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Effects of the antimicrobial peptide LL-37 in combination with hyperthermic preconditioning on the outcome of septic rats

„Effekte des antimikrobiellen Peptids LL-37 in Kombination mit hyperthermer Präkonditionierung auf das outcome im Sepsismodell der Ratte“

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presented by
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Dedicated to my dear wife Mariana

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1. Introduction

1.1. Epidemiology of sepsis

"Sepsis" can be summarized as the systemic response of the organism to severe infection. Over the last decades the incidence of sepsis continues to rise. Sepsis and its complications are the leading causes of death in medical and surgical ICUs (Marshall J.C. et al., 1995; Tran D.D. et al., 1990)

Sepsis is a major health care problem, with a death rate that equals that of myocardial infarction (US National Center for Health Statistics, 2001). Sepsis is a well-documented disease process. Literature relating to its history, incidence, risk factors (Balk R.A. 2000; Bone R.C. 1995; Zanetti G. et al., 1997) and customized probability models using the Simplified Acute Physiology Score II (Le Gall J.R. et al., 1995) or the Sequential Organ Failure Assessment (Moreno R. et al., 1999) for patient outcomes contribute to our knowledge of this complex pathological condition. ICU mortality of all sepsis patients evaluated in three German Intensive Care Units was 35.6%. Overall hospital mortality was 42.6%. Mean ICU length of stay (LOS) was 16.6 days. Survivors stayed on average 4 days longer on ICU than nonsurvivors. One of the studies focused on the epidemiology of sepsis in Germany and estimated between 44,000 and 95,000 severe sepsis cases per year. A recently published European epidemiological study classified 24% of infectious patients as severely septic according to the ACCP/SCCM (the American College of chest Physicians/Society of Critical Care Medicine) criteria (Alberti C. et al., 2002). Severely septic patients generally require expensive intensive care (Edbrooke D. et al., 1999; Edbrooke D.L. et al., 1999; Klepzig H. et al., 1998). The mean direct ICU costs of care were 23297 ± 18631 Euros per septic patient and 1318 Euros per day. In comparison, average daily charges being paid for an ICU patient by the health care system in Germany are 851 Euros (Moerer O. et al., 2002). A recent survey conducted

by the publicly funded Competence Network Sepsis (SepNet) reveals that severe sepsis and/or septic shock occurs in 110 of 100,000 inhabitants (75,000 cases/ year) and sepsis in 116 of 100,000 inhabitants (79,000 cases/ year) in Germany annually (SepNet). This illness is responsible for approx. 60,000 deaths and ranges as the third most frequent cause of death after acute myocardial infarction. Direct costs for the Intensive Care of patients with severe sepsis amount to approx 1.77 billion Euros. This means that around 30% of the budget in Intensive Care is used to treat severe sepsis.

In the United States of America Angus, Derek C. MD et al. have linked all 1995 state hospital discharge records (n = 6 621559) from seven large states with population and hospital data from the U.S. Census, the Centers for Disease Control, the Health Care Financing Administration, and the American Hospital Association. They defined severe sepsis as documented infection and acute organ dysfunction using criteria based on the International Classification of Diseases, Ninth Revision, and Clinical Modification. Mortality was 28.6%, or 215,000 deaths nationally and also increased with age, from 10% in children to 38.4% in patients older than 85 years. The average costs per case were 22,100 US dollars, with total annual costs of $16.7 billion nationally. Costs were higher in infants, nonsurvivors, intensive care unit patients, surgical patients, and patients with multiple organ failure (Angus D.C. et al., 2001).

Padkin and coworkers analyzed the data of ninety-one adult general intensive care units in England, Wales, and Northern Ireland between 1995 and 2000. They found that 27.1% of adult intensive care unit admissions met severe sepsis criteria in the first 24 h in the intensive care unit. And mortality of ICU admission with severe sepsis in the first 24 h was between 35.0%-47.3%, and the 28-day mortality rate was 41.3%. (Padkin A. et al., 2003).

Taken together a rise of sepsis incidence during the next decades can be expected due to the increasing use of invasive monitoring, an increase of

antimicrobial resistance and due to the aged and immunosuppressed population. Thus sepsis is a major health care problem now and in the future.

1.2. Definitions of Sepsis

In 1991, the American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM) convened a "Consensus Conference" in an attempt "to provide a conceptual and a practical framework to define the systemic inflammatory response to infection, which is a progressive injurious process that falls under the generalized term 'sepsis' and includes sepsis-associated organ dysfunction as well" (1992a). The 1992 statement from the ACCP/SCCM Consensus Conference introduced the term Systemic Inflammatory Response Syndrome (SIRS) to describe the host response to critical illness of either infectious or noninfectious etiology (e.g. ischemia, pancreonecrosis, and trauma). SIRS is considered to be present when patients have more than one of the following clinical findings:

a) Body temperature, >38°C or <36°C;
b) Heart rate, >90 min^{-1};
c) Hyperventilation evidenced by a respiratory rate of >20 min^{-1} or a $PaCO_2$ of <32 mm Hg;
d) A white blood cell count of >12,000 cells μL^{-1} or <4,000 μL^{-1} and more than 10 % immature cells.

Bone et al. defined sepsis as SIRS plus infection, "severe sepsis" as sepsis associated with organ dysfunction, hypoperfusion, or hypotension, and "septic shock" as sepsis with arterial hypotension, despite adequate fluid resuscitation. These general definitions are now widely used in practice and serve as the basis for numerous clinical trial inclusion criteria.

Similarly to the TMN classification for cancer diseases (by Pierre Denoix, 1946), Levy, Mitchell M. et al. developed a classification scheme for sepsis—

called PIRO—that stratifies patients on: √) their **P**redisposing conditions, √) the nature and extent of the insult (in the case of sepsis, **I**nfection), √) the nature and magnitude of the host **R**esponse, and √) the degree of concomitant **O**rgan dysfunction (Table 1.1.). It is important to emphasize that the PIRO concept is not extensively validated yet; extensive testing and further refinement is needed before it can be considered ready for routine application in clinical practice.

- ✓ **P**redisposition – includes all premorbid factors, which have a substantial impact on outcome and the disease process. Furthermore, recently genetic factors were identified to contribute to sepsis incidence and mortality.
- ✓ **I**nfection – the prognosis of the infection may be influenced by the type of pathogens (strains) and the source/extent. For example, in patients with generalized peritonitis the risk of mortality is higher than in those with localized appendicitis. Similarly, there is a different immune host response to gram-negative or gram-positive bacterial invasion.
- ✓ **R**esponse – is the host immune response which is still difficult to characterize. For quantification general symptoms and biologic markers (procalcitonin, IL-6, TNF-α, IL-1β, and many others) may be used (Damas P. et al., 1992; Harbarth S. et al., 2001; Hausfater P. et al., 2002; Panacek EA et al., 1999).
- ✓ **O**rgan – the organ failure score can be used to quantitatively describe the degree of organ dysfunction developing over the course of critical illness (Cook R. et al., 2001; Levy M.M. et al., 2003).

Domain	Present	Future	Rationale
Predisposition	Premorbid illness with reduced probability of short-term survival. Cultural or religious beliefs, age sex.	Genetic polymorphisms in components of inflammatory response (e.g., TIR domain, TNF, IL-1, CD14); enhanced understanding of specific interactions between pathogens and host diseases.	In the present, premorbid factors impact on the potential attributable morbidity and mortality of an acute insult; deleterious consequences of insult heavily dependent on genetic predisposition (future).
Insult infection	Culture and sensitivity of infecting pathogens; detection of disease amenable to source control.	Assay of microbial products (LPS, mannan, bacterial DNA); gene transcript profiles.	Specific therapies directed against inciting insult require demonstration and characterization of that insult.
Response	SIRS, other signs of sepsis, shock, CPR.	Nonspecific markers of activated inflammation (e.g., PTC or IL-6) or impaired host responsiveness (e.g., HLA-DR); specific detection of target of therapy (e.g., protein C, TNF, PAF).	Both mortality risk and potential to respond to therapy vary with nonspecific measures of disease severity (e.g., shock); specific mediator targeted therapy is predicated on presence and activity of mediator.
Organ dysfunction	Organ dysfunction as number of failing organs or composite score (e.g., MODS, SOFA, LODS, PEMOD, PELOD)	Dynamic measures of cellular response to insult – apoptosis, cytopathic hypoxia, cell stress.	Response to preemptive therapy (e.g., targeting microorganism or early mediator) not possible if damage already present; therapies targeting the injurious cellular process require that it be present.

Table 1.1. The PIRO system for staging sepsis. TIR domain- Toll-interleukin 1 receptor. TLR, Toll-like receptor; TNF, tumor necrosis factor; IL, interleukin; LPS, lipopolysaccharides; SIRS, systemic inflammatory response syndrome; CRP, C-reactive protein; PCT, procalcitonin; HLA-DR, human leukocyte antigen-DR; PAF, platelet-activating factor; MODS, multiple organ dysfunction syndrome; SOFA, sepsis-related organ failure assessment; LODS, logistic organ dysfunction system; PEMOD, pediatric multiple organ dysfunction; PELOD, pediatric logistic organ dysfunction. (Levy M.M. et al., 2003)

Human septic shock is characterized by infection and a generalized systemic inflammatory response syndrome (SIRS) ultimately leading to multiple organ dysfunctions (MODS) and death. (1992b; 2003) In order to

identify the pathophysiological mechanisms involved in SIRS, including cardiovascular decompensation, rodent models are widely used. It has been shown in rats that an injection with bacterial lipopolysaccharides (LPS) leads to several characteristic features of human septic shock, including febrile responses (Klir J.J. et al., 1993; Long N.C. et al., 1990) and hypotension. (Bauhofer A. et al., 2001)

1.3. The Immune System

The immunological host defense consists of two major parts: *a)* the adaptive immunity and *b)* the innate immunity. The adaptive immunity exists only in vertebrates and in phylogenetically younger animals/organisms. B- and T-lymphocytes, responsible for the humoral and cellular immune response are the main effectors of it. Both groups of cells express different receptors matching specific antigens. After activation through antigen binding they mediate proliferation and differentiation of effectors cells and so called memory cells. Many types of receptors have been identified –most important are the Toll-like receptors.

At present 13 different Toll-like receptor (TLR) family members are identified (Verstak B. et al., 2007). All of them are type I transmembraneous receptors with a similar extracellular domain, which incorporates a leucin-rich portion (leucin-rich-repeats LRR) and a cytoplasmatic domain, which is similar to the cytosolic part of the IL-1 receptor and is therefore called Toll/IL-1-receptor domain (TIR), except of TLR 9 which is different. This TLR 9 is responsible for signal transduction of bacterial DNA (Gewirtz A.T. et al., 2001). Toll-like receptors are located in the cell membrane of the first defense line of the immunity (such cell as macrophage, neutrophil, dendritic and some epithelial cells). Rapidly after appearance of LPS in the blood it is bound with high affinity to the LPS-binding protein (LBP). The complex LPS-LBP binds to CD14 receptors expressed on the macrophage membrane

(Ulevitch R.J. et al., 1995). Then this complex provokes an immune response by docking to TLR4 through an adaptor molecule (Shimazu R. et al., 1999). This docking triggers a huge release of cytokines and other mediators, which generates the inflammatory reaction.

The innate immunity is an evolutionary old part of the immune system which exists in plants, insects and mammals including human being. It was initially discovered in insects which are devoid of an adaptive immune system and rely only on innate immune reactions for their defense; this immediate response accomplishes many activities including recognition and effector functions. Recognition is mediated by broad specificity, pattern recognition, receptors which recognize many related molecular structures (e.g. polysaccharides, polynucleotides) present in microorganisms but not found in the host. The innate responses include the release of antimicrobial peptides, production of cytokines, acute-phase proteins, complement. This type of immunity plays the central role in the acute phase of an infection to eliminate pathogens. It is not an antigen specific neutralization (Janeway C.A., Jr. 1992). However, innate immunity does not induce an immunologic memory (Medzhitov R. et al., 2000).

The strategy of the innate immune response may not be to recognize every possible antigen, but rather to focus on a few, highly conserved structures present in large groups of microorganisms. These structures are referred to as pathogen-associated molecular patterns, and the receptors of the innate immune system that evolved to recognize them are called pattern-recognition receptors. The best-known examples of pathogen-associated molecular patterns are bacterial lipopolysaccharide, peptidoglycan, lipoteichoic acids, bacterial DNA, double-stranded RNA and glucans. Although these structures are chemically quite distinct, all pathogen-associated molecular patterns have common features (Medzhitov R. et al., 2000). First, pathogen-associated molecular patterns are produced only by microbial pathogens, and not by their hosts. For example, lipopolysaccharide is synthesized only by bacteria; pattern-recognition receptors recognize

lipopolysaccharide, thus alerting the host to the presence of the infecting organism. Second, the structures recognized by the innate immune system are usually essential for the survival or pathogenicity of microorganisms. Third, pathogen-associated molecular patterns are usually invariant structures shared by entire classes of pathogens. For example, all gram-negative bacteria have lipopolysaccharides, and therefore, the lipopolysaccharides pattern-recognition receptor of the host can detect the presence of virtually any gram-negative bacterial infection (Medzhitov R. et al., 2000).

1.3.1. Cytokines and sepsis

The systemic response to infection is mediated by the cytokines, descended from macrophage, that target special organ receptors as a response to injury or infection. The inflammatory response to infection or injury is a highly conserved and regulated reaction of the organism. In case of infection, the organism (*e.g.,* a rat or a human being) produces soluble proteins and lipid pro-inflammatory molecules which activate cellular defenses, and similarly anti-inflammatory molecules are produced to attenuate and halt the pro-inflammatory response. An overview of pro-inflammatory and anti-inflammatory molecules is given in **Table 1.2.**

Pro-inflammatory Molecules		Anti-inflammatory Molecules
TNF-α	Thromboxane	IL-1 ra
IL-1β	Platelet activating factor	IL-4
IL-2	Soluble adhesion molecules	IL-10
IL-6	Vasoactive neuropeptides	IL-13
IL-8 (MIP-2)	Phospholipase A2	Type II IL-1 receptor
IL-15	Tyrosine kinase	Transforming growth factor-β
Neutrophile elastase	Plasminogen activator inhibitor–1	Epinephrine
IFN-γ	Free radical generation	Soluble TNF-α receptors
Protein kinase	Neopterin	Leukotriene B_4-receptor antagonism
MCP-1	CD14	Soluble recombinant CD-14
MCP-2	Prostacyclin	LPS binding protein
Leukemia inhibitory factor (D-factor)	Prostaglandins	

Table 1.2. Partial List of Pro-inflammatory and Anti-inflammatory Molecules modified from (Bone R.C. et al., 1997) TNF= tumor necrosis factor, MIP= macrophage inflammatory protein, MCP= monocytes chemoattractant protein, LPS= lipopolysaccharide

Normally the cytokine response is regulated by the intricate network of pro-inflammatory and anti-inflammatory mediators. The initial inflammatory response is kept in check by down-regulating production and counteracting the effects of cytokines already produced. The picture that emerges from analysis of data from patients with sepsis is that a complex mixture of pro-inflammatory and anti-inflammatory molecules may be present. (Dinarello C.A. et al., 1993; Pinsky M.R. et al., 1993)

Cytokines are proteins of around 20-35 kDa. The cytokines can be empirically divided in two groups: pro-inflammatory and anti-inflammatory. The first cytokines are produced by TH_2-lymphocytes and block activation of

TH_1-lymphocytes and macrophages. Cytokines may have multiple effects: on neuronal signalling, in the immune defense or in wound healing, and their function depends on various milieu factors (Hartung T. et al., 2003).

1.3.1.1. TNF – tumor necrosis factor

The name is originally derived from an observation that a soluble factor released by macrophages after injection of LPS was able to destroy tumor cells. Two distinct forms are described – TNF-α and TNF-β. TNF-α is a protein of 17 kDa with 157 aminoacids. TNF-α is mostly secreted by macrophages and TNF-β by lymphocytes. Both have similar mechanism of action. TNF-α is involved in various sepsis mechanisms and can provoke pyrogen reaction (via PGE_2), enhances the bactericidal activity of macrophages, can be thrombogenic, activates osteoclasts and suppresses LPS sensitivity during sepsis (Gemsa D. et al., 1997).

1.3.1.2. IL-6 – interleukin 6

IL-6 is a 26 kDa pleiotropic glycoprotein. It influences B-cell differentiation to plasma cells and is involved in the activation and maturation of T-cells. Together with other interleukins IL-6 may stimulate hematopoetic precursor cells (e.g. thrombocyte growth factor) (Ibelgaufts H. 1995). It is released by stimulated macrophages, monocytes, fibroblasts and endothelial cells. In acute phase reaction IL-6 is one of the main mediators. It also stimulates ACTH synthesis; the subsequently released glucocorticoids inhibit IL-1 and IL-6 production in a feedback loop.

1.3.1.3. IL-1 – interleukin 1

IL-1 is a polypeptide with a molecular weight of 17.5 kDa. Till now two isoforms IL-1α and IL-1β are known with high affinity to the same receptor. Production of IL-1 is triggered by antigens (bacteria, viruses) and LPS, as

well as by other cytokines synthesized by macrophages, endothelial cells, epithelial cells and B-cells. IL-1 has local and systemic effects: it activates T-lymphocytes to produce other cytokines. IL-1 stimulates the release of prostaglandines, complement factors and cytokines like TNF-α and interferons by monocytes' and macrophages' receptor activations. Additionally, it acts as endogenous pyrogen acting in the hypothalamic thermoregulatory area via prostaglandin release. IL-1 production induces simultaneously an IL-1 receptor antagonist, which counteracts IL-1effects (Ibelgaufts H. 1995).

1.3.1.4. MIP-2 – macrophage inflammatory protein

Two murine MIP (macrophage inflammatory protein) proteins, designated MIP-1 and MIP-2 have been described.

MIP-1 is an acidic protein, which has two variants: MIP-1α and MIP-1β have a length of 69 aminoacids (7.8 kDa). Both MIP proteins belong to the family of chemotactic cytokines known as chemokines. They are the major factors produced by macrophages following their stimulation with bacterial endotoxins. Both proteins are involved in the cell activation of human granulocytes (neutrophiles, eosinophiles and basophiles) and appear to be involved in acute neutrophilic inflammation (Fahey T.J., III et al., 1992; Sherry B. et al., 1992; Widmer U. et al., 1993a).

MIP-2 is a basic protein of approximately 6 kDa. This protein is extremely chemotactic for segmented neutrophilic granulocytes and has also a synergistically effect in combination with G-CSF (granulocytes colony stimulating factor) and M-CSF (monocytes colony stimulating factor). MIP-2 also induces the degranulation of human neutrophils but it does not enhance oxidative metabolism. The two MIP-2 proteins known as MIP-2α and MIP-2β have a high homology to each other (Sherry B. et al., 1992; Widmer U. et al., 1993b; Wolpe S.D. et al., 1989). In man they are represented by IL-8.

