



Article

Construction of Curcumin-Loaded Mesenchymal Stem Cell-Derived Exosomes and Their Mechanism in Inhibiting Pyroptosis During Hepatic Ischemia–Reperfusion Injury

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Abstract

Objective: Hepatic ischemia–reperfusion injury (HIRI) is a common pathological condition in liver surgery and transplantation, and cellular pyroptosis plays a key role in its pathogenesis. However, the clinical application of curcumin is limited by its poor water solubility and low bioavailability. This study aims to develop mesenchymal stem cell (MSC)-derived exosomes loaded with curcumin (Exo-Cur). It also investigates the role and mechanism of Exo-Cur in inhibiting HIRI-related cellular pyroptosis. **Methods:** The preparation of Exo-Cur was optimized using orthogonal experimental design. Its solubility, stability, particle size distribution, and zeta potential were then evaluated. The morphology of Exo-Cur and its uptake in hepatocytes were observed using laser scanning confocal microscopy. The effect of Exo-Cur on HIRI was assessed through hematoxylin and eosin (HE) staining, ALT and AST measurements, TUNEL assay, CCK-8 assay, and lactate dehydrogenase (LDH) assay. Inflammatory cytokine protein levels were quantified by ELISA, and their mRNA expression was assessed by qRT-PCR. Pyroptosis was assessed by Western blot, immunohistochemistry, and flow cytometry. Additionally, protein expression changes in the PI3K/Akt/mTOR signaling pathway were analyzed using Western blot. **Results:** Orthogonal experiments determined that the optimal preparation method for Exo-Cur involves cell density at 95%, a curcumin concentration of 30 µg/mL, and a co-cultivation time of 12 h. Characterization results showed that Exo-Cur maintained its typical cup-shaped structure as well as stable particle size and zeta potential. Additionally, its water solubility and its stability in vitro were significantly improved compared to free curcumin. Further mechanistic studies indicated that Exo-Cur could ameliorate the abnormal morphology resulting from HIRI-induced hepatocyte pyroptosis, reduce the proportion of pyroptotic cells, and significantly downregulate the expression of NLRP3 inflammasome and downstream pyroptosis-related proteins (ASC, C-Caspase-1, GSDMD-N). Pathway analysis revealed that Exo-Cur activates the PI3K/Akt/mTOR axis, a pathway inhibited by HIRI. Moreover, rapamycin, an inhibitor of this pathway, reverses Exo-Cur's anti-pyroptosis effect. **Conclusions:** This study develops an efficient and stable Exo-Cur delivery system, confirming its protective effect

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against HIRI by activating the PI3K/Akt/mTOR axis and inhibiting NLRP3-mediated cellular pyroptosis. This innovative combination of MSC-derived exosomes combined with curcumin overcomes the limitations in clinical application of curcumin, such as poor bio-availability and stability, and offers a novel nanotherapeutic strategy to treat HIRI clinically.

Keywords: curcumin; mesenchymal stem cell; exosomes; pyroptosis; hepatic ischemia–reperfusion injury

1. Introduction

Hepatic ischemia–reperfusion injury (HIRI) is a common pathological process in liver surgery, transplantation, and liver trauma, that may lead to severe liver dysfunction and even multi-organ failure. Clinically, HIRI occurs in approximately 20–30% of patients undergoing liver resection, and its incidence is as high as 40–60% in liver transplant recipients; of these patients, 5–10% develop severe post-operative liver failure, resulting in a mortality rate of over 50% [1]. During ischemia, liver tissue is damaged due to hypoxia. After reperfusion, the excessive production of oxygen free radicals, activation of inflammatory responses, and initiation of cell death pathways further exacerbate tissue injury [2]. In the complex pathophysiology of HIRI, pyroptosis, a form of programmed cell death mediated by inflammatory caspases, involves activation of the NLRP3 inflammasome and the caspase-1, caspase-4, caspase-5, and caspase-11 pathways. This activation leads to cleavage of gasdermin D (GSDMD), resulting in cell rupture and the release of pro-inflammatory cytokines such as IL-1 β and IL-18. Consequently, liver tissue damage and inflammatory responses are exacerbated. Among liver cells, Kupffer cells, resident macrophages, are the first to activate the NLRP3 inflammasome by sensing danger signals in the early stage of HIRI. This stimulation triggers pyroptosis and the release of pro-inflammatory cytokines that initiate the inflammatory response. Simultaneously, hepatocytes, as the main functional cells, undergo extensive pyroptosis driven by the inflammatory microenvironment, directly leading to cell loss and dysfunction. Research evidence has indicated that mice with knockout of Nlrp3, Casp1, or Gsdmd genes exhibit significantly reduced liver injury and inflammation in the HIRI model. Furthermore, myeloid cell- or hepatocyte-specific knockout experiments have confirmed the critical role of Kupffer cells in initiating inflammation and of hepatocytes in mediating damage progression. Recent targeted therapeutic advances, such as the use of NLRP3 inhibitors, caspase-1 inhibitors, and intervention strategies targeting GSDMD, have shown potential in preclinical animal models to alleviate HIRI. These findings highlight the pyroptosis pathway as an important therapeutic target for intervention [3–5]. Therefore, finding effective methods to inhibit cell pyroptosis is of significant clinical importance for alleviating liver ischemia–reperfusion injury.

Curcumin (Cur), a natural polyphenolic compound, exhibits remarkable anti-inflammatory and antioxidant properties and has been widely used in the treatment of various diseases. In liver injury models, curcumin significantly inhibits pyroptosis mediated by the inflammasome through the multi-targeted regulation of inflammatory signaling pathways. Studies have shown that curcumin can directly inhibit the assembly and activation of the NLRP3 inflammasome, downregulate the activity of caspase-1, and reduce the cleavage of GSDMD. This effectively blocks pyroptosis and alleviates inflammatory cell death and hepatocyte damage. Its mechanisms of action include scavenging reactive oxygen species (ROS) and inhibiting inflammatory cascades driven by mitochondrial dysfunction. Moreover, its effects are not limited to specific injury processes [6–8]. However,

the poor water solubility, low bioavailability, and rapid metabolism of curcumin limit its clinical application.

To overcome these challenges, the development of new drug-delivery platforms has become a key research focus. Exosomes are nanosized extracellular vesicles (30–150 nm in diameter) secreted by almost all cell types. They have attracted extensive attention as novel therapeutic and drug-delivery platforms in recent years. Unlike synthetic nanocarriers, exosomes possess inherent biological advantages, including excellent biocompatibility, low immunogenicity, and natural tissue tropism, which refers to their natural tendency to target specific tissues. Moreover, exosomes carry various bioactive molecules, including proteins, lipids, messenger RNAs (mRNAs), microRNAs (miRNAs), and long non-coding RNAs (lncRNAs). By transferring these molecules, they participate in inter-cellular communication, thus regulating the physiological and pathological processes of recipient cells [9].

In the field of drug delivery, exosomes can be engineered to carry small-molecule drugs, proteins, or nucleic acids. This approach overcomes the limitations of traditional drug delivery systems, including poor solubility, low bioavailability, and off-target effects. Notably, mesenchymal stem cell (MSC)-derived exosomes (Exo) offer distinct advantages for regenerative medicine and the treatment of inflammatory diseases. MSCs are multipotent stem cells with potent immunomodulatory, anti-inflammatory, and tissue repair properties. MSC-derived exosomes carry functional molecules that reflect the biological characteristics of MSCs, such as inhibiting excessive inflammatory responses, promoting cell proliferation and tissue regeneration, and regulating cell death pathways. These properties make them ideal candidates for treating inflammatory and degenerative diseases. Studies have confirmed that MSC-derived exosomes can alleviate tissue damage in various inflammatory conditions, such as acute kidney injury, myocardial ischemia–reperfusion injury, and inflammatory bowel disease, highlighting their broad therapeutic potential [10]. The exosomes secreted by MSCs contain abundant anti-inflammatory factors and regulatory molecules, which can inhibit Caspase-1 activation, reduce GSDMD cleavage, decrease the release of pro-inflammatory cytokines such as IL-1 β and IL-18, and suppress pyroptosis [11].

Despite the encouraging progress in exosome-based drug delivery and the therapeutic potential of curcumin and MSC-derived exosomes in inflammatory diseases, there remains a significant knowledge gap in the field of HIRI treatment. Specifically, the application of curcumin-loaded mesenchymal stem cell-derived exosomes (Exo-Cur) in HIRI has rarely been reported. Moreover, the underlying molecular mechanism by which Exo-Cur exerts its protective effect, especially its regulatory role in pyroptosis, remains to be systematically investigated. Although some studies have suggested that MSC-derived exosomes or curcumin alone can inhibit pyroptosis [12], the synergistic effect of combining curcumin with MSC-derived exosomes and the specific signaling pathways involved in pyroptosis inhibition remain poorly understood. Furthermore, the preparation method of Exo-Cur and its physicochemical properties, which are crucial for its therapeutic efficacy, need to be thoroughly characterized. Therefore, this study aims to construct an efficient and stable Exo-Cur delivery system and systematically evaluate its physicochemical properties and cellular uptake efficiency. Additionally, it will explore its protective effect and potential mechanism in inhibiting pyroptosis in a HIRI model. By focusing on the regulatory role of the PI3K/Akt/mTOR signaling pathway in Exo-Cur-mediated pyroptosis inhibition, this study intends to fill the existing knowledge gap and provide a novel and effective nano-therapeutic strategy for the clinical treatment of HIRI.

Therefore, the core objectives of this study are as follows: to construct and optimize a stable Exo-Cur delivery system and to characterize its physicochemical properties and cellular uptake efficiency; to evaluate the protective effect of Exo-Cur on HIRI, including

alleviation of liver injury, inhibition of inflammation, and reduction in pyroptosis, both in vivo and in vitro; to elucidate the mechanism by which Exo-Cur inhibits NLRP3-mediated pyroptosis through activation of the PI3K/Akt/mTOR pathway, thus revealing the molecular basis of its protective effect against HIRI.

