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Original Article

Effect of butylphthalide in treating learning disorders in rats with radiation brain injury

Chao-Wang An[1], Jian-Min Li[1], Yao Liu[2], Ya-Ning Zhao[2], Chang-Xiang Chen[2], Jing Hao[2], Cheng-Jing Xue[1]

[1]*The Neurosurgery of Affiliated Hospital,* [2]*Nursing and Rehabilitation College, North China University of Science and Technology, Tangshan 063000, China*

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Abstract: Objective: We observed the effect of butylphthalide (NBP) in treating learning disorders in rats with radiation brain injury. Method: One hundred and twenty male Wistar rats were randomly divided into control group, model group, low-dose NBP group and high-dose NBP group. Radiation at a dose of 22 Gy was administered to the whole brain using linear accelerator to induce radiation brain injury. HE staining was performed to observe the morphological changes of hippocampal neurons. The expressions of extracellular regulated protein kinases 1/2 (ERK1/2) and growth associated protein-43 (GAP-43) were detected by immunohistochemistry and western blotting. MDA content was determined by thiobarbituric acid (TBA) colorimetry. SOD activity of the brain was measured by xanthine oxidase method. The learning capacity of rats was determined using the shuttle box paradigm. Result: Compared with the model group, the damage of nerve cells in the hippocampus was reduced and the MDA content was decreased of NBP group. The expression level of *phospho-ERK1/2 and* GAP-43 and activity of SOD were all higher in NBP group than in the model group; the shuttle box evaluation showed that NBP group animal active avoidance reaction was significantly increased and passive avoidance latency decreased; the above changes were most significant in the high dose NBP group. Conclusion: NBP has a good treatment effect in learning disorders caused by radiation brain injury in rats, which is related to enhanced activity of ERK1/2 and upregulation of GAP-43.

Keywords: Radiation injuries of the brain, learning, ERK1/2, GAP-43

Introduction

Radiation therapy is used as the first-line treatment for primary and secondary brain tumors and head and neck cancers. However, radiation brain injury (RBI) has become increasingly prevalent due to radiation therapy [1] and mainly manifest as a decline in learning and memory capacities [2] in a dose-dependent manner. Hippocampus is the main site of cognition and learning [3], and RBI-induced learning disorders usually indicate structural and metabolic abnormalities of the hippocampus. At present, there are not available treatments against RBI. It is generally believed that cognitive deficit and neurogenic disorders caused by ionizing radiation play a role in RBI. Thus developing drugs that promote synaptic regeneration and reconstruction is the main concern. Growth associatedprotein-43 (GAP-43) is related to nerve growth and considered the preferred molecular marker of neuronal regeneration and synaptic plasticity. Brain injuries are associated with specific expression of GAP-43 in the central nervous system, which is localized to the cytoplasm and axons of regenerated neurons following damage. GAP-43 plays an important role in guiding axonal growth and regulating the formation of new connections between axons [4]. Extracellular signal regulated kinase 1/2 (ERK1/2) is a member of the mitogen-activated protein kinase (MAPK) family. Once activated, ERK1/2 transmits the signals across the nuclear border via the cascade reaction and contributes to neural plasticity [5]. Butylphthalide (NBP) is a novel type of nerve-protective drug extracted by Chinese researchers from celery seeds. Animal experiments have shown that NBP improves brain energy metabolism and local cerebral blood flow, promotes neurogenesis and reduces neural apoptosis [6, 7]. Some researchers have discovered the treatment effect of NBP on RBI [8]. We built RBI model in rats and observed the treatment effect of NBP for learning disorders in rats. By detecting the expressions of phospho-ERK1/2 and GAP-43,

the mechanism of therapeutic effect of NBP against RIB was discussed.

Materials and method

Grouping and modeling

One hundred and twenty adult male AD rats weighing 250 g-300 g were purchased from Beijing Huafukang Biotechnology Co., Ltd. (license No.: SCXK (Beijing) 2009-0004). The rats were randomly divided into control group, model group, high-dose RBI group and low-dose RBI group, with 30 rats in each group. For each group three time points were set up (7 d, 14 d, 28 d).

