Association between p75 neurotrophin receptor gene expression and cell apoptosis in tissues surrounding hematomas in rat models of intracerebral hemorrhage

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Abstract
Animal models of intracerebral hemorrhage were established by injection of autologous blood into the caudate nucleus in rats. Cell apoptosis was measured by flow cytometry and immunohistochemical staining of the p75 neurotrophin receptor. p75 neurotrophin receptor protein was detected by immunohistochemistry. p75 neurotrophin receptor mRNA was examined by quantitative real-time polymerase chain reactions. At 24 hours after modeling, cellular apoptosis occurred around hematoma with upregulation of p75 neurotrophin receptor protein and mRNA was observed, which directly correlated to apoptosis. This observation indicated that p75 neurotrophin receptor upregulation was associated with cell apoptosis around hematomas after intracerebral hemorrhage.

Key Words: intracerebral hemorrhage; apoptosis; p75 neurotrophin receptor; neural regeneration

INTRODUCTION

Intracerebral hemorrhage (ICH), a type of common stroke in daily life, often results in intracerebral hematomas, in which a space occupying effect, including direct damage by the hematoma, leads to acute injury to brain tissues[1-5]. However, secondary injuries occur thereafter, which exacerbates damage and prolongs the pathological condition, in which cell apoptosis plays an important role[6]. Although many studies of cell apoptosis in the surrounding tissues of ICH have been performed, the molecular mechanism of apoptosis following ICH has not been completely clarified[6-7]. Recent studies show that p75 neurotrophin receptor (p75<sub>NTR</sub>), a member of the tumor necrosis factor receptor superfamily, is widely expressed in various cells in the central nervous system. It also plays a pivotal role in nerve cell survival, differentiation and apoptosis[6-10]. Although it has been shown that nerve growth factor precursor-induced p75<sub>NTR</sub>-mediated expression is involved in promoting cell apoptosis[11-12], few relevant reports are available on the p75<sub>NTR</sub>-mediated apoptosis pathway in ICH. In this study, we show the relationship between p75<sub>NTR</sub> expression and cell apoptosis in tissues surrounding the hematomas of ICH in rats.

RESULTS

Quantitative analysis of experimental animals
A total of 62 Wistar rats were used in this study, which were randomly assigned into two groups, sham (n = 10) and vehicle (n = 52). The vehicle group was divided into four subgroups based on various time points including 6, 24, 72 hours, and 10 days (n = 13). During modeling, 12 rats were excluded from the vehicle group (three rats in each subgroup) because of unsuccessful surgery (supplementary Figure 1 online), which resulted in 50 rats in this study.

Cell apoptosis in tissue surrounding hematomas after ICH
Apoptosis was observed after ICH at various time points. At 6 hours after ICH, apoptosis was 13.34 ± 2.51% and increased by 60% compared with that of the sham surgery group with 8.37 ± 3.13% apoptosis (Student’s t-test; P = 0.001). At 24, 72 hours, and 10 days after ICH, rats showed significant elevation in cell apoptosis rates at 20.50 ± 2.14%, 37.76 ± 4.05%, and 30.03 ± 3.69%, respectively (one-way analysis of variance, Student-Newman-Keuls-q; P < 0.001). From 6 to 72 hours after ICH, the
percentage of apoptosis gradually increased and then decreased gradually up to 10 days after ICH, but remained at high levels (Figure 1).

**P75<sup>NTR</sup> protein expression in tissue surrounding hematomas after ICH**

Hematoxylin-eosin (HE) and immunohistochemical staining demonstrated that P75<sup>NTR</sup> protein expression was elevated at various time points in ICH groups (31.31 ± 3.65, 44.19 ± 6.53, 55.60 ± 6.42 and 46.15 ± 6.60) compared with that of the sham surgery group (18.55 ± 2.41; one-way analysis of variance, Student-Newman-Keuls-; P < 0.01). P75<sup>NTR</sup> expression reached a maximum at 72 hours after ICH (analysis of variance, Student-Newman-Keuls-; P < 0.01) (Figure 2).

