

Original Article

Hypocalcemia in sepsis: analysis of the subcellular distribution of Ca²⁺ in septic rats and LPS/TNF- α -treated HUVECsWencheng He¹, Lei Huang¹, Hua Luo¹, Yang Zang¹, Youzhong An², Weixing Zhang¹¹ Department of Intensive Care Unit, Peking University Shenzhen Hospital, Shenzhen Peking University - The Hong Kong University of Science and Technology Medical Center, No.1120, Lianhua Road, Futian District, Shenzhen, 518000, China² Department of Intensive Care Unit, Peking University People's Hospital, Beijing, 100044, China**Abstract**

Introduction: Hypocalcemia has been widely recognized in sepsis patients. However, the cause of hypocalcemia in sepsis is still not clear, and little is known about the subcellular distribution of Ca²⁺ in tissues during sepsis.

Methodology: We measured the dynamic change in Ca²⁺ levels in body fluid and subcellular compartments, including the cytosol, endoplasmic reticulum and mitochondria, in major organs of cecal ligation and puncture (CLP)-operated rats, as well as the subcellular Ca²⁺ flux in HUVECs which treated by endotoxin and cytokines.

Results: In the model of CLP-induced sepsis, the blood and urinary Ca²⁺ concentrations decreased rapidly, while the Ca²⁺ concentration in ascites fluid increased. The Ca²⁺ concentrations in the cytosol, ER, and mitochondria were elevated nearly synchronously in major organs in our sepsis model. Moreover, the calcium overload in CLP-operated rats treated with calcium supplementation was more severe than that in the non-calcium-supplemented rats but was alleviated by treatment with the calcium channel blocker verapamil. Similar subcellular Ca²⁺ flux was found *in vitro* in HUVECs and was triggered by lipopolysaccharide (LPS)/TNF- α .

Conclusions: Ca²⁺ influx from the blood into the intercellular space and Ca²⁺ release into ascites fluid may cause hypocalcemia in sepsis and that this process may be due to the synergistic effect of endotoxin and cytokines.

Key words: sepsis; hypocalcemia; calcium overload; subcellular redistribution; mechanism.

J Infect Dev Ctries 2020; 14(8):908-917. doi:10.3855/jidc.12341

(Received 25 December 2019 – Accepted 14 May 2020)

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Introduction

Sepsis is a life-threatening disease of organ dysfunction in patients hospitalized in intensive care units (ICUs). Despite advances in the understanding of sepsis pathophysiology and the introduction of novel therapeutic strategies, the in-hospital mortality of sepsis is still very high, which can reach up to 30% for sepsis, 50% for severe sepsis and 80% for septic shock [1]. To date, no definite clinical therapy can reverse the dysregulation of host responses during sepsis, and the primary principles of sepsis management remain early identification, early antimicrobial therapy, and early resuscitation [2-4]. Therefore, the mechanisms underlying the dysregulated host responses during sepsis need further study.

Ca²⁺ acts as a ubiquitous intracellular messenger and coenzyme in the body. Physiological functions and cellular activities, such as muscle contraction, neuronal conduction, cardiovascular function, intestinal motility, enzyme activity, blood clotting, and hormone secretion, rely on normal Ca²⁺ concentrations [5,6]. Without

adequate calcium stores, those physiological processes cannot function properly; with particular Ca²⁺ aberrations, serious clinical consequences such as cardiovascular complications and organ dysfunction can occur [7,8].

Considerable evidence has demonstrated that hypocalcemia is a common calcium disturbance in sepsis patients [9-12]. Normally, the blood Ca²⁺ concentration is tightly regulated by calcium-related hormones such as parathyroid hormone (PTH), 1,25-dihydroxyvitamin D₃, the dynamic change of the PTH and 1,25-dihydroxyvitamin D₃ during sepsis has been investigated in several studies [13-15]. However, no generally acknowledged conclusion has been established, as those studies showed paradoxical results. In contrast to the decrease in blood calcium, many studies have demonstrated that Ca²⁺ overload occurs in various organs during sepsis [16-20], implying that Ca²⁺ influx from the extracellular space may cause hypocalcemia in sepsis. However, an increase in efflux from cellular calcium stores, such as

those associated with the endoplasmic reticulum (ER)/sarcoplasmic reticulum (SR), can also lead to Ca^{2+} overload. To date, the Ca^{2+} flux among subcellular compartments under septic conditions has not been well characterized. As Ca^{2+} is the most important coenzyme and second messenger, increased intracellular Ca^{2+} levels have been suggested to contribute to cellular injury in many diseases [8]. Thus, recognizing the subcellular redistribution of Ca^{2+} and its potential pathogenic effects not only will clarify the cause and consequence of Ca^{2+} dysregulation during sepsis but also may provide a new therapeutic target for correcting Ca^{2+} dysregulation-related dysfunctions in sepsis. Here we examined the dynamic change in Ca^{2+} levels in body fluid and subcellular compartments in major organs of CLP-operated rats, as well as the subcellular Ca^{2+} flux in HUVECs which treated by endotoxin and cytokines, hope to provide a deeper understanding of the Ca^{2+} abnormal in sepsis.