2. Results

2.1. Orthogonal Experiments Determined the Optimal Preparation Method for Exo-Cur

Three-factor, three-level orthogonal experiments were conducted, as shown in Table 1. The total sum of encapsulation rates at different levels of each factor (K value) and the range—the difference between the maximum and minimum K values for each factor (R value)—were calculated. These values allow determination of the degree of influence of each factor on the encapsulation rate. A preliminary analysis of Table 2 showed that the R value for the concentration of curcumin was 8.55, the largest among the three factors, indicating its significant impact on the encapsulation rate. The R value for co-cultivation time was 5.43, indicating the second highest influence, whereas cell density had an R value of 4.63, the lowest among the three. At the optimal levels of each factor, cell density at level 2 (95% confluency) had a maximum K value of 102.30. Curcumin concentration at level 1 (30 µg/mL) had a maximum K value of 103.56. Co-cultivation time at level 1 (12 h) had a maximum K value of 105.04. Further variance analysis confirmed the significance of these factors, and the results in Table 3 showed that all three factors had a significant impact on the encapsulation rate of Exo-Cur. Therefore, the optimal formulation for Exo-Cur is A2B1C1, which corresponds to a cell density of 95% confluency, a curcumin concentration of 30 µg/mL, and a co-cultivation time of 12 h.

Table 1. Level table of orthogonal experimental factors.

Level	Factor		
	A Cell Density (%)	B Concentration (µg/mL)	C Co-Incubation Time (h)
1	85	30	12
2	95	40	24
3	100	50	36

Table 2. Visual analysis table of orthogonal experiment.

Test Number	A Cell Density (%)	B Concentration (µg/mL)	C Incubation Time (h)	Empty Train	Encapsulation Rate (%)
1	1	1	3	1	36.22
2	1	2	1	2	35.51
3	1	3	2	3	28.48
4	2	1	1	3	39.79
5	2	2	3	1	34.60
6	2	3	2	2	27.91
7	3	1	2	2	32.35
8	3	2	1	3	29.74
9	3	3	3	1	26.33
K1	100.21	108.36	105.04	97.15	
K2	102.30	99.85	88.74	95.77	
K3	88.42	82.72	97.15	98.01	
k1	33.40	36.12	35.01	32.38	
k2	34.10	33.28	29.58	31.92	
k3	29.47	27.57	32.38	32.67	
R	4.63	8.55	5.43	0.75	

Table 3. Analysis of variance of orthogonal experiment (* $p < 0.05$, ** $p < 0.01$).

Factor	Class III Sum of Squares	Degree of Freedom	Equal Square	F	<i>p</i> Value	Significance
A	37.34	2.00	18.67	43.86	0.022	*
B	113.70	2.00	56.85	133.56	0.007	**
C	44.30	2.00	22.15	52.03	0.019	*
Error	0.85	2.00	0.43			

2.2. Physicochemical Properties of Exo-Cur

The in vitro solubility detection results showed that the maximum solubility of Exo-Cur and free Cur was 1.69 µg/mL and 1.20 µg/mL, respectively, indicating that the addition of Exo improved the solubility of Cur (Figure 1A). The Exo-Cur and free Cur solutions were dissolved in PBS, protected from light, and incubated in a water bath at 37 °C for 30, 60, 90, and 120 min. Their stability was then measured using a spectrophotometer. The results indicated that the stability of both Exo-Cur and free Cur solutions decreased over time. Between the two solutions, the stability of Exo-Cur decreased to a lesser extent, with long-term stability about 10 percentage points higher than that of free Cur, indicating that the addition of Exo improved the stability of Cur (Figure 1B). Transmission electron microscopy (TEM) images revealed that the morphology and size of exosomes were uniform, presenting a typical cup-shaped structure, with uniform electron density, intact vesicular structure, and clear edges. After curcumin loading, the electron density of exosomes was slightly lower, and the surface appeared rougher; however, their morphology and size did not change significantly, indicating that curcumin loading did not markedly affect the stability of exosomes (Figure 1C). Studies have shown that the Zeta potential of exosomes ranges from approximately 0 to −70 mV, and the potential of exosomes does not change much (10 to −70 mV), indicating that the surface charge was essentially unaffected (Figure 1D). Particle size detection showed that the average particle size of Exo was 76.81 nm, while that of Exo-Cur was 81.70 nm (Figure 1E). Western blot showed that both the Exo and Exo-Cur groups exhibited a consistent and typical exosomal protein expression profile: TSG101, CD81, CD63, and CD9 were all positively expressed, and there was no significant difference in signal intensity between the two; in contrast, Calnexin did not exhibit any significant signal, consistent with the negative standard. Therefore, these results indicate that loading curcumin did not alter the expression of characteristic surface and internal sorting proteins of exosomes (Figure 1F). From the above results, it can be concluded that the basic properties of exosomes were not affected by curcumin loading.

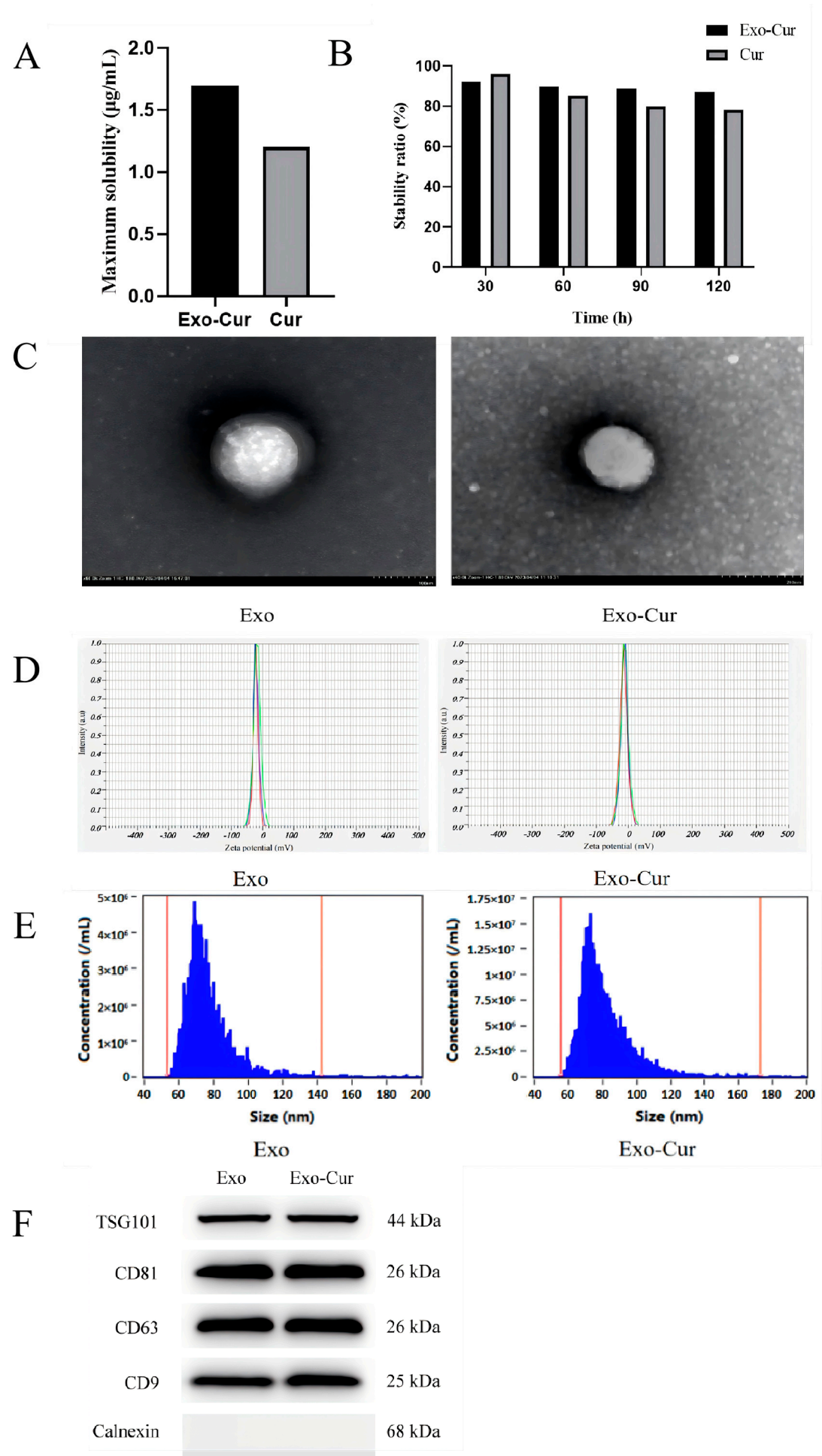


Figure 1. Physical and chemical properties of curcumin-loaded exosomes. (A) Maximum solubility of Exo-Cur and free Cur; (B) Stability ratio of Exo-Cur and free Cur in vitro; (C) Morphology of exosomes; (D) Average particle size distribution diagram; (E) Zeta potential diagram; (F) The expression levels of the marker proteins from EXO and EXO-CUR).

2.3. Hepatocyte Uptake of Exo-Cur

To clarify the uptake of Exo-Cur in hepatocytes, this study labeled the exosomal membrane with PKH26, a red fluorescence dye, and curcumin with Cy2, a green fluorescence dye. Cell nuclei were stained with DAPI, which fluoresces blue. The intracellular fluorescence distribution was then observed using a laser scanning confocal microscope. The red fluorescence signal from Exo was localized predominantly in the cytoplasm of hepatocytes, exhibiting a scattered granular pattern. The green fluorescence signal from curcumin colocalized extensively with the red fluorescence signal of Exo. This colocalization indicates that Exo-Cur was effectively taken up by mouse hepatocytes and localized within the cytoplasm (Figure 2).

