

The changes of hemodynamics and the effect of RHO kinase regulated by butylphthalein on apoptosis in acute myocardial infarction

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Abstract: Background: Cardiomyocyte apoptosis plays a critical role in ventricular remodeling and heart failure following acute myocardial infarction (AMI). The Rho kinase signaling pathway is known to be involved in this pathological process. Butylphthalein, a compound with neuroprotective effects, may exert cardioprotective effects by regulating this pathway. **Objective:** This study aims to investigate the effects of butylphthalein on hemodynamic parameters and cardiomyocyte apoptosis in a rat model of AMI and to explore whether these effects are mediated through the Rho kinase signaling pathway. **Methods:** Fifty SD rats were randomly divided into five groups: Sham, AMI model, butylphthalein, fasudil (a Rho kinase inhibitor) and combination therapy. AMI was induced by ligation of the left anterior descending coronary artery. Two weeks post-surgery, hemodynamic parameters—including left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP) and maximal rate of rise and fall of ventricular pressure ($\pm dp/dt_{max}$)—were measured. Cardiomyocyte apoptosis was assessed using TUNEL staining. The expression levels of Bcl-2, Bax, RhoA, ROCK1 and the p-MYPT1/MYPT1 ratio were detected by Western blot. **Results:** Rats in the AMI model group exhibited significant hemodynamic impairment and increased cardiomyocyte apoptosis. Compared with the model group, butylphthalein treatment significantly improved LVSP and $\pm dp/dt_{max}$, reduced LVEDP, decreased the number of TUNEL-positive cells, upregulated Bcl-2 expression, downregulated Bax expression and suppressed the expression of RhoA/ROCK1 and the p-MYPT1/MYPT1 ratio (all $P < 0.05$). The combination of butylphthalein and fasudil resulted in enhanced therapeutic effects, suggesting a synergistic inhibition of Rho kinase activity. **Conclusion:** Butylphthalein attenuates cardiomyocyte apoptosis and improves cardiac function in rats with AMI, likely through inhibition of the Rho kinase signaling pathway. These findings support its potential as a therapeutic agent for AMI. All experiments were performed on male Sprague-Dawley rats (n=50).

Keywords: Acute myocardial infarction; Butylphthalein; Cell apoptosis; Hemodynamics; Rho kinase

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INTRODUCTION

Acute myocardial infarction (AMI) is a life-threatening condition typically triggered by coronary atherosclerosis, which can be exacerbated by factors such as physical overexertion, emotional stress, excessive food intake, extreme temperature exposure and long-term smoking or alcohol consumption. These factors contribute to the rupture of atherosclerotic plaques, leading to coronary artery occlusion, myocardial ischemia, hypoxia and eventual necrosis (Krittawong *et al.*, 2023). The clinical presentation of AMI is often nonspecific, with symptoms including diaphoresis, agitation, nausea, shock and precordial pain (Raat *et al.*, 2025).

Apoptosis, a form of programmed cell death characterized by cell shrinkage, chromatin condensation and formation of apoptotic bodies, is genetically regulated and plays a pivotal role in various physiological and pathological processes (Gregory, 2023). Emerging evidence indicates that apoptosis is a major mode of cardiomyocyte death following AMI. The extensive loss

of cardiomyocytes reduces the number of functionally viable cells, subsequently triggering ventricular remodeling and contributing to the progression of heart failure (Yan *et al.*, 2024). Therefore, a key research priority in cardiovascular medicine is the development of strategies to protect residual cardiomyocytes after AMI, thereby mitigating left ventricular remodeling and preventing heart failure. Studies have demonstrated that multiple cytokines and signal transduction pathways are involved in the processes of myocardial ischemia, hypoxia and necrosis.

Butylphthalidole (3-n-butylphthalide, NBP) is a novel chemical agent independently developed in China for the treatment of cardio-cerebrovascular diseases. It exhibits pharmacological properties distinct from those of conventional cardiovascular drugs (Hu *et al.*, 2023). As the primary active component of butylphthalein, racemic 3-n-butylphthalide has been shown to exert anti-ischemic effects in both cerebral and myocardial ischemia. Its mechanisms of action include regulation of energy metabolism, mitochondrial protection, inhibition of apoptosis and suppression of free radical generation (An

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et al., 2026). Additionally, butylphthalin increases the levels of nitric oxide (NO) and prostacyclin (PGI₂) in vascular endothelial cells, reduces intracellular calcium concentrations, inhibits glutamate production, enhances antioxidant enzyme activity and alleviates tissue edema (Ma *et al.*, 2024).

Rho is a RAS-related monomer GTPase involved in the regulation of diverse cellular activities. Studies have shown that Rho kinase activity is upregulated following AMI, affecting the structure and function of smooth muscle cells and contributing to myocardial remodeling and heart failure (Chen *et al.*, 2023). Although the role of Rho kinase pathway in AMI has been extensively documented, whether 3-n-butylphthalide inhibits cardiomyocyte apoptosis through modulation of this pathway remains unexplored. Therefore, this study aims to investigate, for the first time, whether NBP attenuates myocardial injury after AMI by regulating the Rho kinase-mediated apoptotic pathway. The findings may provide a novel theoretical basis for the clinical application of NBP in AMI treatment.

