國立臺灣大學醫學院解剖學暨細胞生物學研究所

博士論文

Graduate Institute of Anatomy and Cell Biology

College of Medicine

National Taiwan University

Doctoral Dissertation

心臟保護液在缺血性中風模式中的神經保護作用

Neuroprotective effects of a cardioplegic combination (adenosine, lidocaine, and magnesium) in an ischemic stroke model

王憶嘉

Yi-Chia Wang

指導教授: 謝松蒼 博士

Advisor: Sung-Tsang Hsieh, M.D. Ph.D.

中華民國 111 年 11 月

Nov. 2022

doi:10.6342/NTU202210057

國立臺灣大學博士學位論文

口試委員會審定書

PhD DISSERTATION ACCEPTANCE CERTIFICATE

NATIONAL TAIWAN UNIVERSITY

心臟保護液在缺血性中風模式中的神經保護作用

Neuroprotective effects of a cardioplegic combination (adenosine, lidocaine, and magnesium) in an ischemic stroke model

本論文係王憶嘉（D03446003）在國立臺灣大學醫學院解剖學暨細胞生物學科研究所完成之博士學位論文，於民國 111 年 11 月 7 日承下列考試委員審查通過及口試及格，特此證明。

The undersigned, appointed by the Department of Anatomy and Cell Biology, College of Medicine, National Taiwan University on 07. November, 2022 have examined a PhD dissertation entitled above presented by Wang, Yi-Chia, D03446003 candidate and hereby certify that it is worthy of acceptance.

口試委員 Oral examination committee:

系主任/所長 Director:

Acknowledgement

在研究所的這段經歷，回想起來真的是人生中最緊繃的一段求學歷程。和小時候有標準答案的過程不一樣，在這裡的每一次嘗試都是新的未知的結果，面對這麼多的不確定，很多時候也想要放棄。很感謝謝松蒼老師在這段時間的教導。老師對研究的熱忱與追求真相的努力讓我體會到科學家的精神。老師即便行程滿檔也固定指導我看組織切片及討論實驗中遇到的困境。在分析結果跟整理資料上也總是能帶領我去蕪存菁，整理出符合邏輯的結果。看到老師在科學研究上親力親為與追根究柢的求知態度，讓我也希望在日後的研究上能夠以老師為榜樣，繼續努力。

在研究的過程中特別感謝一起在實驗室打拼的學長、學姊、助理以及暑期學弟妹。有大家一起鼓勵幫忙，除了解決許多研究上的困難與盲點外，在生活中也多了更多的支持。除了研究生的身分，剛進實驗室這個大家庭時我也剛回到醫院這個職場，一開始要平衡兩邊的工作常常覺得力不從心，但實驗室的成員給了我非常大的幫忙，在實驗上、生活上都是很大的動力。

我也特別感謝在臨床工作上老師及同事的支持。有大家的幫忙與包容、我才能夠在這裡完成研究的夢想。

doi:10.6342/NTU202210057

中文摘要

心臟保護液在心臟手術中是常見的藥物。其中 Adenosine, lidocaine 以及鎂離子(ALM)是非去極化心臟保護液體的其中一種配方。目前雖然知道 ALM 對心臟有保護的作用、但這樣的配方是否在神經系統也有保護作用則還沒有定論。因此這一研究探討低劑量的 ALM 是否能在神經系統缺血的時候達到神經保護的效果。我們使用細胞模式以及動物模式來驗證。在細胞模式中我們使用氯化鈷($CoCl_2$)來製造缺氧的環境，並以 SH-SY5Y 細胞株來建立低糖低氧的模式。我們用不同濃度的 ALM 溶液 (1.0 mM adenosine, 2.0 mM lidocaine, and5 mM $MgSO_4$)測試，並發現在. 2.5%, ALM 這個濃度下細胞因為缺氧而死亡的情形有顯著的改善。這個保護效果即使在缺氧持續 1 小時候才開始給予 ALM 也仍有保護的效果。在動物實驗上我們使用暫時性的腦缺血模式(transient middle cerebral artery occlusion)，來探討 ALM 保護液在生物體上的反應。我們用大鼠建立缺血性中風的模型，並隨機分派大鼠到實驗組 (ALM)和控制組(生理食鹽水)，並在腦部灌流停止的狀態下給予藥物。實驗結果顯示腦部缺血壞死的區域在實驗組(ALM)組有明顯的下降(5.0% ± 2.0% vs. 23.5% ± 5.5%, p=0.013)。神經學檢查也顯示和控制組相比實驗組的臨床表現較嚴重 (modified Longa score: 0 [0-1] vs.2 [1-2], p=0.047)。這些保護的效果也反映在血清中的神經細胞損傷標記，實驗組的濃度較低。這些結果提供 ALM 在缺血性中風的治療上可能有治療的潛力。

doi:10.6342/NTU202210057

關鍵字：腺苷，利多卡因，鎂離子，氧化鈷，缺血性腦中風模式，中風

doi:10.6342/NTU202210057

Abstract

Adenosine, lidocaine, and magnesium (ALM) are cardioplegic solutions, which arrested heart contraction in high concentration. Whether low-dose ALM infusion was beneficial to ischemic brain has not been thoroughly investigated. In our study, we examined this issue in cell and animal models. We used cobalt chloride ($CoCl_2$)-treated SH-SY5Y cells to mimic oxygen-glucose deprivation conditions in our cell model. SH-SY5Y cells were incubated with different dilutions of ALM authentic solution (1.0 mM adenosine, 2.0 mM lidocaine, and5 mM MgSO4 in Earle's balanced salt solution). ALM significantly reduced $CoCl_2$-induced cell loss at a concentration of 2.5%. This protective effect persisted even when ALM was administered 1 h after the insult. As for animal model, we chose middle cerebral artery occlusion (MCAO) to mimic ischemic stroke status. We randomly assigned the rats into two groups—the experimental (ALM) and control (saline) groups—and infusion was administered during the ischemia: one hour in transient model and 6 hours in permanent model. In transient MCAO model, 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. In permanent MCAO model, ALM group had longer survival (hazard ratio was 9.95; 95%

doi:10.6342/NTU202210057

confidence interval: 1.61 to 61.9), but the infarction size was not reduced when compared to control group. The survival benefits might come from less brain edema. These results demonstrate the neuroprotective effects of ALM and may provide a new therapeutic strategy for ischemic stroke.

Keywords: adenosine, lidocaine, magnesium, cobalt chloride, middle cerebral artery occlusion, stroke

doi:10.6342/NTU202210057

Table of Contents

doi:10.6342/NTU202210057

Chapter 1. Introduction

Ischemic stroke leads to catastrophic impairment in quality of life, and has been ranked second leading cause of death and disability in the world [1]. Brain is vulnerable to hypoxia, and has limited reserve for regeneration. Though many efforts have been placed in new medication developments, there is still no effective treatments for acute stroke [2].

A combination of adenosine, lidocaine, and magnesium (ALM) was first developed in 1998. The design recipe intended to simulate a hibernating state in order to achieve cardiac protection in cardiac surgery [3]. Since adenosine, lidocaine, and magnesium can cross the blood-brain barrier, the ability of ALM to prevent ischemic-reperfusion injury in the heart might also work in the neuron system. [4,5]. There were evidence in animal models showing ALM's potential in neuroprotection. In a cardiac arrest model on prolonged extracorporeal life support, ALM-treated group had fewer neurological deficits and their brain had lower levels of tumor necrosis factor α [3]. In a traumatic brain injury model, ALM resuscitation fluid increased anti-inflammatory cytokines in brain tissue. Not surprisingly, ALM group also had lower brain injury markers [12].

Compared to traditional cardioplegic solution, which used high-potassium depolarization to arrest heart contraction, high-dose ALM can offer better myocardial protection during asystole [6-8]. On the other hand, low doses ALM have exerted

doi:10.6342/NTU202210057

protective effects in various animal models, including myocardial ischemia, arrhythmia, cardiac arrest, and hemorrhagic and septic shock [9]. The underlying mechanism by which it does this could be partly explained by its ability to lower inflammation, correct coagulopathy, and reduce energy demands [10-12]. 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 systematically examined.

The individual component of ALM have shown some degrees of neuroprotective effects in the cardiac surgery population. Mathew et al. has examined the effect of lidocaine infusion by a randomized, double-blinded clinical trial. The study consisted of 227 patients who had cardiac surgery, and the end-points was postoperative cognitive function at 6 weeks and 1 year after the surgery. Lidocaine or placebo was given by 1 mg/kg bolus followed by a continuous infusion through 48 hours. The study found no improvement of neurocognitive function in the lidocaine group, but the secondary analysis revealed a protective effect of lower dose lidocaine in nondiabetic patients.[13] To further evaluate this phenomenon, a randomized, placebo-controlled clinical trial (Lidocaine For Neuroprotection During Cardiac Surgery, ClinicalTrials.gov Identifier: NCT00938964) has been started to decipher whether perioperative lidocaine could decrease post-operative cognitive dysfunction in non-diabetic patients after cardiac

doi:10.6342/NTU202210057

surgery.[14] Whether combining adenosine and magnesium improve clinical outcome is not known.

