Effects of Shen-Fu injection on mitochondrial function in the intestinal epithelial cells of rats with endotoxemia

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Abstract: Recently, several studies have demonstrated that reactive oxygen species are responsible for inducing multiple organ failure and septic shock. Particularly, mitochondrial dysfunction has been demonstrated in the pathogenesis of multiple organ dysfunction syndrome (MODS). In cytopathic hypoxia, impairment of mitochondrial oxidative phosphorylation decreases aerobic adenosine triphosphate (ATP) production and potentially induces MODS. Shen-Fu (SF) injections are widely used in the treatment of various diseases. SF exhibits cardiovascular protective effects. For example, it can stretch the coronary artery, stabilize blood pressure, regulate IRI, and improve the overall heart function. Clinical studies have demonstrated that SF injections have notable therapeutic effects on septic and hemorrhagic shocks. In the present study, the effects of SF injection on mitochondrial function in the intestinal epithelial cells of rats with endotoxemia were analyzed.

Keywords: Shen-Fu injection; Mitochondrial; Intestinal; Endotoxemia

INTRODUCTION

In recent years, our understanding of the pathogenesis of sepsis has considerably increased. Despite this, severe sepsis, clinically defined as sepsis accompanied with organ dysfunction (Dellinger *et al*., 2008), is associated with a high mortality rate (18–50%; Angus *et al*., 2001; van der Poll and Opal, 2008; Levy *et al*., 2010).

Sepsis is an intricate syndrome. It is caused due to an imbalance between the pro- and anti-inflammatory responses. A systemic inflammatory response is initiated following the release of bacterial lipopolysaccharides (LPSs) or other microbial substances into the lymphatic and circulatory systems. When a signaling cascade is triggered in response to sepsis, a dysregulated systemic response can result in multiple organ failure. Some studies have suggested that in sepsis patients, integrity of the gut is an important determinant of the clinical outcome (Deitch *et al*., 1991; Nieuwenhuijzen *et al*., 1996). For several decades, the focus on metabolic derangements has centered on the hypermetabolism of sepsis, with its attendant failure of adequate tissue oxygenation and alterations in glucose homeostasis. Despite advancements in ICU management, the mortality rate of patients with sepsis and MODS remains high. This may be because the fundamental pathophysiology driving these processes at the cellular and subcellular levels remains poorly understood (Crouser *et al*., 2004). The history of clinical trials in sepsis research has been a story that goes from one failure to another without any apparent loss of enthusiasm, with over 25 unsuccessful clinical trials performed using novel therapeutic agents (Deans *et al*., 2005). This may be because the therapeutic strategies potentially targeted the wrong stages of sepsis pathogenesis. Therefore, there is an urgent need for developing novel treatment strategies to combat the sepsis epidemic.

Recent investigations indicated that during the acute phase of sepsis, following injury to systemic organs, mitochondria are primarily targeted. Therefore, strategies for preventing oxidative stress-induced damage and protecting the mitochondrial membrane integrity need to be developed.

Recently, several studies aimed at targeting the inflammatory signaling pathways in sepsis models have been performed.

In the present study, we used the SF injection that is available for clinical use, has antioxidant properties, and is known to prevent organ damage in some diseases.

The components of Shen-Fu injection (SFI) are extracted from the Chinese traditional herbs. This injection is produced by Ya'an Sanjiu Pharmaceutical Co., Ltd. It is prepared using ginseng (Panax, family: Araliaceae) and aconiti (*Radix aconiti lateralis preparata* and *Aconitum carmichaeli* Debx., family: Ranunculaceae) by using multistage countercurrent extraction and macroporous resin adsorption. The main components of Shen-Fu injection are ginsenoside (0.8mg/mL) and aconitine (0.1 mg/mL). The final product undergoes strict quality control as per the regulations of the China Ministry of Public Health. Thus, performance of the Shen-Fu injection is observed to be relatively stable. In the present study, we analyzed the complex changes occurring in mitochondria present in the intestinal epithelial cells following endotoxemia.

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Further, we evaluated the effects of Shen-Fu injection on the recovery of mitochondrial function in intestinal epithelial cells of endotoxemic rats.

