Additional experiments 3 – Ivabradine effects on arteriolar reactivity

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Introduction

Wei and co-workers[1] have shown that ivabradine is not associated with any effect on arterial reactivity during experimental sepsis. As during sepsis microvascular response to treatments does not reflect that found on larger vessels in a reliable manner (a condition known as loss of coherence)[2] additional experiments were carried out to investigate ivabradine effects on arteriolar reactivity in two rodent models that allow *in vivo* studies of the microcirculation (the dorsal chamber and the cheek pouch models).

Materials and Methods

Experiments were performed on 39 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.[3]

Animal Preparation

The dorsal chamber implantation procedure was performed as described by Endrich and co-workers.[4,5] Briefly, under intraperitoneal anesthesia with a combination of ketamine and xylazine (100 $mg.kg^{-1}$ and 20 $mg.kg^{-1}$, 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, animals were re-anesthetized 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 $IU.ml^{-1}$) 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 electrocardiography (ECG) monitoring (two near front limbs and one near left rear limb; lead I and II). Experiments were performed after 24 hours of catheter and electrodes implantation. MAP, heart rate (HR), and temperature were continuously monitored during the study period. MAP was monitored through the arterial catheter and a blood pressure transducer (TSD104A, BIOPAC Systems, Goleta, CA, USA), while ECG electrodes were connected to an amplifier where analog signal was recorded (Animal Bio Amp, FE 136, AD Instruments, Castle Hill, Australia). Analog pressure and ECG signals were digitized (PowerLab 8/35, AD Instruments, Castle Hill, Australia) and processed using data acquisition software for cardiovascular experiments (LabChart Pro software v. 8, AD Instruments, Castle Hill, Australia). HR was determined from the analysis of surface ECG and expressed as beats per minute (bpm). Rectal and skinfold chamber temperatures were monitored with a beaded type K thermocouple probe (Wavetek 23XT, Wavetek Corporation, San Diego, CA, USA). Body temperature of hamsters was maintained with a heating pad placed near the animal controlled by a rectal thermistor (LB750, Uppsala Processdata AB, Uppsala, Sweden).

The cheek pouch was prepared as described by Svensjö and detailed elsewhere.[6] 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. Monitoring (MAP, HR, and temperature) was performed exactly the same way as described for dorsal chamber experiments. As an exception, femoral artery and vein (rather than carotid and jugular vessels) were catheterized for cheek pouch experiments.

Cecal ligation and puncture (CLP) procedure was performed as described by Rittirsch and co-workers.[7] 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.

Intravital Microscopy – Dorsal Chamber Experiments

Unanesthetized animals were placed in a restraining plexiglass tube attached to an intravital microscope (Ortholux, Leitz, Wetzlar, Germany). 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) 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 v. 1.49 (NIH, Bethesda, MD, USA), a computer-assisted image analysis system, by an investigator blinded to drug treatment. In each animal, 2 arterioles 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 were studied throughout the experiment. Arteriolar

mean internal diameter was measured as the perpendicular distance (in micrometers) between the vessel walls.

Intravital Microscopy – Cheek Pouch Experiments

Intravital microscopy of anesthetized animals was performed exactly the same way as described for dorsal chamber experiments, except for the number of studied arterioles (5 in each animal).

Experimental Protocol – Dorsal Chamber Experiments: [DC] groups

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 dorsal chamber. Included animals were randomly allocated in 3 groups: SHAM [DC] (sham-operated [non-septic] animals fluid resuscitated and treated with saline; n=7), CLP-SALINE [DC] (CLP-operated [septic] animals fluid resuscitated and treated with saline; n=7), and CLP-IVABRADINE [DC] (CLP-operated animals fluid resuscitated with saline and treated with ivabradine [Sigma-Aldrich, St. Louis, MO, USA]; n=7).

After baseline determination of vital signs, animals belonging to SHAM [DC] group were sham-operated and those belonging to CLP-SALINE [DC] and CLP-IVABRADINE [DC] groups were subjected to CLP procedure. Twenty-four hours after CLP or sham operation, animals were fluid resuscitated with intravenous (IV) saline (20 $ml.kg^{-1}$ in 15 minutes) and CLP-IVABRADINE [DC] 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 $mg.kg^{-1}.h^{-1}$; CLP-IVABRADINE [DC] group) was initiated and maintained at a 0.1 $ml.h^{-1}$ infusion rate for 4 hours. After 4 hours of saline or ivabradine infusion, a continuous IV infusion of norepinephrine (0.25 $mcg.kg^{-1}.min^{-1}$) or ivabradine/norepinephrine solution (0.5 $mg.kg^{-1}.h^{-1}$/0.25 $mcg.kg^{-1}.min^{-1}$; CLP-IVABRADINE [DC] group) was initiated and maintained at a 0.1 $ml.h^{-1}$ infusion rate for 1 hour.