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![Figure 1](image1.png)

**Figure 1** Percentage of apoptosis at 6, 24, 72 hours, and 10 days after intracerebral hemorrhage, respectively as determined by flow cytometry. The apoptosis rate is presented in the tables in the lower right quadrants. FITC: Fluorescein isothiocyanate; PI: propidium iodide.

(A–D) Left lower quadrant, annexin V-FITC/PI is the live cell population; lower right quadrant, annexin V-FITC+/PI is the apoptotic cell population; upper right quadrant, annexin V-FITC+/PI+ is the necrotic cell population; left upper quadrant, annexin V-FITC/PI+ is cell debris.

![Figure 2](image2.png)

**Figure 2** Hematoxylin-eosin (HE) and immunohistochemical staining of brain tissue surrounding hematomas after intracerebral hemorrhage (ICH).

(A) The yellow arrow indicates neuronal apoptosis as shown by darkly stained cytoplasm and chromatin margination around the hematoma. The blue arrow shows the infiltration of neutrophils, suggesting that inflammation occurs around the hematoma after ICH (HE staining, × 400).

(B) After ICH, brain tissue edema around the hematoma occurs as indicated by light staining and cell numbers decrease close to the hematoma, (HE staining, × 200).

(C) Perivascular edema, suggesting vascular edema after ICH (HE staining, × 400).

(D) P75 neurotrophin receptor is mainly expressed in the cytoplasm of neurons in the control (HE staining, × 400).

(E–H) Immunohistochemical staining at 6, 24, 72 hours, and 10 days after ICH, respectively (× 400).
p75<sup>NTR</sup> gene expression as determined by quantitative real-time PCR (qRT-PCR)
p75<sup>NTR</sup> mRNA expression was elevated at 6, 24 and 72 hours after ICH (1.17 ± 0.29, P = 0.03; 2.36 ± 0.70, P < 0.001; and 5.04 ± 1.52, one-way analysis of variance, Student-Newman-Keuls-q, P < 0.001, respectively) with significant differences compared with that of the sham surgery group (0.83 ± 0.35). However, there was no significant difference between 72 hours and 10 days (4.39 ± 1.50) after ICH (P > 0.05). Although at 10 days after ICH, p75<sup>NTR</sup> mRNA expression remained significantly higher than that of the sham surgery group (Student-Newman-Keuls test, P < 0.001).

Relationship between p75<sup>NTR</sup> mRNA and cell apoptosis
To explore the relationship between p75<sup>NTR</sup> mRNA and cell apoptosis, each ICH group was compared with the sham surgery group. A correlation analysis of the p75<sup>NTR</sup> mRNA level and apoptosis ratio was performed, which revealed a positive correlation between p75<sup>NTR</sup> mRNA and the apoptosis ratio except at 6 hours after ICH (P = 0.110; Table 1). The linear regression equation was invalid at 6 hours after ICH (P = 0.110), but valid at 24, 72 hours, and 10 days (P = 0.001, P < 0.001, and P < 0.001, respectively).

The regression coefficient (b) at 24 and 72 hours after ICH is the highest.

![Table 1 Parameters and coefficients of correlation between p75 neurotrophin receptor (p75<sup>NTR</sup>) mRNA and cellular apoptosis](https://example.com/table1.png)

**DISCUSSION**

Recent studies show that p75<sup>NTR</sup> is widely expressed in various cells in the central nervous system. It plays a pivotal role in nerve cell survival, differentiation and apoptosis<sup>[6-10]</sup>. Our previous study indicated that p75<sup>NTR</sup> expression increased after ICH, with a positive correlation to apoptosis<sup>[6,7,13]</sup>. Because our previous study was carried out with clinical patient specimens, the experimental group was not investigated at various time points after ICH due to ethical reasons, resulting in no observations of p75<sup>NTR</sup> expression and its time-dependent alterations after ICH. Thus, it was necessary to use an animal model to explore time-dependent alterations of p75<sup>NTR</sup> after ICH.

