



Mesenchymal stem cells' exosomes alleviate chronic visceral pain through Nrf-2-mediated oxidative stress pathway in rats

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Abstract

Introduction: Irritable bowel syndrome (IBS) is a prevalent chronic functional gastrointestinal disorder characterized by visceral hypersensitivity (VH), affecting over 10% of the global population. Current treatments for IBS have notable limitations, highlighting the need for novel therapeutic strategies to address chronic visceral pain and associated comorbidities.

Objectives:

This study aimed to investigate whether bone marrow mesenchymal stem cell-derived exosomes (BMSC-Exos) can alleviate chronic visceral pain in IBS and to explore the underlying molecular mechanisms, with a specific focus on the Nrf-2/HO-1 oxidative stress pathway.

Methods: High-quality BMSC-Exos were isolated and characterized. In vitro, their neuroprotective effects were assessed in oxidatively stressed neurons. In vivo, IBS model rats received intrathecal injections of BMSC-Exos, and their effects on visceral pain sensitivity and anxiety-like behaviors were evaluated. Spinal cord tissues were analyzed to determine modulation of the Nrf-2/HO-1 pathway.

Results: In vitro studies demonstrated that BMSC-Exos effectively rescued oxidative stress-induced neuronal damage. In IBS rats, intrathecally administered BMSC-Exos were internalized by spinal cord neurons, significantly reducing visceral hypersensitivity and anxiety-like behaviors. Mechanistically, BMSC-Exos upregulated the Nrf-2/HO-1 antioxidant pathway, mitigating oxidative stress-induced neuronal damage.

Conclusion: These findings demonstrate that BMSC-Exos alleviate chronic visceral pain and comorbid anxiety in IBS rats, likely through Nrf-2/HO-1-mediated oxidative stress reduction in spinal neurons. These results highlight BMSC-Exos as a promising acellular therapeutic strategy for IBS, offering potential applications for this debilitating disorder.

Keywords: Bone marrow mesenchymal stem cell-derived exosomes, Chronic visceral hyperalgesia, Anxiety mood, Nrf-2/HO-1

1. Introduction

Irritable bowel syndrome (IBS), a functional gastrointestinal disorder affecting 11.2% of the global population,^{27,33} is characterized by chronic visceral hypersensitivity involving both peripheral and central sensitization mechanisms.^{4,10,14,26} These neuroplastic changes contribute to abdominal pain, bloating, and anxiety comorbidities, yet current therapies remain inadequate,

underscoring the need for mechanistic insights and novel therapeutic targets.^{8,21}

Bone marrow-derived mesenchymal stem cells (BMSCs) exhibit antinociceptive potential in chronic pain models by modulating oxidative stress markers, including enhancement of superoxide dismutase (SOD) activity and reduction of reactive oxygen species (ROS).²³ However, clinical translation is hindered by risks of

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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tumorigenicity, immune rejection, and poor cell survival.²⁵ Emerging evidence suggests that BMSC-derived exosomes, nanovesicles enriched with proteins, lipids, and microRNAs, mediate therapeutic effects through paracrine signaling, offering advantages over BMSCs in stability, safety, and scalability.^{5,7,13} Although exosomes alleviate neuropathic pain and inflammation in diverse pathologies, their role in IBS-related visceral pain remains unexplored.^{16,19,22}

Oxidative stress, marked by ROS overproduction, contributes to IBS pathogenesis. The Nrf-2/HO-1 pathway, a critical antioxidant defense axis, is activated under oxidative stress to upregulate SOD and catalase (CAT), thereby reducing malondialdehyde (MDA) and restoring redox balance.^{11,15,24} Although BMSCs demonstrate antioxidant properties, whether BMSC-exosomes modulate this pathway in visceral pain remains unknown.

This study investigated the effects of BMSC-Exos in chronic visceral hypersensitivity using a rat IBS model induced by colorectal distension (CRD) and glutamate-treated PC12 neuronal cultures. Exosomes isolated through ultracentrifugation were evaluated using in vivo intrathecal administration and in vitro co-culture systems. In addition, Nrf-2 knockdown was employed to elucidate the role of the Nrf-2/HO-1 axis in BMSC-Exo-mediated analgesia and anxiolysis. These findings aim to establish exosomes as a novel therapeutic strategy for IBS, bridging the gap between oxidative stress regulation and functional visceral pain management.

2. Materials and methods

2.1. Establishment and evaluation of the irritable bowel syndrome chronic visceral hypersensitivity model

The IBS chronic visceral hypersensitivity model was established in neonatal Sprague-Dawley rats (P8-14) by daily 60 mm Hg CRD for 1 minute using a colon balloon.² Control rats underwent sham procedures without distension. At 6 weeks old, visceral sensitivity was assessed through electromyography (EMG) during 40 and 60 mm Hg CRD.⁶ Electromyography activity was recorded (RM6240BD) during 10-second distensions at 4-minute intervals. Percentage EMG change was calculated as [(CRD – baseline)/baseline] × 100%.

2.2. Histopathological processing of rat descending colon

Descending colons were fixed in 10% formalin for 24 hours, processed through graded ethanol (75%-100% with xylene steps), and paraffin-embedded. Sections (4–7 μm) were cut, mounted, dried, deparaffinized in xylene, rehydrated, and stained with hematoxylin for 5 minutes and eosin for 1 minute. Slides were dehydrated, cleared, and mounted with Permount.

2.3. Behavioral assessment of anxiety-like responses

In the open field test (OFT), rats were individually placed in an open-top transparent arena, and behavior was recorded for 5 minutes. Total distance traveled, time spent in the central zone, and rearing frequency were analyzed. In the elevated plus maze (EPM), rats were positioned at the junction of the open and enclosed arms. The number of entries into open arms, time spent in open arms, and head dips were recorded for 5 minutes.

2.4. Isolation, culture, and characterization of rat bone marrow–derived mesenchymal stem cells

Femurs and tibiae were flushed with PBS to harvest bone marrow. Mononuclear cells were cultured in α-minimum essential

medium at 37°C with 5% CO₂. Nonadherent cells were removed after 24 hours, and the medium was replaced every 2 to 3 days. Immunophenotyping was performed using antibodies against CD73-PE, CD90-APC, CD105-PE-Cy7, CD34-PE, and CD45-APC. According to ISCT criteria, MSC identity was confirmed when ≥95% of cells were positive for CD73, CD90, and CD105 and <5% were positive for CD34 and CD45.

2.5. Isolation, characterization, and tracking of bone marrow mesenchymal stem cell–derived exosomes

Bone marrow–derived mesenchymal stem cells were precultured in serum-free medium for 12 hours, followed by incubation in 1% to 2% Exo-FBS-supplemented medium for 48 hours. Supernatants were processed by ultracentrifugation at 300g for 10 minutes, 2000g for 20 minutes, 10,000g for 30 minutes, and 106,000g for 70 minutes. Exosomes were analyzed by transmission electron microscopy (TEM) (2% uranyl acetate, 100 kV), nanoparticle tracking analysis for size distribution, and Western blot for Syntenin-1, TSG101, and Calnexin (1:1000, Abcam, Cambridge, United Kingdom). PKH26-labeled exosomes (10:5, PBS, 37°C for 30 minutes) were filtered (0.22 μm) and resuspended.

2.6. Intrathecal delivery and systemic pharmacological interventions

A polyethylene-10 catheter (Scientific Commodities Inc, Lake Havasu City, AZ, BB31695-PE/1) was implanted into the L4-L5 intervertebral space. Following a 7-day recovery period, rats received daily intrathecal injections of BMSC-Exos (10, 30, or 100 μg) or vehicle for 3 consecutive days. Concurrent systemic inhibition of Nrf-2 was achieved through intraperitoneal ML385 (10 mg/kg/d) for 5 days.

