Original Article

Mechanism of myocardial damage in burn and blast combined injury

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Abstract: Heart failure due to burn-blast combined injury results in low-emission high-impedance type of systemic hemodynamic disorder, namely significant decreased cardiac output after injury. This study focused on myocardial apoptosis that caused the reduced number of cardiomyocytes, which may lead to decreased heart function. Rat model of sham, burn-blast combined injury (BB) and BB treated by Ac-DEVD-Cho was established, HE staining and transmission electron microscope was used to detected heart injury. And TUNEL staining was employed to measure myocardial apoptosis. Caspase activity assay kits were also used to analyze Caspase-3 and Caspase-12 activation. Left ventricular ejection fraction (LVEF) was measured using color Doppler ultrasound. Cultured myocardial cell H9C2 was treated with serum from sham or BB group with or without Ac-DEVD-Cho administration. Cell apoptosis was analyzed by flow cytometry, and endoplasmic and apoptotic related proteins were detected using Western blot assay. Compared with that in sham group, myocardial injury was obviously observed. Myocardial apoptosis was significantly increased and LVEF was remarkably suppressed. Activity of Caspase-3 and Caspase-12 was also activated. Ac-DVED-Cho administration could inhibit myocardial apoptosis and recover myocardial function. And protect myocardial cell H9C2 from BB serum induced apoptosis. Ac-DEVD-Cho administration serves a critical role in suppressing myocardial apoptosis and maintains myocardial function of rats with BB injury.

Keywords: Myocardial injury, burn-blast combined injury, cell apoptosis, caspase activity

Introduction

Over the past decade, local wars, major incidents, special accident, terrorist attacks happened more frequently and threatened human being around the world. Burn-blast combined injury caused by explosion is the most common disease in those occasions. The burn-blast combined injury has high fatality rate, lasts longer, has complex types of casualties, has patient groups in large scales, and is very difficult to treat [1]. Furthermore, gas explosion in household and accidents and other catastrophic events in manufactury industrial also often result in burn-blast combined injury. Therefore, the study of the pathogenesis and treatment of burn-blast combined injury is meaningful, not only for military to treat battlefield wounded soldiers effectively, but also for peaceful society to maintain social stability and reduce the negative impact of a catastrophic event.

Damage to body from burn-blast combined injury is much severer than damage from simpler burn or blast injury with many complications and high mortality [2]. In the current literature, it is believed that heart failure due to the burn-blast combined injury results in low-emission high-impedance type of systemic hemodynamic disorder, namely significantly decreased cardiac output after injury. Studies have shown that cardiomyocyte apoptosis reduces the number of cardiomyocytes. Apoptosis, also known as programmed cell death, occurs in all living cells, and is regulated strictly by genes. Bcl-2 family members play a crucial role in the regulation of mammalian apoptosis process, and they are positioned upstream of irreversible cell damage. According to their family structure and function, they can be divided into two categories: one is the anti-apoptotic proteins such as Bcl-2, Bcl-xl; the other is pro-apoptotic proteins, such as Bax. Among this family, Bcl-2 and Bax
are the most important two proteins, and they can form heterodimers to inhibit Bcl-2 effect. The ratio between the two proteins determines the strength of the inhibition of apoptosis [3]. When cells are stimulated by medications, nutritional deficiency, or oxidative stress, Caspase 9 and Caspase 8 within the cell are activated through different channels to cut the cell and to further activate Caspase 3, ultimately leading to cell apoptosis. The Caspase-3 is the ultimate performing molecular in various apoptotic pathway [4], such as death receptor pathway, mitochondrial pathway and endoplasmic reticulum stress pathway. Endoplasmic reticulum provides specific environments for intracellular protein synthesis, modification and folding. When stimulated by environmental toxins, hypoxia, viruses and other factors, misfolding could happen in the endoplasmic reticulum. Unfolded protein aggregation and Ca homeostasis cause endoplasmic reticulum stress (ERS) to mediate apoptosis through different pathways. These apoptotic pathways are mediated by CHOP, Caspases-12, and other apoptosis-mediated effector molecule, respectively. During the process, CHOP expression levels were significantly increased in a variety of cells and accumulate in the nucleus. CHOP play an important role in the ERS-mediated apoptosis. Caspase-12 is located in the outer membrane of the endoplasmic reticulum; it is one of the characteristic molecular in ERS-mediated apoptosis pathways [5]. In this study, we tried to understand the important mechanism of endoplasmic reticulum stress-mediated apoptosis pathway involved in heart function declining in burn-blast combined injury.

