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·Original Article·

Acute hyperglycemia together with hematoma of high-glucose blood exacerbates neurological injury in a rat model of intracerebral hemorrhage

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ABSTRACT

Recent evidence suggests that admission hyperglycemia has deleterious effects on the survival and functional outcome of patients with intracerebral hemorrhage (ICH). In this study, we first induced acute hyperglycemia in male adult Sprague-Dawley rats by intraperitoneal injection of 50% glucose (6 mL/kg), and created the ICH model thereafter by delivering autologous whole blood or homologous normal-glucose blood into the right basal ganglia. Twenty-four hours later, we assessed the neurological injury, evaluated the hematoma and brain water content, and investigated autophagy. We found elevations of neurological deficit scores, brain water content, and microtubule-associated protein light chain-3 (LC3) and beclin-1 protein levels, and decreased SQSTM1/p62 levels after ICH with normal-glucose blood (without hyperglycemia). Acute hyperglycemia with ICH of high-glucose blood hematoma was associated with significantly increased forelimb-use asymmetry test scores, brain water content and SQSTM1/p62 protein levels, and evident decreases in the ratio of LC3-II/LC3-I and beclin-1 protein levels. On the other hand, acute hyperglycemia and ICH with normal-glucose blood hematoma only slightly increased the neurological deficit scores and brain water content (P >0.05). In conclusion, the autophagy pathway was activated after ICH, and acute hyperglycemia with hematoma of high-glucose blood exacerbates the neurological injury, and reduces autophagy around the hematoma.

Keywords: intracerebral hemorrhage; hyperglycemia; autophagy

INTRODUCTION

Intracerebral hemorrhage (ICH) accounts for 10–15% of all strokes worldwide and patients with ICH show the worst outcome of all stroke subtypes[1, 2]. Studies have indicated that hyperglycemia may worsen the prognosis of ICH patients in terms of functional recovery at discharge and one-month mortality[3]. However, the causal role and determinant effect of hyperglycemia in ICH remain unknown.

Autophagy has long been considered an important catabolic process that involves the delivery of cytoplasmic material to the lysosome for degradation and plays an important part in many human diseases[4]. Recent research has indicated that autophagy is activated after ICH, and may participate in the pathophysiological process of ICH. He *et al.*[5] observed numerous autophagosomes in the perihematomal area. On the other hand, autophagy can be affected by high glucose levels. Kobayashi *et al.*[6] found that high glucose reduces autophagy in cultured cardiomyocytes. However, the effect of hyperglycemia on autophagy after ICH *in vivo*, and the exact role of autophagy in ICH remain unknown.

In the present study, we used an acute hyperglycemia

rat model of ICH to determine its effects on neurological deficits and brain edema. Specifically, three reliable markers of autophagy were used, microtubule-associated protein light chain-3 (LC3), beclin-1, and SQSTM1/p62.

MATERIALS AND METHODS

Animals and Experimental Design

A total of 89 adult male Sprague-Dawley rats (provided by the Shanghai Laboratory Animal Co., Shanghai, China) weighing 275–325 g were used; they were housed under a 12:12 h light/dark cycle (lights on from 08:00 to 20:00), with free access to food and water. All experiments were approved by the Animal Care and Use Committee of Zhejiang University, and conformed to the guidelines of the “Principles of Laboratory Animal Care” (NIH publication No. 80-23, revised 1996). Utmost efforts were made to minimize the number of animals used and their suffering.

Rats were randomly divided into five groups, (1) ICH model (group I) without acute hyperglycemia, (2) sham (group S), (3) acute hyperglycemia + ICH with normal-glucose blood hematoma (group IG1), which received a 50% glucose (6 mL/kg) injection (intraperitoneal, i.p.) followed by an infusion of homologous blood from another rat with normal blood glucose; (4) acute hyperglycemia + ICH with high-glucose blood hematoma (group IG2), which received a glucose injection followed by an infusion of autologous blood; and (5) hyperglycemia (group G), receiving only a glucose i.p. injection. The glucose was injected 30 min before the ICH. All rats were fasted overnight before the experiments and sacrificed 24 h after the operation.

