Research Paper

Indocyanine Green-001 (ICG-001) Attenuates Wnt/β-catenin-induces Myocardial Injury Following Sepsis

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Abstract

Objective: To investigate the mechanistic pathway of both indocyanine green (ICG)-001 in attenuated endotoxemia-induced cardiac depression through downregulation cardiac Wnt/ β-catenin cell signaling. **Materials and Methods:** Adult (4–6 months) male Albino-Webster mice, their weights ranged from 25 to 30 g, were pretreated with ICG-001 i.p., following cecal ligation and puncture (CLP). Left ventricle (LV) function was assessed using a microcatheter system. Monocyte chemoattractant protein-1 (MCP-1) and cytokines mediators in plasma and myocardium were analyzed by enzyme-linked immunosorbent assay. Further, the cardiac Wnt protein measured by quantitative real-time polymerase chain reaction while β-catenin analysis through Western blotting procedure. The pathological changes and cells injury in the myocardium were examined using hematoxylin and eosin staining. **Results:** CLP mice displayed worse LV function. The exaggerated cardiac depression in CLP mice was associated with higher levels of MCP-1 and cytokines in plasma and myocardium together with greater cardiac levels of cardiac troponin-I and Wnt/β-catenin. Neutralization of sepsis by either ICG-001resulted in improved LV function and reductions in inflammatory mediators. **Conclusion:** Taken together, these data showed that ICG-001 improved LV function following sepsis through downregulation of Wnt/β-catenin and serve as a potential mechanistic pathway ICG-001 in therapeutic cardiac endotoxemia in animal model.

Keywords: Indocyanine green-100, myocardial injury, sepsis

Introduction

Sepsis-induced myocardial dysfunction is one of the common findings increasing the morbidity and the mortality. It is defined as a systemic dysfunction (systolic and diastolic) of the left and right sides of the heart[1] which occurs due to the functional and structural injury in the myocardium with or without lowered cardiac output,[2] and it is characterizing by dilatation of the left ventricle (LV) and reduction in ejection fraction.[3] The occurrence of septic shock and multiple organs dysfunction syndrome in the septic patients is mostly due to myocardial dysfunction which leads to a decrease in the cardiac output. This in turn leads to hypoperfusion of tissues of vital organs, reduction in nutrition supply, and suppression of immunity. Finally, dysfunction of organs takes place.[4] Myocardial injury is a characteristic feature of endotoxemia and septic shock which happens in about 40%–50% of sepsis.[5] In the Intensive Care Unit, about 60% of severe sepsis patients exhibit cardiac dysfunction and the mortality for those patients range from 70% to 90%. In contrast, the mortality in patients who are not showing any sign of myocardial dysfunction due to sepsis is 20%.[6] There is an increase in the mortality of septic patients with left and right ventricles dysfunction.[7] When mice received lipopolysaccharides (LPS), a rapid activation of nuclear factor-κB, with consequent increase of inflammatory cytokines; tumor necrosis factor alpha (TNF- α) and interleukin-1 beta (IL-1α) messenger RNA expression in cardiac cells are greatly ameliorated in toll-like receptor 4 (TLR4)-mutant mice.[8] These studies confirm that TLR4 signaling is partly responsible for the stimulation of proinflammatory mediators in cardiomyocytes during sepsis. Recent biochemical studies and genetic investigations have

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clearly confirmed the Wnts signal in myocardial injury.[9] Regulation of Wnts pathway occurs at different parts is required for adult tissue maintenance, and perturbations in Wnt signaling promote human cells' degenerative pathway.[10] Wnt/β-catenin signaling is regulated at many levels, including by secreted proteins that antagonize the ligand. Among these are secreted Frizzled-related proteins and Wnt inhibitory protein, both of which can bind Wnts, thereby inhibiting interactions between Wnt and Wnt receptors.[11] To understanding the pathway of sepsis related to sepsis-induced cardiac depression, we tested the hypothesis that indocyanine green (ICG)-001 attenuated sepsis-induced cardiac depression through downregulation of Wnt/β-catenin signaling pathway.

Materials and Methods

Experimental animals

Adult (4–6 months) male Albino-Webster mice and their weights ranged from 25 to 30 g obtained from the College of Science, Babylon University. Mice were acclimated for 14 days in a 12:12-h light-dark cycle with free access to water and regular chow diet before the experiments in animal house of Medical College, Kufa University, and this investigation conforms to the Guide for the Care and Use of Laboratory Animals (National Research Council, revised 1996).

