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Combined Effects of Low Level Laser Therapy and Inducers on the Neural Differentiation of Mesenchymal Stem Cells

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ABSTRACT Low level laser therapy (LLLT) improves the therapeutic effectiveness of stem cell therapy for neurological injury through its ability to enhance stem differentiation and protect against neuronal apoptosis through its antioxidation effects. However, the specific mechanisms governing these effects are poorly defined. In this study, we investigated the effects of LLLT on stem cell differentiation at the molecular level, oxidative stress balance, and inflammatory factors to provide theoretical support for its clinical application. Cell viability was assessed via MTT assays. Reactive oxygen species (ROS), total superoxide dismutase (SOD), and total antioxidant capacity (TAC) were used to evaluate oxidative stress levels. Western blot analysis was used to quantitatively investigate protein expression. The levels of secreted proteins and tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) expression were measured by ELISA. Compared to LLLT at 808 nm, LLLT at 635 nm enhanced the proliferation of human umbilical cord-derived mesenchymal stem cells (hUCMSCs). The rates of proliferation markedly increased at a power density 20 mW/cm^2. LLLT enhanced the antioxidant capacity and caused no inflammation in normal cells. Markers of neural precursors were more highly expressed at 808 nm when combined with inducers for 3 d, compared to the more modest increases observed at 635 nm. The expression of neuN on day 7 also increased, most notably when LLLT at 808 nm was combined with cerebrospinal fluid (CSF)/injured cerebrospinal fluid (iCSF). ELISA assays showed that LLLT at 808 nm with CSF/iCSF also increased the differentiation of hUCMSCs into neurons. LLLT at 808 nm combined with inducers promoted the differentiation into neurons and increased the rate of neuronal differentiation.

INDEX TERMS Low level laser therapy, human umbilical cord-derived mesenchymal stem cells, oxidative stress, neuronal differentiation.

I. INTRODUCTION

Due to the aging population, the incidence of traumatic brain injury (TBI) continues to rise [1]. Advances in medical interventions have led to lower levels of TBI associated mortality, but disability amongst the survivors remains a burden on society and families. Over 50% of cranio-cerebral injuries lead to disability [2]. Current TBI treatments include surgical and non-surgical interventions. Stem cell therapy has revolutionized neuronal treatment.

Stem cells, known as ''universal cells'', are characterized by rapid proliferation, multi-directional differentiation, low immunity and self-repair. Mesenchymal stem cell (MSCs) secrete soluble growth factors to stimulate cell proliferation and regulate the inflammatory environment in response to injury. *In vitro*, MSCs regulate oxidative stress and scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS) though the consumption of antioxidants [3].

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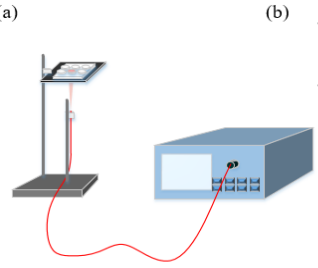
The rapid recovery observed following MSCs transplantation is mediated through paracrine signaling [4]. MSCs can differentiate into any cell type. These cells can be derived from the preimplantation of mammalian embryos or from somatic cells that have been stimulated to undergo reprogramming. MSCs can be extracted from bone marrow, and adipose tissue, but their ability to differentiate is restricted. Examples include human umbilical cord-derived mesenchymal stem cells (hUCMSCs) that can be obtained through non-invasive procedures [5].