The rats in the control group were only anesthetized without radiation. The rats in the model group were conventionally anesthetized. The RBI model was induced by reference to literature [9]. Brain irradiation using 6 MeV electrons was administered once with source-skin distance of 100 cm, the absorbed dose rate of 250 Mu/min and total dose of 22 Gy.

The rats in the NBP groups received intragastric administration of NBP once daily starting 2 weeks before radiation. After radiation, NBP was continuously administered for different durations. According to literature [7], the high dose was set as 160 $mg.kg^{-1}$ and the low dose 80 $mg.kg^{-1}$.

Shuttle box learning

ZH-CSC shuttle box system was used. The rats were placed into the shuttle box at different time points and the test started from 9:00. After 5 min of adaptation, the stimulus (a tone) was given for 5 s, followed subsequently by an electric shock for 20 s. Another round of training began after an interval of 10 s. If the rats succeeded in escaping to the safe zone within 5 s after the stimulus, it was considered active avoidance reaction and the present round of training stopped. Otherwise, 1.5 mA AC electric shock was given for 20 s. If the rats escaped to the safe zone after the second electric shock, it was considered passive avoidance reaction. If the rats still failed, the result was defined as negative for both active and passive avoidance reaction. Each rat received 30 electric shocks, and the passive avoidance latency (PAL) and the number of active avoidance reactions were recorded. The percentage of active avoidance reactions to the total number of trainings was the active avoidance reaction rate (AARR). The higher the AARR and the lower the PAL, the stronger the learning capacity was.

Morphological observation

Five rats were selected from each group at each time point. Brain tissues were harvested after perfusion using 4% paraformaldehyde. The scope of resection was from optic chiasma to transverse cerebral fissure, followed by conventional paraffin embedding. The tissues were cut into coronal sections (thickness 5 μm), which were subjected to HE staining. For each rat, 4 slides were chosen and 4 fields of vision were randomly selected for each slide. The sections were observed under the optical microscope. The number of surviving neurons in each field of vision (40 × 10) was determined, and the average percentage of surviving neurons in each field of vision was calculated (%) using Motic-6.0 image analysis system.

Immunohistochemical anlaysis of phospho-ERK1/2 and GAP-43

At each time point, 5 rats were selected from each group, and brain tissues were harvested and prepared into sections using the same method as in HE staining. After dewaxing and addition of deionized water, the cells were incubated with digestion solution at 37°C in an incubator for 15 min. The cells were washed with PBS for three times, 5 min each time. Endogenous peroxidase was eliminated by incubating the cells with 3% hydrogen peroxide for 15 min. After washing with PBS, the cells were incubated with rabbit polyclonal anti-rat antibodies to phospho-ERK1/2 and GAP-43 (1:300) at 4°C overnight. Next the cells were rewarmed at 37°C for 45 min, washed with PBS and incubated with biotinylated secondary antibodies at 37°C for 40 min. The cells were washed again with PBS and color development was performed by adding DAB substrate. The procedures of counterstaining with hematoxylin, dehydration, transparentization and sealing were performed routinely. Using the optical microscope with the micrometer (40 × 10), the positive cells (brown particles) were counted in the field of vision. For each rat, 4 slides were selected and 4 fields of vision were randomly chosen for each slide.

Table 1. Comparison of AARR across the groups ($\bar{x}$±s, %)

Group	7 d	14 d	28 d
Control group	76.84±4.78	77.36±5.34	77.68±5.53
Model group	45.63±0.90*	32.71±0.65*	34.50±0.78*
Low-dose NBP group	56.23±2.56*,Δ	44.66±2.79*,Δ	50.40±2.16*,Δ
High-dose NBP group	64.71±5.65*,Δ,▲	50.51±5.86*,Δ,▲	69.98±6.26*,Δ,▲

*P<0.05 compared with the control group; ΔP<0.05 compared with the model group; ▲P<0.05 compared with the low-dose NBP group.

Table 2. Comparison of PAL across the groups

Group	7 d	14 d	28 d
Control group	17.68±1.79	17.56±2.55	17.53±3.64
Model group	40.11±1.57*	51.13±1.89*	49.08±1.78*
Low-dose NBP group	34.11±1.57*,Δ	45.52±1.88*,Δ	39.52±1.79*,Δ
High-dose NBP group	28.33±0.87*,Δ,▲	36.53±2.70*,Δ,▲	27.51±1.57*,Δ,▲

*P<0.05 compared with the control group; ΔP<0.05 compared with the model group; ▲P<0.05 compared with the low-dose NBP group.

