Ivabradine Attenuates the Microcirculatory Derangements Evoked by Experimental Sepsis

Marcos L. Miranda, M.D., M.Sc., Michelle M. Balarini, M.D., M.Sc., Daniela S. Balthazar, B.Sc., Lorena S. Paes, M.Sc., Maria-Carolina S. Santos, Ph.D., Eliete Bouskela, M.D., Ph.D.

ABSTRACT

Background: Experimental data suggest that ivabradine, an inhibitor of the pacemaker current in sinoatrial node, exerts beneficial effects on endothelial cell function, but it is unclear if this drug could prevent microcirculatory dysfunction in septic subjects, improving tissue perfusion and reducing organ failure. Therefore, this study was designed to characterize the microcirculatory effects of ivabradine on a murine model of abdominal sepsis using intravital videomicroscopy.

Methods: Twenty-eight golden Syrian hamsters were allocated in four groups: sham-operated animals, nontreated septic animals, septic animals treated with saline, and septic animals treated with ivabradine (2.0 mg/kg intravenous bolus + 0.5 mg · kg^{-1} · h^{-1}). The primary endpoint was the effect of ivabradine on the microcirculation of skinfold chamber preparations, assessed by changes in microvascular reactivity and rheologic variables, and the secondary endpoint was its effects on organ function, evaluated by differences in arterial blood pressure, motor activity score, arterial blood gases, and hematologic and biochemical parameters among groups.

Results: Compared with septic animals treated with saline, those treated with ivabradine had greater functional capillary density (90 ± 4% of baseline values *vs.* 71 ± 16%; $P < 0.001$), erythrocyte velocity in capillaries (87 ± 11% of baseline values *vs.* 62 ± 14%; $P < 0.001$), and arteriolar diameter (99 ± 4% of baseline values *vs.* 91 ± 7%; $P = 0.041$) at the end of the experiment. Besides that, ivabradine-treated animals had less renal, hepatic, and neurologic dysfunction.

Conclusions: Ivabradine was effective in reducing microvascular derangements evoked by experimental sepsis, which was accompanied by less organ dysfunction. These results suggest that ivabradine yields beneficial effects on the microcirculation of septic animals. **(ANESTHESIOLOGY 2017; 126:00-00)**

IN early sepsis, activation of baro- and chemoreflexes increases sympathetic activity in an attempt to maintain organ perfusion.[1,2] In such compensatory response, tachycardia is recognized as an important mechanism to maintain cardiac output. However, some septic patients remain with an elevated heart rate (HR) even after adequate volume resuscitation and restoration of blood pressure. The putative underlying mechanism of such noncompensatory altered chronotropic response is an impairment of the sympathetic nervous system resulting in maladaptive cardiac sympathetic overstimulation, which exceeds the beneficial compensatory effect and leads to persisting elevated HR, considered an early manifestation of septic myocardial dysfunction.[3,4]

Clinical studies have suggested that tachycardia is related to increased mortality in septic patients, indicating that reduction in HR could be considered a therapeutic target in sepsis treatment.[3,5,6] Ivabradine, a selective inhibitor of the pacemaker current in sinoatrial node (also known as I_f current, which has a key role in controlling the rhythmic activity in cardiac pacemaker cells),[7] is capable of lowering HR without unwanted negative inotropic effects, which makes this drug an attractive option to treat tachycardia in critically ill septic patients. Indeed, preliminary findings suggest that its use could improve the outcome of such patients,[8] but several aspects need further elucidation, such as its effects on microcirculatory function.

What We Already Know about This Topic

- Ivabradine is a sinoatrial node inhibitor that lowers the heart rate without loss of inotropy; it has beneficial effects on endothelium, but its microcirculation impact in sepsis is unknown.

What This Article Tells Us That Is New

- In a hamster model of sepsis, ivabradine increased capillary density and arteriolar diameter measured using intravital microscopy, and it reduced renal, hepatic, and neurologic dysfunction. This suggests that ivabradine may be a testable intervention in human sepsis.

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It has already been shown that ivabradine exerts protective effects on endothelial function in nonseptic animal models of endothelial damage.[9,10] This led us to hypothesize that ivabradine could also prevent endothelial dysfunction in septic subjects, leading to reversal of microcirculatory derangements, improvement in tissue perfusion, and reduction of organ failure. As a recent *ex vivo* vasoreactivity study was not able to corroborate this hypothesis,[11] the current videomicroscopy study was carried out to test ivabradine effects in a sepsis rodent model that allows *in vivo* studies of microcirculation. The primary endpoint was the effect of ivabradine on the microcirculation of skinfold chamber preparations, assessed by changes in microvascular reactivity and rheologic variables, and the secondary endpoint was its effects on organ function, evaluated by differences in arterial blood pressure, motor activity score, arterial blood gases, and hematologic and biochemical parameters among groups.

Materials and Methods

Experiments were performed on 28 male golden Syrian hamsters (*Mesocricetus auratus*; 120 to 150 g) housed one per cage under controlled conditions of light (12:12-h light/dark cycle) and temperature (21.0° ± 1.0°C), with free access to water and standard chow. All procedures were approved by the Rio de Janeiro State University Animal Care and Use Committee (Rio de Janeiro, Brazil; protocol number, CEUA/021/2015) and are consistent with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals.[12]

Animal Preparation

The chamber implantation procedure is based on the original model described by Endrich *et al.*[13,14] Briefly, during intraperitoneal anesthesia with a combination of ketamine and xylazine (100 and 20 mg/kg, respectively), a titanium window chamber was microsurgically implanted in a dorsal skinfold, allowing the study of the skin and subcutaneous muscle microcirculation. Six days after chamber implantation (a period that allows recovery of microcirculatory function affected by surgical trauma), animals were reanesthetized, and the left carotid artery was catheterized (polyethylene-50 catheter), allowing continuous mean arterial blood pressure (MAP) and blood sampling. The left jugular vein was also catheterized (polyethylene-10 catheter) for fluid infusion and drug injection. These catheters were filled with heparinized saline solution (40 IU/ml) and tunneled subcutaneously to the dorsal side of the neck where they were attached to the chamber frame with tape. Three wire electrodes were subcutaneously implanted in the back of the animals in good contact with the underlying muscle for surface ECG monitoring (two near front limbs and one near left rear limb; leads I and II). Experiments were performed after 24 h of catheter and electrode implantation.