MSCs can differentiate into bone [6], nerve [7], and fat cells [8] and have the ability to modulate inflammation-associated immune cells and cytokines [9]. Differentiation can be induced by biological factors including chemical antioxidants [10] and exogenous gene expression gene transfections. Chemical antioxidants regulate oxidative stress and as a result, stimulate MSCs to differentiate into mature neurons including neurons and glial cells. However, the toxicity of chemical therapy limits their clinical use [11]. Changes in the cell microenvironment play an important role in stem cell differentiation, and the combination of multiple factors can improve the efficiency of neural differentiation [12]. The differentiation of MSCs into neuronal progenitors is intimately linked to paracrine function, which is regulated by cell-derived growth factors and lymphokines [13]. Brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) are essential for the neural differentiation of MSCs [14]. Basic fibroblast growth factor (bFGF) increases MSC proliferation [15] and induces the differentiation of neural SCs to mature neurons [16]. Mature nerve cells undergo differentiation in response to a range of stimuli, including endothelial growth factor (EGF) [17] and BDNF, the most widely distributed neurotrophic factor. BDNF activates TrkB and P75 which stimulate neuronal growth [18]. Other biological factors promote SC neural differentiation, but their efficiency is low and more potent inducers are required.

Low-level laser therapy (LLLT) uses low-level lasers to the surface of the body to produce clinical benefits. LLLT leads to photochemical reactions, photosensitivity, and light stimulation, leading to changes in the immune response and circulation. LLLT can effectively treat inflammation [19], wound healing [20], neurological injury [21], and pain [22]. LLLT activates photoreceptors that enhance cell proliferation and stimulate the multi-directional differentiation of MSCs. LLLT causes no irreversible tissue damage in mouse models of nerve injury upon continuous treatment for 7 days with a 660 nm laser and energy density of 9 J/cm^2 [23]. Animal behavior, electrophysiological assessments, and inflammation analysis showed that MSCs effectively improve the recovery of lesions in response to light intervention. The photon energy carried by LLLT is limited, but its accumulation increases with photobiomodulation. This promotes the differentiation of neural precursor cells into mature nerve cells [24].

LLLT activates MSCs but its mechanism(s) of action are poorly defined. Gasparyan *et al.* [25] separated MSCs through Transwell filter assays and found that the number of cells in LLLT groups were lower than those of trophic factor groups. Interestingly however, the number of cells in the combined intervention group was higher than that of the trophic factor group. The levels of infrared light migration peaked at 958 nm, indicating that PBM regulated MSCs migration to the injury site. Here, we investigated oxidative stress balance, inflammation, and the neural differentiation of hUCMSCs with LLLT combined with cerebrospinal fluid (CSF) induction.



Radiant exposure [J/cm^2]	Irradance [mW/cm^2]	Exposure time [s]
4	10	400
	20	200
	30	133
	40	100
	50	80
6	10	600
	20	300
	30	200
	40	150
	50	120

FIGURE 1. Optical platform (a) and laser irradiation parameters (b).

II. MATERIALS AND METHODS

A. CELL IDENTIFICATION

Cells were provided by AmCellGene Co., Ltd.. For phenotype analysis, P3 hUCMSCs (1×10^6) seeded into 6-well plates were harvested in phosphate-buffered saline (PBS) and labeled with CD105-FITC, CD90-FITC, CD73-FITC, HLA-DR-FITC, CD14-FITC and CD34-FITC antibodies (BioLegend, America) at 37°C for 30 min. Cells were analyzed by FACs analysis on a BD FACSCanto II flow cytometry (America).

B. EFFECTS OF OPTICAL PARAMETERS ON TEMPERATURE CHANGES AND PROLIFERATION OF MSCs

HMSCs were derived from the umbilical cord of normal deliveries. All donors provided informed consent. HUCMSCs were seeded in 96-well plates (Corning, NY) at a density of 5×10^3. Continuous wave mode light-interference was performed overnight at an energy density of 0, 4, 6 J/cm^2; and a power density of 0, 10, 20, 30, 40, 50 mW/cm^2 at 635 nm and 808 nm (Fig. 1). Temperature was measured using thermography (FLIR, US) before and after irradiation with 6 J/cm^2 at 20 and 50 mw/cm^2. Cell proliferation was assessed via MTT assays at a concentration of 0.5 mg/mL after 1, 2 and 3 d of culture. Absorbances were measured at 490 nm.