In human, the combination of ALM is currently used in cardiac surgery by surgeon's preference. In National Taiwan University Hospital, there were 500 adult cardiac surgeries in 2021, and 98 patients had systemic low-dose ALM infusion during cardiopulmonary bypass. The concern to use ALM intraoperatively is that adenosine may cause bradycardia, which increased the difficulty to wean off cardiopulmonary bypass after the operation. However, there was no adverse drug events reported so far. Permanent stroke happened in 15 patients, which was 2.61% of all cardiac surgical patients in 2021, and the permanent stroke rate in low-dose ALM infusion patients was 1.02%. However, the difference did not reach statistical difference. Moreover, the diversity in patients population, surgeon's technique, the influence of cardiopulmonary bypass, and the effect of anesthesia has made it difficult to evaluate the neuroprotective effect of ALM in cardiac surgery. However, from previous experience we could suggest that low-dose ALM was tolerable in humans, and might not be harmful in heart failure patients if carefully monitored.

Since ALM has never been studied in animal models of ischemic stroke, it is difficult to evaluate the clinical effect of ALM in hypoperfusion status or ischemic stroke. It is almost impossible to standardize the critical clinical setting, infarction size,

doi:10.6342/NTU202210057

and reperfusion time in reality. Thus, we applied a transient middle cerebral artery occlusion model to explore whether ALM is helpful in ischemia-reperfusion brain injury, and analyzed the effect of ALM in permanent middle cerebral artery occlusion model to simulate the condition of malignant stroke. 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.

doi:10.6342/NTU202210057

Chapter 2. Materials and methods

Cell model establishment

Effects of cobalt chloride on the survival of SH-SY5Y cells

The human neuroblastoma cell line, SH-SY5Y, was used in the study. The cells were grown to confluence in tissue culture wells with density $2x10^5/cm^2$ and maintained in culture with fetal bovine serum (FBS). After the cells differentiate in a medium containing FBS for 24 h, we replaced the medium with Earle's balanced salt solution (EBSS), which lacked glucose. Then, the cells were incubated in cobalt chloride ($CoCl_2$) at different concentrations in order to achieve oxygen deprivation via chemical methods. After 24 hours of incubation, cells were collected and analyzed. Cell viability and hypoxic injury were evaluated with alamarBlue cell viability assay and hypoxia-inducible-factor 1 alpha (HIF1α) expression, respectively. We determined the oxygen and glucose deprivation conditions for further study by reliable hypoxic damage.

Cytotoxic profiles of ALM

ALM (1.0mM adenosine, 2.0mM lidocaine, and 5mM $MgSO_4$) was diluted with EBSS solution to 1.25%, 2.5%, 5%, 10%, and 20% to test its cytotoxicity in the SH-SY5Y cell line. We performed an alamarBlue cell viability assay to assess cell survival 24 hours after adding the ALM solution to the cells.

doi:10.6342/NTU202210057

Effect of ALM treatment on cell viability

We added ALM (1.0 mM adenosine, 2.0 mM lidocaine, and 5 mM $MgSO_4$) diluted with EBSS to different concentrations to $CoCl_2$-incubated SH-SY5Y cells for 24 h. We then compared the ALM-treated group with the $CoCl_2$-treated group and determined the most effective therapeutic concentration for cell preservation. In the post-treatment group, we incubated SH-SY5Ycells in 50 μM $CoCl_2$ and subsequently added 2.5% ALM stock (1.0mM adenosine, 2.0mM lidocaine, and 5mM $MgSO_4$) at different time points. The protective effect of ALM was evaluated 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. Sprague Dawley rats (males; 7–8 weeks old; 235–250 g; BioLASCO Taiwan Co. Taipei, Taiwan) were studied for ischemic stroke model. We investigated the effects of ALM treatment on infarction size following embolization.

doi:10.6342/NTU202210057

Anesthetic management for surgical procedure

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 surgery. Rectal body temperature was maintained between 37°C and 38°C using a thermostatically regulated heating lamp. Body weight was recorded at baseline. We placed the rats in the prone position, and plain bupivacaine (0.5%, 0.5ml) was injected subcutaneously at the lateral aspect of the neck and the inguinal area before incision. 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.

Focal ischemia and surgical procedure

Ischemia was induced by middle cerebral artery occlusion (MCAO) using the intraluminal suture technique (Figure 1) [15]. The right common, external, and internal

doi:10.6342/NTU202210057

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 (total filament length 50mm; filament diameter 0.28mm; proximal silicon diameter 0.37+/- 0.02mm; RWD Life science, Co.Ltd).

MCAO model

Ischemia was produced by middle cerebral artery occlusion (MCAO) using the intraluminal suture technique, and the filament was was fixed in ICA region throughout all experiment period before sacrifice. In the transient MCAO model, we left the filament in the ICA for determined ischemic duration and then withdrew it to restore blood flow. All experiments were performed by the same individual (YCW).

Evaluation of MCAO effect on brain

We did transient MCAO model with different ischemic interval: 30 minutes, 60 minutes, 90 minutes, and 120 minutes, permanent MCAO model to determine the ischemic effect on rat brain. Their surgical mortality, image study, clinical behavior, histopathology were compared. The optimal model for ALM experiment should be a

doi:10.6342/NTU202210057

model that provide reliable ischemic injury with low surgical mortality rate.

Neurological examination

The rats were monitored for neurological deficits before surgery and 24 hours 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 [16]. The inter-rater variability using modified-Longa scoring system was low (Figure 2).

Evaluation of the infarction size in the brain

The rats were sacrificed 24 h after MCAO. Before sacrifice, we did 7T brain MRI to evaluate the infarct volume under general anesthesia. Afterwards, we followed an established protocol for euthanasia [17]. Briefly, isoflurane (5%) was provided with oxygen in the gas chamber for 5 min (when the response to the nociceptive stimulus was lost) [17]. 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 [18]. The infarction size was analyzed using imageJ software [19].

doi:10.6342/NTU202210057

Assessment of Brain edema

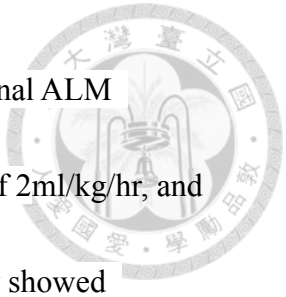
Brain edema was estimated by comparing wet-to-dry weight ratios. Briefly, rats were killed at the end of the experiment by decapitation under deep isoflurane anesthesia. The brain was quickly removed and gently blotted to remove small quantities of adsorbent moisture and was dissected through the interhemispheric fissure into right and left hemispheres. Brain tissue then was weighed with a weighing scale to within 0.1 mg. Dry weight of

the entire ipsilateral and contralateral uninjured hemispheres was determined after the tissue was heated for 3 days at 100°C in a drying oven. Tissue water content was then calculated as %H2O=(1-dry weight/wet weight) x 100%.[20]

Preliminary experiments to determine ALM concentration

We infused ALM at a concentration five times that of the final dose (ALM; 5.0 mM adenosine, 10.0 mM lidocaine, 25 mM $MgSO_4$ in 0.9% normal saline) in three male Sprague Dawley rats through the femoral vein at a flow rate of 2ml/kg/hr. 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

doi:10.6342/NTU202210057



administered a solution diluted to0.2 times the concentration of the final ALM concentration in three male Sprague Dawley rats at an infusion rate of 2ml/kg/hr, 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). The ALM concentration was determined based on the safety profile in preliminary studies. Therefore, we used 1.0 mM adenosine, 2.0 mM lidocaine, and 5 mM $MgSO_4$ 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 $MgSO_4$ 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 key. 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

doi:10.6342/NTU202210057

the femoral vein using a Syringe Pump (Injectomat TIVA Agilia, Fresenius KABI, France). In the transient MCAO model, we maintained the infusion rate at 2ml/kg/hour during the 1-h ischemic insult. After the 1-hour 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. In the permanent MCAO model, we maintained the infusion rate at 2ml/kg/hour for 6 hours. The rats were awake after we placed the filament, so the anesthetic duration was within 30 minutes for each operation. The rats were allowed to recover from anesthesia in cages with free access to food and water.

Enzyme-linked immunosorbent assay (ELISA)

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 metallopeptidase 9 (MMP-9) levels using ELISA (Elabscience) following the manufacturer's protocols.

Statistical analysis

All continuous variables are summarized as mean ± standard deviation (SD), and

doi:10.6342/NTU202210057

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 $P < 0.05$.

doi:10.6342/NTU202210057

Chapter 3. Results

Cell model

Effect of $CoCl_2$ on the viability of differentiated SH-SY5Y cells

To establish a cell model mimicking oxygen-glucose deprivation, differentiated SH-SY5Y cells were exposed to $CoCl_2$ at various concentrations for 24 h, according to previously described protocols [21]. Cells maintained in EBSS medium were used as the control group. As shown in figure 3a, the number of differentiated SH-SY5Y cells was reduced with an alteration of morphology. The cell morphology changed from a fusiform pattern to a round shape. Substantial cell loss was observed when the $CoCl_2$ concentration was > 50 μM.

Expression of HIF1α in $CoCl_2$-treated SH-SY5Y cells

To investigate whether $CoCl_2$ treatment can serve as a surrogate cell model for oxygen-glucose deprivation, we conducted western blotting on cell lysates of $CoCl_2$-treated SH-SY5Ycells. As shown in figure 4, HIF1α expression was upregulated in $CoCl_2$-stimulated SH-SY5Y cells in a dose-dependent manner.

Safety profiles of ALM

To explore the safety profile of ALM, differentiated SH-SY5Y cells were incubated in

doi:10.6342/NTU202210057

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 5). Our results indicate that applying ALM at a concentration < 10% of authentic ALM concentration should be safe.

