Proliferation and differentiation of endogenous neural stem cells and brain functional reconstruction following laser irradiation**☆

Zhang Wei-hong[1], Li Wan-qing[2], Wang Jin-guo[1], Liu Gui-ping[1], Yang Hua-shan[1], Wang Lu-lu[3]

Abstract

BACKGROUND: Numerous studies have shown that the increase of reproductive activity and self-repair capacity of brain endogenous neural cells might be a valuable method to treat ischemia-hypoxia brain damage.
OBJECTIVE: To study the effects of He-Ne laser irradiation on the proliferation and differentiation of endogenous neural stem cells and brain functional reconstruction in newborn rats with hypoxia-ischemia brain damage.
METHODS: Newborn rats aged 7 days were prepared for hypoxia-ischemia brain damage models. From the second day of model induction, rats in the laser treatment group were subjected to He-Ne laser irradiation. Acupuncture point included Baihui (DU20) on the median parietal bone, and *Dazhui* (GV14) between the C_7 and T_1 and the median back. After the second course, the learning and memory ability of rats were tested by Y-type maze test. Then brain hippocampal sections were made and underwent immunohistochemistry for nestin and microtubule-associated protein-2.
RESULTS AND CONCLUSION: In the laser treatment group, the ability of learning and memory were obviously higher than those in the model group (P < 0.05), however, compared with the sham-operated group, the difference was not obviously (P > 0.05). Compared with the sham-operated group, nestin expression in the dentate gyrus was significantly increased in the model and laser treatment groups (P < 0.05), and the increased range was greater in the laser treatment group compared with the model group (P < 0.05). Microtubule-associated protein-2 expression was widely distributed in the cerebral cortex, and darkly stained brown dendrite presented with radiation-shape. Neurons in the hippocampal pyramid and dentate gyrus granular cell layer arranged regularly. Positively stained dendrite presented branch-shape and distributed in the molecular layer. No significant difference was determined between the sham-operated and laser treatment groups. But the microtubule-associated protein-2 expression was significantly weakened in the model group. He-Ne laser irradiation can promote proliferation of endogenous neural stem cells in neonatal rats with hypoxia-ischemia brain damage, induce its differentiation into neurons, thus, achieves reconstruction of learning and memory functions.

INTRODUCTION

Hypoxic-ischemic brain damage (HIBD) continues to be a major problem in perinatal medicine because of the high mortality rate, neurological and intellectual impairment in the surviving infants. How to promote neurological functional recovery after hypoxic-ischemic brain injury in children is the focus of the study.

Recently, neural stem cells have been isolated from various regions and various development periods of the mammalian brain. Central nervous system of mammalian takes part in repairing the injured neuronal web and function. As the application of exogenous neural stem cells transplantation for treatment, nerve damage repair function is not yet satisfactory. Numerous studies have confirmed that cerebral ischemia in the mobilization of the endogenous neural stem cells in attempt to self-repair is obviously insufficient[1]. Therefore, it is the key to through effective interventions activation of endogenous neural stem cells to repair the neonatal hypoxic-ischemic brain damage[2].

Laser medical study found that He-Ne laser irradiation on biological locally has local and long-distance effects[3], and tips to promote ischemia and trauma repair process of the organization. For this reason, our experiments adopted a generally accepted rat model with hypoxia-ischemia, and "*Baihui*" (DU20), *Dazhui* (GV14) points were selected to study the effect of He-Ne laser acupoint irradiation on endogenous neural stem cell proliferation and differentiation after hypoxia-ischemia in neonatal rats, trying to find out the mechanisms and a safe therapeutic method for clinic.

MATERIALS AND METHODS

Design

A randomized controlled animal experiment.

Time and setting

This study was performed at Nursing College of Zhengzhou University, from December 2007 to May 2008.