C. CELL MORPHOLOGY ASSESSMENTS

HUCMSCs in the logarithmic growth phase were inoculated into 6-well plates at a density of 5×10^5 cells/well. Experimental groups included controls, CSF, injured cerebrospinal fluid (iCSF), 635 nm and combined CSF groups.

Cells morphologies were assessed through microscopy after 1, 2 and 3 d of light intervention.

D. DETERMINATION OF INTRACELLULAR ROS, TOTAL SOD, TAC AND NF-κB, IL-1β, TNF-α LEVELS

Cells were seeded into 6-well plates at a density of 5×10^4 cells/well and laser irradiated. Treated cells were then labeled with DCFH-DA (Beyotime Biotechnology, China) in serum-free medium (1:1000 dilution) to a final at concentration of 10 mμ/L at 37°C for 20 min. Cells were washed in serum-free media and absorbances were measured at ex/em 488 nm/525 nm for ROS detection.

After 3 days of laser irradiation, cells were lysed in 200 μL of total superoxide dismutase (SOD) sample solution and cell supernatants were collected and centrifuged at 12000 g for 5 min at 4 °C. Samples were measured 30 min post-incubation at 37 °C. Absorbances were measured at 450 nm to measure SOD.

To measure total antioxidant capacity (TAC), irradiated cells were washed in 200 μL of ice-cold PBS and lysed through ultrasound. Collected supernatants were centrifuged at 12000 g for 5 min at 4 °C and incubated at room temperature for 6 min. Absorbances were measured at 414 nm.

Human nuclear factor kappa-B (NF-κB), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) ELISA kits (mlbio, China) were used to analyze the expression of both transcription factors and cytokines. Supernatants were centrifuged at 1000 g for 10 min to remove particles and polymers. Absorbances were measured at 450 nm.

E. SEMI-QUANTITATIVE DETECTION OF NERVE CELL-ASSOCIATED PROTEINS

Supernatants were removed after laser intervention and dishes were washed in PBS. Cells were trypsin digested (Hyclone, USA) and lysed on ice for 30 min. Lysates were centrifuged at 12,000 rpm for 8 min and total protein concentrations were measured via BCA assay. Samples were denatured in a 100 °C water bath and resolved on SDS-PAGE gels. Samples were transferred to PVDF membranes and blocked in a 5% BSA-0.1% TBST for 1 h to avoid non-specific binding. Membranes were probed with antibodies against GAPDH, neuN and GFAP (ZENBIO, China) at 4 °C overnight and labeled with goat anti-mouse or goat anti-rabbit HRP conjugated secondary antibodies for 1 h at room temperature. Membranes were washed in 0.1%-TBST. ECL luminescence reagent (Beyotime, China) was added to visualize protein bands. Protein expression was semi-quantitated using Image J software.

F. QUANTITATIVE DETECTION OF SECRETED NEURONAL PROTEINS

Following laser intervention, supernatants were collected and protein concentrations were assessed to determine the optimal dilution ratio. The levels of BDNF, GDNF and NT-3 (Elabscience, China) were then assessed. Samples were added dropwise to the solid phase carrier for 2.5 h at room temperature or overnight at 4 °C (100 uL/well). Residual liquid was removed through washing and samples were dried using absorbent paper. Antibodies were diluted 80-fold and added at room temperature for 1 h (100 uL/well). HRP-biotin was diluted 200-fold and added for 45 min. Reaction substrates were then added for 30 min, and the reaction was terminated through the addition of stop solution (50 uL/well). Absorbance's were immediately measured on a 450 nm microplate reader and best fit curves were constructed to assess protein concentrations.

G. STATISTICAL ANALYSIS

The following experimental groupings and statistical methods were set up prior to the experiments according to the data guidelines Published by the American Society for Pharmacology and Experimental Therapeutics [26] and the data analysis methods of the related article [27]. The experiments were to be repeated at least three times and each well was to be tested three times. Data are expressed as mean ± standard deviation ($\bar{x} \pm s$). Data were analyzed using one-way analysis of variance (ANOVA) and two-tailed Student's t-test for comparison between two groups. $p < 0.05$ or $p < 0.01$ were considered to be statistically significant. Data were analyzed using SPSS 25 and plotted using Origin 2017 and Graphpad Prism 8.

