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Effects of antibiotics on the intestinal microcirculation in septic rats

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	Publication 2: Thermostatic tissue platform for intravital microscopy: “the hanging drop” model.	

List of abbreviations

ICU: intensive care unit
SIRS: systemic inflammatory response syndrome
LPS: lipopolysaccharide
LBP: LPS binding protein
TLR: toll-like receptor
NF- κ B: nuclear factor kappa B
MyD88: myeloid differentiation protein 88
NOD: nucleotide-binding oligomerization domain
TNF- α : tumor necrosis factor-alpha
IL-1 β : interleukin-1 β
IL-6: interleukin-6,
IL-12: interleukin -12
IL-10: interleukin-10
IFN γ : interferon gamma
NAG: acetylglucosamine
FA: fatty acids
PMNs: polymorphonuclear neutrophils
CLP: cecal ligation and puncture
CASP: colon ascendens stent peritonitis
MET: metronidazole
IMI: imipenem
TOB: tobramycin
VAN: vancomycin
ERY: erythromycin
IVM: intravital microscopy
MAP: mean arterial pressure
HR: heart rate
FCD: functional capillary density

1. Introduction

Generalized bacterial infections and sepsis continue to be a threat especially for patients with trauma or after major surgery. Septic patients may suffer from disturbances affecting multiple sites, including disturbed microcirculation and usually require monitoring in an intensive care unit (ICU). If not corrected, microvascular dysfunction can lead to global tissue hypoxia, direct tissue damage, and ultimately, organ failure.

Intravenous antibiotic therapy should be initiated as soon as sepsis is suspected. Although the benefit of expedient antibiotic therapy remains unquestioned, little is known about the effects that are unrelated to their antimicrobial property but which the antibiotics may exert upon the septic microcirculation. It can be principally assumed that, following antibiotics therapy, parallel to the antibacterial effect, an improvement of the affected microcirculation also occurs. However, the administration of antibiotics is intended to bacteriolysis and the toxins released may, even if only temporarily, also lead to reinforcement of the alterations in the microcirculation [1]. Similarly, immunomodulating effects, such as the impact on leukocyte activation through antibiotics, are also possible [2].

1.1. Epidemiology of sepsis

Sepsis and septic shock, caused by gram-negative and gram-positive bacteria, fungi, viruses, and parasites, have become increasingly important morbidity and mortality over the past decades. The contribution of gram-positive bacteria and fungal organisms to sepsis has increased in the past, Gram-negative bacteria account for about 60% of cases, Gram-positive for the remainder [3, 4]. Sepsis constitutes a significant consumption of intensive care resources and remains an ever-present problem in the intensive care unit. In spite of considerable progress in intensive care and antimicrobial chemotherapy the lethality due to septic shock still ranges between 30% and 80% [4, 5]. Each year approximately 75,000 patients in Germany suffer from severe sepsis. The length of stay and the cost of laborious therapies lead to high ICU costs. In

Germany severe sepsis has been estimated to generate costs between 3.6 and 7.7 billion Euro annually [6]. In the United States, the septicemia rates more than doubled between 1979 and 1987 causing up to 250,000 deaths annually [7]. In the year 2010, it is estimated that there will be 934,000 new sepsis cases in the United States and in 2020, 1,100,000 [4]. In sepsis, complicated intra-abdominal infections are an important cause of morbidity and are more frequently associated with a poor prognosis [8].

However, an early clinical diagnosis, followed by adequate source control to stop ongoing contamination and restore anatomical structures and physiological function, as well as prompt initiation of appropriate empirical therapy, can limit the associated mortality [8-11].

1.2. Definitions

The term “systemic inflammatory response syndrome” (SIRS) was coined in 1992 by a panel comprised of American College of Chest Physicians and Society of Critical Care Medicine members [9]. They convened to develop consensus definitions of critical illness for the purposes of clinical trial design.

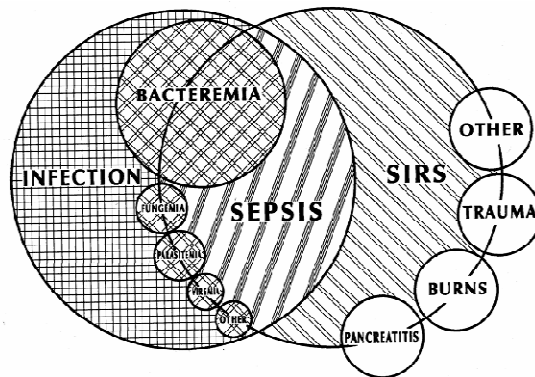


FIGURE 1: The interrelationships between infection, SIRS and sepsis [9].

SIRS describes the host response to critical illness of either infectious or noninfectious etiology, such as burns, trauma, and pancreatitis. SIRS is considered to be present when patients have two or more of the following clinical findings:

- Body temperature higher than 38°C or lower than 36°C

- Heart rate higher than 90/min
- Hyperventilation evidenced by respiratory rate higher than 20/min or PaCO₂ lower than 32 mmHg
- White blood cell count higher than 12,000 cells/ μl or lower than 4,000/ μl.

Infection is defined as the pathological process caused by the invasion of normally sterile tissue or fluid or body cavity by pathogenic or potentially pathogenic microorganism. Bone et al. [9] 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.

Recently, Levy et al. developed a classification scheme for sepsis—called **PIRO**—that stratifies patients on the basis of their *predisposing conditions*, the nature and extent of the *insult* (in the case of sepsis, infection), the nature and magnitude of the host *response*, and the degree of concomitant *organ dysfunction* [11].

Table 1: The PIRO system for staging sepsis [11].

Domain	Present	Future	Rationale
Predisposition	Premorbid illness with reduced probability of short term survival. Cultural or religious beliefs, age, gender	Genetic polymorphisms in components of inflammatory response (e.g., Toll-like receptor, tumor necrosis factor, interleukin 1, CD14); enhanced understanding of specific interactions between pathogens and host diseases	At the present, premorbid factors impact on the potential attributable morbidity and mortality of an acute insult; deleterious consequences of insult depend heavily on genetic predisposition (future)
Insult (infection)	Culture and sensitivity of infecting pathogens; detection of disease amenable to source control	Assay of microbial products (lipopolysaccharide, 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, C-reactive protein	Nonspecific markers of activated inflammation (e.g., procalcitonin or interleukin 6) or impaired host responsiveness (e.g., HLA-DR); specific detection of target of therapy (e.g., protein C, tumor necrosis factor, platelet-activating factor)	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., multiple-organ dysfunction syndrome, logistic organ dysfunction system, Sequential Organ Failure Assessment, Pediatric Multiple Organ Dysfunction, Pediatric Logistic Organ Dysfunction)	Dynamic measures of cellular response to insult – apoptosis, cytopathic hypoxia, cell stress	Response to preemptive therapy (e.g., targeting micro-organism or early mediator) not possible if damage already present; therapies targeting the injurious cellular process require that it be present

This has been conceptually modelled from the TNM classification (tumor size, nodal spread, metastases) which has been successfully used in defining treatment and prognostic indicators in clinical oncology. Further refinements in the definitions and predisposing factors of severe sepsis should improve the understanding and management of severe sepsis and septic shock in the near future.

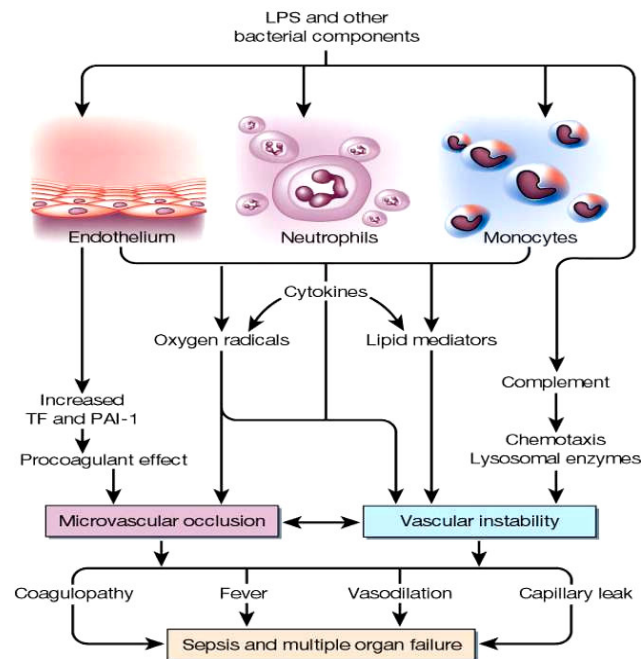
1.3. Pathophysiology

1.3.1. Inflammatory Cascades

When a bacterium enters the body, it is confronted with two lines of defense: a humoral line (complement, antibodies, and acute phase proteins) and a cellular line (monocytes, macrophages, neutrophils and endothelial cells). In the host response to bacteria, mononuclear cells play a key role [12]. Bacterial components (gram-negative bacteria: e.g. lipopolysaccharide (LPS), lipid A, formyl peptides, exotoxins, proteases; gram-positive bacteria: e.g. exotoxins, superantigens, streptococcal pyrogenic exotoxin A (SpeA), enterotoxins, hemolysins, peptidoglycans (PGN), lipoteichoic acid (LTA); and fungal cell wall material) bind to cell receptors on the host's macrophages and activate regulatory proteins [e.g. Nuclear Factor Kappa B (NF- κ B)]. Endotoxin activates the regulatory proteins by interacting with several receptors. The CD receptors pool the LPS-LPS binding protein (LBP) complex on the surface of the cell [13] and then the toll-like receptors (TLR) translate the signal into the cells [14, 15], leading to the eventual production of the proinflammatory [e.g. cytokines tumor necrosis factor-alpha (TNF- α), interleukin-1,-6,-12 (IL-1, IL-6, IL-12) and interferon gamma (IFN γ)] [16-18]. Other agents which can stimulate the same process include exotoxins from gram positive bacteria or products of activation of the complement system such as C5a. At the same time, the body regulates this response by producing anti-inflammatory cytokines (e.g. interleukin-10 [19, 20]) and soluble inhibitors (e.g. interleukin-1 receptor antagonist [21, 22]). The pro-inflammatory cytokines can act directly to affect organ function or they may act indirectly through secondary mediators. The secondary mediators include nitric oxide, thromboxanes, leukotrienes, platelet-activating factor, prostaglandins, and complement. TNF- α and IL-1 (as well

as endotoxin) can also cause the release of tissue-factor by endothelial cells leading to fibrin deposition and disseminated intravascular coagulation (DIC). Then these primary and secondary mediators cause the activation of the coagulation cascade, the complement cascade and the production of prostaglandins and leukotrienes [23, 24]. Clots lodge in the blood vessels which lowers perfusion of the organs and can lead to multiple organ failure [25, 26].

The cumulative effect of this cascade is an unbalanced state, with inflammation dominant over antiinflammation and coagulation dominant over fibrinolysis. Microvascular thrombosis, hypoperfusion, ischemia, and tissue injury result. Severe sepsis, shock, and multiple organ dysfunction may occur, leading to death [12].



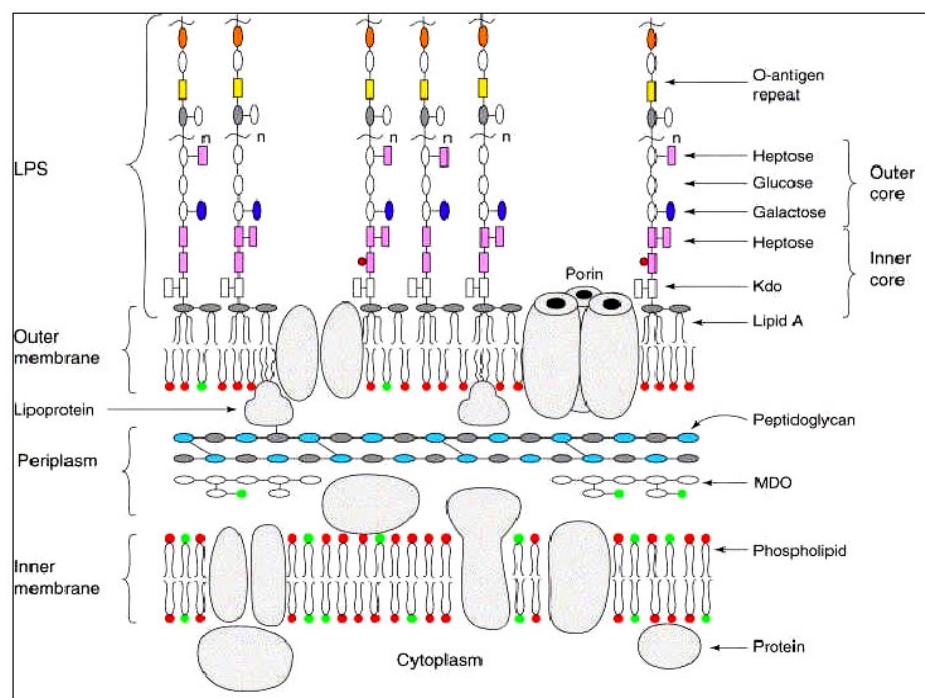
(PAI-1: plasminogen-activator inhibitor-1; TF: tissue factor)

FIGURE 2: Pathogenic networks in shock [12].

1.3.2. Endotoxin

Sepsis is initiated by toxins released from or associated with the infecting organism. Endotoxin are released from the bacterial cells when they multiply but also when bacteria die or undergo lyses [27, 28]. Various endogenous factors like complement

and bactericidal proteins can cause disintegration of bacteria, resulting in the release of LPS [29]. In addition, some antibiotics are known to cause the release of LPS from bacteria [30, 31]. Lipopolysaccharide is an immunogenic glycolipid that is the major component of the outer leaflet of the outer membranes of Gram-negative bacteria [32]. The outer membrane of Gram-negative bacteria is constructed of a lipid bilayer, separated from the inner cytoplasmic membrane by peptidoglycan. The LPS molecule is embedded in the outer membrane and the lipid A portion of the molecule serves to anchor LPS in the bacterial cell wall.



(Kdo: 2-keto-3-deoxyoctonic acid; MDO: membrane-derived oligosaccharides)

FIGURE 3: Components of Gram-Negative Bacterial cell wall [33].

The LPS molecule consists of four different parts [28, 34, 35]:

- **Lipid A** is the lipid component of LPS. It contains the hydrophobic, membrane-anchoring region of LPS. Lipid A consists of a phosphorylated N-acetylglucosamine (NAG) dimer with 6 or 7 fatty acids (FA) attached. Usually 6 FA are found. All FA in Lipid A are saturated. Some FA are attached directly to the NAG dimer and others are esterified to the 3-hydroxy fatty acids that are characteristically

present. The structure of Lipid A is highly conserved among Gram-negative bacteria. Among Enterobacteriaceae Lipid A is virtually constant. Lipid A potently activates the innate immune system, inducing a host response that includes the production of cationic antimicrobial peptides, cytokines, clotting factors, and various immunostimulatory molecules [14, 36].

- The second part of the LPS molecule is the **inner core**, which consists of two or more 2-keto-3-deoxyoctonic acid (KDO) sugars linked to the lipid A glucosamine and two or three heptose (L-glycero-D-manno-heptose) sugars linked to the KDO. Both sugars are unique to bacteria. The smallest LPS molecule produced by gram-negative bacteria under natural conditions is Re-LPS (lipid A with one or two KDO sugars), but longer LPS molecules are more common. The Rd1- and Rd2-LPS serotypes contain a complete inner core and an inner core lacking two heptose sugars, respectively [37].

- The **outer core**, the third part of the LPS molecule, consists of common sugars and is more variable than the inner core. It is normally three sugars long with one or more covalently bound sugars as side chains. LPS serotypes consisting of lipid A and the complete inner and outer core are denoted Ra-LPS, whereas the Rb- and Rc-LPS serotypes only contain a part of the outer core [37].

- The fourth moiety of the LPS molecule is the **O antigen**. This part of the LPS molecule is attached to the terminal sugar of the outer core, extends from the bacterial surface, and is highly immunogenic. It is composed of units of common sugars, but there is a huge interspecies and interstrain variation in the composition and length. In a single LPS preparation, the length of the O antigen may vary from 0 to as many as 40 repeating units, but it generally consists of 20 to 40 repeating units. Each unit is composed of three sugars with a single sugar connected to the first and third sugar of the unit. LPS molecules with O antigen are denoted S-LPS. Colonies from bacteria with O-antigen-containing LPS have a smooth (S) appearance on the plate, while bacteria that express an O-antigen-lacking LPS have a rough (R) appearance [37].

The oligosaccharide O antigens vary from one strain to another, but the R core antigen and lipid are relatively constant across species and strains of gram-negative bacteria.

1.3.3. Receptors

The CD14–LBP complex

In 1990, CD14 (previously known only as a monocyte-specific antigen) was identified as the receptor involved in cellular activation. Activation of host cells was dependent on the presence of LBP and the opsonic receptor CD14 [13]. CD14 plays a key role in initiating cell activation by a group of bacterial envelope components from Gram-negative and Gram-positive microorganisms, as well as mycobacteria [38]. CD14 has been implicated in monocyte activation [39], leukocyte–endothelial cell interactions [40] and regulation of programmed cell death (apoptosis) in both monocytes and endothelial cells [41, 42]. CD14 also exists as a soluble molecule (sCD14) that can be found with two different molecular weights in serum [43]. Membrane bound CD14 (mCD14) is a glycosylphosphatidylinositol-linked molecule anchored in the cell surface, but it is also found in the circulation as sCD14 [44]. The sCD14s have an important role in LPS mediated activation of CD14 negative cells (epithelial and smooth muscle cells) [45].

Toll-like receptors

Although the discovery of CD14 represented a significant step forward in understanding host responses to bacterial components, CD14 lacks a transmembrane signaling domain [46], the involvement of an accessory receptor was proposed. A family of (currently) ten TLRs has been identified with a wide range of ligand specificity including bacterial, fungal and yeast proteins [14, 47, 48]. TLR4 is the LPS receptor [47, 49]. TLR2 is predominantly responsible for recognizing Gram-positive cell-wall structures [50], might be the genuine LPS receptor for LPS from non-enteric Gram-negative bacteria [51], can be activated by cell-wall components of both yeast and mycobacteria [52]. TLR5 is the receptor for flagellin from both Gram-positive and Gram-negative bacteria [53] and TLR9 recognizes deoxycytidylate-phosphate-deoxyguanylate (CpG) elements in bacterial DNA[54]. An additional cell-surface molecule, MD-2, has been identified that is required for activation of TLR4 [55]. Two of the human TLRs (TLR4 and TLR2) have been reported to be associated with CD14 receptor and to mediate recognition of cell wall components from Gram negative and Gram positive bacteria, respectively [50]. TLR4 and TLR2 have been shown to signal

(Toll-interacting protein). There is also an MyD88-independent pathway by which TIRAP/Mal signals through an RNA-dependent protein kinase (PKR) and interferon regulatory factor (IRF)-3. Recently it has been proposed that cells may also be able to respond to LPS by intracellular receptors called NOD proteins (for nucleotide-binding oligomerization domain). Expression of NOD1 and NOD2 confer responsiveness to Gram-negative LPS but not to lipoteichoic acid, which is found in Gram-positive bacteria [12, 58-60].

1.3.4. Cytokines

Cytokines are soluble, low molecular weight glycoproteins which act to regulate both innate and specific immune responses and act as inflammatory mediators. Individual cytokines can be secreted by a variety of cells in response to such insults as infection, trauma, burns, inflammation and hemorrhage. Several cytokines have been implicated in the development of SIRS and sepsis, including TNF- α , IL-1 β , IL-8, IL-6 and IL-10 [61]. Circulating concentrations of those cytokines have been shown to be linked to morbidity and mortality in patients with sepsis [18, 62].

- TNF- α , a cytokine with a relative molecular mass of 17,000 (M_r 17K), is produced by activated macrophages in response to pathogens and other injurious stimuli, and is a necessary and sufficient mediator of local and systemic inflammation [63, 64]. Local increases in TNF- α cause the cardinal clinical signs of inflammation, including heat, swelling, pain and redness. Systemic increases in TNF- α mediate tissue injury by depressing cardiac output, inducing microvascular thrombosis and mediating systemic capillary leakage syndrome. TNF- α amplifies and prolongs the inflammatory response by activating other cells to release both cytokines such as IL-1 and high mobility group-1 (HMG-1) protein, and mediators such as eicosanoids, nitric oxide and reactive oxygen species, which promote further inflammation and tissue injury [65]. Low amounts of TNF- α can contribute to host defence by limiting the spread of pathogenic organisms into the circulation, promoting coagulation to localize the invader, and stimulating the growth of damaged tissues [66].

- IL-1 functions similarly to TNF- α in that it mediates acute inflammatory responses. In vitro, IL-1 induces IL-8 expression in alveolar macrophages, thus indirectly increasing neutrophil chemotaxis into the lung [67]. IL-1 is produced by

monocytes, macrophages, dendritic cells, and a variety of other cells in the body. IL-1 also appears to occupy an important role in sepsis-induced organ dysfunction [68, 69].

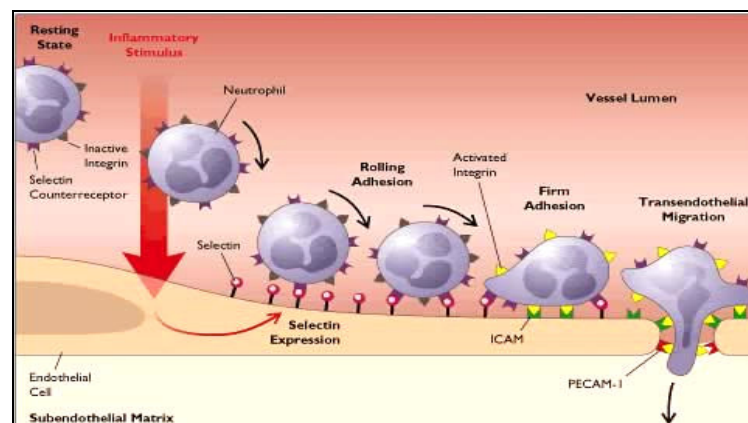
- IL-6 is a cytokine with important prognostic value in sepsis. Although the pathophysiologic role of IL-6 in this syndrome is still controversial, IL-6 has been proposed as an important cytokine biomarker in sepsis. IL-6 augments the cytotoxic potential of neutrophils via selective increase of elastase release [70]. Among proinflammatory cytokines, IL-6 is closely related to the severity of the physiological response to infection and systemic inflammation [71]. Different studies have confirmed that the majority (64-100%) of septic patients have increased circulating levels of IL-6, and that levels are correlated with severity and outcome [72, 73]. Persistently elevated IL-6 levels are associated with both multiple organ failure and death [74]. IL-6 is a valuable outcome predictor in patients with sepsis and septic shock.

- IL-10 is an important immunoregulatory cytokine produced by many cell populations. Its main biological function seems to be the limitation and termination of inflammatory responses and the regulation of differentiation and proliferation of several immune cells such as T cells, B cells, natural killer cells, antigen-presenting cells, mast cells, and granulocytes [75, 76]. IL-10 controls inflammatory processes by suppressing the expression of proinflammatory cytokines, chemokines, and adhesion molecules, as well as antigen-presenting [76]. Both overexpression (e.g., in lymphoma, intensive care unit patients) as well as IL-10 deficiency (e.g., in inflammatory bowel disease, psoriasis) are likely to have a pathophysiological significance [77]. Administration of IL-10 attenuates production of TNF- α in experimental endotoxemia [78].

1.3.5. Leukocyte–endothelial cell interactions

Leukocyte–endothelial cell interaction is essential for an effective defence against bacterial invasion [79]. Leukocytes interact with the vascular endothelium in a three-stage process of rolling, adhesion and migration so that their normally rapid flow through the circulation can be diverted. Since 1985, 3 families of leukocyte-endothelial adhesion molecules have been identified: the selectins, the immunoglobulin gene superfamily, and the integrins [80]. Selectins mediate rolling of leukocytes on the

endothelium of postcapillary venules. Firm adhesion of leukocytes to the endothelial cells as well as leukocyte activation is mediated by receptors of the immunoglobulin gene superfamily. To this family belong 5 molecules that are expressed by endothelial cells: intercellular adhesion molecule-1 and -2 (ICAM-1 and ICAM-2), vascular cell adhesion molecule-1 (VCAM-1), platelet-endothelial cell adhesion molecule-1 (PECAM-1), and the mucosal addressin (MAdCAM-1) [81]. After rolling of the leukocyte on the endothelial surface has arrested its flow, leukocyte integrins are activated by chemokines, chemoattractants, and cytokines. Integrins are transmembrane cell surface proteins. The CD18 or $\beta 2$ integrins are restricted to leukocytes and bind to their counterreceptors of the immunoglobulin gene superfamily [80]. Adherent leukocytes are then able to migrate into the tissues [82, 83]. The endothelial switch from anticoagulant to procoagulant during sepsis is thought to be related to the cytokine-mediated expression of endothelial adhesion molecules and increased tissue factor production by monocytes and endothelial cells [84, 85].



