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Research article

PROTECTIVE EFFECT OF INTERMEDIN ON MYOCARDIAL CELL IN A RAT MODEL OF SEVERE ACUTE PANCREATITIS

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Abstract: Severe acute pancreatitis (SAP) is a common disease with a poor prognosis. Heart failure is one cause of SAP patient death. Intermedin (IMD) is a potent endogenous cardio-protective substance. Administration of exogenous IMD showed beneficial effects in cardiovascular diseases. The aim of this study was to investigate the myocardial damage in SAP and to determine the therapeutic potential of IMD for SAP. Using an SAP rat model, we examined endogenous IMD expression following SAP induction, and determined the effect of IMD on myocardial function, histological morphology, apoptosis-related gene expression, and prognosis. Our results indicated that the cardiac function and histological structure were significantly disrupted in SAP rats. Infusion of exogenous IMD significantly preserved cardiac function and ameliorated myocardial damage. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) revealed that myocardial apoptosis was extensively present in SAP rats, and IMD infusion led to increased expression of the pro-survival factor Bcl-2, but decreased pro-apoptotic factors Bax and caspase-3. In addition, IMD infusion also reversed the change of IMD receptor systems in SAP rat heart tissue. Furthermore, we found that IMD infusion greatly decreased mortality of SAP rats. In conclusion, administration of SAP produced therapeutic effects in SAP through modulating apoptotic and pro-survival gene expression, inhibiting myocardial apoptosis, preserving cardiac function, and

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Abbreviations used: ADM – adrenomedullin; CGRP – calcitonin gene-related peptide; CL – calcitonin receptor-like receptor; IMD – intermedin; MODS –multiple organ dysfunction syndrome; RAMP – receptor-activity-modifying protein; SAP – severe acute pancreatitis; TUNEL – terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

ultimately increasing survival. The current study suggests that IMD may be a useful therapeutic agent for SAP, and provides us an insight for a clinical trial of IMD for treating human severe acute pancreatitis.

Key words: Severe acute pancreatitis, Myocardial damage, Apoptosis, Intermedin treatment

INTRODUCTION

Acute pancreatitis (AP) is a common disease seen in the emergency room. 20% of AP is defined as severe AP (SAP), which has an unpredicTab. course with many complications and poor prognosis [1, 2]. The treatment of SAP has been greatly improved in the past decades, but its mortality is still high, ranging from 15% to 25% [3, 4]. Multiple organ dysfunction syndrome (MODS), necrotic pancreatic parenchyma and bacterial infection have been considered as the major causes of death [5, 6]. Despite extensive research, SAP still remains a challenge in our daily practice [7]. The pathogenesis of systemic complications of SAP is still elusive, and effective therapy is under intensive investigation.

Pulmonary and renal insufficiency, hepatic and gastrointestinal dysfunction, and shock are most frequently observed in SAP. Heart failure in SAP may be part of the primary MODS, or secondary to arrhythmia, cardiac shock, and metabolic dysfunction [1, 5, 8]. Myocardial injury also may contribute to the death of SAP patients [9]. Research on support cardiac function and exploration of cardio-protective agents is of great interest.

Recently, a novel bioactive peptide, intermedin (IMD), has been independently identified by two research groups [10-12]. IMD belongs to the calcitonin gene-related peptide (CGRP) / adrenomedullin (ADM) family and is a potent endogenous cardio-renal-protective substance [13]. Administration of exogenous IMD showed beneficial effects in cardiovascular diseases [12, 13]. We hypothesized that IMD is an endogenous protective factor in SAP and may be used for SAP treatment. To test this hypothesis, we examined IMD expression in an SAP rat model and determined the effect of IMD on myocardial function, histological morphology, apoptosis-related gene expression, and animal survival.

MATERIALS AND METHODS

Animals

Ten-week-old male Wistar rats (weight 160-210 g) were purchased from West China Experimental Animal Center of Sichuan University (Sichuan, China). Rats were permitted 1 week to acclimate to their environment before manipulation. All rats were housed in a climate-controlled room with an ambient temperature of 22-24°C and 12-12 h light-dark cycle. Animals were randomly assigned to control or experimental groups. All surgical procedures were completed in accordance with the guidelines on the care and use of laboratory

animals for research purposes by the West China Hospital Animal Care and Use Committee.

Acute pancreatitis model and experimental groups

Acute pancreatitis was induced using a method previously reported by Mizunuma [14] and Hegyi [15]. Briefly, rats were fasted for 12 h with free access to water prior to surgery. Six percent L-arginine hydrochloride (Sigma Aldrich, St. Louis, MO) was prepared in sterile saline (pH 7.0). The SAP group (n = 50) of rats received intraperitoneal injection of L-arginine solution (4 g/kg) twice with a 1-hour interval [15]. The SAP group was further divided into two groups, SAP alone (SAP, n = 20), and SAP plus IMD (SAP + IMD). The SAP + IMD group also received IMD_{1-53} (Phoenix Pharmaceutical Inc, Belmont, CA) through a mini-pump (100 ng/Kg/h) at the time of SAP induction, as reported previously by Ren *et al.* [16]. All animals were returned to the cages and allowed free access to food and water.

Animals were subjected to measurements of cardiovascular parameters and then sacrificed for tissue collection. Accordingly, the experimental groups were labeled with the combination of treatment and examined time. The following 5 group names are used throughout the manuscript: SAP0h (immediately after the 2^{nd} L-arginine injection), SAP12h, 12h + IMD (12 hours after the 2^{nd} L-arginine injection, without or with IMD infusion), SAP24h, and 24h + IMD (24 hours after the 2^{nd} L-arginine injection, without or with IMD infusion).

