Effects of cococonut water and simvastatin in the treatment of sepsis and hemorrhagic shock in rats[1]

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ABSTRACT

PURPOSE: To evaluate the effects of modified coconut water as fluid of resuscitation combined with simvastatin in hemorrhagic shock and sepsis model in rats.

METHODS: Four groups of Wistar rats with hemorrhagic shock and abdominal sepsis were studied (n=8/group). Rats were bled and maintained at a mean blood pressure 35mmHg for 60min. They were then resuscitated with: 1) saline 0.9%; 2) coconut water+3% NaCl; 3) coconut water+NaCl 3%+simvastatin microemulsion (10 mg/kg i.v.; 4) normal coconut water. At 8h post-resuscitation, blood and lungs were collected for exams.

RESULTS: Clinical scores, TNF-α, IL-1β, liver/kidney proof levels, and lung injury were significantly reduced in coconut water+NaCl 3%+simvastatin group treated rats, comparing with the other resuscitation treatments.

CONCLUSIONS: Resuscitation with coconut water with Nacl 3%+simvastatin had a significant beneficial effect on downregulating cytokines and decreasing lung injury in a rat model of abdominal sepsis and hemorrhagic shock. We also demonstrated that coconut water with Nacl 3%+simvastatin administration clearly made liver and kidney function better and improved clinical score.

Key words: Shock, Hemorrhagic. Sepsis. Resuscitation. Simvastatin. Foods Containing Coconut. Rats.

Introduction

Pathophysiology of sepsis is related to an imbalance between anti-inflammatory and pro-inflammatory substances that will mediate a response to damage body tissues[1]. Despite intensive treatment with aggressive resuscitation, blood pressure control and adequate supply of oxygen, patients with sepsis often persist showing signs of tissue hypoperfusion, which can lead to acidosis and eventually multiple organ failure[2-4]. Researchers have shown that sepsis is characterized by decreased velocity of microcirculatory flow, increased flow heterogeneity, increasing vascular stasis and decreasing perfused capillaries[5,6]. Failure in the microcirculation flow results in shunting of blood from the tissues, leading to deficit in oxygen required for normal cell metabolism[7,8]. Systemic inflammatory mediators and endotoxins disrupt intracellular connections and the signals used by endothelial cells, acting as a unified system. This disruption can result in altered distribution of blood flow in tissues[9].

The hemorrhagic shock and consequent tissue hypoperfusion lead to a reduction of cell oxygenation, metabolic acidosis and hypothermia[10]. The reduction of O2 supply leads to increased anaerobic metabolism and reduction of ATP levels and intracellular calcium, which promote significant changes in cellular function by stimulating the release of pro-inflammatory cytokines. These, in turn, may alter the immune function of macrophages and lymphocytes, causing immunosuppression and increased risk of infection[11].

Some works have studied the composition of coconut water (CW). Santoso *et al.*[12] described the presence of vitamins, sugars, organic acids, fatty acids, amino acids, minerals and electrolytes in the coconut (*Cocus nucifera L.*) water. Aleixo et al[13] determined their selenium content using atomic absorption spectrometry. Another important feature of CW is its antioxidant ability[14-16]. Trace elements and metals in small dosages were determined, leading to believe that CW has potential for use in intravenous hydration or parenteral nutrition solutions supplement[17]. Campbell-Falck *et al.*[18] reported a successful case of intravenous hydration in the Salomon Islands and described its use for this purpose during the II World War.

The electrolyte composition of the 6 months CW resembles more the intracellular fluid then the extracellular, and consists mainly of potassium, calcium, magnesium and chlorine. Sodium is found in much lower concentration than in human plasma[19]. Although CW has already been described as a viable solution for resuscitation, reports of its i.v. use are scarce[20,21],

with the information that it does not interfere in hemostasis mechanisms[22].

Statins inhibit HMG-CoA reductase and have been extensively studied for the prevention of atherosclerosis, inflammatory disease of the vascular endothelium, whose pathogenesis has similarities with the pathogenesis of sepse[23]. Although the effects of statins were initially assigned only to treat hyperlipid levels, many other benefits came to be considered and studied. Statins exert multiple effects on various cells for a number of mechanisms. These pleiotropic effects have been described as antiinflammatory, modifying the interactions between the endothelium and leukocytes[24,25]. Additionally, statins modulate the signaling of inflammatory cells, which in turn reduces the release of cytokines and proteins of acute phase of sepsis[26], and other important antioxidant effects[27].

