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Extracellular vesicles ameliorates sleep deprivation induced anxiety-like behavior and cognitive impairment in mice

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The aim of this research was to explore the therapeutic capabilities of extracellular vesicles (EVs) derived from human umbilical cord mesenchymal stem cells (hUC-MSCs) that had been subjected to heat shock pretreatment, in treating psychiatric disorders induced by sleep deprivation in mice. The EVs were isolated and characterized, while western blotting was utilized to assess the expression of exosomal markers and heat shock protein 70 (HSP70). To evaluate the impact of EV treatment on anxiety-like behavior and cognitive impairment in sleep-deprived (SD) mice, the open field test, plus maze test, and Y-maze task were conducted. Heat shock pretreatment significantly increased the expression of HSP70 in EVs. Administration of EVs from heat shock-pretreated hUC-MSCs improved anxiety-like behavior and cognitive function in SD mice. Furthermore, EV treatment promoted synaptic protein expression, HSP70 expression and inhibited neuroinflammation in the hippocampus of SD mice. Western blotting analysis also revealed that EV treatment reduced the levels of TLR4 and p65 in the hippocampus. EVs from heat shock-pretreated hUC-MSCs have therapeutic potential for sleep deprivation-induced psychiatric disorders by regulating neuroinflammation and synaptic function in mice.

INTRODUCTION

Sleep constitutes an essential biological process for humans, intricately involved in diverse metabolic regulations.[1] The diminishing duration of sleep has increasingly been associated with conditions such as diabetes, obesity, metabolic syndrome, and cardiovascular disease.[2] In recent years, there has been a growing focus on the intimate connection among insomnia, cognitive dysfunction, and anxiety.[3] Mounting evidence indicates that sleep disorders not only serve as potential precursors but also as early symptoms of neurodegenerative diseases.[4] Patients with Alzheimer's disease experience increased sleep interruption and a shift to a sleep-awakening mode,[5] while Parkinson's disease is linked to obstacles in rapid eye movement.[6,7] In addition, disruptions in this delicate equilibrium of sleep often manifest as symptoms of anxiety.[8] Investigations into the neurobiological substrates linking insomnia and anxiety have unveiled shared pathways, neurotransmitter systems, and structural brain alterations.[9] The reciprocal influence between sleep-wake regulation and emotional processing, mediated by intricate neural networks, underscores the intricate nature of this correlation.[10]

Heat shock protein is a highly conservative molecular partner protein induced in a high temperature or stress, which is widely expressed in the nucleus, cytoplasm and endoplasmic mesh, and mitochondria.[11] Heat shock protein 70 (HSP70) is closely related to nervous system diseases, and plays a protective role in most neurological diseases.[12] HSP70 can inhibit oxidative stress and reduce media inflammation mediated in signal transducer and activator of transcription 3 and nuclear factor κB.[13] Research in recent years has found that the reorganization HSP70 can play a brain protection effect through the blood-brain barrier.[14] In our preliminary research, we found that the restructuring mice HSP70 pre-processing can alleviate the cognitive obstacles of mice deprived of sleep and the inflammatory response of the hippocampus tissue, and promote the activation of glial cells.[15]

Heat shock protein such as HSP70 of the mesenchymal stem cells (MSCs) that have been treated with heat shock was increased by raising the thermal transcription factor, thereby enhancing the tolerance of cells to damage.[16] It is worth noting that HSP70 is a key characteristic protein for extracellular vesicles (EVs).[17] Therefore, processing MSCs through heat shock can significantly increase the expression of HSP70 in MSC-EVs. This work thus aims to study the potential therapeutic effects of EVs from human umbilical cord MSCs (hUC-MSCs) post heat-shock treatment against the cognitive impairments in sleep-deprived (SD) mice.

RESULTS

The effects of heat shock treatment on HSP70 expressions of hUC-MSCs

To explore the impact of heat shock treatment on hUC-MSCs, we conducted various analyses to assess the expression of HSP70. Following isolation, hUC-MSCs were induced to undergo osteogenic and adipogenic differentiation for 14 days. Osteogenic differentiation

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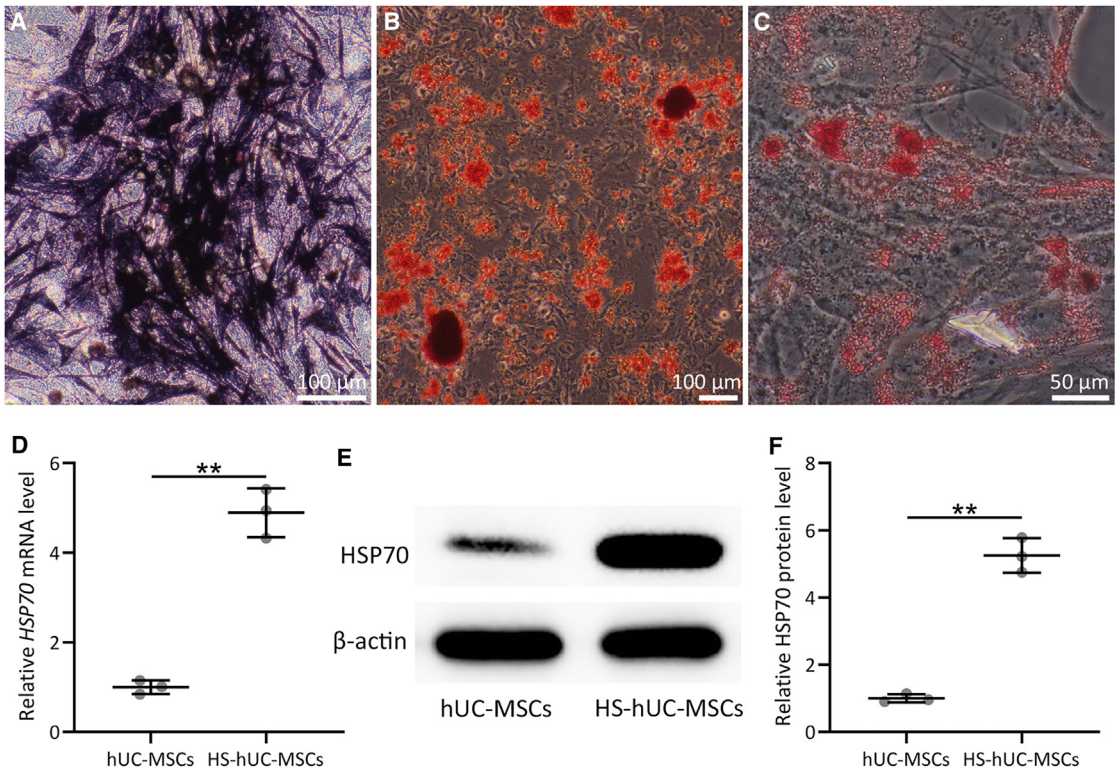

