

Circular RNA ITCH mediates H₂O₂-induced myocardial cell apoptosis by targeting miR-17-5p via wnt/ β -catenin signalling pathway

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Abstract

Cardiovascular disease is a severe threat health worldwide, and circRNAs have been shown to be correlated with the development of cardiovascular disease. Expression of circ-ITCH and miR-17a-5p was evaluated by RT-qPCR. Cell viability was measured using CCK-8. Flow cytometry was applied to measure apoptosis rate. Binding between miR-17-5p and circ-ITCH was detected via luciferase reporter assays. Levels of ATP in cells were examined with ATP testing. Western blot was used to evaluate apoptosis-related proteins and proteins in Wnt/ β -catenin signalling pathway. H₂O₂ induced apoptosis of H9c2 cells and lowered cell viability as well as ATP levels and circ-ITCH expression. After overexpression, circ-ITCH enhanced cell viability and ATP concentration. Meanwhile, apoptosis was inhibited. MiR-17-5p was the target of circ-ITCH as evidenced by luciferase report assays, with higher expression in H₂O₂-induced H9c2 cells. Knockdown of miR-17-5p could promote cell viability and level of ATP and curb apoptosis and p53 and PARP expression. Moreover, overexpressed miR-17-5p could reverse the function of upregulated circ-ITCH. Wnt3a, Wnt5a and β -catenin in Wnt/ β -catenin signalling pathway were increased after H₂O₂ induction. Suppression of Wnt/ β -catenin signalling pathway could initiate the process of injury in H9c2 cells. Circ-ITCH could protect myocardial cells from injuries caused by H₂O₂ by suppressing apoptosis while miR-17-5p played a reverse role, which could upregulate apoptosis and inhibit cell viability via Wnt/ β -catenin signalling pathway.

KEYWORDS

Adenosine Triphosphate, apoptosis, circRNA ITCH, myocardial cell

1 | INTRODUCTION

Cardiovascular diseases are critical human illnesses which are increasing year by year.¹ Coronary arteriosclerosis can interrupt cardiac blood circulation, resulting necrosis of myocardium after ischaemia and hypoxia.^{2,3} H₂O₂ is produced in

ischaemia-reperfusion injury (I/R), contributing to ROS in oxidative stress injury.⁴ Apoptosis of myocardial cells occurs in a variety of heart diseases, such as cardiomyopathy, myocardial ischaemia/reperfusion injury, myocardial infarction and congestive heart failure.^{5,6,7} Thus, it is essential to suppress excessive apoptosis in myocardial cells in cardiovascular diseases.

CircRNAs can regulate miRNAs through the molecular sponge mechanism and are involved with occurrence and development of diseases.^{8,9} There were about 575 circRNAs according to RNA sequencing in adult mouse hearts.¹⁰ Previously, circRNA000203 has been shown to induce its down-stream genes *Colla2* and *GTGF* and promote myocardial fibrosis through sponging miR-26b.¹¹ CircRNA MFACR could absorb miR-652-5p and to inhibited myocardial cell apoptosis and to shrink the myocardial infarction area.¹² CircRNAs have also been shown to play a part in myocardial cell apoptosis in vitro, and to help cardiac repair in vivo, potentially signifying promising therapies for the diseases in the future.¹³⁻¹⁷ CircRNA *ITCH* was widely recognized as a tumour suppressor in various cancers.^{18,19,20} However, the role of circ-*ITCH* in myocardial cells has not been reported previously. In this study, therefore, we evaluated the regulatory role of circ-*ITCH* in H₂O₂-induced myocardial cell apoptosis in vitro as a mimic of I/R and injury.

2 | METHODS

2.1 | Cell culture

H9c2, a rat myocardial cell line, was acquired from ATCC and cultured in DMEM medium (Gibco) containing 10% foetal bovine serum (FBS) and 100 µmol/mL penicillin and streptomycin in a humid incubator at 37°C, 5% CO₂. After confluence of cells reached 80%, cells were digested by 0.25% trypsin and seeded into a 6-well plate incubated with H₂O₂ (0.1 and 1 mmol/L) for 12 hours with a non-treated cell group as a control.¹⁶ Cells with satisfactory well growth were selected for the following experiments.

2.2 | Cell transfection assays and wnt/beta-catenin signalling inactivation

Cells with 1 mmol/L H₂O₂ pretreatment were selected to regulate the expression of circ-*ITCH* and miR-17-5p. circRNA *ITCH* sequence was cloned into pcDNA3.1 plasmid for augmentation. miR-17-5p mimics, NC mimics, miR-17-5p inhibitor and NC inhibitor were obtained from GenePharma. Lipofectamine 3000 (Invitrogen) was used to obtain the following groups of cells, respectively: oe-NC and oe-circ-*ITCH* by transfecting empty pcDNA3.1 and pcDNA3.1-circ-*ITCH* into the cells according to the manufacturer's instructions. Likewise, NC inhibitor, miR-17-5p inhibitor, NC mimics, miR-17-5p mimics and oe-circ-*ITCH* + miR17-5p mimics groups were generated through transfection using Lipofectamine 3000. To study the function of wnt/beta-catenin signalling in cells, we incubated the cells with an inhibitor of the signalling pathway, 1 µmol/L NCB-0846, for

24 hours (Meilunbio). Cells were harvested at 48 hours after transfection or NCB-0846 treatment for the follow-up assays.