The ideal solution for volume replacement in situations of sepsis associated with hemorrhagic shock is not yet well established. In this protocol we studied the effects of modified CW solution, combined with intravenous simvastatin for the volume replacement in rats submitted to a double challenge: sepsis and hemorrhagic shock. These situations are common and deleterious in surgical practice, with high morbidity and mortality.

This study aimed to examine the effects of the modified CW combined with simvastatin as fluid resuscitation in hemorrhagic shock and abdominal sepsis model in rats.

Methods

The institutional Ethics Committee on Animal Use approved this protocol, which was performed at the Nucleus of Experimental Surgery-UFRN, Brazil. Care in the use of animals followed the standards of the Brazilian legislation for the scientific use of animals (Law No. 11794/2008). It was used animal model of hemorrhagic shock and abdominal sepsis to evaluate possible effects of simvastatin and hypertonic coconut water volume replacement in Wistar rats, weighing between 250 and 300g. Rats were fasting for 12 hours before the experiment. They were anesthetized with ketamine (70 mg / kg) and xylazine (10 mg / kg) intramuscular. These drugs were re-administered if necessary until the end of the experiments.

Hemorrhagic shock

After stabilization of anesthesia, monitoring was installed in all animals. The left femoral vein and right femoral artery were

dissected and isolated for the realization and monitoring of fluid loss and replacement. Silicone tubes (F24) were introduced into the femoral artery for mean arterial pressure (MAP) monitoring and in the femoral vein for fluid replacement. The blood was drained through the femoral artery until the MAP stabilize at 35 mmHg. We recorded the volume of collected blood to calculate the fluid volume to be reinfused. MAP was monitored through an Invasive Blood Pressure Monitor. Hemorrhagic shock was maintained for 60 minutes.

Abdominal sepsis and study groups design

After 60 min of shock, for the induction of the sepsis rats remained anesthetized. After shaving and disinfecting the lower quadrants of the abdomen a midline incision of approximately 4cm length was performed. The cecum was identified and the corresponding mesenteric membrane dissected. A cecal ligation was positioned consistently at the distal third, i.e. at 30–40% of the cecum. The perforation of the cecum was performed using a 18G needle, first puncturing the anti-mesenteric side, then continuing the needle penetration through the lumen to the second perforation on the mesenteric side. A small amount of fecal material was then gently extruded from both perforation holes. After repositioning the cecum within the abdominal cavity the abdomen was closed in two layers. Asseptic technique was used. After that the animals received fluid resuscitation intravenously over 10 minutes. The replacement volume was equal to twice the lost blood volume for each rat. We studied four groups (n = 8/group): 1) replacement with saline solution 0.9%; 2) replacement with coconut water + 3% NaCl (CW Na3%); 3) replacement with coconut water + NaCl + 3% sodium simvastatin (CWNa3%S) microemulsion 10 mg / kg i.v.; 4) replacement with normal coconut water (CW). During the period of shock and fluid replacement, the animals were kept in a warm microenvironment at 37°C (Hotplate, Insight, São Paulo, Brazil) and anesthetized by the end of the experiment.

Fluid resuscitation

Coconut water (CW) was obtained from 6 months coconut (*Cocus nucifera L.*) specimens (time from the inflorescence to harvesting the fruits), using sterile technique. CW was harvested immediately before administration to animals and modified appropriately to achieve the 3% NaCl level. We measured the CW pH with pHmeter (Micronal, São Paulo, Brazil), (mean pH 5.8). After that, pH was adjusted to the value 7.4 by using

sodium bicarbonate 10%. Sodium content of CW was adjusted to 3% immediately prior to intravenous infusion, by adding pre-calculated volume of NaCl 10%. Saline solution 0.9% was from B. Braun, Rio de Janeiro, Brazil. We used infusion pump (B. Braun, Rio de Janeiro, Brazil) for fluid resuscitation; the volume was infused for 10 min in constant rate (0,5 mL/min). During the infusion all solutions were heated to 36.5 ° C. The experimental protocol included assessment of clinical parameters, serum, blood tests, imaging and survival 18 hours after resuscitation.