Figure 1. Isolation of hUC-MSCs and the expressions of HSP70 in hUC-MSCs after heat shock treatment

After 14 days of osteogenic differentiation, the hUC-MSCs were stained by alkaline phosphatase (A) and alizarin red S (B). After 14 days of adipogenesis induction, hUC-MSCs were stained with oil red O (C). Quantitative real-time PCR was used to analyze the mRNA expressions of HSP70 from heat shock-pretreated hUC-MSCs (D). Western blotting was used to measure the protein expressions of HSP70 from heat shock-pretreated hUC-MSCs (E) and the expressions were normalized to control (F).

was evaluated using alkaline phosphatase and alizarin red S staining (Figures 1A and 1B), while adipogenic differentiation was assessed using oil red O staining (Figure 1C). In addition, we examined the effects of heat shock treatment on HSP70 expression by analyzing the messenger RNA (mRNA) expression of HSP70 using quantitative real-time PCR (Figure 1D, $p = 0.003$) and the protein expression of HSP70 using western blotting, which was then normalized to the control (Figures 1E and 1F, $p = 0.004$). Our results revealed a significant upregulation of HSP70 expression in heat shock-pretreated hUC-MSCs, as evidenced by the increased mRNA and protein expression compared with the control group.

Upregulation of HSP70 in EVs from heat shock-pretreated hUC-MSCs

To investigate the effects of heat shock on HSP70 expression in EVs derived from hUC-MSCs, we isolated the extracellular vesicles from both non-treated and heat shock-pretreated hUC-MSCs using the protocol established by the International Society of Extracellular Vesicles (ISEV). The isolated EVs were characterized using NTA, which revealed the typical EV morphology and size range of 50–200 nm (Figure 2A). Western blotting was performed to measure the protein levels of HSP70 and the EV markers Alix, CD9, and CD63. The results confirmed the presence of HSP70, Alix, CD9, and CD63 in the isolated EVs, indicating their EV identity (Figure 2B). Furthermore, HSP70 expression in EVs derived from heat shock-pretreated hUC-MSCs was significantly higher than in those from non-treated hUC-MSCs (Figure 2C, $p = 0.002$).

EVs from heat shock-pretreated hUC-MSCs attenuate anxiety-like behavior in SD mice

We next investigated the potential therapeutic effects of EVs derived from heat shock-pretreated hUC-MSCs on sleep deprivation-induced anxiety-like behavior in mice. At a fundamental level, we verified the uptake capacity of mouse hippocampal neuronal cells for EVs. The mouse experiment was divided into four groups, namely mice without any treatment (Control), sleep deprivation model mice (SD), mice treated with EVs secreted by untreated hUC-MSCs after sleep deprivation (SD+EVs), and mice treated with EVs secreted by heat shock-pretreated hUC-MSCs after sleep deprivation (SD+HS-EVs). Mice were subjected to 24-h sleep deprivation using the grid suspended over water method. The SD mice were then injected intravenously with either EVs derived from heat shock-pretreated hUC-MSCs or non-treated hUC-MSCs. The results showed that sleep deprivation significantly decreased the distance traveled (Figure 3A, Control: 18.87 ± 2.53 m/5 min, SD: 9.23 ± 1.79 m/5 min, SD+EVs: 12.94 ± 1.81 m/5 min, SD+HS-EVs: 15.69 ± 2.23 m/5 min) and the number of rearings in the open field test (OFT)

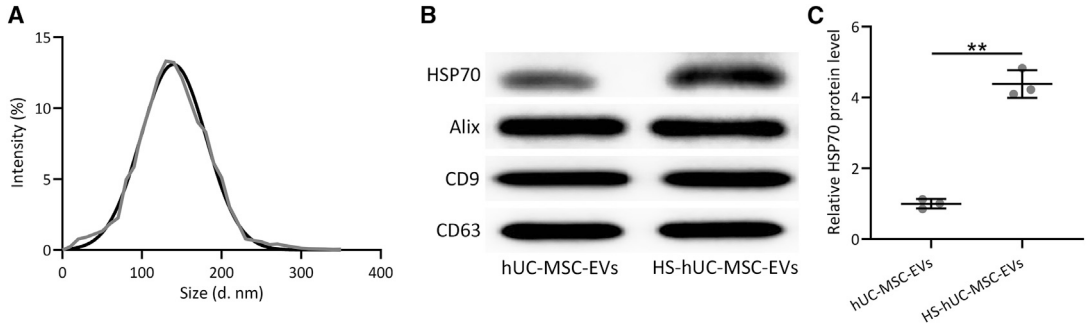

Figure 2. The expressions of HSP70 in extracellular vesicles from heat shock-pretreated hUC-MSCs

(A) Size of the particles in the isolated extracellular vesicles mixture obtained through nanoparticle tracking analysis.

(B) Western blotting was used to measure the protein expressions of HSP70, Alix, CD9, and CD63 from extracellular vesicles isolated from hUC-MSCs or heat shock-pretreated hUC-MSCs.

(C) Comparison of HSP70 protein expressions between extracellular vesicles isolated from hUC-MSCs or heat shock-pretreated hUC-MSCs.

(Figure 3B, Control: 29.55 ± 3.39, SD: 14.65 ± 2.78, SD+EVs: 19.84 ± 3.21, SD+HS-EVs: 25.54 ± 2.92), as well as the time spent in the open arms (Figure 3C, Control: 85.44 ± 7.21 s, SD: 57.73 ± 8.05 s, SD+EVs: 68.14 ± 7.61 s, SD+HS-EVs: 78.35 ± 7.02 s) and the number of entries to the open arms (Figure 3D, Control: 15.15 ± 2.34, SD: 6.08 ± 1.31, SD+EVs: 9.24 ± 1.75, SD+HS-EVs: 12.13 ± 1.37) in the elevated plus maze test, compared with the control group ($p < 0.001$). EVs obtained from untreated hUC-MSCs demonstrated notable therapeutic effects. Nonetheless, the administration of EVs derived from hUC-MSCs subjected to heat shock treatment significantly mitigated anxiety-like behavior induced by sleep deprivation. This was evidenced by increased measures in distance traveled (Figure 3A, $p = 0.025$), number of rearings (Figure 3B, $p = 0.004$), time spent in open arms (Figure 3C, $p = 0.033$), and entries to open arms (Figure 3D, $p = 0.005$) when compared with the vehicle control group. In conclusion, our findings indicate that EVs derived from heat shock-pretreated hUC-MSCs hold promise for therapeutic intervention in sleep deprivation-induced anxiety-like behavior in mice.