MATERIALS AND METHODS

Animal experiments

Adult male Sprague-Dawley rats (SD; 220-240g) were procured. All experiments were approved by the Xuzhou College Institutional Laboratory Animal Care and Use Committee [license number: SYXK (SU) 2007-0037] in accordance with the National Institutes of Health guidelines. The animals were caged in groups of five and had free access to food and water. They were maintained under a 12-h light-dark cycle (lights on at 7:00 am) at 25 °C. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Central Hospital of Xuzhou (Permit Number: 20060828001). Briefly, 96 healthy adult male SD rats were randomly divided into the following groups: untreated control group (Con, n=8), positive-control group (Mod), and therapeutic group (SF). The Con, Mod, and SF groups were further divided into four subgroups: 1, 3, 6 and 12 h (n=8). For inducing anesthesia, chloral hydrate (350mg/kg) was injected intraabdominally at 1-h intervals. The sinistro femoral arteries of rats were cannulated. Systemic arterial pressure and heart rate were recorded continuously. Endotoxemia was induced by slow caudal vein infusion of LPS. In the Mod group, rats were administered an intravenous injection of NS (20mL/kg), followed by LPS. The SF group received SF (20mL/kg), immediately followed by an intravenous infusion of LPS (5mg/kg; obtained from *Escherichia coli* 055:B5; Sigma). LPS was dissolved in buffered isotonic saline at a concentration of 5mg/mL. The dose of LPS and duration of treatment after LPS administration were chosen as per the previously published reports (Galley *et al*., 1997; Svistunenko *et al*., 2006; Levy, 2007). The small intestine samples were collected at each time point after inducing anesthesia and subsequently used for isolating mitochondria. The expression of cytochrome c in mitochondria isolated from intestinal epithelial cells was measured using western blot analysis. Membrane potential were measured using flow cytometry. The ultra microstructure of mitochondria was also observed using electron microscopy.

Measurement of TNF-α and IL-10

Samples stored at-20°C were thawed before use. Serum TNF-α and IL-10 levels were measured using the Rat TNF-α and IL-10 ELISA kit (R&D Systems), according to the manufacturer's instructions. After analyzing the regression equation from standard curve, the samples were diluted 10-fold.

Electron microscopy

At 1, 3, 6, and 12-h post treatment, small intestine samples were obtained and processed for ultrastructure evaluation using electron microscopy. Briefly, tissue slices were rinsed in 0.1M phosphate buffer (pH 7.3). Next, the tissue was fixed using 4% glutaraldehyde and 1% para formaldehyde in 0.1M phosphate buffer (pH 7.3) for 2h at 4°C. The samples were rinsed in phosphate buffer (3 times, 15min each). They were dehydrated using increasing concentrations of acetone (25, 50, 75, 95%) for 10min, each followed by 3 changes of 100% acetone for 20min each. Next, acetone was removed and samples were rinsed with propylene oxide (PO; 2 times, 5min each). The samples were infiltrated using graded PO:EPON mixtures (1:1 and 1:3; 2h each). Finally, the samples were stored overnight in 100% EPON. The tissue slices were flat-embedded into EPON between transparent foils for 24h and subjected to polymerization at 37, 45, and 60°C for 24h each. Finally, ultra thin sections of 60-80 nm were mounted on uncoated copper grids and stained using aqueous solutions of uranyl acetate and lead citrate. The tissue sections were examined using the H-600 Electron Microscope at 80 kV.

Western blotting

After inducing anesthesia, intestinal mucosa was isolated from rats at the aforementioned time intervals. Tissue (5g) was immediately cut into fine pieces and washed thrice to remove blood. Next, 20mL ice-cold isolation buffer [sucrose (0.25mol/L), Na_2EDTA (0.1mmol/L), Tris (0.01 mol/L); pH7.6) was added to the tissue sample. The tissue was homogenized and the mixture was centrifuged at 1,600×*g* for 12min at 4°C. The supernatant was removed and centrifuged at 25,000×*g* for 15min at 4°C twice. The supernatant and pellet were collected separately. To the pellet, 1mL isolation buffer was added. Protein concentration of the supernatant was measured using the Lowry method. Total protein (40g) was resolved using 15% polyacrylamide gel containing 0.1% SDS. Next, the proteins were electro blotted onto PVDF membranes (Bio-Rad) and the membranes were blocked in TBS-T supplemented with 5% nonfat dry milk for 1h at room temperature. After blocking, the membranes were treated with the rabbit polyclonal cytochrome c antibody (1:1000; Santa Cruz Biotechnology) for 12h at 4°C. The membranes were washed in TBS-T once for 15min, followed by 3 times for 5min each. They were incubated with the polyclonal anti-rabbit IgG horseradish per oxidase-conjugated antibody (1:1,000; Amersham Biosciences Corp, Piscataway, NJ) for 1h at room temperature. Subsequently, they were washed and exposed to a mixture of Luminol containing hydrogen peroxide under alkaline conditions (Super Signal West Pico, Pierce-Endogen) for 5min. Chemiluminescence was measured using the Kodak X-OMAT AR film (Eastman Kodak). All experiments were repeated three times.