MAP, HR, and Temperature Monitoring

MAP, HR, and temperature were continuously monitored during the study period. MAP, used as a marker of cardiovascular function, was monitored through the arterial catheter and a blood pressure transducer (TSD104A; BIOPAC Systems, USA), while ECG electrodes were connected to an amplifier where the analog signal was recorded (Animal Bio Amp, FE 136; AD Instruments, Australia). Analog pressure and ECG signals were digitized (PowerLab 8/35; AD Instruments) and processed using data acquisition software for cardiovascular experiments (LabChart Pro software version 8; AD Instruments). HR was determined from the analysis of surface ECG and expressed as beats/min. Rectal and skinfold chamber temperatures were monitored with a beaded type K thermocouple probe (Wavetek 23XT; Wavetek Corporation, USA). The body temperature of hamsters was maintained with a heating pad placed near the animal controlled by using a rectal thermistor (LB750; Uppsala Processdata AB, Sweden).

Intravital Microscopy

Unanesthetized animals were placed in a restraining plexiglass tube attached to an intravital microscope (Ortholux; Leitz, Germany). Moving images of the microcirculation were obtained using a 20× objective (CF SLWD Plan EPI 20x/0.35 Achromat Objective WD 20.5 mm; Nikon, Japan) and a charge-coupled device digital video camera system (SBC-320P B/W Camera; Samsung, South Korea) connected to a video monitor. Acquired microcirculatory images were recorded as video files in digital media for later evaluation. Quantitative off-line analysis of videos was performed using ImageJ version 1.49 (National Institutes of Health, USA), a computer-assisted image analysis system, 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 and the presence of histological landmarks that could facilitate the subsequent return to the same field since the same vessels and capillary fields were studied throughout the experiment. Arteriolar and venular mean internal diameters were measured as the perpendicular distance (in micrometers) between the vessel walls. Arteriolar blood flow velocity was measured by the dual-slit method (a photometric technique used for the measurement of erythrocyte velocity in microvessels) using a commercially available unit (Instrumentation for Physiology and Medicine, USA) modified by Mesquita *et al.*[15] Functional capillary density was considered to be the total length (in centimeters) of spontaneously erythrocyte-perfused capillaries per square centimeter of tissue surface area (cm/cm^2). Erythrocyte velocity in capillaries was assessed by frame-to-frame analysis and determined as the ratio between the distance traveled by a given erythrocyte and the time required for this displacement (expressed as mm/s). One capillary per quadrant of capillary field was studied in each animal during erythrocyte velocity assessment (40 capillaries in total). The selection of these capillaries was

based on two criteria: they should be representative of the mean erythrocyte velocity of their quadrant and have good image quality for reliable analysis.

Cecal Ligation and Puncture Procedure

Cecal ligation and puncture (CLP) was performed as described by Rittirsch *et al.*[16] Briefly, during intraperitoneal anesthesia with ketamine/xylazine (100/20 mg/kg), the cecum was ligated at half the distance between its distal pole and base and punctured once with a 20-gauge needle, followed by extrusion of a small amount of feces to ensure patency. After surgery, hamsters were injected subcutaneously with 50 ml/kg prewarmed (37°C) saline (NaCl 0.9%) and returned to their cage.

For sham-operated animals, the cecum was exteriorized without ligation or puncture.

Arterial Blood Gases, Hematologic and Biochemical Analyses, and Blood Bacteria Count

Arterial blood gases and hematologic and biochemical parameters were used as markers of pulmonary (partial pressure of oxygen [P_{O_2}]), renal (serum urea and creatinine levels), hepatic (total serum bilirubin level), and coagulation (platelet count) function. Blood samples were withdrawn from the arterial catheter and immediately analyzed in a blood gas analyzer (CG4+ cartridge, i-STAT System; Abbott Laboratories, USA) for pH, PO_2, bicarbonate level (HCO_3), base excess (BE), total carbon dioxide content, and arterial lactate concentrations. For hematologic analyses, blood samples were collected in vacutainers containing EDTA. For serum biochemical analysis, blood samples were collected in vacutainers containing clot activator and gel, and serum was separated by centrifugation at 750*g* for 15 min. Hematologic and biochemical analyses were promptly carried out using standard automated analyzers (Pentra Hematology and Clinical Chemistry Analyzers; HORIBA Medical, Japan). For blood bacteria count, serial 10-fold dilutions of blood were prepared in sterile water, and 10 μl of each dilution was plated on MacConkey agar dishes and incubated at 37°C; colony-forming units were analyzed after 24 h.

