

Effects of Txk-mediated activation of NF- κ B signaling pathway on neurological deficit and oxidative stress after ischemia-reperfusion in rats

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Abstract. Ischemic stroke is an extremely mortal cerebrovascular disease, and neuroinflammation and oxidative stress emerge as important traits of ischemic stroke. However, as an inflammation-associated factor, Txk tyrosine kinases (Txk) has been poorly studied in neuroscience research. The aim of the present study was to investigate the role of Txk after ischemia-reperfusion (I/R) *in vivo* and *in vitro*, observe the association between Txk knockdown and neurological deficit and oxidative stress, and to explore whether the process was mediated by the activation of nuclear factor (NF)- κ B signaling pathway. Middle cerebral artery occlusion (MCAO), oxygen and glucose deprivation/reperfusion (OGD/R) model and western blotting have been used to simulate the I/R injury to analyze the expression, and to approximate the localization of Txk, respectively. Brain infarct volume, neurological score, brain water content, apoptosis and oxidative stress assays *in vivo* and apoptosis, cellular viability, the LDH release and oxidative stress assays *in vitro* were observed using a Txk-knockdown lentivirus. Finally, NF- κ B overexpression lentivirus was applied to discuss whether the role of Txk following I/R was regulated by the NF- κ B signaling pathway. The results show that the Txk expression peaked at 24 h after MCAO and 6 h after OGD/R, respectively. Txk molecules gradually entered the nucleus after MCAO and OGD/R. The Txk-knockdown lentivirus resulted in decreased brain infarct volume, neurological score, brain water content, apoptosis and oxidative stress after MCAO *in vivo*. Besides, Txk knockdown decreased apoptosis, LDH release, oxidative stress, and increased cellular viability, after OGD *in vitro*. Finally, NF- κ B overexpression reversed the process of neurological deficit and oxidative stress after Txk regulation

in vivo and *in vitro*. Overall, the present study suggests that Txk potentially regulates apoptosis, neurological deficit, and oxidative stress after I/R, by entering the nucleus. NF- κ B maybe the downstream target factor of Txk.

Introduction

Ischemic stroke is an extremely mortal cerebrovascular disease which has a high recurrence rate (~26%), and usually results in disability, thus becoming a severe health problem worldwide (1). Despite continued advances in therapeutic techniques, the mechanism of cerebral ischemia-reperfusion (I/R) injury is still complex (2); therefore, a clear understanding of the pathophysiology is warranted.

Neuroinflammation and oxidative stress emerge as important traits to be considered in the pathophysiology of ischemic stroke (3). Malondialdehyde (MDA) is an oxidative stress marker, which is also a product of lipid peroxidation reaction (4). During ischemia physiological condition, few free radicals are present, but at the stage of recovery, known as reperfusion, the blood supply of the tissue triggers the 'explosion' of oxygen free radicals, the antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), are involved in the defensive system for protecting against oxidative stress (5,6).

Txk tyrosine kinases (Txk), also known as tyrosine-protein kinases Txk or protein-tyrosine kinase 4 is one of the Tec family of tyrosine kinases that have unique N-termini followed by Src homology 3 (SH3) and Src homology 2 (SH2) protein interaction domains and a tyrosine kinase catalytic domain (7). Txk expression is enhanced in rheumatoid arthritis, where Th1-dominant immunity occurs (8). Modulation of Txk expression by gene transfer or similar modality may lead to the correction of aberrant immunity (9). As an immune-associated protein, Txk has been studied little in the field of stroke.

The nuclear factor (NF)- κ B signaling pathway associated with severe immune deficiencies, whereas dysregulated activation of this pathway contributes to the pathogenesis of various autoimmune and inflammatory diseases (10). NF- κ B signaling pathway is reported to be involved in neurodegeneration and oxidative stress in ischemia/reperfusion (I/R) injury (11). However, the mechanism of Txk and NF- κ B signaling pathway in oxidative stress after I/R are still not fully clarified.

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The present study focused on the role of Txk in I/R injury and Txk knockdown was used to decrease neurological deficit and oxidative stress *in vivo* and *in vitro*. Furthermore, NF- κ B overexpression was conducted to activate NF- κ B expression, which reversed the progress after Txk regulation. The present findings indicated that Txk potentially mediates neurological deficit and oxidative stress through the NF- κ B signaling pathway in I/R injury. These results may provide a new strategy to recede and recover I/R injury.

Materials and methods

Animals. A total of 300 (age, 6-8 weeks; mean age, 7.2 weeks) healthy male adult Sprague-Dawley rats (weighing 250-300 g) were supplied by Liaoning Changsheng Biotechnology Co., Ltd., which were housed in a temperature and light control environment. All animals used in this experiment were cared for in strict accordance with the Guide for the Care and Use of Laboratory Animals (12). All experimental procedures were cared for in strict accordance with the Animal Ethics Committee of Dongyang People's Hospital (permit no. AEWC-20191210A).

Middle cerebral artery occlusion (MCAO) rat model establishment and experimental design. Cerebral I/R was obtained through the MCAO procedure as previously described (13). In brief, the experimental rats were anesthetized with pentobarbital sodium (30 mg/kg, i.p.) until the rats were irresponsive to touch and placed on a heating pad to maintain body temperature. The left common and external carotid arteries were then proximally exposed and ligated after a ventral midline neck incision. A 5-0 nylon monofilament (Beijing Cinontech Co. Ltd.), with the blunt end coated with poly-L-lysine, was inserted into the internal carotid artery and forwarded into the middle cerebral artery origin until a slight resistance was met. Meanwhile, the regional cerebral blood flow was decreased to <20% of the baseline level. Reperfusion was achieved after 1 h of occlusion by slowly withdrawing the nylon monofilament and the blood flow was recovered to 75% of baseline. Sham animals received the same operation without MCAO. All animals were sacrificed after 48 h upon reperfusion (13).

Then, the subjects were randomly divided into six groups (n=6 in each group): (i) Sham group, rats that underwent surgical procedures but had no ischemia; (ii) MCAO group, rats with 60 min of ischemia followed by 6-48 h reperfusion; (iii) Txk-shRNA group, rats receiving intra-cerebroventricular injection of lentivirus-sh-Txk one week before MCAO; (iv) control shRNA group, rats receiving intra-cerebroventricular injection of lentivirus expressing negative control; (v) NF- κ B-oeRNA group, rats receiving intra-cerebroventricular injection of lentivirus-oe-NF- κ B one week before MCAO; (vi) control oeRNA group, rats receiving intra-cerebroventricular injection of lentivirus expressing negative control. Rats were treated according to American College of Laboratory Animal Medicine guidelines (14). The investigators observed the rats daily to ensure animal welfare and to determine the humane endpoints, which included movement, apathy and severe weight loss for 1 h frequently. The rats were sacrificed when they exhibited rapid weight loss (>20%) or were nonmobile and showed signs of deteriorating health, such as hunching, dehydration and labored breathing.

