Isoflurane Enhanced Hemorrhagic Transformation by Impairing Antioxidant Enzymes in Hyperglycemic Rats With Middle Cerebral Artery Occlusion

Qin Hu, PhD; Qingyi Ma, PhD; Yan Zhan, MD; Zhaohui He, MD; Jiping Tang, MD; Changman Zhou, MD, PhD; John Zhang, MD, PhD

Background and Purpose — Because the potential neuroprotective effect of isoflurane is controversial, we attempted to study whether isoflurane after treatment provides neuroprotection in a rat model of hyperglycemia-induced ischemic hemorrhagic transformation.

Methods — Rats received an injection of 50% dextrose (6 mL/kg intraperitoneally) and had a middle cerebral artery occlusion 30 minutes later. Four groups were included: sham-operated, ischemia/reperfusion, isoflurane treatment, and vehicle groups. In the treatment group, after 2 hours of ischemia, 2% isoflurane was administered at the onset of reperfusion. We measured the level of blood glucose at 0, 2.5, 4.5, and 6.5 hours after dextrose injection. Infarct and hemorrhagic volumes, neurological scores, oxidative stress (malondialdehyde, 4-hydroxy-2-nonenal, and nitrotyrosine) and the activities of superoxide dismutase and catalase were measured at 24 hours after ischemia.

Results — Isoflurane had no effects on blood glucose, it failed to reduce infarct, hemorrhage volume, and brain edema, and it enhanced neurobehavioral deficits when compared with the ischemia/reperfusion group at 24 hours after middle cerebral artery occlusion. On the contrary, isoflurane exacerbated these parameters compared with the vehicle group. In addition, it increased the expressions of malondialdehyde, 4-hydroxy-2-nonenal, and nitrotyrosine, and it decreased the activities of superoxide dismutase and catalase compared to the vehicle group.

Conclusions — Isoflurane after treatment worsened physiological and neurological outcomes in this ischemia hyperglycemia-induced hemorrhagic transformation possibly by impairing the antioxidant defense system. 

Key Words: hemorrhagic transformation • hyperglycemia • isoflurane • middle cerebral artery occlusion • oxidative stress

Hyperglycemia has been claimed to be associated with hemorrhagic transformation (HT), which is a major factor limiting the use of tissue plasminogen activator, the only Food and Drug Administration-approved treatment for ischemic stroke.1 Experimental studies have shown that pre-ischemic hyperglycemia dramatically aggravated brain infarct and hemorrhagic conversion in rat middle cerebral artery occlusion (MCAO) in a reperfusion model.2 Hyperglycemia-enhanced HT may be linked to increased inflammatory activity and oxidative stress,2–3 which cause blood–brain barrier disruption and neural cell death. There is currently no effective treatment to prevent HT after ischemic stroke.

As one of the most widely used volatile anesthetics, isoflurane (ISO) has been recognized for its potential neuroprotective properties since the 1980s.4 Some evidence indicated that ISO, administered before or after experimental cerebral ischemia, provided neuroprotection in in vivo and in vitro models.5–8 However, an equivalent number of experiments suggested that ISO exposure during experimental focal or global stroke offered little or no protection and may even worsen histological and functional outcomes.9–11 In addition, the effect of ISO on hyperglycemia-induced HT after stroke had not been tested. Therefore, we attempted to study the effects of ISO after treatment on acute hyperglycemia-induced HT after MCAO in rats.

Materials and Methods

Animal Preparation and MCAO

All experiments were approved by the Institutional Animal Care and Use Committee of Loma Linda University; 131 male Sprague-Dawley rats were purchased from Harlan Laboratories (Indianapolis, IN) and randomly divided into the following groups: sham-operated...
(dextrose [DX]+sham; n=23), ischemia/reperfusion (DX+MCAO; n=31), ISO treatment (DX+MCAO+ISO; n=42), vehicle groups (DX+MCAO+O2; n=28), and additional sham-operated group treated with ISO for testing the effects of ISO on blood glucose of hyperglycemic rats (DX+sham+ISO; n=7). All rats were injected with 50% DX (6 mL/kg) intraperitoneally 30 minutes before MCAO to induce acute hyperglycemia. Anesthesia was induced with ketamine and xylazine (80 mg/kg and 10 mg/kg; intraperitoneally), followed by atropine at a dose of 0.1 mg/kg subcutaneously. During surgery and postoperative period, rectal temperature was maintained at 37.5°C by means of a feedback-controlled heating pad.

