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Original Article

Antifibrotic effects of colchicine on 3T3 cell line ischemia to mitigate detrimental remodeling

[Efectos antifibróticos de la colchicina sobre la isquemia de la línea celular 3T3 para mitigar la remodelación perjudicial]

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Abstract

Context: Acute myocardial infarction (AMI) is one of the clinical manifestations of coronary heart disease caused by the cessation of coronary blood flow. The development of the management of AMI has resulted in a reduction in the mortality rate of AMI patients. Nevertheless, the apparent inflammation triggers detrimental remodeling, so antifibrotic such as colchicine are needed.

Aims: To analyze the impact of colchicine administration on the reduction of ventricular remodeling through the NLRP3 inflammasome, TGF-β, and α-SMA in a 3T3 cell line culture under ischemic conditions.

Methods: The 3T3 cell line culture underwent ischemia treatment using $CoCl_2$ and subsequent treatment with colchicine. This treatment was conducted for 24 hours with a concentration of 300 µM $CoCl_2$, followed by the administration of colchicine at a concentration of 1 µM after the ischemia treatment. The expression levels of NLRP3, TGF-β, and α-SMA were assessed using flow cytometry, and the data were analyzed through one-way ANOVA.

Results: Administration of colchicine for 24 hours following ischemia resulted in a significant decrease in the relative levels of NLRP3, TGF-β, and α-SMA ($p<0.05$) compared to the ischemia group.

Conclusions: *In vitro* administration of colchicine can reduce post-ischemic remodeling, including the NLRP3 inflammasome, TGF-β, and α-SMA.

Keywords: colchicine; inflammasome; ischemia; ventricular remodeling.

Resumen

Contexto: El infarto agudo de miocardio (IAM) es una de las manifestaciones clínicas de la enfermedad coronaria causada por el cese del flujo sanguíneo coronario. El desarrollo del tratamiento del IAM ha dado lugar a una reducción de la tasa de mortalidad de los pacientes con IAM. Sin embargo, la inflamación aparente desencadena una remodelación perjudicial, por lo que se necesitan antifibrinolíticos como la colchicina.

Objetivos: Analizar el impacto de la administración de colchicina en la reducción del remodelado ventricular a través del inflamasoma NLRP3, TGF-β y α-SMA en un cultivo de línea celular 3T3 en condiciones isquémicas.

Métodos: El cultivo de la línea celular 3T3 fue sometido a un tratamiento de isquemia mediante $CoCl_2$ y posterior tratamiento con colchicina. Este tratamiento se realizó durante 24 horas con una concentración de 300 µM de $CoCl_2$, seguido de la administración de colchicina a una concentración de 1 µM tras el tratamiento de isquemia. Los niveles de expresión de NLRP3, TGF-β y α-SMA se evaluaron mediante citometría de flujo, y los datos se analizaron mediante ANOVA unidireccional.

Resultados: La administración de colchicina durante 24 horas después de la isquemia dio lugar a una disminución significativa de los niveles relativos de NLRP3, TGF-β, y α-SMA ($p<0,05$) en comparación con el grupo de isquemia.

Conclusiones: La administración *in vitro* de colchicina puede reducir el remodelado postisquémico, incluidos el inflamasoma NLRP3, el TGF-β y el α-SMA.

Palabras Clave: colchicina; inflamasoma; isquemia; remodelado ventricular.

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INTRODUCTION

Coronary heart disease remains a primary global cause of mortality, morbidity, and hospitalization (Vázquez-Oliva et al., 2018). Globally, there are approximately 32.4 million annual myocardial infarction (MI) cases, making it a leading cause of death. In Indonesia, cardiovascular diseases, including MI, held the top position as the leading cause of death from 2011 to 2014 and 2016, surpassing other noncommunicable diseases (Muflihah et al., 2021).

Acute myocardial infarction initiates an inflammatory response crucial for cardiac repair. However, an excessive inflammatory response can result in adverse ventricular remodeling (Fang et al., 2015). This acute inflammation involves several processes, including complement cascade activation, reactive oxygen species (ROS) production, and formation of the nucleotide-binding oligomerization domain-like receptor family of cytosolic proteins 3 (NLRP3) inflammasome (Jo et al., 2016). NLRP3 is responsible for caspase-1 activation and the release of inflammatory cytokines like IL-1β and IL-18, pivotal mediators in post-AMI (Takahashi, 2019). Therefore, inhibiting NLRP3 can reduce infarction size in postischemic myocardium (Mezzaroma et al., 2021).

