Iranian Journal of Basic Medical Sciences

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Mild hypothermia reduces expression of Fas/FasL and MMP-3 after cerebral ischemia-reperfusion in rats

Jingkun Zhao [1], Shurong Duan [1]*, Jinxia Zhou [2], Ruihong Sun [1], Liming Zhang [1], Desheng Wang [1]

[1] Department of Neurology, The First Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang, China
[2] PuRen Hospital of WuHan, Wu han, China

ARTICLE INFO

Article type:
Original article

Article history:
Received: Jul 11, 2013
Accepted: Dec 9, 2013

Keywords:
Cerebral ischemia/reperfusion
Fas
FasL
Hypothermia
MACO rat
MMP-3

ABSTRACT

Objective(s): To investigate the effects of local mild hypothermia on the expression of Fas, FasL and MMP-3 after cerebral ischemia-reperfusion in rats.
Materials and Methods: Male Wistar rats were divided into sham-operated group (Sham), normothermia group (NT), and hypothermia group (HT). MCAO/R model was established by Longa's method, and reperfusion was allowed after 2 hr occlusion. Mild hypothermia (33±0.5℃) for 6 hr was initiated at the start of reperfusion. Immunohistochemistry was performed to determine expression Fas, FasL, and MMP-3.
Results: Infarct volume was reduced in the hypothermia group (18.43±4.23%) compared with the normothermia group (24.76±5.76%) (P<0.05). In mild hypothermia group, numbers of Fas-positive and MMP-3 positive cells were significantly less than those of normothermia group (P<0.05). Neurological functional scores of mild hypothermia were significantly improved (P<0.05).
Conclusion: Mild hypothermia decreases infarct volume after cerebral ischemia-reperfusion, reduces Fas and MMP-3 expression, but increases FasL in cerebral ischemia-reperfusion rats.

► *Please cite this paper as:*
Zhao J, Duan Sh, Zhou J, Sun R, Zhang L, Wang D. Mild hypothermia reduces expression of Fas/FasL and MMP-3 after cerebral ischemia-reperfusion in rats. Iran J Basic Med Sci 2014; 17:454-459.

Introduction

Cerebral ischemia/reperfusion caused by stroke contributes to worsened neurological outcomes and poor prognosis. Ischemia/reperfusion injury is triggered by a concert of pathological events, leading to inflammation, brain-blood barrier disruption, and neuronal cell death. Current therapeutic strategies for ischemic stroke include pharmacological approaches and neuroprotective modalities (1, 2). Therapeutic hypothermia has been considered a robust neuroprotectant in stroke therapy. The mechanisms underlying neuroprotective action of hypothermia are thought to be multifunctional. Preclinical studies have demonstrated that hypothermia effectively targets a multitude of ischemia-induced pathways including energy depletion, ion shifts, free radical formation, EAA release, and inflammation (3–5). Brain cooling accelerates restoration of ionic homeostasis and inhibits ischemia-induced EAA release (6–8). Moreover, free radical formation and inflammatory responses are suppressed upon hypothermia procedures (7). Hypothermia intervenes in multiple steps of cell death pathway to reduce the ischemic progression following acute stroke (9, 10).

Death receptor-mediated apoptosis plays a critical role in neuronal death following ischemic stroke. Fas (APO-1/CD95) belongs to the tumor necrosis factor receptor (TNFR) superfamily of cell-surface death receptors and is expressed in the nervous system. Fas has been reported to trigger caspase-dependent apoptosis in neuron cells.

Expression of Fas and its ligand have been documented in the brain following ischemia (11), and neutralizing FasL with antibody treatment has been demonstrated to be neuroprotective in experimental *in vivo* models (12, 13). Furthermore, Fas knockout animals have smaller infarct areas than wild-type ones (14, 15). Matrix metalloproteinases (MMPs) can modulate Fas activation by causing proteolytic shedding of FasL from the cell surface (16–18). Several studies have demonstrated that therapeutic hypothermia altered MMP expression in

*Corresponding author: Shurong Duan. Department of Neurology, The First Affiliated Hospital of Harbin Medical University, China. Tel: 86-451-8555-5129; email: duanshurongsj@163.com

stroke (19–21). Moreover, release of soluble Fas ligand (sFasL) has been reported to be reduced in response to hypothermia treatment (22). Although several lines of evidence suggest that hypothermia reduces neuron death through alteration of apoptosis pathway, the mechanism underlying neuroprotective effect of mild hypothermia treatment for ischemic stroke remains sketchy.

