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Application of citrate as a tricarboxylic acid (TCA) cycle intermediate, prevents diabetic-induced heart damages in mice

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ABSTRACT

Objective(s): Higher cellular reactive oxygen species (ROS) levels is important in reducing cellular energy charge (EC) by increasing the levels of key metabolic protein, and nitrosative modifications, and have been shown to damage the cardiac tissue of diabetic mice. However, the relation between energy production and heart function is unclear.

Materials and Methods: Streptozotocin (STZ, 150 mg/kg body weight) was injected intraperitoneally once to mice that had been fasted overnight for induction of diabetes. After diabetic induction, mice received citrate (5 µg/kg) through intraperitoneal injection every other day for 5 weeks. The caspase-3, plasminogen activator inhibitor 1 (PAI1), protein kinase B (PKB), commonly known as AKT and phosphorylated-AKT (p-AKT) proteins were examined to elucidate inflammation and apoptosis in the heart. For histological analysis, heart samples were fixed with 10% formalin and stained with hematoxylin-eosin (HE) and Sirius red to assess pathological changes and fibrosis. The expression levels of marker proteins, tyrosine nitration, activity of ATP synthase and succinyl-CoA:3-ketoacid coenzyme A transferase-1 (SCOT), and EC were measured.

Results: Intraperitoneal injection of citrate significantly reduced caspase-3 and PAI-1 protein levels and increased p-AKT level on the 5th week; EC in the heart was found to be increased as well. Further, the expression level, activity, and tyrosine nitration of ATP synthase and SCOT were not affected after induction of diabetes.

Conclusion: Results indicate that application of citrate, a tricarboxylic acid (TCA) cycle intermediate, might alleviate cardiac dysfunction by reducing cardiac inflammation, apoptosis, and increasing cardiac EC.

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Introduction

Diabetes mellitus has been shown to induce a wide array of chronic complications with cardiac abnormalities (cardiomyopathy) being a prime cause of mortality (1-3). The pathogenesis of diabetic cardiomyopathy affects multiple organ systems as well as cellular organelles such as the sarcostyle, mitochondria, sarcoplasmic reticulum, and myolemma, effectively altering cellular metabolism (4, 5). Human and animal studies have shown that oxidative and nitrative stresses, mediated by reactive oxygen and nitrogen species (ROS and RNS) respectively, are important contributors to cardiac remodeling and abnormal function characteristic of diabetes (6, 7).

Since many diabetic patients exhibit signs of oxidative damage along with excessive ROS production, oxidative injury is believed to be the cause of diabetic complications (7-9). During diabetes development, the level of ROS produced by myocardial cells surpasses the cells ability to process and detoxify ROS, lading to myocardial cell damage. Further, it has been shown that the expression of anti-oxidant enzymes or administration of anti-oxidant compounds together blocked diabetes-induced ROS accumulation in diabetic mice and inhibited the development of cardiomyopathy (10-12). For example, the anti-oxidant protein metallothionein (MT) was found to protect against

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diabetic cardiomyopathy by decreasing intracellular ROS levels (11, 12). In addition, oxidative stress induced by 3-nitro tyrosine (3-NT) modifications in a few key metabolic enzymes including succinyl-CoA:3-ketoacid coenzyme A transferase-1 (SCOT) as well as the α and β subunits of ATP synthase resulted in a loss of energy production in diabetic mice heart (11, 12). Despite the information gathered, the reports about the association between energy level and cardiac performance in diabetic patients are fewer.

The tricarboxylic acid (TCA) cycle is used by all aerobic organisms to synthesize energy source e.g. ATP by metabolizing carbohydrates, fats, and proteins (13, 14). Citrate, an intermediate in the TCA cycle, continues in the TCA cycle via aconitase to regenerate oxaloacetate, which is contained in the foods and fatigue retardants and is able to combine with another molecule of acetyl CoA and continues cycling (14).