Cecal ligation and puncture procedure in mice

Cecal ligation and puncture (CLP) was performed to induce sepsis in mice as previously described.[12] Briefly, mice were anesthetized by i.p., injection of ketamine (Ketamin; DeltaSelect, Dreieich, Germany) and xylazine (Rompun; Bayer, Leverkusen, Germany). The cecum was exposed through a 2-cm abdominal midline incision, and about two-thirds of the cecum was ligated. The ligated part of the cecum was punctured through and through with a 21-gauge needle. After repositioning the bowel, the abdomen was closed in layers, using a 5.0 surgical suture (Ethicon, Norderstedt, Germany). Mice were monitored for various signs of sickness every 4 h for 24 h. Sham surgical operated mice (anesthesia and laparotomy) served as the surgical control group.

Experimental protocol

Mice were assigned to one of the following experimental groups ($n = 8$ in each group): Sham group, vehicle (LPS) group, CLP group, and CLP + ICG-001 group. All treatments were performed in the morning and followed for survival for 24 h. After analysis of cardiac function, the heart tissue and blood were collected and prepared for analysis.

Cardiac function measurements

We assessed cardiac function as described.[13] Briefly, mice were anesthetized with ketamine (i.p.) in dose of (50 mg/kg) postendotoxemia. Animals were laid supine on a heating blanket, and body temperature was maintained at range 37°C ± 0.5°C. The external right carotid artery was exposed, and a microtipped transducer catheter (1.4F, Millar Instrument Inc.) was placed into the artery and then advanced into the LV. The other end of the catheter was connected to an electrostatic chart recorder (model ES 2000, Gould, Cleveland, USA) and pressure volume loops recorded to measure the maximum rate of change in ventricular pressure and ejection fraction using the MPVS-400 system with the aid of *P* van software (Conductance Technologies, San Antonio, TX, and Millar, Houston, TX, USA) was used to measure all data; heart rates, LV end-diastolic pressure (LVEDP), and LV systolic pressure.

Enzyme-linked immunosorbent assay

The samples of blood from mice were centrifuged (in 10,000 RPM, for 10 min), and myocardial tissue was homogenized and treated in phosphate-buffered saline containing 0.5% Triton X100 with a protease inhibitor cocktail. Commercial enzyme-linked immunosorbent assay (ELISA) kits (R and D Systems) were utilized to quantify monocyte chemoattractant protein-1 (MCP-1, TNF-α, IL-1β, and IL-6) in plasma and myocardial tissue and plasma cardiac troponin-I (cTn-I). Samples and standards were prepared according to manufacturer's instructions. Absorbance of standards and samples were determined spectrophotometrically at 450 nm, by a microplate reader (Bio-Rad Laboratories, CA, USA). Obtained data were plotted against the linear portion of a standard curve.[13]

Quantitative real-time polymerase chain reaction

Total RNA was extracted using Trizol-Reagent (Invitrogen, Carlsbad, CA, USA) after homologated myocardial tissue and equal amounts (1 μg) of RNA were reverse transcribed using a RNA PCR kit (Applied Biosystems, Foster City, CA, USA) as described previously.[14] primer sequences (NM-003391) for Wnt [(5' → 3') (CTGACCTGATGCAGACGCAAG), Rev (AGGAGCCACCTGTAGCTCTCATGTA), length (21 bp, 25 bp) amplicon (139 bp)].

Protein preparation and immunoblotting

The myocardial tissue and cell lysates were extracted as described previously.[15] Briefly, the cellular proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto Hybond enhanced chemiluminescence (ECL) membranes (Amersham Pharmacia, Piscataway, NJ, USA). The ECL membranes were incubated with primary antibody protein against β-catenin (Danvers, MA) and antibodies against glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz, CA, USA) as loading controls. Signals were detected using ECL system (GE Healthcare Japan, Tokyo, Japan). Densitometry was performed using a computerized densitometer (Molecular Dynamics, Sunnyvale, CA, USA) and computer-assisted image analysis.

