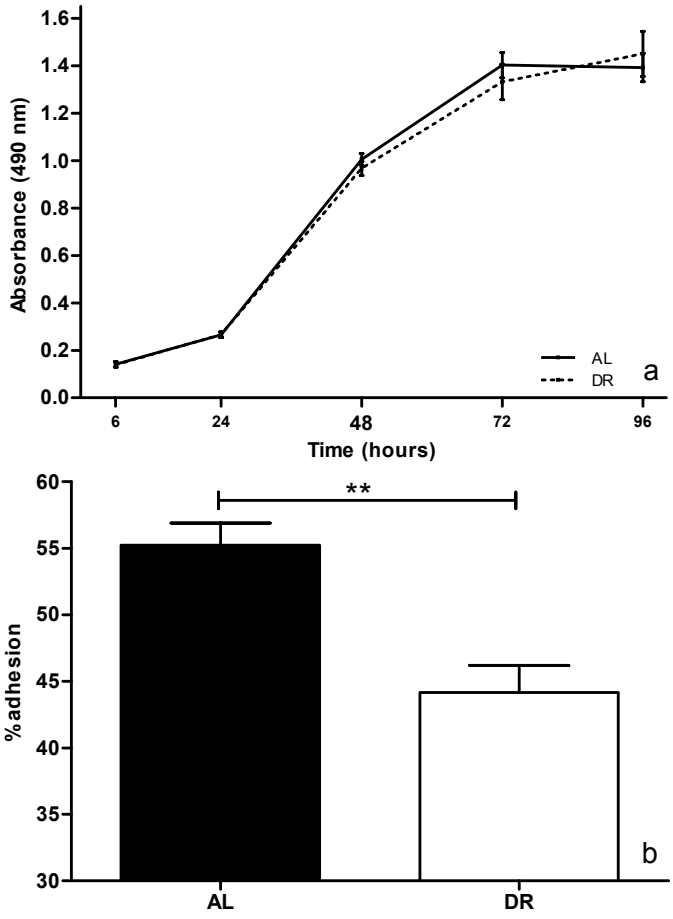

Figure 4: Effect of caloric restriction on *in vitro* tumor cell growth and adhesion. (A).C26 colon carcinoma cells were cultured in medium combined with serum obtained from ad libitum or dietary restriction mice. Dietary restriction did not affect *in vitro* growth of C26 colon carcinoma cells. (B) An *in vitro* adhesion assay we used to determine the effect of serum obtained from dietary restriction mice on adhesion of C26 tumor cells to a HUVEC monolayer. Dietary restriction was associated with reduced adhesion of C26 tumor cells to HUVECs. (**, p=0.0043).

In vitro experiments

To determine the effect of dietary restriction on the in vitro growth rate of C26 colon-carcinoma cells, we evaluated the effect of serum obtained from ad libitum or DR mice on in vitro growth curves of C26 coloncarcinoma cells (Figure 4A). Serum obtained from both groups showed no significant differences on the in vitro growth rates of C26 cells. Next, we evaluated the effect of DR on adhesion of C26 coloncarcinoma cells to endothelial cells in vitro. Therefore we determined the effect of serum from either DR or AL mice on the capacity of tumor cells to adhere to a HUVEC monolayer. C26 cells in the presence of DR serum displayed a significantly (p=0.0043) reduced capacity to adhere to HUVEC as compared to C26 cells in the presence of AL serum (Figure 4B).

Real time Quantitative PCR

The endothelial cell specific adhesion molecule E-selectin has an important role in the process of tumor cell adhesion to endothelial cells. Therefore, we examined the effect of DR on hepatic E-selectin mRNA expression levels. DR resulted in a significant (p=0.0087) reduction of E-selectin mRNA expression (Figure 5).

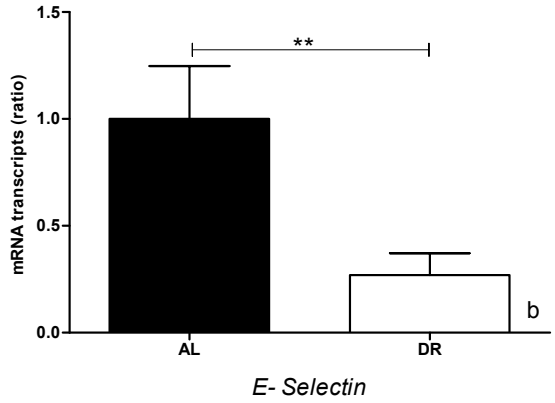

Figure 5: Effect of caloric restriction on hepatic E-selectin mRNA expression. Mice were fed ad libitum or dietary restricted to 70% of the normal daily caloric intake during 14 days prior to surgery. Hepatic tissue was obtained after 14 days of dietary restriction or ad libitum diet. Relative hepatic E-selectin expression level was determined. Dietary restriction was associated with reduced E-selectin expression level. (**, p=0.0087)

DISCUSSION

For most patients with colorectal malignancies surgical resection is the cornerstone of any potentially curative treatment. Surgical trauma results in systemic inflammation as reflected by cytokine release[24,25] and in postoperative cellular immunosuppression[26].

There is emerging evidence suggesting that these surgery induced processes facilitate tumor metastases[27]. Furthermore, surgical procedures induce hematogenic tumor cell dissemination as reflected by increased circulating tumor cells (CTC's) present during surgical procedures[9]. The importance of CTC is underlined by an increased hepatic metastasis rate in CTC positive patients, when compared to CTC negative patients[7]. There are two major schools of thought regarding tumor cell metastasis and extravasation. One the one hand it is believed that CTC arrest in narrow capillaries due to size restriction, on the other hand adhesion of CTC to the microvascular endothelium is considered one of the most important steps[28]. Preventing adhesion of CTC's to the endothelium of distant organs during the perioperative period may be a potential effective treatment to reduce metastasis rates after curative surgery. In the current study we show that preoperative dietary restriction lowers the expression of E-selectin in the liver reduces hepatic tumor load after exposure to CTC.

Selectins mediate tethering, rolling and adhesion of several types of cells. E-selectin, an endothelial cell specific adhesion molecule, is expressed de novo on endothelial cells, such as liver endothelial cells, after transcriptional induction by pro-inflammatory cytokines[29]. These activated endothelial cells express E-selectin, which mediates tumor cell adhesion and subsequent liver metastasis[29]. Here, we demonstrate that dietary restriction [DR) reduces mRNA expression of E-selectin and hepatic tumor load. It is known that tumor cells trigger the induction of E-selectin expression on endothelial cells[30]. We show that serum obtained from DR mice reduces in vitro adhesion of C26 coloncarcinoma cells to HUVEC. Although we do not show a direct correlation, it has already been demonstrated that E-selectin expression plays an crucial role in the process of liver metastasis formation in the murine BALB/c-C26 coloncarcinoma cell model as direct blockage of E-selectin was associated with lower numbers of liver metastasis[31].

The protective effect of DR on HTP was statistically significant after inoculation with 5.0 x10^4cells; while only a trend was observed after injection of 1.0x10^5 cells. These data suggest that if an overwhelming amount of tumor cells has been injected the positive effect of DR on CTC adhesion is blunted. However, this situation is unlikely to be encountered in the clinical situation as the amount of CTC is much lower (1-10 CTC per 7.5 mL blood)[32] than the supra-physiological amounts of CTC used in our model. The model does not fully represent the clinical situation, as a primary tumor is absent and the level of (pre-) and post-operative inflammation may be different. But in this model, where higher concentrations of CTC than ever encountered in the clinical situation are induced, a beneficial effect of DR is observed.

Although a reduced HTP was observed after DR, we cannot rule out that the difference in postoperative calorie intake contributes to this reduction. However, we assume this unlikely as the adhesion assay and hepatic E-selectin mRNA expression level were performed with serum samples obtained directly after DR, thus unaffected by postop-

erative calorie intake. Translation of preoperative DR to the clinical setting also poses a challenge. Although in literature periods much longer of than two weeks 30% DR have been reported[33]. We must take into account that several patients suffering from colorectal disease may be malnourished. Interestingly, a diet consisting of protein restriction without a reduction in calories has been shown to increase maximum longevity in rats and mice as well[34]. Although the magnitude of these increases is around 30–40% of that of DR, neither carbohydrate[35] nor lipid restriction[36,37] exerted these effects. Restriction of proteins could therefore be another way to induce the beneficial effects seen after DR and overcome the problem of reducing calorie intake. In addition, the use of DR mimetics may be a way to overcome the problems associated with DR in surgical patients. A DR mimetic can be loosely defined as any pharmacological intervention that produces beneficial effects of DR without causing or requiring a significant reduction in calorie intake. In clinical practice a DR mimetic might be a powerful addition to standard cancer treatment. One compound that has received considerable attention as DR mimetic is resveratrol, a naturally-occurring polyphenol found in red wine. Resveratrol induces, at doses that can be readily achieved in humans, genomic changes which resemble many of the genetic alterations induced by DR[38]. Furthermore, evidence supporting the use of resveratrol in the treatment of malignancies is emerging[39-41].

The question remains why DR lowers E-selectin expression. We recently reported that DR robustly down regulates the production of proinflammatory cytokines and adhesion molecules in models of renal and hepatic ischemia-reperfusion injury. In addition DR induced the expression of cytoprotective and anti-oxidant genes, leading to a reduced formation of reactive oxygen species[42]. As surgical trauma causes oxidative stress[43,44], the increased protection against oxidative stress and the subsequently reduced inflammatory response, may explain why lower levels of E-selectin are encountered. Microarray analyses are currently being performed, aiming to elucidate how DR induces this protective response. In addition, future experiments need to identify the optimal regimen of DR, in terms of percentage of DR and duration. These should focus on combining the beneficial effects of preoperative fasting, which protects against the side effects of chemotherapy[45,46], with those of DR found in the present study, to a regimen that induces the protection against both.

In conclusion our data demonstrate that preoperative DR is able to reduce hepatic tumor load ten days after inoculation with CTC. This beneficial effect appears to be mediated by reduced vascular E-selectin expression and a subsequent decreased tumor cell-endothelial cell adhesion, as lower E-selectin levels were related to less hepatic metastasis. This may be a mechanism by which DR inhibits hepatic metastasis. Therefore, DR may provide a new strategy in the multimodality treatment of patients with colorectal cancer.

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Part three

Clinical studies

Chapter 7

Preoperative dietary restriction is feasible in live kidney donors

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ABSTRACT

Introduction: Dietary restriction (DR), defined as reduced energy intake without malnutrition, confers protection against renal ischemia and reperfusion injury in animal models. This pilot study investigates for the first time the feasibility of preoperative DR in the clinical setting.

Methods: Live kidney donors were randomized between preoperative DR or ad libitum intake. Seventeen participants were instucted to follow a 30% calorie restricted diet, followed by one day of water-only fasting prior to surgery. Thirteen participants were allowed to eat ad libitum preoperatively.

Results: 94% of the donors adhered to the diet, 31.4% reduction in caloric intake was achieved. Postoperative well-being, appetite and ability to perform daily tasks were not different between both groups. There was no difference in post-transplant graft function of kidneys obtained from DR donors or control donors as determined by serum creatinin levels during the first postoperative month and renograms at post-operative day one.

Conclusions: This study shows that mild dietary restriction is feasible in the setting of live kidney donation. No effect was observed regarding postoperative graft function. Additional studies are warranted to investigate the appropriate regimen of dietary restriction to protecting against ischemia and reperfusion injury, such as increasing the magnitude and/or duration of the reduction in daily caloric intake.

INTRODUCTION

Life-long daily dietary restriction (DR), defined as a reduction in energy intake without malnutrition, is associated with extended longevity[1] in multiple organisms including yeast, worms, flies, mice[2-6] and non-human primates[7]. Long-term DR is able to attenuate damage resulting from oxidative stress by decreasing mitochondrial electron and proton leak and increasing antioxidant defence systems[8-11]. We have recently shown that beneficial effects of long-term DR are induced rapidly and can be tapped for clinically relevant benefits such as protection against ischemia-reperfusion (I/R) injury. After one day of water-only fasting protection against renal I/R injury was achieved. The maximum protection against renal I/R injury in the mouse was induced by both three days of preoperative water-only fasting and two weeks of preoperative reduced (30%) caloric intake. In analogy to long-term DR, we found that short-term DR increased baseline levels of cytoprotective and antioxidant genes[12]. Unbiased transcriptional profiling of kidneys from mice subject to short-term DR revealed a significant enrichment of the signature genes of long-term DR.

Furthermore, DR has been shown to facilitate the functional recovery of ischemically damaged neurons in the brain[13] and to result in a highly significant decrease in infarct volume when compared with ad libitum fed controls[14]. In the heart, DR attenuated the postischemic inflammatory response[15] and similar benefits were found in the retina following ischemia[16].

We hypothesize that dietary restriction of the donor confers protection to the kidney against I/R injury prior to the ischemic insult. Preoperative DR may therefore be a non-invasive way to reduce I/R injury following organ transplantation[17]. Unfortunately, preoperative fasting is currently considered an unwanted necessity as it reduces patient well-being and induces peripheral insulin resistance[18-19]. Furthermore, the concept of reducing I/R injury by slightly longer periods of preoperative fasting or preoperative dietary restriction is novel and so far no clinical studies have been conducted. It is known that humans in general have difficulties to adhere to prescribed diets and it is unknown if people are able to adhere to a diet in preparation for surgery, which causes distress itself. We therefore designed the current pilot study to investigate whether a relatively mild preoperative DR regimen is feasible in the clinical setting and carefully explored the effects of DR on both the donor and recipient.

