Oxygen-glucose deprivation of neurons transfected with toll-like receptor 3-siRNA

Determination of an optimal transfection sequence

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Abstract

Toll-like receptor 3 protein expression has been shown to be upregulated during cerebral ischemia/reperfusion injury in rats. In this study, rat primary cortical neurons were subjected to oxygen-glucose deprivation to simulate cerebral ischemia/reperfusion injury. Chemically synthesized small interfering RNA (siRNA)-1280, -1724 and -418 specific to toll-like receptor 3 were transfected into oxygen-glucose deprived cortical neurons to suppress the upregulation of toll-like receptor 3 protein expression. Western blotting demonstrated that after transfection with siRNA, toll-like ceptor 3 protein expression reduced, especially in the toll-like receptor 3-1724 group. These results suggested that siRNA-1724 is an optimal sequence for inhibiting toll-like receptor 3 expression in cortical neurons following oxygen-glucose deprivation.

Key Words

neural regeneration; brain injury; Toll-like receptor 3; small interfering RNA; primary neurons; ischemia/reperfusion injury; transfection; hypoxia; liposome; immune/inflammatory reactions; RNA interference; grants-supported paper; neuroregeneration

INTRODUCTION

Ischemia/reperfusion is a rapid cascade reaction, which includes recruitment of inflammatory cytokines, neuronal dysfunction, intracellular Ca^{2+} overload, oxygen free radical injury, and excitatory amino acid ty[1-5]. Numerous studies have verified that the inflammation at the ischemic site participates in cerebral ischemia/reperfusion injury[6-7]. Toll protein was originally identified in Drosophila[8]. Toll-like receptors received their name from their similarity to the protein coded by the Toll gene identified in Drosophila[9]. Toll-like receptor 3 (TLR3), an important member of the TLR family, recognizes double-stranded RNA (dsRNA) and polyriboinosinic polyribocytidylic acid[10], activates interferon regulatory factor 3 via TRIF, results in the synthesis and release of interferon-β, interleukin-1, interleukin-6, tumor necrosis factor-a and interferon-indu- cible protein-10, and finally mediates inflammatory reactions[11-12]. However, few reports have addressed whether TLR3 participates in rebral ischemia/reperfusion injury.

Our previous study showed that TLR3 tein expression is upregulated in brain tissues in a rat model of global brain ischemia induced by four-vessel occlusion to simulate cerebral ischemia/reperfusion injury *in vivo* [13]. However, whether TLR3 is involved in oxygen-glucose deprivation *in vivo* remains unclear.

RNA interference (RNAi) is a dsRNA-inducible, sequence-specific RNA-degradation mechanism[14]. The RNAi technique has rapidly become an extremely important tool for identifying drug targets and studying gene function[15]. Viral vector possibly has hidden danger and unknown immunization[16].

This study sought to establish a model of oxygen-glucose deprivation to simulate the pathophysiological process of ischemia/ reperfusion using *in vitro* cultured primary cortical neurons. The rat TLR3 gene was considered a possible drug target. Three pairs of chemically synthesized siRNA were transfected into rat cortical neurons cultured *in vitro* using Hiperfect Transfection Reagent. This study investigated the inhibitory effect of three pairs of siRNA on TLR3 gene expression, and screened an optimal sequence of the TLR3 gene so as to guide clinical studies on ischemic stroke.

RESULTS

Morphology of primary cortical neurons isolated from fetal rats

With an inverted phase contrast microscope, primary cortical neurons appeared round, and transparent. Some cells began to adhere to the wall several hours later, became thin and flat, were uniformly distributed, and had an apparent halo.