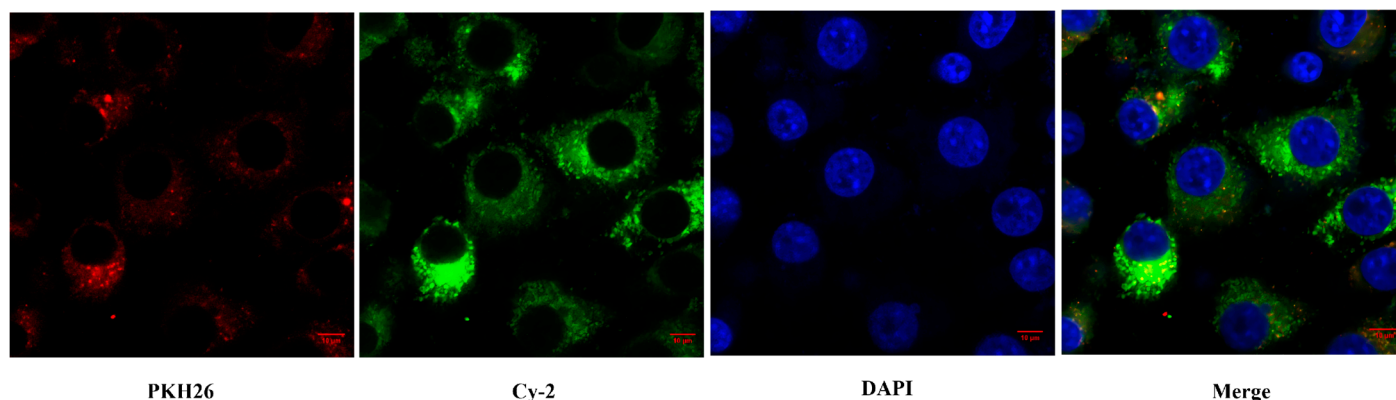


Figure 2. Observation of Hepatocyte Uptake of Exo-Cur Using Laser Confocal Microscopy.

2.4. The Improvement Effect of Exo-Cur on HIRI

HE staining results showed that the Sham group had an intact liver lobule structure, an orderly arrangement of hepatocytes, and no significant degeneration, necrosis, or infiltration of inflammatory cells in hepatocytes. In contrast, the HIRI group exhibited significant pathological changes, including disordered liver lobule structure, widespread degeneration and necrosis of hepatocytes, extensive infiltration of inflammatory cells, and significant dilation and congestion of liver sinusoids. After Exo-Cur treatment, the extent of hepatocyte degeneration and necrosis was reduced, and inflammatory cell infiltration decreased, indicating improvement relative to the HIRI group (Figure 3A). To further validate these results, serum ALT and AST levels were measured. Serum ALT and AST levels were significantly elevated in the HIRI group compared to the Sham group ($p < 0.01$). After Exo-Cur intervention, the levels of ALT and AST were significantly reduced compared to the HIRI group ($p < 0.01$), and the improvement effect of Exo-Cur was better than that of either the curcumin or Exo groups alone (Figure 3B).

To investigate hepatocyte DNA damage caused by HIRI, TUNEL experiments were conducted. The results showed that the proportion of TUNEL-positive cells in the Sham group was minimal. In contrast, the proportion of TUNEL-positive cells in the HIRI group was significantly increased, indicating that HIRI causes extensive DNA damage in hepatocytes. After Exo-Cur treatment, the proportion of TUNEL-positive cells was significantly lower than that in the HIRI group ($p < 0.01$), confirming that Exo-Cur effectively alleviates HIRI-mediated hepatocyte DNA damage (Figure 3C).

The release of lactate dehydrogenase (LDH) is a well-established indicator of the degree of hepatocyte membrane damage. The results showed that LDH release in the HIRI group was significantly increased compared to the Sham group ($p < 0.01$). In contrast, LDH release in the Exo-Cur group was significantly reduced compared to the HIRI group, indicating that Exo-Cur could effectively alleviate HIRI-induced hepatocyte membrane damage (Figure 3D). Results from the CCK8 assay showed that the viability of hepatocytes in the HIRI group was significantly decreased compared to the Sham group. Following Exo-Cur treatment, cell viability significantly increased ($p < 0.01$), indicating that Exo-Cur could promote the proliferation and repair of hepatocytes after HIRI (Figure 3E).

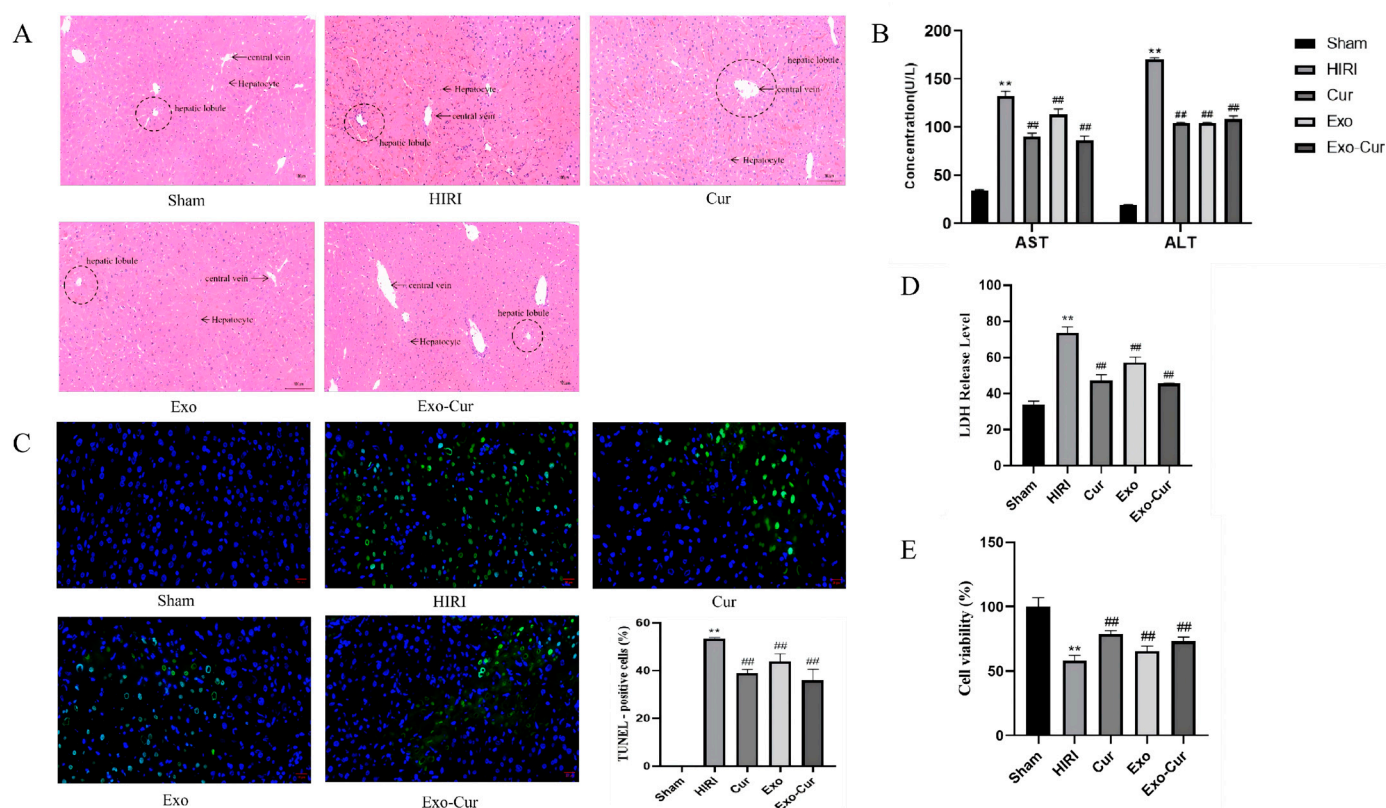


Figure 3. The improvement effect of Exo-Cur on HIRI. (A) HE tissue staining (×200); (B) Serum levels of ALT and AST; (C) TUNEL staining of liver tissue (×400); (D) Effects of different treatment groups on LDH levels; (E) CCK8 assay was used to detect cell viability; Compared with the sham group: ** $p < 0.01$; Compared with the HIRI group: ## $p < 0.01$).

2.5. Exo-Cur Inhibits Inflammatory Response in HIRI Mice

The levels of inflammatory factors in mouse serum were measured using ELISA kits. After liver injury, the expression levels of IL-18, IL-1 β , and TNF- α significantly increased ($p < 0.01$), while Exo-Cur treatment significantly inhibited the expression of these inflammatory factors (Figure 4A). Similarly, following liver injury, the mRNA expression levels of inflammatory factors IL-6, IL-1 β , and TNF- α increased, whereas those of anti-inflammatory factors IL-4, IL-9, and IL-10 significantly decreased. After Exo-Cur treatment, the expression of inflammatory factors decreased. Meanwhile, the expression of anti-inflammatory cytokines significantly increased ($p < 0.01$), indicating a partial restoration of the anti-inflammatory response (Figure 4B).

