Objective: A recent high-throughput sequencing showed that circular RNA Rho-associated kinase 1 (circROCK1) is abnormally highly expressed in sepsis, but whether it is involved in sepsis development remains unclear. The objective of this study was to investigate the biological function of circROCK1 in sepsis-induced myocardial injury and reveal its potential downstream molecular mechanism. Methods: Real-time reverse transcriptase-polymerase chain reaction was applied to detect circROCK1 and miR-96-5p expressions in the serum of septic patients. Spearman correlation analysis examined the correlation between circROCK1 and the clinicopathological characteristics of septic patients. The Cecal puncture and ligation (CLP) method was used to establish an in vivo sepsis model. circROCK1 and miR-96-5p expressions in mice were modified by injection of lentivirus or oligonucleotide. The left ventricular systolic pressure, left ventricular end-diastolic pressure, and the maximum increase/decrease rate of left ventricular pressure were checked. ELISA was applied to detect inflammatory factors levels as well as myocardial injury markers levels. Hematoxylin and eosin staining was performed to observe pathological changes in myocardial tissues, and Western blot examined phosphorylated nuclear factor (NF)-κB and oxidative stress-responsive 1 (OXSR1) expression. Dual luciferase reporter experiment was conducted to confirm the targeting relationship between circROCK1, OXSR1, and miR-96-5p. Results: circROCK1 and OXSR1 were highly expressed in sepsis and miR-96-5p was under-expressed. circROCK1 was positively correlated with serum creatinine, C-reactive protein, procalcitonin, and sequential organ failure assessment scores in septic patients. Silencing circROCK1 could improve the diastolic and systolic function of CLP mice, as well as myocardial damage, reduce myocardial tissue edema and necrosis, and inhibit inflammatory factor level and phosphorylated NF-κB expression. Down-regulating miR-96-5p promoted myocardial injury in CLP mice. Silencing circROCK1 and miR-96-5p inhibited and promoted OXSR1 expression, respectively. Both circROCK1 and OXSR1 had a targeting relationship with miR-96-5p. Conclusion: CircROCK1 promotes myocardial injury in septic mice by regulating the miR-96-5p/OXSR1 axis, and it can be used as a potential target for treating septic myocardial dysfunction.

Keywords: CircROCK1, OXSR1, miR-96-5p, sepsis, myocardial injury
ple-organ injury in septic patients, like lung (Zou et al., 2020), kidney (Shi et al., 2020), and liver injury (Xiong et al., 2021). Circular RNA Rho-associated kinase 1 (circROCK1) is an essential member of the circRNA family and has been shown to be differentially expressed in sepsis (Bao et al., 2019). However, it is not clear whether it has an impact on septic myocardial injury.

This work investigated serum circROCK1 expression level and its clinical effect on septic patients. The effect of circROCK1 on myocardial function and myocardial inflammation in sepsis was investigated in vivo by establishing a mouse model by cecal puncture and ligation (CLP). The results revealed that circRNA promoted myocardial injury in sepsis by sponging miR-96-5p to mediate oxidative stress-responsive 1 (OXSRI) expression.

METHODS

Clinical samples

Healthy people (n=37) were randomly selected from the health examination center of The First Affiliated Hospital of Guangzhou Medical University. From September 2018 to January 2020, blood samples were obtained from patients with sepsis (n=44) and septic shock (n=19) at The First Affiliated Hospital of Guangzhou Medical University. The diagnosis of sepsis or septic shock is based on the International Guidelines for the Management of Sepsis and Septic Shock: 2016. This research was approved by The First Affiliated Hospital of Guangzhou Medical University Institutional Ethics Committee. All subjects and their families had signed the informed consent form.

Diagnostic criteria

Sepsis is diagnosed in reference to International Guidelines for the Management of Sepsis and Septic Shock: 2016, with a Sequential Organ Failure Assessment (SOFA) score of more than 2. Patients diagnosed with sepsis who required adequate hemodynamic resuscitation and sustained hypotension with pressors to maintain arterial pressure of 65 mmHg or higher and serum lactate levels greater than 2 mmol/L (18 mg/dL) were identified as having septic shock.

Exclusion criteria

Exclusion criteria: (1) Under 18 years old; (2) MOF caused by other non-infectious factors; (3) Autoimmune diseases treated with steroids or immunosuppressive therapy; (4) Complications with other diseases that affect blood coagulation; (5) Malignant tumors; (6) Complications with other diseases that may affect this study results; (7) Immunosuppressive drug treatment within the last three months.

