The nutritional reflex : influencing gut barrier function and inflammation

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influencing gut barrier function and inflammation

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The Graduate School VLAG

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Contents

Chapter 1

The inflammatory response and gut barrier function

Introduction

The inflammatory response comprises a complex set of cellular and humoral interactions that can arise in any tissue in response to bacterial infection, trauma or injury[1]. Although a fierce protective immune response is essentially life-preserving, the degree of inflammation has to be carefully managed. An insufficient response promotes infection, whereas an exaggerated inflammatory response causes acute and chronic inflammatory syndromes. Therefore, the proinflammatory response is tightly controlled in order to generate a sufficient fierce response against foreign invaders which is controlled localized by counter-regulatory mechanisms including cytokines, hormones and the autonomic nervous system [2-5].

Clinical syndromes such as sepsis are characterized by an inappropriate and dysregulated inflammatory response that cannot be locally contained[6]. An initial overwhelming inflammatory response leading to tissue damage, increased vascular permeability and shock is followed by a state of immune "paralysis", termed as the compensatory anti-inflammatory response syndrome (CARS), in which patients remain at risk of developing potentially lethal secondary or opportunistic infections[7, 8] (Fig. 1)

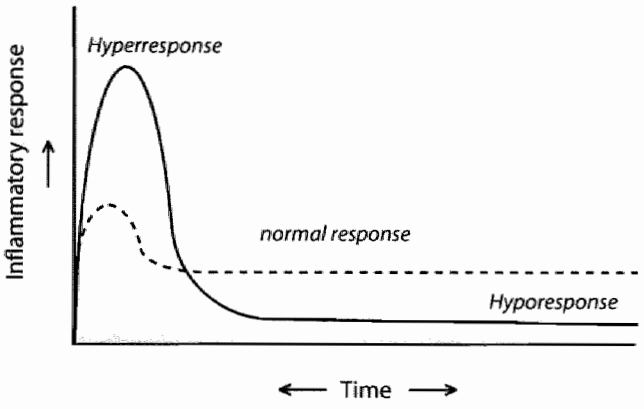

Figure 1:

An initial excessive release of inflammatory mediators upon (microbial) stimuli termed the systemic inflammatory response syndrome (SIRS) is often followed by a compensatory anti-inflammatory response (CARS) in which the immune system does not adequately respond to inflammatory stimuli.

In the last decades, sepsis has become an increasing clinical problem particularly in the elderly with an incidence of approximately 700,000 people annually in the US[9]. Major surgery predisposes for the sepsis syndrome that has become the leading cause of death in non-cardiac ICU's with a mortality of 30-50% (in optimally treated patients). The source of infection remains elusive in many patients, although the bacteria held responsible for this potentially lethal syndrome, frequently originate in the gut. These clinical observations have led to the hypothesis that the gut is the motor or origin of sepsis [10, 11].

It is believed that homeostasis in the gut between bacteria in the intestinal lumen and the gut wall is disrupted by factors such as ischemia, leading to initiation of an unwanted inflammatory response [12, 13]. Dysregulation of this local inflammatory process by multiple

sequential events or dysfunction of counter-regulatory mechanisms may then cause systemic spread of inflammatory mediators and bacterial toxins, leading to tissue damage and organ dysfunction.

In the past decades, therapies have focused on inhibition of the dysregulated hyperinflammatory response, however, such interventions have not been shown to be successful in a clinical setting[14]. Part of this may be attributed to heterogeneous patient groups included, but timing of therapy seems also to be an important factor[15]. In most patients with clinically established sepsis, the inflammatory cascade has been activated to such an extent that anti-inflammatory therapies were started too late. Therefore, simple, safe and effective therapies designed to prevent hyperactivation of the inflammatory response or interventions targeting late mediators in the septic cascade are needed [16, 17].

Recognition of microbial structures and routes of entry

The intestinal lumen is an enormous reservoir of bacteria, bacterial toxins and foreign antigens that need to be strictly separated from the sterile interior of the host to prevent an unwanted and potentially devastating inflammatory response. On the other hand, ingested nutrients have to be absorbed from the intestinal lumen and utilized in order to survive. Consequently, a delicate equilibrium exists within the gastrointestinal tract that is easily disrupted. Preventing invasion of foreign or endogenous threats from the intestinal lumen to the sterile "milieu interieur" of the host is achieved via several mechanisms.

First of all, a physical barrier is formed by the mucus on intestinal cells. Mucus is secreted onto the epithelial surface and lubricates movement of ingested nutrients along the digestive tract. It is secreted by specialized epithelial cells arranged into glands in the mouth and esophagus, and by individual goblet cells in the intestine. Mucus is a viscous material composed of water and glycoprotein. It protects the mucosal cells in the stomach and intestine from autodigestion by digestive enzymes, noxious stimuli and bacterial and fungal invasion (Fig. 2) [18]. Next, the mucosal epithelial cells form a single layer of intestinal immune cells that are "glued" together via tight junctions. Tight junctions consist of a cluster of proteins, including transmembraneous proteins such as claudin and occludin and zonula occludens (ZO) proteins [19, 20]. Zonula occludens proteins are important anchoring sites for intracellular actin filaments such as F-actin that preserve cytoarchitecture[21]. These tight junctions control the paracellular route of entry and can be vigorously disrupted by ischemic damage or release of proinflammatory cytokines such as TNF-α and IFN-γ, leading to loss of intestinal barrier integrity[22-24].

Next to this physical or anatomical barrier, there is also an immunological barrier. The mucosal surface is covered with a layer of secretory immunoglobulin type A (IgA) and secretes proteins such as lipopolysaccharide binding protein (LBP) or small antimicrobial peptides, such as defensins to fence off foreign antigens directly[25, 26]. Furthermore, lymphoid tissue, also termed gut-associated lymphoid tissue (GALT) is present in the intestinal lamina propria and forms clusters of lymphoid follicles in the small intestine, primarily the distal ileum, known as Peyer's patches. The GALT consisting of various immune cells, such as M cells, B and T cells and dendritic cells continuously monitors the mucosa for invading bacteria and are the first line of the immune response to invading dangers from the intestinal lumen[27].

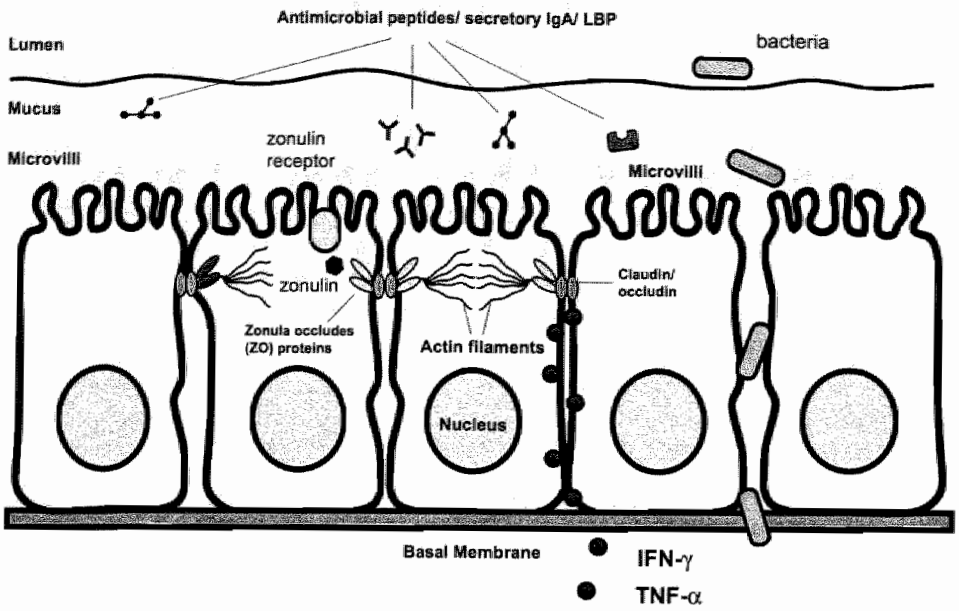

Figure 2:

The intestinal barrier consists of mucus, antimicrobial peptides on mucosal cells and the single layer of intestinal cells connected via tight-junctions. Release of inflammatory cytokines such as TNF-α and IFN-γ can breakdown tight-junction proteins leading to loss of intestinal barrier function.

Besides these intrinsic defense mechanisms, another important external protection is provided by a well-balanced relation within the intestinal lumen between "beneficial" (mostly anaerobic) bacteria and potential pathogenic bacteria such as *Escherichia coli* [28, 29]. Disruption of this balance by fasting, administration of antibiotics or use of antacids, promotes growth of potential pathogens that may be detrimental for the host.

Loss of gut barrier function

The paracellular route of entry in the gastrointestinal tract, controlled by tight junctions is easily affected under pathological conditions leading to translocation of bacteria and bacterial toxins [30-33]. Direct clinical confirmation on the role of gut barrier function loss reflected by bacterial and bacterial toxin translocation is scarce due a lack of appropriate tools to quantify and measure gut barrier integrity. However, there is a lot of circumstantial evidence showing that increased intestinal permeability is involved in development of various clinical inflammatory syndromes. Furthermore, experimental evidence describing the role of gut barrier failure in the pathophysiology of inflammatory diseases is abundant [34-38]. Several conditions are associated with loss of gut barrier function, such as hemorrhage, burns, lack of intraluminal bile salts and starvation [39-43].

One of the important mechanisms leading to breakdown of tight junction proteins in the gastrointestinal tract is release of inflammatory cytokines such as TNF-α and IFN-γ (Fig. 2) [22, 23]. It appears that exposure of intestinal cells to these inflammatory mediators decreases

expression of zonula occludens proteins (ZO-1 in particular) and changes in phosphorylation of occludin[24] (Fig 2). Although these results were obtained in a highly controlled situation in cells in vitro, our group also showed in vivo in a model of systemic hypoperfusion that enhanced levels of proinflammatory cytokines are paralleled by loss of ZO-1 in the ileum[35, 44]. Another regulating pathway of the paracellular route is governed by zonulin. This 47kD protein induces disassembly of tight junctions and subsequently increases intestinal permeability in the acute phase of celiac disease[31, 45]. It has similarities with a protein released by *Vibrio cholerae* which interacts with a specific surface receptor leading to polymerization of actin filaments[46]. It is believed that dysregulation of this conceptual zonulin pathway may be involved in developmental and intestinal disorders leading to subsequent tissue inflammation.

Pathogen-associated molecular patterns and Toll-like receptors

When bacteria or bacterial products invade the sterile host, an inflammatory response is mounted via a receptor-mediated pathway. Bacteria have distinctive characteristics, called pathogen-associated molecular patterns or PAMP's that are recognized by immune cells via Toll-like receptors (TLR's). Toll proteins were firstly identified in flies (Drosophila) as activators of intracellular signaling and share a great homology with the interleukin (IL)-1 receptor[47, 48]. Later, receptors similar to Toll were identified in animals and humans and until now in total 13 mammalian TLR's have been identified that are able to detect a wide range of (micro) organisms[49-55]. Ligands for TLR's include microbial structures such as lipopolysaccharide (LPS), lipoteichoic acid (LTA), peptidoglycans (PGN), bacterial DNA, flagellates, but also non-bacterial components such as zymosan, oxLDL and heat shock proteins trigger intracellular signaling via TLRs[56, 57]. Localization of TLR's on immune cells is partly related to the molecular patterns of their ligands; TLR2, 4 and 6 are located on the cell surface, just as their ligands (LTA, endotoxin and PGN respectively) and recruited to phagosomes upon stimulation. On the other hand, TLR3, TLR7 and TLR9, involved in recognition of nucleic-acid-like structures are intracellularly expressed [51, 58, 59]

Endotoxin or LPS, the major constituent of the lipid bilayer of Gram-negative bacteria[60] is recognized by TLR4 and has since long been regarded essentially involved in development of various inflammatory diseases, given the frequency of Gram-negative infections and increased circulating levels of endotoxin in septic patients [61, 62]. The endotoxin molecule consists of a hydrophilic polysaccharide chain which is covalently bound to the biological active lipid component (Lipid A)[63, 64]. Cellular activation is initiated by shuttleling of LPS-aggregates via a lipopolysaccharide binding protein (LBP)[65, 66]. Next, LBP catalyzes the transfer of endotoxin to CD14, a membrane-bound and soluble glycosylphosphatidylinositol protein without a transmembraneous domain [67, 68]. Subsequently, endotoxin is transferred to TLR4-MD-2 complexes that dimerize and recruit downstream signaling molecules including adaptor molecules such as Myd88 and Mal, kinases such as Interleukin-1 receptor-associated kinases (IRAKs) and TNF-receptor associated factor 6 (TRAF-6) ultimately resulting in phosphorylation of IκB, leading to translocation of nuclear factor kappa B (NF-κB) to the nucleus[69]. This intracellular activation mechanism is not solely activated by LPS-TLR4-MD2 complexes but various TLRs use similar intracellular signaling pathways (Fig. 3).

Next to endotoxin, toxins derived from Gram-positive bacteria such as lipoteichoic acid (LTA) and peptidoglycan (PGN) gained much more attention the last decades, since the number

of Gram-positive infections leading to severe inflammatory syndromes has dramatically increased[70, 71]. LTA and PGN can be considered as the Gram-positive counterparts of endotoxin and are part of the outermembrane of Gram-positive bacteria.

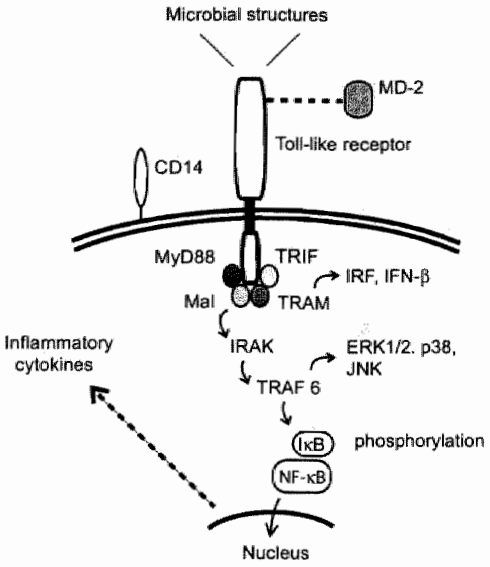

Figure 3:

Microbial structures bind to specific Toll-like receptors (TLRs) on inflammatory cells. Lipopolysaccharide requires the ligand MD-2 for binding and signalling via TLR4. Intracellular signalling is established via adapter molecules such as Myd88, TRIF, Mal or TRAM, kinases such as Interleukin-1 receptor-associated kinases (IRAKs) and TNF-receptor associated factor 6 (TRAF-6) ultimately resulting in phosphorylation of IκB , leading to translocation of nuclear factor kappa B (NF-κB) to the nucleus. This the causes release of various inflammatory cytokines.

Although LTA and PGN initiate an inflammatory response via TLR's similar to endotoxin, experimental evidence that these cell-wall components of Gram-positive bacteria are as toxic as LPS is limited. LTA and PGN supposedly trigger signal transduction via CD14 and an interplay between TLR2 and TLR6 [72, 73]. However, recent evidence suggests that phagocytosis and subsequent activation of nucleotide-binding oligomerisation domain (Nod) molecules is a also an important route[74, 75].

However, release of these Gram-positive derived toxins could not explain the excessive activation of the inflammatory response in sepsis and in the quest to find potential other causes for dysregulation of the host response, it was found that bacterial DNA might play a role. Bacterial DNA is the common denominator of Gram-positive and Gram-negative bacteria and has an immunostimulatory effect on various immune cells, including dendritic cells, monocytes and macrophages[76]. Bacterial DNA, or prokaryotic DNA differs from eukaryotic DNA by an increased number of unmethylated cytosine-guanine motifs (CG), connected via a phosphate molecule [76]. Bacterial DNA and synthetic oligodinucleotides (ODN) containing unmethylated CpG motifs react with cells via TLR9[52]. CpG-DNA structures move into the cell via early endosomes by an unexplained mechanism. Subsequently TLR9 is recruited from the

endoplasmatic reticulum to these CpG-containing structures [77]. Unmethylated CpG motifs then bind to TLR9 leading to a Th1-type immune response via translocation of NF-κB to the nucleus. Bacterial DNA and CpG-ODN have a potent adjuvant effect in vaccination, increases bacterial and parasital killing and is potentially a therapeutic agent in models of allergy and cancer [78-80]. Conversely, CpG-ODN induces a strong inflammatory response in D-galactosamine-sensitized mice leading to a lethal toxic shock, whereas it is considered to be rather non-toxic in unsensitized mice [81].

It has been suggested by some, that TLR ligands such as endotoxin induce a "two-hit" phenomenon together with another stressor leading to hyperactivation of the host inflammatory response [82-84]. This theory would fit in the exaggerated response observed in patients with SIRS or sepsis. However, there is also a substantial amount of evidence showing that sequential exposure to TLR ligands induce a phenomenon called tolerance via suppressor of cytokine signaling (SOCS) proteins [84-86]. The exact underlying mechanisms are not fully elucidated, but dosages and times of administration seem to be of importance.

The inflammatory response

Recognition of bacterial structures by pattern recognition receptors is the first step in the effective host response to attack, kill and clear the invading microorganism(s). Release of proinflammatory mediators by macrophages and neutrophils is central in the initial and rapid immune response. One of the key-mediators released early after exposure to bacterial structures is tumor necrosis factor (TNF) α [87, 88]. This cytokine was discovered as serum factor, released by macrophages after stimulation with endotoxin and caused hemorrhagic necrosis in experimental sarcomas[89]. Interestingly, TNF-α was found to be identical to a humoral mediator described by others and known as cachectin, responsible for cachexia in chronic infection[90].

TNF-α is strongly induced by endotoxin and has a diverse role in the inflammatory response. It activates macrophages and stimulates neutrophils resulting in enhanced phagocytic and cytotoxic activities, adhesion and migration [91, 92]. TNF-α is considered to be pivotal in the pathophysiology of SIRS and the sepsis syndrome since high TNF-α levels are associated with organ failure and death [93, 94]. Release of TNF-α is followed by release of interleukin (IL) 6.

This 21kD glycoprotein has both pro-and anti-inflammatory properties and plays an important role in instigation of the acute phase response [95] Elevated plasma IL-6 levels are not only observed after a microbial trigger but can also occur in the absence of clinically defined infection in trauma patients and following major surgery [96]. Levels of IL-6 also correlate with severity of disease and outcome in sepsis [97]. Next to TNF-α and Il-6 exposure to PAMPs elicts a complex orchestrated network of responses in which complement, (such as complement-factor C5a), chemokines, adhesion molecules (selectins), heat shock proteins (HSP70/ 90) and other (late) cytokines such as Interferons (for example interferon (IFN)-γ) and high-mobility group box 1 (HMGB-1) protein play a role [4, 16, 98-103]. This cascade of responses needs to be tightly regulated since, an exaggerated response causes uncontrolled damage to intestinal tight junctions and host cells, such as liver cells, alveolar cells, tubulus epithelium in the kidney and endothelial cells, leading to organ failure [104-110].

The magnitude of the proinflammatory response is restrained by a powerful anti-inflammatory response. This anti-inflammatory response consists of cytokines including IL-10 and soluble receptors such as soluble TNF receptors [111]. These anti-inflammatory mediators restrict and

localize the inflammatory response and prevent unnecessary damage to the surrounding tissue. On the other hand, exaggerated suppression of the inflammatory response may lead to a hyporesponsive state with potential deleterious effects[112, 113].

Neutralization and clearance of bacterial toxins

An important physiological regulatory mechanism to limit activation of inflammatory cells by bacterial toxins and prevent systemic spread of these microbial products is the binding and detoxification of microbial products by lipoproteins[114]. These lipid transport particles consist of triglycerides or cholesterol esters surrounded by a phospholipid membrane containing apolipoproteins (Fig. 4).

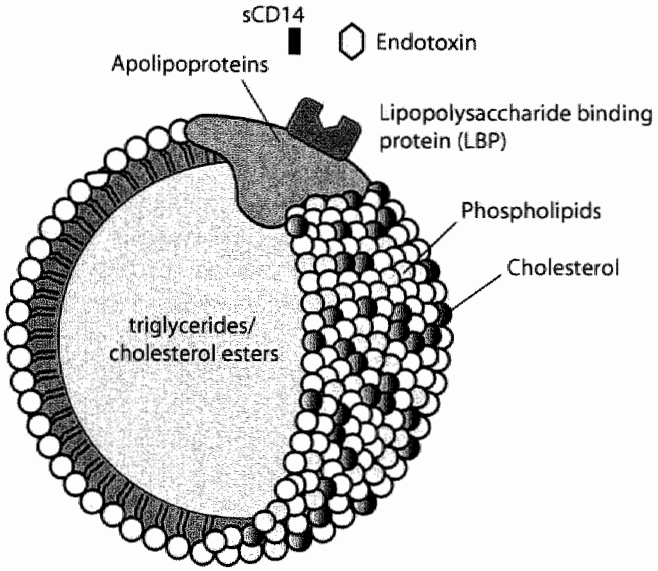

Figure 4:

Lipoproteins consist of triglycerides and cholesterol esters surrounded by phospholipids. In this phospholipids membrane, apolipoproteins are anchored. Bacterial toxins such as endotoxin can bind to triacylglycerol-rich lipoproteins via lipopolysaccharide binding protein (LBP) and apolipoprotein B.

Lipoproteins are categorized based on their density and have specific properties. High-density lipoproteins (HDL) were the first to be recognized to bind endotoxin (LPS)[115-117]. However, later on it has been demonstrated that also other classes of lipoproteins (low-density lipoproteins (LDL), very low-density lipoproteins (VLDL) and postprandially formed chylomicrons) have similar properties[118, 119]. Exogenous administration of (recombinant) lipoproteins in human and animal models significantly reduces the inflammatory response to endotoxin and attenuate endotoxin-induced organ damage[120-123]. In contrast, studies showing that endogenous enhancement of lipoproteins via a lipid-rich nutrition are less clear-cut. Although hypertriglyceridemia does not seem to protect against a bolus injection of endotoxin, preincubation of endotoxin with endogenous lipoproteins clearly attenuated the in vivo response to endotoxin [124, 125]. A difference in kinetics between activation of macrophages by endotoxin and binding of

endotoxin by endogenous lipoproteins is a likely contributor for these differences.
Lipoproteins bind and neutralize bacterial toxins via a complex of proteins. At first it was suggested that endotoxin was bound by lipoproteins via a simple "leaflet insertion model" in which a lipid-lipid interaction between the lipid A part of endotoxin and phospholipids in the outermembrane of lipoproteins reduced toxicity of endotoxin[126]. Later it has been found that LBP is also importantly involved in the binding and transfer of endotoxin towards lipoproteins[127]. The LBP-LPS complex binds to apolipoproteins which are anchored in the outermembrane of lipoproteins [128, 129]. Binding of endotoxin by LBP in the presence of lipoproteins shunts endotoxin away from inflammatory cells, thereby thwarting off initiation of an inflammatory response. (Fig. 5)

A second important function of lipoproteins is the removal of bacterial toxins from the bloodstream via the liver[130-132]. Bacterial toxins, bound and inactivated by lipoproteins, are transported via lymph ducts and blood vessels to the liver in which they are excreted into the bile, via the space of Disse. In this way, unwanted activation of inflammatory cells by circulating bacterial toxins is prevented.

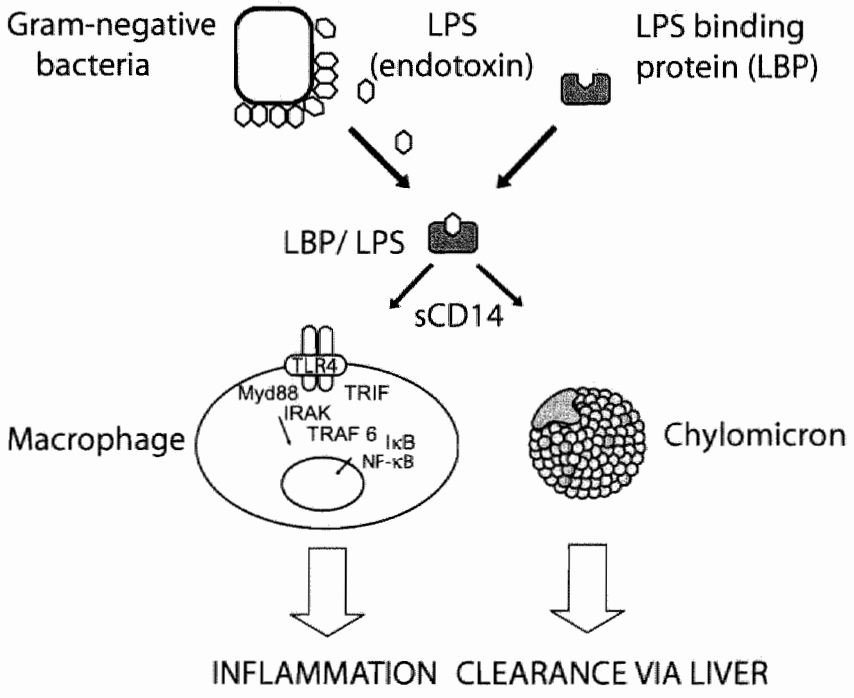

Figure 5:

Lipoproteins can shunt endotoxin away from inflammatory cells by neutralization via LBP and apolipoprotein B, leading to a reduced inflammatory response.

The central nervous system

Excessive release of inflammatory mediators following activation of inflammatory cells by bacterial products is controlled by the central nervous system. Supposedly, inflammatory mediators such as TNF-α, IL-6 and others activate afferent vagal pathways leading to a variety of responses[133-135]. The hypothalamic-pituitary-adrenal signaling pathway (HPA-axis) is activated causing an instantaneous release of serum corticosteroids that leads to inhibition of (excessive) inflammation[136, 137]. In addition, to this afferent or sensory function during inflammation, it was recently identified that there also is an efferent vagal system involved in regulation of the inflammatory response[138, 139] (Fig. 6). Release of acetylcholine, the principal neurotransmitter of the vagus nerve activates nicotinic receptors on macrophages resulting in a decreased release of both early inflammatory mediators (TNF-α) but also late mediators such as HMGB-1 [140, 141]. Intracelullarly, nicotinic receptor activation is associated with activation of the transcription factor STAT3 which is phosphorylated by tyrosine kinase Jak2, leading to the anti-inflammatory effect[142]. Mechanical stimulation of these efferent vagal fibres via direct electrical stimulation or pharmacological stimulation of nicotinic receptors via agents such as CNI-1493 significantly reduces the systemic inflammatory response to endotoxic shock and is called the cholinergic anti-inflammatory pathway [140, 143, 144]. Activation of this neural anti-inflammatory pathway also reduces the inflammatory response and its sequelae during septic peritonitis and following hemorrhagic shock [138, 145, 146]. Although external stimulation of the efferent vagal pathway strongly reduces inflammation, its physiological role remains yet to be understood. Supposedly, vagal afferents are activated by release of cytokines such as IL-1β leading to direct stimulation of the cholinergic anti-inflammatory pathway, however evidence supporting this hypothesis is ambiguous[135, 147-149].

Antiinflammatory therapies

Antibodies and antagonists

Despite ongoing research on development of lethal host responses during sepsis and characterization of the underlying mechanisms, therapeutic options for patients with a fulminant septic syndrome remain limited. One of the many challenges in designing a therapeutic tool for this heterogenous group of patients is timing of treatment. For example, an anti-inflammatory treatment to reduce the hyperactivated inflammatory response would be beneficial in the early phase of sepsis, however, such an effect is not desirable at a later stage when the innate immune system frequently loses the ability to effectively kill invading organisms[150]. The initial stage of activation and the proinflammatory peak evolving shortly after trauma or injury is short-lived, leading to a relatively small therapeutic window. Therefore, anti-inflammatory treatments using antibodies and antagonists have to be started just at the right moment, since initiation of such treatments after such a proinflammatory peak would have devastating effects. For example, animal studies show a beneficial effect of anti TNF-α treatment on endotoxin mortality, however, in the clinical situation in patients with sepsis, TNF-α antagonists were even reported to increase mortality [151, 152]. Part of this may be attributed to the heterogenousity of patient groups, however, timing of treatment

may also be an important contributing factor. This is why late mediators, such as HMGB-1 and macrophage migration inhibition factor (MIF) have gained more attention. Antibodies against some of these factors have proven to be successful in reduction of endotoxin-induced mortality in an experimental setting [100, 153, 154]. However, the clinical relevance of using these antibodies in septic patients needs to be further investigated.

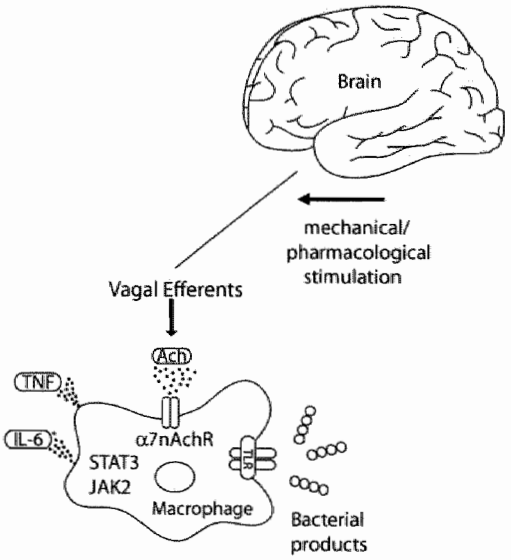

Figure 6:
Mechanical or pharmacological activation of efferent vagal fibres causes increased release of acetylcholine. Acetylcholine subsequently binds to specific nicotinic receptors on the surface of inflammatory cells leading to intracellular signalling via STAT3 and JAK2

Nutrition and endogenous anti-inflammatory mechanisms

Besides inhibition of inflammation via antibodies or antagonist directed against inflammatory mediators or their receptors, stimulation of endogenous compensatory mechanisms is another appealing option. For example the recently discovered cholinergic anti-inflammatory pathway can be stimulated pharmacological or mechanically. Furthermore, the complement cascade can be inhibited by administration of anti-C5a. Previously our group showed that endogenously formed lipoproteins such as LDL, VLDL and chylomicrons effectively neutralize bacterial toxins such as endotoxin via apolipoprotein B on the surface of these lipid transport particles and LBP[128, 129]. The amount of lipoproteins that scavenge bacterial toxins can be upregulated via external administration by an enteral or parenteral route [123, 125, 155]. For example administration of recombinant HDL inhibits the effects of endotoxin in humans and reduce C-reactive protein proinflammatory activity[121, 156].

Administration of probiotics is another way of supporting an endogenous compensatory mechanism that prevents an unwanted inflammatory response. Probiotics are defined as live microorganisms that when ingested exert health benefits on the host through microbial actions. A lot of used strains normally reside in the intestinal lumen, but the growth of these microbes may be altered during disease states[157, 158]. Inhibition of pathogen adhesion, production of antimicrobial metabolites and reduction of inflammation via their DNA are

believed to be important characteristics that explain the beneficial effects of probiotic strains. However, there is a large variability in available strains and it is still elusive what the best strain or set of strains is that can be used in the clinical setting.
Besides administration of exogenous probiotics, endogenous *Lactobacillus* spp, *Bifidobacteria* and other beneficial anaerobes may be enhanced by ingestion of non-digestable food ingredients also known as prebiotics. Many non-digestable oligosaccharides such as inulin and oligofructose are commonly used. It has been shown that administration of prebiotics exert an anti-cancerous effect by stimulating apoptosis[159]. Furthermore, prebiotics combined with probiotic therapy has a synergistic effect and effectively can reduce the inflammatory damage in colitis in patients [160].
The concept that nutrition may be of importance in the reduction of inflammatory complications has been subject of investigation for many years. At first nutrition in the clinical setting was regarded as a means to provide proteins, energy and other essential nutrients to reduce muscle wasting and prevent depression of the immune system by starvation. Gradually, several specific substances such as long chain fatty acids, amino acids and nucleic acids have been added to the nutrional formula, since these ingredients can reduce the inflammatory response in some settings[161, 162]. Despite various clinical trials using this "immunonutrition", there has been much controversy about the effects on inflammation and inflammatory complications in a clinical setting [163-168]. However, there is a general believe that infectious complications can be reduced by immunonutrition, especially in patients undergoing surgery for upper gastrointestinal cancer or trauma.

Aim of this thesis

As outlined in this chapter, the early inflammatory response and subsequent organ damage is pivotal in development of acute and chronic complications following (major) surgery or trauma.
In this thesis we study the effect of preexposure to CpG-DNA on the inflammatory response and intestinal integrity following hemorrhagic shock. Furthermore, the effects of two potential therapeutic interventions on inflammation and gut barrier function are explored in a model of hemorrhagic shock. First, the role of probiotic therapy in preservation of gut barrier integrity following systemic hypotension was analyzed. This study shows that although certain probiotic strains were capable of preserving the inflammatory response, however, there was an unwanted side-effect of increased translocation of *Lactobacillus* spp. Next, we investigated the effects of high-fat enteral nutrition on the inflammatory response and gut barrier function following hemorrhagic shock based on previous in vitro data from our group. The short-term and long-term effects of this lipid-rich nutrition were studied by looking at several inflammatory parameters, markers of gut barrier function and liver damage. Next, the underlying mechanism of protection of high-fat enteral nutrition was assessed in combined models of hemorrhagic shock, bile-duct obstruction and vagotomy.

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Chapter 2

Exposure to CpG-ODN exacerbates gut barrier loss and inflammation following systemic hypotension via an IFN-γ dependent route.

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Abstract

Background: Oligodeoxynucleotides (ODN) containing unmethylated CpG motifs modulate the immune response and affect intestinal inflammation. These effects of CpG-ODN may be of importance during ischemia of the gut, where inflammation and gut barrier function are prominent. Here, we investigate the effect of CpG-ODN during systemic hypotension and identify a role for interferon (IFN)-γ and TLR4 in the found effects

Methods: Control rats and those exposed to CpG-ODN or nonCpG-ODN were subjected to hemorrhagic shock. Inflammatory cytokines were measured in plasma, intestinal permeability and bacterial translocation were determined as marker of gut barrier function. The role of IFN-γ was investigated via anti IFN-γ antibodies and Toll-like receptor (TLR) 4 expression was determined via RT-PCR.

Results: CpG-ODN treatment and hemorrhagic shock significantly augmented IFN-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-6, and nitrite levels following hemorrhagic shock, while plasma IL-10 was reduced. Furthermore, intestinal permeability markedly increased and bacterial translocation was enhanced. These changes were accompanied with enhanced expression of TLR4 expression in the liver. Inhibition of IFN-γ reduced TNF-α ($p<0.05$), IL-6 ($p<0.05$), nitrite ($p<0.05$) and intestinal permeability following hemorrhagic shock and downreglated expression of TLR4 in CpG-treated animals.

Conclusion: CpG-ODN impairs gut barrier function and induces a "two-hit" phenomenon leading to an enhanced inflammatory response and exacerbation of gut barrier loss. We propose that release of IFN-γ following exposure to CpG-ODN primes inflammatory cells via TLR4 leading to increased inflammation and enhanced gut barrier failure following hemorrhagic shock.

Introduction

Bacterial DNA, a common denominator of bacteria is structurally different from eukaryotic DNA by the prevalence of unmethylated cytosine-phosphate-guanine dinucleotides, termed CpG motifs[1]. Both bacterial DNA and synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG motifs are immunostimulatory agents that induce inflammatory mediators such as TNF-α, IL-10, IL-12 and IFN-γ via Toll Like Receptor (TLR) 9 [2] [3] [4].
The Th1-like immune response induced by CpG-ODN has a potent adjuvant effect in vaccination, increases bacterial and parasital killing and is a therapeutic adjuvant in models of cancer [5] [6] [7] [8]. This immune-enhancing property of CpG-ODN may also lead to an unwanted and strong inflammatory response in D-galactosamine-sensitized mice leading to a lethal toxic shock, although it is regarded as nontoxic in normal mice even at high doses [9]. Besides the modulating effect on the systemic immune response, inflammatory processes in the intestine are also affected by bacterial DNA. Recently has been shown that CpG-ODN administration to mice before the onset of DSS-colitis ameliorated colitis and inhibited the induction of proinflammatory cytokines via CD4+ T-cells[10] [11]. The underlying mechanism of these local and systemic effects of CpG-ODN is not fully clear, although release of interferon gamma (IFN-γ) is thought be involved [12] [13].

Although manipulation of the immune response via CpG-ODN opens new therapeutic opportunities, enhancement of the host response is not always desired.
Patients undergoing major surgery for treatment of cancerous disease have a high postoperative morbidity, characterized by inflammatory complications[14]. Although the pathogenesis is not fully understood, an increased inflammatory response and inflammatory damage to the intestine due to systemic hypoperfusion are believed to be of importance [15] [16]. Manipulation of the immune response in such a setting by CpG-ODN may be detrimental and potentially lead to polymicorbial sepsis.
The current study was designed to investigate the effect of CpG-ODN on the inflammatory response and gut barrier function in rats following hemorrhagic shock. Furthermore, we studied the role of IFN-γ in the underlying mechanism of the found effects

Methods

Reagents

CpG oligodeoxynucleotide (ODN) (5'-TGACTGTGAACGTTCGAGATGA-3'+ phosphorothioate backbone [17]) and non-immunostimulatory nonCpG-ODN (5'-GCTTGATGACTCAGCCGGAA-3') was purchased from Eurogentec (Seraing, Belgium) and dissolved in sterile, pyrogen-free saline to a final concentration of 500 μM. A monoclonal antibody directed against rat IFN-γ was kindly provided by Dr. P. van der Meide (University Medical Center Utrecht, the Netherlands).

