


# Protective Effect of Metformin on Sepsis Myocarditis in Zebrafish

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## Abstract

**Purpose:** We found in previous study that metformin could treat sepsis myocarditis in a mouse model. We employed the zebrafish model organism to investigate the effect of metformin on sepsis myocarditis.

**Methods and Results:** Wild-type zebrafish was used to establish a sepsis myocarditis model and combined with image software analysis and cytokine detection, the protective dose of metformin was determined. The results showed that immersion with *Escherichia coli* could cause 75% mortality in zebrafish and make larvae appear as characteristics of severe sepsis myocarditis. Pretreatment with 10 mM metformin for 3 hours could effectively reduce heart congestion and swelling in zebrafish with sepsis myocarditis and increased the heart rate. It could reduce the mortality and prolong the survival time of zebrafish with sepsis myocarditis; Tg(mpx: EGFP) transgenic zebrafish were adopted to explore the number of neutrophils in zebrafish heart before and after metformin protection, and metformin could maintain the number of neutrophils in zebrafish heart; quantitative real-time reverse transcription–polymerase chain reaction showed that metformin could reduce the expression of pro-inflammatory factors, tumor necrosis factor- $\alpha$  and interleukin (IL)-6, and could promote the anti-inflammatory factor, transforming growth factor- $\beta$  and IL-10 expression.

**Conclusion:** We established a zebrafish sepsis myocarditis model and applied metformin in advance to provide a protective effect on the zebrafish heart.

## Keywords

metformin, sepsis, myocarditis, zebrafish

## Introduction

Sepsis is a systemic inflammatory disease caused by infection, accompanied by multiple organ failure. During septic shock, the heart is one of the most vulnerable organs,<sup>1</sup> which is characterized by decreased myocardial contraction and decreased ejection fraction.<sup>2</sup> Sepsis myocarditis is an infection-related cardiovascular disease. This infection is mainly caused by bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus*, *Pneumococcus*, and *Neisseria meningitides*, which is also known as bacterial myocarditis.<sup>3</sup> Research shows that at least 50% of patients with septic shock are diagnosed as septic myocarditis, which is caused by myocardial dysfunction, indicating the poor prognosis of septic shock.<sup>4</sup> Our research group has found metformin could treat bacterial myocarditis in mice<sup>5</sup> not long ago, so we wondered whether it had the same positive effect on sepsis myocarditis in zebrafish.

Metformin is the most basic oral hypoglycemic drug in clinic, which plays an important role in reducing fasting blood

glucose.<sup>6</sup> It is widely used in the treatment of type 2 diabetes by reducing plasma insulin level, reducing insulin resistance, and preventing various complications caused by diabetes.<sup>7</sup> However, in recent decades, metformin has also been found to have a protective effect on cardiovascular disease, which can reduce the incidence of cardiovascular disease in patients.<sup>8-10</sup> There

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are also many cardiovascular-based animal experimental studies of metformin,<sup>11-13</sup> but few of them are used in zebrafish.

Zebrafish, as an excellent vertebrate model, has a variety of advantages over mice or other animals, such as low-cost, transparent in vitro, easy to operate, and so on, and are widely used in human disease research, such as infection, inflammation, tumor transplantation, and immune-related diseases.<sup>14-16</sup> The main blood system of zebrafish is the same as that of the mammal. The innate immune system matures at the somite stage, and macrophages and neutrophils appear at 16 and 26 hours, respectively.<sup>17,18</sup> More importantly, the genetic similarity between zebrafish and human is approximately 87%.<sup>19,20</sup>

The purpose of this study was to establish a zebrafish model of sepsis caused by a bacterial infection, intuitively observe the heart changes of zebrafish during the progress of sepsis, and explore the changes of heart and survival cycle of zebrafish during the progress of sepsis after metformin was given in advance. As zebrafish heart is transparent and visible, the zebrafish is an ideal model to study heart development and function, including cardiovascular development. In this study, it was found that the heart of zebrafish weakened with the development of sepsis appeared congestion and swelling as well as decreased heart rate. After the zebrafish were treated with metformin in advance, the heart of zebrafish was protected to a certain extent when sepsis occurs, such as increased ejection, increased heart rate, reduced congestion, and could maintain the number of neutrophils in the immune cells of the heart, so as to prolong the survival period.

## Methods and Results

### *Zebrafish Maintenance and Care*

Zebrafish were maintained and raised accordingly to the standard scheme.<sup>21-23</sup> Zebrafish were raised on a 14 hours/10 hours light/dark cycle at  $28.5\text{ }^{\circ}\text{C} \pm 0.5\text{ }^{\circ}\text{C}$ . Zebrafish were fed brine shrimp 3 times per day. Zebrafish laid eggs by natural mating in the mating box. After being collected and cleaned, the eggs were transferred to the medium containing methylene blue for culture. Fish eggs were treated with 200- $\mu\text{M}$  1-phenyl-2-thiourea 24 hours after fertilization to inhibit pigment production. Three-day post-fertilization (dpf) wild-type and Tg (mpx: EGFP) transgenic zebrafish larvae were used in this study. All the experiments in this study were approved by the animal ethics committee of Jilin University.