We hypothesized that mild hypothermia applied during ischemia modulates death receptor-mediated apoptosis and investigated the molecular mechanism in the ischemic brain. In the present study, we examined the neuroprotective effects of mild hypothemia on infarction, neurological deficits, and apoptosis in a rat MCAO model.

Materials and Methods

Animals and ischemia model

Adult male Wistar rats weighing 300±20 g were purchased from Vital River Laboratories (Beijing, China) and housed in animal centre of Harbin Medical University. Protocol was approved by the animal ethics committee of Harbin Medical University. A middle cerebral artery occlusion (MCAO) was performed as previously described (23). In brief, animals were initially anesthetized with 10% chloral hydrate. Rectal temperature was maintained and monitored at 37°C with a heating blanket. The right common carotid artery, external carotid artery (ECA), and internal carotid artery were isolated. A nylon suture with its tip rounded by heating on flame was advanced from the ECA into the lumen of the internal carotid artery until it blocked the origin of the middle cerebral artery (MCA). 2 hr after MCAO, reperfusion was performed by removing the suture. MCAO rats were evaluated for neurological damages as previously described (23).

A total of 78 rats were used and divided into 3 groups: Sham-operated group (sham; n=6), normothermia treatment (37±0.5) (NT; n= 36), and hypothermia treatment (33±0.5) (HT; n=36). For sham group, rats were treated in the same manner as MCAO rats without ischemia. Hypothermia was performed as previously described (24). MCAO rats received cooling immediately after ischemia for 6 hr and were subsequently maintained in normal body temperature. For NT group, MACO rats were treated with the same procedures without cooling. Animals were sacrificed at 6 hr, 12 hr, 24 hr, 48 hr, 72 hr, and 2 weeks post-ischemia. Brain tissues were harvested for subsequent experiments.

Infarct volume analysis

Rats were sacrificed by carbon dioxide overdose, followed by perfusion with cold normal saline immediately. The brain was removed, fixed with 4% para- formaldehyde and sectioned into 2mm thick slices. Slices were immersed in 2% 2,3,5-triphenyl tetrazolium chloride (TTC, Sigma) and incubated at 37°C for 20 min. TTC-stained slices were photographed and analyzed by Image-J analysis software (http://rsb.info.nih.gov/ij/). Total infarct volume was calculated by summing the clot area in each section and multiplying by the distance between sections. Lesion area was determined as the percentage of the contralateral hemispheric area.

Neurobehavioral evaluation

Neurological status of each rat was evaluated at indicated time points as previously described (25). Rats were suspended by the tail 1 m above the floor and slowly lowered while observing their posture. Rats were then placed on a flat surface and gently pushed from side to side. Deficits were scored as follows: 0, no deficit; 1, forelimb flexion while suspended by the tail; 2, decreased resistance to lateral push. Animals were also examined by the modified balance beam test (26). Animals were placed on a narrow beam to walk for 60 sec. Deficits were scored based on the scale: 0, steady posture with paws on top of beam; 1, paws on side of beam or wavering; 2, one or two limbs slip off beam; 3, three limbs slip off beam; 4, rat attempts to balance with paws on beam but falls; 5, rat drapes over beam and falls without attempt.

Immunohistochemistry

Brain samples were paraffin embedded and cut into 2 mm thick sections. After deparaffinization, sections were blocked in 1% bovine serum albumin in PBS and incubated with primary antibodies against Fas, FasL, and MMP-3 followed by treating with biotin-labeled anti-IgG secondary antibody. Antibody complexes were detected using the Vector ABC kit (Elite Vectastain ABC kit, Vector Labs) and colorized with 0.05% diaminobenzidine (DAB, Vector Labs). Negative controls were run in parallel using adjacent sections incubated with IgG instead of the primary antibody.

Statistical analysis

The data are expressed as mean ± S.D. Student's *t*-test or Dunnett *t*-test were used to compare the differences between treated groups and control groups, and differences were considered significant at $P< 0.05$.

Results

Mild hyperthermia reduces infarct volume

To evaluate the effects of mild hypothermia on ischemic stroke, MCAO rats were treated with different temperatures and analyzed by a set of histological tests. The mortality of sham, normothermia and hypothermia groups were 0%, 21%, and 13%, respectively. Significant neurological damage was observed in the sham-operated MACO