EVs from heat shock-pretreated hUC-MSCs improve cognitive function in SD mice

Sleep deprivation is known to impair cognitive function in rodents. In this study, we investigated the potential therapeutic effects of EVs derived from heat shock-pretreated hUC-MSCs on sleep deprivation-induced cognitive impairment in mice. To assess cognitive function, the Y-maze test was performed. In this test, the time spent in the novel arm and the spontaneous alternation were measured as indicators of cognitive function. The results showed that sleep deprivation significantly decreased the time spent in the novel arm (Figure 4A, Control: 56.83% ± 5.71%, SD: 28.74% ± 4.81%, SD+EVs: 39.22% ± 4.31%, SD+HS-EVs: 47.75% ± 5.46%) and the spontaneous alternation (Figure 4B, Control: 70.76% ± 8.21%, SD: 44.84% ± 5.49%, SD+EVs: 54.36% ± 7.01%, SD+HS-EVs: 63.29% ± 5.33%), compared with the control group. However, treatment with EVs derived from heat shock-pretreated hUC-MSCs significantly improved the sleep deprivation-induced cognitive impairment, as evidenced by the increased time spent in the novel arm (Figure 4A, $p = 0.007$) and the increased spontaneous alternation (Figure 4B, $p = 0.028$), compared with the vehicle control group. In summary, our results suggest that EVs derived from heat shock-pretreated hUC-MSCs have potential therapeutic effects on sleep deprivation-induced cognitive impairment in mice.

EVs from heat shock-pretreated hUC-MSCs increase synaptic plasticity-related proteins in the hippocampus of SD mice

Subsequently, hippocampal tissues were collected for protein analysis. Western blotting was employed to quantify the protein expressions of crucial synaptic plasticity-related molecules, namely postsynaptic density-95 (PSD95) and brain-derived neurotrophic factor (BDNF). The findings indicated a significant reduction in the expressions of PSD95 ($p = 0.007$) and BDNF ($p = 0.001$) in the hippocampus following sleep deprivation, as compared with the control group (Figures 5A–5C). Conversely, treatment with EVs derived from heat shock-pretreated hUC-MSCs led to a substantial increase in the expressions of PSD95 ($p = 0.017$) and BDNF ($p = 0.002$), relative to the vehicle control group (Figures 5A–5C). This elevation in PSD95 and BDNF expressions within the hippocampus suggests a potential contribution to the observed enhancement in cognitive function.

EVs from heat shock-pretreated hUC-MSCs alleviate sleep deprivation-induced neuroinflammation in the hippocampus of mice by modulating pro- and anti-inflammatory cytokines

This study aimed to investigate whether EVs derived from hUC-MSCs pretreated with heat shock could alleviate sleep deprivation-induced neuroinflammation in the hippocampus, which can lead to cognitive impairment. The protein levels of tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), monocyte chemoattractant protein-1 (MCP-1), and IL-10 in the hippocampus were measured using ELISA. Results indicated that treatment with EVs derived from heat shock-pretreated hUC-MSCs significantly reduced the levels of TNF-α (Figure 6A, $p < 0.001$), IL-1β (Figure 6B, $p < 0.001$), and MCP-1 (Figure 6C, $p < 0.001$) in the hippocampus compared with the sleep deprivation group. Conversely, the level of IL-10 was significantly increased in the EV-treated group compared with the sleep deprivation group (Figure 6D,

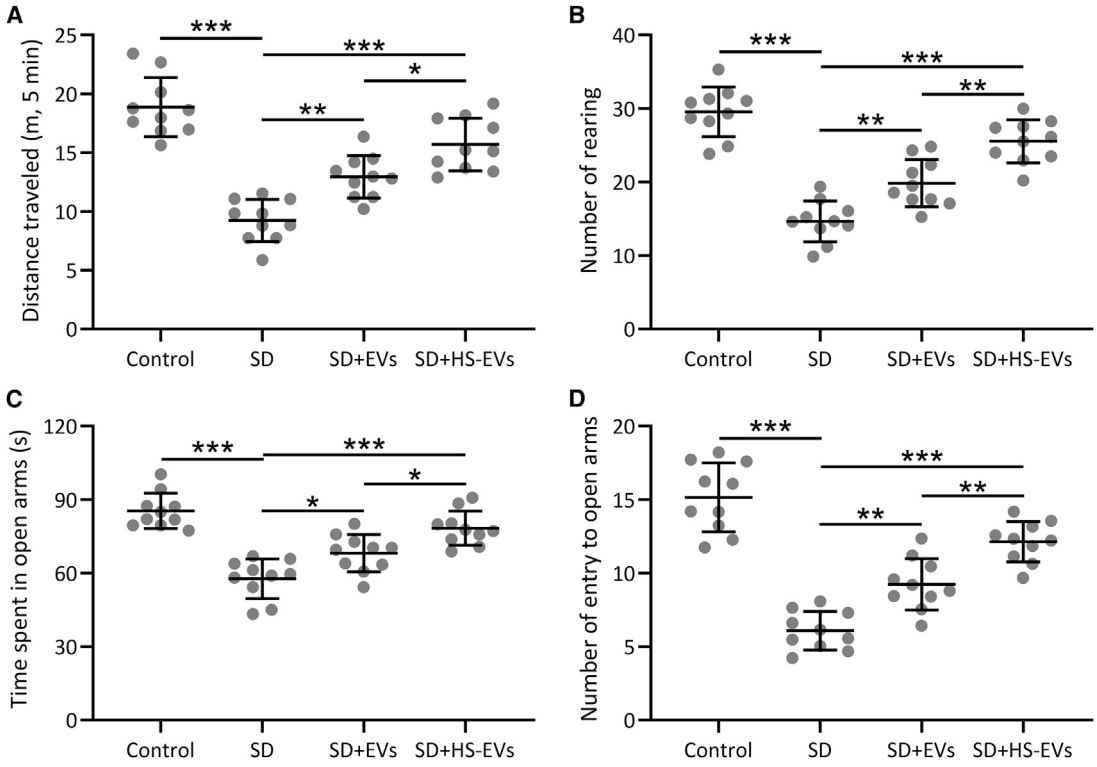

Figure 3. Extracellular vesicles (EVs) from heat shock-pretreated hUC-MSCs ameliorated sleep deprivation induced anxiety-like behavior in mice

Distance traveled (A) and numbers of rearing (B) in the open field test. The time spent in open arms (C) and number of entry to open arms (D) in the elevated plus maze. n = 10 mice for each group. SD, sleep-deprived mice received the treatment of PBS; SD+EVs, sleep-deprived mice received the treatment of EVs isolated from hUC-MSCs without heat shock treatment; SD+HS-EVs, sleep-deprived mice received the treatment of EVs isolated from hUC-MSCs with heat shock treatment.