Attenuation of $CoCl_2$-induced neuronal cell death by ALM

We incubated differentiated SH-SY5Y cells in 50μM $CoCl_2$and ALM at various concentrations (from 0% to 10%) to study whether ALM attenuated $CoCl_2$-induced neuronal cytotoxicity. As shown in figure 6, 2.5% ALM could significantly reduce $CoCl_2$-induced cell loss. We added 2.5% ALM to differentiated SH-SY5Y cells after various durations of $CoCl_2$-treatment (0 to 6 h). There were significant changes in morphology. As shown in figure 7b, neurites lost together with cell numbers decreased in a time-dependent manner. Cell viability was significantly decreased when 2.5% ALM was administered 2 h after oxygen glucose deprivation state began (Fig 7c).

Animal model

Surgical results of permanent MCAO

After permanent MCAO, we did 7T brain MRI to ensure adequate ischemic insults, and calculate the infarction size by staining brain section with 1% 2,3,5-triphenyltetrazolium

doi:10.6342/NTU202210057

chloride (TTC) solution. Among 5 rat, 2 rats died during general anesthesia before MRI examination. One rat died after MRI examination, but before we were ready to sacrifice. The MRI image was shown in figure 8. We chose 1% 2,3,5-triphenyltetrazolium chloride (TTC) stain for infarction size determination because the mortality rate for MRI image was high. The MRI image (T2) of rat brain after permanent MCAO was shown in figure 8.

Surgical results of transient MCAO

We did transient MCAO with various ischemic duration (30, 60, 90, 120 minutes). We had five rats in each group to compare the surgical mortality and infarction size. The infarction size increased with ischemic duration, and was 3.5%±3.0%, 18.5%±5.5%, 27.2%±6.5%. and 30%±6.5%, respectively. One rat died in 90 minutes group, and two rats died in 120 minutes group. Their autopsy showed intracranial hemorrhage. The neurological score in 30 minutes group was not statistically different from normal saline group. Thus we chose 60 minutes as our model for transient MCAO.

ALM-induced reduction in the infarct area after transient MCAO

To examine the therapeutic effects of ALM, rats were randomized into two groups: the experimental (ALM) and control (saline) groups. Treatment was administered during

doi:10.6342/NTU202210057

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 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, Figs 9a and 9b). 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 9c). 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, Figs 9d, 9e, and 9f]

ALM treatment 1 h after MCAO

We examined the therapeutic effect of ALM administered 1h after MCAO. The study

doi:10.6342/NTU202210057

consisted of the experimental (post-stroke 1 h) and control (0.9% normal saline) groups with three rats in each group. After the 1-hour ischemic insult, rats were randomly assigned to the ALM or saline group with an infusion rate at 2ml/kg/hour 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).

ALM increased survival in permanent MCAO model

We did survival analysis in permanent model. We randomly assigned 15 rats to either ALM or control group. Mortality rate before completion of the experiments were as follows: 4 of 15 (26.7%) in ALM group and 3 of 15 (20%) in control group. One rat failed to complete 6 hours infusion due to catheter kinking in ALM group. All the surgical mortality happened within 24 hours after completion of MCAO. These were excluded in our analysis. After emergence from anesthesia, there was no difference in Longa score in ALM and Saline groups. We followed the rats for 5 days, and their survival curve was shown in figure 10A. ALM group had better survival in either Log-rank test (p=0.01) or Gehan-Breslow-Wilcoxon test (p=0.01). The hazard ratio was 9.95. (95% confidence interval: 1.61 to 61.9)

doi:10.6342/NTU202210057

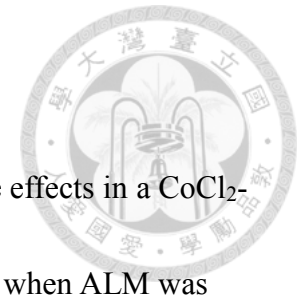
ALM did not decrease infarct area in permanent MCAO model

Infarction size was analyzed with 1% 2,3,5-triphenyltetrazolium chloride (TTC) solution. Infarction size between ALM and control group was not different in permanent MCAO model (figure 10d)

ALM decreased brain edema in non-ischemic brain in permanent MCAO model

Twelve rats, randomly assigned to ALM and saline group respectively, had permanent MCAO surgery, and sacrificed 48 hours later. Mortality before completion of the experiment were 2 premature death (16.7%) before 48 hours in ALM group, and 3 premature death (25%) before 48 hours in Saline group. Two rats in ALM group pulled out their catheter before infusion was complete, and two rats in saline group had catheter dysfunction in saline group. The surgical site had higher water content compared to contralateral hemisphere in either ALM or saline group. ($p<0.001$) However, brain water content in the surgical site showed no difference in ALM and saline groups. On the contrary, brain water content in contralateral hemisphere was significantly lower in ALM group.(ALM 78.89 +/-0.2; Control 79.75 +/-0.2;$p=0.01$, Figure 10c)

doi:10.6342/NTU202210057



Chapter 4. Discussion

Our study has two important findings. First, ALM exerted protective effects in a $CoCl_2$-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. We also found that ALM could not reduce infarction size if brain perfusion was not restored.

Stroke is a leading cause of disability, and ischemic stroke caused by arterial occlusion is responsible for most strokes [22]. The most effective treatment for ischemic stroke is reperfusion therapy using intravenous thrombolysis and endovascular thrombectomy [23]. However, revascularization has a critical time period and thus is not used universally in all patients with ischemic stroke [24]. Other treatment options for stroke include preserving tissue viability (using hypothermia) [25,26], enhancing collateral blood flow [27], controlling edema formation [28], and targeting specific molecules in ischemia-induced pathways [29,30]. 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 $CoCl_2$ 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

doi:10.6342/NTU202210057

clinically in cardiac surgery [31].The neuroprotection potential of ALM has been demonstrated in a rodent traumatic brain injury model [12]. By adjusting its concentration, ALM may have a potential role in acute ischemic stroke treatment through neuroprotection. However, our results showed that ALM could not decrease infarction size in permanent MCAO model. Thus, urgent reperfusion was the key to better outcome in acute stroke. ALM has the potential to decrease ischemic-reperfusion insult in the process.

$CoCl_2$ 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 [32,33]. In the current study, we used $CoCl_2$ 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 [34]. 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 [35]. It produces focal occlusion of a large cerebral artery, as seen in

doi:10.6342/NTU202210057

human stroke, and offers the opportunity to study this phenomenon after reperfusion [36]. Although physiological variables and occlusion conditions can be monitored and controlled using noninvasive methods (such as laser Doppler) to reduce variability [37], 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 [38]. 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 [39]. 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 [40]. It is necessary to include female and older rats to fulfil 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. There was a trend of reduced infarct size in the ALM post-

doi:10.6342/NTU202210057

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. These results may have implications for the treatment of ischemic stroke before reperfusion therapy.

doi:10.6342/NTU202210057

References

1. Campbell BCV, De Silva DA, Macleod MR, Coutts SB, Schwamm LH, Davis SM, Donnan GA (2019) Ischaemic stroke. Nat Rev Dis Primers 5 (1):70. doi:10.1038/s41572-019-0118-8

2. Matei N, Camara J, Zhang JH (2020) The Next Step in the Treatment of Stroke. Front Neurol 11:582605. doi:10.3389/fneur.2020.582605

3. Dobson GP, Letson HL (2016) Adenosine, lidocaine, and Mg2+ (ALM): From cardiac surgery to combat casualty care--Teaching old drugs new tricks. J Trauma Acute Care Surg 80 (1):135-145. doi:10.1097/TA.0000000000000881

4. Santa-Maria AR, Walter FR, Valkai S, Bras AR, Meszaros M, Kincses A, Klepe A, Gaspar D, Castanho M, Zimanyi L, Der A, Deli MA (2019) Lidocaine turns the surface charge of biological membranes more positive and changes the permeability of blood-brain barrier culture models. Biochim Biophys Acta Biomembr 1861 (9):1579-1591. doi:10.1016/j.bbamem.2019.07.008

5. Bynoe MS, Viret C, Yan A, Kim DG (2015) Adenosine receptor signaling: a key to opening the blood-brain door. Fluids Barriers CNS 12:20. doi:10.1186/s12987-015-0017-7

6. Vinten-Johansen J (2013) Adenosine-lidocaine-magnesium non-depolarizing cardioplegia: moving forward from bench to bedside. Int J Cardiol 166 (2):537-538.

doi:10.6342/NTU202210057

doi:10.1016/j.ijcard.2012.09.193

7. Owen CM, Asopa S, Smart NA, King N (2020) Microplegia in cardiac surgery: Systematic review and meta-analysis. J Card Surg 35 (10):2737-2746. doi:10.1111/jocs.14895

8. Francica A, Vaccarin A, Dobson GP, Rossetti C, Gardellini J, Faggian G, Onorati F (2021) Short-term outcome of adenosine-lidocaine-magnesium polarizing cardioplegia in humans. Eur J Cardiothorac Surg. doi:10.1093/ejcts/ezab466

9. Granfeldt A, Letson HL, Dobson GP, Shi W, Vinten-Johansen J, Tonnesen E (2014) Adenosine, lidocaine and Mg2+ improves cardiac and pulmonary function, induces reversible hypotension and exerts anti-inflammatory effects in an endotoxemic porcine model. Crit Care 18 (6):682. doi:10.1186/s13054-014-0682-y

10. Griffin MJ, Letson HL, Dobson GP (2014) Adenosine, lidocaine and Mg2+ (ALM) induces a reversible hypotensive state, reduces lung edema and prevents coagulopathy in the rat model of polymicrobial sepsis. J Trauma Acute Care Surg 77 (3):471-478. doi:10.1097/TA.0000000000000361

11. Conner J, Lammers D, Holtestaul T, Jones I, Kuckelman J, Letson H, Dobson G, Eckert M, Bingham J (2021) Combatting ischemia reperfusion injury from resuscitative endovascular balloon occlusion of the aorta using adenosine, lidocaine and magnesium: A pilot study. J Trauma Acute Care Surg 91 (6):995-1001.

doi:10.6342/NTU202210057

doi:10.1097/TA.0000000000003388

12. Letson HL, Dobson GP (2018) Adenosine, lidocaine, and Mg2+ (ALM) resuscitation fluid protects against experimental traumatic brain injury. J Trauma Acute Care Surg 84 (6):908-916. doi:10.1097/TA.0000000000001874

13. Mathew JP, Mackensen GB, Phillips-Bute B, Grocott HP, Glower DD, Laskowitz DT, Blumenthal JA, Newman MF; Neurologic Outcome Research Group (NORG) of the Duke Heart Center. Randomized, double-blinded, placebo controlled study of neuroprotection with lidocaine in cardiac surgery. Stroke. 2009 Mar;40(3):880-7.