Flow cytometry

Mitochondrial membrane potential was analyzed using the fluorescent probe, rhodamine-123 (Rho-123; Sigma),

in intact cells. Briefly, after treatment, mitochondria were washed twice with PBS and incubated with Rho-123 (10 μg/mL; diluted in PBS) at 37°C for 30 min in the dark. Following incubation, cells were washed twice with PBS and the red fluorescence was immediately measured using the BD FACSCalibur cell analyzer (excitation, 480nm; emission, 525nm) at 37°C.

STATISTICAL ANALYSIS

Statistical analysis was performed using the SPSS 16.0 software. Values represent the mean ±SD. One-way analysis of variance (ANOVA) with repeated measures was used to determine the differences between groups at respective time intervals. A two-sided *P* value ≤0.05 was considered to be statistically significant.

RESULTS

After LPS administration, compared to that of the Con group, the heart rate of rats in the Mod group was significantly higher at 1-3 h (*P*< 0.01, fig. 1); however, it was considerably lower at 6-12h (*P*< 0.01, fig. 1). In contrast, there were manifestly raised in heart rate at any time after SF group vs Con group (*P*<0.01, fig. 1), there were noticeably increased both in Mod group and SF group vs Con group at 30mins and peaking at 30mins (*P*<0.01, fig. 1). Compared to that of the Con group, the mean arterial pressure of both the Mod and SF group rats was significantly lower at 30min (*P*<0.01, fig. 1). However, the mean arterial pressure was significantly higher in the SF group than in the Mod group at 3-12h (*P*<0.01, fig. 1). In the Mod group, the mean arterial pressure increased at 0.5-1h, and subsequently decreased after 1h (fig. 1). In contrast, in the SF and Con groups, no significant differences in the mean arterial pressure at 1-12h were observed (*P*>0.05, fig. 1)

Inflammatory cytokines

As shown in fig. 2, compared to the Con group, the Mod group rats secreted significantly more amounts of pro- and anti-inflammatory cytokines (TNF-α and IL-10; *P*<0.01; fig. 2). In contrast, there was obvious diminution in SF group. The plasma TNF-α level peaked at 3h and gradually decreased by 12h; however, the level was always higher than that observed for the Con group (*P*< 0.01, fig. 2). IL-10 levels were slightly higher in the SF group than in the Con group (*P*>0.05, fig. 2) at 1h. Although IL-10 level peaked at 6h and gradually decreased by 12h, the level was always higher than that observed for the Con group (*P*<0.01, fig. 2). Thus, the SF injection inhibited pro- and anti-inflammatory cytokines (TNF-α and IL-10).

Electron photomicrographs of intestinal mitochondria

Mitochondrial swelling is a normal feature of respiring mitochondria caused by the movement of solutes across the inner mitochondrial membrane during oxidative phosphorylation. We observed the changes in mitochondria after injecting LPS. Representative electron photomicrographs of mitochondria isolated from intestinal epithelial cells have been shown, where A indicates the Con group, B-E represent the Mod group after 1, 3, 6, 12h, and F-G represent the SF group after 1, 3, 6, 12h. Notably, after 1h, compared to the time-matched controls, significant mitochondrial injury was observed in the intestinal tissues of the Mod group rats (fig. 3B). LPS induced high-amplitude mitochondrial swelling, often accompanied by the disruption of membrane integrity (fig. 3C), and necrotic (rather than apoptotic) epithelial cell injury. Thus, at 2h post treatment, LPS treatment caused time-dependent epithelial necrosis as observed in the mitochondrial ultrastructure.