Animals

Healthy male Sprague-Dawley rats, weighing 303-425 grams (average 355 grams) were purchased from Charles River (Maastricht, the Netherlands) and housed under controlled conditions of temperature and humidity. Before the start of the experiments, rats were fed ad libitum with standard rodent chow and had free access to water. The experimental protocol

was performed according to the guidelines of the Animal Care Committee of the University of Maastricht and approved by the committee.

Isolation and stimulation of peritoneal macrophages

Sprague-Dawley rats (n=8) were not exposed (controls), injected with nonCpG (180 μg) or CpG (180 μg) with or without anti IFN-γ (5mg/kg) 18 hours prior to stimulation. Peritoneal macrophages were harvested under sterile conditions, washed five times in RPMI (GIBCO Europe, Paisley, UK) with 1% penicillin/ streptomycin and incubated in 96-well culture plates (Costar, Cambridge, MA) at 2 x 10^5 cells per well for 5 hours with LPS (1 ng/ml), LTA (1 μg /ml) or CpG-ODN (1 μM). After incubation, supernatants were collected and TNF-α was determined by sandwich ELISA [18].

NF-κB assay

Peritoneal macrophages were isolated as described above from rats injected with nonCpG, CpG-ODN or CpG-ODN and anti IFN-γ and stimulated with LPS (10 ng/ml) for 15 minutes. Next, nuclear extracts were isolated and p65 activation was quantified using an oligonucleotide-based ELISA (Active Motif, Rixensart, Belgium) according to the supplier's instructions.

Experimental design and hemorrhagic shock procedure

Rats were allocated to seven groups (n=7 per group) before the start of the experiments. A group of non-exposed rats and rats exposed to CpG by subcutaneous injection of 25 nmoles CpG-ODN in 0.5 ml saline (180 μg) were sacrificed as controls. Non-exposed rats and rats treated with nonCpG and CpG-ODN in equal amounts for 18 hours were subjected to hemorrhagic shock. Additionally, a group of non-exposed and CpG-treated rats were administered antibodies against rat IFN-γ (5 mg per rat). All animals were starved overnight and sacrificed at 4 hours after hemorrhagic shock.

A non-lethal hemorrhagic shock model was used as previously described [19 20]. In short, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p); the femoral artery was dissected and cannulated with polyethylene tubing (PE-10). Mean Arterial Pressure (MAP) and heart rate (HR) were continuously recorded during a 50-minute observation period. At the time of shock (t=0), 2.1 ml blood per 100 gram of body weight was taken at a rate of 1 ml/minute (representing 30-40% of the total blood volume). The severity of the hemorrhagic shock insult as reflected by changes in mean arterial pressure (MAP), heart rate (HR) and hematocrit was similar for all hemorrhagic shock groups and comparable with our earlier data, using the same hemorrhagic shock model [19 20]. At the time of sacrifice (t=4 hours), blood was taken and segments of small bowel were harvested for determination of gut permeability. Plasma was separated by centrifugation, frozen immediately and stored (-20°C) until analysis.

Cytokine analysis

TNF-α concentrations in supernatants of stimulated cells were determined using a sandwich-ELISA [18]. TNF-α, IFN-γ, IL-6 and IL-10 concentrations in arterial blood were determined using standard ELISA's for rat TNF-α and rat IFN-γ (both kindly provided by Hbt, Uden, the Netherlands), rat IL-6 (BD Biosciences, San Diego, CA) and rat IL-10 (Biosource, Camarillo, CA).

Measurement of NO· production

Nitrite (NO_2^-) in rat plasma was measured using Griess reagent. This primary oxidation product of NO· after reaction with oxygen was used as an indicator of NO synthesis.

Intestinal permeability and Microbiological Methods

Intestinal permeability for macromolecules was assessed by measuring translocation of the 44 kD enzyme horseradish peroxidase (HRP, Sigma) by the everted gut sac method as described [20]. Bacterial translocation to distant organs was assessed as described [19] [20]. In short, mesenteric lymph nodes (MLN), the mid-section of the spleen and liver-segment (IV) were collected aseptically in pre-weighed thioglycolate broth tubes (Becton Dickinson (BBL) Microbiology Europe, Maylan, France) in all rats. Tissue-fragments were homogenized and the entire suspension was transferred to agar plates (Columbia III blood agar base supplemented with 5% vol/vol sheep blood (BBL) (duplicate plates) and Chocolate PolyviteX agar (BioMérieux, Marcy L'Etoile, France)). After 48h incubation, colonies were counted, determined using conventional techniques, adjusted to tissue-weight and expressed as number of colony forming units (CFU) per gram tissue.

Statistical analyses

Bacterial translocation data are represented as median and range; all other data are represented as mean ± SEM. A Mann-Whitney U test was used for between-group comparisons. Differences were considered statistically significant at $p < 0.05$.

Results

Preexposure to CpG-ODN enhanced plasma IFN-γ levels after hemorrhagic shock.

Hemorrhagic shock was followed by a rise in circulating IFN-γ levels already after 90 minutes (data not shown) and became more pronounced at 4 hours after shock (1.3± 0.1 ng/ml) in control rats and rats pretreated with nonCpG (0.9±0.04 ng/ml), (Fig. 2). Interestingly, administration of CpG-ODN 18 hours prior to hemorrhagic shock doubled circulating plasma levels of IFN-γ at 4 hours after shock (2.6± 0.5 ng/ml, $^{\#}p<0.05$). In contrast, exposure to CpG-ODN alone resulted in detectable, however low plasma IFN-γ levels (0.03 ng/ml, $p=0.003$) after 18 hours.

Preexposure to CpG-ODN significantly elevated TNF-α, IL-6 and NO release in plasma after hemorrhagic shock, whereas IL-10 was down-regulated.

Hemorrhagic shock typically results in a TNF-α response that peaks at 90 minutes and rapidly fades [20] [21]. As expected, circulating TNF-α was no longer detectable in control rats and nonCpG-pretreated rats at 4 hours after hemorrhagic shock (Fig. 3 A), while circulating IL-6 levels were still detectable at low levels (control: 12±6 pg/ml and nonCpG: 12±5 pg/ml) (Fig. 3 B). Exposure to CpG-ODN alone led to a mild increase of plasma TNF-α levels (14±6 pg/ml) even after 18 hours after exposure, whereas IL-6 was not demonstrable (Fig. 3AB). In contrast, administration of CpG-ODN prior to hemorrhagic shock resulted in elevated TNF-α (68±13 pg/ml, $p<0.001$) and plasma IL-6 levels (171±33 pg/ml, $p<0.005$) compared with control shock rats at 4 hours after hemorrhagic shock. Next, nitrite (NO_2^-) was measured as marker for nitric oxide (NO) release. Administration of CpG-ODN alone led to elevated NO_2^-

levels after 18 hours compared with background levels in non-exposed controls. Hemorrhagic shock by itself enhanced NO_2^- levels (97±7 µM). However, hemorrhagic shock in CpG-ODN rats caused a significant rise in NO_2^- levels (169±38 µM, *p<0.05) (Fig. 3C). Preexposure to CpG-ODN resulted in a significant rise in circulating IL-10 levels (68±12 pg/ml) compared with background levels in controls (13±11 pg/ml, p<0.01), in line with previous in vitro studies[22]. Hemorrhagic shock resulted in a marked increase of plasma IL-10 in non-treated rats (148±13 pg/ml, *p<0.01) and nonCpG treated rats (133±7 pg/ml, *p<0.01) compared to rats not subjected to shock, (Fig. 3D). However, this increase of circulating IL-10 after hemorrhagic shock was not observed in rats exposed to CpG-ODN (62±4 pg/ml). These data show that although exposure to CpG-ODN alone has relatively modest effects, inflammation is strongly aggravated in combination with hemorrhagic shock.

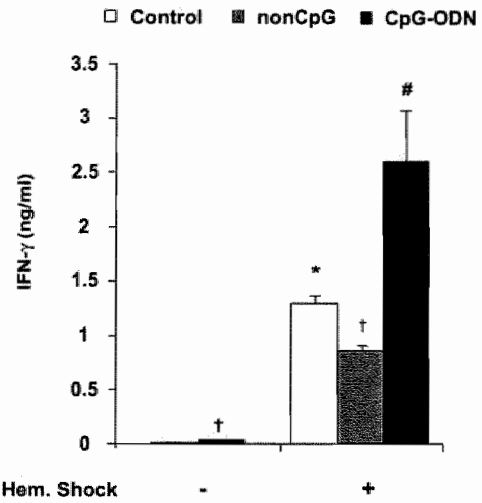

Figure 1:

*Administration of CpG-ODN alone resulted in detectable, significantly increased plasma IFN-γ (34 ± 2 pg/ml, †p=0.003) compared with non-exposed control rats after 18 hours. Hemorrhagic shock caused a rise of circulating IFN-γ levels at 4 hours after shock in control (1298 ± 61 pg/ml, *p<0.01) and nonCpG treated rats (867 ± 37 pg/ml, †p<0.01) compared with non-exposed control rats not subjected to shock. Preexposure to CpG-ODN followed by hemorrhagic shock doubled circulating plasma levels of IFN-γ at 4 hours after shock (2597 ± 469 pg/ml, #p<0.05) compared with control rats and nonCpG treated rats (#p<0.05) subjected to hemorrhagic shock.*

Preexposure to CpG-ODN impairs intestinal barrier function and further enhances hemorrhagic shock-induced intestinal permeability.

Intestinal permeability for the 44 kD macromolecule horseradish peroxidase (HRP) was significantly elevated following CpG-ODN exposure (5.5±0.5 µg/ml) compared with controls (1±0.1 µg/ml, p=0.006), (Fig. 4). As expected, hemorrhagic shock resulted in an increased leakage of HRP (36±3 µg/ ml). Preexposure to CpG-ODN followed by hemorrhagic shock almost doubled leakage of HRP (60±11 µg/ ml, p<0.01) compared with nonCpG treated shock rats (33±1 µg/ ml). In line, exposure to CpG-ODN caused a mild bacterial translocation in all rats with a median total number of 103 colony forming units (cfu)/ gram tissue, whereas bacterial cultures of control rats were sterile (Table 1).

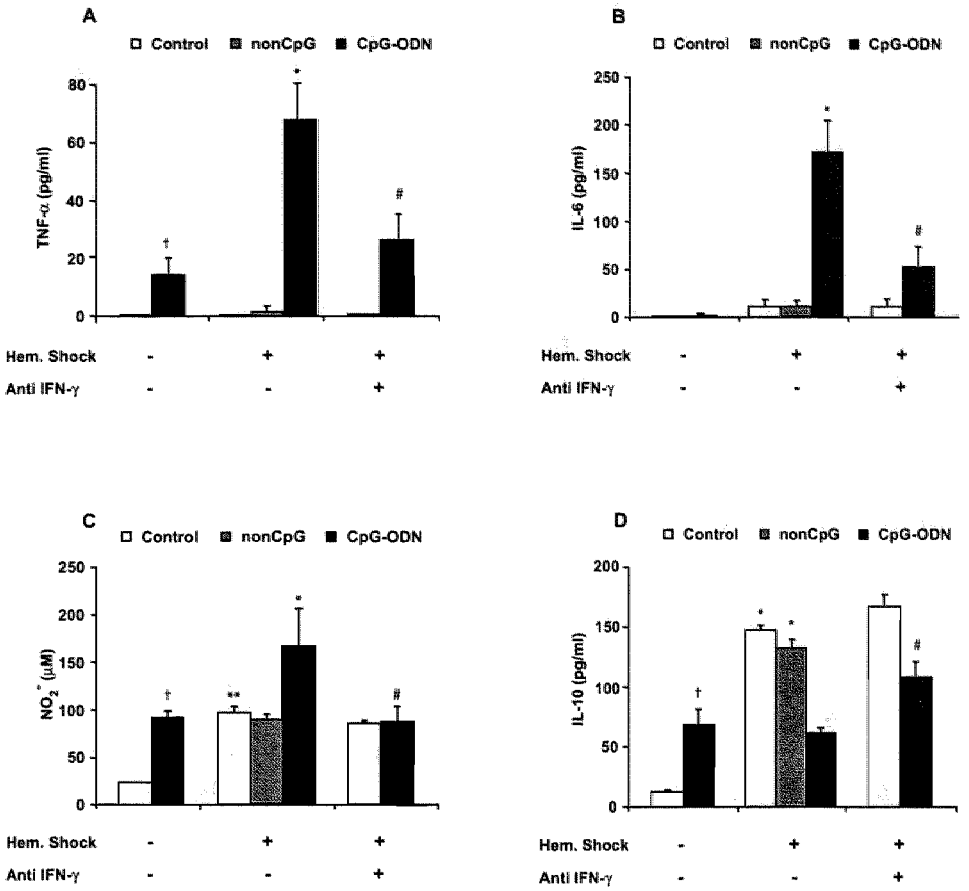

Figure 2:

*A: Administration of CpG-ODN alone enhanced plasma TNF-α (14 ± 6 pg/ml, †p<0.05). CpG-ODN preexposure together with hemorrhagic shock elevated TNF-α (68 ± 13 pg/ml, *p<0.001). Anti IFN-γ reduced TNF-α after hemorrhagic shock in CpG-ODN treated rats (27 ± 8 pg/ml, #p<0.05). B: Preexposure to CpG-ODN strongly enhanced hemorrhagic shock induced IL-6 (171 ± 33 pg/ml, *p<0.01) which was reduced by administration of anti IFN-γ (52 ± 20 pg/ml, #p<0.05). C: Administration of CpG-ODN alone increased nitrite (NO_2^-) (91± 7 μM, †p<0.01). Hemorrhagic shock caused a marked increase in NO_2^- (97± 6 μM, **p<0.01). CpG-ODN preexposure elevated hemorrhagic shock-induced NO_2^- (169 ± 38 μM, p*<0.05). Anti IFN-γ reduced nitrite levels following hemorrhagic shock in CpG-ODN rats (83 ± 12 μM, #p<0.05). D: Administration of CpG-ODN alone enhanced plasma IL-10 levels (68 ± 12 pg/ml, †p<0.006). Hemorrhagic shock caused a marked increase of plasma IL-10 (148 ± 13 pg/ml, *p<0.01) in control and nonCPG treated rats (133 ± 7 pg/ml, *p<0.01). CpG-ODN preexposure together with hemorrhagic shock caused a defective IL-10 response that was restored by administration of anti IFN-γ (109 ± 12 pg/ml, #p<0.01). †/ ** compared with control; * compared with control Hem. Shock; # compared with CpG-ODN-shock;*

Table 1. Bacterial translocation to MLN, spleen and liver at 18 hours after exposure to CpG-ODN and at 4 hours after hemorrhagic shock

Groups	Mesenteric lymph nodes Median (min - max)	Spleen Median (min - max)	Liver Median (min - max)	Total
Control	0 (0)	0 (0)	0 (0)	0
CpG	27 (10–132)†	19 (0-63)†	44 (0-93)†	103†
Control - Hem. Shock	99 (46-164)	72 (0-115)	163 (29-798)	330
CpG - Hem. Shock	248 (177–533)*/‡	275 (28-427)*/‡	320 (19-954)‡	819*/‡
nonCpG - Hem. Shock	80 (52-95)	49 (12-71)	52 (36-70)	165
Control - Hem. Shock + anti-IFN-γ	57 (26 – 69)*	40 (25 – 61)*	58 (19 – 104)*	151*
CpG - Hem. Shock + anti-IFN-γ	135 (74-175)**	70 (38-170)**	131 (18-259)	267**

Results are presented as number of median colony forming units (cfu) /gram tissue (range).

*†p<0.01 compared to normal control; *p<0.05, compared to hemorrhagic shock (Hem. Shock) controls ‡p<0.05 compared to nonCpG controls; **p<0.05, compared to Hem. Shock treated with CpG-ODN.*

As described, hemorrhagic shock caused bacterial translocation to MLN, spleen and liver [19] [20]. Exposure to CpG-ODN prior to hemorrhagic shock more than doubled bacterial translocation (CpG-treated shock rats: total 819 cfu/g vs. non-treated; total 330 cfu/g, $p<0.05$ and nonCpG-treated shock rats: total 165 cfu/g, $p<0.05$). In conclusion, these data indicate that exposure to CpG-ODN impaired gut barrier function and significantly worsened hemorrhagic shock-induced intestinal barrier failure.

Administration of anti IFN-γ reduces TNF-α, IL-6 and NO release in plasma, restored the defective IL-10 response and preserved gut barrier function in CpG-exposed rats after hemorrhagic shock.

The increased IFN-γ levels observed after CpG-ODN administration and the synergistic effect of CpG-ODN preexposure and hemorrhagic shock stimulated us to investigate the role of IFN-γ in the aggravated host response to shock. Anti IFN-γ pretreatment did not significantly affect plasma TNF-α, IL-6, nitrite (NO_2^-) and IL-10 levels following hemorrhagic shock in non-exposed rats (Fig. 3 A-D). However, anti IFN-γ markedly reduced circulating TNF-α plasma IL-6, NO_2^- and the enhanced permeability for HRP (17±0.5 µg/ ml, $p=0.002$) in rats exposed to CpG-ODN 18 hours before hemorrhagic shock (Fig. 3 A-C and Fig. 4). Furthermore, the defective IL-10 response upon hemorrhagic shock in rats preexposed to CpG-ODN was restored to a large extent (109±12 pg/ml, $p<0.01$) (Fig. 3 D). A similar effect was observed with bacterial translocation (Table 1). Bacterial translocation to distant organs was markedly reduced in anti IFN-γ treated rats (**$p<0.01$). In conclusion, it is shown that the enhanced inflammatory response and gut barrier failure caused by CpG-ODN exposure prior to hemorrhagic shock can be effectively reduced by treatment with anti IFN-γ.

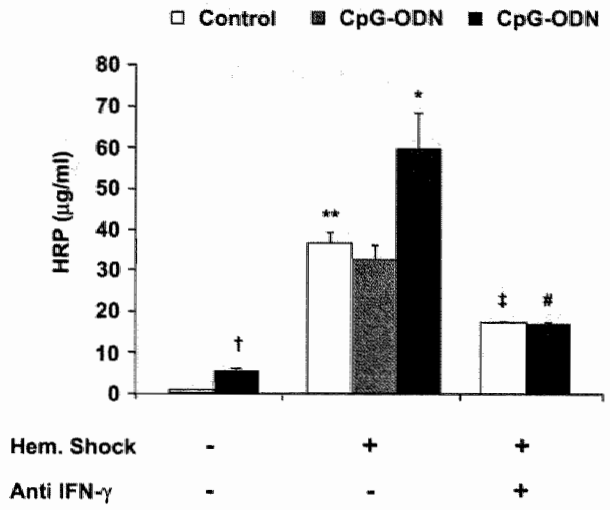

Figure 3:

Preexposure to CpG-ODN followed by hemorrhagic shock enhanced intestinal permeability for horseradish peroxidase (HRP) via an IFN-γ dependent route.

*Exposure to CpG-ODN increased permeability for HRP (5.5 ± 0.5 µg/ml, †p<0.01) compared with control rats (1.0 ± 0.1 µg/ml). Hemorrhagic shock caused a substantial leakage of HRP (36 ± 3 µg/ml, **p<0.01). Preexposure to CpG-ODN followed by hemorrhagic shock strongly aggravated intestinal permeability for HRP (60 ± 11 µg/ml, *p<0.05). Administration of anti IFN-γ markedly reduced permeability for HRP in both control (‡p<0.05) and CpG-ODN treated rats (#p<0.01) subjected to hemorrhagic shock. †/ ** compared with control; * compared with control-Hem. Shock; # compared with CpG-ODN-shock; ‡ compared with control-Hem. Shock*

Preexposure to CpG-ODN enhances expression of TLR4 in vitro and in vivo and increases intracellular signaling via an IFN-γ dependent route.

Endotoxin is a major player in the inflammatory response following hemorrhagic shock[21] [23] and triggers inflammatory cells via TLR4. Our group previously showed that IFN-γ is involved in increased expression of TLR4 in the kidney following ischemia and reperfusion[24]. To further delineate the IFN-γ dependent exacerbating effect of CpG-ODN, we investigated a possible role for TLR4. TLR4 expression was enhanced following 18 hours exposure to CpG-ODN and strongly increased after hemorrhagic shock in the liver, importantly involved in the inflammatory response (Fig. 4A). This enhancement of TLR4 was not observed in nonCpG-treated shock rats and abolished by pretreatment with anti IFN-γ. To measure the value of this enhanced TLR4 expression translocation of NF-κB to the nucleus was determined in peritoneal macrophages. Peritoneal macrophages isolated from CpG-treated rats did not show an enhanced expression before stimulation with LPS compared with nonCpG-treated controls (Fig. 4B). However, stimulation with LPS strongly augmented TLR4 expression and resulted in increased translocation of NF-κB to the nucleus (Fig. 4C) via an IFN-γ dependent route.

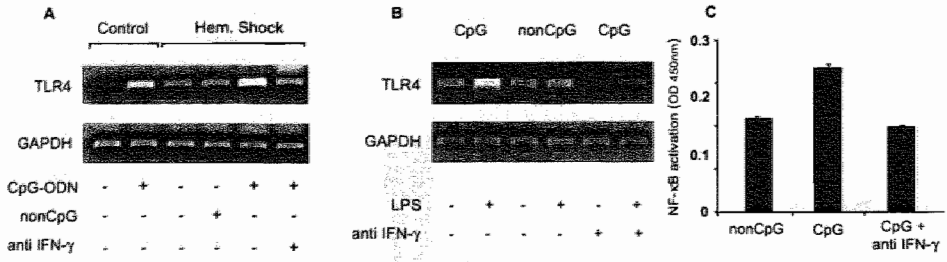

Figure 4:

Exposure to CpG-ODN upregulates expression of TLR4 in macrophages and in the liver leading to increased intracellular signaling via an IFN-γ dependent route.

Cell lysates and nuclear extracts were isolated from peritoneal macrophages of rats exposed to nonCpG, CpG-ODN or CpG-ODN and anti-IFN-γ before and after stimulation with LPS. Exposure to CpG-ODN alone caused enhanced expression of TLR4 in vivo in the liver which was strongly enhanced 90 minutes following hemorrhagic shock in an IFN-γ dependent manner (A). Expression of TLR4 and translocation of NF-κB to the nucleus in peritoneal macrophages following stimulation was markedly enhanced following CpG-ODN treatment (B, C). This enhanced expression of TLR4 and translocation of NF-κB to the nucleus was reduced after administration with anti IFN-γ .

Discussion

Bacterial DNA and synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG motifs are proven immunostimulatory agents. CpG-ODN initiates signal transduction via TLR9 in endosomes, thereby enhancing an IL-18/ IL-12 mediated Th1 type immune response leading to release of inflammatory cytokines such as IFN-γ [25] [26]. The capacity of CpG-ODN to augment a for the host essential inflammatory process opens therapeutic opportunities for treatment of infectious diseases, allergy and cancer [27]. However, an amplified inflammatory response may also lead to increased intestinal damage and exacerbate inflammatory diseases such as colitis or lead to sepsis.

In this study, it is shown in a model of hemorrhagic shock that exposure to CpG-ODN exacerbates the subsequent and relatively low-grade inflammatory response. Deterioration of intestinal barrier function by CpG-ODN treatment alone and in combination with hemorrhagic shock are best explained by the enhanced inflammatory response, since proinflammatory cytokines such as IFN-γ and TNF-α are known to cause gut barrier failure [28] [29]. Also in our study, the magnitude of the inflammatory response significantly correlates with the loss of gut barrier function (data not shown).

Enhancement of the inflammatory response by exposure to CpG-ODN is seemingly in contrast with earlier in vitro studies showing that exposure to CpG-ODN followed by LPS at later time points resulted in a decreased inflammatory response [30] [31] [32]. This hyporesponse was attributed to increased IL-10 levels or induction of tolerance via SOCS-1 or enhancement of Tollip [22] [30] [33] [34]. Interestingly, we did not observe the induction of a hyporesponse

or tolerance but rather a phenomenon known as priming. The discrepancy between the rather mild effects of administration of CpG-ODN without an extra stimulus and the strong IFN-γ dependent synergistic effect of CpG-ODN followed by hemorrhagic shock indicate a priming effect of CpG-ODN. IFN-γ is known to prime macrophages via a TLR4 dependent mechanism, and modulate TLR4 expression in renal epithelial cells[35] [36] [37]. Furthermore, it was recently found that TRIF coupled TLR's such as TLR4 act in synergy with endosomal TLR's such as TLR9[38]. The interferon gamma dependent upregulation of TLR4 may at least in part explain the exaggerated inflammatory response and its sequelae following hemorrhagic shock in CpG-ODN exposed rats, since endotoxin is pivotal in this setting [23]. We now show a strong association at the molecular level between inhibition of IFN-γ, expression of TLR4 and increased translocation of NF-κB to the nucleus in CpG-ODN treated animals and cells suggesting that TLR4 upregulation via IFN-γ is importantly involved in the underlying mechanism of the found effects.

Our data further indicate that exposure to CpG-ODN induces a so-called "two-hit" phenomenon[39] in which reduction of IFN-γ appeared to be protective. Ferrier et al [40] found that repeated stress sessions cause enhanced cytokine expression and disrupt colonic epithelial barrier leading to bacterial translocation. Interestingly this effect requires IFN-γ and depends on presence of CD4+ cells. CpG-ODN seems to be a stress factor that on itself is rather harmless but in combination with another event drives a stronger host response to a normally low-grade trigger.
The fact that anti IFN-γ also prevented hemorrhagic shock-induced gut barrier failure in non-exposed control rats is new and suggests an important role of this lymphokine in the pathogenesis of shock-induced enhanced intestinal permeability. On the other hand, IFN-γ is essential in the host response to bacterial infection[41]. Therefore, further research is needed in order to clarify the exact role of IFN-γ in modulation of these processes.
Besides a sensitizing effect of IFN-γ via priming, the defective IL-10 response following hemorrhagic shock in CpG-ODN treated animals may provide another explanation for the observed events [42]. IL-10 inhibits Th1-type cytokine production and functions primarily to limit the Th1-type inflammatory response [43]. Additionally, TLR-agonists such as CpG-ODN inhibit macrophage responsiveness to IL-10 [44]. In this way, the balance necessary for controlling bacterial infections is dysregulated, favoring an uncontrolled pro-inflammatory response.

In conclusion, we show that exposure to CpG-ODN has until now unrecognized effects following hemorrhagic shock. We identified an important role of IFN-γ in the exacerbating effect of CpG-ODN preexposure on inflammation and gut barrier failure potentially via upregulation of TLR4. Bacterial DNA or CpG-ODN may be a double-edged sword that in addition to the beneficial immuno-stimulatory property in vaccination, allergy and cancer sensitizes the host to intestinal injury by aggravating the inflammatory cascade. CpG-ODN may also exacerbate existing, hidden low-grade inflammatory processes in the gut by sensitizing immune cells via IFN-γ leading to impairment of the intestinal barrier. This phenomenon may contribute to an unwanted increased inflammatory response and disruption of the intestinal barrier leading to infectious complications in patients undergoing surgery or with underlying inflammatory disorders.

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Chapter 3

Strain-specific effects of probiotics on gut barrier integrity following hemorrhagic shock

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Abstract

Probiotic therapy modulates composition of intestinal flora and inhibits the inflammatory response. These properties may be of benefit in preservation of gut barrier integrity after injury or stress. In this study, we examined the effect of two *Lactobacillus* strains selected on their pathogen exclusion properties, on intestinal barrier integrity following hemorrhagic shock. Additionally, responsiveness of the macrophage cell line RAW 264.7 to combined exposure of *Lactobacillus* DNA or oligodeoxynucleotides containing CpG motifs (CpG-ODN) and endotoxin was assessed by measuring TNF-α release.

Rats were administered lactobacilli ($5x10^9$ colony forming units (cfu)) or vehicle for 7 days and subsequently subjected to hemorrhagic shock by withdrawal of 2.1 ml blood/100 gram tissue. Plasma endotoxin, bacterial translocation to distant organs and F-actin in ileum were determined 24 hours later.

L. rhamnosus reduced plasma endotoxin (8±2 pg/ml vs. 24±4 pg/ml, $p=0.01$), bacterial translocation (2 cfu/gram vs. 369 cfu/gram, $P<0.01$) and disruption of F-actin distribution following hemorrhagic shock compared with non-treated controls. In contrast, pretreatment with *L. fermentum* had no substantial effect on gut barrier integrity. Interestingly, DNA preparations from both lactobacilli reduced endotoxin-induced TNF-α release dose-dependently, whereas CpG-ODN increased TNF-α release.

In conclusion, pathogen exclusion properties of both *Lactobacillus* strains and reduction of endotoxin-induced inflammation by their DNA in vitro are no prerequisites for a beneficial effect of probiotic therapy on gut barrier function following hemorrhagic shock. Although pretreatment with *Lactobacillus* spp. may be useful to preserve gut barrier integrity following severe hypotension, thorough assessment of specific strains seems to be essential.

Introduction

In recent years, considerable research has focused on modulation of intestinal flora and the inflammatory response by probiotic bacteria. Probiotics are defined as live microorganisms that when ingested exert health benefits on the host through microbial actions (19). These live microorganisms are a heterogeneous group of microbes in which a variety of *Lactobacillus* spp. and *Bifidobacterium* spp. are frequently used (4, 6, 13, 38). Probiotics have been investigated intensively in inflammatory bowel disease (IBD) and pouchitis in which was shown that such a therapy has a beneficial effect on gut barrier integrity and can ameliorate inflammatory responses (27-30). Although some of these studies show a beneficial effect of probiotic therapy on inflammation and bacterial translocation, there is limited documentation of efficacy of these agents in properly designed controlled trials, thereby complicating practical application of probiotics in a clinical setting (16, 35, 37). Furthermore, the underlying mode of action is not yet fully understood and it remains to be determined whether the described effects are limited to IBD or can also be applied to other disease states in which gut barrier integrity is compromised.

Inhibition of pathogen adhesion and production of antimicrobial metabolites are believed to be important characteristics of viable probiotic strains and selection of those strains is often based on these properties (18, 33). Recently, a novel insight was provided by a study from Rachmilewitz et al. showing that probiotic DNA plays a crucial role in the observed protection of probiotic therapy in experimental colitis via a Toll-like receptor 9 signaling pathway (31). These findings suggest that the effects of probiotics on the host immune response may be based on more than manipulation of intestinal microflora alone and represent systemic modulation of inflammatory processes.
Given these modulatory effects on intestinal microflora and the systemic inflammatory response, we hypothesized that probiotic therapy would be beneficial in preservation of gut barrier integrity following systemic hypotension. It is believed that bacteria and bacterial toxins are able to translocate across the intestinal barrier following severe blood loss (10-12). Subsequently, local activation of inflammatory cells causes release of inflammatory cytokines leading to deterioration of intestinal barrier integrity and increased bacterial translocation (20).

Here, we studied the effect of administration of *Lactobacillus rhamnosus* (LMG P-22799) and *Lactobacillus fermentum* (NumRes2) on intestinal barrier function in a rat model of hemorrhagic shock, by measuring translocation of bacteria and endotoxin and structural damage to the intestinal wall (24, 25). Additionally, we assessed the effect of DNA preparations isolated from both strains and oligodeoxynucleotides containing CpG motifs (CpG-ODN) on TNF-α release by the murine macrophage cell line RAW 264.7 in response to endotoxin.

Methods

Pathogen exclusion properties

Pathogen exclusion was determined using two-week postconfluent Caco-2 cells, cultured in minimal essential medium (MEM) supplemented with 1% sodium pyruvate, 10% fetal calf serum (FCS), and 1% penicillin-streptomycin (all purchased from Gibco, Grand Island, NY). Lactobacilli (*L. rhamnosus* LMG P-22799 and *L. fermentum* NumRes2) and pathogens (*Escherichia coli* E374 (ETEC), *Pseudomonas aeruginosa* LMG21901, *Klebsiella pneumoniae* LMG21902, and *Shigella flexneri* LMG21935) were resuspended in complete MEM (free of penicillin/streptomycin) containing 1% FCS and added to Caco-2 cells. Subsequently, Caco-2 cells were incubated at 37°C for 1 hour, washed to remove non-adherent bacteria and lysed by addition of 1 ml sterile water. The lysed Caco-2 cells were plated on MRS agar and Nutrient Agar to determine the number of adhered lactobacilli and pathogens, respectively. Pathogen exclusion is calculated as percentage of normal adhesion of pathogens to Caco-2 cells.

Oligodeoxynucleotides, genomic DNA and bacterial toxins

Purified, immunostimulatory CpG-ODN (5'-TGACTGTGAACGTTCGAGATGA-3' + phosphorothioate backbone (7) and non-immunostimulatory nonCpG-ODN (5'-GCTTGATGACTCAGCCGGAA-3') (Eurogentec, Seraing, Belgium) were dissolved in sterile, pyrogen-free saline (500 µM). Genomic DNA from both lactobacilli was prepared using a DNA isolation kit (Promega, Madison WI). Purity of DNA was confirmed by measuring UV absorbance at 260/280nm using GeneQuant I (Pharmacia, LKB Biochrom Ltd, Cambridge, England). Lipopolysaccharide (LPS) was removed from DNA preparations using Endotrap 5/1™ (Profos, Regensburg, Germany) after which both preparations contained less than 1 pg LPS per µg DNA.

Cell Culture techniques

The murine macrophage cell line RAW 264.7 was cultured in RPMI supplemented with 10% FCS and 1% penicillin/ streptomycin in 250 ml sterile culture flasks. Cells ($2x10^6$ cells/ml) were washed in RPMI without FCS before the experiment and divided in 2 ml sterile vials (Greiner bio-one, Friekenhausen, Germany) and preincubated for 1 hour with 0.5, 1.5, 4.5 and 13.5 µg/ml CpG-ODN, nonCpG-ODN or DNA preparations from *L. rhamnosus* LMG P-22799 and *L. fermentum* NumRes2. Subsequently, cells were washed in RPMI, plated at $2x10^5$ cells/well (100 µl) in 96-wells polystyrene culture plates (Costar, Cambridge, MA) and stimulated with 10 ng/ml LPS (O55:B5 (Sigma, St. Louis, MO) for 5 hours at 37°C. After incubation, the plate was centrifuged for 3 min at 1500 rpm and supernatants were collected and stored at -20°C. TNF-α was measured in supernatant by sandwich ELISA (14).

Hemorrhagic shock experiment

This study was performed following the guidelines of the Animal Care Committee of the University of Maastricht and this committee approved the protocol. Healthy male Sprague-Dawley rats, weighing 319 – 403 grams (average, 364 grams) purchased from Charles River (Maastricht, the Netherlands) were housed under controlled conditions of temperature and humidity and fed water and chow ad libitum.

Seven days prior to the hemorrhagic shock experiment, rats received *L. rhamnosus* LMG P-22799 or *L. fermentum* NumRes2 ($5x10^9$ colony forming units (cfu)) daily via oral gavage next to standard chow and water (Fig. 1). Non-treated rats received only standard chow and

water prior to the experiments before subjection to (sham) hemorrhagic shock and vehicle-treated rats received 75% maltodextrin, 15% monosodium glutamate, and 10% sodium L-ascorbate 7 days prior to hemorrhagic shock. In total eight groups of n=6 rats per group were included; 1. controls (not fed with lactobacilli, not subjected to hemorrhagic shock, 2. non-treated rats subjected to sham shock, 3 sham shock rats fed with *L. rhamnosus* LMG P-22799 4. sham shock rats fed with *L. fermentum* NumRes2, 5. non-treated hemorrhagic shock rats, 6. vehicle-treated shock rats 7. *L. rhamnosus* LMG P-22799 treated shock rats and 8. *L. fermentum* NumRes2 treated shock-rats.

A non-lethal hemorrhagic shock model was used as previously described (25, 26). In short, rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p); the femoral artery was dissected and cannulated with polyethylene tubing containing heparinized saline (10 IU/ml). Mean Arterial Pressure (MAP) and heart rate (HR) were recorded during a 50-minute observation period. Hemorrhagic shock was induced by withdrawal of 2.1 ml blood per 100 gram of body weight, leading to a dramatic decrease in systemic blood pressure in all shock groups from (97 ± 2 mmHg to 26 ± 1 mmHg). This decrease in blood pressure is known to cause a systemic hypoperfusion of most of the splanchnic organs. Rats recovered spontaneously after 50 minutes and were allowed standard chow ad libitum after six hours. In the sham-shock group, the femoral artery was cannulated, however no blood was withdrawn. At sacrifice (t=24 hours), all rats were anesthetized with sodium pentobarbital (60 mg/kg), tissue was taken aseptically for analysis and plasma was collected in a heparinized pyrogen-free glass tube, separated by centrifugation, frozen immediately and stored (-20°C) until analysis.

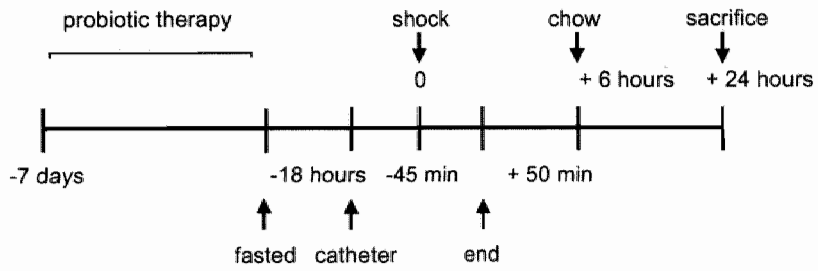

Figure 1:

Experimental design. Probiotics were administered daily by oral gavage, 7 days prior to hemorrhagic shock next to standard chow. Rats were fasted 18 hours before induction of shock; a femoral artery catheter was inserted 45 minutes before and rats were monitored during 50 minutes after which the catheter was removed. Rats were allowed standard chow 6 hours following hemorrhagic shock rats and were sacrificed 24 hours later.