At 6–8 hours after culture, more cells adhered, and some of them had small neurites. At 24 hours, a majority of cells had many neurites, and cell bodies appeared elliptic, pyramidal or stellate, with good refraction (Figure 1A). At 4–7 days, neuronal bodies were plump, with strong refraction and noticeable halation. The neurites became more, thicker, and reticulated, with intact branches and clear background. Glial cells were suppressed by neuronal medium, so all cells had neurites (Figure 1B, C). At 7 days, neurons received oxygen-glucose deprivation. Thus, neuronal neurites and networks were broken. Cell masses were loose, and pyknosis, cell apoptosis and severe cell damage were observed (Figure 1D).
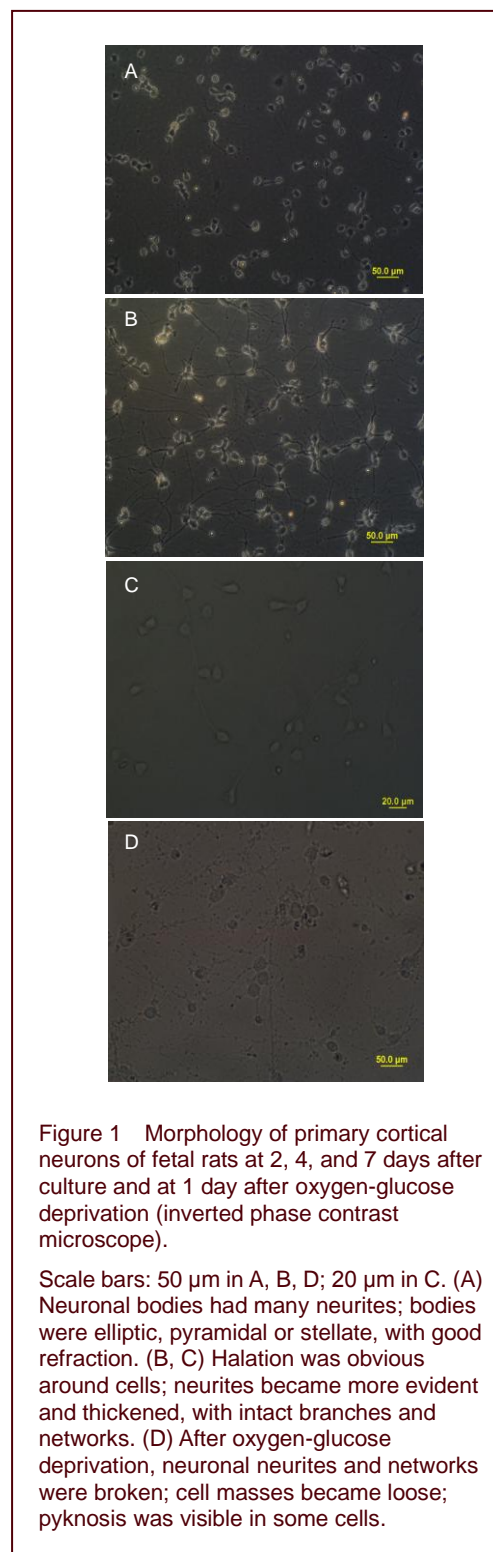
Figure 1　Morphology of primary cortical neurons of fetal rats at 2, 4, and 7 days after culture and at 1 day after oxygen-glucose deprivation (inverted phase contrast microscope).

Scale bars: 50 μm in A, B, D; 20 μm in C. (A) Neuronal bodies had many neurites; bodies were elliptic, pyramidal or stellate, with good refraction. (B, C) Halation was obvious around cells; neurites became more evident and thickened, with intact branches and networks. (D) After oxygen-glucose deprivation, neuronal neurites and networks were broken; cell masses became loose; pyknosis was visible in some cells.

Increased TLR3 expression in neurons following oxygen-glucose deprivation

Experiments were randomly assigned to control and oxygen-glucose deprivation groups. At 7 days after culture, the oxygen-glucose deprivation group was exposed to oxygen-glucose deprivation for 24 hours, and then reoxygenation for 2 hours.

Figure 2   Toll-like receptor 3 (TLR3) expression in neurons exposed to oxygen-glucose deprivation (OGD).

(A) Western blot assay of TLR3 protein. TLR3 protein expression was higher in the OGD group than that of the control. (B) Quantitative analysis of TLR3 protein. Data are represented as the absorbance ratio of TLR3/β-actin. [a]$P <$ 0.05, *vs.* control group. Data are expressed as the mean ± SD (two sample-*t*-test). The experiment was repeated six times.

Figure 3   Effects of different Toll-like receptor 3 (TLR3)-siRNA sequences on TLR3 protein expression in cortical neurons exposed to oxygen-glucose deprivation (OGD).

