

Exogenous cytochrome *c* inhibits the expression of transforming growth factor- β 1 in a mouse model of sepsis-induced myocardial dysfunction via the SMAD1/5/8 signaling pathway

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Abstract. The current study investigated the role of exogenous cytochrome *c* in sepsis-induced myocardial dysfunction (SIMD) using a mouse model and aimed to elucidate its effect on transforming growth factor- β 1 (TGF- β 1) expression during this process. A total of 75 male Kunming mice were randomly divided into the following five group: Normal (N, n=15); sham-operation (SHAM, n=15); sepsis (CLP, n=15); normal saline (NS, n=15); and cytochrome *c* (Cyt_c, n=15). Animals were sacrificed at 0, 6 or 12 h and the samples were analyzed using transmission electron microscopy, histopathological examination, reverse transcription-quantitative polymerase chain reaction, ELISA, protein analysis by western blotting. The SIMD model was developed and a significant downregulation of TGF- β 1 gene expression, in addition to a reduction in the plasma and protein levels of TGF- β 1 as well as the protein levels of TGF- β 1-activated SMAD 1/5/8 were observed in the CLP group. The data from the current study indicate that using exogenous cytochrome *c* as a therapeutic strategy for SIMD is feasible, and may function via the downregulation of TGF- β 1 expression through the SMAD 1/5/8 signaling pathway.

Introduction

Sepsis is a potentially fatal condition and affects millions of individuals worldwide, with a high rate of mortality (1). Cardiac dysfunction is well recognized in sepsis and septic shock (2,3) and sepsis-induced myocardial dysfunction (SIMD) is a key

contributor to morbidity (40%) and mortality (30~60%) in patients with sepsis (4-6). Thus, it is one of the predictors of poor outcome in patients with sepsis (7). At present, the prognosis for patients with SIMD remains poor, thus, a strategy to protect against SIMD is crucial.

The etiopathogenesis of SIMD is yet to be fully elucidated (7,8), however it is known to be characterized by complex mechanisms involving the attenuation of the adrenergic response at the cardiomyocyte level, alterations in the structure and function of atrial ion channels, mitochondrial dysfunction/ineffective oxygen utilization and blunted calcium sensitivity of contractile proteins (9-11). Several studies have indicated that the inflammatory reaction during sepsis is important in the pathogenesis of SIMD (12-14). A number of studies have demonstrated that specific cytokines are associated with SIMD, including tumor necrosis factor- α , peroxisome proliferator-activated receptor- γ coactivator-1 α and β , interleukin (IL)-6, IL-10, IL-1 β , and also transforming growth factor- β 1 (TGF- β 1) (15-18). TGF- β 1 is an immunomodulatory cytokine with a broad range of anti-inflammatory effects, and has been indicated to ameliorate the adverse effects of pro-inflammatory cytokines on myocardial cells (19). As an early inflammatory factor in SIMD, previous studies have hypothesized that TGF- β 1 may have the potential to be used therapeutically for SIMD in humans (18).

Additionally, impaired mitochondrial function has been hypothesized to contribute to SIMD (20). In a model of sepsis, the oxidation of myocardial mitochondrial proteins and lipids were assessed, which led to the suggestion that damage to the outer mitochondrial membrane (OMM) had occurred (21). Disturbance of the OMM leads to permeabilization, and thus, there is a large release of cytochrome *c* (22). Certain studies have indicated that cytochrome *c* oxidase (COX) is inhibited in the septic heart, and one study noted a competitive inhibition of myocardial COX early in sepsis, which progresses to become non-competitively inhibited during the late hypodynamic phase (23,24). Whether exogenous cytochrome *c* has therapeutic value in sepsis and sepsis-associated organ dysfunction remains unclear (11), however, it is clear that cytochrome *c* (endogenous/exogenous) serves an important function in sepsis via the inhibition of COX (23).

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At present, the effect of exogenous cytochrome *c* on SIMD via TGF- β 1 remains to be fully elucidated, thus in the current study, this was investigated using a mouse model.