Endotoxin and Bacterial Translocation

Total circulating endotoxin was determined by a LAL chromogenic endpoint assay (0.001-1 ng/ml), (Hbt, Uden, the Netherlands) following the manufactures instructions.

Mesenteric lymph nodes (MLN), the mid-section of the spleen and a segment (IV) of the liver were collected aseptically in 2 ml pre-weighed thioglycolate broth tubes (Becton Dickinson (BBL) Microbiology Europe, Maylan, France). After weighing, tissue specimens were homogenized and transferred onto agar plates. All agar plates (Columbia III blood agar base supplemented with 5% vol/vol sheep blood (BBL) (duplicate plates), Chocolate PolyviteX agar (BioMérieux, Marcy L'Etoile, France), and Schaedler Kanamycin-Vancomycin agar supplemented with 5%

sheep blood (BBL)) were incubated for 48h, in a 5% CO_2-enriched atmosphere or under anaerobic conditions (Shaedler agar plates). After incubation, the number of colonies on all aerobic plates were counted and adjusted to the weight of the grounded tissue. Colony types were identified to the species level using standard methods. *Lactobacillus* colonies were typed using 16S rRNA sequencing. In short, colonies were resuspended in water and the 16S rRNA gene was amplified using primers 8f (5'-CACGGATCCAGAGTTTGATYMTGGCTC AG-3') and 1510r (5'- GTGAAGCTTA CGGYTACCTTGTTACGACTT-3') (Biolegio BV, Malden, Netherlands). Sequencing of the amplicon was done using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems Inc., Nieuwerkerk aan den IJssel, Netherlands) in combination with various primers. The complete sequenced region was compared to other 16S rRNA sequences in the GenBank, EMBL, DDBJ and PDB databases for strain identification.

Immunofluorescence for F-actin

Frozen sections of ileum (4 µm) were cut and stained for filamentous actin (F-actin). Briefly, slides were fixed in acetone for 10 minutes, and air-dried. Slides were stained for 45 minutes at room temperature with Oregon Green-phalloidin that specifically binds to F-actin. After three washes in PBS, slides were mounted using glycerol-PBS with 1,4-diazabicyclo(2,2,2) octane and 4,6-diamidino(2) phenylindole, and viewed with an immunofluorescence microscope. In total three sections per ileum, from three rats per group were investigated at x600 magnification.

Statistical analysis

Bacterial translocation data are represented as median and range; other data are represented as mean ± SEM. A non-parametric Mann-Whitney U test was used for comparisons between treated and non-treated groups.

Results

***L. rhamnosus* LMG P-22799 and *L. fermentum* NumRes2 inhibit pathogen adhesion in Caco-2 cells.** The effect of both probiotic strains on inhibition of adhesion of various pathogenic bacterial species to Caco-2 cells was tested in vitro. Both *Lactobacillus* strains were able to inhibit adhesion of tested pathogens; *E. coli, P. aeruginosa, K. pneumoniae* and *S. flexneri* (Fig. 2) to a similar extent.

Pretreatment with *L. rhamnosus* LMG P-22799 but not *L. fermentum* NumRes2 reduces circulating endotoxin levels after hemorrhagic shock.
The severity of the hemorrhagic shock insult reflected by changes in mean arterial pressure (MAP), heart rate (HR) and hematocrit was similar for all four hemorrhagic shock groups (data not shown). In line with earlier observations (26), circulating endotoxin was significantly elevated in non-treated and vehicle treated rats subjected to hemorrhagic shock (24±4 pg/ml and 21±1 pg/ml respectively) compared with non-treated rats that underwent sham shock (3±1 pg/ml, $P<0.01$), (Fig. 3). In contrast, endotoxin levels were reduced after hemorrhagic shock in rats pretreated with *L. rhamnosus* LMG P-22799 (8±2 pg/ml, $P<0.01$) compared with non-treated shock and vehicle treated rats.

Interestingly, pretreatment with *L. fermentum* NumRes2 did not reduce endotoxemia caused by hemorrhagic shock (21±3 pg/ml) compared with non-treated or vehicle treated rats.

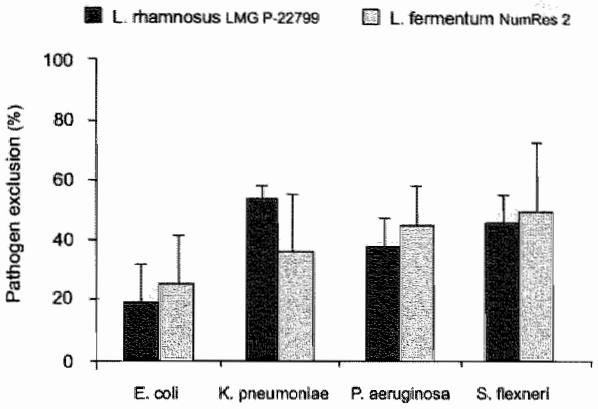

Figure 2:

Lactobacilli and pathogens were added to cultured Caco-2 cells and after a 1 hour incubation period cells were washed and adherent bacteria were determined by culture techniques. Both L. rhamnosus LMG P-22799 and L. fermentum NumRes2 inhibited adhesion of E. coli, K. pneumoniae, P. aeruginosa and S. flexneri with no significant differences between both probiotic strains. Data are presented as percentage of pathogen exclusion, compared to a control situation (e.g. adhesion of pathogen without addition of probiotics).

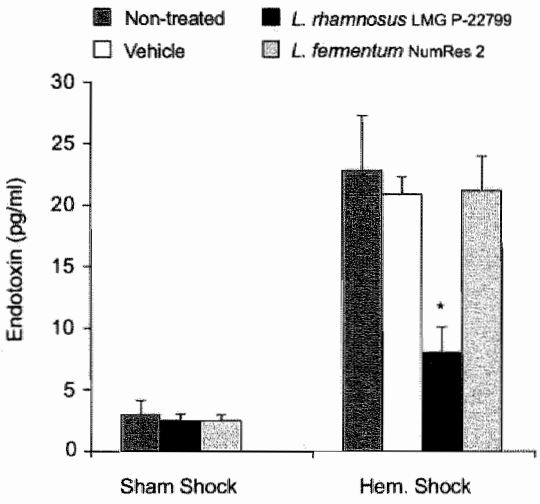

Figure 3:

*Plasma endotoxin was measured 24 hours after (sham) hemorrhagic shock. Endotoxin levels were near detection level in all sham-treated groups and were markedly elevated by hemorrhagic shock in non-treated or vehicle treated rats (non-treated: 24±4 pg/ml and vehicle: 21±1 pg/ml). Pretreatment for 7 days with L. rhamnosus LMG P-22799 strongly reduced endotoxin levels compared with vehicle treated controls (8±2 pg/ml, *P<0.01), whereas pretreatment with L. fermentum NumRes2 had no effect. Data are presented as mean ± SEM.*

***L. rhamnosus* LMG P-22799 reduces bacterial translocation to distant organs.** Hemorrhagic shock typically causes bacterial translocation to mesenteric lymph nodes (MLN), spleen and liver (25, 26). Although pre-treatment with *L. rhamnosus* LMG P-22799 did not significantly reduce bacterial translocation in sham shock rats, this probiotic therapy strongly reduced bacterial translocation to distant organs following hemorrhagic shock (total: 2 colony forming units (cfu)/ gram of tissue) compared with control rats (total: 369 cfu/gram, $P<0.01$) or rats treated with vehicle (total: 292 cfu/gram, $P<0.01$), (Table 1). Overall, the bacteria most frequently found in the cultures of these groups were *E. coli, Enterococcus faecalis* and *S. aureus*. Additionally, *Proteus* spp. and *Lactobacillus* spp. were sporadically cultured.
In rats pretreated with *L. fermentum* NumRes2, significantly less bacteria were found in MLN (15 cfu/gram (0-69), $P<0.01$) and liver (6 cfu/gram (0-13), but bacterial translocation to the spleen was markedly enhanced (185 cfu/gram (30-690), $P<0.05$). Unexpectedly, this increased number of bacteria in spleen was primarily caused by increased bacterial translocation of *Lactobacillus* spp., a phenomenon which was not observed in the other groups. Interestingly, in both intervention groups, the cultured lactobacilli were not identical to the supplemented strain, as determined by 16S-rDNA sequencing (data not shown).

Pretreatment with *L. rhamnosus* LMG P-22799 preserves structural integrity of ileum after hemorrhagic shock. To determine the effect of both *Lactobacillus* strains on structural components of the intestinal barrier, actin filaments were stained in ileum segments using Oregon-Green Phalloidin. F-actin was regularly distributed and a typical fine-meshed pattern was observed throughout the villus in control rats subjected to sham surgery (Fig. 4A). No apparent differences were found in sham shock rats treated with *L. rhamnosus* LMG P-22799 (Fig. 4D) or *L. fermentum* NumRes2 (Fig. 4F). In contrast, hemorrhagic shock caused a significant change of F-actin cytoarchitecture in non-treated and vehicle treated rats (Fig. 4B-C); F-actin distribution was disrupted, the fine meshwork pattern was decreased and F-actin disappeared to a large extent at the mucosal site of intestinal epithelial cells. Pretreatment with *L. rhamnosus* LMG P-22799 markedly attenuated these changes in actin organization caused by hemorrhagic shock (Fig. 4E), whereas treatment with *L. fermentum* NumRes2 did not substantially change actin filament organization after hemorrhagic shock compared with non-treated or vehicle treated rats at the villus top (Fig. 4G).

DNA preparations isolated from *L. fermentum* NumRes2 and *L. rhamnosus* LMG P-22799 reduced the response of RAW 264.7 cells to endotoxin. To determine the contribution of probiotic DNA to the differences found in vivo, RAW 264.7 cells were preexposed with DNA preparations isolated from both probiotic strains and subsequently challenged with endotoxin. Oligodeoxynucleotides containing unmethylated CpG motifs (CpG-ODN) and nonCpG-ODN were included as controls. Stimulation of RAW cells with 10 ng/ ml endotoxin without prior exposure to DNA or immunostimulatory ODN's resulted in detectable TNF-α (3.7 ± 0.1 ng/ml) (Fig. 5). Cells preincubated with CpG-ODN increased TNF-α dose-dependently reaching a maximum TNF-α response at a dose of 13.5 μg/ml (18 ± 1.4 ng/ml). In contrast, preexposure of macrophages with DNA from *L. fermentum* NumRes2 and *L. rhamnosus* LMG P-22799 did not increase responsiveness of macrophages to endotoxin but rather reduced TNF-α release. DNA from *L. fermentum* NumRes2 maximally reduced TNF-α release at a dose of 4.5 μg/ml (2.5 ± 0.5 ng/ml, $p < 0.001$) and DNA from *L. rhamnosus* LMG P-22799 at 13.5 μg/ml (2.1 ± 0.8 ng/ml, $P<0.001$). No significant

differences were found between DNA preparations of both *Lactobacillus* strains.

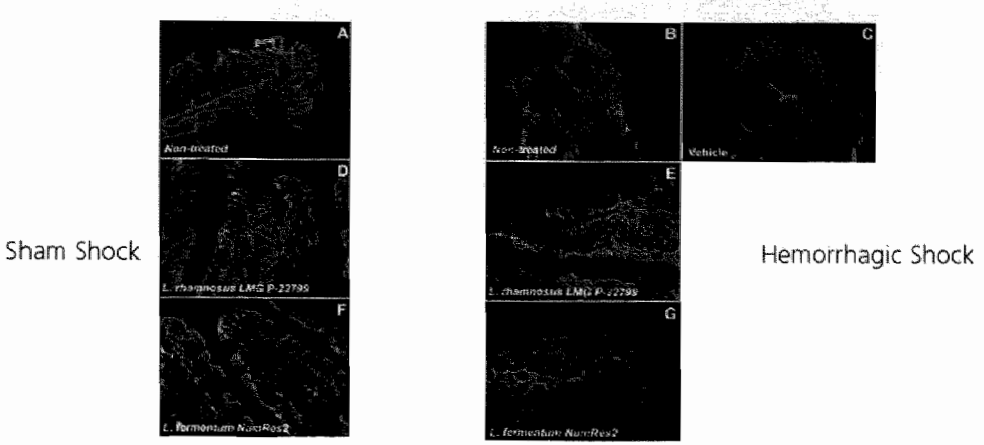

Figure 4:

Pretreatment with L. rhamnosus LMG P-22799 prevents disruption of F-actin filament organization in ileum segments by hemorrhagic shock

Immunolocalisation of F-actin (green) at 600x magnification showed a regular organization throughout the cell in terminal ileum of non-treated and treated rats subjected to sham shock (A, D, F). Hemorrhagic shock markedly disrupted filament organization of F-actin in fasted animals and those that were pretreated with vehicle (B, C). Moreover, tissue was disrupted and disorganized, illustrated by an irregular distribution of nuclei (blue). In contrast, pre-treatment for 7 days with L. rhamnosus LMG P-22799 prevented disruption of cytoarchitecture by hemorrhagic shock (E), whereas L. fermentum NumRes2 had no substantial effect (G). The histology shown is representative for all tissue samples studied (see M&M).

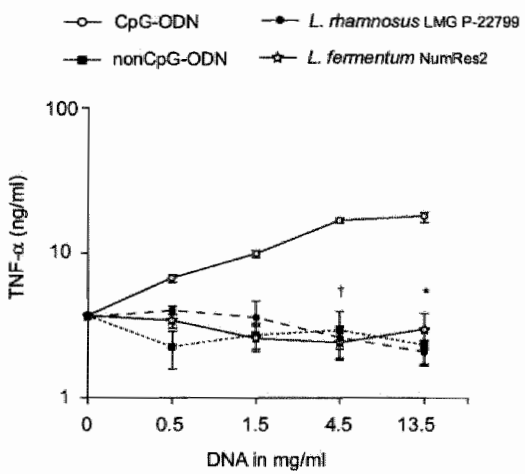

Figure 5:

*DNA from L. rhamnosus LMG P-22799 and L. fermentum NumRes2 suppresses endotoxin induced TNF-α in RAW 264.7 cells. RAW 264.7 cells were incubated with CpG-ODN (open circles), nonCpG-ODN (closed squares), or DNA preparations from L. rhamnosus LMG P-22799 (closed circles) and L. fermentum NumRes2 (stars) at 0.5, 1.5, 4.5 and 13.5 μg/ml, before exposure to 10 ng/ml endotoxin. Prestimulation with CpG-ODN dose-dependently increased TNF-α concentrations in supernatant with a maximum at 13.5 μg/ml DNA (18±1.4 ng/ml). Preincubation with DNA from L. rhamnosus LMG P-22799 reduced TNF-α levels maximally at 13.5 μg/ml (2.1±0.8 ng/ml, *P<0.001) and L. fermentum NumRes2 at 4.5 μg/ml (2.5±0.5 ng/ml, †P<0.001) compared with preincubation with medium (3.7 ± 0.5 ng/ml). There were no significant differences between DNA preparations from both lactobacilli strains. Data are presented as mean ± SEM on a log-scale (y-axis).*

Discussion

Probiotic therapy is increasingly being used in gastrointestinal diseases and inflammatory conditions to inhibit the inflammatory response and reduce intestinal wall damage with equivocal results (21, 35). The diversity of probiotic strains used in various dosages and regimens may contribute to this ambiguity and makes a comparison of their efficacy in disease difficult (37).

In line with studies showing inhibitory effects of probiotic strains such as *Bifidobacterium* and *Lactobacillus* spp. on adhesion of enteric pathogens we show that pathogen adhesion to Caco-2 cells was markedly inhibited by both *Lactobacillus* strains used (i.e. *L. rhamnosus* LMG P-22799 and *L. fermentum* NumRes2) (5, 32). Despite this similar pathogen exclusion property, distinctive differences in effect were found on intestinal barrier integrity loss caused by hemorrhagic shock. The non-lethal hemorrhagic shock model used typically causes an early disruption of gut barrier structure and function, characterized by translocation of bacteria and detectable endotoxemia (25, 26). Interestingly, we now show for the first time that administration of *L. rhamnosus* LMG P-22799 markedly reduced endotoxin and bacterial translocation following hemorrhagic shock. This finding is supported by studies in models of colitis in which other probiotic strains have an ameliorating effect on endotoxin levels and bacterial translocation (28). The effect was specifically related to the bacterium and cannot be attributed to components in the used vehicle as has previously been described (8). The finding that pre-treatment of sham shock rats with *L. rhamnosus* LMG P-22799 did not reduce bacterial translocation to zero is unexplained and may be attributed to the good adhesion properties of this specific strain, leading to a weak translocation of lactobacilli (19).

L. fermentum NumRes2 did not affect plasma endotoxin levels and total bacterial translocation following hemorrhagic shock reflecting the variety in actions between *Lactobacillus* strains and demonstrates that comparison of various strains remains complex (1, 36). An interesting and unexpected finding was that in rats treated with *L. fermentum* NumRes2, bacterial translocation to the spleen was markedly increased and almost all translocated bacteria were *Lactobacillus* spp. These appeared all derived from the host, as 16S-rDNA sequence analyses did not reveal homology to the supplemented strain. In a clinical setting, translocation of lactobacilli occurs rarely and is often regarded as harmless, although bacteraemia with lactobacilli may be detrimental and requires specific antimicrobial treatment (34). The underlying cause of this increased translocation of lactobacilli remains unexplained, however, such an undesired side-effect needs to be avoided.

The effects of *L. rhamnosus* LMG P-22799 and *L. fermentum* NumRes2 on F-actin distribution were in line with the effects found on endotoxin translocation. F-actin is part of the cytoarchitecture of cells and is anchored to tight junctions via zonula occludens (ZO) proteins (15). Tight junction expression is markedly affected by proinflammatory cytokines such as TNF-α (17). In previous studies we showed that hemorrhagic shock causes rapid disruption of ZO-1, paralleled by increased release of inflammatory cytokines (TNF-α and IL-6) (24, 25). The current finding that F-actin distribution is affected even 24 hours after hemorrhagic shock reveals that early disruption of ZO-1 has prolonged consequences for F-actin filament organization in intestinal cells.

The fact that *L. rhamnosus* LMG P-22799 prevented disorganization of actin filaments in intestinal cells is in line with in vitro studies showing that *L. acidophilus* strain LB protects against *E. coli*-induced alterations in F-actin expression in Caco-2 cells (23).

Based on a study from Rachmilewitz et al (31) indicating that the protective effects of probiotics in colitis are mediated by binding of their DNA to TLR9, we investigated the immunomodulatory properties of DNA isolated from both *Lactobacillus* strains. Bacterial DNA or unmethylated CpG motifs are taken up in lysozomes followed by recruitment of TLR9 to these uptake-sites, leading to an inflammatory response via signaling molecules such as Myd88 (22). DNA preparations from both *Lactobacillus* strains used were not immunogenic (data not shown), which is supported by reports on reactivity of DNA from probiotic bacteria in the VSL-#3 compound (21). In line with others we confirmed a strong exacerbating effect of preincubation with CpG-ODN on responsiveness of macrophages to endotoxin (9), whereas *Lactobacillus* DNA preparations markedly inhibited endotoxin-induced inflammation, a previously unrecognized property. It has been shown that probiotic DNA inhibits IL-8 secretion by HT-29 cells to pathogenic bacterial DNA in a competitive manner (21). Next, probiotics also have been shown to inhibit NF-κB in colonic epithelial cells and suppress IL-8 secretion in intestinal epithelia (3, 30). The current data indicate that next to potential competition with bacterial DNA, probiotic DNA induces tolerance to a subsequent trigger with another TLR-ligand dose-dependently. The capacity of probiotic DNA to inhibit endotoxin-induced inflammation may underlie the effect on gut barrier function loss in vivo, since the magnitude of the inflammatory response is directly related to the extent of intestinal damage (2, 24, 25). The optimal dosage of DNA preparations of both strains to inhibit endotoxin induced inflammation is probably different. We administered a single (commonly accepted) dose of both strains that may have been optimal for the *L. rhamnosus* strain, whereas this dose may not have been favorable for the *L. fermentum* strain. A (slight) difference in effect on the inflammatory response in combination with potent pathogen exclusion properties of both strains may have been the cause for the differences in effect of both strains on bacterial translocation following hemorrhagic shock. Further studies are necessary to identify the exact mode(s) of action of probiotics.

In conclusion, the current study shows that two *Lactobacillus* strains with similar pathogen exclusion properties have distinctive, different effects on intestinal barrier integrity loss following hemorrhagic shock. Whereas *L. rhamnosus* LMG P-22799 reduced gut barrier integrity loss caused by systemic hypoperfusion, *L. fermentum* NumRes2 had no substantial effect and even resulted in increased translocation of *Lactobacillus* spp. Furthermore, DNA isolated from both *Lactobacillus* strains suppresses endotoxin-induced inflammation dose-dependently which was previously unrecognized. Our data indicate that certain probiotic strains may be useful in a clinical setting to preserve gut barrier integrity following severe blood loss. However potential useful probiotic strains need to be thoroughly studied using in vitro and in vivo approaches to select suitable strains before applying such therapies in various disease states.

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Chapter 4

Enteral Administration of High-Fat Nutrition Before and Directly After Hemorrhagic Shock Reduces Endotoxemia and Bacterial Translocation

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Abstract

Objective: To determine whether potential enhancement of endotoxin neutralization via high-fat enteral nutrition affects endotoxemia and bacterial translocation after hemorrhage.

Summary background data: Endotoxin and bacterial translocation due to gut barrier failure are important initiating events in the pathogenesis of sepsis after hemorrhage. Systemic inhibition of endotoxin activity attenuates bacterial translocation and distant organ damage. Triacylglycerol-rich lipoproteins constitute a physiological means of binding and neutralizing endotoxin effectively. We hypothesized that enhancement of triacylglycerol-rich lipoproteins via high-fat enteral nutrition would reduce endotoxemia and prevent bacterial translocation.

Methods: A rat model of non-lethal hemorrhagic shock was used. Hemorrhagic shock (HS) rats were divided into three groups: rats starved overnight (HS-S); rats fed with a low-fat enteral diet (HS-LF) and rats receiving a high-fat enteral diet (HS-HF).

Results: Circulating triacylglycerol and apolipoprotein B, reflecting the amount of triacylglycerol-rich lipoproteins, were elevated in HS-HF rats compared with both HS-S rats ($p<0.005$ and $p<0.05$, respectively) and HS-LF rats ($p<0.005$ and $p<0.05$). Circulating endotoxin was lower in HS-HF rats (7.2 ± 10.2 pg/ml) compared with both HS-S rats (29.1 ± 13.4 pg/ml, $p<0.005$) and HS-LF rats (29.9 ± 5.2 pg/ml, $p<0.005$). In line, bacterial translocation was lower in HS-HF rats (incidence 4/8 rats; median 3 (range 0-144) cfu/gram) compared with both HS-S rats (8/8; 212 (60-483) cfu/gram; $p=0.006$) and HS-LF rats (8/8; 86 (30-209) cfu/gram; $p=0.002$).

Conclusion: This study is the first to show that high-fat enteral nutrition, leading to increased plasma triacylglycerol and apolipoprotein B levels, significantly decreases endotoxemia and bacterial translocation after hemorrhage.

Introduction

Lipopolysaccharide (LPS) or endotoxin, a constituent of the outer membrane of Gram-negative bacteria, is an important mediator in the pathogenesis of the sepsis syndrome after major trauma, surgery and hemorrhage [1, 2]. The incidence of sepsis has increased over the years and a further increase is expected due to aging of the population and more complex surgery [3]. Although the pathogenesis of the (late) sepsis syndrome after hemorrhage is not clear, gut barrier failure is considered to play a key role [4, 5]. Several animal studies clearly show that hemorrhagic shock results in gut barrier failure leading to translocation of endotoxin and bacteria [5-9]. Bacterial toxins such as endotoxin can lead to local activation of the inflammatory system and subsequent production (locally) of inflammatory cytokines leading to a further deterioration of the gut barrier and bacterial translocation [10]. Moreover, an increase of systemic endotoxin levels after hemorrhage plays an important role in the development of acute lung injury [8]. This vicious circle of endotoxemia and bacterial translocation and subsequent acute lung injury can be interrupted by interventions that neutralize circulating endotoxin [8, 9]. Several physiological defence mechanisms protect against endotoxemia such as the complement system, the coagulation cascade, the inflammatory response and lipoproteins. Lipoproteins bind and incorporate both Gram-positive and Gram-negative bacterial toxins rapidly, a process which is mediated by lipopolysaccharide binding protein (LBP) and apolipoproteins [11, 12]. Detoxification of endotoxin by lipoproteins prevents endotoxin from initiating an inflammatory response. Triacylglycerol-rich lipoproteins in particular are very potent inhibitors of the bioactivity of endotoxin and protect animals against endotoxin induced lethality [13-16]. Elevation of triacylglycerol-rich lipoproteins, like chylomicrons and very low density lipoproteins (VLDL) would thus induce an increased capacity to inhibit the bioactivity of endotoxin. Physiological elevation of triacylglycerol levels occurs after a fat meal. Chylomicrons, formed in the gut and transported along mesenteric lymphatics, are present locally in the gut in the early postprandial phase. VLDL circulates systemically and is also elevated after enteral feeding [17, 18]. Therefore, high-fat enteral nutrition would theoretically be very effective in order to inhibit the bioactivity of enteric derived endotoxin both locally and systemically after disruption of the gut barrier as occurs following hemorrhagic shock in an early stage. Interestingly, fasting is common in surgical patients most at risk for endotoxemia of enteric origin even though a recent meta-analysis indicates that a "nil by mouth" regime is not beneficial in gastro-intestinal surgery [19]. In animal studies investigating the pathogenesis of the sepsis syndrome, animals are generally fasted overnight before trauma or hemorrhage [5, 6, 8, 20]. Bark et al. [21] reported in rats that brief fasting was associated with significantly increased bacterial translocation following hemorrhagic shock compared with fed animals, indicating the importance of enteral nutrition.

The aim of this study was to induce an increase of triacylglycerol-rich lipoproteins via high-fat enteral nutrition in order to enhance the natural defence mechanism against endotoxin, thereby reducing endotoxemia and bacterial translocation after hemorrhage. In our experiments, we measured circulating triacylglycerol and apoB as indicators of triacylglycerol-rich lipoproteins. Circulating endotoxin and bacterial translocation to mesenteric lymph nodes, spleen and liver were measured as endpoints.

Material and Methods

Animals

The present study was performed according to the guidelines of the Animal Care Committee of the University of Maastricht and this committee approved the protocol. Healthy male Sprague-Dawley rats, weighing 301 – 410 grams (average, 342 grams) purchased from Charles River (Maastricht, the Netherlands) were housed under controlled conditions of temperature and humidity. Before the beginning of the experiments, rats were fed water and chow ad libitum.

Experimental Design

Animals were divided into five groups (n=8 per group). Control rats (C) were starved for 18 hours and sacrificed to assess the effect of fasting alone. Sham shock (SS) rats were starved and the femoral artery was cannulated, but no shock was induced. The hemorrhagic shock groups were either starved 18 hours before the procedure (HS-S), or enterally fed with a low-fat liquid enteral diet (HS-LF) or a high-fat liquid enteral diet (HS-HF) via oral gavage. The exact set up of the procedure is displayed in Figure 1. Blood and tissue samples were taken at 24 hours after onset of shock. The low-fat diet contained: 6.9% (energy-percent) proteins, 75.4% carbohydrates and 16.7% fat. The amount of fat in the low-fat diet was isocalorical to that present in standard rodent chow. The high-fat liquid enteral diet was isocaloric and isonitrogenous to the low-fat diet, but contained 6.9% proteins, 40.9% carbohydrates and 52.2% fat. The protein source was lean milk, the carbohydrate source was a mixture of sucrose and corn starch. The lipid source was vegetable oil with a fatty acid composition of: 8.1% saturated fatty acids (SAFA); 58.9% monounsaturated fatty acids (MUFA), of which oleic acid was the main source (57.4%); 28.2% consisted of polyunsaturated fatty acids (PUFA), of which linoleic acid was the main source (23%), the amount of n-3 and n-6 fatty acids in the high-fat nutrition was less than 5% of the total fat content. The types of carbohydrates and fat used in both diets were identical.

Hemorrhagic shock procedure

Rats were anesthetized with intraperitoneally injected sodium pentobarbital (40 mg/kg). The skin over the left femoral area was shaved and desinfected with povidone iodine solution. The animals were placed in the supine position and allowed to breathe spontaneously. During surgery and throughout the experiment, body temperature was maintained at 37°C with an infrared heating lamp controlled by a thermo analyzer system (Hugo Sachs Elektronik, March-Hugstetten, Germany) connected to a rectal probe. The femoral artery was dissected using aseptic technique and cannulated with polyethylene tubing (PE-10) containing heparinized saline (10 IU/ml). Arterial blood pressure was continuously measured (Uniflow™ external pressure transducer; Baxter™, Utrecht, the Netherlands) and recorded as Mean Arterial Pressure (MAP). Heart rate (HR) was continuously assessed from the instantaneous pressure signal. To keep the arterial catheter patent, it was constantly perfused with physiological saline (3ml/h) via the Uniflow™ system; no heparin was used. After an acclimatisation period of 30 minutes, rats were subjected to hemorrhage by withdrawing blood in quantities of 2.1 ml/100 gram of body weight (representing approximately 30-40% of the circulating volume) at a rate of 1 ml/minute. At 50 minutes after the induction of shock, the catheter was removed and the femoral artery ligated. Six hours after hemorrhage, the rats were allowed access to

standard chow ad libitum. Rats in the sham-shock group were anesthetized and the left femoral artery was cannulated. Sham shock rats were monitored similar as the hemorrhagic shock group, however no blood was withdrawn.
Twenty-four hours after induction of shock, the rats were anesthetized with sodium pentobarbital (60 mg/kg). The skin over de abdomen was shaved and desinfected with povidone iodine. The abdomen was opened via a midline incision, blood samples were taken and mesenteric lymph nodes, the midsection of the spleen and segment IV of the liver were aseptically removed for bacteriological examination.

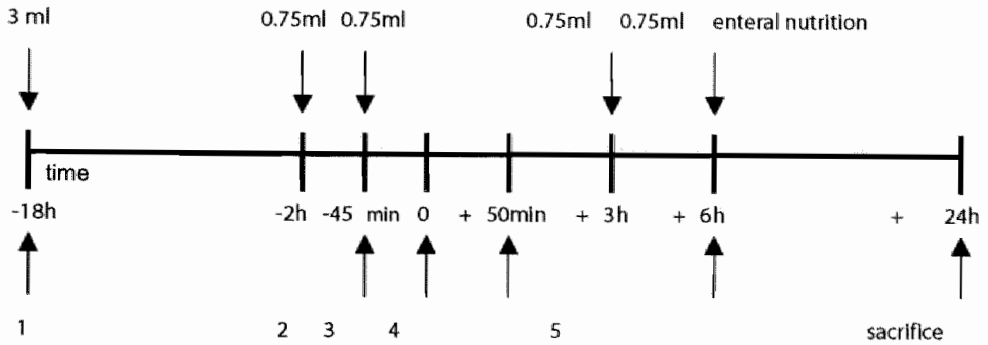

Figure 1:
At -18 hours, rats were starved overnight (1), 45 minutes before withdrawal of blood anaesthesia was given and a femoral artery catheter inserted (2), at t=0 hemorrhagic shock was induced (3); after 50 minutes the femoral artery catheter was removed and the wound was closed (4); after six hours all shock groups were allowed standard chow ad libitum (5); At 24 hours after (sham) shock (t=24h) rats were sacrificed (6). A liquid enteral nutrition (low-fat or high-fat) was administered via gavage in the fed groups (HS-LF and HS-HF), at -18 hours (3 ml), -2 hours (0.75 ml), -45 minutes (0.75 ml), +3 hours(0.75 ml) and +6 hours (0.75 ml).

Plasma samples

Arterial blood samples were collected in heparinized pyrogen-free glass tubes at the time of induction of shock (t=0) and twenty-four hours later. Plasma was separated by centrifugation, frozen immediately and stored (-20°C) until the time of the assay. Hematocrit values were directly measured at the time of shock (t=0) and twenty-four hours later.

Triacylglycerol and apoB

Circulating triacylglycerol was determined using a standard enzymatic assay (Sigma Diagnostics, St. Louis, MO). Levels of apoB were determined using Sandwich-ELISA with a polyclonal antibody against rat apoB, kindly provided by Dr. G. Gibbons, University of Oxford, UK. Briefly, 96-well immunomaxisorp plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with rabbit anti-rat apoB in a concentration of 1 µg/ml. ApoB in plasma was detected with biotin-conjugated rabbit anti-rat apoB followed by peroxidase-conjugated streptavidin. TMB was used as substrate for peroxidase. Plates were read in a microplate reader at 450 nm. As no standard was available, apoB levels were expressed as percentage of pooled plasma of normal healthy male Sprague-Dawley rats with the same weight (300 – 400 gram).

Endotoxin and Bacterial Translocation

Total circulating endotoxin was determined by a chromogenic Limulus Amoebocyte Lysate (LAL) assay (Endosafe, Charles River, Charleston, SC). In short, after thawing heparinized plasma was directly diluted two-fold in pyrogen-free water and subsequently heated for 5 minutes at 75°C, in order to inactivate LPS inhibitors in plasma. LAL-reagent and plasma were incubated for 45 minutes at room temperature. After blocking the reaction with H_2SO_4 an endpoint measurement was used. This assay has an effective range from 0.001-1 ng/ml.
Mesenteric lymph nodes (MLN), the mid-section of the spleen and a segment of the liver were collected aseptically in 2 ml pre-weighed thioglycolate broth tubes (Becton Dickinson (BBL) Microbiology Europe, Maylan, France). After weighing, the tissue specimens were homogenized with sterile grinding rods (Potter S, B.Braun Melsungen, Melsungen, Germany). Subsequently, 500 µl-volumes were transferred onto the following agar plates: Columbia III blood agar base supplemented with 5% vol/vol sheep blood (BBL) (duplicate plates), Chocolate PolyviteX agar (BioMérieux, Marcy L'Etoile, France), and Schaedler Kanamycin-Vancomycin agar supplemented with 5% sheep blood (BBL). Aliquots were spread over the entire surface of the agar. All agar plates were incubated for 48h, in a 5% CO_2-enriched atmosphere or under anaerobic conditions (Shaedler agar plates). After incubation, the colonies were counted on the non-selective Columbia sheep blood agar plates. For determination of the number of colony forming units (CFU) per gram tissue, the number of colonies was counted on all aerobic plates and next adjusted to the weight of the grounded tissue. All different colony types were identified to the species level using conventional techniques

Statistical analysis

Bacterial translocation data are represented as median and range; Mean arterial pressure and heart rate as median with 25^{th} - 75^{th} percentile; other data are represented as mean ± SD. A Mann-Whitney U test was used for between-group comparisons. The Chi-square test was used to compare incidence of translocation. A non-parametric Spearman correlation test was used for bivariate correlations. $P<0.05$ was considered statistically significant.

Results

Hemorrhagic shock procedure

The severity of the hemorrhagic shock insult as reflected by changes in mean arterial pressure (MAP), heart rate (HR) and hematocrit was similar for all three shock groups, i.e. HS-S, HS-LF and HS-HF (figure 2). Directly after induction of shock (t=0) mean MAP-values decreased from 100 (90 – 110) mmHg to 26 (23 -31) mmHg and the HR decreased from 395 (369 – 415) beats per minute (bpm) to 200 (167 – 232) bpm in all three shock groups. Hematocrit was reduced from 42 ± 2.3% at t=0 to 29 ± 2.3% at 24 hours after shock (t=24 hours), $p<0.001$. After 50 minutes both MAP and HR recovered to respectively 68 (59 – 77) mmHg and 339 (313 – 368) bpm. All rats recovered spontaneously after the hemorrhage and no deaths occurred. These data are comparable with those reported by other groups using a similar model of non-lethal hemorrhagic shock [20, 22].

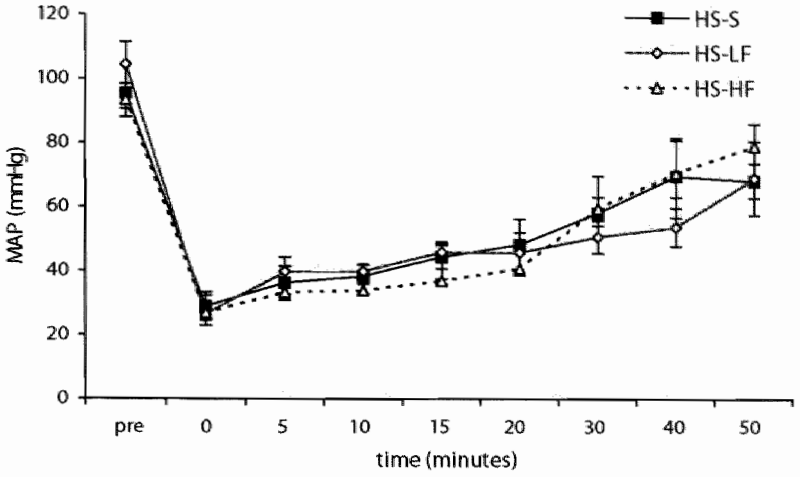

Figure 2:

MAP pressure is represented as median with 25th- 75th percentile. MAP pressure before shock (pre) was 100 (90 – 110) mmHg and decreased to 26 (23 -31) mmHg directly after hemorrhage (t = 0). There was no significant difference in MAP between the groups during the observation period.