In this study, we established animal models of ICH via injection of autologous blood into the rat caudate nucleus to explore brain tissue apoptosis and p75<sup>NTR</sup> expression around the hematoma at various time points. We found that apoptosis began to increase at 6 hours after ICH and as time progressed, the proportion of cell apoptosis gradually elevated and reached a maximum at 72 hours after ICH. A high proportion of cell apoptosis can still be observed at 10 days after ICH. The apoptosis rates are consistent with those of previous studies<sup>[14-18]</sup>. Furthermore, using real-time PCR and immunohistochemistry, we evaluated p75<sup>NTR</sup> at both mRNA and protein levels at various time points of ICH. Compared to the sham surgery group, p75<sup>NTR</sup> mRNA and protein expression increases to a peak value at 72 hours after ICH. p75<sup>NTR</sup> mRNA levels positively correlate to apoptosis, suggesting that p75<sup>NTR</sup> plays an important role in apoptosis after ICH. We also found evidence of secondary brain injury caused by other factors such as nerve cell edema, swelled cell bodies and neutrophilic leukocyte infiltration around hematomas, which indicated an inflammatory response is involved in secondary brain injury. We also found that the level of p75<sup>NTR</sup> expression positively correlates to apoptosis. At various time points, the correlation between p75<sup>NTR</sup> expression and cell apoptosis also varies. Although the apoptosis rate and p75<sup>NTR</sup> expression increase at 6 hours after ICH compared with that of the sham surgery group, the p75<sup>NTR</sup> expression and apoptosis show no linear correlation, suggesting several mechanisms are involved in signal transduction that mediates cell apoptosis in the earliest periods of ICH. At 24 hours after ICH, the association of the p75<sup>NTR</sup> mRNA level and apoptosis is lower than that at 10 days after ICH, inferring there are other mechanisms that contribute to apoptosis at this time point. The apoptosis rate at 24 hours after ICH resulting from increased p75<sup>NTR</sup> expression is higher than that at 10 days after ICH. p75<sup>NTR</sup>-mediated apoptosis reaches a maximum at 72 hours after ICH and is a signaling pathway that mediates apoptosis. However, p75<sup>NTR</sup>-mediated apoptosis decreases at 10 days after ICH.

Based on recent reports and our findings, neurotrophin receptor homolog 2 (NRH2), as another cognate receptor of p75<sup>NTR</sup>, plays an important role during p75<sup>NTR</sup>-mediated nerve growth factor precursor-induced apoptosis signaling<sup>[16-22]</sup>. NRH2 directs apoptosis signaling by regulation of sortilin expression on the cell membrane<sup>[16-19]</sup>, which is a co-receptor of p75<sup>NTR</sup>-mediated apoptosis<sup>[20-22]</sup>. NRH2 possesses a similar DNA sequence to that of the p75<sup>NTR</sup> mRNA. NRH2 has the same transmembrane and intramembrane domains as p75<sup>NTR</sup>, whereas the extramembrane domain of NRH2 is shorter than p75<sup>NTR</sup>. Moreover, NRH2 has no Cys conserved sequences, resulting in an inability to bind to NGF. However, the lack of Cys conserved sequences enhances the binding ability of NGF to TrkA, which indicates that the enhanced binding might be
mediated via its transmembrane domain. In this study, p75<sup>NTR</sup> expression is elevated at 6 hours after ICH compared with that of the sham surgery group, but shows no linear correlation with apoptosis. At 24 hours after ICH, p75<sup>NTR</sup>-mediated apoptosis gradually increases and peaks at 72 hours after ICH. The reason may be that upregulated NRH2 promotes sortilin expression in the membrane to assist p75<sup>NTR</sup>, leading to enhanced apoptosis at 24 hours after ICH. NRH2 and sortilin expression at various time points after ICH needs to be studied further.

