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Reduction of Leukocyte Counts by Hydroxyurea Improves Cardiac Function in Rats with Acute Myocardial Infarction

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background:

This study aimed to decrease leukocytes counts by hydroxyurea (Hu) in an acute myocardial infarction (AMI) rat model and examine its effect on the inflammatory response of myocardial infarction and cardiac functions.

Material/Methods:

AMI was successfully caused in 36 rats, and 12 control rats received sham operation. Rats in the AMI group were then randomly divided into Hu and vehicle group with 18 rats each. Rats in the Hu AMI group received Hu (200 mg/kg) intragastrically while vehicle AMI group received saline. Leukocytes counts, cardiac functions, myocardial tissue morphology, and levels of soluble intercellular adhesion molecule-1 (sICAM), P-selectin and platelet activating factor (PAF) were measured and compared among the three groups four weeks after AMI induction.

Results:

Leukocytes, neutrophils, and leukomonocyte counts in vehicle AMI rats were significantly higher than that of the normal control group ($p < 0.05$). However, Hu treatment decreased their counts significantly ($p < 0.05$). sICAM, P-selectin, and PAF level in vehicle AMI group were significantly higher than those of the normal group, and their level was also decreased by Hu treatment ($p < 0.05$). Echocardiography analysis showed that Hu treatment increased left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) compared to that of vehicle AMI group ($p < 0.05$). Histopathological examination showed that Hu significantly reduced the swelling of the heart muscle fiber in necrotic foci and the number of inflammatory cells infiltrated into myocardial interstitium compared to vehicle AMI group.

Conclusions:


Decrease leukocytes counts by Hu significantly reduced inflammatory reaction and improved cardiac functions in AMI rats.

MeSH Keywords:

Leukocyte Count • Myocardial Infarction • Rats, Wistar

Full-text PDF:

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Background

Acute myocardial infarction (AMI) is a secondary thrombus formation in coronary artery induced by unstable atheromatous plaque rupture and bleeding. The thrombus can further cause coronary artery occlusion, as well as serious and long-term myocardial ischemic and myocardial necrosis [1–5]. Treatment options for AMI include conservative treatment with medicine, thrombolytic therapy in the acute phase, percutaneous coronary intervention, and emergency coronary artery bypass grafting [6–9]. The time window for thrombolysis treatment is short and the success rate of infarct-related vessel recanalization remains low. Other surgical complications include serious residual stenosis and high risk of bleeding [8]. Percutaneous coronary intervention (PCI) has become a routine method in AMI treatment. PCI reduces pain after myocardial infarction and significantly decreases the occurrence of various complications [9]. However, PCI treatment requires a skilled medical team and is an expensive procedure, thus its availability in primary hospitals is limited [8]. Furthermore, 25% of patients have no reflow or slow reflow after infarction-related artery recanalization [10]. Emergency coronary artery bypass grafting also requires high technical skill, and very few medical institutions currently are able to implement this technology [11]. Therefore, new therapeutic measures are needed to improve the success rate of myocardial infarction rescue.

Increased neutrophil count has been found in the peripheral blood of AMI patients [12], probably as a result of stress response and local myocardial necrosis. It has been shown that increased leukocytes counts after AMI can be used in the prognosis of AMI [1,2,13]. AMI patients with significantly elevated leukocytes count have higher risk of various malignant arrhythmias, acute heart failure, cardiogenic shock, and other acute adverse complications [2,14]. In ischemic myocardial tissues, leukocytes, especially neutrophils, are activated and aggregated, and the number of infiltrated leukocytes increases significantly with longer duration of myocardial ischemia [15–17]. In addition, the infiltrated leukocytes can induce microthrombosis in coronary arteries by disrupting the functions of endothelial cells [18–20]. Insufficient microvascular ischemia reperfusion can further aggravate myocardial ischemia and cause extension of the myocardial infarction area [21–24]. Aoki et al. showed that elevated peripheral blood mononuclear cell count serves as an independent predictor for left ventricle remodeling in AMI patients [25]. A study of 7651 patients with acute coronary syndromes showed that WBC counts of >10 000 were associated with increased 30-day and 10-month mortality [26]. Further, neutrophil depletion by antiserum reduces ischemic myocardial injury in dogs [27]. Taken together, these findings show that leukocyte accumulation in response to myocardial ischemia plays an important role in myocardial injury, and that reduced leukocyte content during this process will help reduce myocardial necrosis.