Triacylglycerol and apoB

As expected circulating triacylglycerol levels at the time of shock (t=0) were significantly higher in HS-HF rats (184.5 ± 96.2 mg/dL), compared to both HS-LF rats (18.8 ± 14.8 mg/dL, $p<0.005$) and HS-S rats (23 ± 40.7 mg/dL, $p<0.005$) (Figure 3A). There was no statistical difference between the HS-S group and the HS-LF group. After 24 hours (t = 24 hours) plasma triacylglycerol was still elevated in the HS-HF rats (87.8 ± 23.6 mg/dL), whereas plasma triacylglycerol levels were below detection level in both the HS-LF rats ($p<0.005$) and the HS-F rats ($p<0.005$). As shown in Figure 3B, plasma concentrations of apoB in HS-HF rats at t=0 were higher compared with both HS-LF and HS-S rats. However, statistical significance was observed only between the HS-HF and HS-LF rats ($p=0.006$). After 24 hours circulating apoB levels in plasma were still significantly elevated in the HS-HF group compared with the HS-S group ($p<0.05$), but not with the HS-LF group. As expected, circulating triacylglycerol and apoB levels were significantly correlated at both t= 0 ($r = 0.471$, $p<0.01$) and t = 24 hours ($r = 0.314$, $p<0.05$)

Circulating endotoxin and bacterial translocation

The induced hypertriglyceridemia would potentially increase capacity to inhibit bacterial toxins such as endotoxin and thus preserve gut barrier function. In the systemic circulation endotoxin levels remained near the detection levels in the control groups (C and SS), Figure 4. Circulating endotoxin after 24 hours in the HS-HF group (7.2 ± 10.2 pg/ml) was significantly lower compared with both the HS-S group (29.1 ± 13.4 pg/ml, $p=0.005$) and the HS-LF group (29.9 ± 5.2 pg/ml, $p=0.002$). There was no statistical difference between the HS-S group and the HS-LF group. The bacterial translocation data are represented in Table 1. As expected, cultures from tissues taken from the control group were sterile. In the sham-shock rats, three animals had positive cultures, with low numbers of bacteria. After hemorrhagic shock, bacterial translocation was demonstrated in all animals. The number of bacteria found

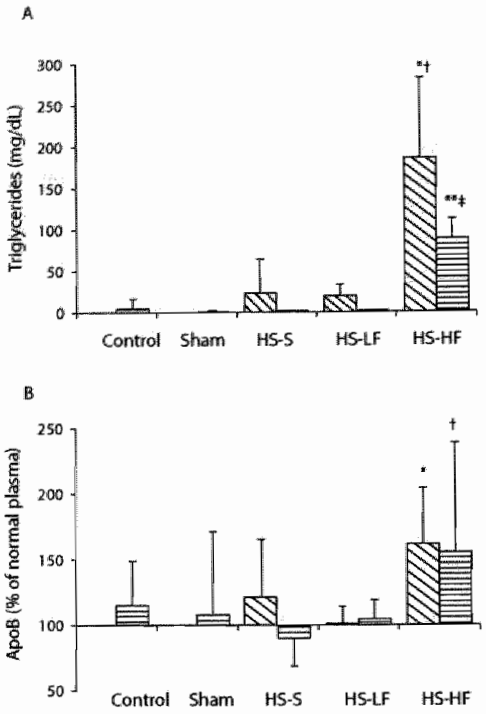

Figure 3:

*A: Triglyceride concentrations in plasma were significantly increased in HS-HF rats at both t=0 (diagonally striped bars) and 24 hours later (horizontally striped bars) compared with both HS-S and HS-LF groups. Values are expressed as mean ± SD. *p<0.005 vs HS-S group at t=0. **p<0.001 vs HS-S group at t=24 hours. †p<0.005 vs HS-LF group at t=0. ‡p<0.001 vs HS-LF group at t=24 hours.*

*B: ApoB in the HS-HF group at t=0 was significantly higher compared with the HS-LF group, *p=0.006. At t=24 hours apoB concentrations in the HS-HF group were significantly higher compared with the HS-S group, †p=0.036. Values are expressed as percentage of the apoB concentration in pooled plasma of healthy rats. Concentration of apoB in Control, Sham-Shock, fasted Hemorrhagic Shock (HS) groups and the HS group fed with a low-fat enteral diet did not significantly differ.*

in mesenteric lymph nodes (MLN), spleen and liver was significantly higher in the HS-S group compared with the sham-shock rats (p=0.001). The median of colony forming units found in MLN of the HS-LF group was not significantly different from the amount of bacteria found in MLN of the HS-S group (212 vs. 86 cfu/gram, p=0.059). In contrast, after high-fat enteral nutrition (HS-HF), sterile cultures were found in 4/8 rats. In addition, the amount of bacteria found in MLN, spleen and liver in the whole group was considerably reduced and significantly lower compared with the HS-S and the HS-LF groups. Overall, the bacteria most frequently found in the cultures were *Escherichia coli, Enterococcus faecalis, Staphylococcus aureus*. Additionally, *Proteus* and *Lactobacillus* spp were sporadically identified. Bacteria were more often found in MLN, than spleen or liver and *Enterobacteriacea* translocated more frequently than *Lactobacillus* spp. Both the species of bacteria and the frequency of translocation to the MLN are comparable with other rat studies investigating bacterial translocation after hemorrhagic shock [20, 23].

In line with our hypothesis, levels of circulating triacylglycerol were inversely related with the total bacterial translocation, r = -0.346, p<0.05 (Figure 5A) and circulating endotoxin levels, r = -0.598, p<0.01 (Figure 5B) in each hemorrhagic shock animal.

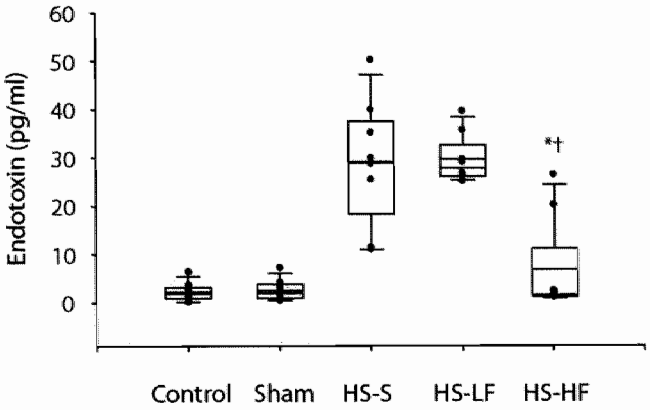

Figure 4:

*Circulating endotoxin is significantly lower in the HS-HF group compared to both the HS-S group (*p=0.005) and the HS-LF group (†p=0.002). Each individual measurement is presented with the 5th and 95th percentile and mean ± SD.*

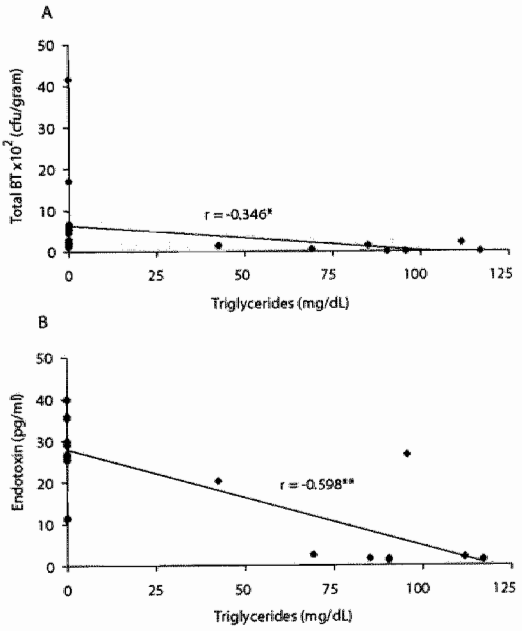

Figure 5:

*A: Circulating triglycerides are inversely correlated with total bacterial translocation. Total bacterial translocation expressed as colony forming units per gram is plotted against circulating triglycerides (mg/dL), r = -0.346, *p<0.05.*

*B: Circulating triglycerides are inversely correlated with the circulating endotoxin level at t = 24 hours. Circulating endotoxin (pg/ml) is plotted against circulating triglycerides (mg/dL), r = -0.598, **p<0.01.*

Discussion

In the present study, we show that administration of high-fat enteral nutrition before and directly after hemorrhage induces an increase in circulating triacylglycerol and apoB concentrations. Interestingly, this study is the first to show that administration of high-fat enteral nutrition was accompanied by reduced plasma endotoxin levels and bacterial translocation. Ulevitch et al.[24] and Tobias et al.[25] proposed in the early 1980's a possible role for high-density lipoproteins (HDL) to bind and inactivate endotoxin. Later, others discovered that hypertriglyceridemia as a result of de novo synthesis in the liver is part of the early response to low-dose endotoxin[26]. This endotoxin-mediated increase in circulating triacylglycerol-rich lipoproteins is considered to have a protective function. In vitro studies showed that triacylglycerol-rich lipoproteins such as VLDL and chylomicrons are potent inhibitors of endotoxin activity[13, 27]. In addition, in vivo studies showed that preincubation of endotoxin with triacylglycerol-rich lipoproteins or repeated intravenous infusions with chylomicrons protect animals against endoxin-induced death[13, 14]. The proposed mechanism for these protective properties of triacylglycerol-rich lipoproteins may be twofold. Firstly, VLDL and chylomicrons can directly inhibit the bioactivity of endotoxin by uptake of endotoxin into these lipoproteins. Secondly, clearance of circulating endotoxin in plasma is enhanced by increased hepatocellular uptake of endotoxin associated with chylomicrons[16]. Triacylglycerol-rich lipoproteins shunt endotoxin away from Kupffer cells towards hepatocytes, thereby decreasing cytokine release and TNF-mediated inflammation. Neutralization of the bioactivity of endotoxin by lipoproteins is mediated in part by LBP, apoA-1 and apoB[12, 28, 29]. LBP functions as a lipid transfer molecule, catalyzing the detoxification of endotoxin. ApoB, the main apolipoprotein of triacylglycerol-rich lipoproteins, is considered to function as a binding site for both endotoxin and LBP.

In our study an increase of triacylglycerols was observed only in the HS-HF rats at both t=0 and t=24 hours. Chylomicrons are primarily triacylglycerol particles and chylomicron formation is a characteristic property of the enterocytes during the postprandial state[30]. Therefore, the majority of triacylglycerol measured at t=0 is probably found in chylomicrons. As chylomicrons are rapidly cleared from the circulation[31], the prolonged hypertriglyceridemia found at t=24 hours may be due to elevated VLDL levels. At the same time, also apoB levels in plasma were enhanced in the HS-HF group. Since the apoB concentration in plasma is known to be strongly correlated with circulating triacylglycerol levels[32] we expected a higher apoB concentration in plasma of HS-HF rats compared to that of HS-S and HS-LF rats. Statistical significance was obtained with the HS-LF group at t=0 and the HS-S group after 24 hours. The absence of a postprandial increase in both triacylglycerols and apoB in the HS-LF group confirms earlier results that the postlipemic response depends on the amount of fat in the administered diet[33]. The hypertriglyceridemia and the elevated apoB levels in the HS-HF rats, which reflect an elevation of triacylglycerol-rich lipoproteins, were accompanied with lower circulating endotoxin levels and reduced bacterial translocation. This effect was specific for the high-fat nutrition, since in the HS-LF group both endotoxin levels and bacterial translocation to MLN, spleen and liver were not significantly different compared with the HS-S group.

A transient endotoxemia, attributed to direct endotoxin translocation, has been described in a different model of hemorrhagic shock with a peak concentration at 150 minutes after onset of shock [10]. The endotoxemia that we observed at 24 hours after shock may be either

the result of direct leakage due to a reduced gut barrier function or derived from translocated bacteria. At this stage, our data do not allow to distinguish between these potential sources of endotoxin. A common hypothesis proposes that endotoxin triggers an inflammatory process locally in the gut resulting in tissue damage, leading to a deterioration of the gut barrier function [9, 10, 34, 35]. Neutralization of the bioactivity of endotoxin in an early stage would thus prevent this loss of barrier function. The importance of endotoxin neutralization after hemorrhagic shock in preserving gut barrier integrity is illustrated by animal studies showing that systemically administered endotoxin inhibitors, i.e. recombinant bactericidal/permeability increasing protein (rBPI) and the endotoxin neutralizing monoclonal antibody, WN1 2225, decrease bacterial invasion into the intestinal wall [8, 9]. We propose that in our study triacylglycerol-rich lipoproteins play a role in neutralizing endotoxin and that this explains the protective effect of high-fat enteral nutrition regarding bacterial translocation. This is supported by the significant negative correlation between circulating triacylglycerol and both total bacterial translocation and circulating endotoxin levels in all hemorrhagic shock rats. At present, it is unclear whether triacylglycerol-rich lipoproteins function locally in the gut or in the systemic circulation. The fact that chylomicrons, apoB and LBP, all essential in endotoxin neutralization, are produced by enterocytes [12, 36-38], is in favor of local endotoxin neutralization by high-fat enteral nutrition.

Opposed to data found in rodents, there is still controversy about the effect of triacylglycerol-rich lipoproteins on endotoxin responsiveness in humans. Van der Poll et al.[39] showed that the in vivo response to endotoxin in humans is not inhibited by hypertriglyceridemia. However, Harris et al. [40] showed that preincubation of endotoxin with triacylglycerol-rich lipoproteins attenuated the inflammatory response evolving from this toxic compound. Moreover, a recent human study from our group showed that postprandial chylomicrons are very potent in neutralizing both endotoxin and lipoteichoic acid in a lipopolysaccharide binding protein (LBP) depending fashion, resulting in reduced cytokine and chemokine secretion[41]. The fact that van der Poll et al. used a bolus injection of endotoxin, creating a sudden increase in endotoxin levels might explain these results, because activation of leukocytes by endotoxin may be more rapid than binding of endotoxin by triacylglycerol-rich lipoproteins[13, 42]. In contrast, our model creates a situation in which endotoxin gradually translocates from the gut lumen into the systemic circulation, resembling the clinical situation. In this setting, exposure of endotoxin to triacylglycerol-rich lipoproteins leading to neutralization may precede the exposure to leukocytes.

In comparison with studies on triacylglycerol-rich lipoproteins and endotoxin neutralization in humans, data on the inhibitory effect of HDL on endotoxin responsiveness are more apparent[43]. As HDL can be up regulated by triacylglycerol-rich lipoproteins via cholesteryl ester transfer protein (CETP) or phospholipid transfer protein (PLTP)[44], triacylglycerol-rich lipoproteins may also indirectly contribute to protection against endotoxin in humans.
In surgery preoperative fasting is currently still a common routine[45], however a recent meta-analysis of controlled clinical trials concludes that there is no clear advantage of keeping patients on a "nil by mouth" regime [19]. Short-term fasting increases the number of coliform bacteria and promotes bacterial adherence to the intestinal mucosa in rats and in a situation of gut barrier failure, these phenomena are thought to promote bacterial translocation[46]. Bark et al. [21] already observed that rats receiving enteral nutrition before hemorrhagic shock

had less bacterial translocation compared with rats which were fasted for 24 hours. Also clinical studies showed the benefit of early postoperative nutrition compared with either fasting[47] or TPN[48-51] . In addition, certain nutrients such as glutamine, arginine, omega-3 fatty acids and nucleic acids have immune-enhancing effects and reduce wound complication, infection and hospital stay when added to the enteral diet [47, 52]. Our data show that a strong additional protective effect can be obtained by peri-shock administration of an enteral nutrition containing a high concentration of fat.

Taken together our current data are the first to show that a simple and relatively short nutritional intervention just before and directly after hemorrhage with a high-fat diet results in improved gut barrier function as reflected by reduced endotoxemia and bacterial translocation after 24 hours. The observed effects seem to be largely dependent on the amount of fat in the enterally administered diet. Whether this can be attributed to early and local scavenging of endotoxin by chylomicrons, leading to less local inflammation or to the prolonged hypertriglyceridemia, which also results in systemical endotoxin neutralization remains to be investigated. This study indicates that further studies on the potential benefit of high-fat enteral nutrition are needed.

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Chapter 5

Pretreatment with high-fat enteral nutrition reduces endotoxin and TNF-α and preserves gut barrier function early after hemorrhagic shock

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Abstract

Gram-negative sepsis is a potentially fatal clinical syndrome characterized by a pro-inflammatory response (TNF-α) to bacterial (endo)toxins and gut barrier function loss. Recently, we found that high-fat enteral nutrition protects against late bacterial translocation in a model of hemorrhagic shock in rats. However, the basis for this protection is unknown.

We hypothesized that the observed protection is due to an early inhibition of endotoxin and the subsequent inflammatory response resulting in a preserved gut barrier function. Sprague-Dawley rats were divided into a group that was starved overnight (HS-S); fed with a low-fat enteral diet (HS-LF) or fed wih a high-fat enteral diet (HS-HF) and subsequently subjected to a non-lethal hemorrhagic shock. Ninety minutes after hemorrhage, arterial endotoxin significantly decreased in HS-HF rats (4.0±0.6 pg/ml) compared to HS-LF rats (10.7±0.9 pg/ml, $p=0.002$) and HS-S rats (15.2±2.2 pg/ml, $p=0.001$). Interestingly, arterial TNF-α was also decreased in HS-HF rats (17.9±10.4 pg/ml) compared to HS-LF (83.5±16.7 pg/ml, $p<0.01$) and HS-S rats (180.9±67.9 pg/ml, $p<0.02$). Loss of tight junction structure (ZO-1) observed in ileum and colon of control hemorrhagic shock rats, was prevented in HS-HF rats. In parallel, intestinal barrier function was preserved in HS-HF rats, evidenced by a reduced permeability to HRP ($p<0.05$), less bacterial invasion and a tenfold reduction of bacterial translocation early after hemorrhagic shock.

This report describes a new strategy to nutritionally prevent endotoxemia, the subsequent inflammatory response and gut barrier failure following hemorrhagic shock. High-fat enteral nutrition requires further evaluation as an intervention to prevent a potentially fatal systemic inflammatory response in patients at risk for sepsis.

Introduction

Gram-negative sepsis is a potentially fatal clinical syndrome, characterized by a proinflammatory response to bacterial (endo)toxin and gut barrier function loss. Although inflammation is essential to fight bacteria and bacterial toxins, hyperactivation may lead to tissue damage which ultimately results in increased vascular permeability and organ injury (1, 2). In the last decade, much research has focussed on modulating the inflammatory response, however, therapeutic interventions with anti-endotoxin or anti-TNF antibodies have been disappointing (3-6). In part this can be attributed to the delay between initiation of therapy and inception of the early pro-inflammatory cytokinemia. Therefore, therapies directed at prevention of endotoxemia and the subsequent early proinflammatory response may be more relevant.
Part of the physiological response to bacterial toxins is an increase in circulating lipoproteins. Both in vitro and in vivo studies showed that lipoproteins can bind and neutralize endotoxin and are potent inhibitors of TNF-α (7-10). A recent study from our group shows that the endotoxin neutralizing capacity of postprandial chylomicrons exceed that of HDL, low density lipoproteins (LDL) and VLDL (11).
Chylomicrons are formed by the gut postprandially and physiologically enhanced by the amount of fat in a meal. We hypothesized that enhancement of chylomicrons via high-fat enteral nutrition would theoretically increase the natural defense against endotoxin, thereby inhibit initiation of the subsequent inflammatory response and prevent tissue damage and gut barrier failure. We have previously shown that high-fat nutrition reduces bacterial translocation at 24 hours after hemorrhage (12). Furthermore, dietary fat has been shown to have an inhibitory effect on alcohol-induced liver injury, in which endotoxin and TNF-α are important mediators (13). However the basis for the observed protective effects remain unknown. Therefore, we investigated in this study the effect of high-fat enteral nutrition on endotoxin, key mediators of inflammation (TNF-α, IL-6) and gut barrier failure early after hemorrhagic shock.

Methods

The present study was performed according to the guidelines of the Animal Care Committee of the University of Maastricht and approved by the committee. Healthy male Sprague-Dawley rats, weighing 300 – 390 grams (average 341 grams) purchased from Charles River (Maastricht, the Netherlands) were housed under controlled conditions of temperature and humidity. Before the onset of the experiments, rats had free access to water and chow.
Animals were divided into three groups (n=8 per group); a group that was starved (HS-S), fed via oral gavage with a low-fat liquid enteral diet (HS-LF) or fed with a high-fat liquid enteral diet (HS-HF). Chow was withdrawn 18 hours before the procedure and the fed groups (e.g. HS-LF and HS-HF) received 3 ml of the liquid diet 18 hours before shock and 0.75 ml at 2 hours and at 45 minutes before hemorrhagic shock. A non-lethal hemorrhagic shock model without resuscitation was used as previously described by Bark et al. (14). This model was chosen to closely simulate the clinical situation since a growing body of evidence indicates that late or hypotensive resuscitation is preferable over aggressive volume resuscitation. At 45 minutes before induction of shock, the left femoral artery was catheterized, followed by a 30 minutes acclimatisation period. Subsequently blood was withdrawn, mean arterial pressure (MAP) and heart rate (HR) recorded and after 50 minutes the catheter was removed and the

artery ligated. Rats were sacrificed and blood and tissue samples were collected at 90 minutes after onset of shock. The composition of both diets is displayed in Table 1. The amount of fat in the low-fat diet was isocalorical to that present in standard rodent chow and the high-fat liquid enteral diet was isocaloric and isonitrogenous to the low-fat diet. Proteins were derived from lean milk, the carbohydrate source was a mixture of sucrose and corn starch. The lipid source was vegetable oil with a fatty acid composition of: 8.1% saturated fatty acids (SAFA); 58.9% monounsaturated fatty acids (MUFA), of which oleic acid was the main source (57.4%); 28.2% consisted of polyunsaturated fatty acids (PUFA), of which linoleic acid was the main source (23%), the amount of n-3 and n-6 fatty acids in the high-fat nutrition was less than 5% of the total fat content. The types of carbohydrates and fat used in both diets were identical.

Hemorrhagic shock procedure

Rats were anesthetized with intraperitoneally injected sodium pentobarbital (50 mg/kg). The skin over the left femoral area was shaved and desinfected with povidone iodine solution. The animals were placed in the supine position and allowed to breathe spontaneously. During surgery and throughout the experiment, body temperature was maintained at 37°C with an infrared heating lamp controlled by a thermo analyzer system (Hugo Sachs Elektronik, March-Hugstetten, Germany) connected to a rectal probe. The femoral artery was dissected using aseptic technique and cannulated with polyethylene tubing (PE-10) containing heparinized saline (10 IU/ml). Arterial blood pressure was continuously measured (Uniflow™ external pressure transducer; Baxter™, Utrecht, the Netherlands) and recorded as Mean Arterial Pressure (MAP). Heart rate (HR) was continuously assessed from the instantaneous pressure signal. To keep the arterial catheter patent, it was constantly perfused with normal saline via the Uniflow™ system; 3 ml/h during the 30 minute acclimatisation period and 1.5 ml/h during the 50 minute observation periode; no heparin was used. Rats were subjected to hemorrhagic shock by withdrawing 2.1 ml blood per 100 gram of body weight (representing approximately 30-40% of the circulating volume) at a rate of 1 ml/minute. At the time of sacrifice (90 minutes), the abdomen was shaved and desinfected with povidone iodine. The abdomen was opened via a midline incision, blood samples were taken and mesenteric lymph nodes, the midsection of the spleen and segment IV of the liver were aseptically removed for bacteriological examination and intestinal biopsies were taken for immunofluorescence, in situ hybridization and determination of gut permeability

Plasma samples

Arterial and portal blood samples were collected in heparinized pyrogen-free glass tubes at the time of induction of shock (t=0) and 90 minutes later (t=90). Plasma was separated by centrifugation, frozen immediately and stored (-20°C) until the time of the assay. Hematocrit values were directly measured at t=0 minutes and t=90 minutes.

Plasma assays

Plasma assays were performed in duplicate in all animals (n=8 in each group). Triacylglycerol was determined using a standard enzymatic assay (Sigma, St. Louis, MO). Total circulating endotoxin was determined by a chromogenic Limulus Amoebocyte Lysate (LAL) assay (Endosafe, Charles River, Charleston, SC). In short, after thawing, heparinized plasma was

directly diluted two-fold in pyrogen-free water and subsequently heated for five minutes at 75°C, in order to inactivate LPS inhibitors in plasma. LAL-reagent and plasma were incubated for 45 minutes at room temperature. After blocking the reaction with H_2SO_4 an endpoint measurement was used. TNF-α and IL-6 concentrations in portal and arterial blood were determined using standard sandwich-ELISA for rat TNF-α (Hbt, Uden, the Netherlands) and rat IL-6 (BD Biosciences, San Diego, CA).

Immunofluorescence

To examine normal distribution of Zonula Occludens protein 1 (ZO-1) we stained ileum and colon sections of healthy control rats fasted for 18 hours, but not subjected to hemorrhagic shock. In total twelve sections from two locations along the gastrointestinal tract (ileum and colon) of four rats per experimental group (i.e. healthy control, HS-S, HS-LF, HS-HF) were investigated for immunofluorescence. Frozen sections of ileum and colon (4 μm) were cut, pre-fixed in cold acetone, allowed to air dry, fixed with 4% paraformaldehyde, and then washed three times with cold PBS. The sections were blocked with 1:10 goat serum and then incubated for 1 hour at room temperature with rabbit anti-ZO-1 polyclonal antibody (Zymed, San Fransisco, CA). As a negative control, sections were incubated with non-immune rabbit serum. Thereafter, sections were washed 3 times with PBS, incubated with Texas Red conjugated goat anti-rabbit antibody (Jackson, West Grove, PA) for 45 min. at room temperature, washed 3 times with PBS, dehydrated in an ascending ethanol series and mounted in DABCO (Sigma) 1:1 in glycerol (90%), containing 4',6-diamino-2-phenyl indole (DAPI) (0.5 μg/ml, Sigma). The distribution of tight junctions was recorded with the Metasystems Image Pro System (black and white charge-couple device camera; Metasystems, Sandhausen, Germany) mounted on a Leica DM-RE fluorescence microscope (Leica, Wetzler, Germany) using 40X and 60X oil immersion objectives. For each tissue section at least 25 randomly chosen microscopic fields were examined at a magnification of 400x and the results were recorded.

Fluorescent in situ hybridization (FISH)

Frozen ileum-sections (4 μm) were fixed on poly-L-lysine coated slides with freshly prepared 4% formaldehyde for 16 hours at 4°C. The hybridization mixture was applied and incubated at 46°C for 12 h. The hybridization mixture contained a probe specific for almost all bacteria (EUB338, 5'-GCTGCCTCCCGTAGGAGT-3')(15) 5'-end labeled with either fluorescein or hydrophilic sulphoindocyanine dyes (Eurogentec, Seraing, Belgium). After hybridization the coverslip was removed, the slides were washed for 10 min at 48° and mounted with DAPI/DABCO:VectaShield (Vector Laboratories, Burlingame, CA), 1:1 in glycerol (90%). Fluorescence was detected by epifluorescence microscopy and photographs recorded with a DCC camera. The images were analysed using programme Q550FW (Leica, Wetzler, Germany). For each tissue section at least 25 randomly chosen microscopic fields were examined at a magnification of 400x and the results were recorded. In total twelve sections of four rats per experimental group (HS-S, HS-LF, HS-HF) were investigated.

Intestinal permeability ex vivo

Intestinal permeability to macromolecules was assessed in five rats of each group (i.e. HS-S, HS-LF, HS-HF) by measuring translocation of horseradish peroxidase (HRP) in isolated segments of ileum as previously described (16, 17). In short, 8 cm segments of the distal ileum were

washed, everted and filled with 1 ml of Tris buffer (125 mmol/L NaCl, 10 mmol/L fructose, 30 mmol/L Tris, pH 7.5) and ligated at both ends. The filled segments were incubated in Tris buffer containing 40 mg/ml of HRP (Sigma, St. Louis, MO). After incubation at room temperature for 45 minutes, the ileum segments were removed from the buffer, blotted to remove excess of HRP and the content was carefully collected in a 1 ml syringe. HRP-activity was measured spectophotometrically at 405 nm after addition of TMB as substrate for HRP

Microbiological methods

In all rats, mesenteric lymph nodes (MLN), the mid-section of the spleen and a liver-segment were collected aseptically in pre-weighed thioglycolate broth tubes (Becton Dickinson (BBL) Microbiology Europe, Maylan, France). After weighing, the tissue specimens were homogenized and transferred to agar plates (Columbia III blood agar base supplemented with 5% vol/vol sheep blood (BBL) (duplicate plates), Chocolate PolyviteX agar (BioMérieux, Marcy L'Etoile, France), and Schaedler Kanamycin-Vancomycin agar supplemented with 5% sheep blood (BBL)). After 48h incubation at anaerobic conditions, the colonies were counted, determined, adjusted to the weight of the grounded tissue and expressed as number of colony forming units (CFU) per gram tissue. Colonies were identified to the species level using conventional techniques.

Statistical analysis

Bacterial translocation data are represented as median and range; all other data are represented as mean ± SEM. A Mann-Whitney U test was used for between-group comparisons. A non-parametric Spearman correlation test was used for bivariate correlations. Differences were considered statistically significant at $p < 0.05$.

Results

The severity of the hemorrhagic shock insult as reflected by changes in mean arterial pressure (MAP), heart rate (HR) and hematocrit was similar for all hemorrhagic shock groups (i.e. HS-S, HS-LF and HS-HF). Directly after induction of shock mean MAP-values decreased from 102 ± 8 mmHg to 27 ± 4 mmHg and the HR decreased from 403 ± 19 beats per minute (bpm) to 199 ± 6 bpm in all groups. Hematocrit was reduced from 43 ± 2% at t=0 min to 35 ± 2% at t=90 min. MAP and HR recovered after 50 minutes to 60 ± 10 mmHg and 304 ± 34 bpm respectively. These data are comparable with our earlier data, using the same hemorrhagic shock model (12).

High-fat enteral nutrition induced an increase in circulating triacylglycerol at both t=0 min (190±25 mg/ dL) and at t=90 min (113±12 mg/dL), (Fig. 1). These elevated levels of triacylglycerol in the high-fat treated group were significantly different from both the HS-LF group (27 ± 10 mg/dL, $p<0.001$, t=0 min and 1 ± 0.6 mg/dL, $p<0.001$, t=90 min) and the HS-S group (9 ± 3 mg/ dL, $p<0.001$, t=0 min and 14 ± 6 mg/ dL, $p<0.001$, t=90 min) and indirectly demonstrate an upregulation of triacylglycerol-rich lipoproteins after high-fat enteral nutrition.

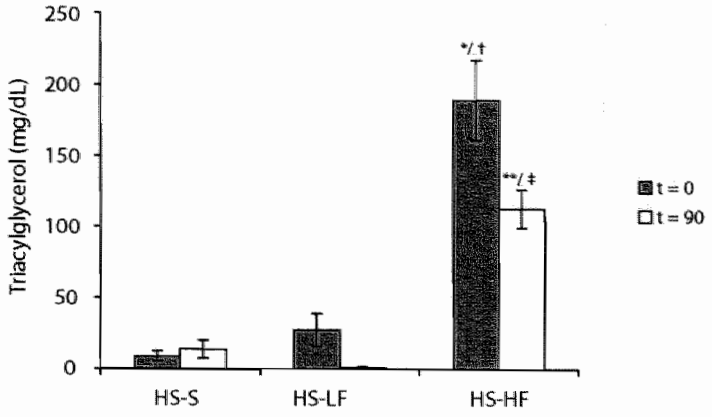

Figure 1:

*Circulating triacylglycerol is significantly higher in the HS-HF group at both t = 0 (grey bars) and at t = 90 minutes (white bars) after hemorrhagic shock. Values are expressed as mean ± SEM. *p<0.001 vs HS-S group at t=0. **p<0.001 vs HS-S group at t=90 minutes. †p<0.001 vs HS-LF group at t=0. ‡p<0.001 vs HS-LF group at t=90 minutes.*

Endotoxin and inflammatory mediators (TNF-α, IL-6)

Next we investigated the effect of administration of high-fat enteral nutrition on endotoxin, TNF-α and IL-6 in arterial and portal blood. As expected, endotoxin was detectable at 90 minutes after hemorrhagic shock in fasted hemorrhagic shock rats (Fig. 2), these data are supported by work of others (18). Endotoxin levels after hemorrhagic shock in fasted rats (arterial: 15.2 ± 2.2 pg/ml, and portal 18.4 ± 3.5 pg/ml) were significantly elevated compared to healthy controls not subjected to hemorrhagic shock (arterial: 0.1 ± 0.1 pg/ml, p=0.001 and portal 2.5 ± 0.7 pg/ml, p=0.001) (data not shown). Endotoxin levels in arterial blood of HS-HF rats (4.0 ± 0.6 pg/ml) were significantly lower compared to HS-LF (10.7 ± 0.9 pg/ml, p=0.002) and HS-S rats (15.2 ± 2.2 pg/ml, p=0.001). Interestingly, there was no statistical difference between the HS-LF group and the HS-S group. The endotoxin concentration in portal blood of HS-HF rats (4.0 ± 0.7 pg/ml) was similarly lower compared to both the HS-LF group (15.4 ± 2.5 pg/ml, p=0.001) and the HS-S group (18.4 ± 3.5 pg/ml, p=0.001), (Fig. 2B).

In line with observations by others (18, 19), elevated TNF-α levels were present in both arterial (Fig. 3A) and portal blood (Fig. 3B) of fasted rats at 90 minutes after hemorrhagic shock. However, TNF-α levels in the HS-HF group (17.9 ± 10.4 pg/ml (arterial) and 11.7 ± 7.3 pg/ml (portal)) were significantly lower compared to the HS-LF group (p=0.008 and p=0.002, respectively) and the HS-S group (p=0.02 and p=0.002, respectively).

In contrast to endotoxin and TNF-α, IL-6 levels were lower in both HS-HF rats (arterial: 5.5 ± 2.1 pg/ml, p=0.02; portal: 4.9 ± 2.4 pg/ml, p=0.004) and HS-LF rats (arterial: 10.1 ± 3.5 pg/ml, p=0.06; portal: 19.8 ± 9.8 pg/m, p=0.05) compared to HS-S rats (arterial: 161 ± 59.8 pg/ml; portal: 177 ± 77.7 pg/ml) (Fig 4). There was no difference between the HS-LF and HS-HF group.

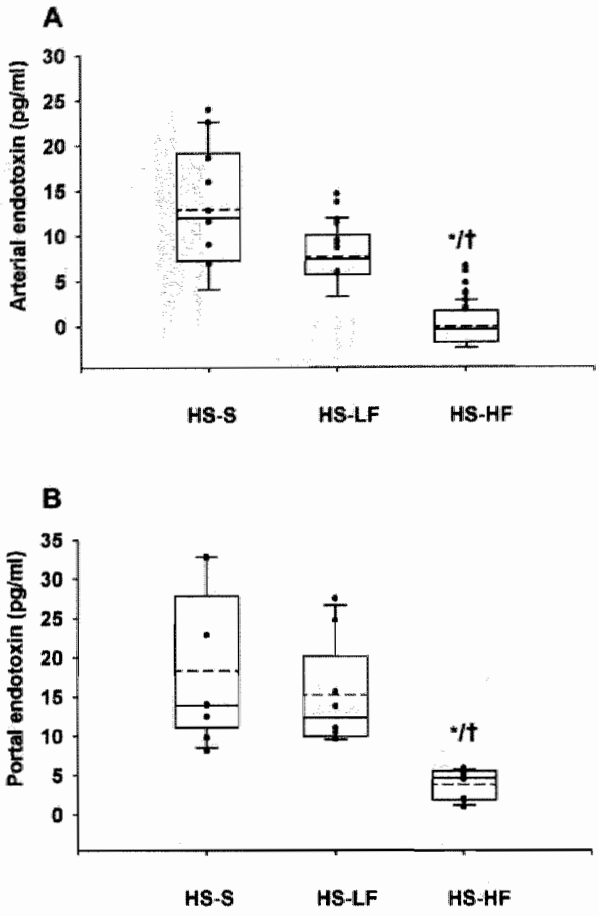

Figure 2:

*Endotoxin in arterial blood is significantly lower in the HS-HF group compared to both the HS-S group (*p<0.001) and the HS-LF group (†p<0.002) (A). Also portal endotoxin was significantly lower in the HS-HF group compared to both the HS-S and HS-LF groups (B). Individual measurements are represented by solid dots with mean (dashed line), median (solid line), 5th, 25th, 75th and 95th percentiles.*

Taken together these data clearly demonstrate decreased endotoxin levels and TNF-α and IL-6 levels in blood of animals treated with high-fat enteral nutrition early after hemorrhagic shock. Most interestingly, triacylglycerol levels at t=0 were significantly and negatively correlated with both arterial endotoxin ($r = -0.77$, $p<0.001$), portal endotoxin ($r = -0.67$, $p<0.001$), arterial TNF-α ($r = -0.59$, $p<0.002$) and portal TNF-α ($r = -0.68$, $p<0.001$).