Methodology

Cecal Ligation and Puncture (CLP) model

Specific Pathogen-Free (SPF) male Sprague-Dawley (SD) rats weighing 200-250 g were randomly separated into four groups: the sepsis group, in which sepsis was established by CLP; the corresponding control group, which received sham laparotomy; the calcium supplement group, which received an injection of 2% CaCl_2 (MP Biomedicals, Burlingame, USA; #MR29229) at 40 mg/kg 4 hours after CLP operation; and the Ca^{2+} channel blocker group, which was treated with verapamil (KL Science, Shanghai, China; #KL18S1109) soon after the CLP operation. All groups received a subcutaneous injection of 4 mL of normal saline for resuscitation soon after the surgery. The CLP-operated rats exhibited lethargy, diarrhea, abdominal swelling, periorbital and perinasal exudates and a mortality rate of 60-80%, while the sham-operated control rats appeared normal and active. The rats were then sacrificed with an overdose of barbital sodium at 6 hours, 12 hours and 24 hours after CLP operation. The blood, urine, ascites fluid, feces, and organs, including the heart, liver, spleen, lungs, kidneys, and brain, as well as peripheral blood mononuclear cells (PBMCs), were sampled for the following experiments. The protocol for the study was approved by the ethics committee of Peking university Shenzhen hospital.

Cell culture and lipopolysaccharide (LPS)/cytokines treatment

Human Umbilical Vein Endothelial Cells (HUVECs; ScienCell, Carlsbad, USA) were cultured in

endothelial cell medium (ECM; ScienCell, Carlsbad, USA) supplemented with 10% fetal bovine serum (ScienCell, Carlsbad, USA), 1% endothelial cell growth supplement (ECGS, ScienCell, Carlsbad, USA), and 1% penicillin-streptomycin (Gibco, New York, USA). Cells were cultured in incubators maintained at 37°C in a humidified environment with 5% CO_2 . Cells were harvested at 80-90% confluency and treated with 10 $\mu\text{g}/\text{mL}$ LPS (Sigma, Burlington, USA), 10 ng/mL human TNF- α (PeproTech, Rocky Hill, USA) and 10 ng/mL interleukin (IL)-6 (PeproTech, Rocky Hill) alone or in combination. Cells were harvested at 3, 6 and 12 hours after treatment initiation.

Measurement of Ca^{2+} levels in body fluid

Blood samples were collected at 6 hours, 12 hours and 24 hours after CLP operation, and serum, urine, and ascites fluid samples were collected at 12 hours and 24 hours after CLP operation. The Ca^{2+} concentration in those samples was determined by the colorimetric determination method with o-cresolphthalein complexone [21]. Samples were assayed in triplicate, and the experiments were conducted according to the protocol of the Quantichrom™ calcium assay kit (BioAssay Systems, Hayward, USA).

Detection of Ca^{2+} in intracellular compartments and flow cytometry assay

We used acetoxymethyl (AM) ester forms of the following organic Ca^{2+} -dyes: Fluo-8 AM (AAT Bioquest; Sunnyvale, USA) for cytosolic Ca^{2+} detection, Rhod-2 AM (AAT Bioquest, Sunnyvale, USA) for mitochondrial Ca^{2+} detection, and Mag-Fluo-4 AM (AAT Bioquest, Sunnyvale, USA) for ER Ca^{2+} detection. All dyes were reconstituted with dimethyl sulfoxide (DMSO) (MP Biomedicals, Burlingame, USA) to a stock concentration of 2.5-5.0 mM and were stored in the dark at -20°C. The stored solutions were diluted with Hanks' buffer with 20 mM HEPES (Thermo Fisher, Massachusetts, USA) (HHBS) before use. To improve the discrimination between cytosolic and mitochondrially localized dye, a small excess of NaBH_4 (Nantong Hongzhi Chemical Co., Ltd. NanTong, China) was added to the Rhod2 AM stock solution to convert Rhod2 AM to dihydroRhod-2 AM before dilution. To prevent the export of the fluorescent indicators by transporters located in cell membranes, 2.5 mM probenecid (AAT Bioquest, Sunnyvale, USA) was added as an inhibitor of organic anion transporters. To identify the target specificity of Mag-Fluo4 AM and Rhod2 AM, ER and mitochondrial tracker dyes were costained with Mag-Fluo4 AM and Rhod2 AM,

respectively, and were then observed under a fluorescence microscope. Single sections of the samples mentioned above were prepared by gentle grinding and were then exposed to the fluorescent Ca²⁺ indicators Fluo-8 AM (5 μM), Mag-Fluo4 AM (2.5 μM) and Rhod2 AM (2.5 μM) sequentially at room temperature for 30 minutes. After incubation, the cells were rinsed three times with HHBS to remove surplus dye. Finally, the resuspended cells were filtered through 300 mesh nickel grids and loaded in a flow cytometer (BD, San Jose, USA) for Ca²⁺ analysis. Fluorescence images of samples labeled with Fluo8-AM and Mag-Fluo-4 AM were acquired at an excitation wavelength of 488 nm, while images of samples labeled with Rhod2 AM were collected at an excitation wavelength of 534 nm. The same illumination parameters were digitally maintained for consistency throughout the experiments.

