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Relationship between NogoA/NgR1/RhoA signaling pathway and the apoptosis of cerebral neurons after cerebral infarction in rats

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Abstract. – OBJECTIVE: The aim of this study was to investigate the effect and the mechanism of the NogoA/NgR1/RhoA signaling pathway on the apoptosis of neurons in cerebral infarction (CI) rats. Our findings might provide references for clinical prevention and treatment of CI.

MATERIALS AND MATERIALS: A total of 60 adult male Wistar rats were randomly divided into 3 groups, including: Sham operation group (Sham group), CI group, and CI + NogoA gene knockout group (CI + NogoA KO group) using a random number table. The model of CI was successfully constructed using suture method in rats of CI group and CI + NogoA KO group blood vessels were exposed in Sham gro 2 days after CI operation, the rats were k and brain tissues were collected. Reverse T scription-Polymerase Chain Reaction (RT-P and Western blotting were us detect t messenger ribonucleic aci RN nd pro tein expression levels of oA/Ng RhoA in brain lesion tissues of r the t roups, respectively. Subse nt damage of brain tis was ed *via* hema-toxylin and eosi E) stainin TC staining was carried o luate the i tion area in each group ermi eoxynucle dyl trans-ferase dUT ick end l g (TUNEL) staining was co ted to meas he apoptosis lev-el of ons i brain tiss of rats in each gro Additi y, the level of Nissl's body in bra su each group was examined by Nissl . Furth ore, the expression lev-el of the elet- ed growth factor (PDGF) i in tis ats in the three groups was me ured *via* unohistochemistry.

RESULTS: The mRNA and protein expres- of the NogoA/NgR1/RhoA signaling athway in brain tissues of rats in CI group creased significantly ($p<0.05$). NogoA KO d significantly reduce the infarction area of b n tissues in rats ($p<0.05$). H&E staining and Nissl's body staining revealed that neurons in the brain tissues of rats showed evident edema and disordered arrangement after CI. Meanwhile, the number of Nissl's body was remarkably reduced. However, after KO of NogoA, brain tissue damage w si alevi d in rats, and the nu of body i eased remarkably at same tim <0.0 According to TUNE g results, ng NogoA could not rev CI-induce poptosis of neurons in brain tis of rats ($p<0.05$). Immunohis ical stain esults demonstrated th expression of P in brain tissues of r n CI group decreased markedly, whereas w significantl evated in rats of CI + NogoA K roup ($p<0.0$

CONCLUSION: The expression of the NogoA signaling pathway was significantly in brain tissues of CI rats. Furthermore, suppressing the NogoA/NgR1/RhoA ling pathway could reduce CI-induced s of neurons in rats.

Key Words:
NogoA/NgR1/RhoA, Cerebral infarction (CI), Neuron, Apoptosis.

Introduction

Cerebral infarction (CI) generally refers to the symptoms of cerebral tissue ischemia, hypoxia, necrosis, neurological dysfunction and even defects caused by an abnormal blood supply in the brain. It is also known as ischemic stroke[1]. Although the current medical technique has developed rapidly, CI is still the main cause of death and disability worldwide[2]. Therefore, it is of great significance to further clarify the occurrence and development of CI for early diagnosis and accurate treatment.

Current studies[3,4] have revealed that varying myelin-related neurite growth inhibitors can suppress the regeneration of damaged axons. For instance, the typical neurite growth inhibitor NogoA is a myelin-derived protein, which can block axonal regeneration and reconnection of damaged axons after stroke[5,6]. NogoA binds to the Nogo receptor

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(NgR1) and activates the intracellular RhoGTP enzyme signaling pathway, thereby inducing growth cone collapse[7,8]. Hence, the design of drugs targeting the NogoA/NgR1/RhoA signaling pathway is of great significance for the prevention and treatment of stroke. However, few studies have elucidated the exact role and mechanism of the NogoA/NgR1/RhoA signaling pathway in CI.

In this research, the messenger ribonucleic acids (mRNA) and protein expressions of the NogoA/NgR1/RhoA pathway in infarcted brain tissues of rats with CI were first detected. Moreover, the effects of NogoA knockout (KO) on neuronal apoptosis and pathological damage in the brain tissues of rats were further verified by KO of NogoA.