To evaluate whether the addition of a TCA cycle intermediate increases energy production in diabetic mice hearts, citrate was injected to diabetic mice model, and apoptotic and inflammatory response makers, energy charge (EC) and cardiac abnormalities were monitored. Our analyses showed that citrate application to diabetic mice partially improved heart function, but the SCOT and ATP synthase activity was not altered. This is the first study to analyze the effects of citrate treatment on diabetic heart damage, and suggests that it may prove to be a useful therapeutic; though, more investigation is needed to prove the efficacy.

Materials and Methods

Diabetic mice models

Friend Virus B-Type (FVB) mice were purchased from Model Animal Research Center of Nanjing University, and all procedures were approved by the Wenzhou Medical University Animal Policy and Welfare Committee (11, 12). After overnight fasting, Streptozotocin (STZ) (Sigma-Aldrich, St. Louis, MO, USA) suspended in 0.1 M sodium citrate buffer (pH 4.5), and was used to treat 8-week-old male mice once via intraperitoneal injection at a concentration of 150 mg/kg body weight (11, 12). Three days after STZ injection, a 5 mm section of the tail was cut and blood was assayed for glucose levels with a Surestep; readings were taken in duplicate for each animal and averaged. STZ-treated animals with a mean nonfasted blood glucose level ≥16.7 mM were classified as diabetic, all others were excluded from the study (11,15,16). Sodium citrate buffer, the STZ vehicle, was used as a control (11, 12).

Three weeks after the induction of diabetes, the mice were segregated into one of two treatment groups at random: one received sterile water and served as the diabetic model group (DM) and one group received sodium citrate (Sigma-Aldrich) dissolved in sterile water and served as the citrate treatment group (DM+Citrate). Mice were received intraperitoneal injections of sodium citrate (5 μg/kg) every other day for 5 weeks (11, 12). Non-diabetic mice also received sterile water and were utilized as the control group (Con).

Histopathological examination

Following the completion of the experimental time course, the hearts were dissected and fixed with a solution of 10% formalin before being embedded in paraffin. Tissue sections of 5 μm thickness were stained with HE and Sirius red (to detect collagen). A computer-assisted image analysis system was utilized to analyze tissue structure and fibrosis as described (11).

Immunoprecipitation (IP) of nitrated proteins with anti-3-NT

Cardiac tissue was collected and then homogenized in lysis buffer (50 mM Tris [Tris (hydroxymethyl) aminomethane]-HCl, pH 7.4, 150 mM NaCl, 1 mM [ethylene diamine tetraacetie acid (EDTA)], 0.1% NP40, 20 mM [dithiothreito (DTT)] with phosphate buffered saline (PBS) (0.02% Tween-20) and a 1% protease inhibitor cocktail before being centrifuged at 15,000 rpm for 15 min. Antibodies were cross-linked with Dynabeads protein A (Thermo Fisher Scientific) according to the manufacturer's instructions. Before incubation with antibody-linked Dynabeads overnight at 4 ˚C, the lysis buffer was pre-cleared with immunoglobulin G (IgG) Dynabeads protein A for 10 min at 4 ˚C. The immunoprecipitated Dynabeads complexes were washed 3 times with PBS (0.02% Tween-20), recovered by re-suspending in loading buffer, and detected by Western blot (12).

Western blot analysis

Western blot assay was used to detect total caspase-3, plasminogen activator inhibitor 1 (PAI1), protein kinase B (PKB) commonly known as Akt, p-Akt, 3-nitrotyrosine, and glyceraldehyde phosphate dehydrogenase (GAPDH). After electrophoresis and transfer, the polyvinylidene fluoride membranes were rinsed briefly in Tris-buffered saline, blocked (5% skim milk or 0.5% bovine serum albumin (BSA)) for 1 hr, and washed 3 times with Tris-buffered saline containing 0.05% Tween 20 for 5 min. The membranes were then incubated with anti-caspase-3 (Abcam, ab2171), anti-PAI1 (Abcam, ab125687), anti-p-AKT (Abcam, EP2109Y), anti-AKT (Abcam, ab8805), or anti-3-nitrotyrosine (Abcam, ab61392) in conjunction with GAPDH (Abcam, ab181602) overnight before being washed as described previously and then reacted with horseradish peroxidase-conjugated antibody for 1 hr. Antigen-antibody complexes were visualized by an electrochemiluminescence (ECL) kit (Biotrand, Crystal

