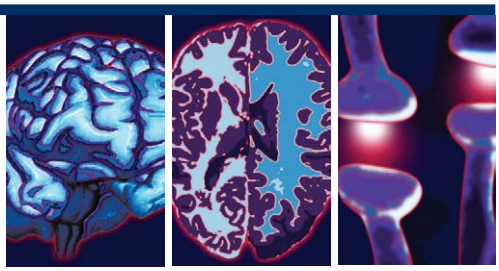

Impact of Combined Adipose-Derived Mesenchymal Stem Cell (ADMSC) and Low-Energy of Extracorporeal Shock Wave Therapy on Protecting Brain Death-Induced Remote Organ Damage in Rat

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ABSTRACT

Objective: This study tested the hypothesis that allogenic adipose-derived mesenchymal stem cell (ADMS) and extracorporeal shock wave (ECSW) therapy could attenuate brain death (BD)-induced remote cardiac organ damage in rat.

Methods: Adult-male SD rats (n=30) were equally divided into group 1 (sham control); group 2 (BD); group 3 [BD + ECSW (0.15 mJ/mm^2/300 impulses applied to skull surface 3 h after BD induction)]; group 4 [BD + ADMSC (1.2 x 10^6 cell) by intravenous injection 3 h after BD induction]; and group 5 (BD-ECSW-ADMSC). Animals were sacrificed by 6 h after BD induction and the heart specimens harvested.

Results: ELISA analysis showed that the circulating level of inflammation (IL-6/MPO/TNF-α) was lowest in group 1, highest in group 2, significantly lower in groups 5 than in groups 3 and 4, and significantly lower in group 4 than in group 3 (all p<0.0001). Flow cytometry demonstrated that circulating inflammatory (Ly6G+) cells and circulating/splenic immune (CD3/CD4+/CD3/CD8+) exhibited an identical pattern whereas Treg+ cells expressed an opposite pattern of inflammatory biomarkers (all p<0.001). The protein expressions

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of inflammatory (IL-1ß/MMP-9/TNF-α/NF-κB/), and apoptotic (cleaved/caspase-3/PARP/mitochondrial Bax), oxidative-stress (NOX-1/NOX-2/oxidized protein) biomarkers exhibited an identical pattern, whereas the protein expressions of mitochondrial integrity (mitochondrial cytochrome C) and anti-inflammatory (IL-4/IL-10) biomarkers exhibited an opposite pattern to inflammatory biomarkers among the five groups (all p<0.0001). The expressions of myocardial inflammatory (CD14+/CD68+) and immune (CD3+/CD4+) cells displayed an identical pattern of inflammatory biomarkers among the five groups (all p<0.0001).

Conclusion: ECSW-ADMSC therapy effectively protected the remote cardiac organ against BD-induced damage.

Keywords

Brain death, Remote organ damage, Inflammation, Immune response

Introduction

Heart transplantation is reserved for patients for severely decompensated heart failure (CHF) [1-4]. Limited donor numbers, graft-versus-host disease and acute and chronic transplant rejection remain unresolved issues. Currently, only brain-dead organ donors supply hearts for transplantation, limiting further the number of available donor hearts.

Of interest, clinical observational studies have established that circulating inflammatory biomarkers were substantially increased in patients after ischemic stroke (IS) [5-10]. Experimental studies have shown that inflammatory activation is markedly increased in animals after acute IS [11-14]. Increased inflammatory reaction and hyper-reactive immune response frequently occur in the circulation [15] and in important vital organs such as liver, heart and kidney after brain death (BD) in animals [11-13,16]. More than 25% left ventricular (LV) dysfunction is observed in the early phase of BD [17], termed "remote organ damage". BD-induced remote organ damage occurs mainly through damage-associated molecular patterns (DAMPs), which are endogenous intracellular molecules released by activated or necrotic cells/tissues, and act as crucial signals that trigger the inflammatory immune response to tissue damage [18]. There is a need to find a new, safe and effective treatment modality that can reduce BD-induced remote organ damage.

Mesenchymal stem cells (MSCs) have capacity for anti-inflammation [19-22] and immunomodulation [23-27]. Our recent studies have shown that adipose-derived mesenchymal stem cells (ADMSC) possess anti-inflammation, anti-apoptosis, anti-fibrosis, anti-oxidative stress and immunomodulation properties in the settings of acute IS, acute ischemia-reperfusion injuries of lung, kidney, ischemic bowel syndrome, acute interstitial cystitis and critical limb ischemia, as well as in sepsis syndrome [19,21,28,29].

Interestingly, extracorporeal shock wave (ECSW) has been shown to improve wound and bone healing by inhibiting inflammation and enhancing angiogenesis [30-33]. ECSW therapy also improves ischemia-related organ dysfunction by enhancing angiogenesis and reduces the generation of oxidative stress, reactive oxygen species (ROS), and sensory pain [34-36]. This study therefore tested the hypothesis that combined ADMS and ECSW therapy could alleviate BD-induced remote cardiac organ damage in rat.

Materials and Methods

■ Ethics

All animal experimental procedures were approved by the Institute of Animal Care and Use Committee at Kaohsiung Chang Gung Memorial Hospital (Affidavit of Approval of Animal Use Protocol No. 2013062803) and performed in accordance with the Guide for the Care and Use of Laboratory Animals [The Eighth Edition of the Guide for the Care and Use of Laboratory Animals (NRC 2011)].

Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-approved animal facility in our hospital with controlled temperature and light cycle (24 °C and 12/12 light cycle).

■ BD induction in the animal model (Figure 1)

The procedure and protocol for BD induction has been described in our recent report [37]. The model is designed to mimic sudden-

onset increased intracranial pressure, as in severe traumatic intra-cranial hemorrhage, resulting in brain-stem failure. Animals were anesthetized by inhalational 2.0% isoflurane on a warming pad at 37°C and placed prone. The scalp, epicranial muscles and pericranium were incised, and a 4F angioplasty balloon catheter introduced into the supradural space through a dental drill burr hole at the paramedian space near the frontal transverse sinus. Each animal was endotracheally intubated with positive-pressure ventilation (180 mL/min) with room air using a small animal ventilator (SAR-830/A, CWE Inc., U.S.A.) at a ventilation rate of 60/min and BD was induced by injecting 0.5 mL of distilled water through the catheter into the supradurally implanted balloon. Heart rate and blood pressure were continuously monitored during the whole procedure.

■ Criteria for identification of BD in animals (Figure 1)

BD manifested as an immediate and significant reduction in systemic arterial blood pressure (SABP) and the power density of low-frequency (LF) components of SABP, and had to include the following criteria [37]:

1. Cessation of spontaneous respiration with complete dependence on mechanical ventilation
2. Irreversible deep coma (lack of responses and reflexes to noxious stimuli elicited by firmly pinching the footpad with forceps)
3. Fixed and dilated pupils without light reflexes regardless of the intensity of light.