2.3 | RT-qPCR

Cells pre-exposed to 0, 0.1 and 1 mmol/L H₂O₂ and the sub-groups under 1 mmol/L H₂O₂ after transfection or treatment with NCB-0846 were all selected for detection of the circ-*ITCH* and miR-17-5p expression with normalization to GAPDH and U6, respectively, by using RT-qPCR methods. Total RNAs were isolated from cells using TRIzol reagent (Invitrogen). Afterwards, High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems) was used for reverse transcription with synthetic primers. Sequences were listed as follows: circ-*ITCH*: forward: 5'-AGCAATGCAGCAGTTT-3'; reverse: 5'-TGTAGCCCATCAAGACA-3'²⁰; miR-17-5p: forward: 5'-GCTGAATTTGTATGGTTTATAGTTGTTA-3'; reverse: 5'-GCACCTTAGAACAAAAAGCACT-3',^{8,9} U6: forward: 5'-ATTGGAACGATACAGAGAAGATT-3'; reverse: 5'-GGAACGCTTCACGAATTTG-3'²¹ GAPDH: forward: 5'-ACCCAGAAGACTGTGGATGG -3', reverse: 5'-TCAGCTCAGGGATGACCTTG-3'. PCR conditions were shown: predenaturation, 95°C, 10 minutes; denaturation, 95°C, 15 seconds; annealing, 60°C, 60 seconds; and extension, 72°C, 30 seconds, 35 cycles. Relative expressions of RNAs were quantified with 2^{-ΔΔCt} methods.

2.4 | CCK-8

This method is applied to measure cellular viability in each group. After incubation with 0, 0.1 and 1 mmol/L H₂O₂, H9c2 cells were explanted into 96-well plate at a cell density of 2 × 10⁵ cells per well and cultured in incubators at 37°C, 5% CO₂. Cells with 1 mmol/L H₂O₂ pretreatment were regulated through transfection assays and generated seven different sub-groups, oe-NC, oe-circ-*ITCH*, NC inhibitor, miR-17-5p inhibitor, NC mimics, miR-17-5p mimics and oe-circ-*ITCH* + miR17-5p mimics groups. All the cells in the seven sub-groups underwent the same process as normal or H₂O₂-treated H9c2 cells. At specific time points (24, 48 and 72 hours), 10 µL CCK-8 was added and the mixture was kept incubated for another hour. A microplate reader (Molecular Devices) was used for detection of optical density (OD) values of cells at 450 nm wavelength.

2.5 | ATP testing

After transfection, cells were rinsed with PBS three times and lysed. Next, lysates were settled and then centrifuged at 4°C, 12 000 g for 5 minutes. Then the liquid supernatant were collected and the ATP concentrations were examined with an ATP Assay Kit (Beyotime).

Luminoskan™ Microplate Luminometer (Thermo Scientific) was used to measure levels of ATP in the cells.

2.6 | Luciferase report assay

3'UTR of circ-ITCH was amplified through RT-qPCR. In order to create a 3'UTR mutation, a Phusion Site-Directed Mutagenesis Kit (Thermo Scientific) was applied. Furthermore, wild type and mutant type of 3'-UTRs were inserted into psiCHECK-2 vectors (Promega) with Lipofectamine 3000 (Invitrogen). *Relative luciferase activities* were checked with dual-Luciferase Reporter Assay System (Promega).

2.7 | Flow cytometry

Cell apoptosis was detected with Annexin V-FITC Apoptosis Detection Kit (Beyotime). After cells were

collected, Annexin V-FITC and PI were applied for staining cells. In the end, CellTrace™ Blue Cell Proliferation Kit (Invitrogen) was used to analyse apoptosis of cells.

2.8 | Western blot

This method was used to semi-quantify the proteins as mentioned below, and cells were harvested and lysed by RIPA Lysis Buffer (Beyotime) to obtain total protein. After that, 40 µg of total protein was measured with BCA protein Assay Kit (Beyotime). Thereafter, proteins were isolated with 8% SDS-PAGE and transferred into Polyvinylidene Fluoride (PVDF) membranes (Invitrogen) later. Next, 8% skimmed milk powder was selected to block membranes for 2 hours and anti-pro-caspase-3 (1:1000; ab183179, Abcam), anti-cleaved-caspase-3 (1:1000; ab49822), anti-p53 (1:1000; ab26), anti-PARP1 (1:1000; ab227224), anti-Wnt3a (1:1000; ab219412), anti-Wnt5a (1:1000; ab227229), anti-β-catenin