2.7. Experimental model construction and intervention strategies

PC12 cells (3 × 10⁵/well) were treated with 15 mmol/L glutamate (Sigma-Aldrich, St. Louis, MO) for 24 hours to induce oxidative stress. Control groups received PBS. For exosome intervention, PKH26-labeled BMSC-Exos were co-incubated during the final 12 hours to enable real-time uptake tracking. Nrf-2 knockdown was achieved using 4 siRNA arms: scrambled siRNA-NC and Nrf-2-targeting siRNAs (positions 1590, 666, and 1194). Silencing efficiency was validated by quantitative real-time polymerase chain reaction (qRT-PCR) at 24 to 48 hours (siRNA sequences are listed in **Table 1**).

2.8. Western blot analysis of protein expression

Proteins from spinal cord tissues were extracted using radio-immunoprecipitation Assay buffer, quantified using the

Table 1
Small interfering sequence.

siRNA	Sense (5'–3')	Antisense (5'–3')
Nfe2l2-Rat-1590	GGAGAGGGAAGAAUAAAG UTT	ACUUUAUUCUCCUCUCCTT
Nfe2l2-Rat-666	CCGAGUACAGUGUCUUAATT	UUAAGACACUGAACUCGGTT
Nfe2l2-Rat-1194	GGUUCAGUGACUCGGAAA UTT	AUUUCCGAGUCACUGAACCTT
Negative control	UUCUCCGAACGUGACACGUTT	ACGUGACACGUUCGGAGA ATT

bicinchoninic Acid assay, denatured at 95°C for 5 minutes, separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene fluoride membranes. Membranes were blocked in 5% nonfat milk for 1 hour at room temperature, then incubated overnight at 4°C with primary antibodies: anti-Nrf2 (1:1000, Proteintech, Rosemont, IL), anti-HO-1 (1:1000, Proteintech), and anti- β -actin (1:7000, ABclonal, Woburn, MA). After washing with tris-buffered saline with Tween-20, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000, Proteintech). Signals were visualized using enhanced chemiluminescence and quantified with ImageJ, normalized to β -actin.

2.9. Quantitative real-time polymerase chain reaction

Total RNA was extracted from spinal cord tissues using TRIzol (Thermo Fisher, Waltham, MA). RNA purity and concentration were assessed with a Nanodrop spectrophotometer (260/280 nm). Complementary DNA (cDNA) was synthesized from 1 μ g of RNA using the Evo M-MLV Reverse Transcriptase Kit (GenStar, Beijing, China). Quantitative real-time polymerase chain reaction was performed on an Applied Biosystems 7500 system with SYBR Green Master Mix under the following conditions: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Primer sequences are listed in Table 2.

2.10. Immunofluorescence staining

Rats were perfused with 4% paraformaldehyde. Spinal cords were sectioned at 10 μ m thickness, permeabilized with 0.3% Triton X-100 in PBS for 1 hour, blocked with 5% normal goat serum in PBS for 1 hour, and incubated with primary antibodies: rabbit anti-Nrf2 (1:200, Proteintech, 16396-1-AP), mouse anti-GFAP (1:300, CST, Danvers, MA, 3670), rabbit anti-Iba1 (1:10,000, Wako, Osaka, Japan, 019-19741), or mouse anti-NeuN (1:500, Millipore, Burlington, MA, MAB377). After PBS washes, sections were incubated with Alexa Fluor 488 or 594 secondary antibodies (1:500, Abcam, 2 hours), counterstained with DAPI (1 μ g/mL, Sigma-Aldrich), and imaged using a Leica TCS SP5 confocal microscope.

Table 2

Primer used for quantitative real-time polymerase chain reaction detection.

Gene	Sequence (5'-3')
Nrf-2	F: AATTGCCACCGCCAGGACT
	R: TCAACACTTCTCGACTTACCCC
HO-1	F: CAGCATGTCCAGGATTGTTC
	R: CCTGACCTTCTGAAAGTTCCTC
MDA	F: TCTGGACAAACCTGAGCCCTAA
	R: GAACCTTGGACTCCACAGACA
SOD	F: AGGATTAAGTGAAGCGAGCAT
	R: AGCCACATTGCCAGGTCTC
CAT	F: ATTGCCGTCGATTCTCCA
	R: AGGGTCCTTCAGGTGAGTTGT
GAPDH	F: CTGGAGAAACCTGCCAAGTATG
	R: GGTGAAGAATGGGAGTTGCT

2.11. Measurement of spinal cord and cellular antioxidant capacity and neuronal injury

Thoracolumbar spinal cord homogenates (10% wt/vol) were prepared from rats. Protein concentration was quantified using the Coomassie Brilliant Blue G-250 assay (A595 nm). Catalase activity (molybdate method, A007-1-1), SOD activity (WST-1, A001-3), and MDA content (TBA method, A003-1) were measured according to the protocols of the corresponding kits (Nanjing Jiancheng Bioengineering Institute). Neuron-specific enolase (NSE) levels in PC12 cells were determined using an ELISA kit (Shanghai Mbio Co, Ltd, Shanghai, China, ml206088).

2.12. Statistical analysis

All data are presented as mean $\pm$ standard error of the mean (mean $\pm$ SEM). Statistical analyses were performed using GraphPad Prism 8.0.3 (GraphPad Software, San Diego, CA). Comparisons between the 2 groups were conducted using a Student *t* test. For multigroup comparisons involving repeated measures (eg, paw withdrawal threshold data), 2-way analysis of variance (ANOVA) followed by a Bonferroni post hoc test was applied. One-way ANOVA with Tukey post hoc test was used for comparisons among 3 or more independent groups. Statistical significance was set at $P < 0.05$, with exact *P*-values reported for all significant differences.

3. Results

3.1. Neonatal colorectal distension induced chronic visceral hyperalgesia and anxiety-like behaviors in adult rats

To establish an IBS rat model, male Sprague-Dawley (SD) rats were subjected to CRD beginning on postnatal day 8 for 7 consecutive days (Fig. 1A). At 8 weeks old, hematoxylin and eosin (HE) staining was performed on colonic tissues from both control and IBS rats. The results showed that, similar to the control group, the descending colon of IBS rats exhibited no marked edema, ulceration, or inflammatory cell infiltration (Fig. 1B). To evaluate whether the IBS model was successfully established, we measured the amplitude of external abdominal oblique muscle discharges during CRD. Irritable bowel syndrome rats exhibited significantly higher discharge amplitudes than control rats at 40 mm Hg and 60 mm Hg distension pressures (Figs. 1C and D), indicating visceral hypersensitivity in adulthood following neonatal CRD.

Chronic pain is frequently accompanied by negative emotions such as anxiety, which further complicate its management. To assess anxiety-like behavior, we employed 2 standard behavioral assays: the OFT and EPM. In the OFT, no significant difference in total distance traveled was observed between IBS and control rats, suggesting that neonatal CRD did not affect locomotor activity. However, IBS rats spent less time and traveled shorter distances in the central zone compared with controls (Figs. 1E–H). Consistently, in the EPM test, the percentage of time spent in and the number of entries into the open arms were significantly reduced in IBS rats (Figs. 1I–L). These findings suggest that chronic visceral hyperalgesia was associated with increased anxiety-like behaviors.

