

Effect of cold irritation on peripheral white blood cell and ICAM-1, IL-1 β expression of brain tissue in rat

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Abstract

Background: Some studies have showed that hypothermia is a neuroprotective factor for cerebral ischemic injury. Inflammation reaction plays a very important role in pathomechanism of neuron degeneration disease induced by cerebral ischemia. However whether there is relationship between cold irritation and inflammation reaction is not well known. **Objective:** To explore effect of cold irritation on peripheral white blood cell and ICAM-1, IL-1 β expression of brain tissue in rat with cerebral ischemia. **Methods:** Model rats were put into low temperature water (0°) for cold irritation for 5 minutes one time every day for 20 days. MCAO rats were subjected to middle cerebral artery occlusion (MCAO) using an intraluminal suture method with permanent ligation of the ipsilateral common carotid artery. We assessed count of the peripheral white blood cells. The brains of all rats were cut at 1, 3, and 5 days after cerebral ischemia and frozen brain tissues were continuously sliced and stained immunohistochemically with Intercellular Adhesion Molecule-1 (ICAM-1) or Interleukin-1(IL-1) antibody. **Results:** Cold irritation model rats were associated with increased leukocyte at 1 and 3 days post-ischemia, increased ICAM-1-positive vessels at 1, 3, and 5 days, and increased interleukin-1(IL-1) at 3 and 5 days. Vascular pathology of the hippocampus at electron microscope levels showed that the blood vessel has inflammation infiltration at 1, 3 and 5 days. **Conclusion:** These data demonstrate that cold irritation significantly increased endothelial adhesion molecule expression, leukocyte infiltration, and vascular pathology of the hippocampus in rat. The mechanism of neuron injury may be related to the inflammation reaction induced by cold irritation.

Keywords: cold irritation, inflammation reaction, interleukin-1, intercellular adhesion molecule

Introduction

A large number of studies have been conducted in recent years that have consistently shown hypothermia to be an effective means of reducing cerebral ischemic injury in both global and focal models of stroke. Interleukin -1L (IL-1) begins to increase at 15 min and peak at 1-2 h later after transient middle cerebral artery occlusion (tMCAO).¹ Polymorphonuclear leukocytes(PMNLs, or neutrophils), another central component, play an important role in the progress of inflammatory response. Neutrophils appear within hours of focal cerebral ischemia, peaking in 1-2 days later. And then they are replaced by monocytes/macrophages at 3-7 days.² At the same time, adhesion molecules which can be locked on the surfaces of leukocytes and endothelial cells were one key step of leukocyte rolling, margination and transendothelial migration. ² Intercellular Adhesion Molecule-1 (ICAM-1) was expressed in microvessels at 1-3 h, and peaked at 24- 48 h and lasting for about 7 days² in brain of rats with focal cerebral ischemia.

Two hours hypothermia during ischemia can significantly reduce the brain infarct volume of rats at 1, 3 and 7 days following 2 h occlusion of the middle cerebral artery³ (MCA). Inflammation reaction plays a very important role in pathomechanism of neuron degeneration disease induced by cerebral ischemia. While whether cold irritation (0°) shows neuroprotective function or neuron damage in brain of rats is still not known. Whether there is relationship between cold irritation and inflammation reaction is also not known. So the purpose of this study is to explore the effect of cold irritation on brain tissue and peripheral white blood cell and ICAM-1, IL-1 β expression in brain of a rat with cold irritation.

Materials and methods

Experimental Procedures. Animal protocols were approved by the Stanford University Administrative Panel on Laboratory Animal Care. Institutional guidelines were followed in all protocols. All animal experiments

were conducted in accordance with the NIH guide for the care and use of laboratory animals (NIH publication 80- 23). All efforts were made to minimize animal suffering, and only the smallest number of animals were used to generate reliable scientific data.