The sum of power density during the period of the low-frequency (LF; 0.25-0.8 Hz in rat) components in the blood pressure (BP) and heart rate (HR) spectra, along with the average values and of mean BP and HR were computerized [38-40].

■ Animal grouping

To determine the impact of BD on remote organ damage, pathogen-free, adult male Sprague Dawley (SD) rats (n=30) weighing 325-350 g (Charles River Technology, BioLASCO, Taiwan) were utilized in the present study. The animals were randomly divided into five groups: sham-control (SC), BD, BD + ECSW (0.15 mJ/mm^2/300 impulses applied to skull surface 3 h after BD induction)], BD + allogenic ADMSC [(1.2 x 10^6 cell) by intravenous injection 3 h after BD induction], and BD + ECSW + allogenic ADMSC. All animals were euthanized and their

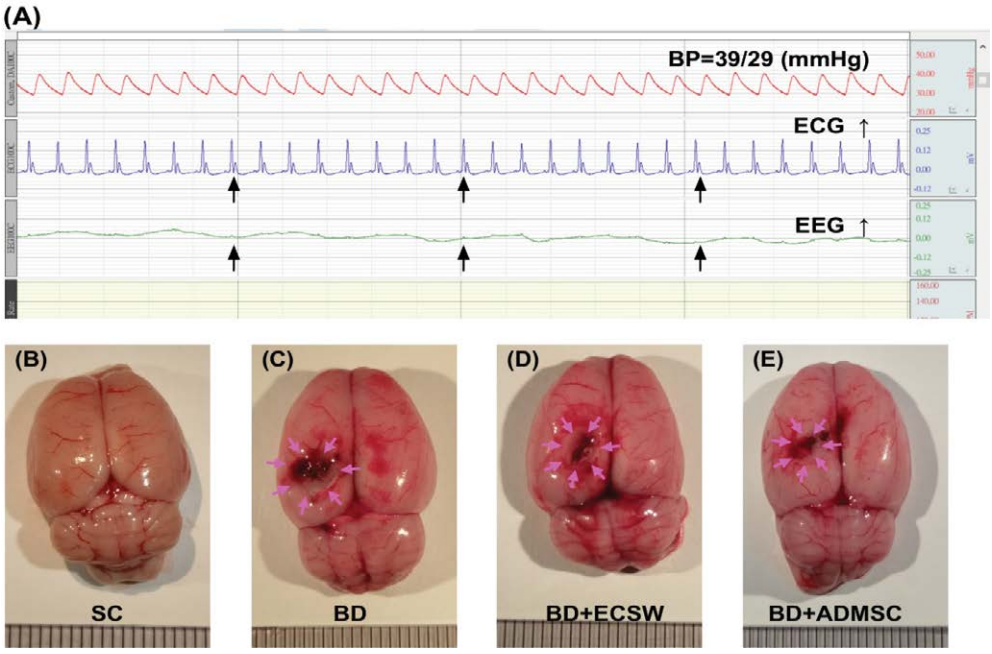

Figure 1: Pathological and electrophysiological findings at 6 h after the BD procedure

A) Illustrating the hemodynamic status (i.e., maximal mean blood pressure in all animals was recorded as 33.2 ± 4.5 mmHg/n=8), mean heart rate = 226.5 ±7.5 beats per minute (larger black arrows), and electrocardiogram recording (small black arrows) in brain death (BD) animals. B to E) Illustrating grossly anatomical picture of brain in sham control (B), BD animal (C), BD animal + ECSW (D), and BD animal + ADMSC (E). The pink arrows indicated the brain damage (i.e., by pressure compression) in BD animals. ECSW = extracorporeal shock wave; ADMSC = adipose-derived mesenchymal stem cell.

brains and hearts were harvested by 6 h after BD induction procedure for individual study.

■ Isolation of adipose tissue from additional 12 SD rate for culturing ADMSCs

The procedure and protocol for ADMSC isolation and culturing have been described in our previous reports [19, 28]. Briefly, animals were anesthetized with inhalational 2.0% isoflurane 14 days before the BD procedure to harvest the adipose tissue surrounding the epididymis. Then 200-300 μL of sterile saline was added to every 0.5 g of adipose tissue to prevent dehydration. The tissue was then cut into <1mm^3 size pieces using a pair of sharp, sterile surgical scissors. Sterile saline (37°C) was added to the homogenized adipose tissue in a ratio of 3:1 (saline: adipose tissue) by volume. Isolated allogenic ADMSCs were cultured in a 100-mm diameter dish with 10 mL DMEM culture medium containing 10% FBS for 14 days.

■ Collection of circulating and splenic blood samples

Blood samples were collected at 6 h after BD induction to measure circulating CD3/CD4$^+$, CD8/CD4$^+$, Treg$^+$ and LY6G$^+$ cells and inflammatory biomarkers by flow cytometry, and plasma levels of inflammatory mediators using ELISA. Additionally, blood samples were collected from the spleen prior to euthanizing the animals for analysis of immune cells (CD3/CD4$^+$, CD8/CD4$^+$, Treg$^+$).

■ Flow cytometric quantification of helper T cells and cytotoxic T cells

The procedure and protocol of flow cytometry for identification and quantification of circulating and splenic immune cells were based on our previous report [27]. Briefly, the peripheral blood mononuclear cells (PBMCs) and splenocytes were obtained from the tail vein using a 27# needle. PBMCs and splenocytes (1.0×10^6 cells) were triple-stained with FITC-anti-CD3 (BioLegend), PE-anti-CD8a (BD Bioscience), and PE-Cy™5 anti-CD4 (BD bioscience). The numbers of CD3$^+$CD4$^+$ helper T cells and CD3$^+$CD8$^+$ cytotoxic T cells were analyzed using flow cytometry (FC500, Beckman Coulter).

■ Immunohistochemical (IHC) and immunofluorescent (IF) staining

The procedure and protocol of IF staining have been described in detail in our previous reports [19, 27]. For IHC and IF staining, rehydrated paraffin sections were first treated with 3% H_2O_2 for 30 minutes and incubated with Immuno-Block reagent (BioSB) for 30 minutes at room temperature. Sections were then incubated with primary antibodies specifically against CD14 (1:300, Bioss), CD68 (1:100, Abcam), CD3 (1:400, Abcam) and CD4 (1:200, Novus Biologicals) while sections incubated with the use of irrelevant antibodies served as controls. Three sections of liver specimen from each rat were analyzed. For quantification, three randomly selected HPFs (200x or 400x for IHC and IF studies) were analyzed in each section. The mean number of positively-stained cells per HPF for each animal was then determined by summation of all numbers divided by 9.