Rats were sacrificed via heart perfusion with 0.9% saline solution. The rats were anesthetized by pentobarbital sodium (30 mg/kg; intraperitoneal), an upper abdominal incision was made, and the heart was exposed. Then, 0.9% saline solution was injected into the apex of heart before cutting open the right auricle and pumped into the systemic circulation until the heartbeat stopped.

Isolation and culture of primary cortical neurons. The primary cortical neurons extracted from embryonic cerebella of 90 Sprague-Dawley rats (16-18 days) were used in the present study as described previously (15,16). The cell suspensions were seeded on six-well plates (concentration: 1.5×10^5 cells per well) and neurobasal medium containing 2% B27 (Gibco; Thermo Fisher Scientific, Inc.) was used for culture under 5% CO₂ in a 37°C incubator, which was supplemented with glutamine (2 mM) and penicillin/streptomycin (50 U/ml) (12). All experimental procedures were cared for in strict accordance with the Animal Ethics Committee of Dongyang People's Hospital (permit no. AEWC-20191210A).

Oxygen and glucose deprivation/reperfusion (OGD/R) model establishment. For the OGD/R model establishment, cells were incubated at 37°C with Earle's Balanced Salt Solution (Sigma-Aldrich; Merck KGaA) without glucose in a 1% oxygen-supplying incubator for 6 h. The cells were then transferred into an incubator at 37°C at normal conditions for another 1-12 h (17). The grouping was similar to that with the rats *in vivo* (17).

Reagents and antibodies. 2,3,5-triphenyltetrazolium chloride (TTC) was purchased from Sigma Aldrich (Merck KGaA). Lipid peroxidation MDA assay kit (cat. no. S0131; Beyotime Institute of Biotechnology), GSH assay kit (cat. no. S0053; Beyotime Institute of Biotechnology), GSH peroxidase (GSH-PX) assay kit (cat. no. S0058; Beyotime Institute of Biotechnology), CAT assay kit (cat. no. S0051; Beyotime Institute of Biotechnology), SOD assay kit (cat. no. S0109; Beyotime Institute of Biotechnology) and cytochrome C activity assay kit (cat. no. K257-100; Biovision, California, USA) were also obtained.

Anti-Txk (cat. no. PA5-98222; Thermo Fisher Scientific, Inc.), anti-NF- κ B (cat. no. BM3940; Boster Biological Technology), anti-interleukin (IL)-1 β (cat. no. ab23437; Abcam), anti-IL-18 (cat. no. ab71495; Abcam), anti-tumor necrosis factor (TNF)- α (cat. no. ab1793; Abcam) and anti- β -actin (cat. no. M01263-2; Boster Biological Technology) primary antibodies, followed by secondary antibodies conjugated to horseradish peroxidase anti-rabbit IgG (H+L) (cat. no. AS014; ABclonal Biotech Co., Ltd.) and anti-mouse IgG (H+L) (cat. no. AS003; ABclonal Biotech Co., Ltd.) were used for western blotting or immunofluorescence (IF).

Txk knockdown and NF- κ B overexpression. The Txk knockdown lentivirus (Txk-shRNA), NF- κ B overexpression lentivirus (NF- κ B-oeRNA) and control lentivirus were designed and chemically synthesized by GenePharma Corporation. The lentivirus vector were stored at -80°C accordingly.

To knockdown Txk *in vivo*, rats were anesthetized and placed in a Kopf stereotactic frame, as previously

Table I. Sequences of Txk.

| Name | Sequence |
|------------------|-----------------------------|
| sh-Txk-1201 | 5'-ATGAAGAGCTTCCAGAAAAA-3' |
| sh-Txk-1442 | 5'-CAATCCTCACTGGTGGAAGG-3' |
| sh-Txk-1746 | 5'-GAAAGACACGCCTTTCAATC-3' |
| Negative control | 5'-AGGTTTTTCAGAACAGGAAAA-3' |
| control-shTxk | 5'-TCTTACCATATGGGGTCAC-3' |

Txk, Txk; sh, short hairpin RNA.

Table II. Sequences of NF-κB.

| Name | Sequence |
|------------------|----------------------------|
| oe-NF-κB-481 | 5'-GGGCTTACCCATGTCTGGAG-3' |
| oe-NF-κB-846 | 5'-TGACAGCAAGCTTCCCAAAC-3' |
| oe-NF-κB-1261 | 5'-ACAGGTCAAGTTCTTTGAGA-3' |
| Negative control | 5'-TGAGACTGAGGCTCTGGCTT-3' |
| control-oeNF-κB | 5'-TGCTGCATTTCAAATCGATC-3' |

NF-κB, nuclear factor-κB; oe, overexpression.

described (18). In brief, a burr hole was bored in the pericranium 0.9 mm lateral to the sagittal suture and 1.9 mm posterior to the coronal suture. A 10-ml microinjection pump (WPI, Inc.) was inserted 3.5 mm deeper into the cortex stereotactically. A 5-ml viral suspension consisting of 1×10^9 genomic copies of Txk-shRNA lentivirus, was ipsilaterally injected into the right lateral cerebral ventricle at a rate of 0.2 ml/min. The needle was taken out after 15 min of injection. To knockdown Txk, cells were subjected to OGD/R treatment, as previously described (19). The NF-κB overexpression lentivirus transfection was performed ahead of Txk-shRNA induction, using the aforementioned method. The sequences of Txk and NF-κB lentivirus are shown in Tables I and II. Negative controls for sh-Txk-1201, 1446, 1742 and control shTxk were used.

