

# The protective effects of reparixin against endothelial ischemia-reperfusion injury

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## Introduction

Acute myocardial infarction (AMI) is a leading cause of death globally.<sup>[1]</sup> Restoration of myocardial blood flow is a critical treatment for myocardial protection.<sup>[2]</sup> There are several myocardial reperfusion techniques including percutaneous coronary intervention, antithrombotic therapy, and coronary artery bypass grafting that have been intensively developed for coronary blood flow restoration.<sup>[3]</sup> Unfortunately, myocardial reperfusion after the ischemic episode usually contributes to the detrimental consequences of acute myocardial ischemia-reperfusion injury (IRI).<sup>[4]</sup> Myocardial IRI induces coronary endothelial dysfunction that in turn promotes myocardial injury.<sup>[5]</sup> Compared to myocardial cells, coronary endothelial cells (ECs) are more susceptible to IRI.<sup>[6]</sup> Exaggerated inflammatory reactions following EC activation are closely associated with oxidative stress during ischemia.<sup>[7,8]</sup> Anti-inflammatory and antioxidant agents may have therapeutic

## ABSTRACT

**Objectives:** Myocardial ischemia is a lack of blood supply to myocardial tissue. Rapid reperfusion therapy is required to prevent myocardial infarction. However, ischemia and reperfusion contribute to myocardial and endothelial injury or ischemia-reperfusion injury (IRI). A pro-inflammatory cytokine interleukin-8 (IL-8/CXCL8) plays an important role in the activation of neutrophil accumulation and promotes endothelial dysfunction. Therefore, inhibition of IRI through the regulation of inflammation using a CXCL8 receptor inhibitor reparixin is an attractive target. The aim of this study is to evaluate the effect of reparixin on endothelial cell viability after IRI.

**Methods:** Human vascular endothelial cells (EA.hy926) were cultured and pretreated with reparixin at concentrations of 0–1 µg/ml. To simulate ischemia, the cells were exposed to simulated ischemia solution for 60 min. Then, the cells were given complete medium as reperfusion followed by treatment with reparixin and incubated for 24 h. Cell viability was tested using MTT assay.

**Results:** Percentages of cell viability of reparixin-treated groups of 0.0625 µg/mL (67.88 ± 7.82% control) and 0.125 µg/mL (84.28 ± 4.68% control) were significantly higher than that of the IR group (44.31 ± 4.64% control) at  $P < 0.05$ . The percentage of cell viability in the 0.125 µg/mL reparixin-treated group was not significantly different compared to the control.

**Conclusion:** Pretreatment and treatment of endothelial cells with reparixin, which is a CXCL-8 receptor inhibitor, demonstrated a protective effect on cell viability after simulated ischemia-reperfusion. However, further studies to investigate the underlying mechanisms are needed.

**Keywords:** Endothelial cells, ischemia-reperfusion injury, reparixin

potential for the treatment of endothelial and myocardial injury. Therefore, new targeted therapies for protecting early endothelial injury have been explored.

Reparixin (reperixin) is a therapeutic inhibitor of CXC-chemokine receptor types 1 (CXCR1) and 2 (CXCR2) or an interleukin-8 (IL-8/CXCL8) receptor inhibitor. It has been shown to reduce polymorphonuclear (PMN) infiltrates and vascular permeability.<sup>[9]</sup> A recent study has reported that reparixin was able to mitigate the postoperative adverse outcomes of cardiac surgery such as hemodynamic instability.<sup>[10]</sup> Moreover, several reports have been shown that reparixin minimized the impact of IRI on graft rejection after organ transplantation including liver, kidney, pancreatic islet, and lung transplantation.<sup>[9,11-13]</sup> However, the role of reparixin in EC dysfunction in myocardial IRI is unknown. The investigation of potential therapeutic of reparixin against EC injury-related IRI by reducing leukocyte accumulation and

vascular leakage is still required. There is currently limited data on the effects of reparixin and its mechanisms of action on the endothelium during IRI. Therefore, this study aims to examine the effect of reparixin on EC viability after simulated IR. This study will help better understand the roles of ECs in IRI and to extend the knowledge in clinically applicable strategies.

## Materials and Methods

### Chemical and reagents

Reparixin was purchased from APEX BIO Technology LLC (Texas, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (Missouri, USA).

### Cell culture

Human EC line EA.hy926 (ATCC® CRL-2922™) was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were incubated with 5% CO<sub>2</sub> at 37°C and were passaged when 80% confluence was reached. The cells at density of 40,000 cells were plated in 96-well plates overnight before performing experiments.

