Additional experiments 1 – Ivabradine effects on microvascular permeability

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Introduction

Hematocrit increase after fluid resuscitation in septic animals (CLP-SALINE group) suggests an increased microvascular permeability, which could explain, at least in part, many microcirculatory and organ dysfunction findings of main experiments. Interestingly, a similar response was not observed in ivabradine treated hamsters which led to the hypothesis that ivabradine effects on vascular endothelial barrier function could reduce microcirculatory leakage in septic animals. Thus, additional experiments were carried out to investigate this hypothesis in a rodent model that allows *in vivo* studies of microvascular permeability, the hamster cheek pouch.

Materials and Methods

Experiments were performed on 21 male golden Syrian hamsters (*Mesocricetus auratus;* 120–150 g) housed one per cage under controlled conditions of light (12:12 hours 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, RJ, Brazil; protocol number CEUA/021/2015), and are consistent with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals.[1]

Animal Preparation

Twenty-four hours before experiments, the cecal ligation and puncture (CLP) procedure (or sham operation) was performed as described by Rittirsch and co-workers.[2] Briefly, under intraperitoneal anesthesia with ketamine/xylazine (100/20 $mg.kg^{-1}$) 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 $ml.kg^{-1}$ of prewarmed (37 ºC) saline (NaCl 0.9%) and returned to their cage. For sham-operated animals, the cecum was exteriorized without ligation or puncture.

The cheek pouch was prepared as described by Svensjö and detailed elsewhere.[3] Briefly, under intraperitoneal anesthesia with a combination of ketamine and xylazine (100 $mg.kg^{-1}$ / 20 $mg.kg^{-1}$, respectively) the cheek pouch was everted and mounted on a microscope stage and an area of about 1 cm^2 was microsurgically prepared for intravital microscopy observations. The cheek pouch was superfused, at a constant superfusion rate of 6 $ml.min^{-1}$, with warm (35º C) bicarbonate buffered salt solution (stabilized with HEPES) continuously bubbled with 95% N_2 and 5% CO_2 to maintain a low oxygen tension (~4 KPa) and a pH of 7.35. A catheter was concurrently inserted in the left femoral vein (polyethylene-10 catheter) for fluid infusion and drug injection.

Intravital Microscopy

Anesthetized animals were placed on an ultraviolet-light intravital microscope (Ortholux II, Leitz, Wetzlar, Germany). Moving images of the microcirculation were obtained using a 40x objective and a charge-coupled device digital video camera system (SBC-320P B/W Camera, Samsung, Seoul, South Korea) connected to a video monitor. Fluorescein-labeled dextran (FITC-dextran 5% solution, Bioflor HB, Uppsala, Sweden; molecular weight 150,000) was injected intravenously (250 $mg.kg^{-1}$) as a macromolecular tracer. The microvascular permeability for large molecules was quantified by counting the number of leaky sites (leaks) in the prepared area (1 cm^2). These sites are defined as visible extravascular spots (diameter >100 µm) of FITC-dextran in post capillary venules seen under fluorescent light.[3]

Experimental Protocol – [AE1] groups

Twenty-four hours after CLP or sham operation, animals were anesthetized and the cheek pouch was prepared. Animals were suitable for experiments if they showed no signs of inflammation and/or bleeding in the preparation.

Included animals were fluid resuscitated with saline (20 $ml.kg^{-1}$ in 15 minutes; intravenously [IV]) and randomly allocated in 3 groups: SHAM-SALINE [AE1] (sham-operated [non-septic] animals topically treated with saline; n=7), CLP-SALINE [AE1] (CLP-operated [septic] animals topically treated with saline; n=7), and CLP-IVABRADINE [AE1] (CLP-operated animals topically treated with ivabradine [Sigma-Aldrich, St. Louis, MO, USA] 10^{-4} M; n=7).

Macromolecular extravasation was induced by 30 minutes of complete occlusion of the cheek pouch circulation by an air inflatable tourniquet placed around the neck of the pouch vascular pedicle (ischemia).[4] The number of leaks was counted 10 minutes after the tourniquet release (reperfusion). In CLP-IVABRADINE [AE1] group, ivabradine was added to the superfusion solution at 10^{-4} M concentration. In saline treated groups, only saline was added to the superfusion solution.

All animals were sacrificed by an IV overdose of ketamine/xylazine (>200/40 $mg.kg^{-1}$) at the end of study period.

Statistical Analysis

Results are expressed as median values and the 5th to 95th percentile ranges for each group. Statistical comparisons of normally distributed variables (assessed by Shapiro-Wilk test) were performed using 1-way ANOVA. When appropriate, Bonferroni method was used for *post hoc* analysis. All statistical analyses were performed using GraphPad Prism 6.03 (GraphPad Software, La Jolla, CA, USA) and the significance level was set as $p < 0.05$ for a two-tailed test.

Results

All animals survived the entire experimental protocol leaving no missing data for statistical analysis. Microvascular permeability (number of leaks) was significantly higher in CLP-SALINE [AE1] group than in any other group (p <0.001; Fig. 1).