(A) TLR3 protein expression after transfection in cortical neurons exposed to OGD (western blot assay). TLR3 protein expression was higher in the OGD group than that in the negative control (NC) group.
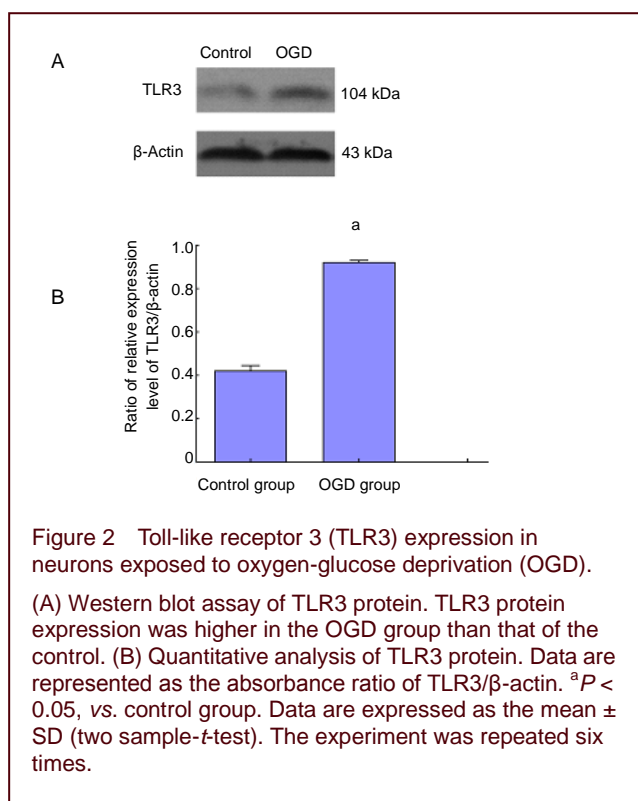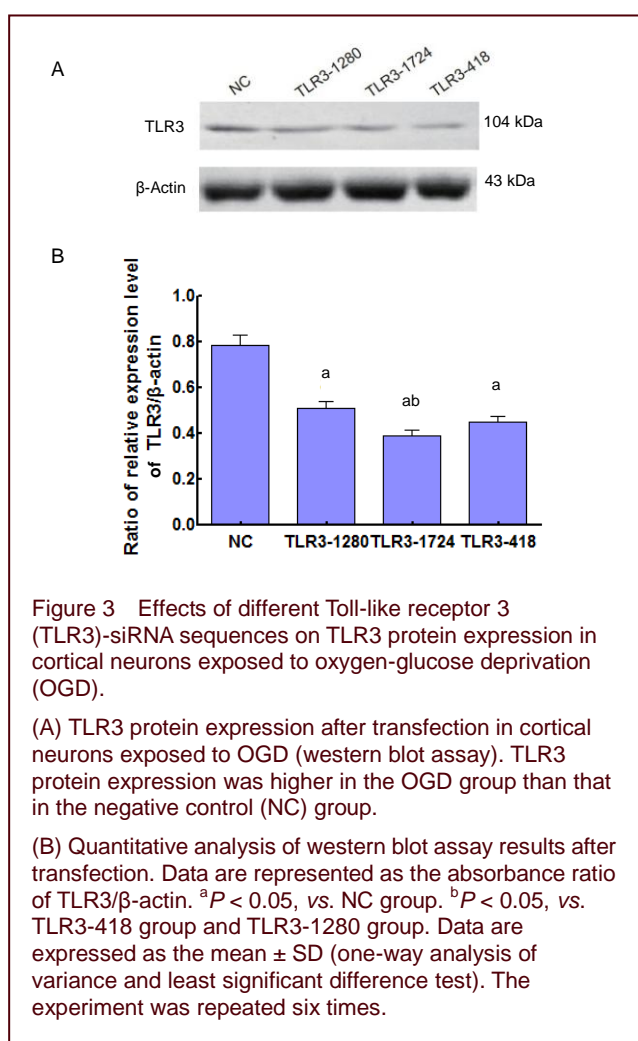
(B) Quantitative analysis of western blot assay results after transfection. Data are represented as the absorbance ratio of TLR3/β-actin. [a]$P < 0.05$, *vs.* NC group. [b]$P < 0.05$, *vs.* TLR3-418 group and TLR3-1280 group. Data are expressed as the mean ± SD (one-way analysis of variance and least significant difference test). The experiment was repeated six times.

At 8 days after culture, TLR3 protein expression was higher in the oxygen-glucose deprivation group than that in the control group ($P < 0.05$; Figure 2).

