Original Article

Effect of oxidative stress on neuronic apoptosis and c-myc expression after cerebral hemorrhage

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Received August 22, 2016; Accepted August 28, 2016; Epub December 1, 2016; Published December 15, 2016

Abstract: Neuron apoptosis after cerebral hemorrhage is one important factor causing late-onset neural damage. Complex mechanisms underlie secondary brain injury after hemorrhage, closely relating with inflammation, oxidative stress and cell apoptosis. C-myc has dual roles of facilitating cell proliferation and apoptosis. This study established a rat cerebral hemorrhage model, on which the correlation between post-hemorrhage oxidative stress and neuron apoptosis was observed, along with the effect of apoptosis gene c-myc expression. Healthy male SD rats were assigned into sham, model, MPEG-SOD groups (N=28). Brain hemorrhage model was generated by autoblood transfusion. 12 h, 1 d, 3 d and 7 d after surgery, neural deficit score (NDS) was measured. Morphology change of brain tissues was observed in addition to water contents. Malonaldehyde (MDA) and superoxide dismutase (SOD) were quantified by spectrometry. Nuclear factor (NF)-κB p65 was measured. Protein level of c-myc was determined by Western blotting, while TUNEL assay described neural apoptosis. Model rats had significantly lower NDS and SOD activity at all time points. Brain tissue water content, MDA level, NF-κB p65, c-myc and TUNEL positive cell were all remarkably elevated (P<0.05) with significant pathology injury. Model rats had significantly lower NDS and SOD activity at all time points. Brain tissue water content, MDA level, NF-κB p65, c-myc and TUNEL positive cell were all remarkably elevated (P<0.05) with significant pathology injury. Such impairment reached the peak at 3 d post-surgery. MPEG-SOD treatment significantly elevated NDS, SOD activity, and suppressed brain tissue water content, MDA level, NF-κB p65, c-myc and TUNEL positive cell (P<0.05), plus remarkably improved pathology injury. Oxidative stress-induced neuron apoptosis after brain hemorrhage might be related with NF-κB activity and activation of apoptosis related gene c-myc.

Keywords: Neuronic apoptosis, oxidative stress, c-myc

Introduction

Cerebrovascular disease is common in clinics, with gradual increase of incidence, morbidity and mortality [1, 2]. Relatively higher mortality existed for post-hemorrhage patients. Aged people are high risk population for cerebral hemorrhage. Major inducing factors include cerebral atherosclerosis, cerebral vascular deformation and hypertension [3, 4]. Currently the post-hemorrhage injury mechanism has not been fully illustrated, nor did effective treatment for improving patient’s prognosis significantly. Post-hemorrhage neuron apoptosis is one important factor causing late-onset neuron injury. A complicated series of mechanism underlies secondary brain tissue injury after hemorrhage, involving inflammation, oxidative stress and cell apoptosis. Oxidative stress is closely correlated with post-hemorrhage neuron apoptosis. Under hypoxia condition, reactive oxygen species (ROS) and NO were activated in neurons, to induce mitochondrial apoptosis pathway. During pathological process of cerebral hemorrhage, abnormal structure/function of mitochondria, oxidative stress injury and increased production of free radicals works as intermediate bridge. Brain tissues are susceptible for ROS-induced injury due to their high oxidative metabolic rate and lower antioxidant levels [5, 6]. Inflammatory mediator induces cell adhesion and aggregation, causing blockade of micro-vessels, activating leukocytes for releasing inflammatory mediators and cytokines. Secondary neuron injury thus occurred in blood-brain barrier under the direction of ROS released from leukocytes [7, 8]. Nuclear factor (NF)-κB has pluripotent regulatory roles...
for mediating inflammatory factor, cytokine, chemotactic factor transcriptional levels such as IL-8 and IL-6, thus playing an important role in cell inflammation and apoptosis. Previous study showed the involvement of NF-κB in pathological process of brain hemorrhage, under which microglia was activated to produce neurotrophic factors and neurotoxic molecules, to release large amounts of free oxygen and cytokines, to activate NF-κB, and participates in neural injury and repair, inflammation, and oxidative stress [9, 10]. C-myc has dual roles of facilitating cell proliferation and apoptosis: under oxidative stress ROS activates NF-κB, which initiates apoptosis related gene c-myc to forming dimers with Max for further binding onto DNA core sequence for inducing neuron apoptosis [11, 12]. This study thus established rat brain hemorrhage model, on which the correlation between oxidative stress post-hemorrhage and neuron apoptosis was observed, in addition to gene expression effect of apoptosis related gene c-myc.

