Neuroprotective Effects of a Cardioplegic Combination (Adenosine, Lidocaine, and Magnesium) in an Ischemic Stroke Model

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Abstract
Adenosine, lidocaine, and magnesium (ALM) are clinically available cardioplegic solutions. We examined the effects of low-dose ALM on ischemic stroke in cell and animal models. Cobalt chloride (CoCl₂)–treated SH-SY5Y cells were used as a surrogate model to mimic oxygen–glucose deprivation conditions. The cells were incubated with different dilutions of ALM authentic solution (1.0 mM adenosine, 2.0 mM lidocaine, and 5 mM MgSO₄ in Earle’s balanced salt solution). At a concentration of 2.5%, ALM significantly reduced CoCl₂-induced cell loss. This protective effect persisted even when ALM was administered 1 h after the insult. We used transient middle cerebral artery occlusion to investigate the therapeutic effects of ALM in vivo. Rats were randomly assigned to two groups—the experimental (ALM) and control (saline) groups—and infusion was administered during the ischemia for 1 h. The infarction area was significantly reduced in the ALM group compared with the control group (5.0% ± 2.0% vs. 23.5% ± 5.5%, p = 0.013). Neurological deficits were reduced in the ALM group compared with the control group (modified Longa score: 0 [0–1] vs. 2 [1–2], p = 0.047). This neuroprotective effect was substantiated by a reduction in the levels of various neuronal injury markers in plasma. These results demonstrate the neuroprotective effects of ALM and may provide a new therapeutic strategy for ischemic stroke.

Keywords Adenosine, lidocaine, and magnesium · Cobalt chloride · Ischemia · Middle cerebral artery occlusion · Stroke

Introduction
Stroke is the second leading cause of death and disability worldwide [1]. Despite the development of new medications, effective treatments for acute stroke are limited [2]. A combination of adenosine, lidocaine, and magnesium (ALM) was first developed in 1998 to mimic the hibernating state of cardiac protection in cardiac surgery [3]. Since adenosine, lidocaine, and magnesium can cross the blood–brain barrier, ALM might exert neuroprotective effects in the ischemic brain [4, 5]. In a cardiac arrest model on prolonged extracorporeal life support, ALM was associated with fewer neurological deficits and lower levels of tumor necrosis factor α in the brain [3]. In a traumatic brain injury model, ALM resuscitation fluid increased anti-inflammatory cytokines in brain tissue and lowered brain injury markers [6].

High-dose ALM can arrest heart contraction and offer better myocardial protection than high-potassium depolarizing solutions [7, 8, 9]. At low doses, ALM exerts protective effects in animal models of myocardial ischemia, arrhythmia, cardiac arrest, and hemorrhagic and septic shock [10]. The underlying mechanism by which it does this could be partly explained by its ability to reduce inflammation, correct coagulopathy, and lower energy demands [6, 11, 12]. The protective effects of ALM on the cardiovascular system have been well elaborated [3]. However, it remains unclear whether the hibernating effects of ALM are beneficial to the brain; this issue has not yet been examined systematically.
Since ALM has never been studied in animal models of ischemic stroke, it is difficult to evaluate the effect of ALM in clinical ischemic stroke. The critical clinical setting, infarction size, and reperfusion time are difficult to standardize for evaluation. Thus, we applied a transient middle cerebral artery occlusion model to explore whether ALM is helpful in ischemia–reperfusion brain injury. We hypothesized that the protective effects of ALM decrease the damage caused by hypoperfusion in an ischemic stroke model and that low-dose ALM infusion decreases infarction size. To the best of our knowledge, this study is the first to use a cell model and then an animal model to explore this issue.

Materials and Methods

Effects of Cobalt Chloride on the Survival of SH-SY5Y Cells

The human neuroblastoma cell line, SH-SY5Y, was used in the present study. The cells were grown to confluence in tissue culture wells with density $2 \times 10^5$ and maintained in culture with fetal bovine serum (FBS). After allowing the cells to differentiate in a medium containing FBS for 24 h, the medium was replaced with Earle's balanced salt solution (EBSS), which lacked glucose. Subsequently, the cells were incubated in cobalt chloride ($\text{CoCl}_2$) at different concentrations to achieve oxygen deprivation via chemical methods. Cells were collected after 24 h of incubation. Cell viability and hypoxic injury were evaluated with alamarBlue cell viability assay and hypoxia-inducible-factor 1 alpha (HIF1α) expression, respectively. Optimal oxygen and glucose deprivation conditions were determined based on reliable hypoxic damage.