Materials and Methods

Animal Grouping and Model Establishment

Sixty male Wistar rats weighing (85.32±7.61) g at the age of 12-14 weeks old were divided into 3 groups, including: Sham operation group (Sham group), CI group, and CI + NogoA gene KO group (CI + NogoA KO group) using a random number table. The model of CI was successfully constructed using suture method in rats of CI group and CI + NogoA KO group. Meanwhile, only blood vessels were exposed in Sham group. No statistically significant difference was observed in basic data among the three groups, such as weeks of age and body weight. The operation was as follows: 1) Rats were first anesthetized and fixed. 2) The left common carotid artery and vagus nerve were separated. 3) The left common carotid artery and the proximal end of the external carotid artery were ligated, respectively. 4) A thread was prepared from a live knot at the proximal end of the left common carotid artery. 5) The internal carotid artery was separated, and an incision was made at the proximal end of the external carotid artery. 6) The bolt was inserted from the incision and pushed forward gradually. When it was pushed forward for about 18 mm, resistance could be clearly felt. This proved that the head of the bolt reached the middle cerebral artery. 7) After 30 min, the bolt was taken out and reperfusion was carried out. 8) Suture and sterilization were then conducted. After 2 days, scoring was performed, and materials were obtained. This investigation was approved by the Animal Ethics Committee of Qingdao Municipal Hospital (Group) Animal Center.

Detection of the Expression of Apoptosis-Related Genes Via Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

1) TRIzol assay (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from tissues of rats in each group. The concentration and purity of extracted RNA were measured by an ultraviolet spectrophotometer. When the ratio of absorbance (A)$_{260}$/A$_{280}$ was between 1.8 and 2.0, RNA samples could be used. 2) Messenger RNAs (mRNAs) were then synthesized into complementary deoxyribose nucleic acids (cDNAs) through RT and stored in a refrigerator at -20°C for use. 3) RT-PCR system was as follows: 2.5 μL 10× Buffer, 4 μL cDNAs, 0.25 μL forward primer (20 μmol/L), 0.25 μL reverse primer (20 μmol/L), 4 μL dNTPs (10 mmol/L), 0.5 μL Taq enzymes (2×10^5 U/L) and 19 μL ddH$_2$O. The amplification systems of RT-PCR were the same. The primer sequences used in this work were shown in Table I.

Detection of Protein Expression Via Western Blotting

Brain tissues of rats in each group were first ground in lysis buffer and subjected to ultrasonic lysis. After centrifugation, the supernatant was collected and was sequentially sub-packaged into Eppendorf tubes. The concentration of extracted protein samples was measured using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) and ultraviolet spectrophotometry. After that, the volume was set constant to the same concentration. After sub-packaging, the samples were placed in a refrigerator at -80°C. The protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto cellulose acetate (PVDF) membranes (Roche, Basel, Switzerland). After incubation with primary antibodies at 4°C overnight, the membranes were incubated with

Table I. Primer sequences.

Target gene		Primer sequence
GAPDH	Forward	5'-GACATGCCGCCTGGAGAAAC-3'
	Reverse	5'-AGCCCAGGATGCCCTTTAGT-3'
NogoA	Forward	5'-TGCTGCCTTTTCTGTTCCTT-3'
	Reverse	5'-AAGGTGCTGGGTAGGGAAGT-3'
NgR1	Forward	5'-GTCCACGAACCCGTAAGGT-3'
	Reverse	5'-ACGATGCTGGATGCTAGTCG-3'
RhoA	Forward	5'-ACGTGTGCTAGCCCCACTGATG-3'
	Reverse	5'-CATCTTTTCCCGATAGGTCCA-3'

goat anti-rabbit secondary antibody for 1 h away from light. Immuno-reactive bands were scanned and quantified using an Odyssey membrane sweeper. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to correct the level of proteins to be tested.

Triphenyl Tetrazolium Chloride (TTC) Staining

1) Fresh brain tissues were first put into a rat brain tissue slice grinder, followed by frozen in a refrigerator for 30 min at -20°C for slicing. 2) Subsequently, brain tissues were cut into 2 mm-thick slices, with no more than 6 slices per tissue. 3) Prepared slices were then placed in fresh TTC solution (2%) and fully contacted the TTC solution for incubation for no less than 0.5 h. 4) After 0.5 h, the slices were taken out and fixed with 4% paraformaldehyde, followed by photography.

Hematoxylin and Eosin (H&E) Staining

Brain tissues obtained in each group were first placed in 10% formalin overnight, dehydrated and embedded in paraffin blocks. Subsequ[illegible] all brain tissues were cut into slices with a[illegible] ness of 5 μm, fixed on glass slides and dri[illegible]r staining. According to relative instructions, sections were soaked in xylene, ethanol at gra[illegible] ent concentration and hemato[illegible]lowed b[illegible] sealing with resin. After dr[illegible]in th[illegible]r, obser- vation and photography w[illegible]conduct[illegible]nder an optical microscope. Fi[illegible]ly [illegible] neurons was observ[illegible]

Terminal De[illegible]otidyl Transferase dUTP [illegible] End Labelin[illegible]UNEL) Sta[illegible]g

Br[illegible]ssues were first si[illegible] baked in an oven at 6[illegible]or 30 [illegible]nd depara[illegible]ined with xylene (5 min[illegible]me[illegible]hen, th[illegible] sections were dehydrated with [illegible]5%, a[illegible]0% ethanol, respective- [illegible]ith [illegible]s f[illegible]ach. Subsequently, the slic- es [illegible]re inc[illegible]with protein kinase K for half an [illegible]ur. After [illegible]ashing with phosphate-buffered [illegible]TdT and luciferase-labeled dUTP [illegible]ere added. After reaction for 1 h at 37°C, the [illegible]tions were incubated with a specific antibody [illegible]ed with horseradish peroxidase at 37°C for 1 h. Next, the sections reacted at room temperature for 10 min, with diaminobenzidine (DAB) as the substrate. After the nucleus was stained with hematoxylin, photography and counting were carried out under an optical microscope.

