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Original Article

Exosomes derived from human placental mesenchymal stem cells in combination with hyperbaric oxygen therapy enhance neuroregeneration in a rat model of sciatic nerve crush injury

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ABSTRACT

Peripheral nerve damage continues to be a significant challenge in the field of medicine, with no currently available effective treatment. Currently, we investigated the beneficial effects of human placenta mesenchymal stem cells (PMSCs)- derived exosomes along with hyperbaric oxygen therapy (HBOT) in a sciatic nerve injury model. Seventy-five male mature Sprague-Dawley rats were allocated into five equal groups. In addition to the control group that received no intervention, damaged animals were allocated into four groups as follows: crush group, exosome group, HBOT group, and Exo+HBOT group. After the last neurological evaluations, tissue samples (sciatic nerve and dorsal root ganglion (DRG)) at the injury side, as well as spinal cord segments related to the sciatic nerve were collected to investigate histological, immunohistochemical, biochemical, and molecular characteristics. We found that the volume of the sciatic nerve, the thickness of the myelin sheath, the densities of nerve fibers and Schwann cells, the numerical densities of sensory neurons and glial cells in the DRG, as well as the numerical density of motor neurons in the anterior horn of the spinal cord, the levels of antioxidative factors (GSH, SOD, and CAT) in the sciatic nerve, as well as the neurological functions (EMG latency and SFI) in the treatment groups, especially the Exo+HBOT group, were significantly improved compared to the crush group. This is while the numerical density of glial cells in the spinal cord, the levels of an oxidative factor (MDA), and pro-inflammatory cytokines (IL-1β, TNF-α, and IFN- γ) considerably decreased in the treatment groups, particularly the Exo+HBOT group, compared to the crush group. We

Abbreviations: PMSCs, Human placental mesenchymal stem cells; HBOT, Hyperbaric oxygen therapy; DRG, Dorsal root ganglion; MSCs, Mesenchymal stem cells; PBS, Phosphate-buffered saline; DMEM, Dulbecco modified Eagle medium; FBS, Fetal bovine serum; IP, Intraperitoneal; H&E, Hematoxylin–eosin; GSH, Glutathione; SOD, Superoxide dismutase; CAT, Catalase; MDA, Malondialdehyde; IL-1β, Interleukin 1 beta; TNF-α, Tumor necrosis factor-alpha; IFN-γ, Interferon-γ; SFI, Sciatic functional index; EMG, Electromyography; ANOVA, One-way analysis of variance.

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conclude that co-administration of PMSCs-derived exosomes and HBOT has synergistic neuroprotective effects in animals undergoing sciatic nerve injury.

1. Introduction

Peripheral nerve damage continues to be a significant issue in clinical settings with no definitive solution currently available [1]. Damage to peripheral nerves happens in approximately 2.2% of trauma instances and is more prevalent in males [2]. These injuries can cause musculoskeletal disorders and disable individuals [3]. There are different reasons why occurs, with motor vehicle crashes being the main cause [4]. Even with notable progress in microsurgery, certain severe nervous system injuries cannot be healed. Failure to properly treat nerve injuries could result in a partial or total loss of sensory, motor, and autonomic functions [5]. After a nerve injury occurs, Wallerian degeneration starts at the point of the axon farthest from the injury [6]. Nonetheless, continuous infatuation negatively impacts microcirculation. Peripheral neural microcirculation plays a crucial part in nerve regeneration, influencing nerve injury and regeneration, as well as blood-oxygen supply, neurotrophic effects, neural conduction maintenance, and axonal transport [7]. Despite major advances in the treatment and care of these patients, research into new and effective treatment protocols remains one of the priorities of healthcare systems worldwide [8].

Multiple research studies have shown the significant promise of mesenchymal stem cells (MSCs) for treating peripheral nerve injuries [9,10]. Nevertheless, these researches have linked the capacity of MSCs to heal the damaged peripheral nerve primarily to the release of paracrine exosomes from these cells, rather than to their ability to differentiate into multiple cell types [11,12]. Exosomes are tiny vesicles released from cells, carrying nucleic acids, lipids, and proteins, crucial for cell differentiation, signaling, tissue functions, and regeneration, such as in the central nervous system. Exosomes are safer and more stable than cells, with longer circulation half-lives, and have the ability to cross the blood-brain barrier, as opposed to cells [13]. Over the past few years, a number of animal studies have shown that exosomes from various cell origins are effective in treating peripheral nerve injuries [14,15]. Nonetheless, selecting the right exosome cell origin is crucial for various therapeutic goals [13]. One of the key sources is human placenta mesenchymal stem cells (PMSCs), which have low immunogenicity and high differentiation potential, in addition to being readily available [16]. In recent studies, we have shown that the use of PMSCs in spinal cord injuries reduces inflammation, inhibits gliosis, oxidative stress, and apoptosis of neurons at the site of injury [17,18].

Given the complexity of the pathological events in peripheral nerve injury, it appears that the best treatment involves multifaceted strategies that can both hinder damage progression and promote healing in the affected area [19].

Hyperbaric oxygen therapy (HBOT) involves giving 100 % oxygen at pressures higher than 1 atm [20]. At present, this mixture is utilized in clinics for treating a variety of illnesses such as diabetic ulcers and spinal cord injury [17,21]. Research indicates that HBOT can improve nerve crush injuries by induction of endogenous neurogenesis via various mechanisms like anti-inflammatory and antioxidant effects, or immunomodulation [22,23].

Considering the advantages mentioned about PMSCs -derived exosomes and HBOT, however, the simultaneous use of these two compounds has not been reported in previous studies.

Therefore, we have presently investigated whether the administration of PMSCs -derived exosomes together with HBOT could synergistically prevent damage progression and induce functional improvement in a model system of rat sciatic nerve injury.

2. Material and methods

All materials used in the present study were purchased from Sigma-Aldrich (St. Louis, MO), except where assigned otherwise.

2.1. Cell culture and extraction of PMSCs-derived exosomes

The steps of collecting, culturing, and validating PMSCs were performed according to our previous study [18]. Briefly, placenta was isolated from pregnant women after cesarean and then moved to the lab for the extraction of PMSCs under aseptic conditions. Following this, PMSCs were separated and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS; MSC110, Excell) and 1 % penicillin-streptomycin, before being incubated at 37 °C with 5 % CO2.

The extraction of PMSCs-derived exosomes was conducted according to our previous studies [17,18]. After PMSCs grew to 80–90 % confluence, they were washed two times with phosphate buffered saline (PBS) and moved to serum-free low glucose-DMEM for 48 h. The liquid was collected and centrifuged at 1.000 rpm for 10 min, then at 2.000 rpm for 10 min, and lastly at 10.000 rpm for 20 min, in order to remove cell debris and apoptotic bodies. Following the addition of five ml of PBS to the isolated cell supernatant, it was subjected to ultracentrifugation at 100,000 rpm for a duration of 70 min. Obtaining the clean exosomes involved a second ultracentrifugation at 120,000 rpm for 70 min. The exosomes that were collected were filtered using a 0.25 μm pore filter from Millipore and kept in a −80 freezer for further evaluation.

2.2. Animals and overall study design

Seventy-five mature male Sprague–Dawley rats were recruited. All procedures were performed according to the US National Institutes of Heath Guide for the Care and Use of Laboratory Animals. The study protocol was approved by the Ethics Committee of Mazandaran University of Medical Sciences. The animals were kept in a standard lab setting with regulated temperature, humidity, and lighting, along with constant availability of food and water. The animals were randomly planned into five equal groups (n = 15). In addition to control group that only a surgical incision was made and closed, sciatic nerve crush injured rats were divided into four groups as follows: crush group, exosome group which received PMSCs-derived exosomes, HBOT group which received HBOT, and Exo+HBOT group which received PMSCs-derived exosomes plus HBOT.