Motor Activity Score Assessment

In the current study, motor activity score was used as a marker of neurologic function. Animals were placed in a 15- × 30- × 15-cm clear plastic container with wood shaving–covered floor for motor activity score assessment by a blind observer. This score was adapted from the study by Chuck *et al.*[17] and consisted of a 6-point ordinal scale ranging from 0 to 5 as follows: 5—animals engaged in locomotion, rearing, head movements, and grooming; 4—animals with normal posture, eyes fully open, head up, and little to no locomotion, rearing, or grooming; 3—animals with eyes partly closed, head somewhat down, and impaired locomotion (including abnormal posture, use of only some limbs, dragging, and/or stumbling); 2—animals with head mostly or completely down, eyes partly closed, flattened posture, and no spontaneous movement; 1—animals with eyes mostly closed and showing loss of righting reflex; and 0—animals with eyes fully closed, body relaxed, and asleep.

Experimental Protocol (Main Experiments)

Animals were suitable for experiments if their baseline MAP, HR, and rectal temperature were within the normal range and if they showed no signs of inflammation and/or bleeding in the skinfold chamber. Included animals were randomly allocated in four groups: sham-operated (nonseptic) animals fluid resuscitated and treated with saline; n = 7 (SHAM), nonfluid-resuscitated nor treated CLP-operated (septic) animals; n = 7 (CLP-CONTROL), CLP-operated (septic) animals fluid resuscitated and treated with saline; n = 7 (CLP-SALINE), and CLP-operated (septic) animals fluid resuscitated with saline and treated with ivabradine; n = 7 (CLP-IVABRADINE; [Sigma-Aldrich, USA]).

After baseline determination of MAP, HR, and microvascular variables, animals belonging to the SHAM group were sham operated, and those belonging to CLP-SALINE and CLP-IVABRADINE groups were subjected to CLP procedure. Twenty-four hours after CLP or sham operation, animals were fluid resuscitated with intravenous (IV) saline (20 ml/kg in 15 min), and CLP-IVABRADINE animals received a 2 mg/kg bolus dose of ivabradine diluted in the fluid resuscitation volume. After fluid resuscitation, a continuous IV infusion of saline or ivabradine solution (0.5 mg $\cdot$ kg^{-1} $\cdot$ h^{-1}) was initiated and maintained at a 0.1 ml/h infusion rate for 4 h.

As shown in figure 1, sequential measurements of MAP, HR, and microvascular variables were performed at four time points: at baseline, just before fluid resuscitation, after 15 min of fluid resuscitation, and after 4 h of saline or ivabradine infusion. Motor activity score was assessed at first, second, and fourth time points after macro- and microhemodynamic evaluations. Blood sampling for arterial blood gases, hematologic and biochemical analyses, and blood bacteria count was performed under sterile conditions at the end of the study period.

Hamsters allocated in the CLP-CONTROL group served as preresuscitation septic controls for arterial blood gases and hematologic and biochemical parameters. To measure these parameters, these animals were subjected to a partial experimental protocol 24 h after CLP procedure: they were not fluid resuscitated nor saline or ivabradine treated, and blood sampling was performed just after the second round of MAP, HR, and microvascular variables measurements.

After arterial blood sampling, all animals were euthanized by an IV overdose of ketamine/xylazine (more than 200/40 mg/kg).

Statistical Analysis

Results are expressed as mean ± standard deviation (SD) of the mean for each group, unless otherwise noted. Sample size

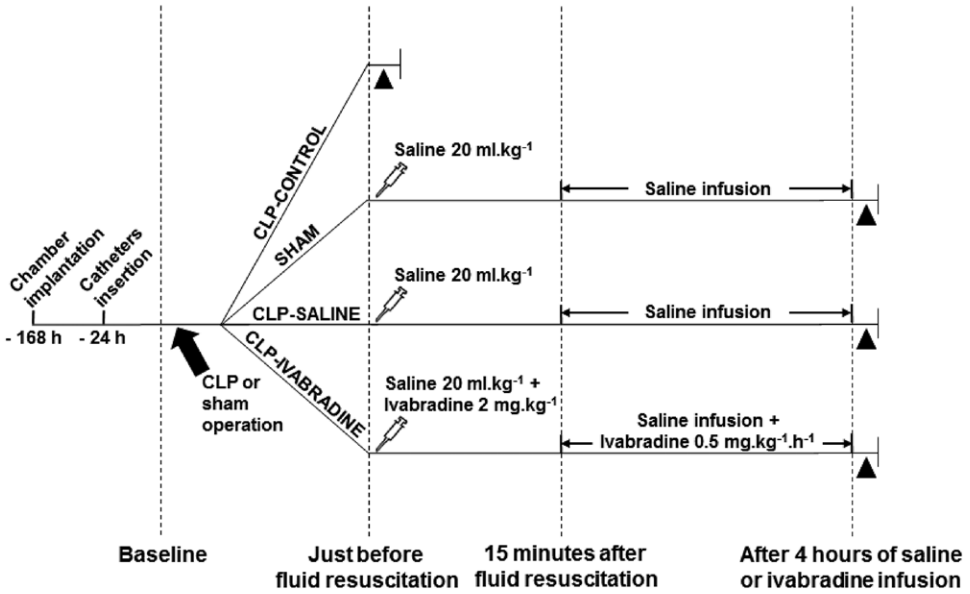

Fig. 1. Schematic representation of the experimental protocol. After baseline determination of mean arterial blood pressure (MAP), heart rate (HR), and microvascular variables, animals were subjected to cecal ligation and puncture (CLP) procedure or sham operation. Sequential measurements of MAP, HR, and microvascular variables were performed at four time points (dashed lines): at baseline, just before fluid resuscitation, after 15 min of fluid resuscitation, and after 4 h of saline or ivabradine infusion. Hamsters allocated in the septic, nonfluid resuscitated nor treated (n = 7; CLP-CONTROL) group were subjected to a partial experimental protocol in which after 24 h of CLP procedure, animals bypassed fluid resuscitation and saline infusion, going directly to blood sampling (*black triangle*) after measurements of the second time point. CLP-IVABRADINE group = septic, fluid resuscitated with saline and treated with ivabradine (n = 7); CLP-SALINE group = septic, fluid resuscitated and treated with saline (n = 7); SHAM group = nonseptic, fluid resuscitated and treated with saline (n = 7).