After baseline evaluations, sequential measurements of MAP, HR, and arteriolar diameter were performed at two time points: after 4 hours of saline or ivabradine infusion and after 1 hour of

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

Experimental Protocol – Cheek Pouch Experiments: [CP] groups

Twenty-four hours after CLP or sham operation, animals were anesthetized, the cheek pouch was prepared, and baseline vital signs were determined. 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 preparation.

Included animals were fluid resuscitated with saline (20 $ml.kg^{-1}$ in 15 minutes; IV) and randomly allocated in 3 groups: SHAM-SALINE [CP] (sham-operated [non-septic] animals topically treated with saline; n=6), SHAM-IVABRADINE [CP] (sham-operated animals topically treated with increasing concentrations of ivabradine; n=6), and CLP-IVABRADINE [CP] (CLP-operated [septic] animals topically treated with increasing concentrations of ivabradine; n=6).

In ivabradine-treated groups, ivabradine was added to the superfusion solution at increasing concentrations (10^{-7} M, 10^{-6} M, 10^{-5} M, and 10^{-4} M) and each concentration was superfused for 15 minutes. In SHAM-SALINE group, only saline was added to the superfusion solution.

After baseline evaluations, sequential measurements of MAP, HR, and arteriolar diameter were performed at the last 5 minutes of superfusion of each ivabradine concentration. 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 means ± standard deviation of the mean (SD) for each group, unless otherwise noted. Statistical comparisons of normally distributed variables (assessed by Shapiro-Wilk test) were performed using 2-way ANOVA for repeated measures and 1-way ANOVA as appropriate. 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.

Dorsal Chamber Experiments

MAP, HR, temperature, and mean arteriolar diameter evolution was similar to that observed in main experiments until initiation of norepinephrine infusion. After that, HR decreased in all groups while MAP increased only in SHAM [DC] group (p=0.001 for SHAM [DC] *vs.* CLP groups; Table 1). Mean arteriolar diameter decreased after initiation of norepinephrine infusion in all three groups without significant differences between arteriolar responses to the catecholamine (p=0.418; Fig. 1).

Table 1 – Heart rate and mean arterial blood pressure after norepinephrine infusion

	SHAM [DC]	CLP-SALINE [DC]	CLP-IVABRADINE [DC]	*p*-value for overall group effect	*p*-value for CLP-SALINE [DC] *vs.* CLP-IVABRADINE [DC]
HR (bpm)	346±49	360±78	238±22	0.001	0.001
MAP (mmHg)	122±15	88±20	89±8	<0.001	>0.999

Heart rate (HR) and mean arterial blood pressure (MAP) were measured after 1 hour of norepinephrine or ivabradine/norepinephrine infusion. Data are presented as means±SD for each group. All p-values were adjusted for multiple comparisons applying the Bonferroni adjustment. bpm = beats per minute; CLP = cecal ligation and puncture procedure; CLP-IVABRADINE [DC] group = septic, treated with norepinephrine plus ivabradine (n=7); CLP-SALINE [DC] group = septic, treated with norepinephrine (n=7); DC = dorsal chamber experiments; SHAM [DC] group = non-septic, treated with norepinephrine (n=7).