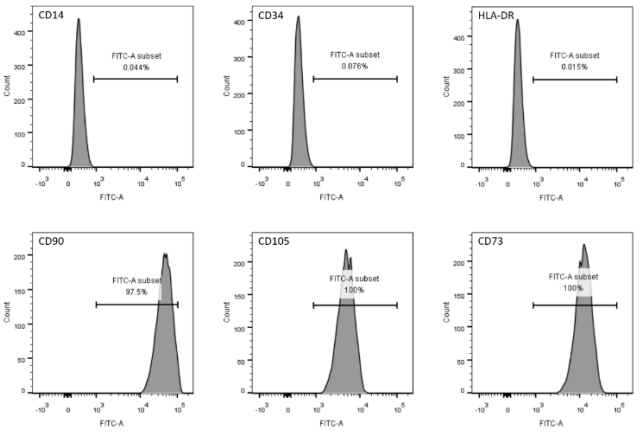


FIGURE 2. Fluorescence-activated cell sorting analysis of cells surface markers.

III. RESULTS

A. IMMUNOPHENOTYPING OF HUCMSCS

As shown in Fig. 2, cells were positive for the MSCs markers CD90, CD105, and CD73, but negative for CD14 (monocyte/macrophage marker), CD34 (hematopoietic/endothelial cell marker) and HLA-DR (MHC-II marker). This confirmed that the cell populations were predominantly hUCMSCs.

B. EFFECT OF OPTICAL PARAMETERS ON TEMPERATURE

The regulation of cell by LLLT is non-thermal [40]. Measurements were performed on cultured cells before and after irradiation (Figure 3). There was no statistically significant

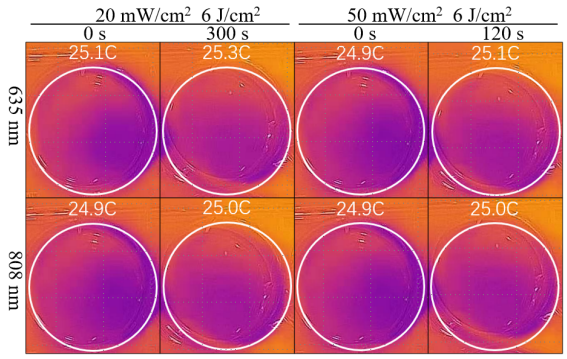


FIGURE 3. Temperature change of cells before and after irradiation.

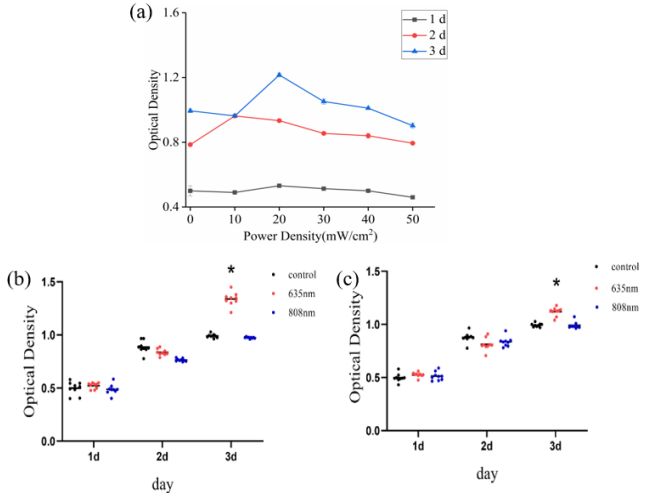


FIGURE 4. Cell proliferation at different power densities (a). Cell proliferation at the indicated wavelengths. Energy densities were (b) 4 J/cm^2 and (c) 6 J/cm^2.

increase in temperature after the plates were restored to room temperature and irradiated with 635 nm and 808 nm lasers.