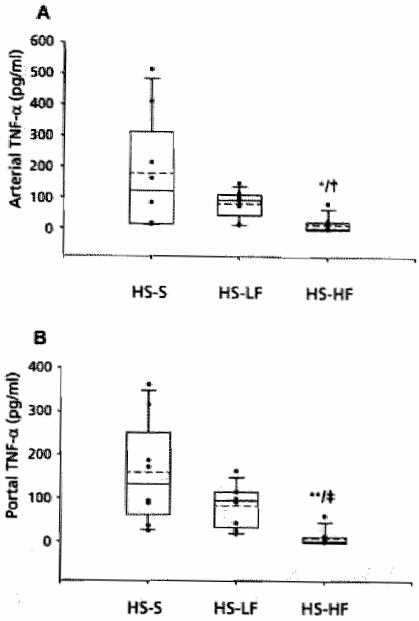

Figure 3:

*Arterial TNF-α is significantly lower in the HS-HF group compared to both the HS-S group (*p =0.02) and the HS-LF group (†p=0.01) (A). Also portal TNF-α was markedly lower in the HS-HF group compared to both the HS-S (**p=0.002) and the HS-LF (‡p=0.002) groups (B). Individual measurements are represented by solid dots with mean (dashed line), median (solid line), 5th, 25th, 75th and 95th percentiles.*

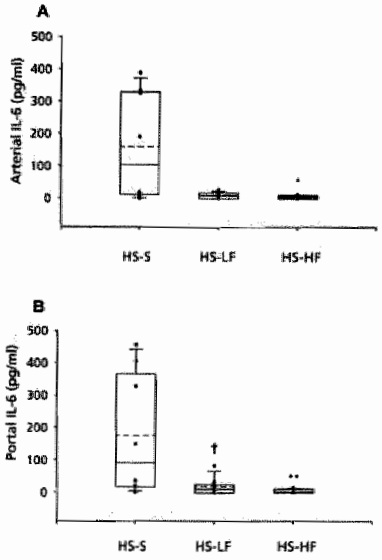

Figure 4:

*Compared with the HS-S group, arterial IL-6 (A) and portal IL-6 (B) were significantly lower in the HS-HF group (*p<0.02 and **p<0.005, respectively). Portal endotoxin was lower in the HS-LF group compared with the HS-S group (†p=0.05). Individual measurements are represented by solid dots with mean (dashed line), median (solid line), 5th, 25th, 75th and 95th percentiles.*

Gut barrier function

An important origin for the observed endotoxemia in this model is the gut. Furthermore, both endotoxin and TNF-α are known to impair the intestinal barrier. Tight junctions in intestinal epithelial cells have an important function in regulating the paracellular route across the intestinal epithelial barrier. In this context, the distribution of the zonula occludens protein 1 (ZO-1), a protein that is part of the tight junction was determined. Figure 5 shows the normal distribution pattern of ZO-1, in ileum (A) and colon (B) of healthy control rats. Following starvation and hemorrhagic shock (HS-S), ZO-1 appeared less distinct, diffusely localized and a loss of protein was observed in several parts of the tissue sections (Fig. 5C, D). In hemorrhagic shock rats fed with a low-fat diet, a similar effect was observed, although to a lesser extent (Fig. 5E, F). However, in rats fed with high-fat enteral nutrition the loss of ZO-1 organization was largely prevented and the tight junction distribution was similar to that observed in healthy controls (Fig. 5G, H).

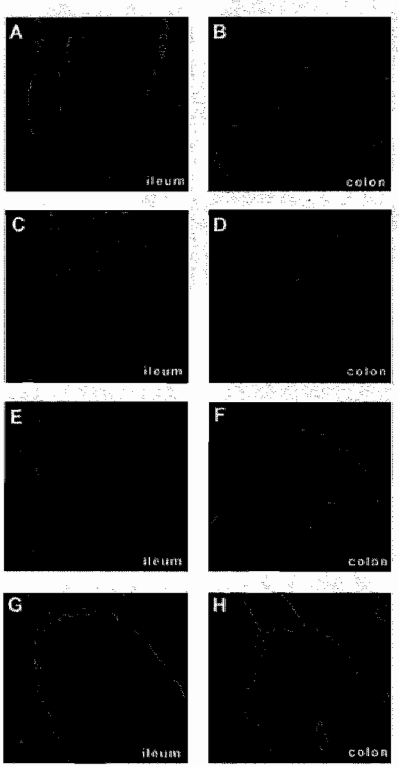

Figure 5:

Immunolocalisation of ZO-1 (red) at a 400x magnification showed a regular distribution in the terminal ileum (A) and colon (B) of control rats that were starved for 18 hours but not subjected to hemorrhagic shock. ZO-1 is localized in the upper part of the enterocytes, showing a normal distribution in association with the cellular surface. Hemorrhagic shock in fasted animals (HS-S) led to a significant loss of ZO-1 in parts of both ileum (C) and colon (D). Moreover, the tissue was disrupted and disorganized, illustrated by an irregular distribution of nuclei (blue). Although tissue sections of HS-S rats showed parts with a normal distribution of ZO-1 the difference compared with the control rats was striking. This pattern was also observed, although to a lesser extent, in ileum (E) and colon (F) of rats fed with low-fat enteral nutrition. In contrast, in animals fed with the high-fat diet (G,H), ZO-1 distribution after hemorrhagic shock was regular, discrete and similar to with tissue-sections of control rats. The histology shown is representative for all tissue samples studied (see M&M).

Next, bacterial invasion was determined by staining bacteria in ileum-sections by FISH. Bacteria were clearly detectable in the intestinal mucosa of ileum in both the HS-S and the HS-LF group (Fig. 6). Median number of bacteria in intestinal mucosa detected by FISH per 100 μm^2 (over all inspected fields) was 8.7 (min: 4.7- max: 16) in the HS-S group and 5.3 (2.7–9.5) in the HS-LF group. However, bacterial invasion was not detected in ileum sections from the HS-HF rats. To assess whether high-fat enteral nutrition reduced intestinal permeability to macromolecules, the permeability of an ileum-segment for HRP was studied ex vivo. As expected hemorrhagic shock resulted in an increased permeability for macromolecules in HS-S rats (20). Interestingly, ileum-segments of HS-HF rats again showed significantly less permeability for HRP (1.1 ± 0.2 µg/ml) compared to ileum-segments of the HS-S group (4.2 ± 1.4 µg/ml, p=0.02) and the HS-LF group (2.6 ± 1.8 µg/ml, p=0.08) (data not shown).

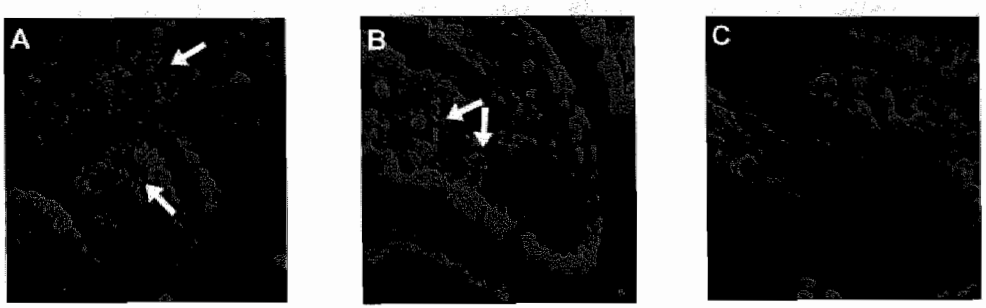

Figure 6:

In situ hybridization for bacteria (bright green; indicated by white arrows) at a 400x magnification showed bacterial infiltration into villi of ileum (A) in starved rats, subjected to hemorrhagic shock. In rats receiving low-fat enteral nutrition before hemorrhagic shock, bacterial invasion was still detected (B). In rats fed with a high-fat enteral nutrition, bacterial invasion could not be detected (C).

Table 1: Composition of enteral nutrition

	Low-fat enteral nutrition (in energy-percent)	High-fat enteral nutrition (in energy-percent)
Protein (milk)	6.9%	6.9%
Carbohydrates	75.4%	40.9%
Fat	16.7%	52.2%

In line, bacterial translocation to MLN, spleen and liver was substantial in fasted rats (HS-S) as early as 90 minutes after hemorrhagic shock, (Table 2). Although administration of low-fat nutrition resulted in a reduction of median colony forming units (cfu) found in MLN, this was not significantly different from the amount of bacteria found in MLN of the HS-S group (80 vs. 41 cfu/gram, p=0.059). In contrast, after high-fat enteral nutrition, sterile cultures were

found in 3/8 rats. In addition, the total number of bacteria found in MLN, spleen and liver was significantly lower compared with the HS-S and the HS-LF groups.
Bacteria most frequently found in the cultures were *Enterococcus faecalis*, *Staphylococcus aureus* and *Escherichia coli*. Bacteria were more often found in MLN, than spleen or liver.
Overall these data clearly show that the reduction of endotoxin, TNF-α and IL-6 levels in blood of high-fat treated rats was accompanied by less structural damage to the gut wall and a functional preservation of the gut barrier.

Table 2. Bacterial translocation at 90 minutes after shock

Groups (n=8)	Translocation Incidence[1]	Mesenteric lymph nodes[2]	Spleen[2]	Liver[2]	Total[2]
HS-S	8/8	80 (39-135)	58 (11-800)	31 (0-929)	189
HS-LF	8/8	41 (5-103)	6 (0-31)†	12 (7-19)†	60†
HS-HF	5/8	5 (0-26)*‡	0 (0-6)*‡	0 (0-33)†	6*‡

[1] Number of rats with bacterial translocation vs total number of rats per group.
[2] Results are presented as number of median colony forming units (cfu) /gram tissue (range). *p<0.05, **p<0.01 compared to HS-LF. †p<0.05, ‡p< 0.005 compared to HS-S.

Discussion

In the present study we demonstrate that administration of high-fat enteral nutrition before hemorrhagic shock decreases post-shock plasma endotoxin, TNF-α and IL-6 levels. Most interestingly the reduced levels of endotoxin, TNF-α and IL-6 in high-fat treated rats were paralled by a preserved gut barrier function. Furthermore, the elevated levels of triacylglycerol in blood after high-fat enteral nutrition, indicative of enhanced triacylglycerol-rich lipoproteins were significantly and negatively correlated with circulating endotoxin and TNF-α on a per rat basis.
Endotoxin is regarded as a pivotal mediator in the pathogenesis of sepsis and multiple organ failure, because administration of purified endotoxin mimics symptoms of Gram-negative sepsis and endotoxin is known to mediate a complex inflammatory response (2, 21). In line with previous rodent studies, hemorrhagic shock in fasted rats resulted in endotoxemia, elevated TNF-α, IL-6 levels and bacterial translocation (14, 19, 22-24). Bacterial toxins such as endotoxin trigger a cascade of inflammatory cytokines in which TNF-α is pivotal in mediating the septic response leading to tissue damage (1, 25). In the current study TNF-α was clearly detectable in blood of HS-S and HS-LF rats but significantly decreased in high-fat treated rats. Circulating IL-6 was elevated in the HS-S rats and significantly lowered in both fed groups. To our knowledge this is the first study to show that a nutritional intervention can abrogate a rise in endotoxin, circulating TNF-α levels and Il-6 early after hemorrhagic shock. Tumor necrosis factor is predominantly produced by macrophages and Kupffer cells, activated by endotoxin or bacteria. As both endotoxin and bacteria were detected after hemorrhagic

shock, the exact trigger of TNF-release could not be established. Goldman et al.(26) showed that TNF-α mediates bacterial translocation and proposes endotoxin as primary initiator for TNF-α production after hemorrhage. Along this line, endotoxemia and the subsequent TNF-α response precede bacterial translocation.

Endotoxin and TNF-α in particular have been shown to impair intestinal barrier function (27-30). We clearly showed a disruption of the tight junctions, indicated by a loss of the tight junction associated protein ZO-1, in fasted and low-fat treated hemorrhagic shock rats. In line with others, showing a decrease of tight-junction expression by elevated proinflammatory cytokine concentrations (29), increased TNF-α levels paralleled loss of ZO-1 protein in the intestine of HS-S and HS-LF rats and low TNF-α levels were accompanied by a preserved ZO-1 distribution in HS-HF rats.

Moreover, intestinal permeability for macromolecules was clearly preserved in HS-HF rats, illustrated by reduced HRP leakage. Also lower numbers of bacteria translocated to MLN, spleen and liver and bacterial invasion into ileum of rats on high-fat enteral nutrition was diminished. Although bacterial translocation and its importance in man remains subject of discussion, we regard this process as an important indicator of gut barrier function in this hemorrhagic shock model.

These data indicate that enteral nutrition potentially reduces the sequelae of endotoxin-mediated cellular processes that may be part of a viscous circle leading to tissue damage. Moreover, it could provide an explanation for our earlier data, showing that high-fat enteral nutrition reduced bacterial translocation and circulating endotoxin at 24 hours after hemorrhagic shock (12).

The molecular target of high-fat enteral nutrition is enigmatic but triacylglycerol-rich lipoproteins are likely candidates to account for the observed effects (8, 31). Binding and detoxification of bacterial toxins by triacylglycerol-rich lipoproteins via lipopolysaccharide binding protein (LBP) and apolipoprotein B is a prominent natural defence against the toxic effects of bacterial toxins (31, 32). Recently our group showed that LBP associates with chylomicrons, facilitating a rapid binding of endotoxin and the endotoxin neutralizing capacity of chylomicrons exceed that of HDL, LDL and VLDL. Furthermore, Kasravi and Harris (33) recently reported that next to direct neutralization of endotoxin by triacylglycerol-rich lipoproteins, chylomicron-bound endotoxin causes tolerance of hepatocytes to inflammatory cytokines, a potential negative regulation of the hepatic response.
However, lipid-rich nutrition also induces several other responses that may explain the beneficial effect. High-fat enteral nutrition indirectly leads to enhanced levels of HDL that next to direct binding of endotoxin exerts potent anti-inflammatory effects (34). Furthermore, release of cholecystokinin octapeptide is induced by intraluminal presence of fat. This intestinal neuropeptide is released in response to fat nutrition and has an anti-inflammatory effect via regulation of NF-κB activity in pulmonary interstitial macrophages (35). To what extent these responses contribute to the observed effects in this study needs to be further investigated.
In conclusion, this study is the first to show that a nutritional intervention via the oral route with high amounts of fat abrogates early endotoxemia and the subsequent rapidly developing inflammatory response after hemorrhagic shock. As a result, gut barrier function is preserved

evidenced by morphologically intact tight-junctions, reduced bacterial invasion, a reduced permeability of the ileum for HRP and lower bacterial translocation to distant organs. The observed effects appear to be specific for high-fat enteral nutrition and are likely to result from an early and efficient scavenging of endotoxin or prevention of endotoxin translocation. It will be of interest to further evaluate the potential of high-fat nutrition as intervention to prevent the potentially fatal inflammatory response after hemorrhage or trauma.

References

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Chapter 6

High fat enteral nutrition attenuates hepatocyte injury in response to hemorrhagic shock

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Abstract

High fat enteral nutrition has been shown to reduce the inflammatory response following hemorrhagic shock in the rat. We hypothesized that this intervention might also ameliorate the remote organ injury to the liver associated with this model.

Male Sprague Dawley rats were either starved fed a low fat diet or a high fat isocaloric isonitrogenous feed prior to a non-lethal hemorrhagic shock by 40% reduction of blood volume. Animals were sacrificed at 90 minutes or 24 hours after injury. Liver cell damage was assessed by histology and long PCR to detect mitochondrial DNA damage. Stress protein expression was measured by western blot and heme oxygenase and Hsp70 mRNA expression by fluorescence detection real time PCR and immunohistochemistry.

Animals fed a low fat diet had the same severity of liver injury compared with starved animals. Animals fed a high fat diet had minimal liver injury and no evidence of mitochondrial DNA damage. Stress proteins including heme oxygenase were significantly elevated in starved and low fat-fed groups compared with healthy or sham hemorrhagic shock controls. The high fat diet group exposed to hemorrhagic shock had a significantly lower expression of stress proteins.

High fat enteral nutrition protects animals from liver injury associated with hemorrhagic shock. This effect is associated with preservation of hepatocellular morphology, attenuation of mitochondrial DNA damage and a reduced stress protein response to injury. It is not yet known whether this effect is mediated by reduction of inflammation and protection of the gut barrier or via a direct effect on the innate immune system.

Introduction

Hemorrhagic shock is a frequent complication in trauma patients, during gastrointestinal bleeding and during some major surgical procedures. Hemorrhagic shock is associated with end organ damage arising from the resultant local and systemic inflammatory response and the low perfusion state. It is believed that this inflammatory response is triggered by bacterial products such as endotoxin that have translocated from the intestinal lumen due to damage to the gut barrier (1). This may occur as a consequence of either ischemia-reperfusion injury to the intestine with complement activation and cytokine release leading to loss of cell junction integrity or from direct leakage due to loss of epithelial cell integrity.
Enteral nutrition may protect the gut by reducing the inflammatory response and the rate of endotoxin and bacterial translocation (2) and may thus reduce end organ damage associated with the hemorrhagic shock model (3). Although the exact mode of action underlying this protection remains to be determined (4), the percentage of fat in enteral nutrition is decisive.
During injury, cells may exhibit autoprotective responses, and increase expression of specific 'stress-proteins'. The expression of heat shock proteins (HSP's) was first observed with hyperthermia, however it is now understood that a variety of stress stimuli, such as inflammatory cytokines (5), ischemia (6), hemorrhage (7), and free radicals (8) stimulate their induction. The HSP family consists of many diverse members which act as cytoplasmic molecular chaperones and are known to exhibit cytoprotective effects against oxidant induced cell injury. The family's most abundant member is HSP70. Pre-induction or overexpression of HSP70 has been shown to be highly effective in reducing tissue injury associated with hemorrhagic shock (9) and endotoxemia (10). Heme-oxygenase (HSP32/HO-1), another HSP, is highly expressed in the liver. The inducible isoform of HO-1, is highly sensitive to oxidative stress and inflammatory stimuli (11). The enzyme catalyses the degradation of heme, to produce CO and biliverdin/bilirubin which have anti-oxidant properties (12). The glucose regulated proteins (Grps) including Grp94 and Grp78 are up-regulated during stress within organelles, and are important in protection against apoptosis (13) (14).
Previous studies using the same model of hemorrhagic shock have clearly demonstrated that enteral supplementation of high fat reduces inflammation and preserves gut barrier integrity (2,15). The current study was undertaken to investigate whether the effects of high fat supplementation were related to reduced hepatocellular injury and an altered hepatic stress protein response.

Methods

Experimental design

The study was performed according to the guidelines of Animal Care Committee of the University of Maastricht. Healthy male Sprague-Dawley rats, weighing 300-410g (mean 338 g) were purchased from Charles River (Maastricht, Netherlands) and were housed under controlled conditions of temperature and humidity.
Animals were divided into 5 groups (n=8 per group). Control rats (C) were starved for 18 hours and sacrificed to assess the effect of fasting alone. Sham shock rats (SS) were starved

and the femoral artery was cannulated, but no shock was induced. The hemorrhagic shock groups were either starved for 18 hours before the procedure (HS-S), or enterally fed with a low fat liquid enteral diet (HS-LF) or a high fat liquid enteral diet (HS-HF) by oral gavage. All hemorrhagic shock groups were sacrificed at 90 minutes and after 24 hours after which blood and tissue samples were taken. The low fat diet contained: 6.9% protein, 75.4% carbohydrate and 16.7% fat. The amount of fat in the low fat diet was isocaloric with that in standard rat chow. The high fat liquid enteral diet was isocaloric and isonitrogenous to the low fat diet but contained 6.9% proteins, 41% carbohydrate and 52% fat.

Hemorrhagic shock procedure.

Rats were anesthetized with intraperitoneal sodium pentobarbital (40mg/kg). Body temperature was maintained at 37°C during surgery and throughout the experiment by infrared heating lamps controlled by a thermoanalyzer (Hugo Sachs Elektronik, March-Hugstetten, Germany) connected to a rectal probe. The femoral artery was dissected and cannulated with polyethylene tubing containing heparinized saline (10 IU/ml). Arterial blood pressure was monitored continuously (Uniflow™ external pressure transducer; Baxter™ Utrecht, Netherlands). Heart rate was assessed continuously from the instantaneous pressure signal. To maintain patency of the arterial cannula, it was perfused constantly with physiological saline (3ml/h) via the uniflow system, no heparin was used. After an acclimatization period of 30 minutes, rats were subjected to hemorrhage by withdrawing blood in quantities of 2.1ml/100g body weight (representing approximately 30-40% of the circulating blood volume), at a rate of 1ml/min. At 50 minutes after the induction of shock the catheter was removed and the femoral artery ligated. Six hours after hemorrhage rats were allowed access to standard chow ad libitum. Rats in the sham shock group were anesthetized and the left femoral artery was cannulated and were monitored identically to the hemorrhagic shock groups but no blood was withdrawn. Twenty four hours after induction of shock rats were anesthetized with sodium pentobarbital (60mg/kg). The skin over the abdomen was cleaned and the liver rapidly frozen for subsequent studies.

Protein extraction

Rat liver tissue stored at –70°C was further cooled in liquid nitrogen before mechanical dissociation using a dismembrator (Braun). Tissue was then dissolved in tissue homogenization buffer and centrifuged to remove debris. The protein concentration of the supernatant was measured using a bichiniconic acid microplate assay (Biorad).

Western blot

Samples (30µg of protein) were denatured and run on 12% SDS PAGE gels using a 5% stacking gel, transferred to nitrocellulose using a semi dry blotter and were blocked with 5% non-fat milk. Primary antibodies against GRP94, GRP 75, HSP 70i, HO-1 and HSP 25 (Stressgen, Vancouver, BC) were used at concentrations of 1:300 for GRP94, 1:500 for cleaved caspase 3 and 1:1000 for all others. The secondary antibodies were anti-mouse HRP (Upstate) or anti rat or rabbit HRP (Santa Cruz). Bands were visualized on film using ECL (Amersham) and were quantitated by densitometry. All membranes were reprobed for β-actin as a control for protein loading.

RNA extraction

Frozen liver tissue was disaggregated in RNA buffer using a ribolyser (Hybaid) and the Fast Green isolation kit according to the manufacturers' instructions. RNA was treated with DNAase1 (Promega) and analysed by spectrophotometry at 260 and 280nm. Ratios varied between 1.98 and 2.01. RNA samples were used as template for a standard PCR reaction using a β-actin primer pair to demonstrate the absence of contaminating DNA.

Fluorescence detection real time PCR for heme oxygenase-1 and hsp70

RNA samples were reverse transcribed using Moloney Murine leukaemia virus reverse transcriptase and random decamers. Primers were designed for rat heme oxygenase-1 and rat hsp70 using Primer design 1.5 (ABPI Prism) (Table 1). 2.5μl cDNA template from each liver was added to 12.5μl Taqman Master Mix (Applied Biosystems), 1.25μl VIC labelled 18s primer and probe mix, 7μl of either HO-1 or HSP-70 primer/probe mix (primers 25μM, probe 5μM) and 1.75μl nuclease-free H_2O and run in an Applied Biosystems ABPI 7000 Taqman real-time PCR machine using Sequence Detector 7.1 software.

Table 1

	Forward	Reverse	Probe
HO-1	GCCTGGCTT TTTTCACCTT	CGAGCACGATA GAGCTGTTTGA	CCGAGCATCGA CAACCCCACCA
HSP70i	CAAGTGCCAG GAGGTCATCTC	CCCGCTTGTG CACGAACT	CTGGACTCTACCACG CTGGCTGAGAAAGA
18SRNA	CGGCTACCAC ATCCAAGGA	CCAATTACAGG GCCTCGAAA	CGCGCAAATTA CCCACTCCGA

Internal control and Long PCR

Total DNA was isolated using the Wizard™ genomic DNA purification kit (Promega), resuspended and quantified using Picogreen reagent (Molecular Probes). Intact mitochondrial DNA was quantified by the Long PCR method which assumes that oxidative damage within the DNA template will interrupt the efficient amplification of the product. A 14.3kb sequence of rat mitochondrial DNA was amplified (TripleMaster™ system Eppendorf) from 25ng of total DNA. Reaction conditions consisted of 1U TripleMaster™ Polymerase mix, 0.25μM dNTP's, 25μM primers (final concentration), 1x Tuning Buffer (supplied with kit) in a 25μl reaction volume. The primers and cycle conditions were derived from Chen et al, 2001 (16). The PCR conditions consisted of an initial denaturation for 1 minute at 94°C followed by 24 cycles of: denaturation at 94°C for 15 seconds, annealing and extension at 68°C for 10 minutes and a final extension at 72°C for 10 minutes. After amplification PCR products were resolved on a 0.8% 1xTBE agarose gel overnight at 40V and were quantified by image analysis (Metamorph® Imaging system v4.0, Universal Imaging Corp, USA).

To correct for mitochondrial copy number a short PCR (104bp) was also run using 25 ng of total DNA. Reaction conditions consisted of 0.6U Taq polmerase (Invitrogen), 1x reaction buffer (supplied with Taq), 3mM $MgCl_2$, 0.1μM dNTP's, 25μM primers, 1x Q solution (Qiagen) in a final volume of 25μl. Primers were Forward 5'GCCCTAGAACTTAACAATTTAACAC-3' and

Reverse 5'-CGGGGTATAATTGGTGGGTA. Cycle conditions were: An initial denaturation step of 95°C for 15 mins; 24 cycles of 94°C 30 secs, 58°C 30 secs, 72°C 30 secs; final extension at 72°C for 2 mins. Products were resolved on a 1.6% agarose gel. Images were also quantified using image analysis.
Quantification of DNA damage was performed using the ratio of the average intensity of Long:Short PCR products. Damage was calculated from the relative amplification of control samples. Prior to sample analysis, linear amplification (and quantitation) was calculated over a number of PCR cycles to determine cycle numbers used for subsequent analysis.

Histology

Formalin fixed tissue was sectioned and stained with haematoxylin and eosin. Sections were examined by a pathologist blinded to the identity of the samples. Tissue injury was scored in three categories indicating the degree of necrosis, cytoplasmic injury and inflammation. Sections were scored 0-1 for no or trivial injury, 2= mild, 3 moderate and 4 severe injury.
Sections were taken of liver tissue at the time of sacrifice, 24 hours after hemorrhagic shock and fixed in methacarn. Antigen retrieval was by microwave heating in 10mM citrate buffer pH6 and endogenous peroxidase was blocked using normal swine serum (NSS) 1:4. Test sections were probed with anti heme oxygenase 1 antibody (Stressgen) 1: 1000 or cleaved caspase 3 antibody (Cell Signalling, Technology) in 1:4 normal swine serum. Control sections were probed with a non-specific antibody rabbit anti mouse IgG (Dako Cytomation, Ely, UK) 1:1000 in 1:4 NSS. The secondary antibody was a biotinylated swine anti rabbit antibody (Dako). Slides were developed using biotinylated horse radish peroxidase and streptavidin (Dako) and the substrate was 3,3'-diaminobenzidine (Sigma, Poole, UK).

Statistics

Results are presented as means and standard deviations. Statistical comparisons were performed using SPSS 11.0 (Chicago, Illinois, USA) by analysis of variance ANOVA.

Results

Stress protein expression in liver tissue

Expression of heme oxygenase-1and Hsp70 mRNA
Levels of mRNA for heme oxygenase-1 were elevated compared with controls in all hemorrhagic shock groups 90 minutes after the onset of shock (Figure 1A). These increases were greatest in the starved and low fat enteral nutrition hemorrhagic shock groups (both $p<0.01$). In samples taken at 24 hours following hemorrhagic shock, persistent elevation of HO-1 expression was evident in the starved hemorrhagic shock group ($P<0.05$) whereas other groups had returned to levels equivalent to controls.
Levels of Hsp70 mRNA were significantly elevated compared with controls at 90 minutes in the starved ($P<0.01$) and low fat enteral nutrition ($P<0.05$) groups only (Figure 1B). The levels of Hsp70 mRNA remained significantly elevated 24 hours after hemorrhagic shock in both of these two groups (both $P<0.05$).

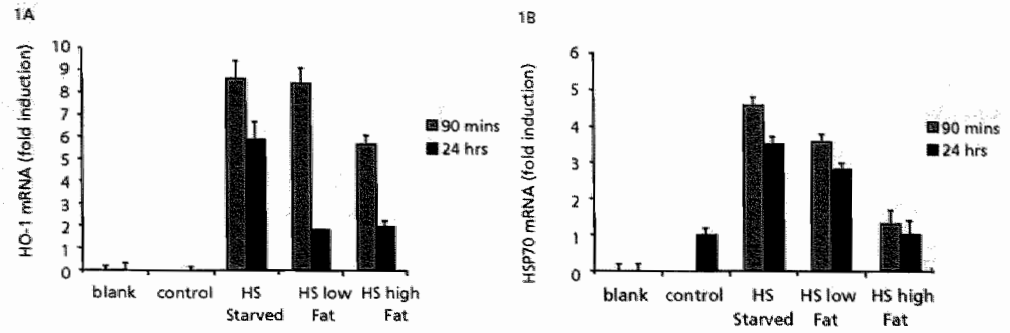


Figure 1:

*Heme oxygenase mRNA expression (A) and Hsp70 mRNA (B) is elevated early after hemorrhagic shock in animals fed a low fat diet or starved, but is not elevated in animals fed a high fat diet. Heme oxygenase and Hsp70 mRNA expression was detected by fluorescence detection real time polymerase chain reaction using specific FAM-TAMRA labelled HO-1 probe and an 18s VIC labelled internal control. Total RNA was isolated from liver tissue 90 minutes and 24 hours after hemorrhagic shock and reverse transcribed using random decamers. Each group represents the mean value for 6 subjects. *P<0.05, †P<0.01 ANOVA compared with control animals.*

Endoplasmic reticulum and mitochondrial chaperones

Protein expression was analyzed only in samples 24 hours after induction of hemorrhagic shock. The endoplasmic reticulum chaperone GRP94 was detected in increased concentrations in the livers of the hemorrhagic shock starved group ($P<0.05$) but was not significantly different from control in any other group Fig 2A. Expression of the mitochondrial chaperone GRP75 was significantly elevated in both the starved hemorrhagic shock group ($P<0.05$) and low fat enteral nutrition hemorrhagic shock ($P<0.05$) groups compared with controls Fig 2B.

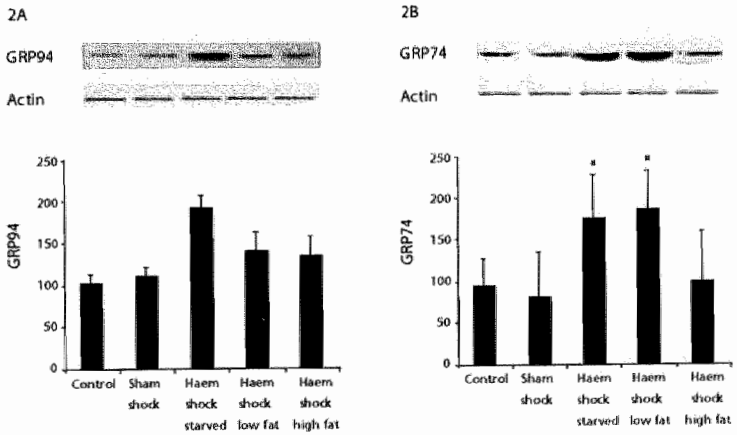
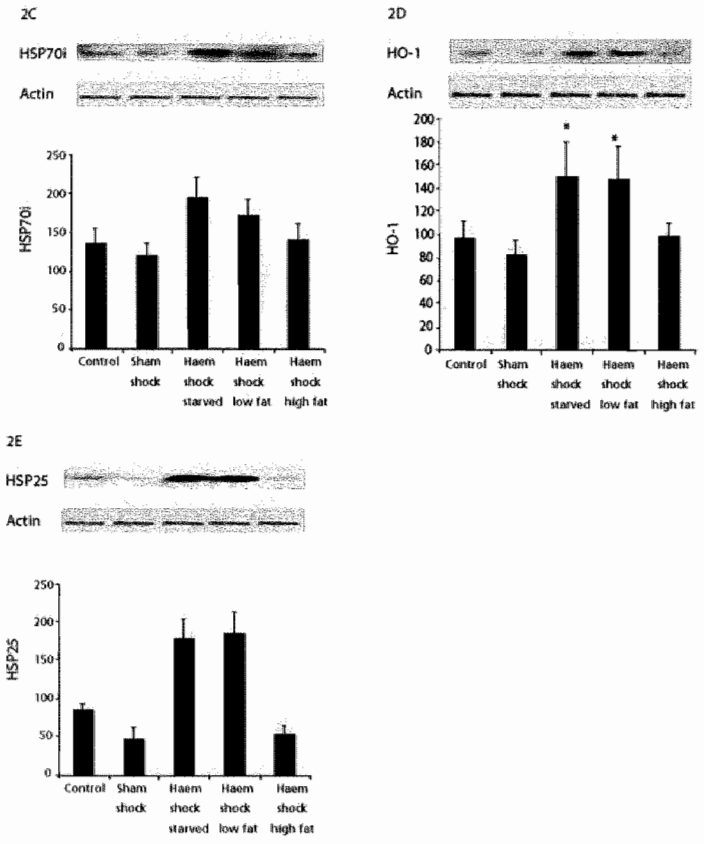


Figure 2:

*Representative western blots, actin loading controls and graphical representation of combined group integrated density values for protein expression of GRP94 (A), GRP 75 (B), HSP70i (C), HO-1 (D) and HSP 27 (E) in rat liver 24 hours after the initiation of hemorrhagic shock. *P<0.05, †P<0.01 ANOVA compared with control animals.*

Cytosolic chaperones

Concentrations of inducible HSP70 were significantly increased in the livers of rats undergoing hemorrhagic shock that were starved ($P<0.05$) Fig 2C. Heme oxygenase-1 expression was increased in the starved-hemorrhagic shock group ($p<0.05$) and the low fat enteral nutrition hemorrhagic shock group ($P<0.05$) but was not significantly increased compared with controls in any other group Fig 2D. Similarly, HSP25 concentrations were increased significantly in the hemorrhagic shock groups that were starved ($P<0.05$) or fed a low fat diet ($P<0.05$) but were not significantly different from control or sham hemorrhagic shock animals in the high fat enteral nutrition hemorrhagic shock group Fig 2E.



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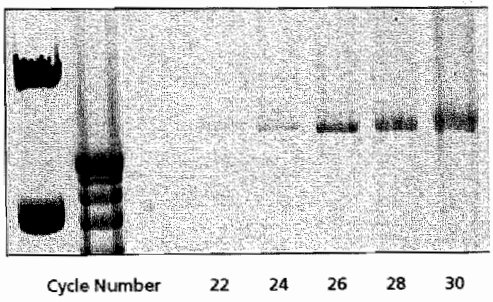
Tissue Injury and Apoptosis in Liver tissue

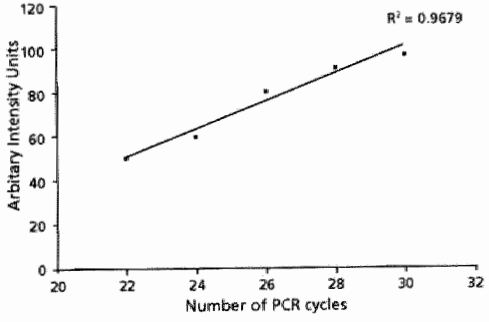
Mitochondrial genomic DNA damage

Genomic DNA damage in the mitochondrion was studied at 90 minutes after induction of hemorrhagic shock. There was a clear linear relationship between band intensity of the 14.3kb mitochondrial genomic DNA and cycle number over the range 22 to 30 amplification cycles (Figure 3A&B). Oxidative injury results in breaks in the genomic mitochondrial DNA leading to a reduction in intensity of the 14.3kb band. Such reduction was evident in both the hemorrhagic shock groups of animals that had been starved or fed a low fat diet (Figure 3C). Preservation of the 14.3kb mitochondrial genomic DNA band was evident in animals fed a high fat diet before hemorrhagic shock (Figure 3C). These results led to a significant reduction in the ratio of the full length mitochondrial genomic DNA fragment to the short PCR internal control in starved ($P<0.006$) and low fat diet ($P<0.025$) hemorrhagic shock groups compared with the high fat hemorrhagic shock group. There was no significant between control and high fat diet hemorrhagic shock groups.

3A



3B



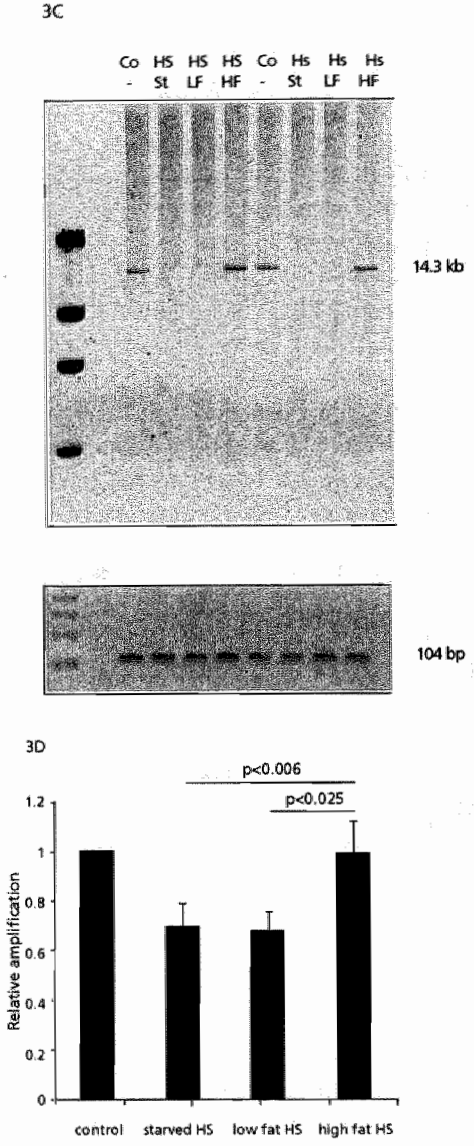


Figure 3:

Mitochondrial DNA injury measured using long PCR 90 minutes after the induction of hemorrhagic shock and in control animals. (A) & (B) gel demonstrating the band intensity of control mitochondrial DNA in relation to cycle number and graphical plot. (C) Gel demonstrating band intensity of mitochondrial DNA for experimental groups (upper panel) and short PCR internal control for mitochondrial DNA loading (lower panel). (D) Relative amplification of mitochondrial DNA (long PCR) relative to internal control (short PCR). Co = control, HS =hemorrhagic shock, St =starved, LF=low fat, HF=high fat.