Statistical analysis

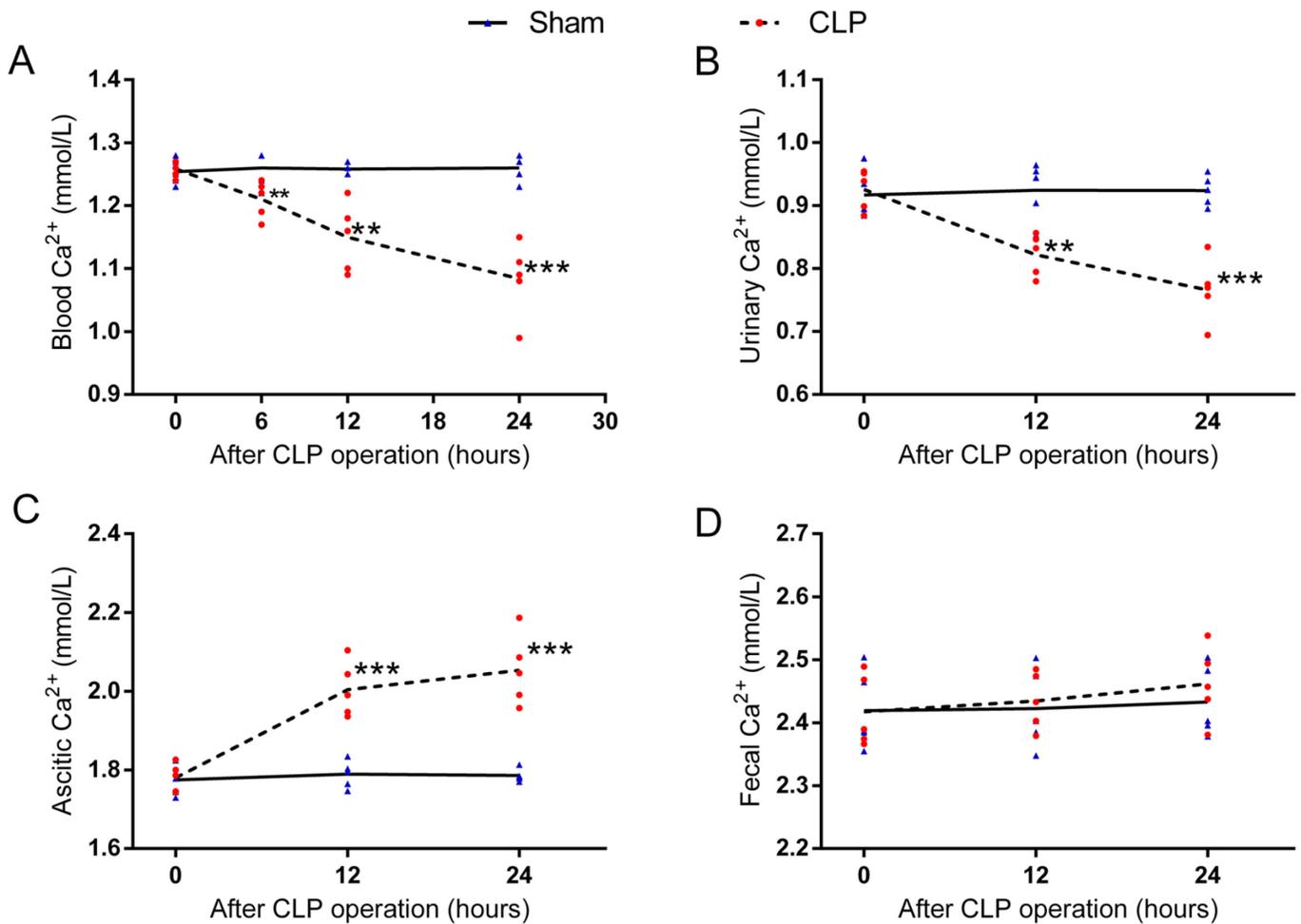
The results are expressed as the means ± standard error of mean (SEMs). Statistical analysis of the data was performed using Student’s t test or Mann-Whitney U test in IBM SPSS 22. A value for p < 0.05 was considered to indicate a statistically significant difference.

Results

The dynamic of Ca²⁺ in CLP-operated rats

To investigate the change in Ca²⁺ levels in body fluids and feces during the sepsis process, we examined the Ca²⁺ levels in blood, ascites fluid, urine and feces at specific time intervals after CLP operation. A decrease in the blood Ca²⁺ concentration from 1.258 ± 0.006 mmol/L to 1.210 ± 0.013 mmol/L was found at 6 hours after operation initiation, decreasing below the normal threshold and reaching 1.084 ± 0.026 mmol/L at 24 hours post CLP operation (Figure 1A). The urinary Ca²⁺

Figure 1. The dynamic change in the Ca²⁺ concentration in body fluids after CLP operation.