was based on previous experience with the sepsis and microcirculation models used. Normally, with these models, it is possible to observe significant differences between groups with the inclusion of five to six animals per group. In this study, a larger number of animals were used, considering the possibility of animal loss due to death during the experimental protocol. Statistical comparisons of normally distributed variables (assessed by Shapiro–Wilk test) were performed using two-way ANOVA for repeated measures and one-way ANOVA as appropriate, whereas Friedman and Kruskal–Wallis tests were used for other variables. When appropriate, an adequate test was used for *post hoc* analysis: Bonferroni method or Dunn multiple comparisons. All statistical analyses were performed using GraphPad Prism 6.03 (GraphPad Software, USA), and the significance level was set as $P < 0.05$ for a two-tailed test.

Results

The average body weight of hamsters was 135 ± 7 g with no significant differences among groups ($P = 0.626$). All animals survived the entire experimental protocol leaving no missing data for statistical analysis.

Microvascular Variables

At baseline, functional capillary density and erythrocyte velocity did not significantly differ between study groups ($P = 0.301$ and 0.125, respectively). In septic groups, functional capillary density and erythrocyte velocity decreased after CLP, while fluid resuscitation partially restored erythrocyte velocity but not functional capillary density (fig. 2). At the end of the microcirculatory study, ivabradine-treated septic animals exhibited significantly better capillary perfusion (functional capillary density and erythrocyte velocity) than those treated with saline (fig. 2; both $P < 0.001$). At baseline, there were no significant differences in arteriolar and venular mean internal diameters and arteriolar blood flow velocity between study groups ($P = 0.812$, 0.189, and 0.681, respectively). CLP elicited a decrease of all three parameters, while fluid resuscitation partially restored them. There were no significant differences in arteriolar and venular immediate responses to CLP and fluid resuscitation between CLP-SALINE and CLP-IVABRADINE groups (fig. 2; all $P > 0.999$). Ivabradine treatment prolonged fluid resuscitation beneficial effects on arteriolar parameters and prevented venular dilatation (fig. 2).

MAP, HR, and Temperature Changes

All animals remained in sinus rhythm throughout the experiment, and extrasystoles were rarely recorded. MAP and HR basal values were not significantly different among the experimental groups ($P = 0.893$ and 0.828, respectively) and were comparable to control values of healthy animals reported by the literature. CLP elicited statistically similar reductions in MAP levels in CLP-SALINE and CLP-IVABRADINE groups and caused an increase in HR (fig. 3; $P > 0.999$, comparing both septic groups). Ivabradine induced a significant