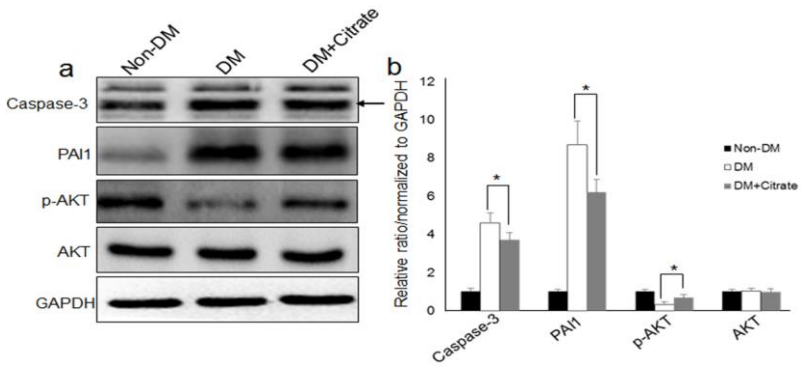

Figure 1. Citrate application prevents diabetes-induced accumulation of pro-inflammatory and apoptotic cytokines in the heart. Caspase-3, plasminogen activator inhibitor 1 (PAI1), p-AKT and AKT levels were assessed by Western blot analysis in cardiac tissues of non-diabetic and diabetic mice with or without citrate application after 8 weeks. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a loading control and the black arrowhead on the right side of the blot indicates Caspase-3 band (A). Semi-quantitative analysis of Caspase-3, PAI1, p-AKT and AKT normalized to GAPDH (B). Data are presented as mean±SEM. Each group included six mice, significant differences between non-DM and DM or DM and DM+Citrate groups were shown (**P*-value<0.05). Non-DM: non-diabetic mice; DM: diabetic mice; DM+Citrate: injection of citrate to diabetic mice

Lake, Ilinois, US) (12), and protein levels were normalized to GAPDH.

Measurement of enzyme activities

SCOT catalytic activity was measured by monitoring the change in absorbance at 313 nm at room temperature, which was an indicator of acetoacetyl-CoA formation. The reaction mixture contains 50 mM Tris·HCl (pH 8.0), 10 mM $MgCl_2$, 0.2 mM succinyl-CoA, 0.1–10 mM lithium acetoacetate, and 4 mM iodoacetamide. The catalytic reaction was started by addition of 300 µg of protein (17).

The production of ADP, which was coupled to NADH oxidation by pyruvate kinase and lactate dehydrogenase, was analyzed via spectrophotometer (340 nm) as an indicator of ATP synthase activity. The reaction mixture, final volume 0.7 ml at 30 ℃, consisted of: 100 mM Tris (pH 8.0), 4.0 mM Mg-ATP, 2 mM $MgCl_2$, 50 mM KCl, 0.2 mM EDTA, 0.23 mM NADH, 1 mM phosphoenol pyruvate, 1.4 unit of pyruvate kinase, and 1.4 unit of lactate dehydrogenase and about 50 to 135 mg proteins (11).

Measurement of EC by high performance liquid chromatography (HPLC)

The sample handling and protein extraction were carried out as described by Manfredi *et al* (18). The gradient elution of HPLC was performed on a 4.6-mm i.d. × 250 mm, 5-µm-particle-size Hypersil C_{18}-ODS column (Elite, Dalian, China) with the buffers (Buffer A: 25 mM NaH_2PO_4 and 100 mg/l tetrabutylammonium, pH 5.0; Buffer B: 10% (v/v) acetonitrile in 200 mM NaH_2PO_4 and 100 mg/l tetrabutylammonium, pH 4.0) at a rate of 1 ml/min. A prepared protein standard was auto-injected, UV- monitored at 260 nm, and peaks distinguished by