Materials and methods

Animal preparation and construction of the SIMD model. A total of 75 male Kunming mice (body weight, 20–24 g) obtained from the Laboratory Animal Center of Chongqing Medical University (Chongqing, China) were randomly divided into the following five groups: Normal (N, $n=15$); sham-operation (SHAM, $n=15$); sepsis by cecal ligation puncture (CLP, $n=15$); normal saline (NS, $n=15$); and cytochrome *c* (Cyt c , $n=15$). Following normal feeding for one week, a sepsis model for the CLP, Cyt c and NS groups was constructed using a previously described CLP procedure (25) in which the cecum is extruded and ligated. In the SHAM group, the cecum was flipped subsequent to opening the abdomen, and no ligation was performed. At the conclusion of the operation, 30 ml/kg saline (Kelun Pharmaceutical Co., Ltd, Sichuan, China) was injected under the skin of the mice in the SHAM, CLP, Cyt c and NS groups, in order to replenish the fluids lost during the operation. Animals in the Cyt c group received a 20 mg/kg intraperitoneal injection of Cyt c (dissolved in 0.15 ml normal saline; Sigma-Aldrich, St. Louis, MO, USA), whereas mice in the NS group received an intraperitoneal injection of an equal volume of normal saline. In every group there were three subgroups, in each of which five animals were sacrificed at 0 (subgroup 1), 6 (subgroup 2) and 12 h (subgroup 3) following a 30-min resting period subsequent to injection, non-injected mice were sacrificed at these time-points without the 30 min resting period. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Chongqing Medical University (Chongqing, China).

Transmission electron microscopy. The mice were anesthetized by 10% chloral hydrate (Sangon Biotech Co., Ltd, Shanghai, China) injection into the *cavum abdominis* (3 ml/kg). The middle lobes of the myocardium were cut using a Paraffin Slicing Machine (Shanghai Medical Equipment Works Co., Ltd., Shanghai, China) into 1–2 mm-cubes and fixed in 2.5% glutaraldehyde (Sangon Biotech Co., Ltd) at 4°C, then the samples were treated with osmium tetroxide (Sigma-Aldrich) and dehydrated in serial concentrations of ethanol (Sangon Biotech Co., Ltd). The samples were then treated with propylene oxide (Befar Group Co., Ltd, Shanghai, China) for 10 min and with a propylene oxide/epoxy resin mixture (Sinopec Group, Beijing, China) for 1 h. These were left overnight with caps removed from the vials to allow for the evaporation of any remaining propylene oxide. Subsequently, the samples were embedded in labeled capsules with freshly prepared resin. Electron photomicrographs were captured using a Hitachi H-7500 Transmission Electron Microscope (Hitachi, Ltd., Tokyo, Japan).

Histopathological examination. Formalin-fixed (Sangon Biotech Co., Ltd), paraffin-embedded (Sigma-Aldrich) myocardial sections (2 μ m thick) were cut and stained with hematoxylin & eosin (H&E; Huayueyang Biotechnology Co.,

Ltd., Beijing, China). The samples were visualized using an electron microscope (H-7560, Hitachi, Tokyo, Japan).

RNA isolation and analysis. A total of 100 mg myocardial mouse tissue was homogenized with 2 ml TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA), with 20 μ l diethyl-pyrocabonate water (Beyotime Institute of Biotechnology, Shanghai, China) per Eppendorf tube, 100 μ l tissue homogenates was added to each tube. Reverse transcription (RT) was completed using the Reverse Transcription System (Promega Corporation, Madison, WI, USA) and treated with DNase I (CWBIO, Beijing, China) to eliminate genomic DNA contamination. RNA was quantified using the RNA 6000 Nano kit and the 2100 Bioanalyzer system (Agilent Technologies, Inc., Santa Clara, CA, USA) with the RNA ladder as the standard.

RT-quantitative polymerase chain reaction (qPCR). The RT-qPCR reaction was performed with a final volume of 25 μ l [2.5 μ l 10X LA Taq buffer (Takara Biotechnology Co., Ltd., Dalian, China); 0.4 mM deoxyribonucleotide triphosphate (Takara Biotechnology Co., Ltd.); 0.4 pM each of the 5' and 3' primers (Takara Biotechnology Co., Ltd); 1.25 μ l 20X SYBR Green I buffer (OPE, Shanghai, China) and 1.25 U LA Taq DNA Polymerase (Takara Biotechnology Co., Ltd.)]. GAPDH was used as internal control to normalize the aromatase mRNA level. The primer sequences used were as follows: GAPDH, F 5'-CAT CAC TGC CAC CCA GAA GA-3' and R 5'-GCT GTA GCC AAA TTC GTT GT-3'; TGF- β 1, F 5'-TGG TGG ACC GCA ACA AC-3' and R 5'-AGC CACTCA GGC GTA TCA G-3'. The thermal cycling profile consisted of an initial 2 min at 94°C followed by 40 cycles of 94°C for 20 sec, 56°C for 20 sec and 72°C for 30 sec. Melting curve analysis was subsequently performed. The experiments were repeated three times with separate samples using a Stratagene Mx3000PTM RT-qPCR system (Agilent Technologies, Inc.).