MATERIALS AND METHODS

Experimental animals

Fifty clean-grade male Sprague-Dawley (SD) rats, aged 14–16 weeks and weighing 320–360 g, were provided by Shanghai SLAC Laboratory Animal Co., Ltd. (License No.: SCXK (Shanghai) 2020-0007). Male rats were selected to eliminate potential confounding effects of hormonal fluctuations associated with the estrous cycle on cardiovascular parameters. The age and weight ranges were chosen to represent young adult rats, which are most commonly used in AMI models and provide a balance between surgical feasibility and translational relevance to adult human populations.

All rats were adaptively fed for 5 days under conditions of room temperature (22 ± 2°C), relative humidity (55 ± 5%) and a 12-hour light/12-hour dark cycle, with free access to standard laboratory chow and water. Rats were group-housed (5 per cage) in standard polypropylene cages (47 × 34 × 20 cm) with corn cob bedding, which was changed twice weekly. Environmental enrichment included nesting material (paper towels) and PVC tunnels for shelter. Animals were acclimatized to handling for 5 days prior to surgery, with each rat being gently handled for 2–3 minutes daily to minimize stress during experimental procedures. This experimental protocol was approved by the Animal Ethics Committee of Liyuan Hospital (Approval No.: TJ-IRB202509063) and all procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition, National Research Council, 2011). All animal experiments are reported following the ARRIVE 2.0 guidelines.

Protocol registration

The study protocol, including the research question, experimental design, sample size calculation, and statistical analysis plan, was prepared prior to the commencement of animal experiments. This study was not registered in a public registry as it is a preclinical mechanistic study rather than a clinical trial. However, the complete protocol is available from the corresponding author upon reasonable request.

Reagents and instruments

Butylphthalin (Wuhan Lanabai Pharmaceutical Chemical Co., LTD.); Pentahydrate (Sigma, No.018k0754); isoproterenol (Shanghai Harvest Pharmaceutical Co., Ltd.); triphenyl tetrazolium chloride (TTC, Sigma); animal ventilator (Chengdu Tai Union Technology co., Ltd; HX-300); biological function tester (Chengdu Tai Union Technology co., Ltd; BL-420E+); Rho kinase inhibitor Fasudil hydrochloride (Shenzhen Haisian, Cat. No.: 30002844); TUNEL apoptosis detection kit (Sangon Biotech (Shanghai), Cat. No.: E607172); Rabbit anti-rat primary antibodies against Bcl-2, Bax, RhoA, ROCK1, p-MYPT1, MYPT1 and GAPDH (Abcam, USA); HRP-labeled goat anti-rabbit secondary antibody (Sangon Biotech (Shanghai), Lot No.: D110058).

Grouping

After the rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (100 mL/L), they were fixed in the supine position, subjected to tracheal intubation and connected to an animal ventilator. In 40 rats, a left thoracotomy was performed obliquely at 5 mm to the left of the sternum to expose the heart. The left anterior descending (LAD) coronary artery was ligated with a 6-0 suture at 2-3 mm distal to its origin, to a depth of 1-1.5 mm and a width of 2-3 mm. Successful model establishment was indicated by myocardial pallor distal to the ligation, weakened cardiac pulsation and ST-segment elevation on the electrocardiogram (Al Ali *et al.*, 2024). The remaining 10 rats underwent thoracotomy with a suture passed under the coronary artery but without ligation, serving as the Sham group.

This study adopted a randomized controlled design. Fifty rats were randomly divided into the following five groups (n = 10 per group) using a random number table generated by SPSS software (version 17.0). Group assignments were concealed in sequentially numbered opaque sealed envelopes. Randomization was performed by an independent investigator not involved in the experiments.: Sham group: Rats underwent thoracotomy with a suture passed under the coronary artery but without ligation and received an equal volume of normal saline via intraperitoneal injection twice daily starting 24 hours after surgery; Model group: Rats underwent LAD ligation to establish the AMI model and received an equal volume of normal saline via intraperitoneal injection twice daily

starting 24 hours after surgery; Intervention group: AMI model rats received 3-n-butylphthalide (2.25 mg/kg) via intraperitoneal injection twice daily starting 24 hours after surgery; FSI group: AMI model rats received Fasudil (10 mg/kg) via intraperitoneal injection twice daily starting 24 hours after surgery; Intervention+FS group: AMI model rats received both 3-n-butylphthalide (2.25 mg/kg) and Fasudil (10 mg/kg) via intraperitoneal injection twice daily starting 24 hours after surgery. All interventions were administered for 2 weeks post-surgery. All data collection and index measurements were performed by experimenters who were blinded to the group assignments (single-blind design).

Sample size justification

Sample size calculation was performed using G*Power software (version 3.1.9.7) based on our preliminary data on LVSP (effect size $f = 0.45$, $\alpha = 0.05$, power = 0.85), which yielded a minimum of 8 rats per group. We included 10 rats per group to account for potential surgical mortality and exclusions.