Animals and Experimental groups. Adult male Sprague-Dawley rats, weighing 200-250 g, were obtained from the Experimental Animal Center of Beijing University, China. The environment condition: air temperature was $22\pm 2^\circ$ and air humidity was 60%. They were maintained under controlled lighting (lights on 07:00–19:00 h) and given free access to water and the commercial laboratory rodent diet. Twelve hours prior to experiment, the rats were fasted, but allowed free access to water. They were randomly divided into three groups (72 rats per group): 1, 3 and 5 days survival. Ice water group, MCAO group and normal group.

Preparing of cold irritation model rats. Model rats were put into low temperature water (0°) for cold irritation for 5 minutes one time every day for 20 days. During the progress of cold irritation rats was ensured keeping their head or nose above water, and at the same time the body of rats were submersed into the cold water. Adding other ice into the cold water so that keeping water temperature. Rats were put mouse cages and permitted access to water and the commercial laboratory rodent diet after cold irritation.

Preparing of Middle Cerebral Artery Occlusion rats. Male Sprague-Dawley rats weighing between 290 and 320 g (Charles River, Wilmington, Del) were anesthetized with 3% halothane by facemask and were subsequently maintained with 1% halothane in 200 ml/ min oxygen and 800 ml/min air. Depth of anesthesia was assessed every 15 min by hind-limb pinch. A thermistor probe was inserted 50 mm into the rectum and rectal temperature was maintained between 36.5° and 37.5° during ischemia. ECG leads were placed to monitor heart rate and respirations. Physiological parameters were monitored every 15 min and maintained in the normal range throughout surgery. The MCA was occluded using an intraluminal suture previously used by our lab.^{4,5} In brief, a midline incision was made in the neck to expose the common carotid (CCA), external carotid (ECA), internal carotid (ICA), and pterygopalatine (PPA) arteries.

The CCA, ECA, and PPA were ligated with a 6-0 silk suture. Ischemia was induced by inserting an uncoated, 30-mm long segment of 3-0 nylon monofilament suture (tip rounded by flame) 19-20 mm from the bifurcation of the CCA to induce ischemia in the arterial territory supplied by the MCA. After 2 h of ischemia, the suture was removed and the animal was allowed to recover. The rats must be fed for 20 days.

Plasma measurements and Counts of WBC. Measurement of the plasma concentration of various metabolites and hormones was carried out, either in trunk blood collected at time of death during ad libitum feeding (leptin) or in tail-tip samples obtained after overnight food deprivation, six at 09:00 h or 30 min after rats were given 2 g/kg glucose in water by gavage.⁶ Six rats per group were respectively anesthetized at 1, 3 and 5 days post-ischemia, counts of the peripheral white blood cells was assessed.

Tissue Fixation. At the completion of the experiment, animals were killed at the specified time points with 10% Chloral Hydrate halothane overdose and prepared for histological analysis. Animals were perfused intracardially with normal saline followed by 4% paraformaldehyde 10% formalin. Brains were quickly removed and sliced into 30-mm-thick coronal sections. Brain slices were then fixed in 10% buffered formalin (pH 7.4) for 1 week. For ICAM- α , IL-1 immunohistochemistry, animals were perfused with normal saline only. Brains were quickly removed and cryopreserved in 20% sucrose/ phosphate-buffered saline (PBS) solution for 24 h. Brains were then sliced into 30- m-thick coronal sections, flashfrozen on dry ice in OCT (Miles) and stored at -70° until use. 25- μ m-thick sections were cut on cryostat from the frozen brain slices and placed on Superfrost Plus slides (Fischer Scientific). Sections were air dried for 24 h and then fixed for 10 min in 75% acetone/25% ethanol prior to use.⁷

Freeze Sectioning. Tissue blocks from five rats were cryoprotected by soaking in a 30% sucrose solution in PB until they sank. Parallel series of 50 μ m thick coronal sections were then obtained on a freezing microtome. For cytoarchitectonic reference, one series of sections was mounted onto gelatin-coated glass slides, air-dried, stained with cresyl violet, dehydrated and coverslipped. Other series of sections from which the present material was taken were soaked in a buffered 20% ethylglycol solution and stored at -20°C .