The sensitivity of RT-qPCR was determined from the Ct values obtained with known quantities of TGF- β 1 and GAPDH standards. The variability between results of different PCR runs was calculated from duplicate samples for all of the targets, and identified an average standard deviation for the threshold cycle (Ct) of the fluorescence signal. Gene expression analysis was conducted using the comparative $2^{-\Delta\Delta C_t}$ method.

Enzyme-linked immunosorbent assay (ELISA). Under anesthesia with chloral hydrate, blood samples were collected via the abdominal aorta in EDTA tubes (Becton-Dickinson, Franklin Lakes, NJ, USA) and were centrifuged at 1,500 \times g for 10 min at 4°C. The resulting plasma supernatant was stored at -80°C until required for analysis, and the concentrations of TGF- β 1 were quantified using a Mouse TGF- β ELISA kit (R&D Systems), according to the manufacturer's instructions.

Protein determination. Myocardial tissue specimens were homogenized in 1 ml ice-cold lysate buffer (CWBIO) consisting of 100 mM Tris-hydrochloric acid; 300 mM sodium chloride; 2% (v/v) Tween-20; 0.4% NP-40; 20% glycerol; protease inhibitor cocktail; and phosphatase inhibitors. The homogenates were centrifuged at 12,000 \times g for 20 min at 4°C and