Inclusion and exclusion criteria

Inclusion criteria: (1) successful AMI model establishment confirmed by ECG (ST-segment elevation > 0.2 mV in at least two leads) and visible myocardial pallor; (2) stable vital signs at 30 minutes post-surgery; (3) recovery of spontaneous breathing within 10 minutes after extubation. Exclusion criteria: (1) failure of model establishment; (2) surgical complications including excessive hemorrhage (>2 mL), severe arrhythmia requiring intervention, or pneumothorax; (3) death within 24 hours post-surgery; (4) signs of infection at the surgical site.

Animal welfare and humane endpoints

Animals were monitored twice daily (8:00 AM and 6:00 PM) by trained personnel throughout the study period. Humane endpoints and criteria for euthanasia were established prior to the study and included: (1) severe respiratory distress (respiratory rate > 120/min or labored breathing for > 30 minutes); (2) inability to eat or drink for > 24 hours; (3) severe weight loss (> 20% of pre-surgery body weight); (4) persistent arrhythmias not responding to observation; (5) uncontrolled infection or severe wound dehiscence; (6) persistent signs of severe pain not relieved by analgesics. Rats meeting any of these criteria were euthanized by sodium pentobarbital (150 mg/kg, intraperitoneal) prior to the scheduled endpoint and excluded from analysis.

Postoperative analgesia was provided with buprenorphine (0.01 – 0.05 mg/kg, subcutaneous injection) administered immediately after surgery and every 8 – 12 hours for the first 48 hours, and thereafter as needed based on pain assessment using the Rat Grimace Scale.

Adverse events

Adverse events were recorded throughout the study. In the AMI model groups, transient arrhythmias (ventricular premature beats or tachycardia) occurred in 12 of 40 rats (30%) within the first 10 minutes after LAD ligation, all of which resolved spontaneously within 2 – 5 minutes without intervention. Surgical mortality within 24 hours post-surgery was 7.5% (3 of 40 rats), attributed to intractable ventricular fibrillation ($n=2$) or severe hemorrhage ($n=1$); these animals were excluded and replaced according to the pre-established protocol. No unexpected adverse events were observed in any group.

Hemodynamics test

The right common carotid artery was dissociated for about 1.5-2.0 cm to ligate distal end (Sorteberg *et al.*, 1998). The proximal end was clipped by an arterial clip. The cannula filled with 1% heparin was connected with the pressure transducer and BL-420E+ biological function recorder. Next, the PE-50 heart catheter was inserted and the arterial clip was released to observe arterial waveform. The following hemodynamic indexes were recorded, including left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), maximal increasing rate of left ventricular pressure (+dp/dtmax), maximal decreasing rate of left ventricular pressure (-dp/dtmax).

Myocardial tissue collection and processing

Immediately following the hemodynamic measurements, the hearts were rapidly excised through a thoracotomy. Hearts from six randomly selected rats in each group were rinsed with pre-cooled normal saline. The atria and right ventricle were then removed and myocardial tissue from the border zone (area at risk) of the infarct was collected. A portion of this tissue was fixed in 4% paraformaldehyde for histological analysis, while the remaining portion was snap-frozen in liquid nitrogen and subsequently stored at -80°C for protein analysis. The hearts from the other six rats in each group were reserved for other subsequent experiments.

TUNEL assay

Paraffin embedded sections were washed, treated by Proteinase K and added with 50 μ L TUNEL to count apoptotic cell number. The samples were further added with 50 μ L converter-POD and 50-100 μ L DAB substrate. Next, samples were re-stained by hematoxylin or methyl green. The nuclei with brown particles was treated as a positive marker. Apoptotic Index (AI) = (Number of TUNEL-positive nuclei / Total number of nuclei) $\times$ 100% (Chen *et al.*, 2013).

Western blot

Myocardial tissue stored at -80°C was collected and total protein was extracted using RIPA lysis buffer (Nie *et al.*, 2017). Protein concentration was determined by the BCA

method. Equal amounts of protein (30 µg) were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% non-fat milk at room temperature for 2 hours. They were then incubated overnight at 4°C with primary antibodies: rabbit anti-rat Bcl-2 (1:1000), Bax (1:1000), RhoA (1:500), ROCK1 (1:1000), p-MYPT1 (1:1000), MYPT1 (1:1000) and GAPDH (1:5000). After washing with TBST, the membranes were incubated with HRP-labeled goat anti-rabbit secondary antibody (1:5000) for 2 hours at room temperature. Protein bands were visualized using an ECL chemiluminescence method and the gray values were analyzed with Image J software. GAPDH was used as an internal control and the relative expression level of the target protein was calculated as the gray value of the target protein divided by the gray value of GAPDH. The activity of p-MYPT1 was expressed as the ratio of p-MYPT1 to total MYPT1.

Statistical analysis

All data analysis was performed on SPSS17.0 software and compared by chi-square test. $P < 0.05$ refers to a significance.