Immunohistochemical Staining

Brain tissue slices were first baked in an oven at 60°C for 30 min and deparaffinized with xylene (5 min × 3 times), followed by dehydration with 100%, 95%, and 70% ethanol, respectively, with 3 times for each. Endogenous peroxidase [illegible] was inhibited by 3% hydrogen peroxide [illegible] and the tissues were sealed with she[illegible]erum for 1 h. Antibodies against platelet-d[illegible]l growth factor (PDGF) were diluted a[illegible]1:200[illegible]) and incubated at 4°C overnight [illegible]owed by [illegible]ing with PBS for 4 times in [illegible]aker. After th[illegible] sections were incubate[illegible]n seco[illegible]y antibo[illegible] Next, the color was [illegible]lev[illegible]d[illegible] diami[illegible]oben- zidine. After col[illegible]velop[illegible]6 sam[illegible] were randomly sele[illegible]from each [illegible]p[illegible] 5 fields of view we[illegible]ly selected[illegible]ch sample. Finally, pho[illegible]graph[illegible] performe[illegible] under an optical mi[illegible]ope (200[illegible]1,400×).

M[illegible] Staining

Paraffin-en[illegible]dded or frozen sections were se- q[illegible]ially staine[illegible]th xylene, anhydrous ethanol, 95[illegible]% and 7[illegible]alcohol, and distilled water. The respec[illegible]ould be based on H&E staining. 2) The sections were then stained with 1% tar violet [illegible]% thionine for 10 min to 1 h. 3) Subsequently, [illegible]ons were rinsed with distilled water. 4) 70% alcohol color separation was performed for several seconds to several minutes. 5) The sections were dehydrated with 70%, 80%, and 95% alcohol, respectively, with 2 min for each. 6) Next, the sections were washed with anhydrous ethanol twice (with 5 min for each time), followed by washing with xylene twice (with 10 min for each time). 7) Finally, the sections were mounted with DPX.

Statistical Analysis

Statistical Product and Service Solutions 22.0 software (IBM, Armonk, NY, USA) was used for all statistical analysis. Measurement data were expressed as mean ± standard deviation. The *t*-test was used to compare the difference between the two groups. $p<0.05$ was considered statistically significant.

Results

MRNA Expression of the NogoA/NgR1/RhoA Signaling Pathway in Brain Tissues of Rats in Each Group

According to RT-PCR results (Figure 1), the mRNA expressions of NogoA, NgR1, and RhoA in brain tissues of rats in CI group were signifi-