Materials and methods

Animals and grouping

All studies adhered to procedures consistent with the International Guiding Principles for Biomedical Research Involving Animals issued by the Council for the International Organizations of Medical Sciences (CIOMS) and were approved by the Animal Care and Use Committee of the First Affiliated Hospital to PLA General Hospital. Rats were randomly divided into normal control group (Sham), the burn-blast combined injury group (BB), or burn-blast combined injury treated with Ac-DEVD-Cho group (Ac-DEVD-Cho), respectively.

Animal model

Rats in each group were weighed, and anesthetized through intraperitoneal injection of ketamine hydrochloride and sumianxin liquid mixture (0.8 ml/kg). After removing the back hair using shaver, the sham and 25% TBSA burn combined blast model was done as described in our previous report [4]. And the rats in Ac-DEVD-Cho group were immediately injected Ac-DEVD-Cho Ringer’s solution (40 mL/kg) via intraperitoneal injection.

Echocardiographic detection

24 h after injury, the rats in sham, BB, and BB treated with Ac-DEVD-Cho group were used for echocardiographic detection, respectively. After anesthetization with intraperitoneal injection of ketamine hydrochloride and sumianxin mixture, rat was fixed on board. The chest area was unhaired using 8% barium sulphide solution, then apex four cavity sections and left ventricular fraction were detected by Color Doppler Ultrasound Diagnostic System (Philips, sonos 7500) with 10 MHz probe (S12-4).

Samples collection

At the indicated time after injury, the rats in different groups were anesthetized with over dose ketamine hydrochloride and sumianxin liquid mixture. Blood was collected from aorta ventrals using 10 ml syringe. Then the serum was obtained after centrifugation and frozen in -80°C refrigerator. And the heart specimens were also collected for further analysis.

Transmission electron microscope analysis

Left ventricular myocardial tissues were fixed by 2.5% glutaral, after washing by 0.1 mol/L phosphoric acid buffer for three times, 1% osmic acid was used to fix tissues. And then the fixed tissues were dehydration using graded alcohol and 100% acetone. After embedding with embedding agent, ultrathin section was gained and dyed by uranyl acetate. The tissue
slices were observed and images were taken using transmission electron microscope.

**Histological staining**

Heart tissues from different groups at indicated times were fixed using 10% formalin solution, after dehydration, the tissues were embedded into paraffin. And then the sections were obtained. Hematoxylin-eosin (HE) staining was used to generally observe the myocardial injury under light microscope. And TUNEL staining as described in our previous report was employed to analyze myocardial apoptosis (Apoptosis and death receptor signaling in diaphragm of burnt rats).

**Caspase activity assay**

After collection the samples of the myocardial tissues in each group or H9C2 cells with different treatment, the samples were lyzed by lysis buffer in Kit. And the caspase 3 and caspase 12 activations were detected using Caspase 3 Activation Assay Kit and Caspase 12 Activation Assay Kit following the manufacturer’s instructions, respectively.

**Cell culture and treatment**

H9C2 cell was cultured in DMEM medium supplied with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 2 mM L-glutamine (Life Technologies, Grand Island, NY, USA) in 37°C incubator with 5% CO₂. After 70~80% confluence in 6-well plate, the cells were treated with 10% serum from sham rats, 10% serum from BB rats, and 10% serum from BB rats combined with Ac-DEVD-Cho. 24 h later, the cells were collected for subsequent analysis.

