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GLUTAMINE AND ALANYL-GLUTAMINE DIPEPTIDE REDUCE MESENTERIC PLASMA EXTRAVASATION, LEUKOCYTE ADHESION AND TUMOR NECROSIS FACTOR-ALPHA (TNF- α) RELEASE DURING EXPERIMENTAL ENDOTOXEMIA

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Glutamine (GLN) appears to be an essential nutrient during organism development and critical illness. The aim of our study was to evaluate the effects of GLN and its generic preparation alanyl-glutamine-dipeptide (DIP) on the microcirculation in endotoxemia in rats and its effects on tonus or aortal rings *in vitro*. Male Lewis rats (n=40) were separated in 4 groups. Group 1 (CON) served as healthy control group while the other groups received an endotoxin bolus i.v. (5 mg/kg lipopolysaccharide, LPS i.v.). In group 3 (LPS+GLN) 0.75 g/kg-1 GLN i.v. before LPS challenge was administered. In group 4 (LPS+DIP) DIP containing 0.75 g/kg GLN was given. Leukocyte-endothelial interactions and mesenteric plasma extravasation were determined at 0, 1 and 2 hours during the experiment by intravital fluorescence microscopy (IVM). Cytokine release (TNF- α , IL-1 β , IL-6, IL-10) was measured by ELISA. GLN treatment reduced leukocyte adherence (-49.7% vs. LPS group, p<0.05) and plasma extravasation (-12.3% vs. LPS group, p<0.05) significantly during endotoxemia compared to untreated LPS animals. In group 4 (DIP+LPS), a decrease of leukocyte adherence (-56.0%) and mesenteric plasma extravasation (-18.8% vs. LPS group, p<0.05) was also found. TNF- α levels were reduced in both GLN and DIP (p<0.05). *In vitro* experiments demonstrated that glutamine agents could attenuate the response to contracting agents in presence of the vascular endothelium, implying nitric oxide pathway. *In vivo*, GLN as well as DIP pre-treatment diminish the detrimental impact of endotoxemia on the mesenteric microcirculation and the TNF- α release, the effects whose clinical importance should be further examined.

Key words: *glutamine, endotoxemia, intravital fluorescence microscopy, microcirculation, leukocyte-endothelial interaction, plasma extravasation, cytokines*

INTRODUCTION

Many tissues of the body are enabled to synthesize glutamine (GLN) out of glutamate and ammonia. This biochemical reaction is catalyzed by the enzyme glutamine synthetase present in the cytosol (1, 2). Because of its manifold participation in the processes of transamination, GLN has been considered as an actual regulator of the aminoacid homeostasis. Additionally, GLN also provides an amide-group for the synthesis of several metabolites, such as the amino-sugars, purines, pyrimidines and nucleotides, which may be of particular importance for the organism development. It is also involved in the synthesis of the important intracellular antioxidant glutathione and is converted into citrulline, which then serves as a precursor for the synthesis of arginine, the substrate for nitric oxide (NO) synthase, to form the potent vasodilator NO (3, 4). For these reasons alone, GLN has been studied extensively over the last decade for its importance in critical illness.

Experimental trauma studies have asserted that GLN represents a conditionally essential amino acid because of the

insufficient re-synthesis during the acute phase of the disease (5). During clinical sepsis, intracellular GLN level can drop more than 50 percent of the normal level while the plasma concentration falls about 20 percent (6). In this context, it has been shown that GLN deficiency may have significant clinical consequences, such as reduced oxygen free-radical defense and altered cytokine response, that may lead to delayed wound healing, intestinal hyperpermeability and bacterial translocation (7). *In vivo* studies show that a breakdown of the microvascular integrity with an increase in permeability is related to the recruitment and adhesion of neutrophils to the endothelium (8). Experimental studies have demonstrated that GLN supplementation enhances immune function, protects microvascular integrity and gut barrier function, and decreases bacteremia (9, 10).

The above evidence suggests that GLN or DIP (synthetic glutamine-containing dipeptide, L-alanyl-L-glutamine) have possible protective effects and may improve intestinal microcirculation by diminishing leukocyte-endothelium interaction and by reducing cytokine liberation. The aim of this study was to determine the effects of GLN and its generic

preparation alanyl-glutamine-dipeptide (DIP) (11) on the mesenteric microcirculation and cytokine release during experimental endotoxemia using intravital fluorescence microscopy. Some possible mechanisms involved were further examined in preparations of the rat thoracic aorta *in vitro*.