14. Bartels K, McDonagh DL, Newman MF, Mathew JP. Neurocognitive outcomes after cardiac surgery. Curr Opin Anaesthesiol. 2013 Feb;26(1):91-7.

15. Gubskiy IL, Namestnikova DD, Cherkashova EA, Chekhonin VP, Baklaushev VP, Gubsky LV, Yarygin KN (2018) MRI Guiding of the Middle Cerebral Artery Occlusion in Rats Aimed to Improve Stroke Modeling. Transl Stroke Res 9 (4):417-425. doi:10.1007/s12975-017-0590-y

16. Xu L, Ding L, Su Y, Shao R, Liu J, Huang Y (2019) Neuroprotective effects of curcumin against rats with focal cerebral ischemia-reperfusion injury. Int J Mol Med 43 (4):1879-1887. doi:10.3892/ijmm.2019.4094

17. Valentim AM, Guedes SR, Pereira AM, Antunes LM (2016) Euthanasia using gaseous agents in laboratory rodents. Lab Anim 50 (4):241-253.

doi:10.6342/NTU202210057

doi:10.1177/0023677215618618

18. Shahjouei S, Cai PY, Ansari S, Sharififar S, Azari H, Ganji S, Zand R (2016) Middle Cerebral Artery Occlusion Model of Stroke in Rodents: A Step-by-Step Approach. J Vasc Interv Neurol 8 (5):1-8

19. Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9 (7):671-675

20. Toung TJ, Hurn PD, Traystman RJ, Bhardwaj A. Global brain water increases after experimental focal cerebral ischemia: effect of hypertonic saline. Crit Care Med 2002;30:644-9.

21. Yoo SY, Yoo JY, Kim HB, Baik TK, Lee JH, Woo RS (2019) Neuregulin-1 Protects Neuronal Cells Against Damage due to CoCl2-Induced Hypoxia by Suppressing Hypoxia-Inducible Factor-1alpha and P53 in SH-SY5Y Cells. Int Neurourol J 23 (Suppl 2):S111-118. doi:10.5213/inj.1938190.095

22. Collaborators GBDS (2021) Global, regional, and national burden of stroke and its risk factors, 1990-2019: a systematic analysis for the Global Burden of Disease Study 2019. Lancet Neurol 20 (10):795-820. doi:10.1016/S1474-4422(21)00252-0

23. Herpich F, Rincon F (2020) Management of Acute Ischemic Stroke. Crit Care Med 48 (11):1654-1663. doi:10.1097/CCM.0000000000004597

24. Vitt JR, Trillanes M, Hemphill JC, 3rd (2019) Management of Blood Pressure

doi:10.6342/NTU202210057

During and After Recanalization Therapy for Acute Ischemic Stroke. Front Neurol 10:138. doi:10.3389/fneur.2019.00138

25. Kuczynski AM, Marzoughi S, Al Sultan AS, Colbourne F, Menon BK, van Es A, Berez AL, Goyal M, Demchuk AM, Almekhlafi MA (2020) Therapeutic Hypothermia in Acute Ischemic Stroke-a Systematic Review and Meta-Analysis. Curr Neurol Neurosci Rep 20 (5):13. doi:10.1007/s11910-020-01029-3

26. Kuczynski AM, Ospel JM, Demchuk AM, Goyal M, Mitha AP, Almekhlafi MA (2020) Therapeutic Hypothermia in Patients with Malignant Ischemic Stroke and Hemicraniectomy-A Systematic Review and Meta-analysis. World Neurosurg 141:e677-e685. doi:10.1016/j.wneu.2020.05.277

27. Ma J, Ma Y, Shuaib A, Winship IR (2020) Improved collateral flow and reduced damage after remote ischemic perconditioning during distal middle cerebral artery occlusion in aged rats. Sci Rep 10 (1):12392. doi:10.1038/s41598-020-69122-8

28. Yao Y, Zhang Y, Liao X, Yang R, Lei Y, Luo J (2020) Potential Therapies for Cerebral Edema After Ischemic Stroke: A Mini Review. Front Aging Neurosci 12:618819. doi:10.3389/fnagi.2020.618819

29. Howell JA, Bidwell GL, 3rd (2020) Targeting the NF-kappaB pathway for therapy of ischemic stroke. Ther Deliv 11 (2):113-123. doi:10.4155/tde-2019-0075

30. Lakhan SE, Kirchgessner A, Hofer M (2009) Inflammatory mechanisms in ischemic

doi:10.6342/NTU202210057

stroke: therapeutic approaches. J Transl Med 7:97. doi:10.1186/1479-5876-7-97

31. Francica A, Tonelli F, Rossetti C, Tropea I, Luciani GB, Faggian G, Dobson GP, Onorati F (2021) Cardioplegia between Evolution and Revolution: From Depolarized to Polarized Cardiac Arrest in Adult Cardiac Surgery. J Clin Med 10 (19). doi:10.3390/jcm10194485

32. Caltana L, Merelli A, Lazarowski A, Brusco A (2009) Neuronal and glial alterations due to focal cortical hypoxia induced by direct cobalt chloride (CoCl2) brain injection. Neurotox Res 15 (4):348-358. doi:10.1007/s12640-009-9038-9

33. Jones SM, Novak AE, Elliott JP (2013) The role of HIF in cobalt-induced ischemic tolerance. Neuroscience 252:420-430. doi:10.1016/j.neuroscience.2013.07.060

34. Tripathi VK, Subramaniyan SA, Hwang I (2019) Molecular and Cellular Response of Co-cultured Cells toward Cobalt Chloride (CoCl2)-Induced Hypoxia. ACS Omega 4 (25):20882-20893. doi:10.1021/acsomega.9b01474

35. Lopez MS, Vemuganti R (2018) Modeling Transient Focal Ischemic Stroke in Rodents by Intraluminal Filament Method of Middle Cerebral Artery Occlusion. Methods Mol Biol 1717:101-113. doi:10.1007/978-1-4939-7526-6_9

36. Larpthaveesarp A, Gonzalez FF (2017) Transient Middle Cerebral Artery Occlusion Model of Neonatal Stroke in P10 Rats. J Vis Exp (122). doi:10.3791/54830

37. Liu F, McCullough LD (2014) The middle cerebral artery occlusion model of

doi:10.6342/NTU202210057

transient focal cerebral ischemia. Methods Mol Biol 1135:81-93. doi:10.1007/978-1-4939-0320-7_7

38. Komatsu T, Ohta H, Motegi H, Hata J, Terawaki K, Koizumi M, Muta K, Okano HJ, Iguchi Y (2021) A novel model of ischemia in rats with middle cerebral artery occlusion using a microcatheter and zirconia ball under fluoroscopy. Sci Rep 11 (1):12806. doi:10.1038/s41598-021-92321-w

39. Liu F, McCullough LD (2011) Middle cerebral artery occlusion model in rodents: methods and potential pitfalls. J Biomed Biotechnol 2011:464701. doi:10.1155/2011/464701

