

Colistin dampens fibrinolysis and endothelial activation during endotoxaemia

A randomised, double blind trial

Christian Schoergenhofer¹; Peter Matzner¹; Marion Mußbacher²; Johannes A. Schmid²; Petra Jilma-Stohlawetz³; Markus Zeitlinger¹; Bernd Jilma¹

¹Department of Clinical Pharmacology, Medical University of Vienna, Vienna, Austria; ²Department of Vascular Biology and Thrombosis Research, Center for Physiology and Pharmacology, Medical University of Vienna, Vienna, Austria; ³Department of Laboratory Medicine, all at Medical University of Vienna, Vienna, Austria

Summary

Colistin electrostatically interacts with lipopolysaccharides (LPS). Pre-clinical studies demonstrated beneficial effects of colistin on LPS-induced coagulation and fibrinolysis. The objective of this trial was to investigate the effects of colistin during experimental endotoxaemia. In this randomised, double-blind, placebo-controlled, crossover trial 16 healthy volunteers received a 2 ng/kg LPS bolus after infusion of 2.5 million IU colistin or placebo. Plasma levels of F1+2 prothrombin fragments, thrombin-antithrombin complexes (TAT), von Willebrand factor antigen levels (vWF), E-selectin, plasmin-antiplasmin complexes (PAP), tissue-type plasminogen activator (t-PA) antigen and activity, plasminogen activator inhibitor-1 (PAI-1) were measured. Infusion of colistin significantly reduced peak concentrations of PAP complexes

by 70 %, t-PA antigen levels by 63 % and t-PA activity by 48 %, while PAI-1 levels decreased numerically by 63 %. Two hours after the LPS bolus F1+2 levels and TAT complexes were slightly reduced in the colistin period, but peak concentrations were similar in both periods. Colistin blunted the LPS induced four-fold increase in soluble E-Selectin levels by ~50 % and the two-fold increase in vWF antigen levels by ~70 %. The LPS-scavenging actions of colistin significantly reduce endothelial activation and fibrinolytic response in the human endotoxaemia model, while the activation of the coagulation system remains largely unaffected.

Keywords

Coagulation, colistin, endotoxaemia, fibrinolysis, endothelial cells

Correspondence to:

Bernd Jilma, MD
Department of Clinical Pharmacology
Medical University of Vienna
Währinger Gürtel 18–20, 1090 Vienna, Austria
Tel.: +43 1 40400 29810, Fax: +43 1 40400 29980
E-mail: bernd.jilma@meduniwien.ac.at

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Introduction

Colistin was introduced to clinical use in the 1950s (1). It's an effective antimicrobial agent against gram-negative and mycobacterial species. Due to its neuro- and nephrotoxicity colistin was replaced by other less toxic antimicrobial substances in the following decades (1, 2). The interest in colistin reemerged with the increase in multi-drug resistant gram-negative bacteria in recent years, most prominently *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae* (1, 3). Furthermore colistin may be used in combination with other antimicrobial substances to break up biofilms (4). Colistin exerts its bactericidal effects by interacting electrostatically with the anionic portion of lipopolysaccharides (LPS), which is located at the outer membrane of gram-negative bacteria (5). It competitively displaces divalent cations Ca^{2+} and Mg^{2+} bridges that stabilise LPS and thereby disintegrates the cellu-

lar membrane (6). This subsequently causes leakage of cellular contents and eventually bacterial death (7).

Colistin may also exert anti-inflammatory effects by binding and neutralising LPS (8). This was shown in various animal models with promising results (8–10), i.e. colistin reduced TNF- α , IL-1 β and IL-6 levels and improved survival in a pneumonia mouse model (8). Furthermore, infusion of colistin reduced the inflammatory response in a human endotoxaemia model (11).

However, endotoxaemia does not only cause an inflammatory response but an intertwined, tissue-factor driven activation of the coagulation system (12). This coagulatory response is counterbalanced by a release of components of the fibrinolytic system demonstrated by increased levels of tissue-type plasminogen activator (t-PA), plasminogen activator inhibitor-1 (PAI-1), plasmin-antiplasmin complexes (PAP) and D-dimers (13). Indeed, colistin prevented the endotoxin-induced disseminated intravascular coagu-

lopathy in a rabbit model (10). Furthermore colistin has prevented the LPS-induced production of inhibitors of fibrinolysis *in vitro* (14).

The endothelium plays a crucial role in the interplay between coagulation, inflammation and fibrinolysis and markers of endothelial activation markedly increased after infusion of LPS (15). This endothelial activation is considered to be largely depending on TNF- α , as blockade of TNF- α almost entirely blunted the LPS-induced increase in E-selectin (16).

