Colchicine-Containing Nanoparticles Attenuates Acute Myocardial Infarction Injury by Inhibiting Inflammation

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Abstract

Purpose Anti-inflammatory therapy is important for reducing myocardial injury after acute myocardial infarction (MI). New anti-inflammatory drugs and their mechanism are necessary to be explored to improve clinical efficacy. We aimed to improve the efficacy of colchicine on attenuating MI injury by nano-drug delivery systems and to investigate the mechanism of anti-inflammatory.

Methods A colchicine-containing delivery system based on calcium carbonate nanoparticles (ColCaNPs) was synthesized. The protection against MI by ColCaNPs was evaluated using an in vivo rat model established by ligating the left anterior descending coronary artery. Macrophage polarization and the levels of inflammatory cytokines were determined using immunohistochemistry, Western blot, and ELISA analysis.

Results ColCaNP treatment showed about a 45% reduction in myocardial infarct size and attenuating myocardial fibrosis compared with groups without drug intervention after MI. Furthermore, ColCaNPs significantly decreased the levels of CRP, TNF-α, and IL-1β in serum and the expression of proinflammatory cytokine in myocardial tissues after MI (p < 0.05). We also found that ColCaNPs notably restrained pyroptosis and inhibited inflammatory response by modulating on M1/M2 macrophage polarization and suppressing TLR4/NFκB/NLRP3 signal pathway.

Conclusion Colchicine-containing nanoparticles can protect against MI injury in a clinically relevant rat model by reducing inflammation. In addition, calcium carbonate nanoparticles can increase the cardioprotective effects of colchicine.

Keywords Acute myocardial infarction · Pyroptosis · Macrophage polarization · Inflammatory response · Colchicine · Nanoparticles

Introduction

Acute myocardial infarction (MI) is a leading cause of morbidity and mortality among patients. In addition to myocardial damage caused by ischemia and hypoxia, post-MI injury is aggravated by excessive local inflammation and pyroptosis, which accelerates extracellular matrix synthesis, leading to myocardial fibrosis, adverse cardiac remodeling, and heart failure. The toll-like receptor 4 (TLR4)/nuclear factor kappa B (NF-κB) signal pathway, a pivotal role in MI, is observed to be markedly promoted in ischemic myocardium [1]. The activation of TLR4 is linked to the promotion of NF-κB which governs the expressions of proinflammatory cytokines and promotes the assembly of NLRP3 inflamasome. Additionally, NLRP3 inflamasome can activate caspase-1, leading to pyroptosis and amplifying the inflammatory response [2]. In view of the important role of
inflammation in myocardial injury, more and more people pay attention to the potential benefits of anti-inflammatory therapy in regulating local inflammation after myocardial infarction, thus accelerating myocardial repair, inhibiting left ventricular remodeling, and improving prognosis. Anti-inflammatory therapy can reduce myocardial injury after acute myocardial infarction and improve atherosclerosis (AS). A previous study showed that the canakinumab targeted the IL-1β/IL-6 pathway to reduce the incidence of cardiovascular events [3]. This study is a milestone and provides clinical evidence for the “inflammation hypothesis” of AS. However, the Food and Drug Administration denied regulatory approval for canakinumab for patients with coronary artery disease [4]. Therefore, the application of canakinumab in the treatment of AS may be limited. Methotrexate has a beneficial effect on AS, as reported by early small-scale clinical studies. But, the CIRT study showed that low-dose methotrexate did not reduce the levels of IL-1β, IL-6, or C-reactive protein, and the incidence of cardiovascular events was similar to placebo [5]. In recent years, the protective effect of colchicine on cardiovascular diseases (coronary heart disease, pericarditis, etc.) has been paid more and more attention. Tardif found that low-dose colchicine led to a significantly lower risk of ischemic cardiovascular events for patients with a recent myocardial infarction [6]. Early initiation of low-dose colchicine after MI greatly reduced the risk of ischaemic CV events [7]. In a randomized trial involving patients with chronic coronary disease, the risk of cardiovascular events was significantly lower among those who received colchicine than those who received placebo. Furthermore, Hou and her colleagues found that colchicine played a crucial role in alleviating the intracellular inflammatory response and NLRP3 inflammasome activation, attenuating the levels of cellular oxidative stress and pyroptosis in endothelial cells [8]. Therefore, colchicine is a promising anti-inflammatory drug for coronary artery disease. However, the therapeutic window of colchicine is narrow, and the high dose of colchicine may produce side effects, which limit its clinical application. Moreover, the protective effects of colchicine on regulating the inflammation response and pyroptosis during MI and the effect of colchicine on the TLR4/NFκB/NLRP3 signaling pathway remain unclear.