1.3.2. Antimicrobial peptides

The activated neutrophil attacks microbes with an array of antimicrobial peptides and proteins, including defensins and cathelicidins, which act much like endogenous antibiotics. The term cathelicidins was introduced in 1995 to encompass bipartite molecules containing both a cathelin domain and a C-terminal antimicrobial peptide domain (Zanetti M. et al., 1995). Such molecules are found in many mammals, including primates, ungulates, rodents, and rabbits (Ritonja A. et al., 1989). Its name, an acronym for cathepsin L inhibitor, saved its modest sequence similarity to certain inhibitors of cysteine proteases. Although the protease-inhibitory properties of cathelin are still unproven, the name is firmly established (Gennaro R. et al., 2000; Zanetti M. et al., 2000). Human and murine neutrophils each contain a single typical cathelicidin: hCAP-18 in humans and CRAMP in the mouse, but in bovine or porcine leukocytes each contain at least 10 different cathelicidins (Zanetti M. et al., 1995). hCAP-18 is stored in the neutrophils' secondary (specific) granules. The proteolytic processing that releases its antimicrobial domain (LL-37) occurs in conjunction with its secretion, and is probably mediated by proteinase 3 (Lehrer R.I. et al., 2002; Sorensen O.E. et al., 2001; Zanetti M. et al., 1995).

1.3.2.1. Mechanism of antimicrobial activity

Antimicrobial activity occurs through several mechanisms (Fig. 1.1). Interactions between the peptide and surface membranes of the target organisms play a key role. The initial binding is thought to depend on electrostatic interactions between the positively charged peptides and the negatively charged molecules at the surface of the target. The biophysical properties of the membrane are perturbed by direct interactions with the peptide. Three main mechanisms have been suggested for peptide permeation of the target cell membrane (van 't H.W. et al., 2001).

1) A barrel stave mechanism involves the formation of transmembrane channels in a voltage-dependent manner with nonpolar domains of the molecules facing the membrane lipids and forming a hydrophilic pore spanning the membrane (Boheim G. 1974).
2) The aggregate channel model involves arrangement of peptides in unstructured clusters in the membrane allowing the dynamic formation of pores for short periods of time and the leakage of intracellular components. Antimicrobial peptides can also enter the intracellular space through this mechanism (Wu M. et al., 1999).
3) A carpet-like mechanism describes covering of the microbial cell membrane by a lawn of antimicrobial peptides (fig. 1.1.). The integrity of the membrane collapses via holes that form by the bending of the lipid bilayer back on itself (He K. et al., 1996; Heller W.T. et al., 2000). Cathelicidins interact with both the inner and outer membrane of Gram-negative microorganisms (Gutsmann T. et al., 1999; Gutsmann T. et al., 2000). The situation for Gram-positive bacteria is less clear; however, several Gram-positive organisms are susceptible to cathelicidins at low concentrations (Bals R. et al., 2003; Turner J. et al., 1998).

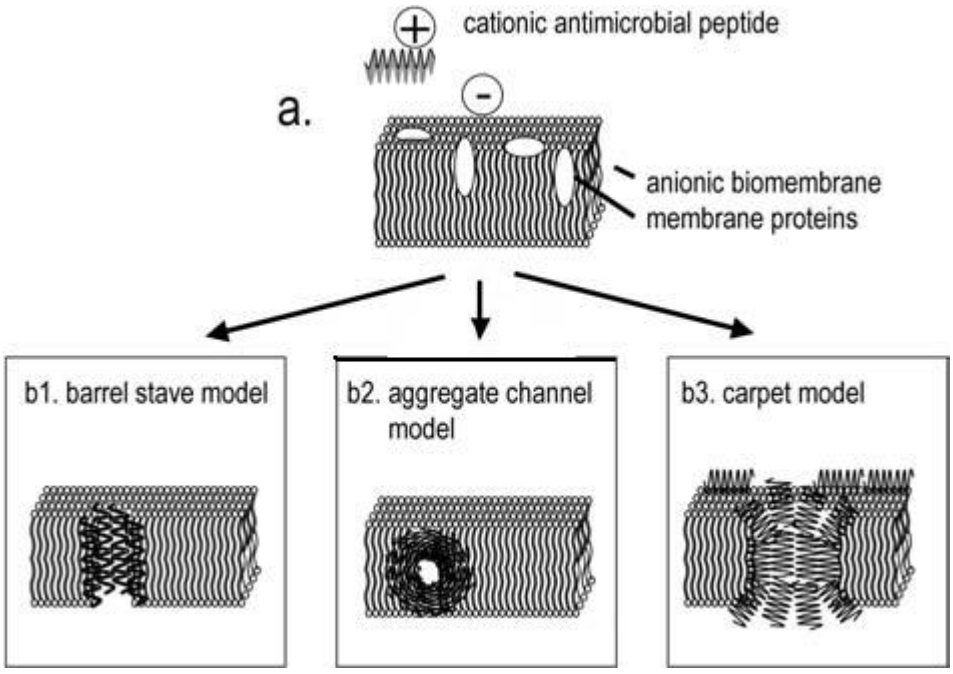


Figure 1.1. Mechanisms of action of antimicrobial peptides. After electrostatic interactions between the negatively charged bacterial wall and the positively charged peptides (a), the peptides associate with the membranes, leading to a destabilization of the membrane and subsequent cell death of the microorganism (b). Several models of action have been developed (as explained in the text): b1, barrel stave model; b2, aggregate channel model and b3, carpet model. (R. Bals and J.et al., 2003)

1.3.2.2. LL-37/hCAP-18 – human antimicrobial peptide

The only known human cathelicidin LL-37 or hCAP-18 (cationic antimicrobial peptide of 18 kDa), was isolated first from bone marrow. (Gudmundsson G.H. et al., 1996; Larrick J.W. et al., 1995) Cathelicidins are usually stored in the granules of neutrophils, moreover they are also produced by various epithelial cells and keratinocytes (Agerberth B. et al., 1995; Dorschner R.A. et al., 2001; Nilsson MF et al., 1999), monocytes, NK cells, B cells, and γ-, δ-T cells (Agerberth B. et al., 2000), and they are induced in keratinocytes in response to inflammatory stimuli (fig. 1.2.) (Bals R. et al., 1998; Frohm M. et al., 1997) or injury (Dorschner R.A. et al., 2001), as an inactive proform (cathelin-like domain C antimicrobial domain). They undergo processing to mature peptides during or after secretion by appropriate proteases. For example, human cathelicidin/hCAP18 is cleaved by elastase (Gudmundsson G.H. et al., 1996) or proteinase 3 (Sorensen O.E. et al., 2001) to liberate its C-terminal antimicrobial domain. This peptide is called “LL-37” because it begins with two leucine residues and has 37 amino acid residues (Agerberth B. et al., 1995; Cowland J.B. et al., 1995; Gudmundsson G.H. et al., 1996; Larrick J.W. et al., 1994; Larrick J.W. et al., 1995).

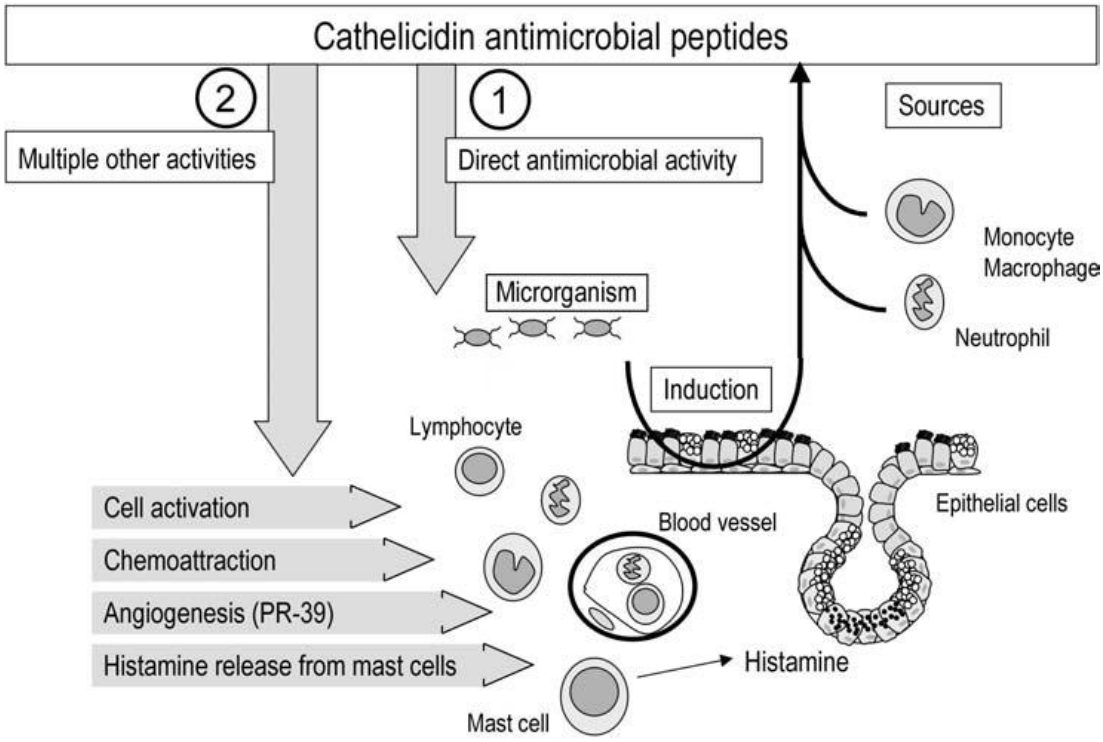


Figure 1.2. Biological functions of cathelicidin antimicrobial peptides. Several cell types secrete Cathelicidins during infection and inflammation. For example, LL-37/hCAP-18 is found in airway surface fluid and originates from epithelial cells as well as professional inflammatory cells, such as macrophages, neutrophils and lymphocytes. The cathelicidin peptides have direct antimicrobial activity. Additionally, they regulate cellular responses including cell proliferation, cell migration of inflammatory cells, release of cytokines and angiogenesis. Cathelicidins are multifunctional peptides that link host defense with inflammation and angiogenesis and activate the adaptive immune system. (Bals R. et al., 2003)

1.3.2.3. Roles of Cathelicidins in Host Antimicrobial Immunity

1.3.2.3.1. *Direct antimicrobial activity.* A recent study found that the cathelin-like domain not only inhibits the protease activity of cathepsin L, a cysteine protease, but also demonstrates potent antibacterial activity (Zaiou M. et al., 2003). The C-terminal antimicrobial peptides of cathelicidins are microbicidal against a broad spectrum of microorganisms, including bacteria, fungi, and parasites, with a wide overlap in specificity, but they exhibit

significant differences in potency from one another (Ramanathan B. et al., 2002; Zanetti M. et al., 1995). Similar to defenses, the mechanism of cathelicidin-mediated microbial killing depends on the formation of ion channels or pores in the microbial cell membrane (Ramanathan B. et al., 2002).

1.3.2.3.2. *Chemotactic activity.* Several cathelicidins are chemotactic for various leukocytes. LL-37 has been found to be chemotactic for neutrophils, monocytes (Agerberth B. et al., 2000; De Y. et al., 2000), and mast cells (Niyonsaba F. et al., 2002). It can induce Ca^{2+} mobilization in phagocytes (De Y. et al., 2000), suggesting that cathelicidins are endogenous activators of phagocytes.

1.3.2.3.3. *Induction of expression or release of inflammatory mediators.* LL-37 enhances the expression of a variety of genes by macrophages (Scott M.G. et al., 2002). It can degranulate mast cells, leading to the release of pro-inflammatory mediators such as histamine and prostaglandins (Agerberth B. et al., 2000; De Y. et al., 2000; Niyonsaba F. et al., 2001). The capacity of LL-37 to chemoattract human peripheral blood T cells (Agerberth B. et al., 2000; De Y. et al., 2000) indicates that it can participate in the recruitment of effector T-cells to sites of microbial infection, thereby contributing to adaptive antimicrobial immunity.

1.3.2.3.4. *Neutralization of bacterial endotoxin.* Killing of bacteria by antimicrobial peptides, phagocytes, and complement system result in the release of bacterial components, such as endotoxin from Gram-negative bacteria and lipoteichoic acid from Gram-positive bacteria. These bacterial components, if allowed to enter the circulation, have a detrimental outcome such as septic shock by inducing the production of high levels of systemic pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6. Many of the cathelicidins, including LL-37, SMAP-29, Bac 2A-NH2 (a linear form of Bac 2A), and indolicidin, bind LPS with high affinity and neutralize its biological

activities (Hirata M. et al., 1994; Larrick J.W. et al., 1994; Larrick J.W. et al., 1995; Niyonsaba F. et al., 2002; Scott M.G. et al., 2002).

1.3.2.3.5. *Angiogenesis and wound healing.* LL-37 can stimulate re-epithelialization of skin wounds, and the wound repair can be inhibited by LL-37-specific neutralizing antibody (Heilborn J.D. et al., 2003; Koczulla R. et al., 2003; Li J. et al., 2000). Thus, cathelicidins are potentially important for wound healing.

1.3.2.3.6. *In vivo roles of cathelicidin.* The participation of LL-37 in host antimicrobial defense has been demonstrated by in vivo studies showing that adenoviral vector–targeted systemic overexpression of LL-37 in mice results in decreased bacterial load and mortality of experimental mice following challenge with *P. aeruginosa* or *E. coli* (Bals R. et al., 1999b; Bals R. et al., 1999a). Furthermore, mice deficient for CRAMP show greatly decreased vascularization during skin wound repair (Koczulla R. et al., 2003; Yang D. et al., 2004).

1.4. Pathophysiology of SEPSIS

The microorganisms – bacteria, initiate the pathophysiology of sepsis. The most important components of the outer membrane are: lipopolysaccharides – LPS, lipid A, endotoxin (in gram-negative invasion) and lipoteichoic acid, peptidoglycan (in gram-positive invasion). Endotoxin binds to the CD14 receptor (on the surface of monocytes, macrophages (Ulevitch R.J. et al., 1995)). The complex CD14-LPS binds to TLR-4 which then triggers a cytosolic cascade resulting in a release of cytokines and other mediators. (Glauser M.P. et al., 1994; Schwandner R. et al., 1999). Lipoteichoic acid mediates its action on cytokine release mainly via the TLR-2 receptor (Verstak B. et al., 2007).

1.4.1. Theory of misbalanced mediator release in sepsis

Immunomodulation is a complex, overlapping network of interactions among factors that work together to overcome severe assaults on the body. Unbalanced expression of them also causes SIRS and MODS. Roger Bone has presented a hypothesis-based explanation for the apparently paradoxical events observed in the critically ill (fig. 1.3). The five stages in the development of multiple organ dysfunction are as follows: 1) local reaction at the site of injury or infection; 2) initial systemic response; 3) massive systemic inflammation; 4) excessive immunosuppression; and 5) immunologic dissonance (Bone R.C. 1996a).

1.4.1.1. *Stage 1.* Prior to development of SIRS or MODS is some insult such as a nidus of infection, a traumatic injury (including a surgical wound). The body's initial response is to induce a pro-inflammatory state in which mediators have multiple overlapping effects designed to limit new damage and to ameliorate whatever damage has already occurred. They destroy damaged tissue, promote the growth of new tissue, and combat pathogenic organisms, neoplastic cells, and foreign antigens. Local levels of both pro-inflammatory and anti-inflammatory mediators can be substantially higher than they are later found systemically (Fukushima R. et al., 1994; Meduri G.U. et al., 1995; Puren A.J. et al., 1995).

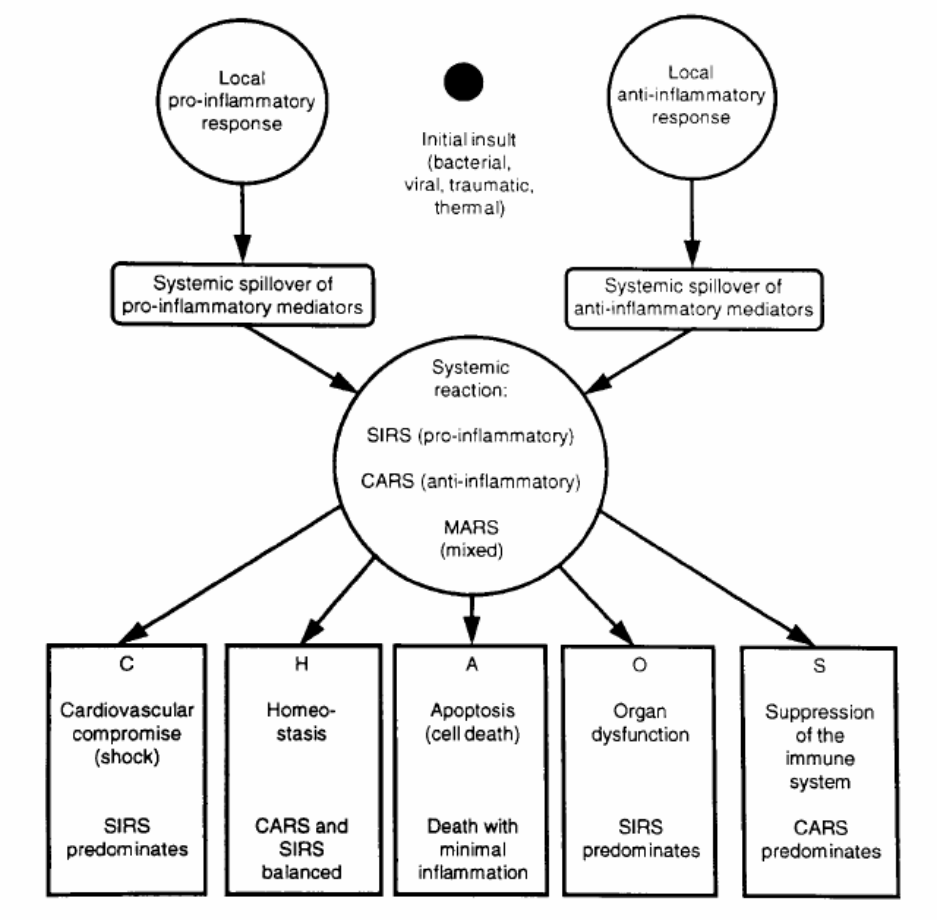


Figure 1.3. New concepts for the clinical sequelae of sepsis, SIRS, CARS, and MARS. (This figure is an adaptation by Bone RC. Sir Isaac Newton, sepsis, SIRS, and CARS. Crit Care Med 1996; 24:1125-28.)

1.4.1.2. *Stage 2.* If the original insult is sufficiently severe, first pro-inflammatory and later anti-inflammatory mediators will appear in the systemic circulation via a variety of mechanisms. The pro-inflammatory mediators help recruit neutrophils, T cells and B cells, platelets, and coagulation factors to the site of injury or infection. (Munoz C. et al., 1991) This cascade stimulates a compensatory systemic anti-inflammatory response, which normally quickly down-regulates the initial pro-inflammatory response. The inflammatory cascade may affect organs, but significant organ dysfunction is rare.

1.4.1.3. *Stage 3.* Loss of regulation of the pro-inflammatory response results in a massive systemic reaction which induces SIRS. The

pathophysiological changes include the following: 1) progressive endothelial dysfunction, leading to increased microvascular permeability; 2) platelet sludging that blocks the microcirculation, causing opportunity to provoke ischemia, which may cause reperfusion injury and induction of heat shock proteins; (Rinaldo J.E. et al., 1990) 3) activation of the coagulation system; and 4) profound vasodilatation, fluid transudation may result in profound shock (Gomez-Jimenez J. et al., 1995; Miyauchi T. et al., 1990). Organ dysfunction or later organ failure results from these changes.