■ Western blot analysis

The procedure and protocol for Western blot analysis were based on our recent reports [19,27]. In details, equal amounts (50 μg) of protein extracts were loaded and separated by SDS-PAGE using acrylamide gradients. After electrophoresis, the separated proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences). Nonspecific sites were blocked by incubation of the membrane in blocking buffer [5% nonfat dry milk in T-TBS (TBS containing 0.05% Tween 20)] overnight. The membranes were incubated with the indicated primary antibodies [cleaved poly (ADP-ribose) polymerase (PARP) (1:1000, Cell Signaling), mitochondrial Bax (1:1000, Abcam), cleaved caspase 3 (1:1000, Cell Signaling), tumor necrosis factor (TNF)-α (1:1000, Cell Signaling), nuclear factor (NF)-κB (1:600, Abcam), interleukin-1β (1:1000, Cell Signaling), myeloid differentiation primary response 88 (MyD88) (1:1000, Abcam) high-mobility group protein-1 (HMG-1) (1:1000, Cell Signaling), IL-34 (1: 500, abcam), IL-10 (1:1000, Abcam), IL-4 (1:500, Abcam), matrix metalloproteinase (MMP)-9 (1:2000, Abcam), γ-H2AX (1:1000, Cell Signaling), cytosolic cytochrome C (1:1000, BD), mitochondrial cytochrome C (1:1000, BD) and actin (1:10000, Millipore)], for 1 hour at room temperature. Horseradish peroxidase-conjugated anti-rabbit immunoglobulin IgG (1:2000, Cell Signaling) was used as a secondary antibody for one-hour incubation at room temperature. The washing procedure was repeated eight times within one hour. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences) and exposed to Biomax L film (Kodak). For the purpose of quantification, ECL signals were digitized using Labwork software (UVP).

■ Oxidative stress reaction in LV myocardium

The procedure and protocol for assessing the protein expression of oxidative stress have been detailed in our previous reports [19,27]. The Oxyblot Oxidized Protein Detection Kit was purchased from Chemicon (S7150). DNPH derivatization was carried out on 6 µg of protein for 15 minutes according to the manufacturer's instructions. One-dimensional electrophoresis was carried out on 12% SDS/polyacrylamide gel after DNPH derivatization. Proteins were transferred to nitrocellulose membranes which were then incubated in the primary antibody solution (anti-DNP 1: 150) for 2 hours, followed by incubation in secondary antibody solution (1:300) for 1 hour at room temperature. The washing procedure was repeated eight times within 40 minutes. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences) which were then exposed to Biomax L film (Kodak). For quantification, ECL signals were digitized using Labwork software (UVP). For oxyblot protein analysis, a standard control was loaded on each gel.

■ Statistical analysis

Quantitative data are expressed as means ± SD. Statistical analysis was adequately performed by ANOVA followed by Bonferroni multiple comparison procedure. Statistical analysis was performed using SAS statistical software for Windows version 13 (SAS institute, Cary, NC). A probability value<0.05 was considered statistically significant.

Results

■ Pathological and electrophysiological findings at 6 h after the BD procedure (Figure 1)

Figure 1 illustrates the pathophysiological findings (i.e., heart rate and blood pressure) in BD animals. The results showed that during the BD period (maintained for 6 h), the mean heart rate was 226.5 ±7.5 beats/min, the mean blood pressure was 33.2 ± 4.5 mmHg and the evoked potential (eV) measured by electroencephalogram (EEG) was 0.42 at baseline to 0.039 at 6h after BD procedure, suggesting the experimental model of BD was successfully created. Additionally, the gross anatomy showed that the pressure applied to the brain had caused the appearance of local ischemia and hemorrhage.

■ ELISA assessment and flow cytometric analysis for determining the circulating levels of inflammatory biomarkers at 6 h after BD induction (Figure 2)

ELISA showed that the circulating levels of TNF-α, MPO, and IL-6, three innate inflammatory mediators, were highest in BD, lowest in SC, significantly lower in BD-ECSW-ADMSC than in BD-ECSW and BD-ADMSC and significantly higher in BD-ECSW than in BD-ADMSC. Flow cytometry showed that the circulating level of Ly6G, another indicator of inflammation, showed an identical pattern to TNF-α among the five groups. These findings suggested that BD generated inflammatory mediators that released into the circulation.

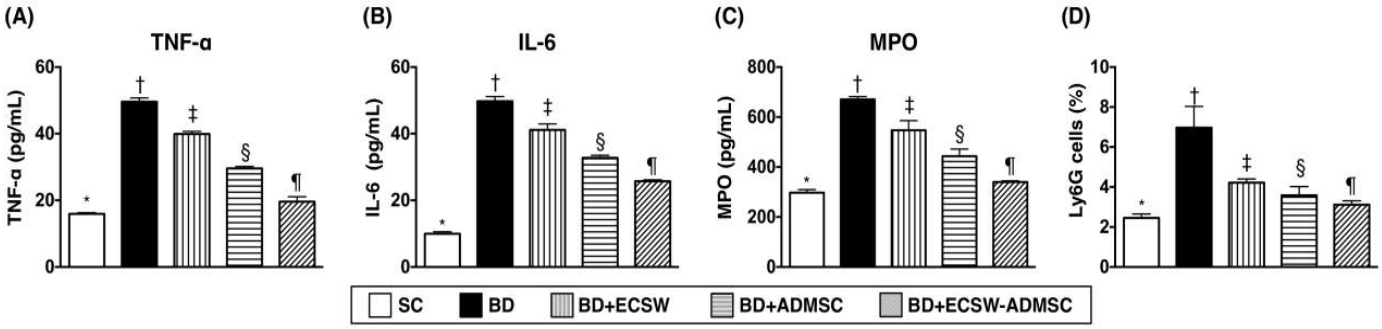

Figure 2: ELISA assessment and flow cytometric analysis for assessment of circulating levels of inflammatory mediators at 6 h after BD induction

A) ELISA analytical result of circulating level of tumor necrosis factor (TNF)-α, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. B) ELISA analytical result of circulating level of myeloperoxidase (MPO), * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. C) ELISA analytical result of circulating level of interleukin (IL)-6, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. D) Flow cytometric analysis of number of circulating level Ly6G+ cells, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.000. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n=6 for each group). Symbols (*, †, ‡, §, ¶) indicate significance at the 0.05 level. SC = sham control; BD = brain death; ECSW = extracorporeal shock wave; ADMSC = adipose-derived mesenchymal stem cell.