The expression of phospho-ERK1/2 and GAP-43 were detected by western blot

Western blot analysis included protein extraction, electrophoresis, membrane transfer, and immune coloration. Respective 5 Five rats at each time point in each group were decapitated under anesthesia. The hippocampal tissue was rapidly frozen in liquid nitrogen and homogenized in ice-cold homogenization buffe. Homogenates were then centrifuged at 800 g for 5 min at 4°C. Then the supernatants were collected and protein concentrations were determined by the Kaumas Bradford method. Samples were stored at -80°C until required. Detection steps: 40 ug protein sample and the volume of the sample buffer were mixed, after boiling would be carried out twelve sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transfered film. The membrane was incubated with *phospho-ERK1/2* monoclonal antibody and GAP-43 monoclonal antibody (Zhongshan Beijing biological company, both 1:2000) overnight at 4°C. The membrane was washed repeatedly with TBST and incubated with corresponding secondary antibody. The antibody was exposed with ECL solution and image analyzer was used to determine the optical density for quantitative analysis.

Determination of MDA content and SOD activity

Five rats were randomly selected from each group at each time point and sacrificed. The bilateral cortical and hippocampal regions were harvested and 0.6 g of the tissue was weighed and homogenized. After addition of relevant solutions with mixing, the tissue was heated in a boiling water bath for 40 min and then cooled. The tissue was centrifuged at 3500 r/min for 10 min, and the supernatant was taken to measure the absorbance at 532 nm and 550 nm, with 1 cm light path and zeroing with distilled water. MDA and SOD contents were calculated according to manufacturer's instruction: MDA content (nmol.mgprot-1) = (A of experimental tube-A of blank tube)/(A of standard tube-A of blank tube) × concentration of standard ÷ protein content; SOD activity (U/mgprot) = (A of control tube-A of experimental tube)/(A of control tube) + 50% × (total volume of the reaction liquid/sample amount ml) ÷ protein content in the tissue.

Statistical analysis

The SPSS 17.0 software, was used for statistical analysis. The measurement data was presented as means ± standard deviation. Repeat measured ANOVA was used to multiple time points parameters. All data was presented as *P*-values less than 0.05 was considered significant.

Result

Observation of the shuttle box

Compared with the control group, AARR of the model group decreased and PAL was prolonged (P<0.05). At 28 d after radiation, AARR and PAL did not show obvious recovery. As compared with the model group, NBP groups showed higher AARR and shorter PAL (P<0.05). Moreover, at 28 d after radiation, AARR and PAL of the NBP groups showed obvious recovery (**Tables 1**, **2**). In order to further explain the role of NBP in the prevention and treatment, we further observed the changes in the morphology of neurons in the hippocampus.

Morphological changes of neurons

Neurons of the control group were normal, with large, round nuclei and distinct nucleoli.

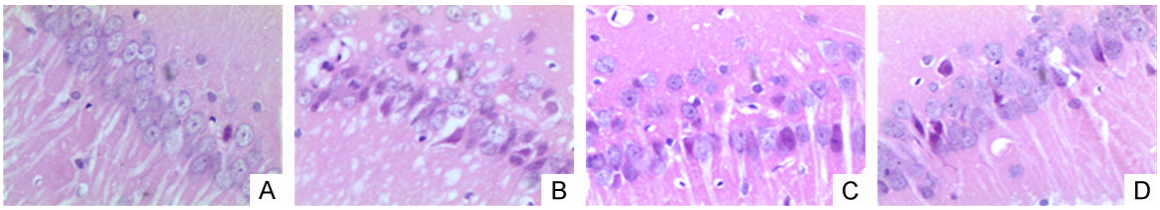

Figure 1. Comparison of morphological changes of neurons across the groups (× 400). A: Control group; B: Model group at 14 d; C: Low-dose NBP group; D: High-dose NBP group.

