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Enhancing the Neuroprotective Synergy of Caffeine and ACSL4 Inhibition via Exosome-Mediated siRNA Delivery

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Abstract

Background: Ischemic stroke, a predominant cause of neurological impairment, involves complex ischemia/reperfusion (I/R) injury where ferroptosis—an iron-dependent cell death driven by lipid peroxidation—plays a critical role. The enzyme ACSL4 is a key facilitator of this process. While caffeine demonstrates neuroprotective potential by modulating adenosine receptors and ACSL4 activity, therapeutic targeting of ACSL4 via siRNA is hampered by the inherent instability and poor delivery efficiency of naked oligonucleotides. **Methods:** An in vitro hypoxia/reoxygenation (H/R) model was established using HT-22 neuronal cells. Bone marrow-derived mesenchymal stem cell (BMSC) exosomes were isolated, characterized via transmission electron microscopy and western blotting for specific markers (CD9, CD63, CD81), and loaded with siRNA targeting ACSL4 (exo-si-ACSL4). Cellular uptake of fluorescently labeled exosomes was confirmed. HT-22 cells subjected to H/R were treated with caffeine (500 μM), transfected with conventional si-ACSL4, or incubated with exo-si-ACSL4, both alone and in combination with caffeine. Assessments included cell viability (CCK-8), ACSL4 expression (RT-qPCR, western blot), inflammatory cytokines (ELISA), and ferroptosis indicators (iron, reactive oxygen species, malondialdehyde, glutathione, mitochondrial membrane potential). **Results:** BMSC-derived exosomes exhibited typical morphological and protein marker profiles and were effectively internalized by HT-22 cells. Exo-si-ACSL4 achieved superior transfection efficiency, reducing ACSL4 mRNA and protein levels by approximately 89% and 87%, respectively, which surpassed the knockdown efficacy of standard siRNA transfection. The combination of exo-si-ACSL4 and caffeine yielded a synergistic protective effect. This combined treatment significantly elevated cell viability, attenuated the release of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), and robustly suppressed ferroptosis hallmarks compared to caffeine or either siRNA delivery method alone. Inhibition was evidenced by reduced lipid peroxidation, decreased intracellular iron and ROS, elevated GSH, and preserved mitochondrial membrane potential. **Conclusions:** This work substantiates that exosome-mediated delivery of si-ACSL4 markedly enhances the precision and efficacy of gene silencing. The synergistic interaction between this targeted molecular intervention and caffeine administration confers pronounced protection against H/R-induced neuronal damage, primarily through the potent attenuation of ferroptosis and inflammation. These findings propose a novel combinatory strategy, integrating a pharmacological agent with an advanced biomimetic delivery system, to overcome central limitations in current nucleic acid therapy for ischemic stroke.

Keywords

Cerebral ischemia/reperfusion injury; ferroptosis; acyl-CoA synthetase long-chain family member 4; extracellular vesicles; gene silencing, neuroprotection; combination therapy

1. Introduction

Cardiovascular disease ranks among the most prevalent chronic conditions, affecting approximately 626 million people worldwide [1]. In 2023 alone, it claimed 19.2 million lives, accounting for one-third of all annual disease-related deaths. Among various types of cardiovascular diseases, ischemic heart disease is particularly prevalent, affecting approximately 239 million people annually. Its age-standardized incidence rate reaches 2,130.2 per 100,000 population [2]. A clinical study involving 288 patients demonstrated that in-hospital mortality rates can rise as high as 13.4% [3, 4]. While reperfusion therapies (e.g., PCI) have successfully improved early survival rates, post-infarction heart failure is emerging as a major driver of late morbidity, mortality, and healthcare costs [5].

During the process of reperfusion for ischemic heart disease, restoring blood flow, while essential, can paradoxically trigger further cardiac damage. This involves complex mechanisms, including inflammation and a specific type of cell death known as ferroptosis. Ferroptosis is an iron-dependent form of regulated cell death driven by the accumulation of lipid peroxides. A key player in this detrimental process is the enzyme ACSL4 (Acyl-CoA Synthetase Long-Chain Family Member 4) [6]. Research confirms that ACSL4 is critically involved in sensitizing cells to ferroptosis by catalyzing the esterification of polyunsaturated fatty acids (PUFAs), such as arachidonic acid and adrenic acid, into phospholipids, making these membrane lipids more susceptible to peroxidation [7]. In the context of myocardial ischemia/reperfusion (I/R) injury, studies have observed overexpression of ACSL4, and its pharmacological or genetic inhibition has been shown to alleviate ischemic damage by countering ferroptosis [8]. The ferroptosis signal flow in this context can be summarized as follows:

