Published in final edited form as:

Neurol Res. 2009 March ; 31(2): 173–178. doi:10.1179/174313209X393591.

Effect of gap junction inhibition on intracerebral hemorrhage-induced brain injury in mice

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Abstract

It has been reported that gap junction contributes to ischemic brain injury and gap junction inhibitors improve neurological outcome in ischemic brain injury models. In the present study, we investigated the effects of gap junction inhibitor, carbenoxolone, on mortality, neurological deficits and brain edema in mice with intracerebral hemorrhage. A total of 80 male CD-1 mice were divided into two parts with two end-points for this study. In part one, animals were divided into four groups: sham, vehicle treatment following intracerebral hemorrhage induction, low-dose carbenoxolone (33 mg/kg) treatment 1 hour after intracerebral hemorrhage induction and high-dose carbenoxolone (100 mg/kg) treatment 1 hour after intracerebral hemorrhage induction groups. Animals were euthanized after 24 hours. In part two, animals were divided into four groups: sham, vehicle treatment 1 hour after intracerebral hemorrhage induction, single high-dose of carbenoxolone treatment at 1 hour after intracerebral hemorrhage induction and three high-doses of carbenoxolone treatment 1, 24 and 48 hours respectively after intracerebral hemorrhage induction. Animals were euthanized after 72 hours. Intracerebral hemorrhage was induced by collagenase injection. Neurological deficits were evaluated using modified Garcia's neurological test, wire hanging and beam balance tests. Brain edema was measured by brain water content. Our results showed that intracerebral hemorrhage produced brain edema and neurological deficits in mice. Carbenoxolone treatment failed to reduce brain edema and neurological deficits. In fact, the high dose of carbenoxolone aggravated neurological deficits and increased mortality 72 hours after the treatment. In conclusion, inhibition of gap junction has no short-term neuroprotective effect on intracerebral hemorrhage-induced brain injury. Further studies are required to assess the long-term effects of gap junction inhibitors in intracerebral hemorrhage models.

Keywords

Intracerebral hemorrhage; gap junction; neuroprotection

INTRODUCTION

Intracerebral hemorrhage is a devastating clinical event with initial mortality greater than 40%. Many survivors of intracerebral hemorrhage are left permanently disabled (Woo and Broderick, 2002). Currently, there is neither an effective therapy to increase survival after intracerebral hemorrhage nor a treatment to improve the quality of life for survivors (D'Ambrosio *et al.*, 2005).

The effects of intracerebral hemorrhage upon brain tissue are biphasic. Initial injury occurs in response to the expanding hematoma imposing sheer forces and mass effect upon the cerebral tissues (Aronowski and Hall, 2005). Intracerebral bleeding leads to increased intracranial pressures and could lead to transtentorial herniation secondary to the mass effect (Badjatia and Rosand, 2005). A later phase involves hematoma-induced apoptotic death of neuronal and glial cells in the surrounding parenchymal rim (Felberg *et al.*, 2002), followed by progressive rupture of the blood-brain barrier and rising brain edema (Qureshi *et al.*, 2001a, b).

The gap junction is an important component of cell-to-cell communication. The structural unit of gap junctions is a connexon or hemichannel, which are made up of six protein subunits, connexins, forming intercellular channels. Connexins have been classified according to their molecular weight and thereby named accordingly: Cx43, Cx37 and Cx26 (De Maio *et al.*, 2002). Gap-junctional channels allow the passage of ions and small molecules with relatively free permeability of molecules up to 1.2 kD. Therefore, most ions and many metabolites such as glutamate and glutamine can diffuse along a concentration gradient between adjacent cells. Even glucose taken up from the blood at specific astrocytic end-feet glucose transporters could spread along the microvascular unit, at least in part by the astrocytic gap junctions (Tabernero *et al.*, 1996).

It is commonly believed that during pathological situations, gap junction channels would continue to allow exchange of intracellular ions and molecules between both dying and viable brain cells. This exchange has been suggested to be either beneficial or detrimental, depending on the nature of the insult (e.g. ischemic or traumatic) (Perez Velazquez *et al.*, 2003). Some evidence suggests that the spreading of apoptotic and necrotic signals from injured and stressed cells to the neighboring viable cells through gap junctions may amplify the extent of injury (Lin *et al.*, 1998; Frantseva *et al.*, 2002b). Others have reported that the blockage of gap junctions through agents such as insulin (Homma *et al.*, 1998), octanol (Frantseva *et al.*, 2002a; Rawanduzy *et al.*, 1997) and carbenoxolone (De Pina-Benabou *et al.*, 2005) could lead to neuroprotective effects in several models of cerebral injury.

To date, no study has assessed the effects of inhibiting gap junction communication on the outcomes of intracerebral hemorrhage. Therefore, in the present study, we investigated whether the gap junction inhibitor, carbenoxolone, is able to protect against the effects of intracerebral injury including brain edema, neurological deficits and mortality in a collagenase-induced mouse model of intracerebral hemorrhage.