### Evaluation of reparixin on cell viability

To determine the toxicity of reparixin on ECs, the cells were treated with the medium supplemented with various concentrations of reparixin (0-1 µg/ml) for 24 h. MTT assay was used to analyze cell viability as previously described.<sup>[14]</sup> Briefly, MTT solution was added to the medium to achieve a final concentration of 0.5 mg/ml and incubated at 37°C for 2 h. After that, the supernatant was gently removed and dimethyl sulfoxide was used to solubilize the purple formazan crystals. The absorbance of the samples was measured using a microplate reader at a wavelength of 570 nm. Cells cultured in the medium were used as the control group.

### Simulating ischemia-reperfusion injury

To simulate ischemia, the cells were incubated with a simulated ischemia solution containing 140 mM NaCl, 6 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 5 mM HEPES, 10 mM 2-deoxy-d-glucose, and 10 mM sodium dithionite.<sup>[15]</sup> To optimize the duration of simulated ischemia, the cells were exposed to the ischemic solution at 37°C in an atmosphere with 5% CO<sub>2</sub> for different durations (0, 15, 30, 60, and 90 min). Cells cultured in complete medium were used as the control group.

To simulate IRI, after simulating ischemia, the ischemic solution was removed and replaced by fresh complete medium to simulate reperfusion. The cells were then incubated at 37°C in an atmosphere with 5% CO<sub>2</sub> for 24 h and 48 h. Following completed simulating IR protocol, cell viability was performed using the MTT assay. After the test, a simulated ischemia

duration of 60 min and reperfusion duration of 24 h was selected to use in subsequent experiments.

### Evaluation of reparixin on cell viability after simulating ischemia-reperfusion

To determine the effect of reparixin, after 1 h of serum deprivation, the cells were pretreated with various concentrations of reparixin (0-1 µg/ml) for 60 min. Simulated ischemia was then induced by incubating the cells with a simulated ischemia solution at 37°C in an atmosphere with 5% CO<sub>2</sub> for 60 min. After removal of the ischemic solution and washing with phosphate-buffered saline, the cells were given complete medium to simulate reperfusion and reparixin was added at the same concentration as the pretreatment; the cells were then incubated at 37°C in an atmosphere with 5% CO<sub>2</sub> for 24 h. Cells cultured in complete medium were used as the control group. The MTT assay was then performed to determine cell viability.

### Statistical analysis

Data are expressed as mean ± standard deviation (SD). All comparisons were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. The statistical tests were performed using GraphPad Prism version 5 software (GraphPad Software, Inc., La Jolla, CA, USA). A *P*-value less than 0.05 was considered statistically significant.

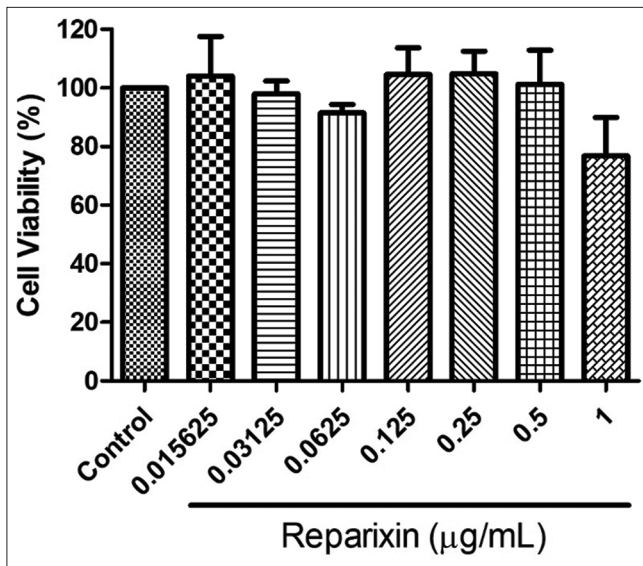
## Results

### Effect of reparixin on cell viability

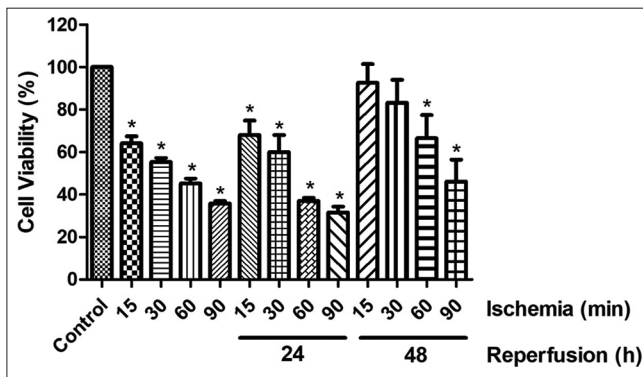
To investigate the toxicity of reparixin, the EA.hy926 ECs were treated with various concentrations of reparixin (0-1 µg/ml) for 24 h before measuring the percentage cell viability using the MTT assay. Figure 1 shows the percentages of cell viability of the treatment and control groups. The result shows that there were no significant differences between the percentages of cell viability of these groups. Therefore, the reparixin concentrations for subsequent experiments were chosen.