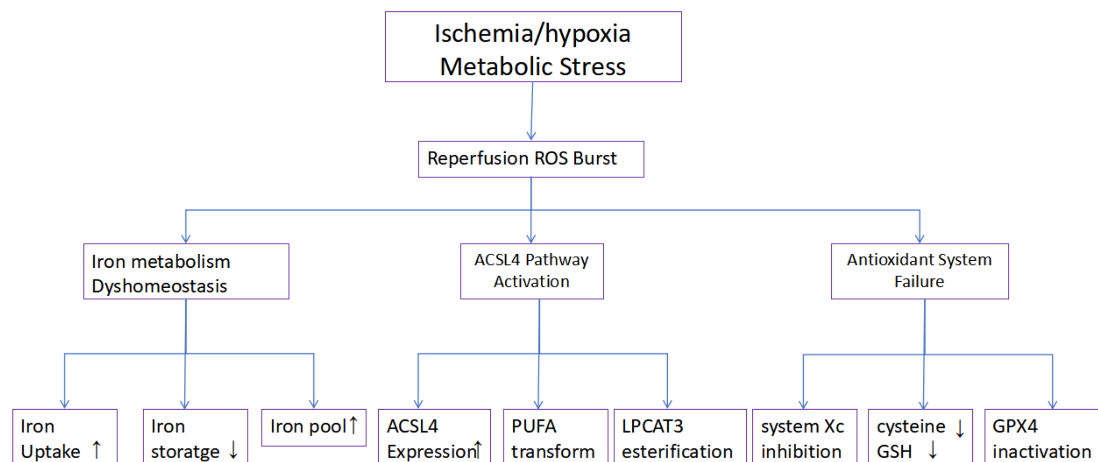

Figure 1. Pathophysiological Mechanisms Linking Metabolic Syndrome to NAFLD Progression Through Lipid Metabolism Dysregulation.

Therapeutic strategies targeting ferroptosis in myocardial ischemia/reperfusion (I/R) injury have gained significant attention, with accumulating evidence supporting the efficacy of ACSL4 silencing. Experimental studies demonstrate that siRNA-mediated knockdown of ACSL4 markedly attenuates ferroptotic cell death and reduces infarct size in I/R models. For instance, research showed that si-ACSL4 decreased lipid peroxidation and improved cardiomyocyte viability by upregulating GPX4 levels [9-11]. Similarly, Studies reported that inhibition of ACSL4 suppressed erastin-induced ferroptosis and reduced inflammatory markers in vivo [12, 13]. Furthermore, several studies suggested that administering si-ACSL4 via nanoparticles restored cardiac function and minimized oxidative damage in a murine I/R

model, highlighting its translational potential [14-17]. Despite these promising results, the clinical application of naked siRNA remains limited due to inherent instability, including susceptibility to nuclease degradation and rapid renal clearance, which compromises bioavailability and targeting specificity. As noted in reviews on nucleic acid therapeutics, unmodified siRNA exhibits a short half-life in circulation, necessitating advanced delivery systems. To address these challenges, exosome-based carriers have emerged as a robust solution. Research confirmed that exosomes encapsulating siRNA significantly enhance cellular uptake efficiency in cardiomyocytes compared to conventional liposomal transfection, owing to their natural membrane composition and low immunogenicity [18-20]. Moreover, multiple researchers engineered cardiac-specific exosomes loaded with si-ACSL4, which not only improved siRNA stability and cardiomyocyte selectivity but also synergized with compounds like caffeine to amplify anti-ferroptotic effects in I/R injury. These findings underscore exosome-mediated delivery as a superior approach to overcome the limitations of traditional siRNA therapy.

