Clinical Hemorheology and Microcirculation 38 (2008) 163–170
IOS Press

Response of the rat intestinal microcirculation to experimental endotoxemia is attenuated by ampicillin but not by its derivative KKP723

M. Gründling [a], Ch. Lehmann [a,b,*], D. Saeger [a], S. Bohnenstengel [c], D. Pavlovic [a], V. Bac [a,c], T. Usichenko [a], K. Meissner [a], M. Wendt [a], O. Hung [b], M. Murphy [b], S. Whynot [b] and U. Lindequist [c]

[a] *Klinik für Anästhesiologie, Ernst-Moritz-Arndt-Universität Greifswald, Germany*
[b] *Department of Anesthesia, Dalhousie University, Halifax, NS, Canada*
[c] *Institut für Pharmazie, Ernst-Moritz-Arndt-Universität Greifswald, Germany*

Received 12 July 2007
Accepted 4 September 2007

Abstract. KKP723 (KKP), a derivative of ampicillin, is a newly developed β-lactam antibiotic. Using an experimental endotoxemia model, the intestinal microcirculation in four groups of animals were evaluated using intravital microscopy (IVM). The groups included were a control group, an endotoxemic group (15 mg/kg i.v. LPS from *E. coli*), an ampicillin (50 mg/kg i.v.) treated endotoxemic group and an endotoxemic group treated with KKP (67.4 mg/kg i.v.). Ampicillin treatment resulted in a significant reduced number of firmly adhering leukocytes in intestinal submucosal venules. KKP treatment did not show this effect on leukocyte activation. We found no changes of the functional capillary density (FCD) of the intestinal wall by treatment with ampicillin or its derivative KKP. The increased leukocyte adherence in the KKP treated LPS animals may be explained by a loss of a possible ampicillin-related anti-inflammatory effect by the biotransformation process. The endotoxemia IVM model is useful to detect effects of antibiotics in an impaired microcirculation.

Keywords: Ampicillin, endotoxemia, microcirculation, leukocyte adhesion

1. Introduction

Systemic inflammation and sepsis continue to be a menace especially for patients with trauma or after major surgery [1]. A disrupted microcirculation is the pathophysiological environment of multiple organ dysfunction syndrome (MODS), a frequently occurring and deadly condition seen in septic patients [2]. If not corrected, it may lead to global tissue hypoxia, direct tissue damage, and ultimately, organ failure and death [3].

[*] Both the first and the second authors equally contributed to the manuscript. Corresponding author: Prof. Christian Lehmann, MD, Department of Anesthesia, Dalhousie University, 1278 Tower Road, 10 West, Victoria General Hospital, Halifax, NS, Canada, B3H 2Y9. Tel.: +1 (902) 473 4344; Fax: +1 (902) 423 9454; E-mail: chlehmann@dal.ca.

Intravenous antibiotic therapy should be initiated as soon as sepsis (systemic inflammatory response syndrome (SIRS) + infection) is suspected. The assumption is that, following antibiotic therapy and parallel to the antibacterial effect, an improvement of the impaired microcirculation also occurs. However, the administration of antibiotics is intended for bacteriolysis. In bacteriolysis the toxins released may, even if only temporarily, also lead to reinforcement of the alterations in the microcirculation [4].

Because of the increased number of infections with antibiotic-resistant bacterial strains the development of new antibiotics is needed. In addition to the results of microbial sensitivity testing, a specific knowledge of the potential effects exerted upon the microcirculation is important in order to detect possible side effects of the new antibiotic substances.

The aim of the present study was to evaluate the effect of a new ampicillin derivative antibiotic KKP723 [5] upon the microcirculation within an experimental animal sepsis model. 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 – the motor of multiorgan failure" [6]).

2. Materials and methods

Animals: A total of 40 male Lewis rats, six to seven weeks old, with a weight between 190–330 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.

Groups: Four groups of animals were studied: control group (control), endotoxemic group (lipopolysaccharide-LPS), ampicillin treated endotoxemic group (LPS + Amp) and endotoxemic group treated with KKP723 (LPS + KKP).

Procedures: Procedures were described previously [7]. Briefly, anaesthesia was induced via i.p. administration of 60 mg/kg pentobarbital and maintained with repeated i.v. injections of 5 mg/kg b.w. pentobarbital. With the animals 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 ± 0.5°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.