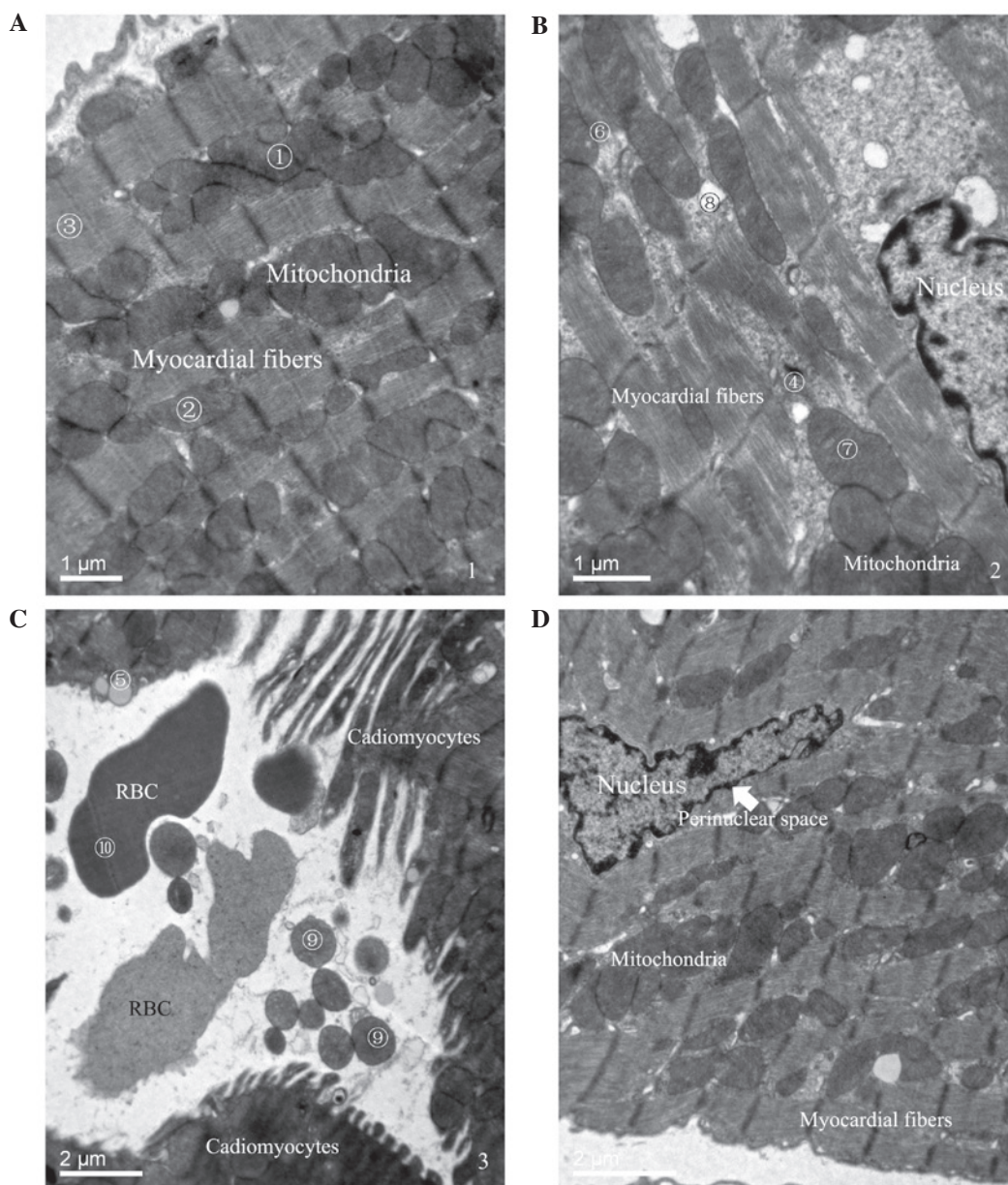


Figure 1. Ultrastructural organization of myocardial tissues. Following electron microscopy, (A) the SHAM group exhibited various characteristics, including (1) a large number of rows of mitochondria, (2) smooth and complete mitochondria cristae, and (3) neat myofibrils (Scale bar, 1 μ m). Isolated myocardial tissues in mice in the (B) CLP (Scale bar, 1 μ m) and (C) NS groups (Scale bar, 2 μ m) demonstrated characteristics of cardiomyocyte damage at 12 h subsequent to the induction of sepsis, including (4) marked exudation of dissolved filaments, (5) increased lipid droplets, (6) mitochondrial disorder and increased swelling of the (7) mitochondria and (8) vacuoles, (9) individual mitochondria and (10) bleeding of red blood cells in the gaps between cells. (D) The Cyt c group exhibited characteristics of protection 12 h subsequent to the induction of sepsis, including no significant increase, swelling or vacuolization of mitochondria, no widening of the perinuclear space of cardiomyocytes and no dissolved filaments (Scale bar, 2 μ m). RBC, red blood cell; SHAM, sham-operation; CLP, sepsis; Cyt c, cytochrome c.

the concentrations of the extracted proteins were determined using the Bicinchonic Acid Protein Assay kit (Sigma-Aldrich). The proteins were then stored at -70°C .

Western blot analysis. Protein samples (50 μ g) were diluted with 2X loading buffer (Beyotime Institute of Biotechnology) and heated in boiling water for 5 min. The proteins were separated by 10% SDS-polyacrylamide gel electrophoresis [10% separating gel and 5% stacking gel (CWBIO); and Protean II xi/XL electrophoresis machine (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and were transferred to a polyvinylidene difluoride membrane (Pall Corp., Port Washington, NY, USA). The membranes were then blocked

with 5% skimmed milk for 1-2 h at 37°C , were incubated with the following primary antibodies for 1-2 h at 37°C : Mouse anti-human IgG2B polyclonal antibody (1:1,000; R&D Systems), mouse anti-human GAPDH polyclonal antibody (1:2,000; R&D Systems), and then washed three times with phosphate-buffered saline with Tween-20 (Sangon Biotech Co., Ltd). Following further incubation with the secondary antibody (goat anti-rabbit/mouse polyclonal IgG; 1:2,000; BIOSS, Beijing, China) for 1 h at 37°C , the membrane was stained by ECL chemiluminescence using BeyoECL Plus (Beyotime Institute of Biotechnology) and visualized using ChemiDoc MP (Bio-Rad Laboratories, Inc.). This experiment was repeated three time using separate samples.

