Crocetin Exerts a Cardio-protective Effect on Mice with Coxsackievirus B3-induced Acute Viral Myocarditis

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Abstract: Previous research has proven that coxsackievirus B3 (CVB3) is broadly considered virus used in the experimental model of animals, which causes myocarditis in humans. To investigate whether there exists a cardio-protective effect of crocetin in an experimental murine model of acute viral myocarditis (AVM). Male BALB/c mice were randomly assigned to three groups: control, myocarditis treated with placebo and myocarditis treated with crocetin (n = 40 animals per group). Myocarditis was established by intraperitoneal injection with CVB3. Twenty-four hours after infection, crocetin was intraperitoneally administered for 14 consecutive days. Twenty mice were randomly selected from each group to monitor a 14-day survival rate. On day 7 and day 14, eight surviving mice from each group were sacrificed and their hearts and blood were obtained to perform serological and histological examinations. Expression of ROCKs, interleukin-17 (IL-17), interleukin-1β (IL-1β), tumor necrosis factor-α (TNFα), RORγt, and Foxp3 was quantified by RT-PCR. Plasma levels of TNFα, IL-1β and IL-17 were measured by ELISA. In addition, protein levels of IL-17 and ROCK2 in cardiac tissues were analyzed by Western blot. Crocetin treatment significantly increased survival, attenuated myocardial necrotic lesions, reduced CVB3 replication and expression of ROCK2 and IL-17 in the infected hearts. ROCK pathway inhibition was cardio-protective in viral myocarditis with increased survival, decreased viral replication, and inflammatory response. These findings suggest that crocetin is a potential therapeutic agent for patients with viral myocarditis.

Key words: crocetin, myocarditis, coxsackievirus B3, inflammation

1 Introduction

In 1995, The International Society and Federation of Cardiology (ISFC), sub part of the World Health Organization explains the term myocarditis is a disease, which occurs due to inflammation in the muscle of the heart and detected via histopathology, immunostaining and immunological study⁵. Cases that mostly concerned with myocarditis are viral in nature²,³. Numerous viruses such as coxsackieviruses group B paroviruses, influenza virus H1N1, adenovirus, echorovirus, yellow fever virus, dengue fever virus, polio virus, rabies virus, hepatitis A and C viruses, are the causative agent for the myocarditis, whereas endomyocardial biopsy technique is used to establish the parvovirus B19 (PVB19) and a diagnostic virus in the determination of myocarditis⁴−⁶. Previous research has proven that CVB3 is broadly considered virus used in the experimental model of animals, which causes myocarditis in humans⁷,⁸.

Myocarditis is one of cardiology’s most difficult clinical troubles, which is characterized by mild dyspnea, acute heart failure, and sudden death⁹. Viral myocarditis is a general type of myocarditis characterized by inflammation of the heart. It is initiated with a viral infection and termed as triphasic disease involving go behind with autoimmune

Abbreviations: CVB3; Coxsackievirus B3, AVM; Acute viral myocarditis, IL-17; Interleukin-17, IL-1β; Interleukin-1β, TNFα; Tumor necrosis factor-α, ISFC; International Society and Federation of Cardiology, PVB19; Parvovirus B19, GIT; Gastrointestinal track, CT=Crocetin, IACUC; Institutional Care and Use Committee, HW; Heart weight, BW; Body weight, LVSP; Left ventricular systolic pressure, HR; Heart rate, LVEF; Left ventricular ejection fraction, LVEDP; Left ventricle end diastolic pressure, Mb; Muscle hemoglobin, cTnI; Cardiac troponin I, CK-MB; MB isoenzyme of creatine kinase

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reaction, which results in cardiac structure and function remodeling\(^{10,11}\).

The most common enterovirus is CVB3 that cause respiratory, and gastrointestinal track (GIT) diseases, and an etiological agent to produce myocarditis. It is a critical condition, if anyone has the risk of heart disease i.e., heart failure and a bad prognosis in acute and chronic myocarditis cases\(^ {12}\). CVB3 quickly multiplies inside the cardiomyocytes of humans and stimulate the pathways of antimicrobial host defense and resulting in heart-reactive autoantibodies being produced. Viral myocarditis pathogenesis is very complicated to understand and has been suggested by several investigators. The main mechanism is the excessive innate immune response that induces inflammation\(^ {13}\). Toll-like receptor 4/nuclear factor \( \kappa B (T L R 4 / N F - \kappa B) \) signaling pathway is directly associated with inflammatory response and several researchers have indicated a positive association between TLR4 activation and viral myocarditis\(^ {14}\).