Given the relationship between inflammatory response, cytokines production, and AMI, we hypothesized that reduced leukocyte count will lead to reduced inflammation, cytokine production, and alleviation of AMI lesions. Here, we decreased leukocyte counts by Hu treatment in a rat AMI model to interrupt the inflammatory reaction process. We also explored the effects of Hu on cardiac functions of AMI rats.

Material and Methods

Experimental animals, AMI model construction and Hu treatment

Fifty male Wistar rats aged 8–10 weeks and weight 180–220 g were purchased from the Experimental Animal Center of Shandong University School of medicine, China. The 50 rats were divided into an AMI model group with 38 rats and a normal control group with 12 rats.

AMI was caused as described [28]. Briefly, rats were anesthetized with pentobarbital sodium (30–40 mg/kg, intraperitoneal injection) and ventilated mechanically. A 3–4-cm transverse incision was made between the third and fourth intercostal spaces. The heart was then exposed and a ligature using a 3-0 Prolene was then placed around the proximal portion of the left anterior descending artery (LAD). A similar sham operation was performed on rats in the normal control group, except no suture of the LAD was performed.

Two out of the 38 rats used for the construction of AMI models died during the process. The remaining 36 AMI rats were randomly divided into the Hu group and vehicle control AMI group (vehicle AMI group) with 18 rats in each group. For rats in the Hu group, 200 mg/kg Hu was administered intragastrically immediately after AMI model construction. Saline was administered intragastrically to the vehicle AMI group.

This study was approved by the Institutional Animal Use and Care Committee of the Shandong University School of Medicine, China.

Measurement of total leukocytes, neutrophils, and leukomonocytes counts

Two milliliters (ml) of venous blood was harvested from the angulus venosus of the rats and collected in tubes containing 0.109 ml sodium citrate as anticoagulant. After mixing fully, the blood samples were used for the determination of total leukocyte, neutrophil, and leukomonocyte counts using a Sysmex xs800i Automated Hematology Analyzer (Sysmex Corporation, Japan).

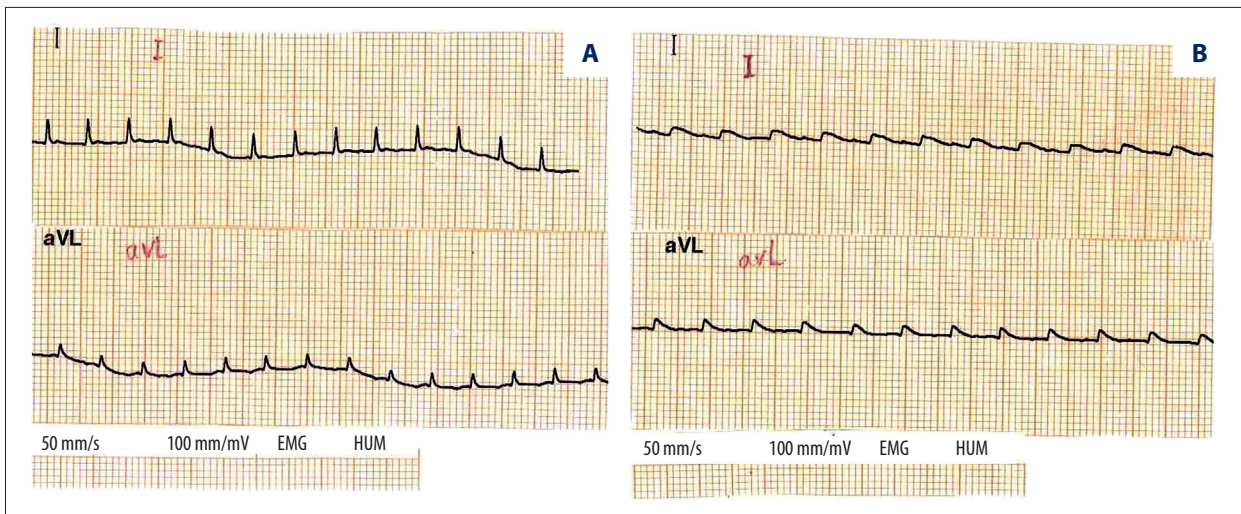


Figure 1. Electrocardiogram (ECG) of rat before (A) and after (B) AMI construction. The elevation of the ST segment on lead I, aVL in Figure 1B compared to Figure 1A indicated that AMI model was constructed successfully.