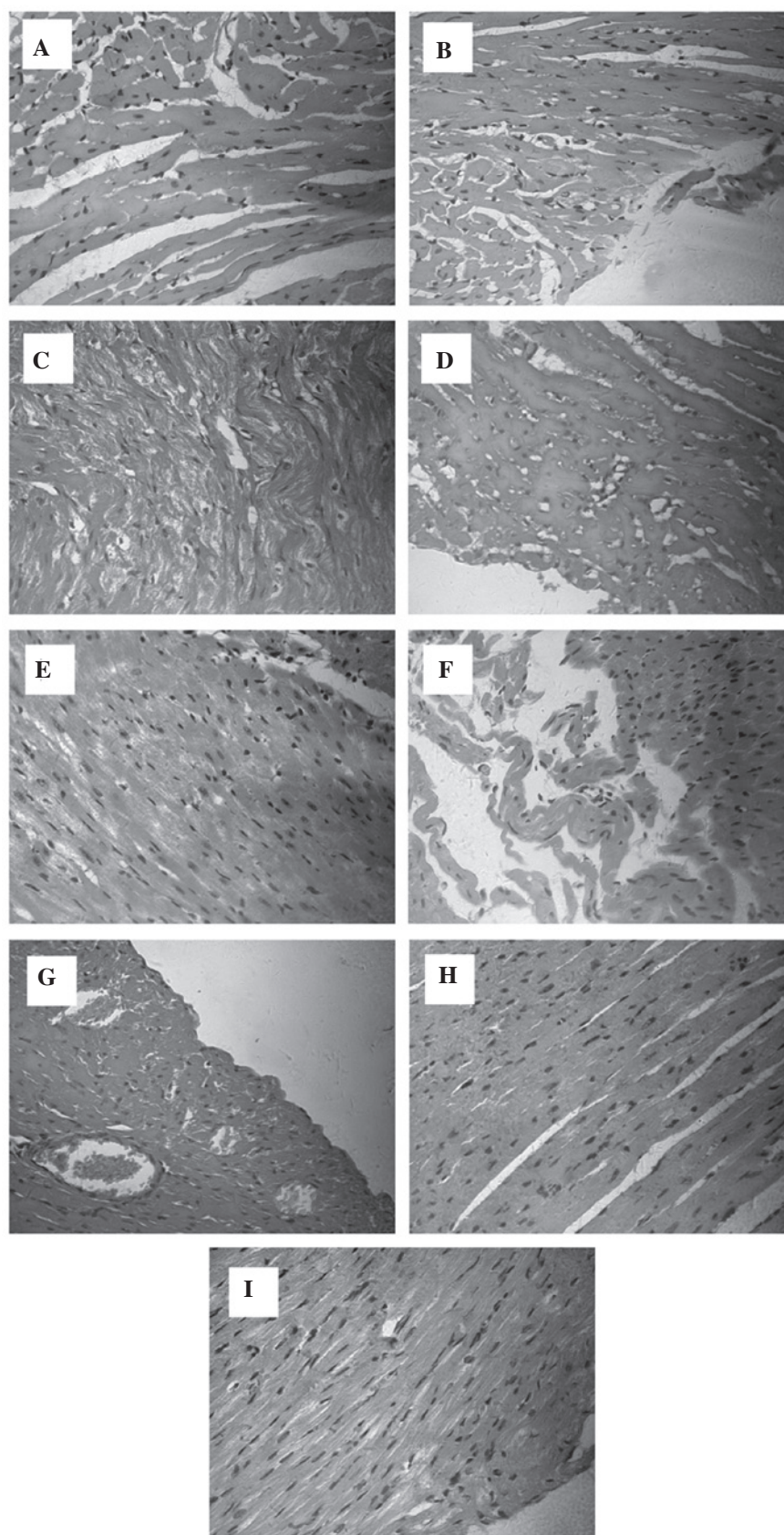


Figure 2. Histopathological analysis of the effects of cytochrome *c* on SIMD was performed at 12 h following injection of normal saline or Cyt*c*. Myocardial tissues from each experimental group were processed for histological evaluation subsequent to H&E staining (magnification, x400). (A and B) The SHAM group exhibited normal histological features, including normal arrangements of myocardial fibers, no infiltration of inflammatory cells in the stroma and no myocardial cell swelling. The CLP group displayed characteristics of acute cardiomyocyte damage and acute inflammation, including (C) tissue edema and (D) focal myocardial necrosis, (E) granular degeneration and (F) myocardial fiber breakage in myocardial cells, (G) myofascial cell hyperplasia and interstitial vascular congestion. The Cyt*c* group demonstrated that cytochrome *c* treatment in part prevents against sepsis-induced damage, and (H) certain parts of the tissues demonstrated normal myocardial histological features, and (I) no clear manifestations of intravascular congestion. SIMD, sepsis-induced myocardial dysfunction; H&E, hematoxylin and eosin; SHAM, sham-operation; CLP, sepsis; Cyt*c*, cytochrome *c*.