METHODS

Study population

The study population consisted of people participating in a live kidney donation program. Donors aged between 18 and 80 years old were eligible for inclusion, provided that they had a body mass index above 18.5 kg/m^2. All participants were approached during their first outpatient visit (five to six months prior to surgery). Approval was obtained from the local medical ethics committee and informed consent was obtained. The dietician instructed participants to keep a precise weighed food record. Single items were recorded in household measurements. Records were kept on two weekdays and one weekend day in one week, approximately four months prior to surgery. These data were, if necessary, completed by a diet recall performed by the dietician. Hereafter, participants were randomized into either the DR group or the control group. This DR regimen is translated from our previous experimental results into a feasible regimen for the clinical setting. It is not directly based on existing experimental data. The DR group aimed to reduce their calorie intake with 30% (relative to the baseline measurements) on day four, three and two before the operation. On the day before surgery, these patients were allowed breakfast followed by 24 hours water-only fasting. The control group was allowed to eat ad libitum and kept a food record form during the four preoperative days. Participants were instructed to record any changes from the preoperative prescribed diet.

Surgical procedure

All donors were prehydrated with intravenous crystalloids from midnight the day before surgery. After endotracheal intubation anaesthesia was carried out according to a protocol for drugs, ventilation, and fluid regimens. At the end of surgery donors received patient controlled analgesia using intravenous morphine.

Data collection

Data were collected prospectively. The following data were obtained from the kidney donor: age, sex, body mass index (BMI, weight (kg)/height^2 (m^2)), cholesterol and fasting blood glucose. Short-term recovery after surgery was measured using the EuroQol[20] questionnaire and visual analogue scores concerning appetite and general well-being. Blood was drawn at 20.00 pm the night before surgery (day -1), six hours after surgery (day 0) and every morning until discharge. Serum levels of C-reactive protein (CRP), leucocytes, insulin, cortisol, urea and creatinine were analysed by the central hospital laboratory using standard methods. Insulin levels were measured at 08:00 pm on the day before surgery to provide an indirect, objective assessment of compliance with the diet. Furthermore, serum albumin levels were determined five to six months and the

day before surgery to estimate the effect of DR on the nutritional status. Complications were recorded for the first 30 days after the operation and defined as events necessitating intra-operative or post-operative interventions or prolonged hospital stay.

The following data were collected from the kidney recipient: age, sex, number of previous transplantations, kidney replacement therapy, graft survival, urea levels and creatinine levels in the first month after surgery. Routinely, all renal transplants were evaluated in the first days postoperatively with 99m-technetium mercaptoacetyl triglycyne renography. All renographs were analysed by two independent observers, blinded to whether the kidney was obtained from a DR or control patient. Peak activity (time until maximum acitivity was observed), end activity (amount of activity 20 minutes after injection of the tracer as a percentage of peak activity), total activity within the first two minutes and a overal grade were noted ranging from zero (no perfusion) to five (excellent transplant function).

Statistical analysis

Categorical data are presented as numbers (percentage) and continuous variables as mean (sd./normal distibution) or median (interquartile distance/no normal distribution). Data were tested for normality using the Shapiro-Wilks test and visual assessment. Continuous data were compared using either the non-parametric Mann-Whitney test or the T-test for parametric data. Dichotomous data were analysed using the chi-square test. Related samples were analysed using the Wilcoxon signed rank test. Repeated measurements were analysed using mixed model analysis. P-values less than 0.05 were considered significant. All the analyses were performed using Statistical Packages for Social Sciences 15.0 (SPSS Inc., Chicago, USA). This is a pilot study and therefore no powercalculations were made.

RESULTS

Study population

Thirty-nine kidney donors were included and underwent randomisation. Figure 1 shows the flowchart of the study. Eventually, 17 patients were analysed in the dietary restriction group and 13 patients in the control group. Baseline characteristics of the study population and surgical procedures are presented in Table 1 and Table 2, and show that there were no significant differences between both groups.

Dietary intervention

Baseline food record forms were detailed enough to calculate the amount of calories consumed. The average baseline intake for the control group was 1863±591 kcal/day,

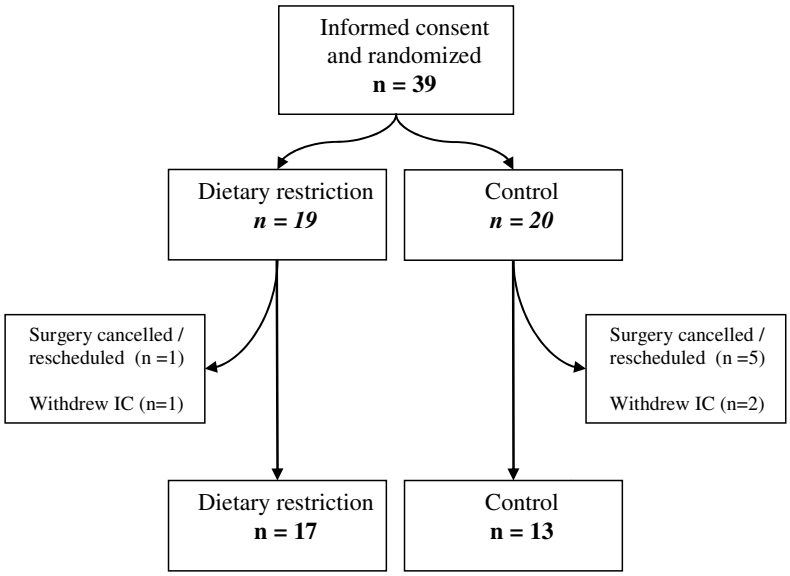

Figure 1: Flowchart of the study. IC = Informed Consent.

Table 1: Baseline characteristics of the study population. Data are presented as means ± standard deviation, unless stated otherwise.

	DR[1] (n = 17)	Control (n = 13)	P-value
Age (Years)	54±9	56±13	0.642
Male : Female ratio (%)	6 (35):11(64)	6 (46):7 (53)	0.711
Height (cm)	171±7.0	166±7.6	0.104
Weight (kg)	74±15	75±13	0.797
BMI (kg/m^2)	25.0±4.0	26.7±3.4	0.812
Weight (kg)			
Onset of study	74±15	75±11	0.812
One day before surgery	74±15	75±12	0.584
Blood glucose[2] (mmol/L)	4.7±0.5	4.6±0.6	0.569
Albumin (g/L)			
Onset of study	46.4±3.1	44.2±3.4	0.106
One day before surgery	46.9±2.0	45.7±2.4	0.199

[1] DR = dietary restricted group.

[2] Blood obtained after a midnight fast at the outpatient department, several months prior to surgery.

which did not differ significantly from their four-day pre-operative intake (1853±675 kcal/day). A 31.4% (29.5% - 33.0%) reduction in calorie intake was achieved in the intervention group. They consumed 1957±408 kcal/day during the baseline measurements and 1322±251 kcal/day preoperatively. Macronutrient composition of the diets (Table 3) did not differ between the two groups. One patient failed to comply with the diet, against 94% who adhered rigorously to it. To provide a more objective means of

Table 2: Baseline characteristics of the surgical procedures. Categorical data are given as number (percentage) and continuous variables as means ± standard deviation. Means between the two groups are compared using the non-parametric Mann Whitney Test.

	DR[1] (n = 17)	Control (n = 13)	P-value
Laparoscopic procedures	17 (100%)	13 (100%)	1.00
Conversion to open approach	Never	Never	1.00
Skin to skin time (min)	199 ± 57	207 ± 55	0.672
Time until warm ischemia	154 ± 53	156 ± 46	0.813
Warm ischemia (min)	6.0 ± 2.1	6.0 ± 1.8	0.942
Cold ischemia (min)	155 ± 47	158 ± 29	0.346
Second warm ischemia (min)	24 ± 7	25 ± 8	0.860
Total ischemia (min)	185 ± 52	189 ± 32	0.295
Blood loss (ml)	97 ± 91	162 ± 139	0.204

[1] DR = dietary restricted group.

Table 3: Macronutrient composition of baseline and preoperative diets. Data are presented as means ± standard deviation.

	Baseline diet[1]	Pre-operative diet[2]
Control (n = 13)		
Protein[3]		16.7 (3.1)
Carbohydrates[4]	48.3 (6.4)	46 (5.0)
Fat[5]	32.2 (5.2)	32.1 (5.5)
DR[#] (n = 17)		
Protein[3]	17.3 (2.9)	18.0 (3.4)
Carbohydrates[4]	49.8 (5.0)	50.3 (5.6)
Fat[5]	30.4 (7.0)	31.4 (6.1)

[1] Mean values of the three day food record form completed several months prior to the operation.[2] Based on food record form filled in prior to surgery or the report of adherence to the prescribed diet.[3] Energy percentage derived from proteins.[4] Energy percentage derived from carbohydrates.[5] Energy percentage derived from fat. [#] DR = dietary restricted group.

diet adherence, insulin levels (Figure 2A) were measured. These data show that the donors of the DR group had low insulin levels (25.5+/-3.6 pmol/L) on preoperative day one (08.00 PM) in contrast with the control group (203.8 +/- 69.0 pmol/L). Serum albumin levels were unaffected by the diet (Table 1).

Postoperative CRP and leucocyte responses of live kidney donors

Surgical tissue trauma leads to an inflammatory response characterized by increased levels of CRP and leukocytes[21-22]. We studied whether this response was affected by preoperative DR. Baseline values of CRP (DR: 2.17 ± 1.63 mg/L vs. control: 2.5 ± 2.2 mg/L, P=0.905) did not differ between both groups. CRP levels increased significantly after the operation and peaked on postoperative day two. No statistically significant

Figure 2: (A) Perioperative insulin levels. These data show that the donors of the DR group had low insulin levels (25.5+/- 3.6 pmol/L) on preoperative day one (08.00 PM) in contrast with the control group (203.8 +/- 69.0 pmol/L) indicative of a fasted state. There were no statistically significant differences postoperatively. Data are presented as means ± standard error of the mean. (B) Perioperative number of circulating leukocytes. The mixed model analysis showed no statistically significant difference (p=0.098) in the number of leucocytes over all the time points measured in de dietary restricted group when compared to the ad libitum group. However, on the first postoperative day, the number of leucocytes was significantly (p=0.02) lower in the dietary restricted group. Data are presented as means ± standard error of the mean.

differences in CRP levels were found between both groups at any of the examined time points. The number of leukocytes increased after surgery in both groups. On the first postoperative day, the number of leukocytes was significantly (P=0.02) lower, but well within the normal range, in the DR group when compared to the control group. However, since a mixed model analysis taking into account all time points did not show significant differences between both groups, this must be interpreted as a trend (Figure 2B).

Postoperative recovery of the donor

By means of visual anlogue scores (VAS), ranging from one to ten, postoperative appetite and general well-being were assessed. There were no significant differences in VAS concerning appetite and well-being between the two groups (Figure 3A and 3B). Preoperative serum levels of cortisol were measured to provide an indication of the stress caused by the diet. In the DR group mean cortisol levels were 206±121 nmol/L and in the control group 185±85 nmol/L, this was not statistically different. In addition, the ability to perform daily tasks was measured by means of the EuroQol questionnaire. Preoperative DR did not influence the postoperative ability to perform daily tasks (Figure 3C). Of the 30 live kidney donors included in the study, 23 (76%) patients had a paid job (13 of the DR group vs. 10 in the control group). There was no statistically significant difference in the number of days before the participants returned to work (DR: 53±28 days vs control: 47±28 days, P= 0.74).

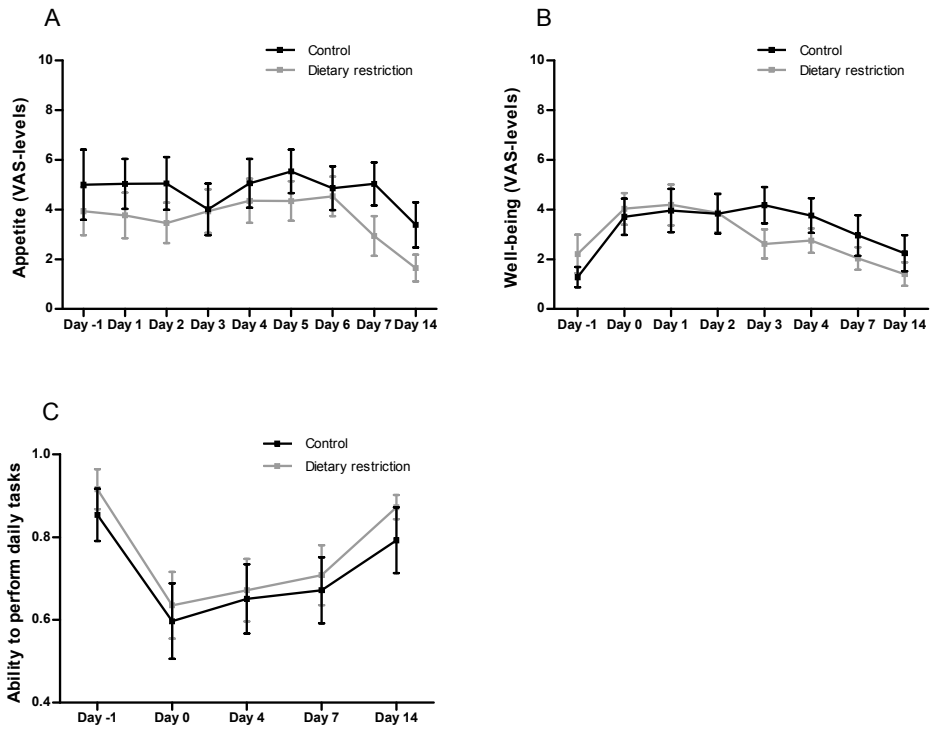

Figure 3: Postoperative visual analogue scores of appetite (A) and general well-being (B) and euroQol questionnaire scores (C). Higher scores indicate less appetite or decreased well-being. Dietary restriction did not influence postoperative appetite and/or well being scores when compared to the controle group. Data are presented as mean, error bars represent standard error of the mean. EuroQol scores indicate the ability to perform daily tasks (walking, getting dressed). Higher scores indicate a better ability to perform these tasks. Dietary restriction did not influence the postoperative ability to perform tasks. Data are presented as means ± standard error of the mean.