Cytotoxic Profiles of ALM

ALM (1.0 mM adenosine, 2.0 mM lidocaine, and 5 mM MgSO₄) was diluted with EBSS solution to 1.25%, 2.5%, 5%, 10%, and 20% to test its cytotoxicity in the SH-SY5Y cell line. After adding the ALM solution to the cells and leaving it for 24 h, we performed an alamarBlue cell viability assay to assess cell survival.

Effect of ALM Treatment on Cell Viability

We added ALM (1.0 mM adenosine, 2.0 mM lidocaine, and 5 mM MgSO₄) diluted with EBSS to different concentrations to $\text{CoCl}_2$-incubated SH-SY5Y cells for 24 h. We then compared the ALM-treated group with the $\text{CoCl}_2$-treated group and determined the most effective therapeutic concentration for cell preservation. The protective effect of ALM was evaluated using the alamarBlue cell viability assay. In the post-treatment group, we incubated SH-SY5Y cells in 50 μM CoCl₂ and subsequently added 2.5% ALM stock (1.0 mM adenosine, 2.0 mM lidocaine, and 5 mM MgSO₄) at different time points. Cell viability was assessed using the alamarBlue cell viability assay.

Animal Preparation

All animal experiments were performed in accordance with the animal protocol approved by the National Taiwan University College of Medicine and College of Public Health Institutional Animal Care and Use Committee (IACUC No. 20180302) and the Animal Welfare Act, National Institute of Health Guide for the Care and Use of Laboratory Animals, and ARRIVE (Animal Research: Reporting in Vivo Experiments) guidelines. Twelve Sprague Dawley rats (males; 7–8 weeks old; 235–250 g; BioLASCO Taiwan Co. Taipei, Taiwan) were studied. We investigated the effects of ALM treatment on infarction size following embolization.

In brief, rats were anesthetized using a 1.5% isoflurane–oxygen mixture by mask, and after a short period of stability, anesthesia was reduced to 1.0% isoflurane for the rest of the experimental period. Rectal body temperature was maintained between 37 and 38 °C using a thermostatically regulated heating lamp. Body weight was recorded at baseline. The rats were placed in the prone position, and bupivacaine (0.5%, 0.5 ml) was injected subcutaneously at the lateral aspect of the neck and the inguinal area to reduce postoperative wound pain. Using an aseptic surgical technique, the right femoral vein was cannulated with PE-50 tubing for vascular access. The animals were housed in separate cages at ambient room temperature during recovery and thereafter until the end of the experiment; they had a 14–10-h light–dark cycle. We prepared meloxicam (1 mg/kg) for oral intake if any rat exhibited nociceptive movements after recovering from anesthesia. We considered a rat to be abnormal if it showed any of the following movements: back-hunching, pacing, licking, biting, scratching, and wound-rubbing or if it refused water or food 2 h after recovery from anesthesia. We did not notice any of these reactions after our procedure during the study; therefore, no rats in our study received meloxicam.

Focal Ischemia and Surgical Procedure

Ischemia was induced by middle cerebral artery occlusion (MCAO) using the intraluminal suture technique [13]. The right common, external, and internal carotid arteries (CCA, ECA, and ICA, respectively) were dissected from the surrounding connective tissue through a lateral neck incision. The filament was placed in the right ECA and gently advanced via the ICA (approximately 20 ± 0.5 mm from the carotid bifurcation) to the middle cerebral artery.
In the transient MCAO model, we left the filament in the ICA for 1 h and then withdrew it to restore blood flow; all experiments were performed by the same individual (YCW).

**Preliminary Experiments to Determine ALM Concentration**

The ALM concentration was determined based on the safety profile in preliminary studies. We infused ALM at a concentration five times that of the final dose (ALM; 5.0 mM adenosine, 10.0 mM lidocaine, 25 mM MgSO4 in 0.9% normal saline) in three male Sprague Dawley rats through the femoral vein at a flow rate of 2 ml/kg/h. They expressed paradoxical breathing during infusion; two of them died shortly after infusion, while one developed asystole during infusion. Therefore, the concentration of the experimental solution was reduced to the current concentration. We also administered a solution diluted to 0.2 times the concentration of the final ALM concentration in three male Sprague Dawley rats at an infusion rate of 2 ml/kg/h, and induced transient MCAO for 1 h. One rat died within 24 h, pathology showed intracranial hemorrhage. The infarct area was smaller in the diluted ALM group, but the difference was not statistically significant (ALM group 6.3% ± 4.0% vs. saline group 14.0% ± 3.9%, p = 0.28). Therefore, we used 1.0 mM adenosine, 2.0 mM lidocaine, and 5 mM MgSO4 in 0.9% normal saline as the final concentration for the animal experiment.