Sampling was conducted at two time points on the 7th and 28th. On day 7, five rats from each group were sampled for biochemical evaluations and another five rats were sampled for pro-

inflammatory cytokines evaluation. Finally, on the 28th day, after the last neurological evaluations, another 5 rats from each group were sampled to evaluate the histology and immunohistochemistry of the sciatic nerve, DRG, and anterior horn of the spinal cord. The behavioral test was also performed one day before the study and then at the end of each week (five times during the study) [19]. Additionally, at the end of the study, the gastrocnemius muscle on the injured side was removed, and its weight was measured and compared between groups.

2.3. Experimental sciatic nerve crushing model

The rats were given xylazine (10 mg/kg) and ketamine (80 mg/kg) via intraperitoneal (ip) injection to induce anesthesia. In sterile conditions, a 2 cm cut was created alongside and directly underneath the right femur bone of the rats. The divider between the vastus lateralis and biceps femoris muscles was dissected without sharp tools to expose the sciatic nerve. The sciatic nerve was carefully removed from the surrounding tissues. A microclamp was used to induce crush injury on the sciatic nerve for a duration of 1 min. In the control group, the sciatic nerve was uncovered and then sealed without any additional actions taken [7]. After the skin was closed with two layers of 4.0 silk sutures and Cefazolin (Loghman Co, Tehran, Iran) was promptly given (50 μg/kg; ip) to avoid infection.

2.4. PMSCs-derived exosomes and HBOT administration

A standard hyperbaric oxygen chamber was used for HBO therapy. The rats received HBOT at 2 atm for 60 min/day 12 h after operation for seven consecutive days [19]. In groups receiving exosomes, upon injury, 200 μg of exosomes were injected through the gastrocnemius muscle in the proximal 2/10 and 3/10 of the calf length where intramuscular nerve endings are concentrated [12,18].

2.5. Histological and stereological evaluations

In the present study, the histological evaluation of the sciatic nerve (including the total volume of the nerve at the site of injury, the numerical density of nerve fibers, and the thickness of the myelin sheath), DRG (including the total volume, and the numerical densities of sensory neurons and glial cells), and the anterior horn of the spinal cord (the numerical densities of motor neurons and glial cells) was performed on the 28th day. To achieve this goal, tissue samples were collected and fixed in 10 % formalin for conservation. Following standard histological protocols, the samples were encased in paraffin and cut using a microtome. Ten equally spaced sections were selected from each sample. Five sections were stained with H&E to examine the volume of the sciatic nerve, numerical density of nerve fibers, and histological changes of DRG and anterior horn of the spinal cord, while the other five sections were stained with toluidine blue to examine the thickness of the myelin sheath.

2.5.1. Stereological assessment of the numerical densities of nerve fibers in sciatic nerve, neurons and glial cells in the DRG and spinal cord

Tissue sections were placed at magnification × 400, then sciatic nerve fibers and other cells that placed within the probe were counted and numerical densities (N_V) were calculated as follows: $N_v = \frac{\sum Q}{\sum P \times h \times \frac{a}{f}} \times \frac{t}{BA}$, where ΣQ: the total number of counted frames; h (μm): the dissector height; Σp: the total number of counted cells within the probe; a/f (mm^2): the probe area; BA (μm): the microtome block advance that set at ten μm; and t (μm): section thickness [24].

2.5.2. Stereological evaluation of the total volumes of the sciatic nerve, DRG, and anterior horn of the spinal cord

The Cavalieri method was utilized to quantify the total volumes. This technique involved overlaying tissue section images onto a grid of points and tallying the points within the tissue region. Following the point calculation, the total volumes were determined in the following manner: $V_{total} = \sum P \times \frac{a}{p} \times t$: that ΣP was counted points; a/p was the area of each point (mm^2), and t was the distance between two consecutive sections (mm) [24].

2.5.3. Stereological evaluation of the thickness of myelin sheath

Tissue sections were examined at a magnification of × 100 in order to determine the average thickness of the myelin sheath. For this purpose, the double line grid (consisting of two parallel lines) was placed randomly over the sampled fields to pinpoint measurement locations. The thickness of the myelin sheath was assessed by employing the orthogonal intercept technique, which involves measuring the distance from the inner membrane to the outer surface of the myelin sheath at various points where a line intersects with the grid. It was designated as orthogonal intercept (oi). The harmonic mean thickness was calculated using a following formula based on an average of 100 measurements: $T = \frac{\pi}{4} \times L_n$: The Ln represents the points where the axon perimeter intersects the test lines in this equation [24].

2.6. Immunohistochemistry

For determination of Schwann cells in the sciatic nerve, immunostaining for S100β antibody was performed on day 28. For this purpose, five sections were chosen from each rat, with evenly spaced intervals. Following deparaffinization, goat serum was applied to all sections to prevent non-specific binding. Afterward, sections were incubated overnight at 4 °C with S100β (1:300 in PBS, Santa Cruz Biotechnology sc-393919) antibody. Subsequently, they were rinsed with PBS and treated with a secondary antibody. In conclusion, diaminobenzidine tetrahydrochloride was included to identify positive responses. A densitometric approach was employed to measure the density of positive reactions.

2.7. Biochemical evaluations

After washing the harvested samples with sterile PBS to remove excess tissue residues, they were immediately frozen at −80 °C for further analysis. To assess the biochemical status, concentrations of glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT), as antioxidant biomarkers, and malondialdehyde (MDA), as oxidant factor, have been investigated [17]. In short, GSH and SOD levels were measured using trichloroacetic and pyrogallol, respectively, with absorptions recorded at 420 nm and 412 nm wavelengths, respectively, via spectrophotometry. Hydrogen peroxide was combined with phosphate buffer to assess CAT activity, and the absorption was measured at 245 nm. In the end, the MDA concentration was determined with thiobarbituric acid and the absorbance was noted at a 520 nm wavelength.

2.8. Determination of pro-inflammatory cytokines

The ELISA technique was employed for assessing the levels of pro-inflammatory cytokines, including IL-1β, TNF-α, and IFN-γ. In brief, 100 mg of tissue was ground in a lysis buffer solution with benzylsulfonyl fluoride (1 mM); Triton X-100 (1 %);

ethylenediaminetetraacetic acid (2 mM); tetrasodium phosphate (2.5 mM); Tris (20 mM); and N-acetyl-L-leucyl-L-leucyl-L-argininal (0.5 μg/ml). Later, the solution was centrifuged at 15,000 rpm for 15 min to separate the cell pellet. Finally, the levels of cytokines were determined using ELISA kits as per the manufacturer's instructions.

2.9. Functional evaluations

2.9.1. Sciatic functional index (SFI)

The SFI test was conducted to evaluate the hindlimb's functional recovery. For this purpose, the rats were positioned in a 15 mm long, 200 mm wide corridor with a 400 mm high ceiling all made of transparent glass. Black paper covered the corridor background to heighten contrast, eliminate extra lighting, and prevent reflections. A 45° angled mirror was positioned under the gallery to capture the reflection of the rats' paw prints. The formula to calculate SFI is as follows: $SFI = -38.3 \times (EPL - NPL/NPL) + 109.5 \times (ETS - NTS/NTS) + 13.3 \times (EIT - NIT/NIT) - 8.8$; where EPL, ETS, and EIT are experimental print length, experimental toe spread, and experimental intermediary toe spread, respectively, and NPL, NTS, and NIT are the same indices for the normal paw. The SFI oscillates around 0 for normal nerve function, whereas SFI around - 100 represents total dysfunction [19].