For prognostic study, we monitored the survival of three groups of rats every 12 h up to 4 days (96 hours): sham (rats received saline), SAP (rats twice received injection of L-arginine), and SAP + IMD (rats twice received injection of L-arginine + IMD infusion) (n = 10 rats/group).

Measurement of parameters for cardiac function

Animals were anesthetized via intraperitoneal injection of 1% pentobarbital sodium (45 mg/kg, body weight) and placed in the supine position on the surgical bench. Following skin preparation, the neck skin was opened and the right common carotid artery was separated from adjacent tissues and received a 0.7 cm-1 cm cut. An arterial duct (PE50, ImagJ-FIJI-Trmk, Japan) was introduced into the arterial lumen to 1.5 cm depth. The arterial pressure was recorded for 30 min via a biomedical recording system (BL-420E, Chengdu, China). Then, the arterial duct was further introduced into the left ventricle, and the following parameters were recorded: heart rate (HR), left ventricular systolic pressure (LVSP), left ventricular diastolic pressure (LVDP), maximum rate of rise of left ventricular pressure (+ Lvdp/dtmax = peak left ventricular dP/dt), and maximum rate of decrease of left ventricular pressure (- Lvdp/dtmax).

Tissue collection and histological analysis

At the end of the recording of cardiovascular parameters, animals were sacrificed with CO_2. The hearts were collected, snap frozen, and stored in liquid nitrogen. Upon histological analyses, the heart were fixed with 10% neutral

buffered formalin, paraffin-embedded, continuously sectioned (4 μm), deparaffinized in xylene, H&E (hematoxylin and eosin)-stained, dehydrated in an alcohol series (80%, 95% and 100%), coverslipped and sealed. The resultant sections were examined under a microscope (Zeiss AxioX-4, Germany) with a digital photography system (Imagepro-plus, ImagJ-FIJI-Trmk, Japan).

Radioimmunoassay (RIA) for IMD protein

Heart tissue was homogenized in a tissue grinder and mixed with 30 μl of aprotinin in 10% EDTA-Na (500 kU/ml). Following centrifugation (3500 rpm at 4°C for 10 min), the supernatants were collected and used for RIA with a commercial kit purchased from Phoenix Pharm (USA), according to the manufacturer's protocol. This kit has 100% cross-reactivity for rat and human IMD, and 32% reactivity with rat and human adrenomedullin and CGRP.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)

Heart tissue was fixed with 4% paraformaldehyde, sectioned, and stained with a commercial TUNEL kit (Boshide BioEngineer INC, Wuhan, China) following the manufacturer's protocol. Briefly, sections were pre-incubated with 3% bovine serum albumin (BSA), then incubated with the TUNEL labeling mix containing calf thymus terminal deoxynucleotidyl transferase (TdT), digoxigenin-11-2'-deoxy-uridine-5'-triphosphate (DIGdUTP), and cobalt chloride in 1X reaction buffer in distilled water. After additional incubations and washes, the resultant sections were examined under a microscope (Zeiss AxioX-4, Zeiss, Germany) with a digital photography system (ImagJ-FIJI-Trmk, Japan).

Lysate preparation and Western blotting

Using lysis buffer containing 20 mmol/l HEPES (pH 7.2), 25% glycerol, 0.42 mol/l NaCl, 1.5 mmol/l $MgCl_2$, 0.2 mmol/l EDTA, 0.5 mmol/l DTT, 0.5 mmol/l phenylmethyl sulfonylfluoride (PMSF) and a protease inhibitor cocktail (10 ml/l; Sigma-Aldrich, St. Louis, MO, USA), whole-cell extracts were prepared from frozen heart tissue. Protein concentration of the extracts was determined by the bicinchoninic acid (BCA) method. Fifty micrograms of protein were separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels (PAG) and electronically transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Richmond, CA, USA). The membrane was blocked in a standard Western blotting procedure. Briefly, the membrane was blocked with 7.5% milk in TBST buffer (20 mM Tris-HCl [pH 7.6], 137 mM NaCl, 0.05% Tween 20), then probed with a primary antibody (rabbit IgG, anti-Bcl-2, anti-Bax, and anti-caspase 3, purchased from Boshide BioEngineer INC, Wuhan China). After washing with TBST buffer, the membrane was further incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:20000, Boshide BioEngineer INC, Wuhan China). The protein bands were visualized using an enhanced chemiluminescence (ECL) detection system (Pierce,

Rockford, IL, USA). GAPDH was used as a loading control and normalization reference for quantification.

Reverse transcription (RT) and quantitative real-time PCR (QPCR)

Trizol (Invitrogen, USA) was used to extract total RNA from heart tissue. Reverse transcription was carried out with a RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas, Shenzhen, China) following the instructions provided by the manufacturer. The resultant cDNA was used as the template for real-time PCR reaction, which was performed in an FTC2000 real-time fluorescence PCR detection system (TBCHNE, USA). The primers and probes for the amplification of specific genes, Bcl-2, Bax, IMD and receptor-activity-modifying proteins (RAMP) are summarized in Suppl. Tab. 1 in Supplementary material at http://dx.doi.org/10.2478/s11658-011-0020-1. The thermal conditions were: 94^{o}C 2 min and 45 cycles of 94^{o}C 20 s, 55^{o}C 30 s, and 60^{o}C 40 s. The concentration of mRNA was quantitated with real-time PCR by normalizing its amplification to the housekeeping gene β-actin. The relative gene expression level was calculated on the basis of ΔCt, which is the difference of threshold cycle (Ct) between the specific gene and β-actin [ΔCt = Ct (specific gene) - Ct (β-actin)].

Statistical analysis

All measurements were performed at least in triplicate and results were expressed as means ± SEM. ANOVA was used for comparisons of more than two groups, and the Student-Newman-Keuls test for comparisons between two individual groups. Log-rank test was used for survival analysis. (SPSS software, version 10.0, SPSS Inc., USA) The differences were considered significant if $p < 0.05$.