(<http://www.hosprract.com/issues/2000/03/etzioni.htm>)

FIGURE 5: Leukocyte–endothelial-cell interactions.

Leukocytic activation is associated with an increased proinflammatory immune response [85, 86], which is caused by an increased release of inflammatory cytokines, including tumor necrosis factor- α , interleukin-1 β and interleukin-8, which initiate generalized neutrophil (polymorphonucleocyte) activation [87]. This release of mediators, together with the generalized activation and sequestration of leukocytes, may contribute to the widespread microvascular injury and subsequent endothelial damage observed in sepsis [88].

1.3.6. Microcirculatory dysfunction in sepsis

Sepsis and multiple organ dysfunction syndromes remain a major problem of intensive care medicine. Clinical observations, assisted by invasive monitoring techniques as well as pathological-anatomical studies, clearly indicate that microcirculatory dysfunction lies at the centre of the pathogenesis [89].

The microcirculation cannot be regarded as a passive vascular conduit for the transport of cells, nutrients, and oxygen into tissue, but rather constitutes a functionally highly active system that dynamically interacts with circulating and tissue-associated cells (i.e. leukocytes, platelets, mast cells, etc.), elaborates mediators, and contributes to local, downstream and even upstream regulation of vascular tone. Damage to this system can affect all participating cellular components, in particular endothelial cells, smooth muscle cells, as well as the constantly changing pool of circulating blood cells. Activation, dysfunction, and injury of microvascular endothelial cells may occur as a result of ischaemia, inflammatory mediators, as well as adherent leukocytes, in particular polymorphonuclear neutrophils (PMNs) with their elaborate armamentarium of inflammatory mediators and proteolytic enzymes [90].

Consequences of microcirculatory dysfunction are (i) the breakdown of endothelial and epithelial barrier function, leading to tissue oedema and uncontrolled inflammatory cell infiltration; (ii) vasodysregulation, leading to the formation of arteriovenous shunts and/or the loss of peripheral resistance with severe macrohaemodynamic consequences; and (iii) disturbance of oxygen transport and utilization by tissue cells [91].

1.3.7. Intestinal microcirculation

Anatomy of intestinal microcirculation

The intestinal wall consists of the muscular, submucosal and mucosal layer [92]. In unfed animals at rest, blood flow to the mucosal layer is about 70–80% of total flow, while the muscular and serosal layers collectively receive 15–25% of organ flow and the submucosal layer is perfused by less than 5% [93]. Of the mucosal blood flow, approximately 60% perfuses the vessels that terminate as end loops and that supply the

epithelial cells in the intestinal villi. The remaining 40% supply flow to the crypts and goblet cells [93]. In the resting or unfed state only 20–30% of capillaries are normally perfused [93].

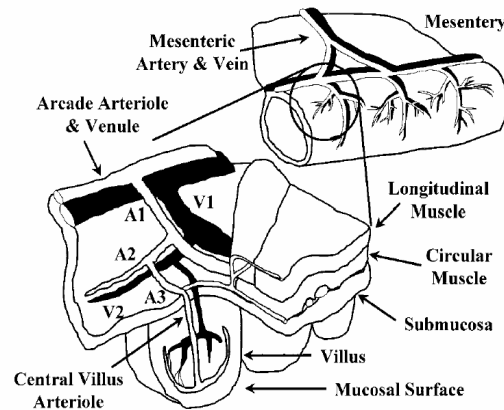


FIGURE 6: Intestinal microcirculation [92].

The intestinal mucosa is the site of nutrient absorption; the submucosa, which consists of glandular cells, produces serous and mucous secretions, as well as newly formed, immature enterocytes; and the muscular layers provide contractile force for intestinal mixing and propulsion of the chime [92]. In addition to vascular perfusion, the organs of the gastrointestinal system contain numerous lymph vessels [94, 95]. The mucosal–submucosal lymph circulation functions to drain the absorbed nutrients and metabolic by-products from the villi during digestion.

The role of intestinal microcirculation in sepsis

The gastrointestinal system anatomically is positioned to perform two distinct functions: to digest and absorb ingested nutrients and to sustain barrier function to prevent transepithelial migration of bacteria and antigens. In pathological conditions, the splanchnic area is reported to be the 'motor' of multiple organ failure [96, 97] and the 'canary' of the body [98]. Impaired splanchnic perfusion occurs as a result of the redistribution of cardiac output from the splanchnic organs to the cardiopulmonary and cerebral circulations [99, 100]. This splanchnic hypoperfusion and resulting damage to the epithelial layer plays an important role in the development of multiple organ

dysfunction owing to enhanced bacterial translocation from the gut and activation of an exacerbated inflammatory cascade [100]. Decreased splanchnic perfusion also leads to the low blood supply to the downstream organs, such as the liver, leading to hepatic dysfunction, which also contributes to multiple organ failure after shock. A fine balance between vasodilators and vasoconstrictors maintains splanchnic perfusion. Increased systemic production of vasoconstrictors such as epinephrine [101], angiotensin II [102], endothelin [103, 104], and thromboxane A₂ [105, 106] has been observed in experimental models of trauma-hemorrhage and sepsis. These vasoconstrictors not only contribute to the increased total peripheral resistance but also act on the splanchnic vessels and reduce their perfusion rate. The reduced production of vasodilators [107] or the attenuated response of the splanchnic vessel to the vasodilators [108, 109] (endothelial dysfunction) is also observed after severe hemorrhagic or septic shock. Both of these factors contribute to the circulatory disturbance. In addition, these effects induce intestinal hypoxia, reduce nutrient supply, increase production of oxygen free radicals, and increase neutrophil accumulation, leading to further damage of the intestinal mucosal barrier and thereby resulting in increased bacterial translocation [110].

Gut barrier function is maintained by a well-balanced intestinal flora, an intact mucosa, and a normal functioning immune system. If one or more of these three protective mechanisms are disrupted, viable bacteria or bacterial products like endotoxin (i.e. LPS) may cross the gut mucosa and spread to the mesenteric lymph nodes or more distant organs, such as the liver and the spleen, a process termed bacterial translocation [111]. Three mechanisms have been suggested to explain the phenomenon of bacterial translocation: altered intestinal barrier function, bacterial overgrowth, and impaired host defence. Although bacterial translocation is widely considered a pathological and potentially harmful phenomenon, translocation of gut bacteria appears to be common early in life and may be important for mucosal antigen sampling in the gut [112]. Mechanistically, in bacterial translocation, bacteria initially attach themselves to intestinal enterocytes, the cell membranes of the latter then rupture, and this allows bacteria to penetrate and reach the basal membrane. Once this has taken place, intestinal lymphatic drainage carries bacteria to the mesenteric lymph nodes from where they can spread to other organs and tissues [113].

1.4. Models of sepsis

Bacterial invasion of the peritoneal cavity due to intestinal leakage after major abdominal surgery frequently leads to organ failure, septic shock, and death [114]. Attempting to decrease the high incidence of morbidity and mortality associated with shock and sepsis, many investigators have used a variety of animal models, not only to clarify the pathogenesis of shock and sepsis but also to identify effective prophylactic and therapeutic strategies. Such models include intravascular infusion of endotoxin [115-120] or live bacteria [116, 121-123], bacterial peritonitis [124-126], cecal ligation and puncture (CLP), and colon ascendens stent peritonitis (CASP) [127, 128].

1.4.1. Endotoxemia

Endotoxin is commonly used in animal models of sepsis, although there is controversy over their relevance to our understanding of human sepsis. When administered to human subjects, endotoxin may mimic many of the features of sepsis [129]. In critically ill patients, increased concentrations of serum endotoxin have been associated with the development of sepsis, disease severity, and mortality [7, 129, 130]. Detectable levels of LPS are identified in up to 75 per cent of patients with sepsis in intensive care setting [131]. Serum endotoxin levels often remain undetectable in more indolent forms of uncomplicated sepsis with the recorded levels being of no prognostic significance [130]. Very high levels are occasionally found in meningococemia and at the start of bactericidal antibiotic therapy [132]. The plausibility of the hypothesis that endotoxin plays a significant role in the pathogenesis of sepsis is supported by many studies that show that antibiotic administration may lead to an sudden release of massive amounts of endotoxin from dead bacteria and an acute hemodynamics worsening [7, 122, 130, 132]. A large intravenous dose of LPS in rats produces rapid cardiovascular collapse and early death [133], whereas lower doses produces a hyperdynamic response with an early increase in cardiac output [117]. In spite of evidences that endotoxin may play an important role in the pathogenesis of sepsis, several authors have expressed concerns that the infusion of endotoxin is not a suitable model to study sepsis [129, 130]. Immunomodulatory therapy regimens targeting TNF- α , IFN γ , IL-1 β , or LPS have failed to improve the outcome of

abdominal sepsis in human patients [128, 134-136]. Moreover, it has been stipulated [129] that endotoxin is only relevant component of gram-negative bacteria; it is however theorized that the other cell wall components also contribute to systemic inflammatory response [137].

Thus, caution is needed in assessing the clinical efficacy of novel therapeutic agents in animal models of endotoxemia [130]. Currently, there is general agreement among researchers that LPS injection may serve as a model for endotoxic shock but not for sepsis [129, 138].

1.4.2. Peritonitis

Peritonitis may be induced in animals in several ways. Bowel can be perforated allowing contamination with gastrointestinal contents, or an inoculum of fecal material or pure bacterial cultures can be instilled into the peritoneal cavity [131]. In early models, segments of intact bowel were isolated and the development of peritonitis was expected. The disadvantage of this method was that the onset of peritonitis was uncontrolled and depended on the timing of gastrointestinal perforation.

To overcome this limitation, the simple and reproducible cecum ligation and perforation (CLP) model was developed and has been used widely in sepsis research [139]. The cecum is ligated distal to the ileocecal valve and perforated using two needle punctures. Needle size can be used to manipulate CLP to give a lethal and non-lethal sepsis [128]. The principal advantage of CLP model is their simplicity. Since sepsis is induced by a straightforward surgical procedure, there is no need to grow and quantify bacteria or in other ways prepare the inoculum. Furthermore, these are models of sepsis due to peritoneal contamination with mixed flora in the presence of devitalized tissue and thus bear an obvious resemblance to clinical problems like perforated appendicitis and diverticulitis [131]. This technique, without fluid resuscitation, promotes rapid onset of shock, while after fluid resuscitation the mortality rate may be reduced with pathophysiological responses resembling those noted in human sepsis [140]. However, some studies even indicated that TNF- α is required for survival after CLP [141, 142].

To improve the understanding of the pathophysiology encountered in sepsis, a new standardized and highly reproducible murine model of abdominal sepsis termed colon ascendens stent peritonitis (CASP) was developed [127]. Bacterial loads of peritoneal lavage, liver, and lung, as well as serum cytokine levels (TNF- α , IL-1 β , IL-10) steadily increased from 6 to 24 h after the CASP procedure. In contrast, continuously low amounts of bacteria and cytokines were found in CLP mice at any point of time.

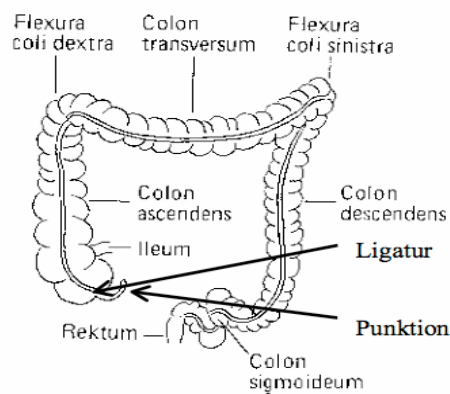


FIGURE 7 : CLP model [139].

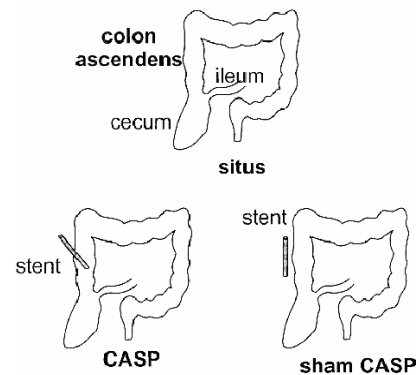


FIGURE 8: CASP model [127].

The CASP model mimics closely the clinical course of diffuse peritonitis with early and steadily increasing systemic infection and inflammation (systemic inflammatory response syndrome). In contrast, CLP reveals a model of intra-abdominal abscess formation with sustained and minor signs of systemic inflammation [143]. Animal models will remain the most appropriate tool for evaluating new therapeutic strategies for the treatment of various diseases. Thus, for evaluation of new treatment approaches for diffuse peritonitis, the CASP model appears advantageous over CLP, whereas for other intra-abdominal infections such as abscesses, the CLP model should be preferred [143].

1.5. Antibiotics

1.5.1. Introduction

Peritonitis is an inflammation of the peritoneum from infected material in the abdominal cavity. It is a serious but rare acute infection, which can lead rapidly to hypovolaemic shock and death. The treatment of patients with intra-abdominal infections must be approached with some urgency because mortality rates for these patients can approach 60% [144]. These types of infection tend to be mixed infections, with gram-positive and gram-negative, aerobic and anaerobic bacteria present. The regimen of choice of antibiotics must therefore be broad in spectrum to cover these potential pathogens. The treatment of intra-abdominal infection is based on surgical treatment, the use of potent antibiotics and when necessary intensive care treatment. The purpose of antimicrobial agents is to limit persistent or residual peritoneal infection following drainage, prevent wound infection, and limit adverse effects of the infection on the host [145]. Antibiotics are effective because of their property of selective toxicity. They are able to attack bacterial cells without having an adverse effect on host cells. This property is made possible by biochemical differences between the cells of the host and the infecting organism. The risk of complications, defined as wound infections, postoperative abscesses and repeat operations, is reduced if appropriate empiric antibiotic therapy is used. A more recent retrospective study of intra-abdominal infection found that initial appropriate antibiotic therapy improved clinical success rates and reduced the length of stay and overall cost of hospitalization [146].

1.5.2. Classification

Antibiotics can be classified as bacteriostatic or bactericidal. Bacteriostatic antibiotics arrest the growth cycle of bacteria, thereby limiting spread of infection and allowing the host immune system to eliminate remaining organisms. In contrast, bactericidal drugs kill all infecting pathogens. Some antibiotics can be used to treat a wide range of infections and are known as 'broad-spectrum' antibiotics. Others are only effective

against a few types of bacteria and are called 'narrow-spectrum' antibiotics. Some antibiotics work against aerobic bacteria that are organisms that need oxygen to live, while others work against anaerobic bacteria, organisms that don't need oxygen.

Antibiotics can be further classified by their chemical structure, activity against specific organisms, or mechanism of action.

Table 2: Classification of antibiotics by chemical structure

Class (chemical structure)	Mechanism of action
B-lactam antibiotics - Penicillins (e.g. Penicillin G, Amoxicillin, Flucloxacillin) - Cephalosporins (e.g. Cefoxitin, Cefotaxime, Ceftriaxone) - Carbapenems (e.g. Imipenem)	Inhibit bacterial cell wall synthesis
Macrolides (e.g. Erythromycin, Azithromycin, Clarithromycin)	Inhibit bacterial protein synthesis
Tetracyclines (e.g. Tetracycline, Minocycline, Doxycycline, Lymecycline)	Inhibit bacterial protein synthesis
Fluoroquinolones (e.g. Norfloxacin, Ciprofloxacin, Enoxacin, Ofloxacin)	Inhibit bacterial DNA synthesis
Sulphonamides (e.g. Co-trimoxazole, Trimethoprim)	Blocks bacterial cell metabolism by inhibiting enzymes
Aminoglycosides (e.g. Gentamicin, Amikacin)	Inhibit bacterial protein synthesis
Imidazoles (e.g. Metronidazole)	Inhibit bacterial DNA synthesis
Peptides (e.g. Bacitracin)	Inhibit bacterial cell wall synthesis
Lincosamides (e.g. Clindamycin, Lincomycin)	Inhibit bacterial protein synthesis
Oxazolidinones (e.g. Linezolid)	Inhibit bacterial protein synthesis

1.5.3. Effects on microcirculation

Antibiotics could and do interfere with various processes that accompany septic conditions and it is not certain that all antibiotics that are used are equally beneficial or whether they could even be deleterious in that respect. In 1975, Rusmin et al. [147] demonstrated that antibiotic administration may induce the release of endotoxin from bacteria retained on intravenous inline filter. Imipenem or imipenem plus tobramycin induced an early increase of total endotoxin from E. coli. Cefuroxime or aztreonam induced much higher endotoxin release causing the formation of filamentous structures in pathogens [148]. More recently, such concerns have been described in terms of a

"therapeutic paradox" [31, 149]. Antibiotic action on microbes in the host can result in the release of bacterial components that will trigger a host proinflammatory response [31, 149-151]. The administration of antibiotics is intended to bacteriolysis and the released toxins may, even if only temporarily, in the same way lead to reinforcement of the alterations in the microcirculation [1]. Furthermore, some antibiotics may have a direct effect on vascular smooth muscle contractility that is seriously impaired in sepsis [152-155]. Various experimental studies examined the effects on blood vessels. For example, neomycin, kanamycin and gentamicin inhibit contractile responses and alter Ca^{++} movements in monkey blood vessels [152]. Streptomycin has been demonstrated to inhibit mechanosensitive conductances in a wide variety of cell types, including muscle [156]. Antibiotics may have effect on cytokine release in human monocytes and lymphocytes. Sulbactam-ampicillin and cefamandole induce IFN γ production, clindamycin TNF and IL-6 release, lincomycin released IL-4 and teicoplanin TNF, IL-1 alpha and IL-6 [157].

Some antibiotics may have unwanted effects on the microcirculation, whereas some antibiotics have immunomodulatory and anti-inflammatory properties. The acceptance of the anti-inflammatory and immunomodulatory aspects of antibiotics came mostly from the inflammatory bowel disease experts, who pondered why antibiotics helped many patients with Crohn's disease despite no obvious infection. Tetracyclines are useful in rheumatoid arthritis by inhibiting the expression of nitric oxide [158]. Gentamycin, an aminoglycoside antibiotic, may exhibit an anti-inflammatory action due to inhibition of neutrophil NADPH oxidase activation [159]. A recent study confirmed that macrolide antibiotics prevent the production of proinflammatory mediators and cytokines [160]. Amoxicillin has been shown to decrease bowel inflammation in ulcerative colitis [161]. Trimethoprim-sulfamethoxazole has recently been shown useful in the treatment of autoimmune diseases, such as rheumatoid arthritis [162]. The beneficial effects of metronidazole in acne vulgaris are attributable to its anti-inflammatory activities rather than its antibacterial ones [163]. Clindamycin is a powerful anti-inflammatory useful in sterile conditions like Fox-Fordyce disease. Clindamycin suppresses the complement-derived chemotaxis of polymorphonuclear leukocytes in vitro, thereby reducing the potential for inflammation [164]. Clarithromycin and roxythromycin may affect the functions of neutrophils in chronic sinusitis by modulating the expression of L-selectin and Mac-1

molecules on neutrophils, thereby attenuating the adhesion of neutrophils [165]. Ciprofloxacin decreases the accumulation of IL-6 [166].

In collaboration with the results of the microbial sensitivity test, a specific knowledge of the potential effects exerted by antibiotic substances upon the microcirculation could very well become a basic factor in the choice of medication to be used within the realm of antibiotic therapy for sepsis.

1.5.4. Metronidazole

Metronidazole (MET), an antiparasitic and antibacterial compound, which was synthesised in 1957 and is widely used for prophylaxis against anaerobic infection after bowel surgery, for treatment of wound abscess, and for treatment of antibiotic-associated colitis caused by *Clostridium difficile* [167]. MET is an important part of combination therapy against *Helicobacter pylori*, a major cause of gastritis and a risk factor for stomach cancer [168]. MET is against virtually all anaerobes with the exception of actinomyces, Propionibacterium acnes, and Lactobacillus species. *C. difficile* resistance is rare, only 1/1,000 strains of *C. difficile* were resistant to MET [169].

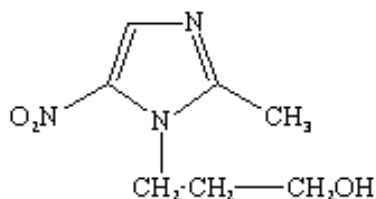


FIGURE 9: Structure of metronidazole [170].

The exact mechanism of MET has not been fully elucidated, but it appears that reduction of metronidazole leads to a polar metabolite that disrupts DNA and inhibits nucleic acid synthesis [171]. MET have anti-inflammatory activities in acne vulgaris therapy [163]. Studies have shown that MET can be effective in relieving symptoms of Crohn's disease or ulcerative colitis. It may be used in combination with other antibiotics or other medications [172].

Molecular formula: C₆H₉O₃N₃

Chemical names: 2-(2-Methyl-5-nitroimidazol-1-yl)ethanol
2-methyl-5-nitroimidazole-1-ethanol
1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole
1-(*beta*-ethylol)-2-methyl-5-nitro-3-azapyrrole

Usual dosing: For *oral* dosage : Adults and teenagers— The usual dose is 7.5 mg/ kg, up to a maximum dose of 1 gram, every six hours for at least seven days. Children—The usual dose is 7.5 mg/kg, every six hours; or 10 mg/ kg every eight hours; For *injection* dosage: Adults and children over 1 week of age—The usual dose is 15 mg/kg one time to start, then 7.5 mg/kg injected into a vein every six hours for at least seven days. Full-term infants—The usual dose is 15 mg per kg one time to start, then 7.5 mg per kg, injected into a vein, every twelve hours starting twenty-four hours after the first dose.

Adverse effects [171]:

- * Frequent: metallic taste, headache, dark urine (harmless).
- * Occasional: peripheral neuropathy (with prolonged use, usually reversible), phlebitis at injection sites, disulfiram-like reaction with alcohol, insomnia, stomatitis.

1.5.5. Imipenem

Imipenem (IMI) was discovered by Merck in 1975. IMI is an intravenous beta-lactam antibiotic developed in 1985. IMI belongs to the subgroup of carbapenems. It is derived from a compound called thienamycin, which is produced by the bacteria *Streptomyces cattleya*. IMI, a broad-spectrum antibiotic, is highly active in vitro against most aerobic and anaerobic gram-positive and gram-negative bacteria isolated from infectious diseases of human beings [173]. It is often kept in reserve for organisms multiply resistant to other beta-lactams.

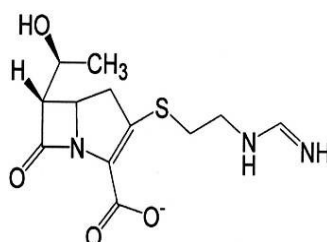


FIGURE 10: Structure of imipenem [174].

IMI inhibits mucopeptide synthesis in the bacterial cell wall, this results in the formation of defective cell walls and osmotically unstable organisms susceptible to cell lysis. Because it is inactivated by a renal dipeptidase, IMI is formulated in combination with a dipeptidase inhibitor—cilastatin [175]. Cilastatin is an inhibitor of dehydropeptidase I. Dehydropeptidase is present on the brush border of proximal renal tubular cells and inactivates imipenem by hydrolyzing the beta-lactam ring. IMI/cilastatin has been shown to be effective in bacteremia, pneumonia, complicated urinary tract infection, pelvic and intra-abdominal infection, skin and soft-tissue infection, and osteomyelitis [176-178]. In numerous studies, both *in vitro* and *in vivo*, endotoxin in various amounts was released when Gram-negative bacteria were exposed to certain antibiotics, such as β -lactam antibiotics, which are commonly used for the treatment of severe sepsis [31]. Treatment of Enterohemorrhagic *Escherichia coli* strains with IMI resulted in less endotoxin release than after treatment with ceftazidime [179]. However, IMI increased leukocyte adherence in the microvasculature when it killed *Staphylococcus aureus* [122].

Molecular formula: C₁₂H₁₉N₃O₄S

Chemical name: 3-[2-(aminomethylideneamino)ethylsulfanyl]-5-(1-formyl-2-hydroxy-propyl)-4,5-dihydro-1H-pyrrole-2-carboxylic acid

Usual dosing: The powder for solution for intramuscular injection is available as imipenem monohydrate 500 mg with cilastatin sodium 500 mg. The powder for solution for intravenous solution is available as imipenem monohydrate 250 mg or 500 mg with cilastatin sodium 250 mg or 500 mg respectively. Adult dosage (expressed in terms of imipenem) is 500 mg to 1000 mg every 6 to 8 hours. The maximum dosage is 4 g/day. Doses should be reduced in renal impairment [175, 180]. Some experts recommend imipenem dosing as high as 25 mg/kg every 6 hours for infants ages 4 to 12 weeks who have severe infections [181].

Adverse Effects [171]:

* Occasional: phlebitis at infusion sites; allergic; nausea, vomiting, diarrhea; eosinophilia; transient transaminitis.

* Rare: drug fever; transient hypotension; seizures.