Activity of soluble intercellular adhesion molecule-1 (sICAM), P-selectin, and platelet activating factor (PAF)

Serum sICAM-1 level was examined by enzyme-linked immunosorbent assay (ELISA) (Dia-clone, France). Serum P-selectin was measured using an ELISA kit (America R&D, USA). Activity of PAF was also analyzed by ELISA kit (Rapid Bio, USA).

Heart function index measurements

Electrocardiography (ECG) was performed before AMI construction and immediately after the successful induction of AMI, with a 12-lead ECG instrument (DECG-03A, Mindray medical international limited, China).

Four weeks after AMI induction, heart function index was measured using a Philips Sonos 5500 multi-function color ultrasonography device (Philips, USA) with an 8-MHz transducer. Tissue Doppler imaging (TDI) and synchronous II lead echocardiography were performed. The following equations were used according to the American Society of Echocardiology (ASE):

$$LVM=1.04 \times [IVSd + (LVEDd + LVPWd)^2 - LVEDd^2] - 14.$$

$$SV=LVEVd - LVEVs.$$

$$CO=SV \times HR. LVEF=(LVEDV - LVEVS) / LVEDV \times 100\%.$$

$$FS=(LVEDd - LVESd) / LVEDd \times 100\%.$$

The definitions are:

LVM – left ventricular mass; IVSd – interventricular septal end-diastolic dimension; LVEDd – left ventricular end-diastolic dimension; LVPWd – left ventricular end-diastolic posterior wall dimension; SV – stroke volume; LVEVd – diastolic left ventricular volume; LVEVs – systolic left ventricular end volume; CO – cardiac output; HR – heart rate; LVEF – left ventricular ejection

fraction; LVEDV – left ventricular end-diastolic volume; LVEVS – left ventricular end-systolic volume; FS – fraction shortening; LVESd – left ventricular end-systolic diameter.

Pathological observations

Four weeks after the successful construction of the AMI rat model and the cardiac function index measurement, hearts of the rats were harvested and fixed in 4% paraformaldehyde solution. The heart samples were embedded in paraffin and continuous slices were obtained. The slices were then stained with hematoxylin and eosin (HE) and changes of infarction areas were observed under a microscope (Olympus BX51, Japan).

Statistical analysis

Statistical analysis was conducted using SPSS 17.0 software, and the results are presented as mean \pm standard deviation ($\bar{x} \pm SD$). Analysis of variance and q-test were used for comparisons between groups. The correlations of various parameters within groups were analyzed by Pearson linear correlation analysis. $P \leq 0.05$ was regarded as a significant difference.

Results

ECG performance in AMI rat model

To verify that the AMI model was successfully constructed, electrocardiograms (ECG) were measured in rats before and after AMI construction. ECG of rats before AMI is illustrated in Figure 1A, which shows that the ST segment was at baseline on lead I and aVL. In comparison, the ECG of AMI rats (Figure 1B) showed significant elevation of the ST segment on lead I and

Table 1. Leukocytes, neutrophils and leukomonocytes counts in AMI rats after Hu treatment.

	n	Leukocytes ($\times 10^9/L$)	Neutrophil ($\times 10^9/L$)	Leukomonocyte ($\times 10^9/L$)
AMI Model group				
Hu group	18	8.32 \pm 1.61*	5.12 \pm 1.31*	2.70 \pm 0.92*
Vehicle group	18	12.54 \pm 1.96	8.56 \pm 1.53	3.87 \pm 1.16
Normal control	12	7.75 \pm 1.72	4.87 \pm 1.25	2.53 \pm 1.04

Values are Mean \pm SD. * Compared with the vehicle AMI group * $P < 0.01$; compared with the normal control group * $P < 0.05$.

Table 2. Level of sICAM, P-selectin and PAF in AMI rats after Hu treatment.

	n	sICAM-1 (ng/ml)	Ps (ng/ml)	PAF ($\times 10^9$)
AMI Model group				
Hu group	18	20.32 \pm 2.61*	7.33 \pm 0.83*	185 \pm 13*
Vehicle group	18	40.58 \pm 3.19	14.52 \pm 1.17	223 \pm 17
Normal control	12	17.68 \pm 2.82	6.92 \pm 0.69	176 \pm 15

sICAM – soluble intercellular adhesion molecule-1 (sICAM); PAF – platelet activating factor (PAF). Values are Mean \pm SD. Compared with the Vehicle AMI group * $p < 0.01$; compared with the normal control group * $p < 0.05$.

