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Impact of citrate pretreatment on ventricular arrhythmia and myocardial capase-3 expression in ischemia/reperfusion injury

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ABSTRACT. Ischemia/reperfusion (I/R) injury often triggers ventricular arrhythmia. Citrate binds calcium ions, forming a soluble calcium citrate complex that may reduce I/R injury by affecting calcium ion concentration. We tested the effects of citrate pretreatment on ventricular heart rate and related factors in a rat I/R model. Fifty male Sprague Dawley rats weighing 350-400 g were randomly divided into equally sized control (A), model (B), and 0.1 M (C), 0.05 M (D), and 0.025 M (E) citrate groups. An I/R model was established by ligating the left anterior descending coronary artery. Serum calcium ion concentration was measured before and after citrate treatment.

Triphenyltetrazolium chloride staining and spectrophotometry were used to determine infarction area and caspase-3 protein levels in myocardial tissue, respectively. Polymerase chain reaction was performed to test myocardial calmodulin (CAM) expression. The frequency of ventricular arrhythmia in group B was significantly higher than in the sham surgery group ($P < 0.05$). Citrate pretreatment resulted in lower and higher frequencies than those observed in the model and control groups, respectively, in a dose-independent manner. The most obvious reduction in ventricular arrhythmia was seen in Group D. Serum calcium ion concentration decreased markedly after citrate treatment ($P < 0.05$), with a specific pattern emerging over time. Infarction area and caspase-3 and CAM levels were significantly lower in the citrate groups compared with the model group ($P < 0.05$). Citrate can reduce myocardial cell apoptosis, alleviating ventricular arrhythmia and protecting the myocardium by reducing serum calcium ion concentration and downregulating caspase-3 and CAM expression.

Key words: Citrate; Ventricular arrhythmia; Caspase-3

INTRODUCTION

Acute myocardial infarction is an important cause of sudden cardiac death. Early acute myocardial ischemia and injury lead to unstable cardiac electrical activity and ventricular repolarization, triggering malignant arrhythmia and sudden death (Fanton et al., 2015; Horvat and Vincelj, 2015). Myocardial ischemia/reperfusion (I/R) may aggravate cardiac dysfunction and myocardial cell injury. Thus, it is important to clarify the mechanisms by which I/R injury can be prevented (Huang et al., 2015; Shekarforoush et al., 2016). Intracellular calcium overload has a leading role in myocardial I/R injury (Gonca and Kurt, 2015; Patil et al., 2015). During I/R, intracellular calcium stores are thought to release Ca^{2+} into the cell, leading to dysregulation of its influx, exacerbating Ca^{2+} overload. Mitochondrial adenosine triphosphate (ATP) production decreases, calcium pump function is restricted, and Na^{+}/Ca^{2+} exchangers as ATP-independent transporters induce elevation of intracellular Na^{+} and acidosis during myocardial ischemia hypoxia. pH and ATP levels then recover during myocardial reperfusion. The resulting pH gradient across the cell membrane activates Na^{+}/H^{+} and Na^{+}/Ca^{2+} exchange and Ca^{2+} influx, leading to intracellular calcium overload. This can cause lack of myocardial depolarization following an action potential, triggering ventricular arrhythmia (Miskolczi et al., 2015; Wu et al., 2015). Mitochondria regulate intracellular calcium levels through a variety of mechanisms. Oxygen free radical production increases during myocardial ischemia hypoxia, resulting in mitochondrial dysfunction and diminished ATP generation. Energy-dependent calcium pumps in membranes of the endoplasmic reticulum, sarcoplasmic reticulum, and cell are therefore blocked. Accordingly, excessive intracellular Ca^{2+} cannot be sequestrated or excluded, finally causing Ca^{2+} overload (Barrabés et al., 2015; Szepesi et al., 2015). Calcium can promote the formation and release of fibrin, thrombin, and blood coagulation factor III by activating platelets in the coagulation process. As an anticoagulant, citrate forms calcium citrate with calcium ions, reducing their concentration in the blood, and inhibiting coagulation. To date, the role of citrate in myocardial I/R has received little attention. This study employed

left anterior descending coronary artery ligation to establish a rat model of coronary I/R. The effect of citrate pretreatment on ventricular arrhythmia, serum calcium concentration, and caspase-3 protein and calmodulin (CAM) expression in the myocardium was measured, providing a basis for the prevention of myocardial I/R injury in clinical practice.