The animals within the four groups (each $n = 9$) were instrumented. Control group received only an equal amount of normal saline. Three groups received 15 mg/kg LPS from *E. coli* (serotype 026:B6, Sigma-Aldrich Chemie, Steinheim, Germany) as i.v. short infusion. One group received 50 mg/kg ampicillin i.v. immediately after LPS-administration ("LPS + Amp"). A second group received 67.4 mg/kg KKP i.v. immediately after LPS-administration ("LPS + KKP"). A third group remained untreated ("LPS"). Subsequent to a two hour observation period, intravital microscopy was performed.

Intravital microscopic investigation was performed upon a 5.0 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.

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 were observed and recorded for 30–60 seconds.

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

Then, the examination of the mucosa was performed through the opening of the intestinal lumen over a length of 2.0 cm antimesenteric with a microcautery knife (Geiger Model-100, Monarch Beach, CA, USA). Here, faeces filled sections were preferred in order 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.

Evaluation of all the video sequences took place off-line on a video monitor. The following parameters were analysed: *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/mm^2), *functional capillary density* (the length of capillaries with observable erythrocyte perfusion in relation to an predetermined rectangular field; [FCD] = cm/cm^2 = cm^{-1}). The analysis of the video sequences was performed in a blinded manner by the investigators.

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-1α, IL-4, IFNγ, MCP1 and GM-CSF (Rat Cytokines 6plex Kit, Bender MedSystems GmbH, Vienna, Austria).

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 error of means are illustrated in the figures.

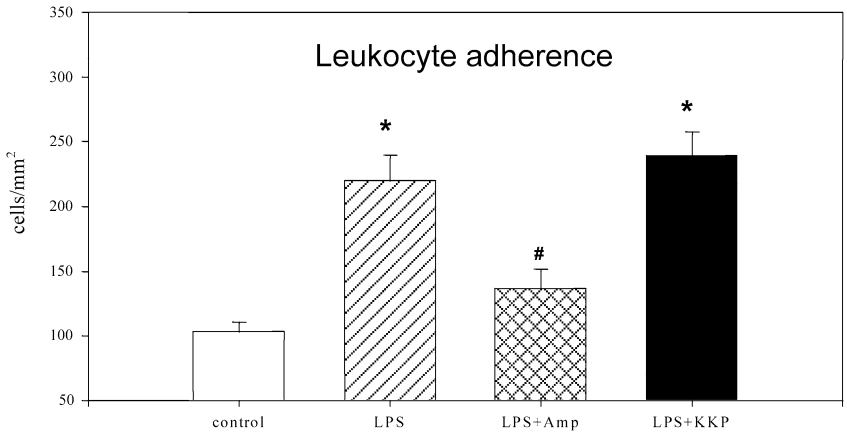

Fig. 1. Leukocyte adherence (cells/mm^2); * = $p < 0.05$ vs. control; # = $p < 0.05$ vs. LPS.

3. Results

The number of firmly adhering leucocytes to the endothelium increased substantially in endotoxin challenged animals compared to the control groups (Fig. 1). Ampicillin administration significantly reduced leukocyte sticking within the V1 venules during endotoxemia. KKP treated LPS animals, as compared with ampicillin treated, demonstrated a significant increased number of leukocytes firmly adhered to the endothelium (Fig. 1).

We found only a slight decrease of the functional capillary density (FCD) of the intestinal wall following endotoxin challenge (Fig. 2). Neither ampicillin nor its derivative KKP723 changed FCD significantly.

Endotoxemia caused a profound cytokine release (Fig. 3). Concentrations of early cytokines (TNF-α, IL-1α) were slightly reduced following administration of both antibiotics. IL-4 levels increased significantly in KKP723 animals.

4. Discussion

The main finding of the present study was that acute ampicillin administration significantly reduced leukocyte adhesion to the endothelium within the intestinal submucosa in the endotoxin-impaired microcirculation. Acute KKP723 administration did not show this effect. We found no changes in the functional capillary density of the intestinal wall by treatment with ampicillin or its derivative KKP723 during endotoxemia.