1.5.6. Tobramycin

Tobramycin (TOB) was discovered from *Streptomyces tenebrarius* in 1967 and introduced into clinical practice in 1970. TOB is an aminoglycoside inhibiting the protein synthesis by irreversibly binding to 30S ribosomal subunit. TOB synergism with cell wall inhibitors is seen because they increase the permeability of the cell. TOB is used to treat various types of bacterial infections, particularly Gram-negative infections. Although aminoglycosides are widely used, their important serious toxicity is a mayor limitation to the usefulness, and the same spectrum of toxicity is shared by all members of the group.

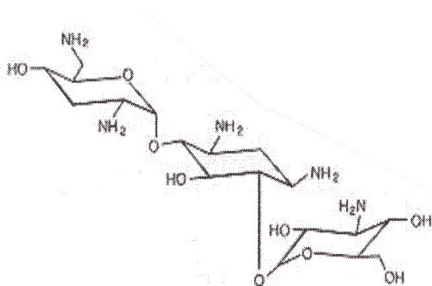


FIGURE 11: Structure of tobramycin [182].

However, aminoglycosides have been associated with low release of endotoxin [183]. TOB induced almost no endotoxin release and morphological changes when bacteria were exposed to bactericidal concentrations [30]. It is recommended for treatment of intra-abdominal infection [8, 184].

Molecular formula: C₁₈H₃₇N₅O₉

Chemical name: 4-amino-2-[4,6-diamino-3-[3-amino-6-(aminomethyl)-5-hydroxy-tetrahydropyran-2-yl]oxy-2-hydroxy-cyclohexoxy]-6 (hydroxymethyl)tetrahydropyran-3,5-diol

Usual dosing: Adult - The once-daily dosing is 7mg/kg. This regimen appeared to be less nephrotoxic than the multiple daily dosing regimen despite significantly higher doses [185].

Adverse effects [171]:

- * Frequent: renal failure (usually reversible).

* Occasional: vestibular and auditory damage (usually irreversible, genetic predisposition in some cases check family for aminoglycoside ototoxicity).

* Rare: neuromuscular blockade.

1.5.7. Vancomycin

Vancomycin (VAN), the first of a series of chemically related antibiotics, was isolated over 40 years ago in the Eli Lilly Company laboratories in the USA from a *Streptomyces* species found in soils obtained from Borneo and India. Since then producers have been found in similar locations all over the World.

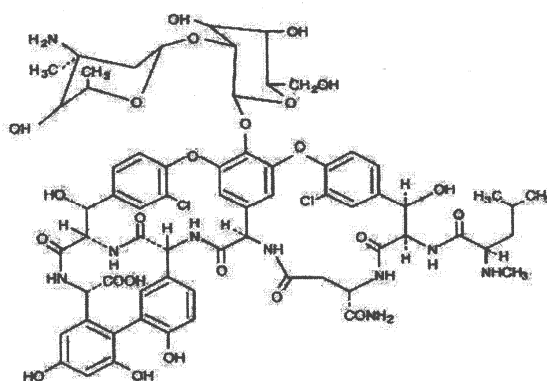


FIGURE 12: Structure of vancomycin [182].

VAN is an glycopeptide antibiotic. The spectrum of activity of glycopeptides covers essentially the Gram-positive organisms and a few anaerobes, and their activity against Gram-negative organisms is most often marginal. Biochemical studies indicate that glycopeptides inhibit the late stages of peptidoglycan synthesis [186]. The main target of VAN is the D-alanyl-D-alanine terminal dipeptide of peptidoglycan precursors, used by bacteria for constructing their cell walls. This prevents the reaction used to link peptidoglycan precursors together from taking place [187]. VAN binds with the substrate, not the enzyme. This is in contrast to the way penicillin inhibits peptidoglycan synthesis. VAN has been the most reliable therapeutic agent against infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) or methicillin-resistant *S. epidermidis* [188, 189]. VAN is used in patients who have

serious allergies to β -lactam agents [188] and prophylactically in cardiac surgery [190, 191]. In 1996 the first MRSA to acquire resistance to vancomycin was isolated from a Japanese patient [192]. VAN interfered with PMNs adherence in vitro [193], downregulated TNF- α production and inhibited TNF mRNA accumulation in LPS stimulated monocytes [194]. However, red man syndrome, which may be due to histamine release, occurs after rapid infusion of VAN [195].

Molecular formula: $C_{66}H_{75}N_9Cl_2O_{24}$

Usual dosing: Adults -The usual daily intravenous dose is 2 g divided either as 500 mg every 6 hours or 1 g every 12 hours. Each dose should be administered at no more than 10 mg/min or over a period of at least 60 minutes, whichever is longer. Other patient factors, such as age or obesity, may call for modification of the usual intravenous daily dose. Pediatric patients -The usual intravenous dosage of VAN is 10 mg/kg per dose given every 6 hours. Each dose should be administered over a period of at least 60 minutes. Close monitoring of serum concentrations of vancomycin may be warranted in these patients.

Adverse effects [171]:

- * Frequent: phlebitis.
- * Occasional: red man syndrome; hypotension and pruritis
- * Rare: neutropenia, fever, eosinophilia, allergy

1.5.8. Erythromycin

Erythromycin (ERY) was discovered in 1952 and is the most widely used macrolide. ERY is produced from a strain of the actinomyces *Saccaropolyspora erythraea*, formerly known as *Streptomyces erythraeus*. ERY prevents bacteria from growing, by interfering with their protein synthesis. It binds to the subunit 50S of the bacterial ribosome, and thus inhibits the translocation of peptides. ERY is most effective against gram-positive bacteria such as pneumococci, streptococci, and some staphylococci [171]. The antibiotic also has some effect on gram-negative bacteria and some fungi. It is used to treat such diseases as pneumonia caused by fungi, and streptococcus and syphilis infections, especially where the patient is allergic to penicillin. ERY may act as a biologic modulator that inhibits IL-8 secretion from exudative cells and thereby blocks the vicious circle of neutrophil recruitment and IL-8

generation in the inflammatory site in chronic sinusitis [196]. ERY may improve otitis media with effusion by inhibiting neutrophil accumulation in the middle ear cavity through modulating the expression of adhesion molecules L-selectin and Mac-1 on peripheral blood neutrophils [197].

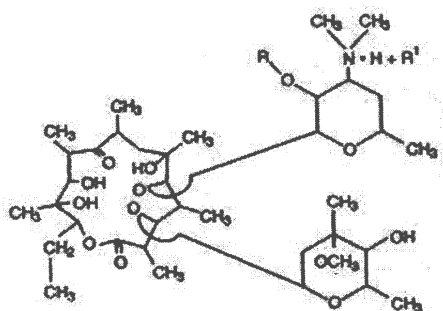


FIGURE 13: Structure of erythromycin [182].

Molecular formula: C₃₇H₆₇NO₁₃

Chemical name: 6-(4-dimethylamino-3-hydroxy-6-methyl-oxan-2-yl)oxy-14-ethyl-7,12,13-trihydroxy-4-(5-hydroxy-4-methoxy-4,6-dimethyl-oxan-2-yl)oxy 3,5,7,9,11,13-hexamethyl-1-oxacyclotetradecane-2,10-dione

Usual dosing: Adults: The usual dose is 250 mg every 6 hours taken one hour before meals. If twice-a-day dosage is desired, the recommended dose is 500 mg every 12 hours. Dosage may be increased up to 4 grams per day, according to the severity of the infection. Twice-a-day dosing is not recommended when doses larger than 1 gram daily are administered; Children: Age, weight, and severity of the infection are important factors in determining the proper dosage. The usual dosage is 30 to 50 mg/kg/day in divided doses. For the treatment of more severe infections, this dose may be doubled.

Adverse effects [171]:

- * Frequent: GI intolerance (oral-dose related); diarrhea; phlebitis with IV administration.
- * Occasional: stomatitis; cholestatic hepatitis (1:1000 especially with estolate salt formulation-reversible); generalized rash.

2. Purpose

The mainstays of clinical sepsis therapy include surgical focus sanitation, supportive intensive care therapy, as well as antibiotics. In collaboration with the results of the microbial sensitivity test, a specific knowledge of the potential effects exerted by antibiotic substances upon the microcirculation could very well become a basic factor in the choice of medication to be used within the realm of antibiotic therapy for sepsis. Clinical studies, by definition, use heterogeneous patient populations and generally have broad entry criteria, making it difficult to separate the effects of genetics, age, disease severity, and antibiotic efficacy on the microcirculation. The monitoring of pharmacological effects on intestinal microcirculation is nearly impossible during acute therapy in patients and requires sophisticated equipment when applied to experimental animals.

This investigation focused on the effect exerted by antibiotics therapy, especially metronidazole, upon the microcirculation, within experimental animal sepsis, in models of colon ascendens stent peritonitis (**CASP**) or in endotoxemia (**LPS**) using intravital microscopy (IVM) and the effects on the release of the cytokines. The intestinal microcirculation was chosen for investigation due to the fact that the intestine serves as a pathologically significant region of circulation in cases of sepsis ; “intestine as the motor of multiorgan failure” [8].

3. Material and Methods

3.1. Material

3.1.1. Experimental animals

A total of 200 male Lewis rats (Charles River Laboratories Germany GmbH, Sulzfeld, Germany), 6–7 week old, with a weight between 200–280 g were used in the investigation. All experimental procedures were performed according to German animal safety legislations. The animals were maintained under 12 hour light/dark rhythmic conditions (temperature: 25°C, humidity: 55–60%) and standard rat chow (Altromin[®], Lage, Germany). All animals had free access to food and water.

3.1.2. Antibiotics

Metronidazole (Metronidazole Delta Select[®]) was obtained from Delta Select GmbH (Pfullingen, Germany); imipenem/cilastatin (Zienam[®]) from MSD Sharp & Dohme GmbH (Haar, Germany); tobramycin (Gernebcin[®]) from Infectopharm Arzneimittel GmbH (Heppenheim, Germany); vancomycin (Vancomycin Abbott[®]) from Abbott GmbH & Co.KG (Wiesbaden, Germany) and erythromycin (Erythromycin Delta Select[®]) from Delta Select GmbH (Pfullingen, Germany).

Dosage:

- Metronidazole:	10 mg/kg	- Imipenem/cilastatin:	20 mg/kg
- Tobramycin:	25 mg/kg	- Vancomycin:	70 mg/kg
- Erythromycin:	5 mg/kg		

3.2. Group selection

3.2.1. CASP group

The animals within the control group ($n = 10$) were laparotomized (sham operation). “Control” group was investigated by intravital microscopy following a 16 hours observation period without antibiotics treatment.

Six groups (each $n = 10$) were CASP operated. One group remained untreated (“CASP”) and the other groups, post 15 hours observation period, received an i.v. short infusion of antibiotics (“CASP + MET”, “CASP + IMI”, “CASP + TOB”, “CASP + VAN”, “CASP + ERY”). Following a total of 16 hours, all groups underwent intravital microscopic investigation.

3.2.2. LPS group

In order to investigate the substance-specific effects independently of the antibacterial effects within a pathologically altered microcirculation, it was necessary to carry out a second experimental series. The animals within the seven groups (each $n = 10$) were instrumented. Six groups received 15 mg/kg b.w. endotoxin from *E. coli* (serotype 026:B6, Sigma-Aldrich Chemie, Steinheim, Germany) as i.v. short infusion. In six endotoxin groups, one group remained untreated (“LPS”), the other groups received antibiotics i.v. immediately after LPS-administration (“LPS+MET”, “LPS+IMI”, “LPS+TOB”, “LPS+VAN”, “LPS+ERY”). Subsequent to a two hour observation period, intravital microscopy was performed. The control group without antibiotics treatment was also subjected to intravital microscopy following two hour observation.

3.3. Method

3.3.1. Operative technique

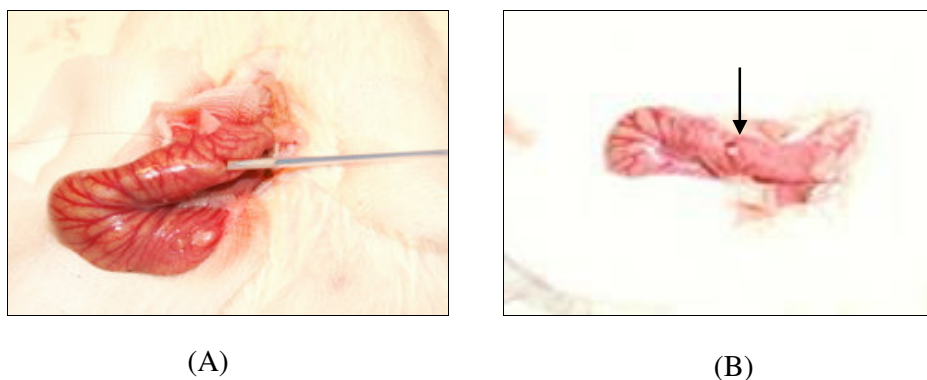


FIGURE 14: CASP surgery. (A) – 16 G stent with cannula is inserted into the ascending colon. (B)- Stent is sutured and cannula is withdrawn.

The method was adapted from the CASP mouse model [127, 143]. CASP and sham operation techniques have been described previously [198].

3.3.2. Anesthesia and monitoring

Animal preparation has been described by our group in detail recently [199-201]. Briefly, all animals were initially anesthetized with 60 mg/kg pentobarbital i.p. (Synopharm, Barsbüttel, Germany) and maintained with repeated i.v. injections of 5 mg/kg b.w. pentobarbital. The arterial line was connected to monitor (Hewlett-Packard Model 66S, Saronno, Italy) via blood pressure system (1DT-XX-1 Safe draw, Becton Dickinson, Singapore) for monitoring mean arterial pressure (MAP), heart rate (HR). Anesthesia and monitoring of MAP and HR were described detailed in our paper [198].

3.3.3. Intravital microscopy

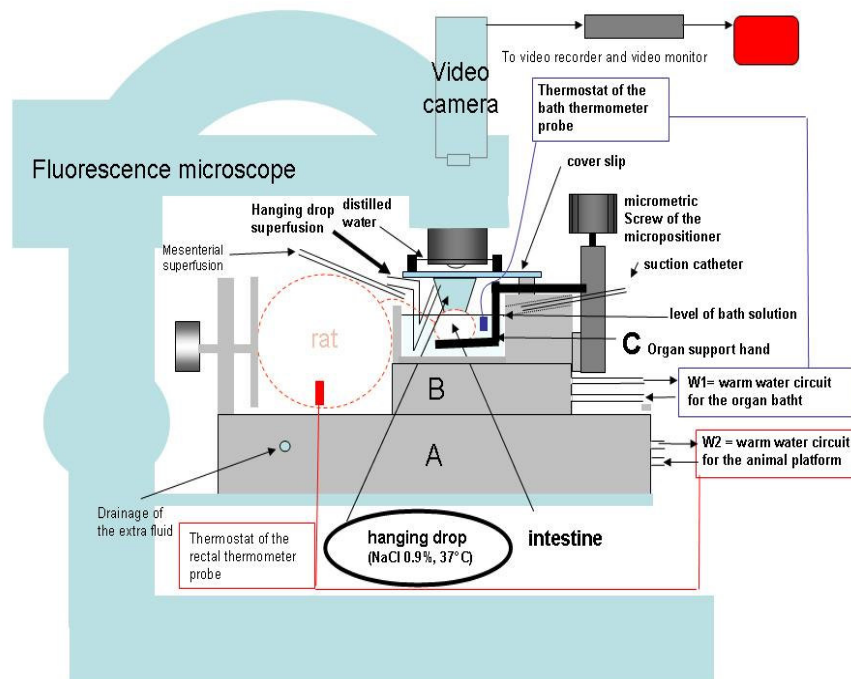


FIGURE 15: Intravital microscopy.

Intravital microscopic investigation was performed upon a 5 cm long segment of the terminal ileum proximal to the ileocaecal valve which had been isolated and held by a supporting device. A cover slip served as a transparent and plane cover. Approximately one square cm of intestinal tract was available for microscopic investigation. Technical devices of intravital fluorescent video microscopy (IVM) were described in detail in our paper [198, 202]. We improved intravital microscopy by the “hanging drop” technique. This technique prevented any tissue quenching, assured undisturbed microcirculation, provided for stable temperature and humidity, and permitted a clear visual field. The advantages of “hanging drop” technique are presented in our publication [202].

Intravital microscopy evaluated and analysed the leukocyte-endothelial interaction and the functional capillary density.

- *Leukocyte-endothelial interaction:* Staining of the leukocytes was performed through the intravenous injection of 200 μ l 0.05% Rhodamin-6G solution (Sigma-Aldrich, Steinheim, Germany) 10 min before using IVM. The microscope was then set to focus upon the submucosa of the prepared intestinal section.

- *Functional capillary density (FCD):* Ten minute before using IVM, 200 μ l of a 5% FITC-albumin solution (Sigma-Aldrich Steinheim, Germany) dissolved in normal saline was then subsequently given in order to facilitate a clearer evaluation of the capillary bed through the resultant amplified contrast of the plasma.

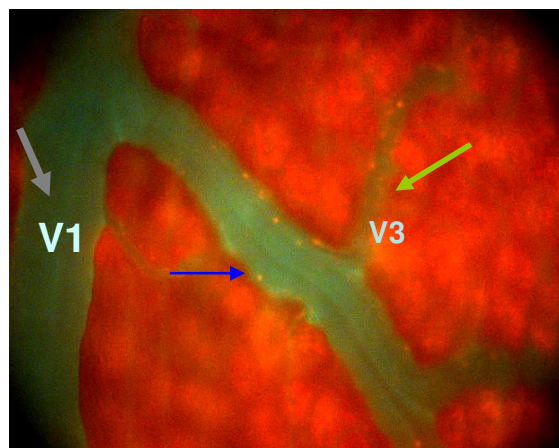
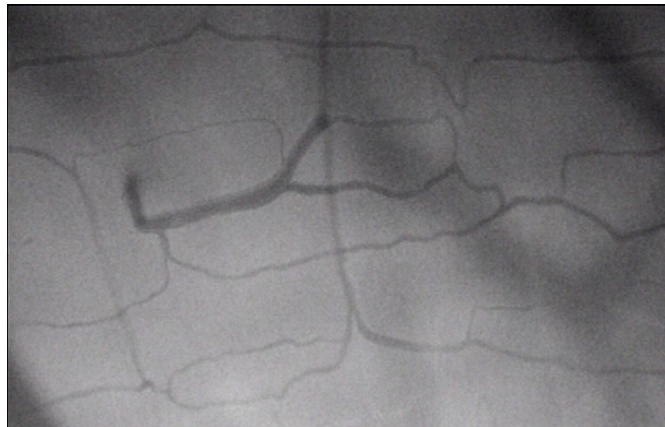


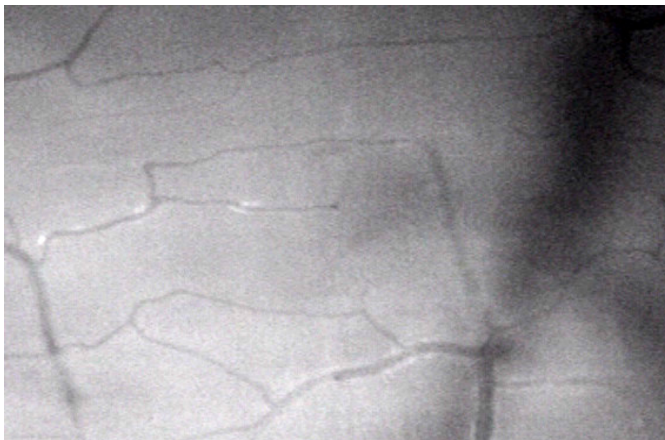
FIGURE 16: Intravital microscopy of the intestinal submucosa of a sham animal 16 hours after sham surgery. Leukocyte - endothelium interaction in collecting venule (V1) and postcapillary venule (V3) stained with Rhodamine-6G.

The following parameters were analysed: *flow of rolling leukocytes* (the number of leukocytes which during an observation period of 30 seconds pass in a rolling motion through a selected vascular diameter; [roller-flow] = cells/min), *adhering leukocytes* (the number of leukocytes which during an observation period stayed immobile for at least 30 seconds to an oblique, cylindrical endothelial surface; [sticker] = cells/mm²). The *functional capillary density* (the length of capillaries with observable erythrocyte perfusion in relation to an predetermined rectangular field; methodology according to Schmid-Schoenbein and Zweifach [203]; [FCD] = cm/cm² = cm⁻¹). The analysis of the video sequences was performed in a blinded manner by the investigators. Evaluation and analysing of leukocyte-endothelial interaction and the functional capillary density have been described detailed in our paper [198].

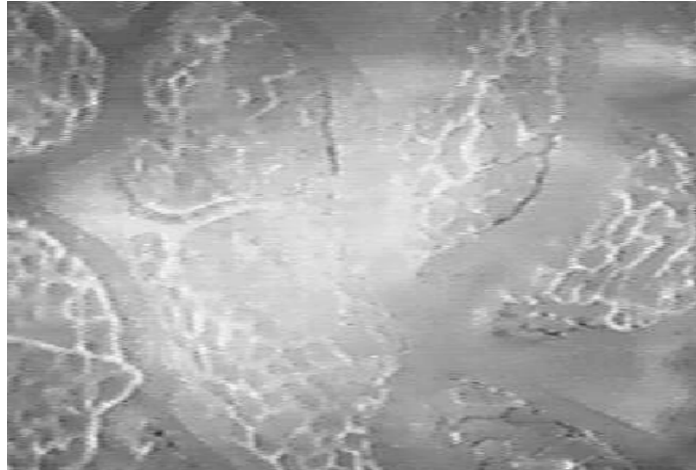
(A)



(B)



(C)



(D)



FIGURE 17: Single frames taken from the video tape registered during intravital microscopy of the terminal ileum in the rat while using FITC and illumination only from above through the fluorescence filters.

- A. Longitudinal muscular layer taken in the normal animal.
- B. Longitudinal muscular layer taken in the septic animal.
- C. Mucosa layer taken in the normal animal.
- D. Mucosa layer taken in the septic animal.

3.3.4. Cytokine determination

At the end of all experiments arterial blood samples (total volume, 1 ml) were drawn to determine release of the cytokines TNF- α , IL-1 β , IL-6 and IL-10. Cytokine levels were determined using Rat-Quantikine ELISA kits (R&D Systems, Wiesbaden, Germany). The method for cytokine determination has been described detailed in our paper [198].

3.3.5. Statistics

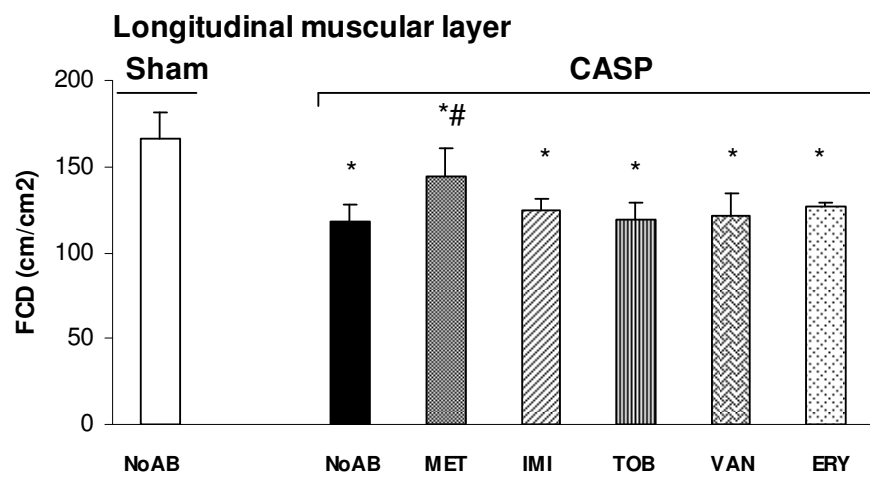
All data shown here are expressed as group means \pm standard error of the mean (SEM). FDR procedure (False discovery rate procedure) was used to determine statistically significant differences between groups, and $P < 0.05$ was considered significant. Statistics has been described detailed in our paper [198] and in publication [204].

4. Results

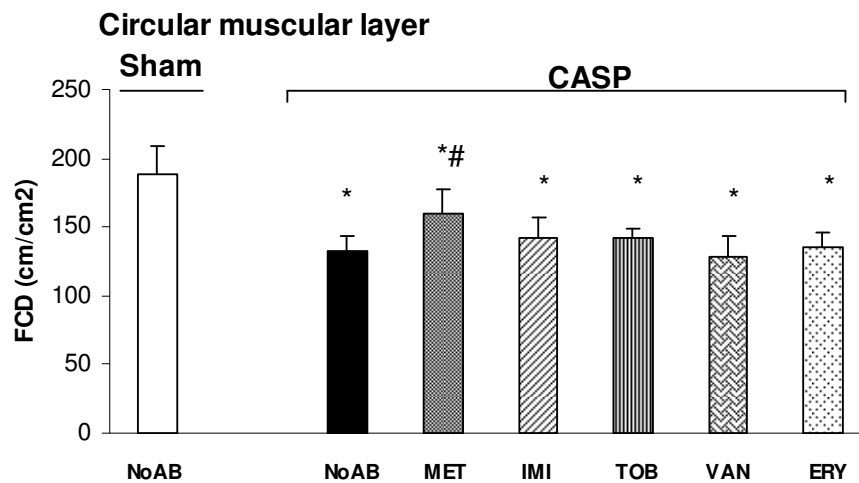
4.1. Section 1 – CASP model

CASP decreased by 30% the number of continuously perfused capillaries in muscular and mucosa layers. In the septic group FCD diminished, while no antibiotic, except metronidazole, improved FCD in muscular and mucosa layers (Fig 18. A - C).