MATERIAL AND METHODS

Experimental animals and groups

Healthy male 10-week-old Sprague Dawley rats weighing 350-400 g were provided by the Animal Experiment Center of Tongji Medical College of Huazhong University of Science and Technology (license SYXK-2013-0025). The rats were kept in a specific-pathogen-free laboratory in accordance with experimental animal standards, and were randomly divided into control (A), model (B), and citrate acid pretreatment (C, D, and E) groups, with 10 individuals in each group. Those in groups C, D, and E received intravenous injections in the tail of 0.1, 0.05, and 0.025 M citrate, respectively, 10 min before reperfusion. The volume injected was 1 mL (according to the clinical dosage equivalent conversion). The rats then received 50 μL physiological saline intubation, with an equal volume of saline given to those in groups A and B.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Wuhan General Hospital of Guangzhou Military.

Drugs and reagents

Citrate acid was purchased from ZsBio (Beijing, China). Triphenyltetrazolium chloride (TTC) and urethane were obtained from Sigma (St. Louis, MO, USA). TTC (0.1 g) was dissolved in 5 mL phosphate-buffered saline (PBS) before use. A caspase-3 kit was supplied by Biobox (Nanjing, China), and a bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Waltham, MA, USA). The QIAamp DNeasy Blood & Tissue Kit was manufactured by QIAGEN (Düsseldorf, Germany). *Taq* polymerase chain reaction (PCR) reagents were provided by Thermo Scientific (Waltham, MA, USA).

Rat model

The rat I/R model was established according to a previously published method (Liu et al., 2014). Rats were administered 5% urethane for abdominal cavity anesthesia, and a 24-guage needle was retained in the caudal vein. The animals were fixed and connected to an electrocardiogram (ECG; lead II configuration) and animal ventilator to control breathing rate (tidal volume, 8 mL/kg; frequency, 70 breaths/min; inspiration:expiration ratio, 1:2). The left common carotid artery was separated to connect the electrophysiological signal recorder. The root of the left anterior descending coronary artery was ligated, whereas control rats underwent threading but not ligation. After 30 min of ischemia, reperfusion was carried out for 2 h, and ECG changes were recorded. Ischemia was deemed to have occurred if, after the left anterior descending coronary artery had been ligated for 5 min, lead II showed ST elevation or increased QRS complex amplitude and broadening fusion with the T wave, arterial blood pressure lowered by >20 mmHg, and cyanosis appeared in the vascular ligation area. Reperfusion was defined as disappearance of the cyanotic area, decrease of the elevated ST segment by >50%

30 min after reperfusion, elimination of the severe atrioventricular block before reperfusion, and mean arterial pressure before ligation <60 mmHg. Rats were euthanized and the heart tissue was collected for determination of serum calcium ion concentration before and after pretreatment, infarction area, capase-3 protein expression, and myocardial CAM levels.

TTC staining for infarction area determination

After being washed in PBS, the left ventricle was frozen at -80°C and cut into 5-mm slices. Sections were incubated in 1% TTC solution, pH 8.5, at 37°C for 0.5 h. The infarction area showed no staining, and normal tissue appeared reddish brown. The Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) was used to calculate infarct size (IS) relative to the left ventricle (IS/LV) and area at risk (IS/AAR), and AAR in relation to the LV (AAR/LV).

