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Original Article

Colchicine attenuates chemical hypoxia-induced pyroptosis through downregulation of nuclear factor kappa B and caspase-1 in cardiomyocytes

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Abstract

Myocardial infarction (MI) is the leading cause of mortality worldwide. During MI, cardiomyocyte necrosis and inflammation are crucial in the post-MI cardiac remodeling process, including pyroptosis. Although colchicine is a well-known anti-inflammatory drug that has been clinically studied in the context of MI, its role in cardiac pyroptosis remains unclear. The aim of this study was to investigate the role of colchicine in pyroptosis in vitro, using $CoCl_2$-induced H9c2 cells. Prior to the primary experiment, the hypoxic model in H9c2 cells was optimized by evaluating hypoxia-inducible factor-1 alpha (HIF-1α) expression and viability in cells exposed to various concentrations of $CoCl_2$ at different time intervals. Subsequently, an in vitro hypoxia model was established by treating H9c2 cells with $CoCl_2$ (600 μM), with or without colchicine (1 μM), for 3 hours. Flow cytometry was used to measure the expression of nuclear factor-kappa beta (NF-κB), interleukin 18 (IL-18), caspase-1, and HIF-1α in pyroptotic cells. Immunofluorescence was used to assess caspase-1 localization and its colocalization with propidium iodide during late-stage pyroptosis. Our data indicated that $CoCl_2$-induced hypoxia significantly upregulated NF-κB, caspase-1, and IL-18 expression, and increased pyroptotic cell death in H9c2 cells. Colchicine treatment attenuated these effects, leading to a marked reduction in NF-κB, caspase-1, and IL-18 expression in hypoxic cells. Colchicine treatment significantly decreased the number of late pyroptotic cells. The protective effect of colchicine was more pronounced in late hypoxia (24-hour) setting compared to early hypoxia (3-hour). These findings suggest that colchicine attenuates cardiac pyroptosis in hypoxic H9c2 cells, as evidenced by the significant downregulation of key proteins involved in this pathway, including NF-κB, caspase-1, and IL-18. This protective effect appeared to be more effective in late hypoxia.

Keywords: Caspase-1, colchicine, HIF-1α, IL-18, pyroptosis

Introduction

Acute myocardial infarction (AMI) is a leading cause of mortality globally, accounting for over nine million deaths annually [1,2]. This disease is characterized by a rapid and irreversible decline in cardiomyocytes function, as these cells lose their regenerative capacity in adults, leading most AMI patients to progress to heart failure and ultimately death [3]. Early diagnosis and prompt treatment of AMI are crucial for improving therapeutic outcomes and reducing hospitalization

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costs [4]. In Indonesia, the prevalence of acute coronary syndrome (ACS), which mostly contribute to the incidence of AMI, reached 1,017,290 people in 2018, with a prevalence of 1.5% in East Java [5].

During the acute phase of AMI, there are complex interplay between the inflammation process and cardiomyocytes death, which further lead to pathological cardiac remodeling [6,7]. The initial inflammatory response after ACS is triggered by the loss of cell membrane integrity due to oxygen deprivation. This stimulates the release of damage-associated molecular patterns (DAMPs) as danger signals, which then bind to pattern recognition receptors (PRRs). This interaction activates nuclear factor-kappa beta (NF-κB), leading to the transcription of Nod-like receptor protein 3 (NLRP3) [8]. Furthermore, NLRP3, together with apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and pro-caspase-1 forms an inflammasome complex [8]. This process activates caspase-1 and pro-inflammatory cytokines (interleukin (IL)-1β and IL-18), which play a role in pyroptosis cell death through pore formation by gasdermin-N [8].

Pyroptosis, a form of programmed cell death, is characterized by the formation of cell membrane pores, the release of pro-inflammatory cytokines, and cell lysis. It serves as a crucial innate immune mechanism by releasing IL-1β and IL-18 [9]. This process is closely linked to NLRP3 activation, which is triggered by reactive oxygen species from oxidative stress, leading to the activation of caspase-1 [9]. Activated caspase-1 cleaves gasdermin D, causing cell membrane rupture and initiating an inflammatory response, while also converting IL-1β and IL-18 precursors into their active forms to further amplifying inflammation [9,10]. Pyroptosis, driven by NLRP3 and caspase-1, contributes to cardiovascular diseases by reducing angiogenesis, destabilizing blood vessel plaques, damaging endothelial cells, and promoting myocardial hypertrophy and fibrosis. These effects make pyroptosis a significant endogenous regulator in cardiovascular pathology [11]. Targeting this pathway using pyroptosis inhibitor has shown positive impact on infarct size reduction and cardiac function improvement following AMI [12].

Colchicine is a potent anti-inflammatory agent that modulates inflammatory processes by inhibiting chemotaxis, adhesion, and mobilization of neutrophils [13]. It affects microtubule assembly that lead to downregulation of numerous inflammatory pathways, including the NLRP3 inflammasome [13]. Mechanistically, colchicine disrupts NLRP3 inflammasome activation and directly or indirectly attenuates NF-κB, which subsequently inhibit IL-1β and IL-18 secretion [14,15]. In the context of AMI, daily dose of colchicine has been shown to significantly reduce cardiovascular events in patients following myocardial infarction by targeting inflammation [16].

The role of colchicine in cardiac pyroptosis, specifically via the NF-κB pathway, has not yet been well established. Furthermore, its effects on cardiac pyroptosis under conditions of early and late hypoxia remain unclear. The aim of this study was to evaluate the effect of colchicine treatment on the expression of critical markers linked to the NLRP3 inflammasome and pyroptosis. The results are expected to provide valuable insights into the potential of colchicine as an inflammatory modulator during the initial response to cardiomyocyte injury.

Methods

Study design

This study utilized an in vitro experimental design to evaluate the effect of colchicine on the expression of critical markers linked inflammasome and pyroptosis using H9c2 cardiac myoblast cell line. A pilot study was first conducted to determine the optimal cobalt chloride ($CoCl_2$) concentration for inducing hypoxia, identifying 600 μM as the most suitable dose. The cells were then exposed to two hypoxia models: early hypoxia (3 h of colchicine exposure) and late hypoxia (24 h of colchicine exposure). Following hypoxia treatment, colchicine (1 μM) was administered for an additional 3 h, with a placebo as a control. After treatment, several biomarkers associated with cell death and activation of the cardiac pyroptosis pathway were assessed.

Cardiac myoblast H9c2 cell culture

The rat cardiac myoblast H9c2 (2-1) was obtained from the European Collection of Authenticated Cell Cultures (ECACC) (88092904; ECACC, UK). H9c2 cells were cultured in Dulbecco's Modified Eagle's medium-high glucose (DMEM-HG) (D6429; Sigma-Aldrich, Merck, Germany) containing

10% fetal bovine serum (FBS) (F2442; Sigma-Aldrich, Merck, Germany) and 1% antibiotic-antimycotic solution (A5955; Sigma-Aldrich, Merck, Germany) in a 5% CO_2 incubator at 37°C.

Cell viability measurement

The cells were plated in 96-well plates at a density of 8×10^3 cells/well and cultured in a 5% CO_2 incubator at 37°C. After 24 h, the cells were treated with serial concentrations of cobalt chloride ($CoCl_2$) at 0, 200, 400, 600, 800, and 1000 µM (C8661; Sigma-Aldrich, Merck, Germany). Cell viability was monitored using the WST-1 reagent (11644807001; Roche, Basel, Switzerland) at 1, 3, 24, and 48 h after $CoCl_2$ addition. The medium from the H9c2 cell culture was aspirated and replaced with 100 µL medium containing 5% WST-1 reagent. The cells were then incubated in a 5% CO_2 incubator at 37°C. After a 20-minute incubation period, the absorbance at 450 nm was determined using a Multiscan Sky High Microplate Spectrophotometer (A51119600C; Thermo Scientific, Waltham, MA, USA).