ICH Model

The rats were anesthetized with pentobarbital (45 mg/kg, i.p.) and placed in a stereotaxic frame. A 1-mm-diameter burr hole was drilled in the skull (0.2 mm anterior and 3.5 mm lateral to bregma) and a needle was lowered into the right caudate nucleus (6 mm below the skull surface). Blood (100 μL) was collected from the right femoral artery and then delivered into the right caudate nucleus at 10 μL/min by a micropump. The needle was left in place for 10 min to prevent blood reflux. Core temperature was maintained at 37°C throughout the surgery, and recovery was assisted using an electric heating pad.

Behavioral Tests

The corner-turn and forelimb-use asymmetry tests were conducted before and 24 h after operation according to the previous description[7].

Corner-Turn Test The rat was allowed to move into a 30° corner, and then turned left or right to get out of the corner. This was repeated 10 times, and the test score was the percentage of right turns.

Forelimb-Use Asymmetry Test The rat was observed in a transparent cylinder (20 cm in diameter and 30 cm in height). Behavior was quantified by analyzing the use of the forelimbs during exploration of the cylinder wall, in terms of the unimpaired (ipsilateral) forelimb (I); the impaired forelimb (contralateral to the blood injection site) (C); and both simultaneously (B). Forelimb-use asymmetry score = $[I / (I + C + B)] - [C / (I + C + B)]$.

Hematoma Assessment

Under pentobarbital anesthesia (45 mg/kg, i.p.), each rat was intracardially perfused with PBS and the brain removed 24 h after operation. Coronal slices (2 mm thick) were prepared, and each slice was digitized. A region of interest was traced around the perimeter of the hematoma, and the hematoma area was quantified using ImageJ software.

Brain Water Content

Rats were decapitated under pentobarbital anesthesia 24 h after operation. The brain was immediately removed and a coronal slice (~4 mm thick) 4 mm from the frontal pole was cut. The slice was divided into hemispheres along the midline, and the cortex and basal ganglia of each hemisphere were dissected out. The cerebellum was also detached to serve as a control. Brain samples were immediately weighed on an electronic analytical balance to obtain the wet weight. The samples were then dried at 100°C for 24 h to obtain the dry weight. The brain water content was calculated as follows: (wet weight – dry weight) /wet weight × 100%.

Western Blot

The brain samples were homogenized with radio-immunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). Protein concentration was determined using a bicinchoninic acid protein assay kit

(Pierce Protein Biology Products, USA). Protein samples (60 μg) were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Merck Millipore, MA). The membranes were blocked with non-fat milk and probed with primary and secondary antibodies. The primary antibodies were rabbit anti-LC3 (1:1 000; Cell Signaling Technology, Beverly, MA), rabbit anti-Beclin-1 (1:1 000; Cell Signaling), rabbit anti-SQSTM1/p62 (1:1 000; Cell Signaling) and mouse anti-β-actin (1:400; Santa Cruz Biotechnology, CA). The secondary antibodies were goat anti-rabbit and goat anti-mouse IgG (MultiSciences Biotech Co., Hangzhou, China). The antigen-antibody complexes were visualized with a chemiluminescence system (Merck Millipore; cat. no: WBKLS0500) and exposed to Kodak X-OMAT film.

Immunohistochemistry

Twenty-four hours after the operation, the rats were anesthetized (pentobarbital, 45 mg/kg, i.p.) and perfused through the heart with 100 mL PBS followed by 120 mL fixative (4% paraformaldehyde in 0.1 mol/L PBS, pH 7.4). The brain was removed and stored in the same fixative at 4°C for 24 h. The fixed brain was cut coronally, ~4 mm from the frontal pole. The 4-μm-thick slices were collected and embedded in paraffin. The paraffin sections were dewaxed, hydrated and immersed in 3% H_2O_2 for 15 min at room temperature to extinguish endogenous peroxidase. The sections were then heated in a microwave in 10 mmol/L sodium citrate buffer (pH 6.0) for epitope retrieval, and washed with PBS. The sections were saturated with 10% goat serum to block nonspecific binding, and then incubated overnight at 4°C with polyclonal rabbit anti-LC3 antibody (1:400; Cell Signaling), monoclonal mouse anti-beclin-1 antibody (1:150; Origene, Rockville, MD) or monoclonal mouse anti-SQSTM1 antibody (1:150; Origene). They were then incubated with the secondary antibody for 60 min. Subsequently, the sections were rinsed, and diaminobenzidine was added for coloration. After hematoxylin counterstaining, dehydration, clearing and coverslipping, the sections were observed under a light microscope (Olympus, CX41).