Ventricular remodeling induces changes in the extracellular matrix within the hard interstitium, decreasing myocardial compliance and leading to fibrosis (Humeres and Frangogiannis, 2019). Fibrosis can occur due to reduced myocardial tissue perfusion, as observed in myocardial ischemia and infarction.

Left ventricular wall fibrosis is a primary change in ventricular remodeling, characterized by irregular and excessive collagen turnover in interstitial and perivascular spaces. This collagen turnover dysregulation primarily affects phenotypically altered fibroblasts, termed myofibroblasts (Gyöngyösi et al., 2017). A crucial stage in fibrosis development is the transformation of fibroblasts into myofibroblasts, which can trigger fibrotic scar formation. These fibrotic scars eventually replace cardiomyocytes, reducing contractility and causing global systolic dysfunction (Travers et al., 2022). The myofibroblast phenotype is marked by the expression of α-smooth muscle actin (α-SMA). Transforming growth factor-β (TGF-β) plays a pivotal role in cardiac pathophysiology, including cardiac repair, hypertrophy, fibrotic remodeling, fibroblast activation, and extracellular matrix deposition (Yousefi et al., 2020).

Current management of acute myocardial infarction patients involves reperfusion via percutaneous coronary intervention, improving blood flow in stenosed or occluded coronary arteries to reduce infarct size. However, detrimental ventricular remodeling can still occur post-AMI (Chacón-Diaz et al., 2022; Xing et al., 2020) by approximately 20%. Mortality rates have declined by up to 5% with pharmacological, mechanical, or combined reperfusion therapies (García-García et al., 2020). Colchicine, an anti-inflammatory agent, functions to prevent heart disease remodeling by inhibiting NLRP3 activation, preventing pore formation via P2X7 receptors, limiting K^+ efflux (Martinon et al., 2006), and reducing TGF-β levels in vitro (Bakhta et al., 2018; Tardif et al., 2019). There has been no scientific research on the mechanism of colchicine in inhibiting cardiac fibrosis *in vitro*. Therefore, this study aims to analyze the effect of colchicine on reducing the expression of NLRP3 inflammasomes, TGF-β, and α-SMA, all of which play a significant role in cardiac fibrosis in ischemia model cell cultures.

MATERIAL AND METHODS

Ischemia and colchicine treatment

The 3T3 cell line was seeded into 6-well plates at a density of 80,000 cells per well and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin (100 U/mL) (Gibco, Grand Island, NY) (Zhao et al., 2015) for 24 hours in a 37°C incubator with 5% CO_2 (Liu et al., 2019). Ischemia treatment involved the addition of cobalt chloride/$CoCl_2$ at a concentration of 600 µM to the 3T3 cell line. Colchicine (Sigma–Aldrich) at a concentration of 300 µM was added to DMEM supplemented (Oates and Antoniewicz, 2023). The study groups included control (healthy cells), colchicine (healthy cells treated with colchicine), $CoCl_2$ (ischemia), colchicine (healthy cells treated with colchicine for 24 hours), and $CoCl_2$ + colchicine (ischemia treated with colchicine for 24 hours). Ethical approval for this research was obtained from the Faculty of Medicine, Brawijaya University, Indonesia (Certificate No. 182/EC/KEPK-S3/07/2023).

Examination of the relative amounts of NLRP3, TGF-β, and α-SMA

The expression levels of NLRP3, TGF-β, and α-SMA were assessed using the Flowcytometry method. The 3T3 cell line suspension was centrifuged at 2500 rpm at 10°C for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 1 mL of PBS, followed by another centrifugation. The cell pellet was then treated with 100 µL of BD cytofix/cytoperm fixative solution (BD554714) and in-

cubated for 20 minutes. Subsequently, 500 µL of BD Perm/Wash buffer (BD554714) was added, and the mixture was centrifuged at 2500 rpm at 10°C for 5 minutes. The supernatant was discarded, and the pellet was stained with intracellular antibodies and incubated for 20 minutes in an icebox. Antibodies used in this study included PE-conjugated NLRP3 (R&D system: IC7578P), AF647-conjugated TGF-β1 (Santa Cruz; 130348), and PE-conjugated α-SMA (Santa Cruz; 53142), followed by the addition of 400 µl of PBS. Samples were analyzed using BD Biosciences FACS CaliburTM flow cytometry with BD CellQuest software PROTM (Rahayu et al., 2022).