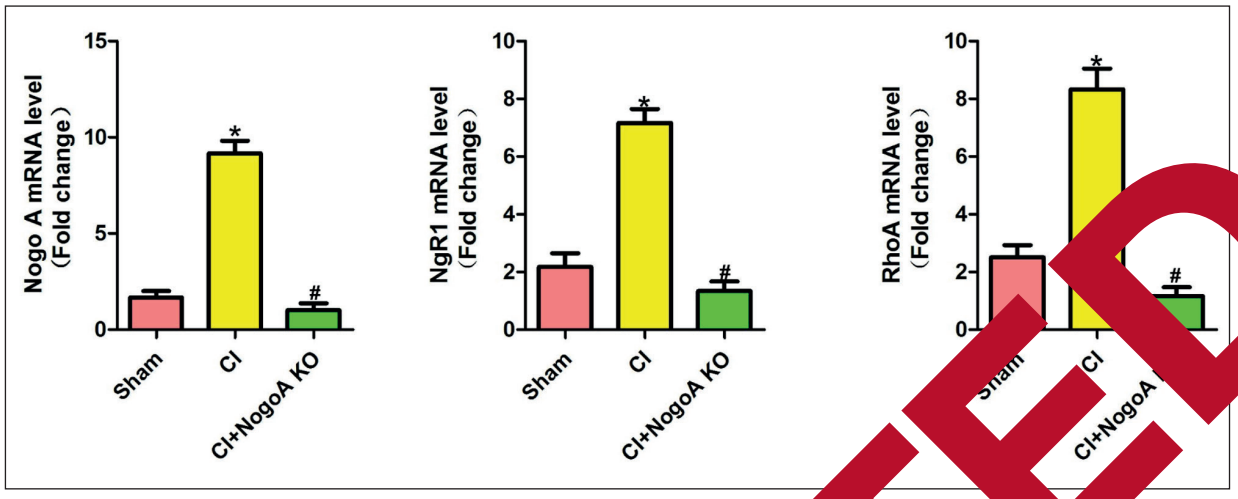

Figure 1. MRNA expression level of the NogoA/NgR1/RhoA signaling pathwa[y] [i]ssues of rat[s] [g]roup. Sham operation group (Sham group), CI group and CI + NogoA gene KO group (CI [N]ogoA [g]roup). #$p<0.$[05] [v]s. Sham group, showing a statistically significant difference.

cantly up-regulated when compared with Sham group ($p<0.05$). After NogoA gene was knocked out in rats, the mRNA expression levels of NogoA, NgR1, and RhoA in brain tissues were remarkably inhibited ($p<0.05$). This indicate[d] [t]he successful induction of the NogoA KO m[odel] rats.

Protein Expression of the NogoA/NgR[1]/RhoA Signaling Pathway [in] Tissues of Rats in Each [G]roup

Western blotting resu[lts] revealed [that] compared with Sham gro[up] [the] prot[ein] levels of NogoA, N[gR1], and [RhoA] in rats of CI group were marke[dly] elevated ([$p<0.]05), whereas were signifi[cantly] duced in CI [+] NogoA KO group ($p<0.05$). (Figure [4]). The above results were consisten[t] with the findi[ngs] at the mRNA level. After t[he] occurrence of CI, [the] expression levels of N[ogoA], NgR[1], and RhoA in the infarction area of b[rain] tiss[ues] of rats increased significantly.

Effect of NogoA KO on CI Area in Experimental Rats

After TTC staining, gray-white CI area appeared brain tissues in the non-infarction area showed red. TTC staining results (Figure [2]) indicated that no evident infarction area was observed in brain tissues of rats in Sham group. However, clear CI appeared in CI group. Meanwhile, certain infarction area appeared in brain tissues of rats in CI + NogoA KO group, which was markedly smaller than that of CI group ($p<0.05$). The above results indicated that inhibit[ing] the NogoA/NgR1/RhoA signaling pathway c[ould] alleviate [CI] caused by middle cerebral artery [is]chemia to [some] extent.

H&E [Staining] Results of Brain Tissues of Rats in Each Group

[Co]mpared with CI group, rats in Sham group [and] NogoA KO group showed the relatively complete structure of the hippocampus, as well as light and uniform cytoplasm. However, the structural integrity of neurons in brain tissues of rats in CI group disappeared. Furthermore, evident pathological changes appeared, such as edema, nuclear shift, and cell necrosis (Figure 4).

Nissl Staining Results in CI Area of Rats in Each Group

As shown in Figure 5, Nissl's body in brain tissues of rats in each group was stained. The results found that the number of Nissl's body in the three groups of rats was (62.33±2.93) *vs.* (18.39±1.93) *vs.* (55.34±2.91), respectively, showing statistically significant differences ($p<0.05$). This suggested that inhibiting NogoA/NgR1/RhoA signals could effectively suppress CI-induced reduction of Nissl's body.

TUNEL Staining Results of Neurons in the Hippocampus of Rats in Each Group

TUNEL staining (Figure 6) illustrated that the positive rates of TUNEL were (2.49±1.23)%, (639.51±3.66)% and (20.67±2.96)% in the three groups, respectively ($p<0.05$). Moreover, Western

