MedicalExpress

ORIGINAL RESEARCH

Protective microcirculatory and anti-inflammatory effects of heparin on endotoxemic hamsters

Marcos L. Miranda,[I] Luiz Felipe M. Prota,[I] Maria Júlia B. Silva,[II] Fernando L. Sicuro,[I] Eliane S. Furtado,[I] Ana Olimpia M.T. Santos,[II] Eliete Bouskela[I]

[I]Laboratory for Clinical and Experimental Research in Vascular Biology – BioVasc, Biomedical Center, Rio de Janeiro State University, Rio de Janeiro, RJ, Brazil [II]Oswaldo Cruz Foundation – Fiocruz, Rio de Janeiro, RJ, Brazil

OBJECTIVE: Apart from its anticoagulant properties, heparin has vasodilator and anti-inflammatory effects that could assist in the reversal of septic microcirculatory changes. This paper investigates the effects of heparin on endotoxemia-related microcirculatory changes and compares them to those observed with the use of recombinant human activated protein C.

METHODS: After skinfold chamber implantation procedures and endotoxemia induction by intravenous *Escherichia coli* lipopolysaccharide administration (2 mg.kg^{-1}), male golden Syrian hamsters were treated with intravenous unfractionated heparin (0.2 mg.kg^{-1}). Intravital microscopy of skinfold chamber preparations allowed quantitative analysis of microvascular variables and venular leukocyte rolling and adhesion. Macrohemodynamic parameters were also analyzed. Endotoxemic hamsters treated with recombinant human activated protein C and non-treated animals served as controls.

RESULTS: Heparin decreased lipopolysaccharide-induced leukocyte rolling and arteriolar vasoconstriction; it also increased survival when compared with non-treated animals, while recombinant human activated protein C decreased leukocyte adhesion. Administration of heparin plus recombinant human activated protein C was associated with a significant attenuation of lipopolysaccharide-induced capillary perfusion deficits.

CONCLUSIONS: Heparin yields protective effects on endotoxemic animals' microcirculation. Those benefits were potentiated when heparin was administered in conjunction with recombinant human activated protein C.

KEYWORDS: sepsis; endotoxemia; microcirculation; heparin; recombinant human activated protein C.

Miranda ML, Prota LFM, Silva MJB, Sicuro FL, Furtado ES, Santos AOMT, Bouskela E. Protective microcirculatory and anti-inflammatory effects of heparin on endotoxemic hamsters. MEDICALEXPRESS. 2014 June;1(3):127-134.

Received for publication on March 3 2014; **First review completed on** March 9 2014; **Accepted for publication on** March 30 2014

E-mail: eliete_bouskela@yahoo.com.br

INTRODUCTION

Sepsis is an infection-related systemic inflammatory syndrome associated with a massive activation of the coagulation cascade, consumption of clotting factors, and disruption of the normal balance between coagulation and fibrinolysis.[1,2] This altered coagulant state is associated with uncontrolled formation of intravascular platelet/fibrin clots in the microcirculation. The so-called microthrombosis is responsible for physical occlusion of capillaries, being an important cause of microcirculatory dysfunction.[3] As sepsis progresses, microcirculatory abnormalities ultimately result in tissue hypoxia, organ failure, and death.[4,5]

Seeing that levels of antithrombotic factors are frequently very low in patients with severe sepsis and septic shock, it could be speculated that correction of those low levels could assist in reversal of the septic procoagulant state, attenuating microvascular perfusion deficits and improving patients' outcome.[6] In this way, numerous clinical trials have already examined the use of antithrombotic drugs such as recombinant human activated protein C (rhAPC), tissue factor pathway inhibitor, and antithrombin III in septic patients. Unfortunately, none of these agents consistently improved the outcome of these patients, but all significantly increased the risk of bleeding.[7]

Unfractionated heparin is the most widely used anticoagulant in clinical practice and is recommended for thromboembolism prophylaxis in septic patients; a vast experience regarding its safety has accumulated.[8,9] Besides its anticoagulant property, heparin has other less studied properties, such as its vasodilator and anti-inflammatory effects.[8,9] Taken together, these properties could be beneficial for microcirculatory function. So, we have hypothesized that heparin could assist in the reversal of septic microcirculatory changes, a decisive step in sepsis treatment, but this subject has been poorly explored in the literature. Strengthening our hypothesis, Dobosz et al.[10] have

DOI: 10.5935/MedicalExpress.2014.03.06

already demonstrated that heparin has a positive influence on organ microcirculatory disturbances accompanying non-infectious experimental systemic inflammatory syndrome. Additionally, in a meta-analysis involving septic patients, heparin was associated with improved 28-day survival.[11]

Aiming to investigate the effects of heparin on endotoxemia-related microcirculatory changes and to compare its effects with those observed with rhAPC (an extensively studied antithrombotic and anti-inflammatory drug), we have carried out this controlled experimental study.

MATERIALS AND METHODS

Experiments were performed on 28 male golden Syrian hamsters (*Mesocricetus auratus,* ANILAB, Animais de Laboratório, Paulínia, SP, Brazil) weighing between 60 and 80 g. Animals were housed one per cage under controlled conditions of lighting (12:12 hours light/dark cycle) and temperature (21.0 ± 1.0°C), with free access to water and standard chow (NUVILAB CR1, Quimtia S/A, Colombo, PR, Brazil). All procedures were approved by the Rio de Janeiro State University Animal Care and Use Committee (protocol number CEUA/060/2010) and are consistent with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and ethical rules specified by the Basel Declaration.