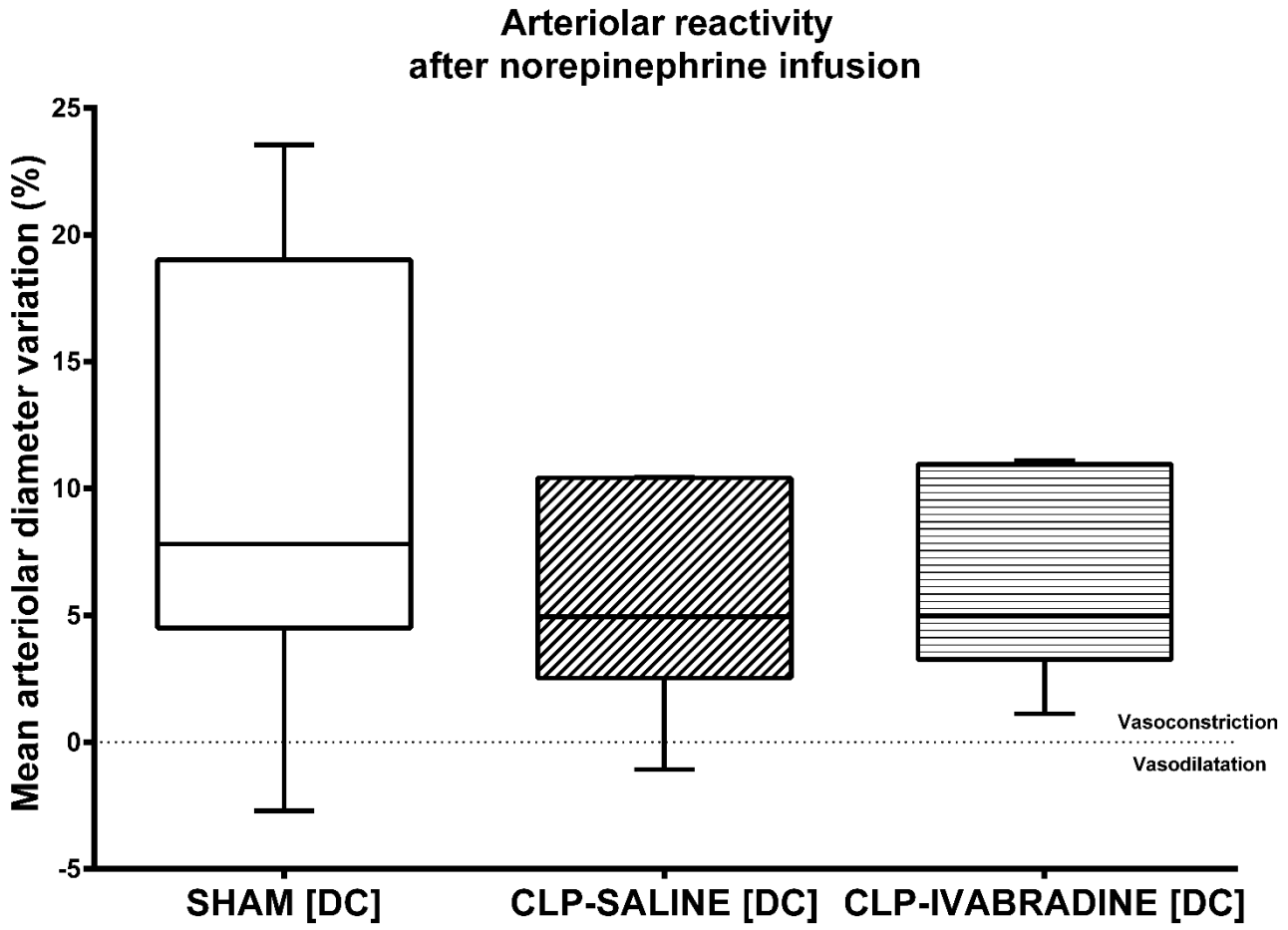

Figure 1 – **Mean arteriolar diameter variation after initiation of norepinephrine infusion (arteriolar reactivity).** Data are given as median values and the 5th to 95th percentile ranges for each group. SHAM [DC] group = non-septic, treated with norepinephrine (n=7); CLP-SALINE [DC] group = septic, treated with norepinephrine (n=7); CLP-IVABRADINE [DC] group = septic, treated with norepinephrine plus ivabradine (n=7). CLP = cecal ligation and puncture procedure; DC = dorsal chamber experiments.

Cheek Pouch Experiments

Topical treatment with ivabradine had no effect on HR or MAP but decreased mean arteriolar diameter in both SHAM-IVABRADINE [CP] and CLP-IVABRADINE [CP] groups as compared with SHAM-SALINE [CP] (p <0.001 for SHAM-SALINE [CP] *vs.* ivabradine groups at 10^{-5} M and 10^{-4} M concentrations; Fig. 2).