C. OPTICAL PARAMETRIC SCREENING

The effects of power density on the proliferation of hUCMSCs are shown in Fig. 4a. Doses of 10, 20, 30, 40, and 50 mW/cm^2 were screened at an energy density 6 J/cm^2. Differences between the experimental groups were small after 1 d, but became more pronounced in experimental groups treated with 10, 20 mW/cm^2 after 2 d. The optical density of the 20 mW/cm^2 group was statistically significantly higher than other groups at day 3, whilst 50 mW/cm^2 groups showed no differences from the controls.

The effects of LLLT on the proliferation of hUCMSCs are shown in Fig. 4. The energy densities assessed were 4 and 6 J/cm^2, repectively. Compared to the control group, changes in the proliferation of hUCMSCs were not obvious on days 1 and 2. However, with photon accumulation, the optical density increased at 635 nm with 4 J/cm^2(Fig. 4b) However, there was no differences were observed between the groups at an energy density 6 J/cm^2 (Fig. 4c).

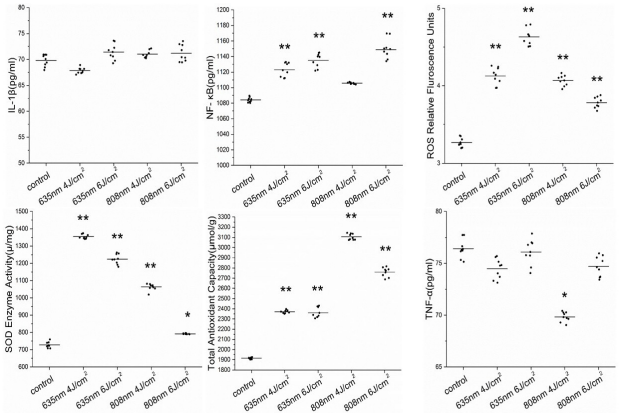


FIGURE 5. Levels of ROS, antioxidants, NF-κB, IL-1β and TNF-α at indicated energy densities.

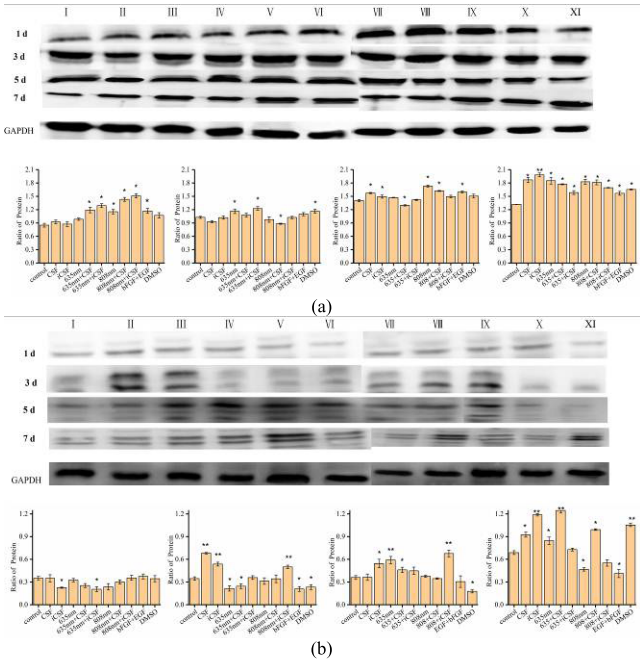


FIGURE 6. Semi-quantitative analysis of neuronal marker expression. (a) Representative gels and semi-quantitative analysis of GFAP and (b) neuN. control (I), CSF (II), iCSF (III), 635 nm (IV), 635 nm + CSF (V), 635 nm + iCSF (VI), 808 nm (VII), 808 nm + CSF(VIII), 808 nm + iCSF (IX), bFGF + EGF (X), DMSO(XI).