Complications

Two complications occurred in the DR group. One live kidney donor developed postoperative epididymitis, which was treated successfully with antibiotics. One donor developed acute tubular necrosis due to a hypotensive period (mean arterial pressure 50 mm mercury) following induction of anesthesia. This resolved spontaneously within days after the operation. In the control group one donor suffered from an iatrogenic colon perforation, which was corrected surgically.

Graft function and survival

Thirty patients received a kidney graft from one of the live kidney donors included in the study. There are no statistically significant differences between the kidney recipients of a “DR graft” or a “control graft” regarding age, sex, number of previous transplantations and kidney replacement therapy (data not shown). Only one graft was lost within

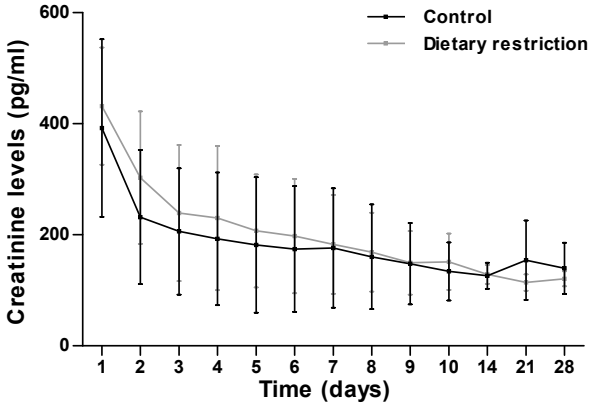

Figure 4: Recipient creatinine levels during the first month of follow up.
Serum creatinine levels are shown for the first month after transplantation. There is no significant difference between both groups (p=0.601) over all time points measured. Data are presented by means of the 95% confidence interval.

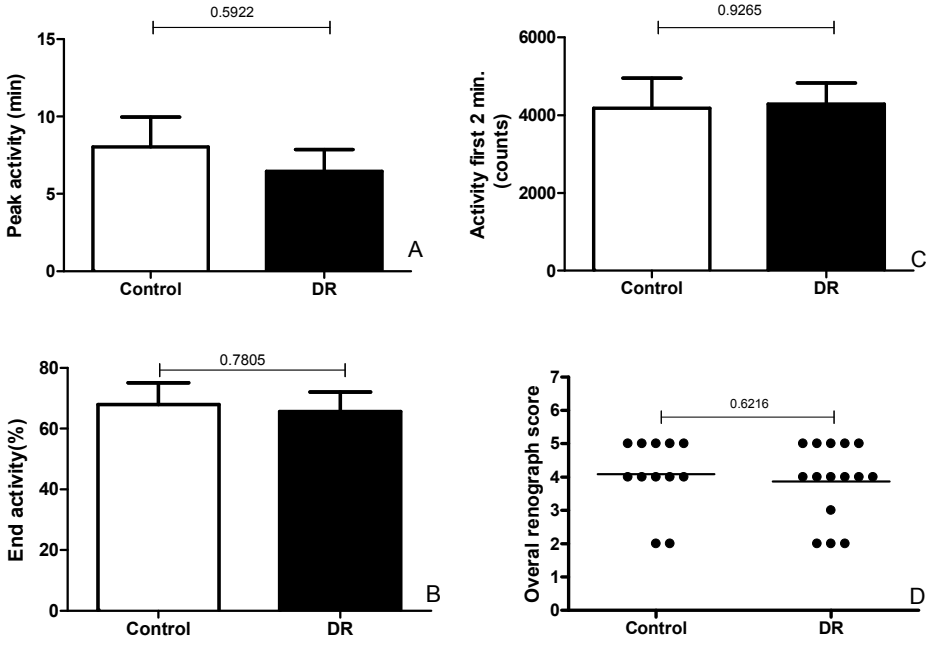

Figure 5: Renograph scores of the transplanted kidneys on postoperative day one.
As shown by means of the time needed to achive peak activity (A), the percentage of end activity (B) and the total amount of counts during the first two minutes (C), there were no significant differences between both groups. The overal score (D) was also not statistically significantly different between both groups. Scoring system renographs: Grade 0 = No function, No perfusion, Grade 1 = Very bad (Perfusion present, no extraction), Grade 2 = Bad (Perfusion present, no extraction, activity increased > 20 minutes after injection of isotope), Grade 3 = Reasonable (Perfusion, extraction and excretion; Peak curve within 10-20 minutes), Grade 4 = Good (Perfusion, extraction and excretion; Peak curve within 5-10minutes), Grade 5 = Excellent (Perfusion, extraction and excretion; Peak curve within <5 min).

the first year after transplantation due to acute rejection after ABO incompatible transplantation (DR group). There were no significant differences in one month creatinine levels of the kidney recipients (Figure 4). Immediate postoperative graft function was measured one day postoperatively using renographs, and showed no significant difference between kidneys obtained from DR or control donors (Figure 5).

DISCUSSION

Ischemia-reperfusion (I/R) injury negatively influences the outcome after kidney transplantation. Preoperative dietary restriction (DR) confers protection against both renal and hepatic ischemia-reperfusion injury (I/R) in mouse models[12,23]. Thus, DR is a potential cost free, non-invasive means to protect kidney grafts against I/R injury. This is the first study that investigates the feasibility of preoperative DR in the clinical setting in live kidney donors. The results show that modest preoperative DR is feasible in the clinical setting and has no measurable effect on postoperative well-being, ability to peform daily tasks and complication rates of live kidney donors.

The preoperative diet used in this study is translated, but not directly based on, the animal studies performed in our laboratory[12] and inspired by existing literature reporting that DR is able to induce protection against ischemia and reperfusion injury[23]. How to convert dietary regimens from experimental to clinical studies remains a point of discussion. More than 24 hours of fasting is in our opinion not applicable in the clinical setting. Patients will become hungry and therefore experience decreased well being in advance of surgery. Extending the number of days with dietary restriction provides more possibilities for errors (people are less likely to completely adhere to the diet), non-compliance, and increases the work load of the dietician. Therefore, we have chosen to combine 24 hours of fasting with 3 days of 30% calorie restriction. However, as our diet failed to demonstrate beneficial effects on postoperative graft function, longer and more extensive dietary regimens may be needed. In this case, additional effort of the dietician is needed and justified by the goal of the study.

Assessing food intake of individuals is usually based on self-report methods. Validity of these methods depends on the accuracy of the participants with recording their dietary intake. The gold standard in assessing validity of reported total energy intake is through doubly-labeled water studies[24]. However, because of the high costs of this method, it is not widely used. Therefore other methods have been designed to measure dietary intake, such as dietary records (a complete and accurate list of all foods consumed, preferably not influenced by the act of recording, in a time period varying from one day to several weeks), diet recall records (a complete recall of all foods and drinks ingested on specified days, may be obtained by a trained interviewer) and food-

frequency questionnaires (reflects food consumption over a designated period of time, mainly provides information about food patterns). We used a three-day dietary record form, accompanied by a dietary recall history if nessecary. Unfortunately, we were not able to determine the amount of underreporting or overreporting, which is a well-documented phenomenon[25]. To minimize underreporting and eating bias, participants were informed about these features and food record forms were collected prior to randomisation. An advantage of our study is the study population. The kidney donors were eager to participate and are very committed, which likely benefits the accurate reporting of intake and a high compliance (94%) with the diet. Compliance was measured subjectively and is diffficult to assess with objective measurements. However, the low insulin levels on the day before surgery at 08.00 pm in the DR group indicate a fasted state, in contrast with the control group where insulin levels were much higher. Collaboration between the surgical and dietetic departments, which was crucial to the success of the study, created no insurmountable logistical problems.

The concept of preoperative DR goes against this current surgical dogma of preoperative carbohydrate loading. Randomized studies, in which glucose was administered either as an infusion or as a carbohydrate-rich drink two to three hours before surgery, reported an increase in patient well-being before and after surgery[26-31]. However, our diet did not impair postoperative well-being. In addition, postoperative appettite and the ability to perform daily tasks were also unaffected by the diet. If anything, DR led to an increase in well-being scores after postoperative day two, although this did not reach statistical significance. Healthy people undergoing a laparoscopic donor nephrectomy have a fast and uneventful recovery, without major complications, which renders it difficult to speculate about complications caused or prevented by the diet. Hypotension after induction of general anaesthesia is a common event which can occur in up to 9% of the patients[32]. We have interpreted the decrease in bloodpressure, which was observed in one patient in the DR group, accordingly, and feel that this has contributed to postoperative acute tubular necrosis. However, we cannot rule out a relationship between DR and postoperative acute tubular necrosis.

The inflammatory response after surgery is partially reflected by increased levels of C-reactive protein and leukocytosis[21-22]. Preoperative dietary restriction did not affect perioperative CRP levels. A trend towards a lower number of leukocytes on postoperative day one was observed, however all values were well within the normal range.

To assess the effect of preoperative DR on renal transplant function, we measured graft function on the first postoperative day by means of renographs and during the first month by serum creatinin levels in the recipient. We acknowledge that this study is not sufficiently powered to draw firm conclusions about the relationship between preoperative DR and renal transplant function, however, we found no measurable effect of DR on renal graft function.

Although this is a pilot study assessing the feasibilty of preoperative DR, the data obtained thus far do not indicate protection against renal I/R injury by this relatively modest DR regimen. The ability of the diet to induce a protective response may have been insufficient. Increasing the length, severity or macronutrient composition of the DR regimen are potential avenues to proceed with preoperative DR. Protein restriction without a reduction in calories has been shown to increase maximum longevity in rats and mice[33]. Although the magnitude of these increases is around 30–40% of that of DR, neither carbohydrate[34] nor lipid restriction[35-36] exerted these effects. Restriction of proteins in combination with calorie restriction could be a more effective way to induce the beneficial effects of DR.

Live donor grafts are the grafts least likely to benefit from DR due to the already very good results. However the results are not uniformly successful. One of the factors independently associated with poorer graft survival of kidneys from live donors is donor age older than 59 years[37]. Experimental studies show that older kidneys are more susceptible to ischemia and reperfusion injury and that DR protects old kidneys against ischemia-reperfusion injury[38-39]. Therefore DR may protect these older kidneys against ischemia and reperfusion injury[17]. The power to demonstrate a difference in renal function between DR and control grafts in our study may have been intrinsically limited, since we have included donors from all ages instead of limiting the study to "older" donors.However, the beneficial effects of DR may not be limited to transplant patients, but could extend to patients at risk for surgically induced I/R injury of the kidney, intestine, liver, heart and/or brain.

In summary, we investigated the feasibility of a mild preoperative diet in elective surgical patients. Our dietary intervention was logistically possible, well adhered to and had no effect on postoperative well-being, appetite and ability to perform daily tasks of the live kidney donors. In the recipient, we found no effect on graft function. This preoperative diet may set the stage for future work aiming at inducing protection against I/R injury by dietary interventions. Increasing the length and/or the severity of the DR regimen are potential avenues to proceed in the setting of clinical transplantation and non-transplant populations.

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Chapter 8

Dietary restriction modifies certain aspects of the postoperative acute phase response

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ABSTRACT

Background: Lifespan extension is achieved through long-term application of dietary restriction (DR) and benefits of short-term dietary restriction on acute stress and inflammation have been observed. So far, the effects of short-term DR in humans are relatively unknown. We hypothesized that short-term DR in humans reduces the acute phase response following a well defined surgical trauma.

Methods: Thirty live kidney donors were randomised between 30% preoperative dietary restriction followed by one day of fasting (n=17) or a four day ad libitum regimen (n=13) prior to surgery. Leukocyte subsets and numbers and serum cytokine levels were determined. Whole blood was stimulated with Lipopolysaccharide (LPS) and cytokine production was determined.

Results: A clear trend towards lower numbers of postoperative circulating leukocytes was observed in the DR group. IL-8 serum levels were significantly higher in the DR group over the first six postoperative days (p=0.018). After LPS stimulation significantly less TNF-α (p=0.001) was produced by blood obtained postoperatively when compared to preoperative blood from the DR group. This was not observed in the control group.