Data analysis

Descriptive data, represented as the mean ± SD for the relative levels of NLRP3, TGF-β, and α-SMA, were subjected to data analysis using one-way ANOVA in SPSS 21 for Windows. Differences between the treatment groups were further evaluated using the Tukey test with a confidence level of 5%.

RESULTS

Relative amount of NLPR-3

The relative amount of NLRP3 in the control group was 17.83 ± 5.23%. However, administration of 300 µM $CoCl_2$ for 24 hours significantly increased the relative amount of NLRP3 in 3T3 cells ($p<0.05$) compared to the control group, reaching 81.25 ± 17.35%. Interestingly, when colchicine was administered to 3T3 cells without prior ischemia, there was no notable change in the relative amount of NLRP3 compared to the controls, with levels remaining at 16.53 ± 2.59%. In contrast, the administration of colchicine after ischemia (following 24 hours of $CoCl_2$ treatment) resulted in a significant decrease in the relative amount of NLRP3 ($p<0.05$) compared to the ischemia group, registering at 16.62 ± 8.49% (Fig. 1).

Relative amount of TGF-β

Within the control group, the relative amount of TGF-β was 13.53 ± 6.07%. In contrast, administration of $CoCl_2$ significantly increased the relative amount of TGF-β in 3T3 cells ($p<0.05$) compared to the control group, reaching 79.64 ± 18.65%. Notably, the administration of colchicine to cells not exposed to ischemia did not significantly change the relative amount of TGF-β compared to the controls, with levels remaining at 11.44 ± 2.96%. However, when colchicine was administered after ischemia (following 24 hours of $CoCl_2$ treatment), it led to a significant decrease in the relative amount of TGF-β ($p<0.05$) compared to the ischemia group, registering at 13.39 ± 7.07% (Fig. 2).

Relative number of α-SMA

The initial α-SMA levels in the control group were 19.89 ± 6.35%. However, the administration of $CoCl_2$ significantly elevated the relative α-SMA levels in cultured 3T3 cells ($p<0.05$) compared to the control, reaching 76.06 ± 14.37%. Providing colchicine to cells not exposed to ischemia exhibited no substantial alteration in the relative α-SMA levels, maintaining at 18.90 ± 6.44%, relative to the control. Conversely, the administration of colchicine after hypoxia-induced a notable reduction in the relative α-SMA levels ($p<0.05$) compared to the ischemia group, measuring 18.71 ± 8.56% (Fig 3).

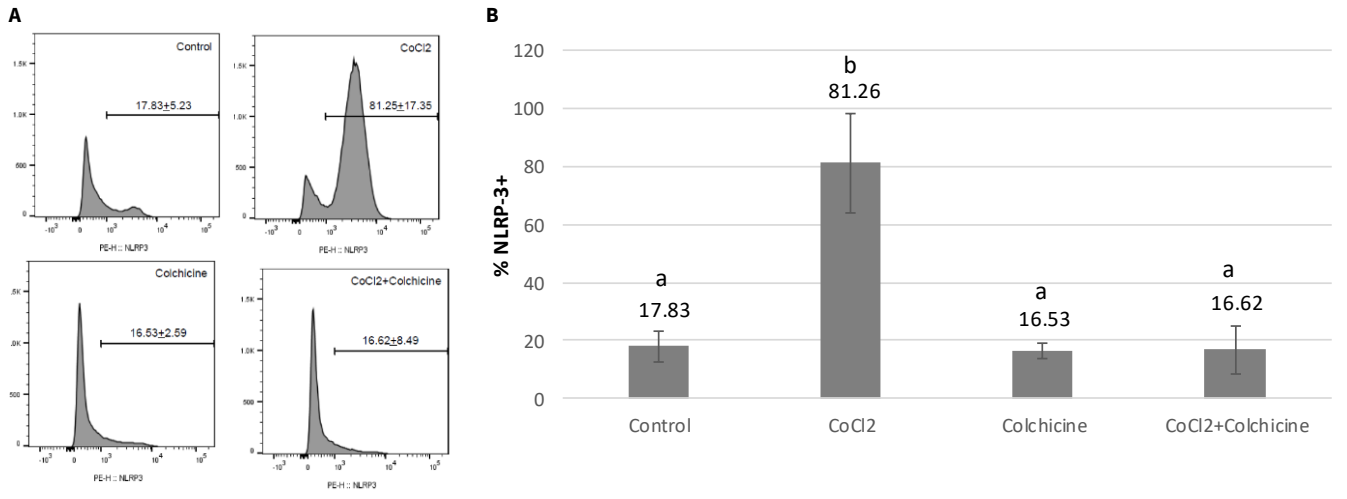
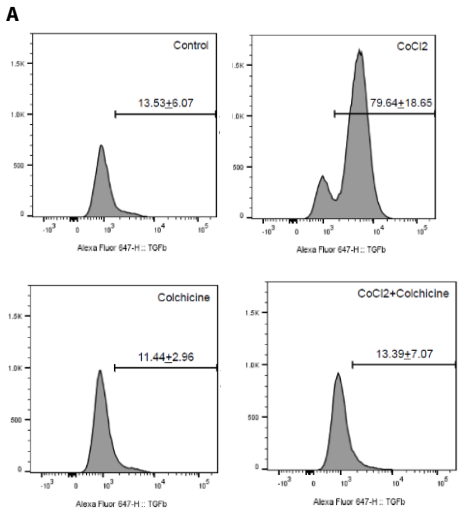