(A)



(B)



(C)

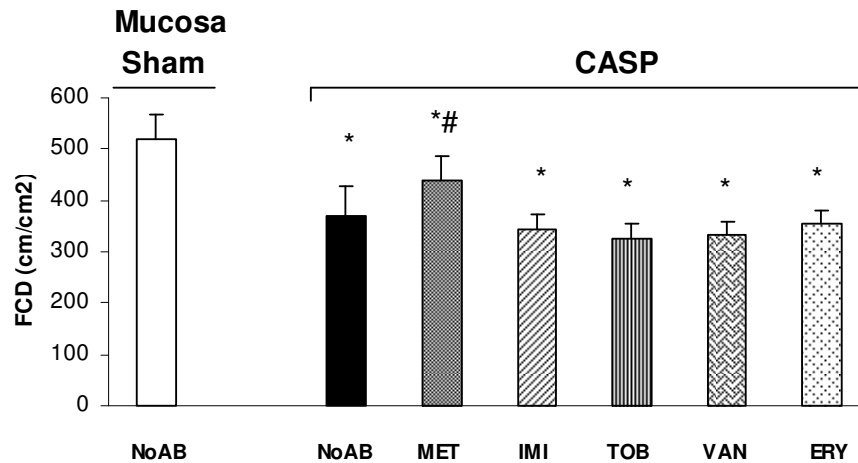


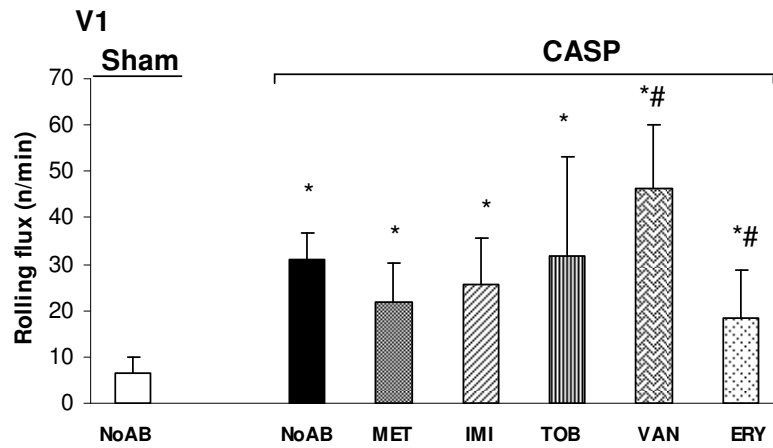
FIGURE 18: Functional capillary density in the CASP model. (A): longitudinal muscular layer; (B): circular muscular layer; (C): mucosa; n= 8-10 rats/group; * = $p < 0.05$ vs. sham; # = $p < 0.05$ vs. CASP

Observations in the CASP-model revealed a frequent increase in leukocyte-rolling within the V1 and V3 venules of the intestinal submucosa (Fig. 19 A and B). Erythromycin decreased leukocyte rolling in V1 as compared to untreated CASP rats ($18,4 \pm 10,4$ vs. $30,8 \pm 5,8$ n/min; $p < 0.05$). The increase in leukocyte rolling flux was not significant within the V3 of the CASP-animals treated with metronidazole in comparison to the sham group. Whereas VAN septic rats increased leukocyte rolling in V1 ($46,2 \pm 14$ vs. $30,8 \pm 5,8$ n/min; $p < 0.05$).

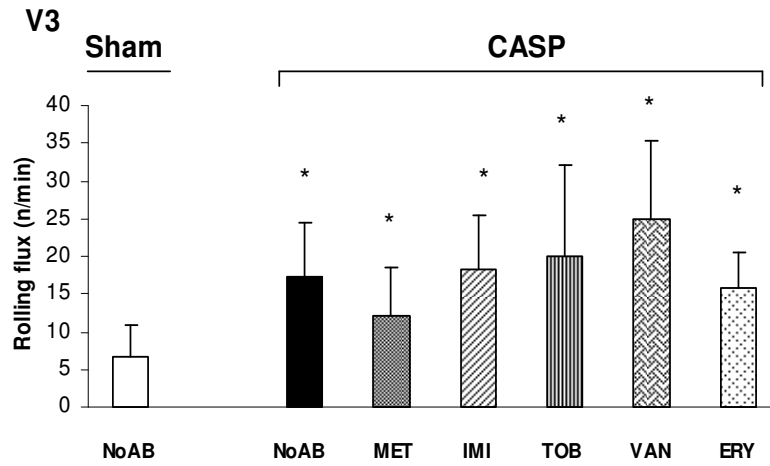
An increase in the number of leukocytes firmly adhered to the endothelium (leukocyte sticking) was registered within the CASP-animals (Fig. 19 C and D). Leukocyte sticking was more pronounced within the V-1 venules of the untreated CASP-animals than within the metronidazole (234 ± 65 vs. 145 ± 20 n/cm²; $p < 0.05$) or erythromycin treated CASP-animals (234 ± 65 vs. 166 ± 32 n/cm²; $p < 0.05$).

TNF- α release in untreated CASP rats was twice as high as in comparison to all antibiotic treated CASP rats, except in CASP rats treated with tobramycin. No antibiotics effect on the release of the cytokines IL-1 β , IL-6 and IL-10 was found in antibiotics treated CASP rats (Fig. 20).

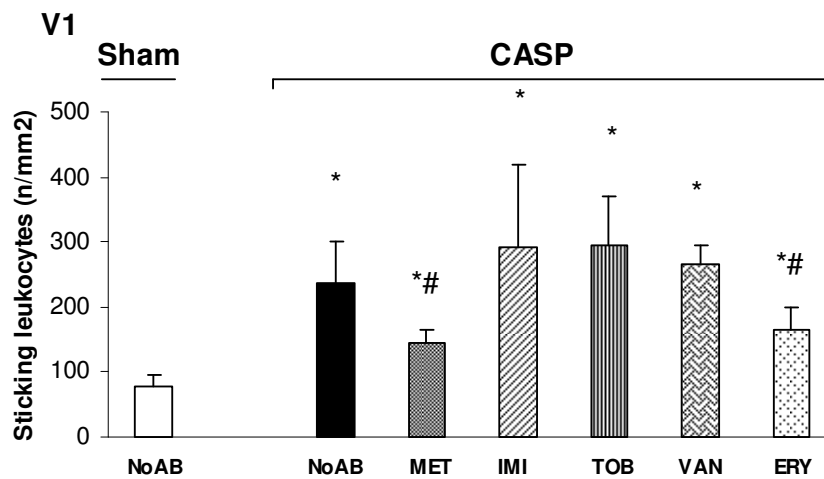
(A)



(B)



(C)



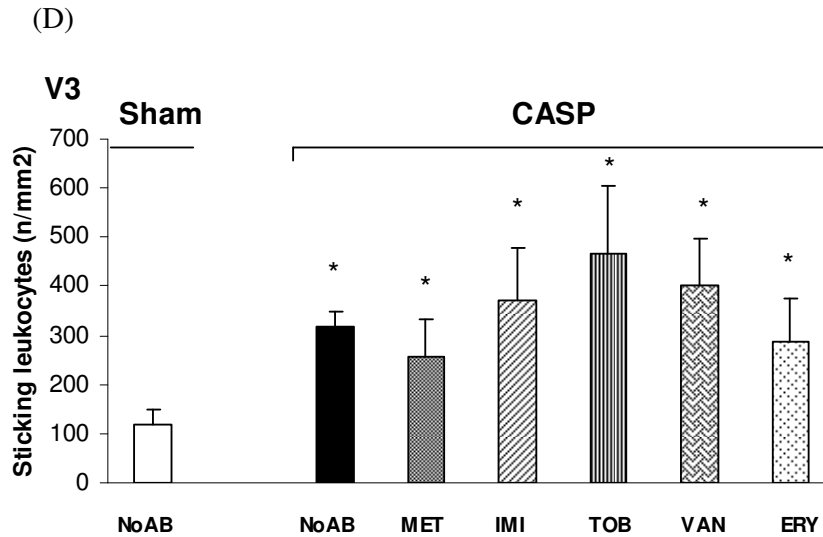


FIGURE 19: Leukocyte adherence within CASP model. (A): V1-rolling; (B): V3-rolling; (C): V1-adhesion; D: V3-adhesion; n= 8-10 rats/group; * = $p < 0.05$ vs. sham.

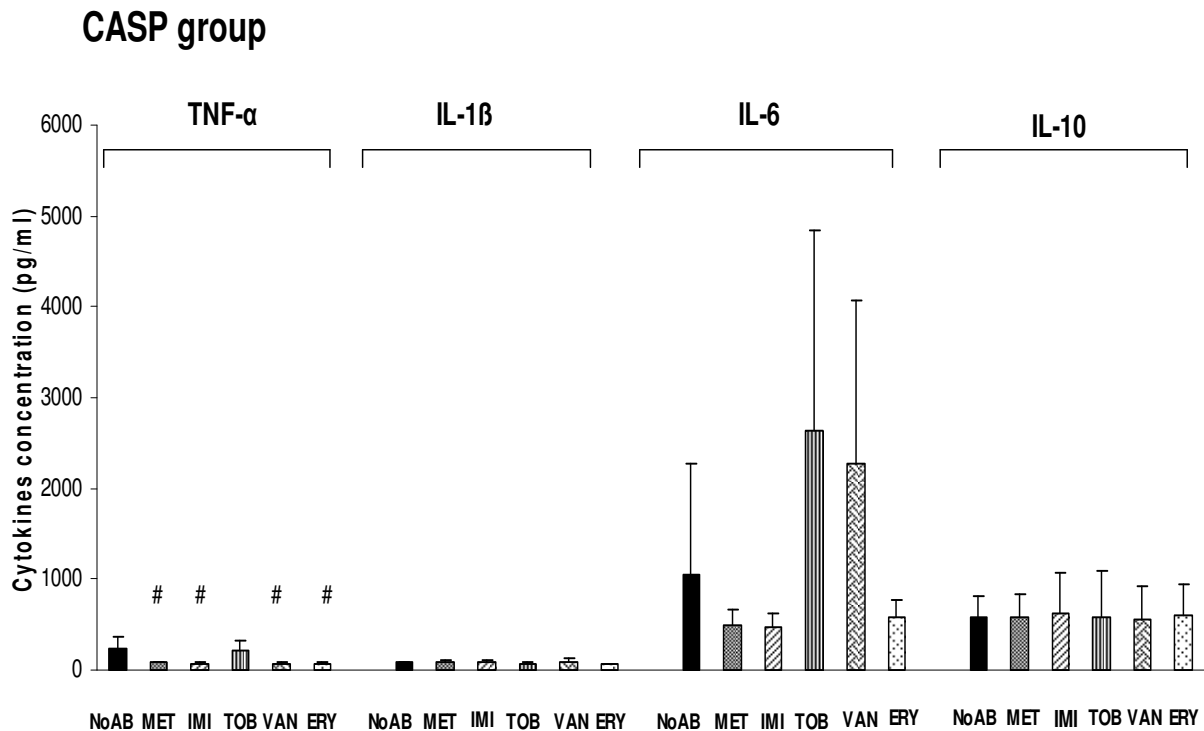
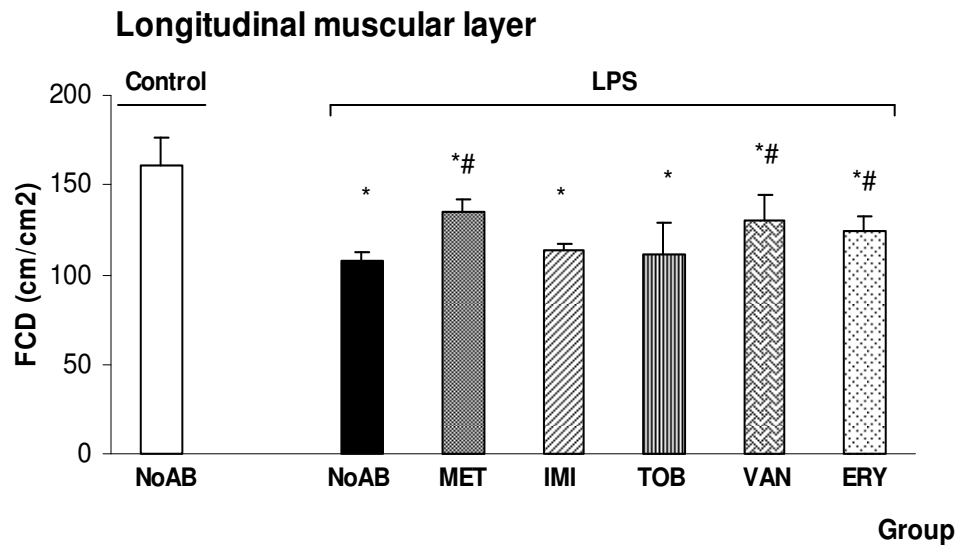


FIGURE 20: Cytokines plasma levels (TNF- α , IL-1 β , IL-6 and IL-10) in CASP rats treated with antibiotics; n = 8-10 rats/ group. Cytokines were measured 2 h after antibiotics infusion at 17h CASP surgery; # $p < 0.05$ vs. CASP

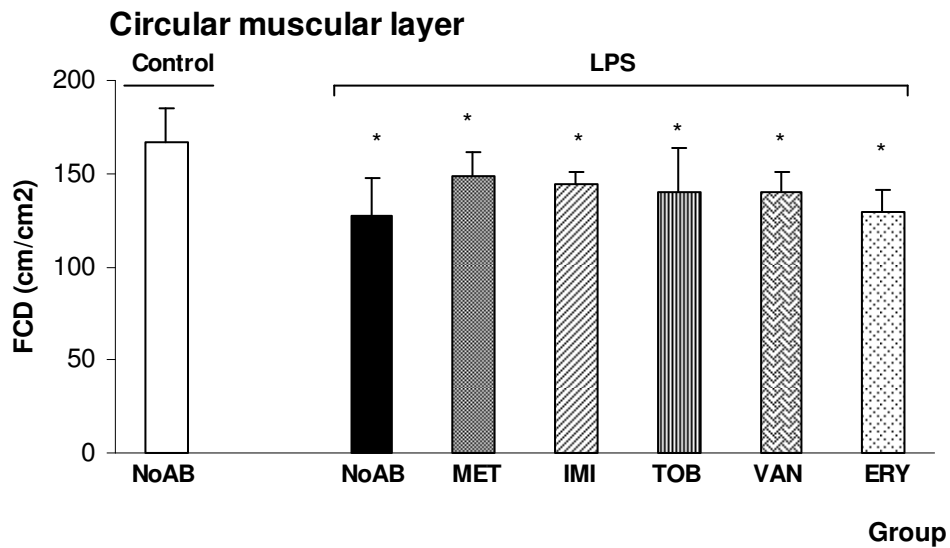
4.2. Section 2 – LPS model

As with the CASP-model, the functional capillary density was also diminished within the endotoxin model. Values for the longitudinal and circular muscle as well as mucosa were significantly lower than those for the control-animals (Fig. 21 A-C). The administration of MET or VAN or ERY led to significantly higher FCD values within the longitudinal muscular layers (Fig. 21 A).

(A)



(B)



(C)

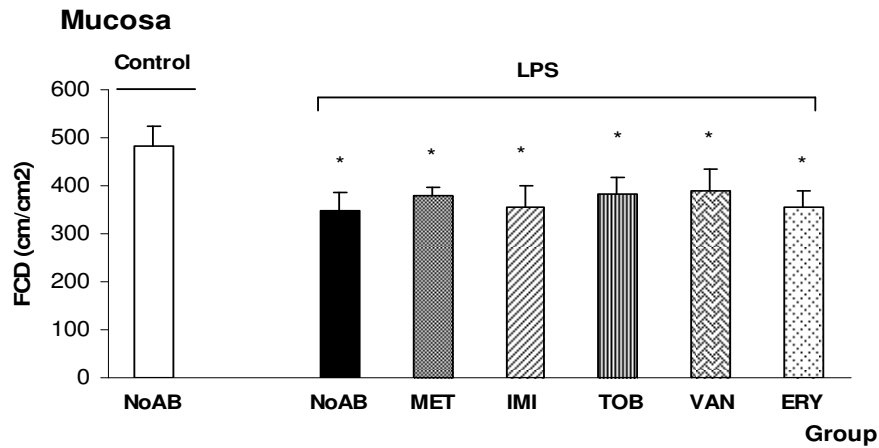
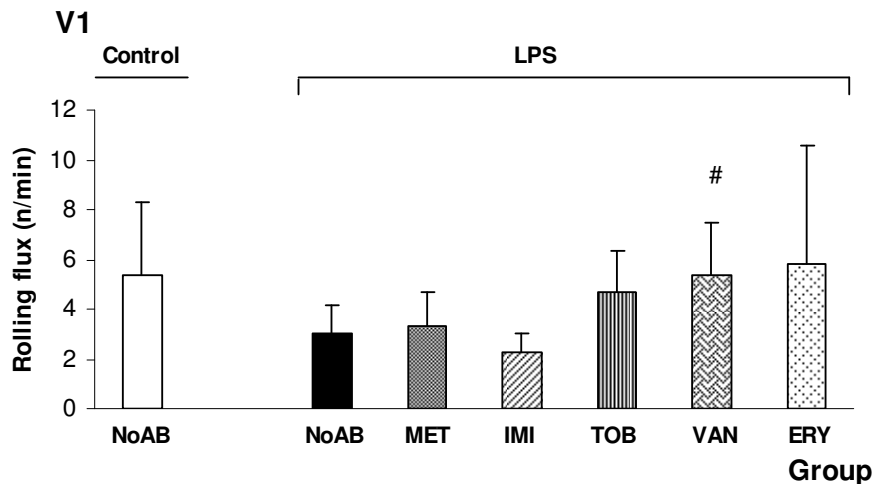


FIGURE 21: Functional capillary density in the LPS model. (A): longitudinal muscular layer; (B): circular muscular layer; (C): mucosa; n= 8-10 rats/group; * = $p < 0.05$ vs. control; # = $p < 0.05$ vs. LPS

In contrast to the CASP-model, the endotoxin-model exhibited no increase in roller flow. The number of rolling leukocytes in the endotoxin animals tended to be lower than those within the control animals whose values were similar to those of the control animals with the CASP-model (Fig. 22 A, B). Leukocyte rolling was significantly increased within the V-1 and V-3 venules of the LPS rats treated with VAN (Fig. 22 A, B). Other antibiotics had no effects on the leukocyte rolling, however leukocyte rolling tended to increase within the V-1 venules in ERY treated LPS rats (Fig. 22 A).

(A)



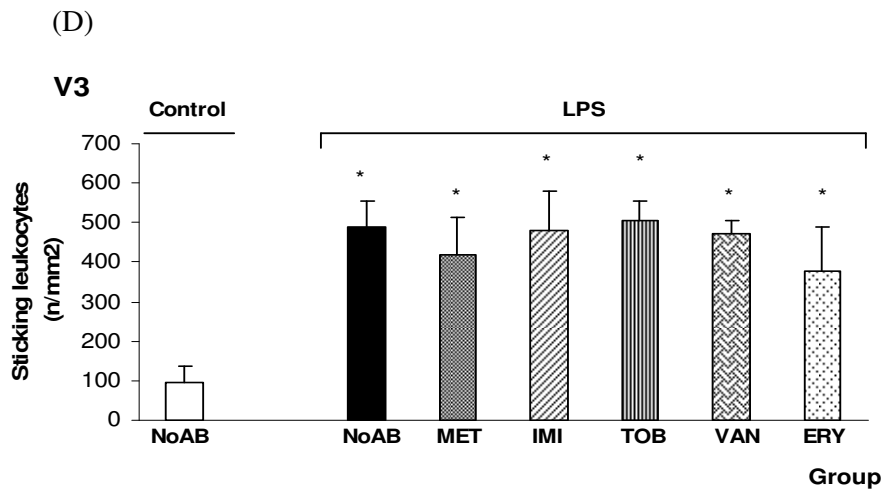
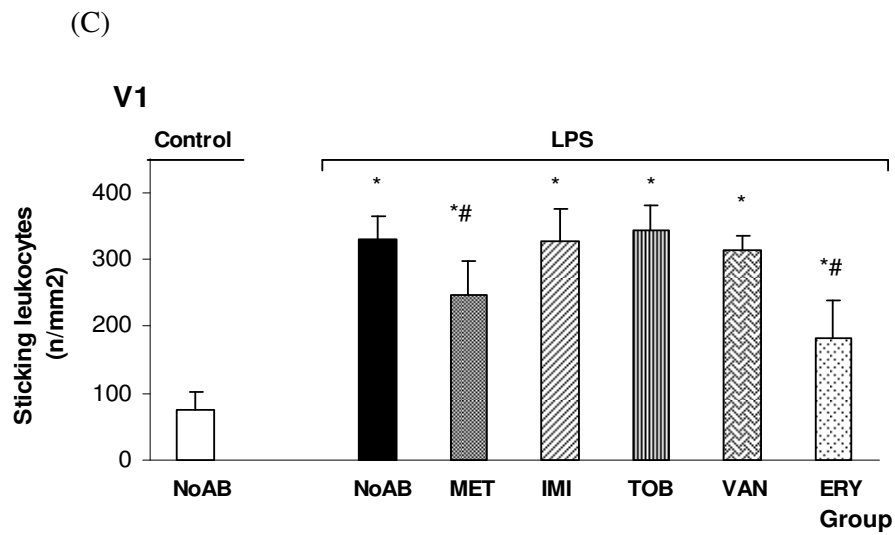
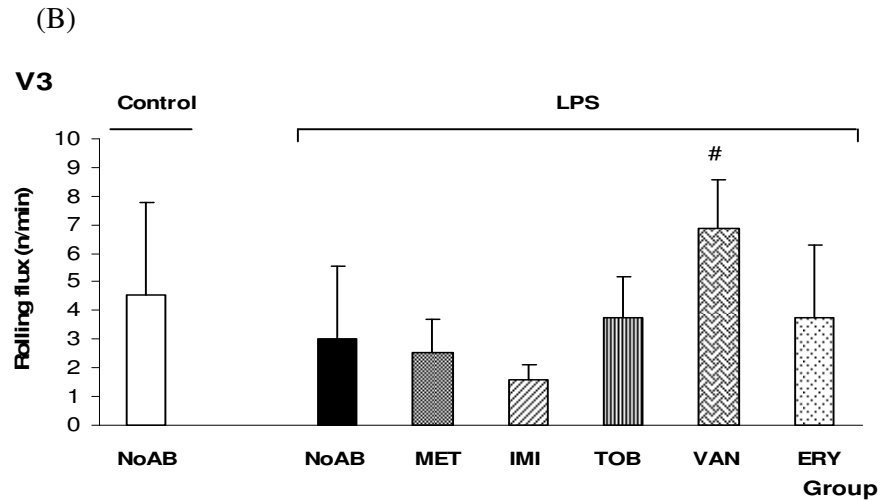


FIGURE 22: Leukocyte adherence within LPS model. (A): V1-rolling; (B): V3-rolling; (C): V1-adhesion; D: V3-adhesion; n= 8-10 rats/group; * = $p < 0.05$ vs. control; # = $p < 0.05$ vs. LPS

The number of sticking leucocytes increased substantially within the endotoxin animals as compared to the control groups (Fig. 22 C, D). Metronidazole and erythromycin significantly reduced leukocyte sticking within the V1 venules of LPS-challenged animals (LPS+MET vs. LPS: 246 ± 51 vs. $330,75 \pm 32,75$ n/cm² and LPS+ERY vs. LPS: $181,6 \pm 57,61$ vs. $330,75 \pm 32,75$ n/cm²). Other antibiotics had no effects on leukocyte sticking within the V1 and V3 venules.