Statistical Analysis

Values are presented as mean ± SD. Statistical comparisons between groups were performed using Student's *t*-test or one-way analysis of variance, followed by the Student-Newman-Keuls test for comparisons across multiple groups. P <0.05 was considered statistically significant.

RESULTS

Plasma Glucose Levels

Plasma glucose markedly increased 30 min after the i.p. injection of 50% glucose. Without any treatment, the plasma glucose levels in the hyperglycemia pre-treatment groups (groups G, IG1, and IG2) returned to normal 24 h after the operation (Fig. 1). After an infusion of 100 μL autologous blood into the right basal ganglia, the plasma glucose levels still fluctuated within the normal range.

Acute Hyperglycemia and ICH with High-glucose Blood Hematoma Exacerbated the Neurological Deficit

There were no statistical differences among the groups prior to operation (Fig. 2). Twenty-four hours after the operation, ICH was associated with neurological deficits (Group I *vs* Group S: corner-turn test score, 92 ± 11.35% *vs* 51 ± 7.38%, P <0.01; forelimb-use asymmetry test

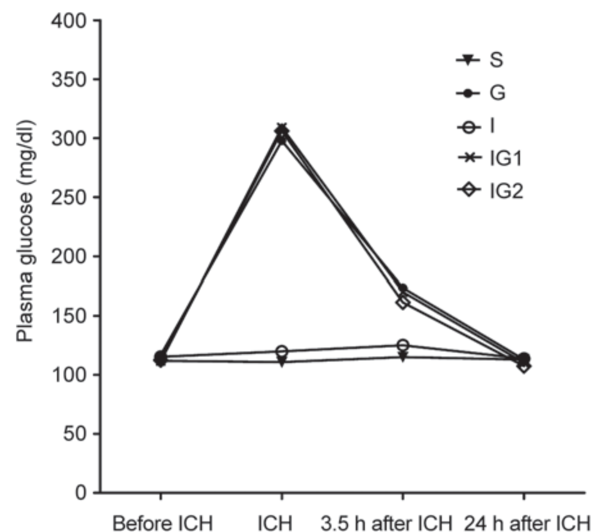

Fig. 1. Plasma glucose levels in groups of sham operation (group S), ICH model (group I) without acute hyperglycemia, acute hyperglycemia + ICH with normal-glucose blood hematoma (group IG1), acute hyperglycemia + ICH with high-glucose blood hematoma (group IG2), and hyperglycemia (group G).

