Hyperglycemia Exacerbates Brain Edema and Perihematomal Cell Death After Intracerebral Hemorrhage

Eun-Chol Song, MD; Kon Chu, MD; Sang-Wuk Jeong, MD; Keun-Hwa Jung, MD; Seong-Hoon Kim, MD, PhD; Manho Kim, MD, PhD; Byung-Woo Yoon, MD, PhD

Background and Purpose—Hyperglycemia has a deleterious effect on brain ischemia. However, the effect of hyperglycemia in intracerebral hemorrhage (ICH) is not well known. We investigated the effect of hyperglycemia on the development of brain edema and perihematomal cell death in ICH.

Methods—Hyperglycemia was induced by intraperitoneal injection of streptozotocin (60 mg/kg) in adult Sprague-Dawley male rats. ICH was induced by stereotaxic infusion of 0.23 U of collagenase into the left striatum. Seventy-two hours after ICH, terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling (TUNEL) staining was performed for perihematomal cell death. We also measured brain water content to evaluate edema formation.

Results—The serum glucose level of the hyperglycemic group was 394.0 ± 180.3 mg/dL (n = 31), and that of the normoglycemic group was 97.5 ± 27.4 mg/dL (n = 31). The size of hemorrhage was similar between groups, without any significant difference (n = 8 in each group). The brain water content of hyperglycemic rats (n = 17) increased in both lesioned (81.0 ± 0.5%) and nonlesioned hemispheres (78.7 ± 0.6%) compared with the normoglycemic group (n = 17; lesioned: 78.9 ± 0.8%; nonlesioned: 77.3 ± 1.1%). In the hyperglycemic group, more TUNEL-positive cells were found in the perihematomal regions (n = 6).

Conclusions—Hyperglycemia caused more profound brain edema and perihematomal cell death in experimental ICH. (Stroke. 2003;34:2215-2220.)

Key Words: brain edema • hyperglycemia • intracerebral hemorrhage • neuronal death • streptozotocin

Hyperglycemia has been associated with increased neuronal damage in animal models of ischemia.1,2 Hyperglycemic or diabetic patients with stroke tend to have a worsened neurological outcome and an increased hemorrhagic transformation rate.3,4 Animal experiments of cerebral ischemia have elucidated mechanisms of the deleterious effects of hyperglycemia on the damaged brain, which include acidosis, release of excitatory amino acids, exaggeration of edema formation, blood-brain barrier (BBB) injury, and hemorrhagic transformation of the infarct.1,5,6 Intracerebral hemorrhage (ICH) represents at least 15% of all strokes in Western populations7 and a considerably higher proportion (20% to 30%) in Asian and black populations.8,9 The prognosis of ICH is poor, often much worse than that of ischemic strokes of similar size.9 Patients with ischemic stroke (up to 30%) will undergo hemorrhagic transformation,10 and a considerable number of ischemic infarct patients after thrombolysis develop hemorrhagic conversion or symptomatic hematoma.11 Brain edema is also usually associated with deterioration in ICH. Many patients can deteriorate because of hematoma expansion or large hematoma and brain edema development.9,12–14 However, the exact mechanism of edema formation after ICH is still unknown. Moreover, there is no animal study showing the effect of hyperglycemia on the brain edema associated with ICH.

To investigate the pathogenesis of ICH, various animal models have been developed.15–17 In the present study we examined the hypothesis that hyperglycemia may exert its effect on the formation of brain edema and associated perihematomal cell death after ICH.

Materials and Methods

Induction of Hyperglycemia and ICH

All the procedures were performed following an institutionally approved protocol in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Sixty-two male Sprague-Dawley rats (Osan, Samtako) weighing 240 to 280 g were used. Rats were grouped as hyperglycemic (n = 31) and normoglycemic (n = 31). Hyperglycemia was induced by intraperitoneal injection of streptozotocin (60 mg/kg; Sigma) 3 days before operation.18 Experimental ICH was induced by the stereotaxic
In brief, after an intraperitoneal injection of 1% ketamine (30 mg/kg; Sigma) and xylazine hydrochloride (4 mg/kg; Sigma), rats were placed in a stereotaxic frame (David Kopf Instruments). A burr hole was made, and a 30-gauge Hamilton syringe needle was inserted into the striatum (location: 3.0 mm left lateral to the midline, 0.2 mm posterior to bregma, 6 mm in depth below the skull).19 ICH was induced by the administration of 1 µL containing 0.23 collagen digestion unit of collagenase type IV (Sigma) over 5 minutes. After completion of collagenase infusion, the burr hole was sealed with bone wax, the wound was sutured, and rats were allowed to recover. Physiological parameters, including mean arterial blood pressure, blood gases, and glucose concentration, were measured during the experiment. During the recovery period, rats were assessed for forelimb flexion and contralateral circling to confirm the procedures. No cases of seizure were observed during the experiments. Rectal temperature was maintained at 37±0.5°C with the use of a thermostor-controlled heating blanket. Free access to food and water was allowed after recovery from anesthesia. Rats were kept in air-ventilated cages at 24°C for the duration of the experiment.