Histological examination

The cardiac tissue samples were fixed in 4% paraformaldehyde for 24 h. Sections 5 μm in thickness were paraffin embedded according to the standard procedure. Then, the samples were

stained with the hematoxylin and eosin (H and E). The degree of heart damage and photographs were obtained from each heart section ($n = 3$ sections per heart) under optical microscopy.

Statistical analysis

Statistical analysis data were performed using StatView Software (Abacus Concepts, USA). Analysis of variance with Fisher *post hoc* test was used to investigate differences between mice, and data differences were confirmed using the Mann–Whitney U-test. Statistically, the present data significance was defined as $P \leq 0.05$.

Results

Indocyanine green-001 improved left ventricle function after cecal ligation and puncture

To investigate the treatment effects of ICG-001 on LV function following sepsis using CLP protocol. The ejection fraction, cardiac output, and LV end-systolic pressure (LVESP) dropped rapidly in CLP and vehicle mice, in addition to elevated the LVEDP measurement, with significantly higher values of rising temperature by that time than in sham group ($P < 0.05$). Furthermore, ICG-001 pretreated groups improved LV functions through increased the ejection fraction, cardiac output, and LVESP beside reduced heart rate and LVEDP ($P < 0.05$) [Table 1].

Effective role of proinflammatory cytokines after cecal ligation and puncture

We next investigated the importance effects of ICG-001 on the local and systemic proinflammatory responses during CLP. At the end of the experiment (24 h after CLP), the levels of inflammatory modulators including (TNF-α, IL-1β, and IL-6) in plasma and myocardial tissue are measured by ELISA according to manufacture protocol. The resulted data showed that all proinflammatory cytokines are increased after CLP and vehicle treatment compared with sham group ($P < 0.05$) in both plasma and myocardial tissue as in Figures 1 and 2.

Table 1: Hemodynamic status of mice was treated with indocyanine green-001 after cecal ligation and puncture

Groups	Sham	CLP	Vehicle	ICG-001
Heart rate (bpm)	424±13	488±11*	481±12*	438±13**
Ejection fraction (%)	62.2±1	34.6±1.3*	32.9±1.9*	51.2±2**
LVEDP (mmHg)	3.1±1.5	7.1±1.3*	7.2±1.5*	4.0±1.3**
LVSP (mmHg)	111.1±1.4	42.3±1.1*	42.1±1.2*	98.1±2.2**
Cardiac output (ml/min)	5.4±1.1*	3.1±1.2*	3.3±1.1*	4.8±1.2**

CLP mice displayed significantly reduced LV function, including developed pressure, ejection fraction, and cardiac output, compared with sham mice and vehicle. Treatment with ICG-001 improved LV function. Data are expressed as mean±SE, n=8 in each group; *P<0.05 versus corresponding sham and vehicle; **P<0.05 versus CLP. CLP=Cecal ligation and puncture, ICG=Indocyanine green, LV=Left ventricle, SE=Standard error, LVEDP=Left ventricle end-diastolic pressure, LVSP=Left ventricle systolic pressure

Indocyanine green-001 and Niclosamide suppress the expression level of monocyte chemoattractant protein-1

Sepsis leads to upstream release of MCP-1 expression in plasma and myocardial tissue. Moreover, the previous results demonstrated that MCP-1 plays a crucial role in the pathogenesis of myocardial injury by different pathways. We assayed the expression of MCP-1 in plasma and myocardial tissue by ELISA. Figures 3 and 4 showed that the levels of and MCP-1 are markedly increased by CLP, whereas ICG-001 treatment attenuates both plasma and cardiac MCP-1 levels ($P < 0.05$).

Indocyanine green-001 attenuated cardiac troponin protein-1 in cecal ligation and puncture

cTn-I is significantly increased in CLP and vehicle-treated mice compared with sham ($P < 0.05$). Pretreatment dosing with ICG-001 significantly attenuated the effects of sepsis on cTn-I ($P < 0.05$) [Figure 5].

Wnt/β-catenin upregulated following sepsis-induced myocardial suppression

Myocardial tissue homogenates with total RNA were extracted, and quantitative real-time-PCR (qRT-PCR) experiments were performed using primers recognizing Wnt, and the RT-PCR was normalized using actin Figure 6. Furthermore, β-catenin investigated by Western blot Figure 7, the Wnt/β-catenin expression in myocardial cells was significantly ($P < 0.05$) increased in CLP and vehicle groups as compared with sham group. The Wnt/β-catenin level of ICG-001 treated groups was significantly ($P < 0.05$) lower than that of CLP group. This indicates the involvement of Wnt/β-catenin in the mechanistic pathway of ICG-001.