KKP723 is a derivative of ampicillin developed by biotransformation with laccase, a common polyphenoloxidase [5]. Ampicillin is considered part of the aminopenicillin family and is roughly equivalent to amoxicillin in terms of spectrum and level of activity. It is indicated for the treatment of susceptible bacterial respiratory tract infections, urinary tract infections, gastrointestinal infections, bacterial meningitis, septicemia and endocarditis. Susceptible organisms include *Enterococcus spp.*, *S. pneumoniae* and non-penicillinase producing *H. influenza*, *E. coli*, *P. mirabilis* and *S. epidermidis*. Ampicillin inhibits bacterial cell wall synthesis by binding to one or more of the penicillin-binding proteins (PBPs) which in turn inhibit the final transpeptidation step of peptidoglycan synthesis in bacterial cell walls, thus

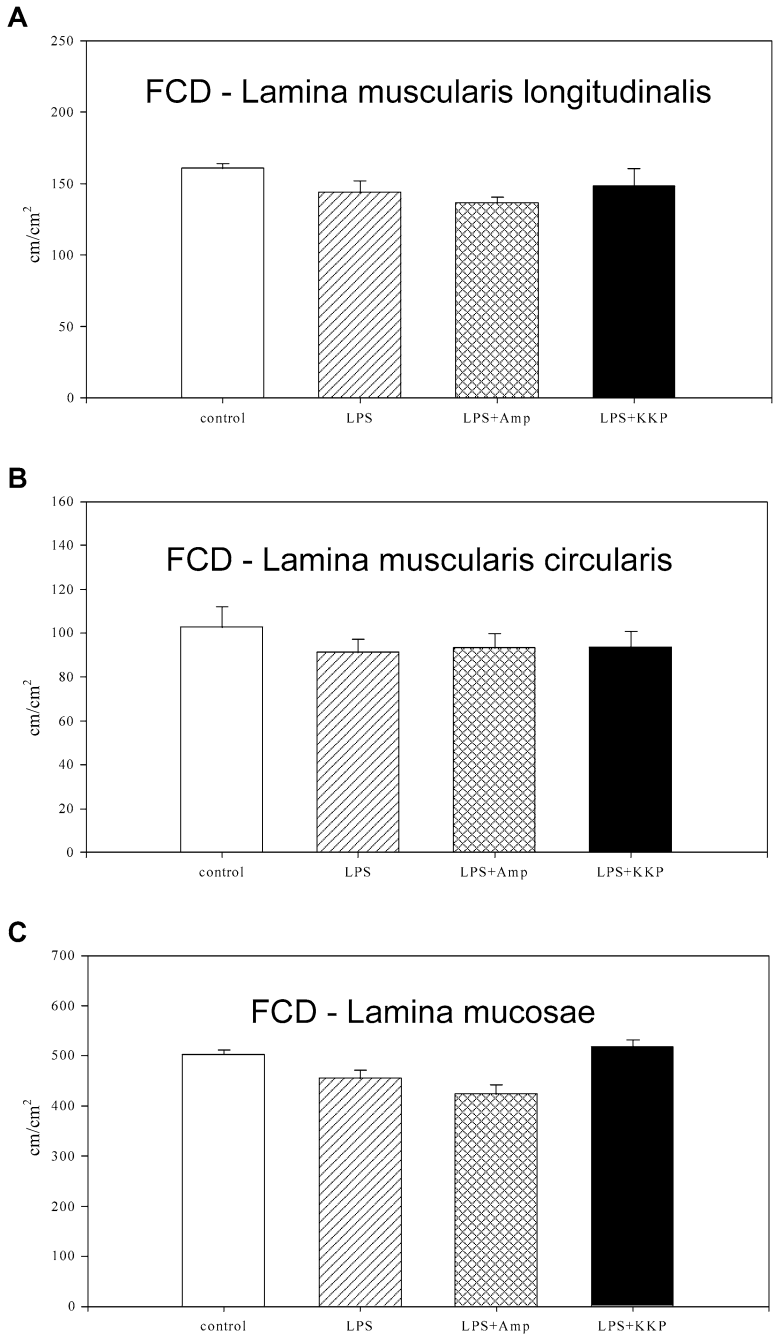

Fig. 2. Functional capillary density (cm/cm^2); A = Lamina muscularis longitudinalis, B = Lamina muscularis circularis, C = Lamina mucosae.

inhibiting cell wall biosynthesis. Bacteria eventually lyse due to ongoing activity of cell wall autolytic enzymes (autolysins and murein hydrolases) while cell wall assembly is arrested [8].