Based on these pre-clinical studies we hypothesised that colistin may have beneficial effects on coagulation activation, the fibrinolytic response and endothelial activation during experimental endotoxaemia. Experimental endotoxaemia is a standard model of acute inflammation in healthy volunteers (17, 18). Therefore in this randomized, placebo-controlled trial we investigated the effects of colistin on the LPS induced activation of coagulation and fibrinolysis in experimental endotoxaemia in healthy volunteers.

Materials and methods

The trial was conducted at the Department of Clinical Pharmacology, at the Medical University of Vienna between September and December 2014 and was performed in accordance with the Good Clinical Practice guideline and the principles set forth in the Declaration of Helsinki. The independent ethics committee of the Medical University of Vienna and the national authority (AGES, Austrian Agency for Health and Food Safety) gave their approval before the trial was initiated. The trial was registered at the EudraCT database with the identifier 2014-00285720. Written and oral informed consent to participate in the trial was obtained from all healthy volunteers before any trial-related activity was performed.

Healthy volunteers

Sixteen male, healthy volunteers aged 19–40 years were included in this trial. Major inclusion criteria comprised normal laboratory parameters, normal medical history and normal findings in the physical examination during the screening visit. Key exclusion criteria were allergies or hypersensitivities to any of the used products, a history of anaphylaxis or severe allergic reactions, coagulopathies or known coagulation disorders, recent participation in trials with investigational drugs or LPS, a body weight <60 or >95 kg, intake of any medication within one week of the first trial day, a clinically relevant illness within three weeks prior to the trial days and liver or kidney dysfunction.

Study design

This was a prospective, investigator- and participant-blinded, single-center, randomised, two-way crossover trial. Subjects were randomly allocated to receive an infusion of 2.5 million IU of colistin or 250 ml 0.9% saline solution as a placebo over 60 minutes (min) followed by a bolus infusion of 2 ng/kg bodyweight LPS (US

Standard Reference Endotoxin *E. coli*, CC-RE- Lot 2) over 1–2 min. Infusion of placebo or colistin was initiated 50 min before infusion of LPS. This dosing schedule was chosen to allow both drugs to reach their maximum effect simultaneously, approximately 1.5 hours (h) after infusion of the LPS bolus (11, 19). After a washout period of 6 weeks subjects received the alternate treatment in a crossover manner.

Trial day

On the trial days subjects reported to the ward in the morning after an overnight fast. Following the infusion of LPS all subjects received a continuous infusion of 100 ml/h 0.9% saline solution for a duration of 8 h. Vital signs, including body temperature, heart rate and blood pressure, were monitored. To alleviate potential LPS-triggered flu-like symptoms paracetamol (500 mg tablet or 1000 mg infusion) was available for all subjects (20). Subjects received a standardised meal 4 h after infusion of LPS. At 8 h after infusion of LPS, subjects were discharged from the study ward. The next morning, 24 h after the LPS bolus another visit was planned.

Laboratory analysis

Blood sampling was performed at pre-defined time-points: at the baseline, as well as 1 h, 2 h, 4 h, 6 h, 8 h and 24 h after the infusion of LPS. Differential blood counts were performed by the accredited central laboratory of the General Hospital of Vienna using the Sysmex XE-2100 / XE-5000 as previously described. To determine the effects of colistin on the activation of coagulation and fibrinolysis in the endotoxaemia model we performed commercially available enzyme linked immunoassays (ELISA) to measure levels of prothrombin fragments F1+2 (F1+2) (Enzygnost® F1+2, Siemens), thrombin-antithrombin complexes (TAT) (Enzygnost® TAT, Siemens), plasmin-antiplasmin complexes (PAP) (PAP Elisa, DRG® International Inc.), plasminogen activator inhibitor-1 (PAI-1) (Technozym® PAI-1 Actibind®, Technoclone), tissue-type plasminogen activator (t-PA) (Technozym® t-PA Combi Actibind®) antigen (Ag) and activity (Act). To investigate the influence of colistin on endothelial activation during endotoxaemia we measured soluble E-selectin by ELISA (sE-Selectin/CD62E Quantikine, R&D Systems) and von Willebrand Factor (vWF) antigen levels (STA LIA test, Diagnostica Stago) (22). All assays were performed according to the manufacturers' instructions.