Histological changes of myocardial tissue in response to sepsis

Serial sections of 4 μm were cut and subjected to H and E staining revealed that sham myocardium mice had normal architecture without changes in erythrocyte leakage and leukocyte infiltration into myocardial tissue with clear myocytes boundaries [Figure 8a]. While myocardial tissue from CLP or vehicle mice after 24 h of sepsis period [Figure 8b-d] showed a marked myocardial injury with the development of contraction

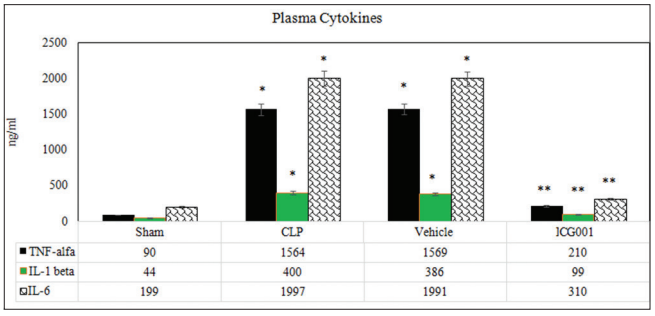

Figure 1: The mean of plasma proinflammatory cytokines (ng/ml) in the four experimental groups 24 h after cecal ligation and puncture. Data are expressed as mean ± standard error, $n = 8$ in each group; *$P < 0.05$ versus corresponding sham, **$P < 0.05$ versus untreated and vehicle.

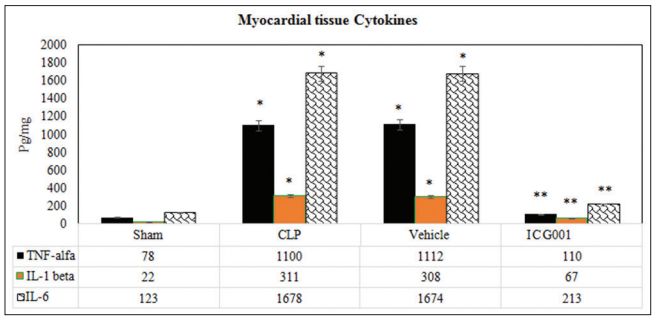

Figure 2: The mean of myocardial tissue proinflammatory cytokines (pg/mg) in the four experimental groups 24 h after cecal ligation and puncture. Data are expressed as mean ± standard error, $n = 8$ in each group; *$P < 0.05$ versus corresponding sham; **$P < 0.05$ versus untreated and vehicle

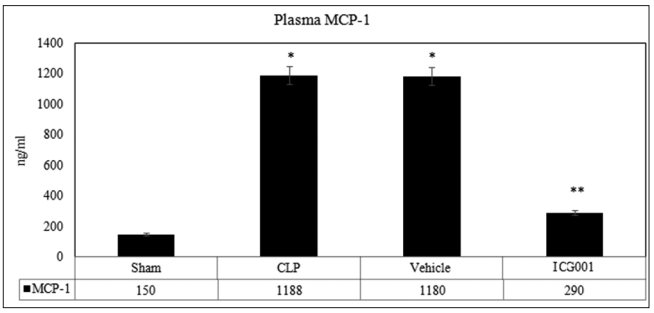

Figure 3: The mean of plasma monocyte chemoattractant protein-1 (ng/ml) in the four experimental groups 24 h after cecal ligation and puncture. Data are expressed as mean ± standard error, $n = 8$ in each group; *$P < 0.05$ versus corresponding sham; **$P < 0.05$ versus untreated and vehicle

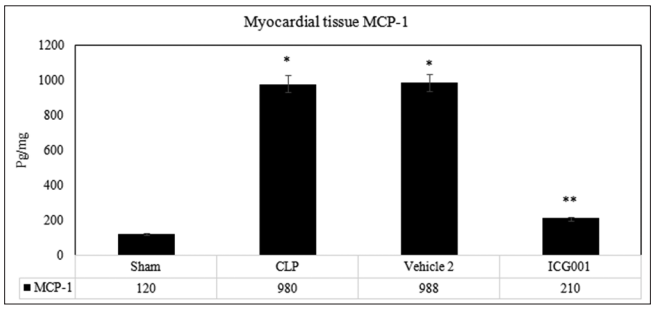

Figure 4: The mean of myocardial tissue monocyte chemoattractant protein-1 (pg/mg) in the four experimental groups 24 h after cecal ligation and puncture. Data are expressed as mean ± standard error, $n = 8$ in each group; *$P < 0.05$ versus corresponding sham; **$P < 0.05$ versus untreated and vehicle