Figure 1. Colchicine reduces the relative amount of NLRP3.

(A) Cultured 3T3 cells treated with colchicine and exposed to NLRP3 antibody. **(B)** The bar graph illustrates the relative amount of α-SMA under ischemic conditions treated with colchicine, showing significant differences between treatment groups. Data represents the mean ± SD, with $p < 0.05$, as indicated by one-way ANOVA (n = 7).



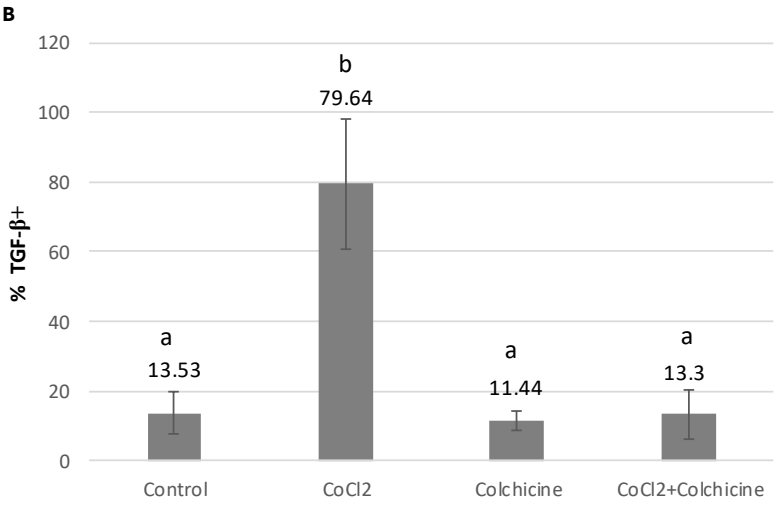
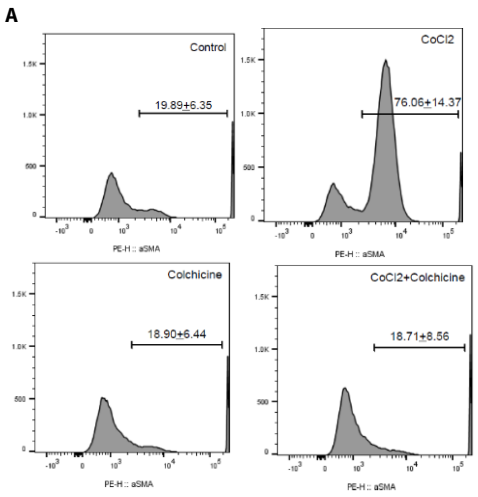


Figure 2. Colchicine lowers the relative amount of TGF-β.

(A) Cultured 3T3 cells treated with colchicine and treated with TGF-β antibody. **(B)** Bar showed that the relative amount of TGF-β under ischemic conditions treated with colchicine significantly differed between the treatment groups. Data are the mean ± SD, $p<0.05$, as indicated using one-way ANOVA (n = 7).