### Effect of simulated ischemia and reperfusion in different durations on cell viability

Figure 2 shows percentages of cell viability after simulated ischemia (0, 15, 30, 60, and 90 min) and simulated IR at 24 h and 48 h. After 15, 30, 60, and 90 min of simulated ischemia, the percentages of cell viability of the ischemia groups were significantly lower than that of the control group. According to simulated IR, after simulated ischemia and 24 h of replenishment with complete medium to simulate reperfusion, the percentages of cell viability of the IR groups were significantly lower than that of the control group. The percentages of cell viability of 60- and 90-min IR were significantly lower than those of the 15- and 30-min IR groups. For simulated ischemia and 48 h reperfusion, only



**Figure 1:** Determination of EA.hy926 cell viability after treatment with different concentrations of reparixin. The results are expressed as mean  $\pm$  standard deviation. Three experiments with independent cell preparations were performed

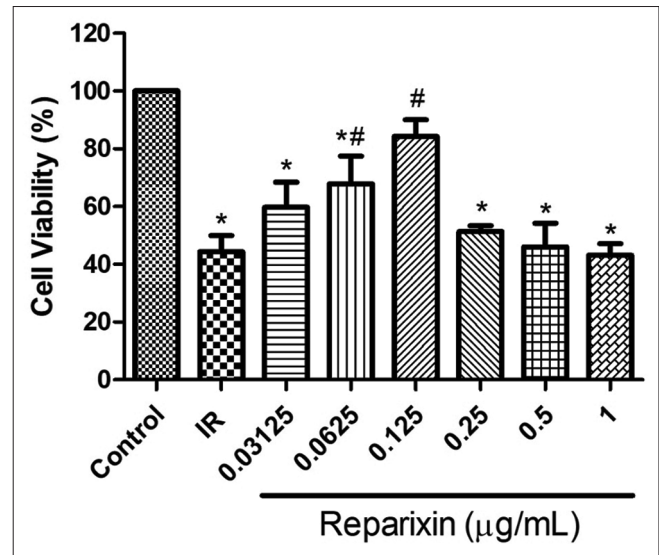


**Figure 2:** Determination of EA.hy926 cell viability after simulated ischemia of variable duration (15, 30, 60, or 90 min) and simulated ischemia with 24 and 48 h of reperfusion. The results are expressed as mean  $\pm$  standard deviation. Three experiments with independent cell preparations were performed. \* $P < 0.05$  versus control group

60- and 90-min IR were significantly lower than that of the control group and there were increases in the percentage of cell viability of all groups. Therefore, the optimal simulated ischemia duration of 60 min with reperfusion at 24 h was selected for subsequent experiments.

### Effect of reparixin on cell viability after simulating ischemia reperfusion

Figure 3 shows percentages of cell viability of the EA.hy926 cells after simulating IR with various concentrations of reparixin (0–1 µg/ml). The percentages of cell viability in the 0.0625 and 0.125 µg/mL reparixin-treated groups were  $67.88 \pm 7.82$  and  $84.28 \pm 4.68\%$  of control, respectively. Comparing to the IR group, they were significantly higher than that of the IR group ( $44.31 \pm 4.64\%$  of control). The percentage of cell



**Figure 3:** Effect of reparixin on EA.hy926 cell viability after simulated ischemic-reperfusion. The results are expressed as mean  $\pm$  standard deviation. Three experiments with independent cell preparations were performed. \* $P < 0.05$  vs. control group. # $P < 0.05$  versus IR group. IR, ischemic-reperfusion

viability in the 0.125 µg/mL reparixin-treated group was the only group that was not significantly different compared to the control group.

### Discussion

Vascular endothelial dysfunction in myocardial IRI is critical in the management of myocardial damage. Therefore, attenuating the impairment of endothelial-myocardial interactions in IRI can preserve EC and myocyte viability. Oxidative stress and the inflammatory response are important in the pathological process of the acute endothelial and myocardial interactive response to IRI. A variety of CXC chemokines are involved in IRI. CXCL8 or IL-8 plays a role in neutrophil-induced myocardial IRI. Coronary EC dysfunction is a consequence of myocardial IRI. Several candidate therapeutic targets for myocardial and endothelial protection have been developed. CXCL8 receptor inhibitors are a therapeutic target for the prevention of reperfusion injury by targeting CXCL8 activity.