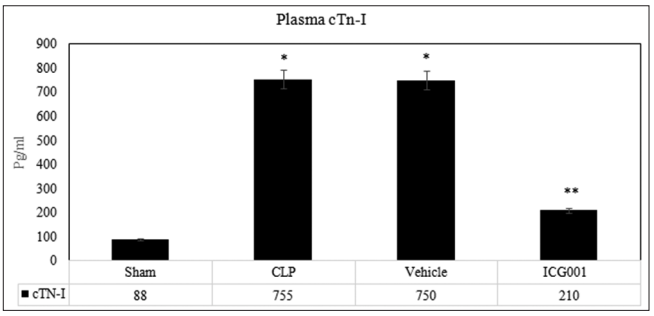

Figure 5: Data are expressed as mean ± standard error, $n = 8$ in each group; *$P < 0.05$ versus corresponding sham; **$P < 0.05$ versus untreated and vehicle

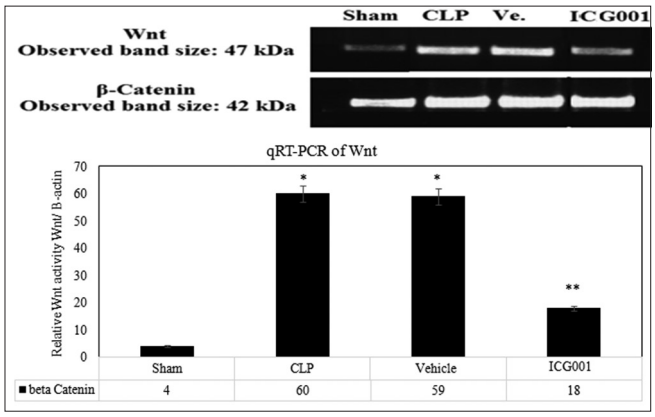

Figure 6: The mean of relative Wnt activity in the four experimental groups 24 h after cecal ligation and puncture. Data are expressed as mean ± standard error, $n = 8$ in each group; *$P < 0.05$ versus corresponding sham; **$P < 0.05$ versus untreated and vehicle

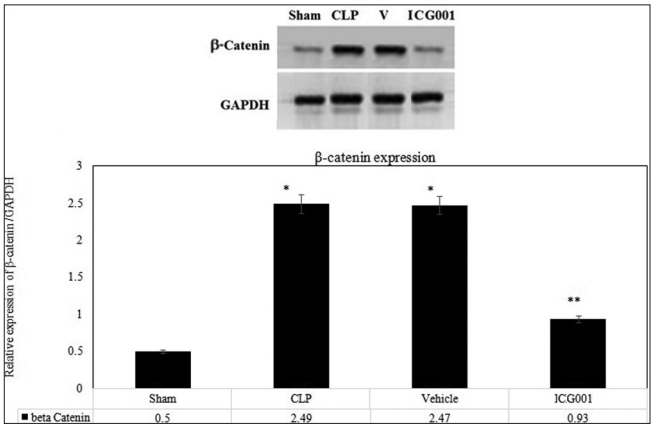

Figure 7: The mean of relative β-catenin activity analysis by Western blot in the four experimental groups 24 h after cecal ligation and puncture. Glyceraldehyde-3-phosphate dehydrogenase was used to normalize the Western blots. Data are expressed as mean ± standard error, $n = 8$ in each group; *$P < 0.05$ versus corresponding sham; **$P < 0.05$ versus untreated and vehicle

bands and polymorphonuclear leukocytes (PMNs) infiltration besides interstitial edema and localized extravasation of red blood cells. While the histological features of the ICG-001 showed mild architectural alterations [Figure 8a-d]. To semi-quantify the difference in cardiac damage, histological sections from all groups were examined and scored according to the protocol of Zingarelli (Zingarelli, Salzman and Szabó) was used [Figure 9]. According to this score system, the following criteria were used:

- Score 0: No damage
- Score 1 (mild): Interstitial edema and focal necrosis
- Score 2 (moderate): Diffuse myocardial cell swelling
- Score 3 (severe): The presence of contraction bands and neutrophil infiltrate

- Score 4 (highly severe): The presence of contraction bands, leukocyte infiltrate, and hemorrhages.