METHODS

Experimental animals

All procedures and methods for these studies were approved by the Animal Care and Use Committee at Loma Linda University and complied with the *Guide for the Care and Use of Laboratory Animals* (<http://research.llu.edu/forms/appendixb.doc>). Male CD-1 mice (34–43 g, Charles River, MA, USA) were housed with a 12 h light/dark cycle, at 24°C constant temperature, and access to water and rat chow *ad libitum*.

Aseptic technique was used for surgery. A total of 80 male CD-1 mice were divided into two parts with two end-points for this study. In part one, animals were divided into four groups: sham, vehicle treatment following intracerebral hemorrhage induction, low-dose carbenoxolone (33 mg/kg) treatment 1 hour after intracerebral hemorrhage induction and high-dose carbenoxolone (100 mg/kg) treatment 1 hour after intracerebral hemorrhage induction groups. Animals were euthanized after 24 hours. In part two, animals were divided into four groups: sham, vehicle treatment 1 hour after intracerebral hemorrhage induction, single high-dose of carbenoxolone treatment 1 hour after intracerebral hemorrhage induction and three high-doses of carbenoxolone treatment 1, 24 and 48 hours respectively after intracerebral hemorrhage induction. Animals were euthanized after 72 hours.

Intracerebral hemorrhage induction

We adopted the collagenase-induced intracerebral hemorrhage model (Rosenberg *et al.*, 1990) as previously described in mice (Tang *et al.*, 2004). Briefly, mice were anesthetized with ketamine (100 mg/kg, intraperitoneal injection) and xylazine (10 mg/kg, intraperitoneal injection) and positioned prone in a stereotaxic head frame (Stoelting, Wood Dale, IL, USA). An electronic thermostat-controlled warming blanket was used to maintain the core temperature at $37 \pm 0.5^\circ\text{C}$. The calvarium was exposed by a midline scalp incision from the nasion to the superior nuchal line, and the skin was retracted laterally with a variable speed drill (Fine Scientific Tools, Foster City, CA, USA). A 1.0 mm burr hole was made 0.9 mm posterior to bregma and 1.45 mm to the right of the midline. A 26-G needle on a Hamilton syringe was inserted with stereotaxic guidance 4.0 mm into the right deep cortex/basal ganglia at a rate 1 mm/min. The collagenase (0.075 units in 0.5 μl saline, VII-S; Sigma, St Louis, MO, USA) in the syringe was infused into the brain at a rate of 0.25 $\mu\text{l}/\text{min}$ over 2 minutes with an infusion pump (Stoelting, Wood Dale, IL, USA). The needle was left in place for an additional 10 minutes after injection to prevent the possible leakage of collagenase solution. After removal of the needle, the incision was closed and the mice were allowed to recover. Sham operation was performed with needle insertion only.

Brain water content

The brain water content was measured as previously described (Yang *et al.*, 1994; Tang *et al.*, 2004). Briefly, mice were euthanized under deep anesthesia. Brains were removed immediately and divided into five parts: ipsilateral and contralateral basal ganglia, ipsilateral and contralateral cortex, and cerebellum. The cerebellum was used as an internal control for brain water content. Tissue samples were weighed on an electronic analytical balance (model AE 100; Mettler Instrument Co., Columbus, OH, USA) to the nearest 0.1 mg to

obtain the wet weight. The tissue was then dried at 100°C for 24 hours to determine the dry weight. Brain water content (%) was calculated as [(wet weight × dry weight)/wet weight] × 100.

Statistical analysis

Quantitative data are expressed as the mean ± standard error of the mean. Statistical significance was verified by analysis of variance (ANOVA) (Dunss's method) for analysis of acute effect (24 hours) and by ANOVA (Turkey test) for analysis of delayed effect (72 hours). $p < 0.05$ was considered statistically significant.

RESULTS

Brain edema

In the 24 hour study, collagenase injection caused a significant rise in brain water content in the ipsilateral basal ganglia of the vehicle treated intracerebral hemorrhage group and low-dose carbenoxolone-treated group when compared with sham animals ($p < 0.05$). The high-dose of carbenoxolone treatment reduced brain edema in the ipsilateral basal ganglia when compared with the vehicle treated intracerebral hemorrhage group and the low-dose treated group. However, this reduction did not reach the statistically significant level.

In the 72 hour study, significant brain edema was found in the ipsilateral basal ganglia of all animals that had undergone intracerebral hemorrhage induction. Neither the low nor high dose of carbenoxolone reduced brain edema (Figure 1) when compared with the vehicle-treated intracerebral hemorrhage group.

Neurological deficits

Neurological deficits were present in all animals after collagenase injection. Neither the low dose nor high dose of carbenoxolone led to any improvement of neurological function when compared with the vehicle treated intracerebral hemorrhage group at 24 and 72 hour time points (Figure 2). In fact, high-dose carbenoxolone (100 mg/kg) given every 24 hours over 3 days worsened the neurological deficit according to performance on the wire hanging and beam balance test (Figure 2B, $p < 0.05$, versus vehicle treated ICH group).