1.4.1.4. *Stage 4.* It is possible that a compensatory anti-inflammatory reaction can be inappropriate, with a resulting immunosuppression - “compensatory anti-inflammatory response syndrome” (CARS) (Bone R.C. 1996b). CARS is the body’s response to inflammation and is more than just immune-paralysis.

1.4.1.5. *Stage 5.* The final stage of MODS is what we have chosen to call “immunologic dissonance” (Kox W.J. et al., 1997). In this stage it is possible to regain organ function if the body can recover its balance. (Bone R.C. et al., 1997)

1.5. Temperature fluctuation – impact on sepsis evolution

1.5.1. Hypothermia and deterioration of immune status

Animal and human studies have shown that intraoperative hypothermia increases the risk of abnormal immune response. (Kurz A. et al., 1996; Sheffield CW et al., 1994; Sheffield C.W. et al., 1994) Intraoperative hypothermia is likely to cause a reduction in peripheral circulation, which may increase tissue hypoxia and make the wound more susceptible to infection, even if contamination levels are low. The process of warming using a warm

air blanket, to prevent hypothermia, is becoming common practice for most major surgery (Melling A.C. et al., 2001).

Both humoral and cellular immunity are adversely affected by lower temperature. In bacterial infections, neutrophil chemotaxis is an essential component of host defense. Hypothermia inhibits both neutrophil chemotaxis and killing via the respiratory burst, and delays induction of pro-inflammatory cytokine production by macrophages. (Kimura A. et al., 2002; Torossian A. et al., 2003) showed that circulating levels of the cytokine interleukin-6 and the chemokine macrophage inflammatory protein-2 were both increased with hypothermia and ameliorated by either G-CSF administration or normothermia. (Gropper M.A. 2003).

1.5.2. Hyperthermia – known effects in sepsis

A potential approach for the treatment of sepsis is to make use of a natural defense mechanism called "the stress response," which is present in virtually all-living cells. The stress response is characterized by the rapid, and almost exclusive, synthesis of intracellular proteins known as heat-shock proteins (HSPs). The stress response can be initiated by a wide variety of different agents, including sodium arsenite, ischemia, several types of metabolic stress, and exposure to a transient period of heat (Lindquist S. et al., 1988; Nover L. et al., 1991). Some results demonstrated the protective effects of the stress response when applied before experimental sepsis. (Buchman T.G. et al., 1993; Buchman T.G. 1994; Rogovein T.S. 1995). And Chu showed that heat stress applied even after the initiation of experimental sepsis is protective against the lethal effects of sepsis. (Chu E.K. et al., 1997)

Heat stress response is an evolutionarily highly conserved response, which is characterized by the production of heat shock proteins (Joslin G. et al., 1991; Ribeiro S.P. et al., 1994) with high homology from worms to human being. These proteins are proposed to have a protective role by taking part in the cellular repair mechanism, by degradation of proteins beyond repair, and

by altering inflammatory cellular functions (Joslin G. et al., 1991; Ribeiro S.P. et al., 1994)). Heat stress administered before sepsis has resulted in cardiac protection, reduction in sepsis-induced acute lung injury, and decreased mortality in animals (Currie R.W. et al., 1993; Meng X. et al., 1996; Villar J. et al., 1993; Villar J. et al., 1994). The mechanism of cardiopulmonary protection by heat in sepsis is unknown but may be related to a decrease in pro-inflammatory cytokine production (Deshpande G.G. et al., 2000).

1.5.3. The nature of heat shock proteins

Heat shock proteins (HSPs) are highly conserved proteins – chaperones which are considered to be an immunity-regulating dangerous signal due to their capability to induce cytokine production (Asea A. et al., 2000; Chen W. et al., 1999; Ohashi K. et al., 2000). Chaperones are proteins involved in repair, transport, folding, and unfolding of various intracellular proteins (Hartl F.U. et al., 2002). It is accepted that heat stress can alter innate immune response by inducing expression of stress proteins, such as HSPs. However, the direct effect of heat stress on the signaling pathway that mediates innate immunity is not clear (Zhou J. et al., 2005).

1.5.3.1. The HSP-70 family

The HSP70 family is various and mainly includes the constitutive cytosolic Hsp70 (or Hsp73) and the stress-induced cytosolic Hsp70 (or Hsp72). HSPs are widely believed to function as molecular chaperones. HSPs also play important roles in antigen presentation, cross-presentation, and tumor immunity (Wallin R.P. et al., 2002). Recently, it has been found that HSR (heat shock response) has anti-inflammatory effects. HSPs reduced mortality in experimental models of septic shock and adult respiratory distress syndrome (ARDS) (Klosterhalfen B. et al., 1997; Villar J. et al., 1994; Wallin R.P. et al., 2002), and can regulate gene expression of pro-inflammatory and anti-inflammatory factors such as TNF-α, IL-1, IL-12,

IL-10, and IL-18 (Snyder Y.M. et al., 1992; Tang D. et al., 2005; Wang X. et al., 2001; Wang Y. et al., 2002).

The 70-kDa heat shock proteins (HSP70s) are expressed both constitutively (Hsc70) and under stressful conditions (Hsp70) in all prokaryotes and eukaryotes (Bukau B. et al., 1998). Members of the Hsp70 protein family play essential roles as molecular chaperones in the cytosol, mitochondria, and endoplasmatic reticulum (Matouschek A. et al., 2000):

- ✓ Their levels were increased in a number of pathological conditions (Dybdahl B. et al., 2002)
- ✓ Are required for protein translocation into endoplasmatic reticulum and mitochondria (Matouschek A. et al., 2000)
- ✓ Are required for uncoating of clathrin-coated vesicles (Gao B.C. et al., 1991)
- ✓ Are activators of the innate immune system (Srivastava P. 2002)
- ✓ Regulate the production of pro-inflammatory cytokines such as TNF-α and IL-6 via CD14 and Toll-like receptor – mediated signal transduction pathway (Asea A. et al., 2000; Asea A. et al., 2002; Vabulas R.M. et al., 2002)
- ✓ Can be the endogenous ligand for the TLRs (Gao B. et al., 2003)

Hotchkiss et al. have reported that hyperthermia can protect mice against lethal effects of endotoxin (Hotchkiss R. et al., 1993). Villar et al. also reported that the induction of heat shock proteins (HSP) reduced mortality rate and histological damage of the lung in a rat model of intra-abdominal sepsis, produced by caecal perforation (Villar J. et al., 1994). In addition, they showed that levels of plasma tumor necrosis factor (TNF), another important mediator of sepsis, decreased in response to endotoxin after heat pretreatment in rats (Ribeiro S.P. et al., 1996). Understanding the biological consequences of fever in septic patients may be important for clinical management. However, fever-producing cytokines, including interleukins IL-

1, IL-6, TNF-α and interferon (IFN) - may stimulate host defense response and regulate immune responses. Furthermore, fever induces HSP that are known as chaperone proteins protecting essential cell components from damage by various noxious stresses (Koh Y. et al., 1999; Lindquist S. et al., 1988).

1.6. The CMRT (clinic modeling randomized trial) concept

In order to investigate the effect of new immuno-modulatory drugs and other biologic active substances a new study type for laboratory animals – Clinic Modeling Randomized Trials – CMRT was established (Table 1.3).

Peritoneal contamination and infection (PCI) was firstly described by Lorenz et al. (1994) (Lorenz W. et al., 1994) and validated by Bauhofer et al. (1998) also from the Lorenz group (Bauhofer A., 1998). Analyzing systematically the clinical situation with multiple interventions CMRTs include: anesthesia, volume substitution, antibiotics, operation, infection with human stool bacteria and analgesia. Further the CMRT concept was standardized as a trial methodology for animals in analogy to the CONSORT statement as concept for clinical trials. (Moher D. et al., 2001)

Table 1.3. (By Torossian A., et al. Anesthesiology 2003)

Clinic modeling trials (CMRT); rationale and characteristics:

- ♦ Modeling clinical trials (scenario and methodology), before or after conducting a clinical trial
- ♦ Modeling clinical complexity is more important than species differences
- ♦ Modeling treatment effects as expected or warranted in the clinical scenario (δ, α and β: e.g. high sample size)
- ♦ Searching for positive and negative results

Modeling the clinical situation	Modeling randomized trials
▪ Developed by clinicians together with theoretical surgeon ▪ Adequate anesthesia (Fentanyl/ Droperidol) ▪ Preoperative antibiotic prophylaxis ▪ Perioperative volume substitution ▪ Operation (laparotomy) ▪ Peritoneal contamination with human stool ▪ Outcome adapted to the clinic: high mortality rate ▪ Postoperative analgesia	▪ Sample size calculation ($\delta = 0.25$, $2\alpha=0.05$, $1-\beta= 0.9$) ▪ Randomized allocation to the groups ▪ Double-blind design ▪ Clinically relevant endpoint (five day mortality) ▪ Evaluation of morbidity ▪ Search for positive and negative results ▪ "Intent to treat" rule ▪ Adequate statistical analysis

2. Aim of the work

Despite advances in medical equipment and the administration of new drugs for treating sepsis in intensive care units, sepsis and its consequences remain one of the main causes of life-threatening clinical complications and is primarily responsible for morbidity and mortality after major surgery or injury.

The human cathelicidin anti-microbial protein, hCAP18 is a component of the innate immune system and has broad anti-microbial activity conferred by its C-terminal fragment LL-37. The antibacterial C-terminus of hCAP18, LL-37 (37 aminoacids), has been shown to exert a broad antimicrobial activity upon both gram-negative and gram-positive bacteria, in order to have synergistic antibacterial effects with the defensine, as well as to be a chemotactic agent for neutrophile, monocytes and T cells.

Hyperthermia (induced hyperthermia) is the situation when the body is no longer capable of sweating; core temperature begins to rise, immediately and swiftly. It was used in cancer treatment and now we hope to see some influence on experimental sepsis in vivo.

Recently, knowledge about a family of protective proteins seems to offer a therapeutic or prophylactic strategy to sepsis. Since first mentioned by Rittosa (Ritossa F. 1962), it is well accepted that living cells, from plant to human, react to hyperthermia and other physiological or pathological stresses by synthesizing a group of highly conserved proteins known as heat shock proteins (HSPs). These proteins protect cells or organisms from damage through a mechanism called *thermotolerance*, or *cross-tolerance*.

The aim of this work was a) to evaluate the effect of the anti-microbial peptide LL-37 as addition to usual antimicrobial therapy; and b) to establish the effect of moderate preoperative hyperthermia (41°C) alone and c) in combination with LL-37 in the development of postoperative abdominal

sepsis using the concept of clinic modeling randomized trials (CMRT) in rats simulating the complex clinical situation.

We hypothesized that perioperative administration of LL-37 as a supplement to general antimicrobial therapy and in combination with heat stress could substantially improve the outcome of septic rats.

3. Materials and Methods

The study was performed with permission of the local animal welfare committee in Giessen, Hessen, Germany. The experiments were done in the Institute for Theoretical Surgery at Philipps University Marburg, Germany.

The CMRT concept (see 1.6.) was applied in combination with the rat peritonitis model of Peritoneal Contamination and Infection (PCI) in all trials.

3.1. Animals

All experiments were carried out in age-matched male rats. Males were selected because they do not have estrogen cycling influences. Sex hormones are proven to have an important influence on the outcome in sepsis. Rats were housed under standard laboratory conditions. They were maintained 23±1°C room temperature with 30-60% environment humidity. Animals were kept up in Microlon-cages (dimensions: 56:34:19 cm), in one ambiance of artificial day-night cycles (12 hour light-dark cycles). International guidelines for animal care were followed (FELASA Working Group on Animal Health et al., 1996). Wistar rats, 220-280g, were obtained from Harlan-Winkelmann (Borchen, Germany) and received a standard rat diet (Altromin R1324, Lage, Germany) and water ad libitum. The animals were allowed to adapt to laboratory conditions for at least 3 days.

3.2. Materials and reagents for sepsis induction

3.2.1. Chemicals

- Fentanyl®, 0.05 %/ 2ml; 10 ml vials, Janssen-Cilag Ltd, Neuss
- Dehydrobenzperidol®, 25 %/2 ml; 10 ml vials, Janssen-Cilag Ltd, Neuss
- Tramundin® 100 vials, Mundipharma GmbH, 65549 Limburg

- LL-37, 1mg/ml (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVP RTES-COOH) chemically synthesized in Peptid laboratory by Dr. Henklein P. works group at the Charité, Berlin, Germany
- Metronidazole 0.5 g IV, 100 ml vials, Serag-Wiessner KG, 95119 Naila, Germany
- Cefuroxime 1500 mg IV, vials, Curasan-Kleinostheim, Germany
- Ringer's Infusion solution, B. Braun Melsungen AG, 34209 Melsungen, Germany
- Second-glue®, UHU GmbH &Co. KG, 77813 Bühl (Baden), Germany
- ELISA assay for cytokine determination, TNF-α, MIP-2 and IL-6 (Fa. BioSurce International, Inc., Camarillo, California, USA), Hsp70 (Stressgen Bioreagents, 350-4243 Glanford Ave Victoria, BC Canada V8Z 4B9)

3.2.2. Plastic and other materials

- Syringes 1 ml, 2 ml, 5 ml B. Braun Melsungen AG, 34209 Melsungen, Germany
- Microlance® 2, needle 0.55*25 mm, Becton-Dickinson S.A., Fraga (Huesca), Spain
- Braunüle® 2.2 mm (orange), B. Braun Melsungen AG, 34209 Melsungen, Germany
- Superior® - heparin coated glass-capillars, Paul Marienfeld GmbH & Co. KG, 97922 Lauda-Königshofen
- Falcon Tubes 16 ml, 50 ml, Genaxxon Bioscience GmbH, 88400 Biberach, Germany
- Eppendorf® -Cups, Eppendorf Vertrieb Deutschland GmbH, 50389 Wesseling-Berzdorf, Germany
- Latex gloves, NOBA Verbandmittel Danz GmbH u. Co KG., 58300 Wetter, Germany
- Vicryl 3/0 SH Ethicon® sewing material, Norderstedt, Ethicon GmbH Germany

3.2.3. Devices

- ELISA reader SLT 340 ATTC ,SLT Lab Instruments, Groeding, Austria
- SLT EasyFit – software for determination of cytokines concentrations, SLT-Labinstruments, Crailsheim, Germany
- Infrared heating lamp 250 Watt (type –ITE; nr. 84273)
- Convection heating system INFORS – HAT (Infors AG; Rittergasse 27, CH-4103 Bottmingen)
- Digital thermometers, Linseis Messgeraete GmbH, 95100 Selb, Germany
- Chronometers, Linseis Messgeraete GmbH, 95100 Selb, Germany

3.2.4. Inoculum

- Standardized human stool suspension “Mix 5”

3.3. Preparation of stool suspension “Mix 5”

For peritoneal contamination and infection a standardized human stool suspension was used. To obtain this suspension we took the stool from three healthy male donors, with the aim to obtain a most similar bacterial spectrum to induce fecal peritonitis. The stool was wrapped immediately after delivery in plastic bags to protect the anaerobic bacteria from room air.

Immediately after collection the obtained probes were mixed carefully with a reduced thioglycolat soup (ratio 1:1) in combination with 10% glycerin from total weight. The reduced thioglycolate solution was prepared a day before usage: 14.5 g thioglycolate, 50 g barium sulfate and 500 ml double distilled water (H_2O). The obtained mixture was treated with nitrogen (N_2) gas overnight to prevent further oxidation. Katalase from bovine liver was added (0.19 mg/100ml; 2000-5000 U/mg).

The thioglycolate solution and the donors’ stool were mixed until a homogenous mixture was obtained. The mixture was distributed in aliquots of 2, 5, 10 ml into sterile plastic cups. Then all cups were frozen at – 80°C.

This procedure was done to obtain a stool suspension for all experiments of this project. This preparation was named “MIX5”

3.4. Study design

All trials were performed on the basis of the CMRT concept, using the rat model of PCI. At first we determined the LD_{50} of the inoculum - “MIX5” and the most effective dose of LL-37. Then we compared different administration schedules of LL-37 in a therapeutical approach. The next step was the investigation of LL-37 prophylaxis. And the last step was the influence of LL-37 as prophylaxis in combination with induced hyperthermia. For each study

and for each animal a standard protocol was used (see tab 3.1). The primary endpoint of all trials was the 120 hour survival rate.

3.4.1. Investigations on Mix-5 and LL-37

These studies were needed to determine the LD_{50} of "MIX5" and the most effective does of LL-37 for further experiments.

3.4.1.1. Determination of a dose response curve of Mix-5 in combination with antibiotics

In this study 34 male Wistar rats were used. All of them were exposed liable to PCI. Each group contained 8 animals. Two animals served as reserve animals for unexpected complications before PCI if needed (for e.g. replacement of allocated ill individuals, anesthesia complications). Intravenous antibiotic prophylaxis was performed with cefuroxime and metronidazole (10 mg/ 3.5 mg kg^{-1}) applied 1 hour before and after PCI.

Mix-5 was diluted with Ringer's solution 1:10 just before administration.

Group A (n=8) – 1.2 ml/kg inoculum

Group B (n=8) – 0.8 ml/kg inoculum

Group C (n=8) – 0.6 ml/kg inoculum

Group D (n=8) – 0.3 ml/kg inoculum

3.4.1.2. Determination of a LL-37 dose response curve

In this study 23 Wistar male rats were used. All of them were exposed liable to PCI. Each group contained 7 animals and 2 additional animals as reserve animals (see a.). For antibiotic prophylaxis was used cefuroxime and metronidazole – (10 mg/ 3.5 mg kg^{-1}). LL-37 was diluted (initial concentration 1mg/ml) with Ringer's solution to 1:4, and administrated IV in the corresponding dose. Administration of AMPs was done one hour before PCI (*time= -1h.)*

Group A (n=7) – control group

Group B (n=7) – 1 mg/kg LL-37

Group C (n=7) – 0.2 mg/kg LL-37

Table 3.1. Study protocol for each animal.

Date: Group:

Study (Name)

(120 Hours surviving of animal after PCI)

Surgeon: Ear mark:

Weight:
Food deprivation: date 14:00 Gender: male ♂

	ml	Hour time	
Anesthesia			Following doses (0.1 ml/kg IV.) time:
1. AB-administration (t =-1 h)			1ml Ringer's addition
Inoculum's administration (t = 0 h)			Stool suspension (1:10 diluted)
2. AB-administration (t =+1h)			1ml Ringer's addition
1. Tramal (t = +1 h)	0.1		
Dead			Date:

NOTES:

Medicaments:
Anesthesia: Fentanyl 0.05 mg/ml + Droperidol 2.5 mg/ml, 3 ml/kg IP
Analgesia: Tramadol-HCl 100 µl SC
Antibiotics: cefuroxime/metronidazole 10/3 mg/kg IV 1 h for and after OP

3.4.2. Main sepsis studies of the project

These studies follow the aim of the proposed work described previously in Chapter 2.