D. CELLULAR ROS, ANTIOXIDANTS, NF-κB AND INFLAMMATION ANALYSIS

The effects of LLLT on hUMSCs ROS, SOD, TAC, NF-κB and inflammatory factors (IL-1β and TNF-α) are shown in Fig. 5. At 635 and 808 nm with energy densities of 4 and 6 J/cm^2, ROS production increased to lower levels. Compared to the control group, LLLT increased the levels of SOD, with SOD production at 808 nm lower than that of the 635 nm treated group. Total antioxidant capacity at 808 nm increased. Laser radiation increased the levels of NF-κB but caused no statistically significant increase in the expression of other inflammatory factors.

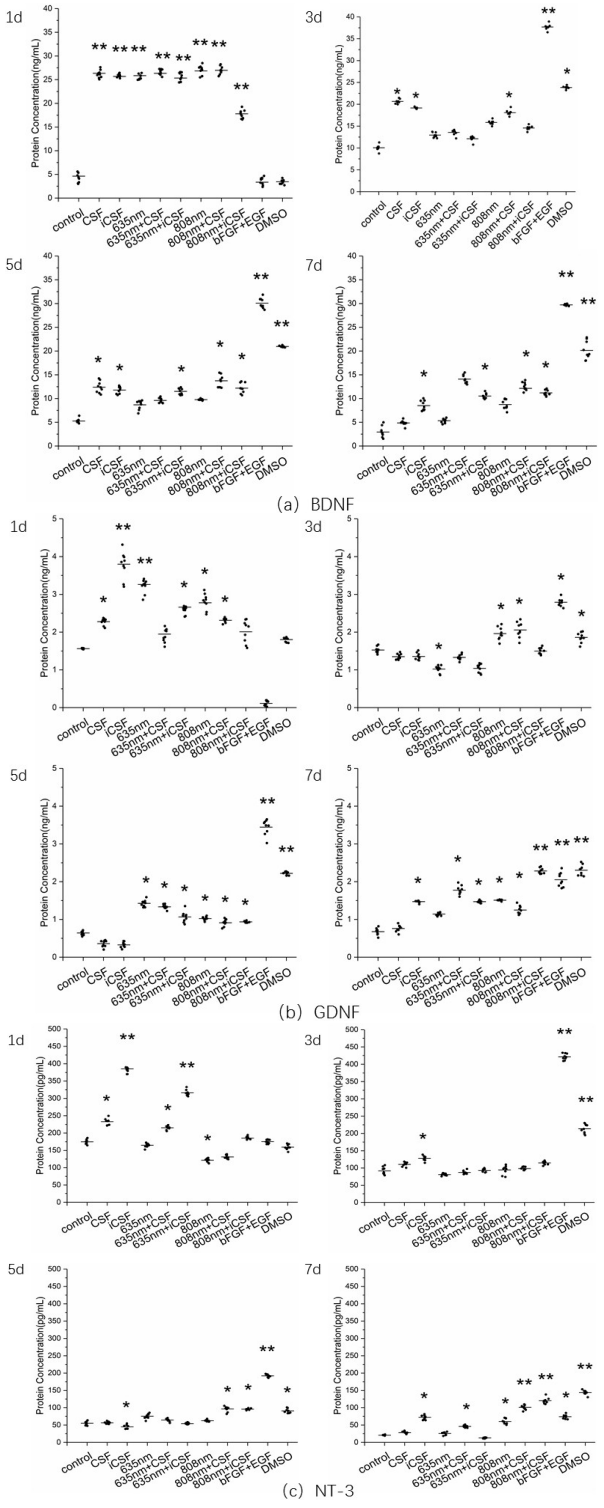


FIGURE 7. Quantitative analysis of secreted protein concentrations.