### *Preparation of Escherichia coli Solution*

*Escherichia coli* strain JM109 was used for this study. Simply, *E. coli* strain JM109 was recovered from lyophilized powder by resuspending in Luria-Bertani (LB) medium and culturing overnight on an LB agar plate at  $37\text{ }^{\circ}\text{C}$ . Then single colony of *E. coli* was picked up and cultured in LB medium to the optical density value (A600) of the medium reached 0.7 in which the colony-forming units (CFU) of the *E. coli* culture were calculated and determined to be approximately  $0.8 \times$

$10^8$  CFU. Finally, the *E. coli* culture was centrifuged and diluted in PBS solution for corresponding concentration. The detailed process could be informed in the published paper.<sup>24</sup>

### *Lethal Dose Experiment of E coli Immersion*

Firstly, 3dpf larvae were sucked randomly from the culture dish into 96-well plate with dropper, 5 pieces for each hole, 4 holes for each group, and 20 pieces in total for each group. Then, the pipette was used to carefully dry the water in each hole in turn and then quickly added 200  $\mu\text{L}$  of  $10 \times 10^8$  CFU/mL *E. coli* solution into each hole in turn to soak for 3 hours. Finally, after the immersion, the bacterial solution was carefully sucked and discarded, washed the larvae with sterile fish water for 3 times, and then transferred them to the new hole to observe the survival and symptoms of the larvae.

### *Protective Dose Experiment of Metformin*

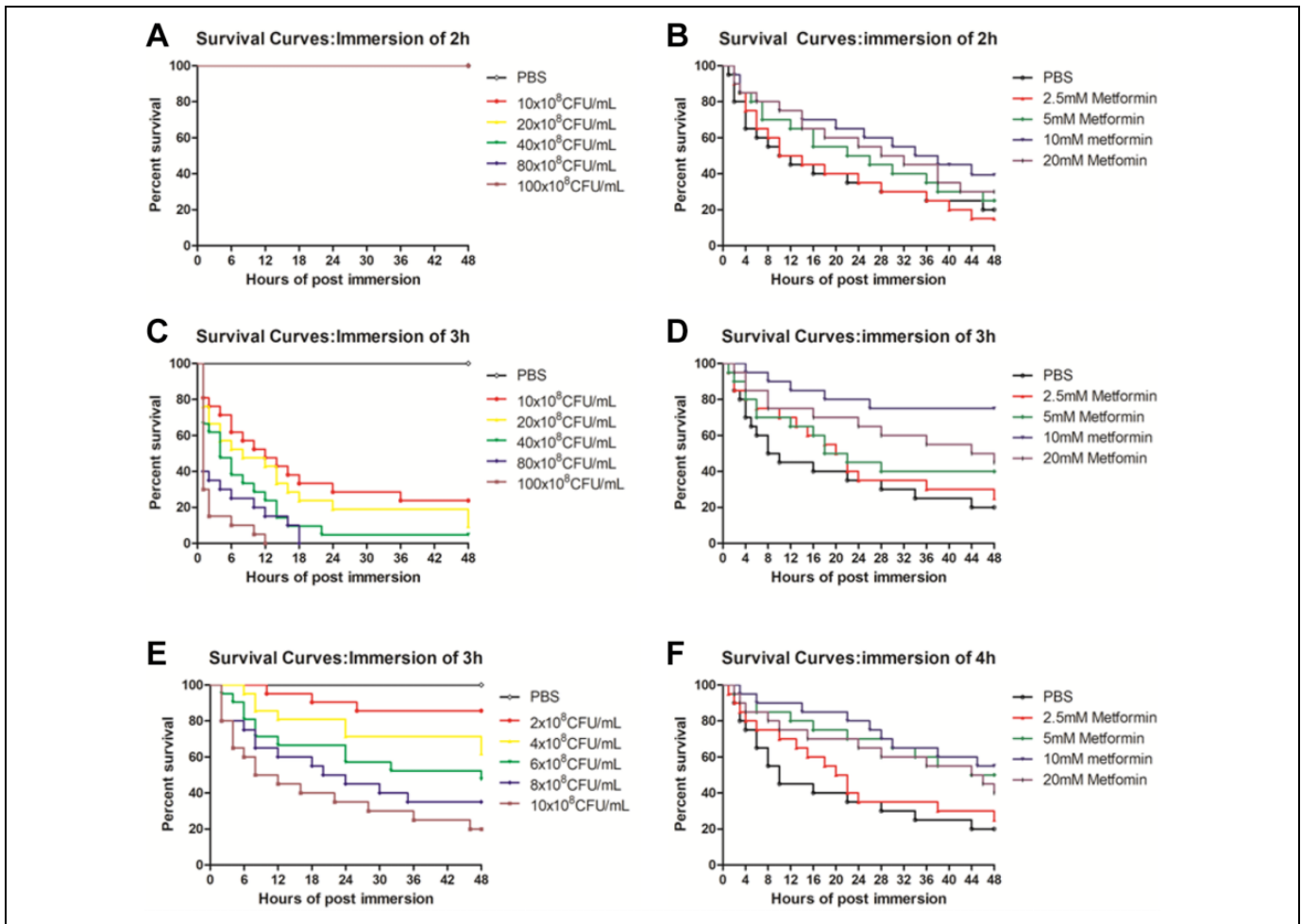
Firstly, 3dpf larvae were sucked randomly from the culture dish into 96-well plate with dropper, 5 pieces for each hole, 4 holes for each group, and 20 pieces in total for each group. Then, the pipette was used to carefully dry the water in each hole in turn, then quickly added 200- $\mu\text{L}$  metformin into each hole in turn and soaked for 3 hours. And after immersion, metformin solution was carefully sucked and discarded, washed it with sterile fish culture water, and then quickly added 200  $\mu\text{L}$  of  $10 \times 10^8$  CFU/mL *E. coli* solution to soak for 3 hours. Finally, after the immersion, the larvae were washed the fish with sterile water for 3 times and transferred it to a plate containing fresh water for culture and observation.