Immunohistochemistry for Light Microscopy.

Prior to beginning the immunohistochemical protocol, a series of three to eight coronal sections that as a group covered a variety of rostrocaudal levels of the brain and brainstem were selected from each brain. Sections included samples from a wide variety of coronal levels across the cerebral hemispheres, thalamus, hypothalamus, mesencephalon, pons, rostral medulla oblongata and cerebellum; the olfactory bulbs were not included. Sections were thoroughly rinsed in PB at 4°C for 48 h. Sections were then pretreated with a 1% hydrogen peroxide solution in phosphatebuffered saline (PBS) solution for 20 min, rinsed, and subsequently blocked with 10% horse serum + 3% bovine serum albumin + 0.5% Triton X-100 in 0.1 M PBS. Sections were incubated for 48 h at room temperature, either in mouse monoclonal IgG 142 (1:400,

Namur, Belgium), or mouse monoclonal IgG CR-50 (1:400, RIKEN, Japan). A biotinylated horse anti-mouse IgG (Pierce, Rockford, IL, 1:200) was used as secondary antibody. ICAM-1 staining was performed on fresh frozen tissue using a murine monoclonal Ab (1A29, Serotec, 1:50). Immunoreagents were diluted in 0.1 M PBS containing 3% normal horse serum and 0.1% Triton X-100. Sections were subsequently incubated in avidin–biotinylated horseradish peroxidase complex (ABC, Vector Laboratories, Burlingame, CA) in 0.1 M PBS for 1 h, and developed with 0.01% H₂O₂ + 0.04% 3,3'-diaminobenzidine tetrahydrochloride (DAB) in acetate buffer pH 6. In some experiments, we enhanced the opacity of the reaction product by including 2.5% nickel sulfate in the developer medium (Ni-DAB). Multiple rinses in PBS were performed between each of the above steps. The specificity of the monoclonal antibodies used is well characterized.⁸ In addition, each experiment included a control section processed without the primary antibodies and this always resulted in the absence of immunostaining. The concentration of primary antibodies was tested and optimized in preliminary experiments. Sections were mounted on gelatin-coated glass slides and air-dried. Some sections were lightly counterstained with cresyl violet.

All sections were finally dehydrated in graded alcohols, cleared in xylene, and coverslipped with DePeX. Ultrathin Re-sectioning and Electron Microscopy. Cells with identifiable cellular morphology and evident Reelin immunostaining were selected (n = 6) from the semithin sections. These cells were photographed and their location recorded on detailed camera lucida drawings. Under a stereomicroscope, the tissue region (-2 mm²) containing each cell was then dissected, flatmounted in Araldite, and re-sectioned in ultrathin (60– 80 nm) sections.⁹ Some ultrathin sections were intensified with lead citrate (0.4%), while other sections were left without intensification. Sections were visualized at 1000–100 000x using a JEOL JEM 1010 transmission electron microscope. Sections were imaged for analysis with a Bioscan digital imaging system (Gatan, Pleasanton, CA, USA). For the purpose of illustration, the regions of interest were directly photographed on film.

Data analysis. From ICAM-1 IL-1-stained tissue, immunopositive vessels were counted in a blinded fashion from four adjacent fields (100×) in each of the four regions described above. Immunopositive vessels were expressed as mean number of vessels per field. All data was collected from single sections taken through the central region of the infarct.

Statistical analysis. Statistical analyses for continuous data were performed using a one-way analysis of variance followed by a multiple comparison procedure (Bonferroni post-hoc test). All data are expressed as mean±S.E.M. P<0.05 was considered significant.

# WBC cells (mean ± S.E.M.)	1d	3d	5d
Normal group	4.25 ± 1.28	4.26 ± 1.22	4.24 ± 1.18
Cold irritaton group	10.21 ± 1.31*	11.02 ± 1.43*	8.01 ± 1.21
MCAO group	11.33 ± 1.83*	11.38 ± 1.92**	8.12 ± 1.27

Table 1. Changes of counts of WBC cells (×10⁹/L) in rats of 1, 3 and 5 days post-ischemia. Counts of the peripheral white blood cells in Cold irritaton group and MCAO group increased at 1 and 3 days post-ischemia, Mean±SEM. n=8. *P<0.05, **P<0.01 vs normal group.