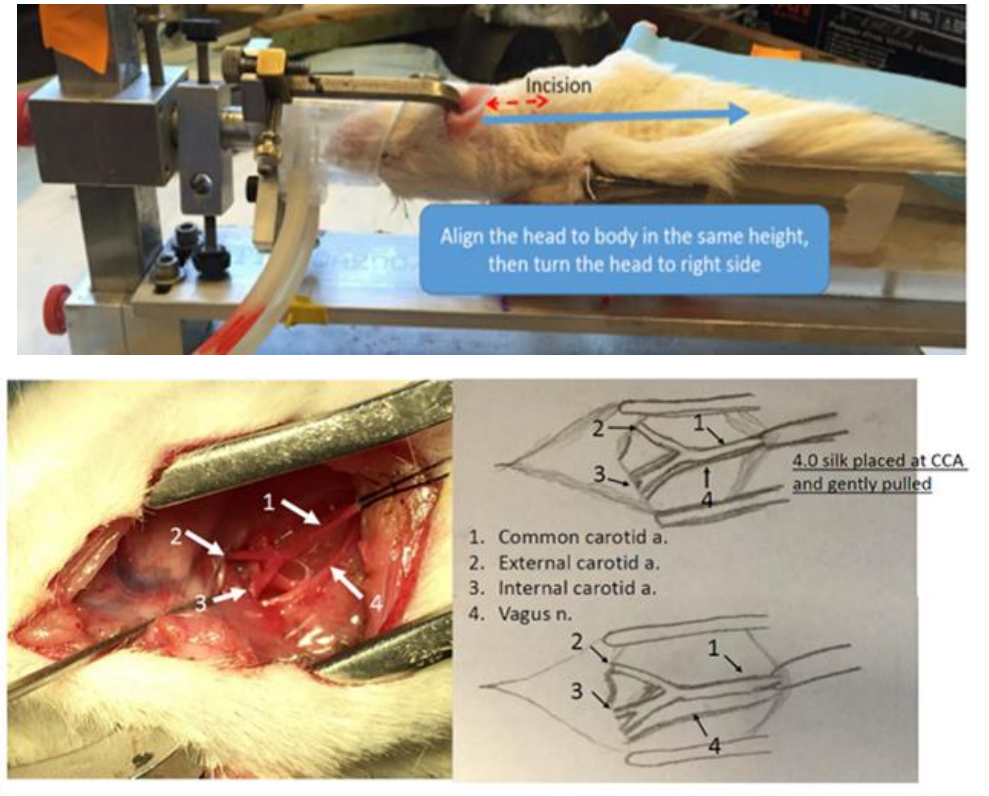
40. Selvamani A, Sohrabji F (2010) The neurotoxic effects of estrogen on ischemic stroke in older female rats is associated with age-dependent loss of insulin-like growth factor-1. J Neurosci 30 (20):6852-6861. doi:10.1523/JNEUROSCI.0761-10.2010

doi:10.6342/NTU202210057

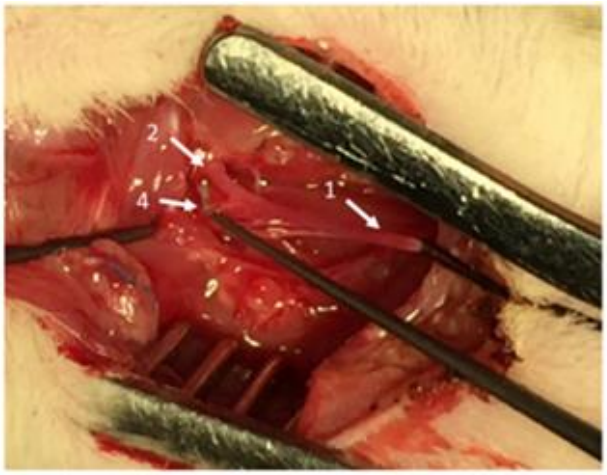
Figure legend

Fig 1 Middle cerebral artery occlusion model

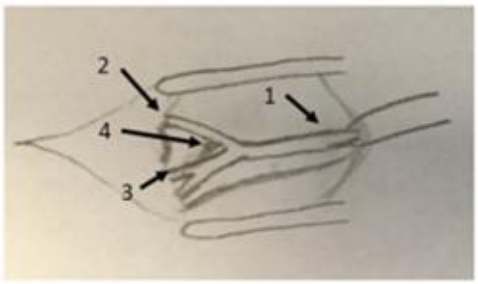
The right common, external, and internal carotid arteries (CCA, ECA, and ICA, respectively) were dissected from the surrounding connective tissue through a lateral neck incision. The filament was placed in the right ECA and gently advanced via the ICA to ICA, MCA bifurcation.

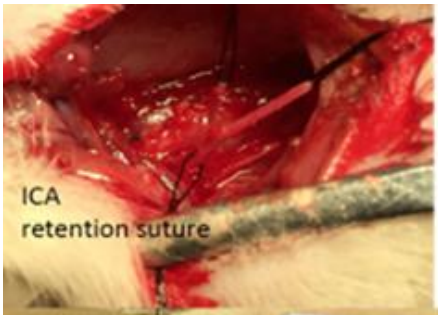


doi:10.6342/NTU202210057



1. Common carotid a.
2. External carotid a.
3. Internal carotid a.
4. Occipital a.





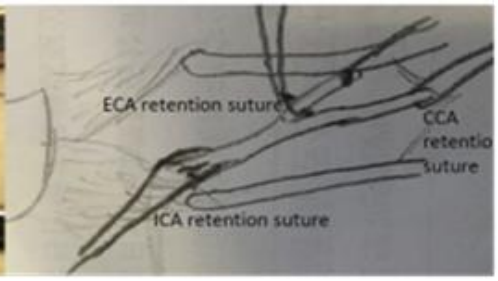
ECA is flipped over and in line with ICA

↓

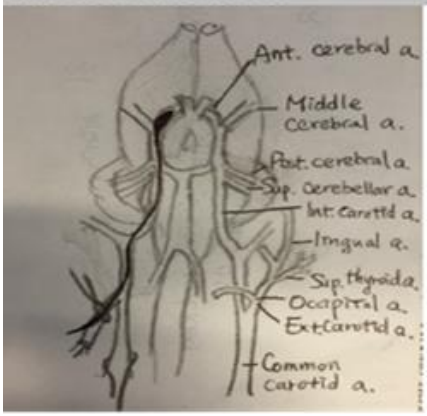
Place a retention suture around ICA

↓

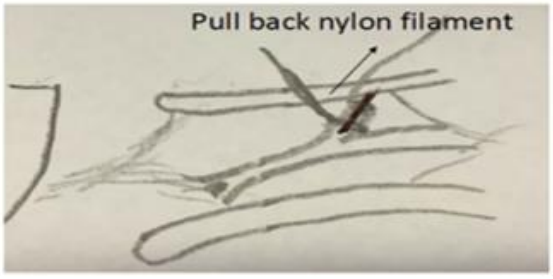
Place a retention suture around ECA



Permanent ischemic stroke model:
Leave the nylon filament in place and close the wound



Reperfusion model:
Withdraw the nylon filament at a desired occlusion time period
Ligate ECA
Release CCA retention suture
Close the wound



doi:10.6342/NTU202210057

Fig 2. Modified Longa score had high consistency among different raters in our study.

Five raters scored 3 different rats who had transient middle cerebral artery occlusion for 1 hour, and the inter-rater variability was calculated.

	Intraclass correlation[a]	95% Confidence Interval
Single measures [b]	0.9765	0.8888 to 0.9994
Average measures[c]	0.9952	0.9756 to 0.9999

Number of subjects (n)	3
Number of raters (k)	5
Model	Raters for each subject are selected at random. One-way random effects model.
Type	Absolute agreement
Measurements	RATER1 RATER2 RATER3 RATER4 RATER5

Longa score	
0	No neurological findings
1	Failure to extend left forepaw fully
2	Circling to the right
3	Falling to the right
4	No spontaneous walking and being depressed
5	Brain death due to ischemia

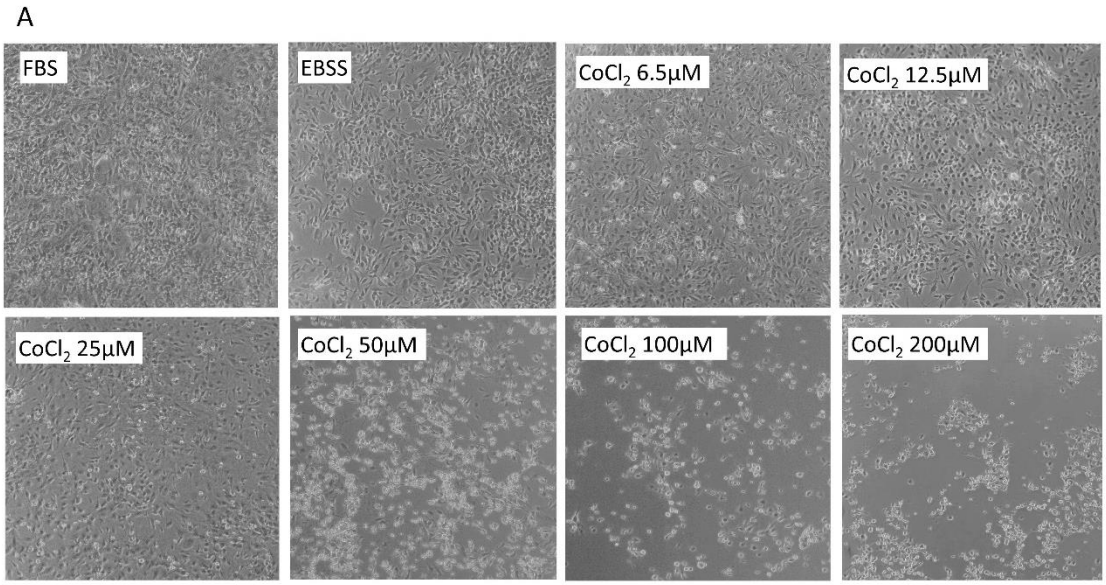
doi:10.6342/NTU202210057

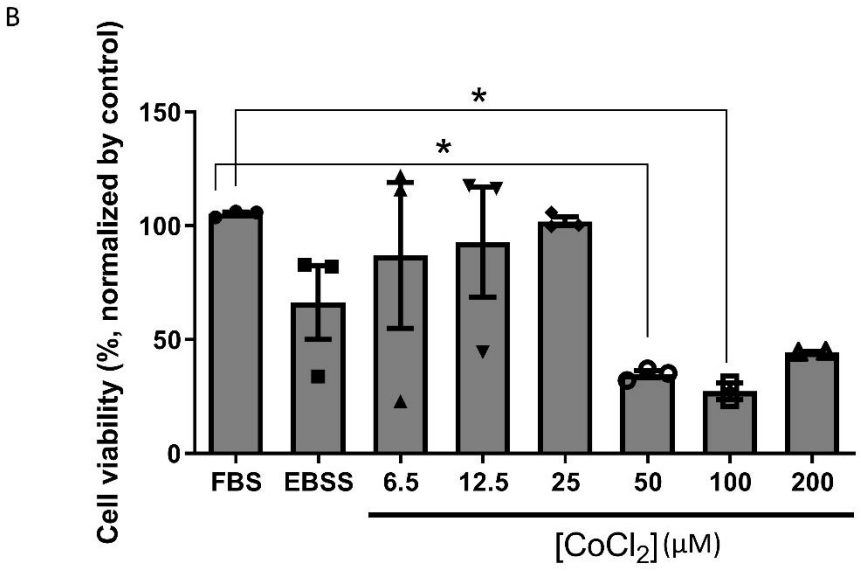
Fig 3 Effects of cobalt chloride ($CoCl_2$) on survival of SH-SY5Y cells of 6.5, 12.5, 25, 50, 100, and 200 μM for 24 hours in comparison with controls of FBS and EBSS