Clinical score

Six and 18 hours after resuscitation, all rats had their clinical score evaluated. Time points for analysis were chosen based on previous data[28]. The score consisted of analyzing the following parameters: 1- presence of piloerection, 2-altered respiration rate, 3-fecal alteration, 4-lacrimation/eyelid changes, 5-contraction of the abdomen, 6-lack of strength when grasping, 7-change in body temperature, 8-alert response (scape after touch), 9-exploration of the environment and 10-compromised locomotor activity. For every parameter present, we gave 1 point, and in the absence of the parameter analyzed, no points were given. Then, the points were computed for each rat. A score of 0 indicated that the rat did not present any clinical alteration, a score between 1 and 3 indicated mild sepsis, between 4 and 7 indicated moderate sepsis and between 8 and 10 indicated severe sepsis.

Laboratory parameters determination

The surviving animals after 18 hours of observation were again anesthetized and blood was harvested for dosages. Serum was separated by centrifugation at 3000 rpm and stored at -40°C for subsequent dosing. Plasma levels of TNF-α and IL-1β were determined using quantitative enzyme linked immunosorbent assay (ELISA) kits, according to manufacturer recomendations (PeproTech, USA). We determined aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea, creatinine, C-reactive protein (CRP) and white blood cell count. Urea and creatinine are indicators of renal function and glomerular filtration. CRP was measured by nephelometry.

Lungs fluorescence imaging ex-vivo.

Imediately after blood collection, rats were injected into the femoral vein with indocyanine green 10mg/Kg, (Ophthalmos, São Paulo Brazil). Ten minutes after injection the lungs and

trachea were harvested for ex-vivo imaging. Optical imaging was performed using FX fluorescence reflectance imaging device (Carestream Molecular Imaging). Filters for excitation and emission were set at 710 nm and 700 nm, respectively. The lungs were placed into the equipment chamber. An imaging protocol (exposure time 20 seconds, 2x2 binning, f-stop 2.8, field of view 120 mm, and focal plane 10 mm) was maintained for all images, and comparative images were taken comparing groups. The optical images of the ex vivo study were evaluated qualitatively by assessing the presence or absence of visibly increased fluorescence in the lungs, comparing CW+Na 3%+ simvastatin group with the fluorescence of saline 0.9% treated group. Quantitative analysis of the lung scans was performed by measuring the fluorescence signal intensity (SI) of the lungs, using the Molecular Imaging software 5.0. A region of interest created by an automated tool was determined around the organ. Mean region of interest signal intensities were expressed as arbitrary fluorescent SI in pixels. Fluorescence grayscale images were colored for depiction purposes according to a color scale set to the highest and lowest levels of mean fluorescence intensity (red and purple indicated maximum and minimum light intensity, respectively). The targets were septic and inflammatory focus in lungs.

Histological evaluation of lung injury

The lungs were removed and immersed in 10% buffered formalin for 48 h and then embedded in paraffin. 4μm sections were stained with hematoxylin and eosin. Pulmonary architecture was evaluated by optical microscopy with BX50 microscope equipped with digital camera DS30 (Olympus, Japan). Two random tissue sections of four different lungs from each group were examined. Lung injury scores were quantified by an investigator blinded to the treatment groups using previous published criteria (Table 1), which gives an overall score between 0 and one[29].

TABLE 1 – Lung injury system (adapted from Matute-Bello *et al.*[29]).

Parameter	Score per field		
	None	1	2
a. Neutrophils in the alveolar space	None	1-5	>5
b. Neutrophils in the interstitial space	None	1-5	>5
c. Hyaline membranes	None	1	>1
d. Proteinaceous debris filling the airspaces	None	1	>1
e. Alveolar septal thickening	None	2x – 4x	>4x

Score = [(20 x A) + (14 x B) + (7 x C) + (7 x D) + (2 x E)]/(number of fields x 100)

Data were stored and statistically analyzed using SPSS 17.0 and GraphPad Prism 7:00 software for Windows, GraphPad Software, La Jolla, California, USA, www.graphpad.com. The results were statistically studied using the method of multiple comparisons by analysis of variance (ANOVA) with repeated measures followed by Tukey and Newman-Keuls tests for comparison between groups. Results were considered statistically significant when $p<0.05$.