**Flow cytometry**

H9C2 cells were cultured in 6-well plates and treated with sham serum, BB serum or BB serum combined with Ac-DEVD-Cho for indicated times. After treatment, cells were harvested using trypsin-EDTA, washed with PBS, resuspended in PBS, and stained with Annexin V and propidium iodide solution (PI; from the BD Apoptosis Detection kit) following the manufacturer’s instructions. Cell apoptosis assay was performed by flow cytometry (FC500; Beckman Coulter, Brea, CA, USA).

**Western blotting**

Total proteins were extracted from tissue or cell samples using RIPA buffer (MACGENE Biotech, Beijing, China), and BCA protein assay kit (Pierce) was used for protein quantification. And then each sample was subjected into SDS-PAGE gel, after transfer, specific primary antibodies and HRP labeled second antibody were used in turn. The bands were subsequently detected using ECL western blotting substrate.

**Statistical analysis**

Data presented are mean ± SEM of a minimum of 3 experiments. Statistical differences were determined using student’s t-test and analysis of variance (ANOVA), followed by Tukey’s or Dunn’s posttest as appropriate.

Statistical significance was set at P<0.05. Data and statistical analysis were performed using Graph Pad Prism version 6.0 for Windows, Graph Pad Software (San Diego, CA).

**Results**

**Heart injury was observed after BB injury**

After the animal model was established, the myocardial samples were collected at indicated times and heart injury was analyzed. The results of HE staining showed that rats in sham group had clear myocardial structure, complete myocardial cell, no congestion in blood vessels, and no inflammatory cell infiltration (Figure 1A); Rats in BB group had a large area of myocardial broken cells, tissue morphology disappearing, edema, and a large number of neutrophil infiltration (Figure 1A). The ultrastructure of myocardial tissue was detected using TEM analysis. And normal cardiac ultrastructure, more organelles contained in myocardial cell cytoplasm, a large number of mitochondria and a small amount of the endoplasmic reticulum were showed in myocardial tissue from sham group (Figure 1B). In comparison with that in sham group, the ultrastructure with visible cardiomyocytes lumps nuclear chromatin condensation, side shift, distributed along the nuclear membrane, nuclear condensation, and apoptotic bodies were observed after BB injury (Figure 1B). It meant that heart injury was occurred after BB injury.
Cell apoptosis in burn-blast induced myocardial injury

Myocardial apoptosis was increased after BB injury

The TUNEL staining was employed to elucidate the myocardial apoptosis in sham and BB groups. As shown in Figure 2A, myocardial apoptosis was significantly increased after BB injury at indicated times. And the activity of apoptosis related proteins, caspase 3 and caspase 12 was also measured. Consistent with the results of TUNEL staining, BB injury still promoted the activity of caspase 3 and caspase 8 compared with that in sham group (Figure 2B). Subsequently, the left ventricular injection fraction, one of the indexes of myocardial function, was analyzed by echocardiographic detection. The fraction was dramatically decreased after BB injury in comparison with that in sham group (Figure 2C). It suggested that BB injury activated caspase activity, induced myocardial apoptosis, and impaired myocardial function.

Administration of caspase inhibitor, Ac-DEVD-Cho, reduced BB injury induced myocardial apoptosis

Above results demonstrated that myocardial apoptosis might serve an important role in BB injury induced myocardial dysfunction. So Ac-DEVD-Cho, the caspase inhibitor was used to reduce BB injury induced apoptosis. After 24 h treatment, the HE staining was done, and the results (Figure 3A, left panel) showed that Ac-DEVD-Cho administration significantly reduced the area of myocardial broken cells, tis-

Figure 1. Heart injury was observed after BB injury. After injury for indicated times, the samples of heart were collected and fixed for further analysis. A. Rats in sham group had clear myocardial tissue structure, complete cardiac cell, no congestion in blood vessels, no inflammatory cell infiltration; rats in BB injury group had a large area of myocardial broken cells, tissue morphology disappearing, edema, and a large number of neutrophil infiltration. B. Rats in sham group had normal cardiac ultrastructure, more organelles contained in myocardial cell cytoplasm. Rats in BB injury group had visible cardiomyocytes lumps nuclear chromatin condensation.
Cell apoptosis in burn-blast induced myocardial injury