Spectrophotometric detection of myocardial capase-3 expression

Heart tissue in the infarction area was isolated to detect protein levels with a BCA assay kit, and caspase-3 protein expression in myocardial tissue was estimated by the colorimetric method, following the manufacturer protocol.

PCR

DNA was extracted from tissues using a QIAamp DNeasy Blood & Tissue Kit, and its concentration determined with a NanoDrop 2000 spectrophotometer (Thermo Scientific). PCR was applied to test target gene expression. Reaction conditions were as follows: 95°C for 5 min, followed by 32 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 60 s, following the manufacturer protocol. β-actin was used as an internal reference. PCR was performed on complementary DNA templates. The following primers were used: CAM forward, 5'-GCT GCC TCT TCA AAA TCG CC-3', and reverse, 5'-CTC TGC ACT GTG TAC CTC GG-3'; β-actin forward, 5'-GAG AGG GAA ATC GTG CGT GAC-3', and reverse, 5'-CAT CTG CTG GAA GGT GGA CA-3'. Products were visualized by electrophoresis on a 1% agarose gel using a Gel Doc EZ gel documentation system (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All statistical analyses were performed with the SPSS 19.0 software (IBM, Armonk, NY, USA). Measurement data conforming to a normal distribution are reported as means ± standard deviations. Chi-square, one-way analysis of variance, and least significant difference tests were applied for comparison of means. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of citrate pretreatment on ventricular premature beat frequency

In comparison with control animals, model rats exhibited a significantly higher frequency of ventricular premature beats ($P < 0.05$). By contrast, in the citrate groups, these

events were less frequent compared with the model group, but more common than in the control group ($P < 0.05$). Group D significantly differed from groups C and E in this respect ($P < 0.05$), indicating that citrate may decrease the frequency of ventricular premature beats in a dose-independent manner, with a concentration of 0.05 M having the strongest effect (Figure 1).

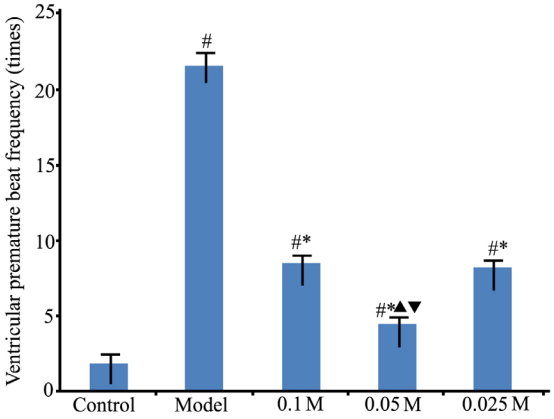

Figure 1. Effect of citrate pretreatment on ventricular premature beat frequency. $^{\#}P < 0.05$ compared with the control group. $^{*}P < 0.05$ compared with the model group. $^{p}P < 0.05$ compared with the 0.1 M citrate group. $^{q}P < 0.05$ compared with the 0.025 M citrate group.

Impact of citrate pretreatment on coupled rhythm and ventricular tachycardia and fibrillation

Citrate pretreatment markedly reduced the frequency of coupled rhythm and ventricular tachycardia compared to the model group ($P < 0.05$). In comparison with concentrations of 0.1 and 0.025 M, administration of 0.05 M citric acid also significantly diminished their frequency ($P < 0.05$; Figure 2). Ventricular fibrillation (sustained for 10.45 s) was observed only one time.