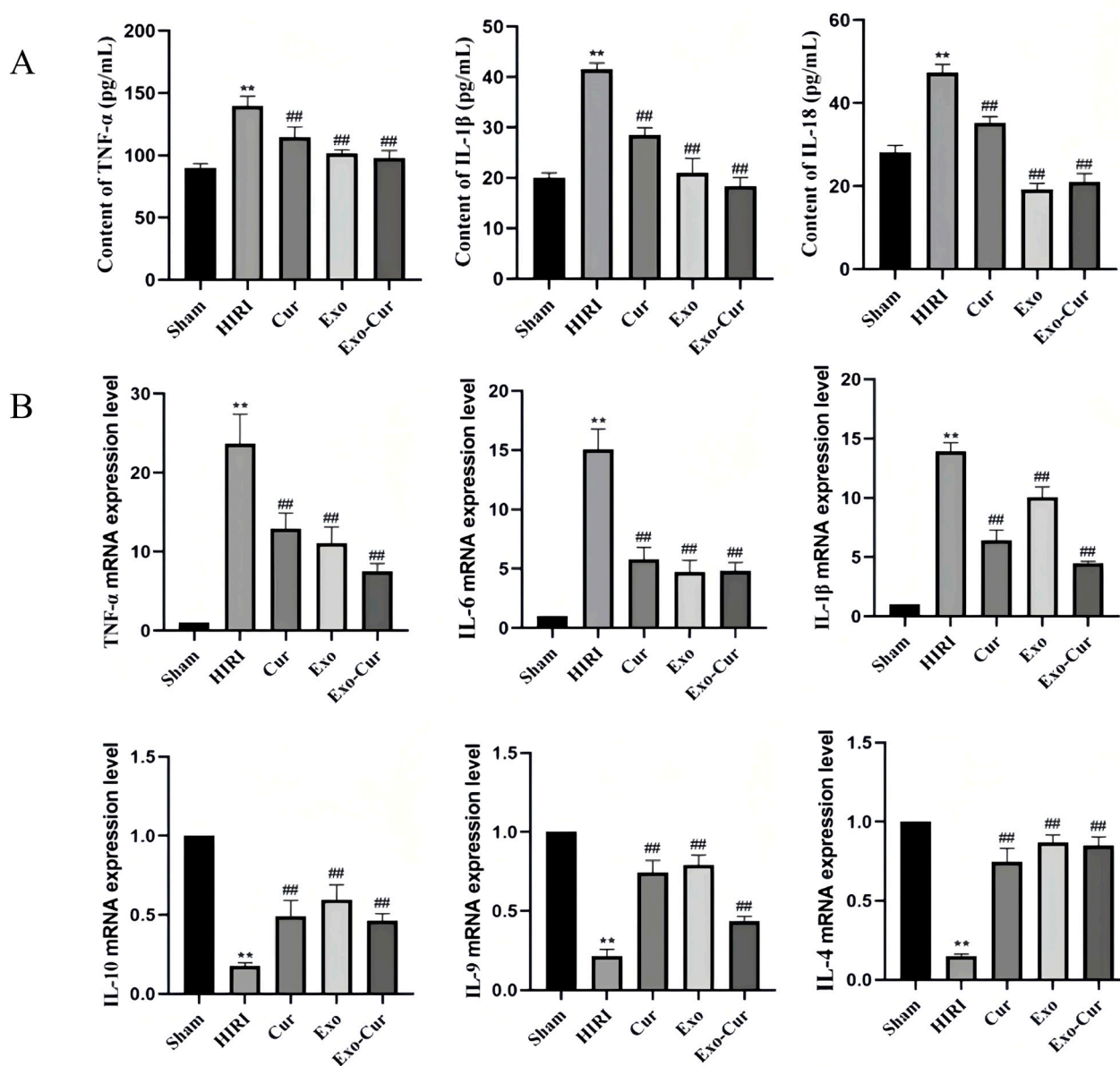


Figure 4. Inhibitory effect of Exo-Cur on the inflammatory response in HIRI mice. (A) Levels of IL-18, IL-1β, and TNF-α in serum; (B) mRNA expression levels of various genes in liver tissue. Compared with the sham group: ** $p < 0.01$; Compared with the HIRI group: ## $p < 0.01$).

2.6. Exo-Cur Inhibits Pyroptosis

Laser confocal microscopy observations revealed that hepatocytes in the Sham group maintained a regular morphology and intact contours, indicative of normal cellular homeostasis. In sharp contrast, hepatocytes in the HIRI group displayed hallmark morphological features of pyroptosis—marked cellular swelling, disrupted membrane integrity, and abundant membrane blebbing—all reflecting the lytic cell death characteristic of pyroptosis, triggered by ischemia–reperfusion stress (Figure 5A). Treatment with Exo-Cur notably ameliorated these pathological changes. This was evidenced by reduced cellular edema, diminished membrane blebbing, and restoration of regular cellular contours, highlighting its capacity to preserve hepatocyte structural integrity against HIRI-induced pyroptotic damage.

To quantify pyroptosis, we performed Cleaved Caspase-1/GSDMD-N dual staining and used pyroptosis-specific markers, followed by flow cytometry analysis. The HIRI group showed a substantial increase in both the number of Caspase-1/GSDMD-N positive

cell clusters and the density of positive cells within these clusters, indicating a robust induction of pyroptosis. This observation is consistent with the morphological evidence of pyroptotic activation after injury. Conversely, Exo-Cur treatment led to a dramatic reduction in the proportion of pyroptotic cells, resulting in a marked decrease in the detection of positive cell clusters. These findings provide strong evidence that Exo-Cur exerts a potent inhibitory effect on HIRI-driven hepatocyte pyroptosis (Figure 5B).

Immunohistochemical staining for NLRP3 showed sparse, uniformly distributed positive signals in the form of brown granules in liver tissues of the Sham group. This reflects basal NLRP3 expression under physiological conditions. Marked upregulation of NLRP3-positive staining in the HIRI group, evidenced by increased signal intensity, indicates activation of the NLRP3 inflammasome, a key molecular event in HIRI pathogenesis. Treatment with Exo-Cur (a combination of Exo and Cur, previously defined) significantly attenuated NLRP3 expression, with a more pronounced inhibitory effect than either Exo or Cur monotherapy alone, indicating a synergistic action of Exo and Cur in targeting NLRP3 (Figure 5C).

Western blot analyses provided additional insights into the molecular basis of Exo-Cur's anti-pyroptotic activity. Compared with the Sham group, HIRI hepatocytes exhibited markedly increased expression of core pyroptosis-related proteins, including NLRP3, ASC, cleaved Caspase-1, and GSDMD-N. These proteins are hallmarks of full NLRP3 inflammasome activation and the downstream execution of pyroptosis. Exo-Cur intervention significantly downregulated the expression of all these proteins, with the most pronounced reduction observed in GSDMD-N, the terminal effector mediating pyroptotic cell lysis. Collectively, these data demonstrate that Exo-Cur effectively attenuates HIRI-induced pyroptosis by suppressing NLRP3 inflammasome activation and the subsequent execution of the pyroptotic signaling cascade (Figure 5D).

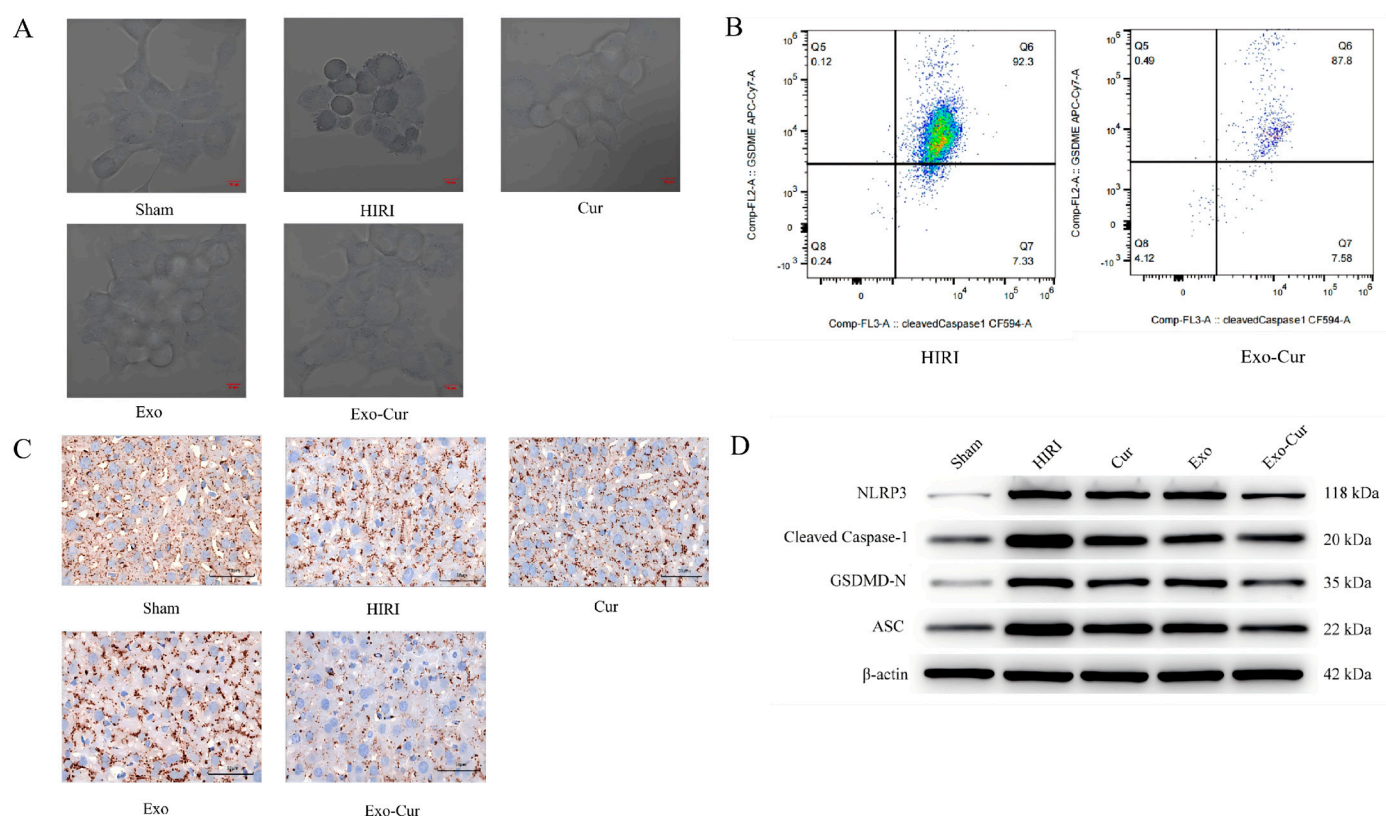


Figure 5. Exo-Cur inhibits pyroptosis of cells. (A) Observation of pyroptotic cell morphology by laser confocal microscopy ($\times 630$); (B) Flow cytometric analysis; (C) Immunohistochemical staining

results of NLRP3 protein in liver tissues ($\times 200$); (D) Expression levels of key proteins involved in pyroptosis in cells.

2.7. Exo-Cur Inhibits Pyroptosis by Upregulating the PI3K/Akt/mTOR Axis

To elucidate the molecular mechanism by which Exo-Cur inhibits pyroptosis and ameliorates HIRI, this study further explored its regulatory relationship with the PI3K/Akt/mTOR signaling axis. Western blot analyses demonstrated that the liver tissues of the Sham group showed basal activation of the PI3K/Akt/mTOR pathway. This was indicated by consistent phosphorylation of PI3K, Akt, and mTOR, along with unchanged expression levels of their total protein counterparts. By contrast, HIRI exposure led to significant reductions in the phosphorylation levels of PI3K, Akt, and mTOR compared to the Sham group. These results indicate suppression of the pathway in response to hepatic ischemia–reperfusion injury, a condition that likely disrupts pro-survival signaling cascades and thereby sensitizes hepatocytes to pyroptotic cell death. The restoration of p-PI3K, p-Akt, and p-mTOR expression in the Exo-Cur-treated group strongly suggests that Exo-Cur reverses the HIRI-mediated suppression of the PI3K/Akt/mTOR axis, providing a mechanistic basis for its anti-pyroptotic and hepatoprotective effects (Figure 6).

