

Additional experiments 2 – Ivabradine effects on dry/wet weight variation of abdominal cavity viscera and on H₂O₂ generation in kidneys and thoracic aortas

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Introduction

Based on ivabradine decreasing effects on reactive oxygen species (ROS) generation^{1,2} and on microcirculatory findings of the main experiments, a fluid-triggered ischemia-reperfusion insult, leading to ROS generation, was suggested as a possible mechanism of the marked differences in renal function among groups. This hypothesis is in agreement with previous studies that have shown that fluid resuscitation can contribute to renal dysfunction due to a reperfusion injury mechanism.^{3,4} Experiments assessing hydrogen peroxide (H₂O₂; a type of ROS) generation in kidneys and thoracic aortas were carried out to investigate this hypothesis.

Hematocrit increase after fluid resuscitation in septic animals (CLP-SALINE group) suggests increased microcirculatory leakage. As increased fluid leakage from the intravascular space results in tissue edema, additional experiments measuring tissue edema on abdominal cavity viscera were also carried out to strengthen this hypothesis. Tissue edema was determined by dry/wet weight variation (a commonly used method for the determination of organ water content and tissue edema).^{5,6}

Materials and Methods

Experiments were performed on 48 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.⁷

Animal Preparation

Twenty-four hours before the experiments, the left jugular vein was catheterized (polyethylene-10 catheter) for fluid infusion and drug injection and the cecal ligation and puncture (CLP) procedure (or sham operation) was performed as described by Rittirsch and co-workers.⁸ 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 °C) saline (NaCl 0.9%) and returned to their cage. For sham-operated animals, the cecum was exteriorized without ligation or puncture.

H₂O₂ Generation

H₂O₂ generation was quantified in microsomal fractions of kidneys and thoracic aortas by Amplex/HRP method (Molecular Probes, Invitrogen, Waltham, MA, USA), which detects the accumulation of fluorescent oxidized products. Due to very small amounts of available protein material, making individual assays impossible, experiments in thoracic aortas were performed in pooled samples from 6 animals (composite sampling). This issue was not observed in kidney experiments as they offered enough material for individual assays. For the preparation of microsomal fractions, tissues were homogenized and the homogenate was centrifuged at 3,000 g

for 15 minutes at 4 °C. Then, the supernatant was centrifuged at 100,000 g for 35 minutes at 4 °C. Pellets were resuspended in 0.5 ml of sodium phosphate 50 mM buffer (pH 7.2) containing sucrose 0.25 M, MgCl₂ 2 mM, aprotinin 5 mg.ml⁻¹, and PMSF (phenylmethanesulfonyl fluoride) 34.8 mg.ml⁻¹ and stored at -80 °C until analysis.⁹ During analysis, microsomal fractions were incubated with sodium phosphate 50 mM buffer (pH 7.4) containing superoxide dismutase 100 U.ml⁻¹ (SOD, Sigma-Aldrich, St. Louis, MO, USA), horseradish peroxidase 0.5 U.ml⁻¹ (HRP, Roche, Indianapolis, IN, USA), Amplex Red 50 uM (Molecular Probes, Invitrogen, Waltham, MA, USA), and EGTA (ethylene glycol tetraacetic acid) 1 mM, in the presence or absence of NADPH (nicotinamide adenine dinucleotide phosphate reduced) 1 mM. Generated fluorescence was immediately measured in a microplate reader (Victor X4, Perkin-Elmer, Norwalk, CT, USA) at 30 °C, using a 530 nm excitation wavelength and 595 nm emission. H₂O₂ production was quantified using standard calibration curves. The specific enzyme activity was expressed in H₂O₂ nanomoles per hour per milligram of protein (nmol.h⁻¹.protein mg⁻¹). Protein concentration was determined by the Bradford method.¹⁰

Dry/wet Weight Variation of Abdominal Cavity Viscera

Abdominal cavity viscera were weighed after removal of contents of the gastrointestinal tract by washing (wet weight). Then, organs were dried to constant weight in a forced-draft oven at 60 °C for 96 hours (dry weight) allowing the calculation of the dry/wet weight variation: $[1 - (\text{dry weight} / \text{wet weight})] \times 100$.

Experimental Protocol – [AE2] groups

Included animals were randomly allocated in 4 groups: SHAM [AE2] (sham-operated animals fluid resuscitated and treated with saline; n=12), CLP-CONTROL [AE2] (non-fluid resuscitated nor treated CLP-operated animals; n=12), CLP-SALINE [AE2] (CLP-operated animals fluid resuscitated and treated with saline; n=12), and CLP-IVABRADINE [AE2] (CLP-operated animals fluid resuscitated with saline and treated with ivabradine [Sigma-Aldrich, St. Louis, MO, USA]; n=12).