**Methodology**

Based on the treatment modality, the rats were assigned to the experimental (ALM; 1.0 mM adenosine, 2.0 mM lidocaine, 5 mM MgSO4 in 0.9% normal saline), or control (0.9% normal saline; 308 mOsm/L) groups using a random table, blinded in consecutively numbered sealed envelopes by another laboratory member who had the
The number in each group was equal. The treatment modality was blinded to the operator (YCW) until all rats were sacrificed and the results collected.

After the filament was placed in the middle cerebral artery, we began to administer the infusion according to the assigned numbers. The treatment was administered through the femoral vein using a syringe pump (Injectomat TIVA Agilia, Fresenius KABI, France). In the transient MCAO model, we maintained the infusion rate at 2 ml/kg/h during the 1-h ischemic insult. After the 1-h infusion, the femoral catheter was removed, and the femoral vein was ligated. The rats were allowed to recover from anesthesia in cages with free access to food and water.

Neurological Examination

The rats were monitored for neurological deficits before surgery and 24 h after MCAO. Neurological deficits were determined using a modified Longa scoring system as follows: 0, no neurological deficit; 1, failure to fully extend the right forepaw; 2, circling to the right; 3, falling to the right; and 4, not walking spontaneously and having a depressed level of consciousness [14].

Evaluation of the Infarction Size in the Brain

The rats were sacrificed 24 h after MCAO. We followed an established protocol for euthanasia [15]. Briefly, isoflurane (5%) was provided with oxygen in the gas chamber for 5 min (when the response to the nociceptive stimulus was lost) [15]. After euthanasia, the rat brains were removed. The cerebrum was coronally sectioned (2-mm thick), and slices were stained with 1% 2,3,5-triphenyltetrazolium chloride (TTC) solution (Sigma-Aldrich®) at 37 °C for 30 min [16]. The infarction size was analyzed using imageJ software [17].

Enzyme-Linked Immunosorbent Assay

After anesthesia but before sacrifice, blood was collected by cardiac aspiration, transferred into tubes containing ethylenediaminetetraacetic acid, and centrifuged twice at 1500× for 20 min. The supernatant, that is, plasma, was analyzed for neuron-specific enolase (NSE), S100B, and matrix metalloproteinase 9 (MMP-9) levels using enzyme-linked immunosorbent assay (ELISA (Elabscience) following the manufacturer’s protocols.

Statistical Analysis

All continuous variables are summarized as mean ± standard deviation (SD), and categorical data are summarized as median and interquartile range. Data were tested for normality using the Shapiro–Wilk normality test or by assessing Q-Q plots of residuals using GraphPad Prism (version 9.3.1 for Windows, GraphPad Software, La Jolla, California, USA). In the cell model, the cell viability test and HIF1α data were averaged for each treatment concentration and analyzed using one-way analysis of variance followed by Dunnett’s post hoc comparison.

In the rodent model, the experimental unit was an individual animal, and the primary outcome was the infarct volume. Sample size calculation for our study was based on alpha level 0.05, power 0.8, and expected 50% difference between the experimental and control groups. Infarction size and plasma NSE, S100B, and MMP-9 levels were compared using the unpaired t-test. Neurological scores were compared using the Mann–Whitney test. Statistical significance was set at P < 0.05.

Results

Effect of CoCl2 on the Viability of Differentiated SH-SY5Y Cells

To establish a cell model mimicking oxygen–glucose deprivation, differentiated SH-SY5Y cells were exposed to CoCl2 at various concentrations for 24 h, according to previously described protocols [18]. Cells maintained in EBSS medium
were used as the control group. The number of differentiated SH-SY5Y cells was reduced with an alteration of morphology, that is, from a fusiform pattern to a round shape (Fig. 1A). Substantial cell loss was observed when the CoCl₂ concentration was > 50 μM.

**Expression of HIF1α in CoCl₂-Treated SH-SY5Y Cells**

To investigate whether CoCl₂ treatment can serve as a surrogate cell model for oxygen–glucose deprivation, we conducted western blotting on cell lysates of CoCl₂-treated SH-SY5Y cells. HIF1α expression was upregulated in CoCl₂-stimulated SH-SY5Y cells in a dose-dependent manner (Fig. 2).

**Safety Profiles of ALM**

To explore the safety profile of ALM, differentiated SH-SY5Y cells were incubated in various concentrations of ALM. The cell viability upon addition of ALM was similar to that of controls when the concentration of ALM was < 10% (Fig. 3), indicating the safety of applying ALM at a concentration < 10%.