In this study, colchicine-containing nanoparticles were designed to improve the efficacy of colchicine on reducing myocardial injury after myocardial infarction and to reduce the adverse reactions of the drug. The mechanism would also be discussed. Compared with free drugs, nano-drug delivery systems have a long blood circulation time which will enable drugs accumulating in lesion more efficiently to improve the drug efficacy [9–11]. The long-term safety of this technology is very important and is still underinvestigated. The liposomal formulation of doxorubicin (known by the commercial name of Doxil®) is the first anticancer nanomedicine approved by the FDA in 1995 for the treatment of some types of cancers, including ovarian cancer. A study published in 2007 showed that pegylated liposomal doxorubicin appeared to be safe as long-term maintenance in ovarian cancer and may be important for a continued response [12]. However, possible emerging long-term side effects such as secondary neoplasia should be considered carefully [13, 14]. Albeit the mechanism of the long-term side effects is unclear, nanomaterials as drug delivery carriers should have good biocompatibility and biodegradability both for themselves and for their degradation products. Calcium carbonate is a common inorganic material in nature. It has good biocompatibility and biodegradability. Amorphous calcium carbonate (ACC) nanoparticles were used here as a pH responsiveness drug carrier to targeted delivery of colchicine to lesions, which is beneficial to reducing the side effect of drugs. The purpose of this study is to explore the myocardial protective effect and potential mechanism of colchicine and its formulations on rats with acute myocardial infarction and to determine whether amorphous calcium carbonate nanoparticles can increase the therapeutic effect of colchicine.

Materials and Methods

Materials

Ammonium bicarbonate (NH₄HCO₃) and dopamine hydrochloride were purchased from Macklin Biochemical Co., Ltd. (Shanghai, China). Amino polyethylene glycol (mPEG-NH₂, Mw = 5000) was supplied by Hunan Huateng Pharmaceutical Co., Ltd. (Hunan, China). Anhydrous calcium chloride (CaCl₂), colchicine (Col), and sodium hydroxide (NaOH) were obtained from Aladdin (Shanghai, China). Evans blue (1%) and 2,3,5-triphenyltetrazolium chloride (TTC, 2%) were purchased from Solarbio, China. Anti-Rat IgG was purchased from Invitrogen (Carlsbad, CA). TUNEL Bright-Red Apoptosis Detection Kit was obtained from Yeasen Biotech Co., Ltd. (Shanghai, China).

Synthesis of Colchicine-Loaded Calcium Carbonate Nanoparticles (ColCaNPs)

The synthesis of ColCaNPs included 3 steps. Firstly, colchicine (8 mg) and CaCl₂ (100 mg) were dissolved in anhydrous ethanol (120 mL). The mixture was transferred into a beaker and covered by parafilm with several pores. The beaker was left in a desiccator along with NH₄HCO₃ powder for 2 days at room temperature. Secondly, dopamine hydrochloride (600 mg) was added into the beaker with stirring under alkaline condition (pH ~ 8.5) for 6 h and then centrifuged. At last, mPEG-NH₂ (20 mg) was added and stirred for 2 h.
The mixture was purified by dialysis using an 8000 D cutoff dialysis membrane against deionized water and freeze-dried to obtain ColCaNPs.

Nanoparticles without colchicine were designated as CaNPs, and nanoparticles without calcium carbonate were designated as ColNPs.

**Characterization of Nanoparticles**

The Fourier transform infrared spectroscopy (FTIR) spectra were detected by a Fourier transform infrared spectrometer (Nicolet iS50, USA). The powder X-ray diffraction (XRD) pattern was collected with an X-ray diffractometer (Rigaku SmartLab, Rigaku, Japan). The hydrodynamic size was measured by dynamic light scattering (DLS) with a nanolaser particle size analyzer (BT-90, Bettersize, China). The morphology was observed using a transmission electron microscope (TEM, HITACHI HT7700 Exalens, Japan).

**Loading Capacity of Colchicine**

The colchicine loading capacity was determined by UV–vis absorbance spectroscopy and calculated by the following equation:

\[
\text{Col loading capacity (\%)} = \frac{\text{total mass of col contained in nanoparticles}}{\text{total mass of nanoparticles}} \times 100\%
\]