Regarding the limitations of this study, we cannot elucidate the exact mechanism that changes p75<sup>NTR</sup> expression at various time points after ICH. Based on studies of apoptosis after ICH, we theorize that the possible mechanisms that trigger p75<sup>NTR</sup>-mediated cell apoptosis are as follows: (1) mast cell-mediated hypersensitivity may be involved in brain injury in the earliest stage of ICH<sup>[23]</sup>; (2) hematomas during early stages of ICH cause increased expression of FasL, thereby initiating Fas/FasL apoptotic signaling pathways<sup>[24]</sup>, and (3) once a higher level of thrombin around hematomas contacts brain tissue, apoptosis is triggered via binding to the thrombin receptor on nerve cells and vascular endothelial cells. Moreover, thrombin-induced neuronal apoptosis signaling pathways may be independent of p75<sup>NTR</sup>, but dependent on reduced cyclin G and cyclin-dependent kinase<sup>[25]</sup>. (4) Reduced local blood flow in brain tissues after ICH results in increased free radicals that activate Ca<sup>2+</sup>/Mg<sup>2+</sup>-dependent endonuclease to trigger apoptosis. In summary, complex pathophysiological mechanisms contribute to brain damage from ICH. Importantly, in the earliest stages of ICH, many factors may be involved in brain damage<sup>[26]</sup>. The mechanism of p75<sup>NTR</sup>-mediated apoptosis after ICH should be investigated further.

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**MATERIALS AND METHODS**

**Design**
A controlled observational cell study.

**Time and setting**
This study was performed at the Laboratory of Histology and Embryology, Xi’an Jiaotong University, China from December 2009 to June 2010.

**Materials**
A total of 62 randomly selected male and female Wistar rats (6-8-weeks-old), weighing 230–250 g, were used for ICH studies. Animals were purchased from the Center for Experimental Animals, Xi’an Jiaotong University, and received humane care in compliance with the guidelines of the First Hospital, Xi’an Jiaotong University and Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China<sup>[27]</sup>.

**Methods**

**Establishment of ICH model and grouping**
Rats were anesthetized, and a midline incision was made over the scalp to expose the skull and bregma. A posterior cranial burr hole (1 mm) was drilled into the right cerebral hemisphere at 2 mm anterior and 3 mm lateral to the bregma (coordinates of the rat right caudate nucleus)<sup>[28]</sup>. With a 27 G needle inserted and advanced 6 mm into the brain, 80 μL autologous blood was injected into the right caudate nucleus at 20 μL/min. The needle remained in place for an additional 10 minutes after injection to prevent back-leakage. After needle removal, the burr hole was sealed with bone wax, the incision was sutured closed, and the animal was allowed to recover<sup>[29]</sup>. Sham surgeries consisted of needle insertion only.

**Specimen collection and pretreatment**
After injection of autologous blood, rats were sacrificed at 6, 24, 72 hours, and 10 days after injury. Control rats were sacrificed at 2 hours after injection. Brains were coronally cut approximately 5 mm on either side of the needle entry site. Brain samples obtained at each time point were divided into trisections according to weight. The first portion was for immunocytochemical analysis and fixed in 4% formaldehyde in phosphate-buffered saline (PBS) at 4°C. The second portion was for flow cytometry and placed in PBS in an ice bath at 4°C. The third portion was for PCR analysis and after decapitation, was immediately placed in enzyme-free cryogenic vials and stored in liquid nitrogen.

**Detection of cell apoptosis in tissue surrounding hematomas after ICH by flow cytometry**
Brain tissues were washed with cold PBS at 4°C and minced using sterile surgical scalpels. Dissected brains were triturated with glass pipettes. Tissues were then forced through a 300 μm nylon mesh to achieve a single cell suspension that was collected and centrifuged at a low temperature at 178 × g for 5 minutes. The supernatant was discarded. Cells were resuspended in cold PBS at 4°C.

Then, the cell suspension at 1–5 × 10<sup>5</sup> cells/mm<sup>3</sup> was centrifuged at low temperature at 178 × g for 5 minutes. The supernatant was discarded. Cells were placed in 500 μL 1× binding buffer with 5 μL annexin V-FITC (Jiankangyuan biological Ltd., Zhuhai, China) and 10 μL propidium iodide (Biosynthesis Biotechnology, Beijing, China), gently mixed at room temperature and then incubated for 15 minutes while protected from light. Stained cells were then analyzed by a FACSCalibur flow cytometer (Becton-Dickerson, NJ, USA). Unstained cells were used as a control. Apoptotic cells were recognized as annexin V-FITC-positive and PI-negative. Results were expressed as the ratio of apoptotic to live cells, with 10 000 events collected.