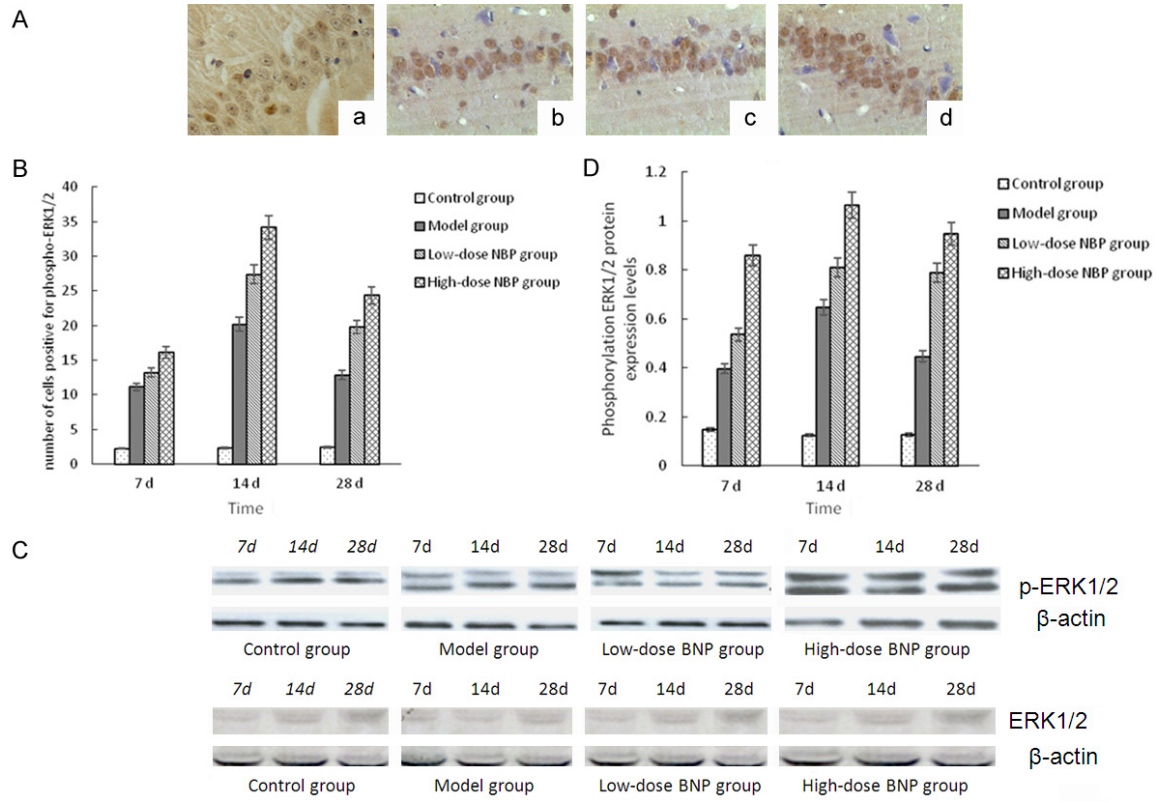

Figure 2. A: Immunohistochemical staining for phospho-ERK1/2 in each group (× 400). a: Control group; b: Model group at 14 d; c: Low-dose NBP group; d: High-dose NBP group. B: Comparison of number of cells positive for phospho-ERK1/2 in each group: The single factor variance analysis results showed that the number of model group at each time point of the phosphorylation of -ERK1/2 positive cells was higher than that of the control group ($P<0.05$); the number of positive cells in NBP group at each time point of the phosphorylation of -ERK1/2 was higher than that of model group ($P<0.05$); the number of positive cells in high dose NBP group at each time point of the phosphorylation of -ERK1/2 was higher than that of low dose group NBP ($P<0.05$). C: Expression of phosphorylated ERK1/2 and total ERK1/2 protein in the hippocampus of rats in each group by Western blot. D: Comparison of expression levels of phosphorylated ERK1/2 protein in the hippocampus of rats in each group: single factor variance analysis results showed that the model group at each time point of the phosphorylation of -ERK1/2 protein level was higher than the control group ($P<0.05$); NBP group at each time point of phosphorylated -ERK1/2 protein levels were higher than those in the model group ($P<0.05$); high dose NBP group at different time points of phosphoric acid -ERK1/2 protein levels were higher than those in the low dose group NBP ($P<0.05$).