Based on the established role of ACSL4 in driving ferroptosis during ischemia/reperfusion (I/R) injury and the limitations of conventional siRNA delivery, this study aims to develop a novel therapeutic strategy using exosome-encapsulated si-ACSL4. The primary objectives are, first, to isolate and purify exosomes from a suitable cellular source, such as mesenchymal stem cells. Second, to thoroughly characterize these exosomes using techniques including Transmission Electron Microscopy (TEM) and Flow Cytometric Analysis to confirm their typical morphology and determine key physical properties like size distribution, polydispersity index, and zeta potential. Third, to load the exosomes with si-ACSL4, creating exo-si-ACSL4 complexes, and to transfect them into target cells under H/R conditions to investigate whether this delivery system can more effectively inhibit ferroptosis and improve cell survival compared to standard siRNA transfection methods. We hypothesize that exosome-mediated delivery will significantly enhance the cellular uptake efficiency and targeting specificity of si-ACSL4. Consequently, we expect that exo-si-ACSL4 will lead to a more potent suppression of ferroptosis, as evidenced by reduced lipid peroxidation and improved cell viability, thereby offering superior protection against H/R-induced injury. This approach seeks to overcome the instability of naked siRNA and maximize the therapeutic potential of ACSL4 inhibition.

2. Methods and Materials

2.1 Cell Model and Treatment

The hypoxia/reoxygenation (H/R) model was established in vitro. HT-22 cells were cultured in hypoxic conditions (1% O2) with serum- and glucose-free DMEM at 37°C for 12 hours, then reoxygenated for 24 hours. Control cells stayed in standard conditions. Caffeine treatments were applied post-hypoxia with concentrations of 50, 100, and 500 μM caffeine (Thermo Fisher Scientific, USA) for 36 hours [21]. The CCK-8 assay was used for cell viability.

2.2 Cell Transfection

The siRNAs used in this study included siRNA#1 (catalog number EMU084681, Sigma-Aldrich Co., USA), siRNA#2 (catalog number 73767, Thermo Fisher Scientific), and si-NC (catalog number SIC001, Sigma-Aldrich). The ACSL4 plasmid (MR215668) and control vector (PS100001) were from Origene Technologies. siRNAs and plasmids were transfected with Lipofectamine 2000; efficiency checked by RT-qPCR and western blot.

2.3 RT-qPCR

RNA was extracted and converted to cDNA for RT-qPCR, using GAPDH as a control to calculate relative expression. The relative RNA levels were calculated using the $2^{-\triangle\triangle CT}$ method. The primers used are listed in Table 1.

Table 1. Primer sequences in this study

Genes	Sequences (5' -> 3')
ACSL4	F: CCTGAGGGGCTTGAAATTCAC
	R: GTTGGTCTACTTGGAGGAACG

2.4 Western Blotting

Proteins were extracted, quantified, separated by SDS-PAGE, transferred to membranes, and analyzed by western blot with chemiluminescence. The antibodies used are as follows: anti-ACSL4 (catalog number 66617-1-Ig, 1:3000,

Proteintech, USA), CD9 (catalog number 20597-1-AP, 1:1000, Proteintech), CD63 (catalog number 25682-1-AP, 1:500, Proteintech), CD81 (catalog number 27855-1-AP, 1:1000, Proteintech), GAPDH (catalog number 10494-1-AP, 1:5000, Proteintech).

2.5 ELISA

Cytokine levels were measured using ELISA kits from Abcam: TNF-α (ab208348), IL-1β (ab197742), and IL-6 (ab222503).

2.6 Ferroptosis Indicators

Levels of iron, Fe2+, ROS, MDA, and GSH were measured using specific commercial kits from Jianglai Bio and Nanjing Jiancheng Bioengineering Institute. Mitochondrial membrane potential was assessed using JC-1 dye and analyzed by flow cytometry.

2.7 Exosome Isolation, Purification, and Characterization

Bone marrow-derived mesenchymal stem cells (BMSCs) were obtained from 4-week-old C57BL/6 mice (purchased from Vital River, Beijing, China), following standard housing conditions and euthanasia protocols as per institutional guidelines [22]. BMSC surface markers (positive: CD90, CD44; negative: CD45, CD34) were analyzed using flow cytometry.

Exosomes from BMSCs were isolated using the PureExo® Exosome Isolation Kit (Applied Biological Materials, Canada), characterized by transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA), and their protein markers were analyzed by western blot. Uptake by HT-22 cells was confirmed using fluorescent staining [23].

2.8 Exosome Isolation, Purification, and Characterization

BMSCs were transfected using Lipofectamine 2000 (Thermo Fisher Scientific). After isolation, 10 µg of exosomes were added to 1 ml of HT-22 cell culture medium.

2.9 Statistical Analysis

Data normality was assessed with the Shapiro-Wilk test. Results are presented as mean ± SD. One-way ANOVA with Tukey's post hoc test was used for group comparisons, with significance set at $p < 0.05$. Analyses were conducted using SPSS 22.0 and GraphPad Prism 10.0.