(A) The blood Ca²⁺ level decreased after CLP operation. (B) The urinary Ca²⁺ level decreased after CLP operation. (C) The ascites fluid Ca²⁺ level increased after CLP operation. (D) The Ca²⁺ level in feces increased slightly after CLP operation. The data for each group were obtained from five independent experiments (**p < 0.01; ***p < 0.001).

concentration showed a trend similar to the blood Ca^{2+} concentration, decreasing from 0.925 ± 0.014 mmol/L to 0.766 ± 0.022 mmol/L (Figure 1B). In contrast, along with an increase in ascites fluid volume in the sepsis group, the Ca^{2+} concentration in ascites was also markedly enhanced, increasing from 1.780 ± 0.016 mmol/L at baseline to 2.096 ± 0.039 mmol/L 24 hours after the CLP operation (Figure 1C). Compared with the level in the sham group, the Ca^{2+} level in the feces was slightly but not significantly higher at the end of the observation period compared with that in the sham group (Figure 1D). The Ca^{2+} concentration in the corresponding samples in the sham group remained within the normal range during the course of the experiment.

Subcellular Ca^{2+} is dysregulated in major organs of CLP-operated rats

To examine the Ca^{2+} flux in subcellular compartments during sepsis, we first examined organelle-specific localization by subjecting HUVECs stained with Mag-Fluo-4 AM and Rhod-2 AM to co-stain with ER and mitochondrial tracker dyes, respectively, and fluorescence microscopy. Mag-Fluo-4 AM and Rhod-2 AM specifically tracked Ca^{2+} in the ER and mitochondria, respectively (Figure 2A). Flow cytometry assays were then performed. Overall, calcium overload was observed in all examined tissues. Tissues with an increased cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) generally exhibited an increased ER Ca^{2+} concentration ($[\text{Ca}^{2+}]_{er}$) and mitochondrial Ca^{2+} concentration ($[\text{Ca}^{2+}]_{mito}$), which started at 6-12 hours and peaked or was maintained at the end of our observation period (24 hours). Among those tissues, the brain, liver and spleen were the most affected, as the intercellular Ca^{2+} fluorescence intensity was more than two times higher than that in the sham group. Other tissues exhibited less than a twofold increase, but the intercellular Ca^{2+} fluorescence intensity remained significantly elevated compared with that in the corresponding tissues from sham group rats, suggesting that calcium overload is a common pathological feature of sepsis.

CLP operation-mediated calcium overload is associated with blood Ca^{2+} influx and can be alleviated by L-type calcium blockers

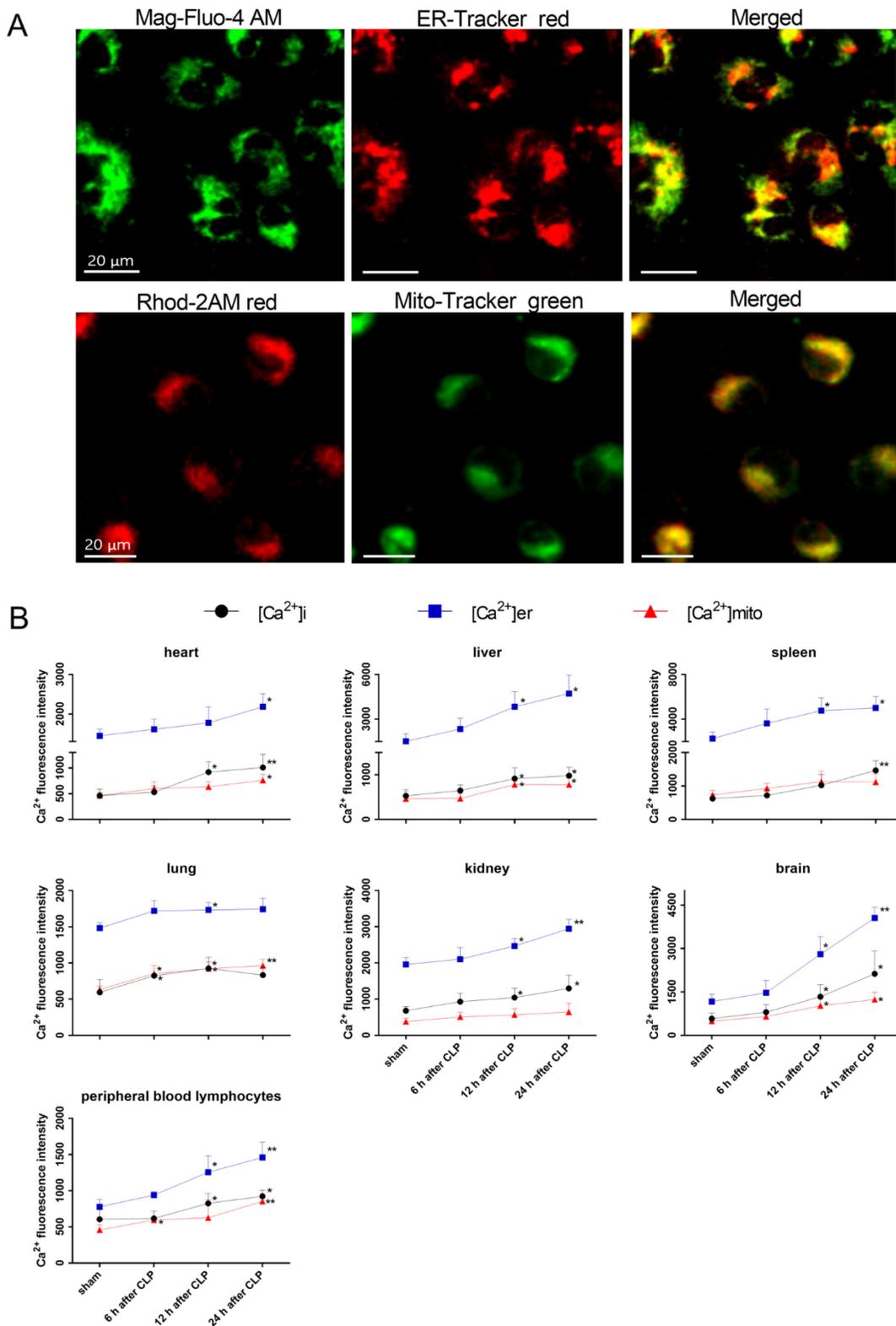
To further investigate whether calcium overload is caused by calcium influx from blood, the blood calcium level was increased by injection with the calcium supplement CaCl_2 4 hours after CLP operation. The dynamic changes in the blood calcium concentration