Neurons of the model group showed degeneration and edema with obscure cell contour. Some neurons were apoptotic and the cell bodies shrank, presenting as polygonal or irregular shape. The morphological changes were less severe in NBP treatment groups, especially the high-dose group (**Figure 1**). The above behavioral and morphological results indicated that NBP had a better control effect on the learning and memory impairment caused by radioactive brain injury. To further clarify whether the mechanism of prevention and treatment of radia-

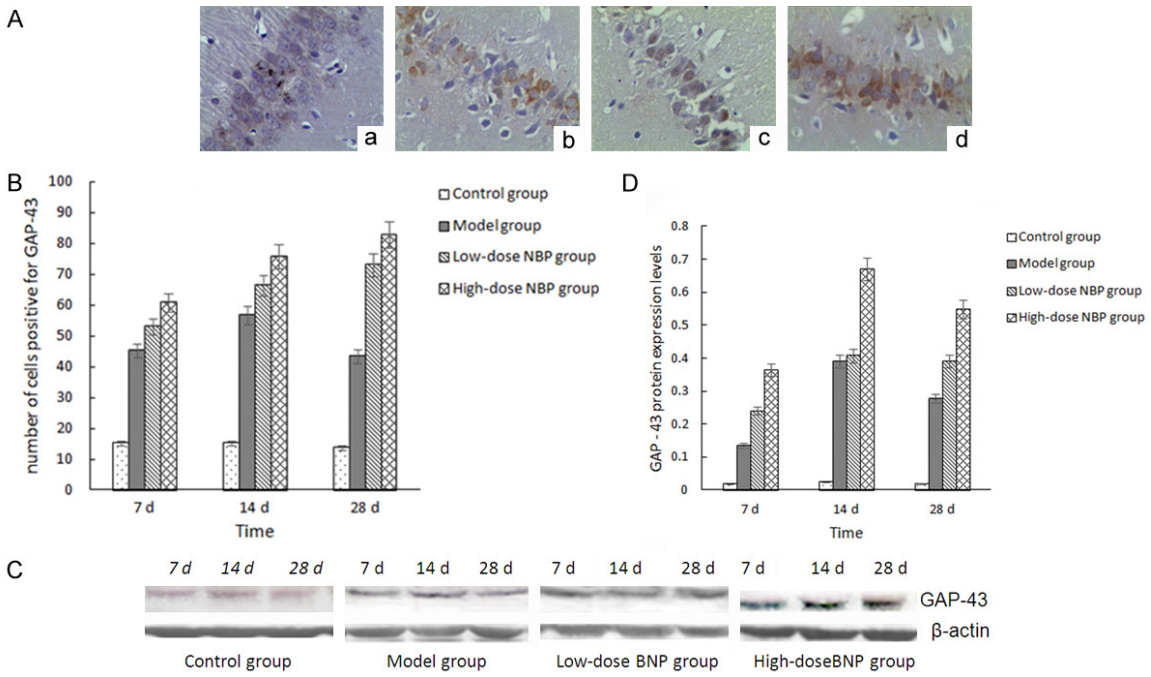

Figure 3. A: Immunohistochemical staining for GAP-43 in each group (× 400). a: Control group; b: Model group at 14 d; c: Low-dose NBP group; d: High-dose NBP group. B: The single factor variance analysis results showed that the number of model group at different time points of GAP-43 positive cells was higher than that of the control group (P<0.05); the number of GAP-432 positive cells in NBP group at different time points P were higher than that of model group (P<0.05); high dose NBP group at each time point of the number of GAP-43 positive cells was higher than the low dose NBP group (P<0.05). C: Expression of GAP-43 protein in the hippocampus of rats in each group by Western blot. D: Comparison of GAP-43 protein expression in the hippocampus of rats in each group: comparison of expression levels of single factor variance analysis results showed that the model group at each time point of the protein level of GAP-43 is higher than that of control group (P<0.05); NBP group at different time points GAP-43 protein levels were higher than those in the model group (P<0.05); GAP-43 protein levels in high dose NBP group at different time points were higher than low phosphorus dose NBP group (P<0.05).

tion brain injury was related to the activation of ERK1/2 and the expression of GAP-43 in NBP. We further examined the changes of phospho-ERK1/2 and GAP-43 in the hippocampus.