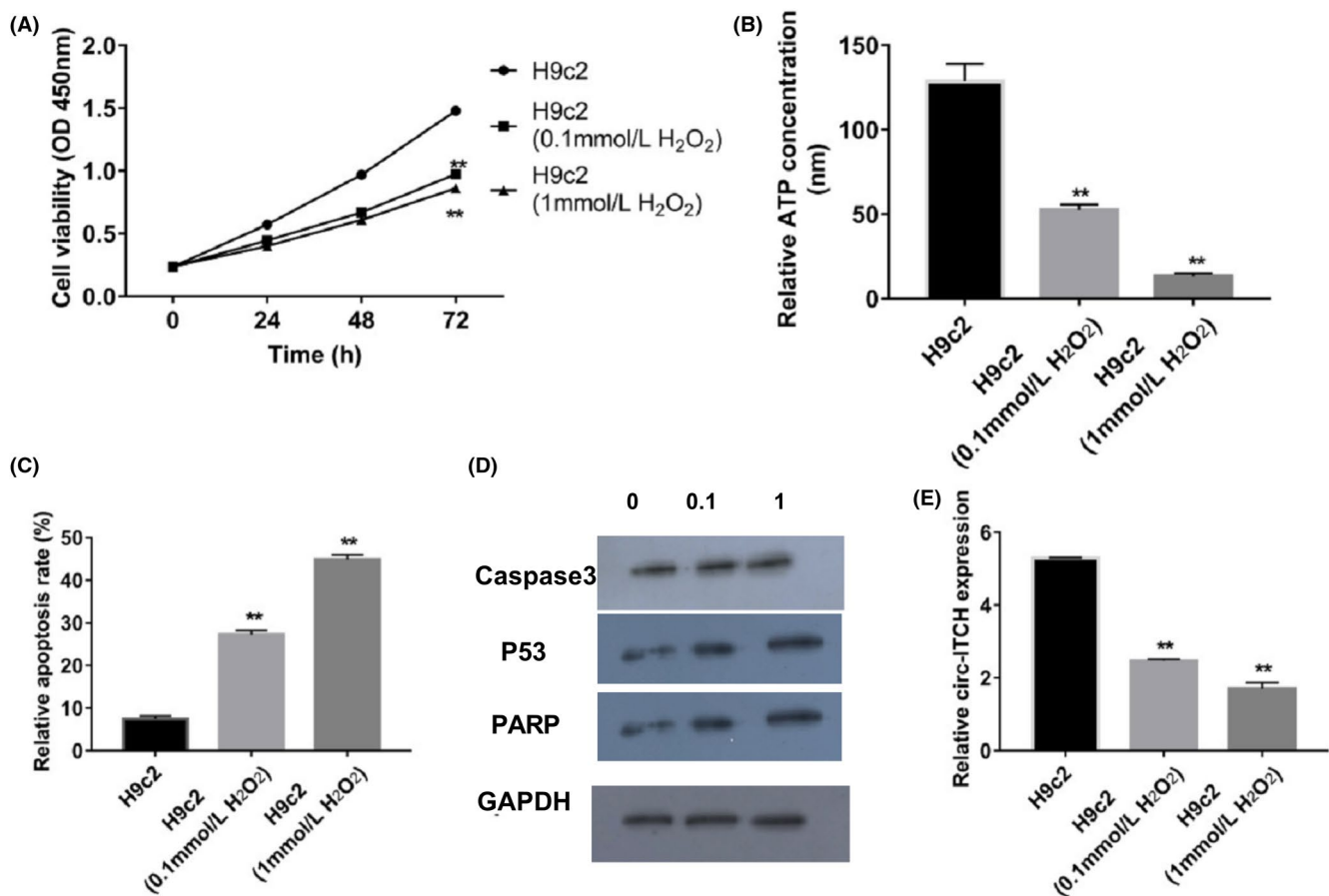


FIGURE 1 H₂O₂ treatment improved apoptosis of H9c2 cells and downregulated circ-ITCH expression. A, Cell viabilities after H₂O₂ treatment were detected by CCK-8, $P < .05$. B, ATP concentrations in H9c2 cells treated with H₂O₂ were analysed with ATP Assay Kit, $P < .05$. C, Apoptosis rate of H9c2 cells were measured by flow cytometry, $P < .05$. D, Caspase 3, p53 and PARP expressions in H9c2 cells treated with H₂O₂ were evaluated by Western blot, $P < .05$. E, Expressions of circ-ITCH in H9c2 cells after H₂O₂ treatment were measured by RT-qPCR, $P < .05$. Student's t test and one-way ANOVA were used