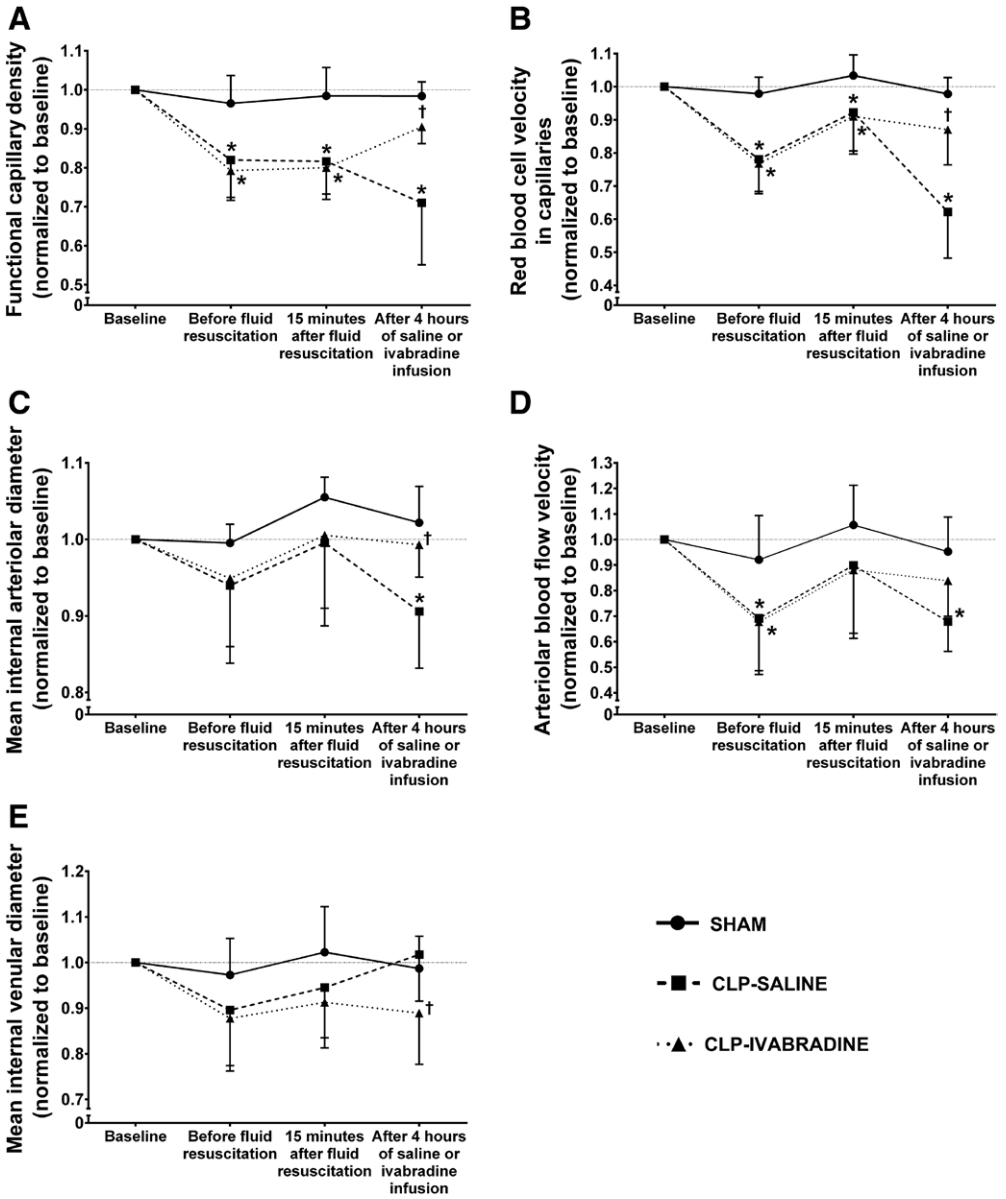

Fig. 2. Functional capillary density (*A*), erythrocyte velocity in capillaries (*B*), arteriolar diameter and blood flow velocity (*C*, *D*), and venular diameter (*E*) evolution during the experimental period. Values (given as mean ± SD) were measured at baseline, just before fluid resuscitation, after 15 min of fluid resuscitation, and after 4 h of saline or ivabradine infusion and are presented as ratios relative to baseline values. *$P < 0.05$ as compared with the nonseptic, fluid resuscitated and treated with saline (n = 7; SHAM) group at the same time point. †$P < 0.05$ as compared with the septic, fluid resuscitated and treated with saline (n = 7; CLP-SALINE) group at the same time point. CLP = cecal ligation and puncture procedure; CLP-IVABRADINE group = septic, fluid resuscitated with saline and treated with ivabradine (n = 7).

HR decrease ($P < 0.001$ *vs.* any other group) without significant effects on arterial blood pressure (except after its bolus dose, when a mild MAP decrease was observed; fig. 3). There were no significant temperature differences between saline- and ivabradine-treated animals ($P = 0.889$).

Arterial Blood Gases, Hematologic and Biochemical Analyses, and Blood Bacteria Count

Arterial blood gases and hematologic and biochemical parameters are presented in table 1. A significant increase in arterial lactate concentration was observed in the CLP-CONTROL group (nonresuscitated septic animals) as compared with the SHAM group ($P < 0.001$). Fluid resuscitation decreased lactate levels in both CLP-SALINE and CLP-IVABRADINE groups ($P = 0.049$ and < 0.001, respectively, as compared with the CLP-CONTROL group), but a major decrease in lactate was observed in ivabradine-treated animals. A marked reduction in arterial pH, HCO_3, and BE was observed in the CLP-SALINE group, which led to significant differences between this group and any other group (all $P < 0.01$). CLP

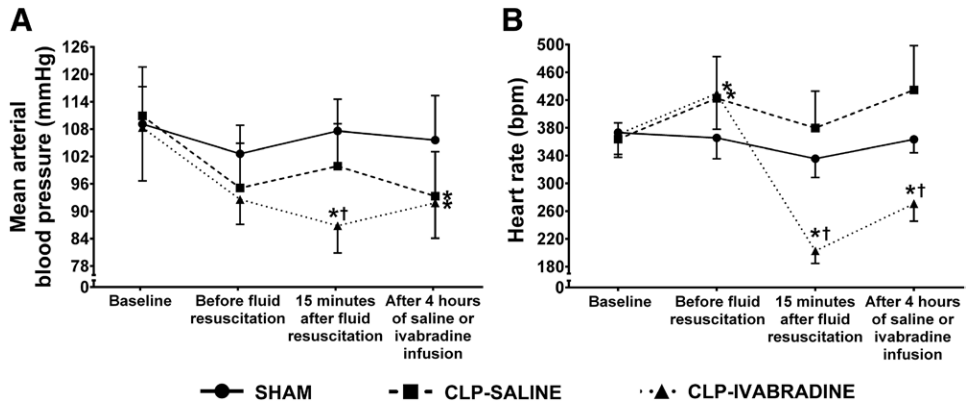

Fig. 3. Mean arterial blood pressure (*A*) and heart rate (*B*) evolution during the experimental period. Values (given as mean ± SD) were measured at baseline, just before fluid resuscitation, after 15 min of fluid resuscitation, and after 4 h of saline or ivabradine infusion. **P* < 0.05 as compared with the nonseptic, fluid resuscitated and treated with saline (n = 7; SHAM) group at the same time point. †*P* < 0.05 as compared with the septic, fluid resuscitated and treated with saline (n = 7; CLP-SALINE) group at the same time point. bpm = beats/min; CLP = cecal ligation and puncture procedure; CLP-IVABRADINE group = septic, fluid resuscitated with saline and treated with ivabradine (n = 7).