Effect of transfection of different TLR3-siRNA sequences on TLR3 protein expression in cortical neurons exposed to oxygen-glucose deprivation

At 5 days after culture, the experiment was randomly divided into four groups: a negative control group, a TLR3-1280 group, a TLR3-1724 group and a TLR3-418 group. That is, the TLR3-siRNA negative control sequence, TLR3-1280 sequence, TLR3-1724 sequence and TLR3-418 sequence were transfected into neurons. The detection was finished at 2 hours after reoxygenation (at 8 days after culture). Western blot demonstrated that TLR3 protein expression was lower in the TLR3-1280 group, TLR3-1724 group and TLR3-418 group when compared with the negative control group ($P <$ 0.05), suggesting inhibitory effects of TLR3-1280, TLR3-1724 and TLR3-418 sequences on TLR3 protein expression. TLR3 protein expression was lower in the TLR3-1724 group when compared with the TLR3-1280 group ($P < 0.05$). No significant difference was detected between TLR3-1724 and TLR3-418 groups, and TLR3-1280 and TLR3-418 groups (Figure 3).

DISCUSSION

TLRs belong to pattern recognition receptors, which can specifically recognize pathogen-associated molecular patterns of a pathogenic microorganism such as bacteria, viruses and protists[17], and induces an innate immune response against pathogens. The inflammatory reaction at the ischemic site was considered an essential factor for aggravating tissue damage[18]. The effects of the TLR family on ischemic injury have been confirmed by many studies concerning the heart[19], liver[20], kidney[21] and lung[22]. However, it remains poorly understood whether TLR participates in cerebral ischemic injury. Hua *et al* [23] confirmed that TLR4 expression was significantly increased following ischemia/reperfusion in a mouse model of global brain ischemia. Their following study verified that the death rate of mice subjected to global brain ischemia/reperfusion was markedly lower in the pretreatment group (pretreatment with the TLR2 agonist Pam3CSK4) when compared with the control group[24]. Moreover, infarct volume and brain edema area was reduced[24], which was consistent with another published study[25]. Stevens *et al* [26] showed that nerve injury was apparently improved in mice pretreated with a TLR9 agonist. Although the mechanisms remain unclear, these

studies indicate that the TLR family is involved in ischemic brain injury. Similarly, whether TLR3 participates in ischemic brain injury requires investigation. Results from this study demonstrated that TLR3 protein expression was 0.420 ± 0.027 in primary neurons cultured *in vitro* under normal conditions, but 0.920 ± 0.010 following oxygen-glucose deprivation, which was significantly higher than the control group. These results indicated that TLR3 participated in oxygen-glucose deprivation-derived neuronal injury. TLR3 are similar to TLR2, TLR4 and TLR9, which can be activated by endogenous substances[27]. Therefore, we assumed that dsRNA released from cells after death and disintegration was TLR3 ligand, which activated downstream signaling pathways, and induced the occurrence of the inflammatory reaction[28]. It remains controversial whether TLR3 is expressed in neurons. Lehnardt *et al* [29] believed that TLR3 was not expressed in neurons, while Peltier *et al*[30] verified that TLR3 was expressed in human neurons, and documented its effects on the immune response. Tang *et al* [31] confirmed that TLR2, TLR3 and TLR4 existed in cortical neurons using the double immunofluorescence labeling method. Prehaud *et al* [32] used the lyssa virus and HSV-1 to infect the human cells NT2-N, and found that TLR3 expression was upregulated. Moreover, Packard *et al* [33] verified TLR3 expression in brain tissues and neurons during cerebral ischemia using a mouse model of middle cerebral artery occlusion and primary cortical neurons, respectively. Results from the present study confirmed that TLR3 possibly participated in oxygen-glucose deprivation-derived neuronal injury *in vivo*, which was identical to a previous test[13].

Specifically suppressing TLR3 expression is a significant step to further study TLR3 function. RNAi is a post-transcriptional process triggered by the introduction of dsRNA, which leads to gene silencing in a sequence-specific manner at the mRNA level[34]. RNAi is rapidly becoming an important method for analyzing gene functions, due to its high stability and specificity, and has also been extensively applied in the study of mammals[35-37]. dsRNA can be harvested by chemosynthesis *in vitro*, or by constructing plasmid expressing siRNA *in vivo*, for inducing RNAi[35, 38-39]. Primary neurons during anaphase were considered as cells hard to transfect. It is technically difficult to transfect siRNA into primary neurons and to reach high transfection efficiency. At present, the transport system is divided into two types: viral transport and non-viral transport. High efficiency transfection of primary neurons commonly utilizes viral vectors and electroporation. To carry genetic material into cells is the instinct of viruses[40-41], however, the viral