3.2. Imbalance of Nrf-2/HO-1 signaling in spinal cord neurons of irritable bowel syndrome rats

Chronic visceral pain is often accompanied by neuronal alterations, and the regulation of the antioxidative stress

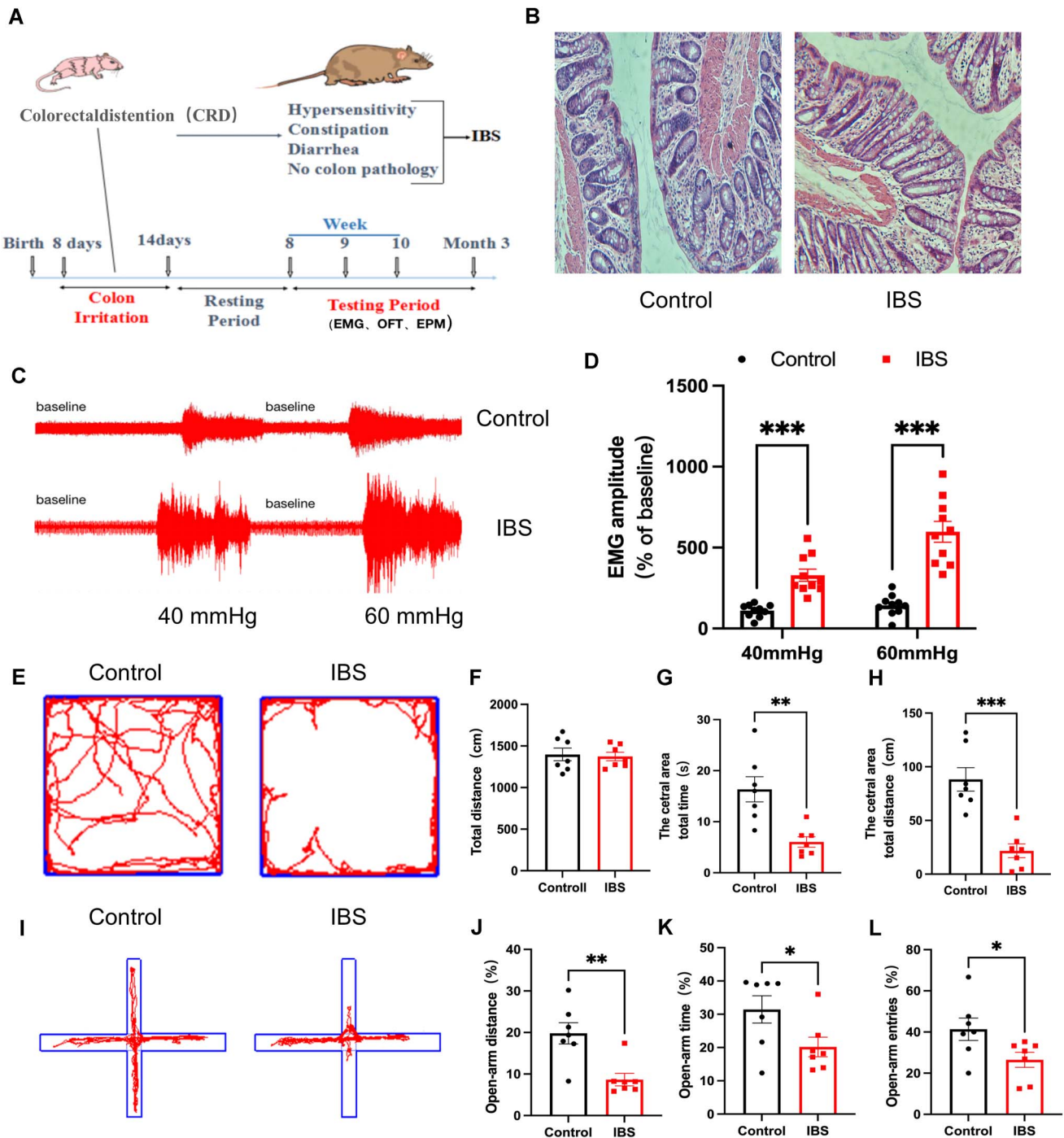


Figure 1. Neonatal rats exposed to CRD developed chronic visceral hyperalgesia and anxiety-like behaviors in adulthood. (A) Experimental design. (B) Hematoxylin and eosin (HE) staining of the descending colon, magnification $\times 100$. (C and D) Representative EMG recordings in control and IBS rats under 40 and 60 mm Hg CRD stimulation, with statistical analysis of EMG amplitudes ($n = 10$, 2-way ANOVA, $F = 52.730$, $***P < 0.001$). (E–H) Representative trajectories in the OFT, statistical graphs of total distance moved, time spent in the central zone, and distance traveled in the central zone in control and IBS rats ($n = 7$, t test, $t = 3.868$, $t = 5.260$, $**P < 0.01$, $***P < 0.001$). (I–L) Representative trajectories in the EPM, statistical graphs of percentage of distance traveled in the open arms, percentage of time spent in the open arms, and percentage of open arm entries in control rats and IBS rats ($n = 7$, t test, $t = 3.762$, $t = 2.230$, $t = 2.267$, $*P < 0.05$, $**P < 0.01$). CRD, colorectal distension; EMG, electromyography; EPM, elevated plus maze; IBS, irritable bowel syndrome; OFT, open field test.

system plays a protective role in the development of both acute and chronic pain. To examine Nrf-2 localization in IBS model rats, double immunofluorescence staining was performed. The results showed that Nrf-2 co-localized with NeuN-positive neurons, but not with GFAP-positive astrocytes or IB1-positive microglia (Fig. 2A). Quantitative analysis

demonstrated that Nrf-2 fluorescence intensity in the spinal cords of IBS rats was significantly lower than in controls (Figs. 2B and C). Western blot analysis further confirmed that protein expression levels of Nrf-2 and HO-1 were markedly reduced in IBS rats compared with controls (Figs. 2D–F). The findings suggest that disruption of the Nrf-2/HO-1-mediated