Now days, novel therapeutic agents or substances that are derived from natural products and have a high therapeutic potential and little side effects, they are used for treating viral myocarditis\(^ {15}\). Crocetin (CT) is a bioactive component of the stigma of saffron (biological name: Crocus sativus L.). The molecular formula of CT \( C_{20}H_{24}O_{4} \) and molecular weight is 328.4 g/mol, as depicted in figure 1\(^ {16}\). It is water insoluble agent and in most of organic solvents in its free-acid form\(^ {16,17}\). Crocetin has various health-promoting activities or pharmacological effects such as anti-apoptotic\(^ {18}\), neuroprotective activity, antioxidative properties, anti-inflammatory, anti-cancer, anti-hyperlipidemic, cardio-protective properties\(^ {19-23}\). Those pharmacological benefits may be attained because of various mechanisms such as enhanced oxygenation in hypoxic tissues, antioxidant benefits, pro-inflammatory mediator inhibition, anti-proliferative activity and apoptosis stimulation in cancer cells\(^ {24-27}\). On the basis of pharmacological effects of CT as cited in literature, in particular, its capability to decrease inflammation and oxidative stress, suggest that it is a suitable agent for ameliorating viral Myocarditis. For that reason, we considered CT might be successfully amending the CXB3-induced acute viral Myocarditis. To check this hypothesis, we examined the impact of CT on acute viral Myocarditis caused by CXB3 and investigated the possible underlying mechanisms of oxidative stress and inflammation by triggering the inflammatory Signaling pathway in animals. Therefore, best and protective drugs to mitigate viral Myocarditis caused by CXB3 should offer opportunities to the pharmaceutical industry for the clinical utilize of CT.

2 Material and Method
2.1 Chemical
All the chemical and reagents used in the current experimental protocol was purchased from Sigma Aldrich USA.

2.2 Cell and virus
Nancy strain (CXB3) was procured from the ATCC (Manassas, VA). The Hep2 cells were procured from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, China. The virus was amplified in Hep2 cells and harvested for the induction of AVM model\(^ {28}\).

2.3 Experimental animal
In the current experimental study, BALB/c mice (20 ± 2 g, 4-5-week-old, sex-male) were procured from the Laboratory Animal Center. All the mice were kept in the standard experimental condition and received the water \( \text{ab libitum} \) and standard food pellets. All experimental studies were approved by the Institutional Care and Use Committee (IACUC).

2.4 AVM mouse model
For the current experimental protocol, the mice were randomly divided into the following groups such as –

- **Group I**: control group
- **Group II**: Myocarditis group treated with placebo
- **Group III**: Myocarditis group treated with crocetin (2.5 mg/kg, body weight)
- **Group IV**: Myocarditis group treated with crocetin (5 mg/kg, body weight)

Intraperitoneal treatment of CVB3 (\( 10^{7} \)), prepared using PBS (titer was estimated with 50% tissue culture infectious dose assay) to induce viral myocarditis in all groups except the control group\(^ {28}\). In the control group, the mice were treated with the equal intraperitoneal dose of PBS. Day 0 consider as the virus inoculation, after the initial inoculation (24 h later), the mice received intraperitoneal injection of crocetin (2.5 and 5 mg/kg) for 14 consecutive days. Meanwhile, the placebo and control group mice received the equal amount of PBS. After 14 days, half of the mice were selected to scrutinize the survival rate during 14 consecutive days\(^ {29}\). We sacrificed the 8 mice from all groups at regular time interval (7 and 14 days) to obtain the plasma and cardiac samples. After that, the heart weight (HW) and body weight (BW) were estimated. For the estimation of heart function index, the ration of HW/BW was estimated.