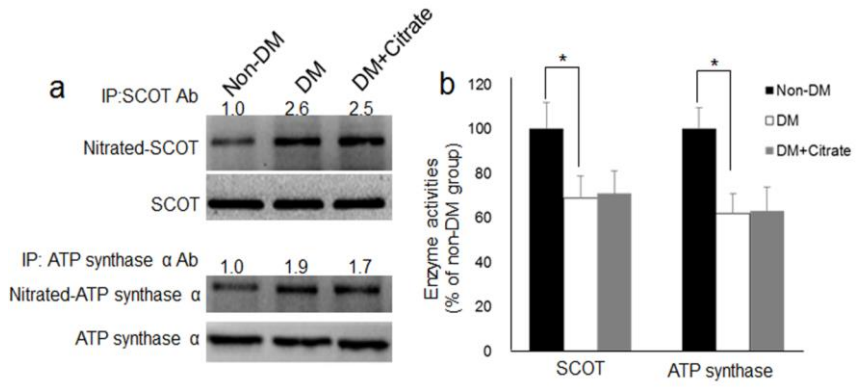

Figure 2. Citrate application did not change diabetes-induced 3-nitro tyrosine (3-NT) generation in succinyl-CoA:3-ketoacid coenzyme A transferase-1 (SCOT) and ATP synthase or their activities. The accumulation of 3-NT in SCOT and ATP synthase α subunits in the cardiac tissue of non-diabetic and diabetic mice with or without citrate application was analyzed after 8 weeks. Diabetic mice heart tissues were analyzed by immunoprecipitation (IP) and Western blot analysis. (A) The numbers on the top of the blots indicate the ratio of the signal density against non-DM group. (B) SCOT and ATP synthase activities were analyzed using cardiac tissue from non-diabetic and diabetic mice with or without citrate application after 8 weeks. Data are presented as mean ± SEM. Each group consisted of six mice, and comparisons were made using two-tailed unpaired Student's *t* tests (**P*-value<0.05). DM: diabetes; Ab: antibody

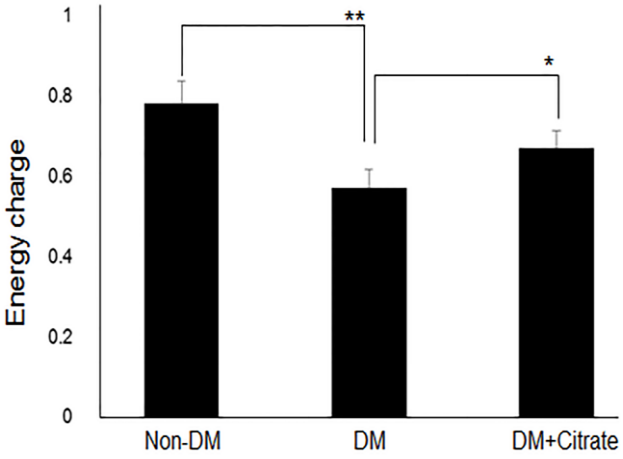

Figure 3. Citrate application rescued the diabetes-induced reduction of energy charge (EC). EC was measured by high performance liquid chromatography (HPLC) using cardiac tissues of non-diabetic and diabetic mice with or without citrate application after 8 weeks. Data are presented as mean ± SEM. Each group consisted of six mice and comparisons were made using two-tailed unpaired Student's *t*-tests (**P*-value<0.05 and ***P*-value<0.01). DM: diabetes

retention times according to the standard (11, 18, 19). EC was calculated based on Atkinson using the following formula: EC = (ATP + 1/2ADP) / (ATP + ADP + AMP) (20).

Statistical analysis

Data were collected from repeated experiments and presented as means±SEM (n≥6). Significant differences between two groups were analyzed using the t-test. Comparisons among multiple groups were calculated by ANOVA with Turkey multiple pairwise comparisons. Statistical significance was established at **P*-value<0.05 and ***P*-value<0.01.