RESULTS

Increase of IMD was observed in SAP rats

IMD has been detected in plasma [17] with an unknown origin. The presence of IMD in diseased myocardium has also been observed [18]. To determine whether IMD level is altered in myocardial tissue in SAP rats, we measured myocardial IMD protein using RIA assay, and IMD mRNA using quantitative PCR. As shown in Fig. 1A, IMD protein was significantly increased at 12 h after SAP induction as compared to the basal level (3860.82 ± 255.62 vs. 2864.33 ± 238.87, $p = 0.012$), and continued to increase at 24 h (4476.52 ± 268.48 vs. 2864.33 ± 238.87, $p < 0.001$). IMD mRNA expression in heart tissue showed a similar increasing pattern at 12 and 24 h after SAP induction (Fig. 1B, see also Suppl. Fig. 1 in Supplementary material at http://dx.doi.org/10.2478/s11658-011-0020-1), as indicated by the significant decrease of Δt value (11.73 ± 0.66 and 13.62 ± 0.74, respectively, vs. basal level 10.23 ± 0.63, $p < 0.001$). These results suggested that myocardial expression of IMD was upregulated in SAP rats, and might play a role in protecting myocardium from SAP-related damage.

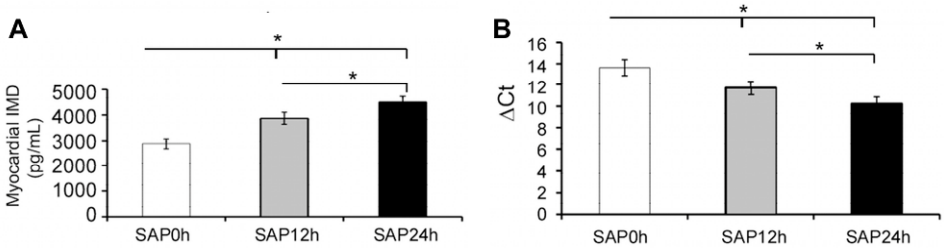

Fig. 1. IMD levels in plasma and heart tissue following the induction of SAP. A – Heart tissues were collected immediately (SAP0h), after 12 h (SAP12h) and 24 h (SAP24h) following SAP induction, and subjected to RIA assay (*, $p < 0.05$). B – Using QPCR, IMD mRNA in myocardial tissue was measured in three groups of rats, SAP0h, SAP12h and SAP24h. See also Suppl. Fig. 1. The bars represent ΔCt values between IMD and β-actin (*, $p < 0.05$, mean ± SEM, n = 9 rats/group).

IMD preserved myocardial function and histological morphology in SAP rats

Since the administration of IMD showed a beneficial effect in myocardial vascular disease [12, 13, 17], we examined whether exogenous IMD was able to enhance the myocardial function and preserve histological morphology in SAP rats. As shown in Tab. 1, five parameters representing the heart function were recorded in five groups of SAP rats. Compared to the basal condition (SAP0h), SAP rats without IMD treatment exhibited a significant decrease of heart function at both 12 h and 24 h after SAP induction (SAP12h and SAP24h). In contrast, the heart function of SAP rats treated with IMD was significantly preserved as compared to that of those without IMD treatment. However, the IMD-treated SAP rats still showed significantly decreased cardiac function as compared to the basal condition. These data suggested that cardiac function impairment was present in SAP rats, and that IMD was capable of preserving heart function significantly.

Tab. 1. Comparisons of parameters for heart function in SAP rats without and with IMD treatment. Data are expressed as mean ± SEM. HR – heart rate; LVSP – left ventricular systolic pressure; LVDP – left ventricular diastolic pressure; +Lvdp/dtmax – maximum rate of rise of left ventricular pressure (peak left ventricular dP/dt); -Lvdp/dtmax – maximum rate of decrease of left ventricular pressure. * – $p < 0.05$, comparison to control (SAP0h); # – $p < 0.05$, comparison between SAP12h and 12h + IMD; † – $p < 0.05$, comparison between SAP24h and 24h + IMD.

Parameters	SAP0h	SAP12h	SAP24h	12h + IMD	24h + IMD
HR	403 ± 11	411 ± 11	415 ± 7*	404 ± 8	404 ± 12
LVSP/KPa	113 ± 6	95 ± 6*	79 ± 7*	107 ± 6#	97 ± 7*†
LVDP/Kpa	2.26 ± 0.37	4.15 ± 0.59*	5.87 ± 0.59	2.89 ± 0.86#*	3.66 ± 1.20*†
+Lvdp/dtmax (mmHg)	6995 ± 709	5584 ± 760*	4125 ± 657*	6329 ± 690#	5484 ± 757*†
-Lvdp/dtmax (mmHg)	4066 ± 741	3218 ± 551*	2676 ± 564*	3757 ± 510#	3412 ± 799†

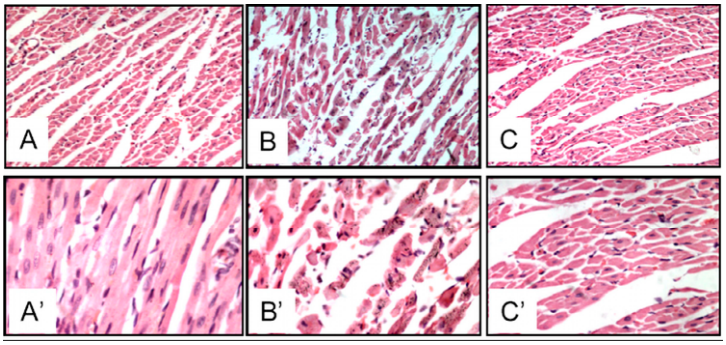

Fig. 2. Myocardial morphology of SAP rats without or with IMD treatment. Representative micrographs of HE-stained sections of rat heart tissue show the histological changes at 24 h after SAP induction. Compared to the basal condition (SAP0h, A/A′), SAP rats without IMD treatment (SAP24h, B/ B′) showed more severe myocardial damage than IMD-treated SAP rats (SAP 24h + IMD, C/C′). Original mgnifications: 100x for A-C and 400x for A′-C′.