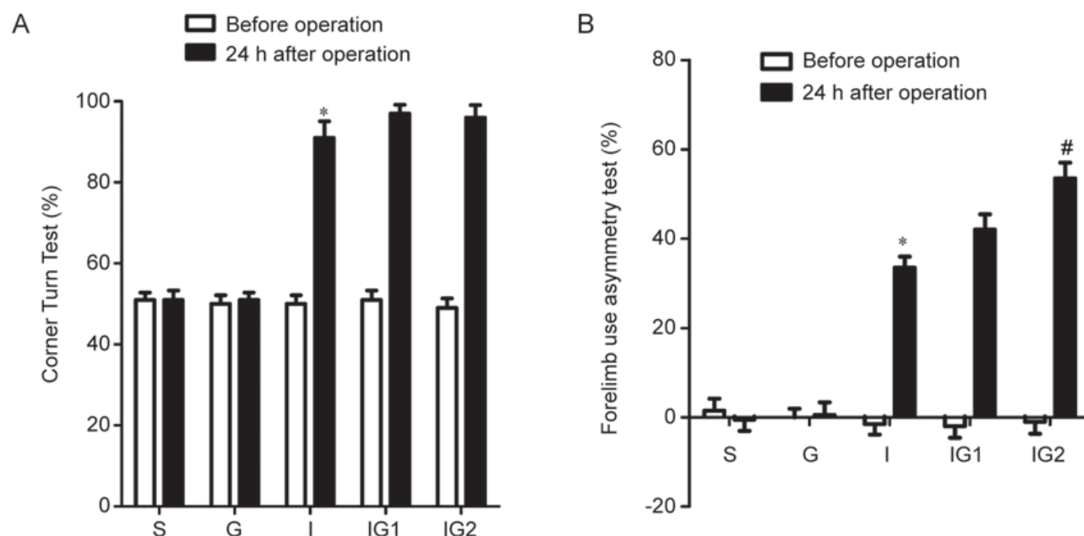

Fig. 2. **Corner-turn test (A) and forelimb-use asymmetry test (B) before and 24 h after the operation. Mean ± SD, *n* = 10. *P <0.01 *versus* group S; #P <0.01 *versus* group I. S, sham group; G, hyperglycemia group; I, ICH model group; IG1, acute hyperglycemia + ICH with normal-glucose blood hematoma group; IG2, acute hyperglycemia + ICH with high-glucose blood hematoma group.**

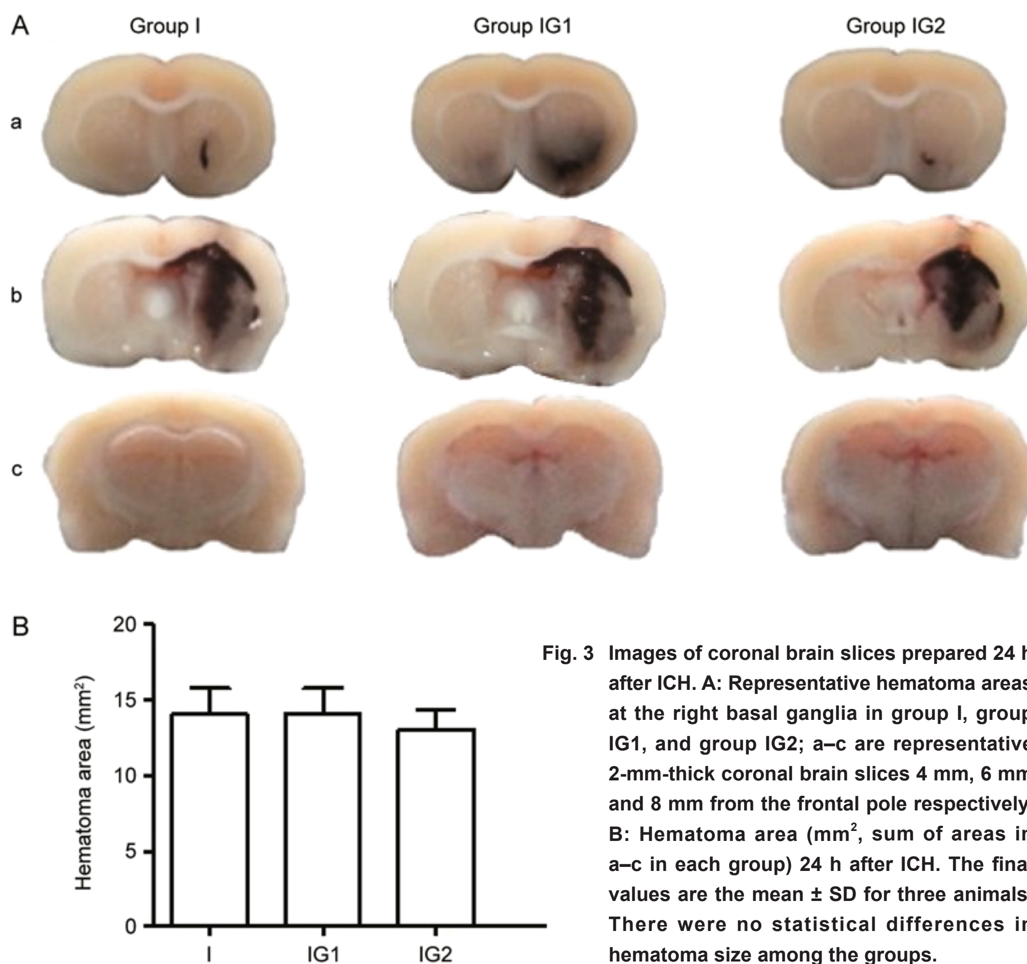

Fig. 3 **Images of coronal brain slices prepared 24 h after ICH. A: Representative hematoma areas at the right basal ganglia in group I, group IG1, and group IG2; a–c are representative 2-mm-thick coronal brain slices 4 mm, 6 mm and 8 mm from the frontal pole respectively. B: Hematoma area (mm^2, sum of areas in a–c in each group) 24 h after ICH. The final values are the mean ± SD for three animals. There were no statistical differences in hematoma size among the groups.**