Endpoints

The main endpoint of this trial were plasma levels of prothrombin fragments F1+2. Secondary endpoints included platelet counts, TAT levels, t-PA antigen levels and activity, E-selectin levels, vWF antigen levels, PAP complex levels and PAI-1 levels. Safety assessments included vital parameters and documentation of adverse events and concomitant medication.

Randomisation and blinding

Block randomisation with block sizes of 8 was performed using an open access randomisation generator (www.randomisation.com). An unblinded study nurse under supervision of an unblinded physician who had access to treatment allocation codes prepared study drugs. These were not otherwise involved in the conduct of the trial. Participants, investigators and laboratory staff were blinded. Both study drugs were not distinguishable from each other by color or physical properties.

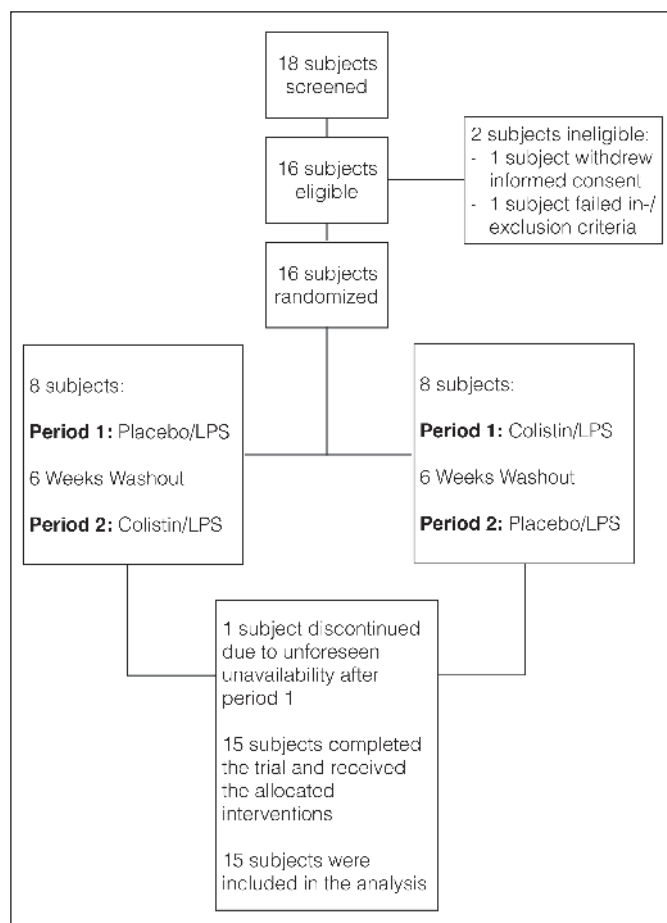


Figure 1: Flow chart.

Table 1: Baseline data and demographics.

Parameter	Means \pm 95% Confidence Intervals
Age [years]	28 \pm 2
Male [n]	16
Height [cm]	184 \pm 3
Weight [kg]	79 \pm 3
Platelets [$\times 10^9/L$]	215 \pm 42

Sample size

The sample size calculation was based on the results of previous trials involving anticoagulants in the human endotoxaemia model, where 10 to 15 subjects were sufficient to detect significant differences in the main outcome parameter (F1+2 levels) between treatment groups in parallel-group studies (23, 24). No data was available on the effects of colistin on prothrombin fragments F1+2. A peak F1+2 concentration of 819 ± 173 pmol/ml was measured in a previous trial (25). Assuming a 25% reduction in this increase a sample size of 12 would suffice to detect a significant difference with a power of 80% and p-value of 0.05. By increasing the sample size to 15 subjects a power of 90% could be reached. Therefore, and to account for potential drop-outs, a sample size of 16 healthy volunteers was chosen.

Statistics

All data are expressed as mean \pm SD or 95% confidence intervals (CI) unless otherwise stated. A repeated measures ANOVA was used for analysis of treatment and period effects on outcome parameters. If significant, (pair wise) post-hoc analysis using non-parametric tests for reasons of robustness were performed, i.e. the Friedman ANOVA and the Wilcoxon test, as appropriate. Eight parameters were tested in total and the p-value of 0.05 was corrected for multiple testing. A two-sided p-value of 0.00625 was considered significant. All statistical evaluations were performed using commercially available statistical software (IBM, SPSS 22).

If measured levels were below the detection limit of the respective assay, the detection limit was imputed (e.g. 2 IU/ml for t-PA activity).