In the present study, the efficacy of reparixin on ECs following IRI was investigated. We assessed vascular EC viability using an *in vitro* model of simulated IRI. The results demonstrated no toxicity of reparixin to ECs in the micromolar range (0–1 µg). Consistently, there was a significant increase in the percentages of cell viability following simulated IRI of 0.0625 and 0.125 µg/mL reparixin-treated groups compared to that of the control group. The data indicated that 0.125 µg/ml of reparixin can minimize EC death following simulation of Ea.hy926 cells with intermittent hypoxia/reoxygenation. The percentage of cell viability did not differ between 0.125 µg/mL reparixin-treated groups and that of the control group. In

addition, higher doses did not provide an additional increase in cell viability. The results are consistent with previous findings in both animal studies and clinical trials. Reparixin or repertaxin has been shown to mitigate tissue damage following transient brain ischemia in rats.<sup>[16]</sup> In addition, an inhibitory effect of repertaxin on inflammation following intestinal ischemia and reperfusion injury in rats has been found.<sup>[17]</sup> In the setting of patients undergoing on-pump coronary artery bypass graft surgery, intravenous reparixin was able to reduce the recruitment of neutrophils and hemodynamic alterations.<sup>[10]</sup>

Reparixin, a noncompetitive allosteric inhibitor of the chemokine receptors CXCR1/2, has attracted attention in targeted drug therapy for some types of cancer and multiple diseases associated with inflammation.<sup>[18,19]</sup> The CXCL8 chemokine receptors CXCR1 and CXCR2, known as CXCL8 receptors, are both expressed on ECs.<sup>[18]</sup> The mechanism of CXCL8-CXCR1/2 signaling in IRI has been explored previously. CXCL8 is initially produced by endothelial and EA.hy926 cells in response to several stimulatory factors.<sup>[20,21]</sup> A previous study found that CXCL8-induced neutrophil activation was involved in acute myocardial IRI.<sup>[22]</sup> Under myocardial ischemia-reperfusion conditions, neutrophils migrate and accumulate in the coronary vascular endothelium, arterial wall, and around cardiomyocytes in the ischemic and reperfused myocardium. Neutrophils produce toxic agents such as free radicals and proteases and also promote the production and release of inflammatory factors.<sup>[23]</sup> de Oliveira *et al.* revealed that reparixin, as antineutrophil therapeutic strategy, not only reduced neutrophil influx into the liver but also suppressed the increase in serum concentrations of TNF- $\alpha$ , IL-6, and CCL3 in a mouse model of liver IRI.<sup>[11]</sup> Similarly, Bertini *et al.* found that repertaxin protected organs against reperfusion injury by inhibiting PMN infiltration.<sup>[24]</sup> Although the present study did not demonstrate the effect of CXCR1/2 inhibitor on reperfusion injury mediated by neutrophils, it is possible that reparixin is able to increase the viability of ECs through blocking CXCR1/2 using an *in vitro* model of IRI. It was previously demonstrated that increased expression of CXCR2 occurs in aortic ECs under hypoxic conditions.<sup>[25]</sup> In addition, blocking CXCR1/2 could inhibit the increase of intracellular free calcium during the reperfusion period.<sup>[26]</sup>

Although it has been reported that CXCL8-induced stimulation of neutrophil is associated with IRI in many cell types, our study was the first showing the role of CXCL8 receptor inhibitor in the mitigation of endothelial injury using the *in vitro* model of IRI. Our result may imply, at least in part, that endothelial IRI occurs through the receptor of CXCL8 on ECs directly. However, further investigation of the expression and roles of CXCL8-CXCR1/2 on ECs as well as the relative contribution of the ligand and their receptors to normal and pathological conditions is necessary. Exploring whether the CXCL8 plays a major role in chemotaxis of neutrophils into the ECs is also concerned. These findings will be fundamental for

pharmacological development of selective CXCR1/2 blockers for the prevention of endothelial IRI in various situations, for example, in AMI reperfusion therapy.

## Conclusion

The present study demonstrates that reparixin can protect ECs against IRI using the *in vitro* cell viability model.

## Authors' Declaration Statements

### Ethics approval and consent to participate

Not applicable.

### Availability of data and material

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

### Competing interests

The authors declare that they have no conflicts of interest with the contents of this article.

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## Authors' Contributions

Conceptualization: TN, PT; Methodology: TN, PT, DS; Validation: PT, TN; Formal analysis: PT, TT, DS, TN; Investigation: TT, TP, SM, PT, TN; Resources: TN, PT; Data curation: PT, TP, DS; Writing-Original Draft: TN, PT; Writing-Review & Editing: PT, TN, DS; Visualization: PT, TN; Supervision: TN; Project administration: TN; Funding acquisition: TN.

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