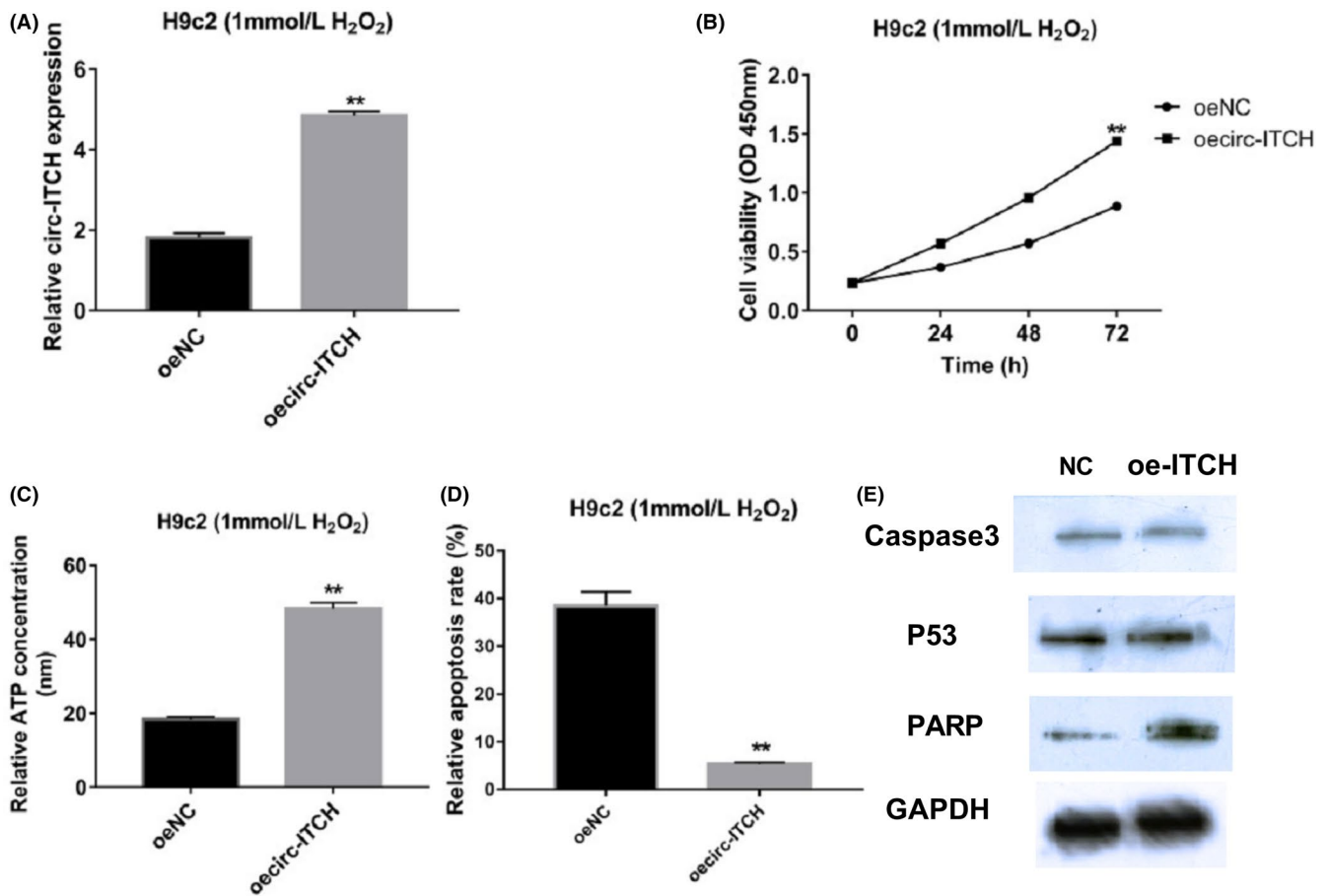


FIGURE 2 Overexpression of circ-ITCH reduced apoptosis in H₂O₂-treated H9c2 cells. A, Levels of circ-ITCH after overexpressed transfection were validated through RT-qPCR, $P < .05$. B, CCK-8 was applied to measure cell viabilities of H9c2 cells with upregulated circ-ITCH, $P < .05$. C, ATP Assay Kit was used for checking ATP concentration, $P < .05$. D, Flow cytometry was for examining apoptosis rate of H9c2 cells with overexpressed circ-ITCH, $P < .05$. E, Western blot was applied to detect expressions of caspase 3, p53 and PARP, $P < .05$. Student's t test and one-way ANOVA were used

(1:1000; ab32572) and anti-GAPDH (1:2000; ab181602) were incubated with PVDF membranes at 4°C overnight. Afterwards, membranes were rinsed by Pierce™ Protein-Free T20 (PBS) Blocking Buffer (Thermo Scientific) three times and incubated with secondary antibodies - Goat Anti-Rat IgG H&L (HRP) (ab97057) at room temperature for 2 hours. Finally, BeyoECL Moon (Beyotime) was used for testing protein.

2.9 | Statistical analysis

All data were analysed using SPSS 19.0 and displayed as mean \pm SD. All experiments were repeated three times, and the differences in the mean values between or among the groups were evaluated using Student's t test and one-way ANOVA was applied for the repetitive results inside each group. $P < .05$ was considered to reveal statistical significance.

3 | RESULTS

3.1 | H₂O₂ induced apoptosis of myocardial cells and downregulated level of circ-ITCH

After cells were treated with different concentrations of H₂O₂, (0, 0.1, 1 mmol/L), the viability of cells decreased as H₂O₂ concentrations increased (Figure 1A). Increasing concentration of H₂O₂ induced declining ATP in H9c2 cells (Figure 1B). After that, apoptosis rates were checked, revealing that death of myocardial cells was induced by H₂O₂ in a concentration-dependent fashion (Figure 1C). As H₂O₂ concentration rose, cleaved caspase 3, PARP and p53 proteins were activated (Figure 1D). Circ-ITCH expression was observed to be downregulated in the H₂O₂ groups. The higher the concentration (1 mmol/L), the lower the expression of ITCH (Figure 1E). Based on the above results, cells with H₂O₂ (1 mmol/L) treatment were chosen for further experiments.

3.2 | Overexpression of circ-ITCH deterred cellular apoptosis in H9c2 cells with pretreatment of H₂O₂

After upregulating circ-ITCH in H9c2 cells with 1 mmol/L H₂O₂ pretreatment, RT-qPCR validated the activated circ-ITCH expression in the transfected group (Figure 2A). Besides, upregulated circ-ITCH induced increased viability of H9c2 cells (Figure 2B). Meanwhile, concentrations of ATP were increased in the overexpressed The circ-ITCH group (Figure 2C). Apoptosis results displayed that upregulation of circ-ITCH could reduce apoptosis rate (Figure 2D). Cleaved caspase 3, p53 and PARP were inactivated by overexpression of circ-ITCH (Figure 2E).

