ЗАГАЛЬНА ХІРУРГІЯ (ВСІ ХІРУРГІЧНІ ДИСЦИПЛІНИ)

UDC 616.831-005.1:599.323.41
DOI https://doi.org/10.32782/2415-8127.2024.70.1

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Cisterna magna double-injection model of hemorrhagic stroke in experimental rats for the study of communicating hydrocephalus

Objectives. The cisterna magna double-injection model of hemorrhagic stroke (CMDIM) was tested in this research to see its validity in provoking communicative hydrocephalus. **Background**. Subarachnoid hemorrhage (SAH) is a devastating disease resulting in high mortality and is a common cause of chronic post-hemorrhagic hydrocephalus (PHH), which affects up to 20% of the survivors. The occurrence of hydrocephalus after SAH is a crucial factor in predicting a poor prognosis, including damage to the brain parenchyma, a prominent cause of disability that, if sufficiently severe, may also lead to patient mortality. In the case of PHH, the mechanisms leading to pathogenesis are poorly understood. Small animal models in basic and preclinical sciences constitute an integral part of testing new hypotheses before translation to clinical practice. **Methods**. Experimental animals were divided into two groups. The first group (control group – CG) was without surgery. In the second group, a 0.15 ml blood injection into cistern magna was followed by a 0.15 ml blood injection 48 hours later. The surgery was performed in sterile conditions under general anesthesia; experimental animals in the surgical group were positioned supine, and blood was taken from the lower third on the ventral aspect of the tail. After this, the rat was turned prone, and the head was fixed in a stereotactic frame**.** Under magnification of surgical loups, an incision was made in the suboccipal region followed by dissection of neck region muscles. Gentle flexion of the rat head allowed by not rigid head fixation gave the possibility to widen the space between the occipital bone and C1 lamina for better cisterna magna visualization. After meticulous hemostasis, the incision was closed using a stapling device. The second surgery was performed with the same steps, except for more proximal puncturing (above the lower third on the ventral aspect of the tail) of the tail artery. We defined hydrocephalus as ventricular volume on histological evaluation, which was > +3 SDs above the mean in control animals. **Results.** Thirty-seven operations were done on 20 rats with 20% posthemorrhagic postoperative mortality. Hydrocephalus in the surgical group occurred in 45% of rats, according to the histological investigations. **Conclusion**. Based on the findings, CMDIM is effective in generating posthemorrhagic hydrocephalus with acceptable mortality.

Key words: subarachnoid hemorrhage, hydrocephalus, experimental hemorrhagic stroke, Wistar rats.

Гаврилів Тарас Степанович, нейрохірург, Комунальне некомерційне підприємство «Обласний клінічний центр нейрохірургії та неврології» Закарпатської обласної ради; асистент, Ужгородський Національний Університет, Медичний факультет, Кафедра неврології, нейрохірургії та психіатрії, taras.havryliv@uzhnu.edu.ua, https://orcid.org/0000-0003-0271-8983, м. Ужгород, Україна

Модель геморагічного інсульту в експериментальних щурів у вигляді подвійної ін'єкції великої потиличної цистерни для вивчення арезорбтивної гідроцефалії

Вступ. Субарахноїдальний крововилив (САК) є підтипом геморагічного інсульту, з високими показниками смертності та частою причиною хронічної постгеморагічної гідроцефалії (ПГГ), яка вражає до 20% тих, хто вижив. Виникнення гідроцефалії після САК є одним з ключових факторів у прогнозуванні негативних результатів лікування, включаючи пошкодження паренхіми головного мозку, що є основною причиною інвалідності, яка також може призвести до смерті пацієнта. У випадку комунікантної постгеморагічної гідроцефалії, патогенетичні механізми останньої недостатньо вивчені. Моделі експериментальних тварин у фундаментальних і доклінічних науках є невід'ємною частиною перевірки нових гіпотез перед впровадженням їх у клінічну практику. **Мета.** У даному дослідженні була протестована модель геморагічного інсульту з подвійною ін'єкцією аутологічної крові у велику цистерну щоб визначити валідність моделі у продукуванні комунікативної гідроцефалії. **Методи.** Піддослідних тварин (30 щурів лінії Вістар) розділили на дві групи. У першій групі (контрольна) операції не виконувались. У другій групі після ін'єкції 0,15 мл. крові у велику потиличну цистерну, слідувала ін'єкція крові 0,15 мл через 48 годин. Оперативні втручання проводили під загальним знеболенням, піддослідних тварин хірургічної групи позиціонували на спину, і проводився забір крові з нижньої третини вентральної сторони хвоста. Після цього щура повертали, а голову фіксували в стереотаксичній рамці. За допомогою бінокулярних луп виконувався розріз у потиличній ділянці з наступним розсіченням м'язів шиї. Легка флексія голови, завдяки нежорсткій фіксації голови, давала можливість розширити простір між потиличною кісткою та дужкою С1 для кращої візуалізації великої потиличної цистерни з наступною ін'єкцією аутологічної крові. Друге оперативне втручання було виконано з тими ж етапами, за винятком більш проксимальної пункції (вище нижньої третини на вентральній стороні хвоста) хвостової артерії. Гідроцефалія верифікувалася гістологічно як об'єм шлуночків головного мозку, який був > +3 стандартних відхилень вище середнього значення у контрольних тварин.