$p < 0.001$). In addition, western blotting showed that the protein expression of HSP70 in hippocampus homogenate of mice treated with exosomes secreted by heat-shocked hUC-MSCs after sleep deprivation was significantly increased (Figures S1A and S1B). These results suggest that EVs derived from heat shock-pretreated hUC-MSCs could increase the expression of HSP70 in hippocampus homogenate of mice after sleep deprivation and have the potential to alleviate sleep deprivation-induced neuroinflammation in the hippocampus through the modulation of both pro-inflammatory and anti-inflammatory cytokines.

EVs from heat shock-pretreated hUC-MSCs inhibit TLR4 and p65 expressions in the hippocampus of SD mice

To investigate the potential mechanism by which EVs from heat shock-pretreated hUC-MSCs alleviate sleep deprivation-induced neuroinflammation, we measured the protein expressions of Toll-like receptor 4 (TLR4) and p65 in the hippocampus using western blotting. Our results showed that treatment with EVs from heat shock-pretreated hUC-MSCs significantly inhibited the expressions of TLR4 and p65 in the hippocampus of SD mice compared with the sleep deprivation group (Figures 7A–7C, $p = 0.015$ and $p = 0.011$). These findings suggest that EVs from heat shock-pretreated hUC-MSCs may alleviate sleep deprivation-induced neuroinflammation in the hippocampus by suppressing the expression of TLR4 and p65.

DISCUSSION

Sleep disturbance could lead to memory cognitive impairment, seriously affect human health, and induce neurodegenerative diseases.[18] The inflammatory response and oxidative stress in the nervous system are involved in the occurrence of cognitive impairment.[19] MSC-EVs have great potential in the treatment of neurodegenerative diseases, especially related cognitive impairments, but how to precondition MSCs to enhance the therapeutic effect of MSC-EVs is a great challenge.[20] HSP70 has neuroprotective function and could prevent and treat neurodegenerative diseases.[21] Our previous study found that recombinant HSP70 pretreatment could improve cognitive impairment in SD mice, as well as the inflammatory response and activation of microglial cells in the hippocampus. Heat shock pretreatment of MSCs could significantly increase the expression of HSP70 in MSC-EVs and thus enhance the therapeutic potential of MSC-EVs. Therefore, this project uses a mouse model of sleep deprivation to study the effect of heat shock treatment of EVs obtained from hUC-MSCs on the improvement of cognitive function in mice for the first time.

Our results showed that the expressions of HSP70 in hUC-MSCs were significantly upregulated by heat shock treatment, as evidenced by quantitative real-time PCR and western blotting analyses. Furthermore, EVs isolated from heat shock-pretreated hUC-MSCs also exhibited increased HSP70 expression compared with those from non-heat

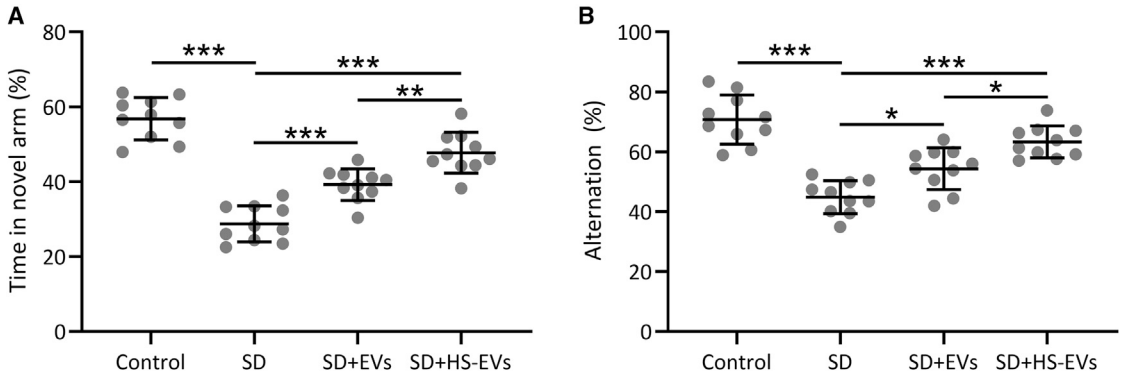

Figure 4. Extracellular vesicles from heat shock-pretreated hUC-MSCs ameliorated sleep deprivation induced cognitive impairment in mice

Time spent in the novel arm (A) and spontaneous alternation (B) in Y-maze test. n = 10 mice for each group. SD, sleep-deprived mice received the treatment of PBS; SD+EVs, sleep-deprived mice received the treatment of EVs isolated from hUC-MSCs without heat shock treatment; SD+HS-EVs, sleep-deprived mice received the treatment of EVs isolated from hUC-MSCs with heat shock treatment.