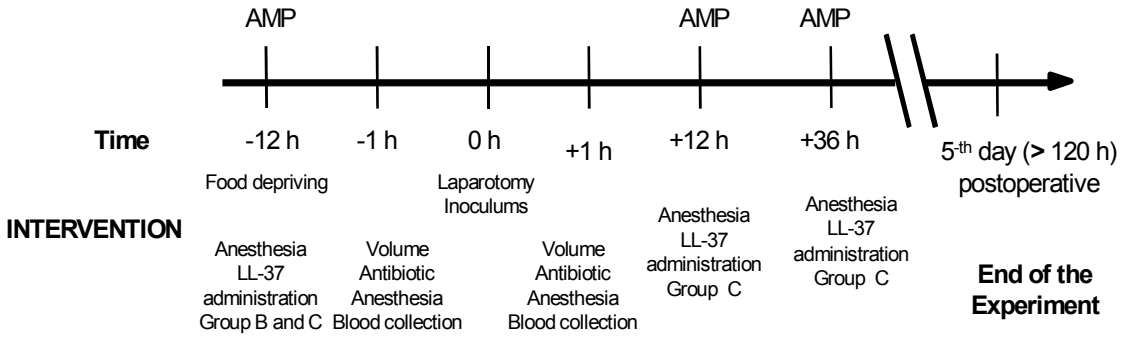
3.4.2.1. Study 1 – LL-37 prophylaxis (1x) versus prophylaxis and treatment (3x)

For this study 32 male Wistar rats were used. All of them were exposed liable to PCI. Each group contained 10 animals plus 2 additional animals as reserve animals (see a.). For antibiotic prophylaxis was given cefuroxime and metronidazole – (10 mg/ 3.5 mg kg^{-1}). LL-37 was diluted (initial concentration 1mg/ml) with Ringer's solution 1:4, and administrated in a dosage of 0.5 mg/kg. All rats were inoculated with 0.6 ml/kg Mix-5 diluted 1:10 with Ringer's solution. Randomly selected, from half of the rats of each group, blood was taken at time point *t = -1h* and *t = +1h.*

Group A (n=10) – control group

Group B (n=10) – 1x LL-37 (*time t = -12h*)

Group C (n=10) – 3x LL-37 (*time t = -12h; t = +12h; t = +36h*)



A)	Control group
B)	LL-37 administration (12h, before OP time)
C)	Three times LL-37 administration (12h, before OP time; 12h and 36h after OP time)

Figure 3.1. Prophylaxis with LL-37 1x versus 3x administration in the PCI model. Schematic representation of the study schedule.

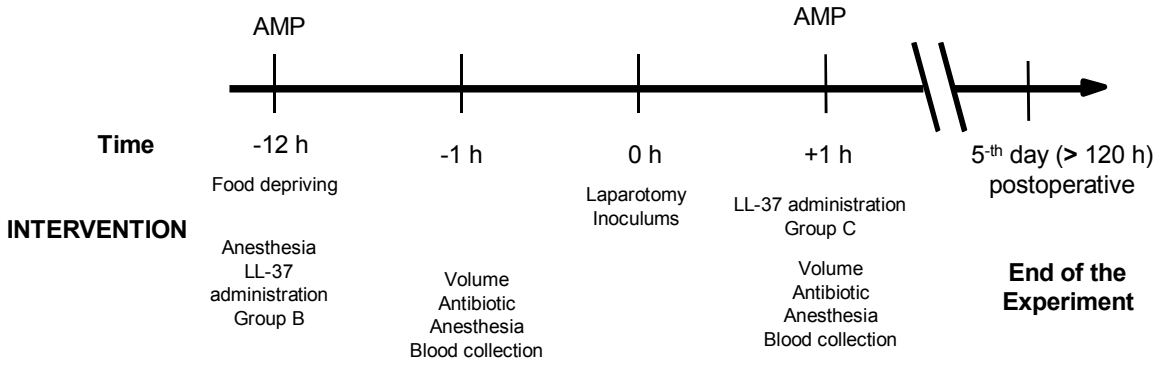
3.4.2.2. Study 2 –prophylactic versus therapeutic administration of LL-37

In this study 62 Wistar male rats were used. All of them underwent PCI. Each group contained 20 animals plus 2 additional animals as reserve (see a.). For antibiotic prophylaxis was used cefuroxime and metronidazole – (10 mg/ 3.5 mg kg^{-1}). LL-37 was diluted (initial concentration 1mg/ml) with Ringer's solution at 1:4 and administrated IV – 0.5 mg/kg. In each group the inoculum dose was 0.6 ml/kg, which was diluted at 1:10 with Ringer's solution. In half of the population of each group, blood was taken at time point *t = -1h* and *t = +1h.*

Group A (n=20) – control group

Group B (n=20) – preoperative administration of LL-37 (*time t = -12h*)

Group C (n=20) – postoperative administration of LL-37 (*time t = +1h*)



A)	Control group
B)	LL-37 administration (12h, before OP time)
C)	LL-37 administration (1h, after OP time)

Figure 3.2. Prophylactic versus therapeutic administration of LL-37 in the PCI model. Schematic representation of study route.

3.4.2.3. Study 3 – the influence of induced hyperthermia prophylaxis in combination with LL-37 administration

In this study 90 Wistar male rats were used. All of them underwent PCI. Each group contained 22 animals. Two additional animals were reserve animals. For antibiotic prophylaxis was used cefuroxime and metronidazole – (10 mg/ 3.5 mg kg^{-1}). LL-37 was diluted (initial concentration 1mg/ml) with Ringer's solution to 1:4 and administrated – 0.5 mg/kg. The groups with induced hyperthermia one day before were exposed to induced hyperthermia for 1 hour at ~ 41°C ± 1°. The dose of inoculum for each group was 0.6 ml/kg, which was diluted at 1:10 with Ringer's solution. In half population of the groups, blood was taken at time point *t = -1h* and *t = +1h.*

Group A (n=22) – Control group

Group B (n=22) – 1x LL-37 (*time t = +1h*)

Group C (n=22) – Only hyperthermia-exposed animals (day before PCI)

Group D (n=22) – Hyperthermia-exposed animals (day before PCI) in combination with 1x LL-37 (*time t = +1h*)

Tab. 3.2. Hyperthermia protocol.

Date: . 06 .2005 **Group: ____**

Protocol for induced hyperthermia in rats

(24 hours before OP; ~41˚C 1 h)

Ear mark: _____

Weight:____Anesthesia _____ ml; Time ____

Gender: male ♂

	Time - hour	Temperature
Start hyperthermia		
41˚C reached		
End of hyperthermia		
Administration of 3ml Ringer's (Sc.)		

NOTES:

Medicaments:

Anesthesia: Fentanyl 0.05 mg/ml + Droperidol 2.5 mg/ml; 3 ml/kg i.p.

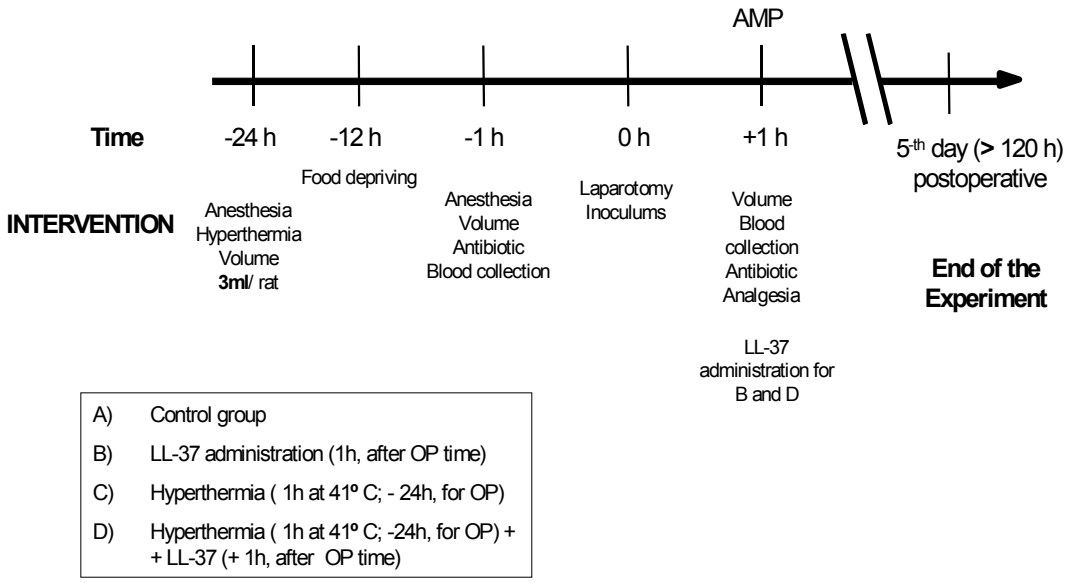


Figure 3.3. The influence of induced hyperthermia in combination with LL-37 prophylaxis in the PCI model. Schematic representation of study route.

3.5. Conduction of the studies

Male Wistar rats, 220-280 g, (obtained from Charles River, Sulzfeld, Germany) were assigned by simple random permutation to the study groups using earmarks. Animals were weighed the day before the experiment and marked with earmarks. Rats were housed under standard laboratory conditions as described previously.

The rats were deprived of food 12 hours before operation and PCI. About one and a half hour before surgery, all animals were anesthetized with 3 ml/kg mixture of Fentanyl 0.001% and Droperidol 2.5% (both Janssen-Cilag, Neuss, Germany), given intraperitoneally (IP). The anesthesia dose was calculated to maintain spontaneous ventilation. After 10-15 min (*time t = -1h*) a tail vein was cannulated in anesthetized animals and an antibiotic prophylaxis was given intraveneously, - cefuroxim and metronidazole - 10 mg/ 3.5 mg kg^{-1}.

Before operation animals received supplemental anesthesia, with 0.1 ml/kg of anesthesia mixture. Then the animals were fixated supine on the operation plate with adhesive strips. Using an antiseptic technique a 2 cm (hypogastrium) midline incision was performed (hypogastrium) and 0.6 ml/kg standardized human stool inoculum (stool concentrate diluted 1:10 in Ringer's solution) was injected into the pelvic region (*time t = 0h*) for peritoneal contamination and infection (PCI). The inoculum dose was established in the first study (standardization of inoculum see 3.4.1.) After inoculum administration the wound was closed in two layers with Vicryl 3-0 suture.

One hour after operation (*time t = +1*) the second antibiotic application was administered (cefuroxime and metronidazole - 10 mg/ 3.5 $mgkg^{-1}$).

For postoperative analgesia, one hour after intervention (*time t = +1*) 20 mg/kg, tramadol was administrated s.c.

After the operation the tail vein catheter was removed and the rats were returned to the cages single or twosome to prevent subsequent cannibalism

of weak rats. Mortality was checked daily on two or three occasions. The primary study end point was the 120 hours survival rate after PCI. The survivors were sacrificed by inhalation of carbondioxide (CO_2).

3.5.1. Additional interventions in the studies

AMP application: In the groups with three times administration of AMP, LL-37 was given intravenously (0.5 mg/kg) one day before or after PCI. LL-37 was diluted (initial concentration 1mg/ml) with Ringer's solution to 1:4. For AMP application no additional anesthesia was performed. Animals were fixated in a special mechanical device (Plexiglas tube) to facilitate tail vein puncture.

Blood collection: About 1.3 ml blood was withdrawn through puncture of venous plexus in the retro-orbital space. The puncture was done with heparin coated glass capillaries and collected in 2 ml Eppendorf cups. Blood sampling was performed always under anesthesia. Each blood sample withdrawn was substituted by IV application of about 2 ml Ringer's solution.

Hyperthermic preconditioning: One day before PCI the hyperthermia groups were subjected to induced hyperthermia. Before the procedure all animals were anesthetized with intra-peritoneal (IP) administration of fentanyl and droperidol (the same dose and mixture as above). About 15 min after anesthesia animals were placed under an IR (infrared) heating lamp. A digital thermometer was inserted 2-3 cm into the rectum for continuous core temperature measurement throughout the heating period. When the core temperature reached 40° - 40.3°C (approx. after 10-12 min) animals were placed for one hour in a convection heating box "INFORS – HAT" to adjust to the desired temperature. After another 8-10 min the core temperature reached 41°C ± 0.6°C. The elevated body temperature was maintained for one hour. Then the animals were transferred to room temperature and 3 ml of Ringer's solution were injected subcutaneously (s.c.) for rehydration. After the heating procedure animals were housed under standard conditions.

3.6. Bioassays – ELISAs

For systemic cytokine analysis blood was collected at two time points of the experiments: “-1h” – one hour before OP (before the first antibiotics administration), “+1h” – one hour after OP (before the second antibiotics and LL-37 administration). For blood collection heparin-coated glass-capillars were used. About 1.3 ml blood was collected, eppendorf tubes were placed in water/ice bath at ~ 0°C and centrifuged at + 4°C for 10 min at10.000 x G. The obtained plasma was transferred into new eppendorf cups and immediately frozen at – 40°C till analysis.

We have determined the following parameters: TNF-α, MIP-2, HSP70 and IL-6. Each assay contains 96 vial standard plates for ELISA reader analysis. The measurements were done with a SLT 340 ATTC ELISA Reader at a wave length of 450 nm and the calculations were performed with the SLT EasyFit software. Measurements were performed as described in detail in the ELISA-kit booklets of the manufacturer.

3.7. Statistical evaluation

The primary endpoint was the 120-hour survival rate. The sample size was calculated with the formula of Friedman et al. (Friedman L.M. et al., 1985) estimating a treatment difference in the mortality rate of 20-50% (δ = 0.2-0.5), an α-error of 0.025 and a power of the test 1-β = 0.9. During the course of the experiments we decreased the delta (δ = 0.2), which resulted in an increased sample size. Randomization was done with random tables.

$$N = \frac{\left(Z\alpha\sqrt{2\bar{p}(1-\bar{p})} + Z\beta\sqrt{p_1(1-p_1)+p_2(1-p_2)}\right)^2}{(p_2-p_1)^2}$$

***N** – total sample size (subjects) with* $\bar{\rho} = (\rho_1 + \rho_2)/2$

***Zα** – the critical value witch corresponds to the significance level α*

***Zβ** – the value of the standard normal value not exceeded with probability β*

***p1** – the event rates in the first intervention group and*

***p2** – in the second group*

(Friedman L.M. et al., 1985)

Mortality rates were analyzed with the Chi-square test. Kaplan-Meier survival curves were plotted and analyzed with the log-rank test. Cytokine data were analyzed with the non-parametric Kruskal-Wallis ANOVA-Test. Only in the case of significant results in the global test, post-Hoc-Tests were performed including a Boferroni correction. All performed tests were analyzed using the SPSS statistic software package for windows (Bühl A. et al., 2000). Cytokine data were given as mean ± SEM (standard error mean). ***P*** levels below 0.05 were considered as significant in all tests used.

4. Results

4.1. Results of preliminary studies

4.1.1. Dose response curve of inoculum MIX-5

In this study the $LD_{50}$of “MIX5” was determined. A standard dose of the antibiotics, cefuroxime and metronidazole 10 /3.5 mg/kg respectively was administered with a variable dose of the “MIX5” suspension. Four groups of 8 animals each were investigated with dosages of 1.2; 0.8; 0.6; 0.3 ml/kg in a 1:10 dilution. The primary endpoint was the 120 h mortality rate. (See Figure 4.1.1.)

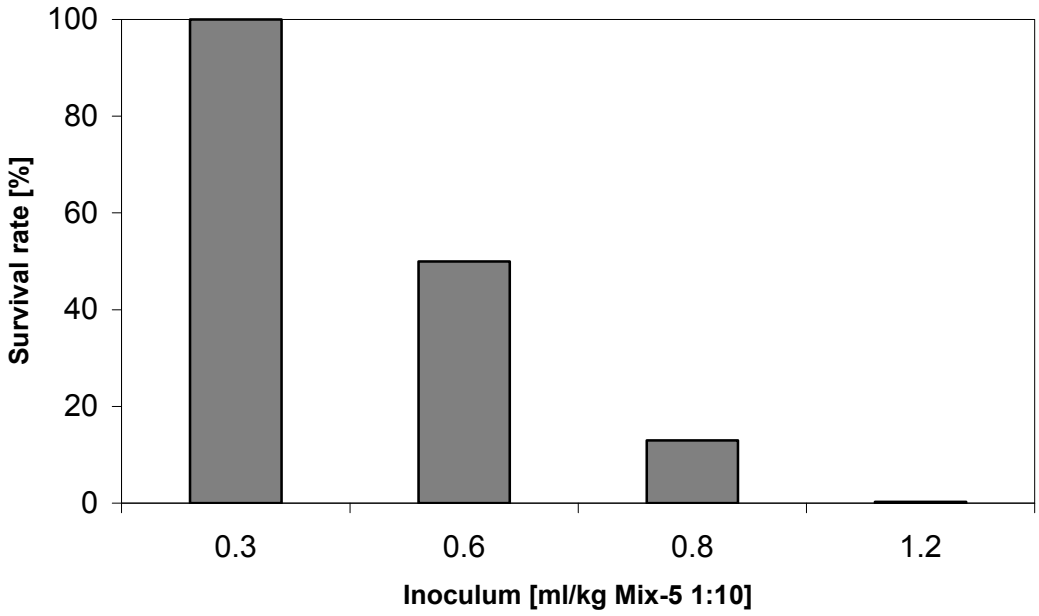


Figure 4.1.1. MIX-5 dose response curve

With a concentration of 1.2 ml/kg no rat survived. A concentration of 0.8 ml/kg resulted in a mortality below the LD_{100} with 13% survivors. Further reductions of the inoculum quantity up to 0.6 ml/kg led to a survival of 50 %.

On a concentration of 0.3 ml/kg all animals survived. The LD_{50} of MIX-5 (0.6 ml/kg) was used for all further experiments.

4.1.2. LL-37 dose response curve

The application schedule of LL-37 was optimized in combination with the antibiotics (cef/met). With the LD_{50} dosage of "MIX5" we investigated two different dosages of LL-37. The animals were exposed to the same treatment as in previous experiments. In addition, at the first antibiotic administration (-1h, before OP) LL-37 was IV injected in a dosage of 0.2 and 1 mg/kg. The antibiotic administration was in the same amount as described before. The control group received the same treatment without antimicrobial proteins (see Figure 4.1.2). We used three groups with seven animals per group.

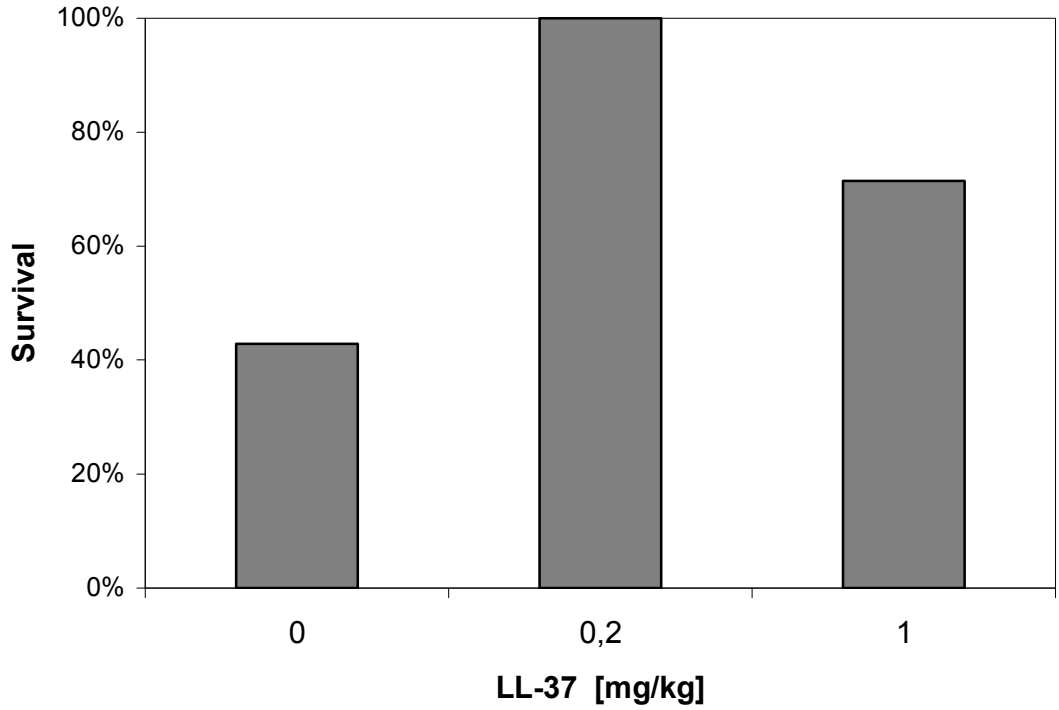


Figure 4.1.2. Survival of rats after prophylactic LL-37 application

Unexpectedly, in the low concentration group of 0.2 mg/kg LL-37 all rats survived. In the high dose group with 1 mg/kg LL-37 a mortality of 29% was obtained. In the control group the mortality was 57%.