Hypoxia-inducible factor-1α (HIF-1α) detection by flow cytometry

To optimize the hypoxia model cell, H9c2 cells were plated in 6-well plates at a density of 2.5×10^5 cells/well and cultured in a 5% CO_2 incubator at 37°C. After 24 h, cells were treated with $CoCl_2$ (0, 200, 400, 600, 800, and 1000 µM). HIF-1α expression in the cells was detected with a specific antibody using fluorescence-activated cell sorting (FACS). Briefly, cells were collected at 1, 3, 24, and 48 h after $CoCl_2$ addition. The cell suspension was centrifuged at 2500 rpm for 5 min at 10°C. The supernatant was discarded after centrifugation, and the cells were treated with 50 µL of IC Fix Buffer (00–8222–49; Thermo Fisher Scientific, Waltham, MA, USA) and incubated on ice for 20 min. Subsequently, 500 µL of 1× perm buffer (00-8333-56; Thermo Fisher Scientific, Waltham, MA, USA) was added. The suspension was then centrifuged at 2500 rpm for 5 min at 10°C. The cell pellet was subjected to intracellular staining using antibody HIF-1α (sc-13515; Santa Cruz Biotechnology, Dallas, TX, USA) for 20 min. Following staining, the cells were analyzed using a flow cytometer (BD FACS Calibur, San Jose, CA, USA) and the data were visualized using FlowJo version 10 software (BD Bioscience, Vancouver, Canada).

Hypoxia induction and colchicine treatment

The hypoxic model cell was induced based on the best results from the previous step. Cells were plated in 6-well plates at a density of 2.5×10^5 cells/well in a 5% CO_2 incubator at 37°C. After 24 h, the cells were exposed to 600 µM $CoCl_2$ with or without 1 µM colchicine (C3915; Sigma-Aldrich, Merck, Germany) for 3 h (early hypoxia) and 24 h (late hypoxia).

Measurement of IL-18, caspase-1, NF-κB, and pyroptosis

After 3 h of treatment, cells were harvested as described previously. Subsequently, the cell suspension was centrifuged, followed by incubation with IC Fix buffer, 1× perm buffer, and antibodies. The antibodies used were IL-18 (MBS9462213; MyBioSource, San Diego, CA, USA), Caspase-1 (sc-392736; Santa Cruz Biotechnology, Dallas, TX, USA), RELA/NF-κB p65 (sc-8008; Santa Cruz Biotechnology, Dallas, TX, USA), and FAM-FLICA Caspase 1 Assay kit (MBS258406; MyBioSource, California, San Diego, CA, USA)). After 20 min of incubation, the cells were analyzed using a flow cytometer (BD FACS Calibur, San Jose, CA, USA) and the data were visualized using FlowJo version 10 software (BD Bioscience, Vancouver, Canada).

Immunofluorescence detection of pyroptosis

To assess the cellular localization of caspase-1 during the final stage of pyroptosis, immunofluorescence was conducted to detect caspase-1 and propidium iodide colocalization. Briefly, H9c2 cells were plated on 24-well plates with coverslips at a density of 8×104 cells/well in a 5% CO_2 incubator at 37°C. After 24 h, the cells were exposed to 600 µM $CoCl_2$, with or without 1 µM colchicine. After 3 and 24 h of treatment, cells were stained using the FAM-FLICA Caspase 1 Assay kit (MBS258406; MyBiosource, San Diego, CA, USA). The pyroptotic cells were visualized using confocal laser scanning microscopy (CLSM FV-1000, Olympus, Tokyo, Japan).

Data analysis

Data are presented as mean±SD and were analyzed using one-way analysis of variance (ANOVA), followed by multiple comparison tests. All statistical analyses were performed using the

GraphPad Prism software (GraphPad, La Jolla, CA, USA). Statistical significance was set at $p<0.05$.

Results

Effect of $CoCl_2$-induced hypoxia on cell viability and HIF-1α

The $CoCl_2$ is a well-established hypoxia-mimetic chemical compound that induces a hypoxia-like state in vitro [17]. To investigate whether $CoCl_2$ elicits cardiomyocyte cytotoxicity, H9c2 cells were treated with various concentrations of $CoCl_2$, and subsequently, cell viability and HIF-1α expression were monitored at distinct time intervals (**Figure 1**). To obtain the most representative in vitro model of hypoxic cardiomyocytes in H9c2 cells, a cell viability assay was conducted along with the measurement of HIF-1α levels in each treatment subgroup. H9c2 cells were treated with serial concentrations of $CoCl_2$ (0–1000 μM) and harvested at various time points (1, 3, 24, and 48 h). An optimized in vitro model of hypoxic cardiomyocytes was defined as subgroup treatment with a high concentration of HIF-1α with the highest cell viability. Our data indicated that $CoCl_2$ treatment significantly induced cellular expression of HIF-α and cell death in a dose-dependent manner (**Figure 1A**). In particular, the number of HIF-1α-positive cells was higher in H9c2 cells treated with 600 μM $CoCl_2$. In contrast, the cell viability in this subgroup was still above 80% (**Figure 1B**). Therefore, this subgroup (600 μM $CoCl_2$) was selected as a representative hypoxic model of H9c2 and used for subsequent experiments. Additionally, two time points as representatives of early and late hypoxia were selected.

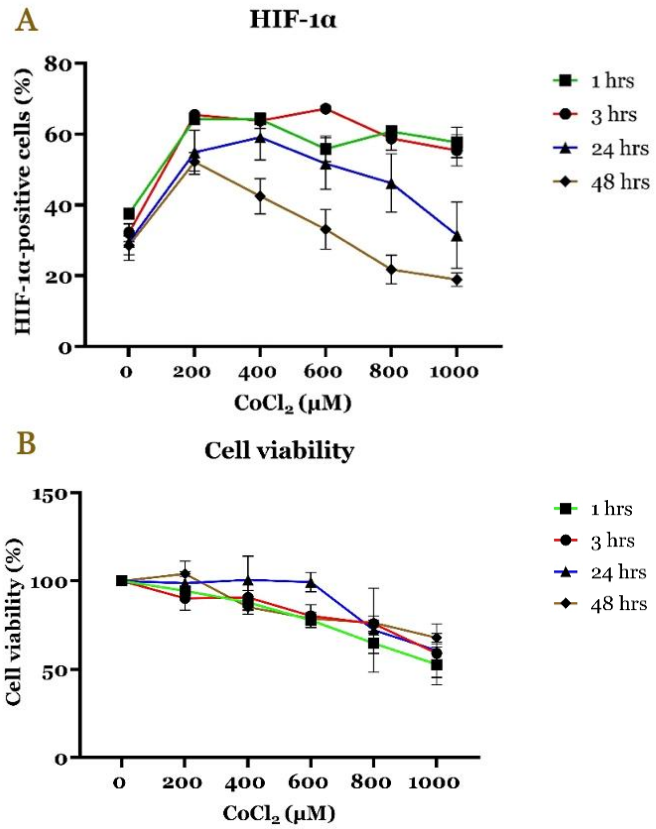

Figure 1. Effect of cobalt chloride ($CoCl_2$) on hypoxia-inducible factor-1 alpha (HIF-1α) and cell viability of H9c2. (A) HIF-1α protein expression was detected by flow cytometry in H9c2 cells under hypoxic conditions with $CoCl_2$ (0, 200, 400, 600, 800, or 1000 μM) at different time points (1, 3, 24, or 48 h). (B) Cell viability assays were performed using the water-soluble tetrazolium 1 (WST-1) method to assess the percentage of living $CoCl_2$-induced H9c2 cells at different time points. Data are presented as mean ± standard deviation of at least four independent experiments.