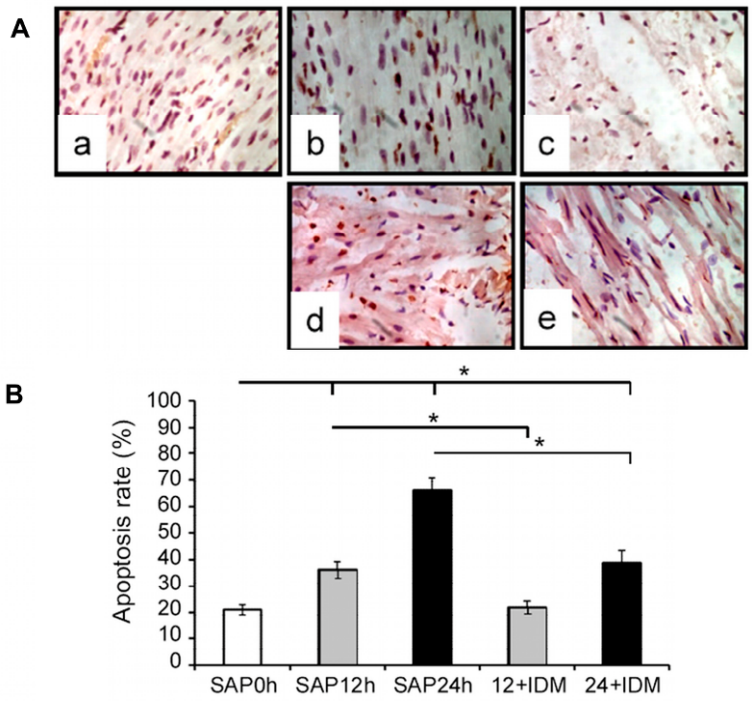

Fig. 3. Apoptosis in myocardial tissue of SAP rats without or with IMD treatment. A – Representative micrographs of TUNEL-stained sections of rat heart tissue show the apoptosis at 12 h (b and d) and 24 h (c and e) after SAP induction without IMD (b and c) or with (d and e) treatment. Compared to the basal condition (SAP0h, a), SAP rats without IMD treatment (b and c) showed more apoptotic myocytes than IMD-treated SAP rats (d and e). Original magnifications: 200x. B – Quantitative results for the apoptotic cells in five groups of rats (*, $p < 0.05$, mean ± SEM, n = 9 rats/group).

To confirm myocardial damage in SAP rats, we further performed histological analysis of hearts from SAP rats without or with IMD treatment at 24 h after SAP induction. As shown in Fig. 2, while the myocardial tissue showed a relatively normal morphology immediately after SAP induction (Fig. 2A and A′), SAP rats without IMD treatment exhibited remarkable structural and cellular (Fig. 2B) changes: disruption and separation of myofiber, endothelial and capillary damage, cellular edema and intensive infiltration (Fig. 2B). In contrast, the myocardial damage in the IMD-treated group was mild (Fig. 2C): relatively normal structure, as well as less endothelial/capillary damage, cellular edema, cellular edema and infiltration. These results indicated that the myocardial damage was present in SAP rats, which may be underlying the decrease of heart function. IMD preserved myocardial function and histological morphology in SAP rats.

Administration of IMD reduced myocardial apoptosis in SAP rats

While necrosis may be one mechanism of myocardial death in SAP, it was found that myocardial cells undergo apoptosis under oxygen depletion and other detrimental conditions [19, 20]. To determine whether myocardial apoptosis occurs in SAP rats and whether IMD has a protective effect on myocardial cells, we examined myocardial apoptosis using TUNEL staining. The results indicated that intensive myocardial apoptosis was present in SAP rats (Fig. 3A, b and c), which could be inhibited by IMD administration (Fig. 3A, d and e). The quantitative results (Fig. 3B) indicated that a significant increase of the myocardial apoptosis rate was observed at 12 h after SAP induction, and continued increasing (up to 65.88 at 24 h). SAP rats with IMD treatment showed a significantly reduced number of apoptotic cells as compared to rats without treatment at the same time points (SAP12 + IMD vs. SAP12h, $p = 0.012$; SAP24h + IMD vs. SAP24h, $p = 0.019$). These data suggest that extensive myocardial apoptosis was present in SAP rats and IMD may mediate a protective effect through inhibiting myocardial apoptosis.

IMD inhibits myocardial apoptosis through regulating pro- and anti-apoptotic factors

Hypoperfusion and oxygen depletion cause activation of the apoptotic cascade in cardiomyocytes [19]. To determine whether IMD administration influences the apoptotic cascade, we examined gene expression for the pro-survival factor Bcl-2, and the pro-apoptotic factor Bax (Fig. 4). Our QPCR results indicated that Bcl-2 was decreased (Fig. 4A) but Bax was increased (Fig. 4B) in SAP rats without IMD treatment. Surprisingly, it appeared that IMD treatment was able to completely prevent the gene expression of Bcl-2 and Bax.

In addition, to confirm whether the gene expression results in a corresponding alteration at the protein level, we measured proteins Bcl-2, Bax, and caspase-3, using Western blotting (Fig. 5A). The quantification of band density was performed and normalized with GAPDH as a loading control. The protein level

changes of Bcl-2 and Bax following SAP induction and IMD treatment were consistent with mRNA data (Fig. 5, B and C). Caspase-3 showed a similar tendency as Bax [20]. Collectively, these results suggest that shifting the apoptotic cascade to a pro-survival direction may underlie the beneficial mechanisms of IMD.