2.9.2. Electrical activity of muscles and nerves

An electromyography (EMG Latency) test was conducted to evaluate muscle reaction to nerve stimulation. To achieve this goal, the rats were anesthetized before the sciatic nerve was uncovered. Next, the stimulating electrode was attached to the upper section of the nerve (close to the gluteal muscles) while the receiving electrode was attached to the gastrocnemius muscle. Following nerve stimulation, muscle activation rate was measured and EMG latency was determined in milliseconds [25].

2.10. Statistical analysis

Data analysis was conducted using SPSS software (IBM SPSS Statistics, version 23) and the creation of graphs was done with Prism software (GraphPad Prism 8.0.2.263). ANOVA with Tukey's post hoc test was employed to examine relationships among groups. In SFI test, the results were analyzed by repeated-measures ANOVA followed by the multiple comparison method of Bonferroni. Outcomes are reported as mean ± standard deviation (SD). P-values <0.05 were considered significant.

3. Results

In all cases, the control group (without any interventions) achieved noticeably superior outcomes compared to the other groups. Hence, we presented the results comparing the findings among the remaining four groups: Crush, Exosome, HBOT, and Exo+HBOT.

3.1. Combination of PMSCs-derived exosomes and HBOT attenuated muscle atrophy after sciatic nerve crush

In the present study, to evaluate the effect of crushing the sciatic nerve on the volume of the gastrocnemius muscle and then the effects of administration of PMSCs-derived exosomes and HBOT in preventing damage, the weight of the muscle on the side of the damage was measured and compared at the end of the study. The comparison of muscle weight at the injury side indicated that exosome, HBOT, and Exo+HBOT groups had a greater weight in comparison with crush group ($p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively) (Fig. 1).

3.2. Combination of PMSCs-derived exosomes and HBOT inhibited the spread of damage in the sciatic nerve after crushing and caused its recovery

Different aspects of the sciatic nerve, such as histological changes, levels of pro-inflammatory cytokines, and antioxidant status properties were examined, and the findings from each section are reported as follows.

3.2.1. Histological characteristics in the sciatic nerve

In Fig. 1A, toluidine blue staining images of the sciatic nerve at the site of injury are shown. When analyzing the total volume of the sciatic nerve at the site of injury, exosome, HBOT, and Exo+HBOT groups showed significantly larger volumes compared to the crush group ($p < 0.05$, $p < 0.05$, and $p < 0.0001$, respectively). Additionally, when comparing the outcomes between different treatment groups, it was found that the Exo+HBOT group had a higher volume compared to both the exosome and HBOT groups (both, $p < 0.05$) (Fig. 1B).

The evaluation of myelin sheaths thickness at the lesion site revealed that the exosome, HBOT, and Exo+HBOT groups had significantly thicker sheaths compared to the crush group ($p < 0.01$, $p < 0.05$, and $p < 0.0001$, respectively). Furthermore, the Exo+HBOT group exhibited significantly thicker myelin sheaths compared to the exosome and HBOT groups ($p < 0.05$ and $p < 0.01$, respectively) (Fig. 2C).

Comparing the numerical density of nerve fibers, it was noted that the exosome, HBOT, and Exo+HBOT groups had significantly higher numbers than the crush group ($p < 0.01$, $p < 0.05$, and $p < 0.0001$, respectively). Moreover, Exo+HBOT group had significantly more nerve fibers in comparison with the exosome and HBOT groups ($p < 0.05$ and $p < 0.01$, respectively) (Fig. 2D).

3.2.2. Immunohistochemical evaluation of Schwann cells in the sciatic nerve

Micrographs of immunohistochemistry against S100β, as a Schwann cells marker, are shown in Fig. 3A. During the quantitative analysis, it was discovered that the exosome, HBOT, and Exo+HBOT groups displayed notably increased levels of S100β-positive cells in comparison to the crush group ($p < 0.05$, $p < 0.01$, and $p < 0.0001$, respectively). Additionally, when comparing Schwann cell density among the different treatment groups, it was observed that the Exo+HBOT group had notably higher density compared to both the

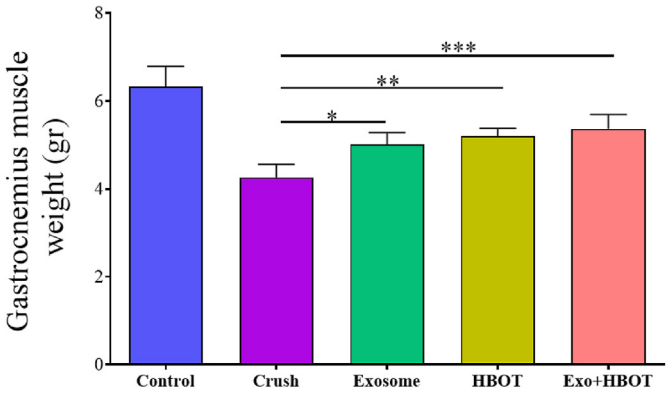

Fig. 1. The impact of PMSCs-derived exosomes in combination with HBOT on muscle mass. The study measured the weight of the gastrocnemius muscle on the injured side to assess the impact of crushing the sciatic nerve and the effectiveness of treating with PMSCs-derived exosomes and HBOT in preventing damage. Mean ± SD. *p < 0.05; **p < 0.01, ***p < 0.001.