shock-treated hUC-MSCs, as confirmed by western blotting. The results of this study suggest that heat shock treatment could upregulate the expression of HSP70 in hUC-MSCs, which could potentially enhance their therapeutic potential by preserving their differentiation capacity. These findings are consistent with previous studies that have shown the beneficial effects of heat shock treatment on stem cells.[22] For instance, a study found that heat shock treatment improved the survival and proliferation of human adipose-derived stem cells.[23] Similarly, another study demonstrated that heat shock treatment promoted the survival and neuroprotective effects of bone marrow-derived MSCs.[24] The results of this study also suggest that HSP70-containing EVs may have potential therapeutic applications in the treatment of various diseases. Our study investigated the therapeutic effects of EVs derived from heat shock-pretreated hUC-MSCs on anxiety-like behavior and cognitive function in SD mice. The results showed that treatment with EVs derived from heat shock-pretreated hUC-MSCs significantly attenuated sleep deprivation-induced anxiety-like behavior and improved cognitive function in mice. These findings are consistent with previous studies that have reported the beneficial effects of MSC-derived EVs on anxiety-like behavior and cognitive function in animal models. For example, various studies have reported that treatment with EVs derived from MSCs attenuated anxiety-like behavior and improved cognitive function in mouse model of Alzheimer's disease.[25,26] Other studies have found that treatment with EVs derived from human MSCs improved cognitive function in a mouse model of traumatic brain injury.[27,28] In conclusion, the results of this study provide further evidence for the beneficial effects of heat shock treatment on stem cells, as well as the potential therapeutic applications of HSP70-containing EVs. Considering that clinical sleep disorder-related diseases are generally not serious enough to require intracranial administration, we chose tail vein injection. It is true that tail vein injection will result in only a small number of extracellular vesicles being enriched into the brain. Therefore, in this study, mice were injected for 7 consecutive days. The dosage is based on the literature and the long-term experience of the research group. However, there is still a long way to go before stem cell exosomes can be used in clinical applications. This is due to the current inability of regulatory agencies to establish appropriate quality control programs for cell exosomes. In addition, further studies are needed to elucidate the underlying mechanisms and optimize the delivery and dosage of EVs for clinical applications.

We investigated the mechanisms underlying the therapeutic effects of EVs obtained from heat shock-treated hUC-MSCs by assessing the expression levels of PSD95 and BDNF in the hippocampus of SD mice. Our findings showed that EV treatment significantly increased the expression levels of both PSD95 and BDNF in the hippocampus when compared with the control group. Furthermore, the EVs from heat shock-pretreated hUC-MSCs effectively suppressed the expressions of pro-inflammatory cytokines, such as TNF-α, IL-1β, and MCP-1, while upregulating the expression of the anti-inflammatory cytokine IL-10 in the hippocampus of SD mice. In addition, treatment with EVs from heat shock-pretreated hUC-MSCs also inhibited the expressions of TLR4 and p65 in the hippocampus of SD mice, suggesting a potential anti-inflammatory effect of EVs. These findings are consistent with other studies demonstrating the therapeutic effects of MSC-derived EVs in ameliorating cognitive impairments and neuroinflammation. In a rat model, injecting MSC-EVs into the vitreous humor 24 h after retinal ischemia resulted in notable improvements in functional recovery and reduction in neuroinflammation and apoptosis.[29] Another finding demonstrates that MSC-EVs have the ability to hinder the upregulation of pro-inflammatory mediators such as TNF-α and nitric oxide, which are typically elevated in chronic inflammatory conditions such as Alzheimer's disease and other neurodegenerative disorders.[30] These studies suggest that MSC-derived EVs have the potential to modulate the immune response and promote neuroplasticity, making them a promising therapeutic approach for cognitive disorders.

While the primary focus is on the increased expression of HSP70 in EVs and its impact on sleep deprivation-induced psychiatric disorders, it is important to consider other molecular constituents of EVs that may contribute to their therapeutic effects. Proteomic analysis could unveil key proteins involved in neuroprotection and cellular regulation, such

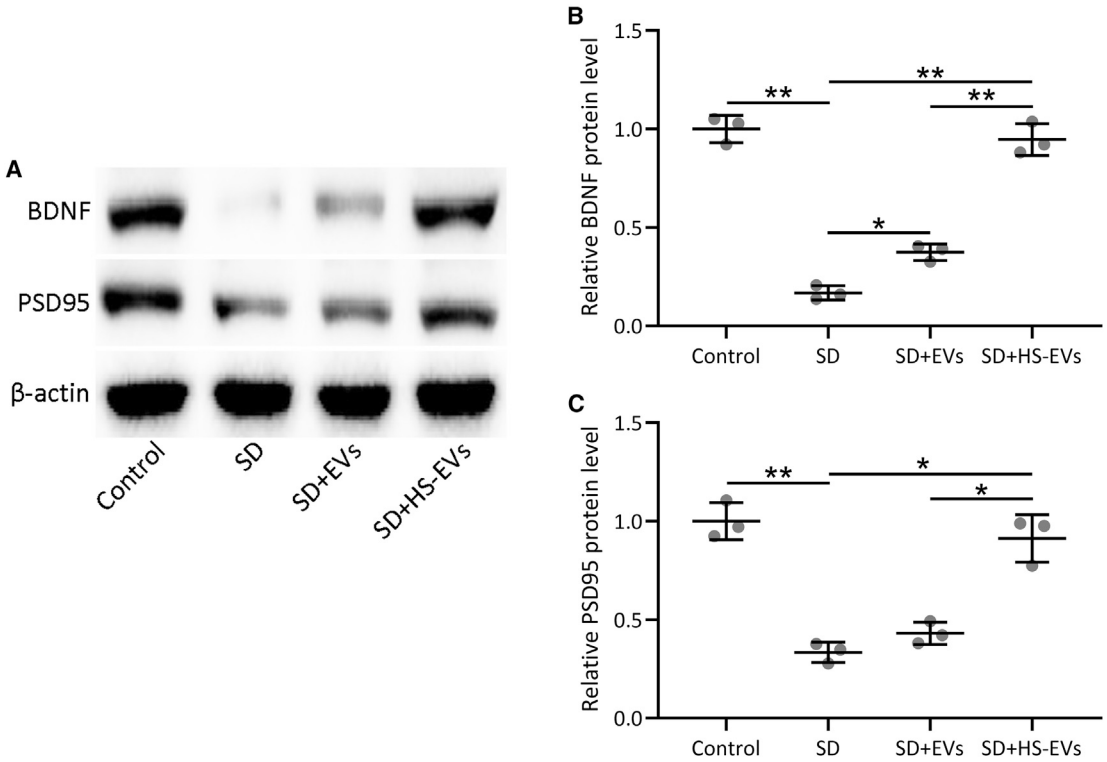

Figure 5. Extracellular vesicles from heat shock-pretreated hUC-MSCs promoted the expressions of PSD95 and BDNF in the hippocampus from sleep-deprived mice

Western blotting was used to measure the protein expressions of PSD95 and BDNF in the hippocampus homogenate (A). Quantitative analysis of PSD95 and BDNF (B and C). n = 3 repeats from mixed 8 mouse tissue homogenate. SD, sleep-deprived mice received the treatment of PBS; SD+EVs, sleep-deprived mice received the treatment of EVs isolated from hUC-MSCs without heat shock treatment; SD+HS-EVs, sleep-deprived mice received the treatment of EVs isolated from hUC-MSCs with heat shock treatment.

as growth factors (e.g., BDNF), anti-inflammatory cytokines (e.g., IL-10), and proteins associated with synaptic plasticity (e.g., PSD95).[16,31,32] Furthermore, the RNA cargo of these EVs, including mRNA and non-coding RNA species, may play pivotal roles in modulating gene expression and cellular functions.[33] Exosomal markers like Alix, CD9, and CD63, identified through western blotting, are indicative of the vesicles' exosomal identity and may facilitate specific cellular interactions.[34] The holistic understanding of these molecular constituents within EVs is crucial for unraveling their comprehensive therapeutic mechanisms and advancing targeted strategies for sleep-related cognitive disorders.

