Low dose cyclophosphamide rescues myocardial function from ischemia-reperfusion in rats

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Abstract

Objectives: The effect of low dose of cyclophosphamide (CP) protecting cardiac function from ischemia-reperfusion injury was studied on rats. The premise is that CP inhibits immune and inflammatory process, thereby limits I/R injury and improves myocardial function. Methods: Open chest rats were submitted to 30 min of ischemia followed by 3 h, 12 h or 24 h of reperfusion. Rats were divided into sham group, I/R group and CP group, and each group included 3 time-point subgroups (3 h, 12 h and 24 h; n = 8 for each subgroup). A total of 750 mg/m 2 cyclophosphamide was intraperitoneally administrated in CP group and saline was given to I/R group. A polyethylene tube was inserted into the left ventricular cavity to detect left ventricular systolic pressure (LVSP) and ±dp/dt max. At the end of the experiment, hearts were harvested for histopathological assessment and infarct size determination. Results: Compared with I/R group, rats treated with low dose CP showed a significant recovery in myocardial function with improved LVSP (88 ± 11 vs 69 ± 11 mmHg of 3 h; 92 ± 11 vs 64 ± 14 mmHg of 12 h; 90 ± 11 vs 64 ± 14 mmHg of 24 h; p < 0.01 respectively). The ±dp/dt max also had the similar trends. The myocardial infarct size was reduced in CP group compared to that in I/R group; the infiltration of polymorph nuclear leukocytes (PMNs) in myocardium was decreased in CP group. The histopathological damage score was also attenuated. Conclusions: These findings suggest that low dose CP rescues cardiac function from ischemia-reperfusion injury by alleviating histopathological damage in rat myocardium.

Keywords: Ischemia; Reperfusion; Hemodynamics; Cyclophosphamide; Myocardial stunning

1. Introduction

Although therapeutic outcomes have markedly improved with the widespread application of coronary artery bypass graft surgery (CABG) or percutaneous coronary intervention (PCI), myocardial ischemia-reperfusion (I/R) injury remains a major clinical problem associated with morbidity and mortality. Myocardial I/R injury may be broadly considered as the conversion of reversible to irreversible cell injury on reperfusion of a previously ischemic area [1]. Endothelial injury may play a critical role in the pathogenesis of myocardial I/R injury by setting the stage for adherence of neutrophils to the vascular endothelium and subsequent development of inflammatory component of the I/R; and suppression of polymorph nuclear leukocytes (PMNs) infiltration or application of scavengers of reactive oxygen metabolites diminishes I/R damage and potentially offers myocardial protection [2]. Cyclophosphamide (CP) is a well-known immunosuppressant with inhibition of immune and inflammatory process. The effect of a single dose of CP on acute myocardial infarction in conscious rats has been studied [3]. The survival rate of rats subjected to coronary ligation was significantly increased after the intraperitoneal injection of 100 mg/kg of the drug; and the populations of T lymphocytes and PMNs [4] have been decreased in blood after CP administration. We hypothesized that intraperitoneal administration of low dose CP might have myocardial protective effects and improvement of myocardial function on rats suffering I/R injury through suppression of PMNs or some other mechanisms.

2. Materials and methods

The Animal Care and Use Committee of Zhejiang University approved all procedures. All animals received humane care in compliance with the guidelines in the Principles of Laboratory Animal Care formulated by the
2.1. Model