MCAO was performed as reported previously. The external carotid artery was isolated and coagulated, a 4-0 nylon suture with a round tip was inserted into the internal carotid artery through the external carotid artery stump, occluding the MCA for 2 hours. Blood was obtained from the tail artery for analysis of glucose level before, during, and after the operation.

ISO After Treatment
In the treatment group, 2% ISO carried by 30% O2 and 70% medical air was administered for 1 hour immediately after ischemia via a chamber. In the vehicle group, rats were placed in the chamber only with 30% O2 and 70% medical air, in which the total concentration of O2 was ~45% (Supplement 1, http://stroke.ahajournals.org).

2,3,5-triphenyltetrazolium Chloride Staining and Evaluation of Infarction Volume
As studied previously, 2,3,5-triphenyltetrazolium chloride monohydrate staining was performed to determine the infarct volume at 24 hours after MCAO (DX+MCAO+ISO, n=8; other groups, n=6). The possible interference of brain edema with infarct volume was corrected by standard methods (whole contralateral hemisphere volume−nonischemic ipsilateral hemisphere volume) and the infarcted volume was expressed as a percentage of the whole contralateral hemisphere.

Spectrophotometric Assay of Hemoglobin
Rats were anesthetized and subjected to complete transcardiac perfusion to remove intravascular blood. The brain was minced and homogenized in 0.1 mol/L phosphate-buffered saline to give 10% (wt/vol) homogenates. The homogenates were then made into a standard curve and used to determine the concentration of hemoglobin and malondialdehyde (MDA) (DX+MCAO+O2, n=5; other groups, n=6).

Cerebral hemorrhage was quantified with a previously described spectrophotometric assay with some modifications. A standard curve was obtained using a “virtual” model of hemorrhage. Incremental volumes of homologous blood (0, 2, 4, 8, 16, 32 μL) were added to the perfused brain. After homogenization and centrifugation, Drabkin reagent (1.6 mL; Sigma) was added to 0.4 mL supernatant aliquots and optical density was measured at 540 nm with a spectrophotometer (Spectronix 3000; Milton-Roy). Hemorrhage measurements of the samples were performed and compared with this standard curve to obtain data in terms of hemorrhage volume (μL).

Neurological Deficits
Twenty-four hours after MCAO, a neurological examination was performed by a blinded investigator as previously described with modifications. The scores given to each rat at the completion of the evaluation was the summation of all 7 individual test scores. (Supplement 2).

Brain Water Content
Animals were anesthetized and decapitated 24 hours after MCAO, as described in our previous study (DX+MCAO+ISO, n=6; other groups, n=5). Brain samples were immediately weighed on an electronic balance (model AIE 100; Mettler Instrument) to obtain wet weight and then dried in a gravity oven at 120°C for 48 hours to obtain the dry weight. Brain water content was then calculated as (wet weight−dry weight)/wet weight.

Measurement of MDA Content
MDA content was measured with the thiobarbituric acid reaction. The method was used to obtain a spectrophotometric measurement of the color produced during the reaction of thiobarbituric acid with MDA at 535 nm; estimated MDA level was expressed as nmol/mg protein.

Western Blot
The brain samples were collected at 24 hours after MCAO (DX+sham, n=6; DX+MCAO, n=7; DX+MCAO+ISO, n=8; DX+MCAO+O2, n=7). Proteins of the ipsilateral hemisphere were extracted by homogenizing in 0.1 mol/L phosphate-buffered saline. Western blotting was performed as described previously using mouse anti-4-hydroxy-2-nonenal (ab48506) and mouse antibodies anti-3-nitrotyrosine (sc-32757).

Activities of Superoxide Dismutase and Catalase
The brain tissue supernatants came from the same used for Western blot and the blood samples were taken from the heart before euthanizing the rats (n=6 for each group). Superoxide dismutase (SOD) activity was measured after the reduction of nitrite by a xanthine-xanthine oxidase system (ab65354), and catalase (CAT) activity was assayed by measuring absorbance at 570 nm according to the protocol (ab83464).