**Animal Models and Experiment Design**

Male Wistar rats were purchased from the SiPeiFu Animal Center (SPF grade) and used for experiments when they were 12–14 weeks of age, weighing 280–320 g. The rats were fed adaptively for 1 week before experiments. Rats were divided into six groups according to different drug interventions: without drug intervention (MI), CaNPs, Col, ColCaNPs, ColNPs, and sham operation (Sham). Each group consisted of 5 animals. The MI group was induced by ligating the left anterior descending (LAD) coronary artery of rats, but did not receive any treatment. The sham group underwent thoracotomy without coronary artery ligation. For drug interventions, colchicine and its formulations were injected at a colchicine equivalent dose of 2 mg kg\(^{-1}\) into the caudal vein at 30 min, 48 h, or 96 h, respectively, after ligation of LAD. The blood was collected from the venous plexus of the anterior inner canthus before and 7 days after MI. One week later, the rats were sacrificed, and the heart, kidney, and liver were taken out for relevant tests. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

**Induction of the MI Rat Model**

All the rats were anesthetized with isoflurane and after orotracheal intubation with a 22G venous catheter for controlled ventilation (Minivent, Harvard Apparatus) with controlled stroke (10 μL g\(^{-1}\)) and frequency (150 min\(^{-1}\)). After left thoracotomy and muscular dissection, ligation of the LAD was performed 2 mm below the left auricle with an 8–0 silk and a smooth catheter was applied to the artery to obtain ischemia for 45 min. The ischemia was visually confirmed by the change in myocardial color turning into white and was assayed using a II lead electrocardiogram, then followed by reperfusion obtained by the catheter removal. Muscle and cutaneous plans were sutured with silk 6–0. Sham-operated animals were subjected to the same surgical procedure without LAD ligation.

**Blood Analysis**

The blood sample was centrifuged for 10 min at 5000 rpm, and then, the serum was stored at – 80 °C for the subsequent analyses. The LDH were measured based on the protocol. IL-1β, TNF-α, CRP, alanine aminotransferase (ALT), and creatinine (Cr) were detected using ELISA kits (MDL Company) on the basis of the manufacturer’s instructions.

**Measurement of Myocardial Infarct Size**

Myocardial infarct size was determined by the TTC method. All slices were incubated for 15 min in TTC (2%) at 37 °C in the dark for pathological examination. Images were captured with a microscope (ZEISS, Germany). TTC-stained area (red, noninfarct area) and non-TTC-stained area (white or pale, infarct area) were analyzed with an Image-Pro Plus image analysis software (Version 4.1, Media Cybernetics, LP, USA). The infarct size was calculated as the proportions of infarct myocardial to the whole myocardial tissues.

**Histological Analysis and Immunohistochemistry**

Briefly, fixed myocardial tissues were firstly dehydrated and embedded in paraffin. The kidney and liver transversely sectioned were performed with H&E staining for morphologic examination. Masson’s trichrome staining was also
performed to detect myocardial fibrosis. Sections were observed using an OLYMPUS microscope (Japan).

We used the number of CD11c cells to document the M1 polarization and used the number of CD206 cells to document the M2 polarization. Paraffin-embedded heart tissue sections were stained with CD206 (1:100; Proteintech Group) or CD11c (1:200; Abcam) to identify the CD206/CD11c antigen expressed by macrophages. Anti-rat IgG was used as a secondary antibody to detect CD206/CD11c + cells.

TUNEL Staining

TUNEL staining was performed based on the standard protocol. Then, cells were stained with a TUNEL kit on ice according to the manufacturer’s instructions. TUNEL-positive cells were counted under fluorescence microscopy (DM3000, Leica, Mannheim, Germany).

Western Blots

Rat tissues and cardiomyocytes were harvested and then split using RIPA protein extraction reagent (MDL, China). Proteins were transferred onto PVDF membranes, then incubated with the specific primary antibodies and the secondary antibodies. Protein lysates from cells were separated by 10% SDS-PAGE and then transferred onto NC membranes (Sigma). After blocking for 1 h, the membranes were incubated overnight at 4 °C with specific antibodies. Then, the secondary antibody (MD912577, MDL) was added and the membranes were maintained at room temperature for 1 h. The signals were determined by Immobilon Western chemiluminescent HRP Substrate (Millipore). β-actin was acted as a loading control. The primary antibodies were used as follows: anti-TLR4 (MDL, cat. no. MD2073-020), anti-p-NFκB (Abcam, Ab219800), anti-NLRP3 antibody (MDL, cat. no. MD6251-020), anti-c-caspase-1 antibody (Affinity, Af4005), anti-GSDMD (Abcam, Ab219800), anti-cleaved GSDMD (Elabscience, e-ab-67333), anti-cleaved IL-1β antibody (Abcam, Ab254360), anti-TGF-β1 (MDL, cat. No. MD7237), anti-IL10 (MDL, cat. No. MD292-020).