Materials

A total of 40 healthy Wistar rats of 7 days old, weighting 12–16 g, irrespective of genders, were provided by the Experimental Animal Center of Henan Province (Qualified certificate No. 410116). The animals were randomly divided into sham-operated group (n=12), model group (n=12), and laser treatment group (n=16). The study was performed in accordance with ethical guidelines for the use and care of animals[4].

He-Ne laser multifunction therapeutic apparatus was produced by Guilin Electronic Instrument Factory. Y-type maze was produced by Zhangjiagang Bio-Medical Instrument Factory. Mouse anti-rat Nestin monoclonal antibodies were purchased from Wuhan

[1]Department of Basic Courses, Nursing College of Zhengzhou University, Zhengzhou 450052, Henan Province, China; [2]Department of Life Sciences, Zhengzhou Teacher College, Zhengzhou 450044, Henan Province, China; [3]Henan Eye Institute, Zhengzhou 450000, Henan Province, China

Zhang Wei-hong☆, Doctor, Lecturer, Department of Basic Courses, Nursing College of Zhengzhou University, Zhengzhou 450052, Henan Province, China zwhong306@zzu.edu.cn

Correspondence to: Wang Lu-lu, Master, Henan Eye Institute, Zhengzhou 450000, Henan Province, China

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Boster Biological Engineering Co., Ltd. Rabbit anti-rat MAP2 antibody monoclonal antibody, sp-9000 high-sensitivity kit, DAB chromogenic kit, purchased from Beijing Zhongshan Biological Engineering Co., Ltd.

Methods

Preparation of HIBD models

Preparation of HIBD was referenced internationally recognized method[5], that is, after left common carotid artery ligation in neonatal rats, they inhaled a low concentration of oxygen (volume fraction of 8% of the O_2, the volume fraction of 92% in N_2). Rats in the sham-operated group did not receive deligation of the left common carotid artery, but exposed to the air.

Interventional procedure

From the second day of model induction, rats in the laser treatment group were subjected to He-Ne laser irradiation, at 632.8 nm wavelength, 4 mW laser power, 3 mm the diameter of light spot, 56.62 mV power density, 33.975 J/cm^2 energy density, 10 minutes once, once a day, 10 days as a course, totally 2 courses, with an interval of 2 days between the two courses. Acupuncture point included *Baihui* (DU20) on the median parietal bone, and *Dazhui* (GV14) between the C_7 and T_1 and the median back[6-7].

Learning and memory testing

After the second course, the learning and memory ability of the rats were tested by Y-type maze test. 10 times of continuous electric shocks to test, 9 times of the correct response shall be to reach the learning standards, then record the number of the formation of conditioned reflex of the animals, as indicators of learning ability. After 24 hours rest, the above experiment was repeated for the first two tests, and then the number of errors of the animals that occurred was recorded, served as indicators of memory ability. In the sham-operated group and model group, the learning and memory ability of the rats were measured at the same time.

Tissue collection and immunohistochemical staining

At 22 days after ischemia-hypoxia, rats were abdominally anesthetized with 100 g/L hydrated ammonium aldehyde and sacrificed, performed with aortic cannlation of left ventricle to rapidly perfuse 100 mL saline in 37 ℃, and fixed with 40 g/L paraformaldehyde buffer (pH 7.2–7.4, 4 ℃) for 30 minutes. The left hemisphere tissue was fixed with 40 g/L paraformaldehyde buffer (4 ℃) for 12 hours, routinely dehydrated, embedded with paraffin, cut into serial coronal sections of hippocampal formation from coronal surface of optic chiasma with the thickness of 5 μm. One section was selected from every 5 sections, and 10 sections were selected from each sample. Paraffin sections were routinely dewaxed, dealt with 3% H_2O_2 for 10 minutes, repaired with hypertensive antigen, blocked with 10% normal goat serum for 20 minutes, added with antibody Ⅰ, incubated in wet box at 4 ℃ for overnight, washed with PBS, added with biotinylation antibody Ⅱ, incubated at 37 ℃ for 20 minutes, washed with PBS, incubated with horseradish peroxidase labeled chain enzyme synthetic prime at 37 ℃ for 20 minutes, colorized with DAB, mildly restained with sappan wood, routinely dehydrated, cleared and fixed with Neutral gum. Normal goat serum instead of primary antibody in the controlled experiment, and immunohistochemical staining was performed simultaneously.