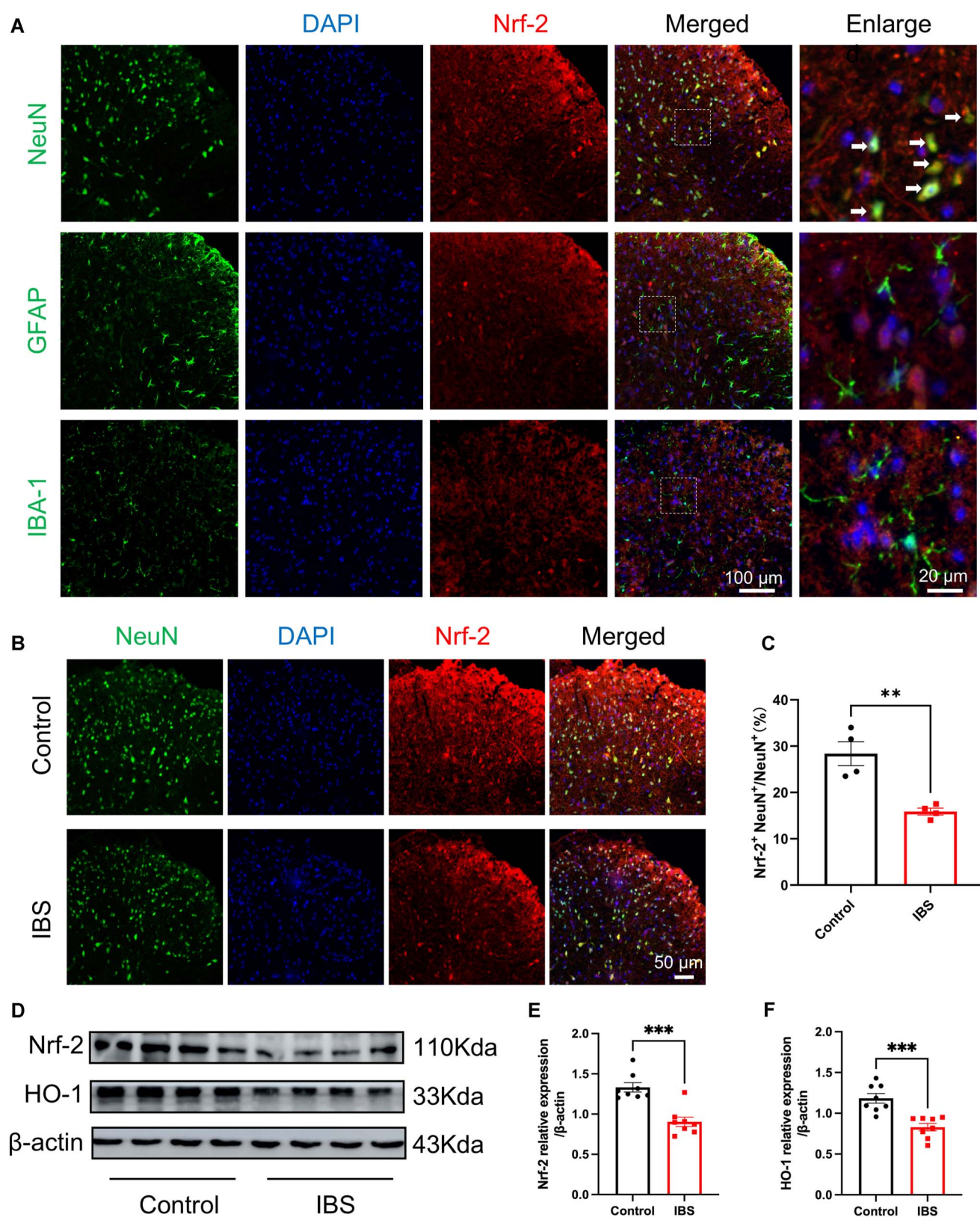


Figure 2. Imbalance of the Nrf-2/HO-1 signaling pathway in spinal cord neurons of IBS rats. (A) Immunofluorescence (IF) showing predominant Nrf-2 expression (red) in neurons (NeuN) in the spinal cord, with no apparent co-localization with astrocytes (GFAP) or microglia (Iba-1). DAPI was used for nuclear staining (blue). Scale bar: 100 μ m (log-mag) and 20 μ m (high-mag). (B and C) Representative IF images and quantitative analysis of Nrf2 in the spinal cord of control and IBS rats ($n = 4$, t test, $t = 4.646$, $**P < 0.01$; scale bar: 50 μ m). (D–F) Representative images and statistical graphs of Nrf-2 and HO-1 protein expression in the spinal cord of control and IBS rats ($n = 8$, t test, $t = 5.198$, $t = 4.723$, $***P < 0.001$). IBS, irritable bowel syndrome.

antioxidative stress pathway in spinal cord neurons may be involved in IBS pathophysiology.

3.3. Bone marrow mesenchymal stem cell-derived exosomes reversed oxidative stress damage in neurons *in vitro*

In this experiment, 3-week-old male SD rats served as bone marrow donors. Bone marrow-derived mesenchymal stem cells were successfully obtained by adherent culture, expanded to the third and fourth passages, and exhibited the characteristic spindle-shaped morphology (Fig. 3A). To ensure purity, flow cytometry analysis was conducted, confirming positive

expression of CD73, CD90, and CD105, and negative expression of hematopoietic markers CD34 and CD45 (Figs. 3B–F). These results verified the successful isolation and identification of BMSCs.

Bone marrow mesenchymal stem cell-derived exosomes, isolated by ultracentrifugation, displayed the characteristic cup-shaped morphology under TEM (Fig. 3G). Nanoparticle tracking analysis showed that most BMSC-Exos ranged from 30 to 200 nm in diameter (Fig. 3H). Western blot confirmed the presence of classical exosome markers TSG101 and Syntenin, while Calnexin expression was absent (Fig. 3I). Together, these findings demonstrated that the extracted vesicles met the established criteria for classical BMSC-Exos.

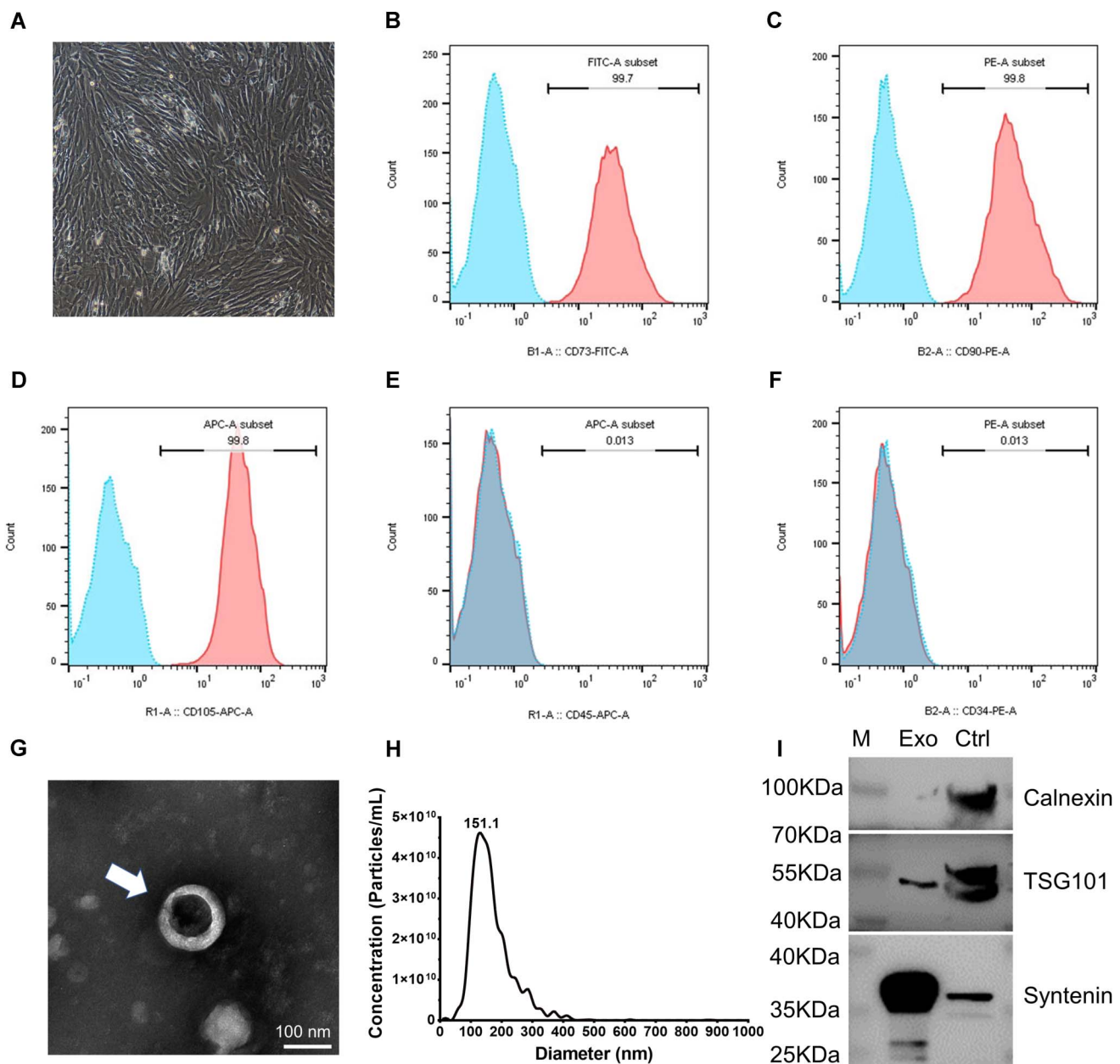


Figure 3. Characteristics of BMSCs and their derived exosomes. (A) Morphological characteristics of rat bone marrow mesenchymal stem cells under the microscopy, magnification $\times 100$. (B–F) Flow cytometry histograms showing fluorescence intensity with antibodies against surface markers (red area) compared with isotype controls (blue area). CD73 (99.7%), CD90 (99.8%), and CD105 (99.8%) were positive, whereas CD34 (0.013%) and CD45 (0.013%) were negative. (G) Representative TEM image of exosomes (arrowheads). Scale bar, 100 nm. (H) Size distribution of isolated BMSC-Exos. (I) Western blot analysis of protein expression in BMSC-Exos with 293T cells as control. BMSC, bone marrow mesenchymal stem cell; TEM, transmission electron microscopy.