3. Results

3.1 Exosomes characterization

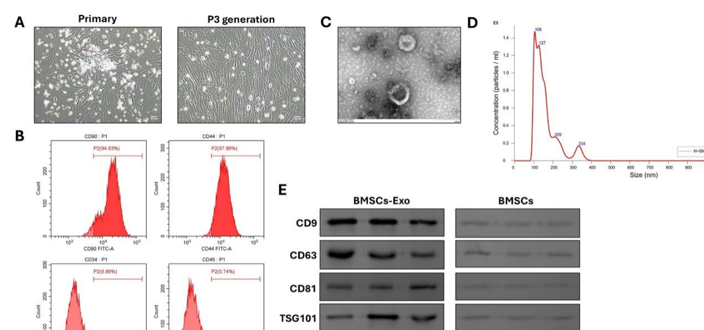

Figure 2. Identification of BMSCs with morphology. (A) markers CD90, CD44, CD45, and CD34 by flow cytometric analysis (B) TEM with characteristic morphology of BMSC-derived exosomes (C) and exosomal markers CD9, CD63, CD81, and TSG101 by western blotting (D).

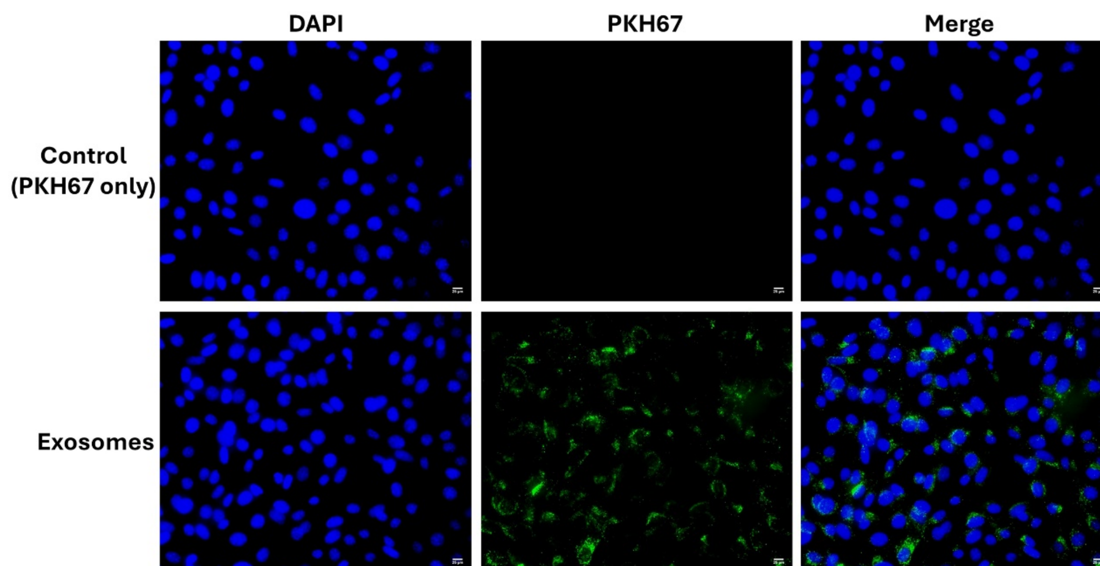

Figure 3. PKH67 fluorescent staining for uptake of exosomes by HT-22 cells.

Firstly, we aimed to amplify the synergistic effect of ACSL4 inhibition and caffeine by utilizing exosomes to deliver siRNA into HT-22 cells. Exosomes, small vesicles released by cells, are known for their ability to efficiently deliver cargo such as siRNA into target cells, offering advantages over direct transfection methods [24-26]. After isolating exosomes from BMSCs, we confirmed their identity through various analyses. As shown in Figure 2, BMSCs exhibited typical morphology, and flow cytometry confirmed the expression of positive markers CD90 and CD44, and the absence of negative markers CD45 and CD34. TEM analysis revealed the typical morphology of BMSC-derived exosomes, and exosomal markers were confirmed.

PKH67 fluorescent staining confirmed the successful uptake of exosomes by HT-22 cells (Figure 3). We then transfected BMSCs with si-ACSL4, isolated the resulting exo-si-ACSL4, and co-cultured it with HT-22 cells under H/R conditions alongside caffeine treatment.