MATERIAL AND METHODS

We conducted two independent sets of experiments: *in vivo* experiments using i.v. administration of GLN and DIP in endotoxemic rats and *in vitro* experiments testing GLN and DIP effects on smooth muscle tension in rat aortal ring preparations.

In vivo experiments

1. Animals

After obtaining approval by the State Animal Care Committee, 40 male Lewis rats were used in the experiments (body weight: 250±50 g, Charles River Laboratories, Sulzfeld, Germany).

2. Anesthesia and experimental protocol

The detailed procedure was described previously (12). Briefly, Anesthesia was induced via intraperitoneal administration of 60 mg/kg⁻¹ pentobarbital (Pentobarbital Natrium, Fagron, Barsbüttel, Germany). The animals were divided into four groups (n=10 per group). Group 1 served as healthy control group (CON). We induced endotoxemia in the groups 2, 3 and 4 by an i.v. administration of 5 mg/kg lipopolysaccharide (LPS) from *Escherichia coli*, serotype O111:B4 (Sigma-Aldrich, Steinheim, Germany); the group 2 was therefore designated as a group (LPS). The glutamine preparations were administered immediately before LPS in a pre-treatment fashion. In group 3 (LPS+GLN) we administered 0.75 g/kg GLN (3% GLN in saline solution) i.v. before LPS challenge. In the group 4 (LPS+DIP) 4.46% DIP in saline solution (1.12 g/kg ALA-GLN containing 0.75 g/kg GLN) was administered. Leukocyte-endothelial interactions and mesenteric plasma extravasation were determined at 0, 1 and 2 hours during the experiment by intravital fluorescence microscopy (IVM); leukocytes were stained with intravenously administered rhodamine 6G (0.2 mL of 0.05 g%) 15 minutes before IVM started and plasma stream was marked with FITC-albumine (Sigma, Deisenhofen, Germany). To quantify plasma extravasation across mesenteric venules 50 mg/kg FITC-albumin was injected 10 minutes before the experiment, as previously described (13). All administered fluids, including intraarterial flush, were calculated to guarantee that all of the animals received an equal amount of intravenous fluids. Blood samples were drawn at the beginning and the end of the experiment in order to determine cytokine release and blood gas parameters. The duration of each experiment, including induction of anesthesia, did not exceed 240 min.

3. Laboratory analysis

Blood samples (0.55 ml) were taken at the beginning and the end of the experiments for arterial blood gas analysis (ABL 330, Radiometer, Hamburg, Germany). In addition, 280 µl plasma was fractionated and stored at -70°C for cytokine analysis (TNF-α, IL-1β, IL-6, and IL-10) using Rat-Quantikine ELISA kits (R&D Systems, Wiesbaden, Germany).

In vitro experiments in aortal rings

The method was described previously (14). Briefly, for the experiment rat thoracic aortas were isolated, and cleaned from surrounding tissue. After a period of stabilization (60 minutes) the vascular muscle (2 mm long aortal rings) was stretched to its

optimal length. The Krebs solution (at 37°C, pH 7.4, gassed with 95% O₂/5% CO₂) was exchanged every 20 min.

Contractility protocols

The effects of GLN and DIP agents on the passive tonus (at resting tension) or active (following pre-contraction elicited with phenylephrine, 5x10⁻⁷M) isometric tension of aortal rings were examined (n=10 animals per protocol). In all protocols cumulative dose response effects were performed for each agent. In addition the effects of preincubation (30 min) with GLN or DIP (each 5x10⁻⁵ M) were examined: for their stimulatory effects on smooth muscle contraction, phenylephrine or 5-HT dose-response tests were performed; or for their inhibitory properties on smooth muscle contraction, following pre-contraction elicited with phenylephrine 5x10⁻⁷M, cumulative dose response to sodium nitroprusside (SNP, NO donor) or minoxidil (ATP-dependent potassium channel activator) were performed.

Substances

Following substances were purchased by Sigma-Aldrich (Deisenhofen, Germany), L-phenylephrine, Na-nitroprusside, 5-HT and minoxidil. Glutamine agents (Fresenius, Bad Homburg, Germany) were the same that were used for the *in vivo* experiments. Dilutions were made in the Krebs solution immediately before experiments.