Colchicine downregulates HIF-1α expression in chemical hypoxia-induced H9c2

HIF-1α is a known biomarker of cellular hypoxia that can further induce cellular responses related to metabolic stress, including cell death. A flow cytometry analysis of H9c2 cells exposed to chemical hypoxia was conducted to examine the effect of colchicine on HIF-1α expression (**Figure 2**). Our results indicated that colchicine had a positive impact on the reduction of HIF-1α expression in both settings (early and late hypoxia). $CoCl_2$ exposure significantly elevated HIF-1α expression in H9c2 cells in early (58.85±6.6% vs 32.27±4.9%, $p<0.001$) and late (62.90±4.8% vs 48.98±3.2%, $p<0.05$) hypoxia compared to control group. Further analysis also showed that colchicine treatment markedly downregulated HIF-1α expression in early (58.85±6.6% vs 42.53±3.9%, $p<0.05$) but not in late (62.90±4.8% vs 49.16±6.8%, $p>0.05$) hypoxia compared to colchicine-treated hypoxic H9c2 cells (**Figure 2**).

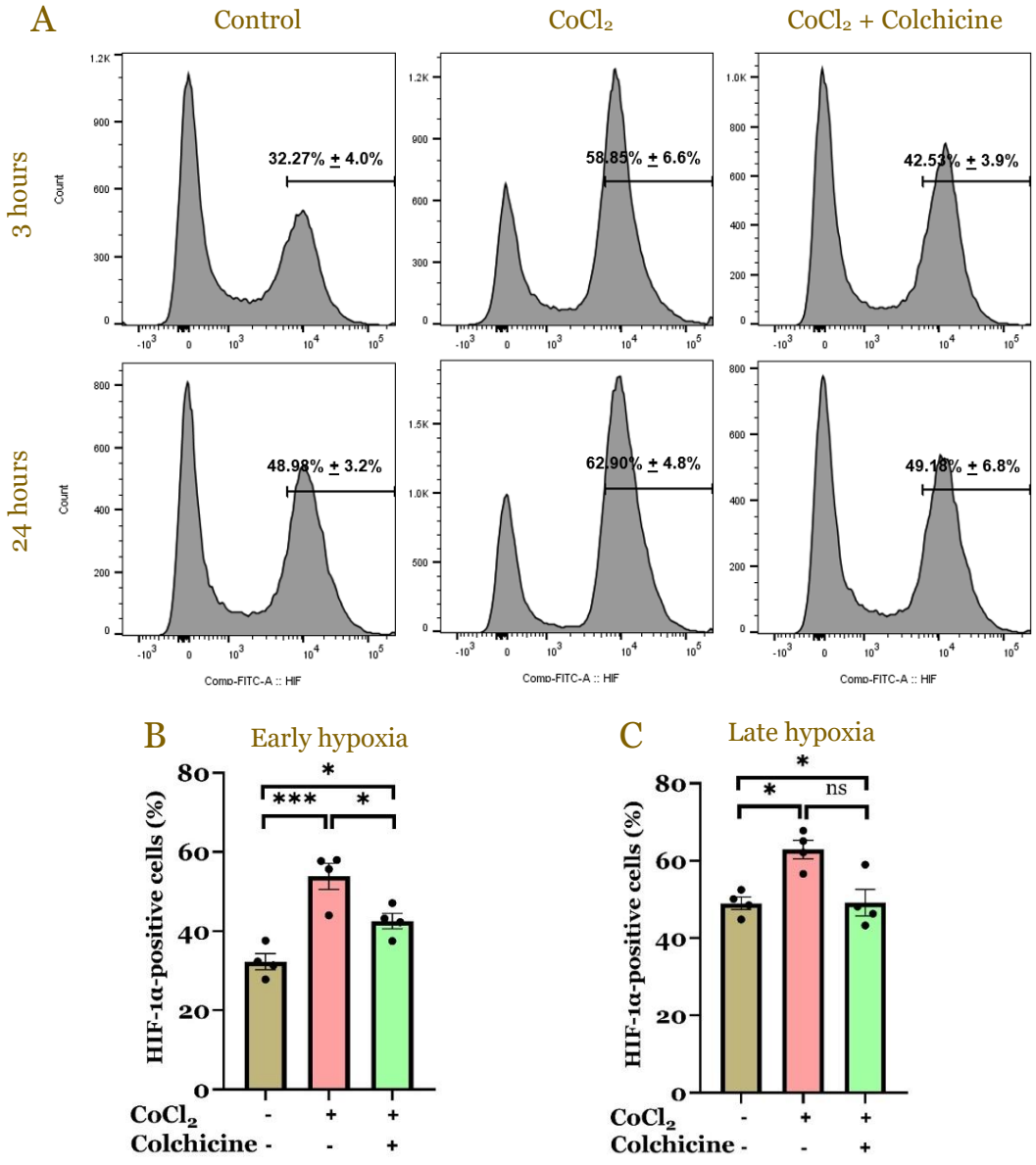

Figure 2. Effects of colchicine on hypoxia-inducible factor-1 alpha (HIF-1α) expression in cobalt chloride ($CoCl_2$)-induced H9c2 cells. The experimental protocol involved exposing cells to 600 µM $CoCl_2$, with or without 1 µM colchicine, for 3 h (representing early hypoxia) and 24 h (representing late hypoxia). HIF-1α expression in these cells was quantified by flow cytometry. The results graphically illustrated the proportion of cells expressing HIF-1α. Data are expressed as the mean values ± standard deviations derived from four independent experiments. Statistical significance was determined using one-way ANOVA with multiple comparison testing, where $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, and $^{****}p<0.0001$.

Colchicine inhibits nuclear factor-kappa B (NF-κB) upregulation in chemical hypoxia-induced H9c2

NF-κB is recognized as a key modulator of inflammasome activation and subsequent cardiac pyroptosis in myocardial infarction models [18]. To examine the effect of colchicine on NF-κB expression, flow cytometry analysis was conducted on H9c2 cells that were exposed to chemical hypoxia (**Figure 3**). Our findings revealed that colchicine effectively reduced NF-κB expression in both early and late hypoxic conditions. Exposure to $CoCl_2$ significantly increased NF-κB expression in H9c2 cells during early (68.43±2.50% vs 46±1.73%, $p<0.0001$) and late (75.35±2.65% vs 52.85±3.78%, $p<0.05$) hypoxia compared to that in the control group. Additional analysis demonstrated that colchicine treatment substantially decreased NF-κB expression in early (75.35±2.65% vs 55.5±3.69%, $p<0.001$) and late (75.35±2.65% vs 63.85±2.49%, $p<0.01$) hypoxia compared to colchicine-treated hypoxic H9c2 cells (**Figure 3**).