3.2 Transfection Efficiency

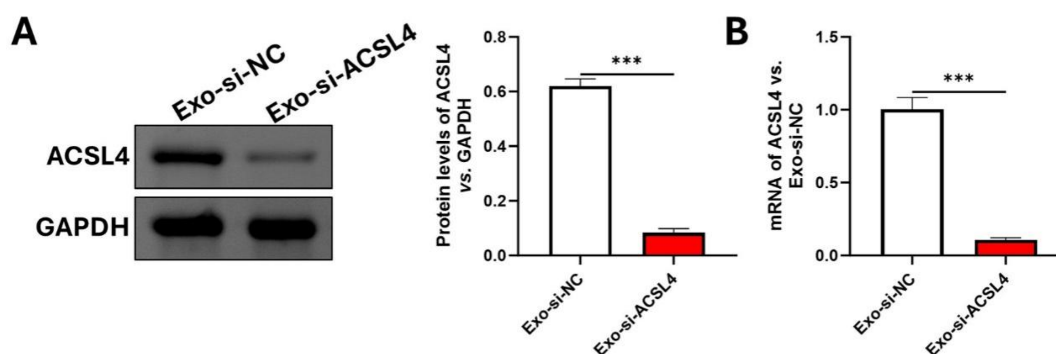

Figure 4. Expression of ACSL4 by western blotting (A) and RT-qPCR (B). Experiments are repeated in triplicate (n=3). ***P<0.001, **P<0.01, *P<0.05.

The results from both qPCR and western blot analyses provide compelling evidence for the highly efficient transfection achieved by exosome-mediated delivery of si-ACSL4. The qPCR data demonstrated an approximately 89% reduction in ACSL4 mRNA levels in the Exo-si-ACSL4 treated group compared to the negative control, while western blot analysis showed a parallel 87% decrease in ACSL4 protein expression. This coordinated and substantial reduction at both transcriptional and translational levels strongly confirms the successful cellular uptake and functional delivery of the siRNA cargo by the exosome vehicle. The marked knockdown efficiency indicates that exosome-mediated transfection was highly effective.

3.3 Exosomes Enhance the Synergistic Effect of ACSL4 Inhibition and Caffeine in Protecting Against H/R-Induced Neuronal Damage

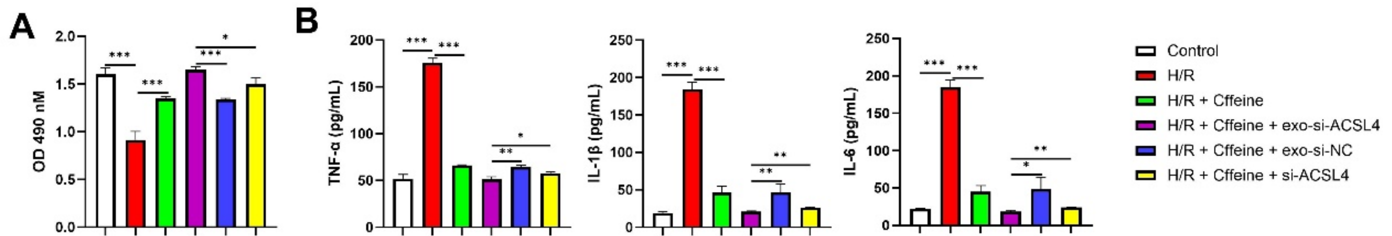

Figure 5. Cell viability by CCK-8. (A) measured at 72 hours post-treatment following the establishment of the H/R model. Cytokine levels by ELISA (B) after reoxygenation. Cells were treated with caffeine (500 μM), transfected with siRNA/si-NC, as well as exo-siRNA or exo-NC. Experiments are repeated in triplicate (n=3). ***P<0.001, **P<0.01, *P<0.05.