Histology

Histology of hemotoxylin and eosin sections, 24 hours after initiation of hemorrhagic shock, showed virtually no evidence of necrosis, cytoplasmic injury or inflammation in the livers of the sham hemorrhagic shock group (Figure 4B) compared with controls (Figure 4A). The most severe liver injury was seen in animals who underwent hemorrhagic shock after being starved

Figure 4C&F or being fed a low fat enteral nutrition diet Figure 4D&F. The animals fed a high fat diet before hemorrhagic shock had no evidence of histological injury and were comparable to controls Figure 4E&F.

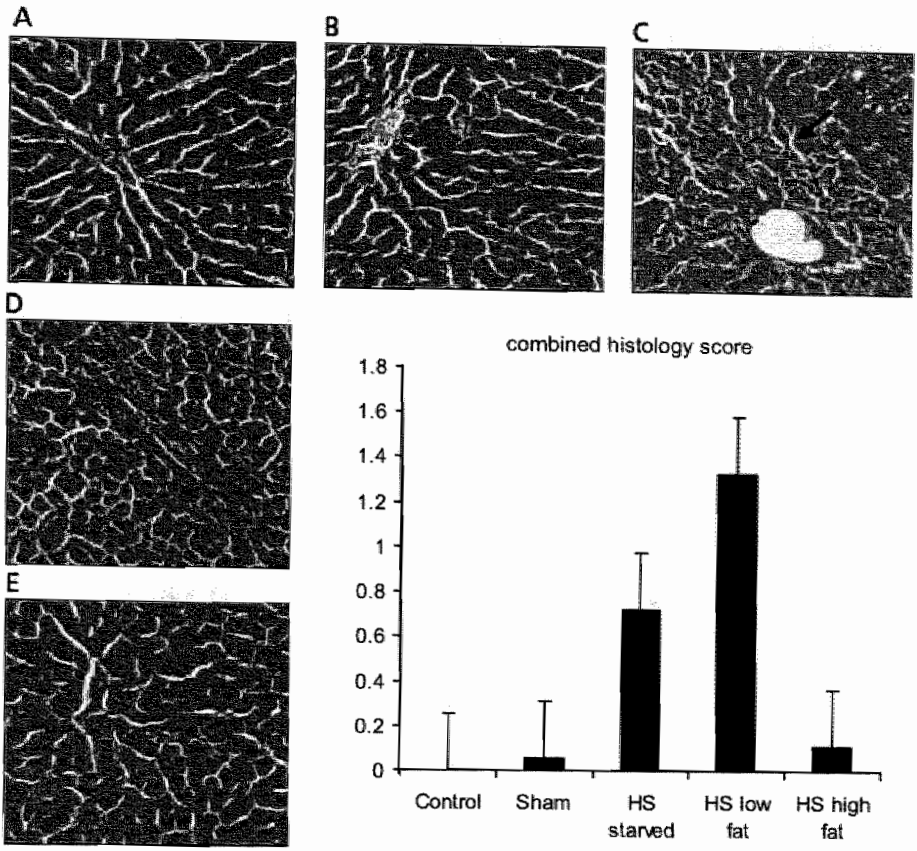


Figure 4:

Mitochondrial DNA injury measured using long PCR 90 minutes after the induction of hemorrhagic shock and in control animals. (A) & (B) gel demonstrating the band intensity of control mitochondrial DNA in relation to cycle number and graphical plot. (C) Gel demonstrating band intensity of mitochondrial DNA for experimental groups (upper panel) and short PCR internal control for mitochondrial DNA loading (lower panel). (D) Relative amplification of mitochondrial DNA (long PCR) relative to internal control (short PCR). Co = control, HS =hemorrhagic shock, St =starved, LF=low fat, HF=high fat.

No evidence of apoptosis was detected in any cell group by either Western blot or immunohistochemistry using cleaved caspase 3 (Data not shown)

Immunohistochemistry for heme oxygenase-1 demonstrated increased cytoplasmic staining in the livers of starved and low fat enteral nutrition hemorrhagic shock groups compared with both controls sham shock animals and non-specific isotype control antibody sections (Figure 5). Again the animals who underwent hemorrhagic shock after being fed a high fat enteral nutrition diet had no evidence of increased HO-1 expression (Figure 5).

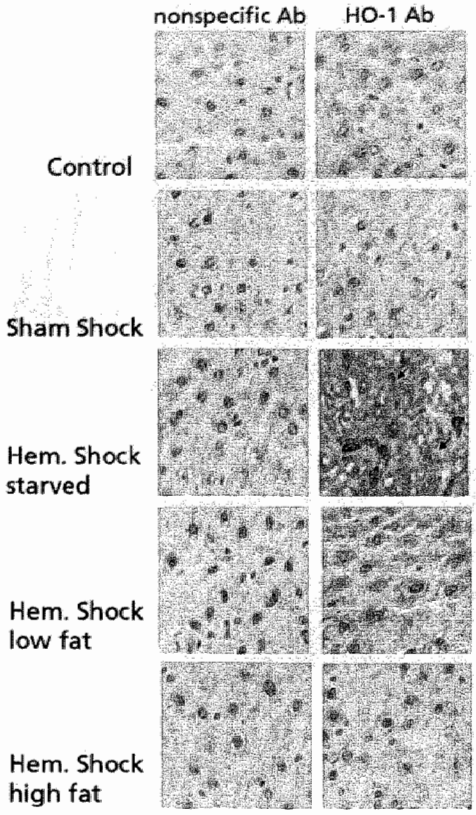


Figure 5:

Heme oxygenase-1 expression in rat liver sections (right hand panels) and non specific antibody controls (left hand panels) from controls rats (A), sham shock (B), hemorrhagic shock starved (C), hemorrhagic shock low fat enteral nutrition (D) and hemorrhagic shock high fat enteral nutrition (E) 24 hours after the initiation of hemorrhagic shock. Increased staining for HO-1 (black arrows) is evident in the rats from the hemorrhagic shock starved and low fat enteral nutrition groups. The hemorrhagic shock high fat enteral nutrition group has staining equivalent to control and sham animals and which is equivalent to expression in non-specific antibody controls.

Discussion

Hepatocellular dysfunction occurs after trauma and hemorrhagic shock. Hemorrhage induced hepatocellular dysfunction is associated with an increase in oxidative stress (17), an up-regulation of pro-inflammatory cytokines (18); (19); (20) and an increase in circulating endotoxin (21); (2;4). Previous experiments using the rat model of hemorrhagic shock have demonstrated that administration of high fat enteral nutrition just before and after hemorrhage markedly inhibited the inflammatory response and protected against intestinal injury (4,15). The current study was designed to test the hypothesis that enteral nutrition using a high fat diet would provide protection of the liver from the remote effects of endotoxaemia and inflammation.

In this study animals that were starved before undergoing hemorrhagic shock had significantly increased concentrations of all stress proteins measured. In this model it is likely that the

increase in stress protein expression relates to the degree of injury to the liver tissue. This is certainly supported by the pattern of histological injury in the livers which was greatest in the groups exhibiting elevated stress protein expression. Furthermore the evidence from the differences in mitochondrial genomic DNA damage also supports the concept that stress protein expression reflects tissue injury in this model. It may not be surprising that the increased levels of stress proteins could not confer protection against the damage observed in both starved and low fat groups. Whilst overexpression or pre-induction of HSP's give rise to protection against trauma (9,10,19), the time over which protein levels were increased in the current study may have been too late to avoid hepatocellular injury. Alternatively, it could be argued that in the absence of a stress response the injury to the liver might have been more severe. Indeed, sections of liver in this study showed evidence of inflammation and necrosis with apoptosis being relatively lacking. This may simply reflect the time point studied (24 hours after induction of hemorrhagic shock) or the techniques used to look for apoptosis.

Animals fed a high fat diet had no evidence of a stress protein response within the liver and expression of heme-oxygenase RNA was equivalent to controls. This contrasted with the observed effects in animals fed an isocaloric isonitrogenous low fat diet where there was evidence of
HO-1 RNA and protein expression. It seems reasonable to conclude that the fed state is protective compared with the starved state but that a greater level of remote organ protection can be achieved by feeding a high fat diet. Furthermore, in this model, feeding a high fat diet does not induce stress proteins resulting in hepato-protection, rather protection of the liver is mediated via prevention of inflammation and cell stress and that this resulted from feeding a high fat diet.
The mechanism of this protection is not clear from the present study. It may be as suggested by earlier work from our group that maintenance of intestinal barrier function and inhibition of the local and systemic inflammatory response results in a reduced stress response in the liver (15). Alternatively other work in humans has suggested that feeding a high fat diet enhances the lipaemic response to sepsis and can negate the effects of translocated endotoxin through the formation of chylomicron/lipopolysaccharide complexes which can be internalised and degraded by hepatocytes (22-26). Certainly whichever the mechanism, the lack of mitochondrial DNA damage found in the high-fat fed animals would suggest that the mechanism of protection in these animals was due to a lack of oxidative stress or an inhibition of stress stimuli such as inflammatory cytokines upon the tissue rather than the induction of a protective intracellular response.

In conclusion this study has demonstrated that hepatic tissue damage associated with hemorrhagic shock results in a stress protein response, which may limit, but fails to prevent, tissue injury. Furthermore high-fat enteral feeding abrogates the remote effects of hemorrhagic shock upon the liver. Further work is required to understand the exact mechanisms which underpin this protection however high fat enteral nutrition may be considered as part of a future viable therapeutic strategy to overcome remote organ injury after trauma.

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Chapter 7

High-fat enteral nutrition reduces endotoxin, TNF-α and gut permeability in bile duct-ligated rats subjected to hemorrhagic shock.

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Abstract

Background/ Aims: Cholestatic patients are prone to septic complications after major surgery due to an increased susceptibility to endotoxin and hypotension. High-fat enteral nutrition reduces endotoxin after hemorrhagic shock. However, it is unknown whether this nutritional intervention is protective in biliary obstruction. We investigated the effect of high-fat enteral nutrition on endotoxin, TNF-α and intestinal permeability in cholestatic rats subjected to hemorrhagic shock.

Methods: Bile duct-ligated (BDL) rats were fasted or fed with low-fat or high-fat enteral nutrition before hemorrhagic shock. Blood and tissue samples were taken after 90 minutes.

Results: Plasma endotoxin decreased after hemorrhagic shock in BDL-rats fed with high-fat nutrition compared to fasted ($p<0.01$) and low-fat treated rats ($p<0.05$). Additionally, circulating TNF-α was reduced in BDL-rats pretreated with high-fat nutrition compared to fasted rats ($p<0.01$). The increased intestinal permeability to macromolecules was reduced by high-fat enteral nutrition, whereas bacterial translocation did not significantly change. Simultaneously, tight junction distribution in ileum and colon was disrupted in non-treated BDL-rats but remained unchanged in high-fat pretreated BDL-rats.

Conclusion: High-fat enteral nutrition protects against endotoxin-mediated complications independently of intraluminal bile. These results provide a potential new strategy to prevent endotoxin-mediated complications in cholestatic patients undergoing major surgery.

Introduction

Major surgery in patients with obstructive jaundice is associated with a high postoperative morbidity, characterized by septic complications (1, 2). Gut-derived endotoxin and systemic hypotension are considered to be important aetiological factors in these events (3, 4). Factors contributing to endotoxin translocation in biliary obstruction are absence of intraluminal bile salts, impaired clearance of endotoxin by Kupffer cells and a decreased gut barrier function (5-7). Systemic hypotension further enhances endotoxin translocation leading to release of inflammatory mediators and gut barrier failure (8). As a result, endotoxin and the subsequent inflammatory response have been important therapeutic targets in biliary obstruction (9-11). Although various experimental studies evaluated potential therapeutic interventions and elucidated mechanisms underlying the increased postoperative morbidity, no effective clinical therapy has been reported thus far that effectively reduces the high complications in cholestatic patients after major surgery (12-14).

A recent study from our group showed that high-fat enteral nutrition reduces endotoxemia and preserves gut barrier function after hemorrhagic shock (15, 16). Although this simple nutritional intervention would also be advantageous for the prevention of endotoxin-mediated complications in combined biliary obstruction and systemic hypotension, a potential drawback might be that bile contributes to the protection of high-fat enteral nutrition. Dietary fat strongly increases secretion of bile salts, which are potent inhibitors of endotoxin (17, 18). More importantly, an intact bile secretion is essential for formation of chylomicrons after high-fat nutrition. These triacylglycerol-rich lipoproteins effectively neutralize endotoxin and protect animals against endotoxin-induced mortality (19, 20).

In this study we investigated the effect of high-fat enteral nutrition on endotoxemia, the subsequent inflammatory response and gut barrier function after hemorrhagic shock in bile duct ligated rats. In this way we aimed to further elucidate the mechanism of protection of high-fat enteral nutrition and to assess a potential role of high-fat enteral nutrition in reducing the harmful sequelae of endotoxin-related events after systemic hypotension in biliary obstruction.

Material and Methods

Animals

This study was performed according to the guidelines of the Animal Care Committee of the University of Maastricht and approved by the committee. Healthy male Sprague-Dawley rats, weighing 280 – 400 grams (average 339 grams) purchased from Charles River (Maastricht, the Netherlands) were housed under controlled conditions of temperature and humidity. Before the start of the experiments, rats were fed ad libitum with standard rodent chow and had free access to water.

Experimental Design

The setup of the experimental protocol is displayed in Figure 1. One week before hemorrhagic shock, bile duct ligation (BDL) was performed. Rats were anesthetized with i.p. sodium pentobarbital (40 mg/kg). A midline incision was made and the common bile duct was dissected, double ligated and cut in aseptic conditions. After recovery rats had free access to

water and standard chow again.
BDL-rats subjected to hemorrhagic shock (BDL-HS) were divided into three groups. Fasted (n=9); fed with low-fat enteral nutrition (n=10) and a group fed with high-fat enteral nutrition, (n=10).
BDL-controls (n=8) were fasted for 18 hours and sacrificed at t=0. Non-ligated rats (NL) were fed with high-fat nutrition and sacrificed at t=0 to study normal lipid absorption (n=13). Non-ligated rats fasted for 18 hours served as controls for the bile duct ligation. Bilirubin and alkaline phosphatase levels were measured in plasma of rats in all groups to evaluate the effect of bile duct ligation. High-fat or low-fat nutrition was administered via oral gavage at 18 hours before shock (3 ml) and at 2 hours and 45 minutes before shock (0.75 ml). The low-fat diet contained: 6.9% (energy-percent) proteins, 75.4% carbohydrates and 16.7% fat. The amount of fat in the low-fat diet was isocaloric to that present in standard rodent chow. The high-fat liquid enteral diet was isocaloric and isonitrogenous to the low-fat diet, but contained 6.9% proteins, 40.9% carbohydrates and 52.2% fat.

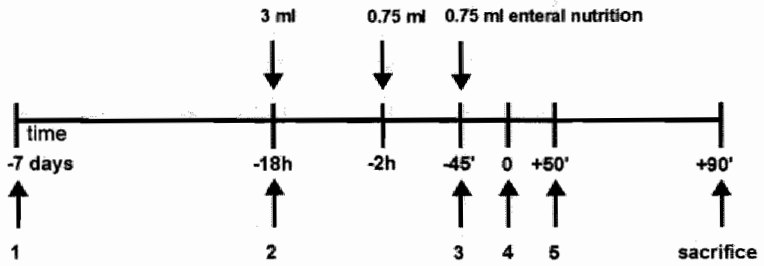


Figure 1:

Seven days before hemorrhagic shock, bile duct ligation was performed (1). At -18 hours before hemorrhagic shock, rats were starved overnight or fed via oral gavage (2), 45 minutes before withdrawal of blood anaesthesia was given and a femoral artery catheter inserted (3), at t=0 hemorrhagic shock was induced (4); after 50 minutes the femoral artery catheter was removed and the wound was closed (5); 90 minutes after t=0 rats were sacrificed. A liquid enteral nutrition (low-fat or high-fat) was administered in the fed groups, at -18 hours (3 ml), -2 hours (0.75 ml) and -45 minutes (0.75 ml).

Hemorrhagic shock procedure

Hemorrhagic shock was induced as previously described (15). In short, rats were anesthetized with i.p. Sodium pentobarbital (50 mg/kg); the femoral artery was dissected and cannulated with polyethylene tubing (PE-10) containing heparinized saline (10 IU/ml). At the time of shock (t=0), 2.1 ml blood per 100 gram body weight was taken at a rate of 1 ml/minute. Mean Arterial Pressure (MAP) and heart rate (HR) were recorded during a 50-minute observation period. Previously we observed that a steady-state is reached after this time-point and that the rats recovered spontaneously. At sacrifice (t=90), blood was taken and segments of small bowel were harvested for immunofluorescence and determination of gut permeability.

Plasma samples

Arterial blood samples were collected in heparinized pyrogen-free glass tubes at t=0 and t=90. Plasma was separated by centrifugation, frozen immediately and stored (-20°C) until analysis. Hematocrit was directly measured at t=0 and t=90.

Plasma assays

Bilirubin and alkaline phosphatase were determined using standard enzymatic assays (ABX diagnostics, Montpellier, France) in a Cobas Fara autoanalyzer (Roche, Basel, Switzerland). Triacylglycerol was determined using a standard enzymatic assay (Sigma, St. Louis, MO). Total circulating endotoxin was measured by a chromogenic Limulus Amoebocyte Lysate (LAL) assay (Endosafe, Charles River, Charleston, SC), as previously described (16, 21). TNF-α concentrations in arterial blood were determined using a standard sandwich-ELISA for rat TNF-α (kindly provided by Hbt, Uden, the Netherlands).

Immunofluorescence

To examine normal distribution of tight junctions we stained for Zonula Occludens protein 1 (ZO-1) which is highly associated with tight junctions. Sections of ileum and colon of four rats per experimental group (i.e. healthy control, BDL-control, fasted BDL-HS rats, low-fat treated BDL-HS rats and high-fat treated BDL-HS rats) were investigated for immunofluorescence. Photomicrographs representative for each group are displayed. Frozen sections of ileum and colon (4 µm) were pre-fixed in cold acetone and subsequently fixed with 4% paraformaldehyde. Rabbit anti-ZO-1 polyclonal antibody (Zymed, San Francisco, CA) was used as a primary antibody and negative controls were incubated with non-immune rabbit serum. Texas Red conjugated goat anti-rabbit antibody (Jackson, West Grove, PA) was used as secondary antibody. Next, slides were dehydrated in ascending ethanol series and mounted in DABCO (Sigma) 1:1 in glycerol (90%), containing 4', 6-diamino-2-phenyl indole (DAPI) (0.5 µg/ml, Sigma). Distribution of tight junctions was recorded with the Metasystems Image Pro System (Metasystems, Sandhausen, Germany) mounted on a Leica DM-RE fluorescence microscope (Leica, Wetzler, Germany). Photomicrographs were recorded at a 400x magnification.

Microbiological methods and intestinal permeability ex vivo

In all rats, bacterial translocation to mesenteric lymph nodes (MLN), spleen and liver was determined as previously described (15, 16). In short, mesenteric lymph nodes (MLN), the mid-section of the spleen and a liver-segment (IV) were collected aseptically in pre-weighed thioglycolate broth tubes (Becton Dickinson (BBL) Microbiology Europe, Maylan, France). Sample collection was identical for each animal. After weighing, all tissue specimens were homogenized and the entire suspension was transferred to agar plates. After 48h incubation, colonies were counted on all aerobic plates, identified to the species level using conventional techniques and next adjusted to the weight of the grounded tissue; sterile cultures were counted as "0".

Intestinal permeability for macromolecules was assessed in all rats by measuring translocation of the 44 kD enzyme horseradish peroxidase (HRP) in isolated segments of ileum by the everted sac method as previously described (16, 22, 23).

Statistical analysis

MAP and HR are represented as median (25^{th}- 75^{th} percentile); all other data are represented as median + 95% confidence interval for median. A non-parametric Mann-Whitney U test was used for comparisons between groups and where appropriate adjusted for multiple comparisons by a Bonferroni correction. Differences were considered statistically significant at $p < 0.05$.

Results

Hemorrhagic shock procedure in bile duct-ligated rats

Bilirubin and alkaline phosphatase were increased in BDL-rats (149 (141-157) and 306 (284-318) mg/dL, respectively) compared to non-ligated rats (1 (0.5-5) and 136 (103-191) mg/dL, respectively) with no differences the four groups of BDL-rats. Directly after hemorrhagic shock (t=0) mean MAP-values decreased from 89 (83 – 93) mmHg to 29 (25 -34) mmHg and mean HR decreased from 330 (310 – 345) beats per minute (bpm) to 224 (192 – 231) bpm in all three shock groups. After 50 minutes both MAP and HR spontaneously recovered. Hematocrit decreased from 43 (41-43) % at t=0 to 33 (32-35) % at t=90 in all hemorrhagic shock groups. Changes in mean arterial pressure (MAP), heart rate (HR) and hematocrit were similar for all hemorrhagic shock groups.

Influence of nutrition on triacylglycerol levels

First we assessed whether bile duct ligation resulted in a malabsorption of triacylglycerol after high-fat enteral nutrition. As expected, high-fat enteral nutrition resulted in elevated triacylglycerol levels in non-ligated (NL) rats compared to fasted NL-controls, Figure 2. Lack of intraluminal bile resulted in malabsorption of dietary lipids, illustrated by the significantly reduced triacylglycerol levels in BDL-rats fed with high-fat nutrition (47 (19–56) mg/dL) compared to non-ligated rats fed with high-fat nutrition (192 (89–263) mg/dL, $p<0.001$) before hemorrhagic shock. Plasma triacylglycerol concentrations in BDL-rats receiving high-fat enteral nutrition were significantly higher compared to fasted ($p<0.001$) or low-fat treated BDL-HS rats ($p<0.01$), or control BDL-rats ($p<0.001$).

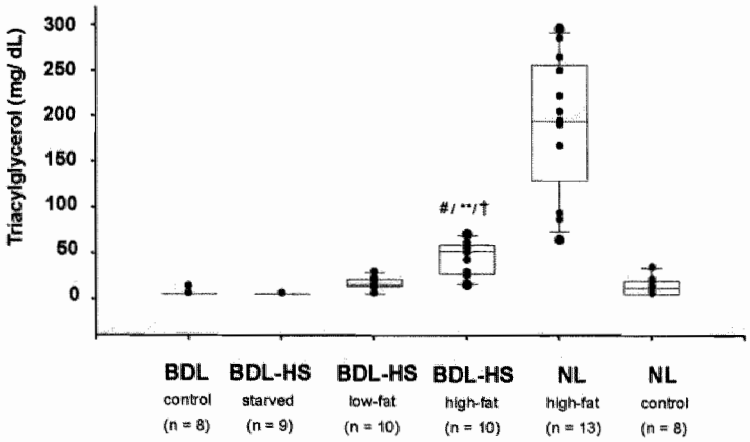


Figure 2:

*High-fat enteral nutrition in non-ligated controls (NL) significantly increased plasma triacylglycerol compared to fasted non-ligated rats, *p<0.001. Bile duct ligation caused a fat malabsorption illustrated by significantly lower plasma triacylglycerol levels compared with NL controls fed with high-fat nutrition, #p<0.001. High-fat nutrition in BDL-rats led to significantly elevated triacylglycerol levels compared to low-fat pretreated BDL-rats (**p=0.006, (Bonferroni corrected)) and fasted BDL-rats (†p<0.001, (Bonferroni corrected)) before hemorrhagic shock. Plasma triacylglycerol levels in bile duct-ligated (BDL) rats that were fasted or that received low-fat nutrition were near detection level and comparable with those of non-ligated normal controls (NL-control) before hemorrhagic shock (HS).*

Endotoxin and TNF-α

Hemorrhagic shock resulted in a systemic endotoxemia in fasted BDL-rats at t=90 (32 (29–36) pg/ml) compared to BDL-controls (2 (1–3) pg/ml, p=0.001), Figure 3. In high-fat pretreated BDL-HS rats endotoxin concentrations were significantly decreased (13 (9-26) pg/ml) compared to fasted (p=0.003) and low-fat pretreated rats (p=0.03). Endotoxin levels were significantly decreased in low-fat pretreated rats compared to fasted rats (27 (18-31) pg/ml, p<0.03).
In line, plasma TNF-α was elevated after hemorrhagic shock in fasted BDL-HS rats (222 (84-258) pg/ml) compared to BDL-control rats (2 (2-97) pg/ml, p=0.002), Figure 4. Circulating TNF-α was significantly lower in BDL-HS rats fed with high-fat nutrition (33 (22-105) pg/ml) compared to fasted BDL-rats (p=0.006).

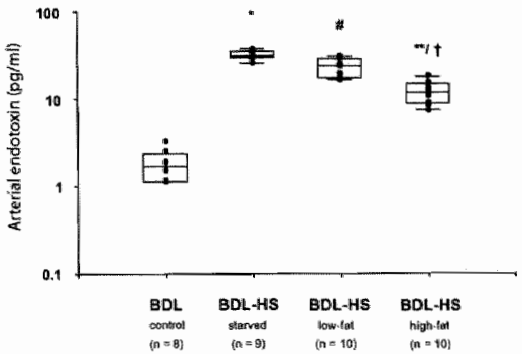


Figure 3:

*Hemorrhagic shock (HS) resulted in a clear endotoxemia in fasted bile duct-ligated (BDL) rats compared to BDL-control rats, *p=0.001. Circulating endotoxin is significantly lower in the high-fat pretreated BDL-HS group compared to both the fasted (**p=0.003, (Bonferroni corrected)) and the low-fat pretreated group (†p=0.03, (Bonferroni corrected)). In BDL-HS rats fed with low-fat nutrition, endotoxin levels were significantly decreased compared to fasted BDL-HS rats (#p=0.024, (Bonferroni corrected)). Individual measurements are represented by median (solid line), 5th, 25th, 75th and 95th percentiles. A logarithmic Y-axis is used.*

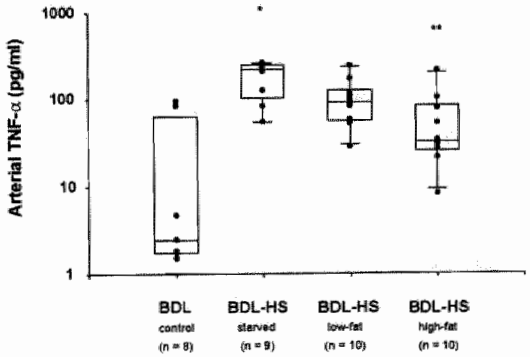


Figure 4:

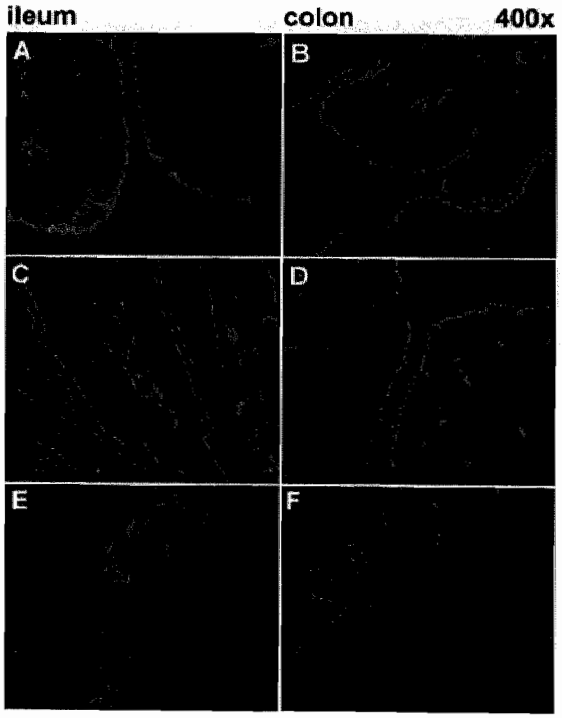
*Hemorrhagic shock (HS) resulted in a clear TNF-α response in fasted bile duct-ligated (BDL) rats compared to BDL-control rats, *p=0.002. Circulating TNF-α was significantly lower after hemorrhagic shock in the high-fat pretreated BDL-HS rats compared to the fasted BDL-HS group (**p=0.006, (Bonferroni corrected)). Individual measurements are represented by median (solid line), 5th, 25th, 75th and 95th percentiles. A logarithmic Y-axis is used.*

Intestinal barrier function

Gut barrier function was determined by measurement of functional and structural indicators. Distribution of the tight junction associated Zonula Occludens protein ZO-1 was assessed in ileum and colon. Figure 5 shows a normal distribution pattern of ZO-1, in ileum (A) and colon (B) of healthy control non-ligated rats. Bile duct ligation did not change ZO-1 staining (Fig. 5C, D). However, bile duct ligation followed by hemorrhagic shock resulted in a dramatic loss of ZO-1 protein in ileum and colon of fasted and low-fat pretreated rats (Fig. 5E, F). In contrast, the loss of ZO-1 distribution was largely prevented in rats fed with high-fat enteral nutrition and was similar to that observed in control rats (Fig. 5I, J). In conclusion, pretreatment with lipid-rich nutrition clearly preserved tight junction distribution in bile duct ligated rats early after hemorrhage.

Next, we assessed intestinal permeability for the macromolecule horseradish peroxidase. As expected, intestinal permeability was increased in fasted BDL-rats subjected to hemorrhagic shock compared to BDL-controls ($p=0.002$), Figure 6. Leakage of HRP was strongly reduced in BDL-rats fed with high-fat enteral nutrition (0.9 (0.6-1.3) µg/ml) compared to both fasted BDL-HS rats (7.3 (6.8-8.7) µg/ml, $p=0.003$) and low-fat pretreated BDL-HS rats (2.2 (1.5-3.9) µg/ml, $p=0.003$).

Finally, bacterial translocation to mesenteric lymph nodes (MLN), spleen and liver was determined. As expected, bacterial cultures from non-ligated controls were sterile. In line with others (7, 24) we observed bacterial translocation in all bile duct ligated animals (Table 1). Hemorrhagic shock was associated with higher median numbers of bacterial translocation compared with BLD-control rats ($p<0.05$). High-fat enteral nutrition before hemorrhagic shock was associated with reduced median numbers of translocated bacteria to MLN however there were no statistical significant differences compared with BDL-rats that were fasted before hemorrhagic shock.



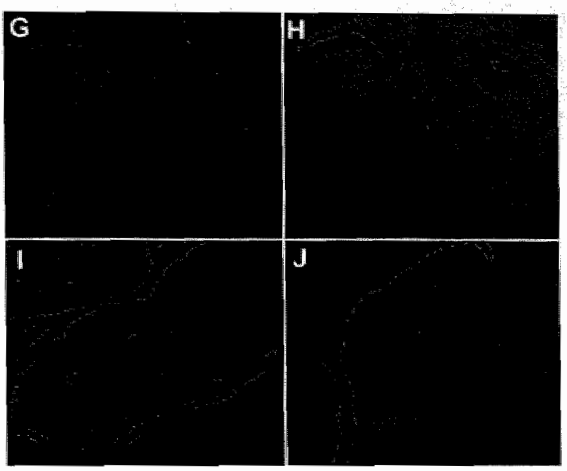


Figure 5:

Immunolocalisation of ZO-1 (red) at a 400x magnification showed a regular distribution in the terminal ileum (A) and colon (B) of control rats that were starved for 18 hours but not subjected bile duct ligation and hemorrhagic shock. ZO-1 is localized in the upper part of enterocytes, showing a normal distribution in association with the cellular surface. Bile duct ligation did not change ZO-1 protein staining (ileum C and colon D) but bile duct ligation in fasted rats subjected to hemorrhagic shock led to a significant loss of ZO-1 in parts of both ileum (E) and colon (F). Moreover, the tissue was disrupted and disorganized. Also in tissue sections of BDL-rats fed with low-fat nutrition before hemorrhagic shock there was a clear ZO-1 protein loss in ileum and colon (G, H), although to a lesser extent compared to the BDL-HSS group. In contrast, in animals fed with the high-fat diet, ZO-1 distribution after hemorrhagic shock was regular, discrete and similar to that of control rats (I, J).

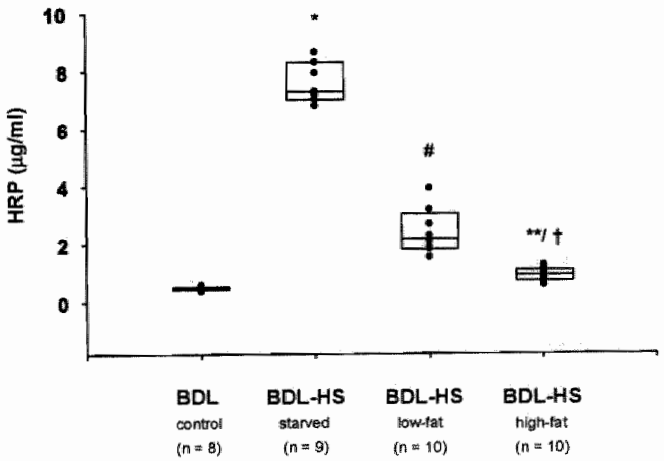


Figure 6:

*Leakage of horseradish peroxidase (HRP) was significantly increased in fasted bile duct ligated (BDL) rats after hemorrhagic shock (HS) compared to BDL-control rats, *p=0.002. In BDL-HS rats fed with high-fat enteral nutrition before hemorrhagic shock, HRP leakage is strongly reduced compared to both the fasted (**p=0.003, (Bonferroni corrected)) and the low-fat treated BDL-HS rats (†p=0.003, (Bonferroni corrected)). In BDL-HS rats fed with low-fat nutrition, leakage of HRP is significantly decreased compared to fasted BDL-HS rats (#p=0.003, (Bonferroni corrected)). Individual measurements are represented by median (solid line), 5th, 25th, 75th and 95th percentiles.*

Table 1. Bacterial translocation in bile duct ligated rats at 90 minutes after shock

Groups	Translocation Incidence[1]	Mesenteric lymph nodes[2]	Spleen[2]	Liver[2]
NL-control	0/8	0	0	0
BDL-Control	8/8	59 (54-111)	106 (25-443)*	741 (201-1367)*
BDL-HS	9/9	324 (156-4184)	350 (95-5262)†	2468 (5-10299)†
BDL-HS	10/10	216 (33-1285)	230 (16-659)	1571 (26-4497)
BDL-HS	10/10	170 (56-798)	235 (122-689)‡	2741 (23-15634)

[1] Number of rats with bacterial translocation vs total number of rats per group.
[2] Results are presented as median colony forming units (cfu)/ gram tissue (range). Only positive cultures have been included. *p<0.01 compared with NL-control; †p<0.05 compared with BDL-control. ‡ in one animal sterile cultures were found NL = non-ligated; BDL = bile duct ligation; HS = hemorrhagic shock

Discussion

Systemic hypotension and biliary obstruction are important factors that contribute to the increased endotoxin-mediated postoperative complications in jaundiced patients. In the last decade, various approaches have been used to target endotoxin and the subsequent inflammatory response in biliary obstructed patients or animals, such as oral bile salts (17, 25, 26), lactulose (27) and preoperative drainage (12, 13). However, there is still no effective therapy to prevent the common postoperative septic complications (28). Recently we showed that high-fat enteral nutrition is effective in reducing endotoxin, TNF-α and gut barrier failure after hemorrhagic shock (15, 16). The present study was designed to further elucidate the mechanism of protection of high-fat enteral nutrition and to investigate the potential beneficial effects of high-fat enteral nutrition on endotoxin-related events in combined bile duct ligation and surgical hypotension.

Bile duct ligation resulted in a substantial malabsorption of dietary triacylglycerol. A slight increase of triacylglycerol was observed in high-fat pretreated BDL-rats at both t=0 and t=90 compared to the fasted and low-fat pretreated groups. However, these levels were less than 20% of those found in plasma of non-ligated controls fed with the high-fat diet (NL-HF). Furthermore, these low concentrations of triacylglycerol were comparable with plasma levels of non-ligated rats fed with low-fat nutrition in which we did not find a significant protection after hemorrhagic shock (16). The current data are in line with earlier studies showing that bile duct ligation results in a strongly reduced fat absorption (29). Although bile is crucial for micellular solubilisation and a normal fat-uptake, hydrolysis of dietary triacylglycerol also occurs in vivo without involvement of bile salts(30). This explains that triacylglycerol uptake is not completely abolished in high-fat pretreated cholestatic rats.

A significant reduction in systemic endotoxin levels was observed after hemorrhagic shock in BDL-rats fed with high-fat enteral nutrition compared to fasted BDL-rats or BDL-rats fed with low-fat enteral nutrition. Interestingly, this reduction of plasma endotoxin appeared to be independent of the presence of intraluminal bile salts and not directly affected by malabsorption of triacylglycerol. Endotoxin triggers a cascade of responses of which the inflammatory response and release of TNF-α in particular, is most prominent (31, 32). In this study TNF-α was significantly reduced in high-fat pretreated rats compared to fasted controls. This is likely due to the decreased plasma endotoxin since Goldman et al. (33) showed that endotoxin is the primary trigger for TNF-α release after hemorrhage.

Endotoxin and primarily TNF-α have been shown to impair intestinal barrier function (34-37). Disruption of the intestinal barrier facilitates endotoxin translocation leading to a vicious circle of ongoing endotoxin translocation, TNF-α release and gut barrier failure. In the present study bile duct ligation followed by hemorrhagic shock resulted in a disruption of tight junctions in fasted BDL-rats. In line with others, showing a decrease of tight-junction expression by elevated proinflammatory cytokine concentrations (36), increased TNF-α levels paralleled loss of ZO-1 protein in the intestine of fasted and low-fat pretreated BDL-rats after hemorrhagic shock, whereas low TNF-α levels were accompanied by a preserved ZO-1 distribution in high-fat pretreated rats. The structural changes in gut wall integrity were accompanied in part by functional changes in intestinal permeability. A clear reduction in permeability for HRP was observed in high-fat pretreated BDL-rats compared to both the control fed and the fasted group.