### **Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction Analysis**

Total RNAs were harvested by using the Trizol reagent per the manufacturer's instructions (Invitrogen). Reverse transcription of equal amounts of RNA was done with a first-strand complementary DNA (cDNA) synthesis kit (Invitrogen) with random hexamers as primers. The quantitative real-time reverse transcription polymerase chain reaction experiment was done with an ABI 7500 Real-Time PCR System (Applied Biosystems). Each cDNA sample of each target gene (tumor necrosis factor [TNF]- $\alpha$ , IL-6, transforming growth factor [TGF]- $\beta$ , and IL-10) was detected in triplicate and results were normalized by glyceraldehyde 3-phosphate dehydrogenase. All primer designs were obtained by Primer Express software (Applied Biosystems) and purchased from Sangon Biotech.

### *Neutrophil Count*

Tg(mpx: EGFP) transgenic zebrafish were used in the experiment, due to the neutrophils labeled with green fluorescent protein convenient for the count, and then the number of cardiac neutrophils in different groups of zebrafish was analyzed.



**Figure 1.** Survival curve of zebrafish in different groups. A, Survival curves,  $n = 20$  fish per group. The fish were soaked in different concentrations of *Escherichia coli* solution for 2 hours,  $10 \times 10^8$  CFU/mL,  $20 \times 10^8$  CFU/mL,  $40 \times 10^8$  CFU/mL,  $80 \times 10^8$  CFU/mL, and  $100 \times 10^8$  CFU/mL, respectively. B, Survival curves,  $n = 20$  fish per group. The fish were soaked in different concentrations of *E. coli* solution for 3 hours,  $10 \times 10^8$  CFU/mL,  $20 \times 10^8$  CFU/mL,  $40 \times 10^8$  CFU/mL,  $80 \times 10^8$  CFU/mL, and  $100 \times 10^8$  CFU/mL, respectively. C, Survival curves,  $n = 20$  fish per group. The fish were soaked in different concentrations of *E. coli* solution for 3 hours,  $2 \times 10^8$  CFU/mL,  $4 \times 10^8$  CFU/mL,  $6 \times 10^8$  CFU/mL,  $8 \times 10^8$  CFU/mL, and  $10 \times 10^8$  CFU/mL, respectively. D, Survival curves,  $n = 20$  fish per group. The fish were first soaked in different concentrations of metformin for 2 hours, second, soaked in  $10 \times 10^8$  CFU/mL *E. coli* for 3 hours. E, Survival curves,  $n = 20$  fish per group. The fish were first soaked in different concentrations of metformin for 3 hours, second, soaked in  $10 \times 10^8$  CFU/mL *E. coli* for 3 hours. F, Survival curves,  $n = 20$  fish per group. The fish were first soaked in different concentrations of metformin for 4 hours, second, soaked in  $10 \times 10^8$  CFU/mL *E. coli* for 3 hours.

### Image Acquisition and Analysis

When the images of different groups were collected during the experiment, the zebrafish were adopted with the side-lying posture to make the eyes coincide as much as possible. After adjusting the posture, the zebrafish heart were recorded with the Olympus microscope (cs-st-v1.18) and the supporting software (cellSens standard) and then used to analyze the heart expansion area, heart rate, and neutrophil number.

### Data Analysis

All data were statistically analyzed by GraphPad Prism 5 software, and the data were expressed by mean  $\pm$  SE. Independent

sample  $t$  test was used to compare the differences between the groups, with  $P < .05$  being statistically significant.

## Results

### Determination of the Lethal Dose of *E. coli* Immersion

We firstly soaked 3dpf wild-type zebrafish in different concentrations of *E. coli* for 2 hours. During the experiment, we found that these concentrations of *E. coli* for 2 hours would cause zebrafish to bend in different degrees, but after being transferred to fresh fish water, the bending phenomenon would gradually recover and straighten, which could not cause zebrafish death. Results were shown in Figure 1A, zebrafish in different concentration groups did not die.