Nrf-2 is a transcription factor that regulates the cellular antioxidant response by promoting the expression of a variety of antioxidant genes, including HO-1. Malondialdehyde, superoxide dismutase, and catalase are 3 commonly used biomarkers of oxidative stress. Malondialdehyde, a product of lipid peroxidation, is widely applied to evaluate the degree of lipid oxidation within cells. Superoxide dismutase and catalase are 2 critical intracellular antioxidant enzymes that scavenge superoxide radicals and hydrogen peroxide, respectively. Activation of the Nrf-2/HO-1 pathway enhances the expression of antioxidant enzymes such as SOD and CAT while reducing MDA production, thereby improving the cellular capacity to resist oxidative stress.

To examine whether BMSC-Exos could rescue neuronal oxidative stress, PC12 cells were treated with 15 mmol/L glutamate for 24 hours to simulate oxidative stress injury, while control cells were treated with an equal volume of PBS. Subsequently, different concentrations of BMSC-Exos (10 μ g/mL, 50 μ g/mL, and 100 μ g/mL) were co-cultured with PC12 cells for 12 hours, and the incorporation of BMSC-Exos into cells was observed by fluorescence microscopy. The results showed that PKH26-labeled BMSC-Exos were incorporated into PC12 cells in a concentration-dependent manner (Fig. 4A). ELISA results further demonstrated that NSE levels were increased in glutamate-treated neurons, indicating neuronal injury (Fig. 4B). These findings confirmed that BMSC-Exos were successfully internalized into oxidative stress-damaged neurons in vitro.

To clarify whether the effect of BMSC-Exos was mediated by the Nrf-2 signaling pathway, specific siRNA sequences targeting Nrf-2 transcription were designed and synthesized (Fig. 4C). Three siRNA sequences were screened in vitro, and qPCR analysis revealed that siRNA-Nrf-2-1590 was the most efficient in reducing Nrf-2 expression in PC12 cells compared with siRNA-NC, siRNA-Nrf-2-666, and siRNA-Nrf-2-1194 after transfection. Therefore, siRNA-Nrf-2-1590 was selected for subsequent experiments. PC12 cells exposed to 15 mmol/L glutamate for 24 hours were co-cultured with 50 μ g/mL BMSC-Exos for 12 hours. qPCR analysis showed that Nrf-2 and HO-1 mRNA levels were reduced in oxidative stress-injured PC12 cells compared with controls, whereas this reduction was reversed by BMSC-Exos (Figs. 4D and E), suggesting that BMSC-Exos rescued neuronal oxidative stress.

Further intervention experiments demonstrated that compared with the siRNA-NC group, BMSC-Exos failed to reverse the reduction of Nrf-2 and HO-1 mRNA in damaged neurons following Nrf-2 knockdown (Figs. 4D and E); At the same time, MDA levels were significantly increased, while SOD and CAT levels were significantly decreased (Figs. 4F–H). These findings indicated that Nrf-2 knockdown abolished the ability of BMSC-Exos to rescue neurons from oxidative stress. Collectively, these results suggest that BMSC-Exos protect neurons from oxidative stress by regulating the Nrf-2/HO-1 signaling pathway in vitro.

3.4. Bone marrow mesenchymal stem cell-derived exosomes administration improved visceral hypersensitivity and anxiety-like behaviors in an irritable bowel syndrome rat model

PKH26-labeled BMSC-Exos were intrathecally injected into rats at postnatal day 50 (Fig. 5A). Double immunofluorescence staining showed that BMSC-Exos were present in the dorsal horn of the spinal cord bilaterally in IBS rats, co-localizing predominantly with NeuN-positive neurons and minimal with GFAP-positive astrocytes or IBA-1-positive microglia. These

findings indicated that BMSC-Exos were mainly taken up by neurons in the spinal cords of IBS rats (Fig. 5B).

To evaluate the effect of intrathecal BMSC-Exos on visceral nociceptive sensitization in IBS rats, different doses of BMSC-Exos (10 μ g, 30 μ g, and 100 μ g) or PBS (control) were intrathecally injected on postnatal day 50. The amplitude of the external abdominal oblique muscle discharge was recorded. The results showed that 10 μ g of BMSC-Exos had no significant effect, whereas both 30 μ g and 100 μ g significantly reduced the EMG amplitude in IBS rats (Figs. 5C and D). No significant difference was observed between the 30 μ g and 100 μ g doses; therefore, 30 μ g was chosen for subsequent experiments. These findings demonstrated that intrathecal administration of 30 μ g BMSC-Exos alleviated visceral nociceptive hypersensitivity in IBS rats.

Next, the OFT and EPM tests were used to assess anxiety-like behaviors. After 7 consecutive days of intrathecal BMSC-Exos administration, IBS + Exos rats spent significantly more time and traveled longer distances in the central zone compared with IBS + Vehicle rats (Figs. 5E–H). In the EPM test, the percentage of open arm time and entries was also significantly increased in the IBS + Exos group (Figs. 5I–L). These results indicated that intrathecal BMSC-Exos administration alleviated anxiety-like behaviors in IBS rats.

3.5. The antioxidative effect of bone marrow mesenchymal stem cell-derived exosomes is dependent on the Nrf-2/HO-1 pathway

As the Nrf-2/HO-1-mediated antioxidative pathway was suppressed in the spinal cords of IBS rats, we hypothesized that BMSC-Exos exert their therapeutic effects on chronic visceral pain through this pathway. To test this, changes in spinal Nrf-2 and HO-1 protein expression were examined after intrathecal BMSC-Exos administration. Western blot analysis showed that Nrf-2 and HO-1 protein levels were significantly increased in IBS + Exos rats compared with IBS + Vehicle rats (Figs. 6A–C).

ML385, a specific Nrf-2 inhibitor, effectively suppresses Nrf-2 activity and downstream gene expression. Rats were intraperitoneally injected with ML385 for 5 days (postnatal days 50–54) concurrently with BMSC-Exos. Compared with the BMSC-Exos group, spinal Nrf-2 and HO-1 protein expression levels were significantly reduced in the ML385 + Exos group (Figs. 6D–F). These findings suggest that the antioxidative effect of BMSC-Exos is dependent on the Nrf-2/HO-1 pathway.

To determine whether BMSC-Exos mediate visceral nociceptive sensitization and anxiety-like behaviors in IBS rats through the Nrf-2/HO-1 pathway, ML385 was preadministered intraperitoneally for 5 consecutive days from postnatal day 50 to 54, combined with intrathecal injection of 30 μ g BMSC-Exos. The discharge amplitude of the external abdominal oblique muscle was measured by EMG, and anxiety-like behavior was assessed using the OFT and EPM. The results showed that the effect of BMSC-Exos in alleviating nociceptive sensitization in IBS rats was blocked by preinjection of ML385 compared with the treatment group (Figs. 6G and H). In the OFT, central time and central distance were significantly reduced in the IBS + ML385 + Exos group after ML385 administration (Figs. 6I–L). Similarly, in the EPM, preinjection of ML385 significantly reduced the percentage of open arm time and entries in the IBS + ML385 + Exos group (Figs. 6M–P).