and the subcellular redistribution of Ca^{2+} in the heart, spleen, liver and brain were examined at specific time intervals post CLP operation. The blood Ca^{2+} level increased from 1.25 ± 0.01 mmol/L at baseline to 1.27 ± 0.01 mmol/L soon after CaCl_2 treatment; however, it dropped to 1.18 ± 0.015 mmol/L rapidly at the end of the observation period (18 hours) (Figure 3A). On the other hand, the intracellular Ca^{2+} concentration in the calcium supplement group was increased by $156.33 \pm 11.94\%$ in the heart, $161.90 \pm 10.15\%$ in the spleen, $155.88 \pm 10.64\%$ in the liver and $207.80 \pm 19.63\%$ in the brain compared with the corresponding concentrations in the sham group, and these increases were larger than those in the CLP-operated group, which exhibited increases of $58.67 \pm 14.65\%$ in the heart, $78.33 \pm 4.23\%$ in the spleen, $82.24 \pm 3.92\%$ in the liver, and $111.40 \pm 4.84\%$ in the brain compared with the corresponding concentrations in the sham group. However, the calcium disturbances in the blood and intercellular region induced by CLP operation were attenuated by treatment with the L-type Ca^{2+} channel blocker, verapamil (Figure 3A, B); while those treatments had no significant effect on the corresponding sham groups. This result suggests that the extent of calcium overload is associated with Ca^{2+} influx from the blood and that this Ca^{2+} influx process may occur, at least in part, via L-type Ca^{2+} channels.