Statistical Analysis

Results were expressed as the mean ± SD. Graph Pad Prism 6.0 and SPSS 20.0 software were used for statistical analysis. The significance of differences between the two groups was assessed by Student’s t-test or Mann–Whitney U test as appropriate, and ANOVA test or Kruskal–Wallis test was used for comparisons among three or more groups. A P value <0.05 was considered statistically significant.

Results

Synthesis and Characterization of Nanoparticles

The amorphous calcium carbonate was synthesized by a vapor-diffusion process. Colchicine was loaded into ACC by co-precipitation. Poly-dopamine coated on ACC to provide functional sites for mPEG-NH₂ modification. FTIR spectroscopy was used to verify the successful synthesis of ColCaNPs (Fig. 1a). The absorption peaks at 877, 1088, 1494, and 1432 cm⁻¹ were ascribed to the vibration of the carbonate group in ACC. The absorption peak at 1601 cm⁻¹ was attributed to the aromatic ring structure in PDA. The absorption peaks at 843, 963, and 1280 cm⁻¹ belong to PEG vibration peaks. These results verified that the colchicine-containing nanoparticle, ColCaNPs was successfully synthesized. The XRD pattern showed no obvious and sharp peaks in 2θ from 30 to 100 (Fig. 1b), indicating the calcium carbonate was amorphous. The result of particle size distribution showed that the hydrodynamic size of ColCaNPs was 243 nm with a PDI of 0.11. The low PDI illustrated that the nanoparticles had a uniform size. The TEM image revealed that ColCaNPs had a spherical characteristic with a size of about 150 nm (Fig. 1c). The size difference between the results of TEM and DLS would be due to the different sample preparative methods. The colchicine loading capacity was 4.3%.

ColCaNPs Reduced Myocardial Infarct Size and Myocardial Fibrosis in MI Rats

In order to determine the therapeutic effect of Col and ColCaNPs on myocardial infarction, myocardial infarction size and myocardial fibrosis were measured by the TTC method and Masson staining, respectively. As showed in Fig. 2a–b, little infarct damage was found in the sham groups, while the percentage of infarct size in MI groups was more than 10 times higher than that in sham groups (P < 0.01), indicating that the MI model was successfully established. There was no statistically significant difference in myocardial infarct size between CaNPs treatment and MI groups. The MI rats that received treatment showed a significant decrease in infarct size. The decreased percentage was 24%, 45%, and 38% for Col, ColCaNPs, and ColNPs, respectively. More importantly, ColCaNPs can further reduce myocardial infarction size than Col.

Masson staining showed increased accumulation of collagen fibers in the MI and CaNPs group which revealed myocardial fibrosis 7 days post I/R injury, while little collagen fiber was detected in Sham groups. The degree of myocardial fibrosis was lower in Col and Col-containing
nanoparticles (ColCaNPs and ColNPs) than that in the MI and CaNPs group. Compared with MI, CaNPs did not significantly reduce the infarct size and myocardial fibrosis (Fig. 2b–c). Colchicine and the Col-containing nanoparticles can significantly reduce myocardial fibrosis.

**ColCaNPs Inhibit the Inflammatory Response in the Peripheral Blood**

Inflammatory cytokines have been shown to be the critical indicators during myocardial infarction. Colchicine is a broad-spectrum anti-inflammatory drug. In order to verify the effect of colchicine and colchicine-containing nanoparticles on the inflammatory response in MI, the serum contents of IL-1β, TNF-α, and CRP were determined. At the baseline, there were no significant differences among different groups in IL-1β, TNF-α, and CRP. As shown in Fig. 3, the levels of IL-1β, TNF-α, and CRP in the MI group were significantly higher than baseline, indicating that MI modeling was successfully established. CaNPs had no influence on IL-1β, TNF-α, and CRP. However, colchicine markedly reduced the levels of IL-1β and CRP, while had no effect on TNF-α. Compared with colchicine, ColCaNPs evidently reduced the levels of TNF-α and CRP, but not IL-1β. There was no significant difference in IL-1β, TNF-α, and CRP between MI and CaNPs. Therefore, CaNPs in this research were only acting as a drug carrier which could not reduce the inflammatory reaction. In addition, compared with ColNPs, the levels of IL-1β and CRP in the ColCaNP treatment group decreased more remarkably.