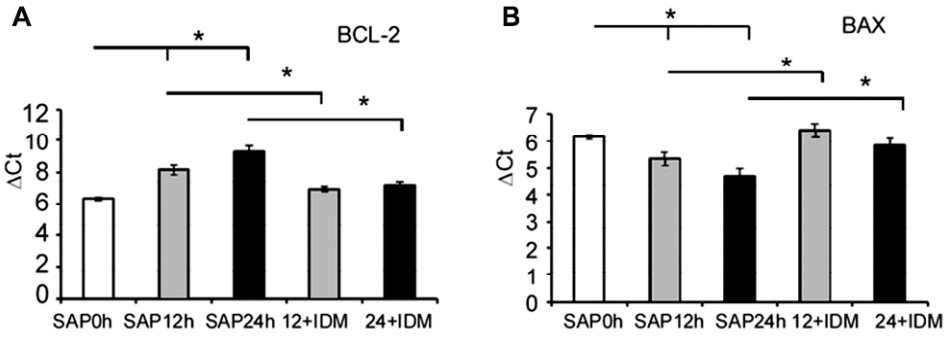

Fig. 4. Gene expression of Bcl-2 and Bax in myocardial tissue of SAP rats without or with IMD treatment. Using QPCR, Bcl-2 (A) and Bax (B) mRNA in myocardial tissue were measured in five groups of rats, SAP0h, SAP12h, 12h + IMD, SAP24h and 24h + IMD. The bars represent ΔCt values between a specific gene and β-actin gene (*, $p < 0.05$, mean ± SEM, n = 10 rats/group, 3 replicates/PCR reaction).

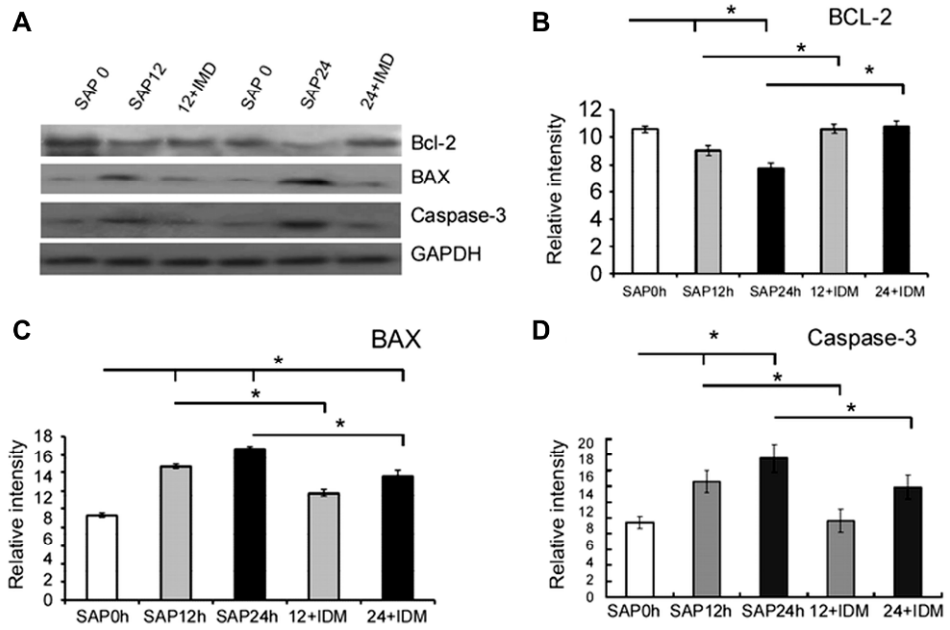

Fig. 5. Bcl-2, Bax and caspase-3 protein in myocardial tissue of SAP rats without or with IMD treatment Using Western blotting (A), Bcl-2 (B), Bax (C) and caspase-3 (D) proteins in myocardial tissue were determined in five group of rats, SAP0h, SAP12h, 12h + IMD, SAP24h and 24h + IMD. The bars represent the relative band density in which each specific protein was normalized with GAPDH (*, $p < 0.05$, mean ± SEM, n = 10 rats/group).

Expression of receptor-activity-modifying proteins (RAMPs) in SAP rats without or with IMD treatment

IMD interacts with a receptor complex consisting of calcitonin receptor-like receptor (CL) and RAMPs. Three subsets of RAMP have been defined, RAMP1, RAMP2, and RAMP3, which have differential expression levels in different tissues and distinguish signaling transduction activity [18, 21, 22]. To determine the expression of RAMPs in heart tissue, we examined their mRNA expressions using QPCR. Our results indicated that RAMP1 and RAMP2 in rat heart tissue were significantly increased at 24 h following SAP induction, and their levels were changed in SAP rats with IMD treatment (Fig. 6 A and B). The expression of RAMP3 did not change in SAP rats, nor was it impacted by IMD administration (Fig. 6C). These results suggested that RAMP1 and RAMP2 may be involved in an IMD-mediated protective mechanism in a cooperative manner with the increase of endogenous IMD, and infusion of exogenous IMD appeared to inhibit the upregulation of RAMP1 and RAMP2 in the heart of SAP rats.