SPSS 17.0 software was used for data analysis. All measurement data were subjected to normality testing (Shapiro-Wilk test) and homogeneity of variance testing (Levene's test). Data that followed a normal distribution and met the homogeneity of variance assumption were expressed as mean $\pm$ standard deviation ($\bar{x} \pm s$). Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) and pairwise comparisons between groups were conducted using Tukey's post-hoc test. Data that did not follow a normal distribution or exhibited heterogeneity of variance were expressed as median (interquartile range) and comparisons among multiple groups were performed using the Kruskal-Wallis H rank sum test. A P -value < 0.05 was considered statistically significant. No outliers were excluded from the analysis.

RESULTS

General observations of rats following AMI induction

Following model establishment, the general condition of rats in each group was monitored daily, including mental status, locomotor activity, food and water intake and respiratory pattern. One week post-surgery, rats in the Experiment group exhibited hair loss, gradually reduced food and water intake, sensitivity to cold, decreased activity and increased heart rate. Some rats presented with nasal and oral hemorrhagic discharge. By two weeks post-surgery, these symptoms had worsened, with some animals showing complete anorexia, immobility, dyspnea and audible wheezing.

The microscopic findings of the rats

In the Sham group, the myocardial fibers were arranged

neatly and compactly, with a clear structure. Cross-striations were visible in the cytoplasm, the nuclei were oval-shaped and no significant pathological changes were observed. Compared with the Sham group, the Experiment group showed a thinning of the left ventricular wall, a distinct infarct area and destruction of the myocardial structure in the infarct zone. Myocardial cells exhibited vacuolar degeneration, accompanied by cell rupture, loss of nuclei and diffuse infiltration of inflammatory cells.

Compared with the Experiment group, the myocardial pathological changes were significantly attenuated in the Intervention, FS and Intervention +FS groups. In the Intervention and FS groups, the thickness of the left ventricular wall was improved, the size of the infarct area was reduced, the outline of the myocardial cells was relatively intact and the surrounding inflammatory cell infiltration was decreased. The myocardial structure improvement was most pronounced in the Intervention +FS group, where the left ventricular wall thickness approached that of the Sham group, the infarct area was markedly reduced and only a few scattered inflammatory cells were observed (Fig. 1).

Comparison of hemodynamics in rats

Compared with the Sham group, the Experiment group exhibited significantly decreased LVSP, $+dp/dt_{max}$ and $-dp/dt_{max}$, along with a significantly increased LVEDP ($P < 0.05$). This indicates that myocardial infarction severely impaired both the systolic and diastolic pump function of the rat hearts. Compared with the Experiment group, the Intervention, FS and Intervention +FS groups all showed significantly increased LVSP, $+dp/dt_{max}$ and $-dp/dt_{max}$ and significantly decreased LVEDP ($P < 0.05$). Among these, the improvement in various hemodynamic parameters was most pronounced in the Intervention +FS group. Specifically, LVSP, $+dp/dt_{max}$ and $-dp/dt_{max}$ in the Intervention +FS group were significantly higher and LVEDP was significantly lower, than those in the Intervention group ($P < 0.05$). These results suggest that 3-n-butylphthalide can effectively improve cardiac function in rats with AMI (Table 1).

Cardiomyocyte apoptosis in rats

TUNEL staining results showed that the apoptotic rate of cardiomyocytes in the Sham group was extremely low. Compared with the Sham group, the apoptotic rate of cardiomyocytes in the Experiment group was significantly increased ($P < 0.05$). Compared with the Experiment group, the apoptotic rates of cardiomyocytes in the Intervention, FS and Intervention+FS groups were all significantly reduced ($P < 0.05$). Furthermore, the apoptotic rate of cardiomyocytes in the Intervention+FS group was significantly lower than that in the Intervention group ($P < 0.05$) (Fig. 2 and Fig. 3).

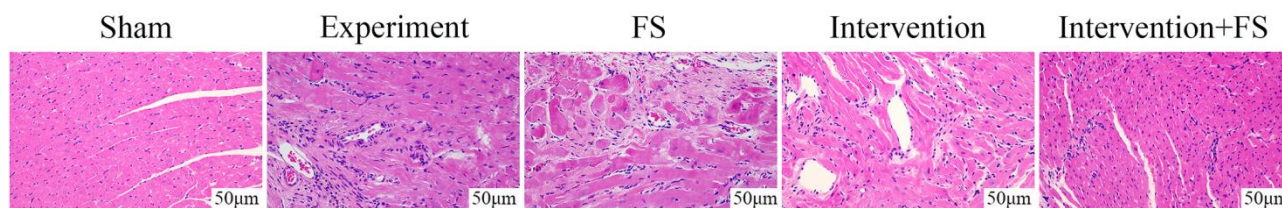


Fig. 1: Pathological changes in rat myocardial tissue across groups (HE Staining).