**ColCaNPs Stimulated Macrophage Polarization to M2 Phenotype**

Macrophage polarization plays an important role in the regulation of inflammatory response. M1 macrophages secrete proinflammatory factors such as IL-1β, which can promote inflammatory response and tissue damage. M2 macrophages secrete IL-10, TGF-β, and other anti-inflammatory factors to inhibit the inflammatory reaction. In this study, the effect
of colchicine and its formulations on macrophage polarization was investigated by immunohistochemistry and Western blot where CD11c + cells were regarded as M1 macrophages and CD206 + cells as M2 macrophages. As shown in Fig. 4a, CD11c + cells and CD206 + cells were significantly decreased in the Sham group. Compared with the MI group, CD11c + cells decreased significantly and CD206 + cells were significantly increased when rats are treated with Col and its formulations. In particular, the decreasing of CD11c + cells and the increasing of CD206 + cells in the ColCaNP group were the most obvious compared with ColNPs or Col groups.

As shown in Fig. 4b–f, compared with the sham group, the expressions of IL-10 and TGF-β significantly decreased and the expression of cleaved IL-1β markedly increased in the MI group. These results indicated that the MI model was successfully established. Compared with the MI group, IL-10 and TGF-β did not change remarkably in rats treated with Col or CaNPs. However, the expression of cleaved IL-1β decreased and the expression of TGF-β increased.

Fig. 2 Colchicine and the Col-containing nanoparticles reduced myocardial injury and myocardial fibrosis. a TTC staining of the myocardium. The infarct size of each rat was assessed by the TTC assay, n = 5. Red stain-viable area; white stain-infarct region; b proportions of infarct myocardial to the whole myocardial tissues (infarct area/whole heart area) × percentage. Values are mean ± SD (n = 5 in each group). Col group compared with the MI group *p < 0.05, compared with the CaNP group ′p < 0.05, compared with the Col group ″p < 0.05, compared with the sham group. 1p < 0.05; ColNPs compared with MI group p < 0.05 (superscript letter “a”), compared with the CaNPs group p < 0.05 (superscript letter “b”); ColCaNP group compared with the MI group *p < 0.05, compared with the CaNP group ′p < 0.05, compared with the Col group ″p < 0.05, compared with the sham group. 1p < 0.05; ColCaNP compared with the MI group *p < 0.05 (superscript letter “a”), compared with the CaNP group ′p < 0.05 (superscript letter “b”), compared with the Sham group p < 0.05 (superscript letter “c”); c representative images of Masson staining 7 days after I/R, n = 5; scale bar = 100 μm.
when rats are treated with colchicine-containing nanoparticles. Moreover, compared with Col treatment, the expression of TGF-β further increased in rats treated with ColCaNPs, but not with CaNPs. And, cleaved IL1β in Col and Col-containing nanoparticles group was significantly decreased. These meant that colchicine plays an anti-inflammatory role by regulating macrophage polarization, and colchicine-containing nanoparticles increased the polarization of M2 macrophages.

**ColCaNPs Inhibit Pyroptosis Processes**

Acute myocardial infarction ischemia–reperfusion injury leads to myocardial cell death, excessive scar formation, and poor ventricular remodeling through inflammation caused by pyroptosis. Pyroptosis is a kind of programmed cell death mainly mediated by caspase-1, which is characterized by the increase of cell membrane permeability and the extracellular release of inflammatory cytokines. In order to evaluate the effect of colchicine and its formulations on pyroptosis, TUNEL staining, LDH, and the level of cleaved caspase-1 (C-Caspase-1), GSDMD, and cleaved GSDMD, which are the hallmark of pyroptosis, were determined. As shown in Fig. 5, the TUNEL-positive cell, LDH, C-Caspase-1, GSDMD, and cleaved GSDMD level were markedly elevated in MI, and these effects were significantly reversed by colchicine-containing nanoparticles (ColCaNPs and ColNPs). Colchicine decreased the expression of C-caspase-1, GSDMD, and cleaved GSDMD (Fig. 5d–f), but did not decrease TUNEL-positive cells and LDH (Fig. 5a–c).

Caspase-1 activation causes the transformation of IL-1β into their active forms, which in turn triggers further myocardial damage in rats subjected to MI. The release of cleaved IL-1β, which could initiate pyroptosis, was significantly inhibited by colchicine and colchicine-containing nanoparticles (Fig. 4c and e). Taken together, these results demonstrated that ColCaNPs inhibit pyroptosis processes, and CaNPs increase the anti-pyrolytic effect of colchicine.