**Attenuation of CoCl₂-Induced Neuronal Cell Death by ALM**

To study whether ALM attenuated CoCl₂-induced neuronal cytotoxicity, we incubated differentiated SH-SY5Y cells in 50 μM CoCl₂ and ALM at various concentrations (from 0 to 10%). Cell viability quantified using the alamarBlue test showed that 2.5% ALM could significantly reduce CoCl₂-induced cell loss (Fig. 4). We added 2.5% ALM to differentiated SH-SY5Y cells after various durations of CoCl₂ treatment (0 to 6 h). There were significant changes in morphology; that is, neurites were lost and cell numbers decreased in a time-dependent manner (Fig. 5B). Cell viability was significantly decreased when 2.5% ALM was administered 2 h after oxygen glucose deprivation began (Fig. 5C).
ALM-Induced Reduction in the Infarct Area After Transient MCAO

To examine the therapeutic effects of ALM, we performed MCAO in rats. Rats were randomized into two groups: the experimental (ALM) and control (saline) groups. Treatment was administered during the 1-h ischemic insult. Animals were sacrificed 24 h later for examination, and body weight and rectal temperature were within normal limits in both the ALM and saline groups. We measured neurological deficits 24 h after recovery from anesthesia using the modified Longa score. No animal died during surgery. However, one rat in the control group was excluded because the femoral catheter kinked within 1 h, and the treatment was not completed. Hence, the analysis was conducted on six experimental and five control rats. The infarction area was significantly reduced in the ALM group compared to the control group (ALM group: 5.0% ± 2.0% vs. saline group: 23.5% ± 5.5%, \( p = 0.013 \), Fig. 6A and B). The modified Longa score was significantly higher in the control group 24 h after MCAO surgery (ALM group: 0.0, [0–1] vs. saline group: 2.0, [1, 2], \( p = 0.047 \), Fig. 6C). Furthermore, we assessed the plasma levels of neuronal injury markers by measuring the concentrations of NSE, S100B, and MMP-9, using ELISA. Neuronal injury marker levels were significantly lower in the ALM group compared with the saline group (ALM vs. saline; NSE (ng/ml): 0.13 ± 0.08 vs. 0.36 ± 0.08, \( p = 0.02 \); S100B (pg/ml): 75.28 ± 5.1 vs. 123.8 ± 19.81, \( p = 0.03 \); MMP-9 (ng/ml): 7.06 ± 0.57 vs. 9.98 ± 0.94, \( p = 0.03 \), Fig. 6D, E and F).

ALM Treatment 1 h After MCAO

We examined the therapeutic effect of ALM administered 1 h after MCAO. The study consisted of the experimental (post-stroke 1 h) and control (0.9% normal saline) groups with three rats in each group. After the 1-h ischemic insult, rats were randomly assigned to the ALM or saline group with an infusion rate at 2 ml/kg/h for 1 h. The rats recovered from anesthesia after the infusion was completed and were sacrificed 24 h after MCAO. The infarct area was smaller in the experimental group; however, the difference was not statistically significant (ALM group: 4.2% ± 3.0% vs. saline group: 14.0% ± 3.9%, \( p = 0.12 \)).
**Discussion**

Our study has two important findings. First, ALM exerted protective effects in a CoCl₂-induced hypoxic cell model, and the protective effect persisted even when ALM was administered 1 h after the ischemic insult. Second, ALM infusion during ischemia decreased the infarction size in the transient MCAO model.