Adult male Sprague-Dawley rats (250–275 g, provided by Shanghai Laboratory Animal Center, Chinese Academy of Sciences) were anesthetized with chloral hydrate (400 mg/kg) through intraperitoneal injection. The animals were placed in a supine position with their paws and upper central incisors taped to the operating table. An endotracheal tube was inserted by way of the mouth into the trachea. Artificial respiration was maintained through the respirator with an inspiratory/expiratory ratio of 1/2, a frequency of 65 strokes/min, and a tidal volume of 15 ml to maintain normal arterial PaO₂, PaCO₂, and pH. The middle skin incision was extended from the sternal angle down to the xiphoid. The left pectoral muscles were dissected longitudinally to expose the left 3rd and 4th ribs. A parasternal incision was made to open the chest by dissecting the intercostal muscles between the left 3rd and 4th ribs and kept open by a stent without cutting ribs. Coronary artery ligation was achieved with a gab occluder fixed onto the left anterior descending coronary artery (LAD). A 7-0 silk suture was passed underneath the LAD (2–3 mm inferior to the left auricle) and tied. Reperfusion was induced through releasing the tie. Significant ECG changes, including widening of the QRS complex and elevation of ST segment, and color changes of the area at risk were considered indicative of successful coronary occlusion and reperfusion. The chest was closed in layers, and the respirator weaned when the animal recovered spontaneous breathing [5].

2.2. Experimental protocol

Rats were subjected to intraperitoneal injection of 750 mg/m² cyclophosphamide (Jiangsu Hengrui Medicine Co. Ltd., NanJing, China) or saline before surgery, followed by 30 min of left ventricle normothermic ischemia, and 3 h, 12 h or 24 h of reperfusion (n = 8 in each subgroup). In sham group, saline was intraperitoneally injected before surgery. The silk suture crossed without ligation and the rat did not receive ischemia-reperfusion. In I/R group, saline was intraperitoneally injected before surgery, and the rat received ischemia-reperfusion. Eighty-four rats have been subjected to surgery, and all surviving 72 rats were assigned to three groups. Three rats died during anesthesia, five rats died during surgery (three in I/R group, two in CP group), four rats died after surgery (two in I/R group, two in CP group). Hemodynamic parameters were detected at 0 h, 3 h, 12 h and 24 h at the end of reperfusion. The animals were sacrificed by bleeding from polyethylene tube, and hearts were excised for assessment of infarct size and later for histopathological study (Fig. 1).

2.3. Hemodynamic assessment

At the end of reperfusion, the rats were anesthetized again, and a polyethylene tube (PE 50) was inserted into the left ventricular cavity via the right common carotid artery [6]. The pressure was transduced and amplified by a pressure transducer (XingHangXinYe Co. Ltd., Beijing, China). Left ventricular systolic pressure (LVSP) and \( \pm d{p/dt}_{max} \) were recorded and programmed by using a biotic signal collection and processing system (MedLab-U/4cs, MeiYi Co. Ltd., Nanjing, China).

2.4. Assessment of risk area and infarct size

After recording hemodynamic parameters, the chest was reopened under artificial respiration. The coronary artery was again briefly occluded through ligation of the tie that remained at the site of the previous occlusion. Immediately 2 ml Evans blue solution (1%) was infused through the catheter into the left ventricular cavity to delineate the ischemic area (underperfused and then reperfused area). When heart beating weaned slowly, the heart was excised. After removal of the right ventricle and atria, the left ventricle was cross-sectioned from the apex to the atrioventricular groove into seven specimens about 1.5 mm in thickness with a steric heart mould [6]. These slices were incubated with 2% triphenyltetrazolium chloride (TTC) solution (pH 7.4) for 15–20 min at 37 °C in a dark room. Then ischemic but viable (TTC-stained) and infarct (TTC-unstained) zones within the underperfused and then reperfused area (Evans blue-unstained) and the nonischemic area (Evans blue-stained) were photographed and programmed by using the analysis software (Image J 1.36, Macintosh, National Institute of Mental Health (NIMH)). The risk area was determined by the percentage of the Evans blue-unstained area to the whole left ventricle, the infarct area was determined by the percentage of the TTC-unstained area to the Evans blue-unstained area. The ability to distinguish viable from nonviable myocardial tissue via the exposure of myocardial dehydrogenase enzymes to TTC has been demonstrated by others as an accurate method of quantifying infarct size [5].