Animal preparation

The chamber implantation procedure has been described previously by Endrich et al.[12] in details. Briefly, under anesthesia with sodium pentobarbital (90 $mg.kg^{-1}$ intraperitoneal injection; Hypnol 3%, Syntec, Cotia, SP, Brazil) animals' dorsal hair was shaved and depilated with a commercial hair-removing solution. After that, the dorsal skin of the back was lifted away from the animal, creating a skinfold. Then, this skinfold was sandwiched between two titanium frames and one of its layers was microsurgically excised in a circular area of 15 mm in diameter. The remaining layer, consisting of epidermis, subcutaneous tissue, and thin striated skin muscle (*panniculus carnosus* muscle) was covered with a removable circular cover glass incorporated into one of the metal frames, creating the window chamber. After a recovery period of 6 days, animals were re-anesthetized and the left carotid artery catheterized (polyethylene-50 catheter) allowing continuous hemodynamic monitoring and blood sampling. The left jugular vein was also catheterized (polyethylene-10 catheter) for fluid infusion and drug injection. These catheters were tunneled under the skin, exteriorized at the dorsal side of the neck, filled with heparinized saline solution (40 $IU.ml^{-1}$), and attached to the chamber frame with tape. Experiments were performed on awake animals after 24 hours of catheter implantation.

Hemodynamic monitoring

Mean arterial blood pressure (MAP) was continuously monitored during the experimental period through the arterial catheter and a pressure transducer. Analog pressure signals were digitized (MP100 Data Acquisition System, BIOPAC Systems, Goleta, CA, USA) and processed using data acquisition software for hemodynamic experiments (AcqKnowledge Software v. 3.5.7, BIOPAC Systems, Goleta, CA, USA). Heart rate (HR) was determined from the pressure trace and expressed as beats per minute (bpm).

Intravital microscopy

The unanesthetized animal was placed in a restraining plexiglass tube attached to the stage of an intravital microscope (Ortholux, Leitz, Wetzlar, Germany) equipped with an epifluorescence assembly (100-W HBO mercury lamp with filter blocks, Leitz, Wetzlar, Germany). The body temperature of the hamsters was maintained with a heating pad placed near the animal, which was controlled by a rectal thermistor (LB750, Uppsala Processdata AB, Uppsala, Sweden). Moving images of the microcirculation were obtained using a 20x objective (CF SLWD Plan EPI 20x/0.35 Achromat Objective WD 20.5 mm, Nikon, Tokyo, Japan) and a charge-coupled device digital video camera system (SBC-320P B/W Camera, Samsung, Seoul, South Korea) resulting in a total magnification of 800-fold at the video monitor. Microcirculatory acquired images were recorded as video files in digital media for later evaluation. Quantitative off-line analysis of videos was performed using Cap-Image 7.2, a computer-assisted image analysis system (Dr. Zeintl Biomedical Engineering, Heidelberg, Germany[13]) by an investigator blinded to drug treatment. In each animal, 2 arterioles, 2 venules, and 10 capillary fields were chosen, taking into account the absence of inflammation or bleeding in the microscopic field. The presence of histological landmarks that could facilitate the subsequent return to the same field was also noted, because the same vessels and capillary fields were studied throughout the experiment. Arteriolar and venular mean internal diameters were measured as perpendicular distance (in micrometers) between the vessel walls. Functional capillary density (FCD) was considered to be the total length (in centimeters) of spontaneously red blood cell-perfused capillaries per square centimeter of tissue surface area ($cm.cm^{-2}$).

Evaluation of leukocyte-endothelial interactions

After *in vivo* staining of leukocytes with rhodamine 6G (0.15 $mg.kg^{-1}$ intravenously [IV]; 0.4 ml; Sigma-Aldrich, St. Louis, MO, USA), leukocyte-endothelial interactions were assessed by intravital fluorescence microscopy. According to their interaction with the microvascular endothelium, leukocytes were classified as passing, rolling, or adhered. Passing leukocytes were defined as white blood cells traversing an observed venular segment without sticking contact (adherence) to the endothelium lining. Rolling leukocytes were defined as white blood cells moving along the endothelial lining with a velocity significantly slower than surrounding erythrocytes. The number of rolling leukocytes was expressed as a percentage of the number of passing leukocytes. A leukocyte was considered to be adherent to the venular endothelium lining if it remained stationary for more than 30 seconds. Adherent cells were counted in a 100 μm venular segment and the number of adherent leukocytes was expressed as the number of adherent cells per field. Cell counting was performed off-line by an investigator blinded to treatment. One venule was studied in each animal, and a single period of 60 seconds was analyzed for all cell counts.

Experimental protocol

Animals were suitable for experiments if their baseline hemodynamic variables were within the normal range.

Animals with signs of inflammation and/or bleeding in the chamber were excluded from the study.

At the beginning of the experiment, animals were given 30 minutes to adapt to restraining plexiglass tube before baseline variables were measured. Immediately after baseline determination of hemodynamic and microcirculatory parameters and evaluation of leukocyte-endothelial interactions, endotoxemia was induced by an IV injection of 2 mg.kg^{-1} of *Escherichia coli* serotype 055:B5 lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO, USA) diluted in normal saline (total volume of 0.2 ml). After endotoxemia induction, hamsters were randomly allocated to one of four study groups: (1) LPS (n = 7) – no further treatment after LPS injection; (2) rhAPC (n = 7) – one hour after LPS injection, a continuous IV infusion of rhAPC solution (24 μg.kg^{-1}.h^{-1}) was initiated and maintained at a 0.05 ml.h^{-1} infusion rate for 5 hours; (3) HEP (n = 7) – one hour after LPS injection, a single IV bolus of unfractionated heparin (0.2 mg.kg^{-1}; 0.1 ml) was administered; (4) rhAPC/HEP (n = 7) – one hour after LPS injection, a single IV bolus of unfractionated heparin (0.2 mg.kg^{-1}; 0.1 ml) was administered and a continuous IV infusion of rhAPC (24 μg.kg^{-1}.h^{-1}) was initiated and maintained at a 0.05 ml.h^{-1} infusion rate for 5 hours.