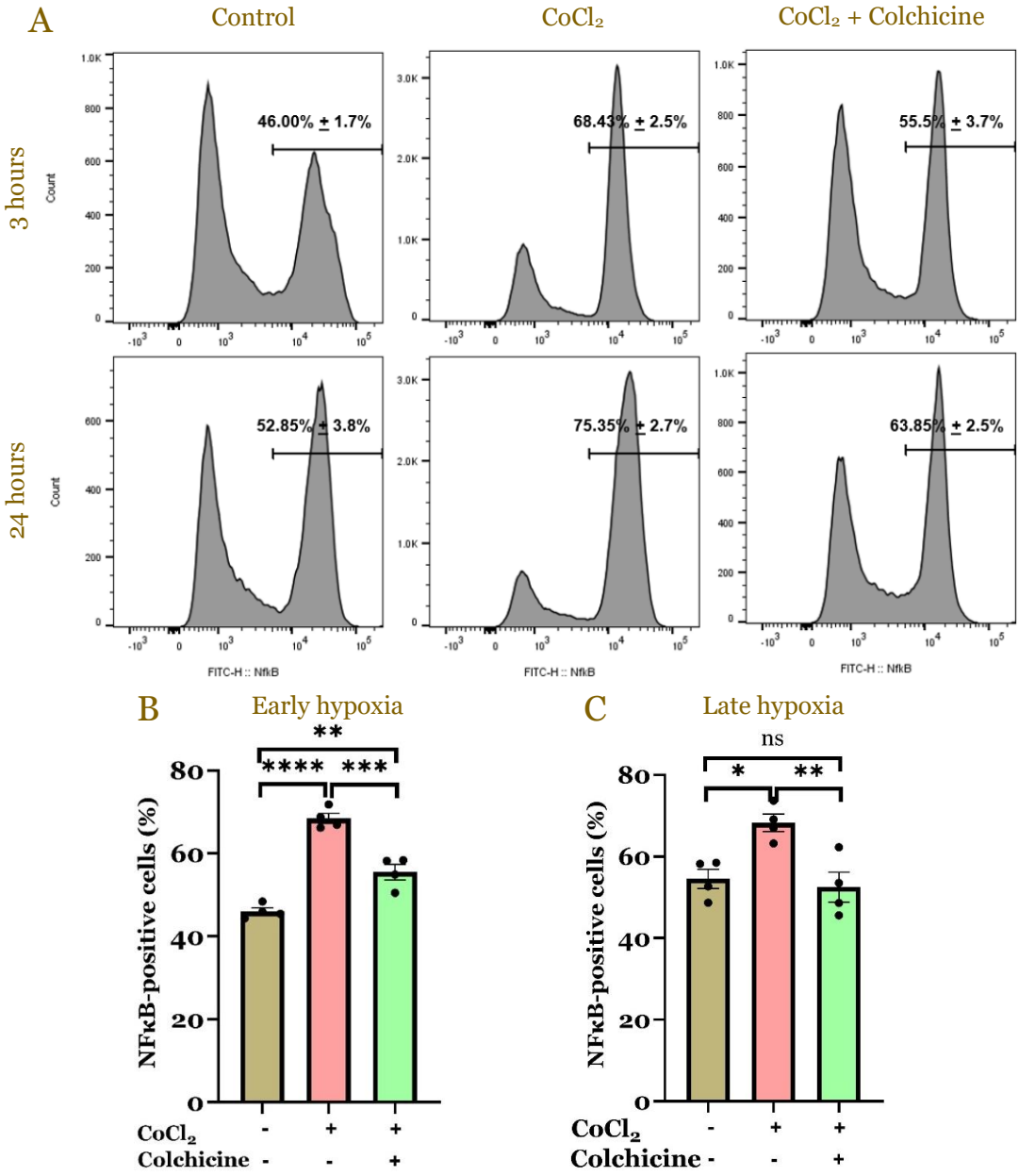

Figure 3. Effects of colchicine on nuclear factor-kappa B (NF-κB) expression in cobalt chloride ($CoCl_2$)-induced H9c2 cells. The cells were treated with 600 μM $CoCl_2$ in the presence or absence of 1 μM colchicine for 3 h (early hypoxia) and 24 h (late hypoxia). Flow cytometry was used to quantify NF-κB expression. The percentage of NF-κB (p65)-positive cells is presented in the graph. Data are expressed as the mean ± standard deviation from four independent experiments. Statistical analysis was conducted using one-way ANOVA with multiple comparison tests; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, and $^{****}p<0.0001$.

Colchicine mitigates the increase in caspase-1 induced by $CoCl_2$ exposure

Research has demonstrated that the elevation and stimulation of caspase-1 play a role in cardiac pyroptosis [19]. To investigate the influence of colchicine on H9c2 cells under hypoxic conditions, flow cytometry was employed to quantify caspase-1 positive cells (**Figure 4**). Our findings revealed that colchicine effectively reduced caspase-1 expression in both early and late hypoxia scenarios. Exposure to $CoCl_2$ led to a significant increase in caspase-1 expression in H9c2 cells during early (62.55±5.42% vs 34.4±3.81%, $p<0.05$) and late (68.25±4.38% vs 54.53±4.71%, $p<0.0001$) hypoxia when compared to the control group. Additional analysis revealed that treatment with colchicine substantially decreased caspase-1 expression in both early (62.55±5.42% vs 47.45±4.99%, $p<0.01$) and late (68.25±4.38% vs 52.5±7.29%, $p<0.01$) hypoxia in comparison to hypoxic cardiomyocytes treated with colchicine (**Figure 4**).