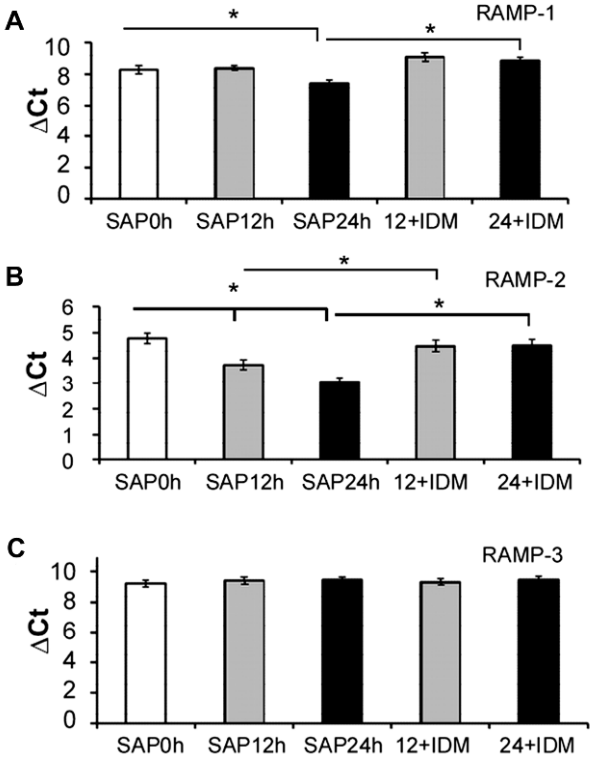

Fig. 6. Gene expression of RAMP1, RAMP2 and RAMP3 in myocardial tissue of SAP rats without or with IMD treatment. Using QPCR, mRNA expression of RAMP1 (A), RAMP2 (B) and RAMP3 (C) in myocardial tissue was measured in five groups of rats, SAP0h, SAP12h, 12h + IMD, SAP24h and 24h + IMD. The bars represent the ΔCt value between IMD and β-actin (*, $p < 0.05$, mean ± SEM, n = 10 rats/group, 3 replicates/PCR reaction).

IMD administration increased the survival of SAP rats

Lastly, to determine whether IMD administration exhibits a therapeutic effect in terms of prognosis of SAP rats, we monitored three groups of rats: sham SAP, SAP without treatment, and SAP with IMD treatment up to 96 hours. Encouragingly, IMD increased the survival rate of SAP rats (Fig. 7, 90% in IMD group, vs. 60% in SAP group, $p = 0.043$), suggesting that IMD may be a promising therapeutic agent for SAP.

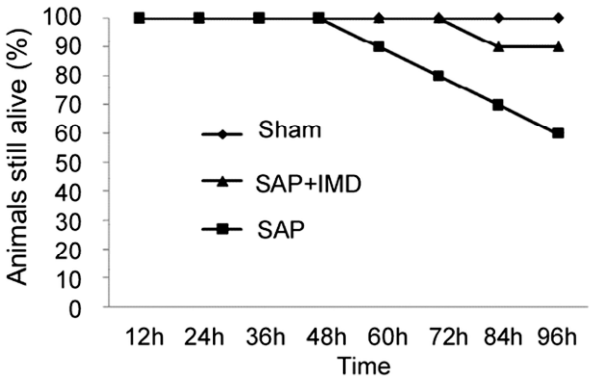

Fig. 7. Survival of SAP rats without or with IMD treatment. Survival of three groups of rats – sham rats, SAP rats without treatment, and SAP rats with IMD treatment – was monitored every 12 hours. The survival percentage in each group was calculated and plotted in a curve (n = 10 rats/group, $p < 0.05$).

DISCUSSION

The primary goal of this study was to investigate the myocardial damage and therapeutic potential of IMD in a rat SAP model. Encouragingly, our data showed that IMD was an endogenous factor that increased in SAP rats and exogenous IMD infusion resulted in beneficial effects such as modulating apoptotic and pro-survival gene expression, inhibiting myocardial apoptosis, and most importantly, decreasing mortality. To our knowledge, we for the first time showed the beneficial effects of IMD in severe acute pancreatitis from the molecular mechanism to the prognosis. Our data suggested that IMD is a promising agent for the treatment of SAP.

Because of the urgent and dynamic course of SAP, it has been challenging to study SAP in patients. In the present study, we used a well-characterized animal SAP model in which pancreatitis is induced by L-arginine injection [15, 19]. Multiple organ dysfunction syndrome (MODS) is one of the major causes of patient death. While other system failures have been extensively studied [24], the cardiac damage and intervention for preservation of heart function have been relatively less emphasized in the management of SAP [8]. Therefore, we focused on the evaluation of cardiac change in the present study. Our results indicated that cardiac function was significantly impaired at 12 h after the induction of

SAP. The heart rate was increased without statistical difference, but the cardiac contractility was greatly comprised, as indicated by the significant decrease of systolic ventricular pressure and the speed of pressure change. At 24 h after SAP induction, heart rate was significantly increased and contractility was further exacerbated. The administration of exogenous IMD successfully preserved cardiac functions at 12 h and 24 h after SAP induction. However, IMD could not completely prevent the cardiac damage, since SAP rats with IMD infusion still showed a significant decrease of cardiac function at 24 h. These data collectively suggested that cardiac impairment was present in SAP rats, which could be partially but significantly corrected by infusion of IMD.

To determine the underlying mechanisms of cardiac impairment and cardio-protective effect of IMD in SAP rats, we examined cardiac histological changes and myocardial apoptosis at two time points (12 h and 24 h after SAP induction). Consistent with the decrease of cardiac function, structural disruption and cell death were observed in the SAP rats at 12 h and further potentiated at 24 h. The TUNEL staining results indicated that apoptosis occurred in SAP rats. It appeared that IMD's cardio-protective effect was achieved through modulating the expression of apoptosis-related factors. It was generally thought that the cardiovascular protective effect of IMD is derived from its vasodilating function, which attenuates vasoconstriction and ischemic damage [11, 13, 24, 25]. In addition, it has been shown that deprivation of oxygen and ischemic reperfusion resulted in apoptosis of cardiomyocytes [23, 26]. Taken together, we speculate that enhancement of blood flow to the heart may be responsible for the reduction of myocardial apoptosis following IMD treatment. Further study of blood flow change in the coronary artery following IMD administration is necessary to address this possibility.