Table 1. Arterial Blood Gases and Hematologic and Biochemical Parameters

	SHAM	CLP-CONTROL	CLP-SALINE	CLP-IVABRADINE	*P* Value for Overall Group Effect	*P* Value for CLP-SALINE *vs.* CLP-IVABRADINE
Arterial lactate (mmol/l)	1.2 ± 0.3	2.7 ± 0.7	2.0 ± 0.4	1.4 ± 0.1	< 0.001	0.08
pH	7.40 ± 0.03	7.39 ± 0.07	7.22 ± 0.02	7.35 ± 0.04	< 0.001	< 0.001
HCO_3 (mmol/l)	33.3 ± 4.0	23.3 ± 6.2	15.7 ± 3.5	26.7 ± 2.5	< 0.001	< 0.001
BE (mmol/l)	9 ± 5	−1 ± 6	−12 ± 3	1 ± 3	< 0.001	< 0.001
TCO_2 (mmol/l)	34 ± 7	24 ± 8	16 ± 5	28 ± 3	< 0.001	0.006
PO_2 (mmHg)	62 ± 6	90 ± 7	104 ± 18	81 ± 12	< 0.001	0.011
Hematocrit (%)	44.0 ± 0.9	48.4 ± 3.6	49.5 ± 2.6	42.8 ± 2.8	< 0.001	< 0.001
Leukocyte count (n/mm^3)	12,475 ± 4,232	4,840 ± 1,242	2,475 ± 1,431	5,200 ± 1,219	< 0.001	0.263
Platelet count (n/mm^3)	269,750 ± 46,557	369,200 ± 45,882	295,000 ± 65,054	266,750 ± 45,110	0.003	> 0.999
Serum glucose (mg/dl)	121 ± 46	40 ± 17	13 ± 4	42 ± 11	< 0.001	0.247
Total serum bilirubin (mg/dl)	0.05 ± 0.01	0.09 ± 0.05	0.14 ± 0.03	0.08 ± 0.01	< 0.001	0.001
Serum urea (mg/dl)	37 ± 9	44 ± 15	194 ± 64	37 ± 11	< 0.001	< 0.001
Serum creatinine (mg/dl)	0.3 ± 0.1	0.3 ± 0.1	0.6 ± 0.4	0.2 ± 0.1	< 0.001	< 0.001
Serum chloride (mEq/l)	110 ± 3	104 ± 3	113 ± 4	112 ± 5	0.001	> 0.999

Blood sampling for arterial blood gases and hematologic and biochemical parameters was performed at the end of the study period. Data are presented as mean ± SD for each group. All *P* values were adjusted for multiple comparisons applying the Bonferroni adjustment.

BE = base excess; CLP = cecal ligation and puncture procedure; CLP-CONTROL = septic, nonfluid resuscitated nor treated (n = 7); CLP-IVABRADINE = septic, fluid resuscitated with saline and treated with ivabradine (n = 7); CLP-SALINE = septic, fluid resuscitated and treated with saline (n=7); HCO_3 = bicarbonate level; PO_2 = partial pressure of oxygen; SHAM = nonseptic, fluid resuscitated and treated with saline (n = 7); TCO_2 = total carbon dioxide content.

elicited an increase in PO_2 and a decrease in leukocyte count and serum glucose (all $P < 0.03$ for SHAM *vs.* septic groups). Fluid resuscitation elicited an increase in serum chloride (all $P < 0.03$ for CLP-CONTROL *vs.* any other group). Hematocrit was significantly higher in both CLP-CONTROL and CLP-SALINE groups than in other groups ($P = 0.029$ and 0.004, respectively, *vs.* SHAM; $P = 0.004$ and < 0.001, respectively, *vs.* CLP-IVABRADINE). Platelet count was significantly higher in the CLP-CONTROL group than in SHAM ($P = 0.008$) and CLP-IVABRADINE ($P = 0.006$) groups. Total serum bilirubin, serum urea, and serum creatinine were significantly higher in the CLP-SALINE group than in any other group (all $P < 0.008$). The bacterial load in blood samples obtained from CLP animals was significantly higher than that in the SHAM group (all $P < 0.01$). There were no statistical differences in blood bacterial count among septic animals (all $P > 0.999$).

Motor Activity Score

At baseline, there were no significant differences in the motor activity score between study groups (all animals scored 5). CLP elicited a significant decrease of this parameter, while ivabradine treatment partially restored it, which led to a significant difference between CLP-SALINE and CLP-IVABRADINE groups at the end of the study (fig. 4; $P = 0.036$).

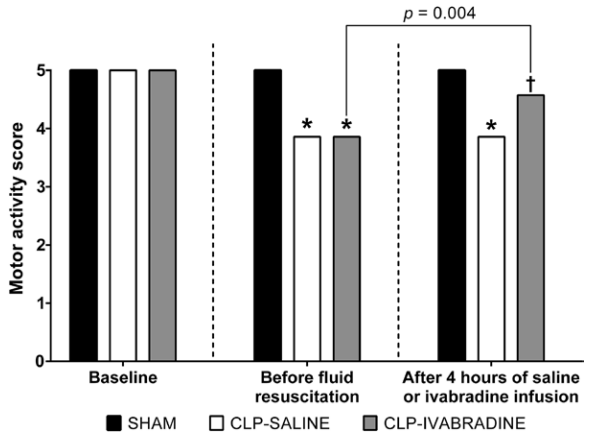

Fig. 4. Motor activity score evolution during the experimental period. Values (given as medians) were measured at baseline, just before fluid resuscitation, and after 4 h of saline or ivabradine infusion. *$P < 0.05$ as compared with the nonseptic, fluid resuscitated and treated with saline (n = 7; SHAM) group at the same time point. †$P < 0.05$ as compared with the septic, fluid resuscitated and treated with saline (n = 7; CLP-SALINE) group at the same time point. CLP = cecal ligation and puncture procedure; CLP-IVABRADINE group = septic, fluid resuscitated with saline and treated with ivabradine (n = 7).