So we extended the soaking time to 3 hours. Results were shown in Figure 1B, and all different concentrations of *E coli* could cause the death of zebrafish. Among them, the  $20 \times 10^8$  CFU/mL group, the  $40 \times 10^8$  CFU/mL group, the  $60 \times 10^8$  CFU/mL group, and the  $100 \times 10^8$  CFU/mL group had 100% mortality within 48 hours, and the  $10 \times 10^8$  CFU/mL group had a mortality of 75% within 48 hours. After being soaked by lethal doses of *E coli*, zebrafish became opaque and curved from transparent and straight, finally ulceration and death occurred over time.

The mortality of *E coli*, we wanted to choose, was at the minimum dose of 75%, so we downregulated the concentration of *E coli*, which were divided into groups of  $2 \times 10^8$  CFU/mL,  $4 \times 10^8$  CFU/mL,  $6 \times 10^8$  CFU/mL,  $8 \times 10^8$  CFU/mL, and  $10 \times 10^8$  CFU/mL for 3 hours. The results were shown in Figure 1C, and the mortalities in 48 hours were 0%, 40%, 55%, 65%, and 80%, respectively. After comparison, immersion with  $10 \times 10^8$  CFU/mL *E coli* for 3 hours was chosen as the minimum lethal dose of 3dpf zebrafish for subsequent experiments.

### Determination of Protective Dose of Metformin

As shown in Figure 1D, we firstly soaked the wild zebrafish of 3dpf in different concentrations of metformin for 2 hours. The results showed that compared with PBS group, the survival time of zebrafish was prolonged by metformin of different concentrations, but the mortality was still very high. The mortalities of 2.5 mM group, 5 mM group, 10 mM group, and 20 mM group were 85%, 75%, 60%, and 70%, respectively.

So we prolonged the pretreatment time of metformin and soaked it for 3 hours. The results were shown in Figure 1E. Metformin in different groups reduced the mortality of zebrafish, and the mortality of 2.5 mM group, 5 mM group, 10 mM group, and 20 mM group were 75%, 60%, 25%, and 55%, respectively. The mortality rate we wanted to choose was between 0% and 30%, that is to say, after metformin immersion treatment, the survival rate could be 70% to 100%. Then, we prolonged the treatment time of metformin again. When the zebrafish were soaked for 4 hours, the results were shown in Figure 2F. The mortalities rates of 2.5 mM group, 5 mM group, 10 mM group, and 20 mM group were 75%, 50%, 45%, and 60%, respectively, which were still high. After comparison, the mortality and survival rate of 10 mM metformin were 25% and 75%, respectively, which could be used as a protective dose for subsequent experiments.

### Effects of Pretreatment With Metformin on Heart of Zebrafish With Sepsis Myocarditis

The zebrafish were pretreated with 10 mM metformin for 3 hours, then soaked with  $10 \times 10^8$  CFU/mL *E coli* for 3 hours. After immersion, we took photos and recorded the heart condition of zebrafish in different groups as shown in Figure 2A, then circled the heart part with software and statistically analyzed the area of zebrafish heart in different groups, which represented the severity of heart swelling and congestion. The

results were shown in Figure 2B. Compared with PBS group, the heart area of zebrafish in *E coli* group was significantly larger ( $P < .05$ ), and the heart area of zebrafish in metformin group was barely changed ( $P > .5$ ); compared with *E coli* group, the heart area of zebrafish in Met + *E coli* group decreased significantly ( $P < .05$ ).

After statistical analysis of heart rate of zebrafish in different groups recorded by video recording, the results were shown in Figure 2C. Compared with PBS group, the heart rate of zebrafish in *E coli* group decreased significantly ( $P < .01$ ); compared with *E coli* group, the heart rate of zebrafish in Met + *E coli* group increased significantly ( $P < .05$ ).