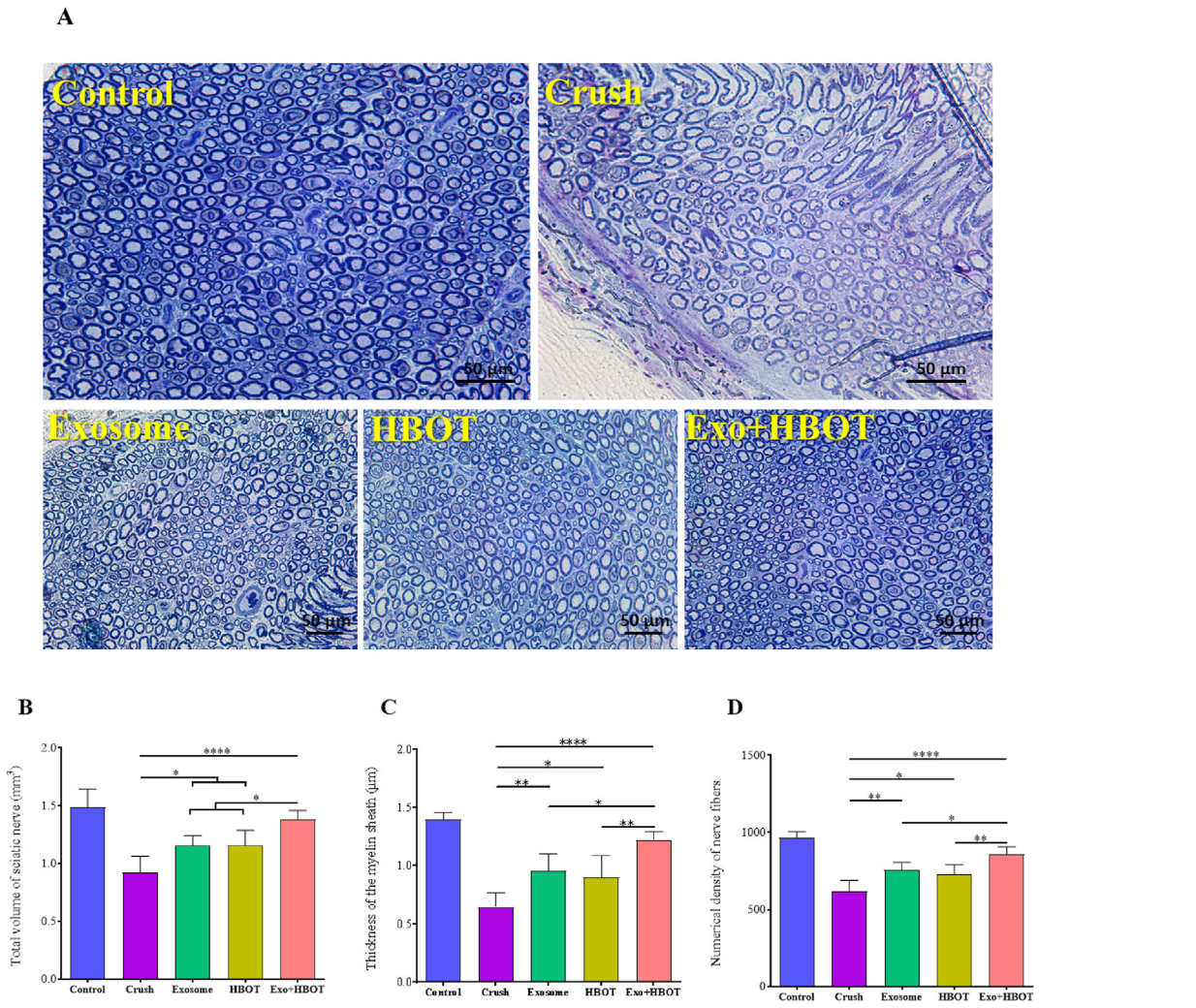

Fig. 2. The impact of PMSCs-derived exosomes in combination with HBOT on histological characteristics in sciatic nerve. (A) Representative micrographs of the sciatic nerve at the site of injury stained by toluidine blue. (B) Total volumes of sciatic nerve at the lesion site were determined by Cavalier's method. (C) Thickness of the myelin sheaths. (D) Numerical density of nerve fibers at the lesion site which was determined by optical dissector method. Mean ± SD. *p < 0.05; **p < 0.01, ****p < 0.0001.

exosome and the HBOT groups ($p < 0.01$ and $p < 0.05$, respectively) (Fig. 3B).

3.2.3. Biochemical evaluations in the sciatic nerve

The results of the evaluation of biochemical biomarkers are shown in Fig. 4. Considering the antioxidant biomarker levels, we observed that the exosome, HBOT, and Exo+HBOT groups compared to the crush group had significantly higher levels of the GSH ($p < 0.05$, $p < 0.05$, and $p < 0.001$, respectively), SOD ($p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively), and CAT ($p < 0.01$, $p < 0.01$, and $p < 0.0001$, respectively). Furthermore, the Exo+HBOT group in comparison with exosome and HBOT groups had considerably higher levels of SOD ($p < 0.01$ and $p < 0.05$, respectively) and CAT ($p < 0.01$ and $p < 0.05$, respectively).

Analysis of the MDA levels showed that the exosome, HBOT, and Exo+HBOT groups had significantly lower concentrations compared to the crush group ($p < 0.05$, $p < 0.001$, and $p < 0.0001$, respectively). In addition, the Exo+HBOT group showed significantly lower MDA concentration than both the exosome and the HBOT groups ($p < 0.01$ and $p < 0.05$, respectively).

3.2.4. Analysis of pro-inflammatory cytokines in the sciatic nerve

The results of the evaluation of pro-inflammatory cytokines using the ELISA method are shown in Fig. 5. According to our findings, the exosome, HBOT, and Exo+HBOT groups compared to the crush group had significantly lower levels of the TNF-α ($p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively), IL-1β ($p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively), and IFN-γ ($p < 0.05$, $p < 0.01$, and $p < 0.0001$, respectively). Additionally, the Exo+HBOT group compared to the exosome and HBOT groups showed significantly decreased levels of TNF-α ($p < 0.001$ and $p < 0.01$, respectively), IL-1β ($p < 0.01$ and $p < 0.05$, respectively), and IFN-γ ($p < 0.01$ and $p < 0.05$, respectively).

3.3. Combination of PMSCs-derived exosomes and HBOT inhibited damage in the DRG

Regarding the changes in the DRG structure, histological changes were investigated and the findings from each section are reported as follows.

3.3.1. Histological characteristics in the DRG

The histological structure of DRG using H&E staining is shown in Fig. 6A. Stereological evaluation of DRG total volume revealed that the exosome, HBOT, and Exo+HBOT groups exhibited significantly greater volumes in comparison to the crush group ($p < 0.05$, $p < 0.05$, and $p < 0.0001$, respectively). Furthermore, when comparing the results between treatment groups, it was found that