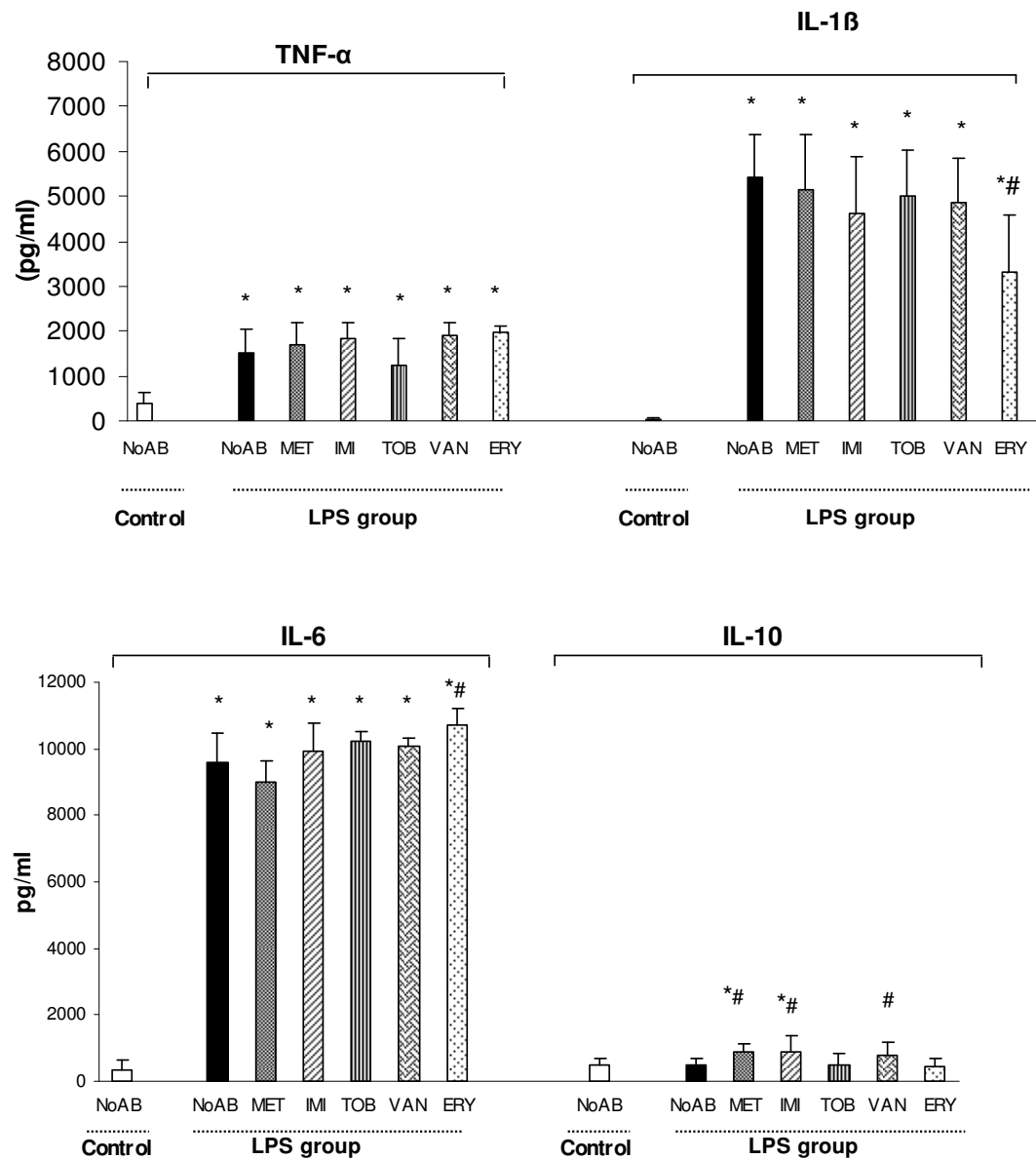


FIGURE 23: Cytokines plasma levels (TNF-α, IL-1β, IL-6 and IL-10) in LPS rats treated with antibiotics; n = 8-10 rats/ group. Cytokines were measured 3 h after antibiotics infusion at 3h endotoxin challenge; * p < 0.05 vs. control; # p < 0.05 vs. LPS

LPS caused a profound cytokine release (Fig. 23). Release of pro-inflammatory cytokines $\text{TNF-}\alpha$, $\text{IL-1}\beta$ and IL-6 was unchanged following administration of antibiotics, except in LPS rats treated with ERY. $\text{IL-1}\beta$ concentrations decreased and IL-6 concentrations increased significantly in ERY treated LPS rats as compared to untreated LPS rats. We observed increase significantly of IL-10 concentrations in LPS animals treated with MET or IMI or VAN.

5. Discussion

Metronidazole, imipenem, tobramycin, vancomycin and erythromycin are frequently used for therapy of peritonitis and sepsis [8, 167, 171, 177, 188]. However, those antibiotics may have harmful or beneficial effects on the intestinal microcirculation.

5.1. Section 1- CASP model

The monitoring of pharmacological effects on the intestinal microcirculation is nearly impossible during acute therapy in patients and requires sophisticated equipment when applied to experimental animals. Some animal studies used models which included intravascular infusion of endotoxin [205, 206] or live bacteria [122, 207], or the CLP model [208-210] for studying the effects of antibiotics on microcirculation and on cytokine release. Endotoxemia models without an infectious focus may serve as a model for endotoxic shock, but not for sepsis. Some animal studies of sepsis used a single strain of bacteria with a single injury or CLP model with abscess formation, and therefore, do not assess whether antibiotics have a similar efficacy across a spectrum of injury severity and systemic inflammatory response syndrome. Previous experiments in mice have shown, that the CASP model of sepsis closely resembles the changes in the septic patient with diffuse peritonitis following anastomosis insufficiency [127, 143, 211].

Therefore, we used **colon ascendens stent peritonitis (CASP)**, a new model of intra-abdominal sepsis that closely mimics the clinical course of diffuse peritonitis with early and steadily increasing systemic infection and inflammation to study the effects of 5 antibiotics (**MET, IMI, TOB, VAN, and ERY**) on the intestinal microcirculation and the release of inflammatory mediators in severe sepsis. Acute administration of metronidazole was associated with an improvement within the parameters of the intestinal microcirculation in septic rats (CASP). Our study have been shown that vancomycin stimulated leukocyte rolling, while erythromycin prevented the increase of leukocyte-endothelial interaction in postcapillary intestinal venules (V1) that occurred within 16h after CASP. TNF- α release in untreated CASP rats was twice as high in comparison to all antibiotic treated CASP rats, except in CASP rats treated with tobramycin. Key findings of the present study are that MET

and ERY were more potent than other antibiotics in improving the intestinal microcirculation in the CASP model.

Beneficial effects of metronidazole on the intestinal microcirculation have been discussed in our publication [198]. Recent research has also shown beneficial effects of metronidazole treatment in serum cytokine changes in Turkish children infected with *Giardia lamblia*. Bayraktar et al. found that TNF-alpha and sIL-2R levels significantly increased in giardiasis cases. IL-1beta, IL-6, IL-8, CRP, and NO levels increased only in the cases associated with allergy. All increased variables significantly decreased following metronidazole treatment and returned to normal levels [212]. Mittelkotter et al. assessed the risk factors of postoperative infections and antibiotic prophylaxis under everyday clinical conditions; they found that preoperative administration of metronidazole, in addition to a long-acting beta-lactam antibiotic, is strongly advised in elective colon surgery, as the absence of an antibiotic cover against anaerobic colonic flora leads to a significantly higher postoperative infection rate [213]. An other study demonstrated that metronidazole was more potent than amoxicillin/clavulanic acid and anti-tumour necrosis factor in improving the indometacin-induced small bowel inflammation [214].

Leukocyte–endothelial-cell interaction is essential for an effective defence against bacterial invasion [79]. During sepsis a sequential activation of leukocytes can be observed: the selectin-mediated, temporarily adhesion to endothelium (rolling), the integrin-mediated firm endothelial adhesion (sticking) and the transmigration process. In our study, CASP rats had markedly decreased functional capillary density in muscular and mucosa layer; increased numbers of rolling and adherent leukocytes compared with sham-operated control rats in submucosa venules 16h after CASP surgery. These results are similar to previous reported in CLP model [215-220]. Antibiotic action on microbes in the host can result in the release of bacterial components that may affect inflammatory response in various ways, including immunosuppressant effects on leukocyte behaviour [31, 149-151]. Leukocytic activation is associated with an increased proinflammatory immune response [85, 86], which may contribute to the widespread microvascular injury and subsequent endothelial damage observed in sepsis [88].

In CASP model, we demonstrated that IMI, TOB, VAN tended to increase leukocyte sticking; vancomycin stimulated leukocyte rolling, while erythromycin prevented the increase of leukocyte-endothelial interaction in postcapillary intestinal

venules (V1) that occurred within 16 h after CASP. However, those antibiotics had no effects on FCD of the muscular and the mucosa layer of the intestinal microcirculation in CASP rats. In numerous studies, both *in vitro* and *in vivo*, endotoxin was released in various amounts when Gram-negative bacteria were exposed to certain antibiotics, such as β -lactam antibiotics [31, 148]. However, the use of different classes of antibiotics may lead to different levels of circulating endotoxin depending on their specific binding sites and different mechanisms of action. β -lactam antibiotics have generally been found to release greater amounts of endotoxin because their target is the bacterial cell wall. In Penicillin-binding protein (PBP) 2-specific antibiotics (e.g. carbapenems) less endotoxin release was detected *in vitro* than after PBP 3-specific antibiotics (e.g. cephalosporins) [221]. Non- β -lactam antibiotics not acting on the bacterial cell wall should consequently liberate smaller amounts of endotoxin. The majority of the published data confirm this hypothesis [194, 222, 223].

Imipenem is a β -lactam antibiotic that has been shown to induce the conversion of gram-negative bacilli to round, spheroid cells followed by bacterial lysis without extensive endotoxin release [224, 225]. In a rat model of *Escherichia coli* infection, endotoxin activity was approximately 35% lower in the imipenem treated group as compared to cefotaxime treated group [226]. Treatment of EHEC (*Enterohemorrhagic Escherichia Coli*) strains with IMI resulted in less endotoxin release than after treatment with ceftazidime [179]. Rapid killing of *E. coli* by IMI has been associated with less endotoxin release than slow killing by ceftazidime, aztreonam and cefotaxime [227]. Silverstein et al. showed that IMI killed rapidly *E. coli* at 1h. Their study indicated significant differences in host response following IMI therapy in model of live *E. coli* and *S. aureus*; IMI increased leukocyte adherence in the microvasculature when it killed *Staphylococcus aureus* [122]. *In vitro*, preincubation of polymorphonuclear leucocyte (PMN) with the highest dosages of IMI (30 and 60 mg/l) was found to increase phagocytosis evaluated via both cytofluorimeter and chemiluminescence, while no effect was detected on superoxide anion production or on lymphomonocyte tests. In an *in vivo* study, the authors administered imipenem/cilastatin (1500 mg/day) to 15 elderly and diabetic patients, in whom both PMN functions (phagocytosis and superoxide anion production) and lymphocyte tests were studied before and on the 3rd and 7th days of treatment. The drug assimilation did not modify the lymphocyte parameters, whereas it increased PMN superoxide anion production and phagocytosis which were depressed in basal conditions [228]. In

our experiment, with IMI infusion of dosage 20 mg/kg, we did not observe any significant effects of IMI on leukocyte–endothelial-cell interaction, which could be explained by rapidly bacteria killing, releasing low endotoxin in IMI treatment.

Tobramycin is an aminoglycoside inhibiting the protein synthesis by irreversibly binding to 30S ribosomal subunit, and is used to treat various types of bacterial infections, particularly Gram-negative infections. Seklecki et al. using in vitro methods, showed that five aminoglycoside antibiotics (amikacin, kanamycin, gentamicin, tobramycin, and netilmicin) increased granulocyte adherence [229]. One interesting study was designed to determine whether there are similar abnormalities in leukocyte function after exposure to the action of these agents in vivo. Venezio et al. showed that four aminoglycosides (gentamycin, tobramycin, netilmycin, and amikacin) administration does not induce human polymorphonuclear leukocytes dysfunction in vivo [230]. Aminoglycosides have been associated with low release of endotoxin [183]. In vitro, TOB had no endotoxin release when bacteria were exposed to bactericidal concentrations [30]. Holzheimer et al. found TOB did not cause endotoxin release in surgical intensive care patients [231]. Total endotoxin levels after treatment with TOB was less than after treatment with cefuroxime and aztreonam as described in vitro and in vivo by Dofferhoff et al. [148]. TOB had no or low endotoxin release when bacteria were exposed to bactericidal concentrations, which could explain our observation.

In our CASP model, 2h after VAN treatment, we showed, for the first time, that VAN treatment increased leukocyte rolling and tended to increase leukocyte adherence in the venular endothelium of the intestinal submucosa, which may have harmful effects on the inflammatory response. VAN is a glycopeptide antibiotic. The most common untoward effects of intravenously administered vancomycin are anaphylactoid reactions characterized by pruritus, erythema and flushing; the extreme flushing is sometimes called “red-man (neck) syndrome” [232-234]. It has been suggested that these anaphylactoid reactions in patients treated with vancomycin result from a vancomycin-evoked histamine release from mast cells and/or basophils [235-239]. If mast cells are stimulated they release multiple mediators that delineate markers for immunologic and nonimmunologic reactions; histamine and tryptase are the two best known. Kubes et al. showed that histamine induces leukocyte rolling in post-capillary venules, mediators such as histamine are capable of inducing P-selectin expression via an alternate pathway, such that preformed P-selectin is rapidly

translocated to the endothelial surface [240]. Recent publication has been shown that histamine induced leukocyte rolling [241]. Holzheimer et al. found ciprofloxacin and vancomycin were intermediate in endotoxin release in surgical intensive care patients [231]. We suggest that VAN elicited effects on leukocytic activation that also could have involved histamine release. This hypothesis was partially confirmed by our experiments. We found that VAN stimulated an increase of leukocyte rolling in sham rats (data not shown).

Our results showed that erythromycin exerted the most favourable effects in preventing leukocyte rolling and adherence in postcapillary intestinal venules (V1) on intestinal microcirculation as compared to CASP rats treated with other antibiotics. The reduction of leukocyte adherence during sepsis might be a protective mechanism to diminish leukocyte adherence during sepsis and to prevent organ injury. ERY is most effective against gram-positive bacteria such as pneumococci, streptococci, and some staphylococci [171]. ERY prevents bacteria from growing, by interfering with their protein synthesis. ERY belong to macrolides antibiotic group. Many studies have been shown that macrolides have therapeutic benefits on chronic inflammatory airway diseases. Recently, some scientists reviewed anti-inflammatory effects of macrolides in lung disease [242, 243]. ERY may act as a biologic modulator that inhibits IL-8 secretion from exudative cells and thereby blocks the vicious circle of neutrophil recruitment and IL-8 generation in the inflammatory site in chronic sinusitis [196]. ERY may improve otitis media with effusion by inhibiting neutrophil accumulation in the middle ear cavity through modulating the expression of adhesion molecules L-selectin and Mac-1 on peripheral blood neutrophils [197]. Other macrolides antibiotics, such as clarithromycin and roxythromycin may affect the functions of neutrophils in chronic sinusitis by modulating the expression of L-selectin and Mac-1 molecules on neutrophils, thereby attenuating the adhesion of neutrophils [165]. However, for the first time, we showed that erythromycin had beneficial effects in preventing leukocyte rolling and adherence in postcapillary intestinal venules (V1) of intestinal microcirculation, an effect which may be beneficial in therapy of peritonitis. Thus erythromycin may have therapeutic value for various infectious and inflammatory disorders independent of its antimicrobial properties.

The cytokines play an important role in the cascade of the pathological events leading to septic shock. The cytokine kinetics following CASP surgery – as described in the mouse model – showed some differences to CLP experiments. CASP surgery resulted in a steadily increasing systemic inflammatory response with significantly higher plasma cytokine levels as compared to CLP experiments [143]. Further differences in the cytokine response between CASP and CLP were suggested, such as the protective effects of TNF- α in CLP but not in CASP, and, conversely, a greater role for IL-12 and IFN- γ in CASP [143]. Most cytokines could not be detected in plasma except for IL-10 in CLP model have described by Yeh et al. [244]; Ebong et al. have shown low levels of bioactive TNF at the site of infection and no detectable TNF in the plasma of CLP mice [128]. Clinical study reported that only 11 of 43 patients with sepsis had detectable circulating TNF [245]. In another study of 87 patients with sepsis, fewer than 10 percent had measurable TNF- α and IL-1 β [246]. In CASP model, we detected cytokines level in the plasma; however release of TNF α and IL-1 β were low, while IL-6 and IL-10 increased at 17 h after CASP surgery; which could be explained by early and shortly release of TNF and IL- 1 after exposure to bacterial products. We found that TNF- α release in untreated CASP rats was twice as high as in comparison to all antibiotic treated CASP rats, except in CASP rats treated with tobramycin; IL-1 β , IL-6 and IL-10 were not effected by antibiotic treatment.

Matsuda et al. examined the effects of various beta-lactam antibiotics on cell death of human polymorphonuclear neutrophils cocultured with *Escherichia coli* in vitro, they found that levels of TNF-alpha and IL-6 in the supernatants with imipenem was significantly lower than in those with other beta-lactam antibiotics [247]. Rapid bacterial killing produced less TNF release in mononuclear cell [227]. Exposure of filtrates of *P. aeruginosa* treated with imipenem to cells caused low-level TNF-production and nitric oxide, whereas meropenem induced high level of TNF [248]. Vianna et al. showed that antibiotic treatment induced an increase in survival rate and decreased plasma and peritoneal fluid levels of TNF-alpha and IL-6 at 6 and 24 h after CLP as compared with saline-treated animals, they concluded that antibiotic-induced endotoxin release is not a major determinant in the inflammatory response and prognosis in murine models of sepsis [208].

Recent studies have suggested that the beneficial effect of ERY is due to its anti-inflammatory properties. Takahashi et al. investigated effects of ERY in model of diffuse panbronchiolitis (DPB) induced by *Pseudomonas aeruginosa* inoculation, their

results showed that ERY treatment reduced TNF- α , IL-1 β and IL-8 [249]. ERY also inhibited *Pseudomonas aeruginosa*-induced TNF- α production in human whole blood [250]. In vitro, ERY inhibited the production of pro-inflammatory cytokines such as tumour necrosis factor (TNF)- α , interleukin (IL)-1 β and IL-8 [251-253], all of which were found in bronchoalveolar lavage fluid (BALF) of DPB patients. Long-term administration of erythromycin reduced TNF- α when cytokines was induced by lipopolysaccharide treatment [254].

5.2. Section 2- LPS model

To differentiate antimicrobial from anti-inflammatory effects we performed the second series of experiments using LPS model. Protective effects of metronidazole, erythromycin and vancomycin upon the microcirculation were found in the LPS model. It was found that some antibiotics may have immunomodulating effects: MET or IMI or VAN treated LPS rats increased IL-10 levels; while ERY treated LPS rats decreased IL-1 β and increased IL-6 concentration.

Contrary to CASP model, LPS administration led frequently to a decreased roller flow, whereas the increase in the number of firmly adhering leukocytes was more pronounced than in the CASP model. The functional capillary density of the muscularis and the mucosal layer was reduced; similar results were reported in previous studies [255, 256].

In the case of pathologically altered microcirculation caused by endotoxin, beneficial effects comparable to the CASP model could be documented. We assert that ERY showed more beneficial effects than MET and VAN in preventing the leukocyte-endothelial interaction in collecting venules of intestinal microcirculation in LPS model. Leukocyte adherence to the endothelium within the collecting venules of the mucosa (V1) was reduced by 45 % under the influence of ERY; it was reduced by 25% after MET treatment as compared to untreated LPS rat. FCD of longitudinal muscular layer also was improved by ERY treatment. Similar results were reported by Tamaoki et al.; they have shown that erythromycin prevents microvascular leakage, neutrophil recruitment, acute lung injury produced by lipopolysaccharide (LPS) in the rat trachea and lungs [257]. Reactive oxygen metabolites are known to promote leukocyte-endothelial interactions in postcapillary venules [258]. Substantial inhibition of

superoxide generation by human neutrophils *in vitro* was seen after treatment with erythromycin and roxithromycin [259]. This was supported by Villagrasa et al., who demonstrated an inhibition of superoxide generation and elastase release by neutrophils treated with ERY following stimulation with a chemotactic peptide [260].

Another observation in our LPS model was the remarkable decrease of the pro-inflammatory cytokine IL-1 β and increase of IL-6 following ERY treatment. The decrease of IL-1 β by ERY treatment have been described by some previous reports [249, 253]; these also have been discussed in CASP model. However, interesting observation in our LPS experiment was increase of IL-6 following ERY treatment. *In vitro* Bailly et al. showed that erythromycin increased total IL-6 production by human monocytes stimulated with LPS [261]. Our result *in vivo* confirmed the results of Bailly et al. *in vitro*. IL-6 is a pleiotropic cytokine involved in the regulation of the immune response, the acute-phase reaction, and hematopoiesis. Although IL-6 is a potent inducer of the acute-phase response, it has anti-inflammatory properties as well. Thus, IL-6 inhibits the production of LPS-induced TNF- α and IL-1 β by cultured human monocytes and in mice [262-264]; IL-6 belongs to the category of anti-inflammatory cytokines [262, 265]. Barton et al. ascertained that IL-6 increased the survival rate of mice in an LPS-GalN endotoxic shock mouse model [266]. The enhancement of IL-6 production by ERY may be involved in the suppression of IL-1 β synthesis. Some studies *in vitro* [267] and *in vivo* [268] demonstrated that treatment with fosfomycin resulted in the suppression of TNF and IL-1 β release from LPS-stimulated but an increase in IL-6 release. Recently, Hirata et al. reported that administration of clindamycin to LPS-stimulated mice resulted in reductions in TNF- α and IL-1 β concentrations and slight increases in IL-6 and IFN- γ levels in serum [269]. Fosfomycin and clindamycin experiments resembled our experimental results following the administration of ERY. It can be therefore postulated that ERY exerted a positive influence upon the intestinal perfusion not only within septic microcirculation (anti-bacterial effect) but also in a pathogenically independent manner (anti-inflammatory effect).

In LPS model, VAN treatment increased leukocyte rolling, improved FCD of the longitudinal muscular layer and increased IL-10, which may have beneficial effect on intestinal microcirculation. We observed a large number of firmly adhering leukocytes and had minimal number of leukocyte rolling in submucosal venules 2h after LPS administration in untreated animals. VAN treatment led to an increase of

leukocyte rolling in submucosal venules (V1 and V3). In contrast to the CASP model, the increase in leukocyte rolling flow represents a beneficial effect in the LPS model, because the number of firmly adherent leukocytes decreased in the same manner (not significant in our experiment). However effects of the histamine release in vancomycin treatment is also to be taken into account in the interpretation of the increased roller flux. Similar to our results, Capodisaca et al. observed that VAN interfered with polymorphonuclear adherence *in vitro* [193]. Another interesting observation in our LPS model was the remarkable increase of the anti-inflammatory cytokine IL-10 following VAN treatment as also described *in vitro* by Ziegeler et al. [270]. IL-10 was considered to be a cytokine that exhibits potent anti-inflammatory activities [271-273]. Some studies showed that treatment with IL-10 protects mice against endotoxic shock by preventing excessive production of proinflammatory cytokines [19, 78, 274, 275]. Furthermore, Siedlar et al. showed that vancomycin down-regulates LPS- TNF- α production and TNF alpha-mRNA accumulation in human blood monocytes [194]. VAN exposed anti-inflammatory actions in endotoxin model without bacterial infection.

6. Summary

I. The goal of this animal investigation was to evaluate the effects of antibiotic treatments with common antibiotics (metronidazole, imipenem, tobramycin, vancomycin, and erythromycin) upon the intestinal microcirculation in septic and endotoxemic rats.

Metronidazole and erythromycin exerted a positive influence upon the intestinal microcirculation not only in sepsis (anti-bacterial effect) but also in a pathogenically independent manner (anti-inflammatory effect); while vancomycin only displayed anti-inflammatory actions in endotoxin model without bacterial infection. Imipenem and tobramycin had no effect on intestinal microcirculation in septic and endotoxin rats.

The clinical usefulness of studies such as this is in the information that it may offer that may be relevant for avoiding potential side effects as well as for recognizing potential beneficial effects of antibiotics chosen for sepsis therapy – always keeping in mind that the microcirculation plays an essential role in the development of multi-organ failure in the instance of sepsis.

II. Observation under intravital microscope is a sophisticated technique that can be correctly performed if experimental conditions are kept under control i.e. if constant temperature and humidity are assured. Simplified techniques that are often used probably do not guarantee stability of the preparation. This is particularly true if the behaviour of blood vessels is observed. Small temperature and humidity fluctuations may have important influences on the blood vessel motility, and not only its diameter but probably on the other parameters, like leukocyte – endothelium interaction, and other tissue reactions. The described animal and organ bath may assure stable minimal conditions for intravital microscopic observation of the small blood vessels.

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Eidesstattliche Erklärung

Hiermit erkläre ich, daß ich die vorliegende Dissertation selbständig verfaßt und keine anderen als die angegebenen Hilfsmittel benutzt habe.

Die Dissertation ist bisher keiner anderen Fakultät vorgelegt worden.

Ich erkläre, daß ich bisher kein Promotionsverfahren erfolglos beendet habe und daß eine Aberkennung eines bereits erworbenen Doktorgrades nicht vorliegt.