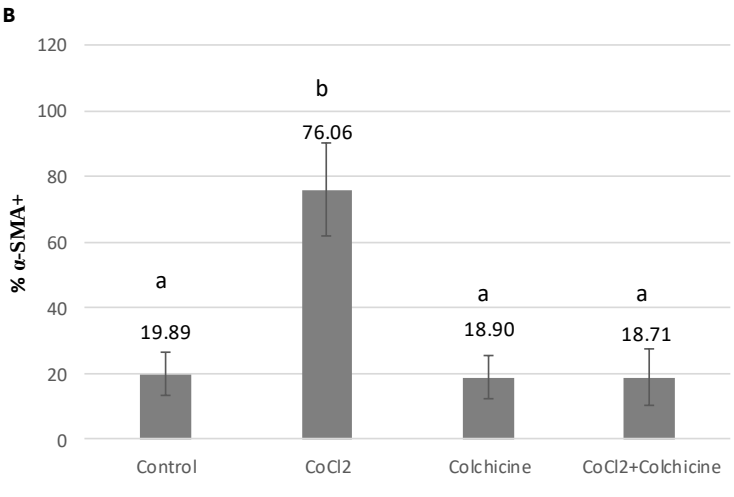


Figure 3. Colchicine lowers the relative amount of α-SMA.

(A) Cultured 3T3 cells treated with colchicine and given an α-SMA antibody. **(B)** Bar showed that the relative amount of α-SMA in ischemia conditions treated with colchicine significantly differed between treatment groups. Data are the mean ± SD, $p<0.05$, as indicated using one-way ANOVA (n = 7).

DISCUSSION

The novelty of this study is that it conducted models of hypoxia using 3T3 fibroblast cell cultures from mice (*Mus musculus*) exposed to $CoCl_2$. Exposure to $CoCl_2$ mimics hypoxia, a condition similar to the effects of oxygen deficiency in the heart, as seen in AMI. This research diverges from previous studies (Fujisue et al., 2017; Li et al., 2022), utilizing mice AMI model via permanent ligation of the left anterior descending coronary artery, additionally, unlike other studies that administered colchicine to human subjects post-myocardial infarction (Tardif et al., 2019) and ST-segment–elevation myocardial infarction (Mewton et al., 2021).

In the study examining the impact of colchicine on ischemic conditions using the 3T3 cell line, 3T3-L1 cells were derived from Swiss 3T3 mouse embryos at the age of 17–19 days and displayed a morphology resembling fibroblast- and adipocyte-like characteristics (Guru et al., 2021; Oates and Antoniewicz, 2023). An apoptotic model using $CoCl_2$ was created to mimic cardiac ischemia-reperfusion injury (IRI) (Ban et al., 2020; Kutryb-Zajac et al., 2022). $CoCl_2$ induces an excessive generation of ROS and depolarizes mitochondrial membranes by activating hypoxia-inducible factor-1α (HIF-1α), thereby triggering various nuclear transcription factors, signaling proteins, cell cycle arrest, and apoptosis, ultimately resulting in tissue damage (Tripathi et al., 2019).

Ischemic conditions can instigate injury that triggers NLRP3 inflammasome activation. In this study, it was demonstrated that administering colchicine under ischemic conditions led to a reduction in NLRP3 levels compared to those not receiving colchicine. This finding aligns with the research by Fujisue et al. (2017), which revealed that short-term colchicine treatment in a mouse model of myocardial infarction can diminish proinflammatory cytokines and NLRP3, thereby enhancing heart function, reducing heart failure, and increasing survival rates. Colchicine, derived from the *Colchicum autumnale* plant, exerts its mechanism of action by intercalating with free α/β tubulin and inhibiting microtubule extension. This action prevents inflammasome activation and hinders neutrophil movement through blood vessels.

Furthermore, colchicine can reduce the expression of L- and E-selectin adhesions, mediating neutrophil rolling and adhesion to the endothelium. It showed that colchicine treatment in a mouse model of myocardial infarction in the short term can reduce proinflammatory cytokines and NLRP3 and improve heart function, heart failure, and survival. Colchicine prevents cardiac dysfunction within 1 week after MI as a result of inhibiting the exaggerated acute inflammatory response, thereby inhibiting the development of heart failure for 4 weeks after MI. Colchicine originates from the *Colchicum autumnale* plant and has a mechanism of action: intercalating with free α/β tubulin and inhibiting microtubule extension, thereby preventing inflammasome activation and blocking the movement of neutrophils through blood vessels. Colchicine can also reduce the expression of L- and E-selectin adhesions, mediating neutrophil rolling and adhesion to the endothelium (Bonaventura et al., 2022).