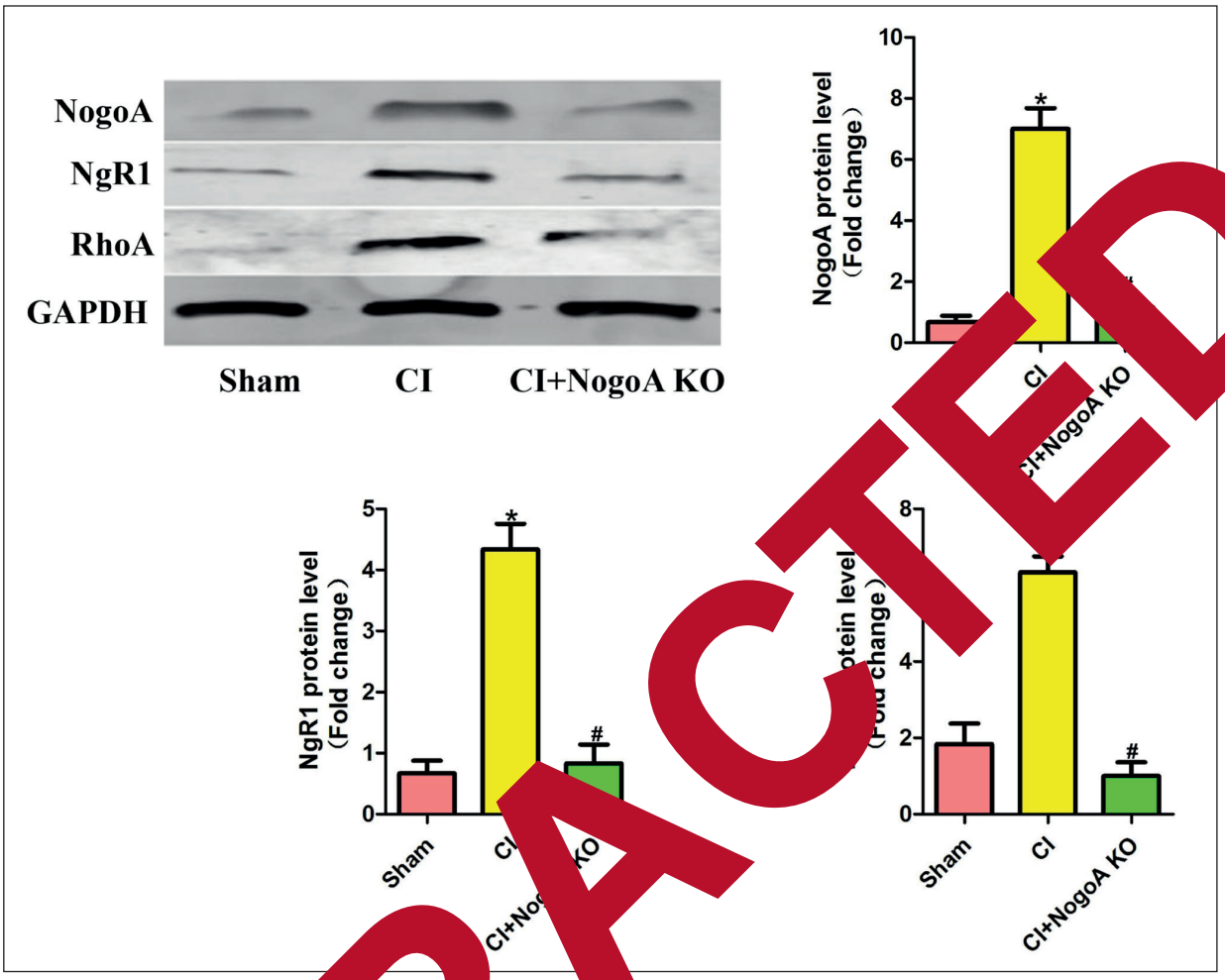

Figure 2. Protein expression l[illegible] of the No[illegible]/NgR1/Rho[illegible] signaling pathway in brain tissues of rats in each group. Sham operation group (Sham gro[illegible]), [illegible] gene KO group (CI+ NogoA KO group). #$p<0.05$ *vs.* Sham group, displaying a statistically [illegible]ican[illegible]e.

blotting dem[illegible]trated [illegible] the ratio of Bax/Bcl-2 in brain t[illegible]es of rats in [illegible] NogoA KO group was s[illegible]icantly lower th[illegible]at of CI group ($p<$[illegible]). The [illegible]e results indicated that NogoA KO [illegible]ific[illegible] reduced the apoptosis of neurons in the [illegible]ampus [illegible]s with CI.

Effect of [illegible] KO on the Expressi[illegible] of PDGF in Brain [illegible]Rats

Ultimately, the expression level of PDGF in [illegible]ain tissues of rats in each group was detected [illegible]mmunohistochemistry. The results manifested that, after the occurrence of CI, the expression of PDGF in brain tissues of the infarction area was significantly suppressed. However, NogoA KO could further up-regulate the level of PDGF ($p<0.05$) (Figure 7).

Discussion

At present, CI is one of the most common cerebrovascular diseases worldwide[9]. Statistics have reported that the morbidity and mortality rates of CI in European and American countries are increasing year by year, especially in the United States[10]. The occurrence and development of CI are closely correlated with genetic alterations. With the development of modern molecular biology and bioinformatics, an increasing number of genes, RNAs, and proteins have been proved to be involved in the occurrence and development of CI[11]. Current researches have indicated that the apoptosis of neurons exerts an important effect on cerebral ischemia-reperfusion injury. Meanwhile, the apoptosis level determines the de-