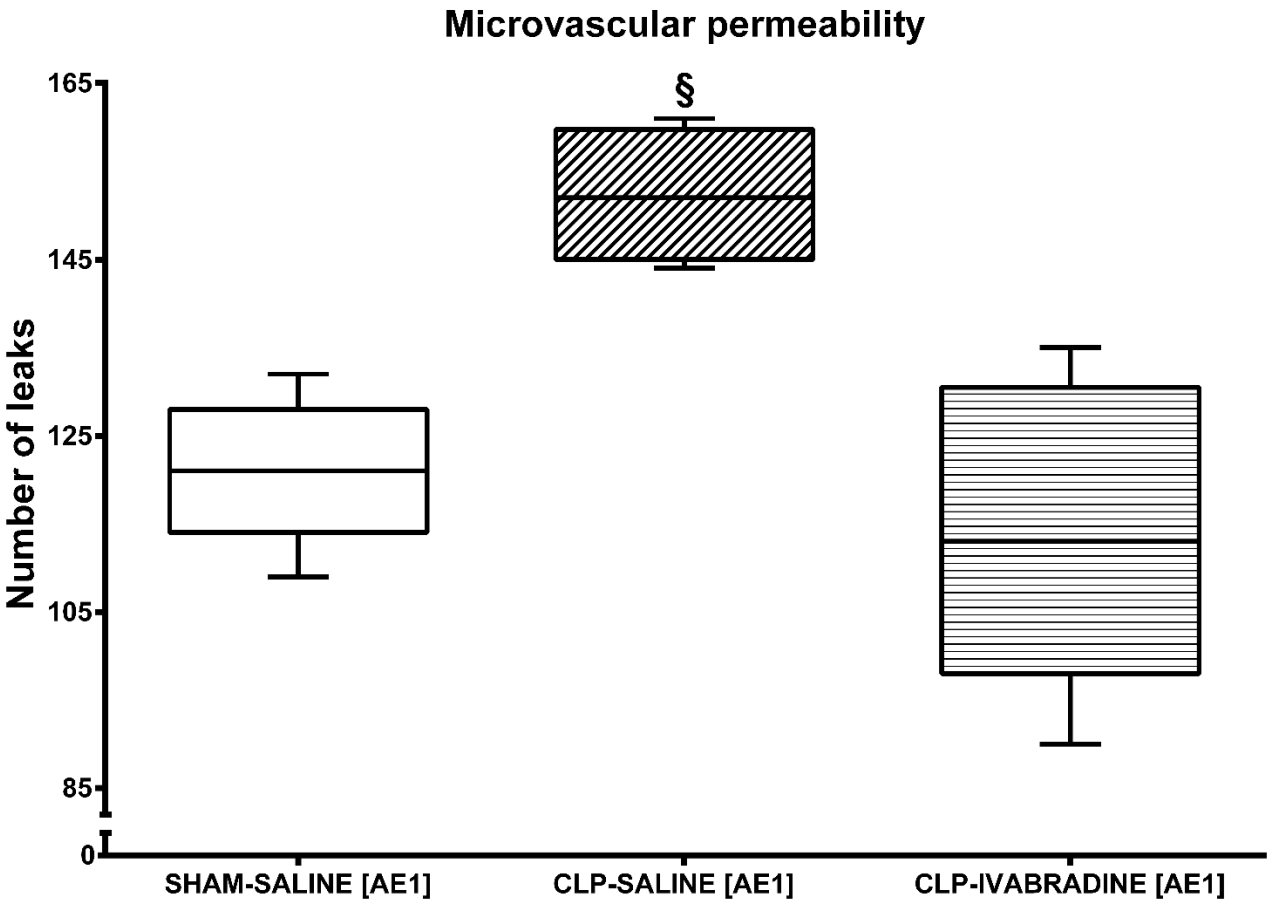

Figure 1 – **Microvascular permeability.** Data are given as median values and the 5th to 95th percentile ranges for each group. SHAM-SALINE [AE1] group = non-septic, topically treated with saline (n=7); CLP-SALINE [AE1] group = septic, topically treated with saline (n=7); CLP-IVABRADINE [AE1] group = septic, topically treated with ivabradine 10^{-4} M (n=7). § p <0.001 as compared with any other group. AE1 = additional experiments 1; CLP = cecal ligation and puncture procedure.

Discussion

The main finding of the current experiment was that ivabradine was associated with less ischemia-induced microvascular permeability in fluid resuscitated septic hamsters when compared with saline treated ones.

Microvascular permeability results are in agreement with hematocrit values observed in the main experiments and could be related to beneficial effects of ivabradine on vascular endothelial barrier function, reducing microcirculatory leakage. Of note, current experiment findings were corroborated by tissue edema results (see Figure 1, Supplemental Digital Content 2, which shows the dry/wet weight variation of abdominal cavity viscera of ivabradine-treated septic animals) and are probably associated with ivabradine decreasing effects on reactive oxygen species generation[5,6] (see Figure 2, Supplemental Digital Content 2, which shows H_2O_2 generation in kidneys of ivabradine-treated septic animals; see Table 1, Supplemental Digital Content 2, which shows H_2O_2 generation in thoracic aortas of ivabradine-treated septic animals).

References

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