Despite the promising findings regarding the therapeutic potential of EVs containing upregulated HSP70 from heat shock-pretreated hUC-MSCs in alleviating sleep deprivation-induced psychiatric disorders in mice, several limitations should be considered. Firstly, the detailed molecular cargo of the EVs, apart from HSP70, remains to be comprehensively characterized. While the study highlights the increased expression of HSP70 and its positive effects, a more in-depth analysis of other bioactive molecules within the EVs, such as specific growth factors, cytokines, and RNA species, would provide a more nuanced understanding of the therapeutic mechanisms. In addition, the lack of a direct comparison with non-heat shock-pretreated EVs from hUC-MSCs poses a limitation, as it would help delineate the specific contributions of the heat shock treatment. Furthermore, while behavioral tests demonstrated improvements in anxiety-like behavior and cognitive function, the study could benefit from a more extensive battery of behavioral assessments to capture a broader spectrum of psychiatric symptoms. We have also not yet conducted longer follow-up and behavioral tests on animals after sleep deprivation and treatment. Finally, the translational relevance of these findings to human subjects requires careful consideration, necessitating further studies to bridge the gap between preclinical mouse models and potential clinical applications. Human sleep disorders are inherently more complex and multifaceted than those modeled in mice. The intricate interplay of genetic, environmental, and lifestyle factors contributing to sleep disturbances in humans is challenging to replicate accurately in animal models. The translation of findings from mice to humans necessitates a careful consideration of these complexities to ensure the therapy's effectiveness and safety in a clinical setting. Addressing these limitations would strengthen the credibility and applicability of the reported therapeutic effects.

In conclusion, this study demonstrated that EVs from heat shock-pretreated hUC-MSCs have a therapeutic effect on sleep deprivation-induced anxiety-like behavior and cognitive impairment in mice. The underlying mechanisms may involve the

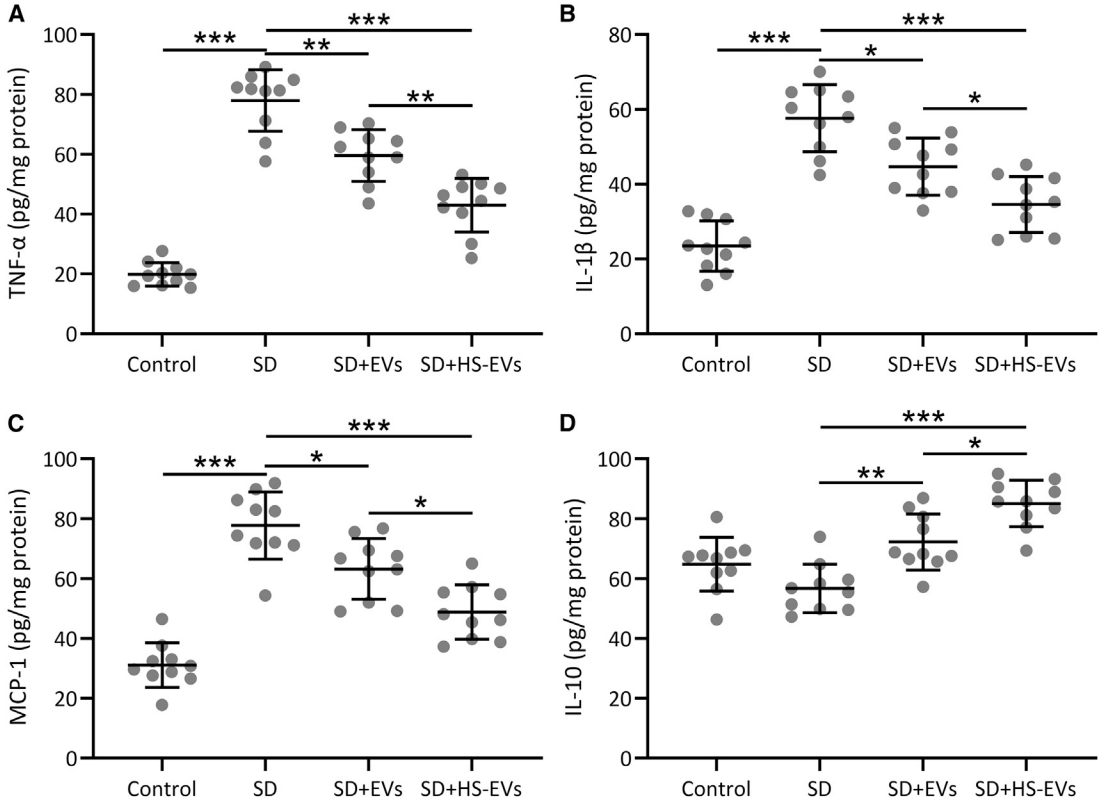

Figure 6. Effects of extracellular vesicles from heat shock-pretreated hUC-MSCs on hippocampal neuroinflammation in sleep-deprived mice

Levels of TNF-α (A), IL-1β (B), MCP-1 (C), and IL-10 (D) were measured in hippocampal homogenates using ELISA. n = 10 mice for each group. SD, sleep-deprived mice received the treatment of PBS; SD+EVs, sleep-deprived mice received the treatment of EVs isolated from hUC-MSCs without heat shock treatment; SD+HS-EVs, sleep-deprived mice received the treatment of EVs isolated from hUC-MSCs with heat shock treatment.

upregulation of PSD95 and BDNF expressions, as well as the suppression of neuroinflammation through the inhibition of TLR4 and p65 expressions in the hippocampus. The results imply that EVs derived from heat shock-treated hUC-MSCs have the potential to be a new therapeutic approach for treating sleep disorders. Nonetheless, additional research is required to fully comprehend the mechanisms behind the therapeutic benefits of EVs in sleep disorders and to enhance the protocols for their clinical use.