E. NEURONAL CELL PROTEIN EXPRESSION

The semi-quantitative analysis of protein expression in both glial and neuronal cells is shown in Fig. 6(a-b). GFAP (50 kD) is a glial cell matrix protein and neuN (molecular weight 46-55 kD) is a nuclear protein in neuronal cells. Positive control groups included EGF with bFGF. The expression of GFAP increased in the 808 nm with CSF/iCSF group on day 1, but neuN decreased. The expression of neuN in the CSF group, iCSF group, and 808 nm with iCSF groups were statistically significantly higher than control groups.

The expression of neuN in the 808 nm with iCSF group was further enhanced on day 5. GFAP was highly expressed to comparable levels across all groups. The expression of neuN was highest on day 7 in the iCSF group, followed by the 635 nm with CSF, and 808 nm with CSF groups.

F. QUANTITATIVE ANALYSIS OF SECRETED PROTEIN EXPRESSION BY ELISA

BDNF, GDNF, and NT-3 were quantitatively analyzed as shown in Fig. 7. The total protein concentrations of the samples were quantified to determine the appropriate dilution ratio. BDNF levels peaked in the CSF group during the initial stages. An upward trend was observed in both the biological factor and chemical induction groups. The levels of GDNF in the iCSF group and 635/808 nm with iCSF group increased, whilst the concentration of biological factors alone group were low. The effects of light intervention were more obvious on day 3, particularly in the 808 nm and 808 nm with CSF groups. Following photobiomodulation, the levels of GDNF increased in the 808 nm with iCSF group to levels comparable to the positive control group.

NT-3 levels were higher in iCSF and 635 nm with iCSF groups during the initial stages, but the effects of 808 nm were less obvious. The levels of NT-3 increased in the 808 nm with CSF or iCSF group on day 5 to levels comparable to the control and chemical induction group. Compared with the 808nm laser intervention, the concentration of NT-3 was higher in the combined intervention group at day 7. NT-3 levels were also higher in the 808 nm iCSF or CSF groups compared to the biological control group at day 7.

IV. DISCUSSION

A number of methods have been described for the differentiation of MSCs. The use of biomaterials provide the opportunity to transdifferentiate MSCs into a neuronal lineage [28]. Polymers of poly l-lactic acid/polycaprolactone fibrous scaffolds can improve MSC differentiate into glial and neural progenitor cells [29]. Mechanical stimuli such as sub-sonic vibration [30] and electric stimulation [31] caused MSCs to differentiate into neural-like cells. However, the differentiation of MSCs induced by LLLT combined with inducers remains less-well defined. The laser produces photochemical and photothermal effects. The key to distinguish between low power laser and high power laser is whether there is the presence or absence of photothermal effects [32]. In cellular experiments [33]and animal experiments [34], some authors have argued that the small temperature increase induced by the laser is not sufficient to explain the LLLT which is consistent with results in this paper. LLLT has a non-thermal effect that does not affect structural changes in the tissue but is sufficient to activate the tissue. LLLT is different from

other light-based treatments because it does not ablate and is not based on heating [35] and is safer than chemical or biological induction and is safer. LLLT has important regulatory effects on cell activity. Photobiomodulation promotes cell proliferation [36] but it's effects on MSCs differentiation are less well characterized [37]. Here, we confirmed that LLLT at 635 nm promotes the proliferation of hUCMSCs and studied the effects of photobiomomodulation on oxidative stress, inflammatory responses and cell differentiation. The energy absorption or transformation of photons is influenced by the spectral characteristics of tissue cells. We screened energy density, power density and wavelengths of three optical parameters. A wavelength of 636 nm could promote the proliferation of ADMSCs, which is consistent with our finding that 635 nm accelerates the proliferation of MSCs [38]. Previous studies showed that the proliferation of MSCs was more obvious at an energy density of 4 J/cm^2 [39]. Power densities were selected through step by step analysis and cell proliferation was enhanced at a density of 20mW/cm^2. The proliferation and differentiation of cells under different states are competitive [37].