Although the exact mechanism of IMD's effect remains unclear, accumulating evidence supports the idea that IMD exerts cardio-protective function through multiple pathways. It has been well established that calcitonin/CGRP family members bind to the CL/RAMP receptor systems, which subsequently initiate multiple intracellular signaling cascades [13]. These pathways synergically enhance cardiac function, including directly increasing myocardial contractility through the PKC, PKA and IP3 pathway, promoting cardiomyocyte growth through the PKC, PKA and MAPK/ERK pathway, and reducing deleterious consequences of oxidative stress in pathological conditions [17]. In the present study, we utilized QPCR to measure the gene expression of the three RAMPs, and it appeared that both RAMP1 and RAMP2 were significantly increased in SAP rats, and exogenous IMD reversed their changes. RAMP3 was not altered in SAP rats, and administration of IMD had no impact on its level either. This is probably due to its lower expression in cardiomyocytes [17]. Thus far, we do not know the mechanism underlying the changes of RAMP expression in SAP rats and how IMD influences their expression. Further studies are needed to elucidate the contribution of the above-mentioned pathways and the roles of RAMP in IMD-mediated beneficial effects in SAP.

From the clinical perspective, the most important indicator for a drug's therapeutic value is its impact on the prognosis. Therefore, to estimate the potential application of IMD in the treatment of SAP, we did a short-term follow-up study of SAP survival after IMD treatment. We could not monitor the SAP rats in a longer period, but a 30% reduction of mortality at day 4 after SAP induction strongly supported the idea that IMD is a promising therapeutic agent for SAP treatment. Our data from this study provided an insight for a clinical trial of IMD for SAP patients.

In addition to L-arginine-induced pancreatitis, several other animal models for pancreatitis have been developed and reported in the literature. The following are a few of the most commonly used models: secretagogue-induced (cholecystokinin or its analog, cerulean), diet-induced (alcohol diet and choline-deficient/ethionine-supplemented diet), bile duct ligation, and pancreatic duct perfusion/injection [27, 28]. Administration of L-arginine induces acute pancreatitis, which has been widely used by researchers, but its mechanism of pancreatitis induction remains unknown. In the current study, we observed that there was a 20% apoptosis rate in the SAP0h group. We do not know whether this is a phenomenon specifically associated with this model or generally present in other SAP models. Regarding the mechanism of apoptosis in the SAP0h group, we speculate that it may be derived from the experimental procedure, because we did not observe many apoptotic cells in the staining of normal rat heart tissue. Apoptosis may occur at one or both stages of the procedure. The first stage was from the first injection of L-arginine to the tissue collection. In this case, the myocardial damage may occur as early as the initiation of SAP induction, and the 20% apoptosis may be an accumulative result of 2 hours. The second stage was from the second injection of L-arginine to the tissue collection, in which the myocardial damage may occur during 1-hour measurement of heart function parameters. Our findings from the current study need to be validated in another model. It is very encouraging that IMD mediated a therapeutic effect in this rat SAP model. However, the following issues remain to be addressed. First, we used an IMD antibody with 32% reactivity with adrenomedullin and CGRP in the RIA assay; this cross-reactivity may produce a false increase if adrenomedullin is increased. When a specific antibody for IMD without cross-reactivity becomes available, it is necessary to use it to confirm the current finding. In addition, there are three bioactive forms of IMD: IMD1-47, IMD8-47 and IMD1-53. Because the roles of IMD1-47 and IMD8-47 are controversial and IMD1-53 has been shown to be cardio-protective [27, 28], we determined the therapeutic potential of IMD1-53 for SAP, but the effects of the other two forms of IMD on SAP remain to be investigated. Lastly, although the serum level of IMD in humans has been reported previously [17], there is no information regarding the IMD level of SAP patients in the literature. As a continuation of the current study, we are collecting samples from SAP patients to expand our study to humans.

Competing interests. The authors declare that they have no competing interests.

Authors' contributions. Xiaodong and Zhi participated in research design. Other authors contributed to the performance of laboratory measurements, data collection and analysis. Xiaodong and Zhi wrote the manuscript.