Since Exo-Cur restores the phosphorylation levels of these signaling proteins, hepatocytes were pre-treated with rapamycin, a specific inhibitor of mTOR, to confirm the pathway dependency of Exo-Cur's biological activity. Subsequent Western blot assays revealed that combined treatment with Exo-Cur and rapamycin abrogated the Exo-Cur-induced upregulation of p-PI3K, p-Akt, and p-mTOR, and reversed Exo-Cur's inhibitory effect on hepatocyte pyroptosis (Figure 6). Collectively, these results support that Exo-Cur exerts its anti-pyroptotic effects in HIRI in a manner dependent on PI3K/Akt/mTOR signaling, underscoring the critical role of this pathway as a therapeutic target for mitigating ischemia–reperfusion-induced hepatic damage.

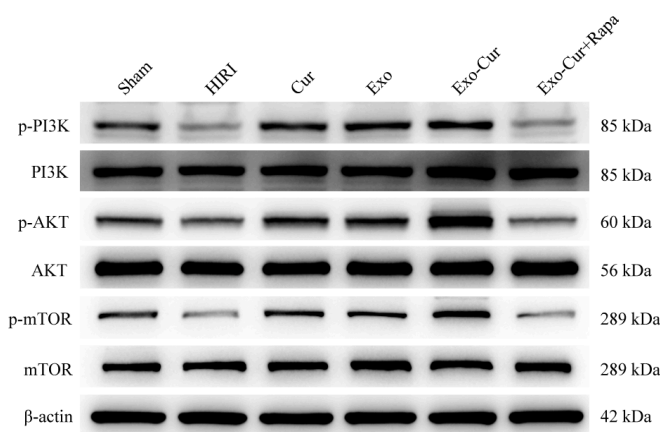


Figure 6. Protein expression levels in the PI3K/Akt/mTOR signaling pathway in different treatment groups.

3. Discussion

This study developed a curcumin-loaded bone marrow mesenchymal stem cell-derived exosome (Exo-Cur) nanomedicine formulation and systematically evaluated its therapeutic effects and molecular mechanisms in a HIRI model. The results indicated that Exo-Cur not only effectively alleviated hepatocyte injury and inflammatory responses but also significantly inhibited NLRP3 inflammasome-mediated pyroptosis. This inhibition was achieved by activating the PI3K/Akt/mTOR signaling pathway, thereby exerting protective effects. The core novel findings of this study are threefold: First, we developed a

MSC-derived exosome-based curcumin delivery system (Exo-Cur) with optimized preparation parameters. This system not only overcomes the inherent limitations of curcumin (poor water solubility, low bioavailability) but also exhibits superior biocompatibility and in vivo stability compared to synthetic nano-curcumin carriers (e.g., gold nanoparticles, liposomes). Second, we clarified that Exo-Cur exerts synergistic protective effects against HIRI by integrating the anti-inflammatory activity of curcumin and the biological regulatory functions of MSC exosomes, and these effects are superior to those of curcumin or exosomes alone. Most importantly, we established a direct causal link between PI3K/Akt/mTOR pathway activation and NLRP3-mediated pyroptosis inhibition in Exo-Cur therapy. This reveals the unique molecular mechanism of Exo-Cur in HIRI protection that has not been reported previously. This finding not only provides a novel nanomedicine strategy for the clinical treatment of HIRI but also enhances our understanding of the complex relationship between programmed cell death and inflammatory regulatory networks.

In terms of formulation development and optimization, this study identified the optimal preparation parameters for Exo-Cur through systematic single-factor experiments and orthogonal experimental design, including cell density, curcumin concentration, and incubation time. Orthogonal analysis revealed that all three factors significantly affected the encapsulation efficiency, ultimately resulting in a formulation with high efficiency and good stability. Furthermore, transmission electron microscopy revealed that the exosomes retained typical cup-shaped morphology after loading with curcumin. They exhibited a mean particle size of approximately 80 nm, consistent with the exosome identification standards published by the International Society for Extracellular Vesicles (ISEV) [13]. The natural phospholipid bilayer structure of exosomes not only encapsulates effectively hydrophobic curcumin molecules but also protects them from rapid degradation and metabolism in the bloodstream, thereby significantly improving the bioavailability and targeted delivery efficiency of curcumin [14,15]. Compared with other nano-curcumin delivery systems, including liposomes and gold nanoparticles, Exo-Cur, an exosome-based curcumin delivery system, offers several distinct advantages. Unlike synthetic gold nanoparticles, which can exhibit cytotoxicity at high doses, MSC-derived exosomes are inherently biocompatible and possess the ability to home to specific tissues [16,17]. Additionally, compared with liposomal curcumin, Exo-Cur demonstrates stronger resistance to serum protein adsorption, thereby prolonging its in vivo circulation time [18]. Recently, using stem cell-derived exosomes as drug carriers has gained attention due to their low immunogenicity, high biocompatibility, and inherent ability to home to specific tissues [19,20]. In this study, Exo-Cur exhibited an approximately 10% greater long-term stability compared to free curcumin in PBS, likely attributable to the stabilizing effect of the exosomal membrane on curcumin. This enhanced stability is consistent with previous findings regarding curcumin-loaded gold nanoparticles, where the nanocarrier encapsulation reduced curcumin degradation and improved the persistence of curcumin both in vitro and in vivo [18]. Furthermore, integrins and adhesion molecules are expressed on the exosomal surface and may mediate interactions with damaged endothelial cells. This interaction thereby enhances accumulation in the ischemic regions of the liver [21]. These characteristics collectively establish Exo-Cur as a promising nanotherapy platform. It not only overcomes the limitations of curcumin itself but also exploits the paracrine functions of exosomes to produce synergistic therapeutic effects, such as improved targeting and enhanced tissue repair.

Pharmacological evaluation showed that Exo-Cur demonstrated significant protective effects in the HIRI mouse model. There was a significant reduction in serum levels of liver enzymes ALT and AST. Additionally, lactate dehydrogenase (LDH) release de-

creased, indicating that Exo-Cur effectively protects the integrity and function of hepatocyte membranes [22]. Importantly, the efficacy of Exo-Cur was significantly greater than that of curcumin or exosomes alone, suggesting synergy between the two. This synergism between curcumin, a plant-derived bioactive compound, and nanocarriers (MSC exosomes) aligns with recent reports that combining phytochemicals with nanovehicles can amplify therapeutic outcomes by enhancing delivery efficiency and activating complementary biological pathways [23]. This synergistic effect may result from both the targeted delivery of curcumin by exosomes and the combined action of bioactive molecules, such as miRNAs and proteins, within them [24]. For instance, MSC-derived exosomes are known to contain various microRNAs with anti-inflammatory and anti-apoptotic effects [24], including miR-124 and miR-181c, which may enhance anti-pyroptosis effects by regulating inflammatory pathways such as NF- κ B [25]. Furthermore, Exo-Cur treatment significantly improved the pathological changes in liver tissue by reducing inflammatory cell infiltration and hepatocyte necrosis. These morphological improvements were highly consistent with the changes in biochemical indicators [26]. Regarding inflammatory regulation, Exo-Cur inhibited the expression levels of pro-inflammatory factors—TNF- α , IL-1 β , IL-6, and IL-18—and promoted the generation of anti-inflammatory factors—IL-4, IL-9, and IL-10—indicating its ability to modulate the immune microenvironment balance in damaged livers [27,28]. This effect is crucial because excessive inflammatory responses during HIRI not only directly cause damage but also activate various cell death pathways, forming a vicious cycle [29,30]. The ELISA and RT-qPCR results of this study confirmed that Exo-Cur precisely regulated the inflammatory network by downregulating pro-inflammatory gene transcription and upregulating anti-inflammatory gene expression [31].

The core finding of this study is the elucidation of how Exo-Cur inhibits pyroptosis and its molecular mechanism. Pyroptosis, as a form of programmed cell death that has received increasing attention in recent years, plays an increasingly well-defined role in HIRI [32,33]. Using laser confocal microscopy, we confirmed that Exo-Cur could significantly alleviate the pyroptotic morphological features of hepatocytes, such as cell swelling and membrane bubble-like protrusions. Flow cytometry quantitative analysis further showed that Exo-Cur treatment reduced the proportion of pyroptotic cells from the significantly elevated levels observed in the HIRI group to near normal levels. Moreover, the effect was superior to that of its individual components, highlighting the advantages of the nanomedicine. Western blot analysis further revealed that Exo-Cur could downregulate the expression levels of key pyroptosis pathway proteins NLRP3, ASC, cleaved Caspase-1, and GSDMD-N. These proteins collectively constitute the core pathway of NLRP3 inflammasome activation: NLRP3 acts as a sensor protein, ASC as a connector protein, and caspase-1 as an effector protein, whose activation leads to GSDMD cleavage and the execution of pyroptosis [25,32].

Pyroptosis can be divided into classical and non-classical pathways based on the activation mechanism. The classical pathway relies on the activation of the NLRP3 inflammasome, leading to the activation of caspase-1. Caspase-1 then cleaves GSDMD to form membrane pores and promotes the maturation of IL-1 β and IL-18. The non-classical pathway is directly activated by intracellular LPS, which in turn activates human caspase-4/5 or mouse caspase-11. This activation induces pyroptosis by cleaving GSDMD and may indirectly activate the classical pathway by triggering the efflux of potassium ions. The efflux of potassium ions then activates the NLRP3 inflammasome. In the context of liver diseases, the activation of both pathways shows pathological specificity. In particular, in the liver ischemia–reperfusion injury model, it is widely accepted that—in the absence of additional LPS stimulation—the classical pathway is the main mechanism mediating hepatocyte pyroptosis. Under this condition, the contribution of the non-classical pathway is relatively limited. Therefore, in studying pyroptosis in liver ischemia–reperfusion

injury, the present study focuses on the classical pathway of the NLRP3-caspase-1-GSDMD axis [34,35]. Notably, the most significant downregulation was observed for GSDMD-N, indicating that Exo-Cur may play a role in the final stages of GSDMD assembly and pore formation. Additionally, TUNEL experiments showed that Exo-Cur could reduce DNA damage, which is common in pyroptosis, as caspase-1 activation can lead to DNA fragmentation [36]. These results are consistent with other studies, which have shown that exosomes can inhibit pyroptosis by delivering anti-inflammatory molecules. These effects have been demonstrated in sepsis and acute liver injury models [24]. Lou et al. reported that MSC exosomes can alleviate liver fibrosis, and the mechanism is partly related to the inhibition of the NLRP3 inflammasome [20]. However, the innovation of this study lies in experimentally establishing a direct link between pyroptosis inhibition and PI3K/Akt/mTOR pathway activation, providing a new perspective for understanding the molecular basis of exosome therapy.