Materials and methods

Experimental animal and grouping

Healthy male SD rats (7 weeks old, body weight 220~240 g) were provided by Laboratory Animal Center, Shandong University (Certificate No. SYXXK-2013-0025) and were kept in an SPF grade facility with food and water provided ad libitum. Animals were randomly assigned into sham, model and MPEG-SOD groups (N=28 each). Autoblood transfusion was performed to establish rat hemorrhage model, on which MPEG-SOD was applied via peritoneal injection. Rats in sham or model group received equal volume of saline. Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Shandong Provincial Hospital affiliated to Shandong University.

Drug and reagent

Malonaldehyde (MDA) and superoxide dismutase (SOD) were purchased from Jiancheng Bio (China). Chloral hydrate and paraformaldehyde were purchased from Kemiou Chem (China). Rabbit anti-NF-κB antibody was purchased from Boster Bio (China). Horseradish peroxidase (HRP)-labelled goat anti-rabbit secondary antibody was purchased from CST (US). C-myc antibody was purchased from Santa Cruz (US). DAB staining kit was purchased from Golden Bridge (China). TUNEL test kit was purchased from Roche (US).

Animal model

Neurological deficit score (NDS) was measured 1 h before surgery to exclude those rats with primary neurological deficits. Brain hemorrhage model was generated by autoblood transfusion as previously described [13]. In brief, rats were fasted 8 h before surgery. After anesthesia in 10% hydrate chloral, rats were fixed in supine position, with head fixed in stereotaxic apparatus. Globus pallidus was located with reference to Rat brain atlas. A micro-loading needle was used to transfuse 50 μL artery blood collected from venous blood vessel (at 25 μL/min speed). With 10-min retention, the needle was slowly retracted, with bone wax to seal the drilling hole. The incision was sutured with sterilization. Equal volume of saline was applied in the same location in sham group.

Drug delivery

MPEG-SOD (100 U) was applied via intraperitoneal injection 20 min before model preparation. Equal volume of saline was given for sham and model group.

Neurological deficit score

Longa 5-grade system was used to evaluate rat neurological function. Successful generation of cerebral hemorrhage was deduced as grade II or above after recovery from anesthesia. At 12 h, 1 d, 3 d and 7 d after surgery, NDS was re-evaluated by Garcia system, with a range from 3 to 18.

MDA and SOD level assay and water content quantification in brain tissues

At 12 h, 1 d, 3 d and 7 d after surgery, rats were sacrificed to extract brain tissues from hemorrhage side (N=7 each). Rat brain tissues were washed in pre-cold saline at 4°C, and were weighted after removing surface water. 10% cortical homogenate was prepared for 10 min centrifugation for collecting supernatants. Protein content was quantified using Coomassie brilliant blue colorimetric method. MDA and SOD levels were quantified using test kit following manual instruction and spectrometry meth-
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Tissue samples (1 mm³) were collected from posterior and anterior side of hematoma puncture site for calculating water content, which was defined as (wet weight - dry weight)/wet weight ×100%.

**HE staining**

Rats were fixed in 4% paraformaldehyde and decapitated for extracting brains. Cerebellum, olfactory bulb and lower brain stem were all removed, with remaining brain tissues immersed in paraformaldehyde. Coronal sections were prepared 1.4 mm posterior of Bregma from paraffin block. Hematoxylin-eosin staining was performed for observation under light field microscope.

**Western blotting for brain expression of c-myc protein**

Rat brain tissues around hematoma were lysed in lysis buffer to collect solution via centrifugation. Protein quantification was measured by BCA kit. Protein samples were separated by SDS-PAGE (8 μL loading volume, 75 V for 25
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Results

General information of NDS

No limb immobility was observed in sham group, which had sensitive response for pain stimuli in limb terminus, thus having NDS at grade 0. Both model and treatment group had paralysis of contralateral limbs, with circled gait toward the hematoma side. Significant round-shaped hematoma can be observed when extracting brain tissues. 4 rats died from infection or subarachnoid hemorrhage plus 4 rats with grade 0 NDS after surgery were excluded from the test cohort. Evaluation of cerebral hemorrhage was shown in Figure 1. No significant behavioral change was found in sham group, while model rats had neurological deficits. Most severe deficits were found at 3 d post-surgery, with statistically significant difference of NDS between model and treatment groups at 12 h, 1 d, 3 d and 7 d post-surgery (P<0.05, Figure 2).