LPS/TNF- α synergistically increases the intercellular Ca^{2+} level in HUVECs

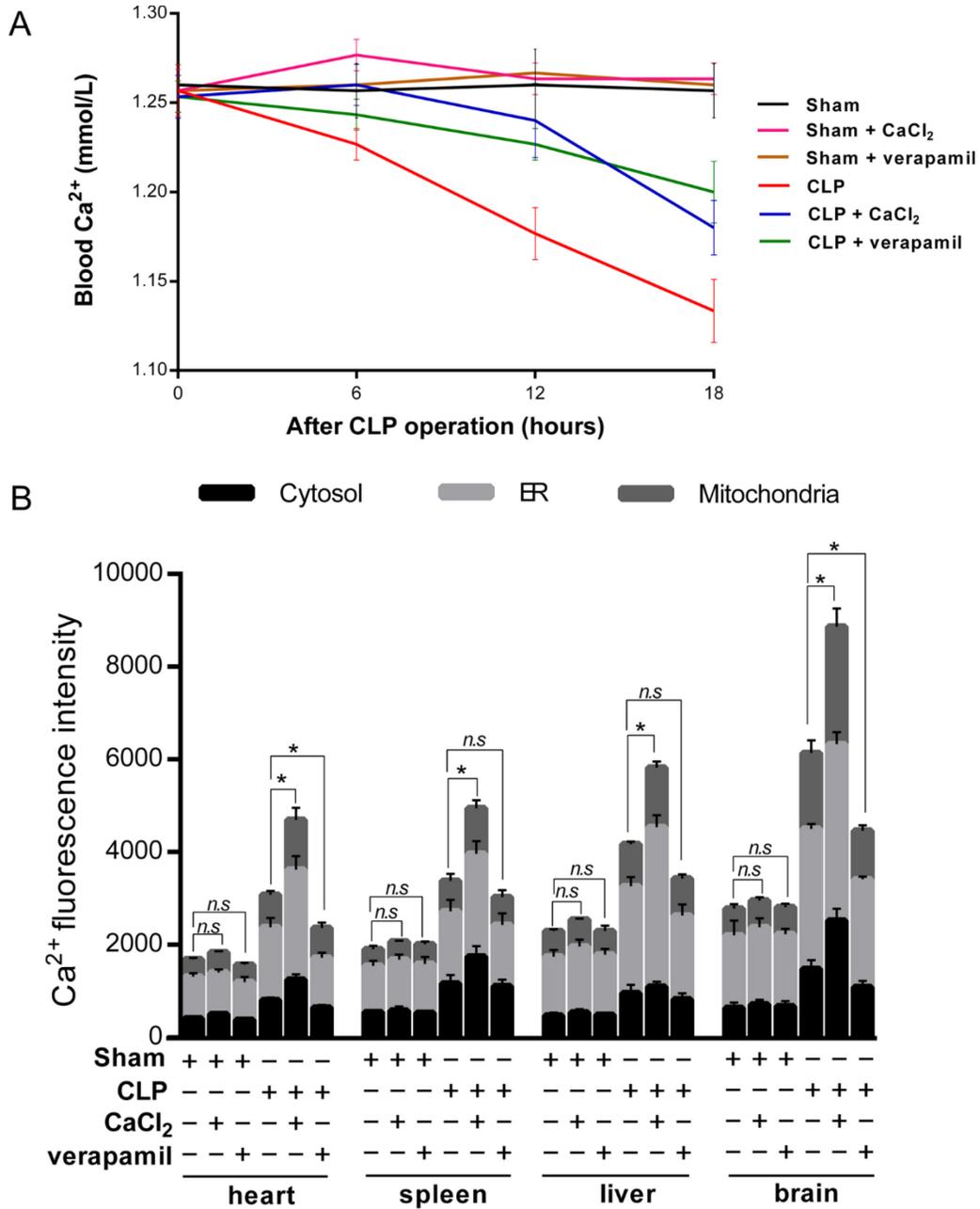
To gain further insight into the potential pathogenic factors involved in calcium influx during sepsis, the redistribution of subcellular Ca^{2+} in HUVECs was examined by treatment with endotoxin and cytokines. Cells were exposed to 10 $\mu\text{g}/\text{mL}$ LPS, 10 ng/mL TNF- α and 10 ng/mL IL-6 alone or in the following combinations: LPS/TNF- α and LPS/IL-6. After exposure to these stimuli, an increase in the Ca^{2+} concentration was found in the cytosol, ER and mitochondria in both the LPS and TNF- α treatment groups but not in the IL-6 treatment group. The increase in $[\text{Ca}^{2+}]_i$ peaked at 3-6 h after exposure to LPS and TNF- α . The concentration then dropped slightly at the end of the observation period but remained higher than that in the control group (Figure 4A). In particular, the increase in the intercellular Ca^{2+} concentration induced by LPS/TNF- α combination treatment was higher than that induced by either LPS or TNF- α treatment alone (Figure 4B). These results suggest that LPS and TNF- α are pathogenic factors that synergistically induce calcium influx into HUVECs.

Figure 2. The subcellular redistribution of Ca²⁺ in various tissues of CLP-operated rats.



(A) Mag-Fluo4 AM and Rhod2 AM costaining with ER-tracker and Mito-tracker, ER and mitochondrial tracker dyes. The [Ca²⁺]_i, [Ca²⁺]_{er} and [Ca²⁺]_{mito} rose synchronously in major tissues of CLP-operated rats. The data for each group are the mean ± SEM of three independent experiments (*p < 0.05; **p < 0.01).

Figure 3. The extent of calcium overload in CLP-operated rats is associated with Ca²⁺ influx from the blood.



(A) The dynamic change in the blood Ca²⁺ concentration in CLP-operated rats after CaCl₂ and verapamil treatment. (B) Verapamil treatment attenuated the calcium overload, while CaCl₂ supplementation aggravated the calcium overload in CLP-operated rats. The data for each group are the mean ± SEM of three independent experiments (*p < 0.05; **p < 0.01; n.s. not significant).