(A) To mimic oxygen-glucose deprivation, differentiated SH-SY5Y cells were exposed to cobalt chloride for different concentrations with an increase in concentrations of cobalt chloride. There was a reduction of cell number and a change in morphology.

(B) The quantitatively viability assay of alamarBlue showed that cobalt chloride at the concentrations higher than 50 μM significantly decreased the viability of differentiated SH-SY5Y cells

doi:10.6342/NTU202210057

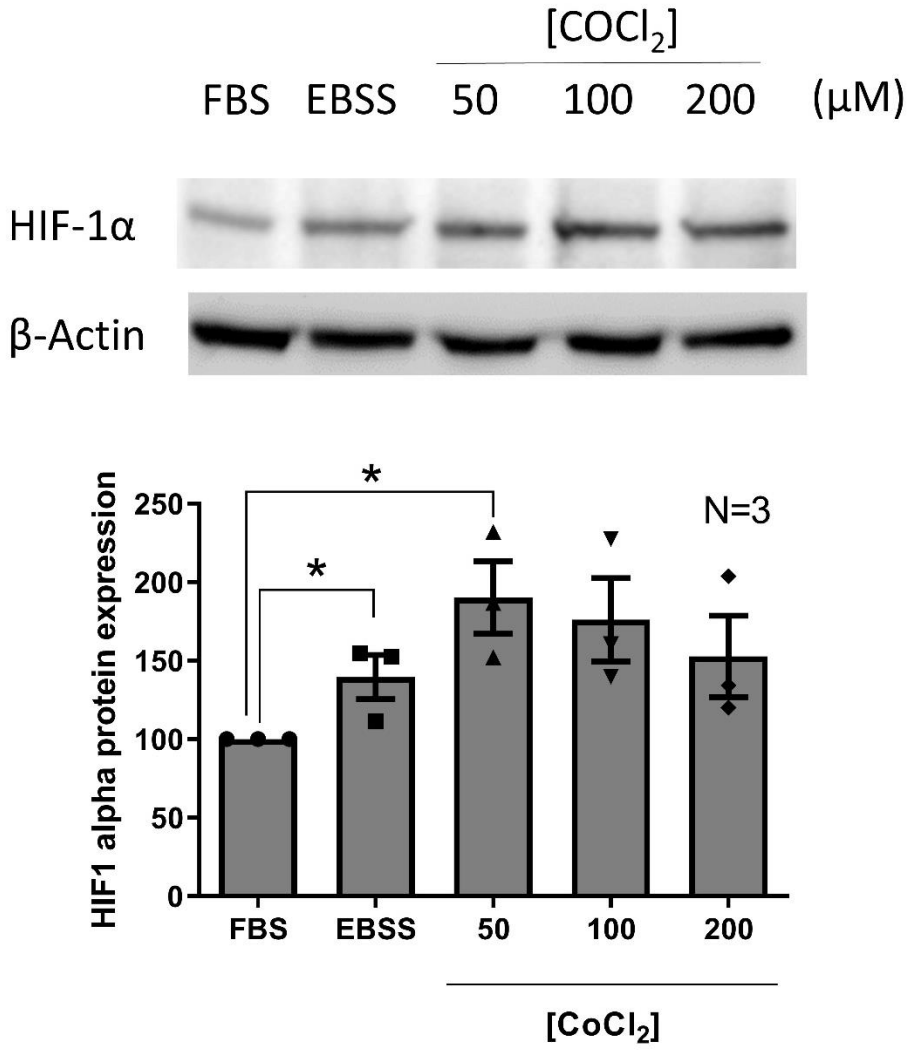




doi:10.6342/NTU202210057

Fig 4 Upregulation of HIF1α protein expression in cobalt chloride-treated SH-SY5Y cells

Differentiated SH-SY5Y cells incubated with glucose deprivation solution and cobalt chloride for 8 hours showed upregulate HIF1α protein expression

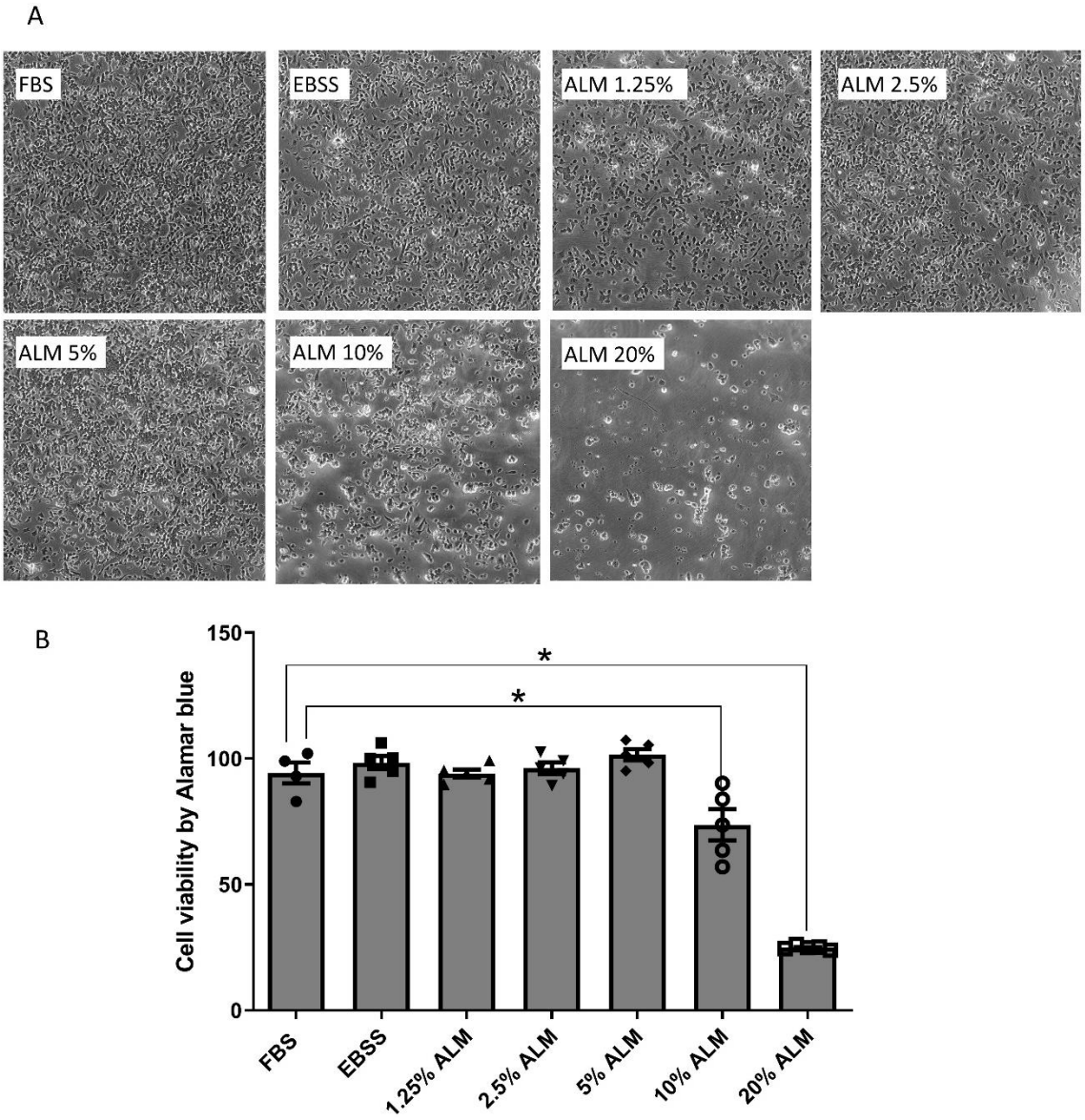


doi:10.6342/NTU202210057

Fig 5 Cytotoxic profiles of ALM

(A) To clarify the toxicity profile for ALM solution, we incubated differentiated SH-SY5Y cells with different concentrations of ALM for 24 hours.

(B) ALM concentration higher than 10% led to significant cell death according to the alamarBlue assay.



Stoke ALM (1.0mM adenosine, 2.0mM lidocaine, 5mM MgSO4) dilutes in EBSS

doi:10.6342/NTU202210057

Fig 6 Effect of ALM-pretreatment on cell viability

(A) We incubated differentiated SH-SY5Y cells with 50 μM cobalt chloride, and added various concentrations of ALM for 24 hours.