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Metronidazole improves intestinal microcirculation in septic rats independently of bacterial burden

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Abstract. To explore the effects of metronidazole (Me) on intestinal microcirculation in septic rats, intravital microscopy (IVM) following 16 hours of colon ascendens stent peritonitis (CASP model) was used. Four groups of animals were studied: control group (sham operation) and CASP group, each with and without Me treatment (10 mg/kg i.v.). In order to investigate the substance-specific effects of Me independently of the antibacterial effects within a pathologically altered microcirculation, a second experimental series with lipopolysaccharide challenge (LPS model) was carried out. The LPS model consisted of the four groups (control animals and LPS animals (15 mg/kg i.v. LPS from *E. coli*) with and without Me). IVM in the LPS experiments was performed following a two hour observation period. Me treated CASP or LPS animals, as compared with untreated, demonstrated significant improvement of functional capillary density (FCD) of the intestinal wall. The increase in the number of leukocytes firmly adhered to the endothelium (leukocyte sticking) in the untreated CASP or LPS animals within the V1 venules of the intestinal submucosal layer, was significantly reduced in the Me treated animals. In conclusion, Me exerts beneficial anti-bacterial and anti-inflammatory effects within the septic microcirculation.

Keywords: Metronidazole, peritonitis, sepsis, endotoxemia, microcirculation

1. Introduction

The mainstays of clinical sepsis therapy include surgical focus sanitation, supportive intensive care therapy as well as antibiotics. Although the benefit of expedient antibiotic therapy remains unquestioned [1], little is known about the effects that are unrelated to their antimicrobial property but which the antibiotics may exert upon the septic microcirculation. It can be principally assumed that parallel to the antibacterial effect, an improvement of the affected microcirculation occurs. Certainly, improvement of microcirculation can be achieved also by other therapies (e.g. [2]). However, the administration of antibiotics is intended to bacteriolysis and the released toxins may, even if only temporarily, in the same way lead to reinforcement of the alterations in the microcirculation [3]. Furthermore, some antibiotics are already known to exert an effect upon vascular tonus [4]. Similarly, immunomodulating effects, such

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as the impact on leukocyte activation through antibiotics, are possible [5–7]. In collaboration with the results of the microbial sensitivity test, a specific knowledge of the potential effects exerted by antibiotic substances upon the microcirculation could very well become a basic factor in the choice of medication to be used within the realm of antibiotic therapy for sepsis.

Intra-abdominal infections rank among the leading causes of sepsis [8]. In the human intestinal tract anaerobic bacteria outnumber aerobic/facultative bacteria by as much as 1000 to 1 [9]. Although anaerobes are relatively noninvasive, antimicrobial therapy of intra-abdominal infections should include agents with toxicity for anaerobic microorganisms, e.g. broad-spectrum antibiotics and/or metronidazole.

This investigation focused on the effect exerted by antibiotic therapy with metronidazole upon the microcirculation within an experimental animal sepsis model (colon ascendens stent peritonitis – CASP). The intestinal microcirculation was chosen for investigation due to the fact that the intestine serves as a pathologically significant region of circulation in cases of sepsis (“intestine as the motor of multiorgan failure” [10]).

2. Materials and methods

2.1. Experimental animals

A total of 64 male Lewis rats, 6–7 week old, with a weight between 200–280 g were used in the investigation. All experimental procedures were performed according to German animal safety legislations. The animals were maintained under 12 hour light/dark rhythmic conditions (temperature: 25°C, humidity: 55–60%). All animals had free access to food and water.

2.2. Group selection

2.2.1. Section 1 – CASP model

Four groups (each $n = 8$) were examined. The animals within the two control groups were laparotomized (sham operation). One group of animals (“Control”) was investigated by intravital microscopy following a 16 hours observation period without any further treatment. At this time point, significant changes in the microcirculation of CASP animals could be observed in preliminary examinations. The other control group (“Control + Me”) received an i.v. short infusion of 10 mg/kg b.w. metronidazole (Delta Select, Pfullingen, Germany) dissolved in normal saline after 15 hours of observation. The antibiotic dosage administered in the rat model was chosen with a view toward approximating the levels achieved in human serum and according to other investigators, which also used metronidazole parenterally (e.g. [11]). One hour later intravital microscopic investigation was performed.

Two other groups of animals underwent CASP procedure, whereby one group remained untreated (“CASP”) and the other group, post 15 hours observation period, received 10 mg/kg b.w. metronidazole (“CASP + Me”). Following a total of 16 hours, both groups underwent intravital microscopic investigation.

At the end of all experiments arterial blood samples (total volume, 1.5 ml) were drawn to determine release of the cytokines TNF- α , IL-1 β , IL-6 and IL-10 (Rat Quantikine ELISA, R&D Systems, Wiesbaden, Germany).

2.2.2. Section 2 – Endotoxin model

In order to investigate the substance-specific effects independently of the antibacterial effects within a pathologically altered microcirculation, it was necessary to carry out a second experimental series. The animals within the four groups (each $n = 8$) were instrumented; two groups received 15 mg/kg b.w. endotoxin from *E. coli* (serotype 026:B6, Sigma-Aldrich Chemie, Steinheim, Germany) as i.v. short infusion. The first group received 10 mg/kg b.w. metronidazole i.v. immediately after LPS-administration (“LPS + Me”). The other group remained untreated (“LPS”). Subsequent to a two hour observation period, intravital microscopy was performed.

The control groups were also subjected to intravital microscopy following two hour observation, whereby one group without metronidazole (“Control”) and another with 10 mg/kg b.w. metronidazole i.v. (“Control + Me”) were examined.

At the end of the experiments arterial blood samples (total volume, 1.5 ml) were drawn to determine release of the cytokines TNF- α , IL-1 β , IL-6 and IL-10.

2.3. Experimental procedures

2.3.1. CASP operation

The method was adapted from the CASP mouse model [12,13]. Briefly, CASP procedure was carried out under anesthesia (60 mg/kg b.w. pentobarbital i.p.; Sigma-Aldrich). Following shaving and disinfection, a 2 cm long median laparotomy was performed above the symphysis. The colon ascendens was luxated from the abdominal wall and perforated with a venous indwelling cannula (16 G, Venflon, Ohmeda, Sweden) approximately 1.5 cm distal to the ileocaecal valve at the antimesenteric site. An approximately 4 mm long, centrally notched segment of the venous indwelling cannula was fixated to the colonic wall with sutures (7/0, Ethicon, Ethicon Norderstedt, Germany). Through careful palpation of the caecum it was possible to check if the intraluminal stent was correctly positioned as well as to fill the stent with faeces. Subsequent to repositioning of the colon ascendens and fluid substitution using 2 ml sterile saline solution, the layers of the abdomen (muscular, skin) were sutured continuously (5/0 Ethicon). Microbiologic examination (16 hours following surgery) revealed predominantly different anaerobic bacteria (e.g. *Bacteroides* spp., *Clostridium* spp.) as well as *E. coli*, *Proteus* spp. and enterococci.

2.3.2. Sham operation

Within the control group for the CASP-operation the stent was not introduced into the intestinal lumen, but instead fixated antimesenteric, externally onto the intestinal wall. The remaining procedural steps were performed just as with the CASP-operation.

2.3.3. Repeat operation for intravital microscopy

Anesthesia was induced via the i.p. administration of 60 mg/kg KG pentobarbital and maintained with repeated i.v. injections of 5 mg/kg b.w. pentobarbital. With the animals positioned in a supine position, polyethylene catheters (PE 50, internal diameter 0.58 mm, external diameter 0.96 mm, Portex, Hythe, Kent, GB) were introduced into the left external jugular vein and right common carotid artery. Arterial blood pressure and heart rate were measured continuously (Hewlett Packard monitor, Model 66S, Saronno, Italy). Tracheotomy was performed in order secure spontaneous breathing. A specially tempered microscopy bench served to maintain a continuous body temperature of $37 \pm 0.5^\circ\text{C}$. Volume substitution was achieved via central venous line using a normal saline solution. The final volume substitution was adjusted to be the same in all groups and the total volume supply stood at 15 ml/kg/h.

Subsequent to central venous and arterial catheterization, tracheotomy as well as shaving and disinfection, median laparotomy was performed from the xyphoid process to the symphysis. The animals were then granted a 15 minute resting phase.

Intravital microscopic investigation was performed upon a 5 cm long segment of the terminal ileum proximal to the ileocaecal valve which had been isolated and held by a supporting device. A cover slip served as a transparent and plane cover. Approximately one square cm of intestinal tract was available for microscopic investigation. Areas of intestinal region not being subjected to examination were covered with gauze, partly immersed in and continuously super-hydrated with normal saline solution maintained at 37°C to avoid dehydration and exposure to ambient air.

Intravital fluorescent video microscopy (IVM) was performed using the following technical devices: epifluorescent microscope Axiotech Vario (Carl Zeiss, Jena, Germany), light source HBO 50 (Carl Zeiss), oculars 10× (Carl Zeiss), lens 20×/0.5 Achromplan (Carl Zeiss), filter type #20 (Carl Zeiss) for examinations with Rhodamin 6G, filter type #10 (Carl Zeiss) for examinations with FITC-albumin, black and white CCD video camera (BC-12, AVT-Horn Aalen, Germany), S-VHS video tape recorder (Panasonic NV-SV120EG-S, Matsushita Audio Video, Germany), black and white monitor (PM-159, Ikegami Electronics, Germany). Within the described configurations, magnifications of ×250 as well as ×500 were achieved.

Initially, staining of the leukocytes was performed through the intravenous injection of 200 µl 0.05% Rhodamin-6G solution. The microscope was then set to focus upon the submucosa of the prepared intestinal section. Five visual fields containing non-branching, grade I stretching venules (V1) over a length of at least 300 µm, as well as yet another five visual fields revealing similar grade III venules (V3) (Fig. 1A,B) were observed and recorded for 30–60 seconds.

200 µl of a 5% FITC-albumin solution (Sigma-Aldrich) dissolved in normal saline was then subsequently given in order to facilitate a clearer evaluation of the capillary bed through the resultant amplified contrast of the plasma. Following focus setting, five video sequences (30 sec) of random fields of the capillaries within the longitudinal musculature were made as well as five fields of the capillaries within the circular muscle (Fig. 1C).

Then, the examination of the mucosa was performed through the opening of the intestinal lumen over a length of 2 cm according to Bohlen et al. [14] antimesenteric with a microcautery knife (Geiger Model-100, Monarch Beach, CA, USA). Here, faeces filled sections were preferred in order to avoid any alterations in heat temperatures along the opposing mesenteric wall. Following flushing with a body temperature, normal saline solution, the intestine was once again lifted and held by the supporting device. Sections of the mucosa directly bordering the mesentery were examined. This guaranteed not only the furthest distance possible from the incision borders but also avoided possible alterations of these mucosal sections brought on by microcauterization. Five, 30 second long video sequences of the mucosa sections, that were chosen at random, were recorded (Fig. 1D).

Evaluation of all the video sequences took place off-line on a video monitor. The following parameters were analysed: *flow of rolling leukocytes* (the number of leukocytes which during an observation period of 30 seconds pass in a rolling motion through a selected vascular diameter; [roller-flow] = cells/min), *adhering leukocytes* (the number of leukocytes which during an observation period stayed immobile for at least 30 seconds to an oblique, cylindrical endothelial surface; [sticker] = cells/mm²), *functional capillary density* (the length of capillaries with observable erythrocyte perfusion in relation to an pre-determined rectangular field; methodology according to Schmid-Schoenbein and Zweifach [15]; [FCD] = cm/cm² = cm⁻¹). The analysis of the video sequences was performed in a blinded manner by the investigators.

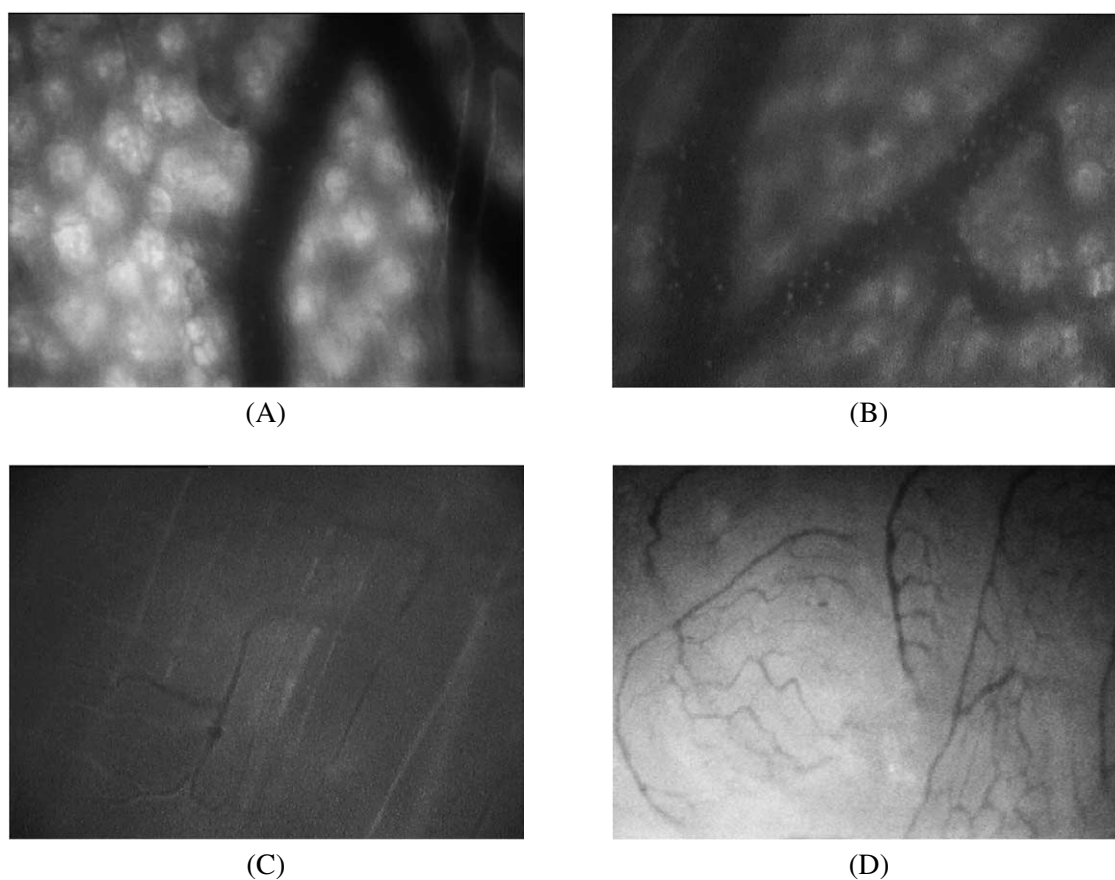


Fig. 1. Intravital microscopy of the intestinal wall. (A) control animal (submucosa, overlying A1 arteriole, beneath V1 venules with adhering leukocytes stained with Rhodamine-6G); (B) animals with 15 mg/kg b.w. LPS (submucosa, adhering leukocytes in V1 and V3 venules, stained with Rhodamine-6G); (C) control animals (muscularis circularis, contrasting of the capillaries with FITC-albumin); (D) control animals (mucosa, contrasting of the capillaries with FITC-albumin).

2.4. Statistics

Statistical analysis and demonstration of the results were performed using the statistical packages SigmaStat/SigmaPlot and SPSS (SSPS Inc. Chicago, IL, USA). A descriptive statistic was initially drawn up (mean value, variance, standard deviation, standard error). Testing for normal distribution was performed according to Kolmogorov–Smirnov.

In cases of normal distribution, single-factor mean value test comparisons were undertaken upon independent test samples (one variable, numerous groups) using single-use variance analysis (analysis of variance – ANOVA). Instances of significance were subjected to post hoc-testing with a corrective *T*-test according to Bonferroni.

In situations where the data were not normally distributed, a non-parametric variance analysis was performed (Kruskal–Wallis) as well as a subsequent corrective Wilcoxon test according to Bonferroni.

The level for significance was set at $p < 0.05$. Mean values \pm standard deviations are illustrated in the figures.

3. Results

3.1. Section 1 – CASP model

Mean arterial pressure of CASP animals was within the normal range but moderately diminished compared to sham operated animals following 16 hours of CASP and during the subsequent observation period (Table 1). Heart rate of all animals were stable throughout the experiments (Table 1).

In comparison to the control group, the functional capillary density (FCD) within the longitudinal and circular muscular layers as well as within the mucosa was decreased significantly in the untreated CASP-animals (Fig. 2). CASP animals treated with Me demonstrated significantly higher values. Observations in the CASP-model revealed a frequent increase in leukocyte-rolling within the V1 and V3 venules of the intestinal submucosa (Fig. 3A,B). The increase in leukocyte rolling flux was not significant within the V-3 venules of the CASP-animals treated with metronidazole in comparison to the controls.

An increase in the number of leukocytes firmly adhered to the endothelium (leukocyte sticking) was registered within the CASP-animals (Fig. 3C,D). Leukocyte sticking was more pronounced within the V-1 venules of the untreated CASP-animals than within the metronidazole treated CASP-animals.

CASP procedure resulted in a significant cytokine release (CASP vs. sham: TNF- α 187.1 \pm 97.5 vs. 9.1 \pm 1.5 pg/ml; IL-1 β 77.8 \pm 19.1 vs. 37.7 \pm 12.1 pg/ml; IL-6 544.1 \pm 262.3 vs. 47.3 \pm 6.3 pg/ml; IL-10 566.9 \pm 253.3 vs. 22.4 \pm 4.9 pg/ml; mean \pm SD; $p < 0.05$). TNF- α release in untreated CASP animals was twice as high as in comparison to metronidazole treated animals (Fig. 6).

3.1.1. Section 2 – Endotoxin model

LPS challenge caused a decrease in mean arterial pressure and an increase of the heart rate. Metronidazole treatment diminished the drop in mean arterial pressure of LPS challenged animals (Table 1).

As with the CASP-model, the functional capillary density was also diminished within the endotoxin model. Values for the longitudinal and circular muscle as well as mucosa were significantly lower than

Table 1
Hemodynamics

	CASP			
	Sham	Sham + Me	CASP	CASP + Me
MAP (0 h)	130 (\pm 5)	137 (\pm 11)	113 (\pm 11)*	113 (\pm 8)*
MAP (1 h)	137 (\pm 9)	139 (\pm 10)	117 (\pm 10)*	117 (\pm 11)*
MAP (2 h)	137 (\pm 5)	141 (\pm 6)	123 (\pm 17)*	123 (\pm 15)*
HR (0 h)	430 (\pm 35)	433 (\pm 15)	442 (\pm 13)	439 (\pm 12)
HR (1 h)	407 (\pm 21)	429 (\pm 9)	437 (\pm 19)	414 (\pm 19)
HR (2 h)	420 (\pm 30)	431 (\pm 12)	444 (\pm 14)	425 (\pm 28)
	LPS			
	Control	Control + Me	LPS	LPS + Me
MAP (0 h)	114 (\pm 16)	120 (\pm 7)	117 (\pm 11)	118 (\pm 14)
MAP (1 h)	124 (\pm 14)	115 (\pm 9)	88 (\pm 14)*	96 (\pm 12)*
MAP (2 h)	129 (\pm 18)	127 (\pm 6)	96 (\pm 14)*	111 (\pm 12)*#
HR (0 h)	416 (\pm 22)	432 (\pm 10)	424 (\pm 37)	428 (\pm 10)
HR (1 h)	424 (\pm 20)	439 (\pm 5)	459 (\pm 29)*	455 (\pm 11)*
HR (2 h)	400 (\pm 47)	437 (\pm 9)	479 (\pm 42)*	453 (\pm 16)*

* $p < 0.05$ vs. Sham/Control, # $p < 0.05$ vs. LPS.

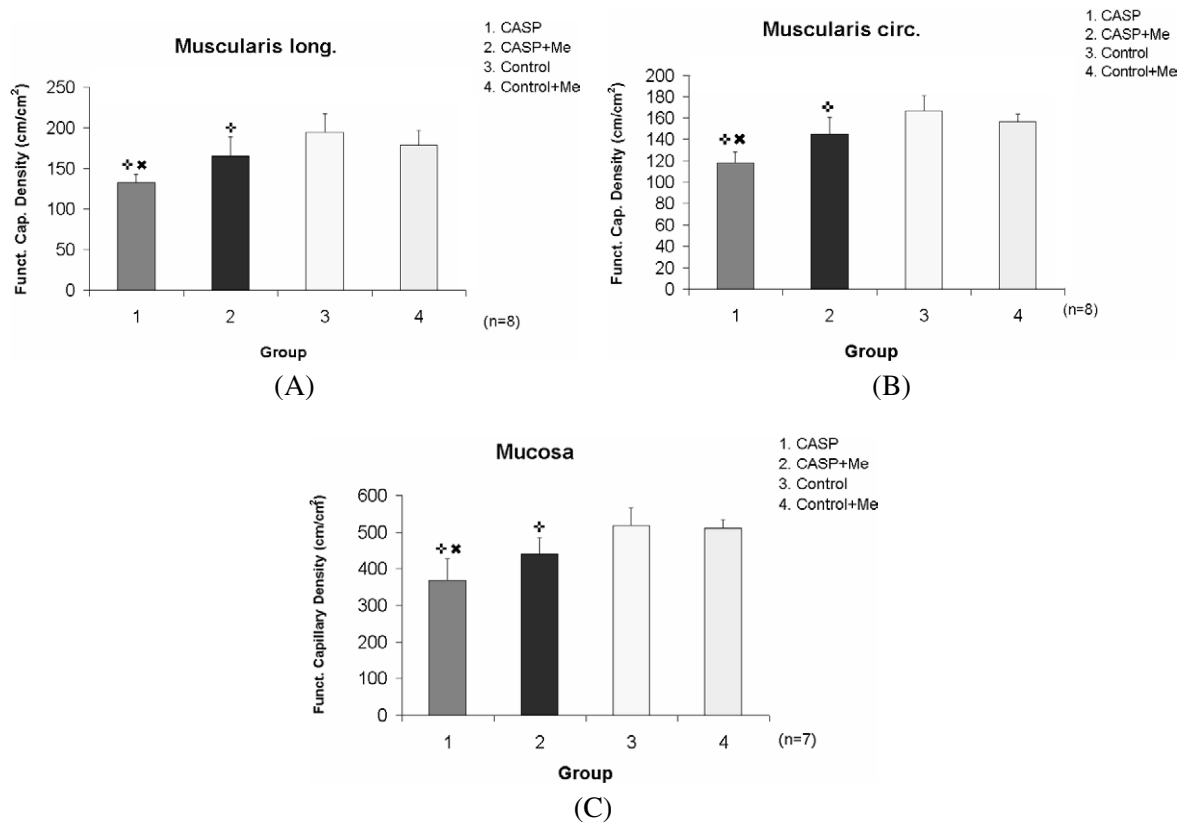


Fig. 2. Functional capillary density in the CASP model. (A) muscularis longitudinalis; (B) muscularis circularis; (C) mucosa; + = $p < 0.05$ vs. control; × = $p < 0.05$ vs. CASP + Me.

those for the control-animals (Fig. 4). The administration of metronidazole led to significantly higher FCD values within the muscular layers.

In contrast to the CASP-model, the endotoxin-model exhibited no increase in roller flow. The number of rolling leukocytes in the endotoxin animals tended to be lower than those within the control animals whose values were similar to those of the control animals with the CASP-model (Fig. 5A,B).

The number of sticking leukocytes increased substantially within the endotoxin animals compared to the control groups (Fig. 5C,D). Metronidazole significantly reduced leukocyte sticking within the V1 venules of LPS-challenged animals.

Endotoxemia caused a profound cytokine release (LPS: TNF- α 1493 \pm 360.6 pg/ml; IL-1 β 4435 \pm 653 pg/ml; IL-6 8993 \pm 158.7 pg/ml; IL-10 266 \pm 111.9 pg/ml; mean \pm SD). Release of pro-inflammatory cytokines TNF- α , IL-1 β and IL-6 was unchanged following metronidazole administration. However, we observed a 3-fold increase of IL-10 concentrations in endotoxemic animals treated with metronidazole (Fig. 6).