Although median numbers of translocated bacteria to MLN was reduced in high-fat treated rats compared with fasted BDL-HS rats there was no significant difference. The presence of bacteria in various organs is a result of a dynamic process of translocation from the gut, survival and clearance of bacteria via the reticuloendothelial system (RES). The reduction of RES clearance function observed in bile duct-obstructed animals leads to impaired clearance of bacteria (7). This apparently contributes to the discrepancy between bacterial translocation and gut barrier structure/ function.

The mechanism(s) responsible for the effect of high-fat enteral nutrition remain unclear. However, the current data show that intraluminal bile is not involved in the beneficial effect of high-fat enteral nutrition. Furthermore, a normal uptake of dietary lipids does not appear to be necessary for the effectiveness of high-fat enteral nutrition, although triacylglycerol-rich lipoproteins such as postprandial formed chylomicrons (38) have been shown to be potent scavengers of endotoxin and inhibitors of endotoxin-induced inflammation (19, 39). Analysis of triacylglycerol levels and plasma endotoxin, arterial TNF-α and HRP leakage shows a positive relation in the shock groups. Theoretically, the slightly enhanced levels of triacylglycerol in the high-fat pretreated BDL-rats may have been sufficient to provide protection after hemorrhagic shock. However, previously protection was not found at the low triacylglycerol levels reached with high-fat enteral nutrition in BDL-rats (15, 16). Based on these data, we conclude that triacylglycerol-rich lipoproteins are not essentially involved in the observed protection, but may rather be an epiphenomenon of lipid nutrition.

High-fat enteral nutrition also induces several other responses independently of the presence of intraluminal bile, which may account for the observed protection. The release of cholecystokinin octapeptide by neuro-endocrine enterocytes is stimulated upon high-fat nutrition. This intestinal neuropeptide has been shown to have an anti-inflammatory effect via regulation of NF-κB activity in pulmonary interstitial macrophages (40). High-fat enteral feeding enhances secretion of surfactant-like particles (SLP) present in the intestinal mucosa (41). These particles appear to be secreted in the intestinal mucosa, are similar to pulmonary surfactant and are thought to be part of a mucosal defense mechanism of the gut (42, 43). It remains to be investigated to which extent these processes contribute to the observed protection.

In summary, the present study shows for the first time that administration of high-fat enteral nutrition decreases endotoxin and the subsequent TNF-α release and simultaneously preserves gut barrier function in cholestatic rats after hemorrhagic shock. These data demonstrate that the protection of high-fat enteral nutrition is independent of the presence of intraluminal bile salts and that a high uptake of triacylglycerol is not essential. The mechanism that underlies the beneficial effect of high-fat enteral nutrition in animals compromised by hemorrhagic shock remains to be further elucidated. This relatively simple nutritional intervention may provide a potential new therapeutic strategy to reduce endotoxin-mediated complications in jaundiced patients undergoing major surgery.

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Chapter 8

Nutritional stimulation of CCK-receptors inhibits inflammation via the vagus nerve.

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Abstract

The immune system in vertebrates senses exogenous and endogenous danger signals via complex cellular and humoral processes and responds with an inflammatory reaction to combat putative attacks. A strong protective immunity is imperative to prevent invasion of pathogens, however, equivalent responses to commensal flora and dietary components in the intestine have to be avoided.

The autonomic nervous system plays an important role in sensing luminal contents in the gut via hard-wired connections and chemical messengers, such as cholecystokinin. Here, we describe that ingestion of dietary fat stimulates cholecystokinin (CCK)-receptors leading to attenuation of the inflammatory response via the efferent vagus nerve and nicotinic receptors. Vagotomy and administration of antagonists for CCK and nicotinic receptors significantly blunted the inhibitory effect of high-fat enteral nutrition on hemorrhagic shock-induced tumor necrosis factor (TNF)-α, interleukin (IL)-6 release. Furthermore, the protective effect of high-fat enteral nutrition on inflammation-induced intestinal permeability was abrogated by vagotomy and administration of antagonists for CCK and nicotinic receptors.

These data reveal a novel neuro-immunological pathway controlled by nutrition that may help to explain the intestinal hyporesponsiveness to dietary antigens and sheds a new light on functionality of nutrition.

Introduction

The immune system in vertebrates senses exogenous and endogenous danger signals via complex cellular and humoral processes and responds with an inflammatory reaction to combat putative attacks(1). Although inflammation is essential to protect the host from invasion of potentially harmful pathogens, an overwhelming inflammatory response leading to tissue damage, increased vascular permeability and organ injury has to be avoided(2, 3). In the gastrointestinal tract, hyperactivation of the immune system to commensal bacteria and dietary antigens is continuously inhibited to maintain homeostasis and to allow absorption and utilization of nutrients(4). Recently, we showed that dietary fat strongly reduced the systemic inflammatory response following hemorrhagic shock indicating a direct interaction between specific food components and the systemic immune response(5, 6).
Ingestion of food triggers a cascade of responses such as initiation of gut contractility and regulation of food intake via hard-wired connections and chemical messengers, such as cholecystokinin (CCK) and PYY_{3-36} (7-10). Besides regulation of metabolism, the parasympathetic nervous system was recently identified to inhibit macrophage activation via the vagus nerve through binding of acetylcholine to α-7 nicotinic receptors located on macrophages(11, 12). Central or peripheral stimulation of this so-called 'cholinergic anti-inflammatory pathway' reduced plasma TNF-α in endotoxic shock and blunted nuclear factor-κB activation after hemorrhagic shock via efferent vagal nerve fibres(13-15). We reasoned that high-fat enteral nutrition, sensed in the gastrointestinal tract activates the parasympathetic nervous system leading to inhibition of the inflammatory response via efferent vagal fibres.

Results and Discussion

To investigate whether a neural based anti-inflammatory pathway is involved in the effect of high-fat enteral nutrition, Sprague-Dawley rats were subjected to (sham) vagotomy, 45 minutes before induction of hemorrhagic shock as described in Methods. Animals were either fasted or enterally fed with high-fat or low-fat nutrition 18 hours, 2 hours and 45 minutes before hemorrhagic shock was induced. Inflammatory mediators and gut barrier function were assessed 90 minutes after shock.

Hemorrhagic shock typically results in systemic release of proinflammatory cytokines such as TNF-α and IL-6(16). In line with our earlier observations, high-fat enteral nutrition (containing 52 en% of fat) strongly reduced hemorrhagic shock-induced TNF-α and IL-6 in rats subjected to sham vagotomy compared with low-fat (containing 17 en% of fat) treated and fasted controls (Fig. 1a-b). These data show that the percentage of fat in the enteral diet is determining for protection since the inflammatory response was only mildly affected in the low-fat treated control group. Vagotomy abrogated the high-fat induced reduction in TNF-α (205 ± 11 pg/ml vs. 5 ± 1 pg/ml (sham), $P<0.01$) and IL-6 levels (80 ± 5 pg/ml vs. 19 ± 9 pg/ml (sham), $P<0.01$) following hemorrhagic shock compared with rats that underwent a sham vagotomy.
Changes in intestinal barrier function were evaluated by determination of bacterial translocation to distant organs, leakage of horseradish peroxidase (HRP) in isolated ileum-segments and plasma endotoxin levels as described in Methods (supporting material).
In line with previous reports (16, 17), the inflammatory response in control shock-rats was

paralleled by bacterial translocation to distant organs (Table 1), an increased permeability for HRP and detectable endotoxin levels (Fig. 1 c-d). Impairment of gut barrier function following hemorrhagic shock is likely caused by proinflammatory cytokines, since application of cytokines such as TNF-α to intestinal cells increased intestinal permeability and decreased inflammatory cytokines prevented loss of intestinal barrier function (18-20). In accordance with high-fat enteral nutrition induced inhibition of the inflammatory response, circulating endotoxin levels, permeability of ileum segments for HRP and bacterial translocation to distant organs were reduced compared with low-fat treated and fasted sham vagotomized rats. Vagotomy reversed this protection of high-fat nutrition and led to elevated plasma endotoxin levels (from: 12 ± 2 pg/ml, to: 28 ± 1 pg/ml, P<0.01), increased leakage of HRP in ileum segments (from: 1.1 ± 0.7 µg/ml to: 2.3 ± 0.5 µg/ml, P<0.01) and increased bacterial translocation (from: 16 colony forming units (cfu)/ gram tissue to: 328 cfu/gram, P<0.01), (Fig. 1c-d, Table 1). Based on these findings we concluded that a parasympathetic neural control mechanism underlies the protective effect of enteral nutrition containing a high percentage of fat.

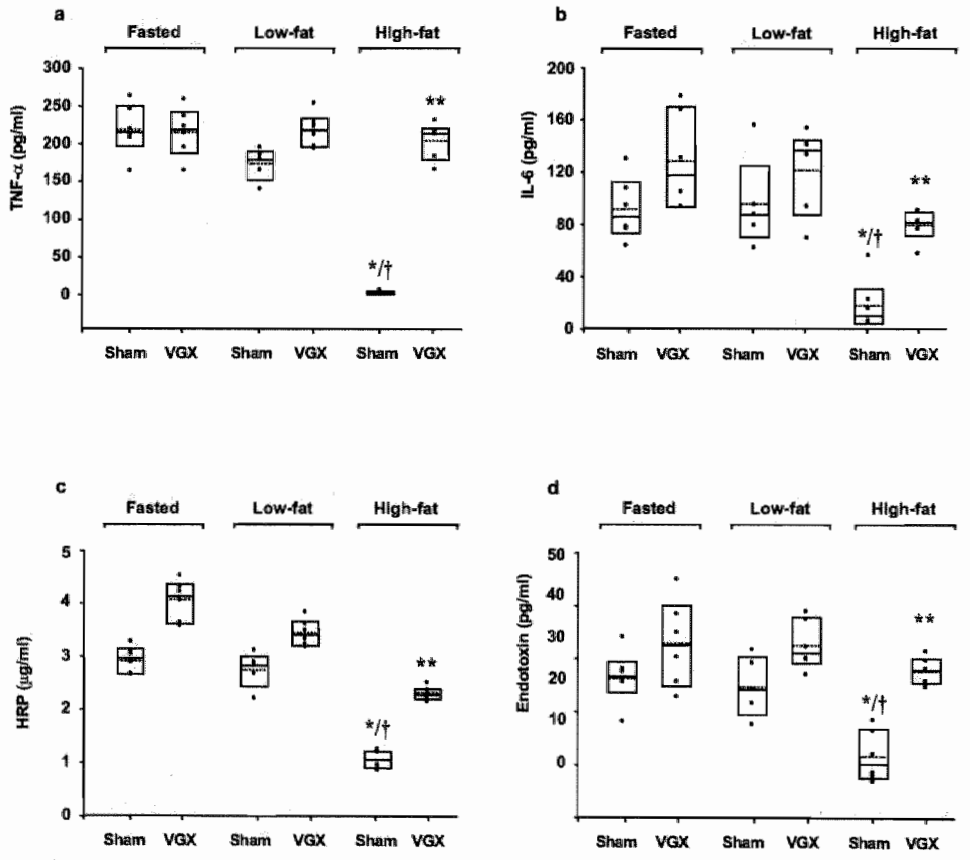
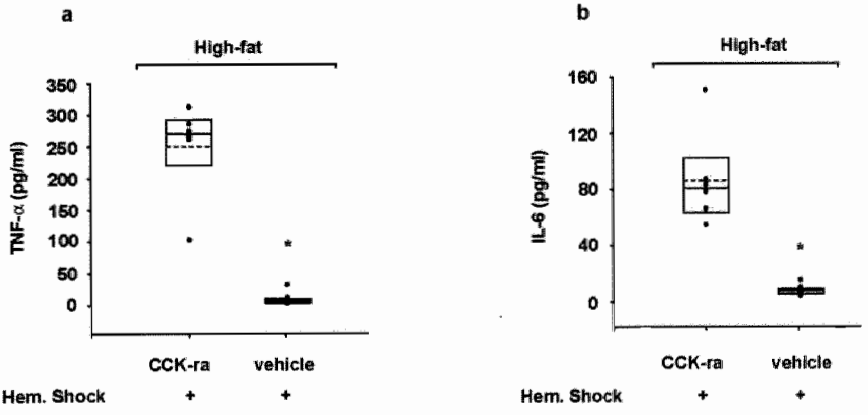


Figure 1:

Inhibition of TNF-α (a), IL-6 (b), leakage of HRP in ileum (c) and endotoxin (d) after hemorrhagic shock by high-fat nutrition is reversed by vagotomy. Data are solid dots, mean (dashed line), median (solid line), 25th and 75th percentiles. Asterisk P<0.01 versus fasted Sham + Hem. Shock; double asterisk P<0.01 versus high-fat treated Sham + Hem. Shock; Dagger P<0.05 versus low-fat treated Sham + Hem. Shock.

Nutrition activates the autonomic nervous system in several ways, based on the nature of its composition. Dietary fat typically results in release of CCK, a potent neuro-endocrine signaling molecule that activates nerve cells via CCK-A and CCK-B receptors (reviewed in (21)). To investigate the role of CCK in the protective effect of high-fat enteral nutrition on the host response to hemorrhagic shock, rats underwent a sham vagotomy and received CCK-A (500 µg/kg) and CCK-B (500 µg/kg) receptor antagonists (22, 23) or vehicle (90% saline, 5% Tween, 5% DMSO) intravenously 25 minutes before hemorrhagic shock. CCK-receptor blockade potentially alters lipid digestion. However, levels of circulating triglycerides in rats treated with CCK-receptor antagonist (187 ± 8 mg/dL) were similar compared to rats treated with vehicle (200 ± 16 mg/dL), indicating that lipid absorption in the acute phase is unaffected.

Administration of CCK-A and CCK-B receptor antagonists enhanced plasma TNF-α (251 ± 30 pg/ml) and IL-6 levels (87 ± 14 pg/ml) following hemorrhagic shock, compared with high-fat treated rats administered vehicle (10 ± 4 pg/ml, $P<0.01$ and 9 ± 1 pg/ml, $P<0.01$, respectively) (Fig. 2a-b). Furthermore, plasma endotoxin was elevated (24 ± 2 pg/ml vs. 13 ± 2 pg/ml (vehicle), $P=0.01$), permeability for HRP was increased (2.2 ± 0.1 µg/ml vs. 1.1 ± 0.1 µg/ml (vehicle), $P<0.01$) (Fig. 2c-d), while more bacteria translocated to distant organs (total 267 cfu/gram (158-837) vs. total 57 cfu/gram (23-217) (vehicle) , $P<0.01$), (Table 1) in animals injected with CCK-receptor antagonists, compared with vehicle treated controls. These findings cannot be attributed to injection of CCK-receptor antagonists, since stimulation of peritoneal macrophages from rats with both receptor antagonists did not trigger TNF-α release (<10 pg/ml, (below detection limit)). Furthermore, injection of CCK-receptor antagonists in rats not subjected to hemorrhagic shock did not elict TNF-α release (13 ± 5 pg/ml) and did not cause bacterial translocation (total: 0 cfu/gram (0-7) or increased leakage of HRP (0.6 ± 0.1 µg/ml) in ileum segments, which is not different from healthy control rats. These data show that high-fat enteral nutrition inhibits the proinflammatory response and prevents loss of intestinal barrier integrity via activation of CCK-receptors.



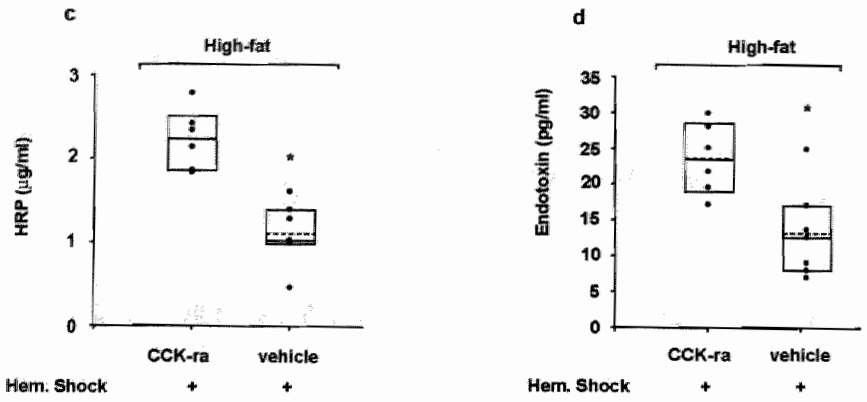


Figure 2:
TNF-α (a), IL-6 (b), leakage of HRP in ileum (c) and endotoxin (d) were increased after hemorrhagic shock in high-fat treated rats injected with CCK-receptor A+B antagonists in high-fat treated rats vagotomized before hemorrhagic shock. Data are solid dots, mean (dashed line), median (solid line), 25th and 75th percentiles. Asterisk P<0.01 versus vehicle treated group.

Table 1:

	High-fat cfu/ gram tissue	Fasted cfu/ gram tissue
Sham-VGX	16 (0-65) *	412 (206-517)
VGX	328 (183-1459)	542 (164-849)
Sham-VGX + CCK-ra	267 (158-837) **	
Sham-VGX + vehicle (CCK-ra)	57 (23-217)	
Sham-VGX + Chlorisondamine	226 (34-1410) **	172 (56-488)
Sham-VGX + vehicle (Chlor.)	22 (5-71)	

All rats were subjected to hemorrhagic shock. Mesenteric lymph nodes, spleen and liver were cultured at sacrifice (90 minutes). High-fat treated or fasted rats were subjected to (sham) vagotomy (VGX) and treated with CCK-receptor antagonists (CCK-ra), chlorisondamine or vehicle where indicated. Total bacterial translocation is expressed as colony forming units (cfu)/ gram tissue. Results are median (range); n=6 per group. Asterisk P<0.05 compared with VGX; Double asterisk P<0.05 compared with vehicle treated.

Stimulation of the parasympathetic nervous system may result in activation of the hypothalamic-pituitary-adrenal (HPA) axis(24). Alternatively, vagal efferent fibres can be stimulated causing inhibition of the inflammatory response via nicotinic receptors. In order to assess activation of the HPA axis, corticosterone levels were measured in plasma. High-fat enteral nutrition enhanced circulating corticosterone after hemorrhagic shock in sham-vagotomized shock animals (19 ± 4 ng/ml) compared with fasted rats (6 ± 4 ng/ml, P =0.046). However, both

vagotomy (9 ± 4 ng/ml, P =0.09 compared with sham vagotomized controls (19 ± 4 ng/ml)) and administration of CCK-receptor antagonists (24 ± 1 ng/ml, P=0.39 compared with vehicle treated rats 22 ± 2 ng/ml) did not significantly affect corticosterone levels in high-fat treated rats. These data are in line with earlier reports showing that vagotomy does not affect corticosterone levels(25). It may well be that the increase in circulating corticosterone is an epiphenomenon caused by stress of oral gavage(26).

Next, we studied whether stimulation of CCK-receptors by high-fat enteral nutrition inhibits the inflammatory response via the anti-inflammatory efferent vagal pathway, by inhibition of peripheral nicotinic receptors using chlorisondamine diiodide. Chlorisondamine diiodide or vehicle (saline) were administered 25 minutes before induction of hemorrhagic shock in a dose (0.125 mg/kg) that blocks only peripheral nicotinic receptors(15, 27). A control (fasted) hemorrhagic shock group that received chlorisondamine was included, because inhibition of nicotinic receptors can cause vasodilatation and hypotension(27). Administration of chlorisondamine at this dose did not cause additional hypotension or changes in heart rate before and just after induction of shock (Fig. 3e). Mean arterial pressure was significantly lower during the 50-minutes observation period compared with vehicle treated controls, however, this did not affect the shock-induced inflammatory response and loss of gut barrier integrity. Chlorisondamine abrogated the inhibitory effect of high-fat enteral nutrition on circulating TNF-α (140 ± 7 pg/ml vs. 63 ± 14 pg/ml (vehicle), P<0.01) and IL-6 (99 ± 12 pg/ml vs. 30 ± 10 pg/ml (vehicle), P<0.05), (Fig. 3a-b). TNF-α and IL-6 levels in these high-fat rats treated with chlorisondamine were comparable with those of chlorisondamine-treated fasted rats. Inhibition of nicotinic receptors in high-fat treated rats via administration of chlorisondamine led to increased bacterial translocation to distant organs (total 226 cfu/gram, P<0.05 vs. total 22 cfu/gram (vehicle)), (Table 1), increased permeability for HRP in ileum segments (2.7 ± 0.2 μg/ml, P<0.01 vs. 1.1 ± 0.2 μg/ml (vehicle)) and elevated plasma endotoxin levels (26 ± 3 pg/ml, P<0.05 vs. 12 ± 3 pg/ml (vehicle)), compared with high-fat treated rats administered vehicle (Fig. 3c-d). These findings indicate that high-fat enteral nutrition inhibits inflammation by stimulation of nicotinic receptors via efferent vagal fibres.

The present study shows that high-fat enteral nutrition stimulates CCK-receptors centrally or peripherally via the afferent vagus nerve leading to inhibition of the inflammatory response via vagal efferents and nicotinic receptors (Fig. 4). Previously, we showed that the beneficial effects of high-fat enteral nutrition on inflammation and intestinal barrier integrity are specific for the amount of lipids in the enteral nutrition, not related to caloric intake and cannot be attributed to formation of endotoxin neutralizing triacylglycerol-rich lipoproteins (5, 6, 16). The finding that high-fat enteral nutrition inhibits inflammation via the vagus nerve provides a functional new mechanism for the interaction between nutrition and the immune response and has widespread implications. It was previously unrecognized that nutrition-induced neuro-endocrine signals such as CCK modulate the immune response via the efferent vagus nerve. From a teleological point of view it is functional that a state of immune-hyporesponsiveness is created during feeding. In this way an unwanted response to temporally present high amounts of dietary antigens, biological toxins and destructive endogenous lysozymes in the gut lumen is prevented, gut barrier function is preserved and homeostasis maintained.

We propose that this neural feedback-loop activated by enteral nutrition is an important player in the thus far largely unexplained state of hyporesponsiveness of the immune system in the intestinal tract to dietary antigens and bacterial toxins.

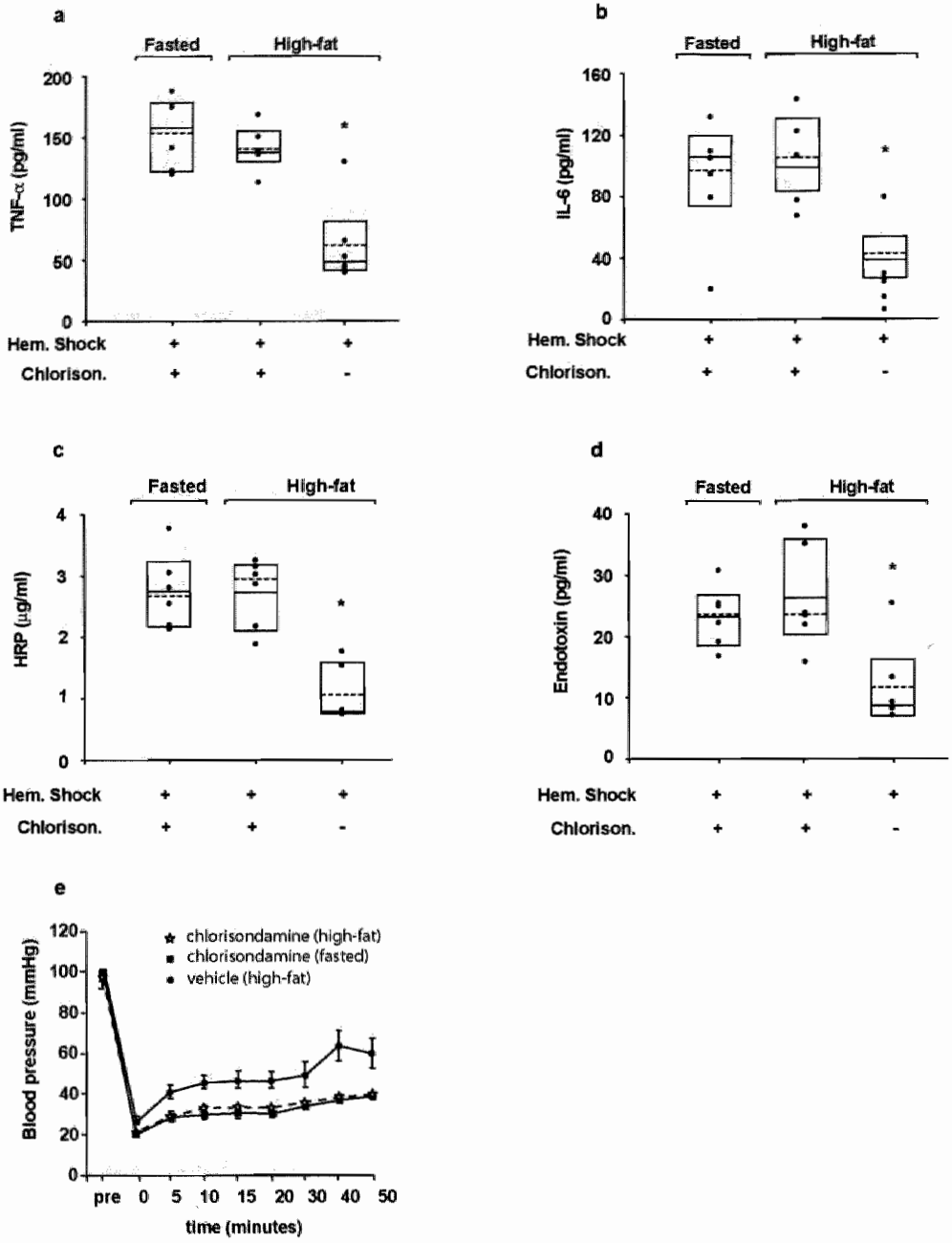


Figure 3:

TNF-α (a), IL-6 (b), leakage of HRP in ileum (c) and endotoxin (d) were increased after hemorrhagic shock in rats injected with chlorisondamine. There were no significant differences between fasted and high-fat treated rats treated with chlorisondamine. Data are solid dots, mean (dashed line), median (solid line), 25th and 75th percentiles. Asterisk P<0.01 versus vehicle treated group. Treatment with chlorisondamine did not affect mean arterial pressure (MAP) before and just after hemorrhagic shock (e), although there was a significant difference in MAP between chlorisondamine and vehicle treated rats from 10 to 50 minutes during the observation period (P<0.05).

Based on our findings, high-fat enteral nutrition is potentially therapeutic in various inflammatory disorders such as sepsis and inflammatory bowel disease (IBD) characterized by an inflammatory response in which TNF-α is prominent and intestinal barrier function is impaired. In light of this, a fasted state could be a risk factor for developing a potentially lethal inflammatory response after trauma or injury.

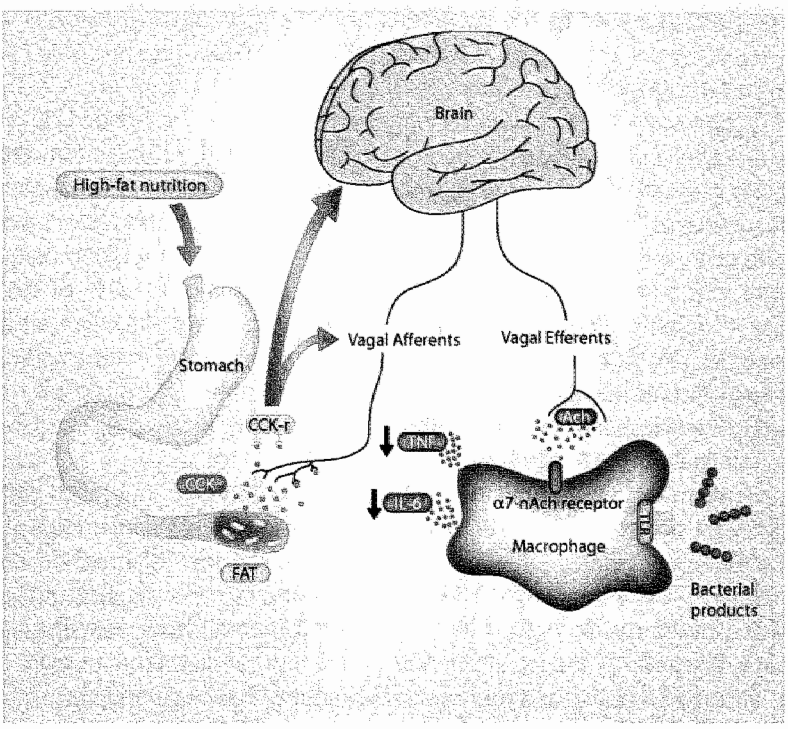


Figure 4:
Dietary fat inhibits the inflammatory response via stimulation of CCK-receptors leading to activation of nicotinic receptors by vagal efferents. Ingestion of high amounts of fat induces release of cholecystokinin (CCK) that binds to CCK-A and CCK-B receptors (CCK-r) located centrally or on peripheral vagal afferents. Activation of CCK-receptors triggers vagal efferents leading to an increase of acetylcholine (Ach), the principal parasympathetic neurotransmitter. Release of inflammatory cytokines such as TNF-α and IL-6 after activation of toll like receptors by bacterial products is inhibited via binding of acetylcholine to nicotinic receptors.

Material and methods

Reagents

CCK-A (Devazepide) and CCK-B (L365, 260) receptor antagonists were a kind gift from ML Laboratories PLC (Liverpool, UK) and dissolved in 90% NaCl, 5% Tween-20, 5% dimethyl sulfoxide (DMSO) to a final concentration of 500 µg/ml. Chlorisondamine diiodide, a nicotinic receptor antagonist was purchased from Tocris Cookson Ltd (Bristol, UK) and dissolved in saline to a final concentration of 0.125 mg/ml.

Animals

Healthy male Sprague-Dawley rats, weighing 290-420 grams (average 360 grams) were purchased from Charles River (Maastricht, the Netherlands) and housed under controlled conditions of temperature and humidity. Before the start of the experiments, rats were fed ad libitum with standard rodent chow and had free access to water. The experimental protocol was performed according to the guidelines of the Animal Care Committee of the University of Maastricht and approved by the committee.

Experimental design and procedures

A non-lethal hemorrhagic shock model was used as previously described (6, 16). In short, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p); the femoral artery was dissected and cannulated with polyethylene tubing (PE-10) containing heparinized saline (10 IU/ml). Mean Arterial Pressure (MAP) and heart rate (HR) were continuously recorded during a 50-minute observation period. At the time of shock (t=0), 2.1 ml blood per 100 gram of body weight was taken at a rate of 1 ml/minute (representing 30-40% of the total blood volume). At 45 minutes before induction of hemorrhagic shock, rats were either subjected to vagotomy or sham vagotomy. In vagotomized animals a ventral cervical incision was made and both vagal trunks exposed. The vagus nerve was ligated at both ends using 4-0 silk suture and divided. In sham-operated animals both vagal trunks were exposed, but the vagus nerve was not ligated and divided.

Before the experiments rats were either fasted (n=18) or fed with low-fat or high-fat enteral nutrition via oral gavage (n=36). The high-fat liquid enteral diet contained 6.9 (energy %) proteins, 40.9 en% carbohydrates and 52.2 en% fat; the low-fat nutrition contained 6.9 en% proteins, 75.4 en% carbohydrates and 16.7 en% fat. The amount of fat in the low-fat diet was isocalorical to that present in standard rodent chow and the high-fat liquid enteral diet was isocaloric and isonitrogenous to the low-fat diet. Proteins were derived from lean milk, and the carbohydrate source was a mixture of sucrose and cornstarch. The lipid source was vegetable oil with a fatty acid composition of 8.1% saturated fatty acids; 58.9% monounsaturated fatty acids, of which oleic acid was the main source (57.4%); 28.2% consisted of polyunsaturated fatty acids, of which linoleic acid was the main source (23%); the amount of n-3 and n-6 fatty acids in the high-fat nutrition was less than 5% of the total fat content. The types of carbohydrates and fat used in both diets were identical. As described before (16), 3 ml was given 18 hours before hemorrhagic shock and 0.75 ml at 2 hours and 45 minutes before hemorrhagic shock via oral gavage. Both fasted and high-fat treated rats underwent vagotomy or sham vagotomy. In order to investigate the role of CCK, animals fed with high-fat nutrition subjected to sham vagotomy were either injected intravenously with CCK-A (500 µg/kg) and CCK-B (500 µg/kg) receptor antagonists (n=6) or vehicle (90% NaCl, 5% Tween-20, 5% DMSO, n=6) at 25 minutes before induction of shock. Potential proinflammatory properties of both CCK-receptor antagonists were investigated by stimulation of peritoneal macrophages isolated from rats (n=3) and injection of CCK-A and CCK-B receptor antagonists in rats not subjected to hemorrhagic shock (n=3). To determine whether the observed effects were specific for stimulation of the cholinergic anti-inflammatory pathway, peripheral nicotinic receptors were blocked by intravenous administration of chlorisondamine at 25 minutes before induction of shock (n=6) in high-fat treated rats, subjected to sham vagotomy. In order to control for the decrease in MAP (from 100 mmHg to 65 mmHg) associated with administration of chlorisondamine, fasted,

sham vagotomized rats treated with chlorisondamine were included as controls (n=6). At 90 minutes after hemorrhagic shock, blood was taken and segments of small bowel were harvested for determination of gut permeability. Plasma was separated by centrifugation, frozen immediately and stored (-20°C) until analysis.

Cytokine analysis

TNF-α, IFN-γ, IL-6 and IL-10 concentrations in arterial blood were determined using a standard ELISA for rat TNF-α and rat IFN-γ (both kindly provided by Hbt, Uden, the Netherlands), rat IL-6 (BD Biosciences, San Diego, CA) and rat IL-10 (Biosource, Camarillo, CA).

Intestinal permeability

Intestinal permeability for macromolecules was assessed by measuring translocation of the 44 kDa enzyme horseradish peroxidase (HRP, Sigma) by the everted gut sac method as described(16).

Microbiological Methods

Bacterial translocation to distant organs was assessed as described (6, 16). In short, mesenteric lymph nodes (MLN), the mid-section of the spleen and a liver-segment (IV) were collected aseptically in pre-weighed thioglycolate broth tubes (Becton Dickinson (BBL) Microbiology Europe, Maylan, France) in all rats. Tissue-fragments were homogenized and the entire suspension was transferred to agar plates (Columbia III blood agar base supplemented with 5% vol/vol sheep blood (BBL) (duplicate plates) and Chocolate PolyviteX agar (BioMérieux, Marcy L'Etoile, France)). After 48h incubation, colonies were counted, determined using conventional techniques, adjusted to tissue-weight and expressed as number of colony forming units (cfu) per gram tissue.

Statistical analyses

Bacterial translocation data are represented as median and range; all other data are represented as mean ± SEM. A Mann-Whitney U test was used for between-group comparisons. Differences were considered statistically significant at $P < 0.05$.

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Chapter 9

Summary & Discussion

Summary and discussion

The inflammatory response is a crucial part of a widespread host response to attack, clear and kill invading microorganisms. Although a strong protective immunity is essential to fence off possible threats, the powerful inflammatory cascade has to be carefully managed and restrained to prevent dysregulation and an exaggerated inflammatory response to relatively harmless stimuli. Especially in the gastrointestinal tract, hyperactivation of inflammatory cells to commensal bacteria or dietary antigens needs to be avoided, since this can cause an unwanted systemic inflammatory response and breakdown of intestinal barrier integrity leading to potentially fatal inflammatory disorders such as sepsis. Patients undergoing major surgery are at risk for development of such inflammatory complications, which form a major cause of postoperative morbidity and mortality. There is an increasing insight in the complex orchestrated inflammatory response to microbial components and the mechanisms that regulate this response. It seems that hypoperfusion of the splanchnic area leading to inflammation and loss of intestinal barrier integrity is an important causative factor. Furthermore, a second event, such as microbial infection, systemic hypotension or another stressor may dysregulate the inflammatory response leading to excessive release of proinflammatory mediators. This is also known as the two-hit phenomenon [1, 2]. Despite increasing insight and ongoing research, identification of patients at risk for development of inflammatory syndromes such as the systemic inflammatory response syndrome (SIRS) or sepsis remains difficult and therapeutic options for these patients are limited.
In this thesis we investigate the role of bacterial DNA in a model of hemorrhagic shock and study two therapeutic interventions that reduce inflammation and preserve gut barrier function.

Hemorrhagic shock causes a systemic inflammatory response, gut barrier loss and spread of bacteria and endotoxin to the systemic circulation and distant organs. This resembles the clinical situation of patients undergoing major surgery in which the splanchnic area is often subject to ischemia due to blood loss during surgery. Although the physiological response to hypoperfusion of the gut (inflammation and gut barrier failure) may be important to trigger the immune system and counter invading microorganisms; a viscous circle of ongoing activation of the inflammatory response and translocation of bacterial toxins needs to be avoided.
In Chapter 2 we investigate the effect of oligodeoxynucleotides containing CpG motifs (CpG-ODN) on gut barrier function and inflammation following hemorrhagic shock. Bacterial DNA and CpG-ODN are proven immunostimulatory agents that initiate signal transduction via TLR9 leading to a Th1 type immune response with release of proinflammatory cytokines such as IFN-γ [3, 4]. The capacity of CpG-ODN to augment a for the host essential inflammatory is shown to enhance vaccination efficacy and improve chemotherapy for treatment of cancerous disease [5, 6]. However, augmentation of the immune response may be detrimental for patients undergoing major surgery for treatment of cancerous disease and potentially lead to polymicrobial sepsis.