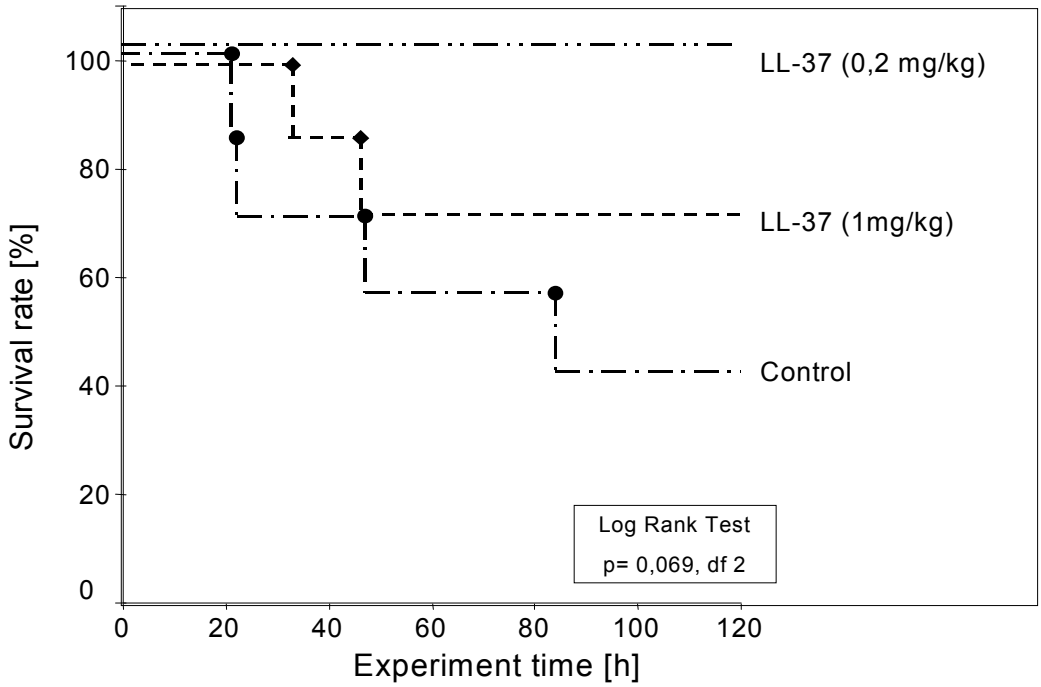


Figure 4.1.3. 120h Kaplan-Meier survival analysis of rats comparing different doses of LL-37 in septic rats, p= 0.069 df 2 in Log Rank test (n=7/group)

We achieved a survival rate of 100% in the 0.2 mg/kg group. However in the 1 mg/kg group survival was with 71% better as in the control group; and without significance in the Kaplan-Meier survival analysis (Figure 4.1.3., Log Rank Test p=0.069, df=2). Based on our results and findings of others (Cirioni O. et al., 2006), we used for further experiments a concentration of 0.5 mg/kg LL-37.

4.2. Survival rates in the main studies

4.2.1. LL-37 prophylaxis (1x) versus prophylaxis and treatment (3x)

The groups were homogenous; mean body weight was 250 ± 30 g. All animals were included in the data analysis.

In the control group we achieved a survival rate of 40%. In the group with antimicrobial protein LL-37 prophylaxis, administrated -12h before PCI survival was increased to 70%. In the group with antimicrobial protein LL-37 administrated three times (at -12h; +12h; +36h in relation to PCI), the survival rate was 67% (figure 4.2.1.). Both groups were not significantly different from the control group p=0.34, df=2 (figure 4.2.2.).

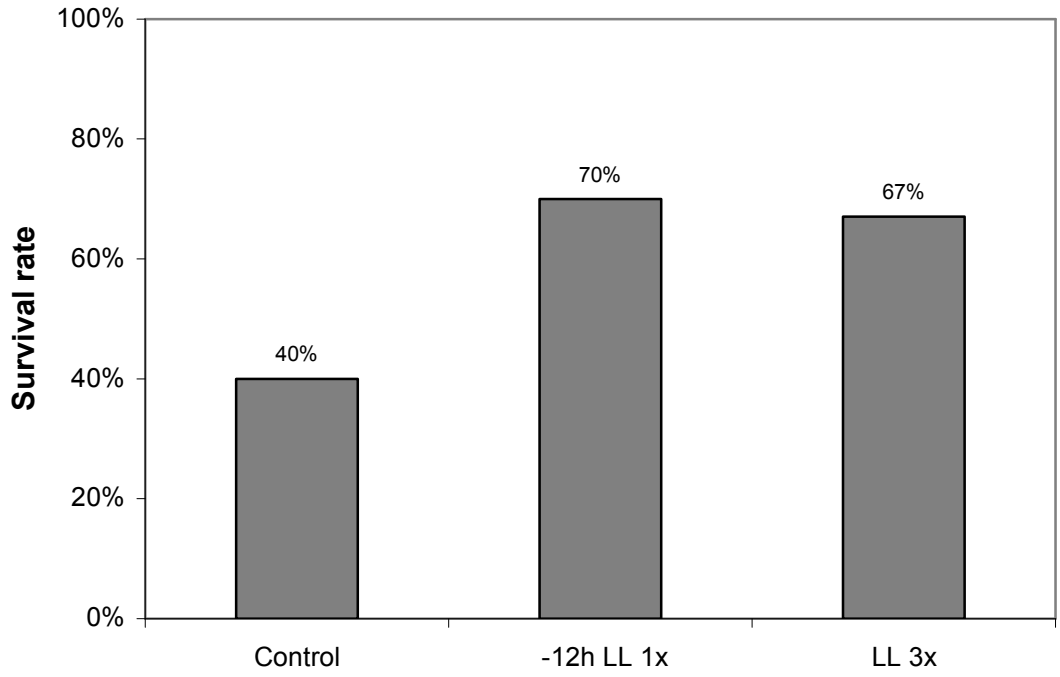


Figure 4.2.1. Survival rate (120h) of rats after: "Control" - PCI without prophylaxis; "-12h LL 1x" - PCI with antimicrobial protein (AMP) LL-37 (0.5 mg/kg) administrated i.v. 12h before contamination; "LL 3x" - PCI with antimicrobial proteins (AMPs) LL-37 administrated i.v. three times (12h before, 12h after and 36h after PCI), (n=10/ group)

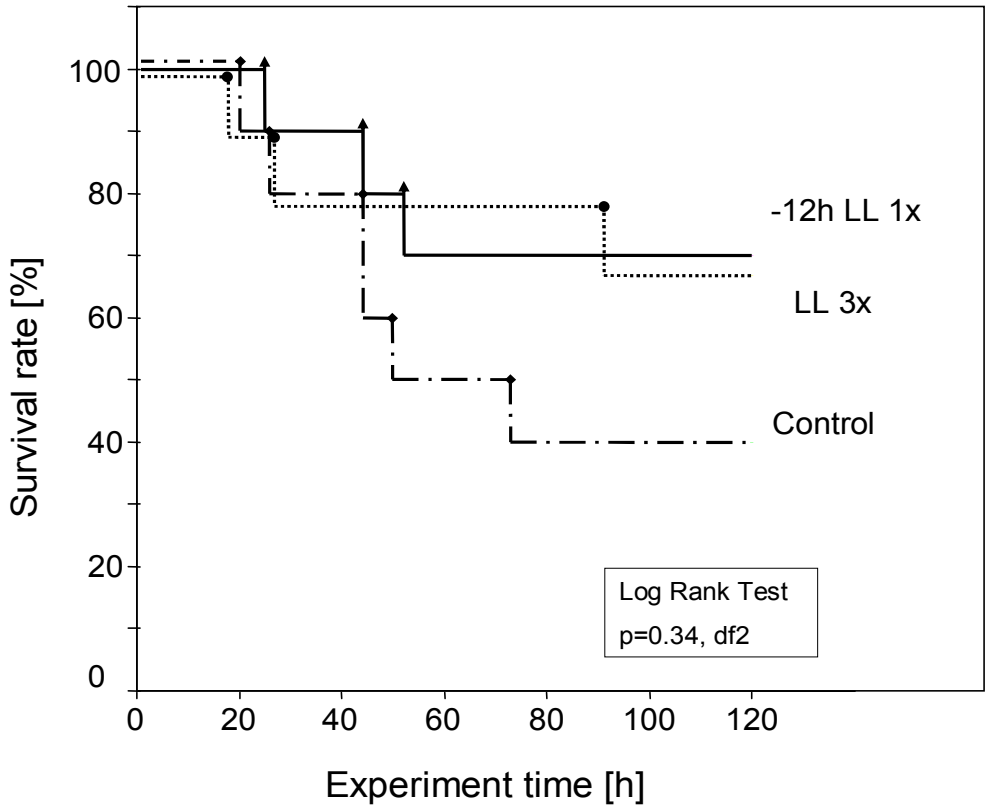


Figure 4.2.2. Kaplan-Meier survival analysis of rats comparing LL-37 prophylaxis 1x versus prophylaxis and treatment 3x with control in septic rats over 120h. Peritoneal contamination and infection (PCI) was performed with standardized human stool.

4.2.2. Prophylactic versus therapeutic administration of LL-37

The groups were homogenous; all animals had a similar body weight of 250 ± 30 g. All animals were included in the data analysis.

We achieved a survival rate of 30% (6/20) in the control group. In the group with antimicrobial proteins (AMPs) LL-37 prophylaxis at 12 hours before contamination, survival was increased up to 55% (11/20). But only PCI with antimicrobial proteins (AMPs) LL-37 administrated i.v. one hour after contamination, (therapeutic administration) was able to improve survival after PCI significantly up to 70% (14/20) (fig. 4.2.3.) (p= 0.0038, df =2; LL-37 therapy versus control in the χ^2 - test p= 0.012, df=1 in Log Rank test) (fig. 4.2.4.)

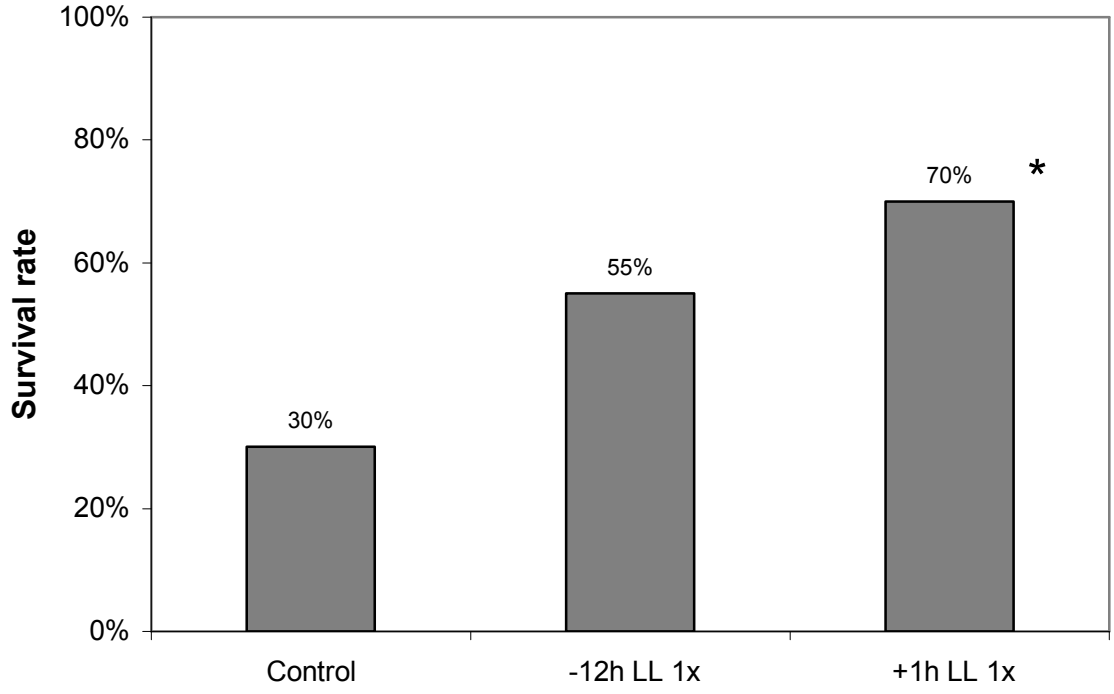


Figure 4.2.3. Survival rate (120h) of rats after: Control - PCI without prophylaxis; "-12h LL 1x" - PCI with antimicrobial proteins (AMPs) LL-37 administrated IV one times 12h before contamination; "+1h LL 1x" - PCI with antimicrobial proteins (AMPs) LL-37 administered IV one times 1h after contamination, (n=20/ group)

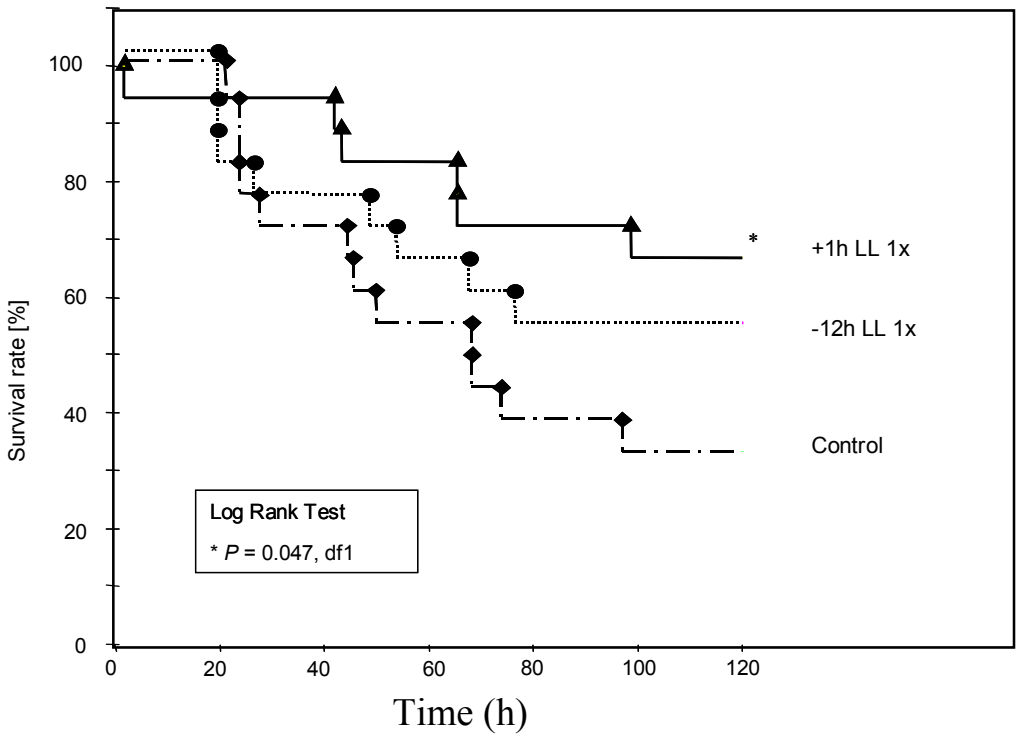


Figure 4.2.4. Kaplan-Meier shows survival analysis of rats comparing LL-37 prophylactic versus therapeutic administration with control group in septic rats over 120h. Peritoneal contamination and infection (PCI) was performed with standardized human stool, AMP was administered in 0.5mg/kg concentration. Statistical analysis reveals a significant difference in the Log Rank test between "+1h LL 1x" and "Control" p=0.012, df1, as well in χ^2 (Chi^2) test p<0.01, df1.

4.2.3. The influence of induced hyperthermia prophylaxis in combination with LL-37 in the PCI model

The groups were homogenous with a similar weight of 250 ± 30 g.

The survival in this experiment was 64% (54/84) at 120 hours (end point of experiment) after peritoneal contamination and infection (PCI). We achieved a survival rate of 38% (8/21) in the control group. Hyperthermia (day before PCI at 41°C during 1 hour) alone improved the survival rate up to 59% (13/22). Approximately the same improvement in the survival rate was obtained with antimicrobial proteins (AMPs) LL-37 administrated IV ones one hour after contamination; survival was slightly increased up to 67% (14/21). Only the combinatory group hyperthermia plus LL-37 was able to improve the survival rate significantly to 90% after PCI (19/21) (figure 4.2.5.). LL-37 therapy combined with hyperthermic preconditioning improved the survival rate versus control: $p = 0.001$, df 1; versus hyperthermia alone $p=0.02$; versus LL-37 therapy $p=0.08$ in the χ^2 (Chi^2) - test. Furthermore the Kaplan-Meier survival analysis revealed significant differences over all groups $p= 0.01$, df=3 in the Log Rank test and a significant difference between control group and hyperthermia in combination with LL-37 administrated IV once. In the Log Rank test 1 hour after contamination we obtained $p < 0.05$, df=1 (figure 4.2.6.)