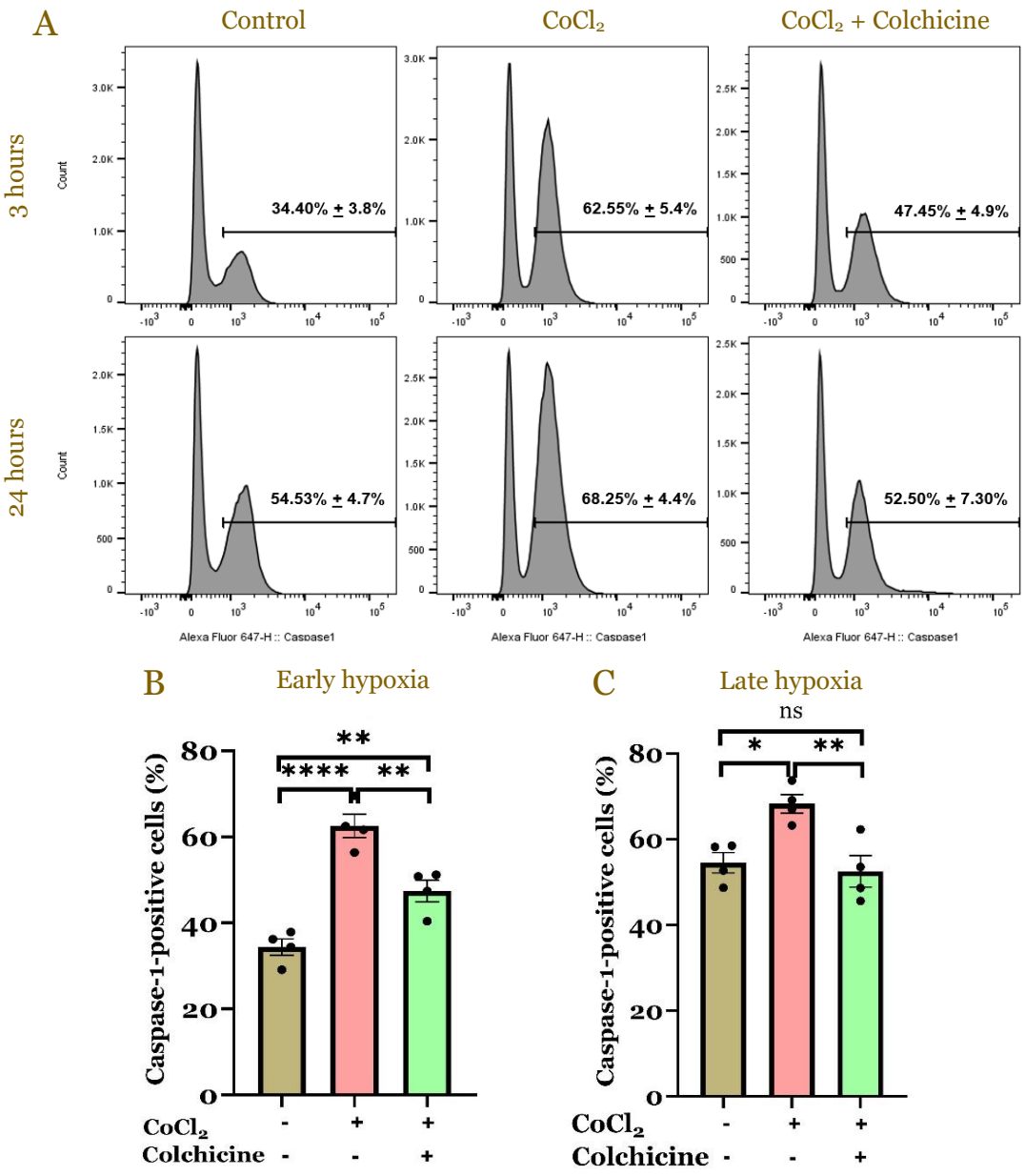

Figure 4. Effect of colchicine administration on caspase-1 expression in H9c2 cells exposed to cobalt chloride ($CoCl_2$). The cells were subjected to 600 µM $CoCl_2$ exposure, with or without 1 µM colchicine, for 3 h (representing early hypoxia) and 24 h (representing late hypoxia). Caspase-1 expression was quantified by flow cytometry. The graph shows the calculated percentage of cells expressing caspase-1. The results are shown as mean ± standard deviation derived from four separate experiments. One-way ANOVA with multiple comparison tests was employed for statistical analysis; $^{*}p<0.05$, $^{**}p<0.01$, and $^{****}p<0.0001$.

IL-18 expression in hypoxic H9c2 cells is suppressed by colchicine

The conventional pyroptotic pathway involves activation of caspase-1, which cleaves and matures IL-18. This mature IL-18 is subsequently released through gasdermin D pores as the final step in pyroptosis [11]. Flow cytometry analysis was performed to examine the effect of colchicine on IL-18 expression, a specific pyroptosis marker (**Figure 5**). This investigation revealed that colchicine effectively diminished IL-18 expression in both the early and late stages of hypoxia. H9c2 cells exposed to $CoCl_2$ exhibited significantly elevated IL-18 expression during early (69.85±1.79% vs 33.58±3.15%, $p<0.01$) and late (70.98±1.99% vs 58.03±2.93%, $p<0.01$) hypoxia compared to the control group. Further examination demonstrated that colchicine treatment markedly decreased IL-18 expression in early (69.85±1.79% vs 50.23±6.22%, $p<0.01$) and late (70.98±1.99% vs 56.53±7.07%, $p<0.001$) hypoxia compared to hypoxic cardiomyocytes treated with colchicine.

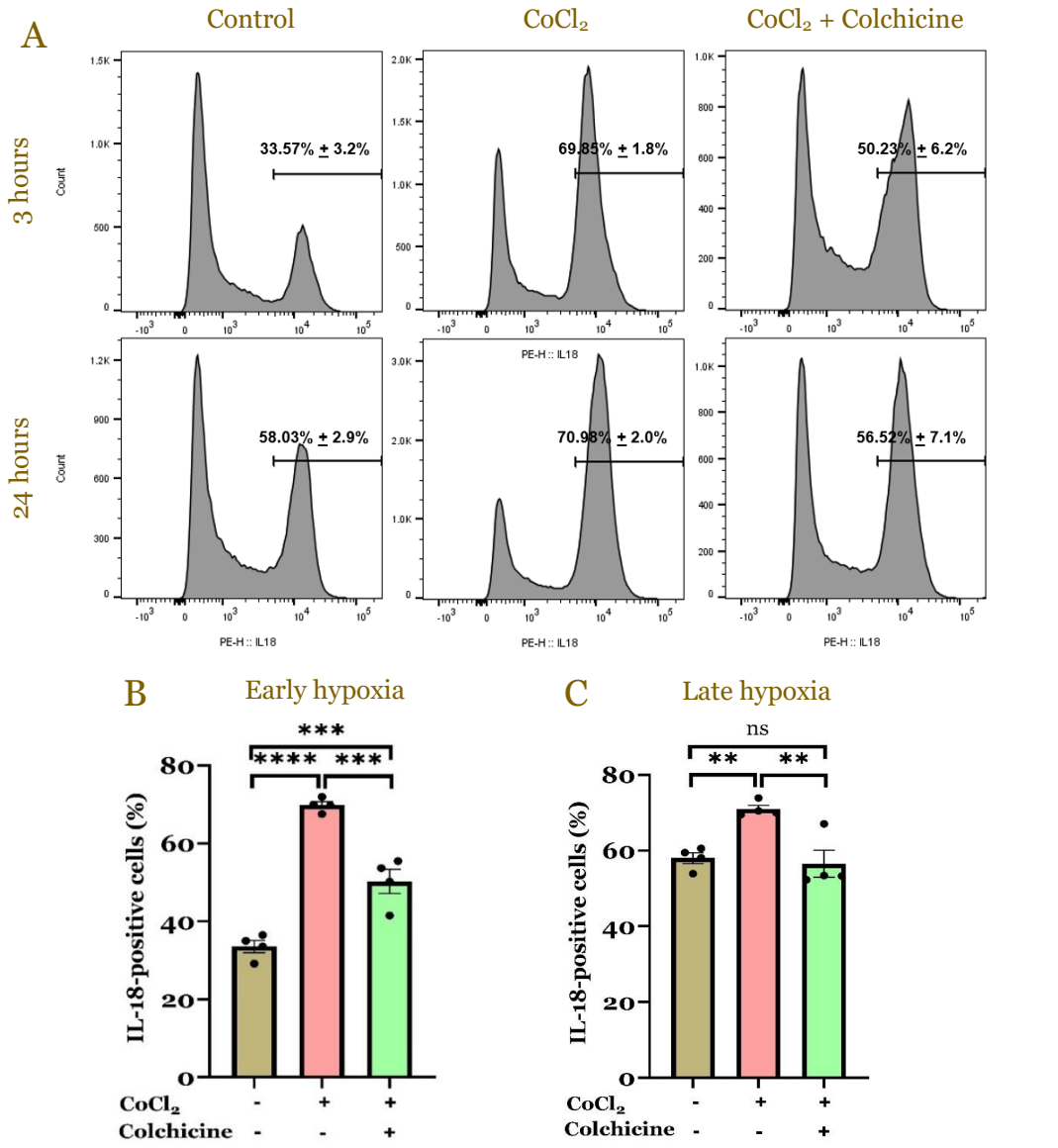

Figure 5. Effects of colchicine on interleukin 18 (IL-18) expression in H9c2 cells exposed to cobalt chloride ($CoCl_2$). The cells were treated with 600 µM CoCl2 with or without 1 µM colchicine for 3 h (early hypoxia) and 24 h (late hypoxia). IL-18 expression was quantified by flow cytometry, and the graph shows the percentage of IL-18-positive cells. Data are presented as the mean ± standard deviation and were derived from four independent experiments. Statistical analysis was performed using one-way ANOVA with multiple comparison tests; $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$.