Results

Between September 23 and September 29, 2014 16 healthy volunteers were screened for participation in this trial. The trial was completed after the last follow-up visit on December 9th 2014. Fifteen healthy volunteers completed the study (► Figure 1). Baseline data and demographics are presented in ► Table 1. One subject missed study period II due to unforeseen unavailability and was excluded from analysis. The anti-inflammatory effects of colistin in the human endotoxaemia model were published recently (26); in essence colistin reduced TNF- α , IL-6 and IL-8.

Coagulation

Infusion of 2 ng/kg LPS increased F1+2 levels several-fold in both periods with slightly higher levels in the placebo period. Overall there was no difference between study periods, although plasma levels of F1+2 seemed to be lower in the colistin group 2 h after infusion of LPS, as confidence intervals did not overlap with means of the other period (► Figure 2).

Plasma levels of TAT increased in both study periods several-fold with similar peak concentrations. There was no difference be-

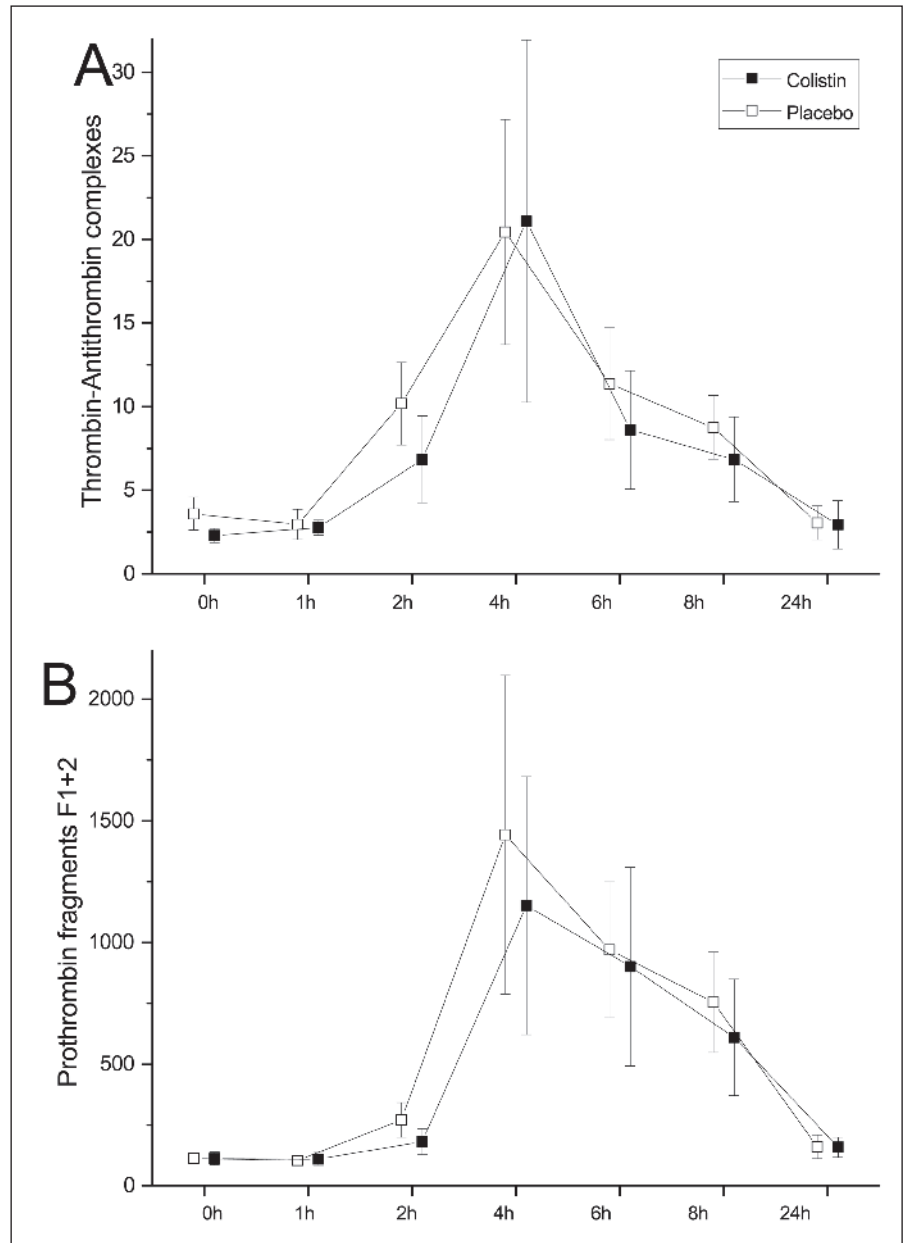


Figure 2: Coagulation specific biomarkers after infusion of an LPS bolus (2 ng/kg) and infusion of colistin or placebo. Presented are means \pm 95% confidence intervals (CI). Prothrombin complexes F1+2 (A) and Thrombin-Antithrombin (TAT) levels (B) are presented. No significant differences between both periods were found. (n=15).

tween both study periods. In the colistin period, TAT levels were lower 2 h after infusion of LPS as indicated by the confidence intervals that did not overlap with means of the other period (► Figure 2).