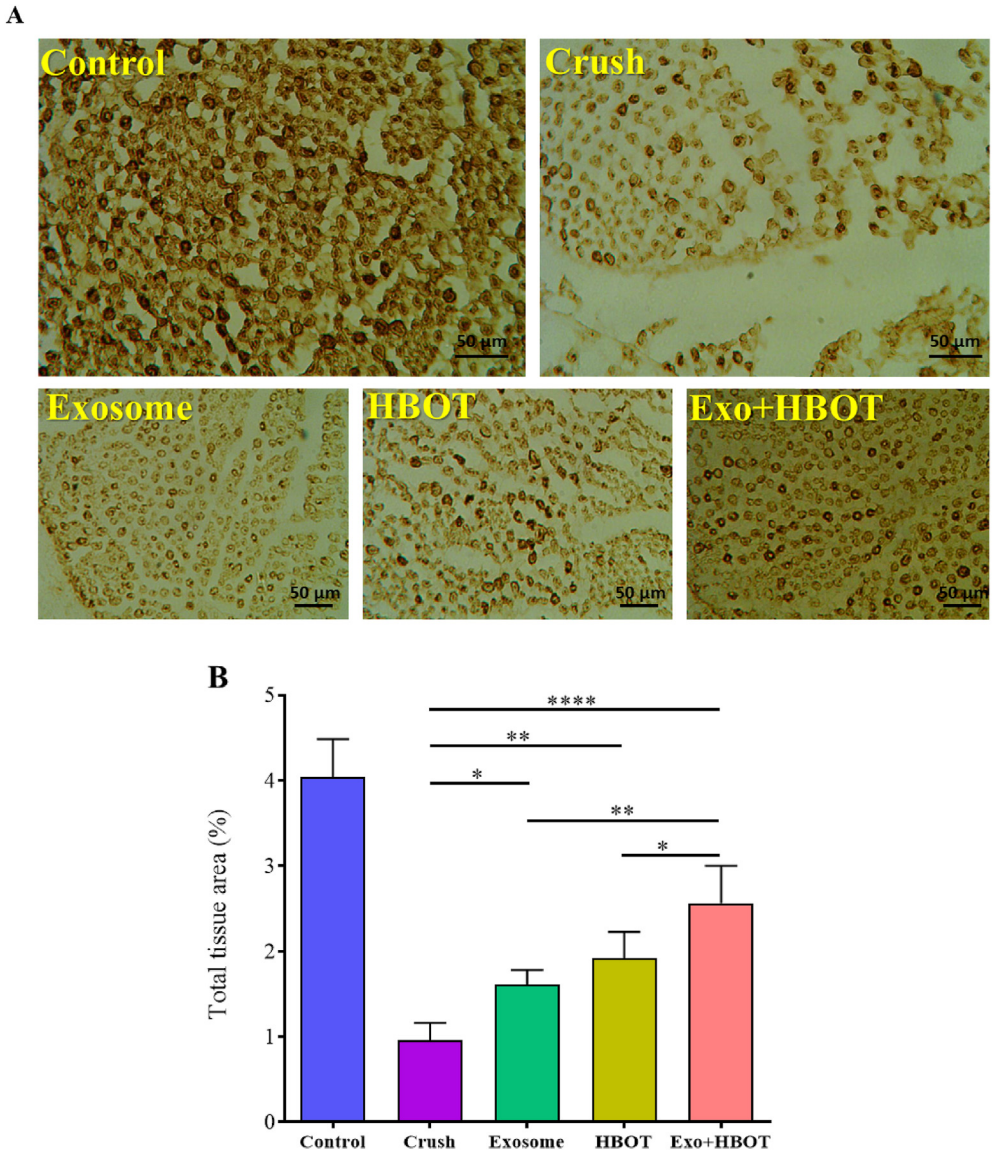

Fig. 3. The impact of PMSCs-derived exosomes in combination with HBOT on the density of Schwann cells. (A) Immunostaining for Schwann cells against S100β antibody. Positive reactions are presented in brown color. (B) Data were analyzed using densitometry. Mean ± SD. *p < 0.05; **p < 0.01, ****p < 0.0001.

the Exo+HBOT group had a higher volume compared to both the exosome and HBOT groups (both, $p < 0.05$) (Fig. 6B).

Comparing the numerical density of sensory neurons, it was noted that the exosome, HBOT, and Exo+HBOT groups had significantly more cells than the crush group ($p < 0.05$, $p < 0.01$, and $p < 0.0001$, respectively). In addition, the Exo+HBOT group had significantly more neurons in comparison with exosome group ($p < 0.05$) (Fig. 6C).

Regarding the numerical density of glial cells in DRG, we observed that the exosome, HBOT, and Exo+HBOT groups had significantly more cells than the crush group ($p < 0.01$, $p < 0.01$, and $p < 0.0001$, respectively). Furthermore, Exo+HBOT group had considerably more cells in comparison with the exosome and HBOT groups ($p < 0.01$ and $p < 0.05$, respectively) (Fig. 6D).

3.4. Combination of PMSCs-derived exosomes and HBOT inhibits damage in the anterior horn of the spinal cord

Regarding the changes in the anterior horn of the spinal cord, histological changes were investigated and the findings are reported as follows.

3.4.1. Histological characteristics in the anterior horn of the spinal cord

In Fig. 7A, H&E staining images of the anterior horn of the spinal cord are shown. When analyzing the total volume of the anterior horn of the spinal cord, the exosome, HBOT, and Exo+HBOT groups showed significantly larger volumes compared to the crush group ($p < 0.05$, $p < 0.01$, and $p < 0.0001$, respectively). Furthermore, when comparing the results between treatment groups, it was found that the Exo+HBOT group had a higher volume compared to the exosome group ($p < 0.05$) (Fig. 7B).

Comparing the numerical density of motor neurons, it was noted that the exosome, HBOT, and Exo+HBOT groups had significantly more cells than the crush group ($p < 0.05$, $p < 0.01$, and $p < 0.0001$, respectively). Furthermore, the Exo+HBOT group had significantly more neurons in comparison with the exosome and HBOT groups ($p < 0.01$ and $p < 0.05$, respectively) (Fig. 7C).

Considering the numerical density of glial cells, we found that the exosome, HBOT, and Exo+HBOT groups had significantly lower cells than the crush group ($p < 0.01$, $p < 0.05$, and $p < 0.0001$, respectively). Furthermore, Exo+HBOT group had considerably

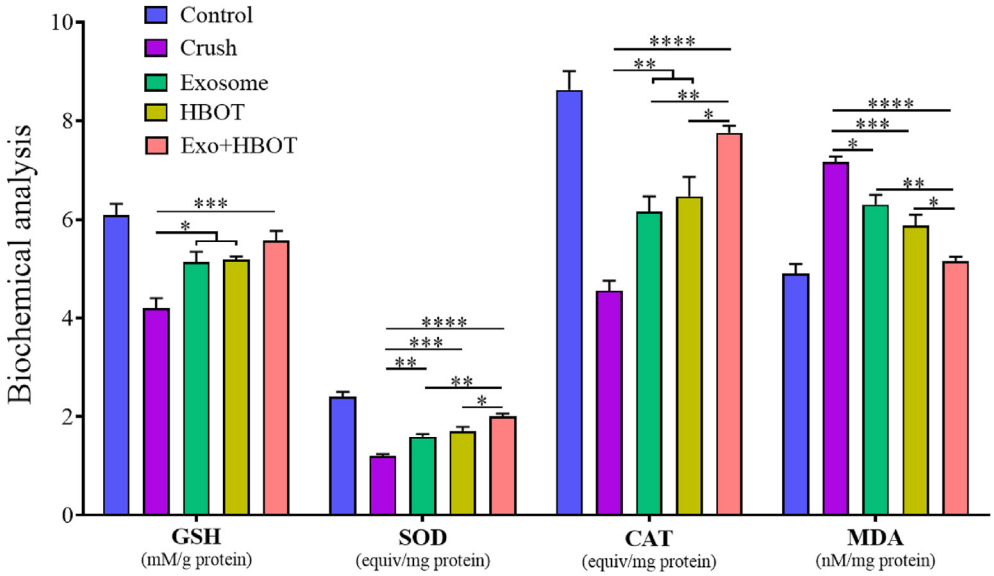

Fig. 4. The impact of PMSCs-derived exosomes in combination with HBOT on biochemical biomarkers levels. Concentrations of antioxidant (GSH, SOD, and CAT) and oxidant (MDA) factors at the lesion site were determined by biochemistry method. Mean ± SD. *p < 0.05; **p < 0.01, ***p < 0.001, ****p < 0.0001.

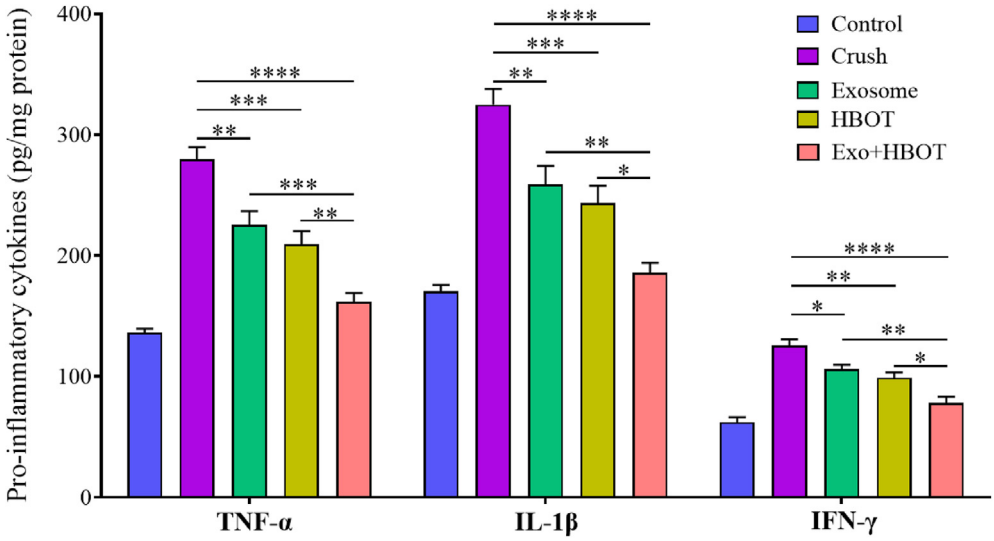

Fig. 5. The impact of PMSCs-derived exosomes in combination with HBOT on pro-inflammatory cytokines levels. The levels for pro-inflammatory cytokines were determined using ELISA method. Mean ± SD. *p < 0.05; **p < 0.01, ***p < 0.001, ****p < 0.0001.

lower glial cells in comparison with the exosome and HBOT groups ($p < 0.05$ and $p < 0.01$, respectively) (Fig. 7D).