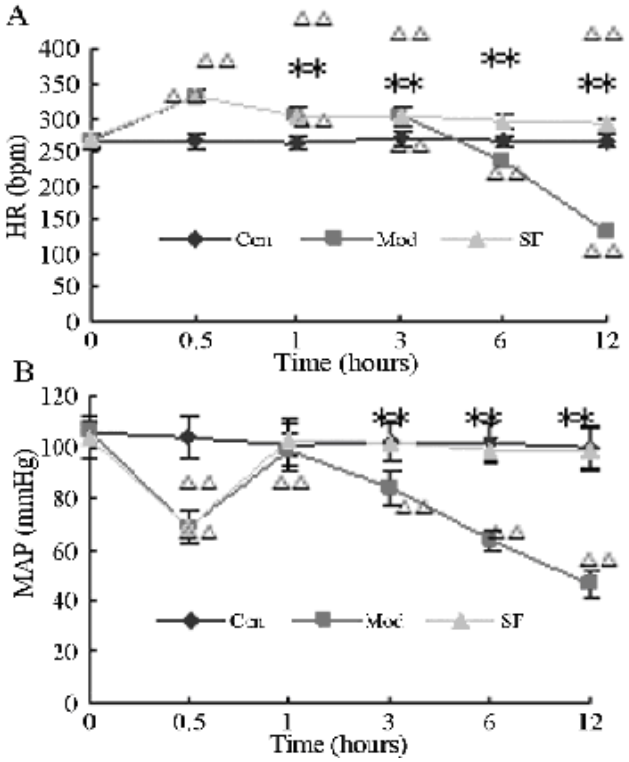
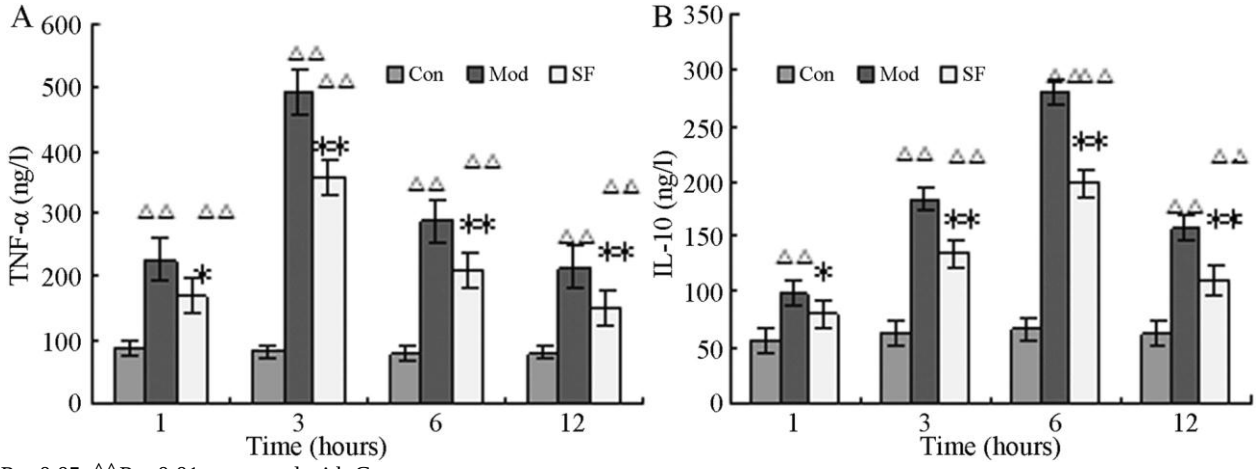


Fig. 1: Heart Rate and Mean Arterial Pressure
$^{>}P<0.05$, $^{>>}P<0.01$ compared with Con group;
* *P*<0.05, ***P*<0.01 compared with Mod group

Cytochrome c

Cytochrome c was the first mitochondrial protein observed to be involved in regulating apoptosis. We measured the differences between the concentration of cytochrome c in the mitochondrial intermembrane space and that in the cytosol as a marker for the endogenous pathway of apoptosis-translocation. Compared to the Con group, rats in the Mod group showed significantly higher cytosolic cytochrome c level at 1h; however, rats in the SF group showed no similar increase. As time increased (1, 3, 6, 12h), the cytosolic cytochrome c levels in the



$^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ compared with Con group;
* $P < 0.05$, **$P < 0.01$ compared with Mod group