3.3 | MiR-17-5p was a target of circ-ITCH and promoted apoptosis of myocardial cells

From the Bioinformatics website, Starbase (<http://starbase.sysu.edu.cn/agoClipRNA.php?source=circRNA&flag=target&clade=mammal&genome=human&assembly=hg19&miRNA=hsa-miR-17-5p&clipNum=1&deNum=0&target=ITCH+>), we searched the miRNA-circRNA interactions and followed this by including the gene name of circ-ITCH. We found that miR-17-5p had a putative binding site with circ-ITCH (Figure 3A). Then, a luciferase report assay was applied for verification of the binding. We noted that luciferase activity

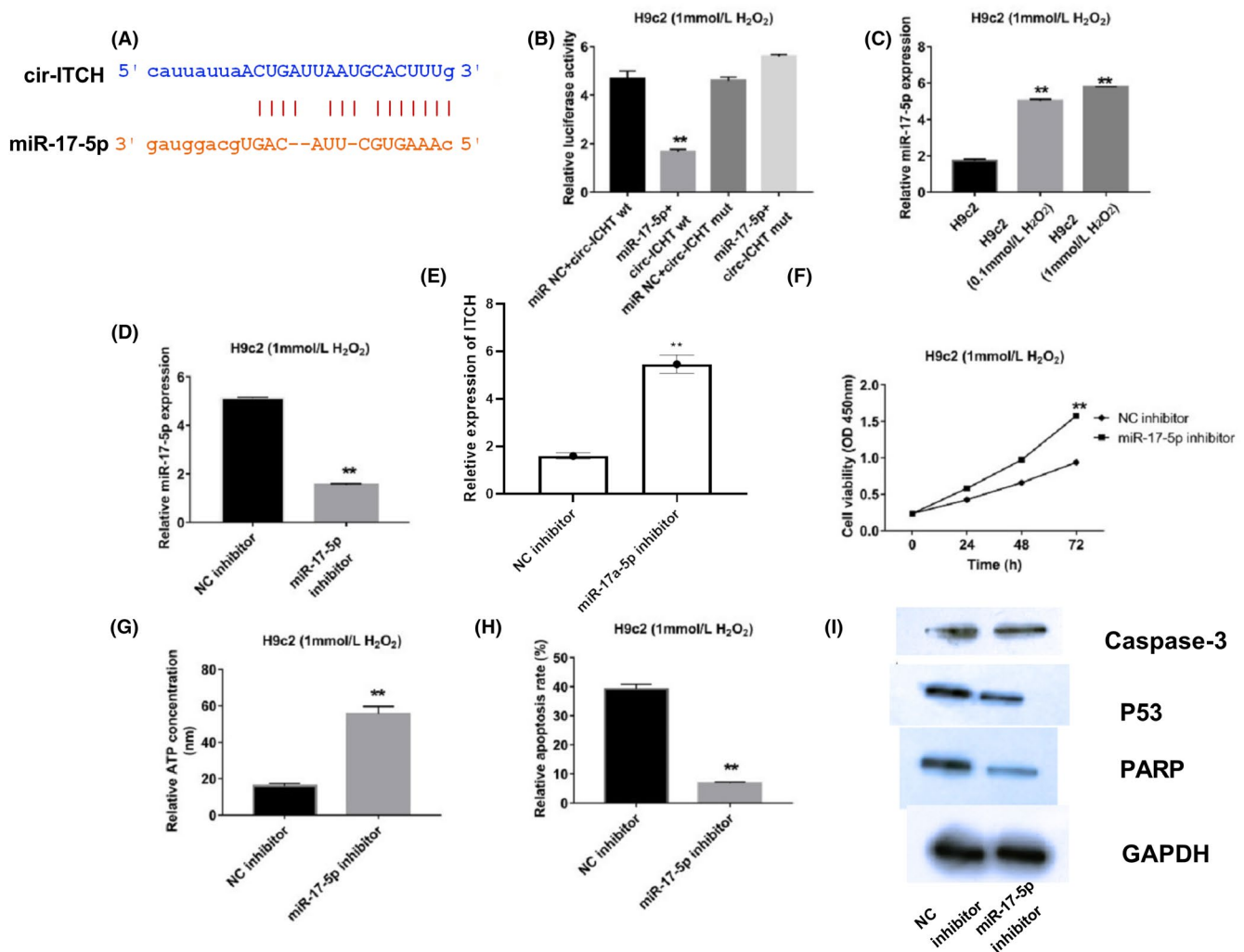


FIGURE 3 MiR-17-5p was the target of circ-ITCH and promoted apoptosis of H₂O₂-induced myocardial cells. A and B, Binding information was acquired from bioinformatics tools (TargetScan) and proved through luciferase report assay, $P < .05$. C, Expressions of miR-17-5p in H9c2 cells treated by H₂O₂ were measured with RT-qPCR, $P < .05$. D, Suppressed miR-17-5p expression was checked through RT-qPCR, $P < .05$. E, Cell viabilities were analysed by CCK-8, $P < .05$. F, ATP concentrations were detected, $P < .05$. G, Apoptosis rate with suppressed miR-17-5p was measured with flow cytometry, $P < .05$. H, Caspase 3, p53 and PARP levels were detected by Western blot, $P < .05$. Student's t test and one-way ANOVA were used