Results

All rats survived until the end of experiments. Coconut water Na3% + simvastatin (CWS) administration prevented several alterations after sepsis induction. Therefore, we investigated the effect of CWS on sepsis severity and mortality using clinical scores. Six hours after CLP, rats presented clear clinical signs of sepsis, and most animals in the sepsis group had severe sepsis. CWS+Na 3% + simvastatin reduced the clinical score 24 hours after CLP and improved the clinical status of CWS treated-animals (Figure 1).
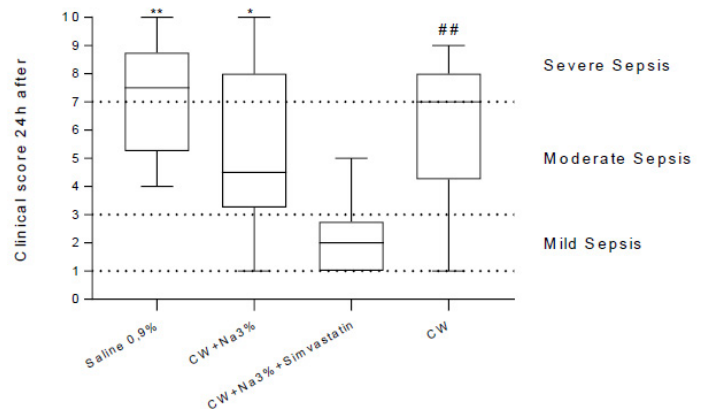

FIGURE 1 – Results of clinical scores of animals and respective groups. CW, coconut water.
*$p<0.05$ compared with CW+Na3%+simvastatin; **$p<0.01$ compared with CW+Na3%+simvastatin; ##$p<0.01$ compared with CW+Na3%+simvastatin.

Serum levels of inflammatory cytokines TNF-α and IL-1β were measured in all groups, and were found to be significantly lower in the coconut water +simvastatin-treated group. Although all groups showed a significant difference ($p<0.05$) compared to the saline treated group, the best results were found in the coconut water +simvastatin-treated group rats (Table 2). More studies in

this area are needed to decipher the mechanism of this particular issue.

TABLE 2 – Interleukin dosage in animals with hemorrhagic shock and abdominal sepsis.

Cytokiness Groups	TNF α (pg/ml)	IL-1β (pg/ml)
Saline 0.9%	545.5±36[ab]	174.9±16[a]
CW Na3%	364.8±42[ab]	86.2±12[a]
CW Na3%S	181.7±16.1[b]	48.1±8[a]
CW	210.1±14.5[a]	70.3±5[a]

*Mean ± Standard deviation.
(1) Measures followed by the same letter differ significantly at p<0.05. CW, coconut water; S, simvastatin.

As for biochemical dosages, the rats treated with CW + Na3% and simvastatin had lower levels of C-reactive protein, AST, ALT, urea and creatinine then in the other groups, with statistically significant differences (Table 3). The same happened with the white blood cell count, where the total leukocytes and neutrophils values of the animals treated with CW + Na3% and simvastatin, were significantly lower than in the other groups. However, as shown in Table 4, the percentage of eosinophils was significantly higher in rats treated with CW + Na3% and simvastatin (p<0.05).

TABLE 3 – Influence of different fluid resuscitation procedures on excretion of PCR, AST, ALT, urea and creatinine.

Biochemicals Groups	C-reactive protein (mg/dL)	AST (mg/dL)	ALT (mg/dL)	Urea (mg/dL)	Creatinine (mg/dL)
Saline 0,9%	9.40 ± 2.2ab	55.2± 6.2ab	62.5± 4.9a	76.8± 4.9ab	3,78± 0,5ab
CW Na3%	4.72 ± 0.39a	39,5± 9.3a	39.3± 5.7a	47.1± 3.4a	2,64± 0,6a
CWNa3%S	2.72 ± 0.46ab	32.1± 2.4ab	26.5± 2.6a	35.3± 2.9ab	1,36± 0,2ab
CW	4.45 ± 0.57b	42.1± 7.4b	36.5± 2.6a	45.3± 4.0b	2,86± 0,3b

*Mean ± Standard deviation.
(1) Measures followed by the same letter differ significantly at p<0.05. CW, coconut water; Na, sodium; S, simvastatin; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

TABLE 4 – Influence of different fluid resuscitation procedures on leukocytes.

Leukocytes Groups	Leuc/µL)	Neutrophils (%)	Eosinophis(%)
Saline 0,9%	12.46 ± 1.26ab	77.37 ± 6.4ab	0.39 ± 0.4a
CW Na3%	8.72 ± 0.39a	54.50 ± 7.0a	4.57 ± 0.5a
CWNa3%S	6.68 ± 0.56a	47.17 ± 6.6ab	5.41 ± 0.6a
CW	8.32 ± 0.53b	57.10 ± 8.5b	3.68 ± 0.7a

*Mean ± Standard deviation.
(1) Measures followed by the same letter differ significantly at p<0.05 (Tukey test).