score, 33.5 ± 7.84% *vs* –0.5 ± 7.98%, *P* <0.01). ICH with or without hyperglycemia increased the corner-turn test scores, which approached 100% (92 ± 11.35% in group I *vs* 96 ± 9.66% in group IG2, *P* >0.05; Fig. 2A). In the IG1 group, the forelimb-use asymmetry test score tended to increase compared with the I group, but with no significant difference (42.0 ± 10.85% *vs* 33.5 ± 7.84%, *P* >0.05; Fig. 2B). In contrast, acute hyperglycemia and ICH with high-glucose blood hematoma (group IG2) resulted in a significantly higher forelimb-use asymmetry test score than group I (53.5 ± 11.07% *vs* 33.5 ± 7.84%, *P* <0.05; Fig. 2B).

Acute Hyperglycemia and ICH with High-glucose Blood Hematoma Exacerbated Brain Edema

There were no statistical differences in hematoma size among the I, IG1, and IG2 groups 24 h after ICH (Fig. 3). Acute hyperglycemia only (group G) did not affect the water content of the ipsilateral basal ganglia compared with the sham group (78.28 ± 0.49% *vs* 78.34 ± 0.51%, *P* >0.05) (Fig. 4). However, 24 h after the ICH operation (group I), edema occurred (79.66 ± 0.52% *vs* 78.34 ± 0.51%, *P* <0.05). When only the serum glucose was elevated (group IG1), the brain water content increased slightly after ICH, compared with the I group (80.12 ± 0.54% *vs* 79.66 ± 0.52%, *P* >0.05). But acute hyperglycemia and ICH with high-glucose blood hematoma (group IG2) exacerbated the edema in the ipsilateral basal ganglia compared with group I (80.80 ± 0.97% *vs* 79.66 ± 0.52%, *P* <0.01; Fig. 4)

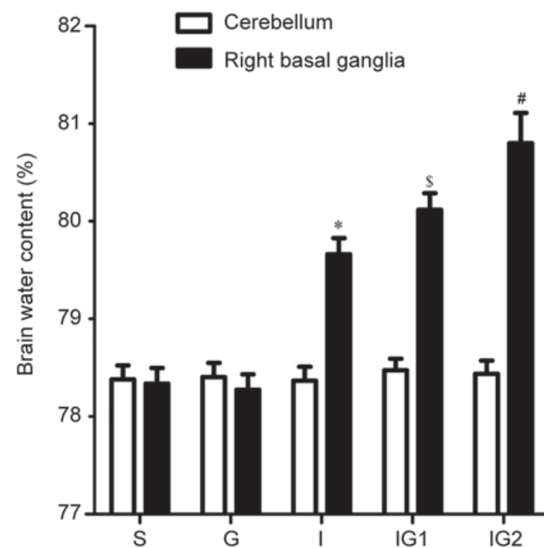

Fig. 4. Water content in the right basal ganglia and cerebellum 24 h after operation. Values are mean ± SD, *n* = 10. **P* <0.01 *vs* group S; $^{\$}$*P* >0.05, $^{\#}$*P* <0.01 *vs* group I. S, sham group; G, hyperglycemia group; I, ICH model group; IG1, acute hyperglycemia + ICH with normal-glucose blood hematoma group; IG2, acute hyperglycemia + ICH with high-glucose blood hematoma group.