3.5. Combination of PMSCs-derived exosomes and HBOT improved neurological functions deficit

In the present research, the EMG latency and SFI were examined to assess neurological functions.

Regarding the EMG latency test, we found that latency of muscle response to a nerve's stimulation meaningfully decreased in the exosome, HBOT, and Exo+HBOT groups compared to crush group ($p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively). Furthermore, the Exo+HBOT group had significantly lower latency in comparison with the exosome group ($p < 0.05$) (Fig. 8A).

Considering the SFI test, we observed that the exosome, HBOT, and Exo+HBOT groups had considerably higher scores in comparison with crush group on days 7 ($p < 0.05$, $p < 0.05$, and $p < 0.01$, respectively), 14 ($p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively), 21 ($p < 0.05$, $p < 0.05$, and $p < 0.01$, respectively), and 28 ($p < 0.05$, $p < 0.05$, and $p < 0.001$, respectively). Moreover, the Exo+HBOT group in comparison with the exosome and HBOT groups had considerably higher scores on days 7 ($p < 0.001$ and $p < 0.01$, respectively), 14 ($p < 0.01$ and $p < 0.05$, respectively), 21 ($p < 0.01$ and $p < 0.05$, respectively), and 28 ($p < 0.01$ and $p < 0.05$, respectively) (Fig. 8B).

4. Discussion

In the present study, we investigated whether simultaneous and separate administration of PMSCs-derived exosomes and HBOT can prevent the spread of damage in the sciatic nerve, DRG, and anterior spinal horn following nerve crush and heal it or not. Overall,

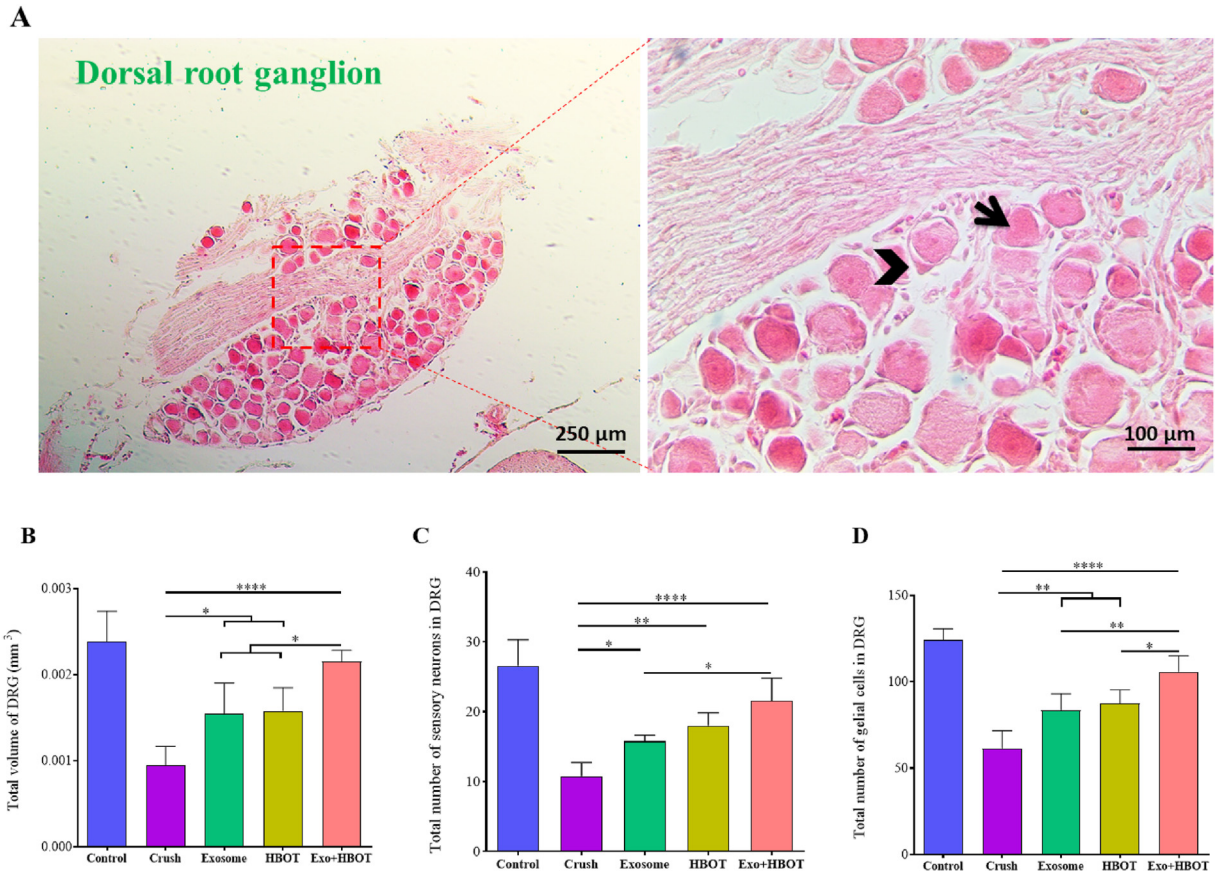

Fig. 6. The impact of PMSCs-derived exosomes in combination with HBOT on histological characteristics in DRG. (A) Representative micrographs of the DRG related to the injured sciatic nerve stained by H&E (arrows: sensory neurons; arrowheads: glial). (B) Total volumes of DRG were determined by Cavalier's method. (C) Total number of sensory neurons in DRG. (D) Total number of glial cells in DRG. Mean ± SD. *p < 0.05; **p < 0.01, ****p < 0.0001.

our findings indicated that administering PMSCs-derived exosomes and HBOT separately can prevent inflammation, oxidative stress, and abnormal alterations in the sciatic nerve, as well as prevent abnormal changes in the ganglion, while also enhancing the survival of motor neurons in the spinal cord's anterior horn. Nonetheless, we noticed that in the combined group that was given both the exosome and oxygen blend, these alterations were more pronounced and impactful. Based on the quantity of assessments conducted in this research, and taking into account that inflammation, oxidative stress, and histological changes are the key elements in the development and dissemination of damage post nerve crush, we will discuss each of these factors in this section [26,27].