Ключові слова: субарахноїдальний крововилив, гідроцефалія, експериментальний геморагічний інсульт, щури лінії Вістар.

Introduction. Subarachnoid hemorrhage (SAH) is a devastating disease resulting in high mortality and is a common cause of chronic post-hemorrhagic hydrocephalus (PHH), which affects up to 20% of the survivors. It is usually caused by impaired cerebrospinal fluid (CSF) flow or drainage and is characterized by pathologic dilation of the cerebral ventricles. Based on clinical and radiographic presentations, PHH lasting two weeks or longer is defined as chronic hydrocephalus (Huo et al., 2011), which commonly requires cerebrospinal fluid shunt surgery but still with a high frequency of poor neurological outcomes and cognitive deficits (Dupont & Rabinstein, 2013; O'Kelly et al., 2009; Woernle et al., 2013). The occurrence of hydrocephalus is a crucial factor in predicting a poor prognosis, including damage to the brain parenchyma, a prominent cause of disability that, if sufficiently severe, may also lead to patient mortality (Phan et al., 2000; Hanley, 2009; Diringer et al., 1998; Bhattathiri et al., 2006). In the case of PHH, the mechanisms leading the pathogenesis are, as yet, poorly understood, and no specific medical treatment for the prevention of chronic hydrocephalus is available. The lack of appropriate models for SAH-associated chronic hydrocephalus partly causes this. Without this detailed understanding, effective treatment is complex (Strahle et al., 2012). Small animal models in basic and preclinical sciences constitute an integral part of testing new hypotheses before translation to clinical practice [1, pp. 2173-2188]. Therefore, the current study evaluated the efficacy of the cisterna magna double-injection model of subarachnoid hemorrhage in experimental rats causing hydrocephalus.

Material and methods. Thirty adult Wistar rats (Biological Research Center, Lithuanian University of Health Sciences, Kaunas, Lithuania), approximately aged 2–6 months, fifteen males and fifteen females, weighing 250 to 500 g., were enrolled in the study. The rats were bred and maintained at the Lithuanian University of Health Sciences animal house under controlled conditions. The rats were housed in individual cages and maintained on a 12-hour light/dark cycle, with free access to food and water; tail marking with a permanent marker was used for rat identification – **Figure 2C**. The experiments were approved by the State Food and Veterinary Service (Vilnius, Lithuania), following European (2010/63/UE) regulations for the care and use of laboratory animals.

Surgical Procedure. The model of SAH was performed as previously described [2, pp. 1-7]. The surgery was performed in sterile conditions under general anesthesia with 3% sevofluran (Baxter, USA) with induction of volatile anesthesia in an induction chamber (Kent Scientific, USA) – **Figure 2A** and additional gas provided via anesthesia mask. Experimental animals in the surgical group were positioned supine, taking approximately 0.15 ml of blood with a 26-gauge needle from the lower third on the ventral aspect of the tail – **Figure 1B** (after holding the tail for 2 minutes in warm water (40 degrees) to achieve artery dilatation – **Figure 1A**). After this, the rat was turned prone, and the head was fixed in a stereotactic frame (individually designed) – **Figure 2B.** Under magnification of surgical loups, an incision was made in the suboccipal region followed by dissection of neck region muscles. Gentle flexion of the rat head allowed by not rigid head fixation gave the possibility to widen the space between the occipital bone and C1 lamina for better cisterna magna visualization – **Figure 1C**. Muscles were retracted with fish hooks secured on frame special fixators, and injection of previously prepared 0.15 ml of blood into subarachnoid space (SAS) via cisterna magna (CM) was performed – **Figure 1D, 1E**. After meticulous hemostasis, the incision was closed using a stapling device. The second surgery was performed in 48 hours with the same steps, except for more proximal puncturing (above the lower third on the ventral aspect of the tail) of the tail artery. Rats in surgical groups were sacrificed after the 20th-day post-induction of SAH by intramuscular injection of 2% pentobarbital sodium (60 mg/ kg body weight) followed by cervical dislocation. Bone rongeur was used to remove the cranium for brain removal; the brain and brainstem were carefully extracted from the cranial vault, placed into a 4% paraformaldehyde solution, and stored at four °C for 48 hours.