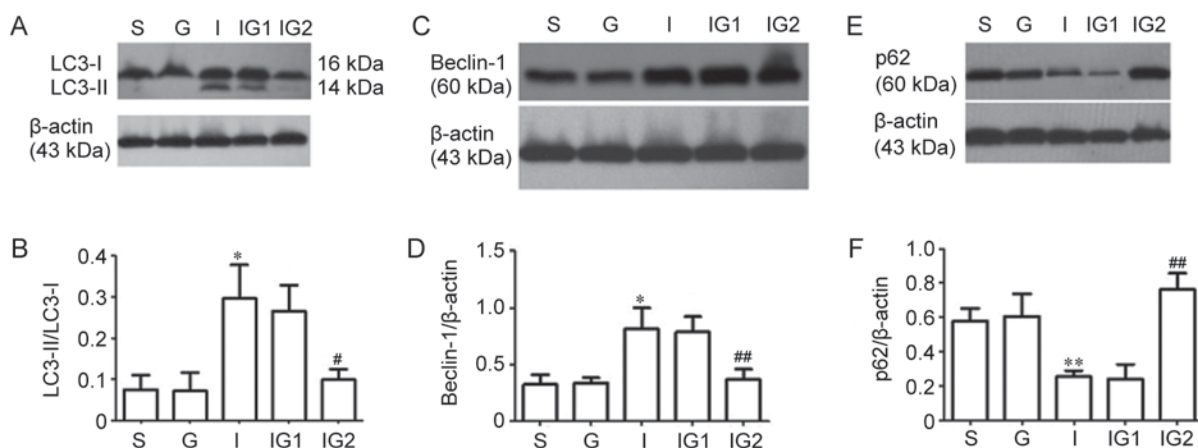

Fig. 5. Western blot analysis showing LC3-I (16 kDa) and LC3-II (14 kDa) (A, B), beclin-1 (60 kDa) (C, D), and SQSTM1/p62 (60 kDa) (E, F) levels in the ipsilateral basal ganglia 24 h after operation. The final values are the mean ± SD for three animals. **P* <0.05, ***P* <0.01 *vs* group S; $^{\#}$*P* <0.05, $^{\#\#}$*P* <0.01 *vs* group I. S, sham group; G, hyperglycemia group; I, ICH model group; IG1, acute hyperglycemia + ICH with normal-glucose blood hematoma group; IG2, acute hyperglycemia + ICH with high-glucose blood hematoma group.

Acute Hyperglycemia and ICH with High-glucose Blood Hematoma Reduced Autophagy around the Hematoma

A group of autophagy-related genes (Atgs) and their encoded proteins (ATGs) have been identified in yeast and fungi. LC3 is a mammalian homologue of yeast ATG8, and LC3-II is specifically associated with autophagosome membranes[8]. Beclin-1 (Atg6) is essential for the recruitment of ATGs to autophagosomes and the induction of autophagosome formation[9]. The SQSTM1/p62 protein is selectively incorporated into autophagosomes through direct binding to LC3, and is efficiently degraded by autophagy. So the level of SQSTM1/p62 is inversely correlated with autophagic activity[10].

Acute hyperglycemia without ICH did not affect the levels of the three markers of autophagy, LC3, beclin-1 and SQSTM1/p62, compared with the sham operation group. However, at 24 h after ICH the LC3-II/LC3-I ratio and the beclin-1 protein level in the right ipsilateral basal ganglia increased (29.65 ± 8.11% *vs* 7.48 ± 3.58% and 81.69 ± 18.10% *vs* 32.39 ± 8.65%, respectively, both $P < 0.05$), and the SQSTM1/p62 level decreased (25.60 ± 3.41% *vs* 57.94 ± 7.35%, $P < 0.01$). An increase in serum glucose