Clinical data collection

Clinical information was recorded, including age, gender, body mass index (BMI), and SOFA score. Additionally, Quzhou People’s Hospital clinical laboratory provided routine blood test results (serum creatinine [Scr], white blood cells, albumin, C-reactive protein [CRP], and procalcitonin [PCT]).

Septic animal model establishment

C57BL/6 mice (25±5 g) were kept in cages in a temperature-controlled room with a 12-hour light/dark cycle and a free diet and water. Twenty-four mice were allocated into the sham group (n=12) and the CLP group (n=12) to evaluate the survival rate within 48 hours after surgery. The survival rate of mice after CLP was measured every 2 h for 48 h.

In the following experiment, another 72 mice were used, 12 of which underwent sham surgery and the rest received CLP. These mice were allocated into 5 groups (n=12 for each): Sham group (CLP without ligation and puncture on the cecum), CLP group (CLP surgery), sh-circROCK1 group (injection with 5×10⁷ sh-circROCK1 lentiviral vector particles via tail vein one week before CLP), sh-NC group (injection with 5×10⁷ sh-NC lentiviral vector particles via tail vein one week before CLP), miR-96-5p antagonist group (injection with 10 μg miR-96-5p antagonist via tail vein one week before CLP), and NC antagonist group (injection with 10 μg NC antagonist via tail vein one week before CLP). sh-circROCK1/NC lentiviral vectors and miR-96-5p/NC antagonist were provided by GenePharma (China). The cardiac function of mice was monitored 12 h after CLP, myocardial tissue and serum samples were collected from 6 mice in each group, and myocardial tissues from the remaining 6 mice were stained with hematoxylin and eosin (H&E). miR-96-5p antagonist sequence: 5’-TAACACT-GTCTGTGTAACGATGT-3’.

CLP procedures: Midline laparotomy was performed, and a 3 cm dissection was performed to expose the cecum. At the site designated for high severity, the cecum was ligated with a 4-0 silk thread and pierced at two sites 1 cm apart with a 20-gauge needle. The cecum was then gently pressed to squeeze a small drop of feces. After laparotomy, all mice were resuscitated by subcutaneous injection of normal saline (37°C, 50 ml/kg). The sham operation was performed with the same procedure but without ligation and puncture of the cecum.

Heart function detection

All mice were anesthetized by intraperitoneal administration of pentobarbital sodium 12 hours after CLP. The right carotid artery was exposed, from where a catheter was inserted into the left ventricle. A multi-channel data acquisition and processing system (RM6240BD, Chengdu Instrument) was utilized to monitor left ventricular systolic pressure (LVSP) and maximum rate of increase/decrease in left ventricular pressure (±dp/dt). The myocardial homogenate was centrifuged, and the supernatant was collected. Tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-17 levels were measured using ELISA kits. Creatine kinase-MB (CK-MB, E006-1-1) and cardiac troponin I (cTnI, H149-2) content in serum were measured. All kits were purchased from Nanjing Jiancheng Bioengineering Institute.

HE staining

The myocardial tissue of mice was fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 4 μm slices. The slices were deparaffinized with xylene, infiltrated with gradient alcohol, and stained with HE. At last, the tissue structure was observed microscopically.
Table 1. RT-qPCR primer sequences

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Forward: 5’-ATGGGGAAGGTGAAAGATGC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-TTACCTCTGAGGCGCATGTG-3’</td>
</tr>
<tr>
<td>U6</td>
<td>Forward: 5’-CTCGGTGCAGCACATATACT-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-AAGCGTTACAAATTTGGGTGC-3’</td>
</tr>
<tr>
<td>CircROCK1</td>
<td>Forward: 5’-TCCAAATGCTGCCCCAAAGCC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-GGTCTCGTCCATCACCTCA-3’</td>
</tr>
<tr>
<td>miR-96-5p</td>
<td>Forward: 5’-ATGCCTTTCACACTTGGTG-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-TCACCGCCTTCGGCGTACA-3’</td>
</tr>
<tr>
<td>OXSR1</td>
<td>Forward: 5’-AAAGAGCTTGTGGCACC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-GCCCTGTGGCTAGATCA-3’</td>
</tr>
</tbody>
</table>

Real-time reverse transcriptase-polymerase chain reaction (RT-qPCR)

Total RNA in mouse myocardial tissue and human serum samples was isolated using TRIZOL RNA extraction reagent (Invitrogen, USA). PrimerScript real-time kit (TAKARA, Japan) was applied for reverse transcription. Finally, RT-qPCR was detected by SYBR Premix Ex Taq II reagent (Applied Biosystems, USA). Using U6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal references for miRNA and mRNA, respectively, relative gene expression was calculated by $2^{-ΔΔCt}$. The primer sequence was shown in Table 1.