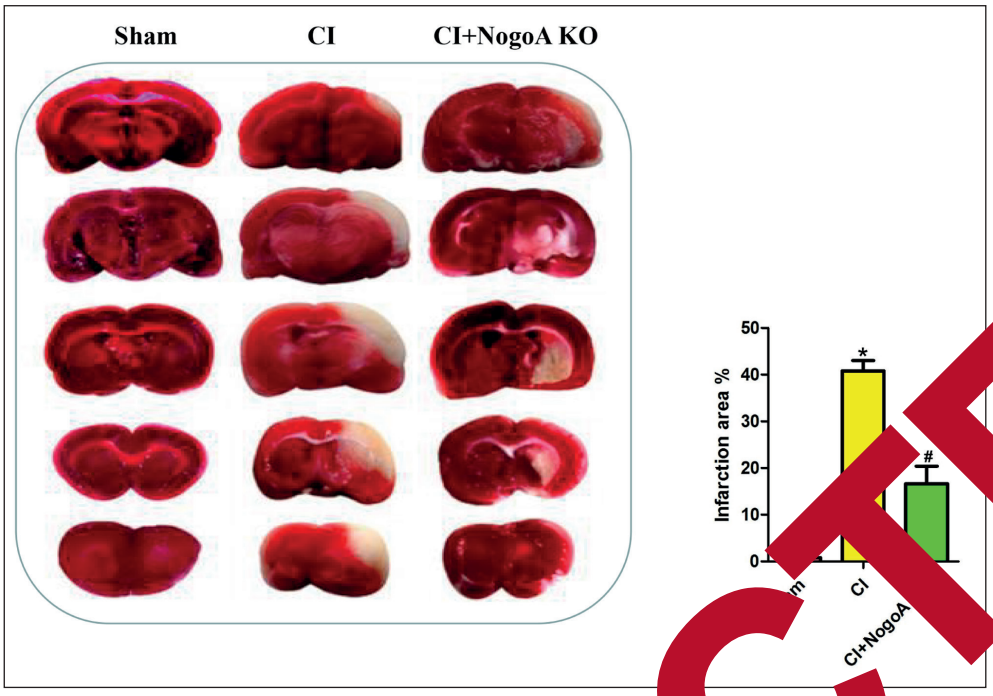

Figure 3. Effect of NogoA KO on CI area in experimental rats. Sham operation group (Sham group), CI group and CI + NogoA gene KO group (CI+ NogoA KO group). $^{\#}p<0.05$ *vs.* Sham group, displaying a statistically significant difference.

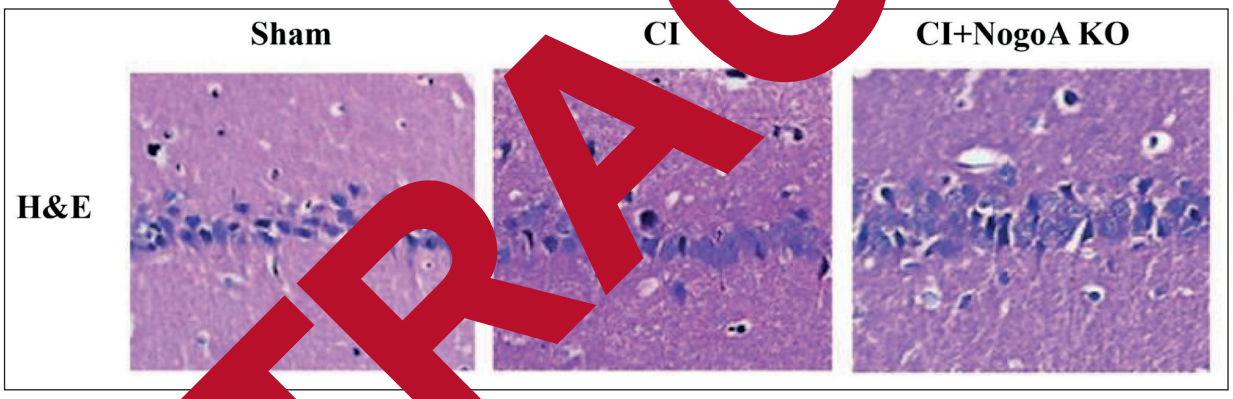

Figure 4. H&E staining results of brain tissues of rats in each group (200×). Sham operation group (Sham group), CI group and CI + NogoA gene KO group (CI+ NogoA KO group).