The fluorescence signal intensity (SI) of the lungs was measures in Saline and CW Na3%+ simvastatin groups. Fluorescent images (Figure 2) showed signal intensity markedly higher in the saline group rats compared to animals in the CW Na3%+ simvastatin group. In Table 5 is shown that corresponding quantitative SI of lungs fluorescence in saline group animals were significantly higher compared to the CW Na3%+ simvastatin group after intravenous injection of 10 mg/kg ICG.
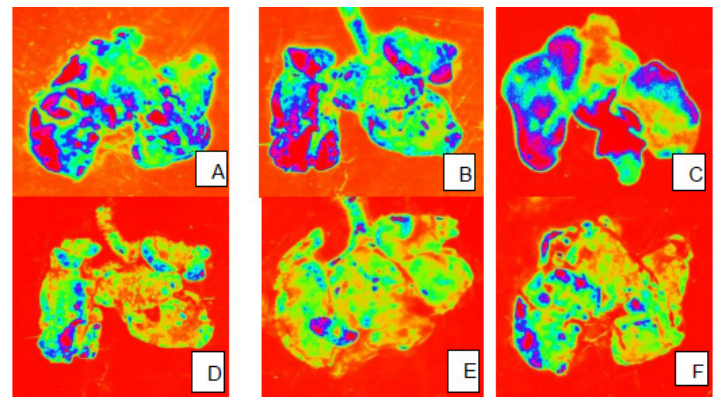

FIGURE 2 - Comparisons between groups — The fluorescence of the lungs on postcontrast indocyanine green (ICG) ex-vivo images was markedly higher in group rats treated with saline (**A,B,C**) compared to rats of the group treated with coconut water +Na3% + simvastatin (**D,E,F**).

TABLE 5 – Influence of different fluid resuscitation procedures on Fluorescence signal intensity (arbitrary units) after intravenous injection of 10 mg/kg ICG.

Groups	Signal intensity (arbitrary unit)
Saline 0,9%	230±12.1
CWNa3%S	117±8.3*

Mean ± Standard deviation. * Measures differ significantly compared with saline group at p<0.05 (Tukey test). CW, coconut water; Na, sodium; S, simvastatin.

Histopathology

As shown in Figure 3, histopathological changes and lung injury were evident in lung tissues from rats with CLP-induced sepsis and shock treated with saline, compared to rats treated with coconut water. Light microscopic analysis of lung tissue from animals with sepsis and shock, treated with saline 0.9% showed thickened alveolar septum with increased cellularity. These animals developed increased neutrophils and a prominent increase in mononuclear and interstitial cells in alveolar septum. Alveolar edema was also present in this group. It is clear that histological damage and leukocytes infiltration were ameliorated after coconut water + Na3% + simvastatin treatment and was accompanied with a declined histology scores. There was no increase in neutrophils in alveolar septum, and minor cells or edema in the alveolar spaces (Figure 3). So, lung biopsy, done in animals at 18 h after treatment with coconut water 3% (Na) + simvastatin, showed minor changes in lung architecture by light microscopy. Histopathologic scores are summarized in Table 6.
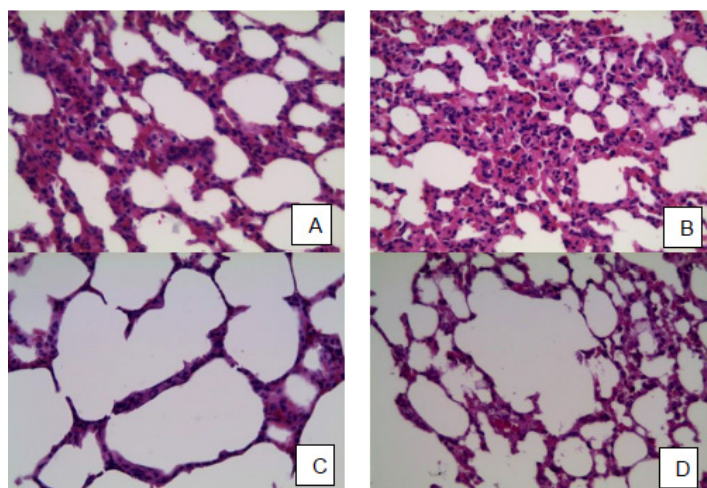

FIGURE 3 - Representative H&E stains of inflamed lungs of saline treated rats (**A, B**) and coconut water + simvastatin treated rats (**C, D**). Magnification: x200. Alveolar and interstitial spaces with both increased neutrophils and a prominent increase in mononuclear and interstitial cells. Thickened alveolar septum with increased cellularity are shown in A,B.