**Morphometric Measurement of Hemorrhage Volume**

Rats were assigned to 2 experimental groups: a normoglycemic group (control; n=8) and a hyperglycemic group treated with streptozotocin (n=8). Three days after the injection of streptozotocin, serum glucose level was obtained by tail vein sampling before and after (measured daily for 2 days) the induction of ICH. Seventy-two hours after the operation, rats were anesthetized by the same methods and killed by decapitation. The brain was removed immediately and fixed with 4% phosphate-buffered paraformaldehyde. Fixed brains were cut coronally through the needle entry site and after (measured daily for 2 days) the induction of ICH.

Digital photographs of the serial slices were taken, and the size of hemorrhage was measured with an image analyzer program (Image Pro-Plus, Media Cybernetics). Total hematoma volume was calculated by summing the clot area in each section and multiplying by the distance between sections.

**Measuring Brain Water Contents**

Rats were assigned to 2 experimental groups: one group consisting of 17 normoglycemic rats and the other group consisting of 17 hyperglycemic rats. Two days after operation, the brains were harvested, and the cerebellum and the brain stem were removed and then divided into 2 hemispheres along the midline. The wet weight of the hemispheres was measured. The hemispheres were incubated in an oven at 100°C for 24 hours to obtain the dry weight. Water content was expressed as percentage of wet weight; the formula for calculation was as follows: (wet weight−dry weight)/(wet weight)×100.

**TUNEL Assay**

Rats used for terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining were killed with an overdose of sodium pentobarbital at 72 hours after collagenase injection (hyperglycemia: n=6; normoglycemia: n=6). The TUNEL procedure for in situ detection of DNA fragmentation was performed.21,22 In brief, air-dried sections were postfixed in 4% paraformaldehyde for 15 minutes at room temperature. After they were washed 3 times in Tris-HCl (pH 7.7), sections were treated with 2% H2O2 for 10 minutes at room temperature to quench endogenous peroxidase activity. Sections were then incubated with terminal deoxynucleotidyl transferase enzyme solution containing 25 mmol/L Tris-HCl (pH 6.6), 200 mmol/L potassium cacodylate, 0.25 mmol/L bovine serum albumin, 2.5 mmol/L CoCl2, 40 µmol/L biotinylated-16-dUTP, and 500 U/mL terminal deoxynucleotidyl transferase (Boehringer-Mannheim) at 37°C for 1 hour. Sections were dipped in 300 mmol/L NaCl and 30 mmol/L sodium citrate for 15 minutes at room temperature to terminate reactions. After sections were washed 3 times in Tris-HCl (pH 7.7) and subsequently blocked with PBS (pH 7.4) containing 10% normal goat serum and 0.3% Triton X-100, biotinylated-16-dUTP was visualized by the avidin-biotin method with 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.005% H2O2. Cell density counts were performed on sections lightly counterstained with toluidine blue stain. A single axial section through the center of the hemorrhagic lesion was analyzed. Square sampling regions (300×300 µm) were used for cell counting. Eight sampling regions were placed along the periphery. The TUNEL-positive cells were identified and counted. Total counts in these sampling regions were converted into cell densities for quantification and comparison between treatment groups.

**Behavioral Testing**

Behavioral testing was performed by 2 individuals blinded to the assignment of rats (total n=28) before and 3 days after ICH with the use of the modified limb placing test, which is a modified version of a test previously described in the literature.23 The test consists of 2 limb-placing tasks that assess the sensorimotor integration of the

### Figure 1. Mean serum glucose level after ICH induction. Values are mean±SD. *P<0.01 compared with normoglycemic group.