Western blot

Total protein in the myocardial homogenate was extracted using a protein extraction kit (Beyotime, China), and later a bicinchoninic acid kit (Nanjing Jiancheng Bioengineering Institute) was utilized to determine the protein concentration. Next, the protein was loaded for electrophoresis, and protein gel was transferred to a polyvinylidene fluoride membrane (Millipore, USA), which was sealed with 0.1% Tween-20 in Tris-buffered saline (5% skim milk) at room temperature for 1 hour, and later combined with the primary antibody p-NF-κB p65 (ab86299, Abcam), NF-κB p65 (#8242, Cell Signaling), OXSR1 (H00009943-M09, Abnova), and GAPDH (ab181602, Abcam) overnight at 4°C. Next, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (ab205718, Abcam) to evaluate protein immunoreactivity. The density of protein bands was analyzed by Image-Pro Plus version 6.0 (Media Cybernetics, USA).

Dual luciferase reporter experiment

The circROCK1 or OXSR1 3’untranslated region sequence containing miR-96-5p binding site was inserted into the pmirGLO vector (Promega, USA), and the wild type (WT) luciferase reporter plasmids were named circROCK1-WT and OXSR1-WT. After the binding sites were mutated, mutant type (MUT) luciferase plasmids (circROCK1-MUT and OXSR1-MUT) were constructed. HEK293 cells were transplanted into 48-well plates at 3×10⁴ cells/well and transfected with 2 µL of plasmids and 100 nM miR-96-5p mimic or mimic-NC (GenePharma, China). After 48-hour transfection, the dual luciferase reporter system (Promega; E1910) was utilized to detect firefly and renilla luciferase activities.

Data analysis

Values expressed as mean ± standard deviation (S.D.) were analyzed by GraphPad Prism software 8.01 (GraphPad Software, USA). The Student’s t-test was applied to compare two groups. Spearman correlation analysis examined the relationship between circROCK1 and the clinicopathological characteristics of septic patients. *P<0.05 was considered statistically significant.

Table 2. Clinicopathological characteristics of subjects

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Healthy subjects (n = 37)</th>
<th>Septic patients (n = 63)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>57.64±6.27</td>
<td>53.78±4.93</td>
</tr>
<tr>
<td>Gender</td>
<td>male (14)/female (23)</td>
<td>male (39)/female (24)*</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.27±0.79</td>
<td>20.98±1.07</td>
</tr>
<tr>
<td>Scr (mg/dL)</td>
<td>0.91±0.17</td>
<td>1.54±0.24*</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>42.97±4.78</td>
<td>31.47±3.59*</td>
</tr>
<tr>
<td>WBC (×10⁹/L)</td>
<td>8.49±2.03</td>
<td>17.56±4.12*</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>11.04±2.39</td>
<td>47.83±8.83*</td>
</tr>
<tr>
<td>PCT (mg/mL)</td>
<td>0.79±0.12</td>
<td>8.74±2.41*</td>
</tr>
<tr>
<td>SOFA score</td>
<td>3.97±1.24</td>
<td></td>
</tr>
</tbody>
</table>

Note: Scr: serum creatinine; WBC: white blood cells; CRP: C-reactive protein; PCT: procalcitonin; SOFA: sequential organ failure assessment. Data expressed as mean ± S.D., combined with student’s t-test or chi-square test; in contrast to healthy subjects, *P<0.05.
RESULTS

Clinical and pathological characteristics of subjects

37 healthy subjects (14 males, 23 females, average age: 57.64±6.27 years old) and 63 septic patients (39 males, 24 females, average age: 53.78±4.93 years old) were recruited. The clinicopathological characteristics of the two groups were detailed in Table 2. There were no significant differences in age or BMI between healthy subjects and sepsis patients. There were significant differences in gender, Scr, albumin, white blood cells, CRP, and PCT.

circROCK1 is highly expressed in sepsis and is related to sepsis clinicopathology

circROCK1 in sepsis was examined. circROCK1 expression in the serum of septic patients was higher than that in healthy subjects (Fig. 1). The clinicopathological

Table 3. The correlation between circROCK1 and clinicopathological characteristics of sepsis