METHODS

Identification of hUC-MSCs

hUC-MSCs at passage 3 were subjected to fluorescence-activated cell sorting and flow cytometry analysis to evaluate the expression of CD29, CD105, Sca-1, CD34, and CD45. The hUC-MSCs were identified based on their surface marker expression pattern, with positive expression for CD29, CD105, and Sca-1, and negative expression for CD34 and CD45. Thus, cells with these surface marker characteristics were identified as hUC-MSCs.

Differentiation of hUC-MSCs

The differentiation capacity of hUC-MSCs was evaluated using adipogenic and osteogenic medium (Gibco, Life Technologies, NY). To determine the differentiation potential of hUC-MSCs into adipocytes and osteocytes, oil red O and alizarin red S staining were utilized, respectively.

Heat-shock-pretreated hUC-MSCs

To subject hUC-MSCs to heat shock conditions, cells at passage 3 were first collected and the culture medium was replaced to remove the nonadherent cells. Cells were then placed in a water bath set to 42°C for 1 h. After the heat shock treatment, the cells were incubated for 48 h at 37°C in a humidified atmosphere with 5% CO_2. The control group included UC-MSCs incubated under typical conditions without heat shock pretreatment.

EVs preparation

The EVs were prepared from hUC-MSCs using the protocol established by the ISEV. To begin, hUC-MSCs were passaged onto 100 mm culture dishes and grown to a density of 70%–80%. The MagCaptureTM Exosome Isolation Kit was utilized to isolate and prepare EVs. The hUC-MSCs were first pretreated in a serum-free medium for 48 h, and then the medium was collected and centrifuged at 1,000 × *g* for 30 min to remove larger EVs. The resulting clear supernatant was filtered through a 0.22 mm

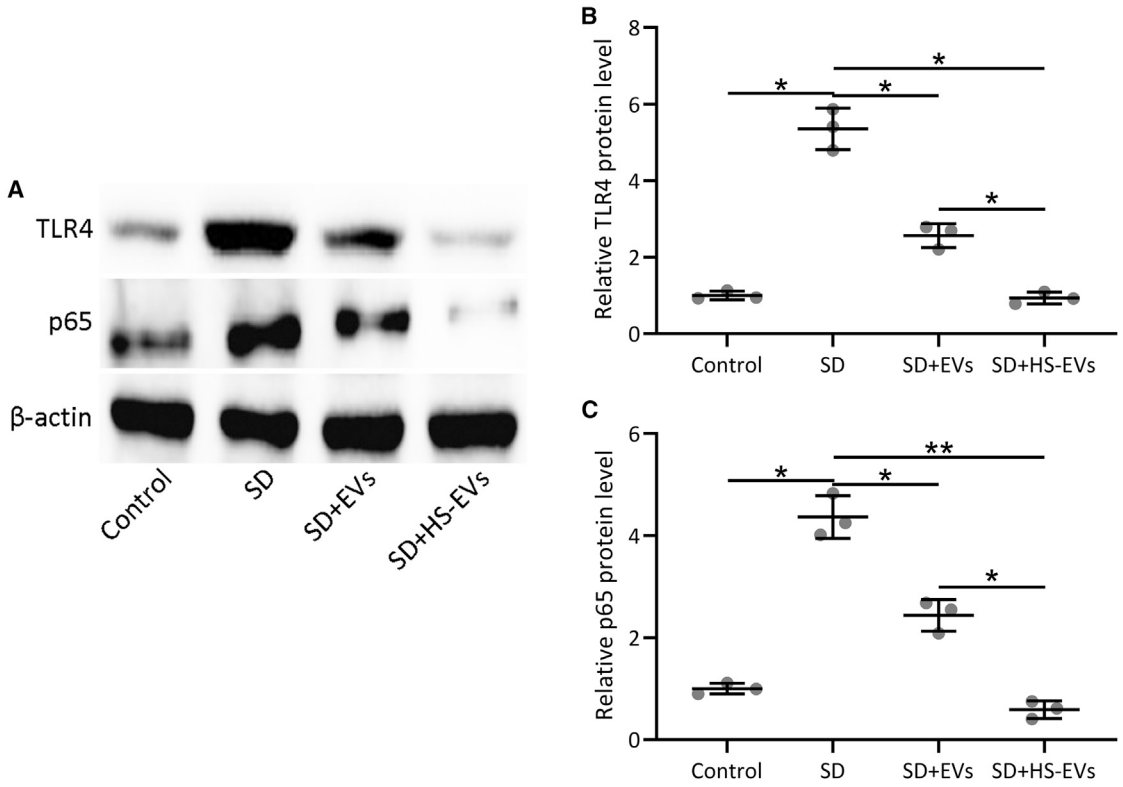

Figure 7. Effects of extracellular vesicles from heat shock-pretreated hUC-MSCs on hippocampal TLR4 and p65 expression in sleep-deprived mice

Protein expressions of TLR4 and p65 were measured in hippocampal homogenates using western blotting (A). Quantitative analysis of TLR4 and p65 (B and C). n = 3 repeats from mixed 8 mouse tissue homogenate. SD, sleep-deprived mice received the treatment of PBS; SD+EVs, sleep-deprived mice received the treatment of EVs isolated from hUC-MSCs without heat shock treatment; SD+HS-EVs, sleep-deprived mice received the treatment of EVs isolated from hUC-MSCs with heat shock treatment.

membrane and concentrated using the Vivaspin-20 Concentration Kit. Gradient centrifugation was employed to collect the hUC-MSCs EVs. The supernatant was subjected to centrifugation at 100,000 × *g* and 4°C for 70 min. The pellet was resuspended in 1 mL of phosphate-buffered saline (PBS), followed by another round of centrifugation at 100,000 × *g* and 4°C for 70 min. The supernatant was removed, and the pellet was resuspended in 50–100 μL of PBS. Finally, the prepared EVs were stored in a −80°C refrigerator.

The cellular uptake of EVs

The HT-22 cell line was procured from the American Type Culture Collection (Rockville, MD). The cells were cultured in 24-well plates using high-glucose DMEM medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum, 50 U/mL penicillin, and 50 mg/mL streptomycin. The cultivation was conducted at 37°C in an atmosphere containing 5% CO_2. Upon reaching 70%–80% confluence, the cells were harvested for subsequent analyses. EVs derived from heat shock-pretreated hUC-MSCs were labeled with Dil and incubated with HT-22 cells. The distribution and intensity of fluorescence were then examined using confocal laser microscopy to assess the uptake of EVs by HT-22 cells.