Mortality

In the 24 hour study, low-dose carbenoxolone produced 100% (6/6 mice) survival rate, whereas both vehicle group and high-dose carbenoxolone group produced equal survival rate of 86% (6/7 mice). No significant difference was detected between groups. In the 72 hour study, carbenoxolone treatment decreased survival rate in a dose-dependent manner (singleinjection group 50% (6/12mice), multi-injection group 43% (6/14 mice) and vehicle group 87.5% (7/8 mice). These marked differences between groups did not reach a statistically significant level possibly due to the limited number of animals in groups (Figure 3).

We also tested the effect of octanol, another gap junction inhibitor, on survival rates in our intracerebral hemorrhage model. We found that 83% (5/6 mice) of mice survived 24 hours

after a single injection of octanol (2 mg/kg) 1 hour after intracerebral hemorrhage induction and only 33% (2/6 mice) survived 24 hours after a single injection of octanol (6 mg/kg) 1 hour after intracerebral hemorrhage. These survival rates are lower than those of vehicle treated groups described above.

DISCUSSION

Our study demonstrated that blockage of gap junctions through application of carbenoxolone did not reduce brain edema and neurological deficits after intracerebral hemorrhage in mice. In fact, the high dose (100 mg/kg) of carbenoxolone aggravated neurological deficit after 72 hours and also increased overall mortality. Additionally, we found that treatment with octanol, another gap junction blocker, led to an increased mortality in intracerebral hemorrhage mice.

The neuroprotective effect of gap junction inhibition has been demonstrated in several *in vitro* and *in vivo* models of neuronal injury. Frantseva *et al.* (2002a) demonstrated that carbenoxolone and octanol significantly decreased cell death 48 hours after induction of ischemia in organotypic hippocampal slices in glucose-free medium. De Pina-Benabou (2005) demonstrated that carbenoxolone added to organotypic hippocampal slice cultures before, during, or 60 minutes after onset of oxygen glucose deprivation, led to markedly decreased activation of caspase-3 and reduced neuronal death. De Pina-Benabou *et al.*, using an intrauterine hypoxia-ischemia model, determined that immediate carbenoxolone post-treatment ameliorated long-term developmental deficits and markedly decreased rate of mortality of new born pups (De Pina-Benabou *et al.* 2005). Using the middle cerebral artery occlusion model in rats, Rawanduzu *et al.* (Rawanduzu *et al.*, 1997) demonstrated decreased mean infarction volume with octanol pre-treatment.

Other reports have also shown beneficial effects of gap junction communication in neuroprotection. Blanc *et al.* (1998) reported astrocyte-astrocyte coupling in organotypic hippocampal slice culture produced neuroprotection against oxidative injury by suppressing the intraneuronal reactive oxygen species accumulation and stabilizing calcium homeostasis. Similarly, the neuroprotective effect of gap junction communication during glutamate insult in cortical neuron-astrocyte cocultures has been demonstrated (Ozog *et al.*, 2002). Cx43 gap junction knockout mice experienced increased ischemic insult after middle cerebral artery occlusion (Nakase *et al.*, 2003).

Our study did not find any neuroprotective effect of gap junction inhibitors against brain injury within 72 hours of intracerebral hemorrhage in mice. The increased mortality and neurological deficits in the treatment groups might result from the toxicity of high dose of gap junction inhibitors. The long-term effect of gap junction inhibition in intracerebral hemorrhage is still unknown and requires further study.

ACKNOWLEDGEMENT

This work was supported by National Institutes of Health (grant no. NS052492) to Jiping Tang.

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A 24-hour study

B 72-hour study

Figure 1:

Brain water content. In the 24 hour study (A), there was a marked increase in ipsilateral basal ganglia water content in animals receiving collagenase injection compared with animals in sham group ($p < 0.05$). Carbenoxolone treatment did not attenuate brain edema ($p > 0.05$, ICH: intracerebral hemorrhage). In the 72 hour study (B), marked increase in water content in ipsilateral basal ganglia was observed in mice after collagenase injection ($p < 0.05$, versus sham). Neither the single injection nor repeated injections of carbenoxolone (100 mg/kg) were able to significantly reduce brain edema ($p > 0.05$)

Figure 2:

Neurological function tests. In the 24 hour study (A), significant neurological deficits were observed in all animals receiving collagenase ($p < 0.05$, versus sham). However, no differences were observed between vehicle treated group and low and high dose carbenoxolone treated group ($p > 0.05$). The data of wire hanging and beam balance tests are represented as the average of three trials per animal. In the 72 hour study (B), three injections of high-dose carbenoxolone (100 mg/kg) worsened neurological deficits as evaluated by wire hang and beam balance testing ($n=6$; $p < 0.05$, versus vehicle treated intracerebral hemorrhage group). The data of wire hanging and beam balance tests are represented as the average of three trials per animal (ICH: intracerebral hemorrhage)

Figure 3: Mortality. Treatment of carbenoxolone caused a dose-dependent decrease in the survival rate of mice after intracerebral hemorrhage (ICH). Specifically, by 72 hours after intracerebral hemorrhage induction, 87.5% of vehicle treated mice survived, while 50% of the animals receiving a single injection of carbenoxolone (Carb100*1) survived, and only 43% of the animals receiving three injections (Carb100*3) survived