At the mechanistic level, this study systematically demonstrated for the first time the central role of the PI3K/Akt/mTOR signaling pathway in the pyroptosis inhibition mediated by Exo-Cur. The results indicated that HIRI inhibited the activation of the PI3K/Akt/mTOR pathway, as evidenced by a significant reduction in the levels of p-PI3K, p-Akt, and p-mTOR. Exo-Cur treatment effectively reversed this inhibition, resulting in a significant increase in the expression levels of these phosphorylated proteins. After treatment with the mTOR inhibitor rapamycin, the protective effect of Exo-Cur was completely abolished, confirming the necessity of this pathway [33,37]. The PI3K/Akt/mTOR pathway is a key intracellular survival signaling axis involved in regulating cell proliferation, metabolism, autophagy, and inflammatory responses [38]. Studies have shown that this pathway can negatively regulate pyroptosis through various mechanisms. First, Akt activation can inhibit the NF- κ B signaling pathway, thereby reducing the transcription of genes such as NLRP3 and pro-IL-1 β . Second, mTOR activation can induce autophagy, which clears damaged mitochondria (mtROS) and inflammasome components; this process thereby inhibiting NLRP3 assembly [39]. Zhong et al. found that NF- κ B limits inflammasome activation by clearing damaged mitochondria [36]. Additionally, the PI3K/Akt pathway can enhance antioxidant defenses by activating transcription factors such as Nrf2, alleviating oxidative stress, which is an important trigger for NLRP3 activation [40]. In this study, Exo-Cur may regulate this pathway via multiple mechanisms: curcumin itself is known to activate Akt, and MSC exosomes may also carry Akt-related regulatory molecules [41]. For instance, some studies have reported that miR-21 in exosomes can indirectly activate Akt by inhibiting PTEN [42]. However, this study indicates that the synergistic effect of Exo-Cur may also involve exosome-mediated intercellular communication. This communication potentially regulates the PI3K/Akt/mTOR activity of recipient cells, directly or indirectly, by delivering signaling molecules. This multi-target action may explain the advantages of Exo-Cur compared to individual components. Future studies could further elucidate the specific contributions of exosomal cargo, including proteins and nucleic acids, in this process [43].

Although this study achieved significant results, some limitations need to be addressed in future research. First, we used a mouse HIRI model, which is widely applied in the study of hepatic ischemia–reperfusion injury but may not fully simulate human hepatic ischemia–reperfusion injury. Human HIRI may involve more complex inflammatory networks and individual differences, especially in the context of chronic liver disease. The differences in immune responses, metabolic pathways, and gene expression between animal models and humans may limit the direct clinical translation of the results of this study [44]. Secondly, the challenge of exosome heterogeneity has not been fully resolved. Different batches of MSC exosomes may exhibit variability in size, composition, and function, which may affect the consistency and reproducibility of Exo-Cur [45]. Although we

characterized the formulation using a zeta potential measurement and transmission electron microscopy, more in-depth multi-omics analyses (such as proteomics and miRNA sequencing) may help ensure the quality of the formulation [46]. Additionally, this study primarily focused on the PI3K/Akt/mTOR pathway; however, pyroptosis may also be regulated by other signaling pathways, such as the TLR4/NF- κ B or MAPK pathways, and the roles of these pathways were not fully explored in this study [47]. For example, TLR4 signaling has been shown to induce pyroptosis in HIRI [48]; however, this study did not assess the potential interactions of these pathways. Another limitation is that we did not systematically evaluate the dose-dependent effects and long-term safety of Exo-Cur. Although short-term experiments showed that Exo-Cur had good tolerance, the potential immunogenicity, off-target effects, or toxicity associated with long-term use of exosomes were not addressed in this study. Furthermore, the *in vivo* distribution, metabolism, and clearance mechanisms of exosomes have not been studied in detail, which may affect their targeting efficiency and optimization for clinical applications [49]. For instance, exosomes may be rapidly cleared by the mononuclear-macrophage system, affecting their accumulation in target tissues [50]. Finally, the mechanistic studies were primarily based on inhibitor experiments, which confirmed the necessity but require further validation for sufficiency using gene knockout or overexpression methods [51]. Although these limitations do not diminish the main conclusions of this study, they highlight areas for future research, including developing humanized models, optimizing exosome engineering strategies, and conducting systematic preclinical toxicological evaluations [44,52].

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4. Materials and Methods

4.1. Exosome Preparation

When BMSCs reached optimal confluence and viability, the culture medium supernatant was collected and subjected to gradient ultracentrifugation. The supernatant was

first centrifuged at 500× g and 4 °C for 10 min to remove dead cells. Subsequently, centrifugation was performed at 1500× g and 4 °C for 15 min to eliminate larger particles and cell debris. Subsequently, the sample was centrifuged at 3000× g and 4 °C for 25 min to further clarify the supernatant by removing residual contaminants. After filtration through a 0.22 µm microporous filter, the sample was subjected to high-speed centrifugation at 100,000× g and 4 °C for 2 h [53]. The supernatant was discarded, and the precipitate containing Exo was resuspended in 200 µL of PBS buffer.

4.2. Exo-Cur Preparation and Extraction

Curcumin was loaded onto BMSCs by the co-incubation method. Dissolve Cur in DMSO, ensuring that the final DMSO concentration is ≤0.1%, to prepare a 1 mg/mL stock solution. Filter the solution using a 0.22 µm microporous filter. Then, dilute it to the desired concentration before adding it to BMSCs. Culture the cells at 37 °C in an atmosphere containing 5% CO₂. After incubation, collect the culture medium supernatant and perform gradient ultracentrifugation to obtain Exo-Cur.

4.3. Encapsulation Rate Measurement

This experiment was conducted using UV-Vis spectrophotometry to determine the encapsulation efficiency of Exo-Cur. First, the maximum absorption wavelength of curcumin in the selected solvent, such as anhydrous ethanol, is determined through wavelength scanning and typically falls near 430 nm. This wavelength is then used as the detection wavelength. An accurately prepared standard solution of curcumin is prepared. The absorbance of a series of standard solutions at different concentrations is then measured at the selected wavelength. A standard curve is constructed to ensure good linearity ($R^2 > 0.999$). Next, to verify the accuracy of the method, a spiked recovery test is performed on the blank Exo supernatant. The results confirm that the sample matrix shows no interference and that the recovery rate falls within the acceptable range of 95% to 105%. During sample measurement, the Exo-Cur sample is subjected to ultracentrifugation, and the absorbance of the supernatant is directly measured at the characteristic wavelength using the blank Exo supernatant as a reference. Subsequently, this absorbance value is used in the standard curve to calculate the mass of free curcumin. Finally, based on the total amount used during preparation and the measured mass of free curcumin, the encapsulation efficiency is calculated using the formula:

$$EE\% = [(Total\ dosage - Mass\ of\ free\ curcumin\ in\ supernatant) \div Total\ dosage] \times 100\%.$$

The entire procedure should be conducted under a light-protected environment. Additionally, the blank control group and sample treatment conditions should be maintained consistently.

4.4. Animal Model Construction and Grouping

4.4.1. Determination of the Sample Size of Experimental Animals

The complete experimental procedure was reviewed and approved by the IACUC of Guilin Medical University (Approval Code: GLMC202203291, 14 March 2022). The sample size was calculated using Power Analysis and Sample Size (PASS) 21.0 software. Using serum ALT activity as the primary outcome measure, the significance level (α) was set at 0.05 (two-sided), and statistical power ($1-\beta$) was set at 0.9. Based on the effect size reported in previously published studies, the minimum sample size for each group was calculated to be 10 mice. To improve the statistical power and resource utilization, it was ultimately determined that each group would consist of 12 mice, with a total of 60 mice in five groups.

4.4.2. Grouping and Quantity of Experimental Animals

SPF C57BL/6J mice (6–7 weeks old, weighing 19–22 g) were purchased from Henan Sikebeisi Biotechnology Co., Ltd. (Anyang, China, License No. SCXK2020-0005). Throughout the experiment, the mice were housed in a standard SPF animal facility with environmental temperature controlled at approximately 23 °C, relative humidity at 60%, and a 12-h light/12-h dark cycle maintained, with free access to food and water. Before the experiment began, all mice were acclimatized for 1 week. During the surgical procedure, the mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). Their body temperature was monitored and maintained at 37.0 ± 0.5 °C using a rectal temperature probe. After stable anesthesia was achieved, an incision was made along the midline of the abdomen to expose the liver. A vascular clamp was then applied to occlude the vascular pedicles supplying the left and middle lobes of the liver, inducing local ischemia in these lobes for 60 min. Subsequently, the vascular clamp was released to restore blood flow, and reperfusion was performed for 6 hours, thereby establishing a HIRI model. The sham surgery group underwent only the laparotomy without clamping the hepatic blood flow, serving as a control for the surgical procedure without ischemia–reperfusion. The protocol of 1-h ischemia followed by 6-h reperfusion was designed based on a widely adopted classic model in this field. This protocol induces significant liver injury and inflammatory response while ensuring animal survival. Continuous monitoring and maintenance of body temperature during the procedure were implemented to eliminate the potential protective effect of hypothermia on ischemic injury. This ensured the consistency and comparability of the experimental results.

A total of 60 mice were randomly divided into five groups of 12 mice each ($n = 12$): the sham operation group, the HIRI group, the HIRI + Cur group, the HIRI + Exo group, and the HIRI + Exo-Cur group. Here, Cur refers to curcumin, Exo to exosomes, and Exo-Cur to curcumin-loaded exosomes. During the reperfusion phase, mice in the HIRI + Cur group received a tail vein injection of 100 µL curcumin solution at a concentration of 100 µg/mL. Mice in the HIRI + Exo group received 100 µL exosome solution at the same concentration, while those in the HIRI + Exo-Cur group received 100 µL curcumin-loaded exosome solution at 100 µg/mL. Mice in the sham operation and HIRI groups were injected with 100 µL of PBS via the tail vein. No unexpected adverse events occurred, and no animals were euthanized during the study.