Twenty-four hours after CLP or sham operation, animals were fluid resuscitated with intravenous (IV) saline (20 ml.kg⁻¹ in 15 minutes) and CLP-IVABRADINE [AE2] animals received a 2 mg.kg⁻¹ 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.kg⁻¹.h⁻¹) was initiated and maintained at a 0.1 ml.h⁻¹ infusion rate for 4 hours. Half of the animals in SHAM [AE2], CLP-SALINE [AE2], and CLP-IVABRADINE [AE2] groups (18 animals; 6 in each group) were sacrificed by an IV overdose of ketamine/xylazine after 15 minutes of fluid resuscitation and kidneys and thoracic aortas were removed for H₂O₂ generation analysis. After 4 hours of saline or ivabradine infusion, remaining animals were sacrificed and abdominal cavity viscera and thoracic aortas were removed for tissue edema determination and H₂O₂ generation analysis, respectively. Hamsters allocated in the CLP-CONTROL [AE2] group served as pre-resuscitation septic controls for tissue edema and H₂O₂ generation experiments. These animals were sacrificed 24 hours after CLP procedure (they were not fluid resuscitated nor treated with saline or ivabradine) and equally distributed in each experiment (6 animals for tissue edema determination and 6 animals for H₂O₂ generation analysis).

Statistical Analysis

Results are expressed as median values and the 5th to 95th percentile ranges for each group, unless otherwise noted. 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. Thoracic aorta experiments were carried out in pooled samples, leaving no biological variance to be used to calculate a p -value. Thus, no statistical inference was performed for this variable.

Results

All animals survived the entire experimental protocol leaving no missing data for statistical analysis. H_2O_2 generation in kidneys and dry/wet weight variation of abdominal cavity viscera were significantly higher in CLP-SALINE [AE2] group than in any other group ($p=0.001$ for CLP-SALINE [AE2] vs. CLP-IVABRADINE [AE2] when comparing H_2O_2 generation and $p=0.017$ for CLP-SALINE [AE2] vs. CLP-IVABRADINE [AE2] when comparing tissue edema; Figs. 1 and 2, respectively). H_2O_2 generation in thoracic aortas was higher in CLP-SALINE [AE2] group than in CLP-IVABRADINE [AE2] in both available time points (Table 1).