TABLE 6 – Histological scores.

Groups	Scores
Saline 0.9%	0.78±0.04ab
CW Na3%	0.30±0.02b
CWNa3%S	0.23±0.01a
CW	0.51±0.04ab

*Mean ± Standard deviation.
(1) Measures followed by the same letter differ significantly at p<0.05. CW, coconut water; Na, sodium; S, simvastatin.

Discussion

The hemorrhagic shock causes tissue damage and increases hypoxia and proinflammatory activity. A rapid resuscitation with adequate fluids is essential to minimize damage and improve tissue perfusion. The present study examined an alternative to reduce the damage caused by hypovolemia using normal and modified coconut water, which showed positive results compared to resuscitation with 0.9% saline. In the present study, we compared the effects 0.9% saline, modified coconut water (Na 3%) and water coconut 3% + Na + simvastatin, and normal coconut water on serum cytokines, biochemical and white blood cell count in rodent model with hemorrhagic shock and abdominal sepsis. Experimental models of sepsis and hemorrhagic shock has often been employed in other estudies[30-32].

From the molecular point of view, coconut water demonstrated to have a downregulating effect in the inflammatory response of TNF-α and IL-1β in serum. This is particularly important since there are not studies so far considering the role of i.v. coconut water in the cytokine response after shock and sepsis. In this regard, this is the first time we could encounter such a direct association with i.v. coconut water treatment of shock+ sepsis and lower TNF-α, IL-1β response then in controls.

TNF-α is considered the main inflammatory agent in cases of hemorrhagic shock and plays a crucial role in the release of other pro-infamatory cytokines (IL-1β, IL-6), leading to excessive and auto-destructive inflammation[33]. Farias *et al.*[34] demonstrated a statistically significant reduction in the expression of pro-inflammatory cytokines in rats treated with modified coconut water volume replacement compared with saline and fresh blood. In the present study, we found that treatment of rats with sepsis and shock with coconut water modified with sodium 3% + simvastatin reduced serum cytokines, biochemical parameters and peripheral leukocytes. This reduction was statistically higher than the other fluids replacement treatments.

Simvastatin has shown immunomodulatory effects, independent of significant reduction in hyperlipidemia[35]. These pleiotropic effects include anti-inflammatory action[36], improvement in endothelial and microvascular function, ischemia/reperfusion[37] and sepsis[38]. For all these properties, simvastatin acted synergistically with CW to improve the parameters studied in rats with shock and sepsis. In this study we used simvastatin microemulsion intravenously, based on the fact that this presentation developed in-UFRN Dispersed Systems Laboratory, has particles in nanoscale, with potential i.v. use without adverse effects. The results obtained in this study with the CW infusion

are probably related to their physicochemical properties. A few studies have shown scientific evidence of coconut water use in medical practice, justified by its content of sugars, vitamins, minerals, amino acids, organic acids and fatty acids essenciais[39]. In addition, CW has significant amount of antioxidants that protect the body against oxidative stress caused by hypoxia inherent to hemorrhagic shock[16].

The indocyanine green (ICG) has been extensively used for decades in the fields of ophthalmology for retinal imaging and cardiology to study cardiac output. ICG has a well documented safety profile[40,41]. We used a higher dose for rodents as described for patients in order to compensate the short blood half life of ICG in rodents (1.5 - 2.3 min) as opposed to patients (3 - 4 min)[42,43]. The relatively high ICG dose provided a prolonged contrast enhancement and allowed sequential imaging of lungs without additional contrast injection.

Our results showed that inflamed and normal lung tissues differed significantly in normalized fluorescence. On the basis of the different temporal behavior of fluorescence intensity in septic rats, it is more likely that vasodilation in the inflamed lungs was present and new microvessels were formed, leading to a faster inflow of ICG contrast agent. Fluorescence imaging is limited to superficial organs and tissues, because the strong scattering and absorption of light in biologic tissue restricts the penetration depth of photons to a few millimeters. As fluorescence in vivo was not detectable with our device in lungs of rats *in vivo*, we decided to perform *ex vivo* imaging, and representative images were recorded in inflamed lungs. A way to further improve the specificity of this method is to use disease-specific contrast agents in combination of a marker for neovascularization.

Conclusions

The volume replacement with coconut water modified with Na 3%, combined with simvastatin had a positive influence in the treatment of rats with sepsis + hemorrhagic shock model. The findings may have significant therapeutically implications in the clinical setting.

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