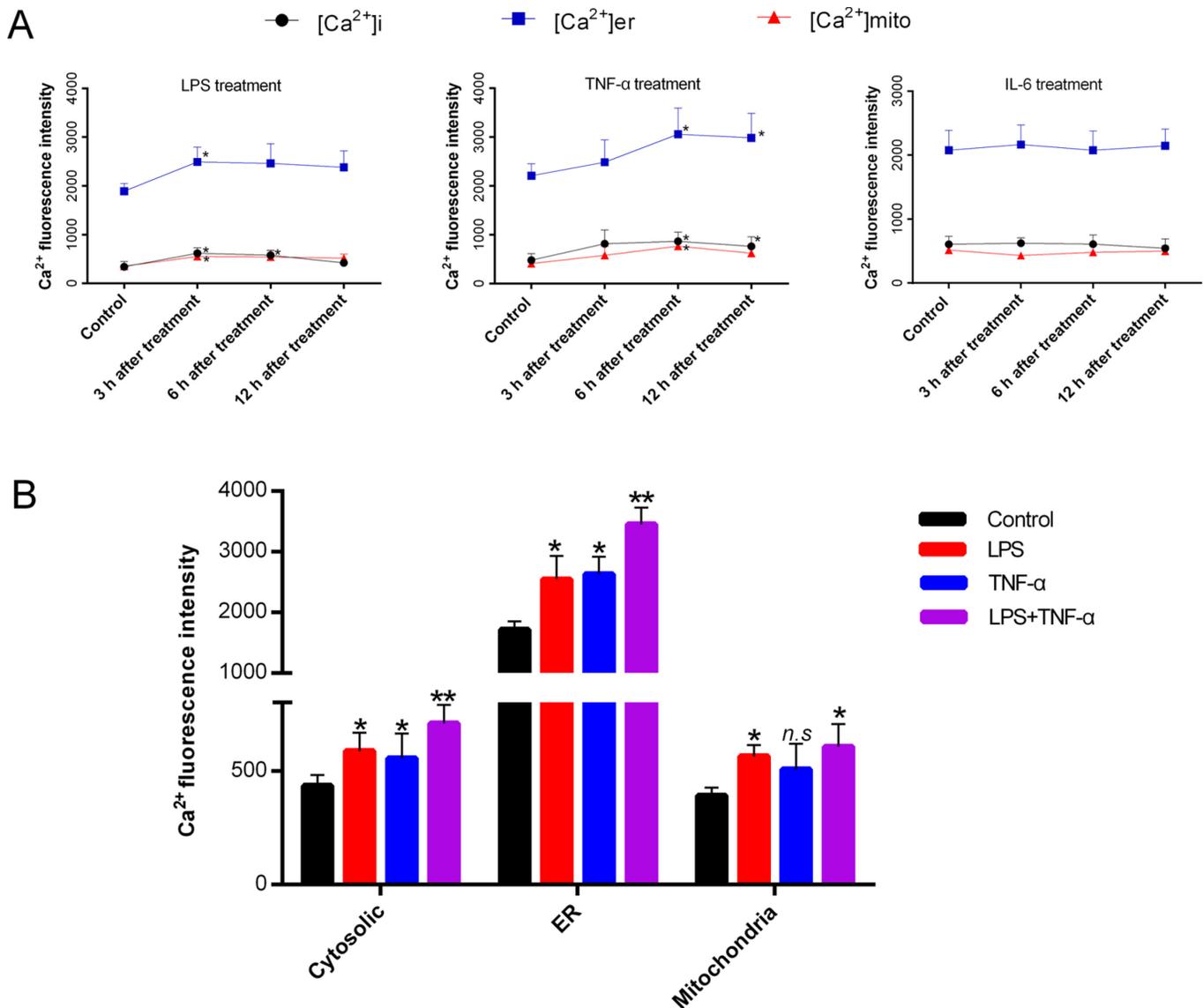
Discussion

Sepsis patients are at particular risk of hypocalcemia [22]. Here, we examined the subcellular Ca²⁺ redistribution in major organs during septic injury in CLP-operated rats as well as in HUVECs treated with endotoxin and cytokines. This study is the first to detail the intercellular calcium flux in major organs in sepsis, which may provide a deeper understanding of calcium metabolism dysregulation in sepsis.

Elucidating the cause and mechanism of hypocalcemia in sepsis is important because many clinical studies have shown that hypocalcemia is associated with poor outcomes in critically ill patients

[14,23-26]. Hypocalcemia in septic patients is likely not merely a blood calcium deficiency but is rather likely connected to Ca²⁺ dysregulation-related signaling events in sepsis because some studies have reported that calcium supplementation did not improve the outcomes of septic patients with hypocalcemia [27-29]. However, whether the change in Ca²⁺ in the blood is a protective mechanism of the body or simply a consequence of metabolic dysregulation remains to be established. Illustrating the direction of blood Ca²⁺ flux will help us understand the cause and consequence of hypocalcemia in sepsis. The abdomen is the second most common source of sepsis [30,31], and an increase in ascites fluid

Figure 4. LPS and TNF-α have a synergistic effect on triggering increases in the intracellular Ca²⁺ concentration in HUVECs.



(A) Effect of LPS and TNF-α treatment on the intracellular Ca²⁺ concentration in HUVECs. (B) LPS and TNF-α synergistically triggered an intracellular Ca²⁺ elevation in HUVECs. The data for each group are the mean ± SEM of three independent experiments (*p < 0.05; **p < 0.01; ^{n.s.} not significant).

is common in sepsis, particularly in patients with peritonitis, liver injury or heart failure. We examined the Ca^{2+} concentration in excretions and found that the Ca^{2+} concentration in ascites fluid was markedly increased after CLP operation. A similar result was reported by Fredrik *et al.*—the total calcium levels in ascites fluid were increased by 56% in endotoxemic pigs [32]. Although diarrhea also frequently occurs in CLP-operated rats, we did not find a significant difference in the Ca^{2+} concentration in feces between the CLP-operated and sham groups, possibly due to the short duration of the CLP-induced sepsis model. Finally, the Ca^{2+} level in the urine also dropped during the experiment, probably due to the low blood Ca^{2+} concentration. Taken together, these findings prompted us to conclude that the increase in Ca^{2+} release into ascites fluid is a reason for hypocalcemia during sepsis.