REFERENCES

1. Waldthaler, A. Schutte, K. and Malfertheiner, P. Causes and mechanisms in acute pancreatitis. **Dig. Dis.** 28 (2010) 364-372.
2. Harper, S.J. and Cheslyn-Curtis, S. Acute pancreatitis. **Ann. Clin. Biochem.** 48 (2011) 23-37.
3. McKay, C.J. and Imrie, C.W. The continuing challenge of early mortality in acute pancreatitis. **Brit. J. Surg.** 91 (2004) 1243-1244.
4. Gravante, G., Garcea, G., Ong, S.L., Metcalfe, M.S., Berry, D.P., Lloyd, D.M. and Dennison, A.R. Prediction of mortality in acute pancreatitis: a systematic review of the published evidence. **Pancreatology** 9 (2009) 601-614.
5. Isenmann, R., Rau, B. and Beger, H.G. Bacterial infection and extent of necrosis are determinants of organ failure in patients with acute necrotizing pancreatitis. **Brit. J. Surg.** 86 (1999) 1020-1024.
6. Takeda, K., Matsuno, S., Sunamura, M. and Kobari, M. Surgical aspects and management of acute necrotizing pancreatitis: recent results of a cooperative national survey in Japan. **Pancreas** 16 (1998) 316-322.
7. Pandol, S.J., Saluja, A.K., Imrie, C.W. and Banks, P.A. Acute pancreatitis: bench to the bedside. **Gastroenterology** 132 (2007) 1127-1151.
8. Darvas, K., Futo, J., Okros, I., Gondos, T., Csomos, A. and Kupcsulik, P. [Principles of intensive care in severe acute pancreatitis in 2008]. **Orv. Hetil.** 149 (2008) 2211-2220.
9. Yegneswaran, B., Kostis, J.B. and Pitchumoni, C.S. Cardiovascular manifestations of acute pancreatitis. **J. Crit. Care** 26 (2011) 225.e11-8.
10. Chang, C.L., Roh, J. and Hsu, S.Y. Intermedin, a novel calcitonin family peptide that exists in teleosts as well as in mammals: a comparison with other calcitonin/intermedin family peptides in vertebrates. **Peptides** 25 (2004) 1633-1642.
11. Takei, Y., Hyodo, S., Katafuchi, T. and Minamino, N. Novel fish-derived adrenomedullin in mammals: structure and possible function. **Peptides** 25 (2004) 1643-1656.
12. Takei, Y., Inoue, K., Ogoshi, M., Kawahara, T., Bannai, H. and Miyano, S. Identification of novel adrenomedullin in mammals: a potent cardiovascular and renal regulator. **FEBS Lett.** 556 (2004) 53-58.
13. Roh, J., Chang, C.L., Bhalla, A., Klein, C. and Hsu, S.Y. Intermedin is a calcitonin/calcitonin gene-related peptide family peptide acting through the calcitonin receptor-like receptor/receptor activity-modifying protein receptor complexes. **J. Biol. Chem.** 279 (2004) 7264-7274.
14. Mizunuma T, K.S. and Kishino Y. Effects of injecting excess arginine on rat pancreas. **J. Nutr.** 114 (1984) 467-471.
15. Hegyi, P., Rakonczay, Z., Jr., Sari, R., Gog, C., Lonovics, J., Takacs, T. and Czako, L. L-arginine-induced experimental pancreatitis. **World J. Gastroenterol.** 10

(2004) 2003-2009.
16. Ren, Y.S., Yang, J.H., Zhang, J., Pan, C.S., Yang, J., Zhao, J., Pang, Y.Z., Tang, C.S. and Qi, Y.F. Intermedin 1-53 in central nervous system elevates arterial blood pressure in rats. **Peptides** 27 (2006) 74-79.
17. Bell, D. and McDermott, B.J. Intermedin (adrenomedullin-2): a novel counter-regulatory peptide in the cardiovascular and renal systems. **Br. J. Pharmacol.** 153 Suppl 1 (2008) S247-262.
18. Zhao, Y., Bell, D., Smith, L.R., Zhao, L., Devine, A.B., McHenry, E.M. Nicholls, D.P., and McDermott, B.J. Differential expression of components of the cardiomyocyte adrenomedullin/intermedin receptor system following blood pressure reduction in nitric oxide-deficient hypertension. **J. Pharmacol. Exp. Ther.** 316 (2006) 1269-1281.
19. Cobelens, P.M., van Putte, B.P., Kavelaars, A., Heijnen, C.J. and Kesecioglu, J. Inflammatory consequences of lung ischemia-reperfusion injury and low-pressure ventilation. **J. Surg. Res.** 153 (2009) 295-301.
20. Yang, K.M., Pyo, J.O., Kim, G.Y., Yu, R., Han, I.S., Ju, S.A., Kim, W.H. and Kim, B.S. Capsaicin induces apoptosis by generating reactive oxygen species and disrupting mitochondrial transmembrane potential in human colon cancer cell lines. **Cell. Mol. Biol. Lett.** 14 (2009) 497-510.
21. Nagae, T., Mukoyama, M., Sugawara, A., Mori, K., Yahata, K., Kasahara, M., Suganami, T., Makino, H., Fujinaga, Y., Yoshioka, T., Tanaka, I. and Nakao, K. Rat receptor-activity-modifying proteins (RAMPs) for adrenomedullin/CGRP receptor: cloning and upregulation in obstructive nephropathy. **Biochem. Biophys. Res. Commun.** 270 (2000) 89-93.
22. Jia, Y.X., Yang, J.H., Pan, C.S., Geng, B., Zhang, J., Xiao, Y., Zhao, J., Gerns, H., Yang, J., Chang, J.K., Wen, J.K., Tang, C.S. and Qi, Y.F. Intermedin1-53 protects the heart against isoproterenol-induced ischemic injury in rats. **Eur. J. Pharmacol.** 549 (2006) 117-123.
23. Gottlieb, R.A., Burleson, K.O., Kloner, R.A., Babior, B.M. and Engler, R.L. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. **J. Clin. Invest.** 94 (1994) 1621-1628.
24. Sargent, S. Pathophysiology, diagnosis and management of acute pancreatitis. **Br. J. Nurs.** 15 (2006) 999-1005.
25. Yang, J.H., Jia, Y.X., Pan, C.S., Zhao, J., Ouyang, M., Yang, J., Chang, J.K., Tang, C.S. and Qi, Y.F. Effects of intermedin(1-53) on cardiac function and ischemia/reperfusion injury in isolated rat hearts. **Biochem. Biophys. Res. Commun.** 327 (2005) 713-719.
26. Holleyman, C.R. and Larson, D.F. Apoptosis in the ischemic reperfused myocardium. **Perfusion** 16 (2001) 491-502.
27. Pan, C.S., Yang, J.H., Cai, D.Y., Zhao, J., Gerns, H., Yang, J., Chang, J.K., Tang, C.S. and Qi, Y.F. Cardiovascular effects of newly discovered peptide intermedin/adrenomedullin 2. **Peptides** 26 (2005)1640-1646.
28. Grossini, E., Molinari, C., Mary, D.A., Uberti, F., Caimmi, P.P. and Vacca, G. Intracoronary intermedin 1-47 augments cardiac perfusion and function in anesthetized pigs: role of calcitonin receptors and beta-adrenoreceptor-mediated nitric oxide release. **J. Appl. Physiol.** 107 (2009) 1037-1050.