Table 1: Comparison of hemodynamic parameters among rat groups. ($\bar{x} \pm s$)

Group (n=10)	LVSP (mmHg)	LVEDP (mmHg)	+dp/dtmax (mmHg/s)	-dp/dtmax (mmHg/s)
Intervention	106.03±10.96*#	8.23±1.77*#	4101.1±291.1*#	3232.7±251.8*#
Experiment	95.35±6.11*	14.56±1.08*	4001.2±345.7*	3021.2±279.3*
Sham	124.96±16.29	6.39±1.38	4473.6±294.3	3775.1±284.9
FS	103.77±8.25*#	9.54±1.37*#	4230.5±300.7*#	3211.3±242.8*#
Intervention+FS	115.81±11.07#&	7.21±1.37#&	4443.8±310.6#&	3588.2±263.7#&

* P<0.05, compared with Sham group; # P<0.05, compared with experimental group; & P<0.05, compared with Intervention group.

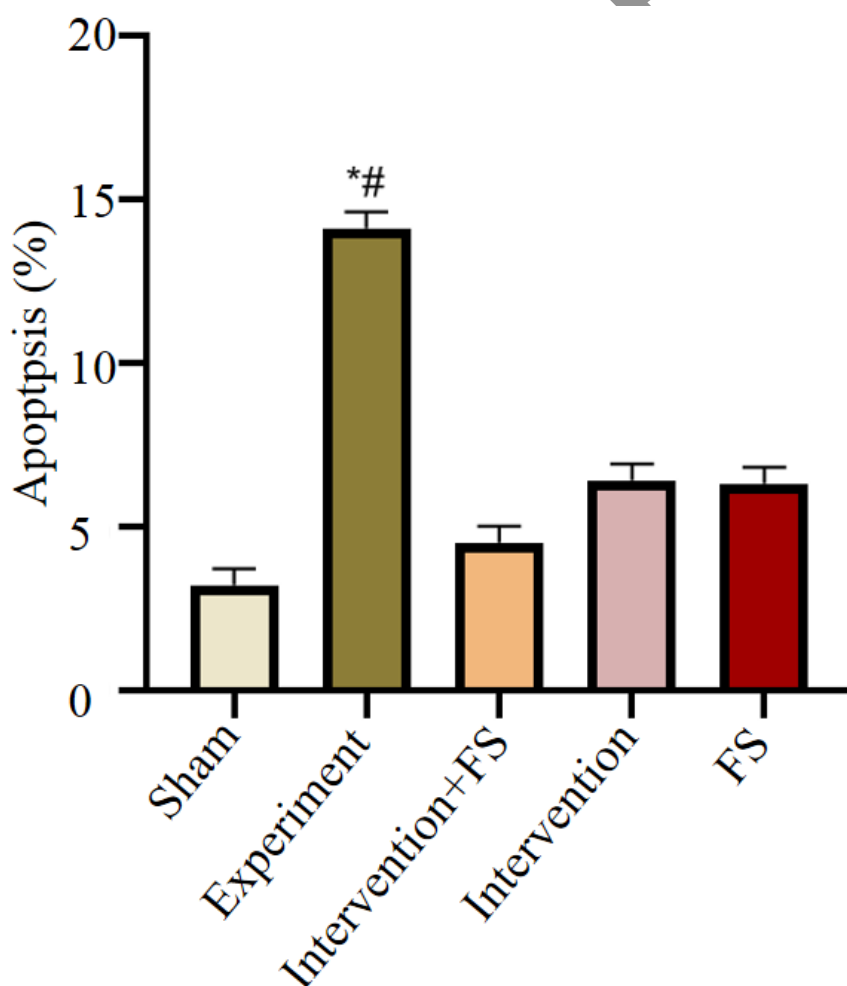


Fig. 2: Cardiomyocyte Apoptosis Index. * P < 0.05, compared with Sham group; # P < 0.05, compared with experimental group.