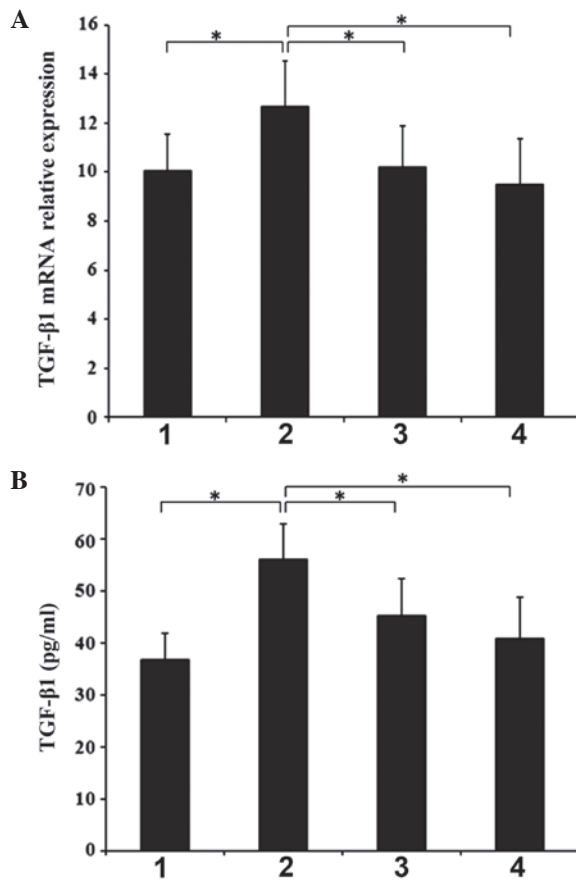


Figure 3. (A) Effects of cytochrome *c* on gene expression of TGF- β 1 in SIMD. (B) Effects of cytochrome *c* on protein plasma levels of TGF- β 1 in SIMD. Myocardial tissues and plasma were harvested at 6 and 12 h subsequent to intraperitoneal injection of cytochrome *c*, and levels of TGF- β 1 gene expression and protein production were measured by RT-qPCR and ELISA, respectively. 1, SHAM 6-h group; 2, CLP 6-h group; 3, Cyt c 6-h group; 4, Cyt c 12-h group. All results are presented as the mean \pm standard deviation from three independent experiments; * P <0.05 between groups denoted by horizontal lines. TGF- β 1, transforming growth factor- β 1; SIMD, sepsis-induced myocardial dysfunction; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SHAM, sham-operation; CLP, sepsis; Cyt c, cytochrome *c*.

Statistical analysis. Data are presented as the mean \pm standard deviation. SPSS, version 10.0 (SPSS, Inc., Chicago, IL, USA) was used for data analysis. TGF- β 1 and SMAD1/5/8 expression were compared using analysis of variance. Comparison between groups was assessed with the Student-Newman-Keuls method, P <0.05 was considered to indicate a statistically significant difference.

Results

Ultrastructural organization of myocardial tissues. Myocardial tissues from mice in the SHAM group exhibited the following characteristics: i) A large number of rows of mitochondria; ii) smooth and complete mitochondrial cristae; and iii) neat myocardial fibers (Fig. 1A). Isolated myocardial tissues in mice in the CLP 12-h group exhibited the characteristics of cardiomyocyte damage (Fig. 1B and C), including marked exudation of dissolved filaments; increased lipid droplets; mitochondrial disorder and increased swelling of the mitochondria and vacuoles; individual mitochondria; and bleeding of red blood cells in the gaps between cells.