Next, oxidative stress levels in the spinal cord were assessed. Malondialdehyde content was determined using the TBA kit, while the activities of SOD and CAT were measured using the WST-1 kit and ammonium molybdate kit, respectively. The

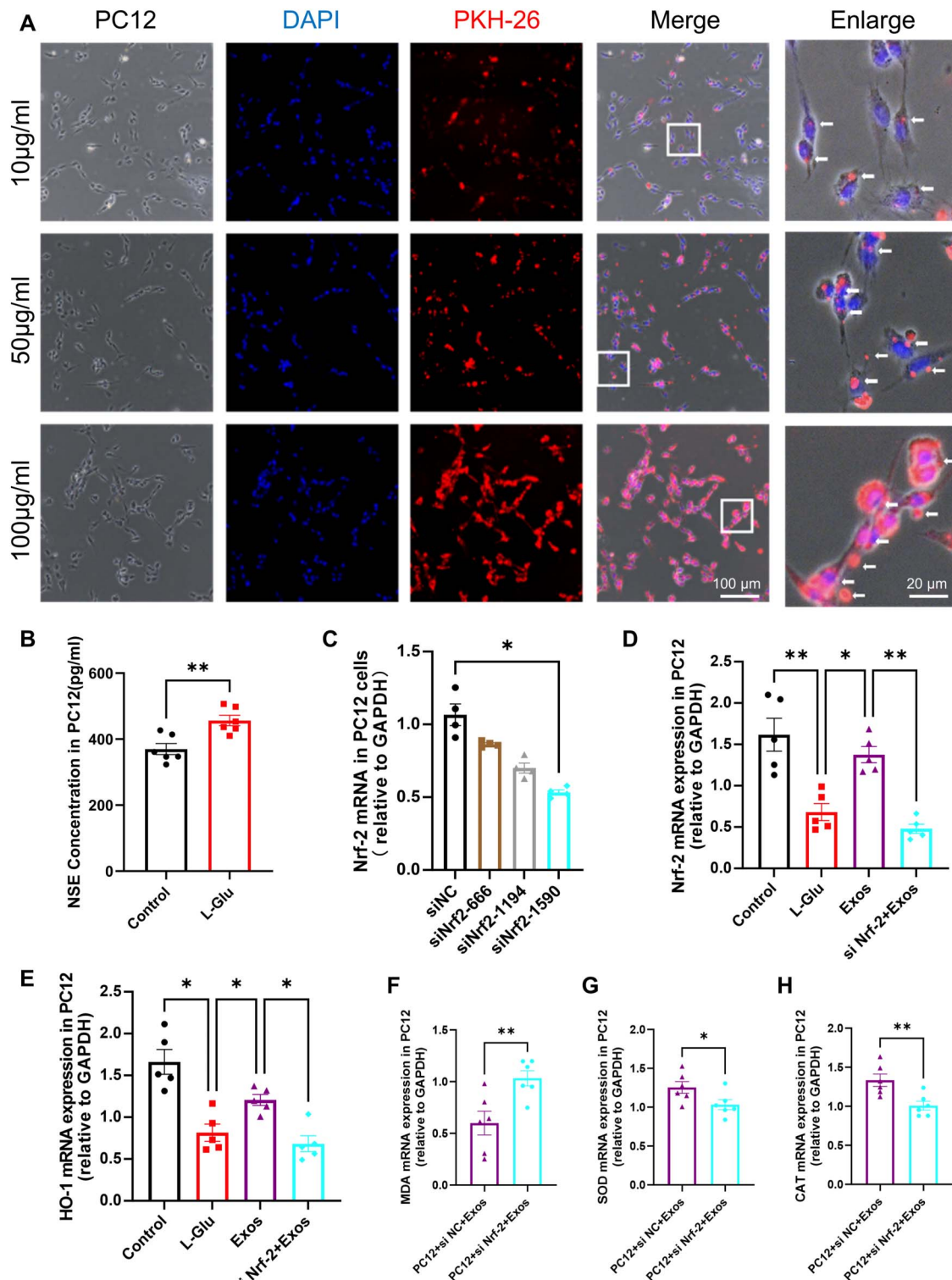


Figure 4. BMSC-Exos reversed oxidative stress-induced neuronal damage in vitro. (A) IF showing co-localization of different doses of PKH26-labeled BMSC-Exos (red) with PC12 cells. DAPI was used for nuclear staining (blue). Scale bar: 100 μ m (log-mag) and 20 μ m (high-mag). (B) Quantification of NSE expression in PC12 cells by ELISA ($n = 6$, t test, $t = 3.758$, $**P < 0.01$). (C) Efficiency ranking of candidate Nrf-2 siRNA at 10 nM ($n = 4$, 1-way ANOVA, $F = 23.312$, $*P < 0.05$). (D and E) Statistical graphs of Nrf-2 and HO-1 mRNA expression in PC12 cells across groups ($n = 5$, 1-way ANOVA, $F = 18.886$, $*P < 0.05$, $**P < 0.01$). (F–H) Statistical graphs of MDA, SOD, and CAT levels in PC12 cells in the si-NC + Exos and si-Nrf-2 + Exos groups ($n = 6$, t test, $t = 3.118$, $t = 2.243$, $t = 3.346$, $*P < 0.05$, $**P < 0.01$). BMSC, bone marrow mesenchymal stem cell; CAT, catalase; IF, immunofluorescence; MDA, malondialdehyde; NSE, neuron-specific enolase; SOD, superoxide dismutase; TEM, transmission electron microscopy.

results showed that MDA levels were significantly increased, whereas SOD and CAT activities were markedly decreased in the IBS group, indicating an imbalance of oxidative stress in the spinal

cord of IBS rats. Intrathecal administration of BMSC-Exos significantly reduced MDA levels and increased SOD and CAT activities. However, this effect was abolished by preinjection of