Fig. 2: TNF-α and IL-10

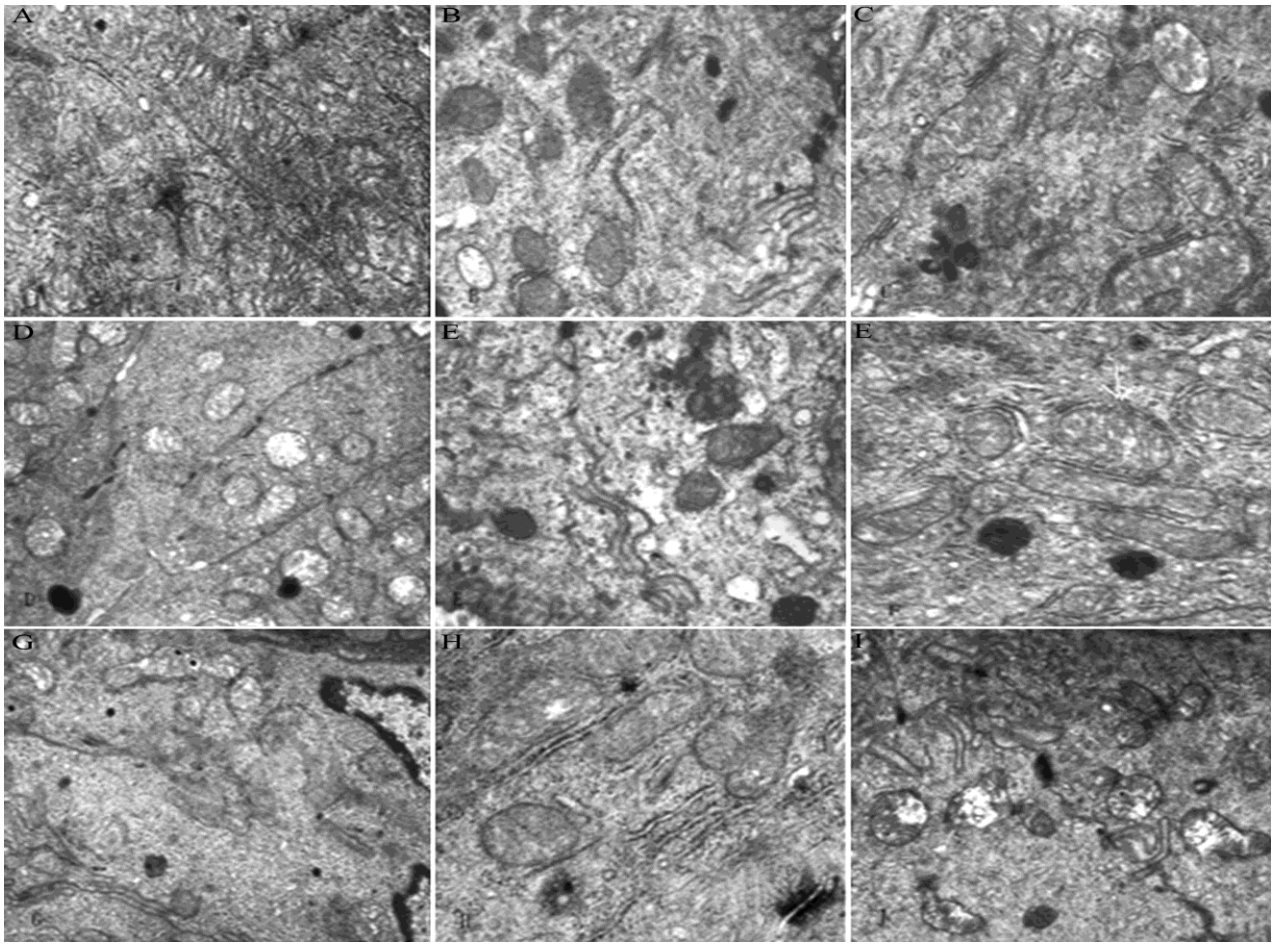


Fig. 3: Representative electron photomicrographs of intestinal mitochondria from A: Con group, B to E: Mod group 1, 3, 6, 12h and F to G: SF group 1, 3, 6, 12h. Namely, significant mitochondrial ultra structural injury was observed in the intestinal tissues of Mod group animals at 1 hrs (fig. 3B) relative to time-matched controls. LPS induced high-amplitude mitochondrial swelling, often accompanied by the disruption of membrane integrity (fig. 3C), necrotic, rather than apoptotic, epithelial cell injury, LPS treatment caused time-dependent epithelial necrosis by 2h post-treatment as evidenced by mitochondrial ultra structural.

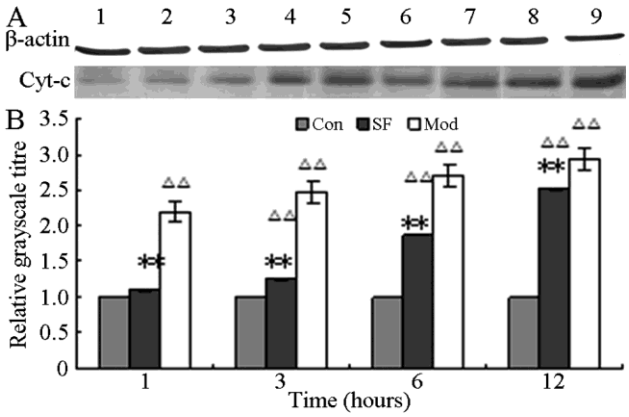


Fig. 4: The cytochrome c relesed from mitochondria after being injectioned by LPS, The cytochrome c relesed from mitochondria elevated significantly (P<0.01) when Mod group compared with Con group at different time points: 1, 3, 6, 12h; cytochrome c relesed from mitochondria didn't increased notably (P>0.05) while SF group compared with Con group at 1h, but cytochrome c relesed from mitochondria increased significantly (P<0.01) at 3, 6, 12h. The cytochrome c relesed from mitochondria decreasd significantly (P<0.01) when SF group compared with Con group at 1, 3, 6, 12h. ($^{\triangle}P$<0.05, $^{\triangle\triangle}P$<0.01 compared with Con group; *P<0.05, **P<0.01 compared with Mod group).