Western blotting. RIPA buffer (cat. no. P0013K; Beyotime Institute of Biotechnology) containing protease inhibitor was used to extract total protein from the cells and the tissue, and the supernatant was collected following centrifugation (12,000/rpm). A total of 40 μg protein from each brain sample using the bicinchoninic acid (cat. no. P0012; Beyotime Institute of Biotechnology) method was subjected to 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. In brief, after removing the combs, the target protein and marker were added and the gel was run at 80V, and then the sample was separated to the bottom of the gel at 120V. The target protein and marker were then electrotransferred to a PVDF membrane at 250 mA for 1 h and blocked with 5% skim milk for 1 h at 37°C. Subsequently, the membranes were incubated overnight in primary antibody (Txk, 1:2,000; NF-κB, 1:3,000; IL-1β, 1:1,000; IL-18, 1:1,000; TNF-α, 1:2,000; β-actin, 1:5,000) at 4°C. Then, the membrane

was washed with 10X TBS-0.1% Tween 20 (TBST) three times and incubated with the secondary antibody (1:5,000) for 1 h at 37°C. The membrane was rinsed three times again with TBST and developed with developing liquid (horseradish peroxidase and alkaline phosphatase; cat. no. P0019; Beyotime Institute of Biotechnology). All data were detected with the ChemiDoc™ Touch Imaging System and analyzed with the Image lab 3.0 software (Bio-Rad Laboratories, Inc.).

IF staining. The expression level of Txk was detected by IF staining as previously described (13). In brief, paraffin-embedded tissue fraction (5-mm thickness) was dewaxed with xylene, then dehydrated using graded concentrations of alcohol, and incubated with 3% H₂O₂ inhibitor, endogenous peroxidase. After blocking in 10% goat serum for 10 min at room temperature, the tissues were incubated with the primary antibody of Txk (1:100) in blocking solution overnight at 4°C. Then, the slides were washed in PBS and HRP-labeled anti-rabbit IgG was applied for 30 min at 37°C, followed by a PBS wash. Finally, images were acquired with a Nikon EclipseNi inverted microscope (400x magnification; TE2000; Nikon Corporation).

Acridine orange (AO)/propidium iodide (PI) staining. AO/PI staining was conducted as previously reported (20). The apoptotic ratio was counted and shown as the ratio of cells with positive ethidium bromide staining.

TUNEL staining. Tissue fraction was determined by TUNEL assay. The TUNEL assay was performed according to the manufacturer's instruction manual (cat. no. C1091; Beyotime Institute of Biotechnology). Images were captured by fluorescence microscopy at x400 magnification.

Infarct volume evaluation. Animals were anesthetized and sacrificed. The whole brain was then rapidly removed, frozen for 15 min at -20°C in an adult rat brain matrix (RWD Life Science) and then cut into 3 coronal sections. Cryosections were stained with 2% TTC for 15 min at room temperature in the dark, followed by fixation with 4% paraformaldehyde at 4°C for 48 h.

Neurological deficit score assessment (Longa Score). The neurological score was evaluated 24 h after reperfusion using a 5-point rating scale: Point 0, no deficit; point 1, failure to extend the left forepaw; point 2, decreased grip strength of the left forepaw; point 3, circling to left by pulling the tail; point 4, spontaneous circling; and point 5, no movement (21).

Brain water content assay. Rats were decapitated under deep anesthesia and brains were immediately removed. Each brain was divided into two halves, infarction and contralateral. The wet weight (WW) was weighed on an electronic balance. Brain tissue was dried in the oven at 100°C for 24 h to determine the dry weight (DW). Brain water content was calculated as [(WW-DW)/WW] x100%.

Cell viability and LDH assays. The viability of neurons was measured by MTT assay. Briefly, the neurons (2,000 cells/well) were cultured in 96-well culture plates for 24 h. Then, MTT