<table>
<thead>
<tr>
<th>Parameters</th>
<th>circROCK1 low expression (n = 31)</th>
<th>circROCK1 high expression (n = 32)</th>
<th>P</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>54.32±5.79</td>
<td>56.43±3.87</td>
<td>0.478</td>
<td>0.237</td>
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<tr>
<td>Gender (male/female)</td>
<td>male (18)/female (13)</td>
<td>male (15)/female (17)</td>
<td>0.327</td>
<td>0.379</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>19.72±0.84</td>
<td>20.37±0.77</td>
<td>0.782</td>
<td>0.113</td>
</tr>
<tr>
<td>Scr (mg/dL)</td>
<td>1.22±0.13</td>
<td>1.94±0.36</td>
<td>&lt;0.001</td>
<td>0.639</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>31.37±2.78</td>
<td>29.25±4.03</td>
<td>0.174</td>
<td>-0.335</td>
</tr>
<tr>
<td>WBC (×10⁹/L)</td>
<td>15.79±3.47</td>
<td>17.63±4.55</td>
<td>0.264</td>
<td>0.341</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>39.84±6.29</td>
<td>54.27±5.91</td>
<td>&lt;0.001</td>
<td>0.749</td>
</tr>
<tr>
<td>PCT (ng/mL)</td>
<td>5.74±1.63</td>
<td>10.93±2.80</td>
<td>&lt;0.001</td>
<td>0.538</td>
</tr>
<tr>
<td>SOFA score</td>
<td>2.69±0.74</td>
<td>5.27±0.63</td>
<td>&lt;0.001</td>
<td>0.673</td>
</tr>
</tbody>
</table>

Note: Scr: serum creatinine; WBC: white blood cells; CRP: C-reactive protein; PCT: procalcitonin; SOFA: sequential organ failure assessment. Data expressed as mean ± S.D., combined with student’s t-test and spearman correlation analysis.
relationship between circROCK1 and sepsis was subsequently examined. circROCK1 was positively correlated with Scr, CRP, PTC, and SOFA scores in sepsis (Table 3).

Silencing circROCK1 improved myocardial injury in septic mice

A CLP mouse model was established to further examine the role of circROCK1 in sepsis. First, the survival rate of CLP mice within 48 hours was evaluated. The survival rate of CLP mice within 48 hours after surgery was 41.2%, while the mice in the Sham group did not die (Fig. 2A). After CLP, circROCK1 expression in the myocardial tissue of mice was elevated; after sh-circROCK1 injection, circROCK1 expression was reversed (Fig. 2B). Later the cardiac function of the mice was checked. After CLP, LVSP and +dp/dt max were reduced, while -dp/dt max, LVEDP, cTnI, and CK-MB were elevated. After sh-circROCK1 injection, the changes in the above indicators were reversed (Fig. 2C). HE staining revealed that myocardial necrosis and interstitial edema appeared in the myocardial tissue of CLP mice, and these pathological changes were attenuated after silencing circROCK1 (Fig. 2D). Additionally, CLP surgery elevated inflammatory factors TNF-α, IL-1β, and IL-6 levels in the myocardial tissue of mice. After silencing circROCK1, inflammatory factor levels were reduced (Fig. 2E). Western blot results implied that CLP elevated phosphorylated NF-κB expression in mouse myocardial tissue. After silencing circROCK1, phosphorylated NF-κB expression was reduced (Fig. 2F). Based on these findings, silencing circROCK1 improves myocardial injury in septic mice.

Silencing miR-96-5p promoted myocardial injury in septic mice

In this work, miR-96-5p was under-expressed in the serum of septic patients (Fig. 3A). Subsequently, miR-96-5p in CLP mice was down-regulated by injection of miR-96-5p antagomir (Fig. 3B). Next, the impact of miR-96-5p on myocardial injury in septic mice was examined. After down-regulating miR-96-5p, LVSP and
+dp/dt\textsubscript{max} decreased, whereas -dp/dt\textsubscript{max}, LVEDP, cTnI, and CK-MB elevated in CLP mice (Fig. 3C). HE staining indicated that down-regulating miR-96-5p aggravated myocardial necrosis and edema in CLP mice (Fig. 3D). Additionally, down-regulating miR-96-5p further elevated inflammatory factors TNF-α, IL-1β, and IL-6 in the myocardial tissue of CLP mice and promoted phosphorylated NF-κB expression (Figs. 3E, F).