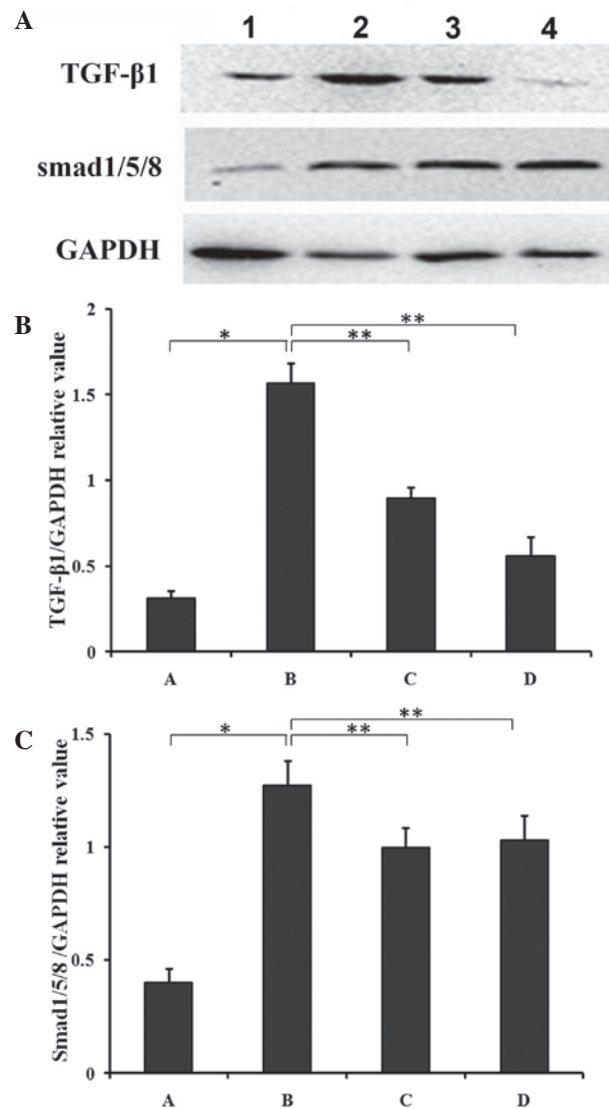


Figure 4. Effects of cytochrome *c* on protein expression of TGF- β 1 and SMAD1/5/8 in SIMD. (A) The myocardial tissues were harvested at 6 and 12 h subsequent to intraperitoneal injection of cytochrome *c* and underwent western blotting. The protein expression levels of (B) TGF- β 1 and (C) SMAD1/5/8 in the different groups were quantified relative to GAPDH expression. 1, SHAM 6-h group; 2, CLP 6-h group; 3, Cyt c 6-h group; 4, Cyt c 12-h group. All results are presented as the mean \pm standard deviation from three independent experiments. * P <0.05 and ** P <0.01 between groups denoted by horizontal lines. TGF- β 1, transforming growth factor- β 1; SIMD, sepsis-induced myocardial dysfunction; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SHAM, sham-operation; CLP, sepsis; Cyt c, cytochrome *c*.

Samples from the Cyt c 12-h group, however, demonstrated characteristics of protection (Fig. 1D), including no significant increase, swelling or vacuolization of mitochondria; no widening of the perinuclear space of cardiomyocytes; and no dissolved filaments. The results from isolated myocardial tissues suggest that cytochrome *c* treatment may protect against SIMD, as certain areas of the tissues exhibited normal myocardial histological features.

Histopathological examination. Histological assessment of the effects of cytochrome *c* on SIMD was conducted at 12 h. Myocardial tissues (n=5) from each experimental group were

processed for histological evaluation with H&E staining (magnification, $\times 400$). As presented in Fig. 2A and B, myocardial tissues from the SHAM group exhibited normal histological features; these features included the myocardial cell arrangement, which consisted cross-striations or a coarse linear pattern, interconnected into a network of oval-shaped nuclei centrally located within the cytoplasm-rich myocyte. Isolated myocardial tissues from the CLP group displayed characteristics of acute cardiomyocyte damage and acute inflammation, including tissue edema (Fig. 2C) and focal myocardial necrosis (Fig. 2D), vacuole degeneration (Fig. 2E) and myocardial fiber breakage (Fig. 2F), myofascial cell hyperplasia and interstitial vascular congestion (Fig. 2G). These alterations were observed in all myocardial tissues from this group. Furthermore, the data from isolated myocardial tissues in the Cytc group suggested that cytochrome *c* treatment in part prevented sepsis-induced damage (Fig. 2H and I).

Effects of cytochrome *c* injection on TGF- β 1 gene expression. RT-qPCR demonstrated that the expression levels of TGF- β 1 were significantly higher in the CLP 6-h group than in the SHAM 6-h group ($P < 0.05$) (Fig. 3B). In addition, the level of TGF- β 1 was observed to be significantly downregulated compared with the CLP 6-h group following 6 and 12-h Cytc treatment ($P < 0.05$).

Effects of cytochrome *c* on protein plasma levels of TGF- β 1. The ELISA demonstrated that TGF- β 1 levels in the plasma were significantly higher in the CLP 6-h group than those in the SHAM 6-h group ($P < 0.05$) (Fig. 3B). Cytochrome *c* treatments for 6 and 12 h were also observed to significantly downregulate the expression of TGF- β 1 compared with the CLP 6-h group ($P < 0.05$).

Effects of cytochrome *c* on protein expression of TGF- β 1 and SMAD1/5/8. Western blot analysis demonstrated that the protein levels of TGF- β 1 and the TGF- β 1-activated SMAD1/5/8 were significantly higher in the CLP 6-h group compared with the SHAM 6-h group ($P < 0.05$) (Fig. 4). In addition, cytochrome *c* treatment for 6 and 12 h significantly downregulated the expression of TGF- β 1 ($P < 0.01$) (Fig. 4B) and SMAD1/5/8 ($P < 0.01$) (Fig. 4C) compared with the CLP group.