Figure 2. Myocardial apoptosis was increased after BB injury. A. After injury for indicated times, the samples of heart were collected for TUNEL staining assay. And cell apoptosis was obviously observed after BB injury compared with those in sham group. B. The activity of caspase 3 and caspase 12 in heart samples was also detected using Caspase Activation Assay Kit. After BB injury, the activity of caspase 3 and caspase 12 was significantly increased. The asterisk (*) indicates a significant difference (P<0.05). C. The left ventricular ejection fraction was also measured by echocardiographic detection. The results indicated that BB injury dramatically reduced left ventricular ejection fraction. The asterisk (*) indicates a significant difference (P<0.05).

Sue morphology disappearing, edema, and the number of neutrophil infiltration. TEM observation also indicated the cardiac ultrastructure including nuclear condensation was alleviated after Ac-DEVD-Cho treatment compared with that in BB group (Figure 3A, middle panel). Finally, the BB injury induced myocardial apoptosis was also obviously suppressed after Ac-DEVD-Cho administration (Figure 3A, right panel). Subsequently, the activity of caspase 3 and caspase 12 was measured, and the results demonstrated that Ac-DEVD-Cho administration could significantly reduce BB injury induced the activity of caspase 3 and caspase 12 (Figure 3B). ER stress related proteins GRP78, CHOP and apoptosis related proteins Bcl-2, Bax were also detected using Western blot assay. Compared with those in sham group, BB injury resulted in upregulating the expression of GRP-78, CHOP, and Bax (the proapoptotic protein) (Figure 3C), and downregulating anti-apoptotic protein Bcl-2 expression; however, Ac-DEVD-Cho administration could reverse the proteins expression induced by BB injury (Figure 3C). And the quantitative analysis of the proteins expression was shown in Figure 3D. It meant that Ac-DEVD-Cho administration might alleviate BB injury induced ER stress and myocardial apoptosis. After that, we detected the left ventricular systolic function. And the left ventricular ejection fraction, fractional shortening results showed that, AC-DEVD-administration can significantly recover left ventricular contractile function damaged by BB injury. Compared with the sham group, BB injury reduced left ventricular function.
Cell apoptosis in burn-blast induced myocardial injury

In order to further identify the function of BB injury on myocardial apoptosis, serum from rats in BB or sham groups was used to treat myocardial cell line H9C2. And Ac-DEVD-Cho was also used to inhibit caspase activity. After treatment for indicated times, cells were collected for flow cytomtery assay. The results indicated that BB serum treatment remarkably promoted H9C2 cell apoptosis in comparison

**Figure 3.** Administration of caspase inhibitor, Ac-DEVD-Cho, reduced BB injury induced myocardial apoptosis. After BB injury, the rats were further treated with or without caspase inhibitor Ac-DEVD-Cho for 24 h. A. The heart samples were collected and fixed for HE staining, TEM detection and TUNEL analysis. B. The activity of caspase 3 and caspase 12 in heart samples was also detected using Caspase Activation Assay Kit. After Ac-DEVD-Cho administration, the activity of caspase 3 and caspase 12 was significantly reduced. The asterisk (*) indicates a significant difference (P<0.05). C. Western blot assay was also employed to detect the related proteins expression, BB injury increased GRP78, CHOP, and Bax expression, and reduced anti-apoptotic protein Bcl-2 expression. Ac-DEVD-Cho administration reversed the proteins expression induced by BB injury. D. The quantitative analysis of protein expression was done, and GAPDH was used for loading control. The asterisk (*) indicates a significant difference (P<0.05) compared with that in sham group, and the heart shape (♥) indicates a significant difference (P<0.05) compared with that in BB injury group. E. The echocardiographic detection was also done for the rats in sham, BB, and BB treated with Ac-DEVD-Cho group.
Cell apoptosis in burn-blast induced myocardial injury