Statistical Analyses
Data were expressed as the mean±SEM. Statistical differences among groups were analyzed by using 1-way analysis of variance followed by the Turkey method. Mortality was analyzed by Fisher exact test. P<0.05 was considered statistically significant.

Results
Physiological Data
The glucose levels in all groups during surgery were significantly higher than the level at the time point of dextrose injection. ISO had no effects on the blood glucose level (Figure 1).

ISO Deteriorated the Outcomes of Hyperglycemia Induced HT 24 Hours After MCAO
Hyperglycemia induced extensive HT in ischemic territories in MCAO rats 24 hours after ischemia (Figure 2A). Compared with the vehicle, ISO after treatment for 1 hour significantly exacerbated the infarction ratio (from 0.33±0.01 to 0.44±0.02; P<0.05) and hemorrhage volume (from 6.29±0.35 to 7.44±0.56 μL; P<0.05). In addition, in vehicle group, 45%...
O2 significantly reduced the hemorrhage volume (from 6.29 ± 0.35 to 4.28 ± 0.61 μL; P < 0.05) when compared with DX + MCAO group (Figure 2).

The mortality was also higher in the ISO after treatment group than in the others: DX + sham, 0% (0/23); DX + MCAO, 22.58% (7/31); DX + MCAO + ISO, 33.33% (14/42); and DX + MCAO + O2, 17.86% (5/28) (Figure 3A). Neurological deficits were worsened 24 hours after MCAO. Animals in the DX + MCAO + ISO group exhibited more severe neurobehavioral deficits compared to the vehicle group (9.91 ± 0.46 versus 13.39 ± 0.50; P < 0.05), but not DX + MCAO group (Figure 3B). In addition, ISO increased the brain water content in the ischemic hemisphere at 24 hours after MCAO compared with the vehicle group (0.8284 ± 0.0028 versus 0.8102 ± 0.0016; P < 0.05; Figure 3C).

Taken together, ISO after treatment increased the infarct, hemorrhage volume, brain edema, and mortality, and it worsened the neurobehavioral deficits in the hyperglycemia-induced HT model in rats when compared to the vehicle group. And the same results were also observed in the female rats (data not shown).

ISO Increased the Oxidative Stress and Impaired the Activities of SOD and CAT in Hyperglycemia Rats After MCAO

The MDA content was greatly increased in the ischemic brain tissue 24 hours after MCAO. The content in the ISO-treated group was much higher than that in DX + MCAO + O2 group (0.219 ± 0.024 versus 0.145 ± 0.019 nmol/g protein; P < 0.05; Figure 3D). Consistent with the MDA content, the Western blot revealed a significant increase of 4-hydroxy-2-nonenal (Figure 4A, B) and nitrotyrosine (Figure 4C, D) at 24 hours after ischemia; this high level of 4-hydroxy-2-nonenal and nitrotyrosine was enhanced by ISO after treatment (P > 0.05 versus DX + MCAO; P < 0.05 versus DX + MCAO + O2; Figure 4).

At 24 hours after MCAO, SOD activity of the infarct area was sharply more decreased than that of DX + sham group (123.4 ± 3.81 versus 144.8 ± 5.46 U/mg protein; P < 0.05; Figure 5A). ISO after treatment strongly decreased the SOD activity compared to the vehicle group in the brain (112.7 ± 3.90 versus 144.8 ± 5.46 U/mg protein; P < 0.05; Figure 5A) and in the blood plasma (11.06 ± 0.65 versus 16.54 ± 0.69 U/mg protein; P < 0.05; Figure 5B) but showed no statistic difference compared with DX + MCAO group. We also found a significant decrease of CAT activity in the brain tissues. (6.79 ± 0.82 versus 14.31 ± 0.89 U/g protein; P < 0.05; Figure 5C) but not in the blood plasma (5.38 ± 0.63 versus 10.00 ± 1.15 U/g protein; P > 0.05; Figure 5D).