Histological Investigations. For all neuropathological analyses, 10μm thick coronal sections were cut (Leica Microsystems LM3050S) and mounted on poly-L-lysine-coated slides. The tissue sections were subjected to hematoxylin and eosin (H & E) staining. The presence of ventricle wall dilatation was evaluated. The size of the

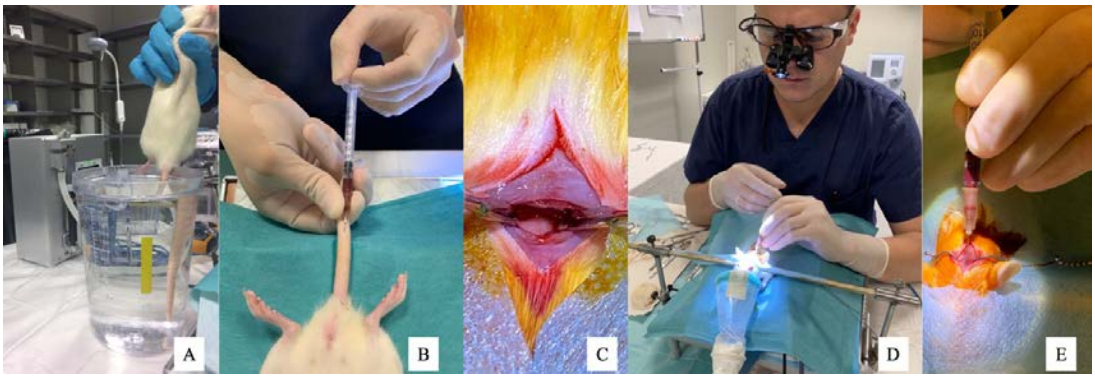

Fig. 1. Surgical steps. Tail holding for 2 minutes in warm water to achieve artery dilatation (A); tail artery puncture (B); cisterna magna visualization (C); injection of previously prepared 0.15 ml of blood into cisterna magna (D, E)

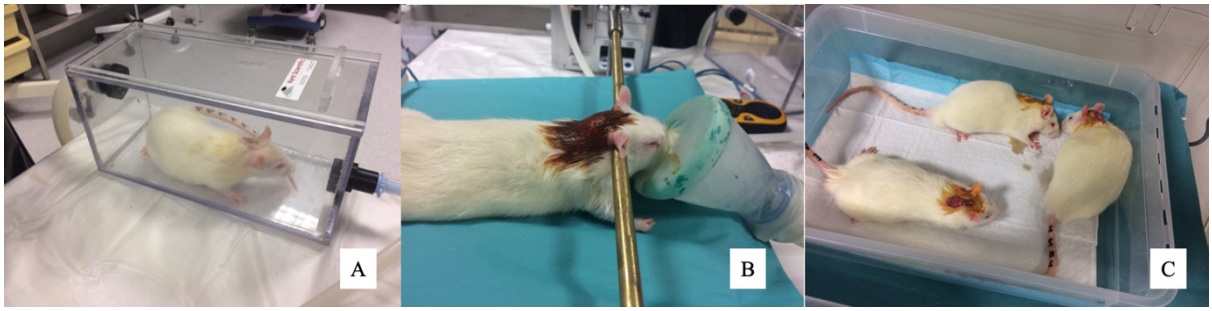

Fig. 2. Positioning, anesthesia, and identification of experimental animals. General anesthesia with 3% sevoflurane with induction of volatile anesthesia in an induction chamber (A); head fixation in a stereotactic frame (B); tail marking with a permanent marker for rat identification (C)

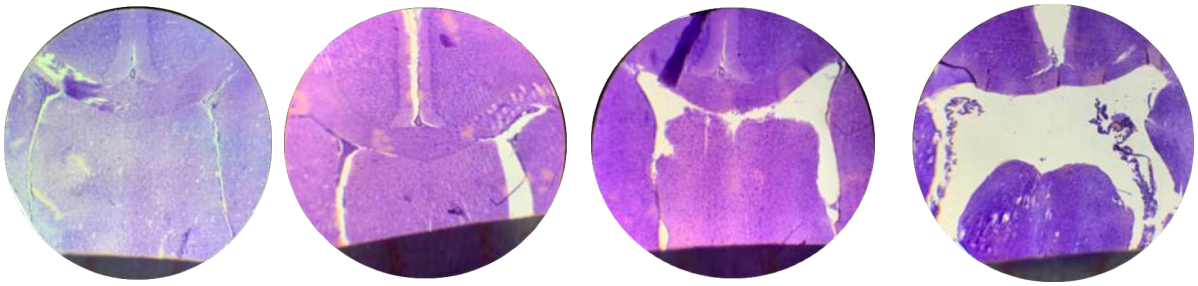

Fig. 3. Histological photomicrographs with hematoxylin and eosin staining of SAH animals with progressive ventricular dilatation (left to right). Scale bar=32 μm

lateral ventricle was determined by the lateral ventricle index, which was calculated on the Nissl stained slice. Specifically, lateral ventricle index equaled lateral ventricle volume divides the area of the brain slice at the level of the preoptic chiasm [3, pp. 547–550]. Hydrocephalus was defined as ventricular volume over +3SD of the mean in sham and control animals.