Stroke is a leading cause of disability, and ischemic stroke caused by arterial occlusion is responsible for most strokes [19]. The most effective treatment for ischemic stroke is reperfusion therapy using intravenous thrombolysis and endovascular thrombectomy [20]. However, revascularization has a critical time period and thus is not used universally in all patients with ischemic stroke [20]. Other treatment options for stroke include preserving tissue viability (using hypothermia) [21, 22], enhancing collateral blood flow [23], controlling edema formation [24], and targeting specific molecules in ischemia-induced pathways [25, 26]. However, these treatments do not show consistent clinical benefits [1]. Developing safe and effective treatments remains a major challenge in experimental and clinical neuroscience. In this study, ALM showed cytoprotective effects in SH-SY5Y cells exposed to CoCl₂ as a surrogate model of oxygen and glucose deprivation. Furthermore, infarction size was reduced in an ischemia and reperfusion rodent model of transient MCAO. ALM is already being used clinically in cardiac surgery [27]. The neuroprotection potential of ALM has been demonstrated in a rodent traumatic brain injury model [6]. By adjusting its concentration, ALM may have a potential role in acute ischemic stroke treatment through neuroprotection.
Fig. 6 Effect of ALM on a rodent stroke model of transient middle cerebral artery occlusion (MCAO). A The infarct areas were measured 24 h after MCAO by 1% 2,3,5-triphenyltetrazolium chloride (TTC) solution. B Infarction area was significantly larger in saline group than that in the ALM group. C Neurological deficits were measured with modified Longa score before MCAO and 24 h after MCAO before sacrifice. The modified Longa score was the same in ALM and saline groups before surgery, and saline group scored higher than ALM group 24 h after MCAO procedure. D Plasma level of neuron specific enolase (NSE) quantified with ELISA was higher in the saline group than in the ALM group. E Plasma level of S100B quantified with ELISA was higher in the saline group than in the ALM group. F Plasma level of metalloproteinase 9 (MMP9) quantified with ELISA was higher in the saline group than in the ALM group.
CoCl₂ has been used to induce hypoxic conditions in vivo and in vitro because it activates HIF1α, causes mitochondrial damage, and increases reactive oxygen species generation following ischemia [28, 29]. In the current study, we used CoCl₂ to mimic hypoxic conditions and demonstrate the cytoprotective properties of ALM. Hypoxia in the human brain causes damage to the neuronal model, along with astrocytes, oligodendrocytes, and pericytes [30]. However, the current cell model of SH-SY5Y cells did not exhibit the effects of ALM on neuroinflammation after ischemia given the lack of astrocytes and oligodendrocytes in the culture system. Further studies are required to expose different cells to ischemia to examine the cell preservation effects and mechanisms (such as on neuroinflammation) of ALM.

The MCAO model is the most widely used model for mimicking human focal ischemic stroke [31]. It produces focal occlusion of a large cerebral artery, as seen in human stroke, and offers the opportunity to study this phenomenon after reperfusion [32]. Although physiological variables and occlusion conditions can be monitored and controlled using noninvasive methods (such as laser Doppler) to reduce variability [33], blood flow to the posterior cerebral artery and branches of the ICA may be obstructed to different degrees during the procedure, leading to variable infarction areas and sizes [34]. In addition, different histological staining methods could contribute to the inconsistency in infarct size. One percent TTC is a marker of tissue dehydrogenase and mitochondrial dysfunction and may overestimate infarct size [35]. To decrease the interference of drawbacks by the MCAO model, we assigned our treatments randomly and kept them blinded until analysis.

Our study has some limitations. First, only young male rats were used in this study because of the concern that estrogen could influence infarct volume in female rats following MCAO [36]. It is necessary to include female and older rats to fulfill clinical needs. Furthermore, we only tested our hypothesis at one time point after ischemia. It remains unknown whether a longer ischemic duration with larger infarct areas will benefit from ALM infusion. This hypothesis should be tested in future studies. During MCAO, hemodynamic parameters such as blood pressure or central venous pressure were not recorded. These parameters are important in acute stroke and should be controlled in future studies [37]. There was a trend of reduced infarct size in the ALM post-stroke study, but the difference did not reach statistical significance. Such a trial will require an increase in sample size. Additional factors such as the concentration and duration of treatment influenced the outcomes. A large-scale experimental design incorporating different concentrations, treatment durations, and time points for therapy is necessary. Nevertheless, the present study showed the feasibility of the proof-of-concept as a foundation for future studies.

**Conclusion**

Low-dose ALM decreased the brain infarct area in a stroke model of transient MCAO. Furthermore, the neuroprotective effect of ALM was substantiated by the reduction in the plasma levels of various neuronal injury markers. These observations suggest the clinical potential of ALM in the treatment of ischemic stroke, warranting further investigation.

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**Author Contribution** All authors contributed to the study conception and design. Yi-Chia Wang performed the experiments and wrote the manuscript with support from Sung-Tsang Hsieh. Chen designed and directed the project of the oxygen–glucose deprivation model and aided in the interpretation of the results. The first draft of the manuscript was written by Yi-Chia Wang, and all authors commented on the previous versions of the manuscript. All authors read and approved the final manuscript.

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**Data Availability** The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

**Declarations**

**Ethics Approval** All animal experiments were performed in accordance with the animal protocol approved by the National Taiwan University College of Medicine and College of Public Health Institutional Animal Care and Use Committee (IACUC No. 20180302).

**Consent to Participate** Human subjects were not involved in this study.

**Consent to Publish** This manuscript does not contain individual person's data.

**Competing Interests** The authors declare no competing interests.

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