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NEW SCHEME FOR THE PREPARATION AND USE OF ARTIFICIAL CEREBROSPINAL FLUID

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This study aimed to explore a set of simplified schemes for the preparation and application of artificial cerebrospinal fluid (ACSF) to improve the experiment efficiency and neurosafety of ACSF. We prepared ACSF into parts A and B, according to the cerebrospinal fluid (CSF) data in rabbits. They were mixed in equal volumes to form ACSF, continous foaming with mixture gas (95% O_2 and 5% CO_2). Sampling inspection showed the chemical stability of ACSF in the three months after preparation. However, it needed to be kept continous foaming, as pH is correlated to the solubility of CO_2. We further improved the application scheme by sealing the foamed ACSF in infusion bags filled with mixture gas, which could keep the pH stable for 24 hours. It was helpful in promoting the progress of clinical and experimental research relating to ACSF.

Key words: *artificial cerebrospinal fluid, blood-brain barrier, cerebrospinal fluid, chemical stability,* bicarbonate buffer, oxygenation, carbon dioxide

INTRODUCTION

The cerebrospinal fluid (CSF) derives from blood, but different blood in its composition. Blood components are isolated by the blood-brain barrier (BBB), except water. They are selectively secreted to form CSF through the choroid plexus (ChP), by protein channels as claudin-2 (CLDN2), whose expression is affected by melatonin (1, 2). It plays an important role in stabilizing the environment in central nervous system (3).

The solute transport is an important way for neurones to clear toxic molecules and metabolites, and absorb nutrients, influencing the pathophysiological process of the central nervous system (4). It is mainly dependent on the circulation of CSF in the subarachnoid space, ventricles and perivascular space (PVS) (5, 6). The composition of CSF is extremely complex, but 99% of them is water, and the other 1% consists of proteins, ions, neurotransmitters and glucose (7, 8).

Artificial cerebrospinal fluid (ACSF) is a transparent liquid prepared with chemical materials, which simulates the CSF in adults in the main chemical indexes following sodium (Na^+), potassium (K^+), magnesium (Mg^{2+}), calcium (Ca^{2+}), chlorine (Cl^-), phosphorus (P), bicarbonate (HCO_3^-), glucose (Glu), pH (pondus hydrogenii), osmotic pressure, *etc*. ACSF has been confirmed to avoid brain injuries caused by normal saline, lactated Ringer's solution, and other clinical liquids (9-12). Therefore, it has been used in clinical and research works in neuroscience for decades.

Since the first successful preparation of ACSF, there have been many preparation methods. However, the formulation principle is relatively unanimous, involving [Na^+] (145.5 mol/L), [K^+] (2.8 mol/L), [Mg^{2+}] (2.2 mol/L), [Ca^{2+}] (2.3 mol/L), [Cl^-] (128.5 mol/L), [HCO_3^-] (23.1 mol/L), [$H_2PO_4^-$] (1.1 mol/L), [Glu] (0.61 g/L), osmotic pressure (289.0 mosm/L), and pH (7.3) (13). Due to the unstable chemical properties of Glu and bicarbonate solutions, the prepared ACSF must be used on the day of preparation and kept continous foaming to stabilize the pH (14, 15). The preparation of ACSF not only takes up much experimental time each day but also requires rigorous conditions such as equipment and a sterile environment. It has therefore troubled experimenters for many years. There are ready-made ACSF products without Glu available, which need to be preserved at 4ºC, but these face the same issues. It has been found that significant changes in pH happen a short time after opening the package at room temperature. As a result, it is important to discuss the simplification of ACSF preparation and application for improving the efficiency of experiments, making it worthy of further study.

MATERIALS AND METHODS

Instruments and reagents

Electronic analytical balance (XSE1050u, Mettler, Toledo, Worthington, OH, USA); full automatic blood gas analyzer (ABL800, Radiometer, Denmark); biochemical analyzer (C16000, Abbott, Abbott Park, IL, USA); osmotic pressure measuring apparatus (SMC-30C, Tianhe Medical Treatment Instrument Co., China); ultra-micro ultraviolet-visible spectrophotometer (ND5000, BioTeke, China); electric-heated thermostatic water bath system (HHS model, Shanghai Boxun Industrial Co., China); acidometer (PHS-3C, Lei-ci, China); purified water equipment