Bacterial lysis is a typical capacity of many bactericidal antibiotics. During bacterial lysis a lot of different toxins are released. The extent of toxin release varies between the specific antibiotics [9].

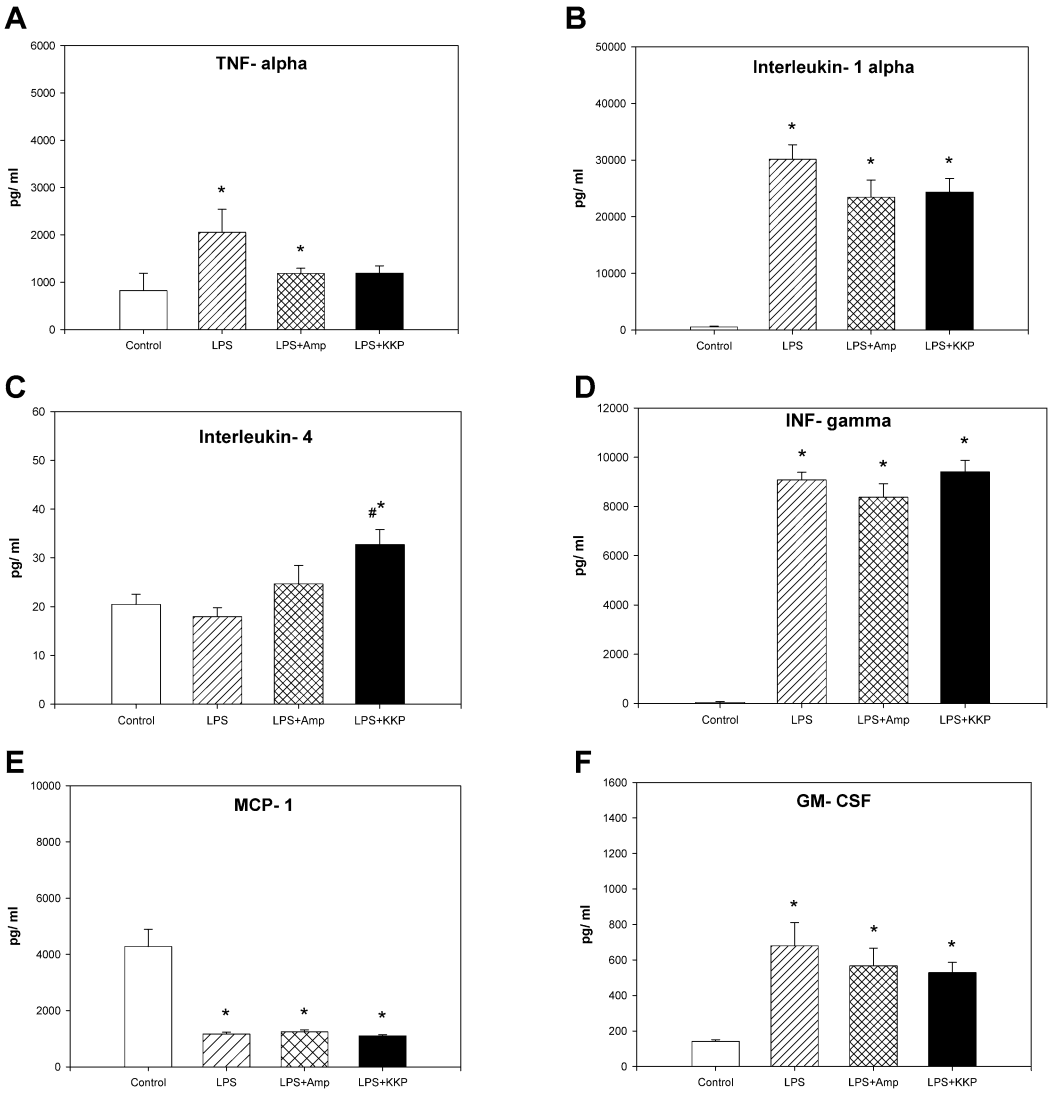

Fig. 3. Cykine release pg/ml; A = TNF-α, B = IL-1α, C = IL-4, D = IFNγ, E = MCP1 and F = GM–CSF; * = $p < 0.05$ vs. control; # = $p < 0.05$ vs. LPS.