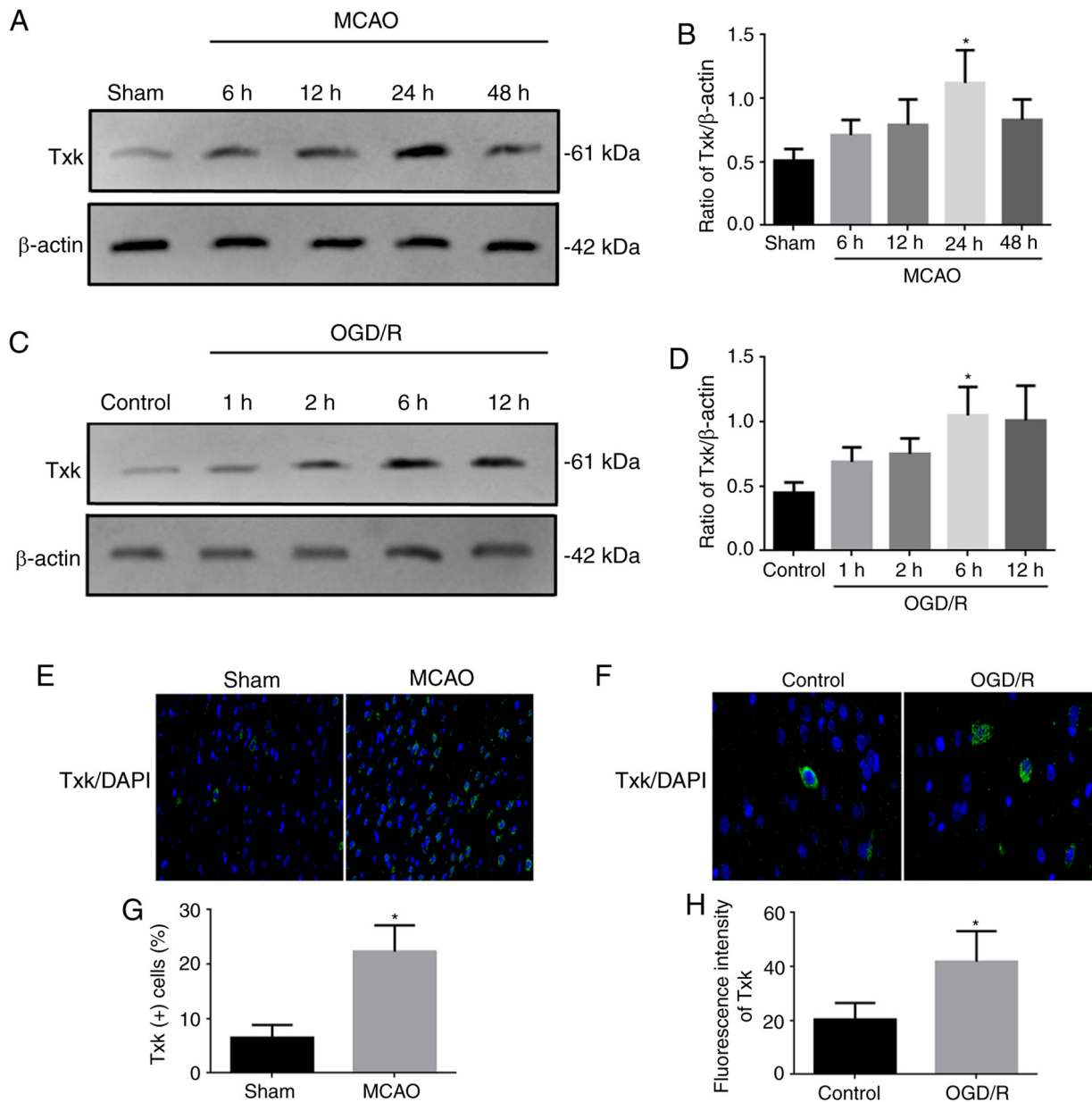


Figure 1. Western blotting and immunofluorescence results. (A) Western blot analysis of Txk expression in sham and MCAO 6-, 12-, 24- and 48-h groups. (B) Quantification of Txk in different groups. (C) Western blot analysis of Txk expression in control and OGD/R 1-, 2-, 6- and 12-h neurons. (D) Quantification of Txk in different neurons. (E) Immunofluorescence assay of Txk in sham and MCAO rats. Magnification, x400) (F) Immunofluorescence assay of Txk in control and OGD/R neurons. Magnification, x1000 (G) Txk-positive cells in sham and MCAO rats and (H) fluorescence intensity in control and OGD/R neurons. Protein level was normalized to β -actin. * $P < 0.05$, MCAO 24 h vs. sham, MCAO 6, 12 and 48 h groups; OGD/R 6 h vs. control, OGD/R 1 and 2 h groups; MCAO vs. sham; OGD/R vs. control; $n = 6$ per group. All data are represented as mean \pm standard error. Txk, Txk tyrosine kinase; MCAO, middle cerebral artery occlusion; OGD/R, oxygen and glucose deprivation/reperfusion.

(5 mg/ml) was added into each well at 37°C for 4 h. After that, MTT was removed and 150 μ l DMSO was added into the well to solubilize purple formazan. Then, absorbance at 570 nm was read using a microplate reader (PLUS 384; Molecular Devices, LLC). LDH cytotoxicity detection kit (cat. no. C0017; Beyotime Institute of Biotechnology) was used as previously described (22).

Oxidative stress parameters and mitochondrial dysfunction detection. For detecting oxidative stress parameters in rat ischemic hemispheres tissue or neurons, lipid peroxidation MDA, GSH, GSH-PX, CAT, SOD and cytochrome C were purchased and operated according to the manufacturer's instructions.

Statistical analysis. Data are all expressed as mean \pm standard. Data statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, Inc.). Differences were analyzed using unpaired t-test, one-way ANOVA and multiple comparisons were analyzed using Tukey's test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of Txk at different time points and its approximate localization in cells after I/R or OGD/R injury. The expression of Txk at different time points after MCAO or OGD/R and its approximate localization was examined by