Expressions of phospho-ERK1/2 and GAP-43

Immunohistochemical detection for phospho-ERK1/2 and GAP-43: Phospho-ERK1/2 and GAP-43 were mainly expressed in the nuclei, showing as small brown particles (**Figures 2A**, **3A**). The control group had a few cells positive for phospho-ERK1/2. Compared with the control group, the model group had more cells positive for phospho-ERK1/2 at 7 d and 14 d; the number of positive cells decreased at 28 d, but it was still higher than that of the control group (P<0.05). Compared with the model group, the NBP treatment groups had higher number of cells positive for phospho-ERK1/2 at all time points, especially in the high-dose group (P< 0.05). The control group had a few cells positive for GAP-43. Compared with the control group, the model group showed an obvious increase of cells positive for GAP-43 at 7 d and 14 d; the number of positive cells decreased at 28 d, but it was still higher than that of the control group (P<0.05). Compared with the model group, NBP treatment groups had a significant increase of GAP-43-positive cells at each time point until 28 d, especially in the high-dose group (P<0.05) (**Figures 2B**, **3B**). Western blot analysis forphospho-ERK1/2 and GAP-43*:* the IOD value of the protein bands after -actin was reflected by the value of the protein bands (**Figures 2C**, **3C**). Compared with the control group, the expression of 7D in the model group, 14 d time and ERK1/2 phosphorylation levels of GAP-43 increased, 28 d decreased, but still higher than the control group (P<0.05); compared with the model group, the expression level of each time point in group NBP, the phosphorylation of ERK1/2 and GAP-43 further

Table 3. Comparison of MDA content and SOD activity across the groups ($\bar{x}\pm s$)

Group	MDA content (μmol/g)			SOD activity (U/mg)		
	7 d	14 d	28 d	7 d	14 d	28 d
Control group	71.32±2.16	71.51±2.08	71.46±2.12	85.26±4.32	84.56±5.42	86.46±5.30
Intermittent hypoxia group	74.86±2.42*	79.84±2.50*	75.24±2.42*	76.42±4.86*	70.67±6.70*	78.32±6.22*
Low-dose NBP group	71.62±1.88*,△	76.41±1.94*,△	72.32±1.98*,△	79.82±5.26*,△	76.20±6.86*,△	80.12±5.76*,△
High-dose NBP group	69.82±2.06*,△,▲	73.12±1.92*,△,▲	70.56±2.32*,△,▲	83.12±5.64*,△,▲	80.62±5.86*,△,▲	84.10±6.02*,△,▲

*P<0.05 compared with the control group; △P<0.05 compared with the model group; ▲P<0.05 compared with the low-dose NBP group.

increased, high expression continued to 28 d, especially changes in the high dose group (P<0.05, **Figures 2D**, **3D**). The results showed that NBP could improve the brain radiation injury in rats and promote the expression of hippocampus ERK1/2 activity, GAP-43. In order to analyze the mechanism of the changes of the NBP regulation, we observed the changes of MDA and SOD activity in the intermediate metabolites of oxygen free radicals.

MDA content and SOD activity in each group

Compared with the control group, the model group showed a significant increase in MDA content and a reduction in SOD activity. MDA content further increased over time, while SOD activity declined (P<0.05). Compared with the model group, NBP treatment groups showed a significant reduction in MDA content and an increase in SOD activity (P<0.05), especially in the high-dose group (P<0.05, **Table 3**).

Discussion

RBI is a common complication after radiation therapy for craniofacial and cervical cancers. According to clinical reports, the probability of cognitive deficit in survivors receiving whole-brain radiation reaches 50%-90%. The decline in learning and memory capacity usually presents as the symptom [10], in a dose-dependent manner. Study [3] shows that after radiation, the level of n-naeetyl aspartate (NAA) in the brain will decline, and ultrastructural damage of the brain usually occurs in a dose-dependent manner. Therefore, how to prevent the damage caused by ionizing radiation to patients' learning and memory capacity is a hot topic. Our results indicated that the rats of the NBP treatment groups suffered less severe learning disorders and the recovery of the learning capacity was faster. In the high-dose NBP group, AARR and PAL remained stable at 28 d, without showing continuous decline as in the model group. This indicated good preventive effect of NBP against learning disorders in rats with RBI, which is consistent with the findings by Chen and Zhang [8, 12].