Main outcome measures

The results of Y-type maze test and nestin and microtubule-associated protein-2 (MAP-2)-positive cells of the rats in each group were observed. The numbers of immune positive cells of each section were counted under a microscope. Five adjacent sights were randomly selected from one brain section to calculate arithmetical average value of positive cells. Nestin-positive cells in the dentate gyrus were counted.

Design, enforcement, and evaluation

This study was designed by Zhang Wei-hong, Li Wan-qing and Wang Lu-lu, performed and evaluated by Wang Jin-guo, Liu Gui-ping and Yang Hua-shan. All authors have accepted regular professional training. The double blind method was not adopted.

Statistical analysis

All data were analyzed using SPSS 10.0 software. Results were expressed as Mean ± SD. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Quantitative analysis of the experimental animals

All 40 rats were involved in the final analysis.

Learning and memory ability

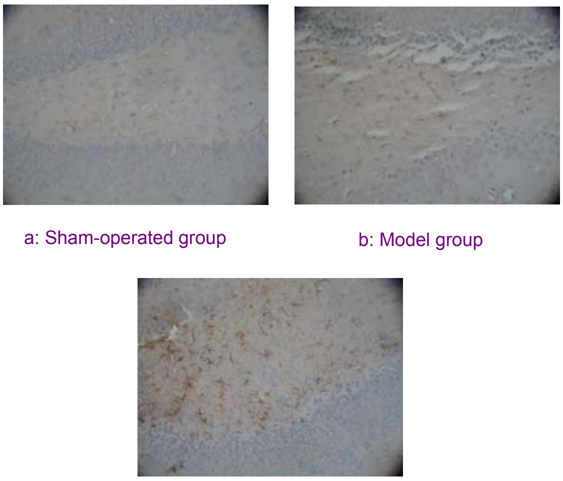
The learning and memory ability of rats in the laser treatment group were obviously higher than those in the model group ($P < 0.05$). However, compared with the sham-operated group, the conditioned reflex number and error frequency of the model group was greater ($P < 0.05$). There were no significant differences between the sham-operated and laser treatment groups. Learning and memory ability of the rats in the three groups were shown in Table 1.

Table 1 Learning and memory ability of rats in the three groups ($\bar{x}\pm s$)

Group	*n*	Learning ability	Memory ability
Sham-operated	12	24.20±4.53	16.80±3.49
Model	12	36.20±6.16	27.60±5.68
Laser treatment	16	23.30±4.97	16.90±3.44
F		24.135	26.990
P		0.000	0.000

Nestin immunohistochemical staining

Immune positive neurons of nestin in each group were mainly located in hippocampal CA1, CA2 and CA3 areas, and dentate gyrus, *etc*. Under an optic microscope, immune positive cells were presented with brown yellow. In the dentate gyrus, there were a lot of nestin-positive cells (Figure 1).



a: Sham-operated group b: Model group

c: Laser treatment group

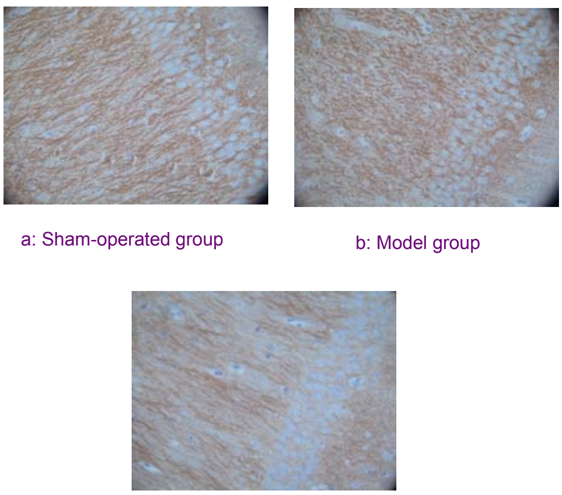
Figure 1 Nestin expression in the dentate gyrus in each group (Immunohistochemical staining, ×400)

Numbers of Nestin immune positive cells in the model group were markedly increased than that of the sham-operated group (P < 0.05), which increased more obviously in the laser treatment group (P < 0.05, Table 2).