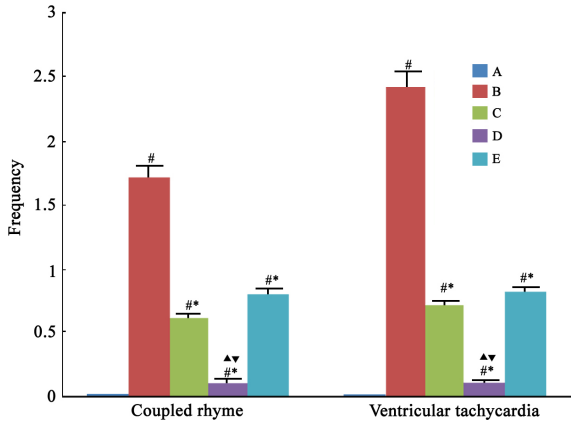

Figure 2. Impact of citrate pretreatment on coupled rhythm and ventricular tachycardia and fibrillation. $^{\#}P < 0.05$ compared with the control group. $^{*}P < 0.05$ compared with the model group. $^{p}P < 0.05$ compared with the 0.1 M citrate group. $^{q}P < 0.05$ compared with the 0.025 M citrate group.

Impact of citrate pretreatment on serum calcium ion concentration

After treatment with citrate for 5 min, serum calcium ion concentration in groups C, D, and E fell significantly, by 12.17, 16.81, and 11.25%, respectively ($P < 0.05$). After 15 min, these concentrations were 8.45, 14.11, and 7.96% lower than before citrate treatment, respectively, compared to which, they did not obviously differ. Interestingly, after 30 min, serum calcium ion concentration increased, and the level in group D was significantly different from those in groups C and E ($P < 0.05$). Our results demonstrated that the 0.05 M treatment exerted the most striking effect, with maximum chelation occurring after 10 min (Figure 3).

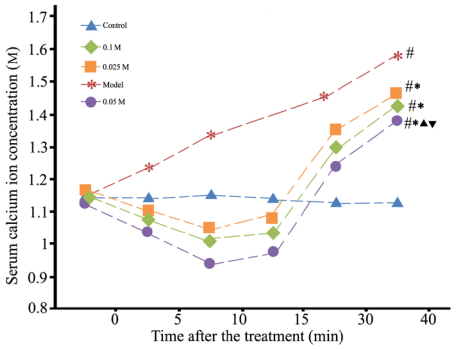

Figure 3. Impact of citrate pretreatment on serum calcium ion concentration. #P < 0.05 compared with the control group. *P < 0.05 compared with the model group. ▲P < 0.05 compared with the 0.1 M citrate group. ▼P < 0.05 compared with the 0.025 M citrate group.

Impact of citrate pretreatment on IS/LV, IS/AAR, and AAR/LV

The IS/LV, IS/AAR, and AAR/LV measurements of rats with I/R injury in the citrate treatment groups were significantly lower compared to those in the model group. In addition, IS/LV and AAR/LV values were markedly lower in the 0.05 M treatment group than in groups C or E ($P < 0.05$), suggesting that this citrate concentration has the strongest impact on IS/LV, IS/AAR, and AAR/LV (Figure 4).

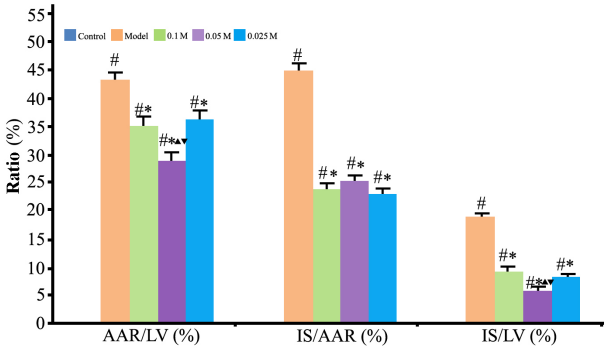

Figure 4. Impact of citrate pretreatment on infarct size (IS) relative to the left ventricle (IS/LV) and area at risk (IS/AAR), and AAR in relation to the LV (AAR/LV). #P < 0.05 compared with the control group. *P < 0.05 compared with the model group. ▲P < 0.05 compared with the 0.1 M citrate group. ▼P < 0.05 compared with the 0.025 M citrate group.