Results

Citrate application partially reversed diabetes-induced apoptotic and inflammatory response markers

Monitoring of whole-blood glucose levels 0, 4, and 8 weeks after STZ injection showed no obvious differences between STZ and STZ+citrate injected diabetic mice (data not shown). Western blot analysis showed an increase of apoptotic and inflammatory response makers (Caspase-3 and PAI1), as well as a reduction in p-AKT level in diabetic mice cardiac tissue after 8 weeks of diabetes challenge. However, the total AKT level was unchanged in comparison with non-diabetic mice in cardiac tissue (Non-DM) (Figure 1). Citrate injection partially reversed diabetic-induction of caspase3, PAI1, and p-AKT, while no changes were observed in total AKT level in the heart (Figure 1). These data indicate that citrate application in diabetic mice partially reduced the apoptotic and inflammatory responses in cardiac tissue of diabetic mice.

Effects of citrate on diabetes induced 3-NT generation in diabetic heart

An increase of 3-NT levels in SCOT as well as the α and β subunits of ATP synthase was observed as a result of citrate injection. Further, there was a significant decrease in EC observed in the cardiac tissue of diabetic mice compared to control mice. To understand whether citrate application changes the 3-NT levels of SCOT and ATP Synthase, total heart proteins were immunoprecipitated by SCOT and ATP synthase α specific antibodies. Precipitates were further probed with 3-NT specific antibody to detect the amount of 3-NT modifications. The data showed that 3-NT levels were significantly greater in cardiac tissue of diabetic mice compared to control mice. Further, citrate application was found to have no change on 3-NT levels (Figure 2A). Interestingly, the precipitated proteins were found to contain similar amounts of total SCOT and ATP synthase α proteins in the cardiac tissue of three groups (Figure 2A).

Effects of citrate on EC in diabetic heart

Activities of SCOT and ATP synthase were observed to be significantly reduced after diabetes induction in wild-type mouse cardiac tissue; their activities were not preserved by citrate application (Figure 2B).

The EC value, a useful predictor of mitochondrial activity, is a reflection of the concentration of ATP and the conversion efficiency of high-energy phosphate bonds among ADP, AMP, and ATP. After 8 weeks, the EC values in heart tissues of three groups were measured. The results showed that citrate injection to diabetic mice partially reversed the observed decrease in EC value due to diabetes (Figure 3).

Citrate application partially reversed cardiac pathological changes caused by diabetes

To further analyze the effects of citrate on diabetes-induced heart damage, histopathological analyses were performed. HE staining showed significant cardiac disorder in the hearts of diabetic mice after 8 weeks, including increased disorganization of myocardial structure, myocardial discontinuation and fibrosis (Figure 4A). Sirius red staining showed significantly increased accumulation of collagen in diabetic mice after 8 weeks (Figure 4B). Citrate application was found to partially rescue the diabetes-induced pathological changes in the cardiac tissue (Figure 4).

Discussion

Diabetes mellitus is a principal risk factor for the development of cardiovascular complications, which now accounts for the mortality of 80% of diabetic individuals (1-4). The data presented in this study indicated that citrate application increased EC and partially eliminated the apoptotic and inflammatory responses in the cardiac tissue of diabetic mice.