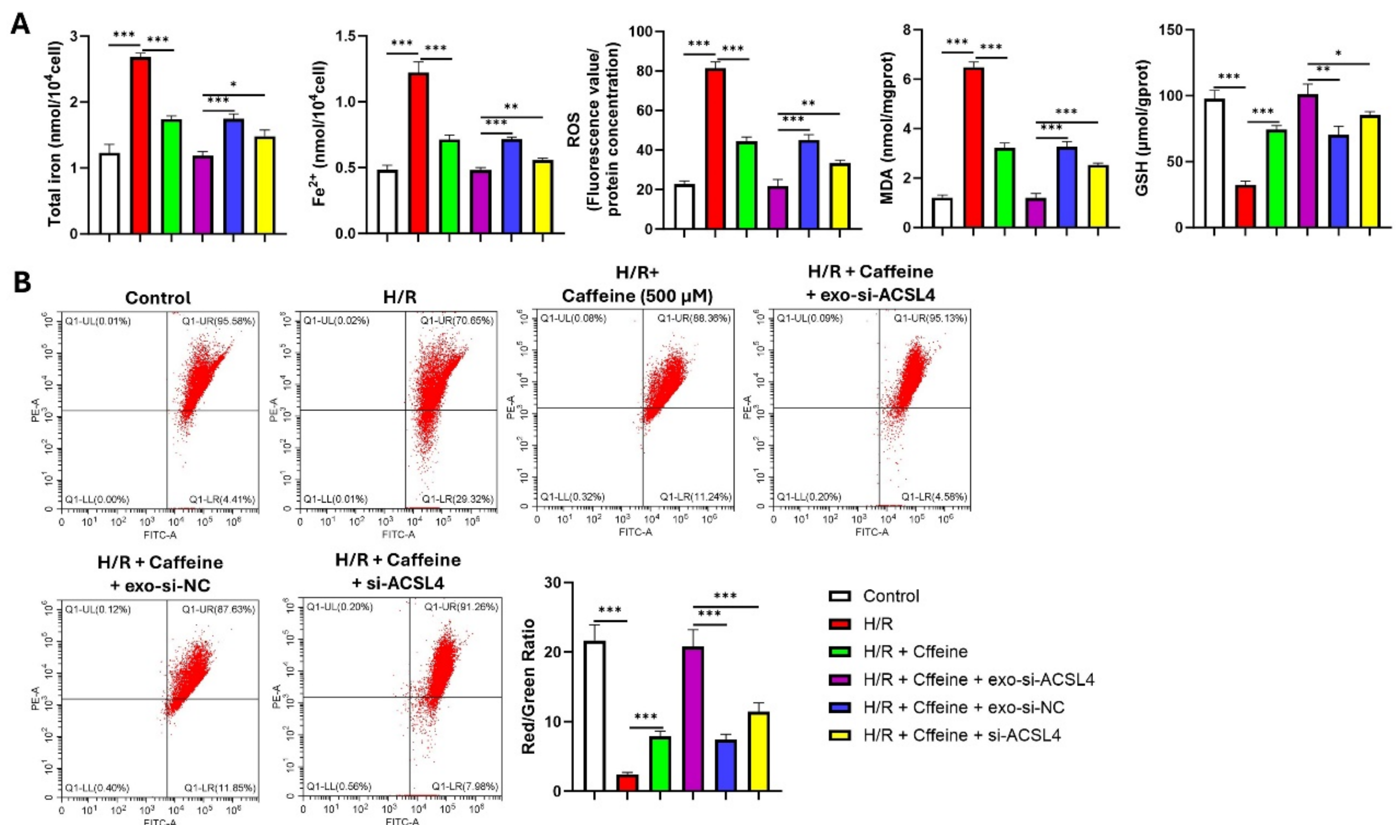

Figure 6. Cellular iron, Fe2+, ROS, MDA, and GSH levels. (A) and mitochondrial membrane potential (B) after reoxygenation. In the measurement of mitochondrial membrane potential, red fluorescence represents high potential, while green means low potential. Cells were treated with caffeine (500 μM), transfected with siRNA/si-NC, as well as exo-siRNA or exo-NC. Experiments are repeated in triplicate (n=3). ***P<0.001, **P<0.01, *P<0.05.

We then transfected BMSCs with si-ACSL4, isolated the resulting exo-si-ACSL4, and co-cultured it with HT-22 cells under H/R conditions alongside caffeine treatment. As shown in Figures 4-6, exo-si-ACSL4 significantly reduced ACSL4 expression in HT-22 cells. Moreover, treatment with exo-si-ACSL4 further enhanced the protective effects of caffeine, leading to increased cell viability, reduced inflammatory response, and inhibited ferroptosis, compared to using si-ACSL4 without exosomes. These findings suggest that exosome-mediated delivery of siRNA is a more effective strategy for enhancing the synergistic protective effects of ACSL4 inhibition and caffeine, offering a promising approach for improving outcomes in H/R-induced neuronal injury.