■ Flow cytometric analysis of circulating and splenic immune reaction (Figure 3)

Flow cytometry showed that the circulating and splenic levels of CD3+/CD4+ and CD3+/CD8+ cells, two indicators of immune cells, were highest in BD, lowest in SC, significantly lower in BD-ECSW-ADMSC than in BD-ECSW and BD-ADMSC and significantly higher in BD-ECSW than in BD-ADMSC. Additionally, the circulating and splenic levels of Treg+ cells, an immune regulatory T cell, exhibited an opposite pattern to CD3+ cells among the five groups.

■ Protein expressions of inflammatory and anti-inflammatory biomarkers in left ventricular (LV) myocardium by 6 h after BD induction (Figure 4)

Protein expressions of TNF-α, NF-κB, IL-1ß, MMP-9 and IL-6, five indicators of inflammation, were highest in BD, lowest in SC, significantly lower in BD-ECSW-ADMSC than in BD-ECSW and BD-ADMSC and significantly higher in BD-ECSW than in BD-ADMSC. The protein expressions of IL-4, IL-10 and IL-34, three indicators of anti-inflammation, displayed an opposite pattern to inflammation among the five groups.

■ Protein expressions of oxidative-stress and DNA damaged biomarkers in LV myocardium by 6 h after BD induction (Figure 5)

The protein expressions of NOX-1, NOX-2 and oxidized protein, three indicators of oxidative stress, were highest in BD, lowest in SC, significantly lower in BD-ECSW-ADMSC than in BD-ECSW and BD-ADMSC and significantly higher in BD-ECSW than in BD-ADMSC. Additionally, the protein expression of γ-H2AX, an indicator of DNA-damage, exhibited an identical pattern to oxidative stress among the five groups.

■ Protein expressions of apoptotic, anti-apoptotic and mitochondrial damaged biomarkers in LV myocardium by 6 h after BD induction (Figure 6)

Protein expressions of mitochondrial Bax, cleaved caspase 3, and cleaved PARP, three indicators of apoptosis, were highest in BD, lowest in SC, significantly lower in BD-ECSW-ADMSC than in BD-ECSW and BD-ADMSC, and significantly higher in BD-ECSW than in BD-ADMSC groups. Additionally, protein expression cytosolic cytochrome C, an indicator

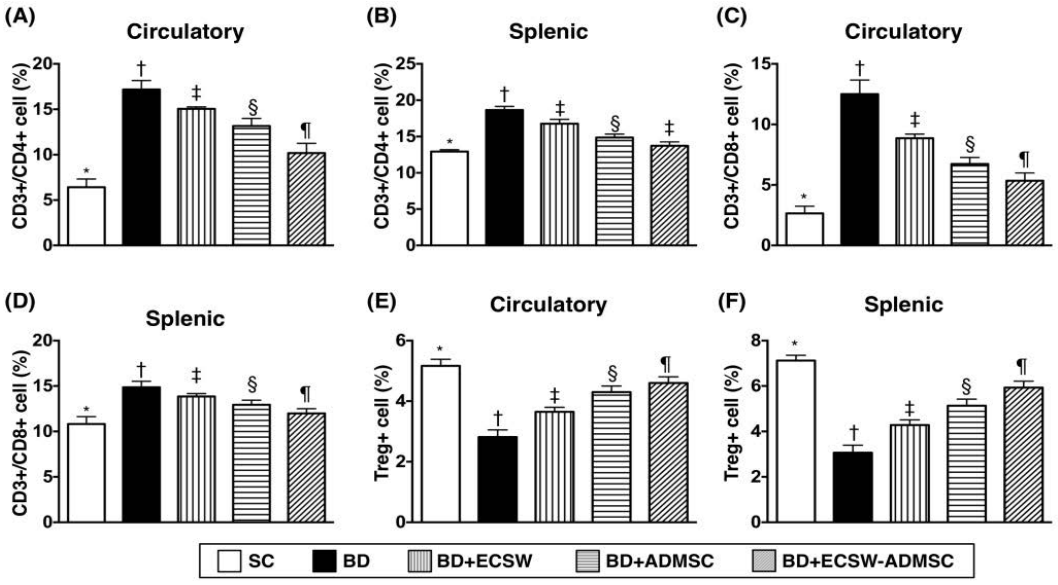

Figure 3: Flow cytometric analysis of circulating and splenic immune reaction

A) Flow cytometric analytical result of circulating number of CD3+/CD4+ cells, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. B) Flow cytometric analytical result of splenic number of CD3+/CD4+ cells, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.001. C) Flow cytometric analytical result of circulating number of CD3+/CD8+ cells, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. D) Flow cytometric analytical result of splenic number of CD3+/CD8+ cells, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.001. E) Flow cytometric analytical result of circulating number of T-reg+ cells, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. F) Flow cytometric analytical result of splenic number of T-reg+ cells, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n=6 for each group). Symbols (*, †, ‡, §, ¶) indicate significance at the 0.05 level. SC = sham control; BD = brain death; ECSW = extracorporeal shock wave; ADMSC = adipose-derived mesenchymal stem cell.