Conclusions: A relatively short pre-operative dietary restriction regimen was able to modify certain aspects of the postoperative acute phase response. These data warrant further studies into the dietary conditions that improve stress resistance in humans. (Dutch Trial Registry Number: NTR1875)

INTRODUCTION

Dietary restriction (DR), defined as reduced food intake without causing malnutrition, is associated with extended longevity[1] and improved resistance against various stressors in multiple organisms[2,3]. Stress resistance is defined as the ability of an organism to withstand and/or reduce damage caused by acute or chronic stressors such as surgery, ischemia-reperfusion injury and toxic agents. Beneficial effects of DR in non-human primates on improved health have been reported[4]. In humans, effects of DR on longevity are not yet known, but the benefits on general health are clear[5-7].

Although lifespan extension is achieved through long-term application of dietary restriction, benefits of short-term dietary restriction on acute stress resistance have been observed[8,9].

The mechanisms leading to the beneficial effects of DR are presently unknown. However, several potential mechanisms, such as those leading to increased resistance against oxidative damage[10-14], have received considerable attention. We have recently shown that short-term DR reduces the inflammatory response after surgically induced acute oxidative stress[15], whereas others have found reduced serum cytokine levels after surgery[16,17]. The effect of short-term DR on acute stress resistance in humans is largely unexplored, however tumor necrosis factor alpha (TNF-α) levels are lower and well-being is improved in asthma patients after short term DR[18]. Surgical trauma causes oxidative stress[19,20] and provokes an acute phase reaction. This response is characterized by increased levels of pro-inflammatory cytokines such as interleukin-6 (IL-6) and TNF-α, increased levels of C-reactive protein (CRP) and leukocytosis[21,22]. In addition, surgery decreases the number of circulating B- and T-cells, which may lead to a temporary impairment of cellular immunity[23,24]. We hypothesized that short-term DR increases acute stress resistance in humans and therefore reduces the postoperative acute phase response.

METHODS

Study population

Thirty people participating in a live kidney donation program were randomized between the diet group and the control group. People aged 18 to 80 years old with a body mass index above 18.5 kg/m^2 were eligible for inclusion. Approval was obtained from the medical ethics committee of the Erasmus MC in Rotterdam. Age, sex, body mass index (BMI, weight(kg)/height(m)^2), medical history and use of medications were collected. Complications were recorded until 30 days postoperatively and defined as events necessitating intra-operative or post-operative interventions or prolonged hospital stay.

Nutritional intervention

Our study aimed to reduce pre-operative calorie intake. Based on our previous experiments[25] calorie intake was reduced with 30% on day four, three and two prior to surgery (relative to the baseline measurement). The day before surgery, patients were allowed breakfast followed by 24 hours water-only fasting. Seventeen patients were randomized to the intervention group. The control group was allowed to eat ad libitum but fasted overnight prior to surgery. They kept a food record form during the four pre-operative days to enable calculation of the ingested calories.

Surgical procedure

Anaesthesia was carried out according to a protocol for drugs, ventilation, and fluid regimens. A laparoscopic nephrectomy was performed in all patients. All operations started at 08.00 A.M. Operating time and peroperative complications were recorded.

Sample collection

Peripheral venous blood samples were obtained at 08.00 P.M. the evening before surgery, six hours after surgery and at 09.00 A.M. every postoperative day until discharge. Samples for cytokine assays were collected in plastic tubes (8.5 mL, BD Biosciences Vacutainer® SST II PlusSan Jose, California,USA). Serum levels of CRP (BD Biosciences Vacutainer® SST II Plus) and leukocyte counts (BD Biosciences Vacutainer®) were measured routinely and analysed by the central hospital laboratory. Samples for immuno-phenotyping were collected at 08.00 P.M. the evening before surgery and at 09.00 A.M. on postoperative day one. Blood was collected in plastic tubes (BD Biosciences Vacutainer®) with lithium heparin.

Cytokine analysis

Total white blood cell count was measured in all samples. Cytokines (Interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, TNF-α and interferon-gamma (INF-γ)) were measured using commercially available cytometric bead arrays (CBA Human Inflammation Kit, BD Biosciences™ and CBA Human TH1/TH2 Cytokine Kit, BD Biosciences™) according to the manufacturers protocol. These cytokines were measured to provide an indication of the acute phase response and the ratio between typical T helper cell type-1 (Th1) and T helper-2 (Th2) cytokines. Measurements were performed on a FACS Calibur (BD Biosciences) and analysis on FCAP Array™ Software (BD Biosciences). Assay sensitivity was five pg/mL for all cytokines.

Immuno-phenotyping

Total number of B-cells (CD19+), T-cells (CD3+), CD3+/CD4+ T-cells, CD3+/CD8+ T- cells, natural killer cells (CD3-/CD16+/CD56+) and human leucocyte antigen-DR

(HLA-DR) expression on T-cells (CD3+/HLA-DR+) was determined according to a standard diagnostic screening protocol using truecount tubes™ (BD Biosciences).

LPS stimulation tests

For *in vitro* stimulation, heparinized blood was stimulated with 50 ng/mL LPS (*Escherichia coli*/ 026;B6, Sigma, St. Louis, MO, USA) for 24 h at 37.5°C. Subsequently, plasma was stored at −80°C. Levels of TNF-α, IL-1β, IL-10 and IL-6 were determined by means of the CBA kit. Due to high levels, IL-8 was determined by enzyme linked immuno sorbent assay (ELISA, Invitrogen, Carlsbad, California,USA) according to the manufacturers protocol. Due to logistical reasons the assays were performed in 9 DR and 8 control patients.

Statistical analysis

Categorical data are presented as number (percentage) and continuous variables as mean (standard deviation/normal distibution) or median (interquartile distance/no normal distribution). Means between two groups were compared using either the non-parametric Mann-Whitney test or the t-test for parametric data. Dichotomous data were analysed using the chi-square test. Related samples were analysed using the Wilcoxon signed ranks test. Repeated measurements were analysed using mixed model analysis. P-values less than 0.05 were considered significant. All analysis were performed using Statistical Package for the Social Sciences 15.0 (SPSS Inc., Chicago, USA).

RESULTS

Study population and surgical procedure

Baseline characteristics did not differ significantly between both groups (Table 1). In the DR group one patient was treated for epididymitis after surgery and a second had acute tubular necrosis due to peroperative hypotension; renal function restored within days. In the control group one procedure was complicated by an iatrogenic perforation of the colon which was surgically corrected. This patient developed peritonitis and was therefore excluded from the study.

Nutritional intervention

All patients adhered to the study protocol without reporting adverse events. Baseline energy intake of the groups was 1863±591 kilocalories (kcal)/day (control) and 1957±408 kcal/day (DR). Calorie intake in the control group was comparable during the four-day pre-operative period (1853±675) kcal/day). In the DR group the preoperative intake was reduced with 31.4% (29.5%-33.0%) to 1322±251 kcal/day. Macronutrient composition of the diets did not differ between both groups (Table 2).

Table 1: Baseline characteristics of the study population

	DR[1] (n = 17)	Control (n = 13)	P-value
Age (Years)	54±9	56±13	0.642
Male : Female ratio (%)	6 (35):11(64)	6 (46):7 (53)	0.711
Height (cm)	171±7.0	166±7.6	0.104
Weight (kg)	74±15	75±13	0.797
BMI (kg/m^2)	25.0±4.0	26.7±3.4	0.812
Weight (kg)			
Onset of study	74±15	75±11	0.812
One day before surgery	74±15	75±12	0.584
Blood glucose[2] (mmol/L)	4.7±0.5	4.6±0.6	0.569

[1] DR = dietary restricted group.

[2] Blood obtained after a midnight fast at the outpatient department, several months prior to surgery.

Table 2: Macronutrient composition of baseline and preoperative diets. Data are presented as means ± standard deviation.

	Baseline diet[1]	Pre-operative diet[2]
Control (n = 13)		
Protein[3]		16.7 (3.1)
Carbohydrates[4]	48.3 (6.4)	46 (5.0)
Fat[5]	32.2 (5.2)	32.1 (5.5)
DR[#] (n = 17)		
Protein[3]	17.3 (2.9)	18.0 (3.4)
Carbohydrates[4]	49.8 (5.0)	50.3 (5.6)
Fat[5]	30.4 (7.0)	31.4 (6.1)

[1] Mean values of the three day food record form completed several months prior to the operation.[2] Based on food record form filled in prior to surgery or the report of adherence to the prescribed diet.[3] Energy percentage derived from proteins.[4] Energy percentage derived from carbohydrates.[5] Energy percentage derived from fat. [#] DR = dietary restricted group.

C-reactive protein

Baseline values of CRP (DR: 2.17 ± 1.63 mg/L vs. control: 2.5 ± 2.2 mg/L, p=0.905) did not differ between both groups. CRP levels peaked on postoperative day two in both groups. No statistically significant differences in CRP levels were found between both groups at any of the examined time points.

Leukocytes

The baseline number of leukocytes (DR: 6.99 ± 1.52 x 10^9/mL vs. control: 7.35 ± 2.23 x 10^9/mL, p=0.616) did not differ between both groups. On the first postoperative day, the number of leukocytes in the control group was significantly (p=0.02) higher than the DR group. After performing a mixed model analysis, which takes into account serial measurements, the overall number of leukocytes was not significantly different

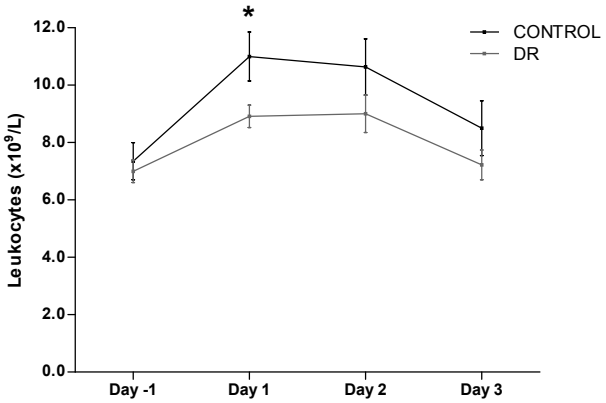

Figure 1: Number of leukocytes on preoperative and postoperative days for both the dietary restricted and control group. Data are presented as mean, the error bars represent the standard error of the mean. An asterix (*) indicates a significant difference (p=0.02).

(p=0.098).In addition, the lower leukocyte count in the DR group, which was seen only on postoperative day one, was still within the normal range. Altogether, this difference must be regarded as a trend (Figure 1).

Immuno-phenotyping

On day one before surgery, B-cells (CD19+), T-cells (CD3+), CD3+/CD4+ T-cells, CD3+/CD8+ T-cells and natural killer cells (CD3-/CD16+/CD56+) did not differ between both groups. HLA-DR expression on T-cells (CD3+/HLA-DR+) was also unaffected by dietary restriction (Figure 2). Postoperatively, there were no significant differences between both groups in absolute lymphocyte numbers. HLA-DR expression on T-lymphocytes was also comparable between both groups. The surgical procedure induced a significant decrease in absolute numbers of B- and T- lymphocytes. This was unaffected by the study diet. However, in the calorie-restricted group the number of natural killer cells decreased significantly postoperatively ($p<0.001$) which was not observed in the control group (p=0.08).

Cytokine analysis

IL-1ß, IL-2, IL-4, IL-5, IL-12p70, and TNF-α levels did not exceed the detection limit of the test in both groups at any time point measured. Serum levels of IFN-γ, IL-10, IL-6 and IL-8 are presented in Figure 3. IFN-γ levels were not affected by the surgical intervention nor by DR. IL-8 serum levels peaked at 6 hours after surgery in both groups, and were significantly higher in the DR group, when compared to the control group, over the six perioperative days (p=0.018). When the postoperative IL-8 levels were corrected for their preoperative value, no differences with regard to the postop-