Colchicine mitigates hypoxia-induced cardiomyocyte pyroptosis

To investigate the impact of colchicine on cardiomyocyte pyroptosis, flow cytometry was used to identify pyroptosis markers in H9c2 cells. Additionally, immunofluorescence was used to detect caspase-1 and propidium iodide colocalization, providing insights into the cellular localization of caspase-1 during the final stage of pyroptosis. Early and late pyroptosis were differentiated based on propidium iodide expression or signal. Cells expressing only caspase-1 were categorized as early pyroptosis, whereas those positive for both caspase-1 and propidium iodide were classified as late pyroptosis. Flow cytometry analysis (**Figure 6**) demonstrated that $CoCl_2$ treatment did not significantly alter the population of cells positive for caspase-1 alone or both caspase-1 and propidium iodide (**Figure 6B** and **6C**), making it challenging to assess the effect of colchicine in the setting of early hypoxia. However, during late hypoxia, $CoCl_2$ exposure significantly increased the late pyroptotic cell population (49.3±2.1% vs 17.0±3.8%, $p<0.0001$) compared to the control group, while colchicine treatment notably reduced this effect (49.3±2.1% vs 38.2±1.5%, $p<0.001$). Furthermore, based on the immunofluorescence approach, both 3 hours and 24 hours $CoCl_2$ treatment significantly induced early and late pyroptosis compared to the control group. Consistently, colchicine treatment significantly reduced the number of cells undergoing early and late pyroptosis in both settings of hypoxia (**Figure 7** and **Figure 8**).

Discussion

Our investigation demonstrated that the induction of chemical hypoxia using $CoCl_2$ activated the pyroptosis pathway in H9c2 cells. This activation was proven by a significant increase in caspase-1, IL-18, and pyroptotic cell counts. $CoCl_2$ effectively stabilized HIF-1α and HIF-2α under normoxic conditions. Cobalt ions (Co2+) are known to substitute for iron ions (Fe2+) in prolyl hydroxylases, which are enzymes that link oxygen levels to HIF degradation in normoxic environments [20]. The initial response to hypoxia involves activation of the AMP-activated protein kinase pathway, which enhances glycolysis and increases phosphofructokinase-1 and pyruvate kinase activity. This response is transient, and prolonged hypoxia results in HIF stimulation. HIF is a transcription factor comprising an alpha subunit (HIF-1α, 2α, and 3α) and a beta subunit. HIF-1α, the most extensively studied subunit, is considered the primary regulator of hypoxia due to its role in maintaining homeostasis during this condition [20,21].

A previous study indicated that HIF-1α contributed to pyroptosis by producing inflammatory mediators such as IL-1β and activating NLRP3 [22]. This study demonstrated that the HIF-1α inhibitor significantly reduced HIF-1α levels and NLRP3 inflammasome activation, along with its downstream targets, such as IL-1β and IL-18, in middle cerebral artery occlusion mice at 6 and 24 h [22]. Furthermore, HIF-1α resulted in a loss of cellular membrane integrity, as indicated by elevated LDH levels, which decreased following HIF-1α inhibitor administration [22]. $CoCl_2$ is known to induce various cellular effects, including cell shrinkage, chromatin condensation, nuclear fragmentation, G2/M phase prolongation, cytochrome c release, mitochondrial transmembrane potential disruption, voltage-dependent anion channel upregulation, and caspase activation [23]. $CoCl_2$ can stimulate extracellular signal regulated kinase (ERK1/2) and phosphatidylinositol 3-kinase (PI3K) signaling pathways, leading to increased HIF-1α translation [23]. Additionally, Co2+ enhances HIF-1/2 transcription by activating various transcription factors such as specificity protein 1 (Sp1), NF-κB, related transcription enhancer factor-1 and nuclear factor of activated T cells through Akt activation [22-24].

The primary contrast between CoCl2 exposure for 3 and 24 h centers on the advancement of pyroptosis in H9c2 cells. During the initial phase of hypoxia (3 h), no notable alterations were observed in the proportion of cells that underwent early or late pyroptosis. In contrast, the extended hypoxia phase (24 h) resulted in a marked increase in the number of cells in late pyroptosis when compared to the control group. This observation indicates that pyroptosis becomes more evident as hypoxic conditions persist. A study has demonstrated that exposing cardiomyocytes to 300 μM $CoCl_2$ for 24 hours led to a notable rise in NF-κB and HIF-1α concentrations [25]. Another study confirmed that treating cardiomyocytes with 100 μM $CoCl_2$ resulted in a substantial increase in HIF-1α nuclear translocation, as evidenced by the immunofluorescence technique [26]. Additionally, Western blot analysis revealed a marked elevation in HIF-1α and NF-κB levels in H9c2 cells subjected to hypoxia induced by $CoCl_2$ [27].

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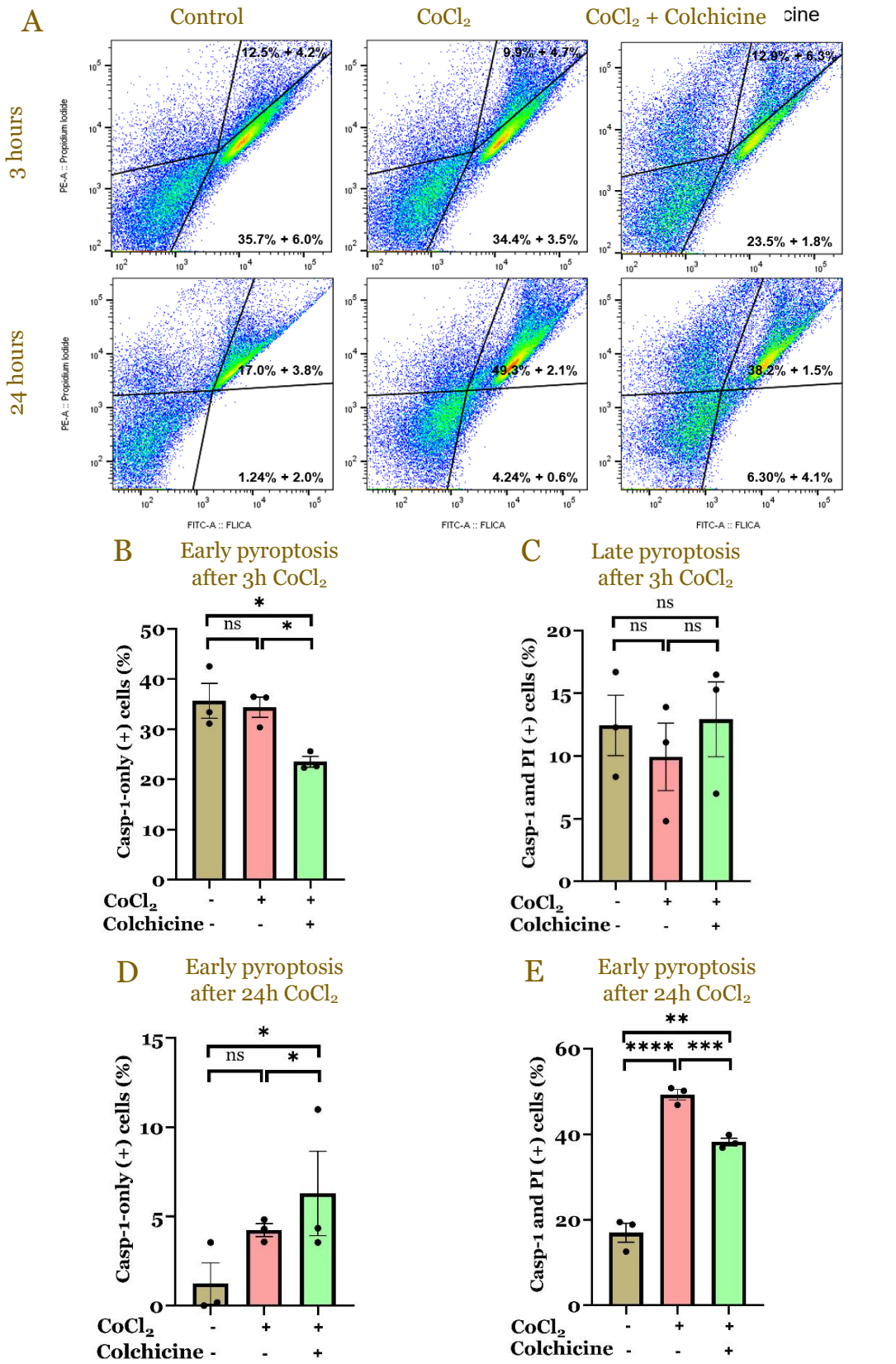