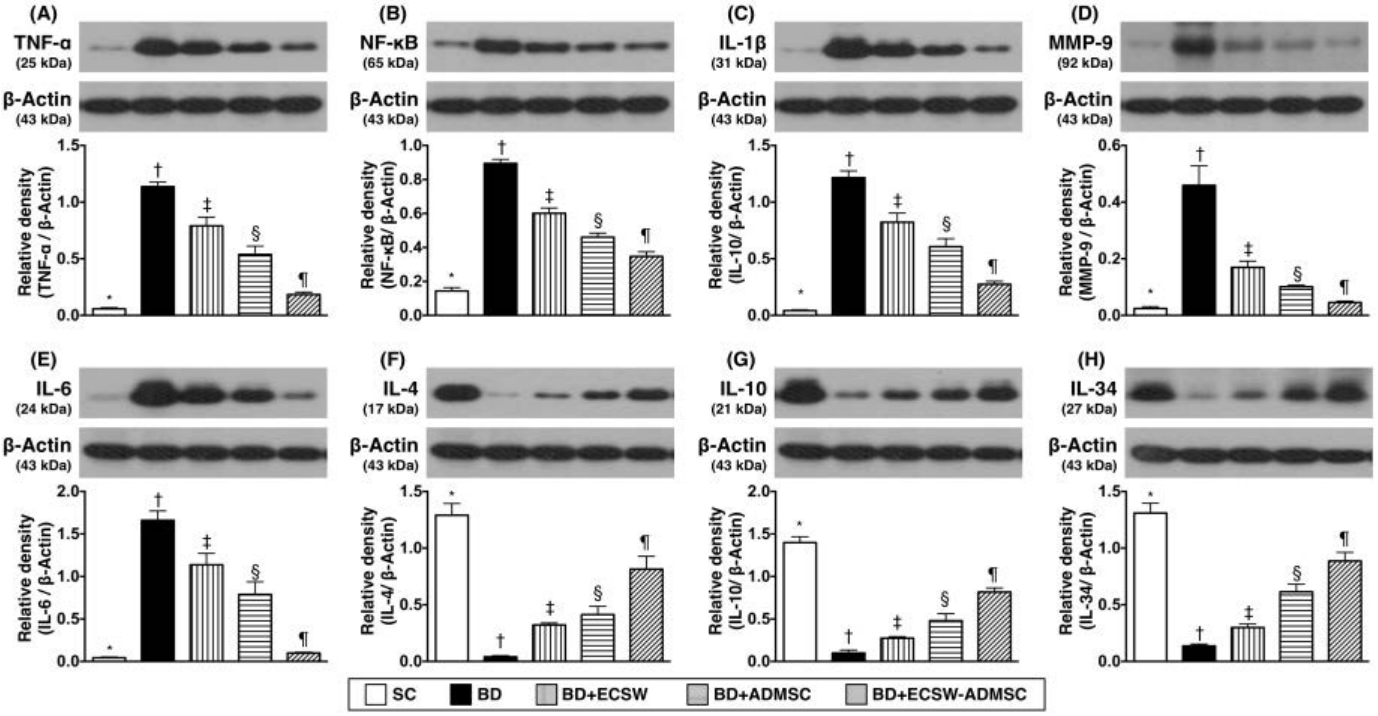

Figure 4: Protein expressions of inflammatory and anti-inflammatory mediators in left ventricular myocardium by 6 h after BD induction .
A) Protein expression of tumor necrosis factor (TNF-α), * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. B) Protein expression of nuclear factor (NF)-κB, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. C) Protein expression of interleukin (IL)-1ß, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. D) Protein expression of matrix metalloproteinase (MMP)-9, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. E) Protein expression of IL-6, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. F) Protein expression of IL-4, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. G) Protein expression of IL-10, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. H) Protein expression of IL-34, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n=6 for each group). Symbols (*, †, ‡, §, ¶) indicate significance at the 0.05 level. SC = sham control; BD = brain death; ECSW = extracorporeal shock wave; ADMSC = adipose-derived mesenchymal stem cell.

of mitochondrial damage, showed an identical pattern to apoptosis among the five groups. Protein expression of mitochondrial cytochrome C showed an opposite pattern to apoptosis among the five groups.

■ Cellular expressions of inflammation in LV myocardium by 6 h after BD induction (Figure 7)

IF staining showed that the cellular expressions of CD14 and CD68, two indicators of inflammation, were lowest in SC, highest in BD, significantly lower in BD-ECSW-ADMSC than in BD-ECSW and BD-ADMSC, and significantly higher in BD-ECSW than in BD-ADMSC.

■ Immune cell infiltration in LV myocardium by 6 h after BD induction (Figure 8)

IHC staining demonstrated that the cellular expressions of CD3 and CD4, two indicators for immune cells, were lowest in SC, highest in BD, significantly lower in BD-ECSW-ADMSC than in BD-ECSW and BD-ADMSC, and significantly higher in BD-ECSW than in BD-ADMSC.

■ Protein expression of damage-associated molecular patterns (DAMPs) -inflammatory signaling biomarkers in LV myocardium by 6 h after BD induction (Figure 9)

The protein expression of HMGB1, an essential component of DAMPs, was lowest in SC, highest in BD, significantly lower in BD-ECSW-ADMSC than in BD-ECSW and BD-ADMSC, and significantly higher in BD-ECSW than in BD-ADMSC. Additionally, protein expression of TRL-2, TLR-2 and MyD88, three downstream signals for DAMPs, exhibited an identical pattern to DAMPs among the five groups.

Discussion

Growing data have shown that BD commonly causes remote organ damage, particularly of heart

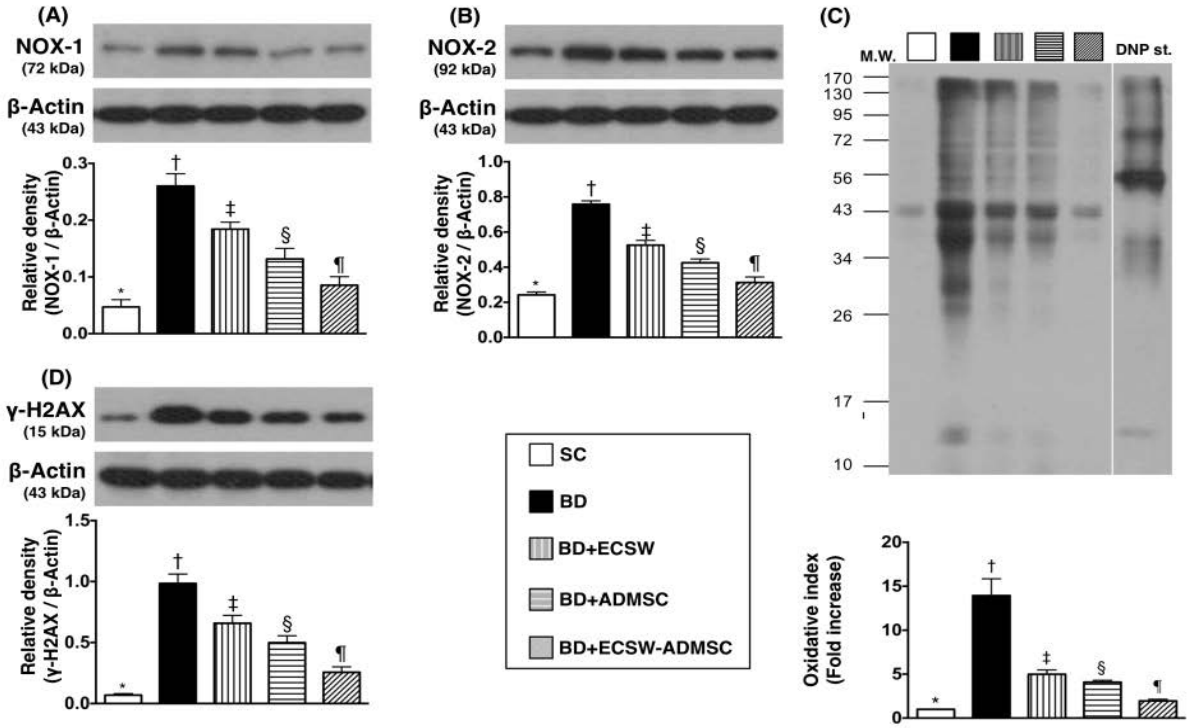

Figure 5: Protein expressions of oxidative-stress and DNA damaged biomarkers in left ventricular myocardium by 6 h after BD induction

A) Protein expressions of NOX-1, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. B) Protein expression of NOX-2, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. C) Oxidized protein expression in lung parenchyma, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. (Note: left and right lanes shown on the upper panel represent protein molecular weight marker and control oxidized molecular protein standard, respectively). M.W = molecular weight; DNP = 1-3 dinitrophenylhydrazone. D) Protein expression of γ-H2AX, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n=6 for each group). Symbols (*, †, ‡, §, ¶) indicate significance at the 0.05 level. SC = sham control; BD = brain death; ECSW = extracorporeal shock wave; ADMSC = adipose-derived mesenchymal stem cell.