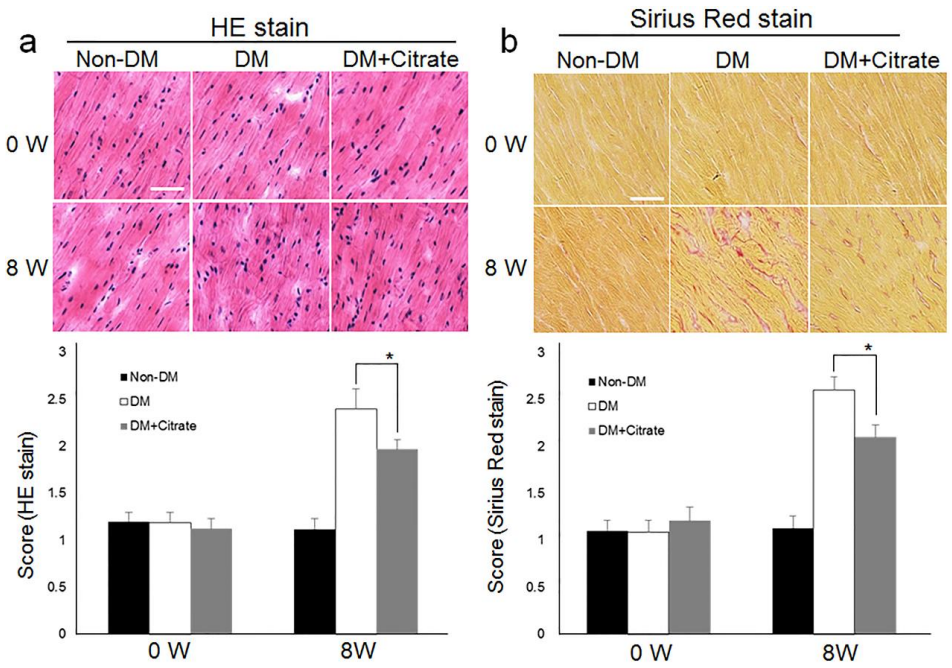

Figure 4. Citrate application supplementation inhibits diabetes-induced cardiac abnormalities. Cardiac tissue was analyzed histopathologically by staining with hematoxylin-eosin (HE) (A) and Sirius red (B), followed by examination utilizing light microscopy and semi-quantitative analysis using a computer-image analysis system. Scale bars = 50 μm. Data are presented as mean ± SEM. A total of 30 images were taken from each group, and significant differences between DM and DM+Citrate groups are shown (**P*-value<0.05). DM: diabetes

Additionally, citrate injection partially protected the cardiac tissue from diabetes-induced damage as noted by decreased cell death, fibrosis, and collagen accumulation.

Several hypotheses have been advanced to explain diabetic cardiovascular complications (21), with the free radical hypothesis being one of the most prominent. Nitrative damage is known to play an important role in diabetes-induced cardiac abnormalities by inducing an inflammatory response. From previous studies, it is known that inflammation is associated with an up-regulation of protein nitration at tyrosine residues (22), yielding 3-NT, which has been used as an index of nitrative stress-induced protein damage (11, 12, 15). MT, a family of metal binding proteins, is able to eliminate superoxide, and when overexpressed in the cardiac tissue of mice was able to prevent protein nitration induced by diabetes (11, 12, 15). Furthermore, MT overexpression in mice cardiac tissue reduced the levels of 3-NT in SCOT as well as ATP synthase, and protected the heart function of mice against diabetic-damage (11, 12). In our analyses, citrate injection did not change nitration levels of SCOT and ATP synthase or their enzymatic activities in the cardiac tissue of diabetic mice (Figure 2). However, citrate addition increased EC, reduced inflammatory and apoptotic responses, which may have resulted in protection of the cardiomyocytes from diabetes. The possible interpretation is that more TCA cycle intermediate in the cells increased the products of the reaction.

ATP is synthesized mainly via glycolysis and TCA cycle (14). Therefore, we supplied citrate, a TCA cycle intermediate, to analyze the connection between energy status and cardiac damaged induced during the period of diabetes. The results suggest that activation of the TCA cycle might be an important therapeutic target for protecting the heart from diabetic stress. Citrate is a molecule, which can be easily obtained from daily food e.g. fruits and drinks. However, citrate addition is also able to activate lipid metabolism, so that balancing cellular citrate level is also important for health (14). In this study, only citrate-mediated EC increase was measured, and further experiments are required to analyze effect of citrate application in other processes including lipid metabolism to test the optimized concentration of citrate for diabetic-induced heart damage therapy.

Even though further studies are required, the results identified in our analyses are useful and important for using cheap nature components like citrate, which enhanced EC in heart under diabetes, and further protected cardiomyocyte damage.

Conclusion

These results showed that citrate application did not change the SCOT and ATP synthase activities in the heart of diabetic mice, but it reduced apoptotic and inflammatory responses, while increased EC in the heart. In addition, citrate application protected cardiac abnormalities induced by diabetes. In this reason, citrate could be considered as a potential

therapeutic component for diabetes induced heart disease.

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