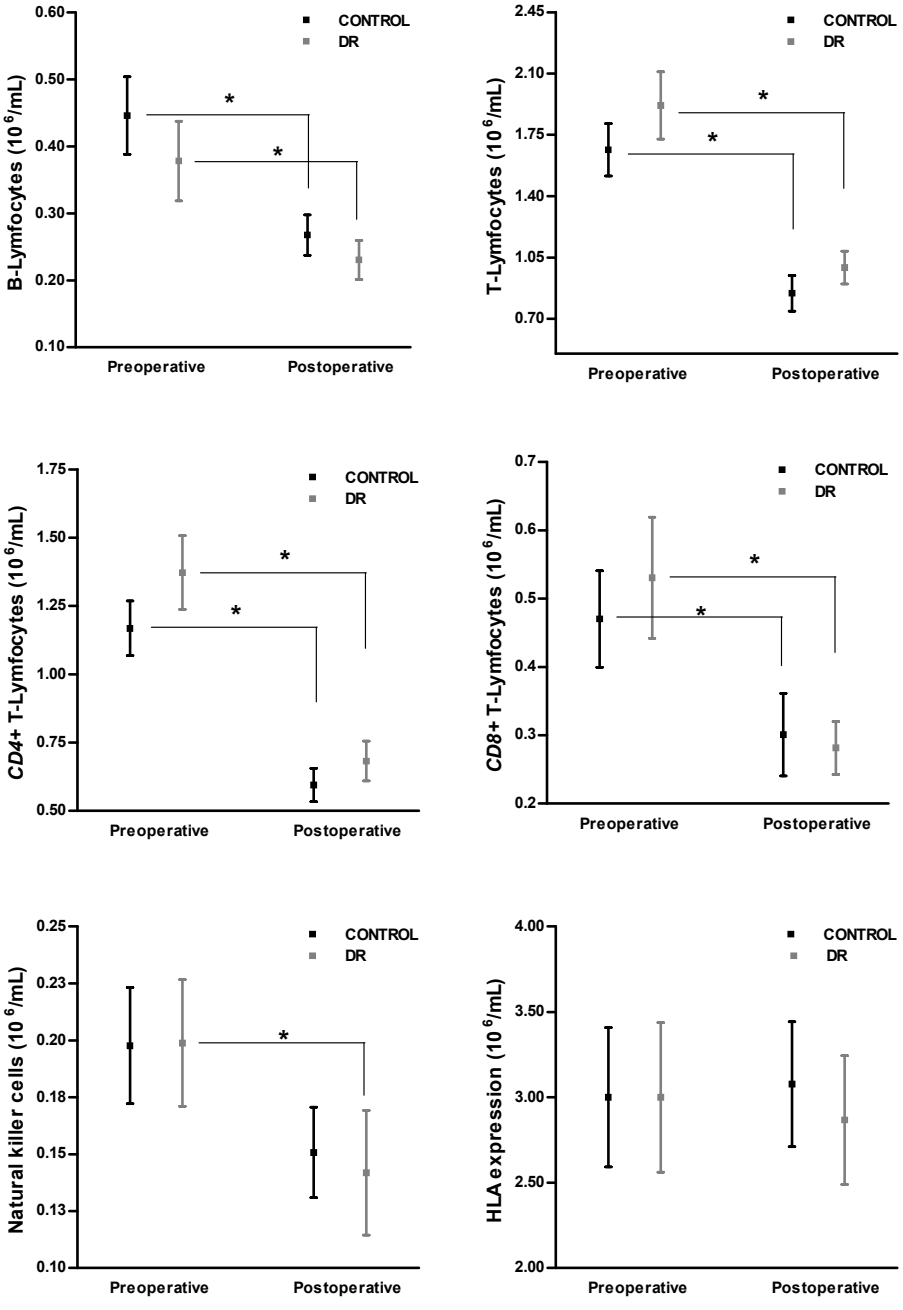

Figure 2: Immuno-phenotyping for both the dietary restricted and control group. Data are presented as means, the error bars represent the standard error of the mean. An asterix (*) indicates a significant difference between the pre- and postoperative values. No statistically significant differences were found between the number of cells between the two groups, both pre- and postoperatively.

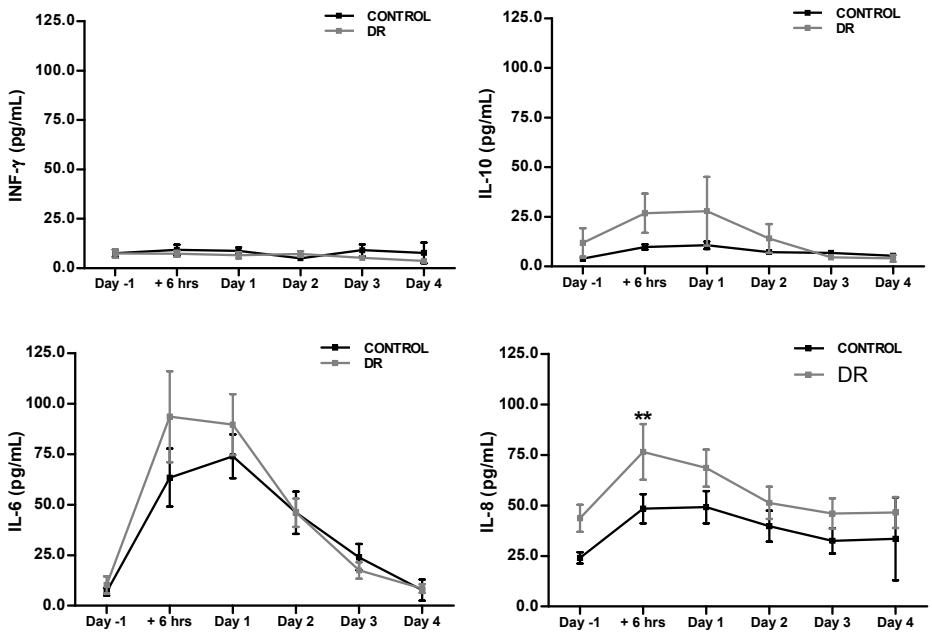

Figure 3: Perioperative cytokine levels for both the dietary restricted and control group. There are no significant differences in the levels of IFN-γ, IL-6 and IL-10. The levels of IL-8 are significantly higher in the DR group, when compared to the control group (p=0.018).

erative response were observed (data not shown). This indicates that the diet induced significantly higher IL-8 levels pre-operatively, but did not affect the magnitude of the postoperative response. IL-6 levels increased after surgery during the first postoperative day in both groups, after which they returned to normal on postoperative day four.IL-10 levels showed a similar pattern. No significant differences between both groups were observed. The ratios between pro-inflammatory and anti-inflammatory cytokines (IL-6/IL-10 and IL8/IL-10) did not differ significantly between both groups (data not shown).

LPS stimulation tests

In 17 patients (9 DR vs. 8 controls) LPS stimulation tests on whole blood samples, obtained before and after surgery, were performed (Figure 4). There were no significant differences between the two patient groups in cytokine levels in unstimulated samples (data not shown). Cytokine levels in the unstimulated preoperative samples were not statistically different from the unstimulated postoperative values in both groups (data not shown). After stimulation with LPS, cytokine levels increased significantly in all samples tested. No statistically significant differences between both experimental groups were found, although we observed a consistent trend in the DR group, where all cytokine levels tended to be lower after stimulation. In the DR group TNF-α levels were

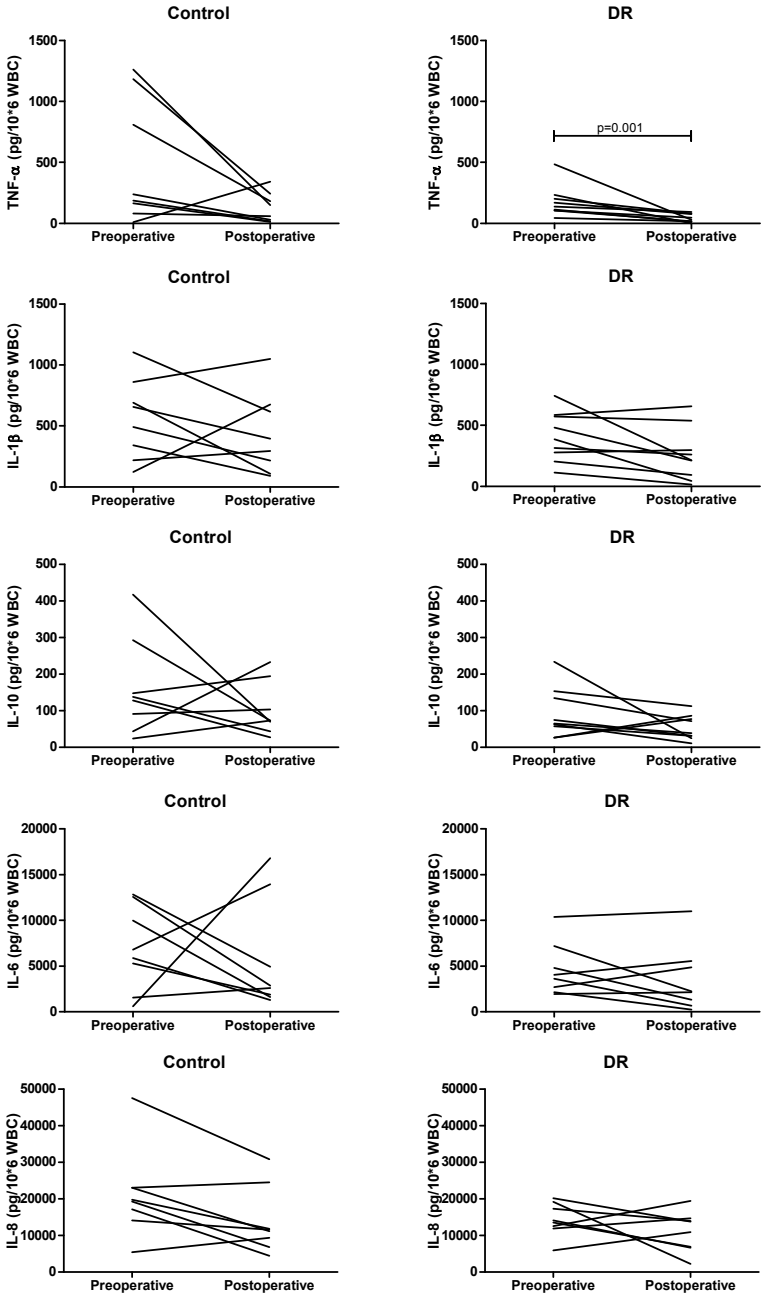

Figure 4: Whole blood stimulation with LPS of pre- and postoperative samples of both the dietary restricted and control group. Lines represent the cytokine response after stimulation with LPS of individual patients before and after surgery. All cytokine values are expressed as: picogram/1*10^6 white blood cells. Tumor necrosis factor alpha production upon LPS stimulation is significantly reduced postoperatively in the DR group (p=0.001).

significantly lower after stimulation (p=0.001) in samples obtained postoperatively when compared to preoperative stimulated samples.

DISCUSSION

We hypothesized that short term DR is able to improve acute stress resistance in humans, and reduce the acute phase response after surgical trauma. We designed a DR regimen involving three preoperative days of 30% DR and one day of fasting. In the DR group we observed a trend towards lower numbers of postoperative leucocytes while IL-8 serum levels were significantly higher. In postoperative whole blood samples from the DR group, TNF-α levels were significantly lower after stimulation with LPS when compared to preoperative samples, which was not the case in the control group.

The preoperative diet we applied in this study is based on our previous studies[25] and reports that DR is able to improve resistance to acute stressors such as ischemia and reperfusion injury of the liver[9], and the toxic side effects of chemotherapy[8]. We studied live kidney donors, who are a healthy, homogenous patient group undergoing a standardised operation, rendering them a suitable study population.

Leukocyte numbers are known to increase after surgical trauma and peak on the first postoperative day[24]. Conventional (open) surgery causes more surgical stress than laparoscopic procedures, and induces higher leukocyte peak values[24]. The peak in total leukocytes on postoperative day one was significantly lower in the DR group when compared to the control group. However, as the mixed model analysis over all timepoints was not significantly different between both groups, this can only be interpreted as a trend. If anything, DR did not lead to increased numbers of leukocytes. As differences in T-cell, B-cell or NK-cell number could not have accounted for this increase it should be due to increased levels of other cell types, most likely granulocytes.

In accordance with previous reports[26-29], serum levels of IL-1β, IL-2, IL-4, IL-5, IL-12p70 and TNF-α were undetectable during to pre- and postoperative period. IFN-γ levels were unaffected by the operation[26,29], whereas IL-10 levels increased shortly after surgery[26]. IL-6 serum levels are often reported as markers of surgical trauma[30,31]. IL-6 levels in our study are similar to those previously reported after laparoscopic surgery, and lower when compared to major open surgery[26].

We hypothesized that the acute phase response, as measured by increases in IL-6 and CRP serum levels, would be attenuated in the DR group as the magnitude of the acute-phase response is directly proportional to the degree of surgical stress[30,31]. Laparoscopic donor nephrectomy in this study was followed by a fast and uneventful recovery, without major complications in both groups. The relatively mild surgical trauma induced by laparoscopic nephrectomy was reflected by low levels of both CRP

and IL-6 and were therefore unlikely to be influenced by DR. This raises the question if live kidney donors are a suitable study population to test our hypothesis, as the surgical trauma is relatively mild. Preoperative dietary modifications might therefore be more beneficial in patient populations undergoing more extensive surgery.

IL-8 levels were significantly higher in the DR group, both pre- and postoperatively. These increased levels were not correlated with infectious complications after surgery, as has been reported before[32]. In obese men following DR for eight weeks, circulating IL-8 levels also increased with 30%[33]. A slight increase in IL-8 may desensitize against the proinflammatory effects of subsequent high IL-8 levels[34]. Mice over producing IL-8 exhibit decreased exudation of neutrophils into body cavities in response to acute inflammatory stimuli[35]. We therefore speculate that the preoperative rise in IL-8 levels by DR reduces its subsequent pro-inflammatory effect in the postoperative period. This is partially supported by our finding of a trend towards lower numbers of postoperative peripheral leukocytes in the DR group.

In line with previous reports[23,36], a decrease in the number of B- and T-lymphocytes following surgery was observed, which was similar in both groups. Although the number of NK cells was significantly reduced in the DR group, the absolute mean numbers were 0.14 $*10^9$ cells/L (DR) and 0.15 $*10^9$ cells/L (control). This is very unlikely to have a clinically relevant impact, as the values are well within the normal range.