As shown in Figure 1, sequential measurements of hemodynamic and microcirculatory parameters were performed at five time points: at baseline and after 1, 3, 6, and 24 hours of LPS injection. Sequential evaluations of leukocyte-endothelial interactions were performed at two time points: at baseline and after six hours of LPS injection.

Survival analysis

After the intravital microscopy phase of the experiments, animals were returned to their individual cage in the *vivarium* with free access to water and standard chow and monitored for survival three times per day for 7 days. After 7 days, surviving animals were euthanized by a lethal dose of pentobarbital.

Statistical analysis

Results are expressed as means ± standard deviation of the mean (SD) for each group, unless otherwise noted. Microvascular diameters and FCD data are presented as ratios relative to baseline values. All hemodynamic and microcirculatory measurements were compared with the baseline of the same group and between groups at the same time point. Statistical differences within and between groups were determined by Friedman and Kruskal-Wallis tests, followed, when appropriate, by Dunn's multiple-comparisons test for *post hoc* analysis. Survival curves were obtained using the Kaplan-Meier procedure, and the Mantel-Cox log-rank test was applied for determination of significant differences between study groups. All statistical analyses were performed using GraphPad Prism 6.03 (GraphPad Software, La Jolla, CA, USA) and the significance level was set as $p < 0.05$.

Ethical adherence: All procedures were approved by the Rio de Janeiro State University Animal Care and Use Committee (protocol number CEUA/060/2010) and are consistent with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and ethical rules specified by the Basel Declaration.

RESULTS

The average body weight of the hamsters did not differ significantly between study groups. All animals survived the intravital microscopy phase of the experimental protocol and underwent survival analysis.

Hemodynamic alterations

Hemodynamic parameters are presented in Table 1. MAP and HR basal values were not significantly different among experimental groups and were comparable to control values for reported data on healthy hamsters.[14] Systemic administration of LPS elicited statistically similar reductions of MAP levels in all study groups (Table 1; 1 hour). MAP was comparable among study groups until 24 hours. At this time point, MAP was significantly higher in the HEP group than in the LPS group. HR was comparable among study groups at each time point (Table 1).

Microcirculatory parameters

- Arteriolar and venular diameters: At baseline, there were no significant differences in arteriolar and venular mean internal diameters between study groups. Arteriolar mean internal diameter was significantly reduced by endotoxemia in LPS and rhAPC groups at 6 and 24 hours when compared to baseline. These groups had comparable arteriolar diameters at each time point (Figure 2). Arteriolar diameter was wider in heparin treated groups

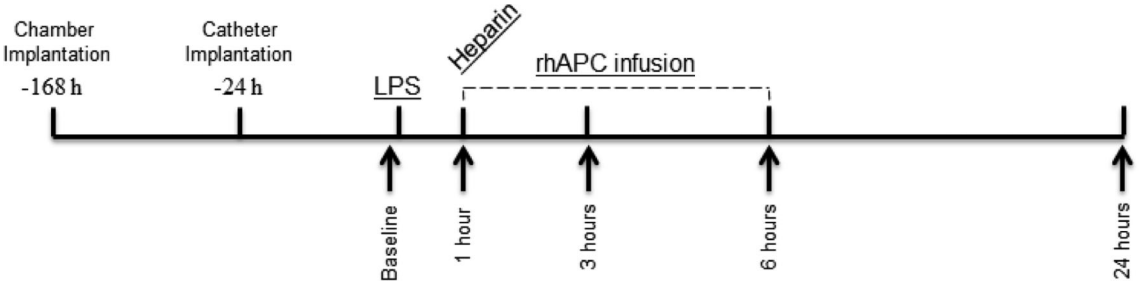

Figure 1 - Schematic representation of the experimental protocol after baseline determination of hemodynamic and microcirculatory parameters LPS was administrated. Sequential measurements were performed after one (1 hour), three (3 hours), six (6 hours), and twenty-four (24 hours) hours of LPS injection (arrows). Sequential evaluations of leukocyte-endothelial interactions were performed at baseline and after six hours of LPS injection.

Table 1 - Mean arterial pressure and heart rate evolution during the experimental period

		LPS	rhAPC	HEP	rhAPC/HEP
Mean arterial pressure (mmHg)	Baseline	110.6 ± 8.3	110.2 ± 2.9	106.3 ± 3.4	110.0 ± 7.8
	1 hour	81.4 ± 11.0*	81.0 ± 11.0*	80.3 ± 10.6*	81.4 ± 5.4*
	3 hours	70.9 ± 10.4*	81.7 ± 7.2*	74.5 ± 4.4*	70.7 ± 4.0*
	6 hours	72.0 ± 7.4*	75.0 ± 9.4*	71.5 ± 5.1*	68.8 ± 6.4*
	24 hours	49.6 ± 34.1*	75.2 ± 3.7*	80.5 ± 6.0*#	77.0 ± 7.3*
Heart rate (bpm)	Baseline	423.6 ± 25.5	417.0 ± 21.7	422.2 ± 22.2	426.2 ± 35.3
	1 hour	429.3 ± 45.3	410.3 ± 19.4	405.2 ± 11.0	414.3 ± 20.3
	3 hours	402.4 ± 53.5	404.5 ± 10.3	404.5 ± 9.0	409.8 ± 9.0
	6 hours	379.4 ± 56.4	413.3 ± 18.3	405.0 ± 13.6	417.8 ± 13.2
	24 hours	392.7 ± 49.3	406.5 ± 26.7	413.0 ± 16.0	401.2 ± 27.0

Data are presented as means ± SD for each group. LPS, endotoxemic animals (n = 7); rhAPC, endotoxemic and rhAPC treated animals (n = 7); HEP, endotoxemic and heparin treated animals (n = 7); rhAPC/HEP, endotoxemic and rhAPC + heparin treated animals (n = 7). * p < 0.05 vs. group baseline; # p < 0.05 vs. LPS group at the same time point.