Tissue hematoma after surgery

No significant tissue hematoma was observed in brain tissues of sham group. Model group had brain hematoma after surgery, with reaching the peak at 3 d and gradual decrease. MPEG-SOD treatment group had significantly

TUNEL assay for neural tissue apoptosis

Paraffin sections were de-waxed and rehydrated. Proteinase K was added for digestion at room temperature, followed by H$_2$O$_2$-methanol blocking. Tissue slides were rinsed in PBS twice, and in neutral buffers (1 h at room temperature). Hydrogen peroxidase was used for room temperature incubation for 30 min, followed by DAB development and methyl-green counter-staining. Tissue sections were dehydrated, and were observed under light field microscope. Image-pro plus system (Media Cybernetics, IPP imaging analysis software corp, US) was used to analyze total number of positive cells observed under light field microscopy (10 randomly selected fields, 400× magnification).

Statistical methods

SPSS20.0 software was used for statistical analysis. Measurement data were firstly tested for normality. Those fitted normal distribution were presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare means among multiple groups, followed by LSD test in paired comparison. A statistical significance was defined when P<0.05.
lower water content of brain tissues compared to model group at all time points (P<0.05, Figure 3).

Tissue morphology of rat brain

Using HE staining and light-field microscopic observation, no significant change was observed in sham group, which had regular arrangement and intact morphology, along with abundant cell number and normal structure. Model group at all time points had irregular neuron arrangement, cell swelling at different grades, accompanied with inflammatory cell infiltration and gill proliferation. The most severe pathological damage occurred at 3 d after surgery. MPEG-SOD treatment alleviated brain damage at different time points (Figure 4).

MDA and SOD levels of brain tissues

Compared to sham group, model rats had significantly elevated MDA at all time points after surgery, plus lowered SOD level (P<0.05). MPEG-SOD treatment remarkably depressed MDA content and increased SOD level compared to those in model group (P<0.05, Figure 5).
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Figure 5. MDA and SOD level of brain tissues. A: Sham group; B: Model group; C: MPEG-SOD group. *, $P<0.05$ compared to control group; #, $P<0.05$ compared to model group.
NF-κB p65 protein expression assay by IHC

Positive expression of NK-κB p65 protein locates in both nucleus and cytoplasm of cells, as shown by brown granules. Sham group had fewer positive expression of NK-κB p65 in brain tissues, while model group had elevated expression 12 h after surgery, and reached the peak at 3 d, with persistent positive expression until 7 d. MEPG-SOD treatment had lowered NK-κB p65 expression compared to model group at all time points (Figure 6).

TUNEL for neuron apoptosis

Most TUNEL-positive cells locate inside neuronal nucleus as shown by brown yellow color. Positive rate of TUNEL in sham group was relatively lower compared to model group. The ratio of TUNEL positive cell reached a peak at 3 d.
after surgery, followed by gradual decrease. MPEG-SOD group had significantly lower TUNEL positive percentage compared to model group at all time points (Figures 7 and 8).

**Western blotting for c-myc protein expression in brain tissues**

At all time points after surgery, c-myc protein expression levels in rat brain tissues from model group were significantly higher than those of sham group (P<0.05). Model rats had peaked c-myc protein expression at 3 d post-surgery, whilst MPEG-SOD group had lower c-myc protein expression compared to model group at the same time point (P<0.05, Figures 9 and 10).

**Discussion**

Cerebral hemorrhage is common pathological change in clinics, and severely affects patient’s life quality. The alleviation of cerebral hemorrhage injury is of critical for decreasing mortality rate. This study utilized an autologous transfusion method to generate rat brain hemorrhage model, on which the effect of post-hemorrhage oxidative stress on neuron apoptosis was observed. The component of tail artery blood used in this experiment was similar to those of hematomata in clinics. Caudal nucleus in rats is similar to the common site of brain hemorrhage in humans, in addition to its ease for localization. The volume of blood transfusion was determined by the ratio of human and rat brain volume, as 50 μL intracranial blood transfusion in rats was comparable to 40 ml of intracranial hemorrhage in humans. Four rats were excluded due to perioperative infection and subarachnoid hemorrhage, plus 4 rats with grade 0 of post-op NDS. After primary cerebral hemorrhage, brain edema, intracranial hypertension, inflammatory response and cell apoptosis may present due to the release of vasoactive substances from peripheral brain tissues and extension of hematomata [14, 15]. Secondary brain edema can cause brain metabolism disorder, which is an important factor causing brain tissue damage. Brain edema can occur at 1--2 h after hemorrhage, and reaches the peak at 2--3 d followed by gradual alleviation. Study showed consistent time course between peak of post-hemorrhage mortality and brain edema formation [16, 17]. This study thus selected four different time points: 12 h, 1 d, 3 d and 7 d after surgery,
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and investigated the effect of oxidative stress on cell apoptosis after brain hemorrhage. In our results, model rats had brain edema and neurological disorder. With elongated time, neurological deficit was further aggravated and reached the peak at 2 d–3 d, followed by gradual alleviation. Tissue swelling and necrosis to different extents was observed, accompanied with inflammatory cell infiltration and glial cell proliferation. 3 d after surgery, pathological damage reached the peak. MPEG-SOD group had significantly lower water content of brain tissues at all time points, accompanied with decreased brain tissue damage, suggesting that the alleviation of secondary brain edema could help to reduce brain tissue injury post-hemorrhage, and to improve neurological disorder.