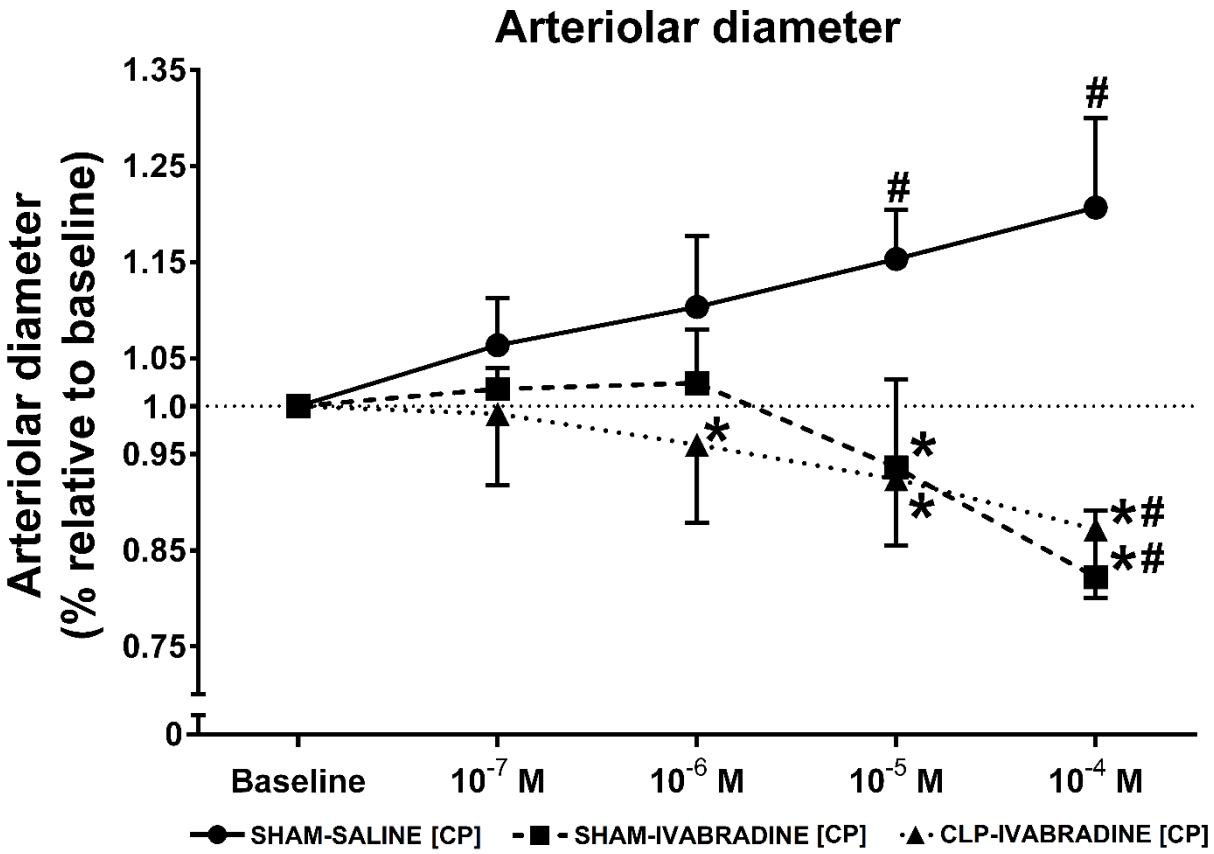

Figure 2 – **Arteriolar response to increasing concentrations of ivabradine.** Data are given as means ± SD. SHAM-SALINE [CP] group = non-septic, topically treated with saline (n=6); SHAM-IVABRADINE [CP] group = non-septic, topically treated with increasing concentrations of ivabradine (n=6); CLP-IVABRADINE [CP] group = septic, topically treated with increasing concentrations of ivabradine (n=6). * p <0.05 *vs.* SHAM-SALINE [CP] group at the same time point; # p < 0.05 *vs.* group baseline. CLP = cecal ligation and puncture procedure; CP = cheek pouch experiments.

Discussion

The main finding of this series of microcirculatory studies was that ivabradine was not associated with any effect on arteriolar reactivity during experimental sepsis. Furthermore, ivabradine had no direct vasodilatory effect on arterioles and induced vasoconstriction in high tissue concentrations.

Norepinephrine studies were carried out employing the same protocol used on main experiments, which allows comparisons between results from both studies. For instance, the same dose of ivabradine was able to attenuate microcirculatory derangements evoked by sepsis on main experiments without any effect on arteriolar reactivity on the current experiments, showing that changes in arteriolar reactivity are not associated with observed ivabradine beneficial microcirculatory effects. Interestingly, some animals showed vasodilatation in response to norepinephrine, which may be explained by considerable variation in individual microvascular response to vasopressors.[2]

The cheek pouch model was chosen for microvessel reactivity investigations because it allows the observation of direct vascular effects of drugs, without affecting systemic parameters (such as heart rate, mean arterial pressure, or cardiac output) as treatments are topically administered in sub-systemic doses, obviating any confounding systemic effects on drug response. The findings of cheek pouch experiments suggest that ivabradine beneficial microcirculatory effects are not related to a direct arteriolar vasodilatatory mechanism and corroborate the hypothesis that ivabradine acts, at least in part, by increasing cardiac output and/or by attenuating endothelial damage.

In main experiments, arteriolar vasodilatation was observed in CLP groups in response to fluid resuscitation, probably due to increased cardiac output and/or shear stress.[8] Considering the results of main and additional experiments, it is possible to speculate that after initial fluid-induced arteriolar vasodilatation ivabradine effects on microcirculatory leakage may have contributed to intravascular retention of the administered fluid, prolonging the beneficial macro and micro-hemodynamic effects of fluid resuscitation.

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