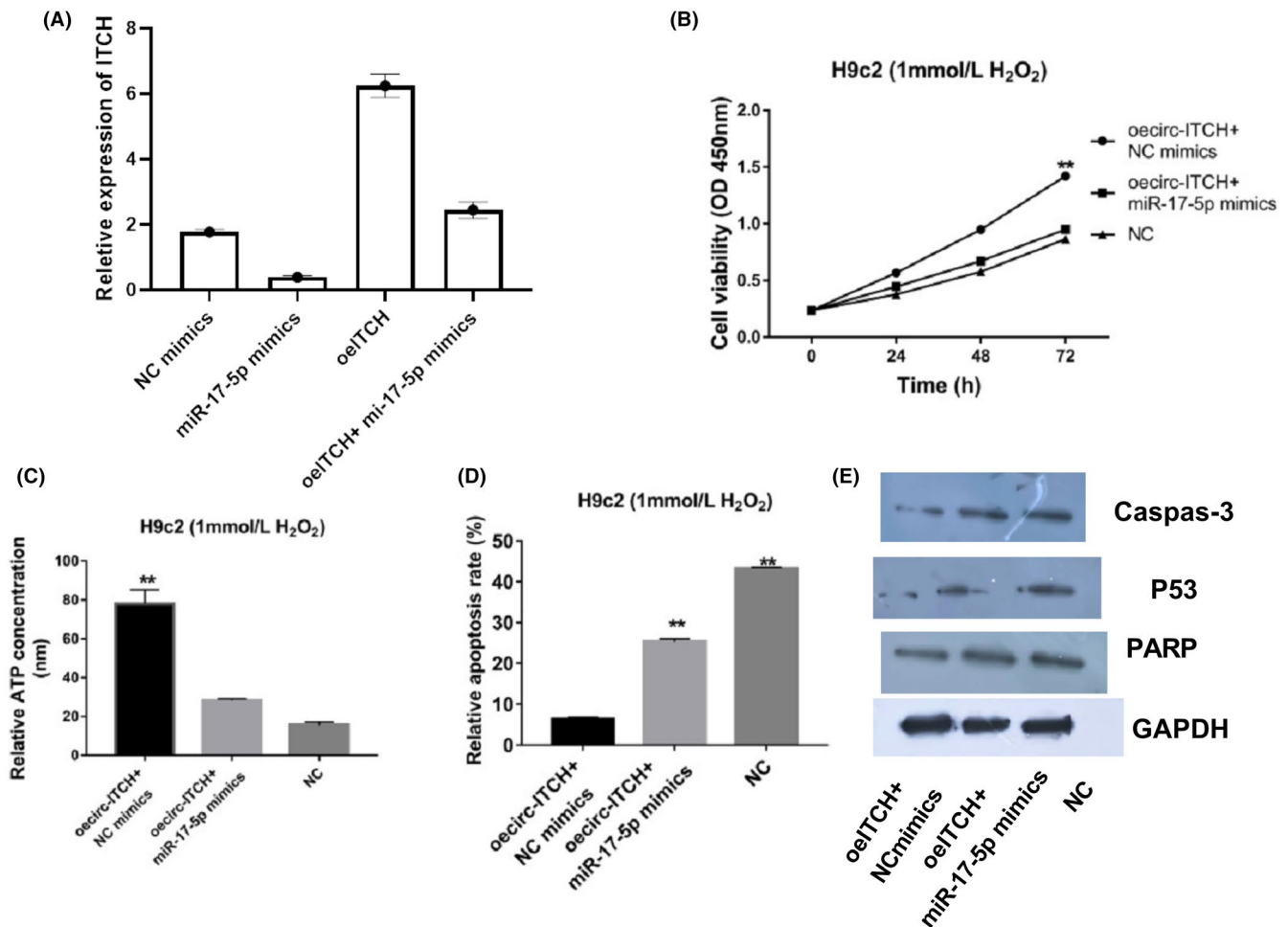


FIGURE 4 Overexpression of miR-17-5p downregulated protection of circ-ITCH to H₂O₂-induced myocardial cells. A, Circ-ITCH expressions with mimics of miR-17-5p were examined with RT-qPCR, $P < .05$. B, Cell viabilities were detected by CCK-8, $P < .05$. C, ATP Assay Kit was applied to check ATP concentrations in H9c2 cells with transfected circ-ITCH and miR-17-5p, $P < .05$. D, Flow cytometry was used to check apoptosis rate in H9c2 cells with transfected circ-ITCH and miR-17-5p, $P < .05$. E, Western blot was used for measuring levels of caspase 3, p53 and PARP, $P < .05$. Student's t test and one-way ANOVA were used

significantly declined when miR-17-5p mimics and wild type of circ-ITCH were co-transfected into the cells, indicating the exact binding between them (Figure 3B). Therefore, miR-17-5p changes were evaluated which revealed that H₂O₂ could induce miR-17-5p expression in H9c2 cells in a dosage-dependent fashion (Figure 3C). Since the 1 mmol/L group was more significantly different than the 0.1 mmol/L group, the former group was selected for the follow-up assays (Figure 3C). Afterwards, RT-qPCR validated the downregulation of miR-17-5p after interference assay in H9c2 cells (Figure 3D). Low viability of cells and ATP concentrations increased with the interference of miR-17-5p (Figure 3E,F). Apoptosis rate dropped as miR-17-5p was suppressed (Figure 3G). Furthermore, cleaved caspase 3, p53 and PARP expressions were lower with inhibition of miR-17-5p (Figure 3H).

3.4 | Overexpression of miR-17-5p counteracted protective effect of circ-ITCH in H₂O₂ induced myocardial cells

After detections of miR-17-5p and circ-ITCH were made individually correlation between circ-ITCH and miR-17-5p was examined. First, expression of circ-ITCH was checked, which indicated that mimics of miR-17-5p could repress level of overexpressed circ-ITCH. Meanwhile, mimics of miR-17-5p could reverse high cell viabilities and ATP concentration caused by high levels of circ-ITCH. Suppressed apoptosis rate with overexpression of circ-ITCH could be enhanced with upregulated miR-17-5p. Moreover, low expression of caspase 3, p53 and PARP with upregulated circ-ITCH were seen associated with overexpression of miR-17a-5p (Figure 4).