Statistical analysis

Data analysis was performed with statistical software package (SPSS 15.0 for Windows, SPSS Inc., Chicago IL, USA). Cytokine release, white blood cell count and plasma extravasation data were expressed as group medians, 10th, 25th, 75th and 90th percentiles. Cytokine release data were analyzed using One-Way Analysis of Variance (ANOVA) followed by the Bonferroni *post hoc* test. Plasma extravasation, white blood cell count, macrocirculation, blood gas and rectal body temperature were analyzed by a Two-Way ANOVA (repeated measures in the factor of time) followed by the Newman-Keuls multiple comparison tests. For *in vitro* experiments, the data were fit to non-linear regression (Hill-Langmuir equation was applied) and the results compared using Two-Way ANOVA that was followed by Bonferroni *post hoc* test. The P<0.05 was taken to be statistically significant.

RESULTS

Macrocirculation and temperature

Heart rate (HR), rectal body temperature (RBT) and mean arterial pressure (MAP) did not differ significantly in all 4 groups at baseline (-0.5 h and 0 h). In the control group, HR (average of approx. 400 min⁻¹) and RBT (approx. 38°C) did not change during the observation period of 120 min (Fig. 1A,C,D). MAP significantly increased after the beginning of the experiment to values averaging between 130 and 140 mmHg (Fig. 1B). LPS administration was associated with a significantly elevated HR (approx. 450 beats.min⁻¹) and RBT, compared to the animals of the control group (p<0.05). In all groups with endotoxemia the MAP values decreased consistently after 30 min of observation, median MAP values never falling below 90 mmHg.

Leukocyte-endothelial interactions

In the untreated LPS group, we observed a significant increase in the number of adherent leukocytes (leukocyte