In contrast to the relatively mild acute phase response induced by laparoscopic nephrectomy, LPS is a major stimulus to the immune system. Stimulation of preoperative whole blood samples showed that dietary restriction itself did not influence the reactivity to LPS. In postoperative samples, TNF-α levels were significantly lower after LPS stimulation in the DR group, when compared to the stimulated preoperative samples. Since TNF-α is important in the acute phase response, this observation supports our hypothesis that DR attenuates the postoperative inflammatory response. And although not significant, there was a clear trend towards a lower production of all other cytokines after LPS stimulation in de DR group.

Although several parameters of the acute phase response were blunted by preoperative DR, we are not able to draw firm conclusions with regard to the clinical consequences. As mentioned before, live kidney donors undergo a relatively mild surgical procedure, with a very low complication rate, which makes it difficult to speculate about the clinical implications of our findings. Furthermore, the immunological parameters were obtained over a relatively short perioperative period. This prevents the detection of changes occurring at later time points. Anesthesia is known to influence postoperative immune function up to several days after surgery[37]. The anesthetic regimen in our study was rigorously adhered to and similar in both groups. Therefore, the differences between the immunologic parameters are very likely to be caused by the preoperative dietary intervention. Differences in postoperative food intake may influ-

ence the measured indices. We have registered the patients' postoperative appetite using a visual analogue score (data not shown) and found no significant differences. It is therefore unlikely that differences in postoperative calorie intake influenced the outcome. Finally, since the kinetics of onset of the beneficial effects induced by DR are not know in humans, the length and severity of our dietary intervention may have been insufficient to induce the maximal beneficial response.

The possible relevance for the clinical situation warrants further investigation as preoperative DR may activate protective mechanisms that induce increased resistance to the post surgical inflammatory response. Human studies on stress resistance and nutrition are sparse, and our study provides important information that corroborates with experimental evidence that calorie restriction is able to blunt the inflammatory response.

In summary, we hypothesized that short-term DR increases acute stress resistance in humans and reduces the acute phase response following surgical trauma. We found evidence to suggest that a relatively short nutritional intervention was able to induce beneficial changes in the acute phase response after a mild surgical intervention. We feel that our data warrant more clinical studies to define the relevant dietary conditions and patient populations which might benefit from them. Furthermore, other parameters, which provide additional insight into whether the post-operative stress response was ameliorated, need to be investigated.

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Part four

Summary, discussion and future perspectives

Chapter 9

Summary, discussion and future perspectives

SUMMARY AND DISCUSSION

In the beginning of the twentieth century a non-invasive way to prolong the life- and health span of various animal species, including non-human primates, was found: dietary restriction (DR). DR may be performed by various regimens such as calorie restriction (CR), fasting, and alternate day fasting (ADF). CR refers to an intervention in which the total daily amount of calories provided to an animal or organism is limited to a certain percentage of the animals' normal daily intake. ADF regimens involve a "feast day" on which food is consumed ad libitum that alternates with a "fast day" on which food is withheld. Fasting is abstinence of all food with ad libitum access to water. Although studies investigating the effect of DR in humans on lifespan are lacking there are reports which suggest that DR might be of benefit in humans as well. In addition, long-term interventions (e.g., months, years) are not amendable in the clinical setting. The overall aim of this thesis was to examine whether the beneficial effects of long-term DR could be induced by short-term nutritional interventions. Furthermore, the feasibility of DR in the clinical setting was investigated.

In **chapter 2** we reviewed the literature on short-term DR interventions and protection afforded against ischemia-reperfusion (I/R) injury in animal models. We concluded that overnight fasting as well as slightly longer periods (up to 4 days) protect against I/R. The effects of preoperative fasting on I/R injury were investigated, as it was suggested that donor nutritional status may affect outcome after liver transplantation. In addition, it was suggested that starvation of donors, due to prolonged stay in the intensive care unit, may adversely affect the transplanted liver[1]. In contrast to this hypothesis, fasting for one to four days protected rat livers from cold preservation injury and resulted in higher survival rates after orthotopic liver transplantation in rats[2] Preoperative DR regimens also protected other organ systems such as brain, heart, liver, and retina against I/R injury in animal models. The proposed protective mechanisms of DR include upregulation of cytoprotective molecules such as heat shock proteins and hemeoxygenase-1[3].

Overall, these animal studies suggest potential uses for DR in the clinical setting, however there are several drawbacks that need attention. Clinical trials have demonstrated adverse effects of the fasted state for surgical patients[4-6]. In addition, randomized clinical trials demonstrated that preoperative administration of carbohydrate-rich drinks improves insulin sensitivity and increases patient well-being[7-12]. Furthermore, restricting preoperative dietary intake may be detrimental in already malnourished patients. Therefore, future research should focus on the role of specific macronutrient components and DR mimetics or agents that may impinge on (some) of the pathways induced by DR. However, to pharmaceutically mimic the effects of DR, the mechanisms by which DR exerts is beneficial effects need to be defined.

In **chapter 3** we sought to elucidate the mechanisms of protection induced by fasting against hepatic I/R injury in mice. Mice were fasted for 72 hours or fed ad libitum prior to 75 minutes of partial (70%) hepatic ischemia, followed by 6 and 24 hours of reperfusion. It was found that fasting decreased hepatocellular I/R injury. In addition, baseline mRNA expression levels of hemeoxygenase-1 and the mitochondrial antioxidants superoxide dismutase-2 glutathione peroxidase-1 and glutathione reductase were significantly upregulated in livers from 72 hours fasted animals when compared to ad libitum fed animals. Six hours after reperfusion, expression levels of these genes were also significantly higher compared to control mice. After reperfusion p-selectin and interleukin-6 were significantly reduced in the fasted mice, and superoxide radical generation and neutrophil influx were significantly attenuated in the fasted group compared to the ad libitum fed group. We conclude that the protective effect of short-term fasting against hepatic I/R injury is induced by increased baseline expression of mitochondrial antioxidant enzymes and the stress response gene heme-oxygenase-1 (HO-1).

It is known that steroids have anti-inflammatory properties and that they are capable of improving the outcome after liver transplantation. In **chapter 4** we hypothesized that the protection imposed by fasting is mediated by increased levels of corticosterone, induced by the stress of food deprivation. C57BL/6 mice were fasted for 1, 2, or 3 days after which serum corticosterone levels were determined. Fasting significantly increased serum corticosterone levels. To prevent corticosterone production, mice underwent a bilateral adrenalectomy (ADX) or sham operation ten days prior to fasting and subsequent renal I/R injury. Mice subjected to ADX exhibited a higher mortality rate after renal I/R injury compared with sham-operated mice. However, ADX mice subjected to sham I/R injury demonstrated similar high mortality rates as ADX mice that underwent renal I/R injury. Therefore, these experiments did not address our hypothesis that the protection afforded by fasting is due to increased corticosterone levels, but showed that mice without adrenal glands do not withstand subsequent surgery. We therefore designed a new set of experiments. Mifepristone, a glucocorticoid receptor antagonist, blocks the downstream signaling of the glucocorticoid receptor. The use of mifepristone enables controlled studies on the effects of corticosterone on renal I/R injury without the need for a bilateral ADX. Mifepristone was administered daily during the 3-day fast, prior to renal I/R injury, while control mice received PBS at the same time-points. The mifepristone regimen did not abolish the protection afforded by fasting on renal I/R injury. Survival rates and kidney function were similar in both groups. Therefore, our results indicate that fasting-induced protection against renal I/R injury is mediated by corticosterone/glucocorticoid receptor-independent pathways. In chapter

3 we demonstrated that fasting increases baseline expression levels of mitochondrial antioxidant enzymes and the stress response gene HO-1. We showed that mifepristone treatment did not interfere with the upregulation of antioxidant defense systems. It would be of interest to investigate whether an agent that exactly mimicks the kinetics of corticosterone production during fasting is able to increase the expression of cytoprotective genes, for instance by corticosteroid administration.

Next, we investigated the role of ghrelin in the protection against renal I/R injury. In **chapter 5** C57BL/6 mice were fasted for one, two, or three days after which acylated ghrelin levels were determined. Fasting significantly increased acylated ghrelin levels in serum. To mimic the increased ghrelin levels induced by fasting, ad libitum fed mice were injected with acylated ghrelin or PBS prior to renal I/R injury. In contrary, to block the effects of fasting induced ghrelin production mice were injected with a growth hormone secretagogue receptor antagonist[13] or a vehiculum prior to renal I/R injury. Administration of acylated ghrelin or ghrelin receptor blockade did not affect renal function after I/R injury. However, it has been shown that treatment with acylated ghrelin improves renal function after I/R injury in a similar model[14]. These conflicting results can be explained by the time-point of administration as we administered acylated ghrelin prior to renal I/R injury, while Takeda et al.[14] administered ghrelin both before and after I/R injury. Although we cannot exclude a protective effect of ghrelin treatment on renal I/R injury, our data demonstrate that increased levels of acylated ghrelin induced by fasting and ghrelin receptor signaling do not mediate its protection against renal I/R injury.

In **chapter 6** the therapeutic use of dietary restriction is extended. For most patients with colorectal malignancies surgical resection is the cornerstone of any potentially curative treatment. Surgical trauma results in systemic inflammation as reflected by cytokine release[15,16] and in postoperative cellular immunosuppression[17]. There is emerging evidence suggesting that these surgery-induced processes facilitate development and outgrowth of tumor metastases[18]. Postoperative inflammatory responses facilitate metastasis formation of circulating tumorcells by increasing the expression of adhesion molecules. We have shown that two weeks of dietary restriction reduces the expression of adhesion molecules and protects against surgically induced inflammation. DR might therefore beneficially interfere with surgery-induced inflammation and subsequent adhesion of circulating tumorcells. BALB/c mice were subjected to two weeks of 30% dietary restriction prior to inoculation with tumor cells in the liver. Hepatic tumor load was scored after ten days as a percentage (tumor surface/total liver surface) on H&E stained sections. We found that DR reduces hepatic tumor load and mRNA expression of E-selectin. Furthermore serum obtained from DR mice reduced *in vitro* adhesion of

C26 colon carcinoma cells to human vascular endothelial cells. Although we do not show a direct correlation, it has been demonstrated that E-selectin plays an crucial role in the process of liver metastasis formation in the murine BALB/c-C26 colon carcinoma model as direct blockage of E-selectin was associated with lower numbers of liver metastasis[19]. The question remains why DR lowers E-selectin expression. We recently reported that DR robustly down regulates the production of proinflammatory cytokines and adhesion molecules in models of renal and hepatic ischemia-reperfusion injury[20]. In addition DR induced the expression of cytoprotective and anti-oxidant genes, leading to a reduced formation of reactive oxygen species[20]. As surgical trauma causes oxidative stress[21,22], the increased protection against oxidative stress and the subsequently reduced inflammatory response induced by DR, may explain why E-selectin expression is reduced.

So far, therapeutic effects of short-term preoperative dietary restriction have been observed in animal models only. Therefore, in **chapter 7** we describe a pilot study which investigates whether a relatively mild preoperative DR regimen is feasible in the clinical setting and explored the effects of DR in surgical patients. Live kidney donors were randomized between preoperative DR and ad libitum food intake. Seventeen participants were instructed to follow a 30% calorie-restricted diet, followed by one day of water-only fasting prior to surgery. Thirteen participants were allowed to eat ad libitum preoperatively. We show that modest preoperative DR is feasible in the clinical setting and has no measurable effect on postoperative well-being, ability to perform daily tasks and complication rates of live kidney donors. We performed this study in live kidney donors since they were eager to participate and very committed, which likely benefits the accurate reporting of food intake. Although compliance was measured subjectively and is difficult to assess with objective measurements, compliance with the diet was high (94%) in this motivated group.

We hypothesized that a preoperative diet in live kidney donors would enhance the resistance of the kidney against ischemia and reperfusion injury, leading to better graft function in the recipient. To assess the effect of preoperative DR on renal transplant function, we measured graft function on the first postoperative day by means of renography and during the first month by serum creatinine levels in the recipient. We found no effects of DR on renal graft function. We acknowledge that this study is a pilot study and not sufficiently powered to draw definite conclusions about the relationship between preoperative DR and renal transplant function. As our diet failed to demonstrate beneficial effects on postoperative graft function, longer and more extensive dietary regimens may be needed. This preoperative diet may set the stage for future work aiming at inducing protection against surgical trauma and I/R injury by dietary interventions.

In **chapter 8** we investigated the effect of preoperative DR on the postoperative immune response in the patients described in chapter 7. We hypothesized that short-term DR in humans reduces the acute phase response following a well defined surgical trauma. Before and after surgery, leukocyte numbers and serum cytokine levels were determined. Whole blood was stimulated with lipopolysaccharide (LPS) and cytokine production was determined. Leukocyte numbers are known to increase after surgical trauma and peak on the first postoperative day[23]. The peak in total leukocytes on postoperative day one was significantly lower in the DR group when compared to the control group. However, as a mixed model analysis over all time-points was not significantly different between both groups, this should be interpreted as a trend. The acute phase response, as reflected by increased IL-6 and CRP serum levels, was not attenuated by DR. Furthermore, IL-8 serum levels were significantly higher in the DR group over the first postoperative days. A slight increase in IL-8 may desensitize against the proinflammatory effects of subsequent high IL-8 levels[24]. Mice overproducing IL-8 exhibit decreased exudation of neutrophils into body cavities in response to acute inflammatory stimuli[25]. We therefore speculate that the preoperative rise in IL-8 levels by DR reduces its subsequent pro-inflammatory effect in the postoperative period. Stimulation of preoperative whole blood samples showed that dietary restriction itself did not influence the reactivity to LPS. In postoperative samples, TNF-α levels were significantly lower after LPS stimulation in the DR group, when compared to the stimulated preoperative samples. Since TNF-α is important in the acute phase response, this observation supports our hypothesis that DR attenuates the postoperative inflammatory response. Overall, these data suggest that a relatively short nutritional intervention induces beneficial changes in the acute phase response after a mild surgical intervention. Therefore, our data warrants further clinical studies to investigate the relevant dietary conditions and patient populations which might benefit from them.