Platelet counts transiently decreased by approximately 20% 6 h after the LPS bolus in both study periods. However, there was no significant difference between colistin and placebo (Suppl. Figure 1, available online at www.thrombosis-online.com). Platelet counts normalised after 24 h.

Fibrinolysis

Infusion of LPS increased PAP levels ~4.7-fold in the placebo period, but only 1.7-fold in the colistin period. Colistin significantly

lowered PAP complex levels ($p < 0.001$, ► Figure 3) after infusion of LPS. The peak PAP concentration was approximately 70% lower in the colistin period compared to placebo.

Colistin significantly reduced t-PA antigen levels after infusion of the LPS bolus ($p < 0.001$, ► Figure 3). In the placebo period the peak concentration of t-PA activator antigen was 25.4 ± 14.9 ng/ml (mean \pm SD) 2 h after the LPS bolus. This increase was almost completely blunted by colistin and the peak concentration was 7.1 ± 1.8 ng/ml (mean \pm SD). In both study periods, tissue-type plasminogen activator activity increased 2 h after infusion of LPS. No significant differences were detected between study periods (placebo: 4.0 ± 3.1 IU/ml vs colistin: 6.9 ± 6.6 IU/ml) (data not shown).

Infusion of LPS increased PAI-1 levels ~20-fold in the placebo period and ~6-fold in the colistin period after 4 h. In the colistin