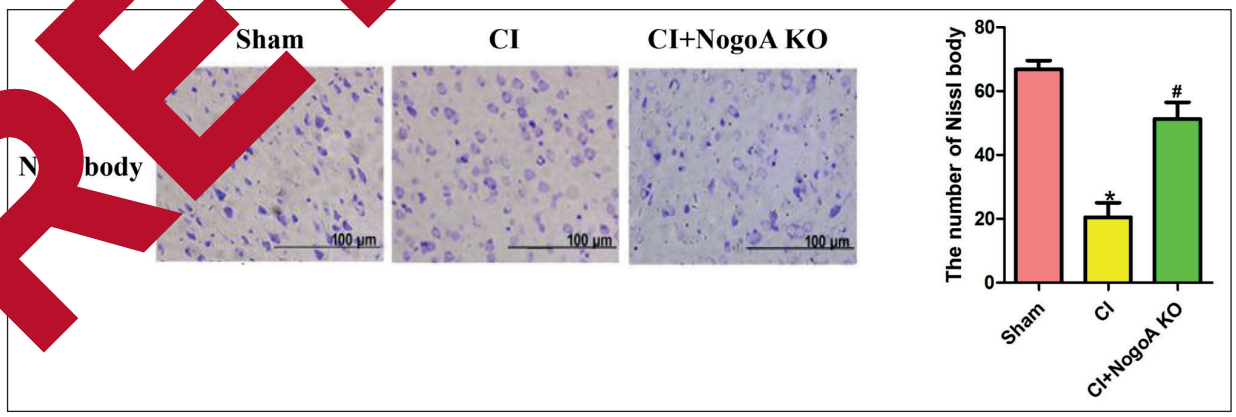

Figure 5. Nissl staining results in CI area of rats in each group (200×). Sham operation group (Sham group), CI group and CI + NogoA gene KO group (CI+ NogoA KO group). $^{\#}p<0.05$ *vs.* Sham group, with a statistically significant difference.

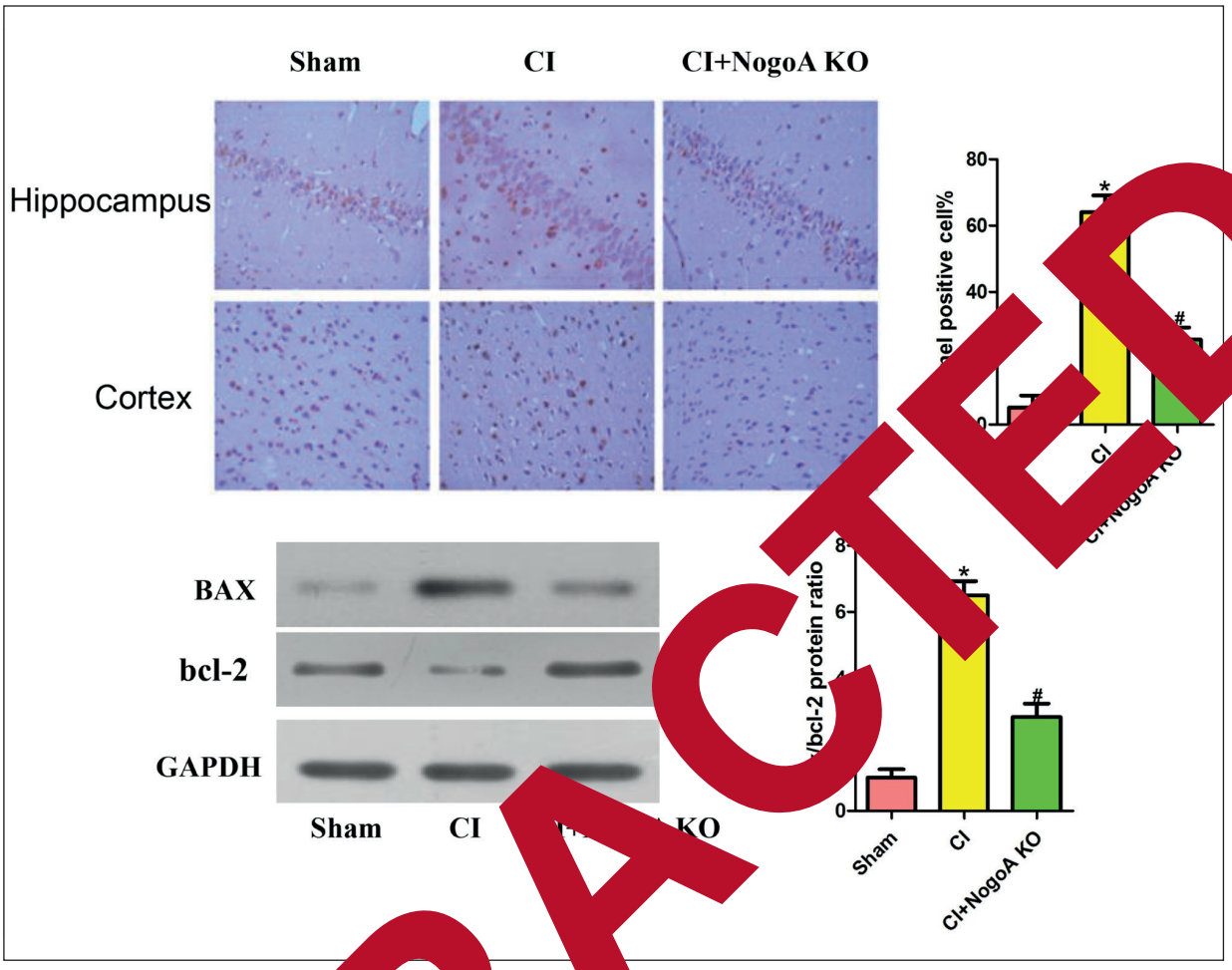

Figure 6. TUNEL staining res[illegible] neuron[illegible] the hippoc[illegible]pus of rats in each group (200×). Sham operation group (Sham group), CI group and CI + Nog[illegible]e KO [illegible] NogoA KO group). $^{\#}p<0.05$ *vs.* Sham group, with a statistically significant difference.