FUTURE PERSPECTIVES

Translation of the results of preoperative DR from animal studies to the clinical setting poses a challenge. We have demonstrated the feasibility of a preoperative diet consisting of three days 30% DR and one day of fasting in humans. In literature periods much longer than two weeks of 30% DR in humans have been reported[26], but not prior to surgery. Surgical patients may be malnourished and may not tolerate and/or respond to DR similarly as healthy animals or humans. Therefore, future research should not be limited to healthy animals, but investigate the effect of preoperative DR in mouse models of cancer or other pathologies such as hepatic steatosis. Steatotic livers are susceptible to I/R injury, and may be protected by preoperative DR. In addition, it

allows to investigate the effect of DR and chemotherapy on tumor growth. Further research should also focus on the role of specific macronutrient components in the protection induced by DR.

A diet consisting of protein restriction without a reduction in calories has been shown to increase maximum longevity in rats and mice[27]. Although the magnitude of these changes is around 30–40% of that of DR, neither carbohydrate[28] nor lipid restriction[29,30] exerted these effects. Restriction of proteins could therefore be another way to induce the beneficial effects seen after DR and overcome the problem of reducing calorie intake. We have recently performed experiments in mice showing that a protein free diet protected against both renal and hepatic I/R injury. Future experiments will be designed to investigate the role of preoperative restriction of essential aminoacids such as methionine, as it has been demonstrated that restriction of this essential aminoacids increases lifespan as well[31].

As mentioned earlier, the use of DR mimetics may be a way to overcome the problems associated with DR in surgical patients. A DR mimetic can be defined as any pharmacological intervention that produces beneficial effects of DR without causing or requiring a significant reduction in calorie intake. One compound that has received considerable attention as DR mimetic is resveratrol, a naturally-occurring polyphenol found in red wine. Resveratrol induces, at doses that can be readily achieved in humans, genomic changes which resemble many of the genetic alterations induced by DR[32]. Resveratrol treatment decreases hepatic I/R injury by significantly increasing glutathione reductase, Cu/Zn-superoxide dismutase, and catalase activities[33]. Furthermore, evidence supporting the use of resveratrol in the treatment of malignancies is emerging. Resveratrol exerted cytotoxic effects on neuroblastoma cells and on the human cholangiocarcinoma SK-ChA-1 cell line[34]. In addition, resveratrol treatment suppressed the growth rate of subcutaneous neuroblastomas[35]. It has been demonstrated that the anticancer activity of this compound is mainly due to induction of apoptosis[36]. However, studies investigating the effect of resveratrol on life-span have conflicting results.

We were unable to demonstrate major beneficial effects of preoperative DR in the clinical setting. For future trials the following topics need to be considered. The ability of a diet to induce a protective response may have been insufficient, as the magnitude of the diet was not comparable to three days of fasting in mice. Increasing the length and severity of the DR regimen, or altering the macronutrient composition are potential avenues to proceed preoperative DR in humans. Furthermore, the laparoscopic donor nephrectomy in our study was a relatively mild surgical intervention which was followed by a fast and uneventful recovery, without major complications. The relatively mild surgical trauma induced by laparoscopic nephrectomy was reflected by low levels

of both CRP and IL-6 which makes a measureble effect of DR unlikely. This raises the question if live kidney donors and the mild surgery-induced trauma are a suitable population to study our hypothesis. Preoperative dietary modifications might be more beneficial in patients undergoing more extensive surgery. In addition, live donor grafts are the grafts least likely to benefit from DR due to the already very good results. However, the results are not uniformly successful. One of the factors independently associated with poorer graft survival of kidneys from live donors is a donor age older than 59 years[37]. Experimental studies show that older kidneys are more susceptible to ischemia and reperfusion injury and that DR protects old kidneys against ischemia-reperfusion injury[38,39,40]. The power to demonstrate a difference in renal function between DR and control grafts in our study may have been intrinsically limited, since we have included donors from all ages instead of limiting the study to "older" donors.

The beneficial effects of DR may not be limited to transplant patients, but could be extended to patients who have suffered spinal cord injury or are diagnosed with malignant disease. Plunet et al.[41] showed that DR is also be effective when applied after surgical induction of cervical spinal cord injury in rats. DR resulted in a 50% reduction in lesion volume, improved regeneration, and improved behavioral recovery. As the onset of I/R injury due to trauma is unpredictable and therefore not amenable to planned nutritional interventions, this is particularly interesting.

It has been shown that preoperative fasting in mice protects normal cells against the toxic side effects of chemotherapy, whereas tumor cells were more sensitive to the therapy. This enabled the administration of very high doses of chemotherapy, without the side effects as normally seen with this regimen[12]. These data are supported by the case history of ten patients who voluntarily fasted prior to different chemotherapy regimens and reported fewer side effects[42].

For the near future, it is necessary to strengthen and confirm our findings that preoperative DR induces protection against oxidative damage, by applying DR in a large animal model. Pigs resemble the human situation and future experiments should determine whether preoperative DR also induces its beneficial effects against I/R injury in a pig model. If DR works in both mice and pigs, the likelihood of DR working in humans increases. Hereafter, research should focus on identifying the underlying mechanisms that induce protection on one hand and the optimal macronutrient composition to protect against I/R injury on the other. Microarray analysis can be used to investigate DR mechanisms as these analyses identify differences in gene expression patterns between DR and control livers. There is a group of candidate genes which could play a pivotal role in the effects of DR. In 2000, Lin et al[43] proposed that sirtuins, homologues of the

yeast Sir2 protein, are critical mediators of the effects of DR. They mimicked calorie restriction in yeast by physiological or genetic means and showed a substantial extension in life-span. This extension was not observed in strains mutant for SIR2. It would be interesting to investigate if sirtuins play a role in inducing protection against I/R injury.

In conclusion, preoperative DR is a promising non-invasive strategy with therapeutic benefits in various experimental settings. Future research is needed to specify the role of DR or its mimetics in the clinical setting.

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Chapter 10

Nederlandse samenvatting

Aan het begin van de twintigste eeuw ontdekte men dat calorische restrictie (CR), het reduceren van de dagelijkse inname van calorieën zonder tekorten aan vitaminen en mineralen te veroorzaken, levensverlengend werkt. Dit fenomeen is beschreven bij vele verschillende diersoorten, waaronder primaten. Zowel de gemiddelde als de maximale levensduur bleek bij deze dieren toe te nemen. Daarnaast bleek CR te leiden tot minder schade geïnduceerd door vrije zuurstofradicalen, zowel *in vitro* als *in vivo*[1]. Er zijn verschillende vormen van CR die deze gunstige effecten kunnen induceren. Allereerst kan men de dagelijkse hoeveelheid aan calorieën beperken tot een bepaald percentage van de normale dagelijkse inname. Daarnaast kan men om de dag vasten. Hierbij wordt de periode van vasten gevolgd door eenzelfde periode waarin men onbeperkt mag eten, de totale inname van calorieën hoeft hierbij niet verminderd te zijn. Hoewel er tot op heden geen onderzoek is gedaan naar de effecten van CR op levensverlenging van de mens, zijn er aanwijzingen dat CR ook in mensen werkt. De bevolking van Okinawa (Japan) consumeert gemiddeld 20% minder calorieën per dag en staan bekend om hun hoge levensverwachting en lage risico op ouderdomsziekten[2].

In dit proefschrift onderzochten wij of een kortdurend dieet bescherming kan induceren tegen oxidatieve schade (door vrije zuurstofradicalen) bij proefdieren. Bovendien werd de mogelijkheid bestudeerd om een CR-schema toe te passen op mensen.

Hoofdstuk 2 biedt de uitkomsten van een literatuurstudie naar de effecten van het kortdurend diëten op de bescherming tegen vrije zuurstof radicalen. Vrije zuurstofradicalen spelen een belangrijke, nadelige rol bij orgaan transplantaties. Gedurende een orgaantransplantatie is er een periode van ischemie en vervolgens recirculatie van het donororgaan. Tijdens de ischemische periode is het orgaan losgekoppeld van de bloedcirculatie waardoor er zuurstofgebrek ontstaat. Na de implantatie van het orgaan in de ontvanger vindt er recirculatie (reperfusie) van bloed plaats. Direct na reperfusie moet de stofwisseling in het orgaan zich herstellen, waardoor er kortdurend een relatieve overvloed aan zuurstof aanwezig is, wat leidt tot de vorming van schadelijke vrije zuurstofradicalen. Dit noemen we ischemie en reperfusie (I/R) schade. In de jaren negentig ontstond het idee dat de voedingstoestand van een donor een belangrijke invloed heeft op de weerstand van het donororgaan tegen I/R schade[3]. Men dacht dat het orgaan van een ondervoedde donor slechter bestand was tegen I/R schade. Uit onze literatuurstudie bleek echter dat enkele dagen vasten in een ratmodel voorafgaand aan een orthotope levertransplantatie, leidde tot betere resultaten. Het "gevaste" orgaan was beter bestand tegen I/R schade. Dit kan mogelijk veroorzaakt worden door veranderingen in het hemoxygenase-1 (HO-1) systeem. Niet alleen de lever, maar ook de hersenen, het hart en de retina bleek men te kunnen beschermen tegen I/R-schade door kortdurende diëten.

Voordat deze voedingsinterventies toegepast kunnen worden in de humane situatie, is het belangrijk om enkele kanttekeningen te plaatsen. Er zijn onderzoeken die

beschrijven dat het nadelig is voor patiënten om nuchter te zijn voor een operatie[4-6]. Daarnaast zijn er gerandomiseerde studies die laten zien dat het innemen van een koolhydraatrijke drank voor de operatie leidt tot betere insulinegevoeligheid en welbevinden na een operatie[7-12]. Tevens is het onbekend of een pre-operatief dieet veilig kan worden toegepast in ondervoedde patiënten. Vervolgonderzoek naar de bijdrage van verschillende voedingscomponenten aan het beschermende effect is daarom nodig. Wellicht kan men slechts één voedingscomponent weglaten en op die manier, zonder calorische restrictie, dezelfde bescherming induceren. Daarnaast zou het wenselijk zijn om met een medicijn het effect van een preoperatief dieet te induceren. Hiervoor is het belangrijk om te weten via welke mechanismen een preoperatief dieet beschermt tegen I/R-schade.

In **hoofdstuk 3** onderzochten we in een diermodel de mechanismen die ten grondslag liggen aan de door vasten geïnduceerde bescherming tegen I/R-schade van de lever. Om dit te onderzoeken werden muizen gedurende drie dagen te vasten gezet of normaal gevoerd voorafgaand aan de inductie van I/R-schade in de lever. Vervolgens werd gedurende 75 minuten de bloedstroom naar 70% van de lever onderbroken waarna reperfusie plaatsvond. Zes en 24 uur later onderzochten we de lever. Pre-operatief vasten leidde tot een vermindering van I/R-geïnduceerde leverschade. Daarnaast was er na vasten sprake van een toegenomen expressie van verschillende beschermende genen, zoals HO-1 en enkele mitochondriale antioxidanten (superoxide dismutase-2, glutathione peroxidase-1 en glutathione reductase), in vergelijking met de controlegroep. Dit verschil bleef bestaan tot zes uur na reperfusie. Naast deze toegenomen hoeveelheid van beschermende genen, was er bij de dieren die gevast hadden ook sprake van een mildere inflammatoire respons (P-selectine en interleukine 6), minder infiltratie van ontstekingscellen en minder radicaalvorming. Wij concluderen dat de toegenomen expressie van HO-1 en antioxidanten door het dieet veroorzaakt zijn en een belangrijke rol spelen in het mechanisme van de beschermende werking van vasten. Echter, het is niet duidelijk welke processen ten grondslag liggen aan deze verhoogde expressie.

Het is bekend dat steroïden ontsteking kunnen remmen en dat het gebruik ervan een gunstige werking heeft op de uitkomst van een levertransplantatie. Wij veronderstelden in **hoofdstuk 4** dat de beschermende werking van vasten geïnduceerd wordt door een toename in corticosteron (CS) spiegels, ten gevolge van de stress van het vasten. Muizen werden te vasten gezet gedurende één, twee of drie dagen, waarna wij de concentratie van CS in het bloed bepaalden. Vasten leidde tot een significante stijging van CS. Aangezien CS wordt geproduceerd door de bijnieren werden vervolgens, om de toename van CS tijdens vasten te voorkomen, de bijnieren verwijderd (adrenalectomie,

ADX). Tien dagen hierna induceerden wij renale I/R-schade en we bestudeerden de overleving. Nier I/R schade werd geïnduceerd door het plaatsten van een klem gedurende 37 minuten op de bloedtoevoer naar de nier. Er was sprake van een toegenomen mortaliteit in de ADX groep vergeleken met de controlegroep (geen ADX). Echter, indien we tien dagen na de ADX een sham I/R procedure toepasten was de mortaliteit identiek. Concluderend bleek dit model ongeschikt voor het testen van de hypothese.