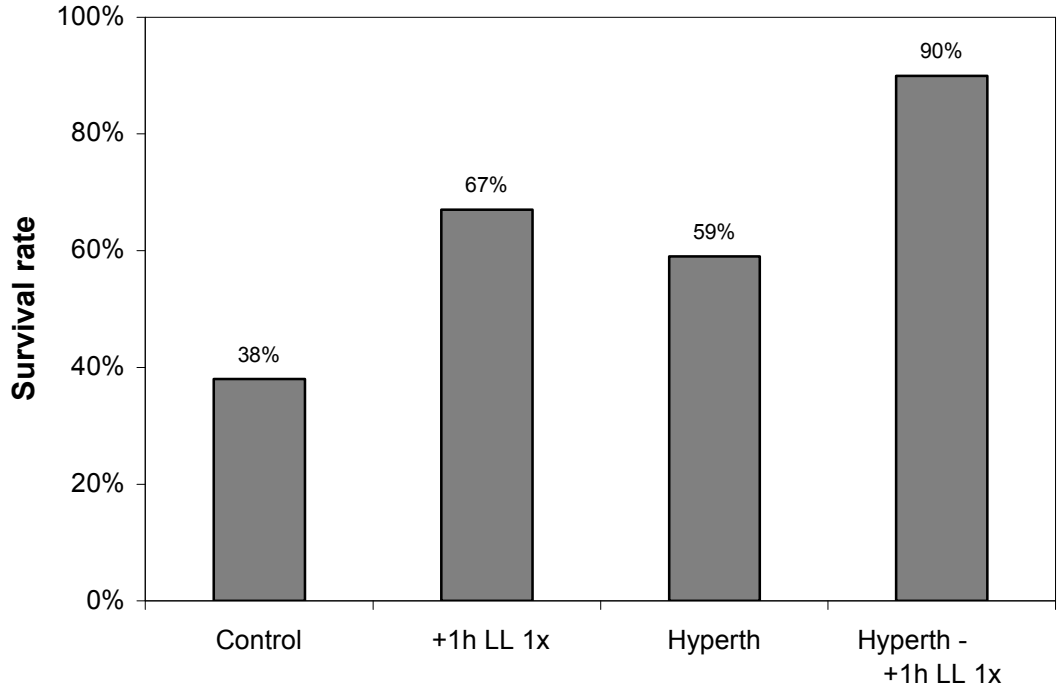


Figure 4.2.5. Survival rate (120h) of rats after: "Control" - PCI without prophylaxis (n=21); "+1h AMP 1x" - PCI with antimicrobial proteins (AMPs) LL-37 administrated IV one times 1h after contamination (n=21); "Hyperth" - induced hyperthermia in rats 24 hours before PCI at 41°C during 1 hour (n=22); "Hyperth - +1h AMP 1x" - induced hyperthermia in rats 24 hours before PCI at 41°C during 1 hour in combination with PCI with antimicrobial proteins (AMPs) LL-37 administrated IV one time 1h after contamination, (n=21/ group)

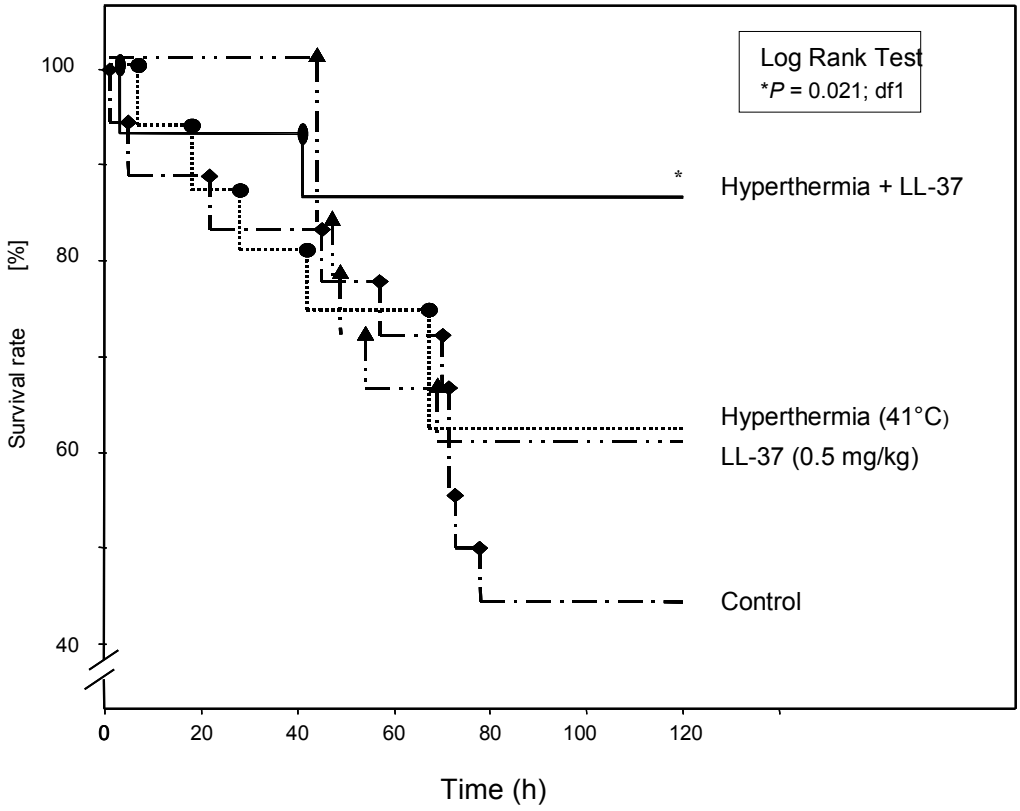


Figure 4.2.6. Kaplan-Meier survival analysis of rats comparing induced Hyperthermia versus AMP once one hour after contamination versus induced Hyperthermia in combination with AMP administration ones one hour after contamination with control in septic rats over 120h. Peritoneal contamination and infection (PCI) was performed with standardized human stool, AMP (LL-37) was administered in 0.5 mg/kg concentration. Induced hyperthermia was performed 24 hours before PCI at 41°C during 1 hour. Statistical analysis reveals a significant difference in the Log Rank test between "Hyperth +1h LL 1x" and "Control" p=0.001, df1, as well in χ^2 (Chi^2) test p<0.01, df1.

4.3. Cytokine expression in the studies

4.3.1. Levels of cytokines after LL-37 administration

In all trials the basal level (before PCI) of the plasma cytokines IL-6, TNF-α and the chemokine MIP-2 were at the detection limit of the assays. One hour after PCI they increased in the plasma of the rats.

The ONEWAY ANOVA test for TNF-α revealed a high significance of $p< 0.001$, df7. Increasing TNF-α levels were expected in the control group after PCI compared to the control group before PCI ($p< 0.05$), df1. Prophylactic administration of LL-37 before PCI did not change the increase of TNF-α after PCI ($p< 0.05$, df1). Changes in other groups were not statistically significant in Post-Hoc tests. (Fig. 4.3.1.)

Data obtained from different groups one hour before PCI were pooled since animals were treated identically. In comparison to TNF-α, MIP-2 showed more statistical significant differences. In the ONEWAY ANOVA test the significance was obtained at a level of $p< 0.001$. A visual comparison of the two cytokines obtained the same tendency of increase after the same intervention. The post-hoc test showed significance between “Control -1h” and “Control +1h” $p<0.05$. Subsequent significant results were obtained with the Post-Hoc test (df =1): “Control -1h” *$p < 0.001$ versus “-12h AMP +1h”; # $p < 0.05$ “Control +1h” versus “+1h AMP 1x +1h”; ^ $p < 0.05$ “-12h AMP -1h” versus “-12h AMP +1h”; *$p <$ 0,001 “+1h AMP 1x -1h” versus “-12h AMP +1h”. (Fig. 4.3.2.)

For IL-6 determination we did not have enough plasma to analyze all experimental groups. In addition, in more than half of the samples in which IL-6 measurement was possible the levels were at the detection limit of the assay. For this reason the IL-6 results are not depicted here.

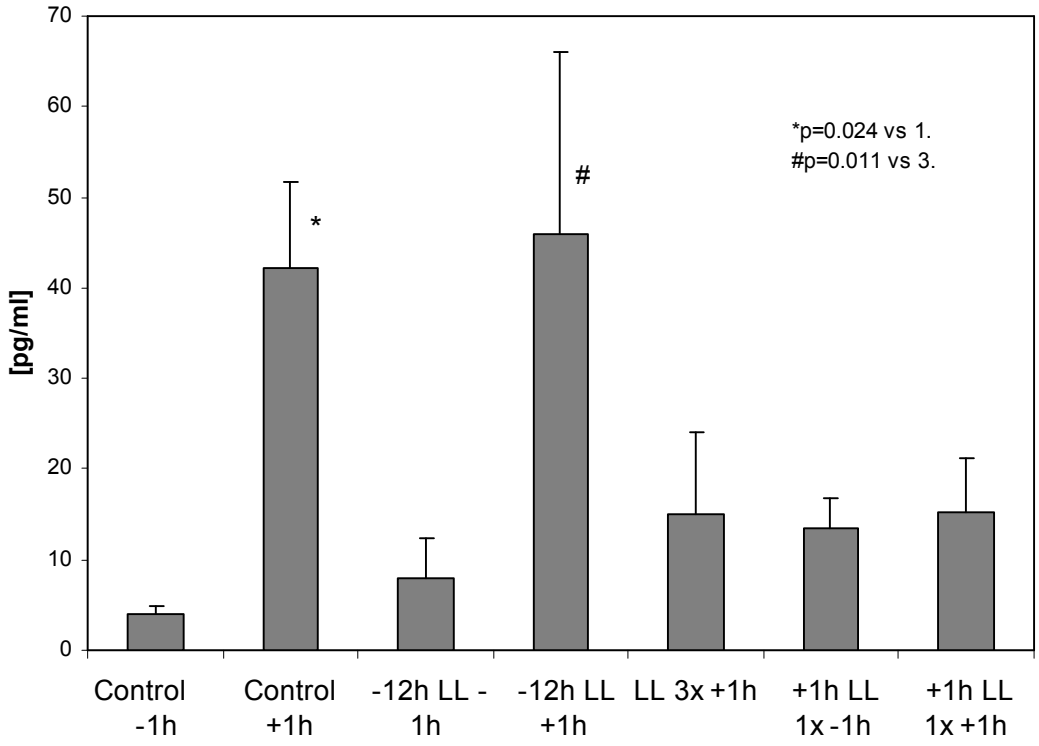


Figure 4.3.1. Value of TNF-α of septic rats with LL-37administration. Peritoneal contamination and infection (PCI) was performed with standardized human stool, LL-37 was administered in a dose of 0.5 mg/kg. Statistical analysis reveals a significant difference in the ONEWAY ANOVA test p=0.001, df7. Post-hoc test: p=0.024 "Control +1h" vs. "Control -1h" and p=0.011 "-12h LL +1h"versus "–12h LL -1h". Data are expressed as means $\pm$ SEM.

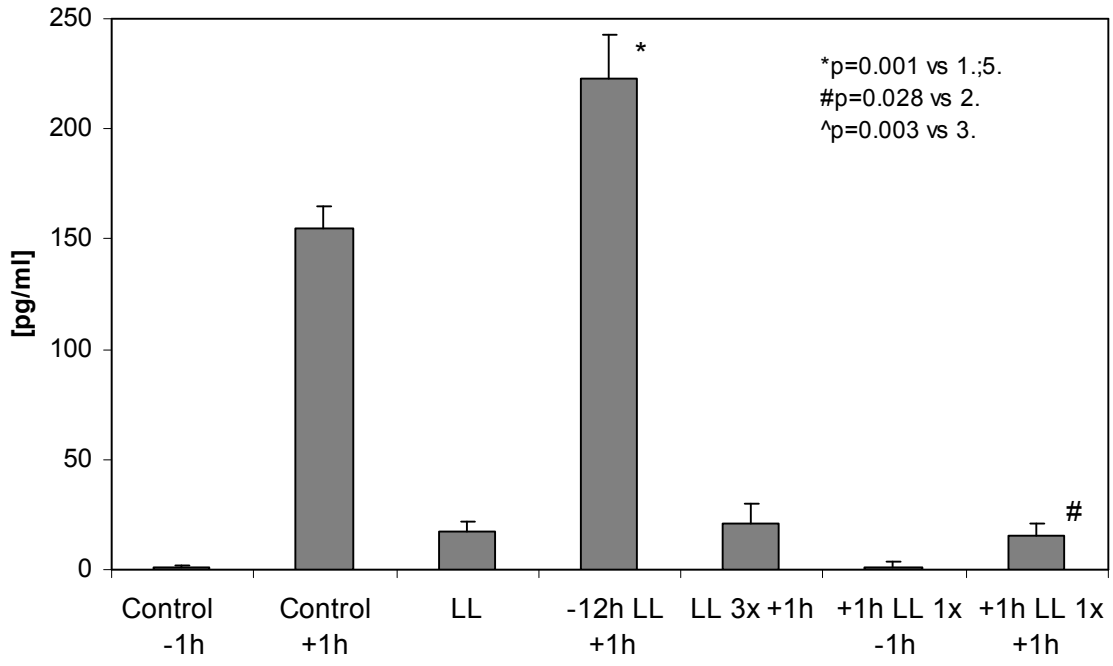


Figure 4.3.2. Value of MIP-2 in AMPs experiments, with control in septic rats over 120h. Peritoneal contamination and infection (PCI) was performed with standardized human stool, AMP was administered in 0.5 mg/kg concentration. Statistical analysis reveals a significant difference in the ONEWAY ANOVA test $p<0.01$. The data are expressed as means $\pm$ SEM.

4.3.2. Cytokines and HSP determination in animals with induced hyperthermia in combination with LL-37 administration

The global statistical test ONEWAY ANOVA demonstrates a moderate significance ($p < 0.05$, df 5). Postoperative levels of TNF-α in the control group were increased but with no statistical significance. We can see a tendency ($p=0.087$, df 1). In other postoperative groups is noted a tendency to diminish the TNF-α plasma levels. The Post-Hoc Boferroni test shows no significance between the groups. The highest postoperative diminishing of TNF-α is noted in the last group "Hyperth +LL 1x +1h". (Figure 4.3.3.)

Preoperative MIP-2 plasma concentration was at the detection limit of the assay. The ONEWAY ANOVA test for MIP-2 was $p < 0.001$, df 5. Subsequent Post-Hoc tests (Boferroni) revealed following results: between "Control -1h" and "Control +1h" $p < 0.001$, df 1; "Control +1h" and "Hyperth +1h" $p < 0.05$, df 1; "Control +1h" and "Hyperth +LL 1x +1h" $p < 0.05$, df 1. In the both hyperthermia groups almost the same postoperative plasma levels were determined, showing a significant reduction of the chemokine MIP-2 in comparison with postoperative levels of the control group. (Figure 4.3.4.)

In comparison with TNF-α and MIP-2, IL-6 has approximately the same levels and showed the same statistical tendency. A performed ONEWAY ANOVA test demonstrates a significance of $p < 0.001$, df 5. And with the Post-Hoc Boferroni test we have obtained $p < 0.005$, df 1 between "Control -1h" and "Control +1h"; and the next statistical significance was $p < 0.005$ in "Hyperth +LL 1x +1h" with "Control +1h". Postoperative plasma levels of IL-6 out of control group had decreasing concentration direction, so in the "Hyperth +LL 1x +1h" the minimal plasma level was depicted. (Figure 4.3.5.)

HSP-70 levels were in the most samples below the detection limit. For this reason they were not depicted here.

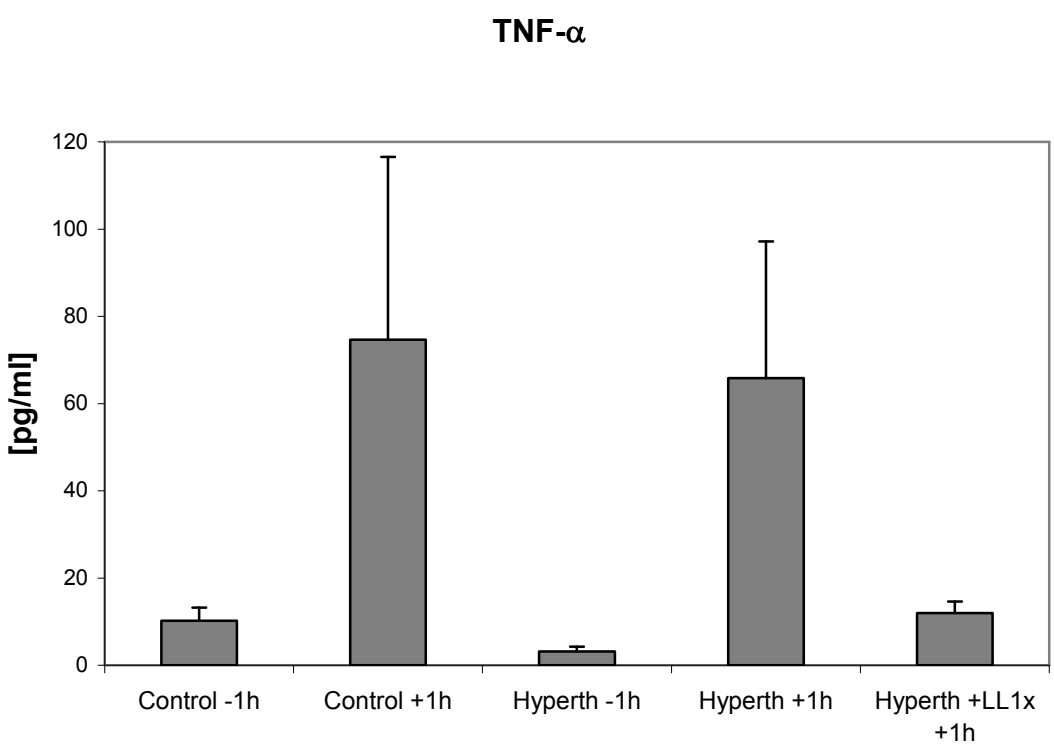


Figure 4.3.3. Value of TNF α in induced hyperthermia in combination with AMPs, with control in septic rats over 120h. Peritoneal contamination and infection (PCI) was performed with standardized human stool, AMP was administered in 0.5 mg/kg concentration. Statistical analysis reveals a significant difference in the ONEWAY ANOVA test $p<0.01$. The data are expressed as means $\pm$ SEM.

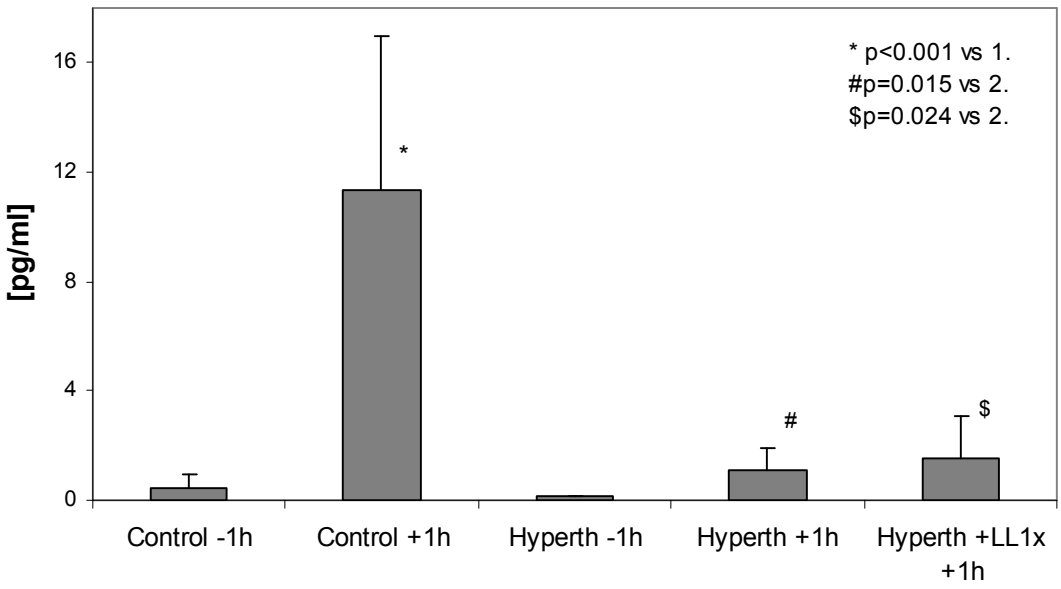


Figure 4.3.4. Value of MIP-2 in induced hyperthermia in combination with AMPs, with control in septic rats over 120h. Peritoneal contamination and infection (PCI) was performed with standardized human stool, AMP was administered in 0,5mg/kg concentration. Statistical analysis reveals a significant difference in the ONEWAY ANOVA test p<0,001. In Post-Hoc Boferroni test "Control -1h" and "Control +1h" p< 0.001, df 1; "Control +1h" and "Hyperth +1h" p < 0.05, df 1; "Control +1h" and "Hyperth +LL 1x +1h" p< 0.05, df 1. The data are expressed as means ± SEM.