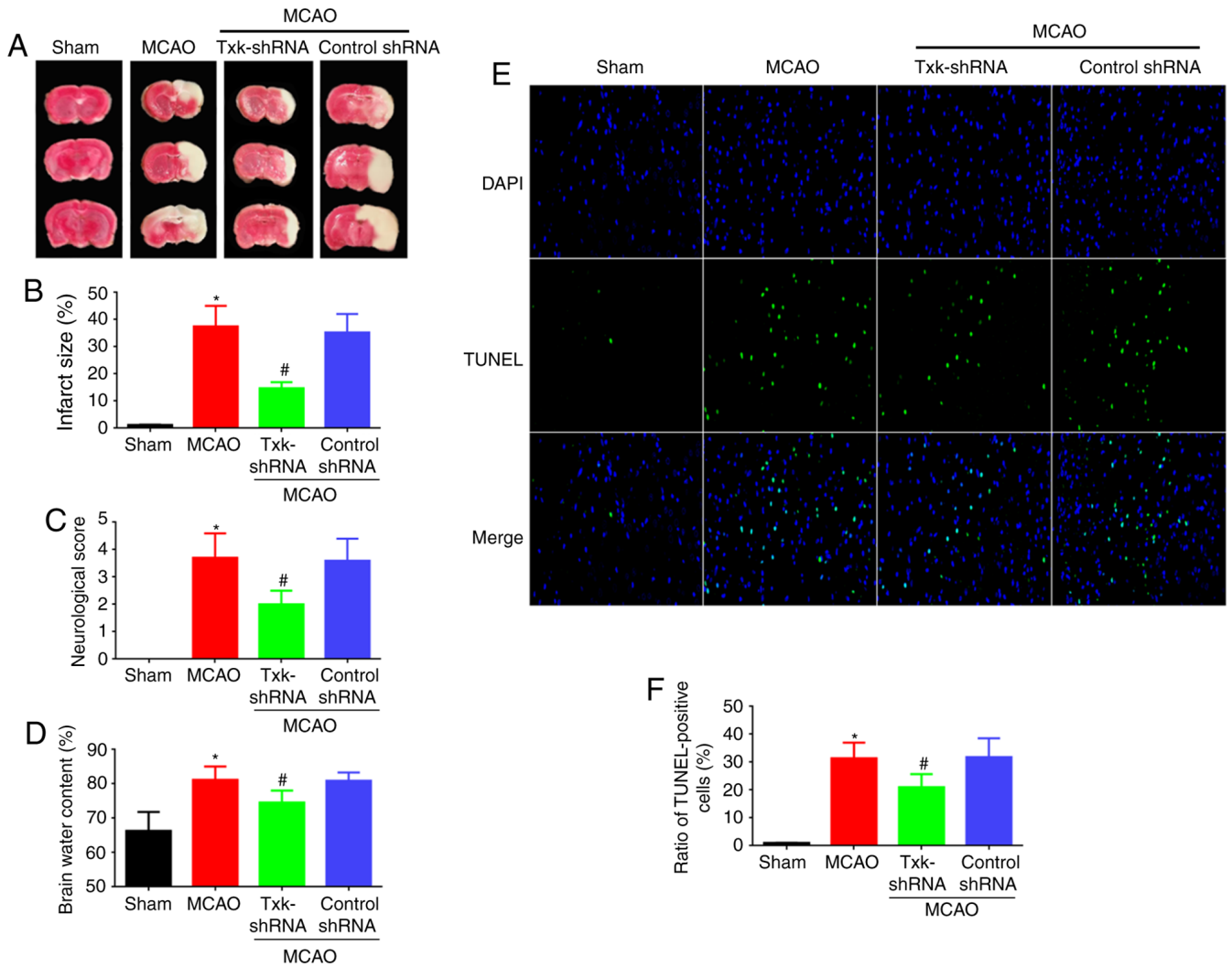


Figure 2. (A) 2,3,5-Triphenyltetrazolium chloride staining, (B) brain infarct volume assay, (C) neurological score assay, (D) brain water content, (E) immunofluorescence assay of TUNEL (magnification, x400) and (F) TUNEL assay of sham, MCAO, Txk-shRNA and control-shRNA groups. *P<0.05, MCAO vs. sham group; #P<0.05, Txk-shRNA vs. MCAO group; n=6 per group. Txk, Txk tyrosine kinase; MCAO, middle cerebral artery occlusion; shRNA, short hairpin RNA.

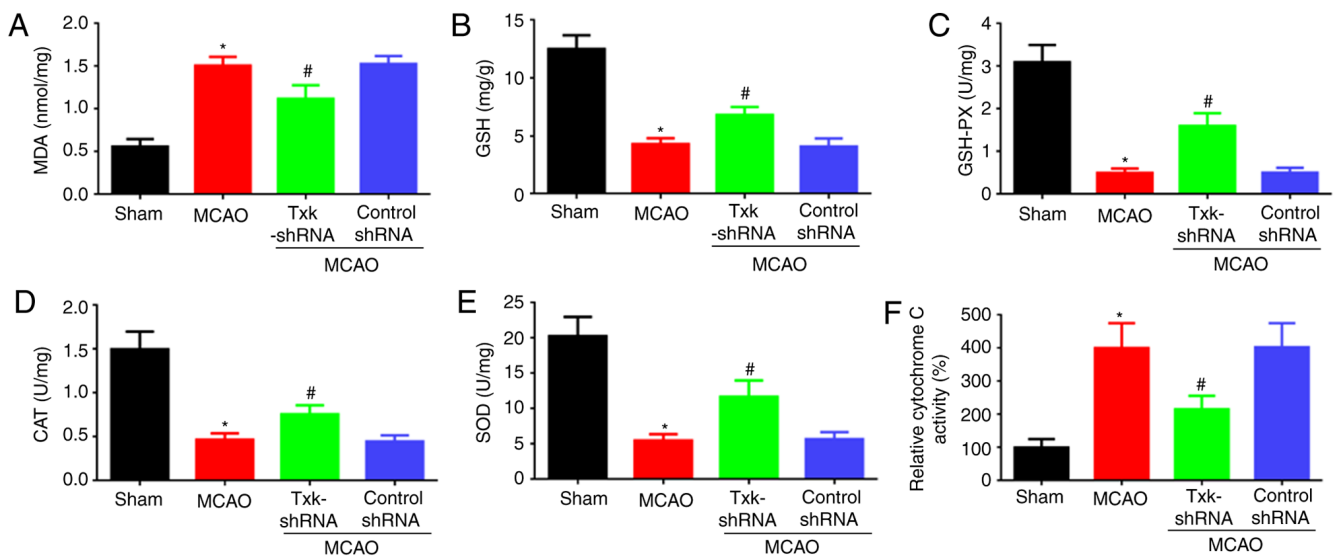


Figure 3. (A) MDA, (B) GSH, (C) GSH-PX, (D) CAT, (E) SOD and (F) cytochrome C activity assays of sham, MCAO, Txk-shRNA and control-shRNA groups. *P<0.05, MCAO vs. sham group; #P<0.05, Txk-shRNA vs. MCAO group; n=6 per group. MCAO, middle cerebral artery occlusion; MDA, malondialdehyde; GSH, glutathione; GSH-PX, GSH peroxidase; CAT, catalase; SOD, superoxide dismutase; shRNA, short hairpin RNA.