### Effects of Pretreatment With Metformin on Cardiac Neutrophils in Zebrafish With Sepsis Myocarditis

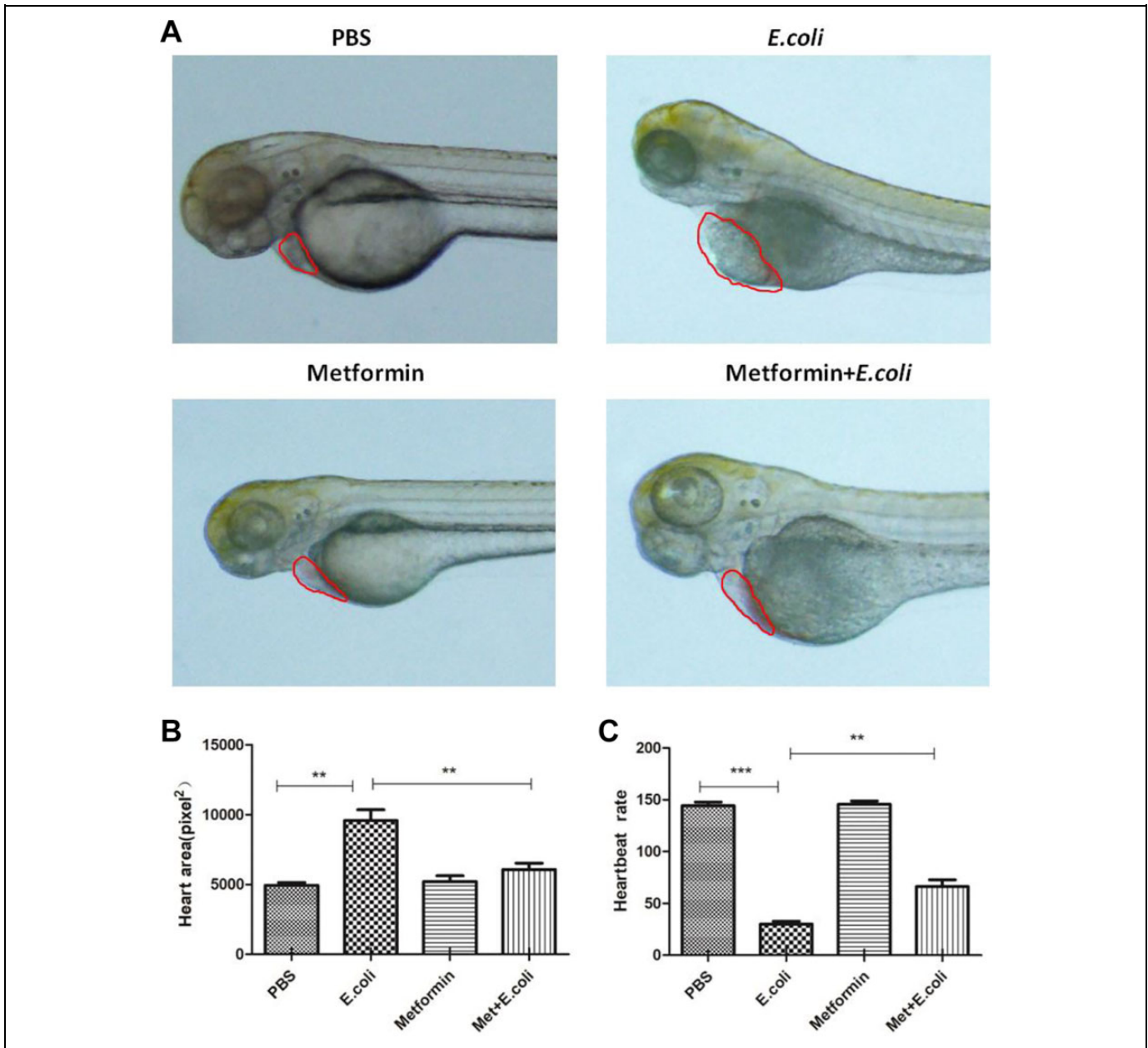
Tg(mpx: EGFP) transgenic zebrafish were pretreated with 10 mM metformin for 3 hours, then soaked with  $10 \times 10^8$  CFU/mL *E coli* for 3 hours. Then statistical analysis was carried out after neutrophils count. The results were shown in Figure 4B. Compared with PBS group, the number of neutrophils in *E coli* group decreased significantly ( $P < .01$ ); compared with *E coli* group, the number of neutrophils in Met + *E coli* group increased significantly ( $P < .05$ ).

### Effects of Pretreatment With Metformin on Cytokines in Zebrafish With Sepsis Myocarditis

After pretreatment with 10 mM metformin for 3 hours, zebrafish then were soaked with  $10 \times 10^8$  CFU/mL *E coli* for 3 hours. Quantitative real-time reverse transcription polymerase chain reaction was used to detect the expression of cytokines in zebrafish. It was found that the expression of TNF- $\alpha$  and IL-6 in *E coli* group was significantly higher than that in PBS group ( $P < .001$ ), while that in Met + *E coli* group was significantly lower ( $P < .05$ ). At the same time, the expression of TGF- $\beta$  and IL-10 in *E coli* group was not significantly different from that in PBS group ( $P > .05$ ), while the expression of TGF- $\beta$  and IL-10 in Met + *E coli* group was significantly higher than that in *E coli* group ( $P < .05$ ,  $P < .01$ ).