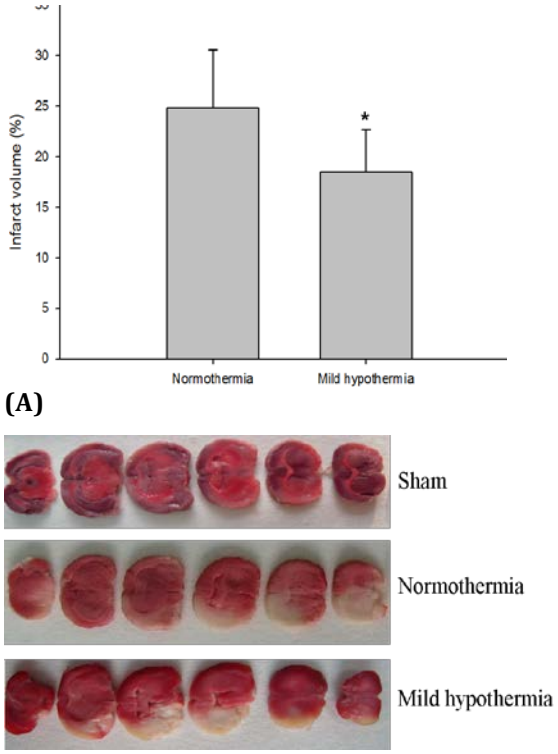

Figure 1. Effect of mild hypothermia on infarct volume in MCAO rats. Infarct areas of sham, normothermia-treated and mild hypothermia were analyzed by TTC staining. (A) Infarct volume was expressed as percentage of infracted region in reference to contralateral hemisphere. (B) Representative image of each experimental group. *P<0.05, hypothermia group compared with normothermia group

rats. The relative percent of infarct volume in normothermia-treated animals was 24.76±5.76%. Mild hypothermia treatment significantly reduced the infarct volume to 18.43±4.23%, compared with the normothermia-treated group (P< 0.01) (Figure 1A). Neuronal damage in rats was evaluated after MCAO by the TTC staining method. Representative images of each experimental group were presented in Figure 1B.

Mild hypothermia improves neurobehavioral outcomes in rats

To investigate the effect of mild hypothermia on neurobehavioral deficits in MACO rats, MACO rats were treated with cooling procedures and examined using two behavioral tests. Mild hypothermia treatment significantly improved neurological outcomes in MACO rats compared with that of the normothermia-treated animals. The scores of balance beam test in normothermia and hypothermia groups were 2.17±0.41 and 0.83±0.75, respectively (P<0.05). Using Berderson test, differences in neurological scores between normothermia (1.83±0.75) and hypothermia groups (0.67±0.52) were significant (P< 0.01) (Table 1).

Table 1. The neurobehavioral deficit scores of normothermia and hypothermia groups

Group	Animal numbers	Balance beam test	Berderson test
Normothermia	6	2.17±0.41	1.83±0.75
Mild hypothermia	6	0.83±0.75*	0.67±0.52*

Results are shown as mean±SD, *P<0.05, Normothermia group compared with hypothermia group

Table 2. Percentage of Fas-positive cells in brain of MACO rat after reperfusion with different brain cooling processes

Reperfusion time (min)	Animal numbers	Normothermia (°C)	Mild hypothermia (°C)
6	4	22.05±4.70	14.20±5.96*
12	4	23.75±8.45	18.65±5.53*
24	4	29.70±6.59	22.90±4.98*
48	4	24.40±6.79	19.95±6.19*
72	4	20.75±4.71	12.35±4.34*

Results are shown as mean±SD, *P<0.05, Hypothermia group compared with normothermia group

Mild hypothermia modulates expressions of Fas and FasL in MACO rats after reperfusion

We further examined the effect of mild hypothermia on expression levels of Fas and FasL using immunohistochemistry. We observed the elevated expression of Fas in the brain of MACO rats after reperfusion. From the immunohistochemistry analysis, the number and intensity of Fas immunoreactivity were higher in normothermia group than in hypothermia group over the designated time frame (Table 2). In addition, ischemia- induced FasL level was significantly higher in hypothermia group than in normothermia group at 6 and 12 hr post reperfusion (P<0.05) (Table 3). Nevertheless, number and intensity of FasL-positive cells were higher in mild hypothermia group than in normothermia group over the designated time frame.