circROCK1 acted as a miR-96-5p sponge in sepsis to mediate ROCK1 expression

After silencing circROCK1, miR-96-5p expression in CLP mice was elevated (Fig. 4A). It is speculated that circROCK1 is available to target miR-96-5p expression. circROCK1 and miR-96-5p have a targeting relationship on \(\text{http://starbase.sysu.edu.cn/}\) (Fig. 4B). Next, a dual luciferase reporter experiment was conducted to further examine the targeting relationship between circROCK1 and miR-96-5p. WT-circROCK1 reduced the luciferase activity in the miR-96-5p mimic group, whereas MUT-circROCK1 had no impact (Fig. 4C). This indicates that circROCK1 competitively binds miR-96-5p in sepsis. OXSR1 has been found to be highly expressed in sepsis and has an important relationship with sepsis development. In this work, OXSR1 expression was elevated in septic patients compared with healthy controls as well as in CLP mouse myocardial tissue compared with the Sham group. After down-regulating miR-96-5p, OXSR1 expression was further elevated (Fig. 4D). Given that, OXSR1 may be a potential target gene of miR-96-5p. On the \(\text{http://starbase.sysu.edu.cn/}\), it was found that OXSR1 and miR-96-5p had potential binding sites (Fig. 4E). Based on dual-luciferase findings, WT-OXSR1 could reduce miR-96-5p luciferase activity, while MUT-OXSR1 had no impact on it (Fig. 4F). Additionally, silencing circROCK1 can inhibit OXSR1 expression in the myocardial tissue of CLP mice (Fig. 4G). These findings indicate that OXSR1 is the target gene of miR-96-5p and is regulated by circROCK1.

DISCUSSION

In recent years, sepsis incidence has risen year by year, but the specific mechanism that affects septic organ injury is still not clear (Font \textit{et al.}, 2020; L’Heureux \textit{et al.}, 2020). In this work, our team found that circROCK1 was highly expressed in the serum of septic patients and indicated a positive correlation with Scr, CRP, PTC, and SOFA scores in sepsis. Additionally, knocking down circROCK1 improved septic myocardial injury by enhancing myocardial contractility, reducing myocardial tissue necrosis and edema, as well as inhibiting the activation of NF-κB signaling pathway and downstream inflammatory factor expression. In terms of mechanism, circROCK1 silencing can ameliorate myocardial injury in sepsis mainly through competitive binding of miR-96-5p-mediated OXSR1 expression.

The CLP mouse model has been widely applied in basic research on sepsis. After CLP, the mice presented myocardial inhibition and inflammatory injury, mainly...
including decreased LVSP and +dp/dt max and elevated -dp/dt max, LVEDP, cTnI, CK-MB, and inflammatory factors. These findings are in line with previous studies (Gao et al., 2021; Liu et al., 2020), revealing that CLP is available to induce severe myocardial dysfunction.

Various circRNAs can regulate inflammatory responses in sepsis, which is vital for understanding sepsis inflammation development. For example, circ_0114428 regulates sepsis-induced renal inflammatory injury by targeting miR-495-3p/cereblon axis (He et al., 2021). The circRNA circVMA21 improves lipopolysaccharide-induced acute kidney injury by targeting the miR-199a-5p/Neurilin-1 axis in sepsis (Li et al., 2021). This work found for the first time that circROCK1 can reduce septic myocardial tissue inflammation mainly by inhibiting NF-κB phosphorylation. NF-κB is an essential inflammatory signaling pathway, which acts in the process of cellular inflammation and immune response (Lawrence 2009; Pfug & Sticheran, 2020).

Organ failure is a common feature of sepsis (Joffre et al., 2020; Black et al., 2020). In recent years, studying the role of circRNA in protecting cardiac function has become a research hotspot. Recently, Qiu and others (Qiu et al., 2021) found that circHIPK3 regulates cardiomyocyte autophagy and apoptosis stimulated by hypoxia/reoxygenation via miR-20b-5p/autophagy related 7 axis. Additionally, Cheng and others (Cheng et al., 2020) found that circular RNA POSTN promotes myocardial infarction-induced myocardial injury and cardiac remodeling through regulating miR-96-5p/Bcl-2/adenosine E1B 19kDa interacting protein 3 axis. In our research, knocking down circROCK1 improved myocardial function and inflammatory injury in septic mice. It is worth noting that circROCK1 has been found to be highly expressed in septic lung tissue, so we speculate that circRNA also has a similar impact on sepsis and other organ failures.

In further research on the mechanism, based on the bioinformatics website and dual luciferase reporter system, circROCK1 was directly bound to miR-96-5p to regulate the NF-κB signaling pathway to reduce the severe response of myocardial injury in septic myocardial tissue inflammation mainly by inhibiting NF-κB phosphorylation. NF-κB is an essential inflammatory signaling pathway, which acts in the process of cellular inflammation and immune response (Lawrence 2009; Pfug & Sticheran, 2020). The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES


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