Intense inflammation at the site of injury in the sciatic nerve is a key destructive factor [28]. Research has indicated that nerve damage triggers an inflammatory reaction and causes the production of pro-inflammatory cytokines like IL-1β, TNF-α, and IFN-γ [19,29]. Inflammation cells and mediators contribute to tissue damage, secondary injury, and also play a part in the regenerative process. Nonetheless, the inability to manage inflammation and its persistent nature can lead to severe tissue damage and eventually cell apoptosis. Presently, we measured the levels of pro-inflammatory cytokines like IL-1β, TNF-α, and IFN-γ in the sciatic nerve. Our results showed that the levels of these cytokines in the treatment groups, especially the combined group, were significantly reduced compared to the crush group. In this regard, Li et al. documented that PMSCs reduce inflammation by modulating Th17/Treg cells [30]. Kulubya et al. reported that PMSCs are a unique resource in the treatment of many diseases, especially neurological, compared to other MSCs, which is due to their anti-inflammatory, neuroprotection, and neuroregeneration [31]. Furthermore, we previously documented that the administration of PMSCs-derived exosomes to rats with spinal cord injury considerably downregulated the expression levels of pro-inflammatory genes such as IL-1β and TNF-α and considerably upregulated the IL-10 [17,18]. On the other hand, Poyrazoglu et al. reported that HBOT can considerably downregulate expression levels of IL-1β and TNF-α genes [32]. Moreover, Alshahrani et al. documented that HBOT significantly reduced the expressions levels of TNF-α and IL-1β, as well as upregulated the IL-10 at the lesion site in the nerve injury [33].

Hence, because of the anti-inflammatory properties of PMSCs-derived exosomes and HBOT, our research findings suggest that combining the two treatments may be more impactful than using either one alone.

Oxidative stress is another major event that occurs in the sciatic nerve after crushing and plays a very destructive role [34]. Oxidative stress triggers the activation of apoptotic proteins which leads to cell death. Therefore, controlling it is crucial in minimizing the extent of harm [7]. Currently, the biochemistry method is being used to measure GSH, SOD, CAT, and MDA to analyze the impact of treatments on biochemical status. In all treatment groups, the levels of oxidative and antioxidant biomarkers showed significant decreases and increases, respectively, compared to the crush group, and these differences were more prominent in the Exo+HBOT group. In this regard, Lu et al. indicated that PMSCs significantly increased oxidative protection enzymes (SOD and CAT) and decreased oxidative damage (MDA) in an animal model of testicular

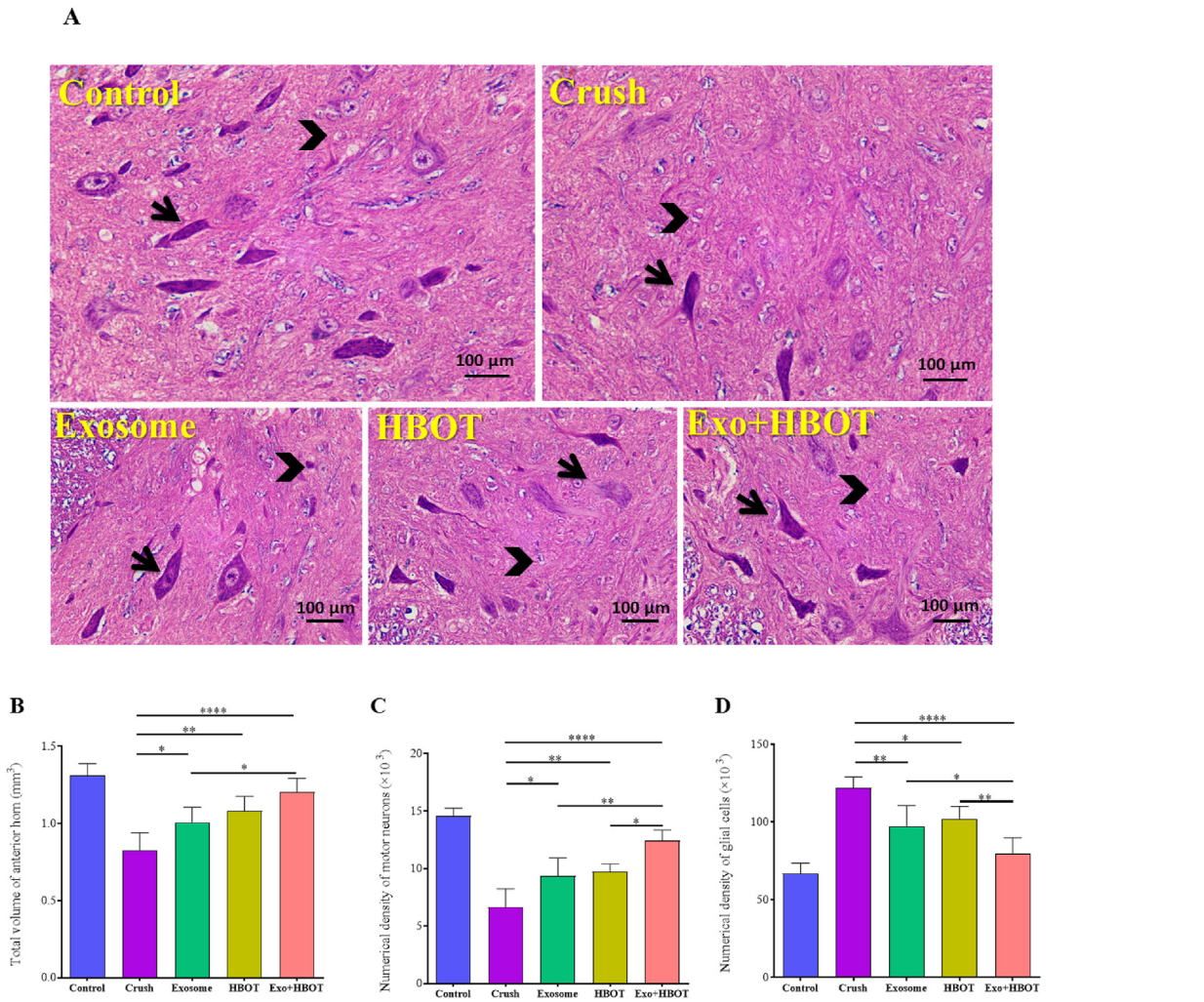

Fig. 7. The impact of PMSCs-derived exosomes in combination with HBOT on histological characteristics in anterior horn of the spinal cord. (A) The photomicrographs of H&E staining from the anterior horn of the spinal cord (arrows: motor neurons; arrowheads: glial). (B) Total volumes of anterior horn were determined by Cavalier's method. (C) Numerical density of motor neurons. (D) Numerical density of glial cells. Mean ± SD. *p < 0.05; **p < 0.01, ****p < 0.0001.

toxicity [35]. In our previous studies, we discovered that the administration of PMSCs-derived exosomes to rats with spinal cord injury considerably reduced oxidant levels and increased antioxidant levels [17,18].

Additionally, HBOT, which is a non-pharmacological and non-invasive treatment, raises the levels of oxygen dissolved in the plasma and oxygen-saturated hemoglobin, resulting in increased oxygen supply to the organs [36,37]. There is a growing amount of evidence suggesting that HBOT not only enhances oxygen supply and neural metabolism, but also has positive effects due to various biological properties, such as anti-oxidative effects [38,39]. In this regard, Kahraman et al. reported that HBOT diminished oxidative stress indicators such as thiobarbituric acid reactive substances and glutathione peroxidase after spinal cord injury [40]. Additionally, Ahmadi et al. [39] as well as Alshahrani et al. [33] reported a significant decrease in levels of oxidant biomarker MDA and an increase in antioxidant biomarkers GSH, SOD, and CAT following HBOT in animals with spinal cord injuries.