Mifepristone, een glucocorticoid receptorantagonist, blokkeert de receptor waar CS aan bindt. Door het toedienen van mifepristone konden we onderzoeken of vasten beschermt tegen I/R schade wanneer het effect van CS geblokkeerd wordt. Gedurende de drie dagen vasten dienden wij mifepristone toe, waarna I/R schade van de nier werd geïnduceerd. Nierfunctie en overleving waren gelijk in de groep die mifepristone kreeg vergeleken met de controlegroep. Wij concludeerden dat CS geen rol speelt in de bescherming tegen renale I/R schade, geïnduceerd door vasten. Omdat wij in hoofdstuk 3 lieten zien dat een verhoging van HO-1 expressie door vasten wellicht een belangrijke rol speelt in het induceren van bescherming, onderzochten wij of mifepristone dit effect teniet kan doen. Dit bleek niet het geval.

Een ander hormoon dat mogelijk een rol speelt in de beschermende werking van vasten tegen I/R schade is ghreline. In **hoofdstuk 5** onderzochten wij of ghreline een rol speelt in deze bescherming. C57BL/6 muizen werden gevast voor een, twee of drie dagen waarna geacyleerd ghreline (ac-Gr) niveaus werden gemeten in het plasma. Vasten leidde tot een significante stijging van deze niveaus. Ook hebben wij ad libitum gevoedde muizen geïnjecteerd met ac-Gr gedurende drie dagen alvorens I/R schade in de nieren te induceren. Het toedienen van ac-Gr aan gevoedde muizen leidde niet tot bescherming tegen nierschade. De onderzoeksgroep van Takeda et al.[13] toonde dit wel aan, maar zij gaven ook ac-Gr na de operatie. Tot slot hebben wij gevaste muizen behandeld met ghrelinereceptorblokkade[14] om het effect van ghreline te neutraliseren voor I/R schade van de nier. Het blokkeren van de ghrelinereceptor had geen effect op de nierfunctie na I/R schade. Hoewel wij een beschermende werking van ghreline op renale I/R schade niet kunnen uitsluiten, suggereren onze resultaten dat ghreline geen rol speelt in het kader van het pre-operatief vasten en de bescherming tegen I/R schade van de nier.

In **hoofdstuk 6** hebben wij een mogelijke uitbreiding van de toepassing van CR onderzocht. Voor veel patiënten met een colorectale maligniteit is een operatie de belangrijkste behandelmogelijkheid. Echter, een operatie leidt tot een ontstekingsreactie gekenmerkt door het vrijkomen van proinflammatoire cytokinen[15-16] en cellulaire immuunsuppressie[17]. Er is steeds meer bewijs dat deze processen de vorming van metastasen kunnen bevorderen[18]. Deze postoperatieve inflammatie vergemakkelijkt tumorcel

adhesie aan het endotheel door de expressie van adhesiemoleculen op het endotheel te verhogen. In eerdere experimenten hebben wij aangetoond dat een pre-operatief dieet de expressie van deze adhesiemoleculen vermindert en beschermt tegen chirurgische inflammatie. Wij stelden de hypothese dat pre-operatieve CR de post-operatieve ontstekingsreactie vermindert en hierdoor de aanhechting van circulerende tumorcellen aan endotheel vermindert. BALB/c muizen werden twee weken op een dieet gezet bestaande uit 30% minder calorieën, alvorens ze te injecteren met tumorcellen. Deze tumorcellen werden in de milt gespoten zodat zij uiteindelijk via de bloedbaan in de lever tot uitzaaiingen uitgroeien. Tien dagen hierna bepaalden wij de hoeveelheid tumor in de lever van de twee groepen; met en zonder CR. Er bleek minder tumor in de lever aanwezig te zijn van muizen die voor de operatie op dieet waren gezet. Ook vonden wij minder expressie van het adhesie molecuul E-selectine in de levers van CR muizen. Dit adhesiemolecuul komt voornamelijk voor op endotheel cellen van bloedvaten. Tevens hebben wij aangetoond dat het serum van muizen die twee weken op dieet hebben gestaan, ertoe leidt dat er *in vitro* minder adhesie is van tumorcellen aan endotheelcellen. Hoewel wij geen direct bewijs leveren dat een dieet leidt tot minder tumor cel adhesie in de lever door een verlaagde E-selectine expressie, is er wel een directe relatie aangetoond in de literatuur die onze hypothese ondersteund[19]. De vraag blijft echter, waarom leidt CR tot een verlaging van de expressie van adhesiemoleculen? Recent hebben wij aangetoond dat CR leidt tot een verminderde productie van inflammatoire cytokinen en adhesiemoleculen in modellen van I/R schade in nier en lever. Daarnaast hebben wij aangetoond dat CR tot een verhoging leidt van enkele enzymen (o.a. HO-1), die beschermen tegen vrije zuurstofradicalen[20]. Andere hebben laten zien dat chirurgische schade ook tot de vorming van vrije zuurstofradicalen[21-22] leidt en dat deze mede verantwoordelijk kunnen zijn voor het ontstaan van de ontstekingsreactie. Concluderend leidt de beschermende werking van CR tegen oxidatieve schade mogelijk tot een reductie van de ontstekingsreactie en daardoor ook tot minder expressie van adhesiemoleculen.

Tot op heden is het merendeel van de gunstige effecten van CR aangetoond in diermodellen. In **hoofdstuk 7** onderzochten wij of een mild preoperatief CR dieet haalbaar is in de klinische setting en wat de effecten ervan zijn in het kader van een niertransplantatie. Hiervoor zijn levende nierdonoren gerandomiseerd tussen een pre-operatief dieet of vrije voedingskeuze voor de operatie. Zeventien mensen kwamen in aanmerking voor een preoperatief dieet dat vier dagen voor de operatie begon. Drie dagen voor de operatie werd de calorische inname verminderd met 30%, en 24 uur voor de operatie mocht men alleen water drinken. Dertien patiënten mochten die vier dagen zelf kiezen wat en hoeveel ze aten. De resultaten van deze studie laten zien dat het gebruikte dieet logistiek haalbaar is zonder dat het welzijn en de dagelijkse werkzaamheden

van deze nierdonoren wordt benadeeld. Een van de moeilijkheden in de kliniek is te zorgen dat mensen zich aan hun dieet houden. Aangezien de door ons onderzochte groep bestond uit mensen die vrijwillig een nier afstaan, beschikten wij over zeer gemotiveerde personen. In onze proefdiermodellen vonden wij dat CR de nier beschermt tegen I/R schade. Analoog hieraan verwachtten wij dat de nieren van CR-donoren een betere nierfunctie zouden hebben dan nieren van gewonen donoren. Om dit te testen hebben wij van alle nieren die getransplanteerd zijn op dag één na de transplantatie de nierfunctie gemeten door middel van nierscans en gedurende één maand de nierfunctie bepaald. Wij hebben in vergelijking met de gewone organen geen verschil in transplantaatfunctie gevonden. Concluderend stellen wij dat het dieet haalbaar is in de klinische setting. Verder onderzoek moet gericht worden op de duur en samenstelling van een dieet dat bij mensen vergelijkbare effecten geeft als in dieren. Daarnaast dient men de achterliggende mechanismen te bestuderen, die leiden tot bescherming. Wanneer deze bekend zijn zou men een mimeticum kunnen ontwikkelen die het diëten in de humane situatie onnodig maakt.

In **hoofdstuk 8** zijn we nader ingegaan op de effecten van een pre-operatief dieet bij post-operatieve inflammatie, in de levende nierdonoren zoals beschreven in het vorige hoofdstuk. Wij veronderstelden dat een pre-operatief dieet de post-operatieve ontstekingsreactie zou verminderen. Voor en na de operatie zijn het aantal en het soort witte bloedcellen bepaald evenals de productie van cytokinen en de waarde van C-reactive protein (CRP). Volbloed van voor en van na de operatie is gestimuleerd met lipopolysaccharide en de hoeveelheid geproduceerde cytokinen is hierin bepaald. We vonden een duidelijke trend tot minder stijging van het leukocytenaantal na de operatie in de dieetgroep. Het is bekend dat het aantal leukocyten na de operatie stijgt en dat de piek hiervan op dag 1 na de operatie ligt[23]. Deze piek was significant lager in de dieetgroep, echter een analyse van alle tijdspunten was niet significant. Interleukine-6 (een belangrijk proinflammatoir cytokine) en CRP waarden waren niet beïnvloed door het dieet. Interleukine-8 (IL-8) waarden waren echter wel significant hoger in de dieetgroep voor de operatie en erna. Een kleine stijging in IL-8 waarden kan beschermen tegen een grotere stijging van IL-8 waarden daarna[24]. Muizen die altijd een overproductie van IL-8 hebben, recruteren minder witbloedcellen op de plaats van een ontsteking[25]. Wij veronderstellen dat de hogere IL-8 waarden in onze dieetpatiënten beschermden tegen de hogere waarden na de operatie. Stimulatie van pre-operatief bloed liet zien dat het dieet de reactiviteit van het bloed op een inflammatoire stimulus niet beïnvloedde. In post-operatief verkregen bloed vonden we wel een verschil. Tumor necrose factor alpha (TNF-α, proinflammatoir cytokine) productie lager was in bloed van dieetpatiënten ten opzichte van de controlegroep. Dit ondersteunt onze hypothese dat een pre-operatief dieet de post-operatieve inflammatie kan verminderen. Met deze studie

hebben wij aangetoond dat een relatief mild pre-operatief dieet effect kan hebben op post-operatieve inflammatie. In toekomstige klinische studies zal er moeten worden onderzocht wat de optimale vorm van het dieet is en voor welke patiënten groepen het inzetbaar kan zijn.

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TM van Ginhoven, T Schepers, H Obertop, CHJ van Eijck. Delayed closure of complex duodenal injuries by a foley ballon catheter duodenostomy. Digestive surgery 2006; 23:150-153

PhD Portfolio

Name PhD student: Tessa Malaika van Ginhoven
Erasmus MC Department: Surgery
Research School: Molecular Medicine

PhD period: 01-07-2007 tot 01-07-2010
Promotor(s): Prof.dr. J.N.M. IJzermans
Dr. R.W.F. de Bruin

1. PhD training

	Year	Workload (ECTS)
General courses		
Laboratory animal science	2008	5.7
Classical Methods for Data-analysis	2009	5.7
Good Clinical Practice	2007	1.5
Presentations conferences		
National conferences	2007	1.0
National conferences	2008	3.0
International conferences	2008	1.0
National conferences	2009	3.0
International conferences	2009	3.0
National conferences	2010	2.0
International conferences	2010	1.0

2. Teaching

	Year	Workload (ECTS)
Lecturing		
Teaching (operating room nurses in training)	2007	0.3
Supervising practicals and excursions, Tutoring		
Supervising anatomy practicals (Medical students)	2008	0.2
Supervising first aid examinations (Medical students)	2009	0.2
Supervising first aid examinations (Medical students)	2010	0.2
Supervising Master's theses		
Bachelor thesis (Nutrition and dietetics)	2007	5.0
Bachelor thesis (Higher Laboratory education)	2008	5.0
Master thesis (Medicine)	2010	5.0

Curriculum Vitae

Tessa Malaika van Ginhoven werd op 13 december 1980 geboren te Dar es Salaam, Tanzania. In 1999 slaagde zij cum laude voor het VWO-eindexamen aan het St. Stanislas college te Delft. Hierdoor kon zij zonder loting beginnen aan de studie geneeskunde (Erasmus Universiteit, Rotterdam). Tijdens haar studie werkte zij als practicumassistent voor de afdeling anatomie. Alvorens aan haar co-schappen te beginnen, heeft zij enkele maanden onderzoek gedaan naar de bijwerkingen van een anti-malaria medicijn aan de Travel Clinic (Dr. D. Overbosch). Haar keuze-co-schap chirurgie volgde zij in het Reinier de Graaf Gasthuis te Delft (Dr. L. Stassen).

Na het behalen van haar artsexamen (cum laude), heeft zij een jaar als ANIOS chirurgie gewerkt in het Reinier de Graaf Gasthuis (Dr. L.P.S Stassen). In deze periode is naast het enthousiasme voor wetenschappelijk onderzoek, ook een passie voor kitesurfen ontstaan. Na de perioden in Delft heeft zij drie maanden als ANIOS op de afdeling chirurgie van het Erasmus MC gewerkt (Prof.dr. J.N.M. IJzermans) alvorens zij begon aan haar promotieonderzoek bij de afdeling experimentele chirurgie (Prof. dr. J.N.M. IJzermans, Dr. R.W.F. de Bruin). In dezelfde periode heeft zij haar zwarte band tae kwon do behaald.

Sinds1 juli 2010 is zij in opleiding tot chirurg in het Erasmus MC te Rotterdam (Opleider: Prof.dr. J.N.M. IJzermans). Vanaf 1 juli 2012 zal zij haar opleiding voortzetten in de Reinier de Graaf Groep te Delft (Opleider: Dr. M. van der Elst).