### Physiological Parameters

<table>
<thead>
<tr>
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<th>Normoglycemic Group</th>
<th>Hyperglycemic Group</th>
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</thead>
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<tr>
<td></td>
<td>Baseline</td>
<td>After ICH</td>
</tr>
<tr>
<td>pH</td>
<td>7.41±0.03</td>
<td>7.40±0.02</td>
</tr>
<tr>
<td>PaO2, mm Hg</td>
<td>109.1±10.2</td>
<td>107.3±8.2</td>
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<td>PaCO2, mm Hg</td>
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<tr>
<td>Rectal temperature, °C</td>
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<td>36.8±0.2</td>
</tr>
<tr>
<td>MABP, mm Hg</td>
<td>88±3</td>
<td>85±3</td>
</tr>
</tbody>
</table>

Values are means±SD. One-way analysis of variance revealed no significant intergroup difference for any variable. ICH indicates intracerebral hemorrhage; MABP, mean arterial blood pressure.
forelimb and the hind limb by checking responses to tactile and proprioceptive stimulation. First, the rat is suspended 10 cm over a table, and the stretch of the forelimbs toward the table is observed and evaluated as follows: normal stretch, 0 points; abnormal flexion, 1 point. Next the rat is positioned along the edge of the table, and its forelimbs are suspended over the edge of the table and allowed to move freely. Each limb (forelimb, second task; hind limb, third task) is pulled down gently, and retrieval and placement are checked. Finally, the rat is placed toward the table edge to check for lateral placement of the forelimb. The 3 tasks are scored in the following manner: normal performance, 0 point; performance with a delay (2 seconds) and/or incomplete, 1 point; no performance, 2 points. Seven points indicates maximal neurological deficit, and 0 points indicates normal performance.

Statistical Analysis
All data in this study are presented as mean±SD. Data from different animal groups were analyzed by the Mann-Whitney U test and unpaired Student t test. The differences were considered significant at probability values <0.05.

Results
Physiological Parameters
All animals survived the experiments. The serum glucose level of the hyperglycemic group (n=31) was 394.0±180.3 mg/dL, and that of normoglycemic group (n=31) was 97.5±27.4 mg/dL (P<0.01, unpaired t test; Figure 1). Other physiological parameters, including mean arterial blood pressure, blood gases, and body temperatures, were not significantly different among experimental groups before, during, or after ICH (Table; unpaired t test).

Size of Hemorrhage
Rats infused with 0.23 U of collagenase developed large hemorrhages in the striatum that were evident by 24 hours (Figure 2). The size of hemorrhage was 8.99±4.51 mm³ (n=8) in the hyperglycemic group and 8.87±3.61 mm³ (n=8) in the normoglycemic group (Figure 2) by 72 hours. There was no significant difference between the sizes of...
hemorrhage between groups \((P=1.00, \text{Mann-Whitney } U \text{ test})\).

Minor intraventricular hemorrhage (IVH) occurred in a few rats \((n=3 \text{ of } 14, \text{ normoglycemic group}; n=3 \text{ of } 14, \text{ hyperglycemic group})\), which did not significantly affect the behavioral test results measured at 3 days after ICH induction (modified limb placing test score: \(6.4 \pm 0.2 \text{ in ICH+IVH group, } n=6\); \(6.3 \pm 0.4 \text{ in ICH only group, } n=22; P=0.95, \text{Mann-Whitney } U \text{ test})\).

**Brain Edema Formation**

Brain water content of the lesioned (left) hemisphere was \(81.0 \pm 0.5\% \text{ in the hyperglycemic group (} n=17) \text{ and } 78.9 \pm 0.8\% \text{ in the normoglycemic group (} n=17) \text{ (} P<0.01, \text{unpaired } t \text{ test})\); brain water content of the nonlesioned (right) hemisphere was \(78.7 \pm 0.6\% \text{ in the hyperglycemic group } \text{ and } 77.3 \pm 1.1\% \text{ in the normoglycemic group (} P<0.01, \text{unpaired } t \text{ test; Figure 3})\). Brain water content of hyperglycemic rats increased in both lesioned and nonlesioned hemispheres compared with the normoglycemic group.