Table 2 Estin-positive cells in dentate gyrus of rats in the three groups ($\bar{x}\pm s$, cells)

Group	n	Number of positive cells in dentate gyrus
Sham-operated	12	20.70±2.61
Model	12	28.00±3.46
Laser treatment	16	39.50±2.78
F		122.361
P		0.000

MAP-2 expression was described in Figure 2.



a: Sham-operated group b: Model group

c: Laser treatment group

Figure 2 Microtubule-associated protein-2 expression in the hippocampal CA1 region in each group (Immunohistochemical staining, ×400)

MAP-2 expression was widely distributed in the cerebral cortex, and darkly brown stained dendrite presented with radiation-shape. Neurons in the hippocampal pyramid and dentate gyrus granular cell layer arranged regularly. Positively stained dendrite presented branch-shape and distributed in the molecular layer. No significant difference was determined between the sham-operated and laser treatment groups. Microtubule-associated protein expression was significantly weakened in the model group.

DISCUSSION

HIBD could result in neonatal morbidity and mortality, some patients with HIBD developed into cerebral palsy and mental retardation. At present, no effective therapeutic strategies have yet been developed to counteract this condition in clinic. Recently, neural stem cells have been isolated from various regions and various development periods of the mammalian brains[8-9]. Central nervous system of mammalian can take part in repairing the injured neuronal web and function. Today the mechanisms of neuronal self-repair following insults to the mammalian brain have been scarce. The limited ability of the mature central nervous system to generate new neural cells limits the self-repair capacity of the damaged brain. However, more and more theories demonstrated that one of most important treatment is to build up the self-restore of brain for HIBD patients. He-Ne laser acupoint irradiation is a non-invasive therapy and has features of soft, painless, and sterile, in addition it has a unique psycho and psychiatric rehabilitation function, so it is a therapeutic tool with widely prospect. Rochkind *et al*[10] research suggested that He-Ne laser irradiation in rat spinal cord can promote the damaged appropriate peripheral nerve repair.

Nestin belongs to a kind of intermediate filament protein, which is an important marker protein in identification of the neural stem cells, and has been widely used as a marker of neural stem cells. Here, we observed that the nestin positive cells in the laser treatment group was significantly increased than that of the other groups, which shows that He-Ne laser acupoint irradiation significantly promoted the neural stem cell proliferation in dentate gyrus with HIBD neonatal rats.

MAP-2 and tubulin binding, involved in the composition of microtubules, was the neuronal structural proteins. As a neuron-specific protein, it was expressed in mature neurons in perinuclear cytoplasm and dendrites, with the assembly of microtubules to form and stabilize the structure of the role of the cytoskeleton. We also observed that there were fewer MAP-2 positive cells, with staining shallower, in the model group, while the He-Ne laser acupoint irradiation promoted the expression of MAP-2 positive cells in HIBD neonatal rat hippocampus and dentate gyrus, indicating that neural stem cells differentiated into neurons in rats with HIBD brain injury, thereby repairing the damaged neural networks to achieve functional recovery. The neural stem cells in dentate gyrus located at the dentate gyrus granule cell layer, cerebral ischemia and hypoxia to stimulate the proliferation of these neural stem cells[11-13] resulting daughter cells migrate into the dentate gyrus granule cell layer, then differentiation into mature granule cells, newborn fiber and hippocampal neurons can establish synaptic contacts, reconstruction or repair of some damaged neural network, so as to achieve the functional recovery. Therefore, we believe that He-Ne laser plays an important role in hippocampal plasticity. This result will provide experimental basis for the prevention and treatment of hypoxic-ischemic brain damage caused by children with mental retardation, cerebral dysplasi by laser acupuncture therapy in clinic.