(HEP and rhAPC/HEP groups) than in the other groups; at 3, 6, and 24 hours, a significant difference was observed between the HEP group and both LPS and rhAPC groups (Figure 2). In terms of venular mean internal diameter, LPS caused slight venodilation, evident at 3 hours ($p < 0.05$ *vs.* baseline). Venular diameters tended to be wider in HEP group and smaller in rhAPC group. These differences faded away in such a manner that at 24 hours there were no significant differences between the venular diameters of the study groups (Figure 2).

- Capillary perfusion (FCD): At baseline, FCD did not significantly differ between study groups. LPS administration decreased FCD in all study groups. FCD was comparable among study groups until 3 hours. From this time point, FCD was significantly higher in the rhAPC/HEP group than in the LPS group (Figure 3).

Evaluation of leukocyte-endothelial Interactions

Treatment with heparin or heparin plus rhAPC reduced the percentage of rolling leukocytes compared with baseline (Figure 4). Endotoxemia significantly increased the number of adhered leukocytes in LPS group. At 6 hours, a smaller number of adherent leukocytes was observed in rhAPC, HEP, and rhAPC/HEP groups compared with the LPS group, although a significant difference was only observed between LPS and both rhAPC and rhAPC/HEP groups (Figure 4).

Seven-day survival

Median survival time after experiments was 2.2, 5.4, 4.7, and 5.8 days for LPS, rhAPC, HEP, and rhAPC/HEP groups, respectively ($p < 0.05$ for LPS *vs.* all other groups; Figure 5).

DISCUSSION

In few pathological states are the connections between coagulation and inflammatory cascades so evident as in sepsis. In this syndrome, inflammatory cytokines, including tumor necrosis factor α, interleukin-1β, and interleukin-6, are capable of activating coagulation and inhibiting fibrinolysis, whereas the procoagulant thrombin is capable of stimulating multiple inflammatory pathways.[15] Heparin may play a positive role in sepsis treatment due to its anticoagulant and anti-inflammatory properties. In fact, in the present study, these effects proved to be beneficial in a validated endotoxemia rodent model that allows *in vivo* evaluation of inflammation and perfusion dysfunction.

The heparin dose used in our study was similar to that capable of preventing ferric chloride-induced venous thrombosis in a murine model of deep vein thrombosis (unpublished data of our group). At this dose, heparin did

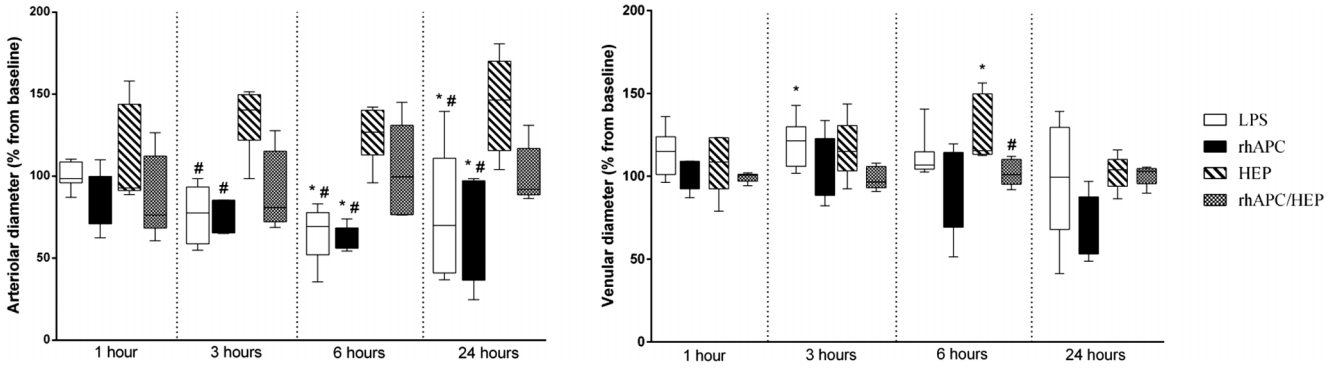

Figure 2 - Evolution of arteriolar and venular mean internal diameters during the experimental period. Values are presented as ratios relative to baseline values. Sequential measurements were performed after one (1 hour), three (3 hours), six (6 hours), and twenty-four (24 hours) hours of LPS injection. LPS, endotoxemic animals (n = 7); rhAPC, endotoxemic and rhAPC treated animals (n = 7); HEP, endotoxemic and heparin treated animals (n = 7); rhAPC/HEP, endotoxemic and rhAPC + heparin treated animals (n = 7). * $p < 0.05$ *vs.* group baseline; # $p < 0.05$ *vs.* HEP group at the same time point.