Results. Thirty-seven surgical interventions were performed on 20 research animals with a general postoperative mortality of 20%. No animals in the control group died.

The introduction of blood samples into SAS markedly increased ventricle size. Among 20 previously healthy animals of the hemorrhagic group, 9 (45%) developed hydrocephalus – **Figure 3**. No significant changes were observed in the CG group.

Discussion. In this experimental study, we evaluated the efficacy of the cisterna magna double-injection model of subarachnoid hemorrhage in experimental rats to study hydrocephalus. The key findings of the research include that rat double-hemorrhage cisterna magna autologous 0.15 ml. blood injection model produces acceptable rates of hydrocephalus induction.

Animal models are essential to studying SAH and its effects [4, pp. 1096–1112]. Among numerous studied animal SAH – models, the most promising are puncture or perforation of a cerebral vessel using a needle or catheter [5, pp. 415–434] and other models using an injection of blood into a cistern. In 411 rat studies, SAH was induced by intracisternal blood injection in 259 (63%) rats (46% single, 17% double) and by endovascular filaments in 173 (33%) rats [6, pp. 250–258].

The cisterna magna is the most common site for blood injection in animal models. A microcatheter or direct puncture may introduce blood into the cisterna magna [7, pp. 1086–1091]. The autologous blood injection into the cisterna magna model is considered the most appropriate model for studying chronic bleeding effects after SAH. To mimic SAH, autologous blood is taken from an artery on the ventral aspect of the tail according to the commonly known anatomical feature of blood vessels on Wistar rats' tails – one ventral artery and two lateral veins [8, pp. 121–125]. The development of delayed injury mechanisms seems mainly to depend on the amount and duration of the subarachnoid blood clot. Direct injection of blood is often the preferred method, given the ability of the investigator to control the initiation, volume, and rate of hemorrhage into the cisterna magna [9, pp. 165–176]. The rat double-hemorrhage model reproduces the time course of the delayed pathophysiological consequences of CVS, which imitates the clinical setting more precisely than other rodent models.

Furthermore, this model is adjustable via various technical considerations or modifications. The rat model has recently become one of SAH's most utilized animal models due to its low cost and ability to use large numbers of animals [10, pp. 538–541]. Adult male Sprague–Dawley or Wistar rats, weighing 250– 500 g, are commonly used for the double-hemorrhage model [11, pp. 325–329; 12, pp. 1190–1197] and ventricular dilation could be found in 42% of the rats [13, pp. 785–791]. This percentage is similar to our results in that 45% of the chronic hydrocephalus induction.

According to several studies in cisterna magna single injection models, mortality rates were 0–16%

[14, pp. 58–64], while cisterna magna double injection models exhibit numbers up to 53% [12, pp. 1190–1197]. In our study, we achieved 20% general postoperative mortality.

Study limitations. The study has several limitations: magnetic resonance imaging and ultrasound are superior in visualization sizes of brain ventricles.

Conclusion. This study shows that the double-injection hemorrhagic model induction of chronic hydrocephalus after repeated punctures of the cisterna magna is feasible in rats. The current model could be applied to study novel prophylactic therapies aiming at reducing the rate of patients requiring permanent CSF diversion after SAH.

Funding. The author states that no funding is involved.

Conflicts of Interest. The author declares no conflict of interest.

Institutional Review Board Statement: The Republic of Lithuania Law undertook experimental procedures involving animals on the care, keeping, and use of experimental vertebrate animals. All experimental procedures were reviewed and approved by the State Food and Veterinary Service of Lithuania according to Directive 2010/63/EU of the European Parliament.

Acknowledgments: Taras Havryliv is grateful to ERASMUS+ for supporting his internship at the Lithuanian University of Health Sciences, where he performed operations using equipment at the Biological Research Center, Kaunas, Lithuania. I want to thank Kestutis Petrikonis (Lithuanian University of Health Sciences, Faculty of Medicine, Department of Neurology, Kaunas) and Rimantas Stakauskas (Biological Research Center, Kaunas, Lithuania) for the opportunity to use their facilities. I also wish to thank Ramunė Grigalevičiūtė for her excellent technical assistance and contribution to the anesthesiological part of the experiment and postoperative care for research animals.

Additional information. Taras Havryliv holds the certificate of Federation of European Laboratory Animal Science Associations (FELASA) and Society of Laboratory Animals (SOLAS) on the education and training of persons carrying out animal experiments – FELASA, category B issued in Humboldt University of Berlin / Berlin Mouse Clinic for Neurology and Psychiatry.

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