4. Discussion

Acute administration of metronidazole was associated with an improvement within the parameters of the intestinal microcirculation in septic rats (CASP). The protective effect of metronidazole upon the

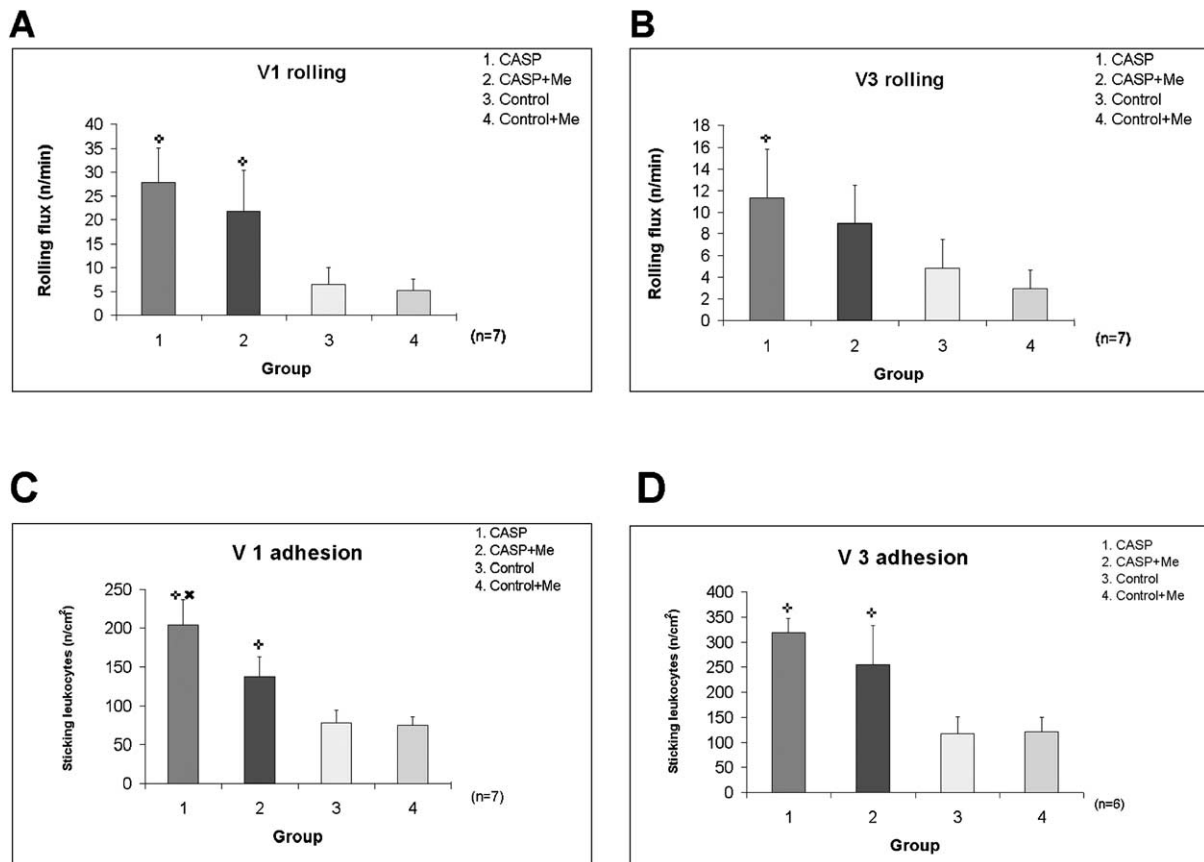


Fig. 3. Leukocyte adherence within CASP model. (A) V1-rolling; (B) V3-rolling (C) V1-adhesion; (D) V3-adhesion; + = $p < 0.05$ vs. control; × = $p < 0.05$ vs. CASP + Me.

microcirculation was documented also in an endotoxin-induced, pathologically altered condition without bacterial burden.

Metronidazole represents an antimicrobial agent with selective toxicity for anaerobic and microaerophilic microorganisms. In combination with broad-spectrum antibiotics it is frequently used in animal models of peritonitis and sepsis, e.g. cecal ligation and puncture (CLP; [16]) or polymicrobial peritoneal contamination and infection (PCI; [17]). Metronidazole and other imidazoles may also modulate leukocyte function. At therapeutic concentrations imidazoles caused a pronounced stimulation of murine lymphocyte transformation [18]. Krehmeier et al. reported, that metronidazole inhibited significantly endotoxin-stimulated TNF- α production of peripheral blood mononuclear cells [19]. For clotrimazole it is known, that it may interact with T-lymphocyte proliferation by blocking intermediate conductance Ca^{2+} -activated potassium channels [20].

Though metronidazole had no influence upon the measured parameters within intact microcirculation, it did prove to have a positive effect in experimental animals with colon ascendens stent peritonitis. Functional capillary density improved within the muscular layers as well as the mucosa. Leukocyte activation (as depicted by the firm adhesion of leukocytes to the endothelium) could be reduced as much as 30%. Rolling behaviour of the leukocytes along the endothelial wall was not significantly affected, although the roller flow means in the V1- and V3-venules tended to lower values. However, a reduction in

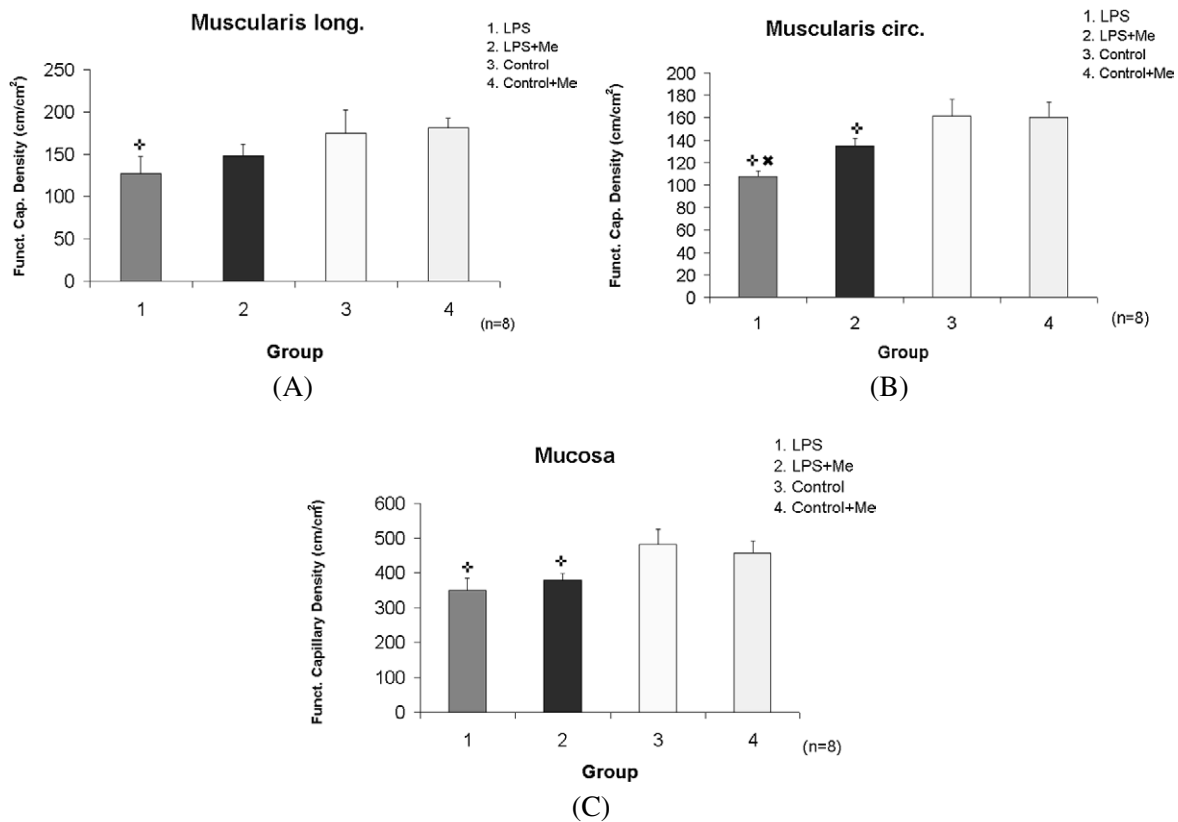


Fig. 4. Functional capillary density in the LPS model. (A) muscularis longitudinalis; (B) muscularis circularis; (C) mucosa; + = $p < 0.05$ vs. control; × = $p < 0.05$ vs. LPS + Me.

firm adhesion could also result in an increase of the roller flow (see LPS results) because of the increased pool of “free-flowing” cells. Thus, a lacking increase of temporarily adhesion may also indicate a overall reduced leukocyte activation in metronidazole treated animals. Furthermore, we found a clear attenuation of TNF- α release by metronidazole as described *in vitro* by Krehmeier et al. [19].

The results would deliver no great surprises in terms of the expected bacterial spectrum [13]. What did, however, remain unclear is whether the effect to the microcirculation was caused by the antibacterial influence of metronidazole and subsequent clinical improvement, or if a pathogen-independent effect of metronidazole was actually responsible. For this reason yet another experimental series was carried out in the absence of bacterial burden.

In the case of pathologically altered microcirculation caused by endotoxin, beneficial effects comparable to the CASP model could be documented. Functional capillary density within the muscular layers of the intestinal wall was improved. Leukocyte adherence to the endothelium within the collecting venules of the mucosa (V1), serving as a marker for leukocyte activation, was reduced under the influence of metronidazole. It therefore can be postulated that metronidazole exerted a positive influence upon the intestinal perfusion not only within septic microcirculation (anti-bacterial effect) but also in a pathogenically independent manner (anti-inflammatory effect).

Similar results were reported by Arndt et al. [21] in two abacterial models investigating the leukocyte–endothelial interaction within the mesentery of rats treated with metronidazole. For the acute model,

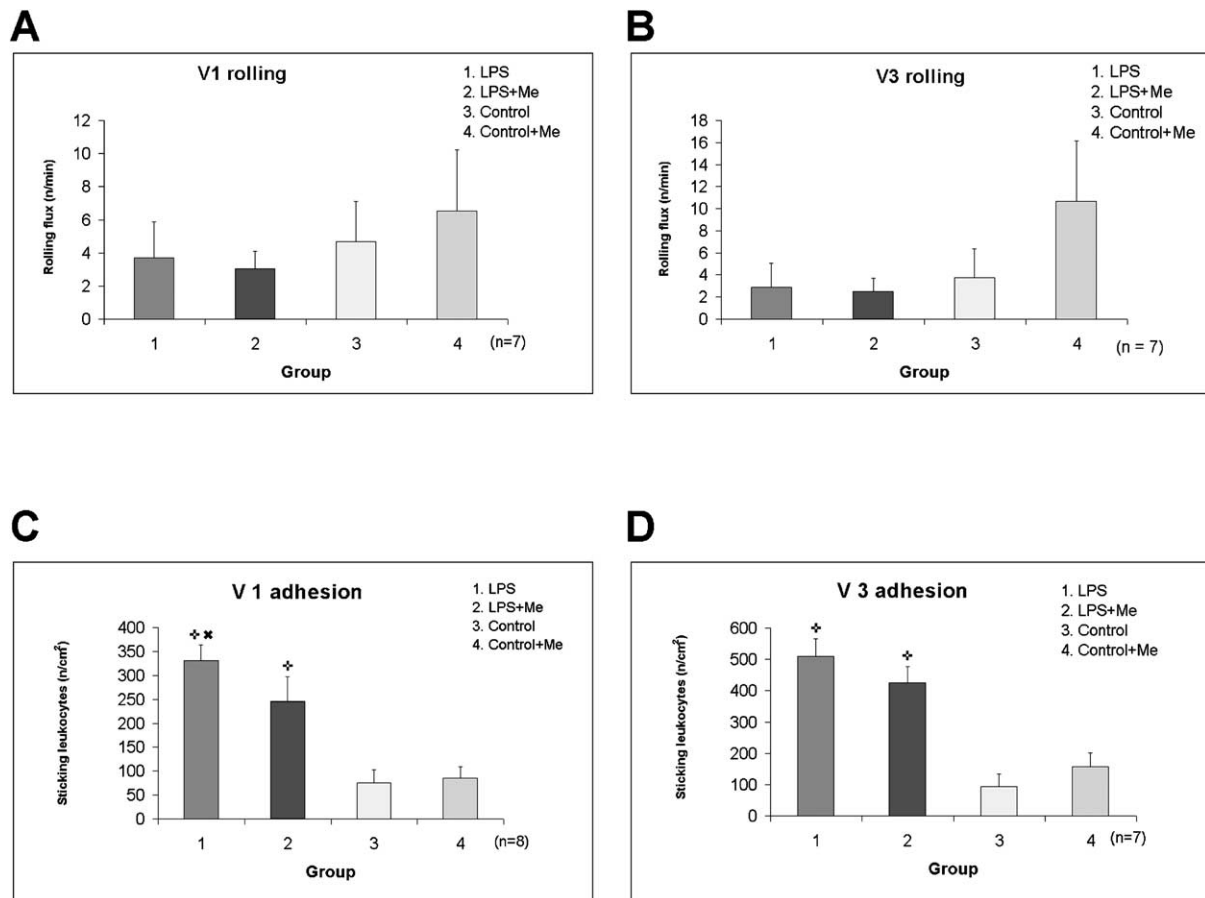


Fig. 5. Leukocyte adherence in the LPS model. (A) V1-rolling; (B) V3-rolling; (C) V1-adhesion; (D) V3-adhesion; + = $p < 0.05$ vs. control; × = $p < 0.05$ vs. LPS + Me.

an inflammatory reaction of the mesentery was achieved through leukotriene- B_4 -superfusion. For the chronic model, an inflammatory intestinal disorder was created by subcutaneous indomethacin application. In both cases the administration of metronidazole resulted in diminished leukocyte activation. In contrast to our experiments with LPS-induced microcirculation disturbances, leukocyte rolling behaviour was also influenced. Interestingly, we observed no stimulation in leukocyte rolling in the presence of LPS burden. We attribute this to the disproportionate increase in leukocyte sticking within the LPS model yielding minimal leukocyte rolling behaviour. Arndt et al. [21] suggested antioxidant properties of metronidazole as mode of action in the abacterial setting. Reactive oxygen metabolites are known to promote leukocyte-endothelial interactions in postcapillary venules [22]. Nakamura et al. [23] reported gut-derived reactive oxygen metabolites generation during intestinal surgery. Thus an antioxidant effect of metronidazole could also be beneficial during the CASP procedure and subsequent intravital microscopy.

Another interesting observation in our LPS model was the remarkable increase of the anti-inflammatory cytokine IL-10 following metronidazole treatment. It was recently shown by Bamias et al. [24] in a M. Crohn mouse model that metronidazole treatment lead to a down regulation of activated intestinal lymphocytes as well as an inhibition of intestinal Th1-cytokine production. IL-10 represents a

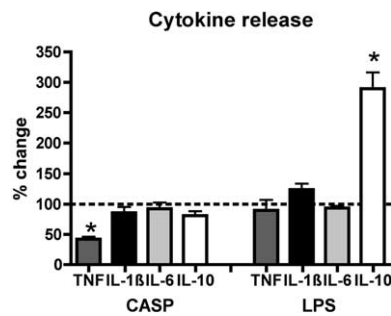


Fig. 6. Relative changes in cytokine release in CASP + Me / LPS + Me animals; * = $p < 0.05$ vs. untreated CASP/LPS animals.

Th2-cytokine. Consequently, metronidazole may shift the immunological response towards a Th2 thymocyte pattern. Although regarding the investigation of Bamias et al. [24] the role of bacterial flora in florid ileitis stood in the forefront, the same results were yielded within a control group of animals, which had not developed (bacterial-induced) ileitis. This confirms the anti-inflammatory characteristics of metronidazole beyond its antibacterial effect. Likewise other studies in colitis models reported that metronidazole treatment significantly lowered intestinal myeloperoxidase activity (e.g. [25,26]). In a cecal ligation and puncture model of abdominal sepsis, metronidazole administration resulted in reduced lung myeloperoxidase concentration [27].

5. Summary

The goal of this animal investigation was to evaluate the effect of an antibiotic treatment with metronidazole upon the microcirculation in septic rats. A significant improvement within the parameters of intestinal microcirculation was indeed observed.

The clinical usefulness of studies such as this is evident by the assistance provided in avoiding potential side effects as well as in recognizing potential beneficially effects of antibiotics chosen for sepsis therapy – always keeping in mind that the microcirculation plays an essential role in the development of multiorgan failure in the instance of sepsis.

The results of the present study may contribute to an extended indication for metronidazole in cases of anaerobic infection, although the clinical impact of metronidazole treatment would have first to be shown in controlled trials in human subjects.

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**Thermostatic tissue platform for intravital microscopy:
“the hanging drop” model**

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Abstract

Intravital microscopy (IVM) imposes the particular problem of the combined control of the body temperature of the animal and control of the local temperature of the observed organ or tissues. We constructed and tested, in the preparation of the rat ileum microcirculation, a new organ support platform. The platform consisted of the organ bath filled with physiological solution, and contained the suction tube, super fusion tube, an intestine support hand which was attached to a micromanipulator, and the thermometer probe. To cover the intestine we used a cover glass plate with the plastic ring which was glued on its upper surface. After a routine procedure (anaesthesia, monitoring and surgery) the intestine segment (2-3cm long) was gently exteriorised and placed on the “hand” of the organ support. A small part of the intestine formed a little “island” in the bath that was filled with physiological salt solution (PSS). The cover glass was secured in place. The PSS from the super fusion tube which was pointed to the lower surface of the cover glass formed a “hanging drop”. Then, the objective of the microscope was immersed into distilled water that was formed by the cover glass plastic ring. The “hanging drop” technique prevented any tissue quenching, assured undisturbed microcirculation, provided for stable temperature and humidity, and permitted a clear visual field.

Key words: intravital microscopy; thermostatic platform; hanging drop;

Introduction

Temperature control in experimental animal research is of paramount importance. The uncontrolled temperature may result in changes of a number of physiological processes and, as a consequence, the experiment would be badly controlled and the obtained results would lose their explanatory power. When doing intravital microscopy (IVM), if measures to preserve temperature and humidity are not taken, cooling and drying of the observed tissue may be very important. The small blood vessels are extremely sensitive to temperature and humidity changes and the results of the observation would be suspect, if not unreliable. The IVM imposes additional problem of the combined control of body temperature of the animal and control of the local temperature of the observed organ or tissues (Nikiforova et al., 1963).

Various methods are used to achieve satisfactory stability of the temperature and humidity in these experiments (Arndt et al., 1998; Casadevall et al., 1999; Embury et al., 1999; Foitzik et al., 1999; Lehmann et al., 2001; Massberg et al., 1998). Control of the animal body temperature is fairly well achieved by the use of the thermostatic platform where the animal is positioned during the experiment. However, control of the temperature of the observed organ or tissue has been a problem (Golub et al., 2003). Below we describe an animal and organ or tissue platform where number of mentioned problems has been solved, including temperature control and maintenance of adequate humidity.

Materials and methods

Platform Description. The platform (Fig 1) consisted of a thermostatic animal platform (A) with the organ bath (B), which were sealed together, and organ support “hand” (C) which position could be controlled by a micropositioner. Both thermostatic animal platform (A) and organ bath (B) were made of Plexiglas and supplied with independent warm water inside circuits (*w1 and w2*) and thermometer probes that were introduced either rectally to the animal, or positioned in the organ bath. This assured that, respectively, the temperature of the animal platform was adjusted after animal temperature, and that the bath solution was kept constant 37°C.

The organ bath (B) was filled with the physiological salt solution (PSS, 0.9% NaCl) and had, attached from above, a suction tube that maintained solution level in the bath. Attached to it was also a super-fusion tube, a “Y” tube, running through the warm Plexiglas block which super-fused the exteriorised intestinal segment in the bath as well the segment between abdomen and the water bath. The super-fusion tube was connected to the PSS container and the “Hotline” pump (Smiths Medical Deutschland GmbH, Kirchseeon, Germany). To cover the intestine we used a histology cover glass with a plastic ring glued onto its upper surface which provided the miniature water pond for immersion of the objective of the microscope.

Preparation of the animal. The following protocols describe the procedures that are possible in the present model and that were used in the experiments that will be described elsewhere. Some illustrative results will be however given in the “results” section.

Animal preparation has been described elsewhere (Birnbaum et al., 2003; Frieling et al., 2005; Lehmann et al., 2004). Briefly, the rats were anesthetized with 100mg/kg thiopental (Trapanal[®]) injected intraperitoneally and the jugular vein and carotid artery cannulated. The arterial line was connected to a HP polygraph (Hewlett-Packard Model 66S, HP GmbH, Böblingen, Germany) via blood pressure system (1DT-XX-1 Safedraw, Becton Dickinson, Singapore) for monitoring mean arterial pressure (MAP), while the jugular line served for fluid resuscitation and, where necessary, infusion of fluorescence agents. The animal that had its abdomen already opened was positioned on the platform on its side, its abdominal overture being as close as possible to the organ bath, and was fixed in that position.

The “hanging drop” (Fig. 2). After gentle exteriorization of the segment of the intestine (3-4cm long), it was placed on the “hand” - intestine support “hand” (c, on the Fig. 1). Its middle part was made a bit higher, and it was partially immersed in the physiological solution that filled the organ bath. The part of the intestine that was to be observed formed a small “island” 2-3mm higher than the level of the bath solution. This was achieved by adjusting bath suction level. The portion of the exteriorized intestine that was close to the animal, but not in the water bath, was covered with cotton wrap that was super-fused with warm solution through the mesenterial super-fusion tube, and prevented it from drying. The other tube (that was to superfuse the hanging drop) was then brought into close approximation with the tissue top which was above the level of the bath solution. The cover glass was then secured in place.

The physiological salt solution coming from the hanging drop superfusion tube that was pointed to the lower surface of the cover glass formed a „hanging drop“ on the lower surface of the cover glass (Fig. 3). With the micropositioner the intestine was driven upwards until the moment that it touched the „hanging drop“ on the lower surface of the cover glass, forming a solution “bubble” linking the serosal side of the intestine to the cover glass (see Fig. 2 for the demonstration and also Fig. 4). As a result, between the intestine and the cover glass remained a 1-2mm space filled with the “hanging drop” of the warm physiological solution (37°C) that was permanently perfusing the tissue (2ml/min). For measuring the temperature of the hanging drop through the experiment, a small temperature probe (commercially available with the HP polygraph) was attached to the lower surface of the cover glass (Fig 3. b and c). In addition, to obtain more precise idea about the local temperature, another probe was introduced in the exteriorised intestinal loop and pushed in to arrive close to the place which is observed (Fig. 3b and c). Then, the plastic ring that formed the little pond above the surface of the cover glass was filled with warm distilled water and the objective of the microscope was lowered into the distilled water until it touched the cover glass. The distance was adjusted so as to allow for the surface of the intestine to be brought into focus by the upward movement of the objective. This assured that the intestine did not touch the cover glass and remained in hanging drop.

Procedures and observations. The ileum segment (terminal ileum) outside of the abdomen was immersed in and permanently super fused with warm PSS (NaCl 0.9%, 37°C). Fifteen minutes before the IVM would start *in vivo* fluorescent marking of the leucocytes with 0,1 ml Rhodamine 6G i.v. (0,05%; Sigma Aldrich GmbH, Steinheim) and staining of the plasma with 1ml/kg bovine FITC-albumin

i.v. (5%; Sigma Aldrich GmbH, Steinheim) was performed. The ileum was examined using IVM, microscope optics: 20x / 0,5; (Achromplan, Carl Zeiss, Jena, Germany). The fluorescence filter set 23 (emission wave length 485-546 and excitation wave lengths 515-530 and 580-630, Zeiss, Jena) were used for two colours registration. The image obtained by a high-resolution video camera (AVT-BC 12, Aalen, Germany) was projected onto a monitor (Ikegami PM-159, Ikegami Electronics Inc., Maywood, USA) and recorded with a Panasonic video recorder (NV-HS8830, Panasonic, UK). With this equipment a 270 fold magnification was achieved. Some pictures (Figure 5C) were taken with the colours digital camera Sony DXC-S500 (purchased from AVT-HORN, Aalen, Germany). After a 15-30 minute stabilization period, first images of the arterioles and venules were recorded, and then, the serosa super-fused with vasoactive agent. Or, some animals were pre-treated with endotoxine (15mg/kg i.v.). 2 hours before the examination. The longitudinal and circular vessels of the intestinal wall, venules of the first and third order, as well as submucosa (after opening the ileum) were examined and sequences of duration of 20 sec per field were registered. All images were analyzed offline (as described previously in: (Birnbbaum et al., 2003; Frieling et al., 2005; Lehmann et al., 2004) using the software package Vlength – Interactive Irregular Shape Length Measurement (Dipl.Ing. H. Düsener, Berlin, Germany). Arteriolar and venular diameters were assessed by 5 independent measurements. After the experiment all animals were killed by overdose of i.v. thiopental.

Temperature measurements. In 3 normal animals, temperature of the hanging drop was measured through the experiment at two places: as mentioned above, the lower surface of the cover glass (Fig 3. b and c) and in the exteriorised intestinal loop (Fig. 3b and c). To investigate the effect of superfusion alone on temperature, the

intestinal segment was then at first locally superfused with warm PSS solution (37°C, 2ml/min). 15 minutes after, the organ bath heating was started and the temperature registered over 60 minutes.

Results

Here we report only the results relevant to the technical description of the model. To maintain the temperature of the organ bath solution at 37°C the temperature of the circulating water had to be significantly higher (up to 45°C), something that depended on the room temperature. The water circulation in the bottom of the organ bath did not produce any visible vibrations. The super-fusion and the bath circulation solution were set at 37°C. The constant flow of the super-fusion solution (2ml/min) that was maintaining the presence and temperature of the „hanging drop“ did not produce any visible disturbances of the obtained pictures. The cover glass that was in contact with the organ bath, which was heated, almost completely closed the organ bath (Fig. 2) while it was constantly receiving warm PSS from below (super fusion of the “hanging drop”). These assured preservation of the warmth and humidity surrounding of the exteriorised intestine and isolated it from the heat loss that was possible through the close vicinity of the cold objective of the microscope. The temperature of the hanging drop was maintained constant, 37°C, through the experiment. As it could be seen on the Fig. 4, superfusion of the limited section of the tissue could not assure maintenance of the normal temperature of the exteriorised intestinal loop and after 15 minutes after the superfusion flow of 2ml its temperature was stable 33°C. Following organ bath heating and animal support platforme heating about 30 minutes were needed to establish stable temperature of the drip and intestinal segment of about 37°C.