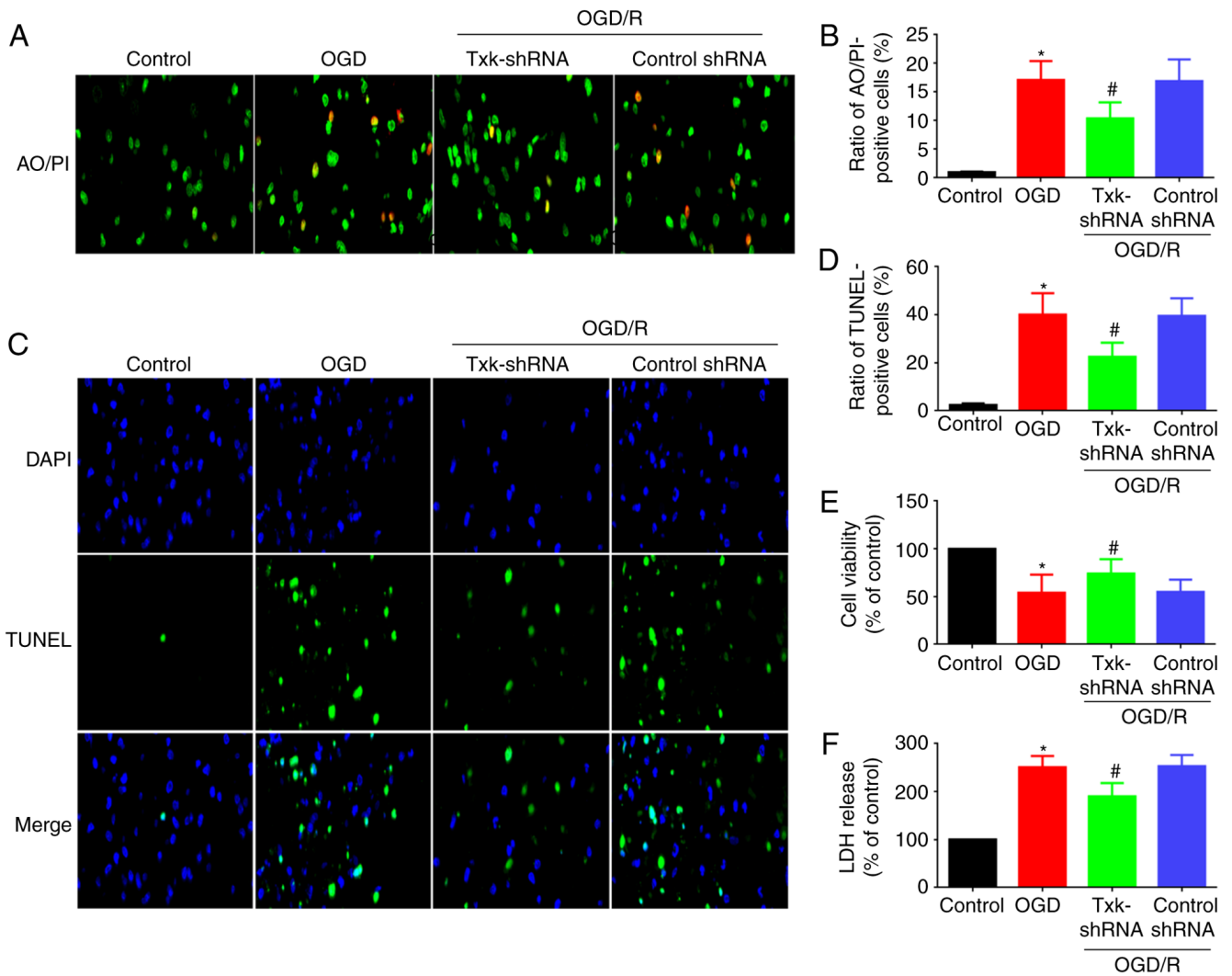


Figure 4. (A) Immunofluorescence assay of AO/PI (magnification, x400), (B) AO/PI-positive cells, (C) immunofluorescence assay of TUNEL (magnification, x400), (D) TUNEL-positive cells (E) cellular viability and (F) LDH release assays of control, OGD/R, Txk-shRNA and control-shRNA groups. * $P < 0.05$, OGD/R vs. control group; # $P < 0.05$, Txk-shRNA vs. OGD/R group; $n = 6$ per group. AO/PI, acridine orange/propidium iodide; Txk, Txk tyrosine kinase; MCAO, middle cerebral artery occlusion; OGD/R, oxygen and glucose deprivation/reperfusion; shRNA, short hairpin RNA.

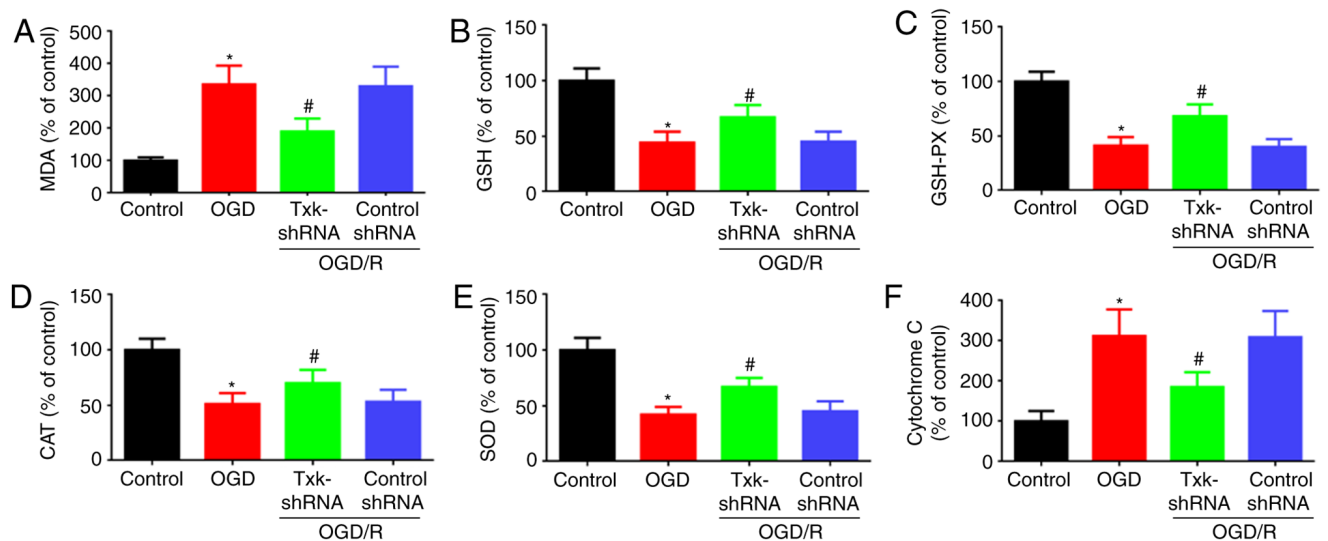


Figure 5. (A) MDA, (B) GSH, (C) GSH-PX, (D) CAT, (E) SOD and (F) cytochrome C activity assays of control, OGD/R, Txk-shRNA and control-shRNA groups. * $P < 0.05$, OGD/R vs. control group; # $P < 0.05$, Txk-shRNA vs. OGD/R group; $n = 6$ per group. MDA, malondialdehyde; GSH, glutathione; GSH-PX, GSH peroxidase; CAT, catalase; SOD, superoxide dismutase; Txk, Txk tyrosine kinase; shRNA, short hairpin RNA; OGD/R, oxygen and glucose deprivation/reperfusion.