Discussion

We did this study for two main reasons. First, hyperglycemia has been claimed to be strongly associated with HT in patients undergoing tissue plasminogen activator therapy, which is the only effective and approved stroke treatment to
date, and this makes the study on hyperglycemia-induced HT after MCAO in rats clinically relevant. Second, as one of the most widely used volatile anesthetics in surgical procedures, ISO has been shown to be neuroprotective in multiple animal works of ischemic brain injury; however, little attention has been given to the possible adverse effects caused by ISO or its metabolites. Therefore, before ISO is used for neuroprotective purposes in stroke patients with hyperglycemia, it is imperative that its effects be examined during relevant circumstances.

In the current study, we found that 2% ISO administered at the onset of reperfusion for 1 hour failed to reduce infarct, hemorrhage volume, or brain edema and failed to improve neurobehavioral deficits when compared with the ischemia/reperfusion group 24 hours after MCAO. In contrast, ISO exacerbated these indicators when compared with the vehicle group. Similar observations were made in female rats in which ISO failed to reduce infarct and hemorrhage volume but increased mortality when compared with MCAO group (data not shown). These results were consistent with a previous study that postischemic ISO application after MCAO in baboons was associated with larger infarction, increased risk of hemorrhagic transformation, and worse neurobehavioral scores. The potential mechanisms for the harmful effect of ISO were not studied in that study. Other studies, however, showed neurotoxicity effect of ISO in neonates and young children attributable to significant neurodegeneration as well as long-lasting postoperative cognitive decline in the elderly. In a similar cerebral ischemia/reperfusion injury model, neuroprotective effect of ISO was demonstrated in mild to moderate ischemic models, at lower ISO concentrations, and after shorter postinjury intervals. Therefore, it is plausible that in our experiment, hyperglycemia-induced HT enhanced ischemic injury and after treatment with ISO resulted in adverse effects, although 2% ISO for 1 hour was popularly used in other models mediating neuroprotection.

How did ISO deteriorate the outcomes of hyperglycemia-induced HT after focal cerebral ischemia? We have found that ISO had no effects on the level of blood glucose (Figure 2A), the inducer of hemorrhagic transformation in our model, which indicates that other perpetrators might play a major role. It had been shown that ISO induced the oxidative stress either by reducing the antioxidant defense mechanism or by generating toxic free radicals such as reactive oxygen species. Thereby, we sought to examine whether there was a role for oxidative stress in mediating the neurotoxicity of ISO after treatment with hyperglycemia-induced HT. In this model, oxidative stress had been massively generated by hyperglycemia and ischemia/reperfusion injury, which seriously disrupted the blood–brain barrier and developed pronounced cerebral edema. It was predictable that further weakened cellular antioxidant defense systems would accel-
erate and widely spread the damage caused by this over- loaded oxidative stress. Oxidative stress usually comes from reactive oxygen species and reactive nitrogen species, including hydroxyl radical, superoxide anion, hydrogen peroxide, nitric oxide, and peroxynitrite, leading to subsequent cell death by promoting DNA fragmentation, lipid oxidation, and protein nitrification.22

SOD and CAT are 2 representative endogenous antioxidant enzymes present in essentially every cell. SOD is considered fundamental in the process of eliminating reactive oxygen species by reducing (adding an electron to) superoxide anion to form hydrogen peroxide, and CAT is responsible for decomposing hydrogen peroxide to H₂O and O₂, consequently removing the deleterious free radicals. It has been demonstrated that reducing activities of SOD and CAT exacerbated neuronal cell injury or edema formation or both after transient focal cerebral ischemia.23,24 In this study, we found that ISO increased the expressions of MDA, hydroxyl-nonenal, and nitrotyrosine, which are markers of lipid peroxidation (MDA and 4-hydroxy-2-nonenal) and protein nitrification (nitrotyrosine), when compared with the vehicle group. We further observed that the activities of SOD and CAT enzymes in ischemic brain tissue were decreased by ISO after treatment. Therefore, we conceptualize that ISO deteriorated the outcomes of hyperglycemia-induced HT after MCAO, possibly by enhancing oxidative stress via impaired activities of SOD and CAT. In 1999, Durak et al25 found that exposure to 2% ISO for 30 minutes for 3 consecutive days could induce renal toxicity in guinea pigs by reducing the activity of antioxidant enzymes. The authors suggested that the increased fluoride concentration, which was one of the metabolites of ISO and one of the most electronegative elements, might inhibit the activity of antioxidant enzymes through making complexes with their cofactor metals such as copper and zinc.25 Kenna and Jones26 also noted that inorganic fluoride from inhaled fluorinated anesthetics might impair antioxidant enzymes by binding tightly to metal cations. A recent study verified that 2% ISO anesthesia in patients decreased the plasma zinc levels, erythrocyte SOD and glutathione peroxidase activities, and trace element levels at 24 hours.19 Therefore, it is highly likely that increased fluoride concentration attributable to ISO after treatment might impair antioxidant defense system by making powerful metal complexes.