4.4.3. Inclusion, Exclusion and n Value Reporting of Experimental Units

The inclusion criteria were specific pathogen-free (SPF) Sprague-Dawley (SD) male mice weighing 20 ± 2 g, with no history of liver disease and showed no abnormalities following a 7-day acclimation period. The exclusion criteria were as follows: (1) death within 48 hours after modeling; (2) serum samples showing hemolysis or RNA extracts with concentrations below 50 ng/µL; and (3) presence of artifacts (such as knife marks or contamination) in pathological sections. All criteria were established prior to the experiment in the protocol. A total of 60 mice were initially included and divided into five groups of 12 mice each. All 60 mice met the inclusion criteria and none met any exclusion criteria; therefore, all were included in the analysis. The n values for the sham operation group, HIRI group, HIRI + Cur group, HIRI + Exo group, and HIRI + Exo-CUR group were all 12 for each analysis item.

4.4.4. Randomization and Control of Confounding Factors

This study employed the random number table method for grouping. Sixty mice were numbered in the order they were captured (1–60). Following the random number table in Medical Statistics (specify author and edition), three random numbers were read sequentially, beginning at the third column of the fifth row. Based on the random number

interval divisions, the mice were assigned to the sham-operated group, HIRI group, HIRI + Cur group, HIRI + Exo group, and HIRI + Exo-Cur group, with 12 mice in each group. The group assignments were sealed and stored by non-experimental personnel. The sealed grouping assignments were unsealed and applied prior to the experimental procedure to preserve allocation concealment.

Confounding factor control strategy: Control measures for potential confounding factors include: (1) Cage and feeding: Mice were randomly assigned to cages, each cage containing four mice. The feeding environment parameters were continuously monitored (temperature maintained at approximately 23 °C, humidity at 60%, and a 12-h light/dark cycle). (2) Modeling and sampling: The modeling procedure was carried out by the same researcher, and the sampling time was consistently scheduled between 9:00 and 10:00 AM to avoid the influence of circadian rhythm. (3) Blinding method: Both serum index measurements and histopathological analyses were conducted using a single-blind method, with investigators blinded to group assignments. Data entry was performed by independent personnel to reduce measurement bias. (4) Reagent mixing: After preparation, all reagents from the same batch were aliquoted, stored protected from light, and aliquoted tubes were randomly selected prior to use to avoid batch differences.

4.4.5. Blinding Implementation and Group Awareness

This experiment was designed using a double-blind method. The specific process was as follows:

(1) Blind setting: The experimental modeling and specimen collection were carried out by Operator A. Specimen testing was done by Operators B and C, and data statistics were performed by Operator D. None of the aforementioned personnel was aware of the animal grouping information. Only the independent third party E had the corresponding relationship of “animal number–grouping–reagent type” and was responsible for reagent preparation, group code preservation, and unblinding procedures.

(2) Cryptographic processing: Third party E marked 60 mice with unique numerical identifiers (1–60) based on the random grouping results. An encrypted “numbered–grouped” correspondence table was established. Third party E prepared solvents in groups according to the corresponding numbers and labeled them with indistinguishable codes. Only Operator A was informed to “inject the mice with the reagents of the corresponding codes according to the numbers”.

(3) Blind execution: Operator A performed surgical modeling, reagent injection, and specimen collection according to the numbers. Operator A labeled the specimens with numbers and sent them to the testing laboratory. Operators B and C completed the laboratory tests and pathological section scoring based on the numbers. The test results were only associated with the numbers. Operator D conducted statistical analysis based on data labeled only by numbers without involving grouping information.

(4) Unblinding process: After all the experimental data were collected, third party E unblinded them and provided the corresponding relationship of “number–grouping” to Operator D to complete the final statistics and analysis of the grouped data. There was no emergency unblinding situation during the experiment.

4.5. Cell Model Construction and Grouping

BMSC cells were cultured in MEM medium, supplemented with 10% serum and 1% penicillin-streptomycin. They were maintained in a 37 °C incubator with 5% CO₂. AML12 mouse hepatocyte cells were cultured in DMEM/F12 medium, supplemented with 10% serum, 1% penicillin-streptomycin, 10 µg/mL insulin, 5.5 µg/mL transferrin, 5 ng/mL selenium, and 40 ng/mL dexamethasone. These cells were also maintained in a 37 °C incubator with 5% CO₂. Hypoxia was induced using cobalt chloride as a hypoxia-mimicking

agent. A working solution of cobalt chloride was prepared at a concentration of 600 μM and applied for 6 hours. After treatment, the medium was removed and the cells were washed with PBS. Normal culture medium was then added to mimic reoxygenation.

AML12 cells were randomly divided into five groups: the Sham group, the HIRI group, the HIRI + Cur group, the HIRI + Exo group, and the HIRI + Exo-Cur group. During the reoxygenation phase, the HIRI + Cur group received a culture medium containing Cur at a concentration of 30 $\mu\text{g/mL}$; the HIRI + Exo group received a culture medium containing Exo at the same concentration; and the HIRI + Exo-Cur group received a culture medium containing Exo-Cur, also at 30 $\mu\text{g/mL}$. The Sham group and the HIRI group were maintained in normal culture medium.

The sample size of this study was initially determined based on preliminary experimental data. A priori sample size estimation was conducted using G*Power software (version 3.1), with effect size $f = 0.4$, α error probability = 0.05, statistical power $(1-\beta) = 0.8$, and five groups. This resulted in a minimum of 8 independent replicates per group. To ensure the reliability of the experiment and account for potential sample loss, the final sample size for each group was established as 10 independent replicates.

Following cell seeding, all AML12 cells were allocated to groups using a computer-generated random number table. This allocation ensured that each group was balanced by evenly distributing the cell passage number, incubator location, and treatment time to reduce selection bias. During the experiment, cell handling, drug administration, and hypoxia/reoxygenation procedures were performed by a single researcher. Subsequent outcome assessments, such as cell viability assays and molecular biology analyses, were conducted by another researcher who was blinded to the group allocation. This approach ensured the objectivity of data collection and result analysis.

4.6. Detection of Serum ALT and AST Levels

After 6 hours of reperfusion, blood was collected from the tail vein of mice and placed in a centrifuge tube. After incubation at room temperature for 30 min, the samples were centrifuged at 3000 rpm for 15 min to separate the serum. A standard calibration curve was established according to the instructions of the alanine aminotransferase (ALT) and aspartate aminotransferase (AST) kits (Elabscience, Wuhan, China). The optical density (OD) of each sample was measured at a wavelength of 510 nm using a microplate reader (ThermoScientific, Waltham, Massachusetts, USA). Serum ALT and AST enzyme activities (U/L) were calculated based on the standard curve provided by the kit. Each sample was assayed in triplicate, and the mean was calculated.

4.7. CCK8 Assay

AML12 cells were seeded at a density of 5×10^3 cells per well in a 96-well plate. After 24 hours of culture, the cells in each group were subjected to their respective experimental treatments. The CCK-8 working solution (Beyotime Biotechnology, Shanghai, China) was prepared by diluting the original solution at a ratio of 1:10 with the appropriate culture medium. Then, 200 μL of the CCK-8 working solution was added to each well and then incubated in the dark for 2 hours. After incubation, the absorbance values at a wavelength of 450 nm were measured using a microplate reader. Cell viability (%) was calculated using the following formula: $\text{Cell viability (\%)} = (\text{optical density (OD) of experimental group} - \text{OD of blank control group}) / (\text{OD of sham control group} - \text{OD of blank control group}) \times 100\%$. Each group had 6 replicates, and the experiment was repeated 3 times.

4.8. Lactate Dehydrogenase Detection

The well plates containing confluent AML12 cells were washed with PBS. The following groups were established using serum-free culture medium: wells with no cells

(background control), control cell wells without drug treatment (sample control), cell wells without drug treatment designated for subsequent lysis (maximum enzyme activity control), and drug-treated cell wells. In the maximum enzyme activity control wells, LDH release reagent provided by the kit (Beyotime Biotechnology, Shanghai, China) was added at a volume equal to 10% of the original culture medium volume. The wells were mixed thoroughly by gentle pipetting and then incubated in the cell incubator for 1 h. Subsequently, 120 μ L of supernatant was taken from each well and transferred to a 96-well plate. Then, 60 μ L of working solution was added to each well, followed by incubation in the dark for 30 min. Finally, the samples were measured at a wavelength of 490 nm.

4.9. ELISA Assay

The levels of TNF- α , IL-1 β , and IL-18 in mouse serum and cell supernatant were measured using ELISA kits (Quanzhou Jiubang Biotechnology, Quanzhou, China) according to the manufacturer's instructions. Standards and samples were added and incubated at 37 °C for 90 min. After washing, the biotinylated antibody was added and incubated at 37 °C for 60 min. Horseradish-peroxidase-labeled streptavidin was then added and incubated at 37 °C for 30 min. Following another wash, the substrate solution was added and incubated in the dark for 15 min, after which the stop solution was added to terminate the reaction. The optical density (OD) was measured at 450 nm using a microplate reader, and the concentrations of each inflammatory factor (pg/mL) were calculated based on the standard curve. Each sample was assayed in triplicate, and the experiment was repeated three times.

4.10. HE Staining

After the mice were sacrificed, the left lobe of the liver was rapidly excised and fixed in 4% paraformaldehyde (Beyotime Biotechnology, China) solution for 24 h. The tissue then underwent graded dehydration, clearing, and paraffin embedding. Subsequently, 5- μ m-thick continuous sections were cut. After dewaxing and rehydration in water, the sections were stained with hematoxylin for 5 min, differentiated in hydrochloric acid alcohol for 30 s, and stained with eosin for 2 min. Following staining, the sections underwent graded dehydration, clearing, and were mounted with neutral mounting medium. The morphology and structure of liver tissue were observed under an optical microscope, and the degree of hepatocyte degeneration, necrosis, and inflammatory cell infiltration was recorded.