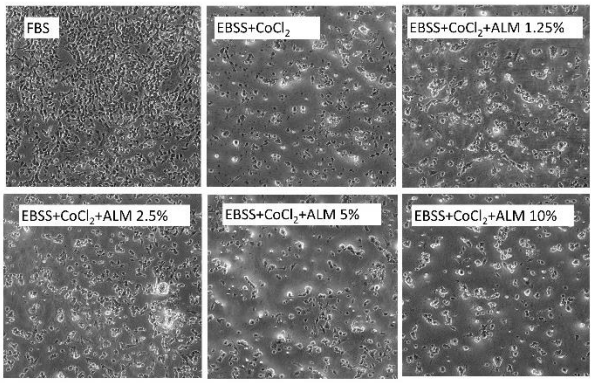
(B) Pretreatment with ALM of the optimal concentration preserved the differentiated SH-SY5Y cells.

(C) The cell viability test of alamrBlue demonstrated that ALM 2.5% solution was the optimal concentration to attenuate cell loss on incubation with EBSS and cobalt chloride.

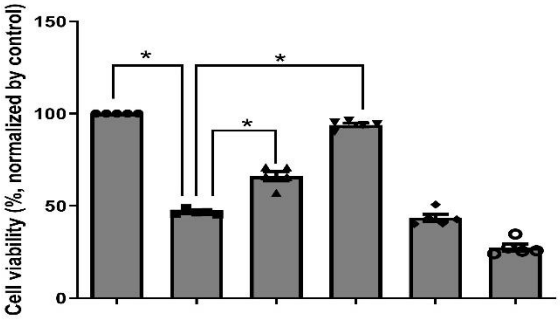
doi:10.6342/NTU202210057

A

B



C



FBS(control)	+	-	-	-	-	-
EBSS (Starvation sol.)	-	+	+	+	+	+
$CoCl_2$ (μM)	0	50	50	50	50	50
ALM (%)	0	0	1.25	2.5	5	10

Stoke ALM (1.0mM adenosine, 2.0mM lidocaine, 5mM MgSO4) dilutes in EBSS

doi:10.6342/NTU202210057

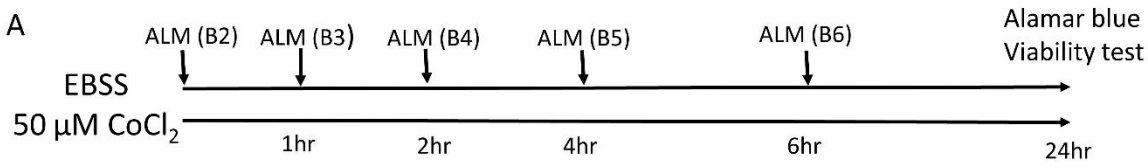
Fig 7 Effect of ALM treatment on the rescue of cell viability after various cobalt chloride incubation duration

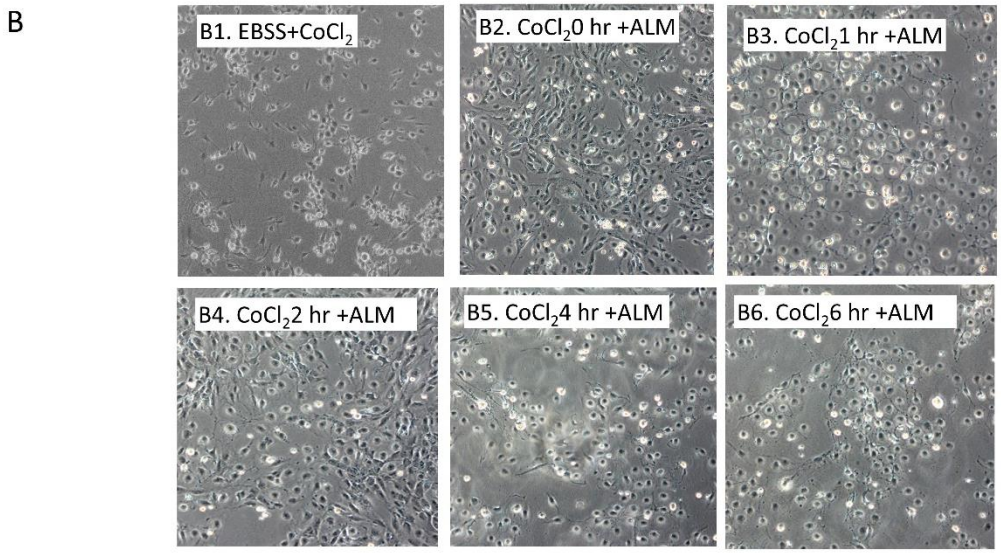
(A) The diagram depicted the study design to assess the effect of ALM after cobalt chloride ($CoCl_2$ 50μM) treatment for various durations (from 1 hour to 6 hours).

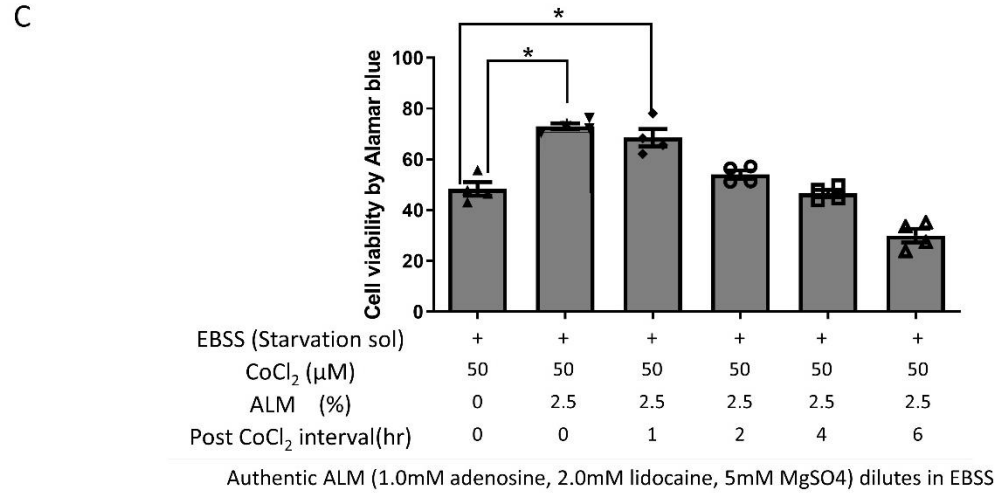
(B) The number of differentiation SH-SY5Y cells decreased if ALM treatment was given after longer cobalt chloride incubation period: (B1) differentiated SH-SY5Y cells with 50 μM CoCl2; (B2) ALM 2.5% given together with CoCl2; (B3) ALM 2.5% given after CoCl2 incubation for 1 hours; (B4) ALM 2.5% given after CoCl2 incubation for 2 hours; (B5) ALM 2.5% given after CoCl2 incubation for 4 hours; (B6)ALM 2.5% given after CoCl2 incubation for 6 hours

(C) The beneficial effect of ALM disappeared if the treatment of ALM was given later than 2 hours after CoCl2 incubation according to the viability alamarBlue test.