Figure 4. Representative Western blots and quantitative analysis of 4-hydroxy-2-nonenal (A and B) and nitrotyrosine (C and D) in ischemic hemisphere of all groups. Isoflurane after treatment intensified the expression of both 4-HNE and nitrotyrosine compared with the vehicle group. Values are the mean±SEM *P<0.05 vs dextrose (DX)+sham; #P<0.05 vs DX+MCAO+O₂. MCA indicates middle cerebral artery; MCAO, middle cerebral artery occlusion; ISO, isoflurane.
One unexpected result from this study was that the vehicle group, in which the oxygen concentration was 45% and delivered at 2 hours after MCAO, ameliorated outcomes, including mild infarct and hemorrhage volume, lowered brain edema and mortality, and improved neurobehavioral deficits when compared with the ischemia/reperfusion group, consistent with the view that normobaric oxygen has neuroprotective effects in acute ischemia stroke and is useful in extending the narrow time window for patients in clinical settings. The one weakness in our experiment was that we did not monitor the physiological parameters such as arterial partial pressure of oxygen, arterial partial pressure of carbon dioxide, and mean arterial blood pressure for the technical constraints (rats were treated in a sealed chamber). In ISO-treated group and the vehicle group, the hyperoxic could increase arterial partial pressure of oxygen and mean arterial blood pressure through vasoconstriction, which may have an acute influence on the effects of hyperglycemia.

Overall, we concluded that ISO after treatment in hyperglycemia-induced HT after ischemia stroke failed to demonstrate any protective value but exacerbated the outcomes by impairing the antioxidant defense system. The current findings implied that ISO may not be an appropriate anesthetic for stroke patients with hyperglycemia.

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Data Supplement

Supplement 1. Schematic for Experiment Design
### Neurological Scores

<table>
<thead>
<tr>
<th>Test Description</th>
<th>Score 0: No movement</th>
<th>Score 1: Slight movement</th>
<th>Score 2: Touch 1-2 sides</th>
<th>Score 3: Touch 3-4 sides</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Spontaneous activity (in cage for 3 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Symmetry in the movement of four limbs</td>
<td>Left limbs no movement</td>
<td>Left limbs minimally extend</td>
<td>Left limbs extend less</td>
<td>Four limbs Complete asymmetry</td>
</tr>
<tr>
<td>3. Forepaw outstretching while held by tail</td>
<td>No outstretching</td>
<td>Slight outstretching</td>
<td>Outstretching Less than right side</td>
<td>Both Symmetrically outstretching</td>
</tr>
<tr>
<td>4. Climb wall (1 min, use feet)</td>
<td>Fall down</td>
<td>Stay but fail to climb</td>
<td>Climb but slip</td>
<td>Normal</td>
</tr>
<tr>
<td>5. Body Proprioception</td>
<td>No response</td>
<td>Weak</td>
<td>Symmetrical</td>
<td></td>
</tr>
<tr>
<td>6. Response to vibrissae touch</td>
<td>No response</td>
<td>Weak</td>
<td>Symmetrical</td>
<td></td>
</tr>
<tr>
<td>7. Beam walking (1 min)</td>
<td>Hug or off</td>
<td>Stand on</td>
<td>Walk less than 20 cm</td>
<td>Walk more than 20 cm</td>
</tr>
</tbody>
</table>