Figure 6. Effects of colchicine treatment on the number of pyroptotic cobalt chloride ($CoCl_2$)-induced H9c2 cells. Cells were treated with 600 µM CoCl2, with or without 1 µM colchicine, for 3 h (early hypoxia) and 24 h (late hypoxia). (A-C) Flow cytometry analysis results demonstrated that $CoCl_2$ treatment did not alter the population of either early or late pyroptotic cells after 3 h of $CoCl_2$ treatment. In late hypoxia, $CoCl_2$ significantly reduced the percentage of late pyroptotic cells compared with in $CoCl_2$-treated cells (A, D and E). Data are presented as mean ± standard deviation from three independent experiments. Statistical analysis was performed using one-way ANOVA with multiple comparison tests; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$.

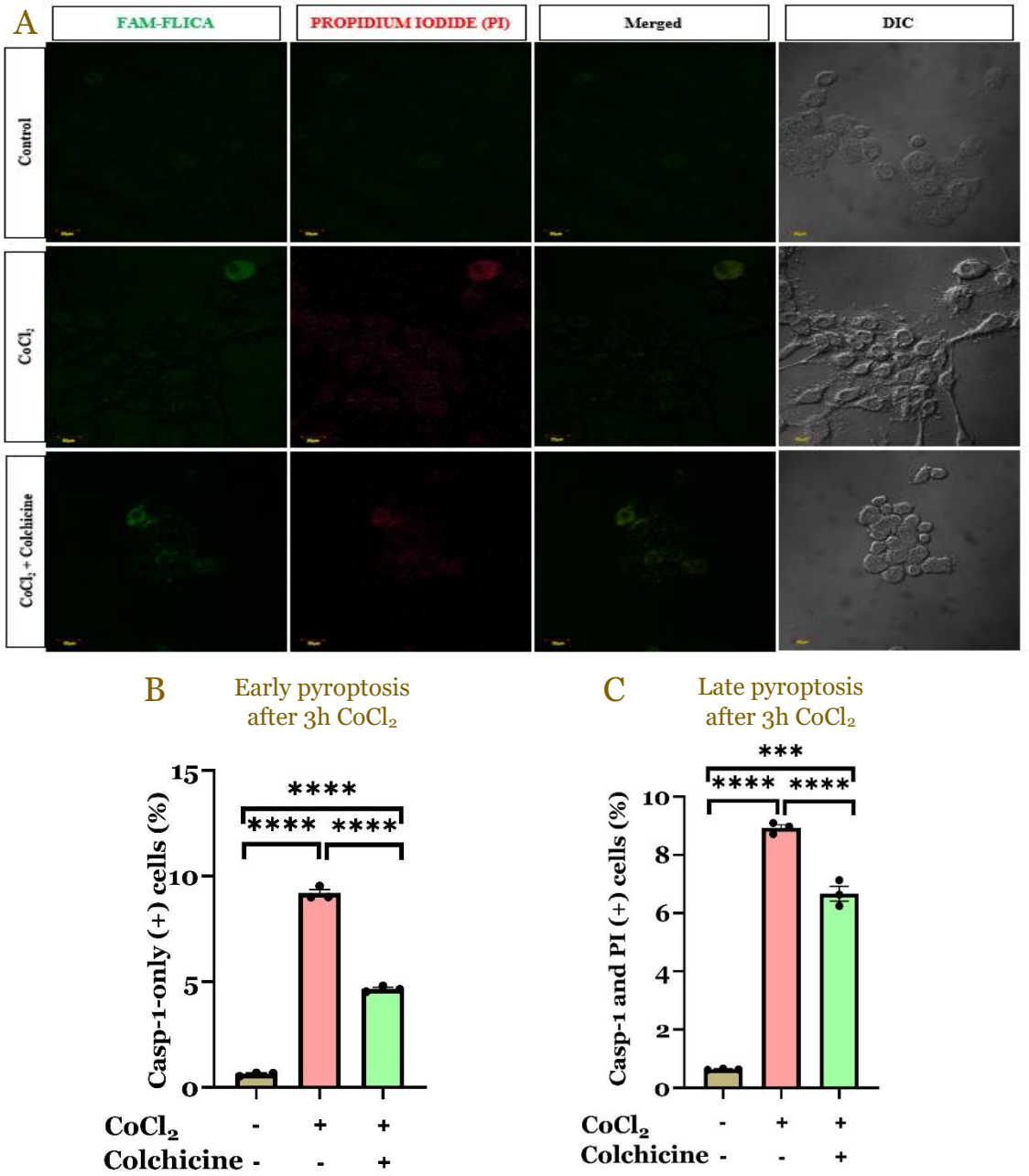

Figure 7. Effect of colchicine on the number of pyroptotic cells after 3 h of cobalt chloride ($CoCl_2$) treatment. The cells were treated with 600 µM $CoCl_2$ with or without 1 µM colchicine for 3 h. (A) Representative images of the early hypoxic cells in each group. (B, C) Quantification of the early and late pyroptotic cells in each group. Green indicates FAM-FLICA/caspase-1, and red indicates propidium iodide. Data are presented as mean ± standard deviation of arbitrary units from three independent experiments. Statistical analysis was performed using one-way ANOVA with multiple comparison tests; $^{***}p<0.001$ and $^{****}p<0.0001$. The scale bar represents 50 µm.

Colchicine interacts with α- and β-tubulin, creating a tubulin-colchicine complex that impedes microtubule formation and hinders its functionality [28]. This interaction causes microtubule depolymerization by obstructing lateral connections between protofilaments [29]. The disruption of microtubule assembly has various implications for inflammatory processes, including the inhibition of inflammasome activity, decreased chemotaxis of inflammatory cells, reduced phagocytosis, and diminished secretion of inflammatory mediators [28]. Colchicine's interference with microtubule function prevents apoptosis-associated speck-like protein containing a caspase recruitment domain from localizing with NLRP3, thereby inhibiting the activation and assembly of the inflammasome complex, including the conversion of pro-caspase-1 to caspase-1 [30,31]. Since activated caspase-1 is crucial for IL-18 maturation, blocking caspase-1 activation impedes the transformation of pro-IL-18 to IL-18 [30-32].