Consequently, this leads to decreased neutrophil degranulation and phagocytosis activity (Otani et al., 2016). In acute coronary syndrome patients, colchicine therapy acts on caspase-1 to suppress monocyte activation due to NLRP3 inflammation (Robertson et al., 2016). Inhibition of the NLRP3 inflammasome reduces infarct size in postischemic myocardial tissue (Tong et al., 2020) due to a decrease in the inflammatory cytokine IL-1β (Bakhta et al., 2018). Notably, NLRP3 was also present in the control group, albeit in relatively lower amounts compared to the ischemia group. Under normal circumstances, inflammasomes are involved in sterile inflammation and play a role in defending against pathogens; however, their overactivation can contribute to the development of diseases (Bonaventura et al., 2022).

This study demonstrates that the relative amount of TGF-β in the ischemia group surpasses that in the normal group. Ischemic conditions induce a pH decrease, leading to the formation of lactic acid, which, in turn, can trigger TGF-β activation in myocardial infarction (MI). Furthermore, this study illustrates that colchicine can potentially to mitigate TGF-β levels. TGF-β plays a pivotal role in reducing cardiac remodeling (Bakhta et al., 2018) by augmenting fibroblast signaling, promoting their transformation into active myofibroblasts, and facilitating the synthesis and storage of fibrin in the extracellular matrix of connective tissue (Shinde et al., 2017). These findings align with Bakhta et al. (2018), who demonstrated that colchicine can effectively reduce TGF-β, thus inhibiting remodeling in the context of myocardial ischemia/reperfusion (I/R) injury. However, the precise mechanism of this inhibition remains unclear. During the early stages of fibroblast cell formation, neutrophils are gradually replaced by anti-inflammatory macrophages, lymphocytes, natural killer cells, dendritic cells, and myofibroblasts, often accompanied by increased IL-10 and TGF-β levels in the infarct area during the repair phase (Scopelliti et al., 2022). Under normal conditions, TGF-β promotes the synthesis and secretion of extracellular matrix proteins, such as collagen I, collagen III, and fibronectin. It also triggers a matrix-maintaining phenotype by suppressing collagenase expression (Dragsbæk et al., 2015; Frangogiannis, 2022; Hanna and Frangogiannis, 2019).

Research indicates that colchicine administered post-ischemia can reduce α-SMA levels. In the tissue healing, fibroblasts require a contractile phenotype characterized by the formation of microfilament bundles and the expression of α-SMA (Shinde et al., 2017). α-SMA triggers a proinflammatory fibroblast phenotype and contributes to matrix degradation, facilitating leukocyte recruitment. The clearance of dead cells and matrix debris from infarcts stimulates anti-inflammatory pathways and activates the TGF-β cascade. This results in the conversion of fibroblasts into α-SMA-expressing myofibroblasts. Activated myofibroblasts secrete substantial amounts of matrix protein, forming a collagen-based scar to safeguard the infarcted ventricle from catastrophic complications, such as cardiac rupture. Additionally, infarcted fibroblasts may contribute to cardiac repair by promoting angiogenesis. Fibroblasts break down α-SMA$^+$ fibers as scar tissue matures, converting them into specialized cells that play a role in scar tissue maintenance. Prolonged activation of fibroblasts and myofibroblasts in the infarct zone may contribute to adverse remodeling and the pathogenesis of heart failure (Venugopal et al., 2022).

Colchicine holds promise as a potential shield against myocardial injury during ischemia by mitigating ventricular remodeling, including the reduction of NLRP3, TGF-β, and α-SMA. Nevertheless, further

research is imperative to assess the short-term side effects of colchicine treatment.

CONCLUSION

Administering colchicine can diminish post-ischemic remodeling, including the NLRP3 inflammasome, TGF-β, and α-SMA, which differs from not adding colchicine in ischemic conditions.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Contribution	Astiawati T	Rohman MS	Wihastuti TA	Sujuti H	Endharti AT	Sargowo D	Oceandy D
Concepts or ideas	x	x	x				
Design	x	x	x				
Definition of intellectual content	x	x	x	x	x	x	x
Literature search	x						
Experimental studies	x	x	x				
Data acquisition	x	x					
Data analysis	x	x	x				
Statistical analysis	x	x	x	x	x	x	x
Manuscript preparation	x	x	x	x	x	x	x
Manuscript editing	x	x	x	x	x	x	x
Manuscript review	x	x	x	x	x	x	x

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