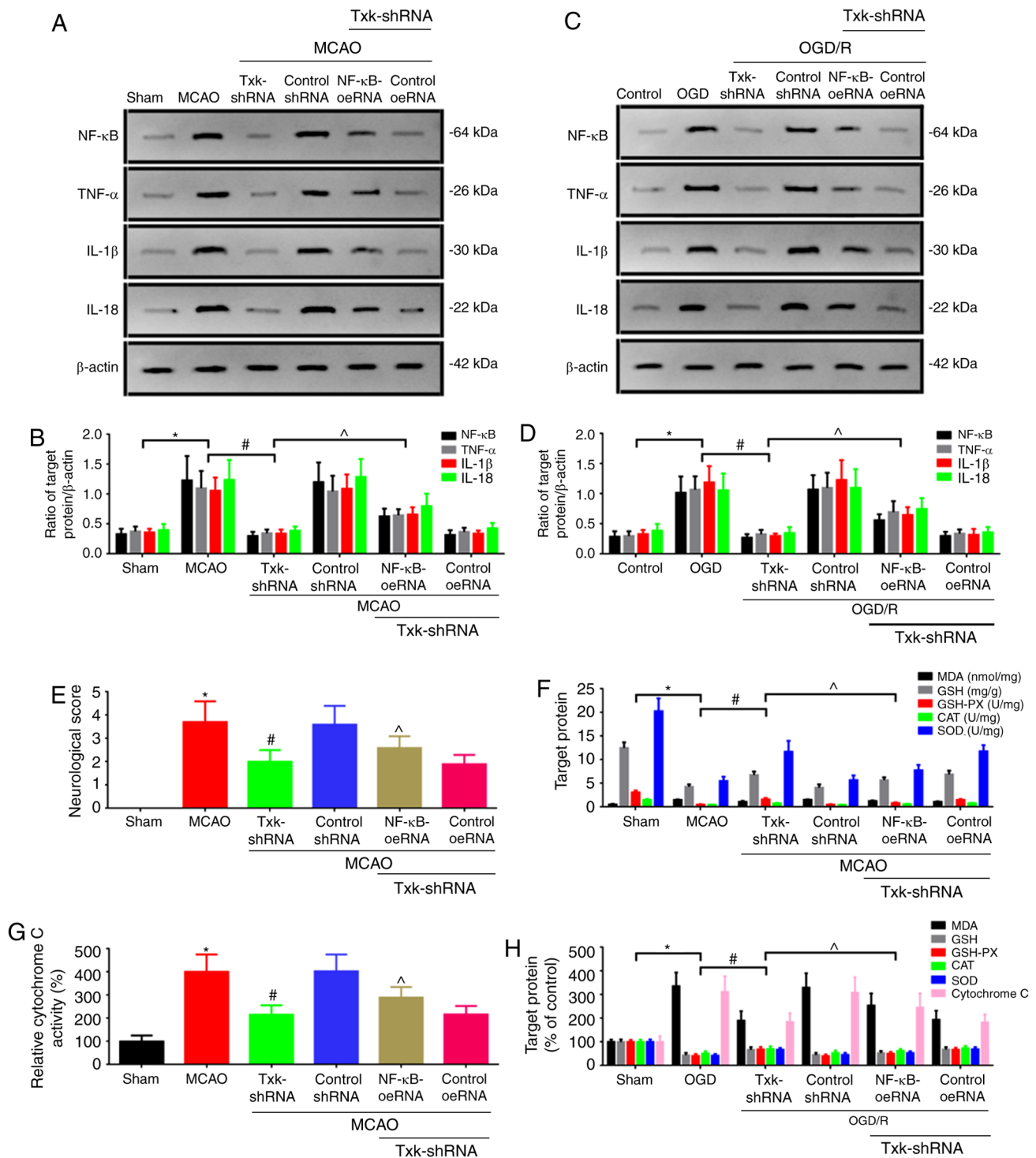


Figure 6. (A) Western blot analysis and (B) quantification of NF-κB, TNF-α, IL-1β and IL-18 expression in sham, MCAO, Txk-shRNA, control shRNA, Txk-shRNA+NF-κB-oeRNA and Txk-shRNA+control oeRNA groups *in vivo*. (C) Western blot analysis and (D) quantification of NF-κB, TNF-α, IL-1β and IL-18 expression in control, OGD/R, Txk-shRNA, control shRNA, Txk-shRNA+NF-κB-oeRNA and Txk-shRNA+control oeRNA groups *in vitro*. (E) Neurological score assay, (F) MDA, GSH, GSH-PX, CAT and SOD assays and (G) cytochrome C activity assay *in vivo*. (H) MDA, GSH, GSH-PX, CAT, SOD and cytochrome C activity assays *in vitro*. Protein level was normalized to β-actin. *P<0.05, MCAO or OGD/R vs. sham or control group; #P<0.05, Txk-shRNA vs. MCAO or OGD/R group; ^P<0.05, Txk-shRNA+NF-κB-oeRNA vs. Txk-shRNA group; n=6 per group. NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; IL, interleukin; MCAO, middle cerebral artery occlusion; OGD/R, oxygen and glucose deprivation/reperfusion; MDA, malondialdehyde; GSH, glutathione; GSH-PX, GSH peroxidase; CAT, catalase; SOD, superoxide dismutase; shRNA, short hairpin RNA; oe, overexpression.

western blotting and IF (Fig. 1). The Txk expression peaked at 24 h after MCAO (P<0.05), compared with the sham, and MCAO 6, 12, and 48-h groups (Fig. 1A and B). In addition, after OGD/R stimulation, the Txk expression peaked at 6 and 12 h (P<0.05), compared with the control group, and

OGD/R 1 and 2-h groups (Fig. 1C and D). Thus, 24 h after MCAO and 6 h after OGD/R was used for the I/R model for the follow-up experiments. The approximate localization of Txk is shown in Fig. 1E and F, the Txk/DAPI-positive cells or fluorescence intensity were increased after MCAO

and OGD/R, and the Txk molecules gradually entered the nucleus (Fig. 1G and H).

Txk knockdown contributes to the neuroprotection against acute stroke attack in rats. In order to knockdown Txk expression, Txk-shRNA was used, and the expression of Txk was decreased after shRNA administration (Fig. S1). To investigate the potential function of Txk on cerebral I/R injury in rats, the impact of Txk on brain infarct volume, neurological score and brain water content was examined (Fig. 2A-D). The Txk-shRNA group showed a significantly decreased brain infarct volume, neurological score and brain water content compared with the MCAO group ($P < 0.05$). Apoptosis was also detected in Fig. 2E, and the TUNEL-positive cells were significantly decreased in the Txk-shRNA group ($P < 0.05$) compared with the MCAO group (Fig. 2F).

Txk knockdown decreases oxidative stress after I/R injury in rats. As Txk knockdown contributed to the neuroprotection against acute stroke attack, the associated oxidative stress and mitochondrial dysfunction parameters were also observed. The level of MDA and cytochrome C, an indirect indicator of mitochondrial function (20) in the MCAO group was obviously increased compared with the sham group ($P < 0.05$); GSH, GSH-PX, CAT and SOD showed contrasting results. MDA and cytochrome C levels were decreased in the Txk-shRNA group ($P < 0.05$) compared with the MCAO group (Fig. 3A-F).