**TUNEL-Positive Cell Density After ICH**

TUNEL staining revealed a high density of positive cells within the hemorrhage lesion itself as well as in the surrounding periphery (Figure 4). Brain sections that were counterstained with toluidine blue showed that the TUNEL-positive cells were not likely to be infiltrating leukocytes (Figure 4). Quantitative analysis showed regional TUNEL-positive cell differences between the groups \((n=6)\). The hyperglycemic group \((923.1 \pm 74.3 \text{ cells per square millimeter})\) showed significantly more TUNEL-positive cells than the normoglycemic group \((301.7 \pm 91.5 \text{ cells per square millimeter}; P<0.01, \text{unpaired } t \text{ test})\).

**Discussion**

Using a rat model of ICH, we have shown that hyperglycemia may cause more profound brain edema. Numbers of TUNEL-positive cells were significantly increased in rats with hyperglycemia. The size of hemorrhage and physiological parameters (except for serum glucose level) were not significantly different between normoglycemic and hyperglycemic rats.

**Figure 4.** Results of TUNEL staining. a, b, Representative section in the perihematomal zone of the hemorrhagic lesion shows the presence of TUNEL-positive stained cells (a, hyperglycemic group; b, normoglycemic group). Counterstaining with toluidine blue indicates that polymorphonuclear leukocytes do not show TUNEL-positive staining. Note the more abundant TUNEL-positive cells in the hyperglycemic group (a). c, Density of TUNEL-positive stained cells per square millimeter. Values are mean±SD; \(n=6\) per group. *\(P<0.01\) compared with normoglycemic group.
In our study brain edema after ICH with hyperglycemia increased in both lesioned and nonlesioned hemispheres, and this may be more attributable to vasogenic edema. Brain edema is generally divided into cytotoxic and vasogenic types, depending on whether the BBB is intact or disrupted. The brain edema of experimental ICH is regarded as a combination of cytotoxic and vasogenic edema. Brain edema forms as a result of BBB disruption and the local generation of osmotically active substances. Brain edema was increased not only in the perihematoma region but also in the more distant structures, even in contralateral basal ganglia, possibly because of increased BBB permeability and diairsis, in rat ICH models and human subjects with ICH.

Our results revealed that hyperglycemia may aggravate brain edema, culminating in neural cell death around the hematoma, with an increase in TUNEL-positive cells. Collagenase itself, used in this study for the induction of ICH, did not cause cell death in vitro (10 to 15 U/mL). Apoptosis is reported to be involved in the pathophysiology of cell death after ICH. However, the triggering events that are responsible for initiating the apoptotic cascade after ICH remain to be defined. The activated blood components (eg, thrombin, iron, and heme) may induce high levels of cytokine activity. Inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) may be involved in hyperglycemia-induced brain edema. Administration of IL-1 and TNF-α induces opening of the BBB and causes vasogenic brain edema. In vitro studies demonstrated that hyperglycemia increases the secretion of IL-1β in cultured human aortic endothelial cells. Mouse uterine epithelial cells cultured in high glucose showed an increase in TNF-α production via an increase in IL-1β secretion. Acute hyperglycemia increases the level of circulating TNF-α in humans; this effect is more pronounced in patients with impaired glucose tolerance.

Previous experimental studies have demonstrated that hyperglycemia worsens acute BBB injury after transient forebrain ischemia in rats and accentuates brain edema. The summarized speculations for hyperglycemia-induced brain injury are as follows. (1) Free radical formation is increased with hyperglycemia-induced brain injury. The free radicals and nitric oxide increase BBB permeability and brain edema. Bradykinin induces bradykinin-mediated brain edema with inflammation. Bradykinin is a potent vasodilator and induces brain edema in animal models; bradykinin B2 receptors play a role in the formation of vasogenic brain edema in a cold injury model in rats, and B2 receptor antagonist reduces vasogenic edema. Bradykinin increases BBB permeability and facilitates extravasation by dilatation of arterial blood vessels. (3) Hyperglycemia exerts its effect on cell death by inducing inflammatory reactions in ICH. (4) Hyperglycemia causes acute elevation of cytosolic free calcium as a result of increased calcium influx. Rising calcium concentration in the cytoplasm causes calcium influx into mitochondria and disrupts mitochondrial membrane potential and normal metabolism, leading to cell death. The calcium channel blocker (S)-emopamil reduces brain edema from collagenase-induced hemorrhage in rats, possibly by reducing calcium entry and sodium exchange.

In summary, our data provide initial evidence that hyperglycemia may aggravate brain edema with neural cell death in an experimental rat ICH model.

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References
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