# OD Mean (mean ± S.E.M.)	1d	3d	5d
Normal group	15.53 ± 1.08	15.67 ± 1.18	15.67 ± 1.12
Cold irritaton group	20.86 ± 1.12*	21.93 ± 1.21*	21.05 ± 1.34*
MCAO group	22.98 ± 1.05**	21.67 ± 1.05*	20.53 ± 1.06*

Table 2. Expression of ICAM-1 in CA1 region of hippocampus in rats of 1, 3 and 5 days post-ischemia. Expression of ICAM-1 in CA1 region of hippocampus in Cold irritaton group and MCAO group increased at 1, 3, 5 days post-ischemia, Mean±SEM. n=8. *P<0.05, **P<0.01 vs normal group.

Results

Changes of counts of WBC. Counts of the peripheral white blood cells in cold irritation group and MCAO group increased at 1 and 3 days after ischemia. There were significantly different compared with normal group (*P<0.05, **P<0.01). But there were not significantly different compared between cold irritation group and MCAO group(P>0.05)(Table 1).

Expression of ICAM-1 in CA1 region of hippocampus. Expression of ICAM-1 in CA1 region of hippocampus increased at 1, 3, and 5 days after ischemia in cold irritation group and MCAO group. The optical density mean (OD Mean) were significantly different compared with normal group (*P<0.05, **P<0.01). But there were not significantly different compared between cold irritation group and MCAO group(P>0.05) (Table 2, Figure 1).

Expression of IL-1 in CA1 region of hippocampus. Expression of IL-1 in CA1 region of hippocampus increased at 3 and 5 days after ischemia, but not at 1 day in cold irritation group and MCAO group. The optical density means (OD Mean) were significantly different compared with normal group (*P<0.05, **P<0.01). But there were not significantly different compared between cold irritation group and MCAO group(P>0.05) (Table 3, Figure 2).

Vascular pathology of the hippocampus at electron microscope.

Vascular pathology of the hippocampus at electron microscope levels showed that there was inflammation infiltration in brain microvessel at 1, 3 and 5 days, and there was a great deal of inflammation infiltration at 3 days. There were angiostegnosis, swelling of endothelial cell mitochondria and micrangium periphery astrocyte cellular (Figure 3).

# OD Mean (mean ± S.E.M.)	1d	3d	5d
Normalgroup	20.76 ± 1.86	20.53 ± 1.67	20.73 ± 1.82
Cold irritaton group	20.98 ± 1.12	21.76 ± 1.1 2*	21.73 ± 1.58*
MCAO group	21.01 ± 1.90	22.45 ± 1.94**	21.82 ± 1.81*

Table 3. Expression of IL-1 in CA1 region of hippocampus in rats of 1, 3 and 5 days post-ischemia. Expression of IL-1 in CA1 region of hippocampus in Cold irritaton group and MCAO group increased at 3,5 days post-ischemia, Mean±SEM. n=8. *P<0.05, **P<0.01 vs normal group