Discussion

In the current study, the main observations were as follows: i) Exogenous cytochrome *c* inhibited the expression of TGF- β 1 within the myocardial tissues from mice with SIMD; and ii) this function may proceed via the downregulation of TGF- β 1-activated SMAD1/5/8 expression.

Mitochondrial dysfunction has been suggested to be involved in the pathogenesis of SIMD (23,26), in the initial dysfunction and subsequent amplification. The mitochondrial oxidative phosphorylation machinery has been demonstrated to be essential for cell function, maintenance and survival (27). Patients with sepsis have been reported to exhibit increased mitochondrial respiratory capacity in peripheral blood immune cells (28), and impaired oxidative

phosphorylation has been suggested to lead to SIMD (27). Failure of the cell to consume ATP and provide adequate ADP at the adenine nucleotide transporter during oxidative stress predisposes it to cytochrome *c* release and the initiation of apoptosis (29). Cytochrome *c* and COX represent the terminal step of the electron transport chain, which is considered to be the rate-limiting step for metabolism in mammals (27). They exhibit unique regulatory features, including allosteric regulation, isoform expression and regulation through cell signaling pathways (23). The role of cytochrome *c* and COX phosphorylation has been investigated in various human diseases, including cancer, asthma, ischemia/reperfusion injury, inflammation and sepsis (27,30). Inflammatory signaling can function as an off-switch, whereas growth factor signaling leads to a general partial inhibition of COX (31). In sepsis, COX has been identified to be competitively inhibited early, which progresses to non-competitive inhibition in the later stages (32). Therefore, the presence of COX activity, and the level of activity, can be used as a predictive biomarker for sepsis-associated mortality in humans (33). During sepsis and septic shock, the endogenous Cytc of myocardial tissues in mitochondrion exudates excess cytoplasm and the density of the mitochondrion markedly decreases, resulting in the reduced inhibition of the functions of mitochondria and heart. Previous studies have demonstrated that the addition of exogenous cytochrome *c* may reverse myocardial competitive COX inhibition, restore the activity of COX and improve cardiac function during sepsis (32). It was reported that exogenous cytochrome *c* repleted cardiac mitochondria, restored heme *c* content and increased the kinetic activity of COX (32,34). Thus, exogenous cytochrome *c* administration is suggested for use in a novel therapeutic strategy for SIMD.

TGF- β is an important cytokine that regulates proliferation, differentiation, apoptosis, embryonic development, angiogenesis, wound healing and other functions in various cell types (35). Members of the TGF- β superfamily, comprising the TGF- β and bone morphogenetic protein (BMP) families, are released in pathophysiological conditions and are the classical activators of SMAD proteins (36). SMAD proteins are the intracellular effectors of TGF- β signaling and are activated by receptors, which results the SMAD proteins forming complexes with each other and translocating into the nucleus, where they regulate transcription (37,38). TGF- β 1 induces formation of a complex of type I and II receptors, which results in the activation of SMAD 2/3 and the SMAD and non-SMAD signaling pathways (39,40). BMPs, including ALK 2/3/6, bind to BMP R-II and activates SMADs 1/5/8 (41). TGF- β 1 activated its downstream signaling (SMADs) and induced greater fibrotic and oxidative stress to atrial compared with ventricular fibroblasts (42). Thus, enhanced expression of TGF- β 1 and other family members in the heart under pathophysiological conditions is an indicator of the activation of SMAD signaling (41). A previous study indicated that TGF- β may suppress the transcriptional activity of genes associated with mitochondrial biogenesis or function (43) and the mitochondria have been demonstrated to modulate TGF- β 1 signal transduction (44). Loss of activity of COX during conditions of stress has been suggested to result in alterations to the mitochondrial membrane potential (45). Thus, TGF- β 1 is hypothesized to increase mitochondrial

oxygen consumption and ATP generation in the presence of diverse energy substrates (46).

The current study observed that exogenous cytochrome *c* was protective against SIMD in a mouse model. Exogenous cytochrome *c* modulation of TGF- β 1 signaling was associated with reduced SMAD1/5/8 protein expression, significantly downregulated expression of TGF- β 1 and SMAD1/5/8.

Together, the observations of the current study support the use of exogenous cytochrome *c* as a novel therapeutic strategy for SIMD. One potential mechanism for this may be via the inhibition of the TGF- β 1/SMAD1/5/8 pathway.

Acknowledgements

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