Ca^{2+} influx into cells from the blood has been suggested to contribute to hypocalcemia in sepsis, as increased intracellular Ca^{2+} concentrations have already been reported in various tissues from sepsis models. Zaloga *et al.* reported a 57% increase in $[\text{Ca}^{2+}]_i$ in lymphocytes of sepsis patients compared to those of healthy control patients or nonseptic critically ill patients ($p < 0.05$) [33]. Baldwin *et al.* reported that HEp-2 cells infected with enteropathogenic *E. coli* exhibited a 4-fold increase in intercellular Ca^{2+} [34]. Our results also showed that calcium overload is common in sepsis. However, Ca^{2+} influx is not the only cause of increased intercellular Ca^{2+} ; Ca^{2+} release from intracellular calcium stores can also lead to calcium overload. Therefore, to illustrate the Ca^{2+} flux between the cytoplasm and calcium stores, the Ca^{2+} concentrations in the ER and mitochondria were examined at the same time as cytoplasmic Ca^{2+} . We found that the Ca^{2+} concentrations in the ER and mitochondria were systemically increased in major tissues of our sepsis model following $[\text{Ca}^{2+}]_i$ elevation and that the extent of calcium overload was enhanced when the blood calcium level was increased by administration of a calcium supplement after CLP operation. However, treatment with the calcium channel blocker verapamil attenuated the calcium overload as well as the hypocalcemia, suggesting that calcium influx from the blood but not release from calcium stores contributes to calcium overload in sepsis. These findings may explain why some studies showed that calcium supplementation during sepsis worsens patient outcomes.

Calcium overload can cause serious consequence. When intercellular Ca^{2+} increases excessively, a number of Ca^{2+} -dependent enzymes, such as proteases,

endonucleases and phospholipases, are activated, ultimately leading to cellular self-degradation and death [35]. Some studies have reported that the breakdown of skeletal muscle proteins occurs during sepsis in association with the activation of calpain, a neutral protease mediated by Ca^{2+} signaling upregulation [36]. In addition, reports indicate that Ca^{2+} signaling causes phosphoinositide-3 kinase (PI-3K) pathway downregulation, which could lead to a decrease in insulin-mediated glucose uptake in skeletal myocytes [37]. Moreover, the persistent increase in intercellular Ca^{2+} is also related to organellar abnormalities. In the basal state of cells, steep Ca^{2+} concentration gradients exist across both the plasma membrane and the ER membrane, as the low permeability of the plasma membrane to Ca^{2+} and the calcium flux among the cytosol, ER, mitochondrial, and endolysosomal system compartments are interconnected and involved in regulating the global intracellular Ca^{2+} concentration [38]. Researchers have reported numerous abnormalities in calcium transport mechanisms at the plasma membrane and within the ER under septic conditions. As the main intracellular calcium store, the ER plays a vital role in maintaining calcium homeostasis in cells. However, the persistent uptake of Ca^{2+} into the ER to reduce calcium overload in the cytosol may lead to the overactivity of chaperones. As a result, many misfolded proteins accumulate in the ER and therefore trigger ER stress. The ER stress signaling pathway plays an important role in apoptosis and inflammation in sepsis [39-41], although the relationship between the Ca^{2+} imbalance and ER stress in sepsis needs further investigation. Mitochondrial Ca^{2+} disturbances were also found in major organs in our sepsis model; although whether the increase in mitochondrial Ca^{2+} was derived from the cytosol or ER is unknown, mitochondria were the first organelles shown to be capable of Ca^{2+} uptake, and calcium overload in mitochondria has been associated with the apoptotic process [42].

If calcium overload is a major factor in the pathophysiology of sepsis, then identifying the potential factors that cause Ca^{2+} influx during sepsis would provide a new therapeutic target for correcting Ca^{2+} dysregulation and improving the outcome. Under septic conditions, the levels of endotoxin or proinflammatory cytokines increase rapidly, and these factors are transported throughout the circulatory system during the acute phase of infection. These stimuli may damage membrane integrity or cause dysfunction of calcium channels, such as calcium pumps or $\text{Na}^+/\text{Ca}^{2+}$ exchangers, leading to uncontrolled

Ca²⁺ diffusion across the cellular membrane. Using HUVECs, a synergistic effect on calcium influx was observed after LPS/TNF- α combined treatment. Although IL-6 is significantly upregulated in septic patients, we did not find that it affected calcium overload in HUVECs. Considering that single-target therapies such as IL-1 receptor antagonists and anti-endotoxin antibodies fail to improve the outcome of sepsis, we speculate that the pathophysiology of sepsis is initiated by various mediators that work synergistically. Thus, further study should be performed to identify other potential factors and the underlying mechanism that cause calcium influx during sepsis.

Conclusion

The cause of hypocalcemia in sepsis is multifactorial. Ca²⁺ influx from the blood into the intercellular region and Ca²⁺ release into ascites fluid take an important role in causing calcium disorder in sepsis, this process may be due to the synergistic effect of endotoxin and cytokines. Giving calcium supplement to septic patients with hypocalcemia should be careful for its potential effect of aggravating calcium overload in tissues.

Acknowledgements

This work was supported by Sanming Project of Medicine in Shenzhen (CN).

Authors' contributions

Wencheng He performed the experiments and wrote the manuscript; Wencheng He, Lei Huang and Hua Luo performed the analysis; Yang Zang assisted with animal experiment; Youzhong An and Weixing Zhang designed experiments; All authors read and approved the final manuscript.

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Conflict of interests: No conflict of interests is declared.