Impact of citrate pretreatment on caspase-3 protein expression in myocardial tissue

As a reference, caspase-3 expression in group A was given a value of 1, with expression in the other groups being determined relative to this. We found that caspase-3 protein expression in the citrate-treated groups was down-regulated compared to that in the model group ($P < 0.05$). As before, 0.05 M treatment resulted in the greatest effect, in this case, in inhibiting caspase-3 expression (Figure 5).

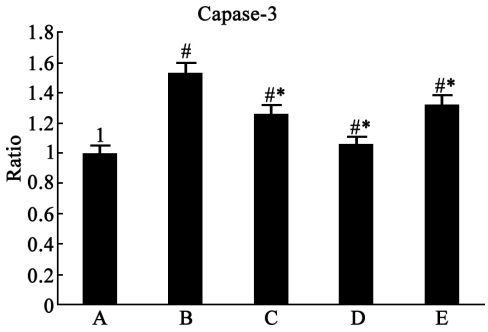

Figure 5. Caspase-3 protein expression in myocardial tissue. #P < 0.05 compared with the control group. *P < 0.05 compared with the model group.

Impact of citrate pretreatment on CAM expression in myocardial tissue

Compared to that in the sham operation group, the expression of CAM in the model group clearly increased, while being markedly reduced in the citrate pretreatment groups (Figure 6). CAM levels were notably suppressed following treatment with 0.05 M citrate, compared to that with 0.1 or 0.025 M ($P < 0.05$).

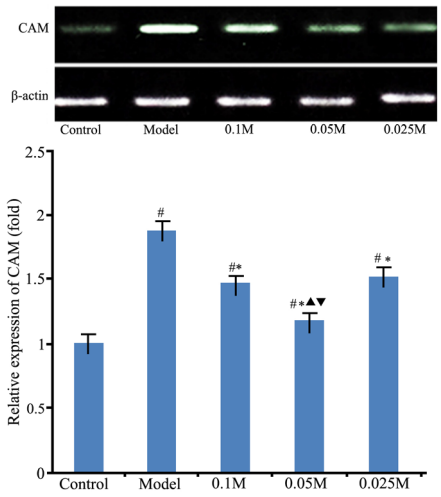

Figure 6. Calmodulin (CAM) expression in myocardial tissue. #P < 0.05 compared with the control group. *P < 0.05 compared with the model group. pP < 0.05 compared with the 0.1 M citrate group. qP < 0.05 compared with the 0.025 M citrate group.

DISCUSSION

Myocardial I/R injury may lead to malignant arrhythmia. Concerning changes in myocardial cell membrane ion channel structure and function, ventricular electrical remodeling is an important cause of arrhythmia following myocardial infarction (Liu and O'Rourke, 2013; Bell et al., 2015). After myocardial infarction, reentry and "abnormal automaticity" are associated with ventricular arrhythmia. Triggered activity involves delayed and early afterdepolarization, including that induced by action potential extension, and open L-type Ca^{2+} channels (Wu et al., 2011; Diez et al., 2013; Neckár et al., 2013; Tang et al., 2013). Calcium overload plays an important role in the pathology of myocardial I/R arrhythmia. Ischemia and hypoxia decrease Na^+/K^+-ATPase activity, increase Na^+/H^+ exchange, and heighten intracellular Na^+ concentration, leading to Na^+/Ca^{2+} exchange and reduced ATP production. This process also suppresses the uptake of Ca^{2+} by the sarcoplasmic reticulum and its release from the cell, resulting in cytoplasmic Ca^{2+} accumulation. Reperfusion supplies high-energy phosphate compounds, and the sarcoplasmic reticulum releases Ca^{2+}. A large quantity of calcium is thus present in the cell, leading to increased amplitude of delayed afterdepolarizations, and ventricular arrhythmia. This study showed that the frequency of ventricular arrhythmia was significantly higher among rats in group B compared with those in the control group ($P < 0.05$). Moreover, citrate treatment resulted in lower and higher frequencies than those observed in the model and control groups, respectively, in a dose-independent manner. The 0.05-M group most obviously reduced ventricular arrhythmia, suggesting that citrate can inhibit this pathology in an I/R rat model.