Txk knockdown contributes to the neuroprotection after OGD/R stimulation. Since the potential function of Txk on cerebral I/R injury in rats was investigated, this process was also studied *in vitro*. The AO/PI assay is shown in Fig. 4A, and the AO/PI-positive cells were significantly decreased in the Txk-shRNA group ($P < 0.05$) compared with the OGD/R group (Fig. 4B). To investigate the apoptotic function of Txk after OGD/R, TUNEL staining was conducted in neurons. The TUNEL-positive cells were significantly decreased in the Txk-shRNA group ($P < 0.05$) compared with the OGD/R group (Fig. 4C and D). In order to obtain more direct evidence, cellular viability and LDH release assays were used to assess OGD/R-induced injury in neurons. The cellular viability was significantly enhanced in the Txk-shRNA group ($P < 0.05$) compared with the OGD/R group (Fig. 4E), and the LDH release showed the opposite result (Fig. 4F).

Txk knockdown decreases oxidative stress after OGD/R stimulation. Since the potential function of Txk on oxidative stress and mitochondrial dysfunction was investigated in rats, this process was also studied *in vitro*. The level of MDA and cytochrome C in the OGD/R group was notably increased compared with the control group ($P < 0.05$); GSH, GSH-PX, CAT and SOD showed the contrary results. MDA and cytochrome C levels were decreased in the Txk-shRNA group ($P < 0.05$), compared with the OGD/R group (Fig. 5A-F).

Txk regulates neurological deficit and oxidative stress after I/R via NF- κ B signaling pathway. In order to investigate Txk-regulated neurological deficit and oxidative stress after I/R via NF- κ B signaling pathway, a method for increasing the NF- κ B level was attempted: NF- κ B overexpression lentivirus

(NF- κ B-oeRNA). NF- κ B-oeRNA was used to enhance NF- κ B expression, and the associated protein, neurological deficit and oxidative stress were then observed *in vivo* and *in vitro* (Fig. 6). As shown in Fig. 6A and B *in vivo*, compared with the MCAO group, the NF- κ B, TNF- α , IL-1 β and IL-18 levels in the Txk-shRNA group was decreased ($P < 0.05$). However, in the Txk-shRNA+NF- κ B-oeRNA group, the NF- κ B, TNF- α , IL-1 β and IL-18 levels were increased ($P < 0.05$) compared with the Txk-shRNA group. The *in vitro* results are shown in Fig. 6C and D. The neurological deficit score, oxidative stress and mitochondrial dysfunction parameters *in vivo* (Fig. 6E-G) and *in vitro* (Fig. 6H) were also detected, which showed the identical tendency with the western blot analysis.

Discussion

Ischemic stroke is characterized by a decrease in cerebral blood flow and deprivation of both oxygen and glucose, which are required to maintain the metabolic demands of the brain (23-24). Focal cerebral ischemia models, such as MCAO and neuron OGD, resemble human ischemic stroke (25,26).

Oxidative stress has been associated with different pathological processes such as neurotoxicity, neuroinflammation, ischemic stroke and neurodegenerative diseases (27,28). During I/R injury, the excessive production of reactive oxygen species, reactive nitrogen species, excitatory amino acid toxicity and inflammatory reaction are implicated in neuronal damage (29).

Txk, a member of the Tec family, whose catalytic activity was essential for its function to enhance Th1 cell function (30), suggested that Txk maybe belong to an immune-associated protein. Txk had been reported that it may be associated with breast cancer, schizophrenia, rheumatoid arthritis and diabetes (8,31-33). The Txk expression level was enhanced in rheumatoid arthritis, where Th1-dominant immunity occurs (8). The modulation of Txk expression by gene transfer or similar modality may lead to the correction of aberrant immunity (9). However, Txk as an inflammation-associated factor had been poorly studied in neuroscience-associated studies. To address these issues, the Txk expression level was detected after MCAO and OGD/R by western blotting and IF staining. The results showed the Txk expression level was increased after I/R injury. Besides, the Txk molecules gradually entered the nucleus for expression; this suggests that Txk may play a biological role after entering the nucleus in ischemic stroke. This may also indirectly prove that Txk lacks PH domain and has a nuclear translocation signal sequence, which is responsible for the nuclear translocation (34).

In order to detect the function of Txk after MCAO, a Txk knockdown lentivirus was used to block the expression level of Txk. In the present study, Txk-shRNA decreased the brain infarct volume, neurological deficit score, brain water content and apoptosis level, which suggested that blocking Txk could decrease infarction and neuron apoptosis, and improve neurological function and encephaledema. Furthermore, Txk-shRNA receded oxidative stress injury and mitochondrial dysfunction through detecting MDA, GSH, CAT, SOD and cytochrome C level, an indirect indicator for mitochondrial function (23). Txk-shRNA decreased oxidative stress injury and mitochondrial dysfunction; this may be due to the association between Txk and inflammation (30).

Furthermore, to verify the function of Txk in neurons, the Txk knockdown lentivirus was used to decrease Txk expression after OGD/R stimulation. The results showed that Txk knockdown could decrease apoptosis level, LDH release, oxidative stress and increase cellular viability; it exhibited the same tendency with the experiments *in vivo*.

NF- κ B is activated in cerebral vascular endothelial cells after cerebral ischemia, which triggers a dramatically increasing production of inflammatory cytokines, leading to an inflammatory cascade reaction and aggravating brain damage (35). NF- κ B inhibition played a key role in reversing ischemic stroke pathologies (36). However, whether the role of Txk after ischemic stroke was regulated by NF- κ B signaling pathway has not been reported yet. However, in the present study, the anti-NF- κ B from Boster was NF- κ B-P65, the other subtype I κ B α was not detected, and the other inflammation signaling pathways or target inflammatory cells are still unclear; further studies are required to explore this further.

In the present study, NF- κ B overexpression lentivirus was used to enhance NF- κ B expression level, and it was observed whether the process could be reversed after Txk knockdown. The results showed that the neurological deficit score, oxidative stress and mitochondrial dysfunction parameters *in vivo* and *in vitro* was reversed after NF- κ B regulation. These results suggest that the regulation of neurological deficit and oxidative stress by Txk was possibly mediated by NF- κ B signaling pathway. The present findings indicated that Txk knockdown significantly regulated apoptosis, neurological deficit and oxidative stress after I/R by entering the nucleus and is modulated through NF- κ B signaling pathway. Taken together, these findings may provide a new strategy to decrease neurological deficit and oxidative stress after I/R injury.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by QX and JW. The first draft of the manuscript was written by QX and all authors commented on previous versions of the manuscript. QX and JW confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animals were cared for in strict accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996), and the experimental design was

approved (approval no. 20200416) by the Ethics Committee of Dongyang People's Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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