## Discussion

Sepsis is the primary cause of death in critically ill patients. Its pathogenesis is complex, involving inflammation, immune response, coagulation dysfunction, and other aspects.<sup>25</sup> When pathogenic microorganisms invade host cells, innate immune cells such as neutrophils, macrophages, and dendritic cells recognize receptors through their surface pattern recognition and combine with pathogen-related molecular patterns to start the immune response of the body, and with the synthesis and release of a variety of pro-inflammatory cytokines, including TNF- $\alpha$ , IL-1, IL-6, interferon, and so on.<sup>26</sup> These pro-inflammatory factors interact with inflammatory mediators to further amplify the body's inflammatory response, leading to sepsis. At the same time, the negative feedback of

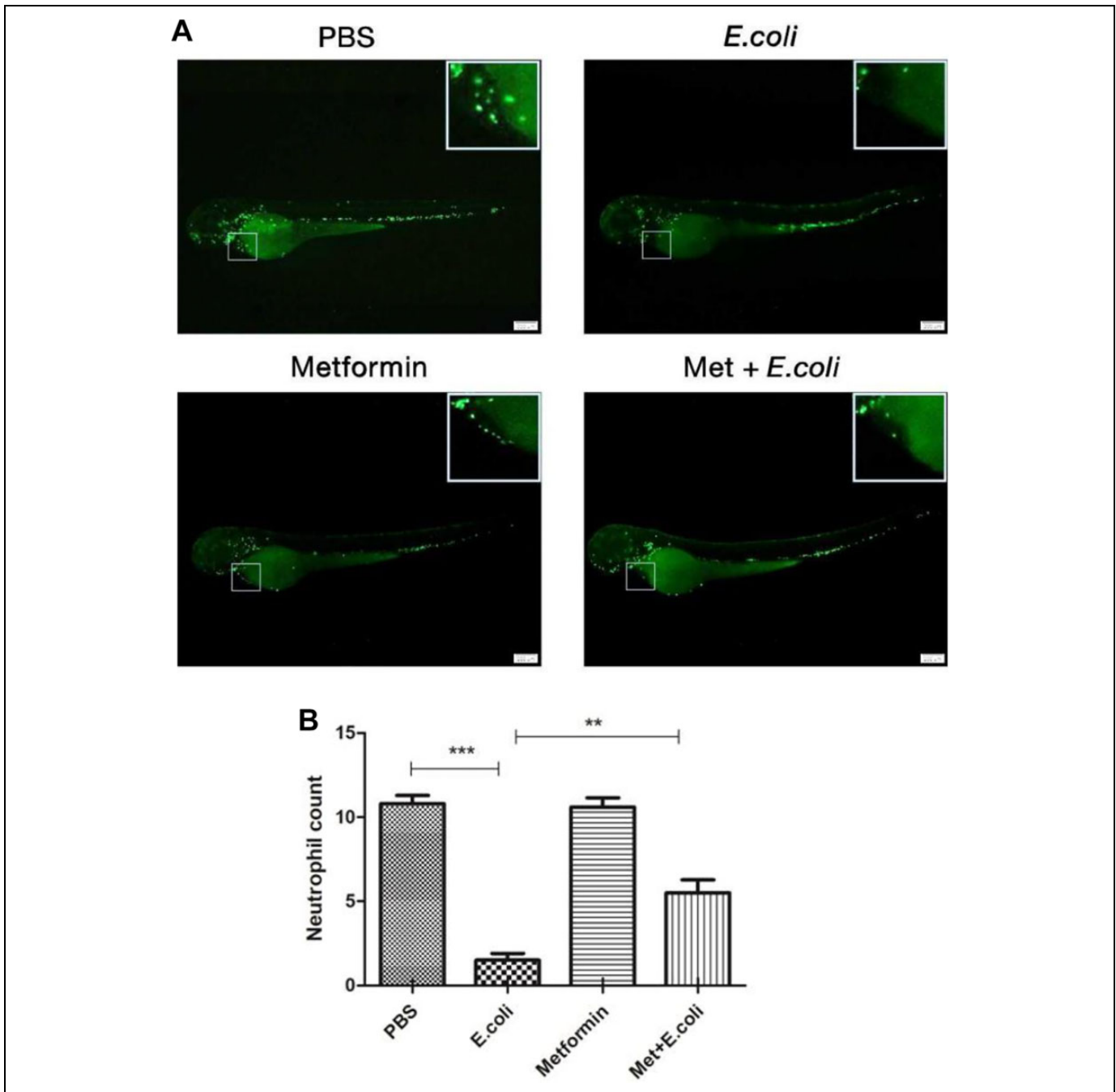


**Figure 2.** Effect of metformin on heart of zebrafish with sepsis myocarditis. A, Effect of metformin on swelling and congestion of zebrafish heart with sepsis myocarditis, the whole hearts were circled by red zones. The area of red zone represented as the severity of myocarditis. B, Effect of metformin on heart area of zebrafish with sepsis myocarditis, the area of red zones calculated by computer software were statistically analyzed (\*\* $P < .05$ ; \*\*\* $P < .01$ ,  $n = 10$ ), (*Escherichia coli* group vs PBS group, Met + *E coli* vs *E coli* group). C, Effect of metformin on heart rate of zebrafish with sepsis myocarditis, the heart rate of zebrafish recorded on video were statistically analyzed (\*\* $P < .05$ ; \*\*\* $P < .01$ ,  $n = 10$ ), (*E coli* group vs PBS group, Met + *E coli* vs *E coli* group).

inflammatory response makes the concentration of anti-inflammatory cytokines, such as IL-4, IL-10, TGF- $\beta$ , and IL-13, increase, thus inhibiting the generation and release of pro-inflammatory cytokines, and preventing the further development of systemic inflammatory response.<sup>27</sup> Under normal circumstances, there is a dynamic balance between pro-inflammatory cytokines and anti-inflammatory cytokines, but in sepsis patients, the balance is often out of balance. On the one hand, inflammatory cells cannot be activated effectively. On

the other hand, anti-inflammatory cytokines are not produced enough, so the body is prone to immune dysfunction, resulting in excessive tissue damage, involving various organs.