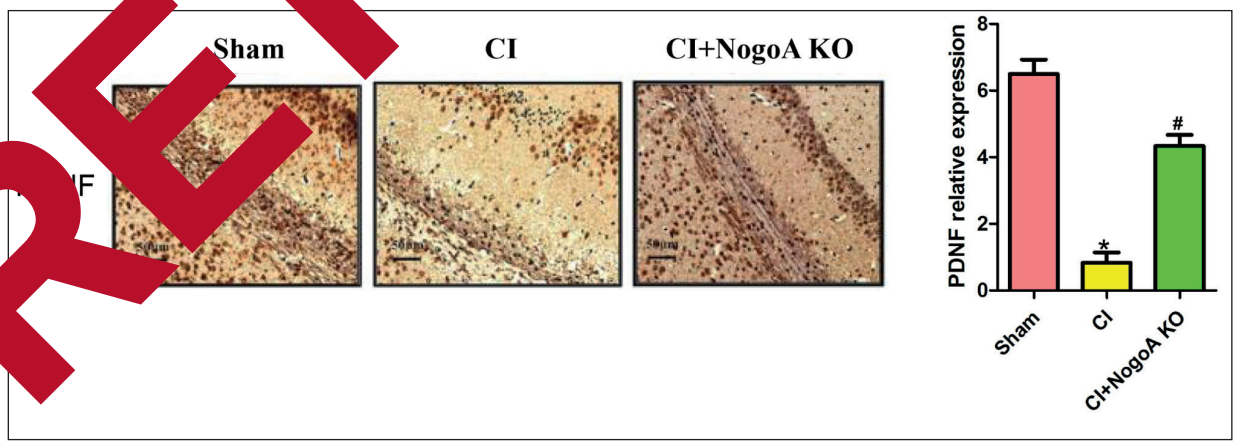

Figure 7. Effect of NogoA KO on the expression of PDGF in brain tissues of rats (400×). Sham operation group (Sham group), CI group and CI + NogoA gene KO group (CI+ NogoA KO group). $^{\#}p<0.05$ *vs.* Sham group, showing a statistically significant difference.

gree and prognosis of disease in a direct way[12]. In this study, the middle cerebral artery of SPF Wistar rats was embolized by a bolt from the carotid artery. This simulated the clinical onset process of CI much better than before. Meanwhile, NogoA was knocked out in rats, revealing the crucial role of NogoA in the occurrence and development of CI.

Apoptosis refers to programmed cell death controlled by genes under physiological or pathological conditions in order to maintain homeostasis[13,14]. In the process of CI, varying apoptosis-inducing signals are activated, thereby triggering edema, apoptosis or necrosis of neurons in the infarction area. In particular, during the first 6 h after CI, neuronal death is mainly manifested as necrosis. Subsequently, necrosis can be replaced by apoptosis. Hence, samples were collected at 48 h after CI in the present work. Meanwhile, the effect of NogoA KO on neuronal apoptosis in rats was further explored[15,16]. In the process of apoptosis, predominant expression levels of Bax and Bcl-2 determine the fate of cells in a direct way. Programmed cell death occurs when the expression of pro-apoptotic gene Bax dominates. H... ever, apoptosis is inhibited when the exp... of Bcl-2 is up-regulated[17,18]. In this study, N... A KO notably increased the level of anti-apop... gene Bcl-2 and inhibited the level of pro-ap... tosis gene Bax, thereby suppr... resulti... from ischemia and hypoxia.

Previous studies hav... orted ... NogoA shows a predominant ... xp... n ... campus and cerebr... rtex ... mals. Moreover, it exerts a ... ffect on t... pair of nervous system i... ell. Zemm... al[5] have demonstrated ... hat N... expressed in adult hippocam... exhibits a ... ue role in rapidly limiti... hysiological syn... plasticity[19]. In addi..., Dele... et al[20] have found that NogoA can ... tio... an important negative regulator for fu... and st... ural plasticity in mature ...onal ... ork.

Conclusions

...ur findings demonstrate for the first time that the NogoA/NgR1/RhoA signaling pathway plays a crucial role in CI in rats. Furthermore, inhibiting the NogoA expression could effectively alleviate CI-induced brain injury and the apoptosis of neurons in rats.

Conflict of Interests

The authors declare that they have no conflict of interest.

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