4.11. TUNEL Experiment

Following the instructions provided with the TUNEL apoptosis detection kit (Servicebio, Wuhan, China), liver tissue paraffin sections were stained. After dewaxing and rehydration in water, the sections were incubated with proteinase K solution at 37 °C for 20 min, followed by washing with PBS. Then, we added the TUNEL reaction solution and incubated the sections in the dark at 37 °C for 60 min. Then, after washing with PBS, we applied DAPI staining solution and incubated the sections at room temperature in the dark for 5 min. Finally, the sections were sealed with anti-fade mounting medium. We observed the sections under a laser confocal microscope. Blue fluorescence indicated cell nuclei, while green fluorescence marked TUNEL-positive cells, representing cells with DNA fragmentation.

4.12. Immunohistochemistry

After dewaxing liver tissue paraffin sections and rehydrating them in water, heat-induced antigen retrieval was performed using citrate buffer (pH 6.0) for 15 min, followed

by cooling to room temperature. The sections were then incubated with 3% hydrogen peroxide (H_2O_2) solution at room temperature for 10 min to block endogenous peroxidase activity. After washing with PBS, the sections were incubated with 5% goat serum blocking solution at room temperature for 30 min. NLRP3 primary antibody (Servicebio, China) was applied, and the sections were incubated overnight at 4 °C. Following washing with PBS, HRP-labeled secondary antibody (Servicebio, China) was added, and the sections were incubated at room temperature for 30 min. DAB chromogenic reaction was performed for color development, followed by hematoxylin counterstaining, gradient dehydration, clearing, and mounting. The sections were observed under an optical microscope, with NLRP3-positive expression indicated by brown granules.

4.13. Immunofluorescence

PKH26 fluorescent dye (Umibio, Shanghai, China) was used to label the Exo-Cur membrane structure. Exo-Cur suspension was mixed with PKH26 staining solution at a final concentration of 2 $\mu\text{mol/L}$ and incubated at 37 °C for 10 min. An equal volume of fetal bovine serum was then added to terminate the staining reaction. The mixture was centrifuged at 4 °C at 100,000 rpm for 70 min to remove free dye, and the precipitate was resuspended in PBS. AML12 cells were seeded in culture dishes suitable for confocal laser scanning microscopy. During the reoxygenation phase, PKH26-labeled Exo-Cur was added and incubated for 12 hours. The culture medium was discarded, and the cells were washed twice with PBS, then fixed with 4% paraformaldehyde for 20 min. Subsequently, the cells were incubated with DAPI staining solution at room temperature in the dark for 5 min to stain the nuclei, followed by sealing with an anti-fade mounting medium. Observations were made under a laser confocal microscope, with red fluorescence (PKH26) tracing the Exo-Cur membrane, green fluorescence (Cy2) tracing curcumin, and blue fluorescence (DAPI) indicating cell nuclei. The distribution and uptake of Exo-Cur within the cells were then analyzed.

4.14. Western Blot

Different groups of cells were mixed with a RIPA to PMSF solution at a ratio of 100:1 and lysed on ice, then centrifuged at 4 °C, 12,000 rpm for 15 min. The protein concentration of the samples was quantified using BCA. The corresponding volumes of lysis buffer and loading buffer were added. The samples were then denatured at 100 °C for 10 min and stored for later use. Electrophoresis was first performed at 120 V for 30 min, then the proteins were transferred at a constant voltage of 80 V for 1.5 h. The pre-cut PVDF membrane was activated by soaking in methanol. The gel was cut according to the required protein molecular weight, and an electro-transfer sandwich was made following the principle of placing the black gel side against the white membrane. The membrane was transferred at a constant current of 250 mA for an appropriate duration with ice packs in the tank for cooling. The membrane was incubated in 5% non-fat milk blocking solution with gentle shaking for 1–2 h. The blocked membrane was washed three times with TBST buffer, each time for 5 min. The membrane was incubated with the primary antibody overnight at 4 °C. The membrane was then washed three times with TBST buffer for 5 min each. The secondary antibody was diluted and the membrane was incubated with shaking for 1–2 h, followed by washing. Protein bands were detected by Western blot using an ultrasensitive ECL kit (Millipore, Burlington, MA, USA). The antibodies used were as follows: NLRP3, ASC, p-PI3K, p-AKT, AKT (Abways, Shanghai, China); PI3K, p-MTOR (Proteintech, Rosemont, IL, USA); C-Caspase-1, GSDMD-NT (Affinity, Cincinnati, OH, USA, USA); β -actin (Servicebio, China); Goat anti-Rabbit IgG (H+L) and Goat anti-Mouse IgG (H+L) (Sera Care, Milford, MA, USA).

4.15. qRT-PCR

Total RNA was extracted using the TRIzol reagent. The TOLOBIO reverse transcription kit was used to synthesize cDNA from total RNA by reverse transcription. The components of the reverse transcription reaction mixture were as follows: 4 μ L 5 $\times$ All-in-one RT buffer, 1 μ L All-in-one Enzyme Mix, x μ L (1 $\text{pg}^{-1}\mu\text{g}$) template RNA, and RNase-free H_2O was added to bring the total volume to 20 μ L. The reverse transcription reaction was carried out at 50 $^\circ\text{C}$ for 15 min, followed by 85 $^\circ\text{C}$ for 5 s. The relative expression levels of target genes were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method, with the β -actin gene serving as the internal reference. The sequences of the primers are listed in Table 4.

Table 4. The primers used in this study.

Gene Name	Forward	Reverse
β -actin	5'-CTCCATCCTGGCCTCGCTGT-3'	5'-GCTGCTACCTTCACCGTTCC-3'
IL-4	5'-AGTTGTCATCCTGCTCTTCTTCTC-3'	5'-ATGGCGTCCCTTCTCCTGTG-3'
IL-1 β	5'-TCGCAGCAGCACATCAACAAG-3'	5'-TCCACGGGAAAGACACAGGTAG-3'
IL-9	5'-TGTCTCTCCGTCCCAACTGATG-3'	5'-GAGTCTTGATTTCTGTGTGGCATTG-3'
IL-6	5'-GAGAGGAGACTTCACAGAGGATACC-3'	5'-TCATTTCACGATTTCCAGAGAAC-3'
IL-10	5'-GGACAACATACTGCTAACCGACTC-3'	5'-TGGATCATTTCCGATAAGGCTTGG-3'
TNF- α	5'-CACGCTCTTCTGTCTACTGAACCTC-3'	5'-CTTGGTGGTTTGTGAGTGTGAGG-3'

4.16. Flow Cytometry

Cell samples were centrifuged at 1200 rpm for 5 min, and the supernatant was discarded. The cells were resuspended in PBS, centrifuged once more at 1200 rpm for 5 min, and the supernatant was discarded. PBS (containing 1% BSA) was then added to the cells, which were subsequently centrifuged at 1200 rpm for 5 min, after which the supernatant was discarded. Next, 50 μ L of PBS (containing 1% BSA) mixed with 2 μ L of GSDME-NT-APC-CY7 (Abcam, USA) was added to the cells, and the mixture was incubated in the dark at 4 $^\circ\text{C}$ for 20 min. After incubation, the cells were resuspended in PBS (containing 1% BSA), centrifuged at 1200 rpm for 5 min, and the supernatant was discarded. The cells were then resuspended in 100 μ L of Fixation/Permeabilization solution and incubated in the dark at 4 $^\circ\text{C}$ for 20 min. Subsequently, 500 μ L of 1 $\times$ Perm/WashTM buffer was added. The cells were then centrifuged at 1200 rpm for 5 min, and the supernatant was discarded. The cells were resuspended in 100 μ L of 1 $\times$ Perm/WashTM buffer containing 1 μ L of cleaved caspase-1-CF594 (CST, USA) and incubated in the dark at 4 $^\circ\text{C}$ for 30 min. After incubation, 500 μ L of 1 $\times$ Perm/WashTM buffer was added, the cells were centrifuged at 1200 rpm for 5 min, and the supernatant was discarded. Finally, the cells were resuspended in 400 μ L of PBS (containing 1% BSA), and flow cytometry was performed to detect cells positive for cleaved caspase-1⁺ and GSDMD-N⁺. These cells were identified as pyroptotic and used to calculate the pyroptosis rate. The experiment was repeated three times, with three samples set for each group.

4.17. Statistical Analysis

Statistical analysis was conducted with SPSS 21.0 software, and all continuous data are presented as mean $\pm$ standard deviation. First, data normality was assessed using the Shapiro–Wilk test. For normally distributed data, comparisons between two groups were performed using an independent samples *t*-test. For comparisons among multiple groups, one-way ANOVA was employed. Levene's test was conducted to assess variance homogeneity. When the ANOVA results were statistically significant ($p < 0.05$), further post hoc multiple comparisons were performed. Specifically, if the variances were homogeneous, Tukey's test was used. If the variances were not homogeneous, the Games-Howell test

was applied. Data that did not follow a normal distribution were analyzed using appropriate non-parametric tests, such as the Mann–Whitney U test for two-group comparisons or the Kruskal–Wallis H test for multiple groups. GraphPad Prism 8.0 software was used for data visualization. A *p*-value of less than 0.05 was considered statistically significant.

5. Conclusions

Overall, this study developed the Exo-Cur nanomedicine and confirmed its protective effect against hepatic ischemia–reperfusion injury by activating the PI3K/Akt/mTOR signaling pathway and inhibiting NLRP3/GSDMD-mediated pyroptosis. These findings not only validate the potential of Exo-Cur as a novel therapeutic strategy but also enhance our understanding of the regulatory networks involved in inflammation and cellular death during liver injury.

In the future, the research scope of cell death-related regulatory pathways can be expanded to systematically explore the interaction mechanisms of the PI3K/AKT/mTOR pathway with TLR4/NF- κ B, MAPK, and other pathways in the regulation of HIRI cell death. This would clarify the synergistic or antagonistic effects of each pathway and enhance understanding of the molecular networks influenced by Exo-Cur in regulating cell death. Additionally, studies investigating the dose-dependent effects and long-term safety of Exo-Cur should be conducted to systematically investigate the immunogenicity, off-target effects, and potential toxicity of exosomes through long-term animal experiments. Furthermore, techniques such as noninvasive imaging can be utilized to analyze in-depth the distribution, metabolism, and clearance mechanisms of Exo-Cur in vivo. These approaches will help optimize targeted delivery strategies to enhance Exo-Cur's enrichment efficiency at the site of liver injury, thereby providing a basis for the formulation of clinical dosing regimens.

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