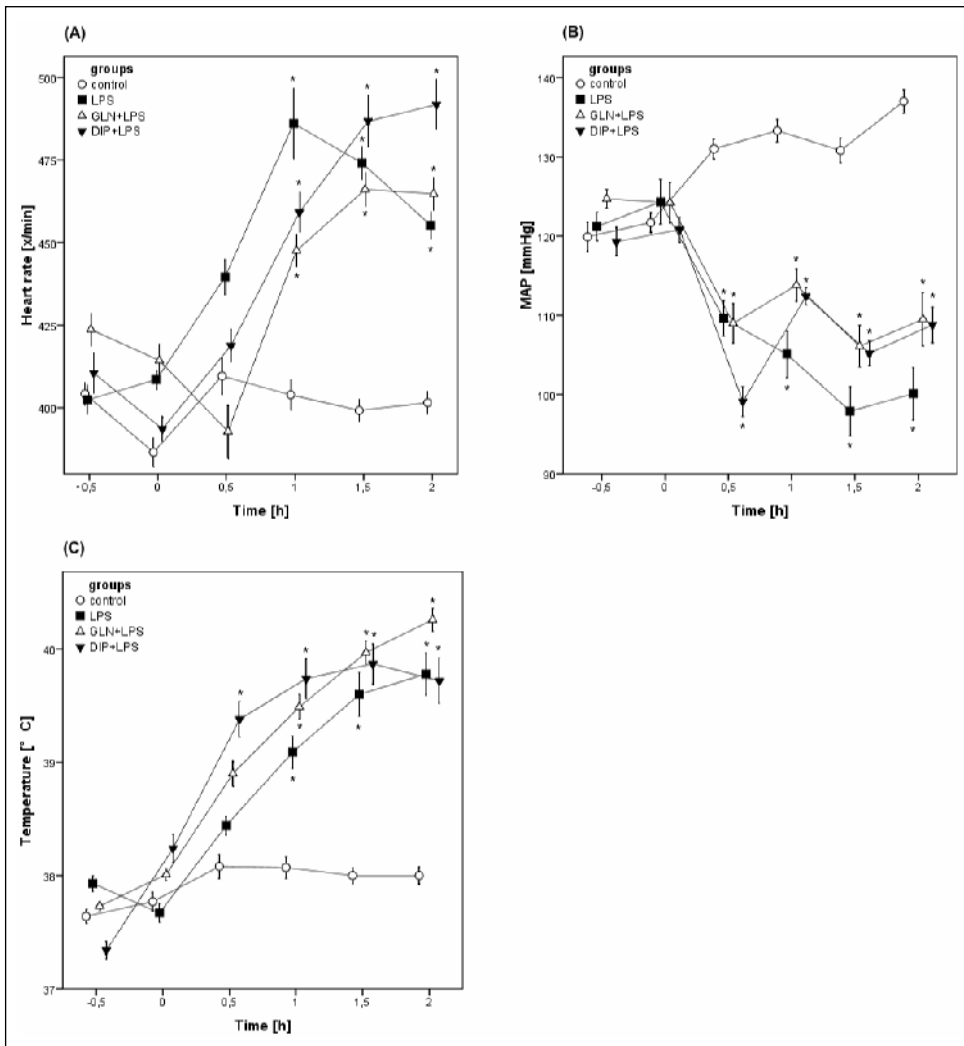


Fig. 1. Macrocirculation and rectal body temperature. Panel A - Heart rate (HR, beats.minute⁻¹), Panel B - Mean arterial pressure (MAP, mmHg), Panel C - Rectal body temperature (RBT, °C); Significances: * p<0.05 for all groups vs. control group; mean±SEM.

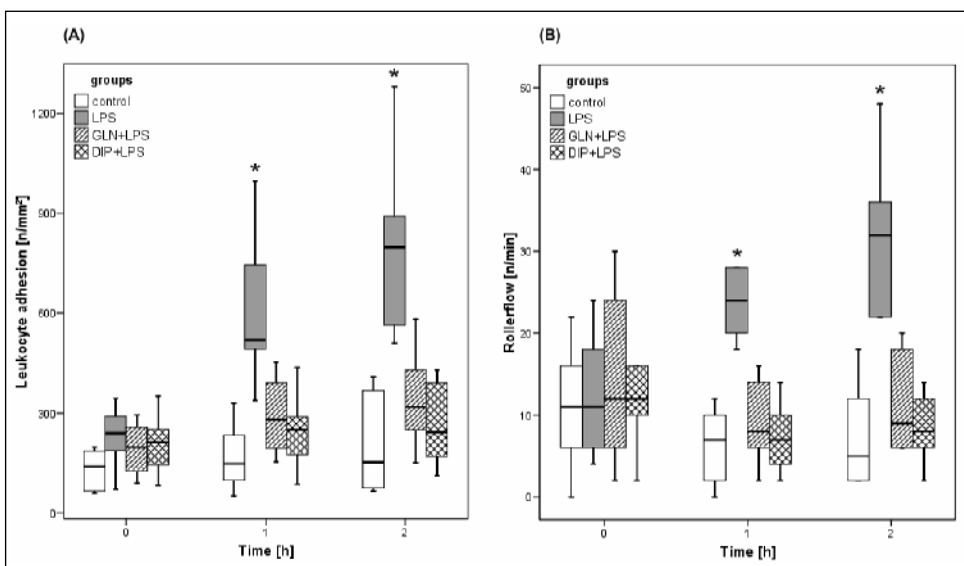


Fig. 2. Leukocyte-endothelial interactions. Panel A - Leukocyte adhesion (n.mm⁻²); Panel B - Rollerflow (n.min⁻¹); Significances: * p<0.05 for LPS group vs. other groups. Median, 10th, 25th, 75th, 90th percentiles.

adhesion) after 1 and 2 hours as compared not only to the baseline (t=0) but also to the controls, GLN+LPS and DIP+LPS groups (p<0.05; *Fig. 2A*). Indeed, the animals of GLN+LPS and the DIP+LPS group did not show a significant increase in leukocyte adhesion following endotoxin administration. The rolling behavior (rollerflow) was unchanged in the control

group during the experiment (*Fig. 2B*). Rollerflow significantly increased following endotoxin challenge, as compared to the initial value (t=0) and the corresponding value of the control group (p<0.05). Dipeptide and glutamine administration reduced the number of the temporarily adherent leukocytes significantly.