Discussion

In the current study, CLP-induced sepsis was associated with a hypovolemic and hypodynamic microcirculation in the hamster skinfold chamber. Saline-based fluid resuscitation temporally and partially normalized many of the microvascular variables affected by experimental sepsis but possibly led to impaired organ function (renal failure in particular). A sustained beneficial microcirculatory effect of fluid resuscitation was only observed in ivabradine-treated animals. Moreover, ivabradine-treated septic animals showed less organ dysfunction than saline-treated ones. Therefore, our key result was that ivabradine treatment attenuated the microcirculatory derangements observed in the setting of fluid-resuscitated sepsis, improving tissue perfusion.

The ivabradine dose used in the current study is considered high compared to that recommended for human use. This is due to the fact that smaller animals tend to have higher drug needs in relation to their body mass because of their higher metabolic rates.[18] Bolus dose (2.0 mg/kg) was chosen based on previously published experimental study that showed that this dose was safe in murines[19]; maintenance dose ($0.5\ mg \cdot kg^{-1} \cdot h^{-1}$) was chosen based on pharmacokinetics calculations to keep a constant serum drug concentration. With this dosing, animals achieved a decrease in HR without significant effects on arterial blood pressure, except for an MAP decrease of limited clinical significance after 15 min of ivabradine administration. Saline volume used for fluid resuscitation (20 ml/kg) was chosen based on that commonly administered during the initial phase of sepsis resuscitation in humans. Previous experience with septic hamsters has shown that these animals can cope with up to 40 ml/kg of saline without major cardiopulmonary repercussions.[20,21]

It is reasonable to speculate that some of our microcirculatory findings could be related to a pure mechanical effect secondary to ivabradine-induced HR reduction.[22] In the septic heart, a state of oxygen supply/demand mismatch deteriorates cardiac efficiency, decreasing stroke volume. In this situation, an elevated HR exacerbates the mismatch, contributing to cardiac dysfunction, while a decrease in HR leads to a decrease in myocardial oxygen consumption and cardiac workload with a parallel increase in diastolic coronary blood flow, which augments myocardial performance and perhaps tissue perfusion.[23] Furthermore, HR reduction may improve diastolic function, resulting in maintained or even increased stroke volume due to better ventricular filling.[8,23] Indeed, an increased cardiac output in ivabradine-treated animals could explain most of arteriolar and capillary findings, while cardiac dysfunction could explain venular dilatation in the CLP-SALINE group; altered venular capacitance has also been described as a consequence of microvascular reactivity impairment in septic subjects.[24] In contrast with the findings of the current study, Wei *et al.*[11] observed nonsignificant reductions in stroke volume and cardiac output in nonfluid-resuscitated septic rats treated with ivabradine when these animals were compared with septic untreated ones. This observation highlights the importance of an adequate preload before attempting to decrease HR during sepsis.

As the current study did not have an experimental group in which HR was corrected by pacing, the possibility that other properties of ivabradine beyond pure HR reduction have had a role as potential mechanisms of the microcirculatory findings cannot be ruled out.[25] Indeed, it has already been shown that ivabradine exerts a marked reduction in vascular oxidative stress and improves endothelial function in nonseptic animal models of endothelial damage.[9,10] Interestingly, capillary recruitment took longer to occur than other microcirculatory changes did, and it was not observed after 15 min of fluid resuscitation (fig. 2). This may be due to the fact that capillary perfusion changes are not entirely explained by changes in arteriolar flow or in macrohemodynamics as capillary obstruction by microthrombi or leukocyte plugs and an increased presence of rolling or adherent leukocytes in venules are also crucial to capillary perfusion and may hamper adequate capillary flow.[14,21,26] Considering that the endothelium plays several regulatory functions on the activity of leucocytes and platelets, one may hypothesize that beneficial ivabradine effects on endothelial function have contributed to the late capillary recruitment.

Hematocrit results provide another evidence that endothelial damage may have been decisive for microcirculatory findings. The hematocrit value has been used as an indirect marker of plasma leakage in the context of endothelial-damaging diseases.[21] Its increase after fluid resuscitation in the CLP-SALINE group, which suggests increased microcirculatory leakage, was not observed in ivabradine-treated animals. This

may be related to beneficial effects of ivabradine on vascular endothelial barrier function, reducing microvascular permeability (fig. 1, Supplemental Digital Content 1, http://links.lww.com/ALN/B334, which shows the number of leaky sites in the cheek pouch microcirculation of ivabradine-treated septic animals) and tissue edema (fig. 1, Supplemental Digital Content 2, http://links.lww.com/ALN/B335, which shows the dry/wet weight variation of abdominal cavity viscera of ivabradine-treated septic animals). Excessive interstitial edema leads to increased distance for oxygen diffusion from capillary to surrounding parenchymal cells with subsequent impairment of tissue oxygen transport, tissue hypoxia, and eventually organ dysfunction. Thus, ivabradine effects on microcirculatory leakage may have contributed to intravascular retention of fluid, prolonging the beneficial microcirculatory effects of fluid resuscitation and reducing fluid-induced organ dysfunction. It should be noted that the blood accumulation in the venous system observed in the CLP-SALINE group animals also promotes leakage in postcapillary venules.[27]

Wei *et al.*[11] have shown that ivabradine is not associated with any effect on arterial vasoreactivity during experimental sepsis, which is also true for arterioles (fig. 1, Supplemental Digital Content 3, http://links.lww.com/ALN/B336, which shows *in vivo* arteriolar responsiveness to norepinephrine in ivabradine-treated septic animals). Furthermore, ivabradine does not have any intrinsic vasodilatory effect and may even induce vasoconstriction in high tissue concentrations (fig. 2, Supplemental Digital Content 3, http://links.lww.com/ALN/B336, which shows *in vivo* arteriolar response to increasing doses of ivabradine). These findings suggest that ivabradine beneficial microcirculatory effects are not related to a direct arteriolar vasodilatory mechanism and corroborate the hypothesis that ivabradine acts, at least in part, by reducing endothelial damage and improving ventricular ejection.