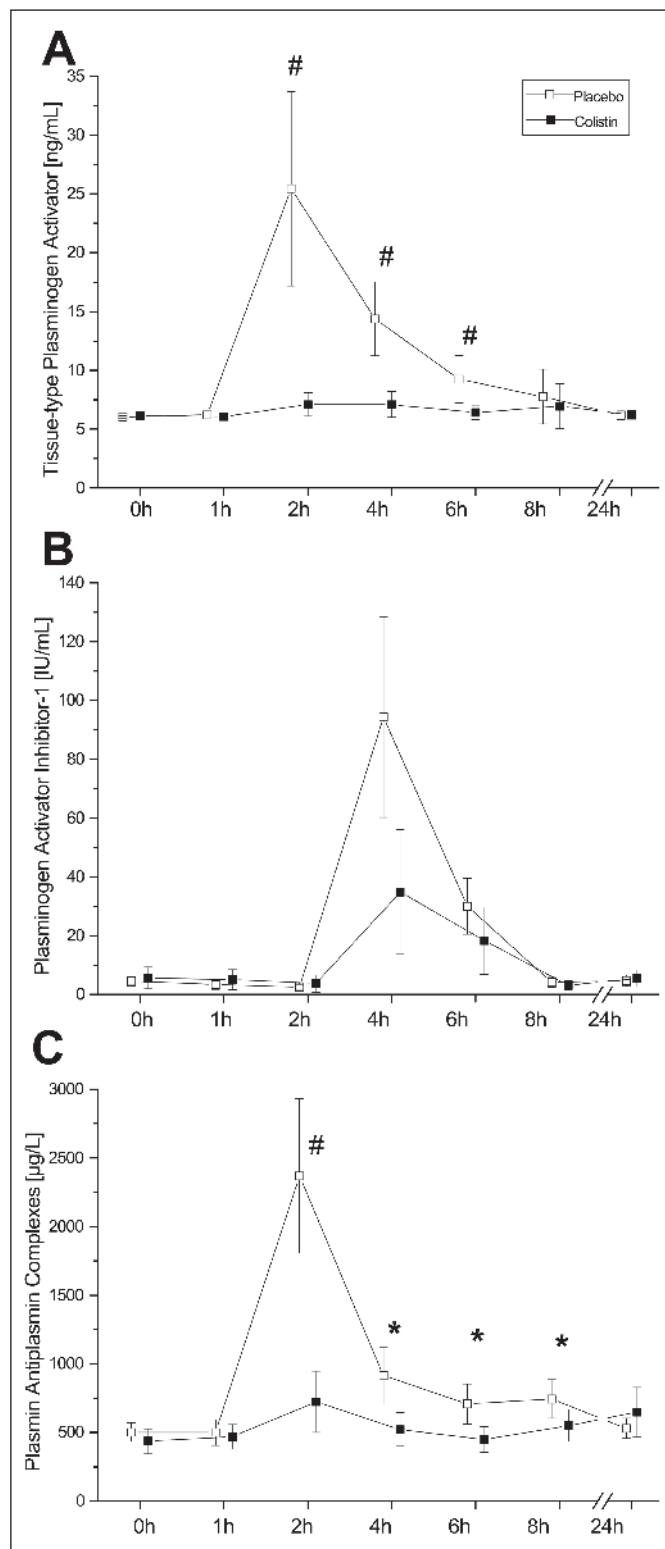


Figure 3: Fibrinolysis specific parameters tissue-type plasminogen activator (t-PA) antigen (A), plasminogen activator inhibitor-1 (PAI-1) (B) and plasmin antiplasmin (PAP) complexes (C) after infusion of 2 ng/kg bodyweight and colistin or placebo. Presented are means \pm 95% CI. Colistin significantly reduced plasma concentrations of t-PA ($p < 0.001$), PAI-1 ($p = 0.02$) and PAI-1 ($p < 0.001$). * $p < 0.05$ # $p < 0.001$ ($n = 15$).

period, PAI-1 levels were numerically lower compared to placebo ($p = 0.02$, ► Figure 3).

Endothelial specific biomarkers

Colistin reduced E-selectin levels compared to placebo 4 h, 6 h, 8 h and 24 h after the LPS ($p = 0.001$, ► Figure 4). E-Selectin increased two-fold in the colistin period and four-fold in the placebo period compared to baseline. Infusion of colistin also decreased the LPS-induced increase in vWF antigen ($p = 0.001$, ► Figure 4). Colistin almost completely abolished the increase in vWF antigen levels. The maximum increase was measured after 24 h, which was 28% higher compared to baseline. In contrast, in the placebo period von Willebrand factor increased two-fold 2 h after infusion of LPS.

Adverse events

Colistin infusion was well tolerated. Serum creatinine did not increase 24 h post-dose. Following LPS injection, many subjects experienced flu-like symptoms. After colistin infusion, five (33.3%) cases of shivering, three (20%) cases of headache, and four (26.7%) cases of joint pain occurred. After infusion of placebo 10 subjects (66.7%) reported shivering, eight (53.3%) reported headache, and five (33.3%) reported joint pain. Two subjects (13.3%) experienced a mild syncope in the placebo period.

Discussion

This placebo controlled trial investigated the actions of colistin on the activation of the coagulation system, the fibrinolytic system and the endothelium in a human endotoxaemia model. The results show that infusion of colistin i) markedly reduces PAI-1, t-PA antigen levels and PAP complexes, ii) reduces soluble E-selectin and vWF, markers of endothelial activation, and iii) slightly delays the onset of the activation of the coagulation system without reducing the magnitude of the activation as assessed by F1+2 levels, TAT complexes or platelet counts.

Colistin binds and neutralises LPS and thereby reduces cytokine levels, such as TNF- α , IL-1 β or IL-6, in animal models (5, 8, 27, 28). These findings were recently confirmed in human endotoxaemia: in the same experimental setup infusion of colistin before the infusion of the LPS bolus at 2 ng/kg bodyweight reduced the LPS-induced increase in TNF- α ~6-fold, in IL-6 ~4-fold and in IL-8 ~3-fold (26). This also translated in a reduced increase in C-reactive protein by ~35% compared to placebo (26). High-density lipoproteins also bind and neutralise LPS and significantly reduced LPS-induced coagulation activation and fibrinolysis (29). Based on the similar mechanism of action we hypothesised to find similar effects of colistin. However, we only detected a slight delay, but no significant reductions of peak concentrations of F1+2 or TAT complexes. This is also reflected by the transient decrease in the platelet count, which was similar in both study periods (► Figure 3). In contrast to that, infusion of colistin almost entirely blunted the activation of the fibrinolytic system. Although colistin