Ca^{2+} is the principal ion responsible for the myocardial cell L-type calcium channel current, which participates in myocardial cell action potential repolarization and the sinoatrial node pacemaker process, activating the sarcoplasmic reticulum to release calcium ions as the main ion flow. It is also involved in myocardial cell excitation-contraction coupling and electrical activity enhancing myocardial contractility (Gonano et al., 2014; Sun et al., 2014). This study revealed that citrate treatment significantly reduced serum calcium ion concentration ($P < 0.05$), which was lowest 10 min after administration, before rising again after 30 min, suggesting that citrate has a strong ability to chelate extracellular calcium.

Extracellular calcium ions produce a current via calcium ion channels, and participate in spontaneous depolarization and overshoot in slow- and fast-response cells, respectively. Apoptosis plays a critical role in I/R injury, and is associated with multiple factors, including oxygen free radicals and intracellular calcium overload (Antzelevitch et al., 2011; Kloner et al., 2011). Triggers of apoptosis induce a caspase cascade, with caspase-3 performing a key function. Apoptosis and caspase-3 overexpression appear in the same region of the ischemic myocardial area (Talukder et al., 2007; Dow et al., 2009). Our results showed that myocardial cell apoptosis increased during the I/R process, but caspase-3 protein expression decreased in the citrate-treated groups. Cell apoptosis predominates in early ischemia, whereas necrosis occurs as the degree of ischemia increases. Intracellular calcium overload and abundant free radical production after reperfusion produce irreversible cell apoptosis and aggravate I/R injury. Citrate intervention can protect myocardial tissue through an anti-apoptotic effect. Myocardial infarct size can be reduced to a certain extent, reflecting alleviation of I/R injury. Our data demonstrated that citrate can significantly reduce the area of myocardial infarction and ischemia, further confirming that such pretreatment relieves myocardial damage in rats with I/R injury. The mechanism behind this may be related to the chelation of extracellular calcium ions.

Citrate intervention can reduce intracellular calcium overload caused by influx after myocardial ischemia, and improve myocardial electrical remodeling. Myocardial ischemia and hypoxia inhibit ventricular muscle cell L-type calcium channel current, reduce peak current, and shorten phase two of the cardiac muscle cell action potential. This further accelerates myocardial repolarization, leading to ectopic unified repolarization of ventricular muscle and triggering ventricular arrhythmia. Peak L-type calcium channel current reduction diminishes the effect of calcium on the sarcoplasmic reticulum, triggers intracellular calcium overload to form concussion afterdepolarization, and induces ventricular arrhythmia by triggered activity. On the other hand, intracellular calcium elevation activates the apoptosis signaling pathway. The area of myocardial infarction is related to the degree of myocardial cell apoptosis. Here, the citrate-treated groups showed significantly reduced infarction areas, caspase-3 protein levels, and CAM expression compared to the model group. This indicates that citrate can protect the myocardium by lowering serum calcium ion concentration and caspase-3 and CAM production, minimizing myocardial cell apoptosis, and relieving ventricular arrhythmia.

The present study investigated the impact of citrate on rats with I/R injury and described the mechanism operating during post-ischemia reperfusion. However, it was limited by the small sample size and lack of an exogenous calcium ion control. Our future research will focus on the role of citrate in the alleviation of myocardial injury, as well as its relationship with calcium ions.

In summary, in a rat model of coronary artery I/R via ligation of the left anterior descending coronary artery, our preliminary results highlight the protective role of citric acid against myocardial damage caused by I/R, which correlated with a reduction in serum calcium ion concentration and decreased expression of caspase-3 and CAM.

Conflicts of interest

The authors declare no conflict of interest.

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