vector may produce an intensive interferon-like immune reaction[42-43], which could induce unknown effects during studies of innate immunity. Moreover, the high cost of construction limits its application. TLR3, as a participant of the innate immune response to inflammation[12, 44], recognizes viral dsRNA, increases TLR3 expression, and affects experimental outcomes, so that viral vectors cannot be used. Moreover, electroporation easily causes neuronal injury and apoptosis during transfection, and its transfection efficiency is not high[45]. Krichevsky and Kosik[46] used TransMessenger transfection reagent to transfect siRNA targeting the MAP-2 gene into primary neurons, which resulted in a transfection efficiency of 40%. Thus, cotransfection of siRNA and plasmid was achieved, which brought hope for transfection into primary neurons by liposomes. Our pilot experiment found that TLR3 protein expression was low in neurons, so it is not plausible to inhibit TLR3 protein expression and to screen effective siRNA sequences. Because TLR3 protein expression is high following oxygen-glucose deprivation, this study screened effective sequences of three pairs of chemically synthesized siRNA under oxygen-glucose deprivation conditions. siRNA specific to the TLR3 gene was transfected into a primary neuron model of oxygen- glucose deprivation, which reached a high protein inhibitory effect.

Due to its nontoxicity, liposomes are non-immunogenic substances and have been considered safe vectors for gene transfection[45]. In the past 10 years, chemically synthesized non-viral vectors have developed rapidly, including cationic lipids, polymers, dendrimers and surfactants[47]. Hiperfect Transfection Reagent from QIAGEN is effective for the transfection of siRNA into primary neurons, showing high transfection efficiency at a low concentration. In 2006, Jeske *et al* [51] used Hiperfect to transfect siRNA into trigeminal neurons, and revealed a decrease in TRPA1 protein expression by 58.1 ± 12.0%. In 2009, Ribeiro *et al* [52] used Hiperfect Transfection Reagent to successfully transfect corpus striatum neurons following oxygen-glucose deprivation, and then inhibited protein expression. In accordance with Kim *et al* [53], this study used Hiperfect Transfection Reagent to transfect primary cortical neurons at 5 days for 24 hours, and then oxygen glucose deprived models were prepared. After reoxygenation, high-efficiency transfection of primary neurons was complete, and an optimal sequence that could inhibit TLR3 gene expression was screened. Western blotting revealed that compared with the negative control group, the ratio of relative expression was 35.13% in the TLR3-1280 group, 50% in the TLR3-1724 group, and 42.70% in the TLR3-418 group. The siRNA sequence

could suppress TLR3 protein expression. TLR3 protein expression was minimal in the TLR3-1724 group when compared with the TLR3-1280 group. The reasons for the different inhibitory effects of siRNA specific to the identical target gene at different target sites may be associated with target site and target sequence structure[54-56]. If target sequences have a stem or loop structure, or with more base pairings, and many hydrogen bonds, will result in a poorer silencing effect[54-56].

In summary, it is feasible to transfect primary neurons using Hiperfect following oxygen-glucose deprivation. This study successfully screened three pairs of effective sequences of TLR3-siRNA, and identified an optimal method to transfect primary neurons by Hiperfect Transfection Reagent. This study paves the way for future studies on the expression and effects of TLR3 and its downstream effects after cerebral ischemia/reperfusion injury, and provides a feasible method for future investigations requiring protein transfection by siRNA after ischemic brain injury.

MATERIALS AND METHODS

Design
A cytological, *in vitro*, comparative observational study.

Time and setting
Experiments were performed in the Laboratory of Neurobiology, Xuzhou Medical College, China from August 2011 to September 2012.

Materials
A total of 20 healthy clean pregnant Sprague-Dawley rats at gestational day 18 were provided by the Experimental Animal Center, Xuzhou Medical College, China (license No. SYXK (Su) 2007-0037). The protocols were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[57].