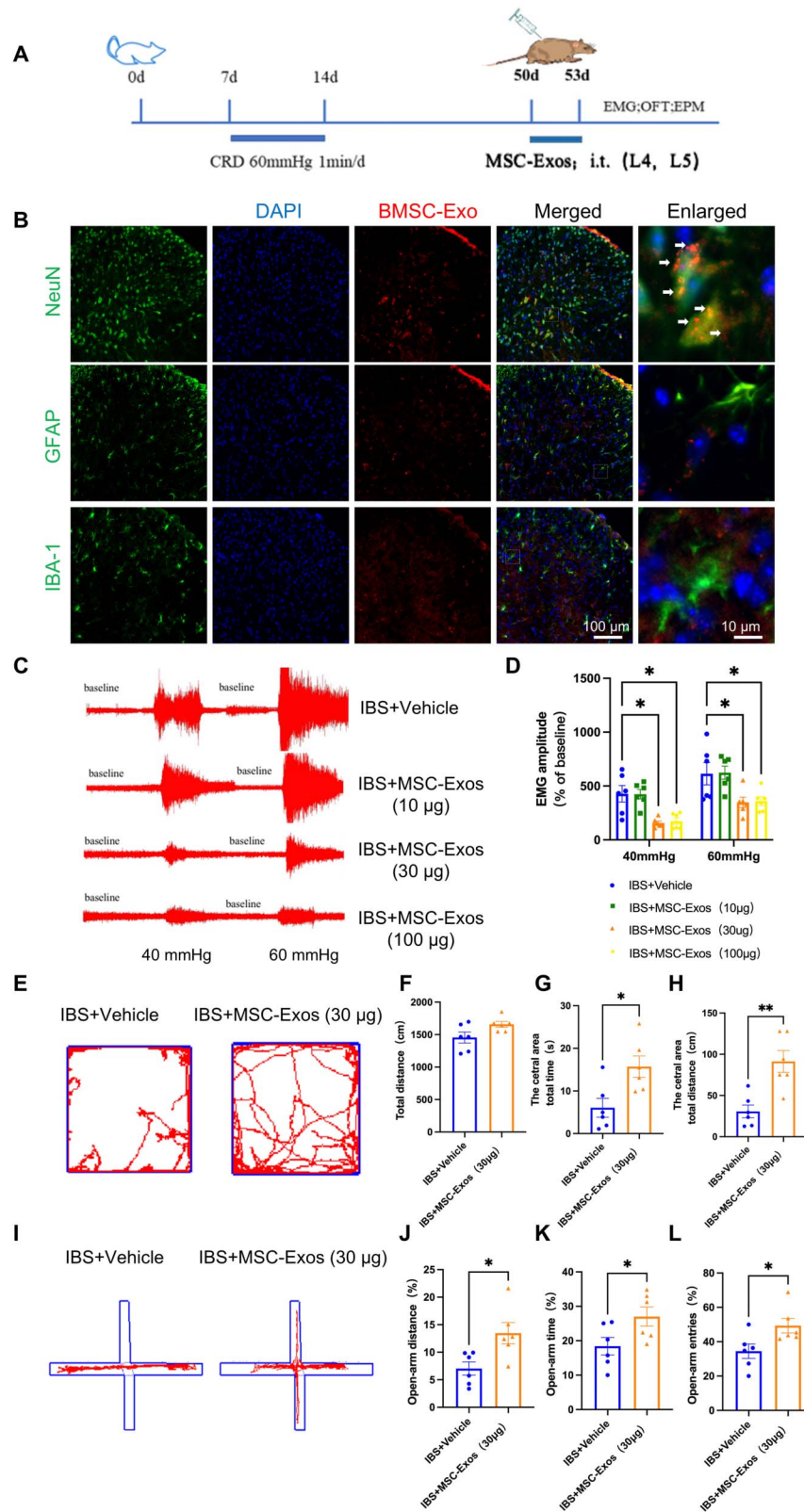


Figure 5. BMSC-Exos administration improved visceral hypersensitivity and anxiety-like behaviors in IBS rats. (A) Experimental design. (B) IF showing predominant localization of BMSC-Exos (red) in neurons (NeuN) of the spinal cord, with minimal co-localization in astrocytes (GFAP) or microglia (Iba-1), DAPI was used for nuclear staining (blue). Scale bar: 100 μ m (log-mag) and 10 μ m (high-mag). (C and D) Representative EMG recordings and statistical analysis of EMG amplitudes under 40 and 60 mm Hg CRD in rats treated with different doses ($n = 6$, 2-way ANOVA, $^*P < 0.05$). (E–H) Representative OFT trajectories and statistical graphs of total distance moved, time spent in the central zone, and distance traveled in the central zone in control and exosome-injected rats ($n = 6$, t test, $t = 2.90$, $t = 3.957$, $^*P < 0.05$, $^{**}P < 0.01$). (I–L) Representative EPM trajectories and statistical graphs of percentage of distance traveled, time spent, and entries into the open arms in control rats and IBS rats treated with exosomes ($n = 6$, t test, $t = 2.830$, $t = 2.287$, $t = 2.494$, $^*P < 0.05$). BMSC, bone marrow mesenchymal stem cell; CRD, colorectal distension; EMG, electromyography; EPM, elevated plus maze; IBS, irritable bowel syndrome; IF, immunofluorescence; OFT, open field test.