2.5. Histopathological analysis

Additional hearts were fixed in 10% formalin and embedded in paraffin. The paraffin-embedded tissues were sectioned and stained with hematoxylin-eosin and analyzed by light microscopy. The histological sections were examined by an observer blinded to the treatment regimen, for the extent of myocardial tissue injury and the intensity of PMNs infiltration (the mean of the absolute number of PMNs from...
The following morphological criteria [8] were used to determine the histopathological damage: score 0, no damage; score 1 (mild), interstitial edema and focal necrosis; score 2 (moderate), diffuse myocardial cell swelling and necrosis; score 3 (severe), necrosis with the presence of contraction bands, neutrophil infiltration and the capillaries were compressed; and score 4 (highly severe), widespread necrosis with the presence of contraction bands, neutrophil infiltration, capillaries compressing and hemorrhage.

2.6. Statistical analysis

All data were expressed as mean ± SD. Analysis was carried out by using the SPSS statistical package (version 13.0).

Univariate analysis was used to test the differences of all measurements. When a significant p value, <0.05, was shown for certain variables, post hoc test (LSD and Bonferroni correction) was followed. A value of p < 0.05 was considered statistically significant.

3. Results

3.1. Improved myocardial function in CP group (Figs. 2 and 3)

Damaged LVSP and ±dp/dt\textsubscript{max} of left ventricle were recorded in I/R and CP groups. LVSP ±dp/dt\textsubscript{max} became worst at 24 h in I/R group, but treatment with CP generated a significant improvement in cardiac performance at all time-points. Compared with I/R group, the LVSP significantly improved (88 ± 11 mmHg vs 69 ± 11 mmHg of 3 h; 92 ± 11 mmHg vs 64 ± 14 mmHg of 12 h; 90 ± 11 mmHg vs 64 ± 14 mmHg of 24 h; p < 0.01 respectively) after treatment with low dose CP. Likewise, +dp/dt\textsubscript{max} and −dp/dt\textsubscript{max} also had the similar trend.

3.2. Myocardial infarct size diminished in CP group (Fig. 4)

Similarly sized risk areas were observed both in I/R and CP groups at each time-point. There were no significant differences among groups in ischemic area, suggesting that the ischemic severity was similar in those groups tested in the present study. In contrast, the infarct sizes were significantly reduced by 14.3% of 3 h, 28.3% of 12 h and 18.4% of 24 h (p < 0.01 respectively) in low dose CP-treated rats compared with I/R group. These results strongly suggest that CP markedly attenuates reperfusion injury as demonstrated by a significant reduction in infarct size.

3.3. Attenuated histopathological damage (Figs. 5 and 6)

Three hours after transient ischemia-reperfusion, myocardial destruction with focal infiltration of PMNs was evident in the left ventricular free wall. Myocardial destruction rapidly progressed after ischemia-reperfusion, and pathological features of the infarct area became apparent at 24 h with widespread necrosis, including the presence of contraction bands, PMNs infiltration, capillaries compressing and a lot of hemorrhage. After low dose CP treatment, the histological features became typical of normal cardiac structure or mild architectural damage, characterized by interstitial edema and localized necrotic areas. The mean histopathological damage in CP group and I/R group scored 1.8 ± 0.5 vs 2.2 ± 0.5 of 3 h; 2.3 ± 0.5 vs 3.3 ± 0.6 of 12 h; 2.8 ± 0.5 vs 3.8 ± 0.5 of 24 h (p < 0.01 respectively).

Of note, the total numbers of adhered and infiltrated PMNs in CP-treated hearts were less than that observed in I/R group (7 ± 8/HPF vs 20 ± 4 /HPF of 3 h; 19 ± 8/HPF vs
4. Discussion

The significant improvement of myocardial function was observed in the group of rats with myocardial I/R, treated with intraperitoneal injection of a single low dose CP. This was along with the demonstration of the preservation of infarct size and histopathological damage. It is unlikely that CP directly stimulates cardiodynamics (i.e., exerted a coronary vasodilation or a positive inotropic effect).