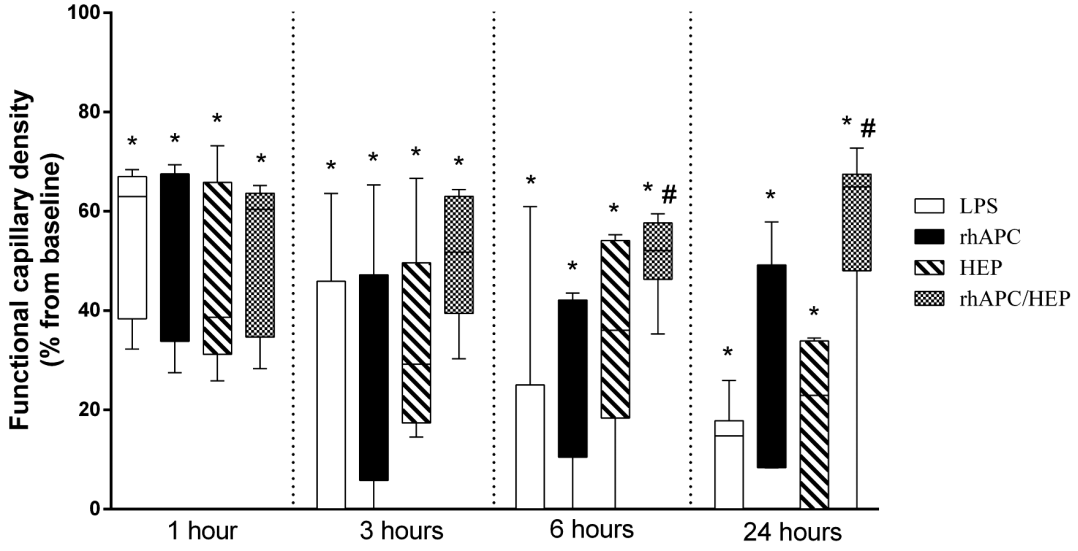

Figure 3 - Functional capillary density evolution during the experimental period. Values are presented as ratios relative to baseline values. Sequential measurements were performed after one (1 hour), three (3 hours), six (6 hours), and twenty-four (24 hours) hours of LPS injection. LPS, endotoxemic animals (n = 7); rhAPC, endotoxemic and rhAPC treated animals (n = 7); HEP, endotoxemic and heparin treated animals (n = 7); rhAPC/HEP, endotoxemic and rhAPC + heparin treated animals (n = 7). * $p < 0.05$ *vs.* group baseline; # $p < 0.05$ *vs.* LPS group at the same time point.

not result in bleeding complications. Whereas rhAPC also has anti-inflammatory and anticoagulant properties and its use has been extensively studied in sepsis syndrome, we considered this drug as a control treatment in our experiment. rhAPC was administered at a dose rate similar to that clinically given in the PROWESS study[15] to patients with severe sepsis (24 $\mu g.kg^{-1}.h^{-1}$). In a previous experimental study of Hoffmann et al.[2], at this dose, rhAPC demonstrated beneficial microcirculatory and anti-inflammatory effects in endotoxemic hamsters without increasing bleeding complications. The endotoxin dose used in our study was adjusted to affect microcirculatory parameters without the induction of severe hypotension. During the initial hours of the experiment, this simulates the hyperdynamic phase of sepsis. After this initial period, non-treated animals (LPS group) were considered to be in endotoxemic shock. Because LPS-induced hypotension is dependent on activation of the inflammatory cascade, it is understandable that substances with anti-inflammatory properties (heparin and rhAPC) could hamper the hypotensive effect of LPS. Some of the anti-inflammatory effects of heparin have already been described: TNF-α inhibition, protection against oxygen free radicals, and blockade of complement activity.[9,10]

Although heparin is largely used for the prevention of blood clotting, it produces additional vascular effects independent of its anticoagulant activity. Tasatargil et al.[8] have shown that heparin causes concentration-dependent vasodilatation in human arteries, and that this action seems to be linked to endothelium-dependent mechanisms, including changes in the generation of nitric oxide and endothelium derived hyperpolarizing factor. Furthermore, Tangphao et al.[16] have shown that heparin is an endothelium-dependent venodilator. The mechanism of heparin-induced venous relaxation involves increased availability of nitric oxide, possibly related to local release of histamine. Sternbergh et al.[17] have demonstrated that heparin has dose-dependent protective effects on vascular endothelial

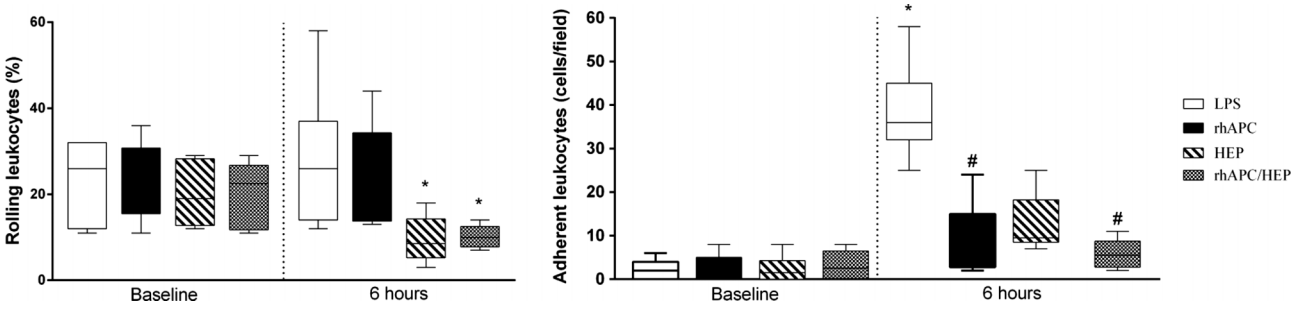

Figure 4 - Sequential evaluation of leukocyte-endothelial interactions. The number of rolling leukocytes is expressed as a percentage of the number of passing leukocytes. The number of adherent leukocytes is expressed as the number of adherent cells per field. Sequential measurements were performed at baseline and after six hours of LPS injection. LPS, endotoxemic animals (n = 7); rhAPC, endotoxemic and rhAPC treated animals (n = 7); HEP, endotoxemic and heparin treated animals (n = 7); rhAPC/HEP, endotoxemic and rhAPC + heparin treated animals (n = 7). * $p < 0.05$ *vs.* group baseline; # $p < 0.05$ *vs.* LPS group at the same time point.