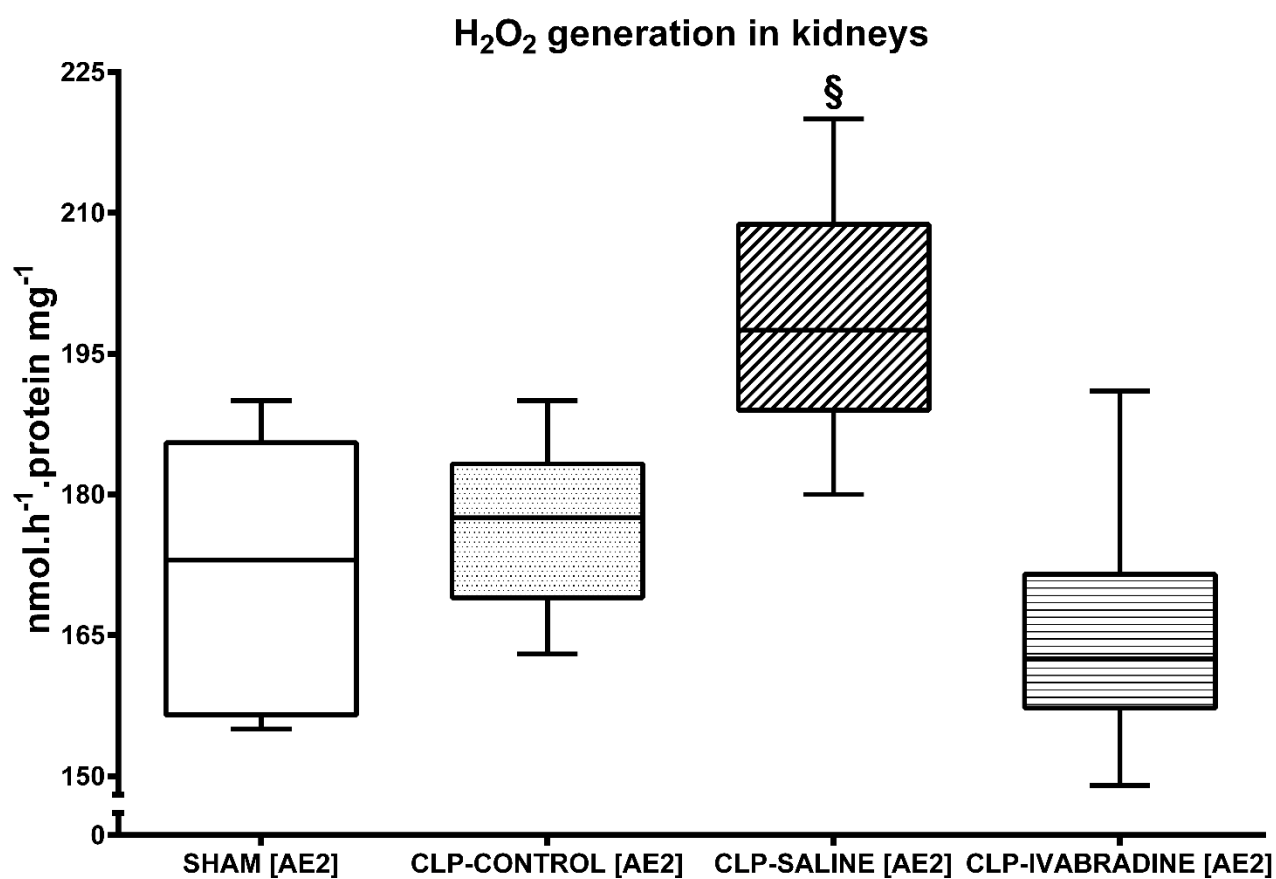


Figure 1 – **H₂O₂ generation in kidneys**. Data are given as median values and the 5th to 95th percentile ranges for each group. SHAM [AE2] group = non-septic, fluid resuscitated and treated with saline (n=6); CLP-CONTROL [AE2] group = septic, non-fluid resuscitated nor treated (n=6); CLP-SALINE [AE2] group = septic, fluid resuscitated and treated with saline (n=6); CLP-IVABRADINE [AE2] group = septic, fluid resuscitated with saline and treated with ivabradine (n=6). § $p < 0.05$ as compared with any other group. AE2 = additional experiments 2; CLP = cecal ligation and puncture procedure.