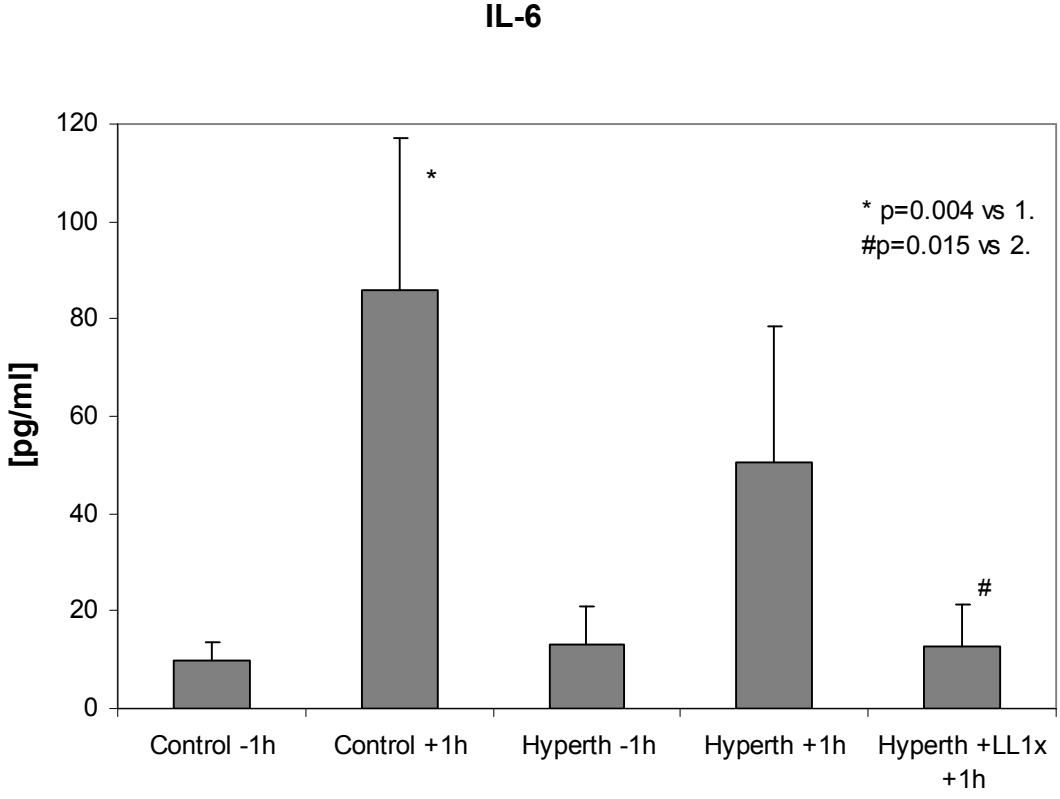


Figure 4.3.5. Value of IL-6 in induced hyperthermia in combination with AMPs, with control in septic rats over 120h. Peritoneal contamination and infection (PCI) was performed with standardized human stool, AMP was administered in 0.5 mg/kg concentration. Statistical analysis reveals a significant difference in the ONEWAY ANOVA test p<0,001. In Post-Hoc Boferroni test p< 0.005, df 1 between "Control -1h" and "Control +1h"; and the next statistical significance was p< 0.005 in "Hyperth +LL 1x +1h" with "Control +1h"The data are expressed as means ± SEM.

5. Discussion

Endotoxemia and shock can occur during antibiotic therapy of sepsis caused by the release of LPS from the outer membrane of destroyed bacteria (Kirikae T. et al., 1997; Shenep J.L. et al., 1985). Once released, the biological activity of LPS is difficult to be neutralized. Thus, a substance that suppresses the actions of LPS could be a reasonable adjunct for preventing/ treating endotoxin shock or sepsis caused by Gram negative bacterial infections. Several substances, including neutralizing antibodies against LPS, LBP (LPS binding protein), or cytokines, have been tested to prevent the cascade of LPS-induced hyper inflammation (Le Roy D. et al., 1999).

However, much attention has recently been focused on low molecular-weight cationic antibacterial peptides that possess both antibacterial and LPS-neutralizing activities. Originally, these peptides were shown to contribute to the host's innate defense system against microbial infections by impairing the membranes of targeted organisms (Gudmundsson G.H. et al., 1999; Hancock R.E. et al., 2000a). Recently, it was reported that a peptide derived from human LL-37, one of the cathelicidin family, could lower serum TNF-α levels and protect mice from lethal endotoxin shock (Kirikae T. et al., 1998). Our results suggest that LL-37 can prevent sepsis, especially in higher doses. Even in lower doses, overall assessment scores improved. On the other hand, higher doses for treating sepsis appeared to have adverse effects and appeared to be toxic to organs (especially cytotoxic) affected by sepsis (Fukumoto K. et al., 2005). Therefore, lower doses of LL-37 can be used to treat sepsis, but higher doses should not be used. This paradox may arise because LL-37 seems to be able to damage the cells of a septic organ directly. At lower doses, its inhibition of LPS-induced TNF-α expression is stronger than any direct damage it causes, but at higher doses the inhibitory action is overridden by the direct damage activity. This is only a hypothesis at present and needs further study to elucidate the exact mechanism involved. From our study, we conclude that the safest use of LL-37 for both prevention and treatment is at lower doses. A dose of 1 mg/ml LL-37 seems to be

already too high, since with this dose a higher mortality was obtained compared to a dose of 0.2 mg/ml. Rats given the highest doses actually died before the rats with the lowest doses did, so higher-dose of LL-37 would appear to be toxic through a mechanism as postulated by Ciornei et al. (Ciornei C.D. et al., 2005; Ciornei C.D. et al., 2006). Further study to determine the safest dose is required before LL-37 can be used clinically (Fukumoto K. et al., 2005).

5.1. Implication of LL-37 in infectious disease - sepsis

Antimicrobial peptides play an important role in innate immunity by acting as effector molecules in host defenses against pathogens. The dominating targets are bacterial membranes, and the killing reaction must be faster than the growth rate of the bacteria. Antimicrobial peptides (AMPs) have emerged as multifunctional effector substances of the innate immune system.

The role of antimicrobial peptides as effectors of innate immunity is widening from solely endogenous antibiotics to multifunctional mediators that provide a first line of host defense, modify the local and global inflammatory response, and activate some immunological mechanisms (Boman H.G. 2003; Hancock R.E. et al., 2000b; Zanetti M. 2004). Previously, researches showed that LL-37 is a potent immune modulator and that it stimulates the expression of a wide variety of genes involved in the innate immunity. It has been demonstrated to be a chemoattractant for human monocytes, T cells, and mast cells. Furthermore, LL-37 is a potent anti-endotoxic agent and induces chemokine production. It also has a variety of other functions, including promotion of histamine release from mast cells, inhibition of tissue proteases, stimulation of wound healing and angiogenesis (Bowdish D.M. et al., 2004)

AMPs are part of the innate immune system of many species and are thought to provide protection against bacteria, fungi and viruses, either by directly killing or binding to bacterial endotoxin and blunting the biological effects of infection (Lehrer R.I. et al., 1999). It has been shown previously that systemic administration of peptide derivatives of LL-37/hCAP-18 blunts the clinical consequences of septic shock in animal models. These effects are mediated by binding of the cationic peptide antibiotics to endotoxin, neutralizing LPS and decreasing the release of TNF-α (Kirikae T. et al., 1998). Evidence that mammalian antimicrobial peptides actually contribute to innate immunity in vivo is based primarily on their expression patterns and in

vitro activity against microorganisms. In vitro, naturally occurring cationic peptides, as well as synthetic analogues block the ability of LPS to stimulate the production of TNF-α, IL-6, and other inflammatory mediators. Moreover, studies in mice have shown that cationic peptides can block endotoxin-induced TNF-α release and reduce the mortality associated with endotoxemia (Kirikae T. et al., 1998; Scott M.G. et al., 1999; VanderMeer T.J. et al., 1995). However, several cationic peptides have been shown to bind LPS (Scott M.G. et al., 2000).

Although cationic peptides often have antimicrobial activity, the key to their therapeutic potential may lie among the other activities attributed to them, such as their ability to alter the inflammatory response (Scott M.G. et al., 2000a). For example, under conditions analogous to those found in vivo, LL-37 is a weak antimicrobial agent. Conversely, it is one of the most potent anti-endotoxic agents among cationic host defense peptides, is directly chemotactic, and induces dramatic changes in the phenotype of monocyte-derived dendritic cells. This indicates a substantial role of cationic peptides in the innate immune response (Scott M.G. et al., 2002).

In a study by De Yang et al. (De Y. et al., 2000), LL-37 was demonstrated to be a chemoattractant for monocytes, neutrophils, and T cells.

In the light of these observations, we investigated the therapeutic potential in our rat PCI model, applying LL-37 once versus three times iv and in a prophylactic versus therapeutic approach. In the PCI model septic shock was induced by intraperitoneal administration of a standardized stool suspension (MIX 5). The PCI model combined with the CMRT concept represents a clinic relevant experimental setting superior to other more simple models like models with single strain bacteria or endotoxemia. In our trials the clinical situation and conditions of randomized controlled trials (for e.g. randomization, blinding, adequate statistics) are modeled (Bauhofer A. et al., 2004; Lorenz W. et al., 1994; Torossian A. et al., 2003). The antimicrobial and endotoxin-neutralizing effects of LL-37 were used in combination with clinically common iv antibiotics.

Our findings clearly show that LL-37 reduces the PCI mediated adverse effects, by down regulation of the pro-inflammatory cytokines response. The pro-inflammatory reaction in the PCI model is most probably induced by microbial compounds like LPS and lipoteichoic acid. In all of the APM studies LL-37 caused inhibition of TNF-α, IL-1 and MIP-2 production in blood. Importantly, intravenous LL-37 produced a significant reduction in TNF-α plasma levels, compared to the control group treated just with standard antibiotics as described before. Antibiotic therapy and LL-37 reduced the plasma levels of LPS to a similar extent and showed similar abilities in blocking TNF-α levels in animals. It is important to notice that prophylactic administration of LL-37 was not as effective with regard to improved survival rate as was therapeutic administration (1x, one hour after contamination). Two key determinants of sepsis survival are bacterial clearance and the inflammatory response to the infection. Inflammatory response to the infection was reduced as demonstrated by pro-inflammatory cytokines. Finally, comparable data were observed when the drugs were administered at 60 minutes after PCI.

The protective effect of LL-37 on animal survival is likely due to both, the ability of this peptide to decrease the levels of endotoxin and pro-inflammatory cytokines in the plasma of septic animals and its bactericidal activity. Additional mechanisms by which LL-37 contributes to host defenses against microbial invasion have been previously described.

We found in our trial that LL-37 prophylaxis 12 hours before infection is not effective, but LL-37 therapy improves survival after sepsis significantly. This is in accordance with the results of a recent study, which demonstrated improved survival after CLP-induced sepsis with LL-37 treatment being superior to conventional antibiotic treatment (Cirioni O. et al., 2006). However, our results extend these findings, in that we routinely use antibiotics in our intra-abdominal sepsis model. This suggests additive or even synergistic effects of antibiotics (cefuroxime/metronidazole) in combination with LL-37. Though the mechanism of interaction needs further investigation. Antibiotics are routinely given for sepsis prophylaxis in clinical

practice and may interact differentially with any other mediator such as cytokines (Bauhofer A. et al., 2004)

The data presented in our study support the notion that prophylactically or therapeutically administration of LL-37 in our PCI model provides protection against bacterial pathogens, such as against peritoneal infection and contamination (PCI). Bals et al. showed that this supports the suggested role of LL-37/hCAP-18 as a host defense molecule activating phagocytes in epithelia and the blood. (Bals R. et al., 1999b)

Thus, it is becoming increasingly clear that peptides are more than simply naturally occurring antibiotics, but, rather, play a complex role in resolving infection, attenuating inflammation, and, when this attempt at resolution is not sufficient, alerting the adaptive immune response (Bowdish D.M. et al., 2004). Characterization of the mechanism of action of these peptides is shedding light on the properties that make them potentially therapeutic agents.

It is worth considering what would permit one to conclusively test the alternative hypotheses whether, e.g., LL-37 is acting as an immune modulator or an antimicrobial in vivo. It was shown that a derivative of LL-37, instilled simultaneously with *P. aeruginosa* into the mouse lung, reduced lung damage and pro-inflammatory cytokine production but not bacterial counts (Scott M.G. et al., 1999a). In an analogous manner, it is well-established that LL-37 treatment can reverse endotoxemia, even in the absence of infection. Therefore, we consider that the general thesis that LL-37 can act as an immunomodulating factor is well established. What is not clear yet is which function of LL-37 dominates in clearing an infection. In conclusion, our study data demonstrate the potential for the therapeutic use of immuno-modulatory, anti-infective peptides, which do not eliminate bacteria directly and therefore, circumvent the issue of antimicrobial resistance (Bowdish D.M. et al., 2005). These agents should find broad use in a variety of modes of administration and treatment regimes and against a range of pathogens.

Conclusion: LL-37 is a multifunctional peptide displaying both antimicrobial activity and a variety of immunological functions related to inflammation, suggesting that LL-37 may regulate both infection and inflammation. Therefore, LL-37 may form a template for the development of new anti-infectious agents. Development of LL-37-based therapies should however, consider the balance between antimicrobial and inflammatory activities. Whether such peptides enhance wound repair, and thus may act as dual agents, is of great importance, but remains to be determined.

The data presented in this study provide evidence that antimicrobial peptides protect against the consequences of bacterial infection (PCI - sepsis), highlighting the role of these substances as part of the innate host defense system. The protective effect resulting from LL-37 immunity modulation suggests new strategies for developing treatment of infections and favorably modifying the host response.

Thus, LL-37 could be an attractive candidate as a therapeutic agent that can be used for endotoxin shock and sepsis caused by gram-negative and gram-positive bacterial infections.

5.2. Hyperthermia – a beneficial factor in sepsis?

Animal and human studies have shown that intraoperative hypothermia increases the risk of wound infection (Kurz A. et al., 1996; Sheffield C.W. et al., 1994). Intraoperative hypothermia is likely to cause a reduction in peripheral circulation, which may increase tissue hypoxia and thus makes the wound more susceptible to infection, even if contamination levels are low. The process of warming using a warm air blanket, to prevent hypothermia, is becoming common practice for most major surgical procedures.

The exogenous application of heat stress triggers a natural cellular defense mechanism called the stress response. In previous studies (Ribeiro S.P. et al., 1994; Villar J. et al., 1993; Villar J. et al., 1994), it has been demonstrated that the stress response is protective when induced 18 hours before intratracheal instillation of phospholipase A_2 and before the production of experimental sepsis by caecal ligation and perforation (Chu E.K. et al., 1997).

The evolutionarily conserved febrile response during infection has been associated with improved survival in vertebrates and invertebrates although its protective mechanism of action and especially that of the thermal component is one of the most poorly understood aspects of inflammation - much of the hyperthermia effects on the immune system have been attributed to the expression of heat shock proteins in several organs, as shown for example after the hyperthermia treatment of mice (39-40°C over 6 hours) (Ostberg J.R. et al., 2005). In our setting 24 hours after hyperthermia pretreatment (41°C) we could not detect increased HSP levels. Probably the duration of heat application of only one hour was not long enough, but the current results demonstrate that preconditioning hyperthermia was associated with increased survival after experimental sepsis (PCI). We did not measure or control glutamine levels in our animals; however it has been shown recently that glutamine-starving monocytes have a reduced thermo-resistance. This is associated with an inhibition of the cytoprotective protein HSP70 in vitro (Pollheimer J. et al., 2005) - temperatures within the range of

physiologic fever influence production or bioactivity of pro-inflammatory cytokines (Blake D. et al., 1994) In addition, apoptosis induced by sepsis-is attenuated (Chen H.W. et al., 2000); - lipopolysaccharide induced hyporesponsiveness of platelets is reduced (Dong H.P. et al., 2005). Fever-range thermal stress promotes lymphocyte-endothelial adhesion, which also suppresses tumor progression (Chen Q. et al., 2006). Metabolic amelioration of lactic acidemia in a rat model of sepsis, possibly preserves organ perfusion, improves oxygen delivery and prevents cellular dysfunction (Deshpande G.G. et al., 2000). Accumulating evidence indicates that hyperthermia specifically enhances polymorphonuclear neutrophils (PMNs). PMNs are recognized as key mediators of inflammation, and may also play a crucial role in mediating the anti-tumor effects of a mild, fever-range whole body hyperthermia (Ostberg J.R. et al., 2005). Thus, therapeutic use of mild (fever-range) whole body hyperthermia (39-40°C) was first reported to be beneficial in a phase I study for patients with advanced solid tumors (Kraybill W.G. et al., 2002). However, although there is strong evidence in animal models of infection supporting therapeutic use of hyperthermia, only a few prospective, randomized trials have been reported in humans. These basically focus on metastases tumor therapy, and the results remain equivocal, with mild to moderate hyperthermia as an adjunctive to radio-/chemotherapy providing substantial benefit, for example in a phase II study in 41 patients with metastatic colon cancer (Hegewisch-Becker S. et al., 2002) and in 16 patients with advanced gastric carcinoma (De Roover A. et al., 2006). However, major clinical trials of therapeutic hyperthermia or hyperthermic preconditioning in a curative approach are lacking to date.

Interestingly, timing of hyperthermia treatment in relation to an infectious insult seems to be a crucial factor: like others before (Gulluoglu B.M. et al., 2002; Hotchkiss R. et al., 1993; Koh Y. et al., 1999; Villar J. et al., 1994), we found an improved (though not significant) survival rate 120 hours after intra-abdominal sepsis in animals with hyperthermic preconditioning (41°C for 1 h) which *per se* could not increase the outcome after sepsis significantly (P = 0.1). So far, timing of hyperthermia may also be of importance, since in one

recent report hyperthermia treatment after sepsis was found to be unfavorable (L'Her E. et al., 2006).

Whether the observed benefits on survival duration are due to potential impacts for energy metabolism or to an anti-inflammatory effect of hyperthermia requires further investigation.

Besides, preoperative warming may be an alternative to the controversial use of prophylactic antibiotics, which avoids the associated risks of allergy and resistance, in clean surgery (Gulluoglu B.M. et al., 2002).

5.3. Additive effects of the antimicrobial peptide LL-37 and hyperthermia in sepsis

In our main trial, neither hyperthermia preconditioning nor LL-37 per se could increase the survival rate after sepsis significant compared to control, but the combination of both modalities revealed additive effects resulting in an improved outcome. The combination was effective as compared with controls (P = 0.001) and with hyperthermic preconditioning alone (P = 0.02), suggesting additive effects. The markedly reduced pro-inflammatory cytokines may have contributed to this. Reduced pro-inflammatory cytokine levels after LL-37 treatment and sepsis have also been reported by others (Cirioni O. et al., 2006a). Hence both modalities may stimulate cellular immune defense mechanisms and alter the cytokine network in our combined trial. LL-37 amplified the beneficial effect of hyperthermic preconditioning on survival and reduced the excessive pro-inflammatory cytokine production, but not beyond that of septic control rats. This is important, since a reduction below the levels in the control animals would be adverse for infection clearance. An adequate immune response is needed. Hyperthermia per se is known to alter innate and adaptive immunity; therefore it was unreasonable to examine a hyperthermic preconditioning only group.