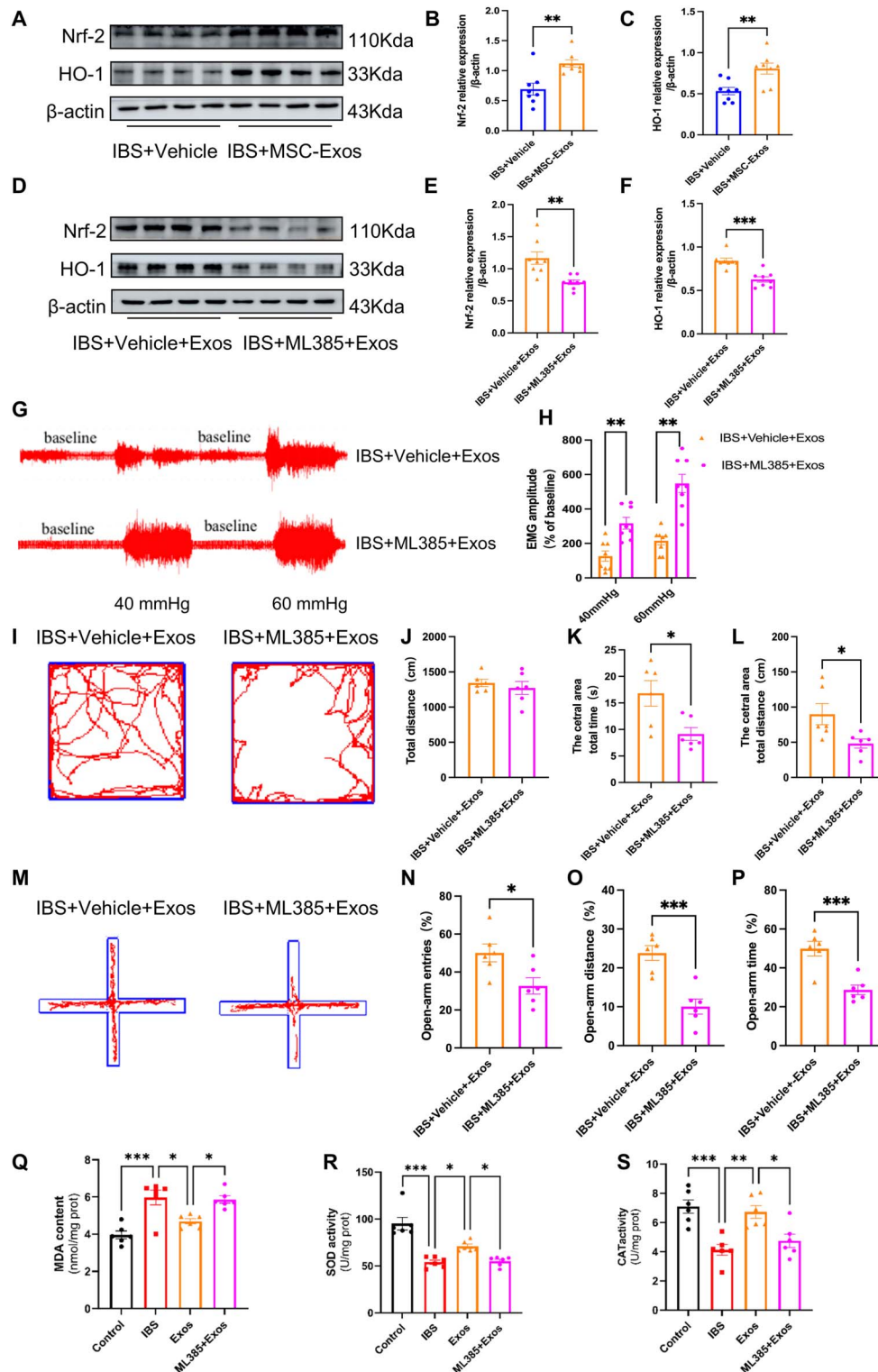


Figure 6. The antioxidative effect of BMSC-Exos is dependent on the Nrf-2/HO-1 pathway. (A–C) Representative images and statistical graphs of Nrf-2 and HO-1 protein expression in the spinal cord of the Model group (IBS + Vehicle) and Treatment group (IBS + BMSC-Exos) ($n = 8$, t test, $t = 3.686$, $t = 3.867$, $**P < 0.01$). (D–F) Representative images and statistical graphs of Nrf-2 and HO-1 protein expression in the Treatment group (IBS + Exos + Vehicle) and Antagonist group (IBS + Exos + ML385) ($n = 8$, t test, $t = 3.655$, $t = 5.044$, $**P < 0.01$, $***P < 0.001$). (G–H) Representative EMG recordings and statistical graphs of rats in the Treatment group (IBS + Vehicle + Exos) and Antagonist group (IBS + ML385 + Exos) under 40 and 60 mm Hg CRD ($n = 8$, 2-way ANOVA, $F = 44.790$, $**P < 0.01$). (I–L) Representative OFT trajectories and statistical graphs of total distance moved, time spent in the central zone, and distance traveled in the central zone by rats ($n = 6$, t test, $t = 0.682$, $t = 2.854$, $t = 2.555$, $*P < 0.05$). (M–P) Representative EPM trajectories and statistical graphs of percentage of entries, distance traveled, and time spent in the open arms ($n = 6$, t test, $t = 2.736$, $t = 5.085$, $t = 4.729$, $*P < 0.05$, $***P < 0.001$). (Q–S) Statistical graphs of MDA content and SOD and CAT activities in spinal cord tissues of the Blank group (Control + Vehicle), Model group (IBS + Vehicle), Treatment group (IBS + Exos), and Antagonist group (IBS + Exos + ML385) ($n = 6$, t test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). BMSC, bone marrow mesenchymal stem cell; CAT, catalase; CRD, colorectal distension; IBS, irritable bowel syndrome; MDA, malondialdehyde; OFT, open field test; SOD, superoxide dismutase.

ML385 (Figs. 6Q–S). These findings suggest that BMSC-Exos enhance the resistance of spinal cord tissue to oxidative stress in IBS rats by regulating the Nrf-2/HO-1 signaling pathway.

Taken together, these results indicate that intrathecal administration of BMSC-Exos significantly alleviates chronic visceral pain sensitization and anxiety-like behaviors in IBS rats, and that these effects are blocked by preinjection of ML385. This finding suggests that BMSC-Exos mitigate chronic visceral pain and anxiety comorbidity through modulation of the Nrf-2/HO-1 signaling pathway.

4. Discussion

This study provides novel evidence for the therapeutic potential of BMSC-Exos in alleviating chronic visceral hyperalgesia and anxiety-like behaviors in a rat model of IBS, mediated through activation of the Nrf-2/HO-1 antioxidant pathway. These findings are consistent with the growing recognition of oxidative stress and central sensitization as critical contributors to IBS pathophysiology, while supporting BMSC-Exos as a promising cell-free therapeutic strategy.

Persistent visceral pain in IBS is increasingly attributed to central sensitization, a state of hyperexcitability in spinal and supraspinal pain-processing circuits. Our neonatal CRD model reproduced this phenomenon, with adult rats displaying exaggerated visceromotor responses and anxiety-like behaviors. These outcomes parallel clinical observations of altered pain thresholds and psychiatric comorbidities in IBS,⁹ reinforcing the interaction between peripheral nociceptive signaling and central neuroplasticity. Oxidative stress, characterized by excessive ROS and reduced antioxidant defenses, has emerged as a key driver of neuronal dysfunction in chronic pain.²⁹ Our data extend this concept to IBS, demonstrating elevated MDA levels and reduced SOD and CAT activities in the spinal cord of IBS rats, suggesting that oxidative stress may impair gut-brain axis communication through both neuronal and nonneuronal mechanisms.

Recent studies highlight neuroimmune interactions as pivotal in maintaining central sensitization. For example, microglial activation in the spinal dorsal horn promotes the release of pro-inflammatory cytokines (eg, IL-1 β , TNF- α), which enhance synaptic plasticity and nociceptive transmission.¹⁸ Although our study focused on neuronal Nrf-2/HO-1 signaling, future investigations should examine whether BMSC-Exos also modulate glial redox balance, given their reported anti-inflammatory effects in neuropathic pain models.³⁰ Furthermore, the gut microbiota, a critical regulator of oxidative stress and visceral sensitivity, may interact with BMSC-Exos to influence outcomes.

The therapeutic effects of BMSC-Exos demonstrated here build upon their established roles in regulating inflammation, oxidative stress, and neuronal repair. Unlike parent BMSCs, exosomes circumvent risks of tumorigenicity and immune rejection, while retaining bioactive cargo such as miRNAs and enzymes capable of modulating recipient cells' function.¹ In vitro, BMSC-Exos rescued glutamate-induced neuronal oxidative damage, corroborating previous findings of their neuroprotective properties in neuropathic pain models.³¹ More importantly, intrathecal administration of BMSC-Exos attenuated both visceral hyperalgesia and anxiety-like behaviors in vivo, underscoring their dual ability to target sensory and affective dimensions of IBS. This dual efficacy contrasts with conventional therapies, such as antispasmodics and antidepressants, which often address symptoms in isolation.

A central finding of this study is the identification of the Nrf-2/HO-1 axis as the primary mediator of BMSC-Exos' therapeutic

effects. Activation of Nrf-2 by BMSC-Exos led to upregulation of HO-1, SOD, and CAT, accompanied by reductions in MDA and behavioral hypersensitivity, consistent with the pathway's role in counteracting oxidative damage.¹² Nevertheless, the observed reduction in total Nrf-2 levels in our study suggests alterations in its cellular pool but does not directly demonstrate decreased transcriptional activity. Moreover, although our findings suggest that the Nrf-2/HO-1 pathway in recipient cells is essential for the protective effects of BMSC-Exos, whether Nrf-2 knockdown influences exosome uptake efficiency remains an important question for future research.

Despite these promising findings, several limitations should be acknowledged. First, the neonatal CRD model replicates visceral hypersensitivity but does not fully capture the heterogeneity of human IBS, which also involves dysbiosis, immune activation, and postinfectious mechanisms.^{3,17,31} Integration of multiomics approaches, such as metagenomics and metabolomics, may elucidate how BMSC-Exos interact with host-microbiota networks. Second, while intrathecal administration was effective for mechanistic studies, it has limited clinical applicability. Alternative delivery routes, such as intravenous or oral administration, which have recently been optimized for exosome-based therapies using nanoparticle encapsulation³² should be evaluated for bioavailability and gut-brain axis targeting. Third, the contribution of specific exosomal cargo, such as miR-23a-3p or miR-27a, to Nrf-2 activation remains undefined. Proteomic or miRNA sequencing of BMSC-Exos could identify candidate mediators, enabling the design of engineered exosomes with enhanced therapeutic specificity. For instance, exosomes enriched with Nrf-2-inducing miRNAs, such as miR-34a, have shown efficacy in neurodegenerative disease models.²⁰ Finally, electrophysiological studies are warranted to determine how BMSC-Exos influence synaptic plasticity in spinal dorsal horn neurons, a critical determinant of central sensitization.

This study demonstrates that BMSC-Exos ameliorate chronic visceral hyperalgesia and anxiety-like behaviors in IBS rats through neuron-specific activation of the Nrf-2/HO-1 pathway. By targeting oxidative stress and central sensitization, BMSC-Exos provide a novel, cell-free therapeutic approach for IBS that addresses both nociceptive and psychiatric comorbidities. Future research should incorporate gut-brain axis components, optimize delivery systems, and apply exosome engineering to advance clinical translation. Such efforts may transform therapies not only for IBS but also for other functional pain disorders associated with redox imbalance and neuroplasticity, including fibromyalgia and migraine.

Disclosures

The authors have no conflict of interest to declare.

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Authors' contributions: Y.L.: draft preparation, experiment design, in vivo experiments, and data analysis. M.L. and X.L.: in vitro experiments and data analysis. C.L. and X.C.: conduct literature review. L.J.: IBS model construct. J.Y., Y.L., L.L., and Y.Z.: data analysis. J.L.: experiment design, supervision, editing, and writing the manuscript.

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