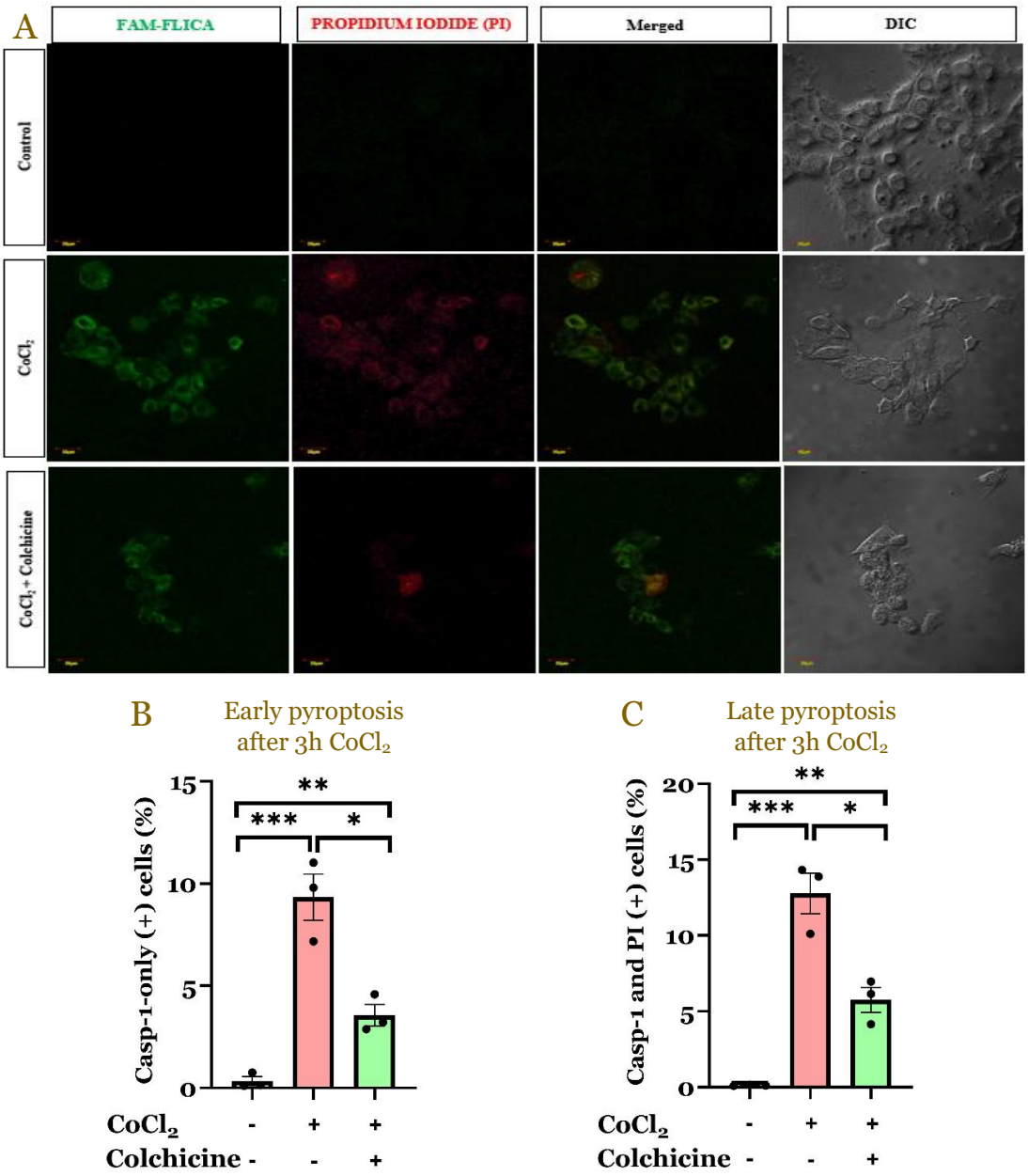

Figure 8. Effect of colchicine on the number of pyroptotic cells after 24 h of cobalt chloride ($CoCl_2$) treatment. The cells were treated with 600 µM CoCl2 with or without 1 µM colchicine for 24 h. (A) Representative images of late hypoxic cells in each group. (B, C) Quantification of the early and late pyroptotic cells in each group. Green indicates FAM-FLICA/caspase-1, and red indicates propidium iodide. Data are presented as mean ± standard deviation of arbitrary units from three independent experiments. Statistical analysis was performed using one-way ANOVA with multiple comparison tests; $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$. The scale bar represents 50 µm.

Activated caspase-1 not only facilitates IL-18 maturation but also cleaves gasdermin D, eliminating the intramolecular inhibition of the gasdermin-N domain [33]. This domain subsequently undergoes conformational changes, oligomerizes, and is incorporated into the plasma membrane to form pores [33]. These pores, measuring 10-15 nm in diameter, trigger pyroptosis by releasing substrates such as IL-1β and IL-18 [7,10,33,34]. Moreover, colchicine can suppress NF-κB in the nuclear fraction by modulating various upstream factors, including reactive oxygen species (ROS), which in turn regulate inflammatory cytokine expression [35]. Colchicine is also known to alleviate oxidative stress by reducing calcium (Ca2+) influx into neutrophils [36].

Altogether, the findings of the present study revealed that colchicine reduced cardiac pyroptosis in H9c2 cells under hypoxic conditions by substantially decreasing the expression of

crucial proteins in this pathway, including NF-κB, caspase-1, and IL-18. This discovery is important because it sheds light on the potential use of colchicine as a therapeutic agent to reduce heart damage during myocardial infarction, specifically by targeting the pyroptosis mechanism. However, this study had several limitations that affected the translatability and clinical relevance of the findings. This primarily relies on H9c2 cells and $CoCl_2$-induced chemical hypoxia, which may not fully replicate the complexity of human cardiomyocytes or true hypoxic conditions. The use of a single colchicine dose without dose-response analysis and the focus on short-term effects limits insights into optimal dosing, long-term outcomes, and adaptive responses. Additionally, the study lacks in vivo validation, functional outcomes, and comparisons with other treatments while providing limited mechanistic insights and exploring potential side effects. These limitations highlight the need for further research to enhance the applicability and comprehensiveness of this study.

Conclusion

Colchicine attenuated cardiac pyroptosis in hypoxic H9c2 cells, proven by the significant downregulation of key proteins involved in this pathway, including NF-κB, caspase-1, and IL-18. Overall, colchicine showed a protective effect in both early and late hypoxia by downregulating key proteins involved in the pyroptosis pathway, with a more pronounced effect on reducing late pyroptotic cells after 24-hour exposure. Further studies are required to evaluate the role of colchicine in animal models of myocardial infarction, particularly in association with the pyroptosis pathway.

Ethics approval

Not required.

Acknowledgments

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Competing interests

All the authors declare that there are no conflicts of interest.

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Underlying data

The project contains the following underlying data: Colchicine attenuates chemical hypoxia-induced pyroptosis through downregulation of nuclear factor kappa B and caspase-1 in cardiomyocytes, https://doi.org/10.6084/m9.figshare.28207961.v2 version 2. Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Declaration of artificial intelligence use

We hereby confirm that no artificial intelligence (AI) tools or methodologies were utilized at any stage of this study, including during data collection, analysis, visualization, or manuscript preparation. All work presented in this study was conducted manually by the authors without the assistance of AI-based tools or systems.

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