Eight animals in each group were included, and seven sections from each animal were evaluated [Figure 8 and Table 2].

Discussion

During sepsis, the inflammatory responses mediate myocardial injury, including LV dysfunction and cardiac pathophysiological changes [Figure 9].[16] The previous studies reported that expression of inflammatory mediators (IL-1β, TNF-α, and IL-6) was higher following myocardial injury and sepsis.[17] It was also found that in *in vivo* sepsis mouse model, LPS induced the MCP-1 expression in both plasma and myocardial tissue.[18] To understand the pathway of sepsis related in the vulnerability to endotoxemic cardiac depression, the present study investigated the two kinds of agents to improve the LV function following sepsis and possible pathway. To the best of our knowledge, there were no published data that discussed the relationship between ICG-100 pathways on improved cardiac function following sepsis by CLP model in mice. A number of published papers have investigated and confirmed that myocardial dysfunction during sepsis is related with inflammatory mediator's expression, including IL-6, TNF-α, and IL-1β.[19] Furthermore, inflammatory cytokines have been upregulation in myocardial dysfunction in clinical aspects after acute injuries caused by sepsis, myocardial ischemia and reperfusion, and burns.[20] In addition, intravenous administration of either TNF-α or IL-1β in animal experiments can evoke a similar process to that caused by sepsis lead to comorbidity and mortality, and this adverse effects of proinflammatory cytokines can be ameliorated by antibodies that antagonize the effects of these molecules.[21-23] Other studies demonstrated that TNF-α also plays an important role in the septic myocardial dysfunction and that TNF-α links TLR4 activation pathway.[24] In the present study, we demonstrated that sepsis increases the levels of inflammatory mediators (IL-1β, TNF-α, and IL-6) in both plasma and cardiac tissue of mice that associated with worse LV function performance through the hemodynamic measurements (heart rate and ejection fraction), and these results are associated with increased the levels of circulating cardiac Tn-I in mice exposed to CLP. Our data suggest that higher levels of expression of myocardial-depressant proinflammatory cytokines in the heart directly attenuated cardiac contractility and induced myocardial injury. These results contribute, in some part, to the mechanism of exaggerated cardiac depression in experimental sepsis mouse model. Interestingly, we observed that pretreatment with ICG-001 results in a greater reduction in cytokines with improvement in LV function, ejection fraction

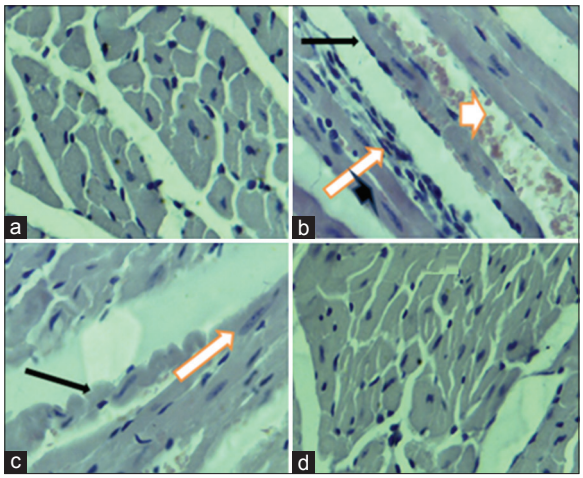

Figure 8: Indocyanine green treatment improved histopathological changes after sepsis. Heart tissue from cecal ligation and puncture demonstrating extensive contraction band change (black arrows) and extensive extravasation of red blood cells (white arrowhead) with margination of polymorphonuclear leukocytes (white arrows) and interstitial edema (black arrowhead [A - sham, B - cecal ligation and puncture, C - Vehicle, D - indocyanine green-001])