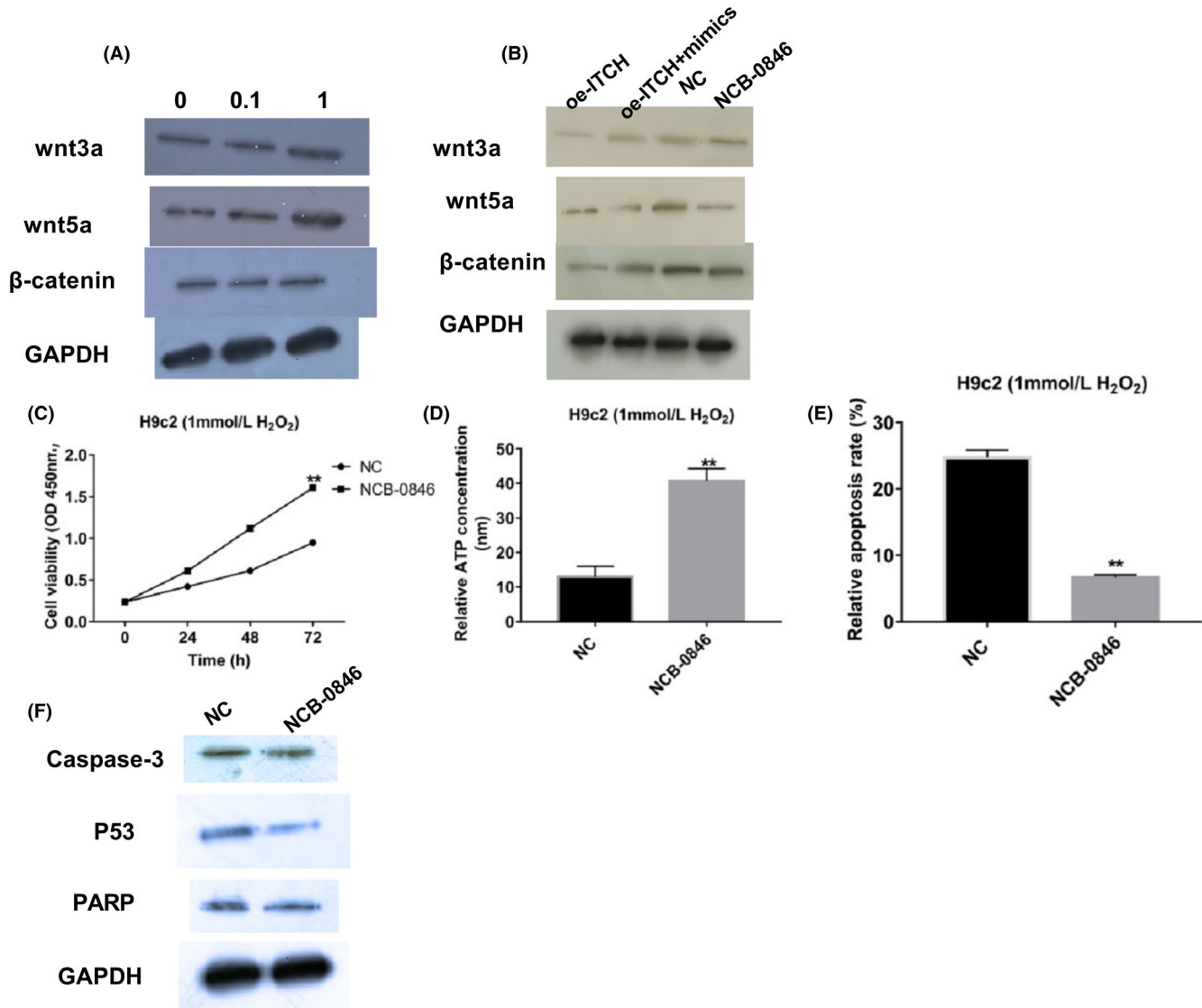


FIGURE 5 Wnt/ β -catenin signalling pathway took part in apoptosis of H_2O_2 -induced myocardial cells. A, Expressions of Wnt3a, Wnt5a and β -catenin in H9c2 cells treated after H_2O_2 were measured with Western blot, $P < .05$. B, Levels of Wnt3a, Wnt5a and β -catenin with transfected circ-ITCH and miR-17-5p and after NCB-0846 treatment were measured using Western blot, $P < .05$. C, Cell viabilities after NCB-0846 treatment were analysed with CCK-8, $P < .05$. D, ATP concentrations with NCB-0846 added were analysed through ATP Assay Kit, $P < .05$. E, Apoptosis rate were evaluated using flow cytometry, $P < .05$. F, Western blot was performed for measuring expressions of caspase 3, p53 and PARP, $P < .05$. Student's t test and one-way ANOVA were used

3.5 | Wnt/ β -catenin signalling pathway participated in apoptosis of myocardial cells

Western blot results showed that H_2O_2 led to higher Wnt3a, Wnt5a and β -catenin protein levels and when H_2O_2 concentration was raised from 0.1 to 1 mmol/L, the changes in Wnt3a, Wnt5a and β -catenin protein expression were more significant (Figure 5A). Therefore, interactions between circ-ITCH, miR-17a-5p and the Wnt/ β -catenin signalling pathway were investigated in cells with 1mmol/L H_2O_2 treatment. Comparing the NC and oe-ITCH groups, the changes showed that overexpression of circ-ITCH could downregulate Wnt3a, Wnt5a and β -catenin. When

comparing the co-transfected group where circ-ITCH and miR-17-5p were both upregulated with the oe-ITCH group, we showed that miR-17-5p mimics could reverse the fall in signalling proteins induced by ITCH overexpression (Figure 5B). In order to confirm that the Wnt/ β -catenin signalling pathway was involved in injury to myocardial cells, NCB-0846, a Wnt/ β -catenin signalling inhibitor was applied to silence the signalling. Wnt3a, Wnt5a and β -catenin all decreased in NCB-0846 group in comparison with the NC group (Figure 5B). Functional assays were adopted to study the regulatory role of wnt/ β -catenin signalling in the myocardial cells, which revealed that cell viability and ATP concentrations were enhanced while apoptosis

rate dropped, accompanied by lower caspase 3, p53 and PARP proteins when signalling pathway was inactivated (Figure 5C-F).