Interstitial edema and impaired tissue perfusion may have contributed to hepatic and neurologic dysfunction in CLP-SALINE animals but may not entirely explain the strikingly negative renal function results observed in this group. The marked differences between CLP-SALINE (fluid resuscitated) and CLP-CONTROL (nonfluid resuscitated) groups suggest iatrogenic injury due to fluid resuscitation. Fluid resuscitation with saline can contribute to renal dysfunction during critical illness whether by fluid or chloride overload or by a fluid-triggered ischemia–reperfusion insult mechanism mediated by reactive oxygen species.[27–31] The reperfusion injury hypothesis for renal dysfunction in CLP-SALINE animals was corroborated by additional experiments, in which ivabradine has effectively prevented fluid-induced H_2O_2 generation (fig. 2, Supplemental Digital Content 2, http://links.lww.com/ALN/B335, which shows H_2O_2 generation in kidneys of ivabradine-treated septic animals; table 1, Supplemental Digital Content 2, http://links.lww.com/ALN/B335, which shows H_2O_2 generation in thoracic aortas of ivabradine-treated septic animals).

Lower pH, HCO_3, BE, and total carbon dioxide content values found in the CLP-SALINE group are indicative of metabolic acidosis. As arterial lactate differences among septic groups do not appear to be clinically significant and do not correlate with the marked depression in BE, it is possible to argue against hypoperfusion as the cause of acidosis in this group. In the same way, chloride overload may not entirely explain the differences observed between groups. Thus, renal dysfunction may be suggested as the cause of acidosis in the CLP-SALINE group.

Besides septic neurologic dysfunction, differences in motor activity score could also be explained by the presence of uremia and hypoglycemia in the CLP-SALINE group. Pulmonary function analysis, as depicted by arterial oxygen partial pressure to fractional inspired oxygen ratio, was biased by PO_2 increase in the CLP-SALINE group. This contradictory change has been previously observed in severely ill septic hamsters and is likely to be related to an adaptive characteristic of the species to different oxygen consumption situations.[14]

Platelet count decrease in SHAM and CLP-IVABRADINE groups as compared with that in the CLP-CONTROL group probably reflects a dilutional effect of fluid resuscitation. Leukocyte count differences observed after sepsis induction are likely to be related to the activation and emigration of neutrophils out of the microvascular bed and to leukocyte sequestration.[14] The development of hypoglycemia after CLP is a common finding during septic states in small animals.[32]

Perspectives and Limitations

If the findings of the current study are replicated in humans, the clinical implication is that ivabradine may improve microcirculatory function of septic patients and perhaps organ function, contributing to sepsis treatment.

Treatment of septic noncompensatory tachycardia with β-blockers has already been associated with better outcomes in septic patients.[3] Clinical and experimental studies have shown that esmolol may enhance intrinsic cardiac contractility and vascular responsiveness to catecholamines during sepsis, preserving microvascular blood flow and reducing norepinephrine requirements.[33,34] The HR-lowering action of ivabradine has been compared directly with β-blockers: while I_f current inhibition decreases HR by prolonging diastole, the negative inotropic action of β-blockers prolongs both systole and diastole.[35] As a result, ivabradine produces a greater prolongation of diastolic time than β-blockers for the same reduction in HR and may be associated with greater diastolic coronary blood flow and ventricular filling.[35] Thus, it is tempting to speculate that ivabradine use in septic subjects may result in better macro- and microvascular parameters than β-blocker use, but that comparison needs formal investigation. Moreover, ivabradine combination with other negative chronotropic drugs should also be tested as administration of both ivabradine (a peripherally acting drug) and a centrally acting drug (such as dexmedetomidine)[14] could further attenuate cardiac and vasomotor sepsis-induced sympathetic activation, improving tissue perfusion.

This study has some limitations. First, even though the CLP model may reproduce many clinical features of sepsis syndrome, results cannot be generalized/translated to the much more complex clinical scenario of human sepsis. Second, the study of the skin and subcutaneous muscle microcirculation may not be representative of microcirculatory changes in organs of crucial importance in the pathophysiology of sepsis, such as splanchnic organs, which limits conclusions. However, the hamster skinfold window chamber model is widely used for microvascular studies in septic animals because septic microcirculatory changes seem to be ubiquitous and to have common pathophysiologic mechanisms, which make them comparable in different tissues and organs.[14,36] Third, the absence of additional biochemical data pertaining to the acid–base status limits conclusions about the acid–base differences observed among groups. Finally, the absence of different types and doses of fluids during the resuscitation phase may limit conclusions as some of the findings may have been related to beneficial effects of ivabradine on saline-induced injury and would not be observed if balanced or colloid solutions were used.

Conclusions

In the current study, ivabradine administration was effective in reducing microvascular derangements evoked by fluid-resuscitated sepsis in the hamster skinfold chamber microcirculation, which was accompanied by better tissue perfusion and less organ dysfunction. These results suggest that ivabradine yields beneficial effects on the microcirculation of fluid-resuscitated septic animals. As the current study discusses an unlabeled and experimental use of ivabradine, further investigations in experimental models closer to human sepsis are required to confirm this benefit.

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Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Miranda: Laboratory for Clinical and Experimental Research in Vascular Biology - BioVasc, Pavilhão Reitor Haroldo Lisboa da Cunha, Rio de Janeiro State University, Rua São Francisco Xavier 524, 20550-013 Rio de Janeiro, Brazil. marcoslmiranda@gmail.com.

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