**ColCaNPs Inhibit TLR4/NFκB/NLRP3 Signal Pathway**

TLR4/NFκB/NLRP3 is an important signaling pathway to regulate inflammatory response after myocardial infarction. The activation of NLRP3 inflammasome is a key link of the inflammatory response, which can induce caspase-1-mediated pyroptosis. To investigate the possible mechanism of the inhibiting effect of ColCaNPs on MI-induced inflammatory response, we used the Western blot analysis to examine the levels of TLR4, p-NF-κB, and NLRP3 protein. As shown in Fig. 6, comparing with the sham group, MI conduced to the upregulation of TLR4, p-NF-κB, and NLRP3. Comparing with MI, the administration of ColCaNPs markedly downregulated the expressions of TLR4, p-NF-κB, and NLRP3. However, colchicine and ColNPs only inhibited the expression of TLR4 and p-NF-κB. And, there were no significant differences in the protein expression of TLR4, NLRP3, and p-NF-κB between MI and CaNPs groups. Therefore, there was synergism in inhibiting TLR4/NFκB/NLRP3 signaling pathway when calcium carbonate and colchicine existed together.
Colchicine and its formulations stimulated M2 macrophage polarization. 

**Fig. 4** Colchicine and its formulations stimulated M2 macrophage polarization. 

- **a** CD11c and CD206 immunohistochemical staining. Scale bar = 40 μm. 
- **b-c** Western blot of TGF-β1, IL-10, and cleaved IL-1β; d TGF-β expression; e cleaved IL-1β expression. f IL-10 expression. Values are mean ± SD (n = 5 in each group). MI group compared with the ColCaNP group, *p < 0.05; compared with the ColNPs group, *p < 0.05 (superscript letter “c”); compared with the Sham group, *p < 0.05 (superscript letter “f”); Col group compared with the ColCaNP group, *p < 0.05
Fig. 5 ColCaNPs inhibit pyroptosis processes. a TUNEL staining. Scale bar=60 μm; b the TUNEL-positive cells; c serum LDH; d Western blot of c-caspase-1, GSDMD and C-GSDMD; e caspase-1 protein expression; f relative protein expression of GSDMD and C-GSDMD. Values are mean±SD (n=5 in each group). MI group: compared with the Colchicine group, p<0.05 (superscript letter “a”); compared with the ColCaNP group, p<0.05; compared with the ColNP group, p<0.05 (superscript letter “c”). ColCaNP group: compared with the CaNP group, p<0.05; compared with the Col group, p<0.05
Safety Evaluation

In order to assess the biosafety of colchicine and its formulations, body weight, H&E staining, liver function (serum ALT level), and renal function (serum Cr level) were measured after drug interventions. As shown in Fig. 7a, there were no significant differences in the body weight of each group (all $P > 0.05$). In the ColCaNPs, ColNPs, and CaNPs, compared with the sham operation group and MI group, the pathological results were normal (Fig. 7b–c), the ALT and Cr were not significantly different, but the ALT level in the MI group is significantly higher than in the sham group, which might be caused by the MI-induced heart failure (Fig. 7d–e). It suggests that colchicine and nanomaterials have good safety and biocompatibility.

Discussion

Here, we reported the beneficial effects of an old drug, colchicine, and its formulations, on cardiac damage in the rat model of myocardial infarction injury, and the underlying mechanisms. Our results showed that colchicine and colchicine-containing nanoparticles significantly reduced the infarct size and myocardial fibrosis. Calcium carbonate nanoparticle could help colchicine regulate the polarization of macrophages, promoted the secretion of anti-inflammatory cytokines, and increased the protein expression of anti-inflammatory factors, like IL-10 and TGF-β1. In addition, calcium carbonate nanoparticle could increase the anti-pyrolytic effect of colchicine by inhibiting LDH secretion and decreasing the expression of TUNEL-positive cells, cleaved caspase-1, and cleaved GSDMD. Moreover, we preliminarily proved that the cardioprotective effect of colchicine-containing nanoparticles was due to the increased effect of colchicine on inhibiting the TLR4/NF-κB/NLRP3 signal pathway.

One hallmark of MI is the acceleration of inflammatory responses. Inflammation plays not only a role in the pathophysiology of myocardial infarction in the acute stage, but also an important role in the determination of the extent of tissue injury and repair after myocardial infarction [15]. The release of inflammatory cytokines is the key step in the development of cardiac inflammation. IL-1β is a prominent and early mediator of inflammation in the infarcted heart. Short-term anti-inflammatory therapy with colchicine was also associated with smaller infarct size and reduced inflammatory response in a pilot study of patients with STEMI [16]. Among patients with a recent myocardial infarction, colchicine at a dose of 0.5 mg daily led to a significantly lower risk of ischemic cardiovascular events [6]. Early initiation of low-dose colchicine after MI greatly reduced the risk of ischemic CV events [7]. In a randomized trial involving patients with chronic coronary disease, the risk of cardiovascular events was significantly lower among those who received 0.5 mg of colchicine once daily than among those who received a placebo [17]. Robertson found that colchicine therapy in acute coronary syndrome patients acted on
caspase-1 to suppress NLRP3 inflammasome monocyte activation [18]. However, a large amount of evidence has confirmed the efficacy of colchicine in cardiovascular diseases, but there are few studies on the specific mechanism. Moreover, in clinical practice, colchicine has side effects, which limit its clinical use. In order to overcome the side effects...
of colchicine, Chen et al. developed an intramyocardial delivery system of colchicine using an injectable, thermosensitive poly(lactide-co-glycolide)-poly(ethylene glycol)-poly(lactide-co-glycolide) polymer hydrogel as a vehicle for the treatment of MI while minimizing its systemic toxicity [19]. The data showed that colchicine, especially colchicine-containing nanoparticles, markedly attenuated the cardiac inflammation by inhibiting the generations of inflammatory cytokines after MI, and the colchicine-containing nanoparticles had great potential as an anti-inflammatory therapy for the treatment of MI. However, in clinical work, intracardiac injection of drugs is risky and difficult to operate.