4 | DISCUSSION

Myocardial ischaemia-reperfusion injury (I/R) refers to aggravated ischaemic myocardial injury caused by restored blood perfusion after ischaemia, and is associated with clinical features such as abnormal heart rate, myocardial systolic dysfunction. This becomes irreversible reperfusion injury and so on.²² With increased applications of thrombolytic therapy, cardiovascular surgery and cardiopulmonary bypass (CPB), reperfusion injuries have become more common than before.²³ Until now, mechanisms of reperfusion injury have included overproduction of reactive oxygen species, Ca²⁺ overload, excessive apoptosis of myocardial cells and inflammatory reactions, in^{24,25} recent years, researchers have revealed that oxidative stress played an important role in development of ischaemic heart disease, and was the main element inducing apoptosis in myocardial cells.²⁶ In this study, H₂O₂ was applied to cause oxidative stress in H9c2 cells. H₂O₂ treatment could decrease cell viability as well as ATP concentrations, but increased apoptosis rate, as evidenced by higher levels of caspase 3, p53 and PARP. Results from those experiments revealed that H₂O₂ treatment lead to increased apoptosis in myocardial cells with suppression of ATP as well as cell viability.

Circular RNA is a type of single-stranded, closed RNA formed by reverse cleavage of the exon at the maturation of the precursor mRNA, whose formation contains intron pairing driving ring formation, RNA-binding protein pairs driving ring formation and lasso-driving ring formation.²⁷⁻²⁹ In cardiac muscle tissues of the heart-failing mouse, lots of misaligned circRNAs were discovered. Among 1163 kinds of circRNAs tested, expressions of 29 kinds of circRNAs were upregulated and 34 kinds of circRNAs were decreased.³⁰ CircRNA-010567 could enhance ischaemia-reperfusion injury of cells through sponging miR-141, indicating that it might be an important regulator in heart failure after myocardial infarction.³¹ Moreover, circRNA HRCR was shown to retard heart failure as well as myocardial hypertrophy by absorbing miR-223 and increasing ARC.³² Circ-ITCH was also detected as a suppressor in many kinds of cancers, such as bladder cancer, ovarian cancer and osteosarcoma.^{33,34} However, research about circ-ITCH is rare in cardiovascular diseases. In this study, circ-ITCH was found to be downregulated after treatment with H₂O₂, which indicated that circ-ITCH might have a role in myocardial cells. Therefore, the functions of circ-ITCH were evaluated. Upregulated circ-ITCH could

increase cell viability as well as ATP concentrations in H₂O₂ treated H9c2 cells. Meanwhile, decreased apoptosis rate and low expression of caspase 3, p53 and PARP reminded that circ-ITCH could repress oxidative stress injury of myocardial cells, suggesting that circ-ITCH might be a protector of myocardial cells.

MicroRNAs have been shown to participate in pathological and physiological processes of cardiovascular diseases, such as cardiac hypertrophy, arrhythmia, myocardial ischaemia and myocardial fibrosis.³⁵ MiRNA-195 overexpression could activate myocardial hypertrophy, accelerating the progression of heart failure.³⁶ High MiR-210 expression in myocardial cells from living mice is associated with upregulating of many kinds of angiogenic factor and suppression of activation of caspase 3/7, indicating that miR-210 could be a potential therapy in cardiovascular diseases.³⁷ MiR-17-5p was a promoter in hypoxia-induced myocardial cells, which was significantly upregulated in AMI patients.³⁸ Moreover, the luciferase report assay proved that miR-17-5p was the target of circ-ITCH and expressions of miR-17-5p were upregulated in H₂O₂ treated H9c2 cells. Furthermore, suppression of miR-16-5p could increase cell viability and ATP levels with reduced apoptosis and decreased expression of caspase 3, p53 and PARP. In addition correlation between miR-17-5p and circ-ITCH revealed that miR-17-5p could restore the protective functions of circ-ITCH in myocardial cells. According to previous studies, Wnt/ β -catenin signalling pathway could accelerate process of cardiovascular diseases.^{39,40} In this study, we also measured proteins in the Wnt/ β -catenin signalling pathway, which revealed that Wnt3a, Wnt5a and β -catenin levels were all upregulated. After Wnt inhibitor, NCB-0846 was applied, levels of Wnt3a, Wnt5a and β -catenin were decreased, which upregulated viability of H9c2 cells as well as ATP concentrations and suppressed apoptosis rate and related protein expression. The results indicated that miR-17-5p took part in the process of oxidative stress injury of myocardial cells through the Wnt/ β -catenin signalling pathway and negative regulation by circ-ITCH.

5 | CONCLUSION

Circ-ITCH could retard the injuries seen in H₂O₂-treated myocardial cells in suppressing apoptosis through absorbing miR-17-5p via negative regulating factors in the Wnt/ β -catenin signalling pathway. This suggests that it could be a potential factors in release from oxidative stress injury to myocardial cells.

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CONFLICT OF INTEREST

None

ETHICAL APPROVAL

Ethical approval was not required for this study.

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