Radiation-induced impairment of learning and memory capacity is closely associated with neurogenic disorders. ERK1/2 is a key regulator of cell regeneration, differentiation and migration, which can be activated by several neurotrophic factors such as brain-derived neurotrophic factors and VEGF. As a result, nerve regeneration is promoted and the neuronal loss due to brain injuries is reduced. Conditional knockout of ERK1/2 would lead to failed axonal growth [13, 14], which suggested the key role played by ERK1/2 in nerve regeneration. GAP-43, an important axon growth factor, will be expressed intensively after brain injuries and react with intracellular signal molecule G protein. This process will increase the reactivity of excitatory amino acid receptors and induce the release of calmodulin, thus promoting axonal sprouting and formation [15]. In recent years, Chinese scholars Huang and Liu et al. have confirmed that traditional Chinese medicine such as ShenxiongHuayu capsule and kidney-tonifying and brain-invigorating pills can enhance neurogenesis and recovery of learning capacity after brain injury. The working mechanism is both associated with enhanced activity of ERK1/2 and upregulation of GPA-43 [16, 17]. Given the close connections of ERK1/2 signaling and GPA-43 to the learning capacity, we detected hippocampal expressions of phospho-ERK1/2 and GAP-43 in rats with RBI and both were significantly upregulated in a dose-dependent manner in the NBP groups; moreover, the high expressions persisted for some time. This indicated that NBP possibly worked by enhancing ERK1/2 activity and upregulating GAP-43.

Ionizing radiation can cause oxidative stress to the brain, leading to neural damage, neurotransmitter release problems and cognitive

deficit [18, 19]. Huo [20] found that whole-brain radiation induced a reduction in the SOD level and an increase in the MDA level, which was accompanied by aggravated hippocampal neuronal damage and cerebral edema. Therefore, ionization-induced radiation injuries can be prevented by anti-oxidation. After NBP treatment in our study, the MDA level showed a significant reduction and SOD activity increased compared with the model group; furthermore, there was an improvement of learning and memory capacity. NBP can inhibit xanthine oxidase system, water solubility and lipid peroxidation system, thus reducing oxidative damage. Besides, NBP inhibits iron-dependent peroxidation in the mitochondrial respiratory chain, thus preventing mitochondrial membrane damage caused by peroxidation and suppressing cascade reactions that amplify the oxidative stress [21-23]. Based on the above results, we believe that the strong anti-oxidative effect of NBP played a vital role. The latest research shows that anti-oxidation is crucial for activating the signaling pathway related to axonal regeneration. For example, Liu [24] reported that H_2O_2 pretreatment inhibited the oxidative stress-induced damage to PC_{12} cells by activating ERK1/2 signaling pathway, thus reducing cell apoptosis. This represents a good evidence of the anti-oxidative effect of ERK1/2. Lu et al. [25] applied VitE to rats with cerebral ischemia and the results of induced axonal reconstruction and repair were observed in the central nervous system, in addition to higher expression of GAP-43 over a longer period of time. This means GAP-43 is involved in the resistance against oxidative stress in the presence of VitE. In this experiment, we detected the changes of MDA content and SOD activity in each group, both of which were significantly increased in a dose-dependent manner; moreover, the high expression and high activity persisted for some time. The MDA content after NBP treatment decreased significantly, also in a dose-dependent manner. We infer that the anti-oxidative effect of NBP in rats with RIB is probably related to enhanced ERK1/2 activity and upregulated GAP-43. This is the molecular mechanism of the neuroprotective effect of NBP against learning disorders in rats with RBI.

Our experiment showed that NBP upregulated GAP-43 by activating ERK1/2 signaling pathway in the hippocampus, thus promoting neurogenesis and improving learning disorders in rats with RBI. NBP possesses multiple pharmacological activities and its protective effect against ionizing radiation remains to be further investigated.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Min-Jian Li, The Neurosurgery of Affiliated Hospital, North China University of Science and Technology, NO. 78 South Jianshe Road, Tangshan 063000, China. Tel: +86+15081978570; E-mail: zhaoynyn5@126.com

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