[17, 37]. Although the underlying mechanism of BD-caused remote organ damage has been extensively investigated [11-17, 37], effective treatments have seldom been reported [37]. One important finding in the present study was that intravenous administration of allogenic AMDSC effectively ameliorated BD-caused remote cardiac organ damage. Interestingly, our recent study demonstrated that allogenic ADMSC therapy significantly reduced the incidence of BD-induced post heart-transplanted rejection [37]. Our current findings therefore corroborate those of our recent study [37]. Another important finding was that ECSW therapy was comparably as effective as ADMSC for alleviating BD-caused remote cardiac damage. Intriguingly, our previous study established that ECSW therapy reduced brain infarct volume in rodents after acute IS [36]. In this way, our current findings are consistent with our previous report [36]. The most important finding was that combined ECSW-ADMSC treatment was superior to either one alone at protecting the heart from BD-caused damage, highlighting a synergistic effect of this combined regimen for protecting remote organs from BD damage. Of particularly important clinical-relevant issue is that this combination may be a potential treatment modality for BD patients to avoid remote cardiac organ damage.

Abundant data has delineated that ischemic stroke (IS) and BD commonly elicit inflammatory reactions and upregulate the immune response [5-17,36]. Links between an increase in inflammatory/immune reaction and tissue/organ damage has been recognized by several previous studies [19,21,27-29,35-37]. An essential finding in the present study was that not only the localized (i.e., within the heart tissue specimen) but also the systemic molecular-cellular inflammatory and immune perturbations have been clearly identified in the rat after BD procedure. Our findings, in addition to strengthening the findings of previous studies [19,21,27-29,35-37], could explain why the

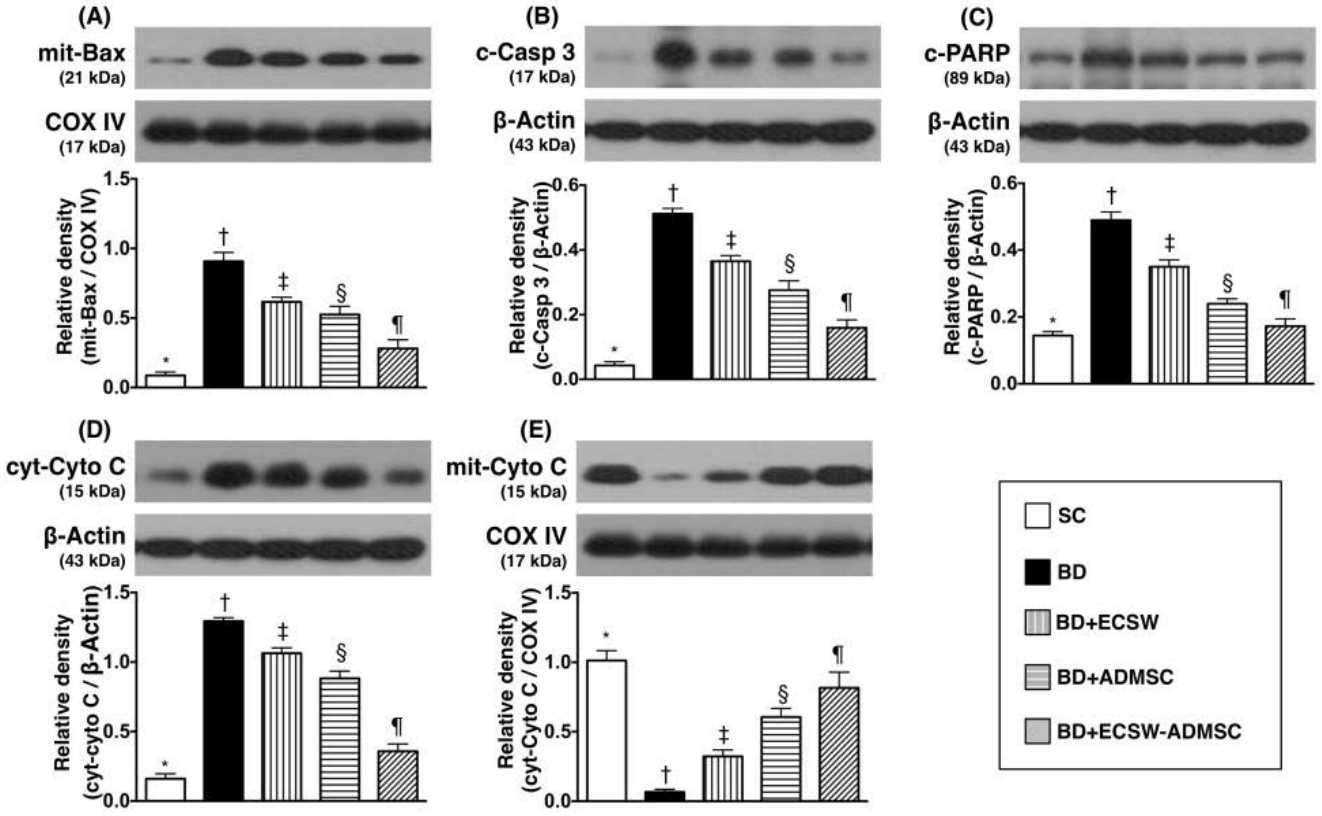

Figure 6: Protein expressions of apoptotic, anti-apoptotic and mitochondrial damaged biomarkers in left ventricular myocardium by 6 h after BD induction.

A) Protein expression of mitochondrial (mit)-Bax, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. B) Protein expression of cleaved caspase (c-Casp) 3, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. C) Protein expression of cleaved poly (ADP-ribose) polymerase (c-PARP), * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. D) Protein expression of cytosolic cytochrome (cyt-Cyto) C , * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. E) Protein expression of mitochondrial cytochrome (mit-Cyto) C, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n=6 for each group). Symbols (*, †, ‡, §, ¶) indicate significance at the 0.05 level. SC = sham control; BD = brain death; ECSW = extracorporeal shock wave; ADMSC = adipose-derived mesenchymal stem cell.

heart organ was damaged after BD procedure in rat. Of importance, ECSW or ADMSC therapy significantly, and combined ECSW-ADMSC more significantly, ameliorated the innate (i.e., inflammation) and adaptive (immune) reactions in SD rat in setting of BD.