Nanoparticle drug delivery systems primarily optimize the biodistribution and cellular uptake of the encapsulated drugs, achieving higher therapeutic efficacy and lower toxicity than traditional treatment [20, 21]. Nanoparticles can be tailored to site-specific and target-oriented delivery of drug molecules so as to modify the drug distribution in the body [22]. In addition, the uptake of nanoparticles by cells is principally endocytosis, whereas small and lipophilic drugs across the cell membrane by simple diffusion. These lead to a reduction in drug dose and adverse side effects and an increase in drug efficacy. Nanomedicine on cardiovascular disease treatment, including anti-inflammation, has made great progress [23–27]. For example, an injectable reconstituted HDL nanoparticle was reported to deliver statins to atherosclerotic plaques tissue, resulting in the enhanced systemic bioavailability and local anti-inflammatory effects [28]. Rosiglitazone-loaded nanoparticles showed an anti-inflammatory effect in a dose-dependent manner, up to 5 times more efficient than the free rosiglitazone, and an increased rosiglitazone uptake which is consistent with the effect shown [29]. Nanoparticles preferentially deliver drugs in inflammatory sites because of the enhanced vascular permeability in inflamed tissues [30]. Our previous study showed that taking advantage of the acidification of intracellular pH by calcium carbonate nanoparticle, the efficacy of the loaded DOX was remarkably improved, and its side effect was significantly reduced [31]. Therefore, in this study, colchicine was encapsulated into calcium carbonate nanoparticles, which, on the one hand, reduced adverse reactions and, on the other hand, increased the therapeutic effect.

The results of our study showed that colchicine could reduce myocardial infarct size and inflammatory factors as previous reports [32, 33]. Nevertheless, colchicine-containing nanoparticles could further reduce myocardial infarct size and the levels of inflammatory factors. The reduction of infarct size by ColCaNPs is about two times higher than that by free colchicine. Furthermore, ColCaNPs, compared with colchicine, could significantly reduce myocardial infarct size, inhibit the inflammatory response. Therefore, the nano-delivery system based on calcium carbonate can help colchicine to play a better anti-inflammatory role. This might be due to the fact that CaNPs were a pH-responsive carrier which can selectively release drugs at lower pH inflammation sites.

Macrophage polarization plays an important role in regulating the inflammatory response. A growing body of evidence shows that altering the inflammatory response by alternative macrophage polarization is protective against complications related to MI. Therefore, macrophage polarization was investigated to explore the possible mechanisms of inflammatory regulation by colchicine and its formulations. We found that the cardiac expression of M1 macrophage was decreased and the expression of M2 macrophage was increased in MI injury rats treated by colchicine and its formulations. Furthermore, IL-10 can reverse cardiac remodeling after myocardial infarction by stimulating M2 macrophage polarization and fibroblast activation [34] and TGF-β mediates M2 macrophage polarization for anti-inflammation and angiogenesis in infarcted areas [35]. In addition, calcium carbonate nanoparticle could greatly enhance the effect of colchicine on M2 macrophage polarization and inhibit M1 macrophage polarization. And, there was synergism in macrophage polarization when calcium carbonate and colchicine existed together.