doi:10.6342/NTU202210057



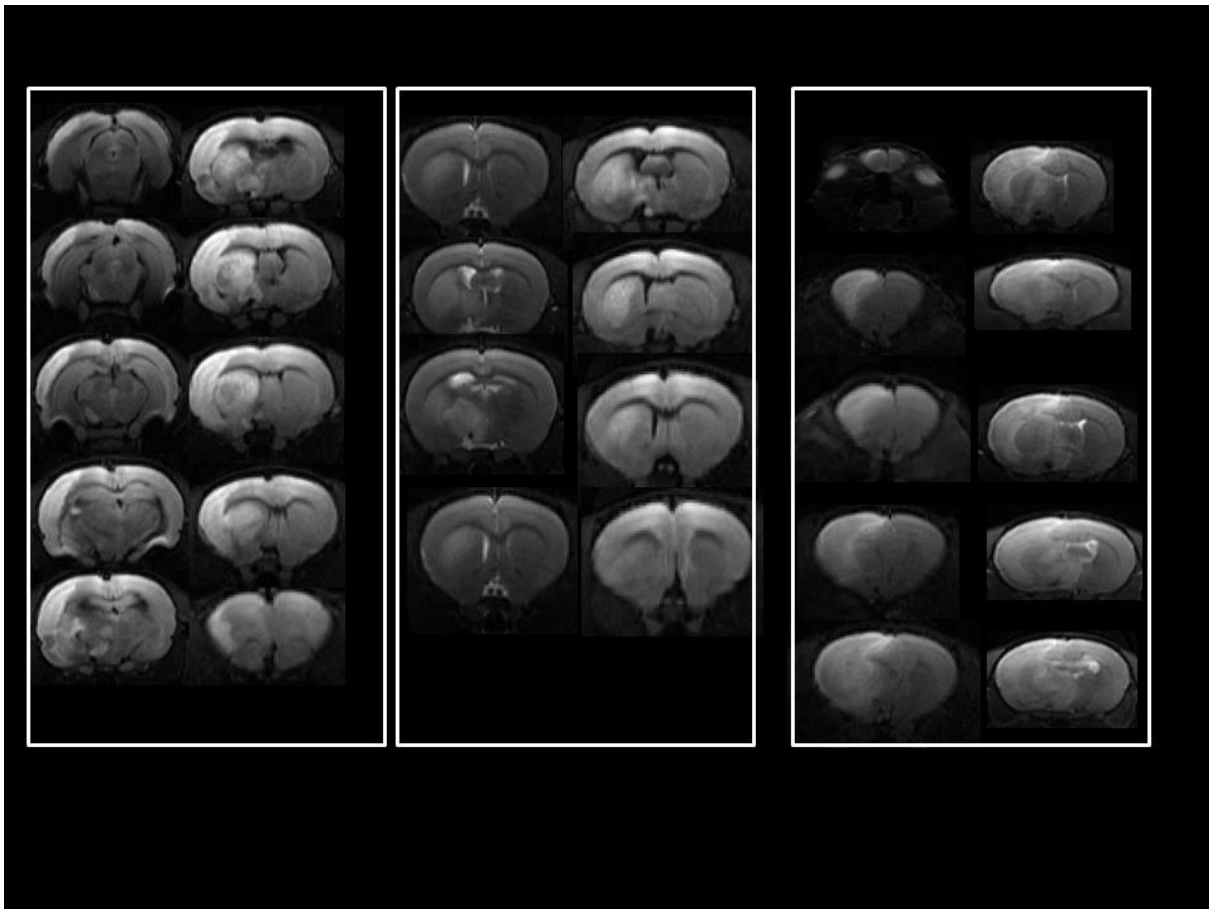




doi:10.6342/NTU202210057

Fig 8. MRI image of brain after permanent middle cerebral artery occlusion.

Three out of five rats successfully had their brain imaged by 7T MRI under general anesthesia. The ischemic side showed lesion in cortex and basal ganglia.



doi:10.6342/NTU202210057

Fig 9 Effect of ALM on a rodent stroke model of transient middle cerebral artery occlusion (MCAO)

(A) The infarct areas were measured 24 hours 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

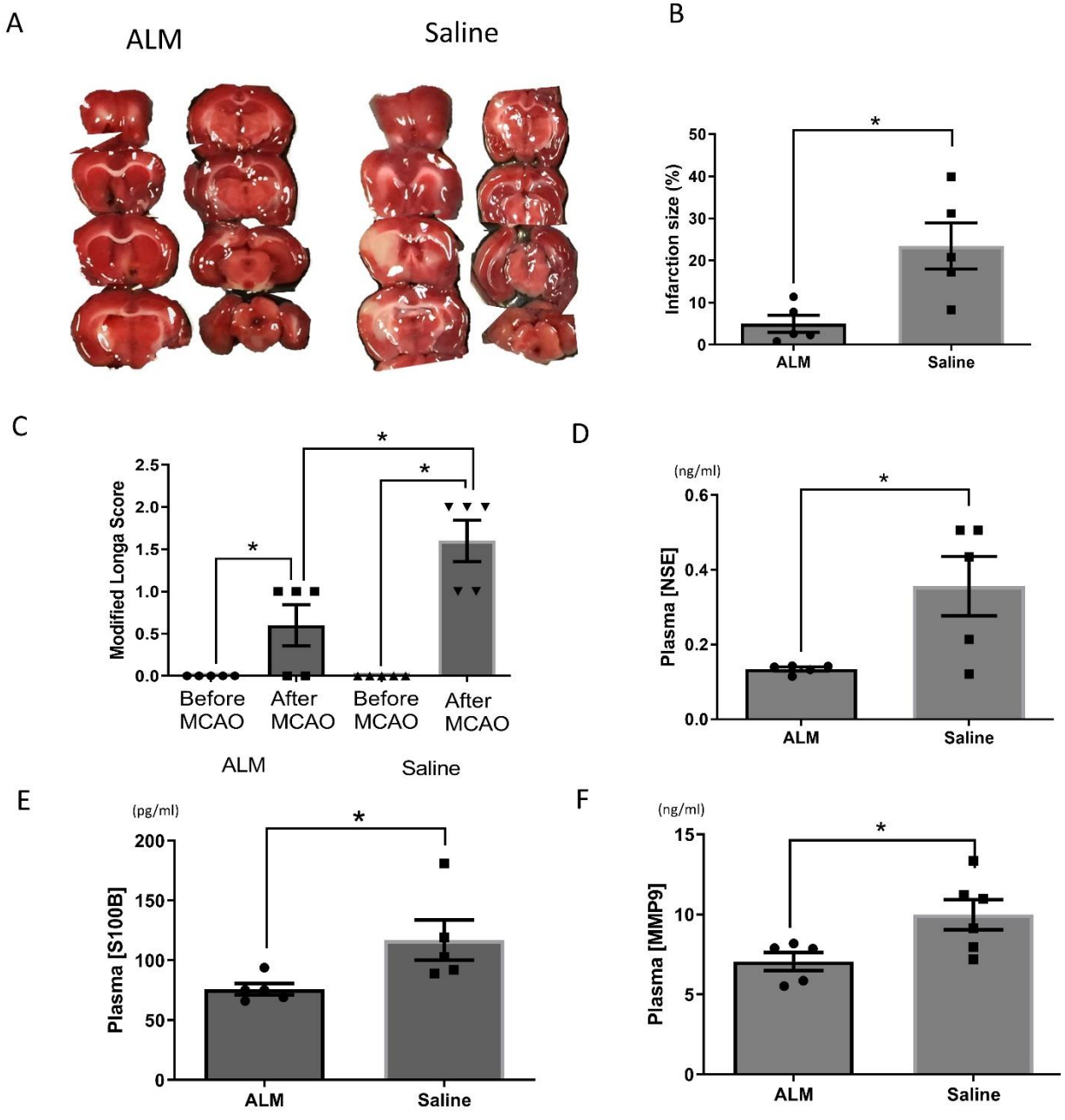
(C) Neurological deficits were measured with modified Longa score before MCAO and 24 hours after MCAO before sacrifice. The modified Longa score was the same in ALM and saline groups before surgery, and saline group scored higher than ALM group 24 hours after MCAO procedure.

(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

doi:10.6342/NTU202210057



doi:10.6342/NTU202210057

Fig 10. Effect of ALM on a rodent stroke model of permanent middle cerebral artery occlusion (MCAO)

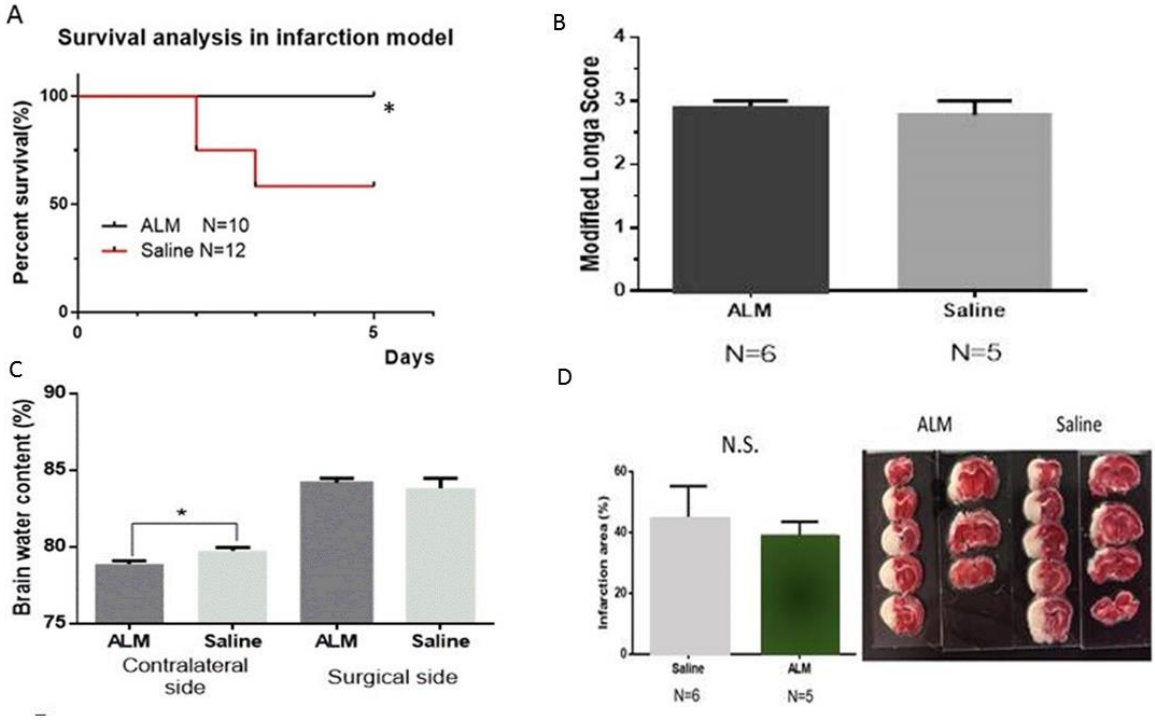
(A) ALM group had better survival than control group

(B) Neurological deficits were measured with modified Longa score before MCAO and 24 hours after MCAO before sacrifice. The modified Longa score was the same in ALM and saline groups after permanent MCAO procedure.

(C) Brain water content measured by wet-dry method showed that ischemic brain edema level was similar in ALM and saline groups. However, ALM group had lower brain edema level in the non-infarction side.

(D) The infarct areas were measured 24 hours after MCAO by 1% 2,3,5-triphenyltetrazolium chloride (TTC) solution. Infarction area was similar in saline group and ALM group

doi:10.6342/NTU202210057



doi:10.6342/NTU202210057