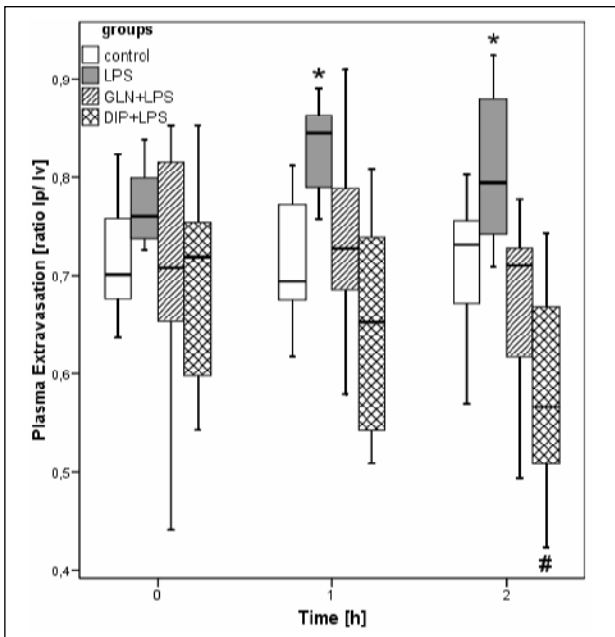


Fig. 3. Plasma extravasation. Fluorescence activity in the venule under the study (Iv) and in contiguous areas of the perivenular interstitium (Ip), expressed as the ratio Ip/Iv; Significances: * $p < 0.05$ for LPS group vs. other groups; # $p < 0.05$ for DIP+LPS group vs. LPS group or control group. Median, 10th, 25th, 75th, 90th percentiles.

Table 1. Cytokine levels.

	Control	LPS	GLN+LPS	DIP+LPS
TNF-α	79 (64-152)	958 (834-1270)	678 (490-1004)	697 (617-1034)
IL-1β	145 (128-173)	1809 (1369-2305)	1856 (1760-2142)	2063 (1416-2526)
IL-6	740 (405-1220)	13034 (12923-13148)	13023 (12917-13144)	12890 (12814-13036)
IL-10	226 (157-434)	496 (401-733)	476 (425-797)	505 (399-604)

Cytokine levels (pg.ml⁻¹); Median (25th, 75th percentiles); following two hours of observation; LPS-lipopolysaccharide; TNF- α -tumor necrosis factor-alpha; IL-interleukin; Significances: * $p < 0.05$ for all groups vs. control group; # $p < 0.05$ Glutamine groups vs. LPS group.

Extravasation

In the untreated LPS group a significant increase in plasma extravasation occurred during the observation time while the extravasation ratio in the control group was constant. In the GLN treated group the extravasation was at the level of the control group. DIP administration in endotoxemic animals significantly reduced the plasma extravasation values after 2 hours in comparison to the control group ($p < 0.05$). (Fig. 3).

Blood gases

The blood gas analyses showed respiratory stability while the glucose and lactate levels in the endotoxemic animals were significantly elevated as compared to the control group ($p < 0.05$; data not shown).