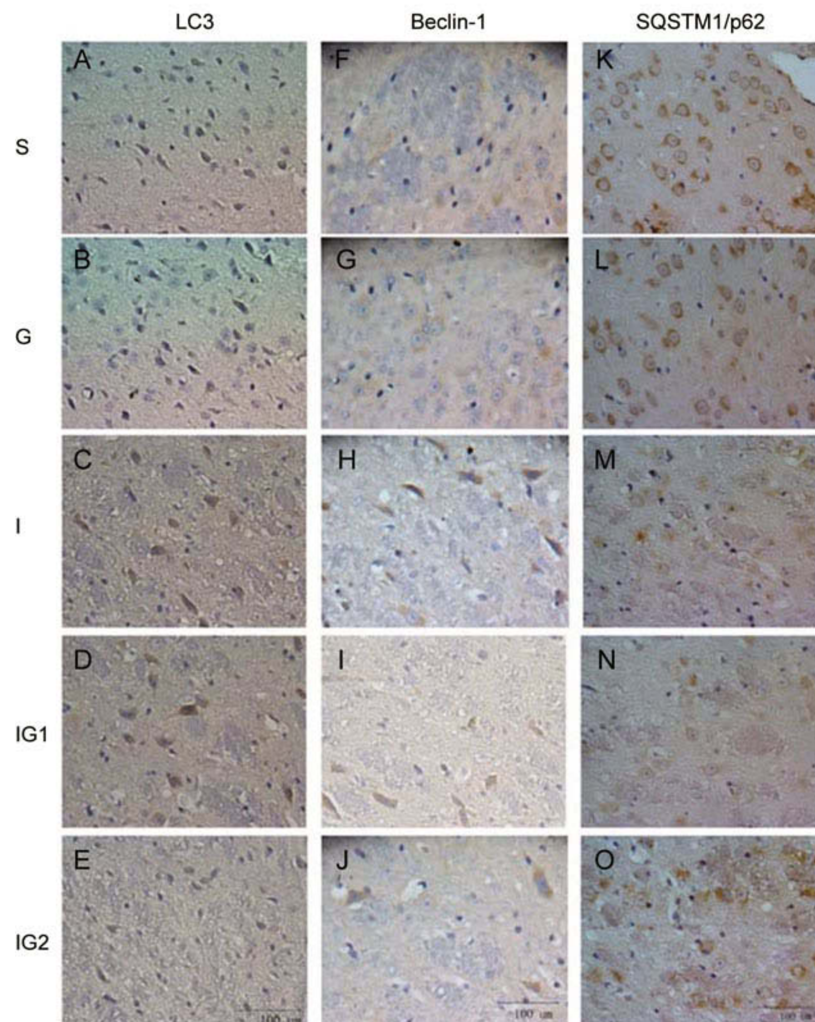

Fig. 6. Sections stained for LC3 (A–E), beclin-1 (F–J) and SQSTM1/p62 (K–O) in the ipsilateral basal ganglia 24 h after operation. Images were captured under a light microscope (×400), scale bars, 100 μm. S, sham group; G, hyperglycemia group; I, ICH model group; IG1, acute hyperglycemia + ICH with normal-glucose blood hematoma group; IG2, acute hyperglycemia + ICH with high-glucose blood hematoma group.

only (group IG1) did not affect the level of LC3, beclin-1 or SQSTM1/p62 after ICH (26.55 ± 6.34% *vs* 29.65 ± 8.11%; 78.79 ± 13.40% *vs* 81.69 ± 18.10%; 24.27 ± 8.46% *vs* 25.60 ± 3.41% respectively, all $P > 0.05$). However, an increase in both serum glucose and ICH hematoma glucose (group IG2) reduced autophagy around the hematoma; the LC3-II/LC3-I ratio and beclin-1 protein levels decreased (9.88 ± 2.57% *vs* 29.65 ± 8.11%; 36.93 ± 9.01% *vs* 81.69 ± 18.10%, both $P < 0.05$), and the SQSTM1/p62 level increased (79.19 ± 9.43% *vs* 25.60 ± 3.41%, $P < 0.01$; Fig. 5).

The staining of LC3- and beclin-1-positive cells was weak in the sham and G groups. However, 24 h after ICH, LC3- and beclin-1-positive cells were deeply stained around the hematoma. Consistent with the results of the Western blot analysis, we found that acute hyperglycemia and ICH with normal-glucose blood hematoma did not markedly change the LC3 and beclin-1 staining compared with the ICH group, but acute hyperglycemia and ICH with high-glucose blood hematoma weakened the staining (Fig. 6).

Moreover, SQSTM1/p62-positive cells were deeply stained in the sham and G groups. However, 24 h after ICH, the p62 staining around the hematoma was weakened. Consistent with the results of Western blot analysis, we found that acute hyperglycemia and ICH with normal-glucose blood did not markedly change the SQSTM1/p62 staining, but acute hyperglycemia and ICH with high-glucose blood deepened it (Fig. 6).

DISCUSSION

In this study, we found that autophagy occurred in the ipsilateral basal ganglia 24 h after ICH. Acute hyperglycemia together with ICH hematoma of high-glucose blood aggravated the neurological deficits and brain edema, and reduced autophagy around the hematoma after ICH.