The “hanging drop” permitted a clear visual field (Fig. 5), permitted a sufficiently large movement of the objective over the tissue of the intestine and assured its

position close to the cover glass. Micrometric movement of the organ support handle and the strategy of first creating of the „hanging drop“, establishing a contact of the tissue with the „hanging drop“ and then moving upward the objective and bringing the tissue in focus, prevented from tissue quenching and assured undisturbed microcirculation particularly during the observation of sensitive ileal mucosa. If relatively fast application of pharmacological agents as super-fusion was to be done, pre-warming of the solution was needed.

Discussion

We describe technical solutions that assured well controlled tissue humidity and temperature, facilitated avoidance of tissue quenching, allowed super fusion with pharmacological agents, and assured a transparent visual field. The use of the “hanging drop” is only originality of the above described organ bath and the other technical details that were used to construct it, closely resemble previously described animal and organ platforms (Golub et al., 2003; Gore et al., 1977; Itoh et al., 1993; Madorin et al., 1999; Neviere et al., 1999).

Regarding observation of the intestinal microcirculation in the rat we improved the previously used organ platforms resolving, to great extent, the number of drawbacks. We used a method that is well known: a large water-filled thermostatic Plexiglas platform, and added to it a organ bath. The animal platform, the organ bath and the bath solution and super-fusion fluid heating required four separate circuits if an adequate temperature was to be achieved. The super-fusion fluid had to be kept at 37°C as well as the solution for the organ bath perfusion, to allow for fast perfusion of the tissue or the entire organ bath, if necessary. Our temperature experiment demonstrated that if only superfusion of the observed field would be used, a perfusion flow of 2ml/minute would be insufficient to maintain normal temperature. However a complete immersion of the tissue segment in warm solution would assure stable and normal temperature of the hanging drop and observed tissue. We did not test how big segment of the intestine not immersed in the warm solution would suffice for significant drop in segment temperature, although it

appeared that the bigger the un-perfused segment the lower temperature was to be expect after relatively short time of exposure to the ambient.

The advantages of the model. Exteriorisation of tissue that is often practiced (Hungerford et al., 2000; Dansker, 1958) and number of other below given references) requires good control of temperature and humidity. The described model assured that the temperature was well controlled. The entire tissue segment was at 37°C and permanently immersed in the physiological solution. The „hanging drop“ assured perfect visibility. The optical way (objective – distilled water – cover glass – „hanging drop“ – tissue) assured extremely high resolution. It is theoretically possible to use very thin glass and achieve very high magnification – as high as the thickness of the living tissue would permit. The cover may be a metal plate with a window with very thin glass. The working distance would depend on the thickness of the glass between objective and the tissue.

An important advantage of the model is that it may permit observation of relatively local effects of the agents. Most often it is needed to observe just local effects of the agents and not to have more general or systemic effects. The proposed model permits this. The entire superfusion (mesenterial superfusion and hanging drop superfusion, see fig 1) drains in the organ bath where, in fact almost entire organ is close into (since it is, from above, covered by the cover slip glass). The drug, if used, is perfused through the hanging drop that covers just small surface of the organ that comes out of the solution just below it. Therefore a local effect could be observed. The solution, nevertheless, drains in the organ bath which has about 40-50 ml and is either strongly diluted or, if needed, could be completely washed with high flow of fresh solution, particularly through mesenterial superfusion.

The most significant advantage of the “hanging drop” model, apart from temperature and humidity control, is that the quenching of the tissue could be completely avoided. This is important since capillary circulation collapses at extremely low pressure (having very low intramural pressure by themselves and almost no wall rigidity). In addition, the organ bath that was about 3 cm higher from the animal support platform permitted for trans-illumination of the tissue from all sides.

Drug administration is often done just locally by superfusion (Atwood et al., 1998; Scalia et al., 1999; Tyml et al., 1998). In the presented model the super-fusion tube allows for the local application of the pharmacological agents directly into the hanging drop. We have described the efficacy of this direct application earlier (Frieling et al., 2005). However, the super-fusion solution had to be pre-warmed.. Higher flow of the PSS in the organ bath assured that the concentration of the agent that might affect the larger segment of tissue, was about 100-1000 times lower than that applied on the tissue under observation.

Fluid level. The level of the liquid in the bath is well controlled since it is lower than the abdominal opening, i.e. the abdominal opening is slightly higher than the wall of the bath so that the bowel “descends” toward the support - organ support hand, Fig. 1 (c). The solution can not be drained out of the bath in the direction of the abdominal opening (please see the figure). If the level of the liquid mounts, it is suctioned automatically. Often, piece of cloth, rat hair, catheter loop may be

interposed and in fact drive some solution over the border and the entire platform has to be in a Plexiglas tray to permit maintenance of the dryness of the working place.

The earlier models, and we suspect probably almost all earlier models, did not permit good temperature and humidity control. How much this affected the results (published over the years by various labs) is hard to say and the experiments that would verify this could be performed. The presented model does not have advantages over other models in better visualisation of the structures. Advantages are better control of humidity and temperature. It is possible that if the temperature and humidity were not controlled that number of capillaries that can be identified would not be the same, as compared to the other models. As we mentioned earlier, this could be verified in carefully conducted experiment. All levels of the bowel wall in the rat could be visualised, However the deeper the focus, the less sharp picture were obtained. The round structures that can be seen in the Fig. 4B represent the basis of the intestinal villi, which are in the interior of the bowel, seen through all thickness of the bowel. To observe the vilus circulation itself the intestine has to be opened and observed from the mucosal side. This is routinely done but in this paper the figures representing the vilus microcirculation are not given.

Visibility and distance. The intravital microscopy of the intestine and of the most of other preparations normally does not use extremely high magnification that would require very short distance. However, the intestinal wall can be brought close to the cover slip or even permitted to touch it slightly. The minimal distance is determined by the coverslip. Very thin cover slips are needed if very short distance is needed, what is seldom the case in the intravital microscopy of the intestine. If on the

contrary longer distance is needed (when using long distance objectives) the depth of the water pond on the upper side of the cover slip has to be augmented, what could be done by increasing the height of its wall. To do this other, thicker plastic ring should be glued on the upper surface of the cover slip.

Disadvantages of the model. The presented model may have a drawback in the fact that it permits for eventual local tissue washout since its great part is permanently immersed in circulating physiological solution. This however has not been examined in depth and disadvantages linked to it are only hypothetical. In addition, the present model needs two small circuits of heated water and impose tubing which some investigators find cumbersome. The set up could be, nevertheless, easily adapted to use electric hot plates that would heat the animal or the organ bath, both supplied with thermometer probes and auto control of the temperature, what would probably simplify the system.

Conclusion. Observation under intravital microscope is a complicated technique that can be correctly performed if correct temperature and humidity are assured. Simplified techniques that are often used probably do not guarantee stability of the preparation. This is particularly true if the behaviour of blood vessels is observed. Small temperature and humidity fluctuations may have important influences on the blood vessel motility, and not only its diameter but probably on the other parameters, like leukocyte – endothelium interaction, and other tissue reactions. The described animal and organ bath may assure stable minimal conditions for intravital microscopic observation of the small blood vessels.

Acknowledgements

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Figure legends

Figure 1. (a) Schematic two dimensional representation of the entire setup (b) Close up of the organ bath to displaying the hanging drop (which is, to permit more visibility, shown un-proportionally large). The animal, its mesenterium and the exteriorised intestine are presented very schematically with the dashed lines. (c) three dimensional representation of the close up with the cover slip lifted, while all other pieces around it are removed (microscope, tubing, warm water circuits, thermostats, micropositioner, and customary video material; all dimensions are given in mm). (See the methods for more explanation.)

Figure 2. Demonstration of the hanging drop (to take a photograph of the animal preparation below the cover glass is relatively difficult and we give only a simulation performed in the lab). A. demonstration of the hanging drop with a cover glass and the finger, B. View from above C. schematic representation of the hanging drop viewed from above.

Figure 3. Close-up photo of the organ bath. The portion of the exteriorised intestine between animal and the segment under observation is displayed uncovered and plastic ring that serves to immerse the objective of the microscope were removed for clarity (a). Normally it is covered with a cotton wrap and aluminium foil (b and c) and superfused with the warm PSS, the same used for superfusion of the intestine under observation. Panels b which is a photograph taken from above, and the scheme c show the exteriorised intestinal loop, organ superfusion catheter, and the position of the temperature probes.

Figure 4. Follow up of the temperature of the hanging drop, interior of the intestine and rectal temperature of the animal during only superfusion of the hanging drop with warm PSS (37°C; 2ml/min) during the first 15 minutes and after heating the organ bath and animal platform (the last 45 minutes) (n=4; mean \pm SD).

Figure 5. Single frames taken from the video band registered during intravital microscopy of the terminal ileum in the rat while using rhodamine and FITC and illumination only from above through the fluorescence filters. The horizontal bar indicates 100microns.

a. Muscular longitudinal layer taken in the normal animal. The capillaries and pre-capillary vessels that run longitudinally on the most superficial layer of the wall of the terminal ileum.

b. Large more superficial venule (V-1) in a control animal.

c. Deep vessels (V-3 venule) with sticking leucocytes (rhodamine stained) taken in the animal pre-treated with endotoxine (15mg/kg i.v. 2 hours before the examination). This is a deep vessel and the marked leucocytes are only in focus. In the background it is possible to see the basis of individual intestinal vili.

Figure 1a

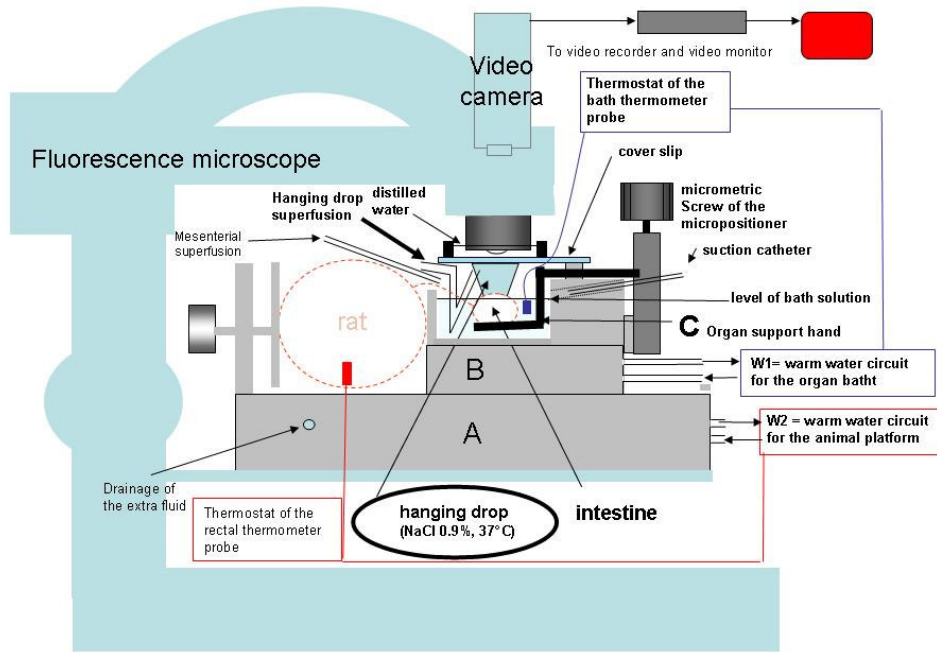


Figure 1b

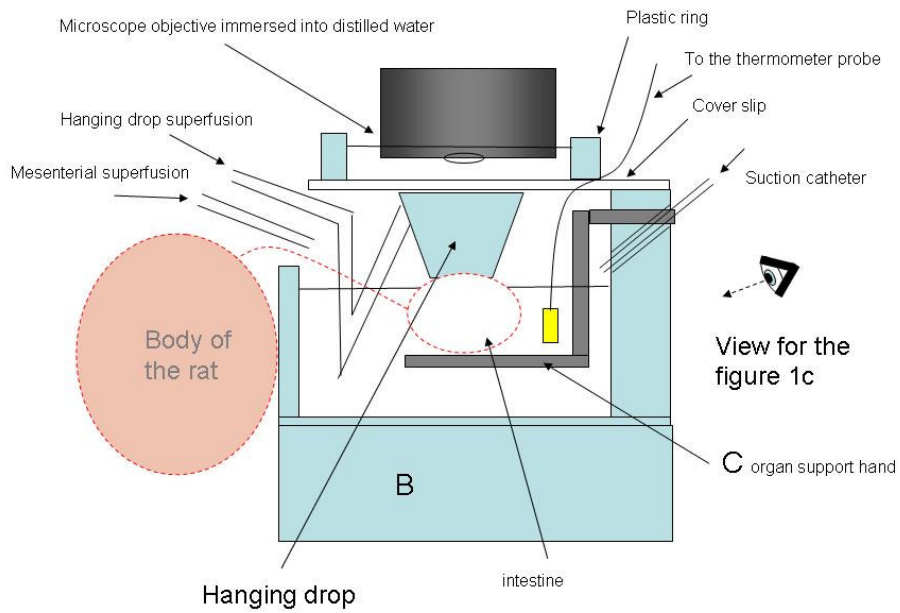


Fig 1 c

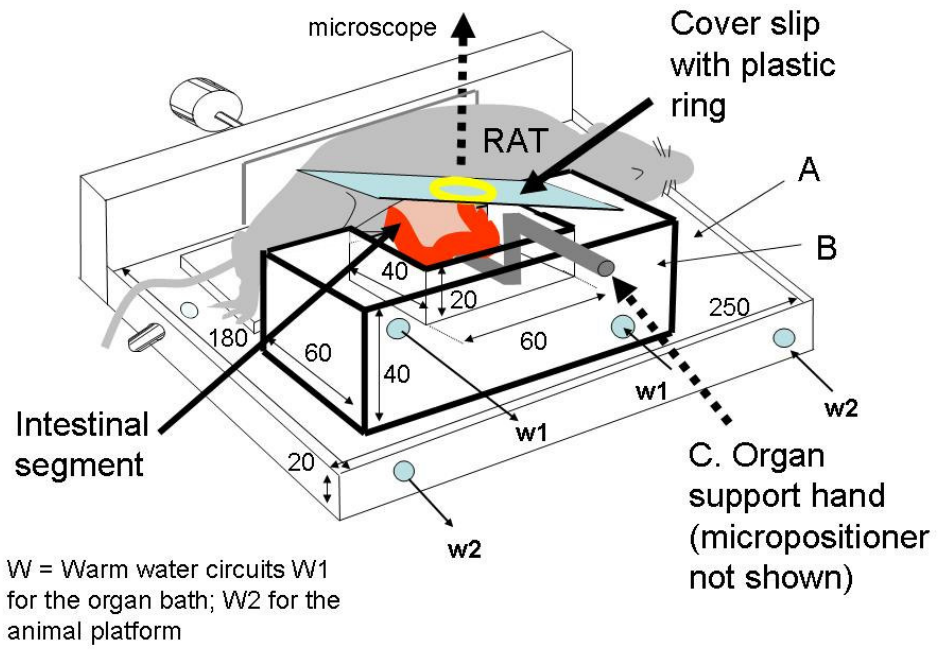


Figure 2, a, b and c

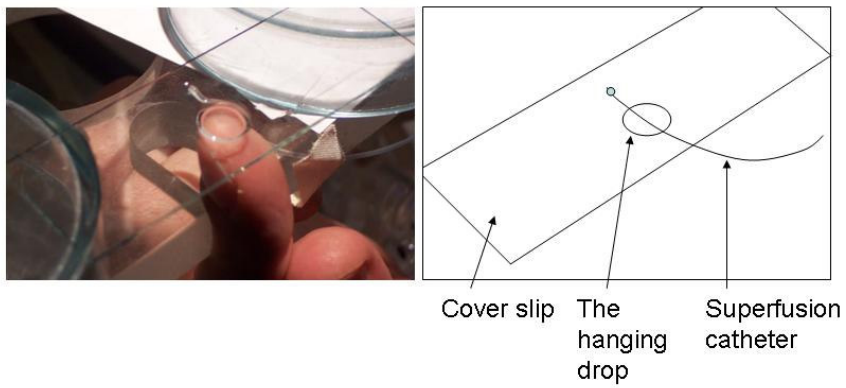
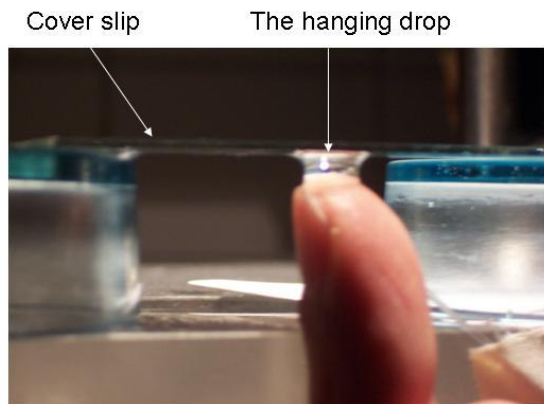


Figure 3a, b and c

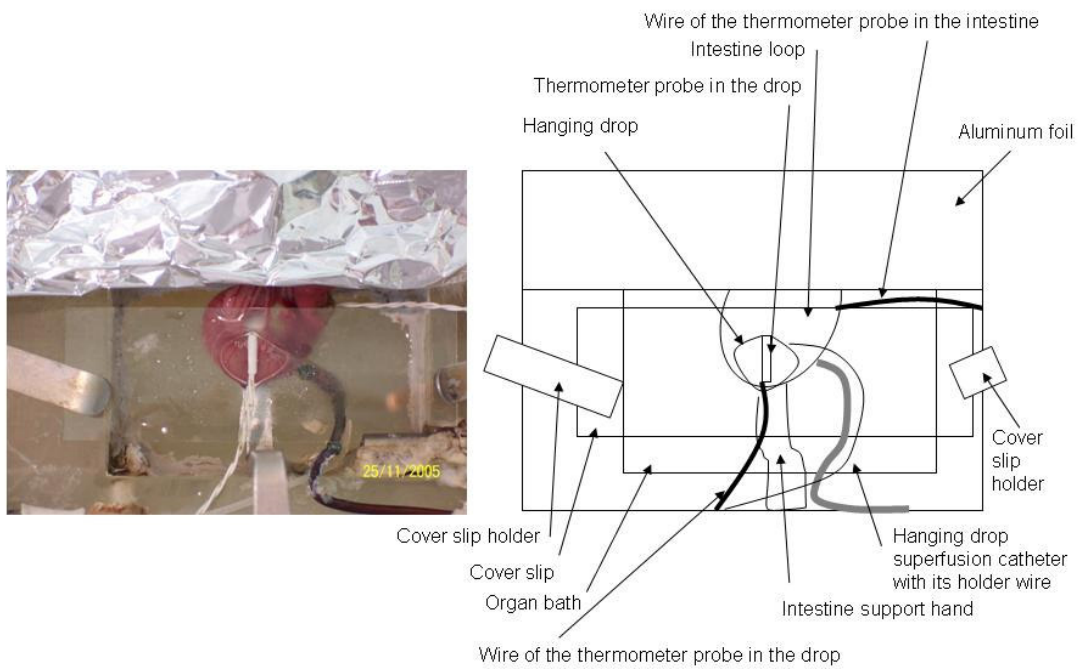
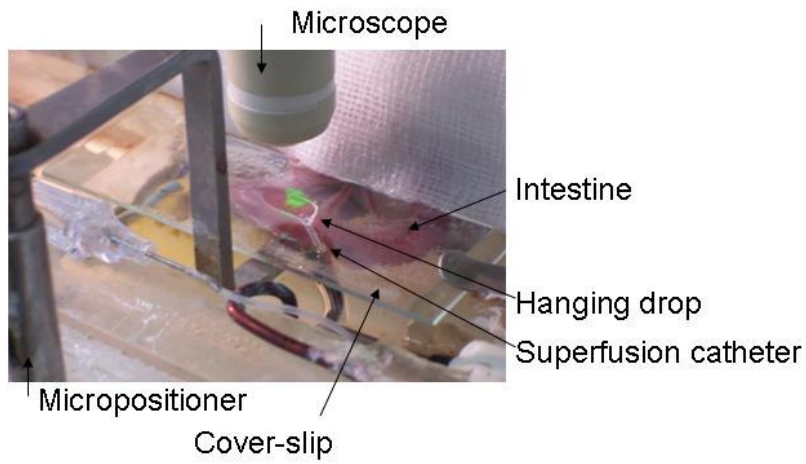


Figure 4

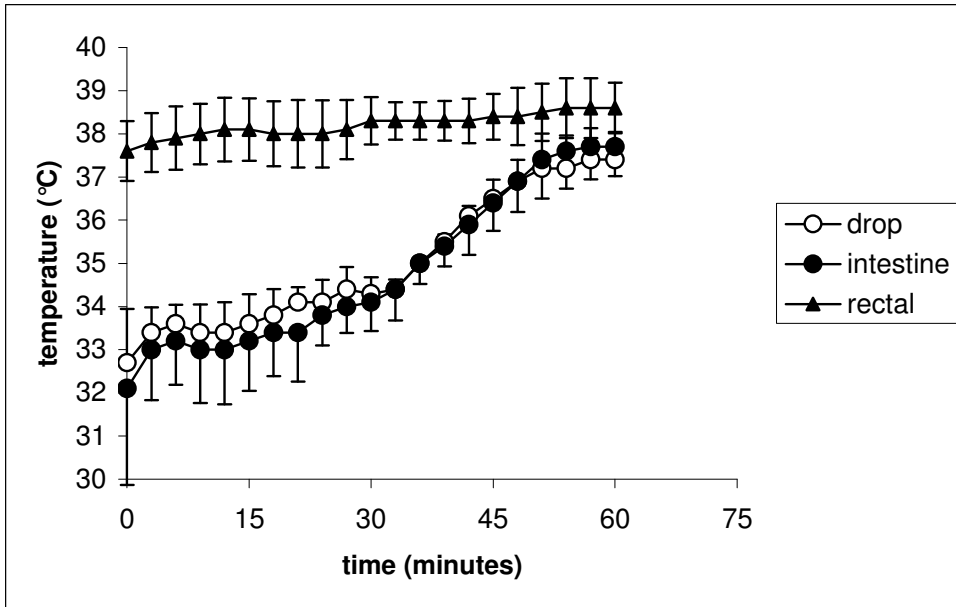
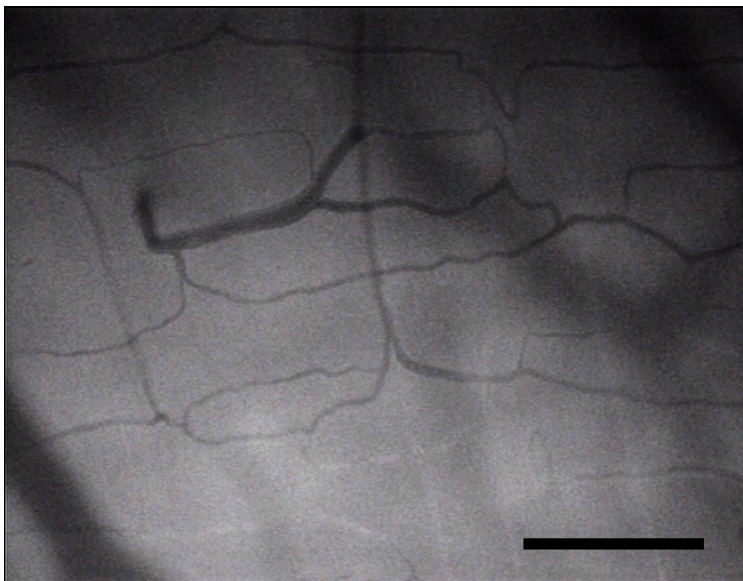
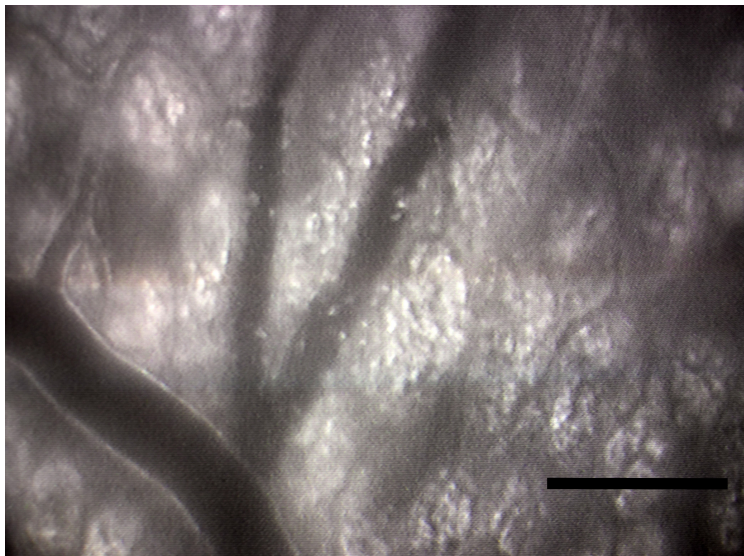


Figure 5a



5b



5c