An association between inflammation and oxidative stress, DNA damage and apoptosis has been well established in previous studies [19,27,28,35-37]. A principal finding in the present study was that oxidative-stress and DNA/mitochondrial-damage biomarkers were remarkably increased in BD compared with SC animals. Additionally, the apoptotic biomarkers were also found to be identically increased with oxidative stress in BD animals. Our findings, in addition to reinforcing the findings of previous studies [19,27,28,35-37], once again explained how BD can cause remote organ damage. Of particular importance, these biomarkers were significantly reduced by ECSW or ADMSC therapy, but more markedly reduced by combined ECSW-ADMSC therapy. In this way, our findings extended those of previous studies [19,27,28,35-37] and could, at least in part, explain why remote cardiac damage was less severe in rat after receiving ESCW-ADMSC treatment.

This study has limitations. First, without titrating the dosages of ECSW and ADMSC for an optimal effect on attenuating BD-induced remote cardiac organ damage, we did not know whether ECSW or ADMSC was superior at protecting the heart from BD injury. Second, although the short-term outcomes were attractive and promising, we did not provide the results of long-term outcome because the study period was only six hours after BD induction.

In conclusion, the results of the present study demonstrated that BD caused remote cardiac organ damage mainly via the inflammatory and immune

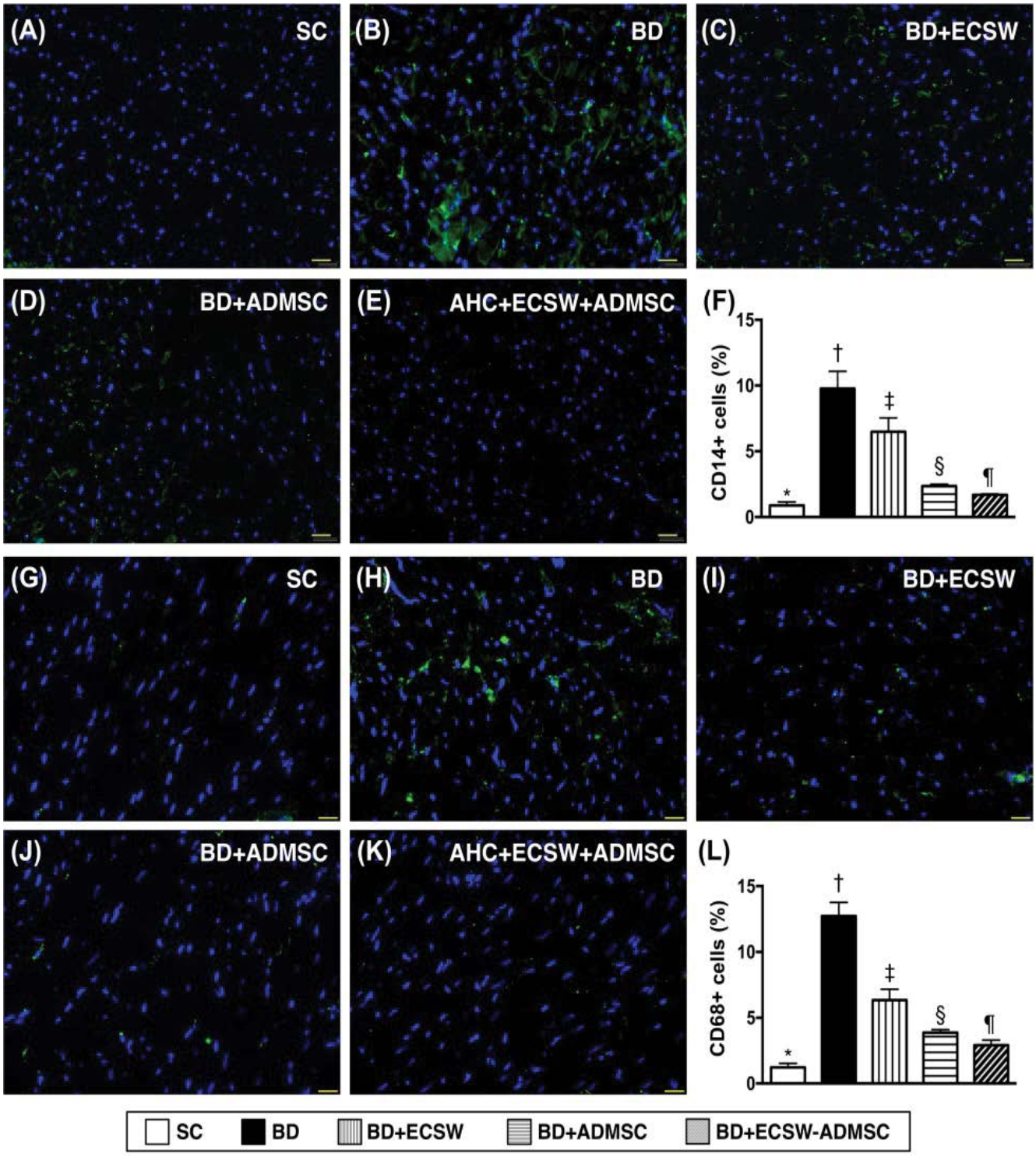

Figure 7: Inflammatory cell infiltrations in left ventricular myocardium by 6 h after BD induction.

A to E) Illustrating IF microscopic finding (400x) for identification of CD14+ cells (green color). F) Analytical result of number of CD14+ cells, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. G to K) Illustrating IF microscopic finding (400x) for identification of CD14+ cells (green color). L) Analytical result of number of CD14+ cells, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. Scale bars in right lower corner represent 20 μm. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n=6 for each group). Symbols (*, †, ‡, §, ¶) indicate significance at the 0.05 level. SC = sham control; BD = brain death; ECSW = extracorporeal shock wave; ADMSC = adipose-derived mesenchymal stem cell.