Con and SF groups also increased, and the cytochrome c released from mitochondria significantly decreased (P< 0.01). These results indicated that the SF injection helped in maintaining the stability and integrity of cell membranes (fig. 4).

Mitochondrial membrane potential

Endotoxins are known to induce antiapoptotic phenotypes in monocytes, which may affect the mitochondrial membrane potential. At 1, 3, 6, and 12h post LPS injection, compared to that for the Con group, the mitochondrial membrane potential decreased significantly (P< 0.01). While examining the time course of changes in mitochondrial membrane potential, a time delay was observed following SF injection, during which the membrane potential decreased (fig. 5, F-I). Therefore, mitochondrial membrane potential was preserved after SF injection. Compared to that of the Con group, mitochondrial membrane potential in the SF group did not decrease considerably (P>0.05) at 1h; however, at 3, 6, and 12h, membrane potential decreased significantly (P< 0.01). At 1, 3, 6, and 12h, compared to that of the SF group, mitochondrial membrane potential decreased significantly (P< 0.01) (fig. 5).

The first figure represents the Con group, 2-5 represent the SF group at 1, 3, 6, and 12h, and 6-9 represent the Mod group at 1, 3, 6, and 12h. Compared to the Con group, at 1h, cytochrome c released from the SF group was not significantly higher. the note is merely light developing. In contrast, at 3, 6, and 12h, cytochrome c released from mitochondria in the Mod and SF groups was significantly higher than that in the Con group, but SF group were decreased significantly.

In figure, A indicates the membrane potential, B-E represent the Mod group at 1, 3, 6, and 12h, and F-I represent the SF group at 1, 3, 6, and 12h.

DISCUSSION

Endotoxemia, sepsis, and septic shock are associated with oxidative stress, consumption of endogenous antioxidants, and mitochondrial damage (Ogilvie *et al*., 1991; Cowley *et al*., 1996; Galley *et al*., 1996, 1997; Yassen *et al*., 1999; Alonso de Vega *et al*., 2002; Brealey *et al*., 2002; Vanhorebeek *et al*., 2005; Svistunenko *et al*., 2006; Levy, 2007). Several studies have demonstrated that inflammatory cytokines are generated during sepsis and are correlated with a negative outcome. The complex regulation of synthesis of proinflammatory cytokines and counter regulatory mediators (such as, anti-inflammatory cytokines and receptor antagonists) is poorly understood. Due to this, the exact role of inflammatory cytokines in sepsis-related morbidity and mortality remains unknown. Recent studies have revealed that apoptosis in septic shock is associated with a poor outcome. The following two apoptotic pathways are mainly involved: In the first pathway, different proapoptotic signals in the mitochondria are integrated, which results in the release of cytochrome c; the second pathway, in addition to mitochondria, also involves the recruitment and release of caspases. The mechanisms underlying mitochondrial damage during acute endotoxemia are presently unknown; however, the ultra structural features, including mitochondrial swelling and membrane damage, and functional manifestations of injuries have provided important insights. Mitochondrial swelling is a normal feature of respiring mitochondria and is caused by the movement of solutes across the inner mitochondrial membrane during oxidative phosphorylation. We observed changes in mitochondrial structure after LPS injection. It was observed that mitochondrial swelling occurred at 1h (fig. 3A). Further, as time increased, mitochondrial damage including high-amplitude swelling, vague or partially ruptured membrane, cristae vacuolization, and release of lysosomal enzymes also increased (figs. 3B-E). However, in the SF group, decreased mitochondrial swelling, cristae vacuolization, and lysosomal enzyme release were observed, as compared to that in the time-matched Con group (fig. 3 F-I).