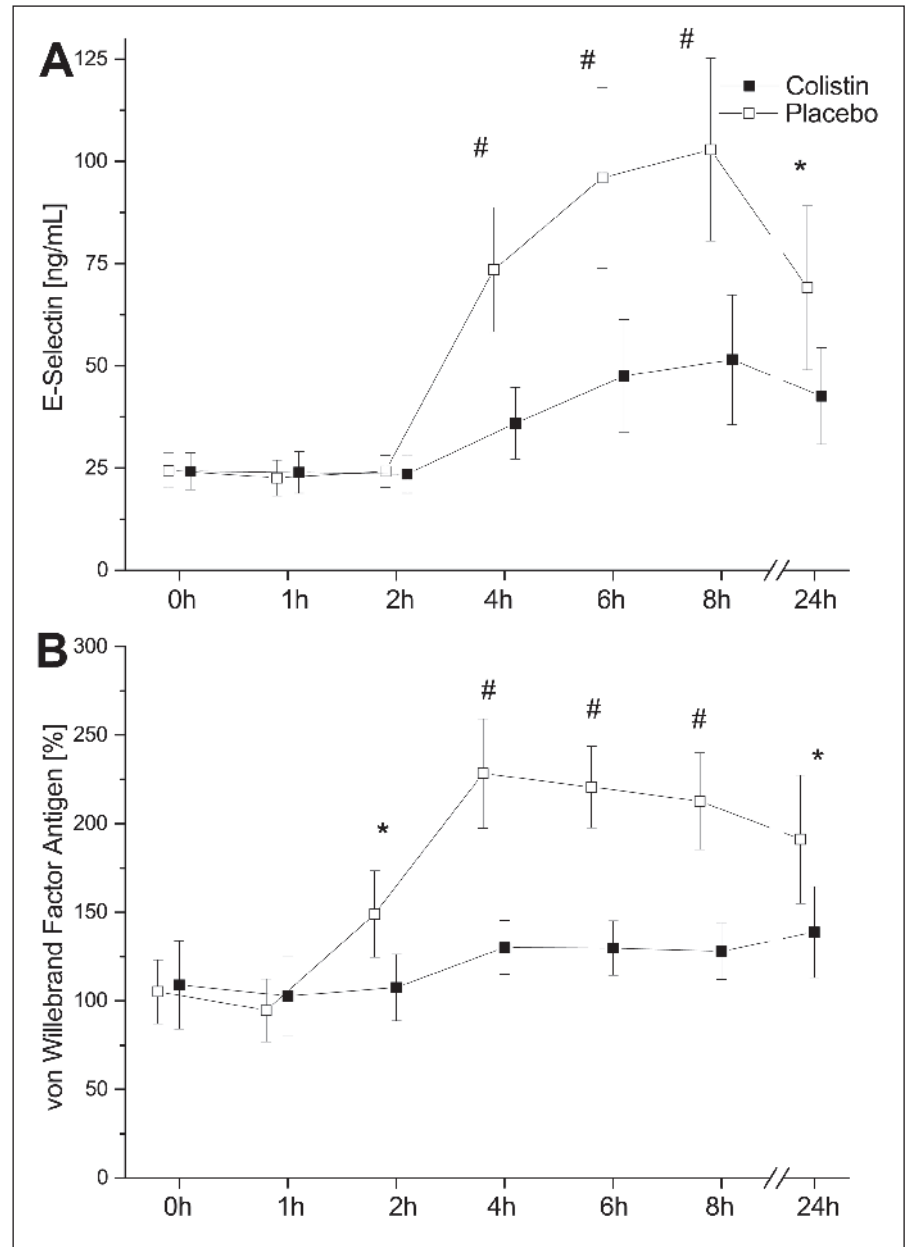


Figure 4: E-selectin (A) and von Willebrand factor antigen levels (B) after infusion of 2 ng/kg bodyweight and colistin or placebo. Presented are means \pm 95% CI. Colistin significantly reduced plasma concentrations of E-selectin ($p=0.001$) and of von Willebrand factor antigen levels ($p=0.001$). * $p<0.05$ # $p<0.001$ ($n=15$).

and high-density lipoprotein both bind to the lipid A portion of LPS, they differ in their activity against LPS, potentially due to differences in molecule size and the processing of LPS (30, 31).

The current understanding of endotoxaemia involves the LPS-binding protein, which forms a complex by binding to the lipid A portion of LPS. These complexes are recognised by CD14 expressed on monocytes and activate them (32). CD14, together with an accessory protein MD-2, facilitates the activation of toll like receptor 4, which downstream activates the NF- κ B pathway with consecutive cytokine production (33, 34). Tissue factor is expressed and possibly transferred to platelets and neutrophils by the formation of microparticles by monocytes, macrophages and endothelial cells (35). The presence of tissue factor subsequently acti-

vates the coagulation system via factor VII and finally the conversion of prothrombin to thrombin (36). Interestingly, infusion of an anti-CD14 antibody did not reduce the generation of F1+2, but reduced PAP and tPA concentrations after infusion of an LPS bolus (37).