Learning and memory ability is a high-level brain function. The limbic system, particularly the hippocampus is not only ischemia and hypoxia vulnerable back, but is a key positision of learning and memory. During the development of brain, hippocampal damage will be caused by abnormal brain function and behavior. So, immature brain with HIBD will inevitably lead to changes in learning and memory function. In this study, the change of learning and memory ability in rats was detected by Y-type maze. In the laser treatment group, the ability of learning and memory were obviously higher than those in the model group. This can explain why laser treatment can improve the ability of learning and memory in neonatal rats after HIBD, which helps to reduce the mental retardation and other neurological sequelae from beginning.

In conclusion, after HIBD, their own capacity of endogenous neural stem cells proliferation and differentiation is limited, while He-Ne laser irradiation can promote proliferation of endogenous neural stem cells in neonatal rats with HIBD, induce the differentiation into neurons, and achieve reconstruction of learning and memory functions.

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激光调控内源性神经干细胞的增殖分化及脑功能重建**☆

张伟宏[1]，李宛青[2]，王金国[1]，刘桂萍[1]，杨华山[1]，王璐璐[3] ([1]郑州大学护理学院基础教研室，河南省郑州市 450052；[2]郑州师范高等专科学校生命科学系，河南省郑州市 450044；[3]河南省眼科研究所，河南省郑州市 450052)

张伟宏☆，男，1974年生，河南省开封市尉氏县人，汉族，2010年郑州大学第一临床医学院毕业，博士，讲师，主要从事新生儿缺血缺氧性脑损伤的基础和临床研究。

通讯作者：王璐璐，硕士，河南省眼科研究所，河南省郑州市 450052

摘要

背景：大量研究表明增强脑内源性神经细胞的增殖能力和自我修复将成为治愈缺血缺氧性脑损伤有价值的方法之一。

目的：观察氦氖激光对新生大鼠缺血缺氧性脑损伤内源性神经干细胞增殖分化及脑功能重建的影响。

方法：7 d龄健康Wistar新生大鼠，建立缺血缺氧性脑损伤模型后第2天开始，激光穴位照射组给予氦氖激光照射。穴位选取顶骨正中的“百会”穴，以及第7颈椎与第1胸椎间、背部正中的“大椎”穴。假手术组和模型组不给予激光照射。于第2疗程结束后，用Y-型迷宫检测各组大鼠的学习记忆能力。随后制备脑海马切片，分别进行巢蛋白和微管关联蛋白2免疫组织化学染色。

结果与结论：①激光穴位照射组大鼠的学习和记忆能力明显高于模型组($P < 0.05$)，但与假手术组相比，无明显差异($P > 0.05$)。②大鼠内源性神经干细胞的表达：与假手术组比较，模型组、激光治疗组齿状回内巢蛋白免疫阳性细胞均明显增多($P < 0.05$)，且激光治疗组增多幅度大于模型组($P < 0.05$)。③神经元特有结构蛋白的表达：激光治疗组大脑皮质微管关联蛋白2表达相当广泛，强阳性染成棕褐色的树突呈条索样、流星样放射状分布，海马各区锥体神经元和齿状回颗粒细胞层神经元排列比较整齐，树突连续阳性染色呈树枝状交叉分布于分子层。假手术组与激光治疗组染色所见无明显差别。模型组微管关联蛋白2表达明显减弱。结果提示激光治疗能够促进缺血缺氧性脑损伤新生大鼠脑内源性神经干细胞增殖，诱导其向神经元方向分化，并达到学习记忆功能的重建。

关键词：氦氖激光；学习记忆；缺血缺氧；神经干细胞；新生大鼠

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