Table 3. Cardiac indexes of AMI rats after Hu treatment.

	n	LVESd (mm)	LVEDd (mm)	LVEF (%)	FS (%)
AMI Model group					
Hu group	18	4.1 \pm 0.4*	6.3 \pm 0.4*	62 \pm 6*	35 \pm 4*
Vehicle group	18	5.4 \pm 0.3	7.4 \pm 0.4	46 \pm 7	27 \pm 6
Normal control	12	2.1 \pm 0.4	4.6 \pm 0.3	84 \pm 5	53 \pm 5

Compared with Vehicle group * $p < 0.01$; compared with the normal control group * $p < 0.05$. Values are Mean \pm SD. LVESd – left ventricular end systolic dimension; LVEDd – left ventricular end diastolic dimension; LVEF – left ventricular ejection fraction; FS – fractional shortening.

avL (≥ 0.5 mv). The ECG result confirmed the success of AMI model construction.

Decrease of leukocytes, neutrophils and leukomonocytes counts by Hu

Leukocytes, neutrophils, and leukomonocytes were measured 4 weeks after AMI construction and Hu administration, and the results are presented in Table 1. Leukocytes, neutrophils, and leukomonocytes in the vehicle AMI group were significantly higher than in the normal control group ($p < 0.05$), but Hu treatment significantly decreased their levels compared to the vehicle AMI group ($p < 0.05$). No significant difference was found between the Hu AMI group and the normal control group ($p > 0.05$).

Decrease of sICAM, P-selectin, and PAF levels by Hu treatment

The level of sICAM (ng/ml), P-selectin (ng/ml) and PAF ($\times 10^9$) measured by ELISA 4 weeks after AMI construction and Hu administration are shown in Table 2. sICAM-1, P-selectin, and PAF level were significantly higher in the vehicle AMI group compared with the normal control group ($p < 0.05$). However, their levels were decreased by Hu treatment in the Hu AMI group compared with the vehicle AMI group ($p < 0.05$). No significant difference was found between the Hu AMI group and the normal control group ($p > 0.05$).

Cardiac function indexes by echocardiography

Cardiac function was examined with echocardiography 4 weeks after AMI induction (Table 3). LVESd and LVEDd levels were significantly higher in the vehicle AMI group than

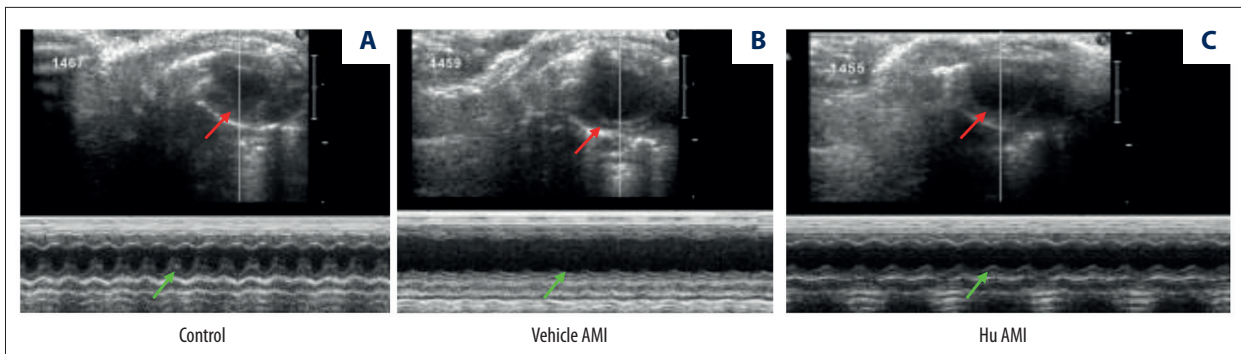


Figure 2. Left ventricular short axis diastolic ultrasonic manifestation. (A) Control group, cardiac muscle thickness (red arrow) and wall motion (green arrow) was normal. (B) Vehicle AMI group, cardiac muscle was thinner (red arrow), and wall motion was reduced (green arrow). (C) Hu AMI group, cardiac muscle was thicker compared to vehicle AMI rat (red arrow), wall motion increased compared to vehicle AMI group (green arrow).