Consequently, even though it can be principally assumed that, following antibiotic therapy, parallel to the antibacterial effect, an improvement of the impaired microcirculation occurs, a reinforcement of the alterations in the microcirculation may result because of the toxin release. This side effect of antibiotics may be of short duration, but this can be long enough to endanger a critically ill patient.

On the other hand, anti-inflammatory (side) effects, such as attenuation of leukocyte activation through antibiotics, are also known. We could show anti-inflammatory effects of metronidazole during chronic sepsis, but also in endotoxemia, i.e. independently of bacterial burden [7]. Similar results were reported by Arndt et al. [10] in two abacterial models investigating the leukocyte-endothelial-interaction within the mesentery of rats treated with metronidazole. Legssyer et al. [11] described in a model of abacterial, LPS-induced pulmonary inflammation a reduced inflammatory response by treatment with azithromycin.

In rats, after cecal ligation and puncture (CLP), elevated serum TNF-α levels and cardiac output were observed following acute treatment with ampicillin in comparison to bacteriostatic antibiotics [12]. The authors suggested that this may be related to the known enhanced microbial toxin release by bactericidal antibiotics. However, the elevated TNF-α levels after CLP in rats treated with bactericidal antibiotics does not increase mortality.

Duez et al. compared the interplay of several antimicrobial agents on the neutrophil respiratory burst in response to formylmethionyl-leucyl-phenylalanine (fMLP), a chemoattractant [13]. In the penicillin family an inhibitory effect has been observed. The authors attributed this inhibitory effect to a direct oxidant-scavenger activity mainly of HOCl. Reactive oxygen metabolites are known to promote leukocyte-endothelial interactions in postcapillary venules and decrease red blood cell deformability [14,15]. Thus an antioxidant effect of ampicillin could be responsible for our results regarding leukocyte activation. Other groups showed similar effects on leukocyte-endothelial interactions with different therapeutic approaches [16–18].

Our results showed a reduced inflammatory response following ampicillin treatment during endotoxemia, that means without bacterial challenge. This anti-inflammatory effect of ampicillin was not to be seen in its derivative, KKP723. The lactase-induced biotransformation has probably resulted in a loss of the anti-inflammatory properties. However, we did not find increased leukocyte activation as a consequence of a possible increased toxin release by KKP723.

5. Summary

The aim of the present study was to evaluate the effects of the new antibiotic KKP723 in the LPS-impaired intestinal microcirculation. The ampicillin derivative KKP723 did not affect leukocyte activation or functional capillary density during endotoxemia. However, it did not show the observed anti-inflammatory effect caused by ampicillin.

The clinical value of microcirculatory studies such as this is that potential adverse as well as beneficially effects of newer and older antibiotics can be recognized – always keeping in mind that the microcirculation plays an essential pathogenic role in the development of multiorgan failure in sepsis. Thus, the results of the present study may add some information about the properties of the new ampicillin derivative KKP723 to be concerned in the further development of the substance.

References

[1] C. Alberti, C. Brun-Buisson, H. Burchardi, C. Martin, S. Goodman, A. Artigas, A. Sicignano, M. Palazzo, R. Moreno and R. Boulme, Epidemiology of sepsis and infection in ICU patients from an international multicentre cohort study, *Intensive Care Med.* **28** (2002), 108–121.

[2] T. Sordia, J. Tatarishvili and G. Mchedlishvili, Hemorheological disorders in the microcirculation during septic shock in rats, *Clin. Hemorheol. Microcirc.* **35** (2006), 223–226.

[3] P.E. Spronk, D.F. Zandstra and C. Ince, Bench-to-bedside review: sepsis is a disease of the microcirculation, *Crit. Care* **8** (2004), 462–468.

[4] I. Ginsburg, The role of bacteriolysis in the pathophysiology of inflammation, infection and post-infectious sequelae, *APMIS* **110** (2002), 753–770.