2.5 Detection of cardiac function index
Left ventricular systolic pressure (LVSP) and heart rate (HR) were estimated via using the Power Lab recording instrument (AD Instruments, Castle Hill, Australia). Left ventricular ejection fraction (LVEF) and left ventricle end diastolic pressure (LVEDP) were detected via transthoracic echocardiography using an Agilent Sonos 5500 ultrasound
2.6 Myocardial damage marker

Myocardial damage marker such as muscle hemoglobin (Mb), cardiac troponin I (cTn I) and MB isoenzyme of creatine kinase (CK-MB) were estimated using the commercially available kits (R&D Systems, Minneapolis, MN).

2.7 Cytokines

Pro-inflammatory cytokines parameters such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-17 (IL-17) were estimated using the commercially available kits (R&D Systems, Minneapolis, MN).

2.8 RNA extraction, cDNA synthesis and PCR quantification

Total RNA from the heart tissue or cells was extracted with Trizol reagent (Invitrogen) according to the manufacturer’s instructions. The first strand cDNA was synthesized with random primer in a 20 μL reverse transcription reaction using 2 μg total RNA. After initial denaturation of 95°C for 1 min, different PCR cycle was performed to amplify β-actin (20 cycles), IL-17 (25 cycles) and ROCK2 (25 cycles), which consisted of denaturation at 95°C for 15 s, annealing at 58°C for 15 s and extension at 72°C for 30 s. The PCR products were run on 2% agarose gel and stained with ethidium bromide. The stained PCR products were photographed and analyzed with ImageJ to obtain densitometric value of each product. Expression of those genes was quantified as relative values versus that of β-actin. A quantitative RT-PCR was performed to detect the CVB3 RNA abundance as well as the mRNA levels of Tnfα, IL-1β, ROrγt and Foxp3, using a SYBR Green I qPCR kit (Biotool Inc., Shanghai, China) run on the ABI QuantStudio Dx machine (Thermo Fisher). GAPDH was used as the internal control to calculate relative values of viral abundance using 2^-ΔΔCt method. The sequences of primers used in this study are illustrated in Table 1.

2.9 ELISA measurement of TNFα, IL-1β and IL-17

Plasma levels of IL-17, often referred as IL-17a, IL-1β and TNFα, were measured with corresponding ELISA kits (R&D Systems) according to the manufacturer’s instructions. In brief, a 96-well plate was coated with a capture antibody overnight at 4°C. On the next morning, the plate was washed and incubated with 100 μL diluted plasma samples (1 in 4 dilutions with PBS) for 2 h at RT. After washing, a detection antibody, which was conjugated with biotin, was added and incubated for 2 h at RT. Then, streptavidin-HRP was used to detect the biotinylated antibodies and to develop a yellowish color for measurement at O.D. of 450 nm.

2.10 Statistics analysis

All experiments were repeated at least three times unless otherwise mentioned. Numeric data are presented as mean ± SEM. The multiple Kaplan–Meier survival data were analyzed with the log-rank test for trend in the GraphPad Prism 7 software. The Bonferroni corrected threshold was computed to determine the differences among groups of survival data. Other data were analyzed with student’s t-test, one-way or two-way ANOVA whenever it was appropriate. Comparisons among the control, placebo and Fasudil groups were performed and a value of p < 0.05 was considered significant.

3 Results

3.1 Detection of cardiac function index

CVB3 infected mice showed declined in the level of HR, LVSP and LVEF, and raised the level of LVEDP compared with the normal control group. Amusingly, treatment with crocetin (2.5 mg/kg and 5 mg/kg body weight) in the CVB3-infected mice significantly improved the level of HR, LVSP and LVEF, and LVEDP (Fig. 1).

3.2 Myocardial damage markers

Untreated CVB3 induced AVB mice showed a significant elevation in myocardial injury marker levels such as cTn I, Mb, and CK-MB (Fig. 2). When CVB3 induced myocarditis mice received a drug crocetin, there was a reduction in the levels of all three myocardial damage markers.
3.3 CT treatment attenuates CVB3-induced myocarditis in mice

The impact of crocetin on CVB3 induced acute virus myocarditis were done by infecting mice with inducing agent CVB3 and further cured with placebo and two doses of crocetin from 0 to 7 days after induction. The study suggests that CVB3 induced AVM animals have viral myocarditis and shows a sign of lethargy, weakness, weight loss, etc and after post induction of 14 days approximately 40% of mice were no more. Treatment with crocetin significantly alleviates the Viral myocarditis in CVB3 induced AVM in mice with the high chance of survival rate and similar result was followed by a smaller loss of body weight (Fig. 3). The score for pathologic myocarditis was also lesser in CVB3 induced AVM mice when treated with crocetin.