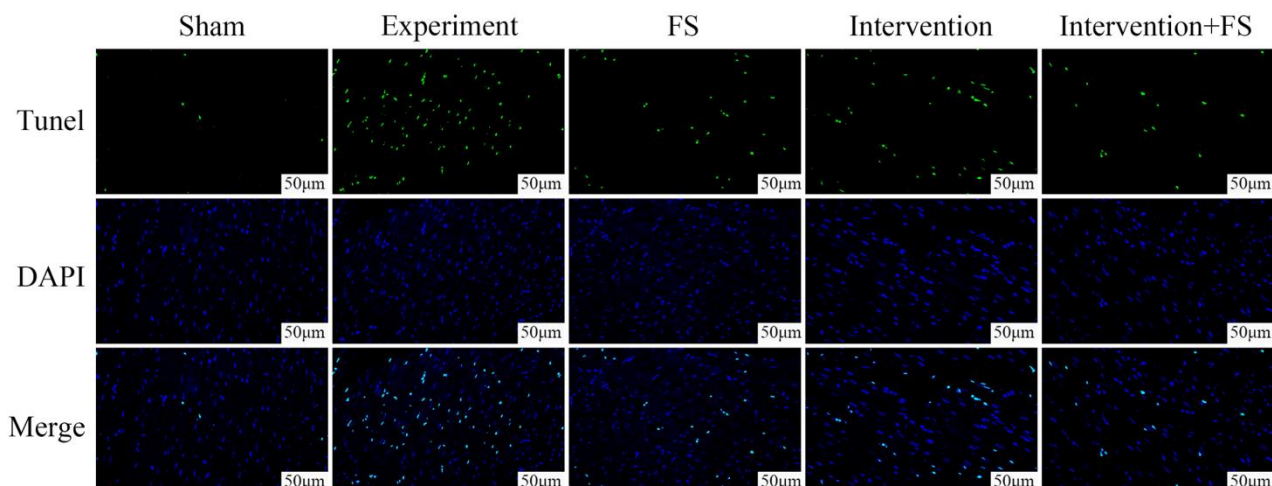


Fig. 3: Representative images of cardiomyocyte apoptosis in each group (TUNEL staining, $\times 400$).

Table 2: Comparison of Bcl-2, Bax, Rho, ROCK1 protein expression and p-MYPT1/MYPT1 ratio in myocardial tissue among rat groups. ($\bar{x} \pm s$)

Protein	Sham	Intervention	Experiment	FS	Intervention+FS
Bcl-2	1.00 $\pm$ 0.13	0.43 $\pm$ 0.07*	0.74 $\pm$ 0.06*#	0.72 $\pm$ 0.03*#	0.85 $\pm$ 0.10#&
Bax	1.00 $\pm$ 0.17	4.35 $\pm$ 0.28*	1.97 $\pm$ 0.23*#	2.07 $\pm$ 0.25*#	1.25 $\pm$ 0.11#&
Rho	1.00 $\pm$ 0.04	4.35 $\pm$ 0.43*	2.57 $\pm$ 0.47*#	2.69 $\pm$ 0.36*#	1.94 $\pm$ 0.27#&
ROCK1	1.00 $\pm$ 0.05	2.57 $\pm$ 0.27*	1.68 $\pm$ 0.18*#	1.75 $\pm$ 0.27*#	1.22 $\pm$ 0.12#&
p-MYPT1/MYPT1	0.12 $\pm$ 0.02	0.75 $\pm$ 0.08*	0.53 $\pm$ 0.08*#	0.59 $\pm$ 0.06*#	0.41 $\pm$ 0.05#&

* $P < 0.05$, compared with Sham group; # $P < 0.05$, compared with experimental group; & $P < 0.05$, compared with Intervention group.

These findings indicate that both 3-n-butylphthalide and the ROCK inhibitor can inhibit cardiomyocyte apoptosis following myocardial infarction and their combined application produces a synergistic effect.

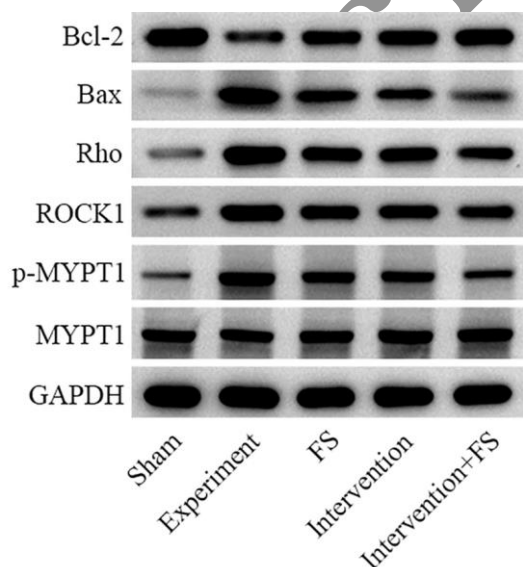


Fig. 4: Western blot bands of Bcl-2, Bax, Rho, ROCK1 and p-MYPT1/MYPT1 protein expression in myocardial tissue among rat groups.

The expression levels of Bcl-2, Bax, Rho kinase and its downstream proteins in myocardial cells

Western Blot results demonstrated that compared with the Sham group, the expression level of the pro-apoptotic protein Bax was significantly increased, while the expression level of the anti-apoptotic protein Bcl-2 was significantly decreased in the myocardial tissue of the Experiment group ($P < 0.05$). Compared with the Experiment group, the expression levels of Bcl-2 protein were significantly increased, the expression levels of Bax protein were significantly decreased and the Bcl-2/Bax ratio was consequently increased in the myocardial tissue of the Intervention, FS and Intervention+FS groups ($P < 0.05$). The upregulation of Bcl-2 and the downregulation of Bax were significantly more pronounced in the Intervention+FS group than in the Intervention group ($P < 0.05$) (Table 2, Fig. 4).

Compared with the Sham group, the expression levels of Rho and ROCK1 proteins, as well as the phosphorylation level of their downstream substrate MYPT1 (p-MYPT1/MYPT1 ratio), were significantly increased in the myocardial tissue of the Experiment group ($P < 0.05$), indicating that the ROCK pathway was activated following myocardial infarction. Compared with the Experiment group, the expression levels of Rho and ROCK1 proteins and the p-MYPT1/total MYPT1 ratio

were significantly decreased in the myocardial tissue of the Intervention group and the inhibitor (FS) group ($P < 0.05$). The inhibitory effect on ROCK1 expression in the Intervention+FS group was comparable to that in the Intervention group ($P < 0.05$). However, the reduction in the p-MYPT1/total MYPT1 ratio was significantly greater in the Intervention+FS group than in the Intervention group ($P < 0.05$), suggesting that the intervention drug (NBP) and Fasudil synergistically inhibit ROCK kinase activity (Table 2, Fig. 4).