We show that exposure to oligodeoxyinucleotides containing CpG motifs (CpG-ODN) before hemorrhagic shock, strongly enhances release of proinflammatory cytokines and results in a defective anti-inflammatory response. In line, permeability for HRP and translocation of bacteria and endotoxin are increased. In this study, we demonstrate that these effects are mediated by IFN-γ probably by priming inflammatory cells via upregulation of TLR4; the augmented inflammatory response was accompanied by an increase in TLR4 expression in the liver. To measure the value of this enhanced TLR4 expression, we investigated whether upregulation of TLR4 expression also caused an increased transcription. In vitro studies showed in peritoneal macrophages that the enhanced expression of TLR4 induced by exposure to CpG-ODN lead to an enhanced translocation of NF-κB to the nucleus. The IFN-γ dependent upregulation of TLR4 may partly explain the exacerbated inflammatory response and enhanced loss of intestinal barrier integrity in CpG-ODN exposed rats, since endotoxin is pivotal in initiation of the inflammatory response following hemorrhagic shock[7]. These data are in line with earlier findings from our group in which we show that TLR4 expression in renal epithelial cells is modulated by IFN-γ [8-10]. Bacterial DNA or CpG-ODN may be a double-edged sword that in addition to the beneficial immunostimulatory property in vaccination, allergy and cancer sensitizes the host to intestinal injury by aggravating the inflammatory cascade. CpG-ODN may also exacerbate existing, hidden low-grade inflammatory processes in the gut by sensitizing immune cells via IFN-γ leading to impairment of the intestinal barrier.

In order to prevent development of gut barrier failure we first studied the effect of probiotic therapy prior to hemorrhagic shock. Probiotics have been investigated intensively in inflammatory bowel disease (IBD) and pouchitis in which is shown that such a therapy has a beneficial effect on gut barrier integrity and can ameliorate inflammatory responses [11-14]. Given these modulatory effects on intestinal microflora and the systemic inflammatory response, we hypothesized that probiotic therapy would be beneficial in preservation of gut barrier integrity following systemic hypotension. In Chapter 3 we show that two different *Lactobacillus* strains (*L. rhamnosus* LMG P-22799 and *L. fermentum* NumRes2) are able to effectively reduce pathogen adhesion in vitro. Interestingly, these two probiotic strains had distinctive, different effects on intestinal barrier integrity loss following hemorrhagic shock. Whereas *L. rhamnosus* LMG P-22799 reduced gut barrier integrity loss caused by systemic hypoperfusion, *L. fermentum* NumRes2 had no substantial effect and even resulted in increased translocation of *Lactobacillus* spp. Besides the capability of probiotic bacteria to prevent pathogen adhesion, probiotic DNA has been shown to play a crucial role in the observed protection of probiotic therapy in experimental colitis via a Toll-like receptor 9 signaling pathway [15]. However, we did not observe any difference on endotoxin induced inflammation in RAW 264.7 cells between bacterial DNA isolated from *L. rhamnosus* LMG P-22799 and *L. fermentum* NumRes2. The optimal dosage of DNA preparations of both strains to inhibit endotoxin induced inflammation is probably different; we administered a single (commonly accepted) dose of both strains that may have been optimal for the *L. rhamnosus* strain, whereas this dose may not have been favorable for the *L. fermentum* strain. A difference in effect on the inflammatory response in combination with potent pathogen exclusion properties of both strains may have been the cause for the differences in effect of both strains on bacterial translocation following hemorrhagic shock. Although further studies are necessary to identify the exact mode(s) of action of probiotics, these data indicate that certain probiotic strains may be useful in a clinical setting to preserve gut barrier integrity following severe blood loss. However, potential useful

probiotic strains need to be thoroughly screened before applying such therapies in various disease states since the risk of translocation of these bacteria forms a serious barrier to use such therapies in a clinical setting.

Besides manipulation of inflammation and gut barrier function via probiotics, several physiological mechanisms exist to oppose release of bacterial toxins or reduce release of inflammatory mediators. Manipulation of these intrinsic defence mechanisms such as the complement system, the coagulation cascade, the central nervous system and lipoproteins may offer a therapeutic option. Based on previous work from our lab and other groups we focused on the endotoxin-neutralizing capacity of lipoproteins to reduce inflammation and prevent gut barrier failure. Lipoproteins have the capacity to bind and neutralize bacterial toxins via lipopolysaccharide binding protein (LBP) and apolipoproteins [16, 17]. Triacylglycerol-rich lipoproteins such as VLDL and chylomicrons are potent inhibitors of endotoxin activity[18, 19]. Furthermore, preincubation of endotoxin with triacylglycerol-rich lipoproteins or repeated intravenous infusions with chylomicrons protect animals against endoxin-induced death[18, 20]. Already in the early 1980's high-density lipoproteins (HDL) were identified to bind and inactivate endotoxin. Later was found that hypertriglyceridemia as a result of de novo synthesis in the liver is part of the early response to low-dose endotoxin [21-23]. This endotoxin-mediated increase in circulating triacylglycerol-rich lipoproteins is considered to have a protective function. From all lipoprotein classes, triacylglycerol-rich lipoproteins have been shown to be very potent inhibitors of the bioactivity of endotoxin and protect animals against endotoxin induced lethality [20, 24, 25]. Theoretically, elevation of these triacylglycerol-rich lipoproteins would thus increase the host's natural defence against bacterial toxins, thereby reducing risk of hyperactivation of inflammatory cells by release of bacterial products. Elevation of chylomicrons, which are postprandially formed in the gut following a high-fat meal, would enforce the ability to scavenge microbial toxins both at the source (the intestine) and in the circulation.

In Chapter 4 and 5 we show that administration of enteral nutrition containing high amounts of lipids strongly reduces translocation of endotoxin and inhibits release of IL-6 and TNF-α compared with an isocalorical, isonitrogenous low-fat diet. Furthermore, the shock-induced increase of intestinal permeability for horseradish peroxidase (HRP) was reduced by high-fat nutrition and bacterial translocation to distant organs decreased. In line, loss of cellular integrity in the intestine due to systemic hypoperfusion, measured by ZO-1 expression, was prevented. These data are the first to show that a simple nutritional intervention using high amounts of dietary fat reduces the inflammatory response and preserves gut barrier function following systemic hypotension. The protective effect of high-fat enteral nutrition on gut barrier function are best attributed to the decreased inflammatory response and the reduced levels of circulating endotoxin, since both endotoxin and primarily TNF-α have been shown to impair intestinal barrier function[7, 26-31].

Given the fact that high-fat nutrition exerts an inhibitory effect on release of proinflammatory mediators, endotoxemia and bacterial translocation it is to be expected that end-organ damage associated with hemorrhagic shock is reduced. In Chapter 6 we investigated whether the effects of high-fat supplementation were related to reduced hepatocellular injury and an altered hepatic stress protein response. During injury, cells may exhibit autoprotective

responses, and increase expression of stress proteins. Reduction of these stress proteins such as heat shock protein (HSP) 70 and Heme oxygenase 1 (HO-1) would suggest decreased cellular damage. In line with the effects on inflammation and gut barrier function, administration of high-fat enteral nutrition significantly reduced hepatocellular damage following hemorrhagic shock. This was evidenced by reduced levels of HSP70 and HO-1 and less disruption of cytoarchitecture in the liver. The lack of mitochondrial DNA damage found in the high-fat fed animals suggests that the mechanism of protection in these animals was due to an inhibition of stress stimuli such as inflammatory cytokines upon the tissue rather than the induction of a protective intracellular response.

Alltogether, these findings suggest that neutralization of endotoxin via triacylglycerol-rich lipoproteins plays a role in the protective effect of high-fat enteral nutrition. This is supported by the significant negative correlation between circulating triacylglycerol and both total bacterial translocation and circulating endotoxin levels in all hemorrhagic shock rats.
However, these data do not correlate with the effect of triacylglycerol-rich lipoproteins on endotoxin responsiveness in humans in which is shown that hypertriglyceridemia does not inhibit the host response to endotoxin[32]. This may be explained by difference in kinetics of binding between endotoxin and lipoproteins and endotoxin and inflammatory cells may be of influence since preincubation of endotoxin with triacylglycerol-rich lipoproteins attenuates inflammation[33]. In our model of hemorrhagic shock endotoxin gradually translocates from the gut lumen into the systemic circulation, resembling the clinical situation. In this setting, exposure of endotoxin to triacylglycerol-rich lipoproteins leading to neutralization may precede the exposure to inflammatory cells.

To investigate involvement of triacylglycerol-rich lipoproteins in the underlying mechanism of protection of high-fat enteral nutrition, a combined model of hemorrhagic shock and bile duct ligation was used. Bile duct obstruction causes malabsorption of fat leading to decreased formation of chylomicrons and other classes of lipoproteins. Theoretically, decreased levels of triacylglycerol-rich lipoproteins in these animals would diminish endotoxin-neutralizing capacity leading to a loss of protection of enteral high fat. Indeed, the level of circulating triacylglycerol-rich lipoproteins following a fat-rich enteral diet was reduced by 80% in bile duct ligated rats (Chapter 7). Remarkably, enteral administration of high-fat nutrition in this setting still reduced the inflammatory response following hemorrhagic shock, reduced intestinal permeability for HRP and bacterial translocation and preserved cellular integrity in the intestine. It is possible that the slightly enhanced circulating triacylglycerol levels that were detectable in bile duct ligated rats fed with high-fat nutrition may have been sufficient to provide protection after hemorrhagic shock. However, in our previous studies, such levels of circulating triacylglycerol found in rats fed with the low-fat enteral diet did not confer protection (Chapter 4-6). Based on these data, we concluded that triacylglycerol-rich lipoproteins may not be essentially involved in the observed protection, but may are rather an epiphenomenon of lipid nutrition. Although the mechanism of protection was as yet unresolved, this study clearly indicated that patients with biliary obstruction and systemic hypotension, that have a strongly increased risk of septic complications would benefit of such a nutritional intervention.

Besides elevation of triacylglycerol-lipoproteins, another potent and specific response is triggered by high amounts of dietary lipids. The neuro-endocrine hormone cholecystokinin

(CCK) is released, leading to gallbladder contraction and satiety via activation of the autonomic nervous system. The role of CCK in regulating food uptake and satiety via the autonomic nervous system has been studied intensively. Recently a novel characteristic of the autonomic nervous system was identified by the group of Tracey et al[34]. Besides the renowned regulation of metabolism, the parasympathetic nervous system also inhibits macrophage activation via the vagus nerve through binding of acetylcholine to α-7 nicotinic receptors located on macrophages[35]. Central or peripheral stimulation of this so-called 'cholinergic anti-inflammatory pathway' via a mechanical or pharmacological route reduces plasma TNF-α in endotoxic shock and blunts nuclear factor-κB activation after hemorrhagic shock via efferent vagal nerve fibres[36-39]. This prompted us to investigate involvement of the autonomic nervous system in the protection provided by high-fat enteral nutrition. In Chapter 8 we studied the role of the autonomic nervous system in the protective effect of high-fat enteral nutrition. This study shows that bilateral vagotomy abrogated the high-fat induced inhibition of the inflammatory response and gut barrier loss. In accordance, circulating endotoxin levels, permeability of ileum segments for HRP and bacterial translocation to distant organs were reduced as well. These results suggest a pivotal role of the autonomic nervous system in the mechanism of protection of high-fat enteral nutrition. Subsequently, we show that the autonomic nervous system is activated by the high percentage of dietary fat via CCK–receptors. Finally, we demonstrate that stimulation of the autonomic nervous system by high-fat enteral nutrition via the neuro-endocrine hormone CCK inhibits the inflammatory response via the anti-inflammatory efferent vagal pathway (Chapter 8). These findings reveal a novel nutrional and neuro-immunological pathway providing a physiological basis for the since long studied close interaction between nutrition and the immune response. It was previously unrecognized that nutrition-induced neuro-endocrine signals are involved in inhibitory effects on the inflammatory response. From a teleological point of view it is functional that a state of immune-hyporesponsiveness is created during feeding. In this way an unwanted response to temporally present high amounts of dietary antigens, biological toxins and destructive endogenous lysozymes in the gut lumen is prevented, gut barrier function is preserved and homeostasis maintained. We propose that this neural feedback-loop activated by enteral nutrition is an important player in the thus far largely unexplained state of hyporesponsiveness of the immune system in the intestinal tract to dietary antigens and bacterial toxins.

Based on our findings, high-fat enteral nutrition is potentially therapeutic in various inflammatory disorders such as sepsis, ileus and inflammatory bowel disease (IBD) characterized by an inflammatory response in which TNF-α is prominent and intestinal barrier function is impaired. In light of this, a fasted state could be a risk factor for developing a potentially lethal inflammatory response after trauma or injury.

In summary, we show that hemorrhagic shock in rats is accompanied with release of proinflammatory mediators, increased intestinal permeability, endotoxin and bacterial translocation and loss of intestinal integrity.We showed that exposure to CpG-ODN prior to hemorrhagic shock causes a two-hit phenomenon leading to an augmented proinflammatory response, a defective anti-inflammatory response and deterioration of shock-induced gut barrier loss. It is to be expected that in this model of combined exposure to CpG-ODN and hemorrhagic shock end-organ damage such as in the liver is enhanced. Certain probiotic strains can inhibit this shock-induced increase in intestinal permeability. However, the underlying

mode of action is unclear and there was an undesirable side-effect of enhanced translocation of *Lactobacillus* spp. Potential useful probiotic strains need to be thoroughly studied using in vitro and in vivo approaches to select suitable strains before applying such therapies in various disease states to prevent unwanted side-effects. We demonstrate in a series of experiments that high-fat enteral nutrition strongly reduces inflammation and preserves gut barrier function following hemorrhagic shock and delineate the underlying mechanism. The mechanism of protection of high-fat enteral nutrition reveals a new neuro-immunological pathway in which ingestion of high amounts of dietary lipids stimulates the autonomic nervous system via the vagus nerve, thereby inhibiting the inflammatory response via nicotinic receptors. Identification of this nutritional and neurogenic feedback mechanism provides a novel insight in the since long studied close interaction between nutrition and the immune system and presents a physiological basis for the cholinergic anti-inflammatory pathway. Understanding this mechanism, the use of nutrition in patients at risk for inflammatory complications such as patients undergoing major surgery becomes pivotal.

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Nederlandse samenvatting

Een ontstekingsreactie is onderdeel van de afweer van het lichaam tegen micro-organismen en weefselschade. Deze reactie moet enerzijds sterk genoeg zijn om de schadelijke effecten van dit soort bedreigingen te voorkomen, anderzijds is een overmatige reactie op relatief ongevaarlijke stimuli ongewenst en schadelijk. Voornamelijk in de darm moet een te hevige ontstekingsreactie op de aanwezige bacteriën en eventuele schade worden voorkomen, omdat ten gevolge hiervan ernstige darmschade kan ontstaan. Die schade kan resulteren in een vicieuze cirkel waarin ontstekingscellen voortdurend worden geactiveerd door bacteriële producten vanuit de darm.

Chirurgische patiënten, die een grote operatie moeten ondergaan, hebben een verhoogd risico op algemene ontstekingssyndromen zoals bloedvergiftiging, ook wel sepsis genoemd. Hoewel de exacte ontstaanswijze van deze ziektebeelden nog onduidelijk is, neemt inzicht in de complex georganiseerde ontstekingsreactie die hieraan ten grondslag ligt toe. Door een verminderde doorbloeding van de darm zoals die voorkomt na grote operaties of ernstig bloedverlies, komen ontstekingsfactoren vrij waardoor er verlies optreedt van darmwand integriteit. Wanneer deze ontstekingsreactie heel hevig en ongecontroleerd verloopt kan dit leiden tot ziektebeelden zoals sepsis. De ontstekingsreactie kan heviger verlopen door meerdere oorzaken, bijvoorbeeld doordat het individu wordt blootgesteld aan opeenvolgende operaties, andere letsels of infectie. Dit wordt ook wel het "two-hit" fenomeen genoemd.
Er is veel onderzoek verricht naar de ontstaanswijze en behandeling van het ziektebeeld sepsis. Daardoor is weliswaar het inzicht in dit ziektebeeld toegenomen, echter het is in de klinische praktijk nog steeds heel moeilijk om patiënten die een hoog risico hebben op sepsis vooraf te identificeren. Daarnaast zijn de behandelingsmogelijkheden voor patiënten met sepsis, of andere soortgelijke ontstekingssyndromen, nog steeds beperkt.

In dit proefschrift zijn twee mogelijke therapieën bestudeerd in een model van verbloedings (hemorragische) shock. Tevens hebben we onderzocht wat het effect is van een tweede letsel (blootstelling aan bacterieel DNA) in het gebruikte diermodel.
Als model voor een grote operatie is een experimenteel model van hemorragische shock gekozen. Hemorragische shock veroorzaakt een verminderde doorbloeding van de organen in de buik, m.n. de darm. Dit leidt tot een meetbare lokale en systemische ontstekingsreactie, een verhoogde permeabiliteit van de darm en verspreiding van bacteriën en bacteriële toxinen via de bloedbaan en lymfe naar organen. Dit is vergelijkbaar met de situatie zoals die wordt gezien bij patiënten die een grote operatie hebben ondergaan. Ook hier ontstaat een algemene ontstekingsreactie en soms verlies van darmwand integriteit door een verminderde doorbloeding (hypoperfusie) van de darmen.
De lichaamseigen reactie op verminderde doorbloeding van de darm (het vrijkomen van ontstekingsfactoren en verlies van darmwand integriteit) is nodig om de afweer te activeren, zodat mogelijk binnendringende micro-organismen kunnen worden gestopt. Echter, de ontstekingsreactie zou beperkt moeten blijven zodat verspreiding van bacteriën, bacteriële gifstoffen en ontstekingsfactoren door het lichaam wordt voorkomen.

In Hoofdstuk 2 is een studie beschreven waarin ratten worden blootgesteld aan kleine stukjes DNA (oligonucleotiden), voorafgaand aan hemorragische shock. Deze kleine stukjes DNA stimuleren het immuunsysteem en lijken op bacterieel DNA aangezien ze zogenoemde CpG motieven bezitten (CpG-ODN). Dit immuunstimulerende effect van bacterieel DNA is sinds

enkele jaren bekend en wordt gestuurd via een specifieke herkenningsplaats (receptor) op ontstekingscellen, Toll-like receptor 9 (TLR9) genoemd. Het activeren van ontstekingscellen door bacterieel DNA leidt tot een bepaald type ontstekingsreactie (Th1) waarbij o.a. de ontstekingsfactor interferon (IFN)-γ vrijkomt. De eigenschap van bacterieel DNA om het afweersysteem te stimuleren verhoogd de efficiëntie van vaccinaties en versterkt het effect van chemotherapie bij kanker. Echter, voor patiënten die een grote operatie ondergaan is extra activatie van het afweersysteem niet wenselijk, aangezien dit kan leiden tot ziektebeelden zoals sepsis.

De resultaten uit deze studie tonen aan dat blootstelling van ratten aan CpG-ODN voorafgaand aan hemorragische shock de ontstekingsreactie versterkt en de natuurlijke ontstekingsremmende factoren (zoals interleukine (IL)-10) vermindert in het bloed. Bovendien verslechtert de door shock aangetaste darmbarrièrefunctie nog verder. Verder laten de experimenten zien dat de effecten van bacterieel DNA worden veroorzaakt door het vrijkomen van de ontstekingsfactor (cytokine) IFN-γ. Dit cytokine is verantwoordelijk voor een verhoogde aanwezigheid van Toll-like receptor 4 (TLR4), op de buitenkant van cellen in de lever. TLR4 is de herkenningsplaats voor endotoxine, het belangrijkste bestanddeel van Gram-negatieve bacteriën. De verhoogde expressie van TLR4 zou een verklaring kunnen zijn voor de versterkte ontstekingsreactie en toegenomen weefselschade gezien bij ratten die blootgesteld werden aan CpG-ODN voorafgaand aan hemorragische shock. Er wordt namelijk gedacht wordt dat endotoxine voor een belangrijk deel verantwoordelijk is voor de ontstekingsreactie en weefselschade na hemorragische shock door activatie van ontstekingscellen via TLR4.
Om te onderzoeken of de verhoogde aanwezigheid van TLR4 ook van belang is, is het experiment in vitro nagebootst met behulp van ontstekingscellen (macrofagen). Deze experimenten laten zien dat ook in vitro de aanwezigheid van TLR4 op macrofagen wordt verhoogd en dat deze verhoogde expressie leidt tot een toegenomen productie van ontstekingsfactoren in deze cellen. Deze toegenomen productie binnen de cel is aangetoond door een verhoogde translocatie van nuclear factor (NF)-κB naar de kern. De toegenomen aanwezigheid van TLR4 op ontstekingscellen leidt theoretisch tot een versterkte reactie op endotoxine. Dit zou deels een verklaring kunnen zijn voor de versterkte ontstekingsreactie die werd gezien na blootstelling aan bacterieel DNA in het hemorragische shock model.
Het effect van bacterieel DNA op de afweer lijkt tweeledig te zijn. Enerzijds heeft versterking van de immuunrespons door bacterieel DNA een gunstig effect bij vaccinatie en chemotherapie, terwijl deze versterkte reactie anderzijds leidt tot meer darmschade en meer ontsteking na hemorragische shock.

In Hoofdstuk 3 wordt een experiment beschreven waarin is bestudeerd of een therapie met probiotica gedurende zeven dagen hemorragische shock geïnduceerd darmbarrièrefalen kan voorkomen en ontsteking kan verminderen. Probiotica worden sinds langere tijd bestudeerd in modellen waarbij de dikke darm is ontstoken (colitis). De resultaten uit deze studies laten zien dat probiotische therapie een gunstig effect heeft op darmbarrièrefunctie en ontstekingsprocessen kan reduceren. Op basis van deze bevindingen is de hypothese ontwikkeld dat probiotische therapie voorafgaand aan hemorragische shock een gunstig effect zal hebben. In Hoofdstuk 3 wordt beschreven dat twee verschillende *Lactobacillus* stammen (*L. rhamnosus* LMG P-22799 en *L. fermentum* NumRes2) in staat zijn om adhesie van pathogenen aan darmcellen in vitro in gelijke mate te verminderen. Echter, de effecten van deze twee probiotische stammen in

het hemorragische shock model zijn geheel verschillend. Toediening van *L. rhamnosus* LMG P-22799 verminderde het verlies van darmwand integriteit veroorzaakt door de shock, terwijl *L. fermentum* NumRes2 hierop geen substantieel effect had en zelfs leidde tot een toegenomen translocatie van *Lactobacillus* spp. Een mogelijke verklaring voor de gevonden effecten is de eigenschap van probiotica om adhesie van pathogene bacteriën te remmen. Recent is echter aangetoond in modellen van colitis, dat DNA van dergelijke gunstige micro-organismen een belangrijke rol speelt via activatie van Toll-like receptor 9. Vervolgens is onderzocht of DNA, geïsoleerd uit de gebruikte *Lactobacillus* stammen, een effect zou hebben op endotoxine-geïnduceerde ontsteking. Er werden geen verschillen gevonden tussen DNA preparaten van beide micro-organismen op het vrijkomen van de ontstekingsfactor tumor necrosis factor alpha (TNF-α) na stimulatie van macrofagen met endotoxine. Het is mogelijk dat verschillen in dosis-effect relaties tussen de gebruikte stammen, de oorzaak is dat er geen duidelijk meetbare verschillen waren. De precieze oorzaak voor de gevonden verschillen tussen beide probiotische bacteriën is echter niet duidelijk en meer onderzoek is nodig om het werkingsmechanisme(n) van probiotica te ontrafelen. De resultaten laten zien dat probiotische stammen die klinisch gebruikt gaan worden zeer grondig moeten worden getest omdat het risico op translocatie van *Lactobacillus* spp moet worden vermeden.

Naast manipulatie van de ontstekingsreactie en darmbarrière-functie door probiotica, zijn er een aantal fysiologische afweermechanismen die bacteriële invasie voorkomen en ontsteking verminderen. Manipulatie van deze intrinsieke afweermechanismen zoals het complement systeem, de stollingscascade, het centrale zenuwstelsel en vorming van vettransportdeeltjes (lipoproteïnen) zou mogelijk therapeutisch kunnen zijn. Eerdere studies hebben aangetoond dat lipoproteïnen endotoxine kunnen neutraliseren, waardoor de ontstekingsreactie wordt verminderd en darmbarrièrefunctie behouden blijft. Lipoproteïnen kunnen endotoxine neutraliseren via het lipopolysaccharide bindings-eiwit (LBP) en dragereiwitten in de buitenmembraan (apolipoproteïnen). Met name de triacylglycerol-rijke lipoproteïnen zoals very-low density lipoproteïnen (VLDL) en chylomicronen remmen de activiteit van endotoxine en beschermen dieren tegen endotoxine geïnduceerde dood. Al in de jaren tachtig werd gevonden dat high-density lipoproteïnen (HDL) endotoxine kunnen binden en inactiveren. Later werd ontdekt dat verhoging van de hoeveelheid triaycylglycerol-rijke lipoproteïnen ten gevolge van nieuw-vorming in de lever, onderdeel is van de vroege reactie op endotoxine. Dit mechanisme werd gezien als beschermend in ziektebeelden zoals sepsis. Triacylglycerol-rijke lipoproteïnen zijn de meest krachtige remmers van de (bio)activiteit van endotoxine. Theoretisch zou verhoging van deze lipoproteïnen de natuurlijke bescherming van het individu tegen bacteriële toxinen verhogen, waardoor het risico op overactivatie van ontstekingscellen wordt voorkomen. In dit kader leidt een toename van chylomicronen, die in de darm worden gevormd na een vetrijke maaltijd, tot een verhoging van de capaciteit om microbiële toxinen te neutraliseren zowel in de bloedbaan als lokaal in de darm.

Hoofdstuk 4 en 5 tonen aan dat toediening van een vetrijke enterale voeding in een model van hemorragische shock de translocatie van bacteriën en endotoxine vermindert en het vrijkomen van de ontstekingsfactoren IL-6 en TNF-α reduceert. Bovendien wordt de shock-geïnduceerde verhoogde intestinale permeabilteit voor grote moleculen, zoals mierikswortel (horseradish) peroxidase (HRP) verminderd en is er minder verlies van integriteit van de darm. De integriteit van de darmwand is gemeten door bepaling van het zonula occludens 1 (ZO-1)

eiwit in dunne en dikke darm. Het ZO-1 eiwit is een belangrijk onderdeel van de opbouw en integriteit van een cel.
De gegevens uit deze studies zijn de eerste die laten zien dat een eenvoudige interventie met een vetrijke voeding de onstekingsreactie en darmbarrièrefalen na hemorragische shock verminderen. Het beschermende effect van vetrijke voeding op darmbarrièrefunctie kan het best toegeschreven worden aan een verminderde ontstekingsreactie, omdat eerder is bewezen dat zowel endotoxine, als TNF-α leiden tot darmbarrièrefalen.

Op basis van de gevonden gunstige effecten van vetrijke voeding op het vrijkomen van ontstekingsfactoren, endotoxemie en bacteriële translocatie valt te verwachten dat orgaanschade geassocieerd met hemorragische shock ook wordt verminderd. In Hoofdstuk 6 is het effect onderzocht van vetrijke enterale voeding op leverschade na hemorragische shock. Als maat voor leverschade zijn een aantal stress-eiwitten bepaald. Tijdens stress, zoals tijdens een grote operatie, reageren cellen met een zelfbeschermingsmechanisme dat o.a. leidt tot verhoogde aanwezigheid van de stress-eiwitten heat shock protein (HSP) 70 en heme-oxygenase 1 (HO-1). Overeenkomstig met de effecten van vetrijke enterale voeding op de ontstekingsreactie en darmbarrièrefunctie, werd er minder leverschade gezien na hemorragische shock in deze groep ratten. De door shock geïnduceerde toename in concentratie van HSP70 en HO-1 was gereduceerd, er was minder micropscopische cel-schade van de lever en bijna geen mitochondrieële DNA schade.
De resultaten uit deze studies tonen aan dat een korte interventie met vetrijke voeding de ontstekingsreactie na hemorrhagische shock vermindert, darmbarrière integriteit behoudt en leverschade vermindert. De significante negatieve correlatie tussen concentraties van triglyceriden in het bloed en de mate van bacteriële en endotoxine translocatie die bestaat (Hoofdstuk 4), doet vermoeden dat neutralisatie van endotoxine door triacylglycerol-rijke lipoproteïnen een rol speelt in de beschermende werking van vetrijke enterale voeding.

Deze gegevens komen niet overeen met studies in mensen, waarbij is gevonden dat een verhoogde concentratie triacylglycerol in bloed de ontstekingsreactie op endotoxine niet remt. Echter, wanneer endotoxine tevoren wordt geïncubeerd met triacylglycerol-rijke lipoproteïnen wordt de ontstekingsreactie op endotoxine wel geremd. Deels kan dit verklaard worden door de snelle binding van endotoxine met ontstekingscellen ten opzichte van de langzame binding van endotoxine met lipoproteïnen. In het model van hemorragische shock wordt aangenomen dat er sprake is van een geleidelijke translocatie van endotoxine vanuit het darmlumen zoals waarschijnlijk ook gebeurd na grote operaties. Het is voorstelbaar dat in de situatie van hemorragische shock, lipoproteïnen die vanuit de darm gevormd worden na een vetrijke maaltijd, endotoxine neutraliseren voordat deze in contact komt met ontstekingscellen.

Om te onderzoeken of triacylglycerol-rijke lipoproteïnen werkelijk betrokken zijn bij het mechanisme onderliggend aan de beschermende werking van vetrijke voeding, is gebruik gemaakt van een gecombineerd model van hemorragische shock en galwegobstructie. Galwegobstructie leidt tot een verminderde opname van vet en een daling van de hoeveelheid gevormde chylomicronen, omdat gal nodig is voor de vet vertering in de darm. In theorie zouden deze verminderde spiegels van triacylglycerol-rijke lipoproteïnen ook zorgen voor een verminderde capaciteit om endotoxine te neutraliseren en zodoende leiden tot een verlies van bescherming van vetrijke voeding.

Zoals verwacht was de hoeveelheid circulerende triacylglycerol-rijke lipoproteïnen sterk verminderd (80%) in de groep vetrijke gevoede ratten met galwegobstructie ten opzichte van de controle groep (Hoofdstuk 7). Echter, in ratten met galwegobstructie verminderde vetrijke voeding wel de ontstekingsreactie na hemorragische shock en zorgde ook voor behoud van de darmbarrièrefunctie. Het is natuurlijk mogelijk dat de licht verhoogde triacylglycerol-rijke lipoproteïnen in de met vetrijke gevoedde galwegobstructie ratten voldoende waren om endotoxine te neutraliseren. Echter vergelijkbare spiegels van deze lipoproteïnen in dieren die vetarme voeding kregen bleken eerder niet beschermend te zijn (hoofdstuk 4-6). Op basis van deze data hebben wij geconcludeerd dat triacylglycerol-rijke lipoproteïnen waarschijnlijk niet in belangrijke mate betrokken zijn bij de beschermende werking van vetrijke voeding. Hoewel het mechanisme dat ten grondslag ligt aan de bescherming van vetrijke voeding tot nog toe onopgehelderd was, bleek uit deze studie dat vetrijke voeding mogelijk ook gunstig kan zijn voor patiënten met een gestoorde galafvloed, die een bekend verhoogd risico hebben op infectieuze complicaties.

Naast verhoogde spiegels van triacylglycerol-rijke lipoproteïnen brengt vetrijke voeding ook andere specifieke fysiologische processen teweeg. Vetrijke voeding leidt o.a. tot het vrijkomen van het neuro-endocriene hormoon cholecystokine (CCK) uit darmcellen na een vetrijke maaltijd. CCK stimuleert het samentrekken van de galblaas en activeert het autonome zenuwstelsel en doet een gevoel van verzadiging ontstaan. De rol van CCK in het proces van voedselinname en verzadiging wordt gereguleerd via het autonome zenuwstelsel en is uitvoerig bestudeerd. Recent is een geheel nieuwe functie van het autonome zenuwstelsel ontdekt. Stimulatie van zenuwbanen van de nervus vagus vanuit de hersenen naar de organen, de zogenaamde efferente zenuwbanen, leidt namelijk tot een remming van de ontstekingsreactie op endotoxine via specifieke herkenningsplaatsen op ontstekingscellen (α-7 nicotine receptoren). Dit wordt ook wel het cholinerge anti-inflammatoire mechanisme genoemd. Vanuit de gestimuleerde zenuwcellen is er een verhoogde afgifte van acetylcholine. Dit acetylcholine bindt aan de α-7 nicotine receptor op macrofagen, waardoor in de cel de productie van TNF-α wordt geremd via signaaleiwitten zoals STAT3 en SOCS-3. Op basis van deze nieuw beschreven functie van het autonome zenuwstelsel hebben wij vervolgens onderzocht of hiermee de beschermende werking van vetrijke voeding op de ontstekingsreactie en darmbarrièrefunctie kan worden verklaard. In Hoofdstuk 8 is het effect bestudeerd van dubbelzijdige doorsnijding van de nervus vagus in het model van hemorragische shock. Deze studie toont aan dat doorsnijding van de vagus in vetrijke gevoede ratten het remmende effect van vetrijke voeding op het vrijkomen van TNF-α en IL-6 volledig voorkomt, terwijl ook het positieve effect op darmbarrièrefunctie teniet wordt gedaan. Deze resultaten tonen aan dat het autonome zenuwstelsel betrokken is bij het beschermende effect van vetrijke voeding op de ontstekingsreactie en darmbarrièrefunctie. In een volgend experiment is aangetoond dat vetrijke voeding niet meer beschermend werkt bij medicamenteuze blokkade van receptoren voor het hormoon CCK. Dit suggereert dat vetrijke voeding het autonome zenuwstelsel activeert via CCK-receptoren. Tenslotte is aangetoond dat het remmende effect van vetrijke voeding op de ontstekingsreactie toegeschreven kan worden aan activatie van het cholinerge anti-inflammatoire mechanisme.

Deze bevindingen laten een nieuw, tot nog toe onbeschreven mechanisme zien dat een fysiologische basis vormt voor de bekende relatie tussen voeding en de afweer. Het was eerder niet bekend dat voedings-geïnduceerde signalen via het autonome zenuwstelsel betrokken

zijn bij de remmende werking van voeding op de ontstekingsreactie. Evolutionair gezien heeft een dergelijk mechanisme een duidelijke functie. Een heftige afweerreactie op (tijdelijk) aanwezige toxische stoffen in voeding of op endogene lysozymen die vrijkomen na een vetrijke voeding moet namelijk worden vermeden. Bovendien kan dit mechanisme een rol spelen in de tot nu toe nog onbegrepen hoge drempel tot activatie van het immuunsysteem in de darm op voedselantigenen en bacteriële toxinen. Op basis van deze bevindingen kan worden geconcludeerd dat vetrijke voeding preventief kan werken voor ontstekingssyndromen zoals sepsis in (chirurgische) patiënten die een groot risico hierop hebben. Tevens impliceren deze bevindingen dat het nuchter houden van chirurgische patiënten het risico op een versterkte ontstekingsreactie na een grote operatie verhoogt.

Concluderend heeft het onderzoek aangetoond dat blootstelling aan bacterieel DNA leidt tot een versterkte ontstekingsreactie en meer verlies van darmbarrièrefunctie na hemorragische shock. Dit effect wordt veroorzaakt door IFN-γ en is geassocieerd met een verhoogde expressie van TLR4 in de lever. Ten tweede is aangetoond dat toediening van bepaalde probiotica voorafgaand aan shock de darmbarrièrefunctie behoudt. Dit effect is echter stam-specifiek en het onderliggende werkingsmechanisme is nog niet duidelijk. Vanwege de verhoogde kans op translocatie van *Lactobacillus* spp moeten probiotische stammen zorgvuldig worden getest alvorens deze in een klinische setting te gebruiken.
Tenslotte hebben we in een aantal studies laten zien dat vetrijke voeding de ontstekingsreactie na hemorragische shock sterk remt, darmbarrièrefunctie behoudt en leverschade vermindert. Dit gunstige effect van vetrijke voeding kan niet worden toegeschreven aan verhoogde spiegels van triacylglycerol rijke lipoproteïnen. Het blijkt dat inname van vet leidt tot activatie van het autonome zenuwstelsel via afgifte van cholecystokine, waardoor het cholinerge anti-inflammatoire mechanisme wordt geactiveerd. Deze bevindingen onthullen een nieuw neuro-immunologisch voedingsmechanisme, waarin specifieke voedselcomponenten in staat zijn de imuunrespons te beïnvloeden via het autonome zenuwstelsel.

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Curriculum Vitae

Misha Luyer was born in Tilburg, the Netherlands on November 18th 1974. After he finished from Cobbenhagen College (high school) in 1993 he attended Medical School at the University of Nijmegen at which he graduated in August 2000. As a medical student he performed a surgical internship in Botsford General Hospital in Detroit, USA in 1997. Also he participated in a research project at Mayo Clinic, Rochester, USA in 1999 supported by the Dutch Heart Foundation.

He worked at the Atrium hospital in Heerlen as a surgical intern before starting in 2001 as a PhD-student at the Department of Surgery, University Maastricht (Prof.dr. W.A. Buurman and Prof.dr. J.W.M. Greve). Two abstracts presented at the conference of the Netherlands Society of Gastroenterology have been awarded with the NESPEN-price. Furthermore an award was given for best presentation at SEOHS (a symposium for experimental research surgical specialities) in 2002 and 2005. In 2003 he received an AGIKO-stipendium from the Netherlands Organisation for Health Research and Development. In January 2005 he started his training as a surgical resident at the Department of Surgery, Academic Hospital Maastricht (Prof.dr. M.J.H.M. Jacobs and Prof.dr. J.W.M. Greve).

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