**HE staining and immunohistochemical staining of p75<sup>NTR</sup>**
Paraffin sections were stained with hematoxylin for 4 minutes, rinsed three times in running water for 2 minutes each rinse, treated with a 1% solution of hydrochloric acid alcohol for 30 seconds and then rinsed as described above. Then, sections were stained with eosin for 10 minutes, rinsed as described, followed by...
graded ethanol dehydration, xylene I and II permeabilization and then mounted with neutral gum. Immunohistochemical procedures were performed according to the protocol for the p75<sup>NTR</sup> immunohistochemistry kit (Promega, Madison, WI, USA). Rat brain sections were dewaxed and rehydrated, rinsed with distilled water and PBS, quenched with 3% H<sub>2</sub>O<sub>2</sub> for 10 minutes at 37°C, blocked with 10% normal goat serum for 3 minutes at 37°C and incubated in goat anti-rat-p75<sup>NTR</sup> polyclonal antibody (1:150; Boaoeng, Beijing, China) at 4°C overnight. Sections were then incubated in biotinylated rabbit anti-goat IgG (1:150; Boaoeng, Beijing, China) for 1 hour at room temperature, washed, incubated in streptavidin peroxidase for 25 minutes at 37°C, colorized with a diaminobenzidine-H<sub>2</sub>O<sub>2</sub> solution, washed, dehydrated in graded ethanol, immersed in xylene and then coverslipped. Control sections were processed with omission of the primary antibody. Three randomly selected × 400 visual fields of each section were examined under a light microscope (Leica, Wetzlar, Germany). The ratio of p75<sup>NTR</sup>-positive cells was calculated as the number of p75<sup>NTR</sup>-positive cells to the total cell number in one field. Results were expressed as the average positive ratio of three visual fields.

**Detection of p75<sup>NTR</sup> mRNA expression by real-time PCR**

P75<sup>NTR</sup> and internal reference β-actin primers were synthesized by Beijing Sanbo Yuanzhi Biotechnology Co., Ltd. P75<sup>NTR</sup> primers sequences were: P<sub>1</sub>: 5'-CCT CAT TCC TGT CTA TTG CTC CAT C-3' and P<sub>2</sub>: 5'-TTC CTC ACC TCC TCA CGC TTG G-3'.

Total RNA was isolated from rat brain using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. PCR reactions were performed in 96 well plates with an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, CA, USA) using SYBR Green to monitor amplification. Reactions volumes were 25 μL containing 200 nM each primer, 5 μL cDNA (3 ng) and 10 μL 2× SYBR Green Master Mix Reagent (Applied Biosystems). PCR was performed at 93°C for 2 minutes, followed by 40 cycles of 93°C for 1 minute and 55°C for 1 minute. PCR reactions were performed in triplicate. Real-time PCR data were analyzed with the manufacturer’s software (Invitrogen). Results were expressed as p75<sup>NTR</sup> mRNA relative expression to β-actin.

**Statistical analysis**

Measurement data were represented with mean ± SD. All data met the conditions of linear regression and were analyzed using SPSS 16.0 (SPSS, Chicago, IL, USA). The Kolmogorov-Smirnov method was used for the data normality test. Student’s t-test was used to compare differences between groups. One-way analysis of variance was used for comparisons among groups with the Student-Newman-Keuls test. The relevance of apoptosis and tested data was evaluated by Pearson correlation analysis. A P ≤ 0.05 was considered significant.

**Author contributions:** Baixiang He provided data and wrote the first manuscript. Gang Bao designed the study and was responsible for obtaining funding. Shiwen Guo provided suggestions for performing this study and reviewed the manuscript. Gaofeng Xu provided a part of the data and integrated the data. Qi Li and Ning Wang statistically analyzed all data. Qi Li also wrote the first manuscript.

**Conflicts of interest:** None declared.

**Ethical approval:** This study was approved by the Animal Ethics Committee of the First Affiliated Hospital of Xi’an Jiaotong University, China.

**Supplementary information:** Supplementary data associated with this article can be found, in the online version, by visiting www.nrronline.org, and entering Vol. 7 No. 8, 2012 after selecting the “NRR Current Issue” button on the page.

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