DISCUSSION

During the pathogenesis of acute myocardial infarction (AMI), coronary artery occlusion disrupts blood flow, leading to insufficient myocardial blood supply, ischemia and subsequent cardiomyocyte injury and apoptosis (Buonpane *et al.*, 2025). Current therapeutic strategies for AMI focus on enhancing the anti-apoptotic capacity of cardiomyocytes. The primary approach to salvage viable myocardium is the restoration of myocardial reperfusion in the shortest possible time. However, due to the nonspecific clinical manifestations of AMI and its rapid progression, patients often present beyond the optimal window for reperfusion therapy, limiting the potential for myocardial salvage. In this context, effectively inhibiting apoptosis of residual cardiomyocytes and delaying ventricular remodeling have emerged as critical strategies for improving clinical outcomes in patients with AMI.

Butylphthalate (3-n-butylphthalide, NBP) is a chemical compound originally extracted from celery seeds. It has been shown to significantly inhibit the expression of adhesion molecule-1 and protease-activated receptor-1 in vascular endothelial cells following ischemia-reperfusion injury, thereby reducing vascular inflammation and thrombosis (Tian *et al.*, 2024). Additionally, NBP promotes neutrophil and inflammatory factor infiltration into ischemic lesions, reduces myocardial infarct size and enhances cellular function and metabolism (Hou and Brenner, 2024). Emerging evidence suggests that Rho kinase activity is upregulated during AMI and participates in the regulation of key signaling pathways involved in myocardial remodeling and heart failure (Miyahara *et al.*, 2022). Therefore, the present study established a rat model of AMI to investigate the effects of NBP on cardiac function, cardiomyocyte apoptosis and the Rho kinase signaling pathway.

In this study, AMI was induced by coronary artery ligation. Rats in the Model group exhibited typical manifestations of heart failure, including lethargy, reduced locomotor activity and respiratory distress. Histopathological examination revealed thinning of the left ventricular wall, disruption of myocardial architecture in the infarct zone and inflammatory cell infiltration, confirming successful model establishment.

Hemodynamic assessment further demonstrated that, compared with the Sham group, the Model group exhibited significantly decreased LVSP and $\pm dp/dt_{max}$, along with significantly increased LVEDP, indicating severe impairment of both systolic and diastolic cardiac function. These findings are consistent with previous reports that AMI induces massive cardiomyocyte necrosis, reduced myocardial contractility, decreased ventricular ejection fraction and elevated LVEDP (Xu *et al.*, 2024, Carberry *et al.*, 2025). Following NBP intervention, the general condition of AMI rats improved, myocardial tissue damage was alleviated and infarct size was reduced. Notably, NBP significantly increased LVSP and $\pm dp/dt_{max}$ while decreasing LVEDP, demonstrating a protective effect on cardiac pump and diastolic function. These results align with studies reporting that NBP improves neurological deficits following cerebral ischemia (Liu *et al.*, 2024), suggesting that NBP may exert multi-organ protective effects through common mechanisms such as anti-apoptosis, anti-inflammation and mitochondrial protection.

Our study further demonstrated that cardiomyocyte apoptosis was significantly reduced in the NBP-treated groups. These findings indicate that while AMI induces substantial cardiomyocyte apoptosis, NBP effectively inhibits this process, suggesting that its anti-apoptotic effect is a key mechanism underlying its cardioprotective properties. Previous studies have shown that both necrosis and apoptosis contribute to cardiomyocyte loss during ischemia, hypoxia and ischemia-reperfusion injury (Bo *et al.*, 2022). A previous study (Pan *et al.*, 2025) reported that cardiomyocyte necrosis leads to extensive cell loss after AMI, a process closely associated with apoptosis. Cardiomyocyte apoptosis has been identified as an independent factor contributing to the progressive expansion of infarct size following AMI (Chen *et al.*, 2025).

Apoptosis is an active, gene-controlled process of cell death. Multiple factors and signaling pathways regulate the expression of apoptosis-related genes and influence apoptotic progression. Studies have demonstrated that cardiomyocyte apoptosis involves various cytokines and signaling molecules, including Rho kinase, Bax and Bcl-2 (Yang *et al.*, 2025). To elucidate the mechanisms underlying myocardial apoptosis in AMI rats, we examined the expression of Bcl-2 and Bax proteins. The results showed that NBP significantly upregulated the anti-apoptotic protein Bcl-2 and downregulated the pro-apoptotic protein Bax, thereby increasing the Bcl-2/Bax ratio. Low Bcl-2 expression is closely associated with cardiomyocyte apoptosis (Li *et al.*, 2021). Bax, which is distributed in the cytoplasm, translocates to the mitochondrial membrane upon activation, where it binds to Bcl-2, alters mitochondrial membrane permeability,

induces mitochondrial damage and triggers apoptosis (Mao *et al.*, 2021).