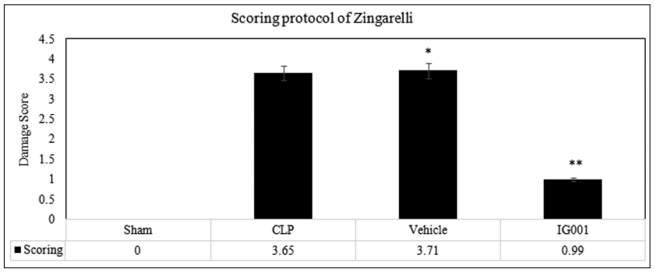

Figure 9: Cardiac damage score. Data are expressed as mean ± standard error, $n=8$ in each group; *$P<0.05$ versus corresponding sham; **$P<0.05$ versus untreated and vehicle

Table 2: The difference in median histopathological grading of abnormal cardiac changes

Categories	Sham		CLP		Vehicle		ICG-001	
	n	Percentage	*n*	Percentage	*n*	Percentage	*n*	Percentage
No abnormality (0)	0	100	0	0	0	0	0	0
Mild (1)	0	0	0	0	0	0	7	87.5
Moderate (2)	0	0	0	0	0	0	1	12.5
Severe (3)	0	0	6	75	7	87.5	0	0
Highly severe (4)	0	0	2	25	1	12.5	0	0
Total	8	100	8	100	8	100	8	100
Median	No abnormality		Severe		Severe		Mild	

CLP=Cecal ligation and puncture, ICG=Indocyanine green

was improved to 53.2% ±1% in ICG-001 treated mice, but it was improved by 49.4% ±1.2%. While the differences become much smaller in other LV functional parameters, such as LVESP, and cardiac output following treatment with ICG-001. MCP-1 is one member of the C-C chemokine family and expressed by many cell types with upregulates the infiltration and migration of monocytes and neutrophils. Many studies demonstrated that antagonism of MCP-1 has been shown to decrease neutrophils recruitment and reduce tissue injury in many animal models of sepsis-induced organs injury.[25] In the present study, we found that MCP-1 levels in plasma and cardiac tissue are significantly higher in the CLP than that in sham mice due to extensive contraction band change, extensive extravasation of red blood cells along with migration of PMNs, and interstitial edema. The overall pathological scoring indicates that the damage score was significantly reduced in ICG-001 treated mice compared with the CLP mice after 24 h sepsis. In normal mammalian cells whether from tissues or organs, the cell cycle must be correctly controlled. Cell cycle regulation in cardiomyocyte is usually disturbed by a series of genetic changes, such as gene amplification, gene overexpression or silence, and gene mutation. These genes include cell-regulated genes such as Wnt/β-catenin that play an important role in pathological changes in the aspects of both atherosclerotic plaque and myocardial injury.[26] Therefore, the role of Wnt/β-catenin as a prognostic indicator can be through the factor that provokes acute myocardial injury changes. It is believed that Wnt/β-catenin pathway may act as a pivotal role in controlling cell injury by integrating different stimuli.[27] If this pathway is correctly regulated in myocardial cell injury, we could target some of its constituents such as Wnt/β-catenin using some targeting inhibitor to reduce its effects. At present, some of these inhibitor molecules in this pathway have been considered as targets for experimental therapy, and some of them are under developmental way.[27] Our study demonstrated that the expression of Wnt/β-catenin is upregulated or it is stably expressed in sepsis-inducible myocardial injury, and deregulation status of Wnt/β-catenin expression is attenuated with ICG-001. We believe that Wnt/β-catenin may be also a potential upregulator of inflammatory mediators.

Conclusion

This work found that Wnt/β-catenin proteins have endogenous expression after sepsis and lead to attenuated LV function. Further, it was found that the expression of Wnt/β-catenin is closely related to the changes of inflammatory mediators, such as IL-1β, TNF-α, and IL-6, further reduced myocardial function, and all of these suggest that Wnt/β-catenin could be a biomarker and a novel target for therapy in patients with sepsis to improved LV function. These results led us to believe that endogenous overexpression of Wnt/β-catenin mediates the expression of MCP-1, led to increased level cTn-I with sequential signal caused myocardial cell injury. Our study showed that there were low levels of Wnt/β-catenin in treated ICG-001 compared with no treated or vehicle mice. We do not know why Wnt/β-catenin expression was suppressed under the effects of ICG-001 and further studies are required to delineate the same.

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Conflicts of interest

There are no conflicts of interest.

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