Quantitative real-time PCR

For quantitative real-time PCR analysis, total RNA was extracted from hUC-MSCs using a TRIzol reagent (Invitrogen, Waltham, MA) following the manufacturer's instructions. The purity and concentration of RNA were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). cDNA was synthesized from 1 μg of total RNA using a reverse transcription kit (TaKaRa, Dalian, China). The mRNA expression of HSP70 was analyzed using the SYBR Green Master Mix (TaKaRa) and the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). The following primers were used for HSP70: forward, 5′-GCATCGAGACTATCGCTAATGAG-3′ and reverse, 5′- TGCAA GGTTAGATTTTTCTGCCT-3′. The expression level of HSP70 was normalized to the housekeeping gene GAPDH using the $2^{\Delta\Delta Ct}$ method.

Animals

The animals used in this study were male C57BL/6 mice (B6 mice) aged 8–10 weeks and weighing 22–25 g. The mouse experiment was divided into four groups, namely mice without any treatment (Control, n = 10), sleep deprivation model mice (SD, n = 10), mice treated with EVs secreted by untreated hUC-MSCs after sleep deprivation (SD+EVs, n = 10), and mice treated with EVs secreted by heat

shock-treated hUC-MSCs after sleep deprivation (SD+HS-EVs, n = 10). The injection dosage was 50 μg/mouse, and the EVs were dissolved in normal saline. The mice were injected with the EVs via the tail vein once a day for 7 consecutive days.

Construction of SD mouse model

An SD mouse model was prepared using a modified multi-platform water environment method. The volume of the sleep deprivation box in the SD group was about 41 × 34 × 17 cm, and 12 small platforms with a diameter of 3 cm were placed, and the water surface in the box was 1 cm lower than the platforms. The mice were placed on the platform for 20 h every day, and then removed to dry their bodies. The mice were then put back into clean and dry cages to rest for 4 h for 7 consecutive days. The sleep control box of the large platform control group adopted a large platform with a diameter of 6 cm. The mice could sleep well on the large platform, and the other conditions are the same as those of the SD group. During the modeling period, the water in the box was changed every day and the mice could eat and drink by themselves, and they were still fed according to groups. The study was approved by the ethics committee of Quanzhou First Hospital Affiliated to Fujian Medical University.

OFT

OFT was conducted using a square device surrounded by a black baffle with a white bottom measuring 50 × 50 cm and a height of 50 cm. A camera was installed on top of the device for recording the behavior of the mice. The open field was divided into 5 × 5 square grids, with the central 9 grids defined as the central area and the surrounding 16 grids as the peripheral area. The experimental environment was kept quiet, with a constant temperature and humidity, and the device was isolated by a shading curtain to prevent external stimulation. During the test, the mice were placed at the same position on the periphery of the open field, and the video recording system was started simultaneously. The test lasted for 10 min, during which the movement track of the mice was recorded synchronously by video. SMART software was used to analyze the movement time and distance of the mice in the central area. The time and distance of activity in the central area were used to determine the anxiety level of the mice.

Elevated plus maze test

The elevated plus maze test device consisted of two open arms and closed arms with a length of 30 cm and a width of 5 cm. The two arms were distributed in an "X" shape, and the outer periphery of the closed arm has a black baffle with a height of 15 cm. The entire setup was 50 cm above the ground, and a video camera was mounted on top and connected to a recording system to simultaneously record the behavior of the mice in the setup. To avoid the influence of external disturbances on the mice, the entire device was isolated by a blackout curtain. The experimental environment is constant temperature and humidity. During the test, the mouse was lightly placed in the central area of the maze with its head facing the open arm, and the video recording system was quickly turned on. Each test lasted 5 min, and the video recording of the mouse's movement trajectory was performed. SMART software was used to analyze the movement time and distance of the mice in the open arms. The shorter the movement time and the shorter the distance of the mice in the open arms, the more anxious they were.

Y-maze task

The Y-maze, a food-rewarding maze task, can detect spatial reference memory and spatial working memory in mice associated with hippocampus and prefrontal cortex brain regions. A 90 × 90 × 76 cm Y-maze was used in this study. The first stage was the training period. The novel arm was blocked by a partition. The mice were placed in the starting arm, and moved freely in the starting arm and other arms for 10 min. After the training, the mice were put back into the cage. After 1 h, the second phase of the experiment was carried out. The second stage was the detection period. The partition of the new and different arms was removed, and the mice were placed in the starting arm and move freely in the three arms for 5 min. The time each mouse stayed in each arm and the number of shuttles within 5 min was recorded by video. In the Y-maze experiment, the time that the mouse stayed between each arm within 5 min and the number of shuttles in each arm within 5 min were recorded.

Western blotting assay

The western blotting assay was conducted by lysing the cells in cold radioimmunoprecipitation assay buffer that contained protease inhibitors. The resulting proteins were separated by 12% SDS polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were then incubated with antibodies against HSP70, Alix, CD9, and CD63 to detect the target proteins.

Statistical analysis

The data were presented as mean ± standard deviation. Before conducting the analysis, the normality of the data was evaluated using four distinct methods: the Anderson-Darling test, D'Agostino-Pearson test, Shapiro-Wilk test, and Kolmogorov-Smirnov test. Once normality was confirmed, the analytical approach involved the use of one-way ANOVA, followed by Dunn's multiple comparisons test. In cases where normality assumptions were not met, the Kruskal-Wallis test was applied, and the analysis was supplemented by Dunn's multiple comparisons test. Unpaired t test with Welch's correction was used to determine differences between groups. One-way ANOVA followed Dunn's multiple comparisons test was employed for comparing multiple groups. Statistical significance was considered to be $p < 0.05$.

DATA AND CODE AVAILABILITY

Data will be made available on request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2024.101207.

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AUTHOR CONTRIBUTIONS

Data curation, Y.L., Z.K., C.S., S.L., and W.X.; investigation, Y.L., Z.K., and W.X.; writing – original draft, Y.L.; writing – review & editing, Z.K., C.S., S.L., and W.X.; validation, Z.K., C.S., and S.L.; supervision, Z.K. and W.X.; investigation, W.X.; project administration, W.X.; resources, W.X.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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