The Rho kinase (RhoA/ROCK) signaling pathway plays a crucial role in myocardial remodeling and the progression of heart failure following AMI (Lu *et al.*, 2023). The present study confirmed that protein expression of RhoA and ROCK1, as well as the p-MYPT1/MYPT1 ratio—a marker of downstream substrate phosphorylation—were significantly increased in myocardial tissue of the Model group, indicating abnormal activation of the Rho kinase pathway. To investigate whether NBP exerts its protective effects through modulation of this pathway, we introduced the specific Rho kinase inhibitor fasudil as a positive control and established a combination therapy group. The results demonstrated that NBP monotherapy significantly reduced RhoA/ROCK1 expression and p-MYPT1 levels, with effects comparable to those of fasudil. These findings provide direct evidence that NBP inhibits Rho kinase pathway activity in the AMI model.

Furthermore, combined administration of NBP and fasudil produced superior outcomes in improving hemodynamic parameters, inhibiting cardiomyocyte apoptosis and increasing the Bcl-2/Bax ratio, along with a synergistic inhibitory effect on ROCK activity. Rho kinase is known to interfere with cell survival and its inhibition may protect cytoskeletal integrity and promote cell survival. Additionally, Rho kinase can reduce Bcl-2 activity, thereby promoting apoptosis and contributing to cardiovascular disease progression.

This study is the first to systematically evaluate the regulatory effect of NBP on the Rho kinase pathway and its downstream apoptosis-related proteins (Bcl-2, Bax) in an AMI model. The results demonstrate that NBP significantly reduces Rho kinase expression and upregulates the Bcl-2/Bax ratio, thereby inhibiting cardiomyocyte apoptosis. These findings expand the current understanding of the cardioprotective mechanisms of NBP, suggesting that it may exert myocardial protective effects through regulation of the Rho kinase-mediated apoptotic pathway. This provides a new experimental basis and theoretical support for the clinical application of NBP in the treatment of AMI.

However, this study has several limitations. First, reverse validation techniques such as Rho kinase overexpression or gene silencing were not employed; therefore, the necessity of Rho kinase in the mechanism of action of NBP could not be definitively established. Second, in addition to regulating the Bcl-2/Bax pathway, Rho kinase is involved in multiple downstream effector branches, including inflammation and fibrosis. Whether NBP exerts its effects through integration of multiple targets requires further investigation. Furthermore, the use of only male rats, while controlling for hormonal variability, limits the

generalizability of our findings to female populations, in whom AMI pathophysiology and outcomes may differ. Future studies incorporating both sexes are warranted. Future studies should combine genetic intervention approaches with more detailed temporal dynamic observations to further elucidate the upstream and downstream networks regulated by NBP in the Rho kinase pathway, thereby providing a more robust theoretical foundation for its clinical translation.

The findings of this study, obtained in a well-established rat model of AMI, provide mechanistic insights that may be translationally relevant to human AMI. The Rho kinase pathway is conserved across mammals and has been implicated in human heart failure and myocardial remodeling. The doses of NBP used in this study (2.25 mg/kg, twice daily) are within the range shown to be clinically safe and effective in human ischemic stroke trials (phase III, 200 mg twice daily), suggesting a reasonable translational window. However, the rat AMI model involves acute ligation of a healthy coronary artery in young animals, which differs from human AMI that typically occurs in aged individuals with atherosclerotic disease. Therefore, further validation in large animal models and in the presence of comorbidities (e.g., diabetes, hyperlipidemia) is needed prior to clinical translation.

CONCLUSION

In conclusion, 3-n-butylphthalide inhibits the Rho kinase pathway by reducing the protein expression of RhoA and ROCK1, as well as the level of their downstream substrate p-MYPT1/MYPT1. Concurrently, it upregulates the anti-apoptotic protein Bcl-2 and downregulates the pro-apoptotic protein Bax. Through these mechanisms, it inhibits cardiomyocyte apoptosis, thereby significantly ameliorating myocardial pathological damage and cardiac dysfunction in AMI rats. These findings provide a new experimental basis for its clinical application.

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Author's contributions

Xi Feng: Research conception and design, experimental operation, data collection and organization, statistical analysis, drafting of the manuscript.

Xiurong Wang: Participation in animal model establishment, hemodynamic measurements, data acquisition and analysis.

Yonghua Zhang: Participation in molecular biology experiments (Western blot, TUNEL staining), data organization and figure preparation.

Huaming Zhang: Overall study supervision, review of experimental design, manuscript revision and final approval, funding support.

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Data availability statement

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethical approval

This study was approved by the Animal Ethics Committee of Liyuan Hospital (Approval No.: TJ-IRB202509063). This study was performed in adherence with the ARRIVE guidelines. See supplementary file for the ARRIVE checklist.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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