4. Discussion

Our findings build upon previous research demonstrating the neuroprotective potential of caffeine. A recent study by Jia et al. provided important evidence that caffeine can ameliorate hypoxia/reoxygenation (H/R)-induced neuronal injury by suppressing ferroptosis, specifically through the inhibition of the ACSL4 pathway. This established ACSL4 as a valid therapeutic target for combating ferroptosis in neuronal cells. In our current study, we aimed to enhance the specificity and efficiency of targeting this pathway.

Exosomes have been increasingly recognized for their potential in targeted drug delivery, particularly in the context of cerebral I/R injury [31]. For example, exosomes derived from preconditioned mice have been shown to protect neurons against I/R injury by delivering specific microRNAs that modulate key signaling pathways [32]. Similarly, astrocyte-derived exosomes transporting miR-34c have been reported to alleviate neuronal injury caused by I/R by targeting the TLR7/NF-κB/MAPK axis [31]. Additionally, exosomes from BMSCs have been found to promote angiogenesis and reduce neuronal damage in ischemic stroke models by upregulating SIRT6 and suppressing Notch signaling [32].

We isolated and characterized exosomes and loaded them with si-ACSL4 to create a targeted delivery system. In our study, we further advanced the field by demonstrating that exosome-mediated delivery of si-ACSL4 significantly enhances the protective effects of caffeine against H/R-induced neuronal injury. Unlike earlier approaches that focused on direct siRNA transfection, our use of exosome-based delivery ensures more efficient and targeted gene silencing, particularly in the context of ferroptosis. This combination of caffeine and exosome-mediated gene regulation is unprecedented and suggests a new direction for integrating neuroprotection with advanced delivery systems in stroke treatment.

5. Limitations and Future Plans

Future studies will build upon our current findings through a multi-faceted research plan. Firstly, we will progress to in vivo validation, investigating the combined neuroprotective potential of caffeine and our engineered exo-si-ACSL4 system in rodent models of ischemic stroke. This will be crucial for assessing therapeutic efficacy in a complex physiological environment. To further optimize our delivery system, we will conduct more rigorous physicochemical characterization of the isolated exosomes. This will include systematically measuring their zeta potential to predict colloidal stability and particle surface charge, and determining the polydispersity index (PDI) to ensure a uniform size distribution, which is critical for consistent cellular uptake and batch-to-batch reproducibility. From a mechanistic perspective, while our current work confirms the successful knockdown of ACSL4, we plan to deepen our investigation into the ferroptosis signaling pathway. We will examine key upstream and downstream markers, specifically by quantifying the expression levels of GPX4 and PTGS2, to conclusively verify that our intervention rescues the endogenous antioxidant defense and suppresses lipid peroxidation. Finally, we aim to explore the broader impact of caffeine on interconnected cell death pathways, such as apoptosis and pyroptosis, to gain a more comprehensive understanding of its full therapeutic potential beyond ferroptosis inhibition.

6. Conclusion

In conclusion, this study successfully validates our initial hypothesis that exosome-mediated delivery of si-ACSL4 can significantly enhance the neuroprotective effects of caffeine by providing a more efficient and targeted approach to inhibit ferroptosis in HT-22 cells under H/R conditions. Our investigation began by establishing a reliable methodology: we first isolated exosomes from BMSCs and confirmed their identity through characteristic morphology and positive marker expression. We then demonstrated that these exosomes could be effectively loaded with si-ACSL4 and efficiently delivered into neuronal cells.

The core of our findings rests on the superior performance of the exo-si-ACSL4 system. As hypothesized, the exosomal delivery led to a dramatic and specific knockdown of ACSL4, as unequivocally shown by both qPCR and western blot analyses, which revealed approximately an 89% and 87% reduction in ACSL4 mRNA and protein levels, respectively. This level of silencing far exceeded what is typically achievable with standard transfection methods, directly confirming our premise that exosomes would enhance uptake efficiency and targeting selectivity. More importantly, this enhanced molecular effect translated into significant functional benefits. The combination of exo-si-ACSL4 and caffeine yielded a powerful synergistic effect, leading to a greater increase in cell viability, a more

pronounced reduction in inflammatory response, and a more robust inhibition of ferroptotic cell death compared to either treatment alone or to si-ACSL4 delivered without exosomes. Therefore, our work provides novel insights by integrating a pharmacological agent (caffeine) with a sophisticated nucleic acid delivery system (exo-si-ACSL4).

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