[5] F. Schauer, U. Lindequist, A. Schäfer, W.D. Jülich, E. Hammer and U. Jonas, Biotransformation von biologisch aktiven Verbindungen aus verschiedenen chemischen Stoffklassen mittels der Enzyme Laccase und Manganperoxidase, *PCT/EP* 01/07152 (2001).

[6] C. Ince, The microcirculation is the motor of sepsis, *Crit. Care* **9** (2005), 13–19.

[7] Ch. Lehmann, V.H. Bac, D. Pavlovic, M. Lustig, S. Maier, F. Feyerherd, T.I. Usichenko, K. Meissner, H. Haase, M. Jünger, M. Wendt, C.D. Heidecke and M. Gründling, Metronidazole improves intestinal microcirculation in septic rats independently of bacterial burden, *Clin. Hemorheol. Microcirc.* **34** (2006), 427–438.
[8] C. Repchinsky (ed.), *Compendium of Pharmaceuticals and Specialties*, Canadian Pharmaceutical Association, Ottawa, 2006, pp. 129–130.
[9] U. Krehmeier, M. Bardenheuer, G. Voggenreiter, U. Obertacke, F.U. Schade and M. Majetschak, Effects of antimicrobial agents on spontaneous and endotoxin-induced cytokine release of human peripheral blood mononuclear cells, *J. Infect. Chemother.* **8** (2002), 194–197.
[10] H. Arndt, K.D. Palitzsch, M.B. Grisham and D.N. Granger, Metronidazole inhibits leukocyte-endothelial cell adhesion in rat mesenteric venules, *Gastroenterology* **106** (1994), 1271–1276.
[11] R. Legssyer, F. Huaux, J. Lebacq, M. Delos, E. Marbaix, P. Lebecque, D. Lison, B.J. Scholte, P. Wallemacq and T. Leal, Azithromycin reduces spontaneous and induced inflammation in DeltaF508 cystic fibrosis mice, *Respir. Res.* **7** (2006), 134.
[12] J.A. Stockwell, Y.C. Huang, Y.F. Su and C.A. Piantadosi, Bactericidal antibiotics increase tumor necrosis factor-alpha and cardiac output in rats after cecal ligation and puncture, *Circ. Shock* **42** (1994), 68–75.
[13] J.M. Duez, A. Pechinot and C. Neuwirth, Modulatory effect of antimicrobial agents or aminoacids on the oxidative burst of polymorphonuclear neutrophils triggered by formylmethionyl-leucyl-phenylalanine (fMLP), *Pathol. Biol. (Paris)* **54** (2006), 37–43.
[14] M. Suzuki, H. Asako, P. Kubes, S. Jennings, M.B. Grisham and D.N. Granger, Neutrophil-derived oxidants promote leukocyte adherence in postcapillary venules, *Microvasc. Res.* **42** (1991), 125–138.
[15] M.B. Yerer, H. Yapislar, S. Aydogan, O. Yalcin and O. Baskurt, Lipid peroxidation and deformability of red blood cells in experimental sepsis in rats: The protective effects of melatonin, *Clin. Hemorheol. Microcirc.* **30** (2004), 77–82.
[16] B. Leithäuser, M. Fassbender, M. Eickhoff, M. Elg, G. Eichner and F.R. Matthias, The direct thrombin inhibitor melagatran counteracts endotoxin-induced endothelial leukocyte adherence and microvascular leakage in the rat mesentery. Rationale for the treatment of inflammatory disorders beyond sepsis?, *Clin. Hemorheol. Microcirc.* **36** (2007), 277–289.
[17] P. Thaveeratitham, W. Khovidhunkit and S. Patumraj, High-density lipoproteins (HDL) inhibit endotoxin-induced leukocyte adhesion on endothelial cells in rats: effect of the acute-phase HDL, *Clin. Hemorheol. Microcirc.* **36** (2007), 1–12.
[18] K. Sun, C.S. Wang, J. Guo, Y.Y. Liu, F. Wang, L.Y. Liu, J.G. He, J.Y. Fan and J.Y. Han, Effect of Panax notoginseng saponins on lipopolysaccharide-induced adhesion of leukocytes in rat mesenteric venules, *Clin. Hemorheol. Microcirc.* **34** (2006), 103–108.