The underlying mechanism by which CP improves myocardial function in I/R injury rats might be partially associated with the decrease in number of PMNs infiltration and it still remains to be further elucidated. It tends to believe that infiltrating PMNs, activated by an ischemic insult, directly contact and injure neighboring myocyte via the release of inflammatory substances [9]. The activation of PMNs in reperfusion areas appears to have detrimental consequences on cardiac function [10]. Minano et al. [11] have previously presented the evidence to demonstrate that CP could induce 'PMNs depletion' of circulation in rats. However, the effect of CP treatment on PMNs infiltration in myocardium during myocardial I/R injury has not been shown previously. For the first time, we presented data to demonstrate that with single low dose CP treatment, the PMNs infiltration in myocardium was decreased in I/R rat hearts. Besides the presence of PMNs infiltration, hemorrhage was presented in I/R group, which almost disappeared in CP group. It has been suggested that PMNs might induce postischemic damage by liberating reactive oxygen metabolites, hydrolytic enzymes, and eicosanoids, which would lead to microvascular injury [12—14]. Hemorrhage was primarily due to damaged vessels, which could accelerate the PMNs infiltration. Interestingly, a previous study has shown an excellent correlation between the extent of
hemorrhage and the infarct size [15]. With previous observation and our findings, we believe that the decrease in number of PMNs in myocardium in rats treated with intraperitoneal injection of CP is associated with the improvements of myocardial function in I/R injury.

It may also be speculated that treatment with CP would probably render protection from myocardial stunning. To the best of our knowledge, a potential myocardial stunning may happen after ischemic injury [16]. At present, the two viable theories regarding the pathogenesis of myocardial stunning are the oxyradical hypothesis and the calcium hypothesis [17]. PMNs are powerhouses of free radical production and can overwhelm the limited extracellular protective mechanisms [18]. Kraemer and Mullane [19] showed that the addition of leukocytes to buffer-perfusing isolated hearts subjected to I/R aggravates the loss of contractility termed myocardial stunning during reperfusion. Reduction of PMNs by CP treatment could contribute to a decrease in production of free radicals, and then myocardial function could be preserved by attenuating ‘myocardial stunning’.

CP has been widely used as an anticancer medicine. After long-term CP treatment, it may cause adverse effects such as marked myelosuppression [20], hemorrhagic cystitis [21], gonadal failure [22], and development of malignancies [23]. However, these side effects are usually caused by a cumulative dose of cyclophosphamide. Although these side effects are common in anticancer treatment with high doses of CP, it is a relatively safe drug at low dose. A single low dose injection of CP before I/R would avoid the cumulative dose [24]. The average nadir white blood cell count is about 3000 cells/mm³ after a single dose of 1 g/m² CP in humans. The approximate times to nadir and recovery of leukocyte counts are 8–14 days, and 21 days, respectively [24,25]. By using single low dose of CP, the short-term toxicity could be mildly reduced and the long-term immunosuppression or myelosuppression could be avoided. The dose (750 mg/m²) we used in this study is similar to the dose clinically used in patients with autoimmune diseases, and patients can usually tolerate well at this dose level. Previous experiments have applied similar doses in myocardial infarct rats [3] and demonstrated that was a relatively safe dose level. Furthermore Itescu et al. [24] had applied CP pulse therapy (0.5–1.0 g/m²) monthly in sensitized cardiac allograft recipients, which proved to be safe and highly effective at least in 12 months. As reported, the rates of systemic fungal infections are similar in patients treated with or without CP. The incidence of cytomegalovirus (CMV) reactivation or disease after transplant was actually lower in CP-treated patients, no other systemic viral or bacterial infections were seen in patients treated with CP, and no malignancies had developed after 53.5 patient-months of follow-up [24].

In summary, this study has demonstrated that low dose CP can protect myocardial function in rats from ischemia-reperfusion injury, with a decrease in infarct size and attenuation of histopathological damage. The findings could help to gain some insight into therapeutic potential of low dose of CP in early reperfusion injury.

References