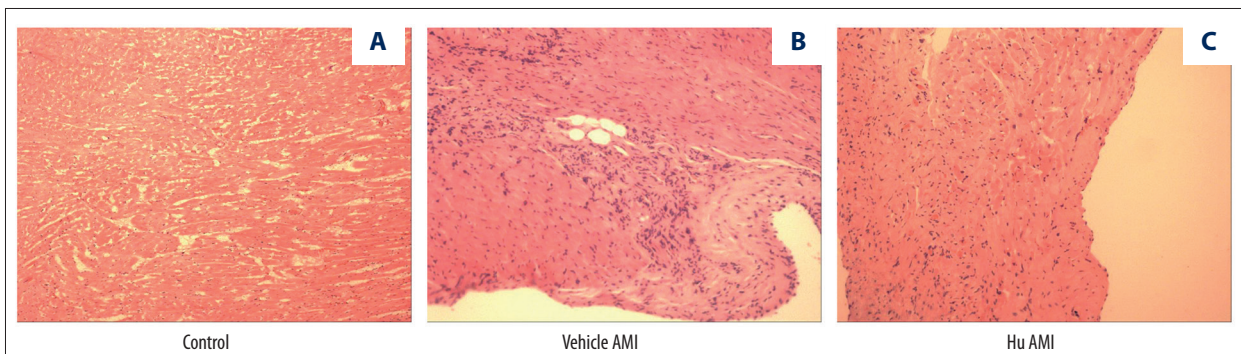


Figure 3. Myocardial tissue morphology under light microscope (200 \times). (A) Control group myocardial tissues and nuclei were uniform myocardial fibers were arranged uniformly. No infiltration of inflammatory cells in myocardial interstitium was observed. (B) Vehicle AMI group; obvious swelling in necrotic foci was observed, and the nuclei were arranged in disorder. There were significant amount of infiltration of inflammatory cells in myocardial interstitium. (C) Hu AMI group; nuclear staining and size of focal necrosis of myocardial tissues and residual cardiomyocytes were uniform, and myocardial fiber tissue showed mild swelling. A small amount of fibroblasts and inflammatory cell infiltration was observed.

in the normal control group ($p < 0.01$), Hu treatment significantly decreased the levels compared with the vehicle AMI group ($p < 0.05$). However, the measurements in the Hu AMI group were still higher than in the normal control group ($p < 0.05$) (Table 3). LVEF and FS percentage was significantly lower in the vehicle AMI group than in the normal control group ($p < 0.05$). Hu treatment significantly increased the percentage compared with the vehicle AMI group ($p < 0.05$), but levels in the Hu AMI group were still lower than in the normal control group ($p < 0.05$) (Table 3).

Figure 2 shows the representative ultrasonic image of rats from the 3 groups. In the normal control group, cardiac muscle thickness (Figure 2A, red arrow) and ventricular wall motion were normal, and there was no obvious segmental motion (Figure 2A, green arrow). In the vehicle AMI group, the cardiac muscle became thinner and the ventricular wall motion in the infarction regional was low (Figure 2B). In the Hu AMI group, cardiac muscle became slightly thicker, and the motion

of the infarction regional was slightly lower than in the vehicle group (Figure 2C).

Myocardial tissue morphology

Myocardial tissue was harvested from rats of the 3 groups and observed under a microscope after HE staining. As shown in Figure 3A, there were no obvious infarcts, and the myocardial nuclei and the myocardial fibers were uniformly arranged and orderly in the normal control group. No infiltration of inflammatory cells in myocardial interstitium was observed (Figure 3A). In the vehicle AMI group, obvious swelling in necrotic foci of the heart muscle fiber was observed, and the arrangement of nuclei was disordered. There was a considerable amount of infiltration of inflammatory cells in myocardial interstitium (Figure 3B). In the Hu AMI group, nuclear staining and size of focal necrosis of myocardial tissues and residual cardiomyocytes were uniform, and myocardial fiber tissue showed mild swelling. A small amount of fibroblasts and inflammatory cell infiltration was observed (Figure 3C).

Discussion

Increased leukocyte count is part of the inflammation process and participates in the pathogenesis of AMI, further affecting the prognosis of AMI patients [29]. Our results show that the level of inflammatory cytokines, amount of leukocyte infiltration into myocardial interstitium, and cardiac function index were all reduced by Hu treatment in a rat AMI model, suggesting that leukocyte reduction through Hu treatment might be a promising preventive and treatment option for AMI.