Figure 4. Ac-DEVD-Cho administration inhibited BB serum induced H9C2 cell apoptosis. The myocardial H9C2 cells were cultured and treated with sham serum, BB serum, or BB serum with Ac-DEVD-Cho for indicated times. A. The cells were collected and cell apoptosis was measured by flow cytometry assay. After BB serum treatment, cell apoptosis was significantly increased in comparison with that in sham group, and Ac-DEVD-Cho administration remarkably reduced BB serum induced cell apoptosis. B. The quantitative analysis was done after sham serum, BB serum, or BB serum with Ac-DEVD-Cho treatment at indicated times. The asterisk (*) indicates a significant difference (P<0.05) compared with that in sham group, and the heart shape (♥) indicates a significant difference (P<0.05) compared with that in BB injury group. C, D. The activity of caspase 3 and caspase 12 was measured. After BB serum treatment, the activity was significantly increased in comparison with that in sham group, and Ac-DEVD-Cho administration remarkably reduced BB serum induced caspase activity. The asterisk (*) indicates a significant difference (P<0.05) compared with that in sham group, and the heart shape (♥) indicates a significant difference (P<0.05) compared with that in BB injury group.

with that treated with sham serum (Figure 4A), and Ac-DEVD-Cho administration significantly reduced BB serum induced H9C2 cell apoptosis (Figure 4A). Subsequent quantitative analysis showed that more than 30% cell apoptosis was observed after BB serum treatment for 6 h or 24 h, and cell apoptotic rates were about 20% and 10% after Ac-DEVD-Cho treatment for 6 h and 24 h, respectively (Figure 4B). The activity of cell apoptosis related proteins, caspase 3 and caspase 12, was measured. Consistent with the results from flow cytometry, the activity of caspase 3 and caspase 12 was significantly increased upon BB serum treatment, and Ac-DEVD-Cho administration remarkably reverses this reaction (Figure 4C, 4D). Taken together, BB serum treatment increased H9C2 cell apoptosis, and Ac-DEVD-Cho administration dramatically reduced BB serum induced cell apoptosis via inhibiting the activity of caspase 3 and caspase 12.

Ac-DEVD-Cho administration reversed the proteins expression induced by BB serum treatment

After indicated treatment, H9C2 cells were collected for Western blot assay. Compared with that treated by sham serum, proteins expression of GRP78, CHOP, and Bax was upregulated and Bcl-2 was downregulated after BB serum treatment (Figure 5A); And Ac-DEVD-Cho administration reversed the proteins expression induced by BB serum at a certain extent (Figure 5A). And quantitative analysis also showed that after BB serum treatment for 6 h or 24 h, the expression of GRP78 (Figure 5B), CHOP (Figure 5C), and Bax (Figure 5E) was significantly
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Increased. Compared with that treated with BB serum, the expression of GRP78 (Figure 5B), CHOP (Figure 5C), and Bax (Figure 5E) was remarkably decreased after BB serum combined with Ac-DEVD-Cho treatment. However, the anti-apoptosis protein Bcl-2 was significantly decreased after BB serum treatment (Figure 5D), and Ac-DEVD-Cho administration successfully reversed Bcl-2 downregulation induced by BB serum (Figure 5D). It suggested that BB serum treatment might regulate ER stress and cell apoptosis related proteins expression, and Ac-DEVD-Cho administration reversed related proteins expression and reduced BB serum induced H9C2 cell apoptosis.

Discussion

With the development of modern weapons, injuries caused by explosion are rarely a single burns, wounds, blast injury, or radiation injury. Rather, they are often caused complicately by two or more injury factors simultaneously or consecutively, and damage body much more severely. The combined effects of various injury factors were shown at different levels; whole body, tissues, organs, cells and submicroscopic structure, and molecular levels, and are also shown in important pathophysiological processes. In many cases, the composite effect of combined injury is “mutually aggravating” effect, making condition more complicated and diseases more difficult to treat [6].