3.4 Crocetin treatment improved survival of CVB3-infected mice during a 14-day follow-up study

We began a scrutiny of the 14-day on a coxsackievirus B3-induced AVM in an animal to test, regardless of whether, treatment with crocetin prevents the contagion of CVB3 in mice (Fig. 4). Administration of crocetin at a dose
level of 5 or 10 mg/kg improved survival relative to animals treated with placebo. 10 mg/kg of crocetin dose was found to be effective and statistically relevant. Only the high dose treatment with crocetin was statistically relevant and within we retained the treatment of crocetin (10 mg/kg) for the remainder of our research.

3.5 Crocetin treatment reduced viral burden in cardiac tissues and plasma levels of β and IL-1α and pro-inflammatory cytokines, TNF-α
Quantitative RT-PCR is used to examine the CVB3 RNA viral genomic in the heart and the pancreas of a mouse and to track the replication of virus. In the acute phase, CVB3 replication was greatly vigorous, as comparatively higher rates of viral RNA were observed in the heart and pancreas on day 7 than those of day 14. Administration of the crocetin significantly declined the viral burden in these two tissues sensitive to CVB3 at both points of time. In mouse heart, the pro-inflammatory cytokines expression (TNFα and IL-1β) were observed. The CVB3 infectivity raised the mRNA levels of both genes on day 7 but on day 14 after viral inoculation the basal level were come back. Therapy of crocetin reversed the elevation of the TNF-α and IL-1β mRNA levels caused by viral infection. Constantly, Crocetin therapy improved the elevated raise level of TNFα and IL-1 in plasma caused by CVB3 in mice (Fig. 5).

3.6 Crocetin treatment inhibited CVB3 infection-induced expression of ROCK2 and IL-17
We performed a semi-quantitative RT-PCR in the tissue of the heart to investigate the underlying mechanism of crocetin management to find out the level of mRNA ROCK1 and ROCK2 in CVB3 induced AVB mice. After the inoculation of virus, there was an elevation in the level of ROCK2 mRNA (Fig. 6). Treatment with crocetin improved the raised level ROCK2 mRNA in CVB infected mice. It is also observed that CVB3 infected mice show a high level of ROCK2 that controls the release of IL-17 (Th17-specific cytokine) with enhanced expression. A significant enhancement was observed in the level of IL-17 mRNA in the cardiac tissue of the group treated with placebo compared with normal control rats. CVB3 induced AVM treated with crocetin declined the level of IL-17 mRNA up to half of the placebo-treated AVB mice on both time points. Elisa was used to assess the levels of IL-17 in cardiac tissue of mice (Fig. 7). The above finding favors the anti-inflammatory activity of crocetin via less IL-17 production and improved ROCK2 effect.

3.7 Crocetin treatment influenced CD4+ T cell populations in the mouse hearts after CVB3 infection
CVB3 induced AVB mouse showed increased ROXpt (Th17-specific marker) gene expression and reduced level of Foxp3 (a regulatory T-cell (Treg)-specific gene) i.e. sub-populations of infiltrated CD4+ T lymphocytes and deter-
mined by using quantitative RT-PCR (Fig. 8). Reduced expression of RORγt gene and elevated levels of Foxp3 mRNA were observed in CD4+ T cells of isolated heart of crocetin-treated AVM animals, which suggest a shift in T cell subpopulations from pro-inflammatory Th17 to anti-inflammatory Tregs.

**4 Discussion**

CVB3 infection may be serious and cause acute viral myocarditis that can lead to dilated cardiomyopathy\(^\text{30}\). Cardiac muscular inflammation is termed as Myocarditis. CVB3 infected viral myocarditis is presented histopathologically via cardiomyocyte necrosis, and lymphocyte or monocyte infiltration\(^\text{31, 32}\). CVB3 infection causes cardiac inflammation and illustrated by elevation in pro-inflamma-
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Fig. 8 showed the effect of crocetin on RORγt and Foxp3 expression in CVB3 mouse myocarditis model. a: RORγt and b: Foxp3. Crocetin = CT, Control = Con, coxsackievirus B3 = CVB3. Values are shown as mean ± SD. #p < 0.05, ##p < 0.01 and ###p < 0.001 compared with control group, *p < 0.05, **p < 0.01 and ***p < 0.001 compared with CVB3 group.