Mitochondrial swelling can occur via two distinct mechanisms. Energy-dependent or the so-called simple osmotic swelling is a consequence of solute accumulation

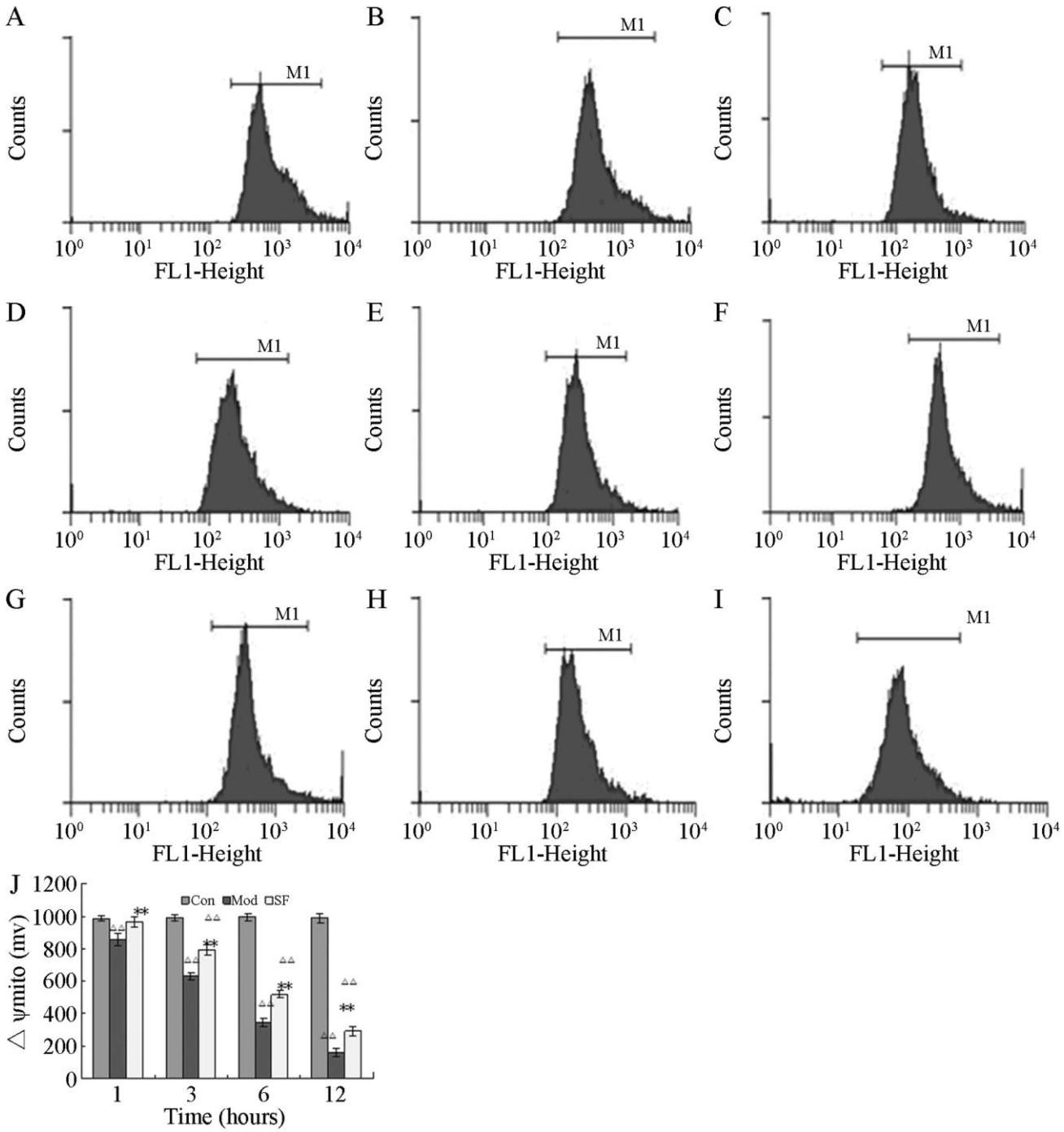


Fig. 5: The membrane potentials of mitochondria after being injectioned by LPS, The membrane potentials decrease significantly (P<0.01) when Mod group compared with Con group at different time points: 1, 3, 6, 12h; membrane potentials didn't step down notably (P>0.05) while SF group compared with Con group at 1h, but membrane potentials reduce apparently (P<0.01) at 3, 6, 12h, The membrane potentials of mitochondria decreasd significantly (P < 0.01) when Mod group compared with SF group at 1, 3, 6, 12h. ($^{\geq}P^{<0.05,\geq\geq}P$<0.01 compared with Con group; *P<0.05, **P <0.01 compared with Mod group).

(usually monovalent cations and anions) that is driven by the proton motive force, or in some cases, by simple concentration gradients. Swelling of this type is not associated with membrane damage, as opposed to colloid osmotic swelling, which occurs when the inner membrane becomes permeable to small molecules and ions but remains impermeable to proteins. This type of swelling occurs when the mitochondrial permeability transition pores open and is essentially driven by the osmotic pressure gradient that exists across the inner

mitochondrial membrane caused by the entrapment of proteins within the matrix. Swelling of this kind can be difficult to reverse, is accompanied by loss of mitochondrial function, and can lead to cell death by necrosis or apoptosis (Crouser *et al*., 2002a).