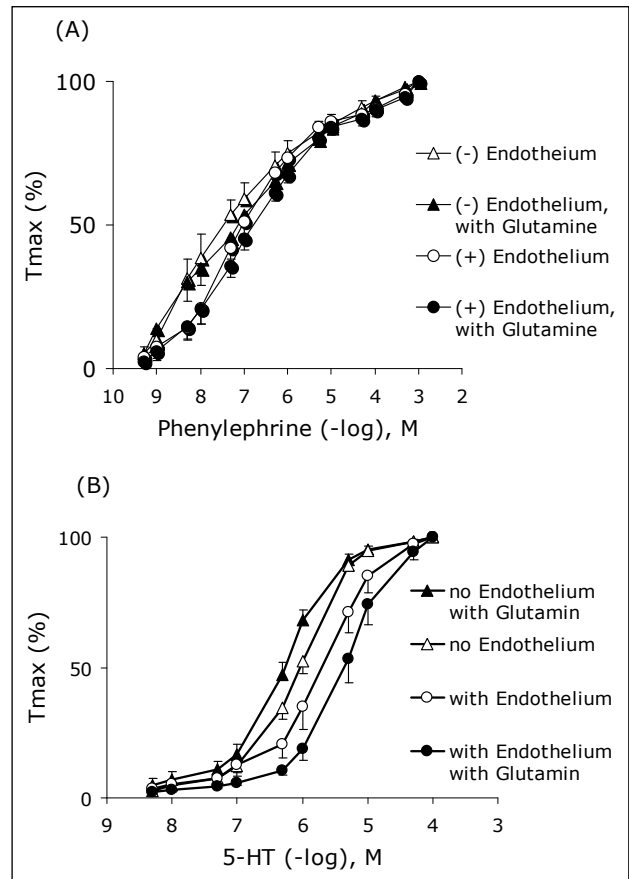


Fig. 4. Dose-response curves. The responses to phenylephrine (Panel A) and 5-HT (panel B) were obtained following 30 minutes preincubation of rat aorta *in vitro* with glutamine preparations. Significances: phenylephrine, controls vs. glutamine with endothelium, $p < 0.05$; for 5-HT differences were not significant; mean \pm SEM.

Cytokine release

At the beginning of the experiment the IL-1 β , IL-6, IL-10 and TNF- α levels did not differ between the 4 experimental groups. Endotoxin challenge resulted in a systemic release of inflammatory cytokines. In the GLN+LPS- and DIP+LPS-group we observed reduced TNF- α levels as compared to the untreated LPS group (Table 1; $p < 0.05$). Neither GLN nor DIP pre-treatment reduced IL-1 β , IL-6 or IL-10 cytokine release.

Aortal contractility

In rat aortal rings, following 30 minutes preincubation with glutamine the dose response curves of phenylephrine or 5-HT were displaced to the right only in preparations with endothelium. In contrast, both glutamine dipeptide agents did not influence dose-response of phenylephrine and of 5-HT and all glutamine preparations and did not influence the relaxing dose-response curves of SNP or minoxidil (data not shown). (Fig. 4)

DISCUSSION

In the present study, it was demonstrated, by using the intravital fluorescence microscopy, that GLN and DIP pre-treatment improved the microcirculation during experimental

endotoxemia by reducing mesenteric leukocyte-endothelial interaction and plasma extravasation, and that GLN and DIP treatment resulted in a reduction of endotoxin-induced TNF- α release. In vitro experiments indicated that some of the effects could partially be explained by nitric oxide regulation mechanisms.

To our knowledge, no studies have yet examined the effects of parenterally administered GLN and DIP on the mesenteric microcirculation during experimental endotoxemia. Nevertheless, beneficial effects of glutamine preparations for the condition have been suggested and several mechanisms proposed, and the most often cited is the effect on oxygen radicals scavenging (15). The ratio of intracellular content of reduced (GSH) to oxidized (GSSG) glutathione is important, because it appears to be the major regulator of the cellular redox potential (15-18). Supplementation of GLN *in vitro* and *in vivo* leads to an increase of GSH levels (15, 19) and thereby to an elevated GSH: GSSG ratio, reducing oxidative stress. However, it was shown that oxidative stress is related to the activation of some inflammatory pathways (20-22), e.g. the nuclear factor- κ B (NF- κ B), resulting in the synthesis of a number of pro-inflammatory cytokines (for example TNF- α) (23) and inducible nitric oxide synthase (iNOS) (24). The *in vitro* experiments of our study indicate that the NO-pathway may be involved, at least partially, in the vascular effects that could be related to what has been observed *in vivo*.

Other *in vitro* studies demonstrated that low GLN concentrations resulted in higher cell adhesion molecule (CAM) expression and greater transendothelial migration of neutrophils (25). It could be shown that GLN administration at levels similar to or higher than physiologic concentrations decreased CAM expressions on endothelial cells and neutrophils (26, 27), which could explain our finding of reduction of leukocyte rolling and sticking after the administration of GLN.

Number of other processes that may be influenced by GLN are closely associated with the inflammatory response (28, 29). It was revealed that GLN is a potent inducer of heat shock protein 72 (HSP72) and 25 (HSP25) expression, or that GLN may mediate cellular protection by an enhanced expression of HSP70 (30, 31). In accordance with the previous studies (9, 32), a significant attenuation of TNF- α release in septic rats with GLN and DIP treatment as compared to the untreated LPS group was observed in the present study.

Taken together, the presented results demonstrate that GLN and DIP pre-treatment have beneficial effects within the microcirculation in a rat model of experimental endotoxemia. Clinical trials are necessary to examine the impact of GLN supplementation upon the microcirculation in human sepsis.

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Conflict of interests: None declared.

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