A potential explanation for the increased survival after hyperthermia plus LL-37 treatment in sepsis is a reduction of the excessive release of the pro-inflammatory cytokine IL-6 and the chemokine MIP-2. MIP-2 enhances PMN recruitment and migration into infected tissues. In this light we can affirm enhanced neutrophil function through hyperthermic preconditioning. Our model is clinically relevant for studying development of postoperative intra-abdominal sepsis and accompanying prophylaxis and treatments. Thus current and previous work indicates that hyperthermic preconditioning may improve host immune response to a subsequent infectious challenge. Furthermore, our results suggest, that administration of LL-37 or its active

motifs, may improve host response to bacterial peritonitis in such patients, however more data on the mechanisms and possible side-effects are needed before it may enter a clinical trial in septic patients. These results of this work are published in Anesthesiology. (Torossian A. et al., 2007).

Conclusion: Our model is clinically relevant for studying the development of postoperative intraabdominal sepsis and accompanying risk factors. This work indicates that postoperative hyperthermia enhances the host immune response to an anti-infectious challenge. Our results suggest that the administration of LL-37 in combination with hyperthermia preconditioning (heat shock or prewarming) may improve the host response to bacterial peritonitis in patients at risk.

6. Summary

Background: We studied the effects of LL-37 prophylaxis or therapy on the outcome after intra-abdominal sepsis and tested whether additional preconditioning with fever-range hyperthermia augments host immune response and improves survival.

Methods: A rat model of peritoneal contamination and infection (PCI) with human stool bacteria was used to simulate clinical trial conditions. In *Trial 1* we compared 1) PCI only; 2) LL-37 prophylaxis (0.5 mg/kg, 12 h before infection) and 3) LL-37 therapy 3 time administration (0.5 mg/kg, -12h before PCI and +12h, +36h after infection), 20 rats/group. In *Trial 2* we compared 1) PCI only; 2) LL-37 prophylaxis (0.5 mg/kg, 12 h before infection) and 3) LL-37 therapy (0.5 mg/kg, 1h after infection), 20 rats/group. In *Trial 3*, using 22 rats/group, we compared 1) PCI only; 2) LL-37 therapy; 3) hyperthermic preconditioning (41°C for 1h, 24 h before infection); and 4) hyperthermia combined with LL-37 therapy (0.5 mg/kg). The primary endpoint was mortality at 120 h. Secondary endpoints were systemic pro-inflammatory cytokine (TNF-α, IL-6, MIP-2) and heat shock protein (HSP)-70 levels.

Results: In Trial 1 we achieved a survival rate of 40% in the control group. In the group with the antimicrobial protein LL-37 administrated once at -12h, before inoculum survival was increased to 70% and to 67% in the group with LL-37 administrated three times (at time point correspondingly to -12h; +12h; +36h in relation to PCI. In both groups survival rates were not significant different to the control. In Trial 2, 30% of the control group compared to 70% of the LL-37 therapy group survived (P=0.038). After LL-37 prophylaxis survival was 55%. Survival rate in trial 3 was 38% in the control group, 67% in the LL-37 therapy group, 59% in the hyperthermia preconditioning group and 90% in the combination group (hyperthermia preconditioned plus LL-37 therapy) (P = 0.01). Hyperthermic preconditioning

plus LL-37 reduced pro-inflammatory cytokine concentrations after sepsis. Compared to controls MIP-2 levels were 1.5 ± 1.5 pg/ml versus 11 ± 6 pg/ml, (P = 0.028) and IL-6 levels were respectively 13 ± 8 pg/ml versus 86 ± 31 pg/ml, (P = 0.015).

Conclusions: In this rat model of intra-abdominal sepsis, LL-37 therapy given alone was more effective in comparison to LL-37 given three times. The later was not successful to improve the outcome significantly. The combination preconditioning and LL-37 therapy was most effective. Additional hyperthermia, initiated 24 hours before contamination, reduced mortality and downgraded the systemic pro-inflammatory cytokine response.

Zusammenfassung

Antimikrobielle Peptide (AMP) sind Bestandteil des angeborenen Immunsystems. Wir haben die Wirkung des antimikrobiellen Peptids – LL-37 als Prophylaxe oder Therapie in Kombination mit hyperthermer Präkonditionierung in septischen Ratten erforscht. Wir untersuchten wieweit die oben beschriebenen Behandlungen die Mortalität septischer Ratten senkt und die Freisetzung pro-inflammatorischer Zytokine reduziert.

Methodik: Wir verwendeten für die Studien das CMRT Konzept (clinic modelling randomised trials) in Ratten, um die komplexe klinische Situation und die Bedingen von klinischen Studien zu simulieren. In dem von uns verwendeten Peritonitismodell wurden die Tiere in Fentanyl/ DHB-Analgesie, nach i.v. Antibiotikaprophylaxe laparotomiert und mit einer standardisierten humanen Stuhlsuspension peritoneal kontaminiert und infiziert (PCI). In Studie I erhielten die Tiere 1) PCI (Kontrolle); 2) eine Prophylaxe mit 0,5 mg/kg von dem antimikrobiellen Peptid LL-37 (12 h vor PCI), 3) 3x LL-37 (12 h vor -; 12h nach- und 36 h nach PCI); 20 Ratten/Gruppe. In Studie II erhielten sie 1) eine PCI (Kontrolle); 2) eine Prophylaxe mit 0,5 mg/kg LL-37 (12 h vor PCI), 3) eine Therapie mit 0,5 mg/kg LL-37 (eine Stunde nach PCI); 20 Ratten/Gruppe. In Studie III erhielten die Tiere 1) eine PCI (Kontrolle); 2) eine Therapie mit 0,5 mg/kg LL-37 (eine Stunde nach PCI); 3) eine hypertherme Präkonditionierung (41°C für 1h, mit 24 h vor PCI) und 4) Therapie mit 0,5 mg/kg LL-37 (eine Stunde nach PCI) in Kombination mit hyperthermische Präkonditionierung (41°C für 1h, mit 24 h vor PCI), 22 Ratten/Gruppe. Der primäre Endpunkt war in allen Studien die 120-Stunden-Überlebensrate; in Studie II und III wurden als sekundäre Endpunkte die Plasmaspiegel von pro-inflammatorischen Zytokinen (TNF-α. IL-6 und MIP 2) und von HSP70 (heat shock protein) bestimmt.

Ergebnisse: In Studie I überlebten in der Kontrollgruppe 40%, mit LL-37 Prophylaxe (-12h LL vor PCI) 70% und nach LL-37 3x (LL 3x) 67%. In Studie

II betrug die Überlebensrate in der Kontrollgruppe 30%, nach LL-37 Prophylaxe (-12h AMP 1x) 55%, und nach LL-37 Therapie (+1h AMP 1x) 70% (p=0,038, vs. Kontrolle). In Studie III betrug die Überlebensrate in der Kontrollgruppe 38%, nach LL-37 Therapie (+1h AMP 1x) 67%, nach hyperthermische Präkonditionierung (41°C für 1h, mit 24 h vor PCI) 59%. Nur durch Kombination der LL-37 Therapie (0,5 mg/kg LL-37 eine Stunde nach PCI) mit der hyperthermischen Präkonditionierung (41°C für 1h, 24 h vor PCI) konnte die Überlebensrate signifikant (*P* = 0,01) auf 90% erhöht werden. Die untersuchten Zytokine konnten durch die LL-37 Therapie und am deutlichsten durch Kombination von LL-37 mit der hypethermen Präkonditionierung gesenkt werden. Der statistische Vergleich zwischen der Kontrollgruppe und der Kombinationsbehandlung ergab einen signifikanten Unterschied für MIP-2 mit *P* = 0,028 und für IL-6 mit *P* = 0,015.

Schlussfolgerung: Die besten Ergebnisse in Form einer gesteigerten Überlebensrate erbrachte in unserem Sepsismodell eine antimikrobielle Therapie mit LL-37 in Kombination mit einer hyperthermen Präkonditionierung. Eine LL-37 Prophylaxe, eine 3-malige Gabe von LL-37 (vor und nach Sepsisinduktion) oder eine alleinige hypertherme Präkonditionierung konnte nicht im gleichen Umfang das Outcome verbessern. Ein möglicher Erklärungsgrund für die positive Wirkung der Kombination von LL-37 und Hyperthermie könnte in der Erniedrigung von pro-inflammatorischen Zytokinen wie dem IL-6, sowie von Chemokinen wie dem MIP-2 liegen.

Rezumat (romanian)

A fost studiat efectul administrării adiționale a LL-37 la terapia antimicrobiana uzuală (cefuroxime şi metronidazole) în infecția intraperitoneală, provocată prin inocularea de masă fecală standardizată la şobolani Wistar (masculi). Proteina antimicrobiană din grupa Cathelicidinelor a fost administrată intravenos cu scop de profilaxie şi terapie. În studiu au fost incluse şi grupe de animale la care s-a utilizat precondiționarea termică (supunerea în prealabil la hipertermie). Scopul studiului a constat în evaluarea influenței terapiei adiționale cu proteina antimicrobiană LL-37 asupra ratei de supraviețuire a animalelor, timp de 120 ore după provocarea infecției intraperitoneale şi monitorizarea răspunsului imun al organismului.

Metode. Pentru simularea condițiilor clinice a fost utilizat modelul deja cunoscut ca CMRT sau şobolan infectat şi contaminat intraperitoneal (ICI) cu mase fecale umane preventiv standardizate. Primul şir de experimente a inclus trei grupe a câte 20 animale fiecare. La prima grupă (de control) s-a efectuat tratament uzual după (ICI). La animalele incluse în grupa a doua li s-a administrat cu scop profilactic proteina antimicrobiană LL-37 (0,5 mg/kg) cu 12 ore înainte de infectare, iar animalelor din grupa a treia proteina LL-37 (0,5mg/kg) li s-a administrat atât profilactic cu 12 ore înainte de ICI cât şi terapeutic, peste 12 şi 36 ore.

În al doilea şir de experimente au fost incluse trei grupe omogene a câte 20 animale fiecare. Prima grupă (de control) a primit tratament uzual după ICI. Animalelor din grupa a doua li s-a administrat cu scop profilactic LL-37 (0,5 mg/kg) cu 12 ore înainte de infectare. Animalele din grupa a treia au primit tratament adițional cu LL-37 (0,5 mg/kg) la interval de o ora după infectare.

În şirul al treilea de experimente au fost incluse patru grupe omogene a câte 21 animale fiecare. Grupa întâia a fost ca de obicei cea de control. Animalele din grupa a doua au primit tratament adițional cu LL-37 (0,5 mg/kg) peste o ora după infectare. Animalele din grupa a treia au fost supuse precondiționării termice (hipertermie T=+41°C, timp de o oră) cu 24 ore înainte de ICI. Animalele din grupa a patra au fost supuse unui tratament combinat, care a inclus precondiționarea termica (similar grupei a treia) şi administrare terapeutică adițională a LL-37 (0,5 mg/kg) la o oră după infectare.

A fost evaluată supraviețuirea animalelor timp de 120 ore de la ICI.

Pentru evaluarea răspunsului organismului la infecție şi efectul tratamentului aplicat, a fost determinată evoluția concentrației citokinelor proinflamatorii (TNF-α, IL-6), chemokinei MIP-2 şi nivelul HSP-70 (heat shock protein) în sângele animalelor.

Rezultate. În primul şir de experimente în grupul de control s-a observat o rată de supraviețuire a animalelor de 40%, în grupa a doua rata de supraviețuire a fost 70%, iar în grupa a treia – 67%. Indicii obținuți în grupele doi şi trei nu sunt statistic semnificativi comparativ cu grupa de control. În şirul doi de experimente s-a observat o rata variabilă de supraviețuire de 30% - în grupa de control, 55% - în grupa a doua cu administrare profilactică a LL-37 şi 70% - în grupa a treia cu administrarea terapeutica a LL-37. Rezultatul obținut în grupa a treia este relevant şi statistic semnificativ (P=0,038) în comparație cu grupa de control.

Şi în şirul al treilea de experimente rata supraviețuirii animalelor a fost diferită în dependență de metoda de terapie adițională utilizată. În grupa de control au supraviețuit 38% animale, în grupa cu administrare terapeutică a LL-37 au supraviețuit 67% animale, în grupa a treia unde s-a folosit precondiționarea termică a animalelor au supraviețuit 59% , iar cel mai surprinzător rezultat a fost obținut în grupa a patra unde a fost aplicată terapia suplimentară combinată, unde au supraviețuit 90% animale, rezultat cu o veridicitate statistică înaltă de 95% sau P=0,01. Aproape toate metodele utilizate în tratamentul sepsisului au contribuit nesemnificativ la diminuarea concentrației citokinelor proinflamatorii. Precondiționarea hipertermică şi administrarea terapeutică a LL-37 a contribuit la diminuarea semnificativă a concentrației citokinelor proinflamatorii după declanşarea sepsisului. Astfel concentrația citokinelor animalelor aflate în stare septică a fost: MIP-2 11±6 pg/ml VS 1,5 ±1,5 pg/ml (cu semnificația statistică P=0,028); IL-6 86±31 pg/ml VS 13±8 pg/ml (cu semnificația statistică P=0,015), unde prima valoare corespunde grupei de control, iar a doua valoare grupei cu tratament adițional combinat.

Concluzii. În această modelare a sepsisului intra-abdominal supraviețuirea animalelor a fost mai înaltă în grupele cu administrare terapeutică a LL-37 într-o singură priză, şi mai scăzută în grupa cu administrare a LL-37 în trei prize. Dar cel mai impresionant rezultat de supraviețuire maximală a fost obținut în grupa unde s-a utilizat tratamentul adițional combinat care include precondiționarea hipertermică şi administrarea terapeutică a LL-37 într-o singură priză. Tratamentul adițional combinat a contribuit la reducerea semnificativă a răspunsului proinflamator al citokinelor, micşorând esențial concentrația lor plasmatică, în aşa mod, contribuind la o scădere importantă a ratei mortalității. Administrarea profilactică a LL-37 nu a adus careva rezultate importante.

7. References

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8. Annex

8.1. List of abbreviation

ACCP	The American College of chest Physicians
ARDS	adult respiratory distress syndrome
CARS	compensatory anti-inflammatory response syndrome
CD14	cluster of differentiation group of cell surface marker protein; a membrane-associated glycosylphosphatidylinositol-linked protein expressed at the surface of cells, especially macrophages
CMRT	clinic modeling randomized trials
CO2	bicarbonate
CRAMP	cathelin related antimicrobial peptide
CRP	C-reactive protein
DNA	Deoxyribonucleic acid
G-CSF	granulocyte colony-stimulating factor
hCAP-18	human cationic antimicrobial peptide of 18 kDa
HLA-DR	human leukocyte antigen-DR
HSP70	70-kDa heat shock protein
HSP	heat-shock protein
HSR	heat shock response
ICU	Intensive Care Unit
IFN	interferon
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IP	intra-peritoneal
IV	intravenous administration
kDa	kilodalton
LBP	Lipopolysaccharid-Binding-Protein
LD_{100}	lethal dose 100 percent

LD_{50}	median lethal dose
LL-37	a peptide which begins with two leucine residues containing 37 amino acid residues
LODS	logistic organ dysfunction system
LOS	length of stay
LPS	bacterial lipopolysaccharides
LRR	leucin-rich-repeats
MCP	monocytes chemoattractant protein
MIP	macrophage inflammatory protein
MODS	multiple organ dysfunction syndrome
NK cells	natural killer cells
OP	Operation
PAF	platelet-activating factor
PCI	peritoneal contamination and infection
PCT	procalcitonin
PELOD	pediatric logistic organ dysfunction
PEMOD	pediatric multiple organ dysfunction
PGE_2	prostaglandin
PIRO	Predisposing, Infection, Response, Organ
s.c.	Subcutaneous
SCCM	Society of Critical Care Medicine
SEM	standard error of the mean
SepNet	Competence Network Sepsis
SIRS	Systemic Inflammatory Response Syndrome
SOFA	sepsis-related organ failure assessment
TIR	Toll/IL-1-receptor domain
TLR	Toll-like receptor
TLR9	Toll-like receptor 9
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor α
TMN	Classification by tumor, metastasis, lymph nod
US	United States

8.2. List of own publications

Die vorliegende Arbeit wurde in folgenden Publikationsorganen in Teilen veröffentlicht:

1. Eugeniu Gurschi, Robert Bals, Hinnerk Wulf, Alexander Torossian, Artur Bauhofer: **Das antimicrobielle Peptid LL-37 verbessert das outcome nach abdomineller Sepsis in der Ratte.** Poster Präsentation; Deutscher Anästhesie Congress 2006 (Leipzig)

2. T. Vassiliou, E. Gurschi, A. Bauhofer, R. Bals, H. Wulf, A. Torossian: **Effektivität einer hyperthermen Präkonditionierung und antimikrobiellen Therapie (LL-37) im Sepsismodell der Ratte.** Anästh Intensivmed 2006; 47: 423-424.

3. Alexander Torossian, Eugeniu Gurschi, Robert Bals, Timon Vassiliou, Hinnerk Wulf and Artur Bauhofer: **Hyperthermic Preconditioning plus LL-37 Therapy Improves the Outcome in Septic Rats.** ASA 2006 Celebration of Research Edition (Chicago USA)

4. Eugeniu Gurschi, Artur Bauhofer, Robert Bals, Hinnerk Wulf, Alexander Torossian: **Untersuchung zur Dosisfindung des antimikrobiellen Peptids LL-37 Prophylaxe einer intra-abdominellen Sepsis in der Ratte.** Poster Präsentation; Deutscher Anästhesie Congress 2007 (Hamburg)

5. Artur Bauhofer, Eugeniu Gurschi, Robert Bals and Alexander Torossian: **The antimicrobial peptide LL-37 and hyperthermic preconditioning improves the outcome in septic rats.** Surgical Infection Society – Europe, 2007

6. Alexander Torossian, Eugeniu Gurschi, Robert Bals, Timon Vassiliou, Hinnerk Wulf, Artur Bauhofer: **Effects of the Antimicrobial Peptide LL-37 and Hyperthermic Preconditioning in Septic Rats.** Anesthesiology. 107(3):437-441, September 2007

8.3. Verzeichnis akademischer Lehrer/ academic teachers

Meine akademischen Lehrer waren die Damen und Herren:

in Marburg:
Bauhofer, Wulf, Lukasewitz, Torossian, Eberhardt

In Chişinău (Moldova):
Ababii, Gurschi, Pîrgari, Gereg, Cazacu, Andriuță, Anton, Batâr, Eşanu, Galeţchi, Ghicavîi, Groppa, Hotineanu, Iarovoi, Lîsîi, Matcovschi, Moldovanu, Palade, Pântea, Roşca, Rudi, Sofroni, Ştefăneț, Ţâbârnă, Tâbârnă, Testemițanu, Vorojbit, Zapuhlih.

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