Cytochrome c was the first mitochondrial protein involved in the regulation of apoptosis. Its biological functions and intramitochondrial location have been previously established. Proapoptotic proteins, such as Bax and Bid, migrate to the outer mitochondrial membrane as a part of the apoptotic signaling mechanism and promote the release of cytochrome c. In our study, compared to that in the Con group, at different time points, the amount of cytochrome c released was significantly higher in the Mod group (P< 0.F4).

The mechanism of enhanced outer mitochondrial membrane permeability to cytochrome c in LPS-treated animals has not established. However, it is known to be associated with the mitochondrial localization of Bax and ceramide, factors known to participate in outer mitochondrial membrane damage *in vitro* (Eskes *et al*., 1998; Siskind *et al*., 2002). In conclusion, endotoxemia causes permeability changes in both the inner and outer mitochondrial membranes and both forms of injury contribute towards the impairment of mitochondrial function. Release of cytochrome c from the mitochondrial intermembrane space into the cytosol is a prominent downstream manifestation of the evolution of apoptotic cell death. Cytochrome c binds to the cytoplasmic protein Apaf-1 via its C-terminus in the presence of ATP, resulting in the formation of an oligomeric complex (Susin *et al*., 1999). This complex recruits procaspase-9 in the presence of dATP, resulting in the activation of caspase-9, the initiator enzyme.

The ultrastructural appearance of mitochondria, particularly the high-amplitude swelling and associated loss of mitochondrial function, suggested that the endotoxin-induced mitochondrial changes were not caused by simple osmotic swelling; however, they were more similar to the MPT-induced changes (Zoratti and Szabò, 1995). Crouser *et al*. (2004) demonstrated that an MPT inhibitor attenuated mitochondrial swelling and normalized the respiratory control of mitochondria in LPS-treated animals. In fact, they were the first to report the abnormal permeability of outer mitochondrial membrane to cyt c during acute endotoxemia. Compared to the time-matched controls, the mitochondrial membrane potential significantly decreased in the LPS-treated animals (P<0.05, fig. 3B-E).

The SF injection decoction was produced using ancient Chinese traditional herbs and was mainly composed of ginseng and aconiti. It has been widely used in China for over 800 years. SF injection for intravenous use is prepared using multistage countercurrent extraction and macroporous resin adsorption. Further, fingerprinting technology was adopted to ensure the quality of the final product (Ji *et al*., 2011). SF injection has been clinically used for the treatment of several diseases, and is well-known for its cardiovascular protective effects. Previous studies demonstrated that SFI exhibits protective effects on cerebral ischemia and hypoxia-induced damages (Wang *et al*., 2012; Yang *et al*., 2013). Additionally, SFI could also alleviate post-resuscitation myocardial dysfunction and lung injury by enhancing energy metabolism (Ji *et al*., 2011; Zhang *et al*., 2012; Guo *et al*., 2016). Several clinical studies have emphasized the notable therapeutic effects of SFI on septic and hemorrhagic shocks (Li *et al*., 2000). In our previous work, we reported that in septic shock patients, the combined use of SF and EGDT improved hemodynamics, reduced organ damage, and shortened the ventilation period and ICU stay (Li *et al*., 2015). In this study, SF injection significantly attenuated the endotoxin-related inflammatory cytokine responses (P<0.05, fig. 2).

Endotoxins are known to induce antiapoptotic phenotypes in monocytes, which may affect the mitochondrial membrane potential. However, in the present study, in the SF group, TNF-related apoptosis was not observed, evident by both cytochrome c release and membrane potential decrease. SF injection was observed to inhibit mitochondrial swelling in the (fig. 3F-I) and in other vital organs during endotoxemia, suggesting that sepsis-induced mitochondrial swelling was a manifestation of MPT (Crouser *et al*., 2002b, 2004).

CONCLUSION

In the present study, we revealed for the first time that in endotoxemia, SF protects the intestinal tissues against mitochondrial injury. The results indicated SF-induced mitochondrial protection via inhibition of inflammatory responses. In the light of these findings, SF injection can dominuted cytochrome c release and simultaneously decrease membrane potential, thereby preserving the mitochondrial function during endotoxemia. Further investigation is required for determining the optimum protective dose and treatment time for SF, which will help in preserving the functions of mitochondria in systemic organs during the acute phase of sepsis.

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