Cytokines production and nuclear factor kappa B (NF-κB) stimulation. There are no appropriate antiviral drugs are present to treat CVB3 infection. Preventive measures are still the main therapy for CVB3-infected viral myocarditis, owing to be deficient in appropriate antiviral medication.

Information in the literature suggests that CT has anti-mutagenic activity, antioxidants, anticancer agents, Antibacterial, antiviral and antidyslipidemia activity. However, this bioactive molecule was not explored earlier for acute viral Myocarditis. Various research studies on animal models suggest its antioxidant, anti-cardiac and anti-inflammatory activity.

We utilized a standard viral myocarditis experimental model on mice infected by CXB3. On this basis of analysis of this study, we suggest that CT potentially consider as a secure and booming therapeutic alternative for myocarditis due to infection with CVB3.

Our findings suggested that the animal model was successfully developed as proof by diminished heart function and improved inflammatory levels Biomarkers and Cytokines. Additionally, there was a reduction in the survival rate of the CXB3-induced mice group. Therefore, the observation of data supports the attenuation of inflammatory response by CT at 2.5 mg/kg and 5 mg/kg, body weight in the CXB3-induced mice group.

An experimental model of AVM in mice explains the involvement of activation of ROCK2 in immune response which was evidenced by PCR and immuno-blotting results. We have also detected many pro-inflammatory cytokines i.e. TNFα, IL-1β and IL-17. Rho-kinase signaling thus plays an essential role in AVM pathogenesis. Our results showed that CT hampering the ROCK2’s CXB3-induced expression in mouse hearts does not inhibit the expression of basal that is reliable with earlier research, indicating that could ROCK2 expression may be enhanced due to inflammatory cytokines. Yet, the exhaustive signaling cascades implicated in this infection would make further investigation possible.

The virus not only direct attacks on cardiac tissue and injured its but also excessively stimulates the immunological responses in case of viral Myocarditis. Increased expression of IL-17 was seen in the placebo group when contrast to the control group, this was steady with current studies revealing the similar involvement of Th17 lymphocytes and their cytokine IL-17. This also implicated in the abnormal immune responses and produces aggravate heart injury in acute viral Myocarditis. In the acute inflammatory response, IL-17 has acted in tandem with TNFα and IL-1, the main macrophage cytokines, to exacerbate inflammation. Along with the monoclonal antibody, Neutralizing IL-17 considerably recovered the survival rate and amended the heart injury in CVB3-infected AVM, thus inhibiting. During CVB3 infection, there was the inequity of Th17/Treg cells. In mice infected with CVB3, an elevation in the infiltration of Th17 and Treg cells, and the serum level of associated cytokines. Furthermore, the defensive effects of crocetin in CXB3-infected mice were directly correlated with the regulation of Th17/Treg imbalance and decreased viral replication of CVB3 during the pathogenesis of viral myocarditis. Crocetin repressed Th17 cells differentiation and facilitated both the delineation and oppressive activity of Treg cells in vivo, therefore potentially showing a pharmacological activity against viral myocarditis. Our findings therefore indicate that Crocetin could be a successful method for the prevention or treatment of viral myocarditis. Many studies have indicated a functional imbalance in Th17/Treg during acute myocarditis caused by CVB3. It has been proposed that Treg and pro-inflammatory Th17 cells play either suppressor or effector roles, during an ailment. As a result, alleviation of proliferation of Th17/Treg cells and function immune system could signify the protective activity through which Crocetin regulates the viral myocarditis.

5 Conclusion

Briefly, we have proven via different activities that crocetin actively shown the anti-CVB3 activity against AVM in vivo. In addition, crocetin also improved the damage of tissue and survival rate in animals against viral Myocarditis. Moreover, elucidation of signaling cascades and molecular mode of action is remains to be confirmed through which crocetin shows antiviral activity against CVB3. Lastly, it is
evidenced that crocetin may be considered superior therapeutic compound in the management of acute viral myocarditis.

Data Availability Statement
The authors confirm that the data supporting the findings of this study are available within the article.

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