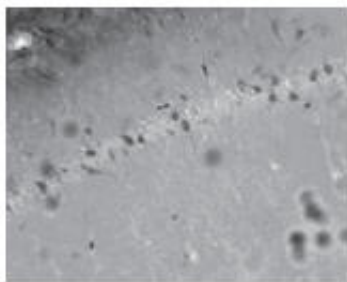


Normal group

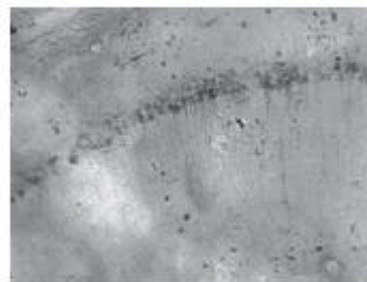
MCAO group

Cold irritation group

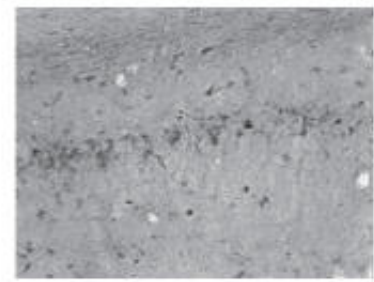
Figure 1. Expression of ICAM-1 in CA1 region of hippocampus in rats of 1, 3 and 5 days post-ischemia. Expression of ICAM-1 in CA1 region of hippocampus in Cold irritation group and MCAO group increased at 1, 3, 5 days post-ischemia, Mean±SEM, n=8. *P<0.05, **P<0.01 vs normal group



Normal group



MCAO group

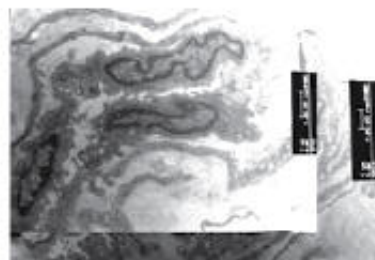


Cold irritation group

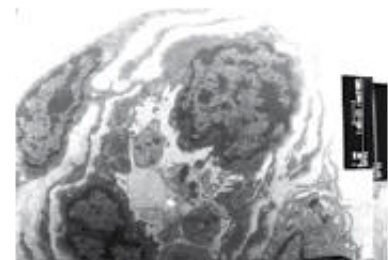
Figure 2. Expression of IL-1 in CA1 region of hippocampus. Expression of IL-1 in CA1 region of hippocampus increased in Cold irritation group and MCAO group. Anti-IL-1-stained×10×10



Normal group



MCAO group



Cold irritation group

Figure 3. Vascular pathology of the hippocampus at electron microscope. Normal group (×5000); Cold irritation group (×8000); MCAO group (×6300). Cold irritation group and MCAO group angiostegnosis and endothelial cell mitochondria was swelling, microvessel periphery astrocyte cellular swelling

Discussion

Hypothermia is a neuroprotective factor against cerebral ischemic injury. This study showed that cold irritation can increase positive expression of ICAM-1 in brain vessels at 1, 3, and 5 days, and increase positive expression of interleukin-1 (IL-1) at 3 and 5 days. Vascular pathology of the hippocampus at electron microscope levels showed that there were a great deal of inflammation infiltration in brain microvessels at 1, 3 and 5 days.

Leukocyte adhesion to endothelium is necessary for initiation of the peripheral immune response. Once activated, leukocytes bind to endothelial ICAM-1 through their CD11/CD18 leukointegrin. ICAM-1 constitutively expressed at low level on vascular endothelium, but during the progress of ischemic insult and subsequent reperfusion, ICAM-1 has been shown to be dramatically upregulated in brain microvasculature.¹⁰⁻¹⁴ ICAM-1 mRNA has been detected at 1 h post-ischemia, reaching peak levels at 10 h after reperfusion.^{15, 16} Several studies have now shown that antagonists to adhesion molecules effectively reduce reperfusion injury after MCAO.^{17, 18} Additionally, ICAM-1-deficient mice have been shown to be resistant to cerebral ischemic injury.^{19, 20} Recently, Mabuchi and colleagues conducted a study which showed that microglia and macrophages, with their IL-1L production, contributed significantly to the expansion of infarct following focal cerebral ischemia in rats.²¹

We explored the effect of cold irritation on the number of peripheral white blood cell and the protein expression of ICAM-1, IL-1fA in brain tissue of rats. Results indicated that cold irritaion can lead to neuron

damage, increase endothelial adhesion molecule expression and enhance leukocyte infiltration in brain of rats. The mechanism of neuron damage may be related to the activation of inflammation reaction induced by cold irritation.

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