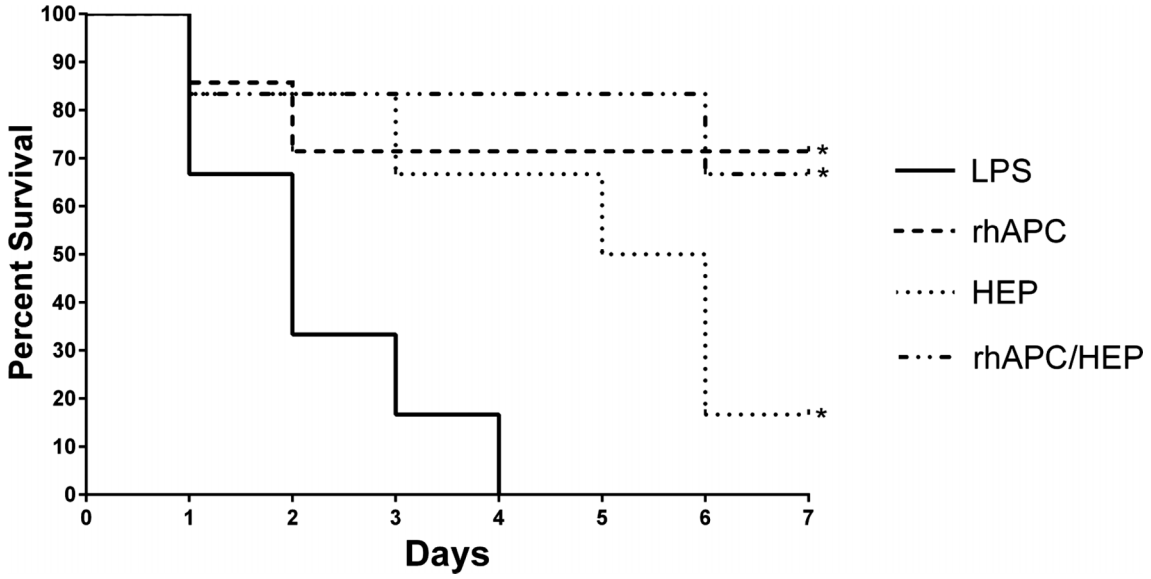

Figure 5 - Kaplan-Meier survival curves. LPS, endotoxemic animals (n = 7); rhAPC, endotoxemic and rhAPC treated animals (n = 7); HEP, endotoxemic and heparin treated animals (n = 7); rhAPC/HEP, endotoxemic and rhAPC + heparin treated animals (n = 7). * $p < 0.05$ *vs.* LPS group.

cell function distinct from its anticoagulant, antiplatelet, or anticomplement activity. Thus, heparin can preserve endothelial-dependent vasodilatation in states of oxidative stress.[17] An inhibitory effect on biosynthesis and release of endothelin-1, a potent endogenous vasoconstrictor, has also been shown.[18] In our study, we have observed that the vasodilator effect of heparin also occurs in venules and arterioles, even during endotoxemia. Surprisingly, rhAPC administration prevented LPS-induced venular vasodilatation but could not prevent endotoxin-mediated decrease of arteriolar mean internal diameter. Apparently, apart from an anti-inflammatory action, an endothelium-mediated vasodilator effect is also necessary to maintain arteriolar diameters during endotoxemia.

Because endotoxemia is a known up-regulator of leukocyte-endothelial interactions and because increased leukocyte-endothelium interactions are related to microcirculatory impairment[5], we have performed intravital fluorescence microscopy to study leukocyte kinetics. Leukocyte migration involves a step-wise process: first leukocytes roll along the vascular endothelium, then they firmly adhere and finally emigrate from the microcirculation to inflamed tissues. Thus, the quantitation of rolling and adherent leukocytes can reliably reflect the extent of the inflammatory response.

To understand the results related to the number of rolling leukocytes, it is important to know that as early as 30 minutes after LPS administration there is a massive decrease in venular leukocyte rolling fraction (about 12% of non-adherent leukocytes are rolling); baseline conditions are reached again 3 hours after LPS (20–30% of non-adherent leukocytes are rolling) and a profound increase in the number of rolling leukocytes occurs 8 hours after LPS administration (about 60% of the non-adherent leukocytes are rolling).[14] When leukocyte-endothelial interactions were assessed (6 hours after LPS administration) there was sufficient time for recovery of the basal number of rolling leukocytes as observed in LPS group. The maintenance of a small fraction of rolling leukocytes in HEP and rhAPC/HEP groups may be related to an anti-inflammatory action of heparin. In our study, rhAPC was not capable of preventing the induction of leukocyte rolling. An opposite behavior was observed regarding leukocyte adhesion. This result might be related to different adhesion molecules involved; while rolling involves the selectin family of adhesion molecules, firm adhesion involves β_2-integrins (expressed in leukocytes) and the endothelial intercellular adhesion molecule-1 (ICAM-1).[19,20] So, the discrepancy between our observations could be attributed to complex cellular interactions found in *in vivo* studies. Heparin and rhAPC may have different and additional actions on these adhesion molecules. The improved results found in rhAPC/HEP group regarding both leukocyte rolling and adhesion favor this hypothesis.

Considering the temporal evolution of functional capillary perfusion, our study showed that administration of rhAPC plus heparin was associated with significant attenuation of capillary perfusion deficits induced by LPS administration. Several factors are related to the microcirculatory impairment observed after endotoxemia induction, such as systemic hypotension, vasoconstriction, stiffness of RBC, increased leukocyte-endothelium interactions, and platelet/fibrin clot formation.[5] Because differences in capillary perfusion between groups cannot be entirely explained by changes in arteriolar mean internal diameter or by macro-hemodynamic changes, we can speculate that anti-inflammatory and antithrombotic activities of both heparin and rhAPC may have been crucial to capillary perfusion differences observed between groups seeing that capillary obstruction by microthrombi and an increased presence of rolling and adherent leukocytes in venules may hamper adequate capillary flow.[2,21,22,23] Thus, reduction of microvascular platelet aggregation and leukocyte plug formation facilitates capillary blood flow, improving tissue perfusion and reducing organ failure.[3]