Methods
Incubation of rat cortical neurons
Pregnant rats were anesthetized with ether, and decapitated. The fetal rats were placed in ice-cold Dulbecco's Modified Eagle's Medium-H (DMEM-H). The heads of fetal rats (both genders) were obtained with aseptic elbow smooth forceps. Bilateral cerebral hemispheres were exposed, obtained and placed in DMEM-H at 5°C. The meninges and blood vessels were peeled off with straight-head microforceps under an anatomical micro-

scope. The cerebral cortex and hippocampus were dissociated, cut into pieces, and digested with trypsin containing 0.25 ethylenediamine tetraacetic acid at 37°C for 15 minutes. During this period, the specimens were stirred twice with a narrow mouthed dropper. Fetal bovine serum (10% (v/v); Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, Zhejiang Province, China) was added to terminate the digestion. The specimens were centrifuged at 1 000 r/min for 3 minutes, incubated in 100 mL of serum-free Neurobasal medium (Gibco, Grand Island, NY, USA), supplemented with 2 mL of B27 and 0.5 mmol/L L-glutamine (Sigma, St. Louis, MO, USA), and stirred to resuspend cells. Cell liquor was filtered with a 200-mesh screen. Cells at a density of $1.5–2 \times 10^5/cm^2$ were incubated in culture flasks and Petri dishes precoated with polylysine (0.05 mg/mL polylysine in aqueous solution) at 37°C in a 5% (v/v) CO_2 incubator (Thermo, Waltham, MA, USA) overnight. Half of the Neurobasal medium was replaced once every 4 days[58].

Establishment of oxygen-glucose deprivation
At 7 days after culture, the Neurobasal medium was replaced by glucose-free Earle's Balanced Salt Solution (Gibco). Neurons were rapidly placed in an anaerobic incubator at 37°C (95% (v/v) N_2 + 5% (v/v) CO_2, oxygen concentration was below 1% (v/v)) for 2 hours. Glucose-free Earle's Balanced Salt Solution was replaced by Neurobasal medium. After 24 hours of incubation in a common incubator, the following experiments were performed[59].

Design and synthesis of the siRNA sequence of the TLR3 gene
Three pairs of siRNA sequences were designed and synthesized by Shanghai GenePharma Co., Ltd. Negative control sequence composition was identical to the above-mentioned siRNA sequence, but it was a missense sequence. Primers are as follows:

TLR3-1280:	Sense: 5'-CCC UCGAGUACAA UAA UAU TT-3'
	Antisense: 5'-AUA UUA UUG UAC UCG AGG GTT-3'
TLR3-1724:	Sense: 5'-GGA GAG GUC UGG GAA AUA UTT-3'
	Antisense: 5'-AUA UUU CCC AGA CCU CUC CTT-3'
TLR3-418:	Sense: 5'-CCC UAU GGA UUC UUG UGG UTT-3'
	Antisense: 5'-AUA UUA UUG UAC UCG AGG GTT-3'
Negative control:	Sense: 5'-UUC UCC GAA CGU GUC ACG UTT-3'
	Antisense: 5'-ACG UGA CAC GUU CGG AGA ATT-3'

Principle of design: (1) length of chemically synthesized oligo siRNA was 21 bp; G + C content in siRNA sequence was between 30% and 60%; (2) four sequential identical bases were avoided; (3) sequences with high G + C content at 5′ end and low G + C content at 3′ end

were removed; (4) all sequences were strictly screened according to BLAST, and homology was excluded[56].

siRNA transfection

At 5 days of culture, primary neurons were randomly assigned to four groups. With Hiperfect Transfection Reagent (QIAGEN, the Netherlands), four groups of 625 ng siRNA were dissolved in 50 μL serum-free medium. After mixing, 6 μL Hiperfect Transfection Reagent was added to 44 μL serum-free medium, and mixed with dissolved siRNA. The specimens were gently mixed, shaken for 10 seconds, and incubated at room temperature for 5–10 minutes. The transfection complex was added to cortical neuron culture flasks drop by drop, mixed, and incubated in a common culture flask. At 7 days, oxygen-glucose deprived neurons in each group were retransfected according to the above-mentioned method in a common culture flask for 24 hours, and then cells were collected[51-53] (Figure 4).