Pyroptosis is a newly identified form of inflammatory cell death triggered by caspase-1. It plays a pivotal role in the pathogenesis of various cardiovascular diseases, especially MI [36]. Prevention or attenuation of cardiomyocyte pyroptosis is necessary to ensure the normal cardiac contraction function after MI. There is direct evidence that cardiomyocyte pyroptosis is a major determinant of the extent of myocardial I/R injury, and the noncanonical inflammamsome pathway may be the major signaling pathway involved in cardiomyocyte pyroptosis [37]. GSDMD-dependent myocardial pyroptosis is confirmed in vivo, and GSDMD deficiency significantly reduced I/R-induced myocardial infarct size. GSDMD acts as the executor of pyroptosis by producing a cleaved GSDMD, which causes pyroptotic cell death by forming membrane pores. Our study showed that colchicine-containing nanoparticles significantly suppressed the TUNEL-positive cells, LDH release, and colchicine and colchicine-containing nanoparticles reduced the cardiac production of several pyroptosis-related proteins, including GSDMD, cleaved GSDMD, and cleaved caspase-1. These results suggest that the protective effects of colchicine and colchicine-containing nanoparticles on the ischemic heart can likely be ascribed to the attenuation of pyroptosis. Pyroptosis is a programmed cell death and is characterized by an intensive inflammatory response. Inhibiting cell death by pyroptosis could limit inflammatory diseases, including atherosclerosis, ischemic heart disease, and myocardial infarction [38–41]. Therefore, inhibiting pyroptosis is beneficial to reduce inflammation.
Suppressing the NLRP3-mediated pyroptosis pathway protects myocardial ischemia–reperfusion injury [42–44]. In the acute phase of MI, TLR4 was stimulated [45], leading to the activation of NF-κB, which promotes the formation of the NLRP3 inflammasome by oligomerization of inactive NLRP3, ASC, and procaspase-1. Numerous studies have highlighted the central role of NLRP3 inflammasome in MI [46, 47]. Inhibition of NLRP3 protects against myocardial ischemia–reperfusion injury [2]. NLRP3 inflammasome activates caspase-1, which leads to the production and secretion of proinflammatory cytokines such as IL-1β and IL-18 on the one hand [48], and induces pyroptosis on the other hand [49]. Dai Y. found that TLR4/NF-κB/NLRP3 signaling pathway activation could induce cell pyroptosis [50]. Another study showed interventions targeted at TLR4/NF-κB/NLRP3 signaling pathway activation could induce cell pyroptosis [50]. Previous studies showed that during MI, TLR4 led to activation of NF-κB. And, the activation of NF-κB in M1 macrophages resulted in the transcription of proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α. Alternatively, the activation in M2 macrophages would result in the secretion of cytokines, like IL-10 and TGF-β1 [51], which would restore damaged cells and promote wound healing. The effects of colchicine on tubulin inhibit the assembly of the NLRP3 inflammasome; promote M2 macrophages to release IL-10, TGF-β1, and other anti-inflammatory cytokines that act to suppress proinflammatory signaling; and promote favorable healing by dampening the growth of vascular smooth muscle cells [52]. Liu found that inhibition of TLR4 and NF-κB could regulate macrophage polarization and improve cardiac function after myocardial infarction [53]. Wang Y. also showed colchicine, at low concentration, reduced the activation of caspase-1, and promoted macrophage M2 polarization and the release of IL-1β [54]. In this study, we found that with the downregulation of TLR4, NFκB, and NLRP3 signal protein expression, the secretion of inflammatory factors decreased and the secretion of anti-inflammatory factors increased after ColCaNP treatment. Thus, we inferred that colchicine-containing nanoparticles might regulate macrophage polarization by inhibiting TLR4/NFκB/NLRP3 signaling pathway, achieving anti-inflammatory and cardiac protective effects (Fig. 8). As a drug carrier, calcium carbonate has little effect on the inflammatory reaction, but there was synergism when calcium carbonate and colchicine existed together in reducing the inflammatory response and regulating macrophage polarization processes by inhibiting TLR4/NFκB/NLRP3 signaling pathway.

**Conclusion**

Colchicine-containing nanoparticles reduced infarct size and attenuated myocardial fibrosis after MI by inhibiting cardiomyocyte pyroptosis and regulating macrophage polarization. The cardioprotective effects of colchicine are due to the inhibition of the TLR4/NFκB/NLRP3 signal pathway in the acute phase of myocardial infarction.
More importantly, calcium carbonate nanoparticles have synergized effects with colchicine in increasing the anti-inflammatory and anti-myocardial pyroptosis. Based on this feature, the colchicine-containing calcium carbonate nano-delivery system has great potential for future clinical translation.

Author Contribution L.W. contributed to the animal experiments. Y.F.P. contributed to the nano-drug synthesis. L.I.S. contributed to the data analysis. C.L. and D.S.X. contributed to the study analysis and data analysis. Z.Q.L., Q.L., and A.Y. contributed to the revision of the manuscript. Y.J.W., L.W., and C.Z.L. contributed to the conception and design of the project.

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Data Availability The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Declarations

Ethics Approval and Consent to Participate All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. This article does not contain any studies with human participants performed by any of the authors.

Informed Consent Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no competing interests.

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