Heparin or/and rhAPC treated animals had increased survival compared with animals in LPS group. This is in consonance with prior studies regarding human sepsis.[11,15] Unfortunately, recent studies concerning the use of antithrombotic agents in septic patients ended in controversial and/or inconclusive results: although benefits were not consistently observed, these agents were associated with significant increase in the risk of bleeding.[7,24] The interest in antithrombotic drugs decreased after the publication of the PROWESS-SHOCK trial in which rhAPC failed to show survival benefit for patients with severe sepsis or septic shock.[24] This resulted in the announcement of rhAPC's manufacturer of a worldwide voluntary market withdrawal, leaving many important unanswered questions about the role of antithrombotics in sepsis treatment. The expressive benefit in mortality observed in our study may be related to the LPS model: endotoxemic animals might be a population with high risk of death or, in other words, those animals that would most benefit from antithrombotic drug therapy.[25] Another hypothesis is that LPS-induced endotoxemia is an inflammation-dependent state in which anti-inflammatory drugs have important benefits on survival rates. Given the number of mechanisms involved, a similar response is not found in human sepsis.[26] Finally, and differently from what happened in human studies, no hemorrhagic events were observed in our study, which may have decisively contributed to our results.

Taken together, our results may suggest that heparin has beneficial microcirculatory and anti-inflammatory effects in endotoxemic hamsters. These benefits were potentiated when heparin was administered in conjunction with rhAPC. It is possible that each of these drugs has different, but additive anti-inflammatory effects, making treatment with both drugs more effective in controlling the inflammatory response. Our results may indicate a path to be followed in development of new drugs for sepsis treatment: a drug or a combination of drugs with potent anti-inflammatory effects and capable of preserving endothelial-dependent vasodilatation could attenuate sepsis-related capillary perfusion deficits, possibly improving patient outcome. Drugs with pleiotropic effects such as antithrombotics have greater therapeutic potential than compounds directed against a single target.[9] Clinical use of these drugs for sepsis treatment has been held back by fear of bleeding, but the development of non-anticoagulant drugs or of agents with mild anticoagulation properties could mitigate this concern.

We are aware that our study has some limitations. Initially, we recognize that the study of the skin and subcutaneous muscle microcirculation may not be representative of microcirculatory changes in splanchnic organs. Given the crucial importance of the splanchnic perfusion in the pathophysiology of sepsis, this could be considered a limitation of the skinfold window chamber model. However, the first reactions after endotoxin administration seem to be comparable in different organs.[14] In the second place, although we know that fluid therapy is recommended for the early management of severe sepsis and septic shock, in our study, animals were not fluid resuscitated because this study was designed to evaluate effects of heparin on microcirculation independently of fluid therapy. Finally, the use of recombinant human proteins (rhAPC) in hamsters may be limited by their even shorter half-lives.[2] However, because we found systemic effects long after drug discontinuation, it could be speculated that rhAPC worked well in this study.

CONCLUSIONS

In our study, heparin was effective in attenuating the inflammatory response, as depicted by decreased leukocyte rolling, reducing LPS-induced arteriolar vasoconstriction at the hamster skinfold window microcirculation, and improved survival. These benefits were potentiated when heparin was administered in conjunction with rhAPC, because treatment with both drugs was associated with marked anti-inflammatory effects and significant attenuation of capillary perfusion deficits induced by LPS administration. Further studies in experimental models closer to human sepsis are required to confirm these results.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Maria das Graças Coelho de Souza for her technical assistance, and Mr. Cláudio Natalino Ribeiro and Mr. Paulo José Ferreira Lopes for their help with animal care.

This study was supported by grants from CNPq (National Council for Scientific and Technological Development, Brasilia, Brazil) and FAPERJ (State of Rio de Janeiro Agency for Research Support, Rio de Janeiro, Brazil).

Author Contributions: Miranda ML analyzed the data and wrote the manuscript. Prota LFM contributed with data analysis and manuscript drafting. Silva MJB designed the study and analyzed the data. Sicuro FL analyzed the data. Furtado ES performed the experiments. Santos AOMT designed the study and performed the experiments. Bouskela E designed and supervised the study and critically revised the manuscript.

Conflicts: Authors report no conflicts of interest.

RESUMO

OBJETIVO: Além de suas propriedades anticoagulantes, a heparina apresenta efeitos vasodilatadores e anti-inflamatórios que podem ajudar na reversão das alterações sépticas da microcirculação. Este artigo investiga os efeitos da heparina sobre alterações da microcirculação relacionadas com endotoxemia e compara-os com os observados com o uso de proteína C humana recombinante ativada.

MÉTODOS: Após os procedimentos de implantação da câmara de dobras cutâneas e indução de endotoxemia por administração intravenosa de lipopolissacarídeo da Escherichia coli ($2\,mg.kg^{-1}$), hamsters sírios dourados machos foram tratados com heparina não fracionada intravenosa ($0{,}2\,mg.kg^{-1}$). A microscopia intravital de preparações de câmara de dobras cutâneas permitiu a análise quantitativa de variáveis microvasculares e venulares, bem como de rolamento e aderência de leucócitos. Parâmetros micro-hemodinâmicos também foram analisados. Hamsters endotoxêmicos tratados com proteína C humana recombinante ativada e animais não tratados serviram como controles.

RESULTADOS: A heparina diminuiu o rolamento de leucócitos e a vasoconstricção arteriolar induzida por lipopolissacarídeo e produziu um aumento de sobrevida em comparação com animais não tratados, enquanto proteína C humana recombinante ativada diminuiu a adesão de leucócitos. A administração de heparina mais proteína C humana recombinante ativada resultou numa significativa atenuação dos déficits de perfusão capilar induzida por lipopolissacarídeo.

CONCLUSÕES: A heparina produz efeitos protetores sobre a microcirculação dos animais endotoxêmicos. Estas vantagens foram potenciados quando a heparina foi administrada em combinação com proteína C humana recombinante ativada.