Figure 4 siRNA transfection method.
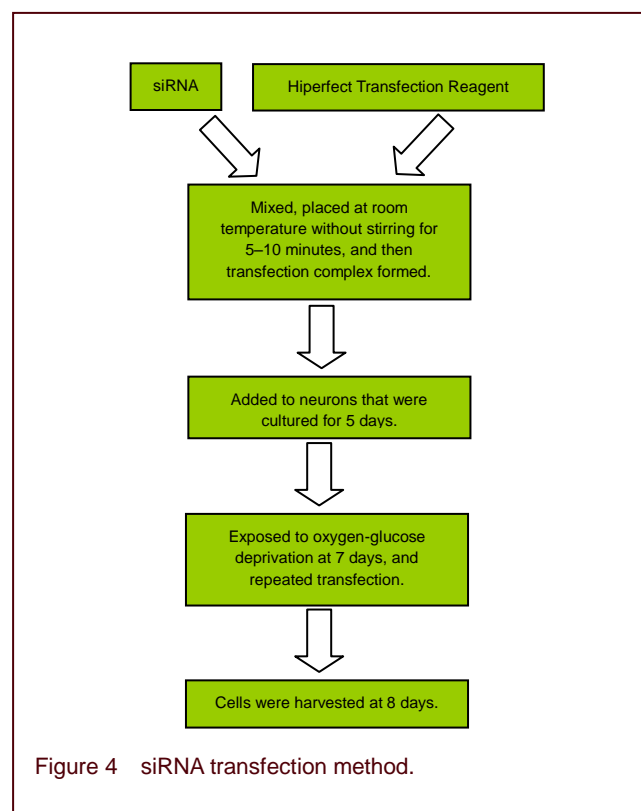
Cell harvesting

Cell specimens were washed three times with PBS after the medium was discarded. Remnant PBS was removed. Cell specimens were lysed with 500 μL ice-cold lysis buffer in each bottle, and shaken on ice. Lysate was transferred to a 1.5 mL Eppendorf tube for centrifugation at 12 000 r/min at 4°C for 15 minutes. The supernatant was placed in an additional Eppendorf tube. The specimens were stored separately at –80°C.

Western blotting

The protein concentration of each sample was determined using a protein concentration kit (the Beyotime Biological Co., Haimen, Jiangsu Province, China) and equal amounts of protein were prepared in radioimmunoprecipitation analysis lysis buffer. After denaturation in a water bath at 100°C for 5 minutes, an equal volume of protein was added to each well. The samples were electrophoresed on a 10% sodium dodecyl sulphate- polyacrylamide gel at 80 V. The proteins were transferred electrophoretically to a nitrocellulose membrane at 360 mA for 2 hours. The membrane was blocked with Tris-Buffered-Saline with Tween containing 50 g/L skim milk at room temperature for 1 hour, and incubated with rabbit anti-rat TLR3 monoclonal antibody (diluted by blocking buffer; Abcam, San Francisco, USA; 1:500) at 4°C overnight. The membrane was washed three times with washing buffer, each for 5–10 minutes, incubated in goat anti-rabbit secondary antibody (1:1 000; Zhongshan Golden Bridge Biotechnology, Beijing, China) at room temperature in the dark for 2 hours, washed with Tris-Buffered-Saline with Tween, visualized with alkaline phosphatase, and photographed. Absorbance values were measured with Image J software (National Institutes of Health, USA).

Statistical analysis

Data were expressed as the mean ± SD, and analyzed with SPSS 13.0 software (SPSS, Chicago, IL, USA). Pictures were drawn with GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). Paired comparison was performed with two-sample t-test. Comparisons among multiple groups were performed by one-way analysis of variance. Paired comparison of intergroup differences was conducted using least significant difference test. A value of $P < 0.05$ was considered statistically significant.

Research background: TLR3-mediated immune inflammatory reaction induced neuronal apoptosis and tissue injury.

Research frontiers: Our previous study found that TLR3 protein expression was upregulated in brain tissues after cerebral ischemia/reperfusion injury, but its precise mechanism remains unclear.

Clinical significance: Results from this study provide a new action target for lessening the immune inflammatory reaction after cerebral ischemia/reperfusion injury.

Academic terminology: TLRs are a class of proteins that play a key role in the innate immune system as well as the digestive system. They are single, membrane-spanning, non-catalytic receptors usually expressed in sentinel cells such as macrophages and dendritic cells, that recognize structurally con-

served molecules derived from microbes.

Peer review: This study established models of oxygen-glucose deprivation in primary neurons cultured *in vitro* and transfected siRNA into neurons with liposome Hiperfect to allow the study of TLR3, downstream protein expression, and its effects following cerebral ischemia/reperfusion injury.

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(Reviewed by Diwakarla S, Raye W, Li X, Bian LG)
(Edited by Wang J, Qiu Y, Li CH, Song LP, Liu WJ, Zhao M)