Acute Hyperglycemia and ICH with High-glucose Blood Hematoma Exacerbated Neuronal Injury after ICH

Admission hyperglycemia, which frequently occurs after ICH, is induced by diabetes mellitus or a stress response. It is considered to be a predictor of poor outcome, and is potentially related to the presence and severity of intraventricular extension[11-13]. Animal studies have also shown that persistent hyperglycemia induced by an i.p. injection of streptozotocin exacerbates cerebral hematoma expansion, and causes more profound brain edema and perihematomal cell death after ICH[14, 15]. Earlier studies found that diabetes or chronic hyperglycemia induces microvasculature lesions, including in the cerebral vessels[16], which may be partly responsible for the deleterious effects of persistent hyperglycemia on ICH. In this study, we used a transient hyperglycemia rat model by i.p. administration of a 50% glucose solution, and without any treatment, the plasma glucose levels of the acutely hyperglycemic rats returned to normal 24 h after operation. Therefore, the potential effects of hyperglycemia-induced lesions in cerebral vessels prior to ICH were excluded and we assumed that the results reflected the effects of acute hyperglycemia on ICH.

Our results showed that acute hyperglycemia and ICH with high-glucose blood hematoma exacerbated the neurological deficits. This is in line with a previous report which showed that admission hyperglycemia is associated with poor functional recovery at discharge after ICH[3].

In addition, recent studies have indicated that pre-ischemic hyperglycemia exacerbates brain damage[17,18] because hyperglycemia worsens blood-brain barrier (BBB) injury after ischemia[19]. In our study, we found that an increase only in serum glucose slightly elevated the neurological deficit scores and brain water content, although the changes were not statistically significant. The deleterious effects induced by acute hyperglycemia may also be associated with the exacerbated breakdown of the BBB after ICH. When the serum glucose and glucose levels in the hematoma were both increased, the neurological deficit scores and brain water content were significantly elevated, which indicated that increased glucose levels in the hematoma play a role in brain injury. Nishikawa *et al.*[20] also found that hyperglycemia increases the production of reactive oxygen species *in vitro*.

Autophagy Pathway Was Activated after ICH, But Acute Hyperglycemia and ICH with High-glucose Blood Hematoma Reduced Autophagy around the Hematoma

Autophagy is essential for homeostasis and cell survival, and has been implicated in many diseases[21-23]. We found that the autophagy pathway was activated after ICH, which is consistent with recent studies[5]. However, it remains controversial as to whether autophagy is harmful

or beneficial after ICH. The answer may depend on the physiological or pathological environment around the cells and the level of autophagy. In myocardial ischemia-reperfusion injury experiments, although the induction of autophagy during the myocardial ischemic phase is protective, further enhancement of autophagy during the myocardial reperfusion phase appears to be detrimental and may induce cell death[24]. Thrombin and iron are confirmed aggravating factors in ICH. Hu *et al.*[25] found that thrombin activates autophagy in the brain. Furthermore, 3-methyladenine, an autophagy inhibitor, exacerbates thrombin-induced cell death in cultured astrocytes, which infers that autophagy is protective. However, a recent report indicated that the intracerebral infusion of iron activates autophagy, and deferoxamine (an iron chelator) significantly reduces autophagy after ICH, which suggests that iron-induced autophagy plays a role in brain injury[5]. Thus, further investigation is required and direct evidence is needed to determine the functional role of autophagy in ICH.

The findings of the present study indicated that an increase in serum glucose alone did not affect autophagy around the hematoma after ICH, but acute hyperglycemia and ICH with high-glucose blood hematoma reduced autophagy. It seems that autophagy around the hematoma after ICH is mainly influenced by local high-glucose blood. Similar results have been found in cultured cardiomyocytes, in which high glucose reduces autophagy compared with normal glucose levels[6]. However, the mechanism of decreased autophagy *in vivo* may also be associated with the secretion of insulin induced by acute hyperglycemia; and Prod'home *et al.* found that insulin inhibits autophagy *in vitro*[26].

Reduced autophagy may only be an epiphenomenon of neurological injury, which is exacerbated by acute hyperglycemia and ICH with high-glucose blood hematoma. Another possibility is that decreased autophagy may cause the death of a mass of cells around the hematoma in other ways, such as apoptosis and necrosis. An increase in necrosis results in the activation of the relevant inflammatory response and the exacerbation of brain edema. However, further interventional experiments are needed to investigate the exact role of autophagy in ICH, and the link between the decrease of autophagy and the exacerbation of neurological injury.

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