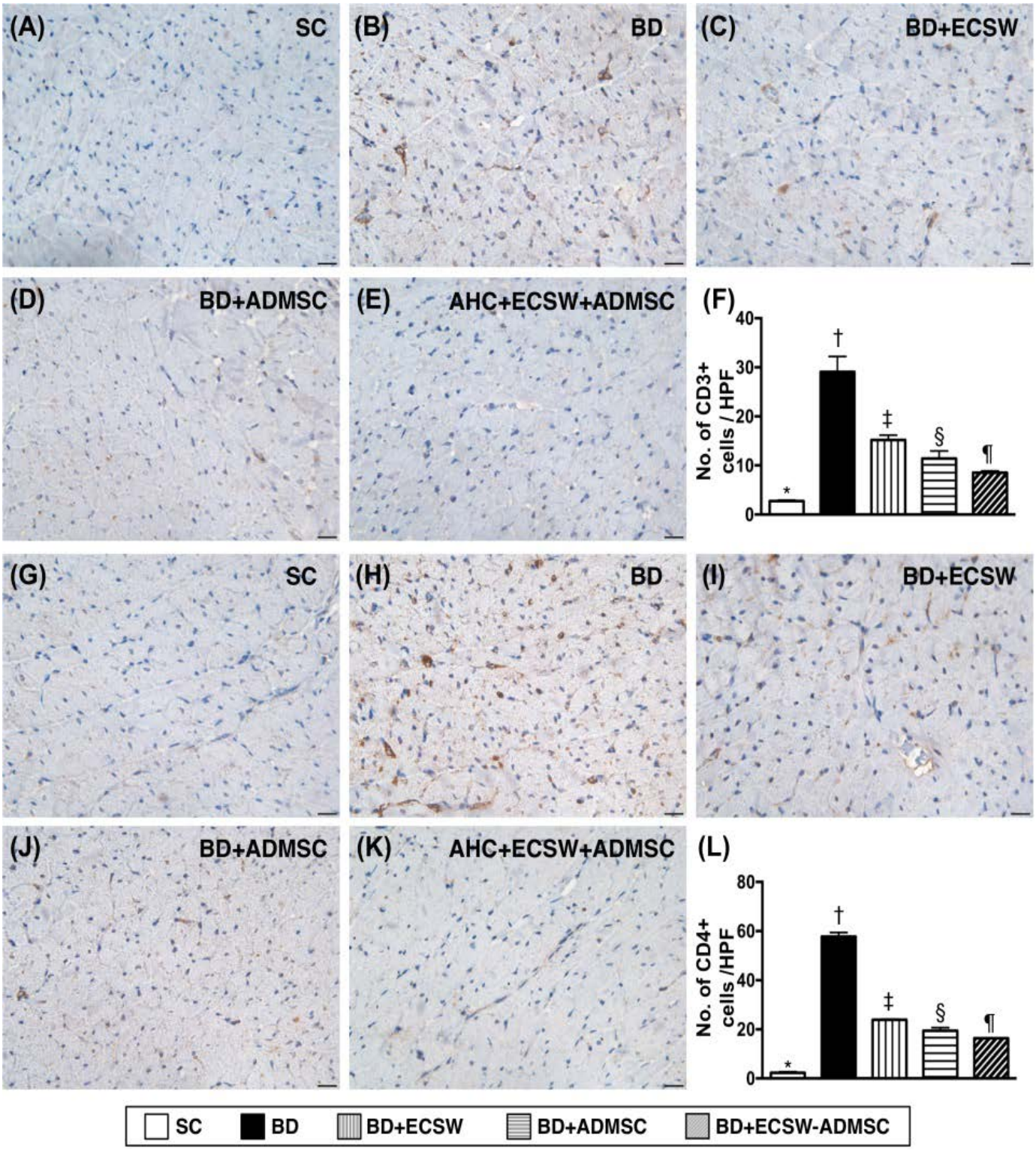

Figure 7: Immune cell infiltration in left ventricular myocardium by 6 h after BD induction

A to E) Illustrating microscopic finding of immunohistochemical (IHC) staining (400x) for identification of CD3+ cells (brown color). F) Analytical result of number of CD3+ cells, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. G to K) Illustrating microscopic finding of IHC staining (400x) for identification of CD3+ cells (brown color). L) Analytical result of number of CD4+ cells, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. Scale bars in right lower corner represent 20 µm. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n=6 for each group). Symbols (*, †, ‡, §, ¶) indicate significance at the 0.05 level. SC = sham control; BD = brain death; ECSW = extracorporeal shock wave; ADMSC = adipose-derived mesenchymal stem cell.

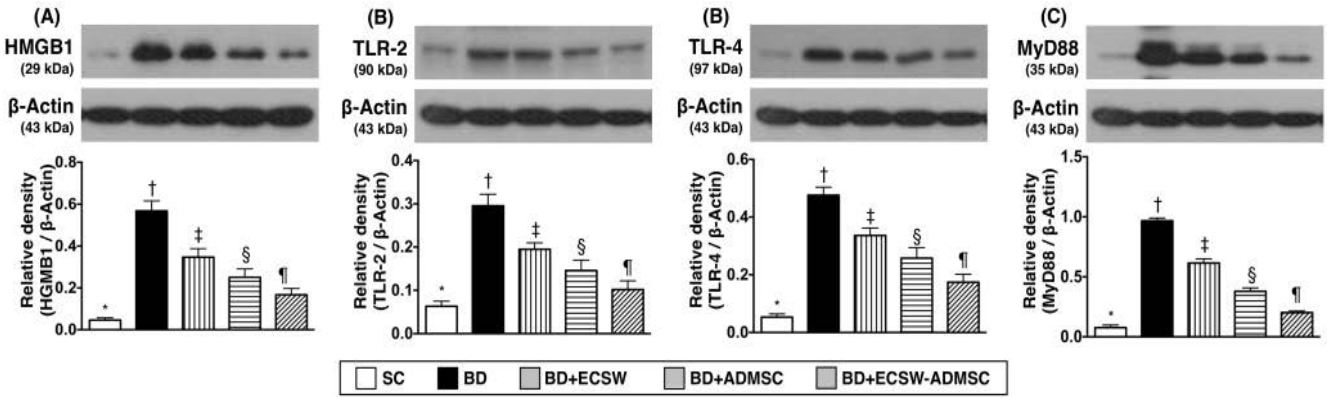

Figure 9: Protein expression of damage damage-associated molecular patterns (DAMPs)-inflammatory signaling biomarkers in LV myocardium by 6 h after BD induction

A) Protein expression of high-mobility group protein-1 (HMG-1), * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. B) Protein expression of tall-like receptor (TLR)-2, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. C) Protein expression of TLR)-4, * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. D) Protein expression of myeloid differentiation primary response 88 (MyD88), * vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n=6 for each group). Symbols (*, †, ‡, §, ¶) indicate significance at the 0.05 level. SC = sham control; BD = brain death; ECSW = extracorporeal shock wave; ADMSC = adipose-derived mesenchymal stem cell.

reactions. Combined ECSW-ADMSC offered a synergistic effect via suppressing the inflammatory/immune molecular-cellular perturbations and protecting the heart from BD-induced damage.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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