Intracellular ROS regulates the self-renewal and differentiation potential of stem cells [40], [41]. Irradiation at 635 and 808 nm led ROS production. Huang *et al.* [42] showed similar effects, in which ROS production in the mitochondria was associated with short-term increases in MMP following PBM (3 J/cm^2, 810 nm, 20 mW/cm^2) delivery to control cells. Mild ROS levels stimulate neural differentiation [43]. SOD can scavenge superoxide anion free radicals, and TAC is used as a comprehensive index of antioxidation. A delicate balance exists between ROS production and cellular antioxidants. Excessive oxidative stress disturbs this balance, leading to high levels of ROS and subsequent tissue damage. After irradiation, the secretion of SOD increases. Other oxides in ROS that are unaffected by SOD can be removed by a variety of antioxidants in TAC. Accordingly, the TAC value of 4 and 6 J/cm^2 was higher than that of the control group and increased cell proliferation was observed. Assis *et al.* [44] also confirmed that PBM increased the expression of SOD. When SOD scavenges ROS, the total antioxidant capacity is high, and cell proliferation is enhanced.

NF-κB is redox-sensitive and activated by ROS [35]. Excessive energy generation leads to the over-activation of NF-κB and enhanced ROS level, which exacerbates cell damage [35]. Moderate levels of mitochondrial oxidative stress result in neuroprotective through NF-κB signaling [45]. Photobiomodulation regulates inflammation and can improve the safety of transplantation. In activated inflammatory cells, PBM can decrease inflammation [46], which is particularly important for neuronal disorders.

Our previous study showed that cells differentiated into nerve-like cells at a 808 nm of 6 J/cm^2, although no effects on cell proliferation were observed [39]. MSCs differentiation is regulated by cell density and cell-to-cell contact [47]. Cellular growth factors also influence differentiation. To guide the differentiation of MSCs into nerve cells, CSF provides biological factors required for cell differentiation. This study prolonged the induction time and further explored the effect of MSC differentiation.

Self-renewal potential as opposed to multi-directional differentiation increased. At higher cell densities, differentiation was stimulated through the combined effects of the paracrine and autocrine systems, leading to enhanced GFAP expression [47]. Western blot analysis showed that the effects of light intervention were less obvious. Cell densities increased at day 3, and GFAP expression was regulated through autocrine and paracrine effects. Semi-quantitative analysis indicated that the intercellular paracrine effects were strong following light intervention and that the differentiation of glial cells was pronounced. LLLT combined with biological inducers therefore promotes the differentiation of neural MSCs and increases their differentiation into neurons.

Upon assessment of the secretory functions of mature nerve cells, we further studied the type of differentiated neuronal cells. GDNF is secreted by glial cells and promotes cell proliferation following light intervention. Para-secretory effects combined with an inducer enhance its expression [47]. In this study, the concentration of BDNF increased in response to photobiological regulation and biological induction at later stages. These data confirmed that BDNF plays an important role in stem cell differentiation [12]. Previous studies showed that the neurotrophins NT-3 and BDNF can develop into neuromuscular synapses [48]. The expression of NT-3 in neurons was more obvious at 808 nm in the inducer groups. With continuous photobiological regulation, the differentiation of MSCs towards cells of neuronal lineage was enhanced.

The detection of related proteins shows that light intervention can promote stem cell differentiation into nerves, but the cycle numbers remain high. In addition, the effects of LLLT on global gene expression now warrant further investigation.

V. CONCLUSION

A low energy density 4 J/cm^2 of 635 nm at 20 mW/cm^2 using optical parameters could effectively promote cell growth. LLLT leads to low-levels of oxidative stress and increases NF-κB expression. The effects of 808 nm were more pronounced, but did not influence the release of pro-inflammatory factors. The combined effects of LLLT at 808 nm and cerebrospinal fluid could improve the efficiency of neural differentiation and the secretion of nutritional factors in MSCs.

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(Hongjun Wu and Ziyang Zang contributed equally to this work.)

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