The activation of the coagulation and the fibrinolytic system are intertwined, but regulated separately during endotoxaemia. Tissue factor drives coagulation activation, while fibrinolysis is mainly mediated by TNF- α (38). Tissue factor pathway inhibitor and active site inhibited factor VIIa dose-dependently inhibited coagulation activation during endotoxaemia without affecting the fibrinolytic or the inflammatory response (39). However, TNF- α may also be involved in the activation of coagulation to a certain

What is known about this topic?

- Colistin covalently binds to lipopolysaccharide (LPS) and thereby may exert beneficial effects during endotoxaemia.
- In animal models colistin prevented LPS-induced disseminated intravascular coagulation.
- Anti-inflammatory effects of colistin were shown in human and animal models of endotoxaemia.

What does this paper add?

- Infusion of colistin blunted the LPS-induced endothelial activation in healthy volunteers.
- Colistin dampened the fibrinolytic response to LPS during endotoxaemia.
- Colistin slightly delayed the activation of coagulation, although its magnitude remained unaffected.

extent. Interestingly, a similar delay in the activation of coagulation was found after blockade of TNF- α during endotoxaemia (16). Thus, we assume that TNF- α is involved in the early phase of coagulation activation, whereas blockade of IL-6 did not reduce endotoxin-induced activation of coagulation in humans (40). A direct LPS-induced effect on tissue factor bearing cells is likely a driving factor at later stages of endotoxaemia.

The activation of coagulation in inflammatory states is counterbalanced by an activation of the fibrinolytic system. In contrast to the largely unaffected activation of coagulation, the response of the fibrinolytic system was blunted by the infusion of colistin. In the placebo period, LPS markedly increased t-PA, PAI-1 and PAP. The observed changes are in line with previous trials using the human endotoxaemia model (12) and to the well-known changes during acute sepsis (36). As discussed above, these effects are mainly attributable to a reduction in TNF- α levels.

Colistin markedly reduced the LPS induced increase in E-selectin and vWF antigen, most likely due to a reduction in TNF- α (26), because TNF- α inhibitors blunt their release during endotoxaemia (16, 41). This endothelium protective effect may prove beneficial for patients suffering from endotoxaemia, as the TNF- α endothelium axis is involved in endothelial barrier disruption, increased vascular permeability and leukocyte transmigration (42). The activation of the endothelium and the consecutive expression of adhesion molecules may be central to the development of sepsis-induced multiple organ failure (43). VWF captures and localises platelets to the vessel wall (44). In sepsis or systemic inflammatory response syndrome endothelial cells release vWF and platelets aggregate, which consecutively causes thrombocytopenia and may lead to consumptive coagulopathy or even a clinical picture resembling thrombotic thrombocytopenic purpura (45, 46). Interestingly, E-selectin and vWF antigen correlated with the Simplified Acute Physiology Score and the Multi Organ Failure Score, which predict mortality, with the severity of systemic inflammatory response syndrome and with mortality (47, 48).

One of the major limitations of the trial is that the LPS bolus was injected after infusion of colistin. In patients this seldom may be the case, as antimicrobial treatment usually does not precede infections. LPS is usually quickly bound to transfer proteins, cleared from circulation by the liver and detoxified (49). LPS was detectable for approximately 30 min after bolus injection (50). Unbound LPS or LPS bound to transfer proteins triggers the inflammatory response (49). We hypothesised that colistin binds and neutralises unbound LPS and thereby reduces its pro-inflammatory actions. Thus, for our model, in order to elucidate colistin's anti-endotoxin effect at its possibly highest magnitude, the presence of colistin in plasma at the time of the LPS injection was necessary.

In certain clinical situations, such as gram-negative gut infections, or abscesses, in times when the gastrointestinal barrier function is impaired and for all infections in which the infectious focus is not immediately resolved by treatment, repetitive bouts of endotoxaemia may occur. In these patients, colistin may provide beneficial effects beside its antimicrobial activity.

Another limitation was that we were only able to detect tissue-type plasminogen activator activity 2h after infusion of LPS, because the activity was below the detection limit of the applied assay at all other time-points.

In conclusion, infusion of colistin markedly reduced the endothelial activation, the fibrinolytic response and in human endotoxaemia, while the activation of coagulation remained unaffected. Thus, in patients with gram-negative sepsis colistin may alter the course of the disease, independent of its antimicrobial activity.

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Conflicts of interest

None declared.

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