Therefore, our research results propose that the combination of PMSCs-derived exosomes and HBOT could have a greater effect due to their anti-oxidative properties compared to using each treatment individually.

Lastly, the third crucial aspect in nerve injury, which is closely related to inflammation and oxidative stress, is destructive histological changes. In the present study, to evaluate this factor, we assessed tissue characteristics of the sciatic nerve, DRG, and the anterior horn of the spinal cord. The findings of the research revealed that there was significant improvement in all cases within the treatment groups that were studied, indicating that the sciatic nerve volume, myelin sheath thickness, nerve fiber count, and Schwann cell count were notably higher than those in the crush group. Additionally, we noted an improvement in motor neuron survival in the treatment groups, along with a reduction in glial cell numbers in the anterior horn of the spinal cord. However, all these cases were significantly better in the Exo+HBOT group compared to the groups that received single treatment.

On the other hand, the most important confirmatory feature for cellular and molecular evaluations in nerve damage is conducting behavioral tests [17,18]. Therefore, in the present study, in order to evaluate the behavioral status, EMG latency and SFI tests were evaluated in the studied groups. The results of both tests showed that the treatment groups had a significantly better condition compared to the crush group, and this improvement was more evident in the Exo+HBOT group. These behavioral findings were

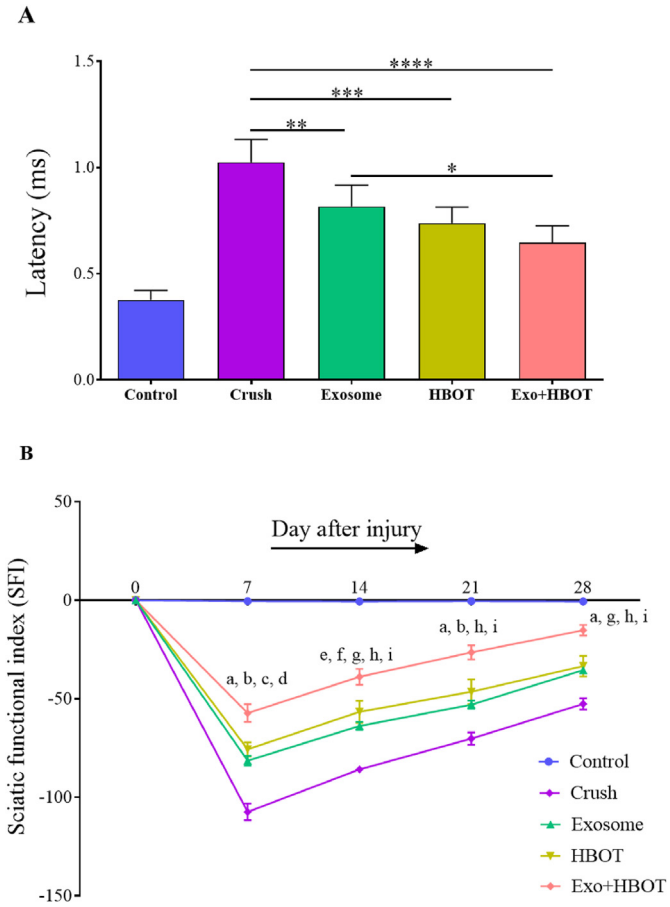

Fig. 8. The impact of PMSCs-derived exosomes in combination with HBOT on neurological functions. (A) EMG latency on day 28 after injury (Millisecond; MS). (B) The sciatic functional index (SFI) test was performed before and days 7, 14, 21, and 28 after injury. Mean ± SD. *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. [a] $p < 0.05$ Exosome and HBOT groups vs Crush group; [b] $p < 0.01$ Exo+HBOT group vs Crush group; [c] $p < 0.001$ Exo+HBOT group vs Exosome group; [d] $p < 0.01$ Exo+HBOT group vs HBOT group; [e] $p < 0.05$ Exosome group vs Crush group; [f] $p < 0.01$ HBOT group vs Crush group; [g] $p < 0.001$ Exo+HBOT group vs Crush group; [h] $p < 0.01$ Exo+HBOT group vs Exosome group; [i] $p < 0.05$ Exo+HBOT group vs HBOT group.

consistent and corroborating with histological, biochemical, and molecular evaluations.

While the treatments in the present study were initiated immediately after injury, maximizing the treatment's efficacy, it is crucial to acknowledge that, in clinical settings, treatment typically begins during the subacute or chronic phases of injury. In the acute phase, the injury is still in its early stages, and the tissue damage has not yet reached its maximum extent. However, in the subacute and chronic phases, tissue alterations such as chronic inflammation, fibrosis, and neuronal degeneration are more pronounced, which can make therapeutic interventions more challenging. Therefore, it is important to consider that the timing of treatment initiation in clinical practice is often delayed, and its efficacy in later phases of injury remains an area of great interest. The impact of PMSCs-derived exosomes and HBOT when administered during the subacute or chronic phases, where the tissue environment is more complex and reparative processes may already be underway, is an important question that requires further investigation. Future studies should explore the therapeutic potential of these treatments when applied at later time points, to determine how their effectiveness might be influenced by the progression of injury and the established tissue changes. Such investigations could help to better translate these promising therapeutic strategies into clinical practice, where immediate post-injury treatment is not always feasible.

On the other hand, although the results of this study indicated that the combination of PMSCs-derived exosomes and HBOT can have significant therapeutic effects in reducing inflammation, oxidative stress, and tissue alterations following nerve injury, it is important to acknowledge the clinical limitations of HBOT. Currently, facilities equipped with HBOT chambers are relatively rare, and access to this treatment can be challenging, especially for patients in intensive care units. For critically ill patients, administering HBOT may not be feasible due to the need for specialized equipment and close monitoring. These practical limitations highlight the difficulties of translating this therapy into clinical settings.

5. Conclusion

In general, we discovered that the separate administration of PMSCs-derived exosomes and HBOT had neuroprotective effects. Nevertheless, the outcomes indicated that the combined administration of both substances resulted in synergistic effects. Therefore, although the combination of PMSCs-derived exosomes and HBOT shows promising results in laboratory settings and animal models, translating this treatment into clinical practice requires the availability of specialized facilities and overcoming practical challenges, such as limited access to this technology and the logistical difficulties of providing medical care. Further research is needed to explore alternative approaches or improve access to HBOT for patients in various clinical conditions.

Ethics approval

The use of human placenta samples was approved by the ethics committee of Mazandaran University of Medical Sciences, and written informed consent was obtained before clinical sampling. The methods were carried out in compliance with the ARRIVE guidelines. All experimental protocols were approved by Ethics Committee based on NIH Guide for the Care and Use of Laboratory Animals (Ethic no: IR.MAZUMS.4.REC.1402.18342 and 18343).

Data availability

Data will be made available on request.

Authors contributions

F.T.A. contributed to the study design, data acquisition and analysis, provided financial support, as well as drafting of the manuscript. A.J. and H.M. designed molecular and biochemical assessments and analyses. F.A. designed histological and immunohistochemical assessments and analyses. F.M. K. designed functional assessments and analyses. A.R. designed stereological assessments and analyses. M.O. and M.A.A. designed histological and behavioral assessments and analysis. D.N. supervised the study, and contributed to the study concept and design, interpretation of data, and editing and final approval of the manuscript. All authors reviewed and commented on the manuscript and approved the final manuscript.

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Declaration of competing interest

The authors declare no conflicts of interest.

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