REFERENCES

1. Dempfle CE. Coagulopathy of sepsis. Thromb Haemost. 2004; 91(2):213-24.
2. Hoffmann JN, Vollmar B, Laschke MW, Inthorn D, Fertmann J, Schildberg FW, et al., Microhemodynamic and cellular mechanisms of activated protein C action during endotoxemia. Crit Care Med. 2004;32(4):1011-7.
3. Mariscalco MM. Unlocking (perhaps unblocking) the microcirculation in sepsis. Crit Care Med. 2006;34(2):561-2.

4. Trzeciak S, Cinel I, PhillipDellinger R, Shapiro NI, Arnold RC, Parrillo JE, et al. Resuscitating the microcirculation in sepsis: the central role of nitric oxide, emerging concepts for novel therapies, and challenges for clinical trials. Acad Emerg Med. 2008;15(5):399-413.
5. Santos AO, Furtado ES, Villela NR, Bouskela E. Microcirculatory effects of fluid therapy and dopamine, associated or not to fluid therapy, in endotoxemic hamsters. Clin Hemorheol Microcirc. 2011;47(1):1-13.
6. Koestenberger M, Cvirn G, Gallistl S, Baier K, Leschnik B, Muntean W. Drotrecogin alfa (activated, Xigris) in combination with heparin or melagatran: an in vitro investigation. J Thromb Thrombolysis. 2004; 18(1):5-10.
7. Minneci PC, Deans KJ, Cui X, Banks SM, Natanson C, Eichacker PQ. Antithrombotic therapies for sepsis: a need for more studies. Crit Care Med. 2006;34(2):538-41.
8. Tasatargil A, Golbasi I, Sadan G, Karasu E. Unfractioned heparin produces vasodilatory action on human internal mammary artery by endothelium-dependent mechanisms. J Cardiovasc Pharmacol. 2005; 45(2):114-9.
9. Young E. The anti-inflammatory effects of heparin and related compounds. Thromb Res. 2008;122(6):743-52.
10. Dobosz M, Mionskowska L, Hac S, Dobrowolski S, Dymecki D, Wajda Z. Heparin improves organ microcirculatory disturbances in caerulein-induced acute pancreatitis in rats. World J Gastroenterol. 2004; 10(17):2553-6.
11. Davidson BL, Geerts WH, Lensing AWA. Low-dose heparin for severe sepsis. N Engl J Med. 2002;347(13):1036-7.
12. Endrich B, Asaishi K, Götz A, Messmer K. Technical report – a new chamber technique for microvascular studies in unanesthetized hamsters. Res Exp Med. 1980;177(2):125-34.
13. Klyscz T, Jünger M, Jung F, Zeintl H. Cap image – a new kind of computer-assisted video image analysis system for dynamic capillary microscopy. Biomed Tech. 1997;42(6):168-75.
14. Hoffmann JN, Vollmar B, Inthorn D, Schildberg FW, Menger MD. A chronic model for intravital microscopic study of microcirculatory disorders and leukocyte/endothelial cell interaction during normotensive endotoxemia. Shock. 1999;12(5):355-64.
15. Bernard GR, Vincent JL, Laterre PF, LaRosa SP, Dhainaut JF, Lopez-Rodriguez A, et al. Efficacy and safety of recombinant human activated protein C for severe sepsis. N Engl J Med. 2001;344(10):699-709.
16. Tangphao O, Chalon S, Moreno HJ, Abiose AK, Blaschke TF, Hoffman BB. Heparin-induced vasodilation in human hand veins. Clin Pharmacol Ther. 1999;66(3):232-8.
17. Sternbergh WC, Makhoul RG, Adelman B. Heparin prevents postischemic endothelial cell dysfunction by a mechanism independent of its anticoagulant activity. J Vasc Surg. 1993;17(2):318-27.
18. Imai T, Hirata Y, Emori T, Marumo F. Heparin has an inhibitory effect on endothelin-1 synthesis and release by endothelial cells. Hypertension. 1993;21(3):353-8.
19. Chandra A, Enkhbaatar P, Nakano Y, Traber LD, Traber DL. Sepsis: emerging role of nitric oxide and selectins. Clinics. 2006;61(1):71-6.
20. Li X, Klintman D, Weitz-Schmidt G, Schramm R, Thorlacius H. Lymphocyte function antigen-1 mediates leukocyte adhesion and subsequent liver damage in endotoxemic mice. Br J Pharmacol. 2004; 141(4):709-16.
21. Yeh YC, Sun WZ, Ko WJ, Chan WS, Fan SZ, Tsai JC, et al., Dexmedetomidine prevents alterations of intestinal microcirculation that are induced by surgical stress and pain in a novel rat model. Anesth Analg. 2012;115(1):46-53.
22. Turek Z, Sykora R, Matejovic M, Cerny V. Anesthesia and the microcirculation. Semin Cardiothorac Vasc Anesth. 2009;13(4):249-58.
23. Cepinskas G, Wilson JX. Inflammatory response in microvascular endothelium in sepsis: role of oxidants. J Clin Biochem Nutr. 2008; 42(3):175-84.
24. Ranieri VM, Thompson BT, Barie PS, Dhainaut JF, Douglas IS, Finfer S, et al., Drotrecogin alfa (activated) in adults with septic shock. N Engl J Med. 2012;366(22):2055-64.
25. Abraham E, Laterre PF, Garg R, Levy H, Talwar D, Trzaskoma BL, et al. Drotrecogin alfa (activated) for adults with severe sepsis and a low risk of death. N Engl J Med. 2005;353(13):1332-41.
26. Opal SM, Laterre P-F, Francois B, LaRosa SP, Angus DC, Mira JP, et al. Effect of eritoran, an antagonist of MD2-TLR4, on mortality in patients with severe sepsis: the ACCESS randomized trial. JAMA. 2013; 309(11):1154-62.