Why does leukocyte accumulation exacerbate tissue injury in AMI? The underlying mechanism might be as follows: first, leukocyte accumulation and activation increases the viscosity of peripheral blood; second, leukocyte accumulation causes capillary plugging and the no-reflow phenomenon, thus leading to coronary artery occlusion; third, migrating leukocytes release proinflammatory mediators, such as lipoxygenase products, free radicals and hydrolytic enzymes, which causes cardiovascular toxicity and myocardial ischemia [30]. The activated neutrophils in the infarct adhere to vascular endothelial cells, affecting the dilation of the coronary artery, increasing the resistance of blood vessels, and further aggravating myocardial ischemia. A feedback loop exists between AMI and leukocytes level: increase in leukocytes promotes the development of the AMI, and the further development of AMI can in turn induce leukocyte accumulation [29].

There are several methods to lower leukocyte counts. The first is through leukocyte reduction filter. Leukocytes depletion from the systemic circulation by filtering during cardiopulmonary bypass reduces the expression of neutrophil adhesion molecules [31]. This reduction leads to decreased leukocyte deposition and interaction with the vascular endothelium, and thereby decreases neutrophil-mediated injury [31,32]. A study showed that 27% of capillaries were plugged after perfusion with whole blood, whereas only 1% capillary had no-reflow with leukocyte-depleted blood [33]. However, leukocyte depletion filters are costly and its benefits need confirmation from large-scale randomized prospective studies [34]. Due to the foreseeable difficulty in applying leukocyte filters in rats, we did not consider leukocyte filters as a method of leukocyte reduction in our experiments.

The second way to reduce leukocyte counts is by drugs used in leukemia treatment. Busulfan, also called myleran, is a bifunctional alkylating agent that is widely used as an alternative to total body irradiation in conditioning therapy for hematopoietic stem cell transplantation [35]. Despite its short half-life (2–3 h) and quick turnover *in vivo*, orally administered Busulfan causes hepatotoxicity and inhibits hematopoiesis [35]. Due to the decreased immunity in AMI rats, we did not consider Busulfan as a leukocyte reduction reagent. Hu is another antileukemic drug that targets ribonucleotide reductase, thus inhibiting DNA synthesis. Hu selectively inhibits cells at the S phase and is used

in the treatment of chronic myelogenous leukemia. Hu is administered orally and is readily absorbed [36]. Hu distributes rapidly and widely in the body, and concentrates in leukocytes and erythrocytes. Hu has a half-life of 1.5–5 h, and is probably metabolized and also eliminated through the renal pathway. More than 80% of Hu will be secreted 12 hours after administration [36]. Based on these advantages, we chose Hu for leukocyte reduction purposes in our AMI rat. ONO-AE-248, another drug candidate, is a selective prostaglandin E2 (PGE2) receptor type 3 (EP3) agonist [37]. However, ONO-AE-248 has a short half-life, is still under clinical development, and is costly, thus it was not considered by us. Metformin was also found to improve cardiac function in a nondiabetic rat model of post-MI heart failure [38], but it requires long-term administration (12 weeks).

Intercellular adhesion molecule-1 (ICAM-1), also called CD54, is an immunoglobulin (Ig)-like cell adhesion molecule [38]. ICAM-1 is expressed in several cell types, including leukocytes and endothelial cells. ICAM-1 is also induced in atherosclerotic lesions and is involved in their progression [39,40]. Therefore, monocyte counts and serum ICAM-1 level can be used as a marker for coronary atherosclerosis [40]. P-selectin (Ps) is a member of the selectin family of adhesion molecules that is expressed in platelets and is stored on the membrane of alpha granules and on the Weibel-Palade bodies of endothelial cells. P-selectin mediates the adhesion of leukocytes to stimulate endothelial cells and to activate platelets [41]. Platelet-activating factor (PAF) is a phospholipid mediator and mediates many leukocyte functions, including platelet aggregation [42]. Ps and PAF function together to promote the adhesion and accumulation of leukocyte to endothelial cells and the subsequent release of inflammatory mediators. Our ELISA analysis showed that ICAM-1, Ps and PAF were all increased in AMI rats, indicating the role of these inflammatory factors in the pathogenesis of AMI. Further, Hu treatment significantly decreased their level, concomitant with an improvement of cardiac function in AMI rats. However, the exact molecular mechanism by which these inflammatory factors contribute to AMI and how Hu down-regulated their expression is unknown.

Conclusions

Decrease leukocytes count by Hu treatment in AMI rats can reduce inflammatory reaction significantly and improve heart functions after AMI, suggesting that leukocyte reduction through Hu treatment might be a promising preventive and treatment option for AMI. Our experimental study provides new insight and clinical guidance for the treatment of AMI.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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