MPEG-SOD is one product of SOD coupled with MPEG. With relatively longer half-life, it can exert neuroprotective roles via effectively decreasing content of free radicals, decreasing cell edema, improving micro-circulation, and inhibiting lipid peroxidation [18, 19]. Under physiological condition, body can maintain a homeostatic status between oxidation and antioxidation. Such balance, however, is breached in cerebral hemorrhage, as body’s oxidative level goes beyond anti-oxidative potency, thus enhancing oxidative stress level and aggravating brain injury. MDA level can reflect lipid peroxidation response and cell injury level, while SOD is the primary agent inside body to clear free radicals and to block related cell damage [19]. This study showed elevated MDA level and decreased SOD in model rats at all time points, with the peak reaching at 3 d post-surgery. Compared to model group, MPEG-SOD group had decreased lowered MDA level and elevated SOD level, indicating that SOD could alleviate cerebral hemorrhage injury via inhibiting body oxidative stress level and improving anti-oxidative enzymatic activity, possibly via mitochondrial ATP-sensitive potassium channel and mitochondrial membrane stability [18].

NF-kB signal pathway is involved in cellular hypoxia/ischemia and acclimation of ischemia. Both in vivo and in vitro studies showed the participation of NF-kB in pathology of cerebral hemorrhage, whose secondary injury is also related with NF-kB activation. 2 h after brain hemorrhage, NF-kB is activated and reaches the peak level at 2 d post-hemorrhage [20]. Cytoplasmic NF-kB normally has no activity as it exists in the dimer form with binding onto inhibitory protein IκB. The activation of NF-kB facilitates IκBα expression, for further feedback regulation on NF-kB expression [20]. IκB degradation can be induced by IL-1 or LPS signal. The release and activation of NF-kB lead to nuclear localization for binding onto specific κB sequence, which facilitates transcription of inflammatory mediator and cytokines. Production of large amounts of pro-inflammatory factors and mediators further causes inflammatory response. In this study, 12 h after surgery, positive expression of NF-kB p65 increased from 12 h after surgery, and reached the peak at 3 d post-op. 7 d after the surgery, NF-kB p65 positive expression is still visible. MPEG-SOD group had lower NF-kB positive expression rate compared to model group at the same time point, suggesting that the activation of NF-kB in peri-hematoma tissues after brain hemorrhage can be activated by hypoxia/ischemia, and activation of inflammatory cells, microglia and neurons, further aggra-

![Figure 10. Relative expression of c-myc protein in brain tissues.](image-url)
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vating injury and causing secondary brain tissue damage. During critical steps of early phase initiation of acute inflammatory injury, both post-phosphorylation degradation of IkB, and release plus translocation of NF-κB from complexes are possibly involved [21]. MPEG-SOD may protect hemorrhage brain tissues via alleviating cell edema, improving microcirculation, decreasing expression of inflammatory factors, suppressing NF-κB activation, and inhibiting adhesion between leukocytes and endothelial cells.

Previous studies showed certain correlation between number of TUNEL-positive cells and c-myc protein expression after cerebral damage [21, 22]. This study found significantly elevated TUNEL-positive cell in rat brain hemorrhage model group. TUNEL positive level reached the peak at 3 d after surgery, followed by gradual decrease. Expression of c-myc protein in brain tissues is consistent with TUNEL-positive cells. Both TUNEL-positive cell number and c-myc protein expression were significantly lowered in MPEG-SOD group compared to model group at the same time point, suggesting that ROS could activate NF-κB under post-hemorrhage oxidative stress condition, further activating apoptotic related gene c-myc, which might further facilitate oxidative stress response and induce neuron apoptosis. MPEG-SOD might protect neurons via inhibiting c-myc expression of NF-κB activation.

Conclusion
Neuron apoptosis induced by post-hemorrhage oxidative stress might be related with activation of NF-κB, and initiation of apoptosis related gene c-myc.

Acknowledgements
This work was supported by Shandong Province Clinical Medical Research platform of early Alzheimer’s disease prevention and control fund (No. 2013KJHM180405).

Disclosure of conflict of interest
None.

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