Table 3. Percentage of FasL-positive cells in brain of MACO rat after reperfusion with different brain cooling processes

Reperfusion time (min)	Animal numbers	Normothermia (°C)	Mild hypothermia (°C)
6	4	38.75±14.02	55.05±24.56*
12	4	59.10±9.16	69.45±13.65*
24	4	46.00±21.33	50.78±14.49
48	4	36.10±8.84	40.78±18.36
72	4	33.40±8.76	37.95±12.77

Results are shown as mean±SD, *P<0.05, Hypothermia group compared with normothermia group

Table 4. Percentage of MMP-3 positive cells in brain of MACO rat after reperfusion with different brain cooling processes

Reperfusion time (min)	Animal numbers	Normothermia (°C)	Mild hypothermia (°C)
6	4	63.80±22.70	48.75±19.76*
12	4	46.00±11.85	36.60±15.67*
24	4	46.75±12.89	36.90±16.48*
48	4	42.50±19.20	29.95±19.20*
72	4	32.20±12.82	26.50±5.79

Results are shown as mean±SD, *P<0.05, Hypothermia group compared with normothermia group

Mild hypothermia alters expressions of MMP-3 in brain of MACO rats after reperfusion

As FasL level is associated with the activity of MMP-3, we next determined the expression of MMP-3 in brain tissues of MACO rats after reperfusion undergoing different temperature treatments. Expression of MMP-3 was absent in sham group (data not shown). Level of MMP-3 was significantly increased in response to reperfusion with its peak at 6 hr post-reperfusion (Table 4). Ischemia- induced MMP-3 expression was significantly lower in hypothermia group than in normothermia group at 6, 12, 24, and 48 hr post reperfusion (P<0.05).

Discussion

In the present study, we demonstrate that mild hypothermia ameliorates neurological deficits in rat undergoing MACO and reperfusion. The improvement is associated with a decrease in Fas expression and elevated expression of FasL in comparison with those of normothermia treatment. Moreover, our results show that level of MMP-3 is relatively lower in ischemic/reperfusion rats treated with mild hypothermia compared with that of normothermia-treated ones. According to clinical observations the window of thrombolytic therapy is considered to be 3 hr; the experimental model was designed to have animals undergo ischemia for 2 hr followed by reperfusion (27).

Ischemic stroke is associated with significantly high mortality and disability. Cerebral ischemic cascade is complex with spectrum of modulatory molecules, signaling pathways, and proteins that are involved in maintaining the micro-environment in aspect of fate of neuron cells. Despite reperfusion restoring blood flow, it also leads to secondary brain damages resulting from edema, inflammation and oxidative stress. Mild hypothermia has been demonstrated to be a robust treatment for cerebral ischemic injury, pre-clinically and clinically (2, 18). Brain cooling is thought to manipulate metabolic stores and decelerate biological and biochemical reactions responding to ischemia (28, 29). Several studies have reported that therapeutic hypothermia inhibits ischemia-induced apoptosis with results of decreased apoptotic cells and biochemical events associated with the intrinsic and extrinsic pathways (30–34). In addition, hypothermia treatment leads to resistance of cell to apoptotic stimuli including Fas ligation (35, 36).

Our findings are consistent with previous studies in which both Fas and FasL were found localized in neurons and astrocytes of peri-ischemic areas, whereas the expressions were absent in sham-operated animals (14, 22). It has been documented that Fas expression is increased in 30 min after ischemia and reaches a peak at 6 hr post ischemia (22, 37, 38). It has been reported that MMPs cleave FasL to form sFasL that binds to Fas triggering apoptosis (22, 39). Our data show that mild hypothermia upregulates FasL expression in response to ischemia/ reperfusion. Given ameliorated neurobehavioral outcomes, it is suggested that elevated FasL in animals undergoing mild hypothermia remain membrane bound. The finding is supported by further experiments focusing on MMPs expression in ischemic brain.

MMPs have been documented to be upregulated in response to cerebral ischemia (40, 41). They are involved in neuroinflammation, cleavage, and activation of other MMPs and shedding of death receptors. Increasing evidence has shown that MMP-3 plays a critical role in modulating inflammation and cell death (42). MMP-3 initiates inflammatory procedures in a Parkinson's model with production of TNF-α and other cytokines in microglial cells (43, 44). LPS-induced inflammation leads to increased IL1β stimulation and astrocyte expression of MMP-3 (45). A recent study has reported that MMP-3 is involved in neuron apoptosis after ischemia/reperfusion through FasL shedding (22). Our data demonstrate an increase in the expression of MMP-3 parallel with worsened neurobehavioral outcome in ischemia/reperfusion animals. In addition, the elevated MMP-3 expression was suppressed by mild hypothermia and resulted in improved neurologic deficits, suggesting the absence of MMP-3 may lead to an improvement in neuron death.

Conclusion

We demonstrate that mild hypothermia modulates expression of the Fas and FasL that are involved in apoptotic pathway. Mild hypothermia decreases MMP-3 activation leading to improved neurobehavioral outcomes due to less sFasL release.

Acknowledgment

The present study was supported by the Heilongjiang Province Nature Science Fund (D200938), and Heilongjiang Province Health Department Fund (2006-052).

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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