For one thing, the heart needs a lot of energy to maintain the contraction of the heart muscle, for another, the heart pumps blood for the whole body, which leads to the heart being an organ easily involved in the development of sepsis. When bacterial sepsis occurs, with mitochondrial damage, apoptosis or necrosis can be induced by various mechanisms.<sup>28,29</sup> Our

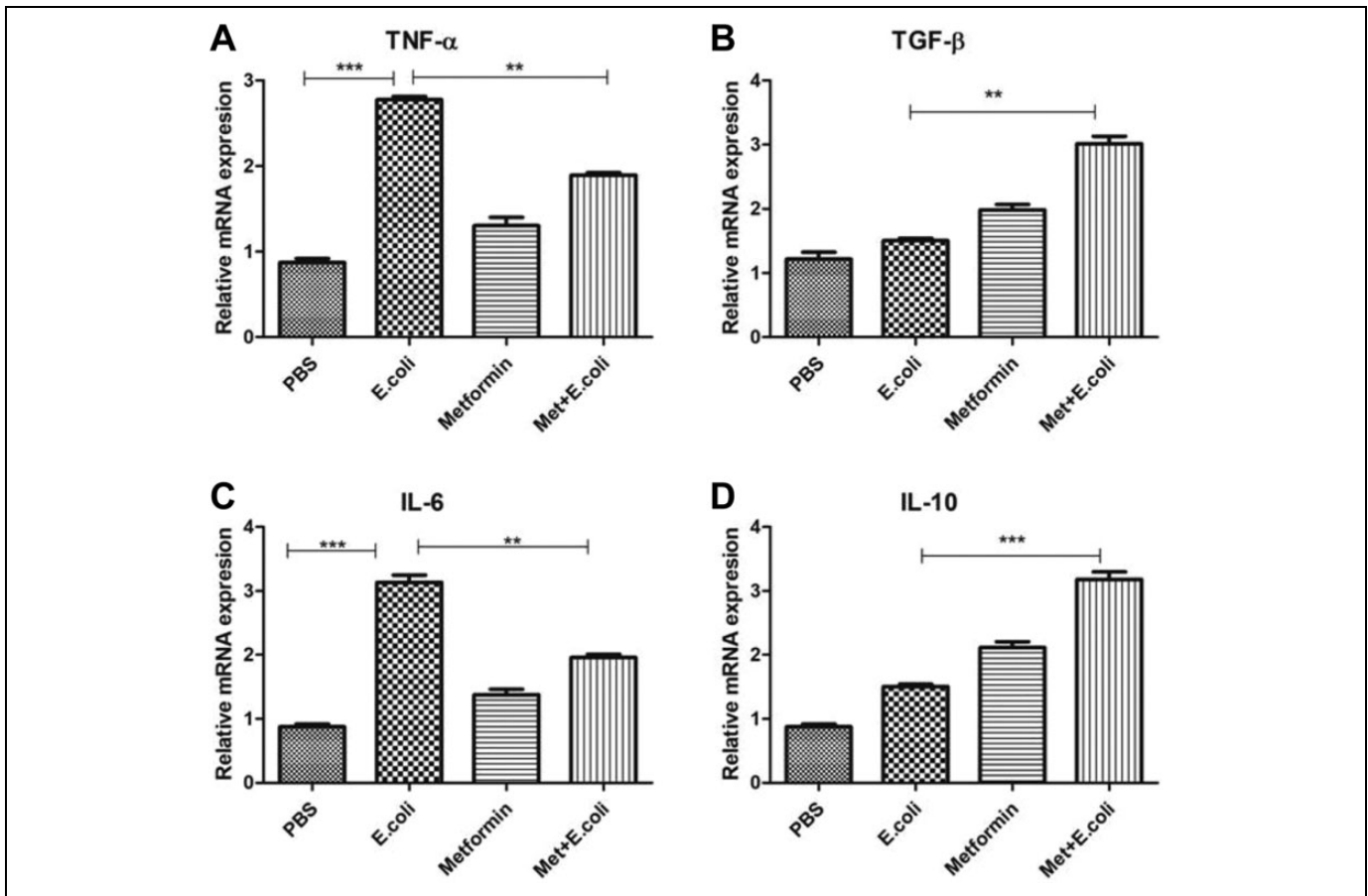


**Figure 3.** Effect of metformin on cardiac neutrophils in zebrafish with sepsis myocarditis. A, Cardiac neutrophils of different groups of zebrafish recorded by repeated experiments with Tg (mpx: EGFP) transgenic zebrafish, the neutrophils labeled by green fluorescent protein could be visually counted. And the cardiac neutrophils were amplified and shown in the upper right corner of each group image. B, Statistical analysis of neutrophils in different groups of hearts (\*\* $P < .05$ ; \*\*\* $P < .01$ ,  $n = 10$ ; *E coli* group vs PBS group, Met + *E coli* vs *E coli* group).