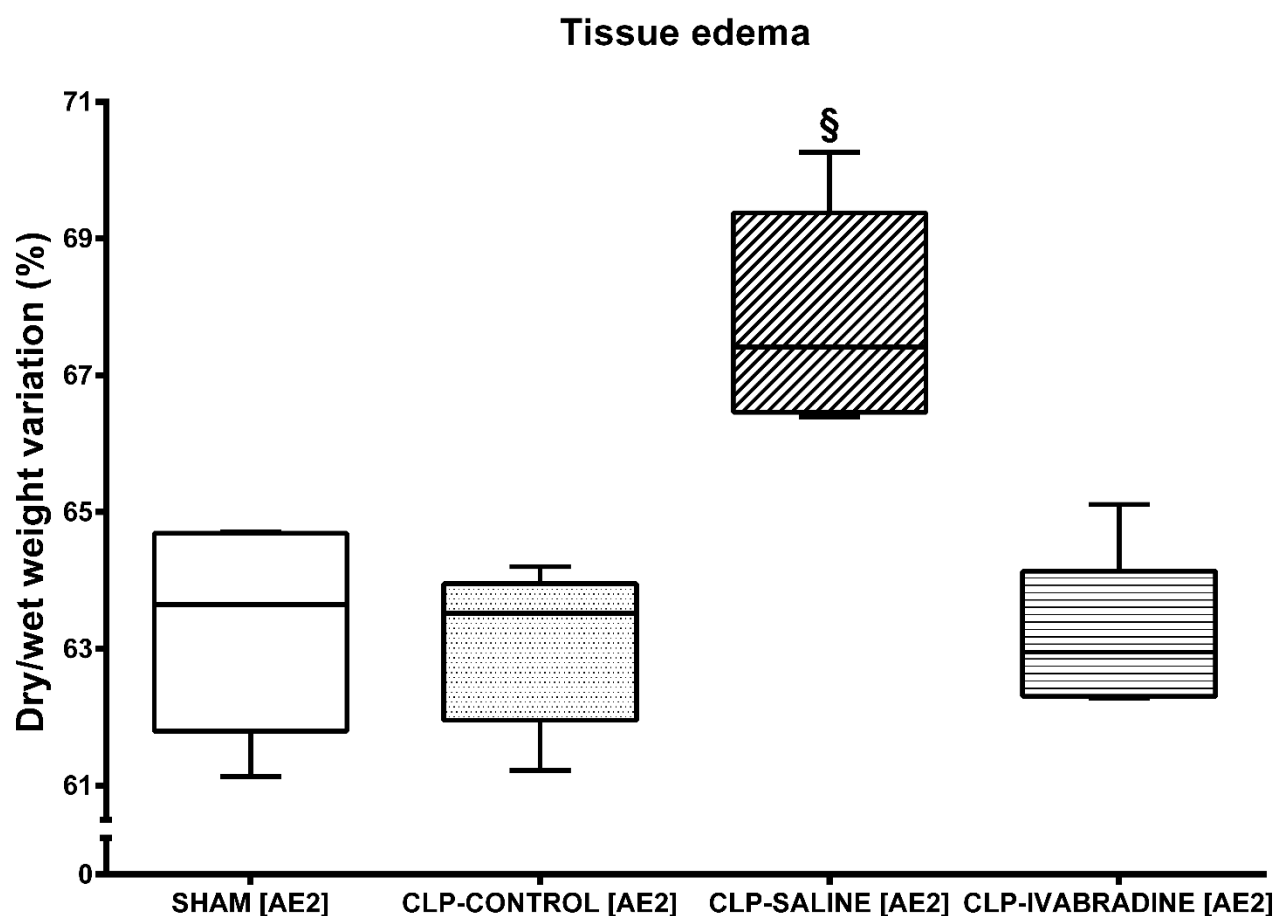


Figure 2 – **Dry/wet weight variation of abdominal cavity viscera**. Data are given as median values and the 5th to 95th percentile ranges for each group. SHAM [AE2] group = non-septic, fluid resuscitated and treated with saline (n=6); CLP-CONTROL [AE2] group = septic, non-fluid resuscitated nor treated (n=6); CLP-SALINE [AE2] group = septic, fluid resuscitated and treated with saline (n=6); CLP-IVABRADINE [AE2] group = septic, fluid resuscitated with saline and

treated with ivabradine (n=6). § $p < 0.05$ as compared with any other group. AE2 = additional experiments 2; CLP = cecal ligation and puncture procedure.

Table 1 – Hydrogen peroxide (H_2O_2) generation in thoracic aortas (in $nmol \cdot h^{-1} \cdot protein \cdot mg^{-1}$)

	SHAM [AE2]	CLP-CONTROL [AE2]	CLP-SALINE [AE2]	CLP-IVABRADINE [AE2]
No resuscitation	N/A	36	N/A	N/A
15 minutes after resuscitation	55	N/A	50	23
4 hours after resuscitation	35	N/A	40	32

Data are presented as the result of pooled samples from 6 animals for each group and time point. AE2 = additional experiments 2; CLP = cecal ligation and puncture procedure; CLP-CONTROL [AE2] group = septic, non-fluid resuscitated nor treated; CLP-IVABRADINE [AE2] group = septic, fluid resuscitated with saline and treated with ivabradine; CLP-SALINE [AE2] group = septic, fluid resuscitated and treated with saline; N/A = not applicable; SHAM [AE2] group = non-septic, fluid resuscitated and treated with saline.

Discussion

The main findings of these experiments were that ivabradine was associated with less renal and systemic H_2O_2 generation and less tissue edema in abdominal cavity viscera in ivabradine-treated fluid resuscitated septic hamsters when compared with those treated with saline.

Current H_2O_2 findings corroborate the hypothesis that fluid resuscitation has led to reperfusion injury in CLP-SALINE animals during main experiments, as mitochondrially-derived ROS are key mediators generated during ischemia-reperfusion.¹¹

Ivabradine decreasing effects on ROS generation^{1,2} may explain CLP-IVABRADINE [AE2] results, while a decreased H_2O_2 generation may, at least in part, explain renal function results (in main experiments) and current tissue edema findings. ROS can directly disrupt microcirculatory

structures, such as the endothelial glycocalyx. Continuous glycocalyx degradation and shedding leads to loss of integrity of adherens junctions and increased paracellular permeability with subsequent impairment of endothelial barrier function. Increased endothelial permeability results in fluid leakage from the intravascular space and tissue edema. Accumulation of water in tissues finally leads to tissue hypoxia due to increased diffusion distances between functional capillaries and tissue cells in combination with poor oxygen solubility and transport in tissue water. This pathophysiologic mechanism may help to explain several sepsis-associated organ dysfunctions.¹²

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