The effects of BB injury on hemodynamics have not been well studied in the current literature. It is now believed that heart failure and pulmonary vascular injury caused by burn-blast combined injury could directly result in low-emission high-impedance type of systemic hemodynamics disorder, i.e., significantly decreased cardiac output and increased systemic vascular resistance and pulmonary vascular permeability after injury. The shock are followed by sympathetic-adrenergic and renin-angiotensin system hyperthyroidism, pulmonary vasoconstriction, increased capillary hydrostatic pressure, and increased peripheral vascular resis-
Apoptosis, also known as programmed cell death, occurs in all living cells, subject to strict regulation by genes. When cells are stimulated by medications, nutritional deficiency, or oxidative stress, Caspase 9 and Caspase 8 within the cell are activated through different channels to cut the cell and to further activate the cell are activated through different channels. Caspase 9 and Caspase 8 within the cell are activated through different channels to cut the cell and to further activate the cell are activated through different channels. These apoptotic pathways are mediated by CHOP, Caspases-12, and other apoptosis-mediated effector molecule, respectively [11]. Under normal physiological conditions, it is located in the cytoplasm, with lower level of expression. But when stressed, CHOP expression levels were significantly increased in a variety of cells and accumulated in the nucleus. CHOP plays an important role in the ERS-mediated apoptosis. Studies have shown that during endoplasmic reticulum stress, CHOP over expression can cause cell cycle arrest and apoptosis, CHOP under expression can cause decreased apoptosis. In mechanism, CHOP can cause apoptosis in a variety of ways [12]. First, it can reduce
the expression level of anti-apoptotic factor Bcl-2, promote the shift of pro-apoptotic factor Bax protein from the cytoplasm to the mitochondria, and increase the transcription rate of pro-apoptotic factor Bim to induce apoptosis. Caspase is a class of cysteine protease having the same sequence; it is an important mediator of apoptosis. Under normal circumstances, Caspase-12 is located in the outer membrane of the endoplasmic reticulum, and is one of the characteristic molecular in ERS-mediated apoptosis pathways [13]: When Caspase-12 is deficient, ERS-mediated apoptosis is partially suppressed. During endoplasmic reticulum stress, Caspase-12 can be activated by multiple mechanisms. After the activation of Caspase-12, it can cut Caspase-9 precursor, leading to Caspase-9 activation, and thus Caspase-3 activation and mediated apoptosis. This pathway does not involve the mitochondrial cytochrome C/Apaf-1 pathway.

In this study, we observed by transmission electron microscopy that after burn-blast combined injury, myocardial cell apoptosis had significant changes, especially endoplasmic reticulum showed significant expansion, ultrastructure was severely damaged, which is a pathological basis of ERS. Furthermore, in terms of molecular mechanisms, among many ERS molecules, we chose GRP78, CHOP, and Caspase-12 proteins in three different pathways and measured their expression levels. GRP78 is an important regulatory molecule for endoplasmic reticulum protein synthesis and secretion [14], and is responsible for monitoring the endoplasmic reticulum homeostasis. It is involved in nascent protein recognizing and folding and preventing abnormal protein accumulation, and is also involved in calcium ion transport. Therefore, GRP78 is an aniconic molecule of ERS. Its increased expression level indicates endoplasmic reticulum homeostasis and ERS activation. In this study we found that, compared with the control group, myocardial GRP78 protein expression level in burn-blast combined injury group was significantly increased, suggesting that the combined injury activated GRP78 expression, and further myocardial ERS. In addition, as mentioned earlier, CHOP, Caspase-12 represent two unique ERS apoptotic pathways. This study found that, after the burn-blast combined injury, myocardial Caspase-12 active substance expression and myocardial CHOP expression were markedly higher than the control group indicating cardiomyocytes CHOP, Caspase-12 proteins and their corresponding apoptotic pathways were activated at different degrees after the burn-blast combined injury.

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Disclosure of conflict of interest

None.

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