research group reported that metformin could treat bacterial myocarditis by activating PKC $\epsilon$ -IRF4 signal pathway in mitochondria of cardiomyocytes in mice.<sup>5</sup> It was found that lipopolysaccharide (LPS) could induce myocardial injury through MAPK/JNK and NF- $\kappa$ B signaling pathway when LPS stimulated mouse cardiomyocytes H9c2. Metformin can protect cardiomyocytes by inhibiting p38MAPK and JNK phosphorylation and then inhibiting activation of MAPK/JNK

signaling pathway and NF- $\kappa$ B signaling pathway.<sup>30</sup> In addition, metformin can reduce the level of Caspase-3 and enhance the expression of Bcl-2 protein in Adriamycin-induced myocardial toxicity experiment of albino rats, indicating that metformin has antiapoptotic effect.<sup>31</sup>

Our group established a mouse model of septic peritonitis induced by intraperitoneal injection of *E coli* in the process of drug screening and found that intraperitoneal injection of a



**Figure 4.** Expression of cytokines in different groups. A, Expression of TNF- $\alpha$  in different groups, qRT-PCR showed that metformin could significantly reduce the expression of TNF- $\alpha$  (\*\* $P < .05$ ; \*\*\* $P < .01$ ; *E coli* group vs PBS group, Met + *E coli* vs *E coli* group). B, Expression of IL-6 in different groups, qRT-PCR showed that metformin could significantly reduce the expression of IL-6 (\*\* $P < .05$ ; \*\*\* $P < .01$ ; *E coli* group vs PBS group, Met + *E coli* vs *E coli* group). C, Expression of TGF- $\beta$  in different groups, qRT-PCR showed that metformin could significantly enhance the expression of TGF- $\beta$ . (\*\* $P < .05$ ; \*\*\* $P < .01$ ; *E coli* group vs PBS group, Met + *E coli* vs *E coli* group). D, Expression of IL-10 in different groups, qRT-PCR showed that metformin could significantly enhance the expression of IL-10 (\*\* $P < .05$ ; \*\*\* $P < .01$ ; *E coli* group vs PBS group, Met + *E coli* vs *E coli* group). IL-6 indicates interleukin-6; IL-10, interleukin-10; PBS, phosphate-buffered saline; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; TGF- $\beta$ , transforming growth factor- $\beta$ ; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

small amount of *E coli* allowed mice to become resistant to lethal *E coli*. This resistance could be obtained 2 hours after injection of a small amount of *E coli*, and all mice injected with lethal doses of *E coli* could survive. This suggested that a small amount of *E coli* injection caused some preconditioning in mice.<sup>24</sup> In addition, when metformin was given to mice with bacterial sepsis by our research group, it was found that the survival period of mice could also be prolonged.<sup>5</sup> So we studied whether zebrafish could gain some resistance to the next lethal dose of *E coli* by giving a certain amount of metformin firstly.

In our current experimental study, we have established a lethal *E coli* zebrafish model. In the process of immersion of lethal *E coli*, zebrafish had a series of inflammatory reactions after being infected. The most obvious was the change of the heart area of zebrafish, with swelling and congestion, slow ejection as well as significantly reduced heart rate (Figure 2). After pretreatment with metformin experiments, we found that

pretreated with 10 mM metformin for 3 hours could reduce the mortality of zebrafish and prolong the survival time of zebrafish (Figure 1). Then we took photos and video records of zebrafish's heart and used software to analyze and count the differences between different groups (Figure 2). It was found that pretreated with metformin for 3 hours could also reduce the symptoms of sepsis myocarditis caused by the subsequent lethal dose of *E coli*, such as congestion, swelling, and heart rate slowing. Surprisingly, it could maintain the number of neutrophils in the heart to participate in the inflammatory response to remove pathogens (Figure 3). After that, we tested the inflammation-related factors and found that metformin could reduce the expression of TNF- $\alpha$  and IL-6 and promote the expression of TGF- $\beta$  and IL-10 (Figure 4). This may indicate that metformin could maintain the number of neutrophils in the heart and regulate inflammatory cytokines to improve the heart disease of zebrafish, so as to achieve a protective or therapeutic effect.

In conclusion, we established a model of zebrafish sepsis myocarditis, and metformin could be used to intervene in advance to protect the heart of zebrafish. This suggests that we will have more options to prevent the occurrence and development of sepsis myocarditis. However, our experiment was based on zebrafish as a whole, and cell experiments are needed to further study the mechanism of metformin protecting sepsis myocarditis.

### Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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