(zsyc-2000L, Shanghai Water Environmental Protection Science and Technology Co., China); nano gas foaming disk (cylindrical, 18 × 30 mm); 75% alcohol; mixture gas (5% CO_2 and 95% O_2); potassium chloride (Sinopharm Chemical Reagent Co., Ltd., China, GB/646-93, AR, 500 g); sodium chloride (Aladdin, China, C111533, 500 g, AR, 99.5%); D-(+)-glucose (Aladdin, China, G116300, 500 g, AR); magnesium chloride·$6H_2O$ (Meilunbio, China, MB0404, 500 g); sodium dihydrogen phosphate·$2H_2O$ (Meilunbio, China, MB2662, 500 g); sodium acetate (Wuxi Yatai United Chemical Co., Ltd., GB/T694-1995, AR, 500 g); calcium chloride (Sinopharm Chemical Reagent Co., Ltd., China, 031/0106000055C148-2017, AR, 500 g); and sodium bicarbonate (Meilunbio, China, MB011115, 500 g).

Animals

The research protocol was approved by the Animal Care and Use Committee of the First Affiliated Hospital of Fujian Medical University and in accordance with the guidelines of the National Institute of Health.

Twenty male New Zealand rabbits (2.5 – 3.0 kg body weight) were used in this study. All the animals had free access to tap water and food and were housed in a room with 25ºC temperature-controlled and light rhythm.

Acquisition of cerebrospinal fluid samples from awake rabbits

This was improved based on the method of trans-cutaneous cisterna magna puncture (16). The animal was placed in a rabbit hutch to limit its activity. Under asepsis, the operator held the rabbit's head with the left hand and gently patted its neck to relax it. The operator punctured the occipital trochanter at the midpoint and the spinous process of the first cervical spine toward the tip of the nose with a 1-ml syringe (injection needle, 0.45 × 16 RWSB). The needle was inserted to a depth of 1.2 cm and then the plunger was pulled back after getting a sense of breakthrough. 0.5 – 1.0 ml of clear CSF was obtained and sealed for analysis (*Fig. 1*).

The qualified CSF sample was clear without blood contamination and more than 0.5 ml volume.

Cerebrospinal fluid assessment

The obtained CSF samples were sealed and analyzed within five minutes. The items included [Na^+], [K^+], [Mg^{2+}], [Ca^{2+}], [Cl^-], [Glu], [P], [Fe], [Cr], [BUN], [Pro] (x-protein), [Lac^-] (Lactate), [TCO_2] (Total of CO_2), [BE] (buffer excess), [HCO_3^-], PO_2, PCO_2, HB (hemoglobin), pH, and osmotic pressure.

Artificial cerebrospinal fluid production

ACSF was produced into parts A and B, in reference to the ACSF95 (Otsuka Pharmaceutical Co., Tokyo, Japan), according to the CSF of rabbits. Part A was an acidic electrolyte solution containing glucose, and part B was an alkaline electrolyte solution containing sodium bicarbonate.

Part A contained D-(+)-Glu 738.66 mg, sodium chloride 7.012.8 mg, calcium chloride 155.40 mg, magnesium chloride·$6H_2O$ 162.64 mg, and sodium acetate 377.34 mg per liter. The pH was adjusted to 4.0 with appropriate hydrochloric acid.

Part B contained sodium bicarbonate 2.184.26 mg, potassium chloride 223.65 mg, and sodium dihydrogen phosphate·$2H_2O$ 62.40 mg. The pH was adjusted to 8.0 with appropriate hydrochloric acid.

Solutions were fully dissolved with water for injection at 25ºC, filtrated by negative pressure suction with 0.2-μm filter membrane, poured into glass bottles (500 ml) sealed with rubber plugs and aluminum edges, and marked. They were stored at room temperature after being sterilized in 105ºC high-pressure steam for 40 minutes. They were mixed in equal volumes to form ACSF and continous foaming by mixed gases (5% CO_2 and 95% O_2) at 25ºC before use.

Stability assessment of artificial cerebrospinal fluid

We randomly selected five samples from parts A and B within one week (initial) and after three months (long-term) after production, respectively. The pH of A and B were respectively measured at 25ºC. The ACSF with continous foaming had the following items detected: [Na^+], [K^+], [Mg^{2+}], [Ca^{2+}], [Cl^-], [Glu], [P], pH, PO_2, PCO_2, [TCO2], [BE], [HCO_3^-], and osmotic pressure. The results were compared with the CSF data in rabbits.

The pH change in foaming artificial cerebrospinal fluid

We randomly selected five samples of A and B, respectively, in 100 ml each and mixed them evenly at room temperature (25ºC). The ACSFs were divided into four groups: the non-foaming group, fully foaming group, continous foaming group, and 38ºC foaming group, 50 ml in each group. The evolution of

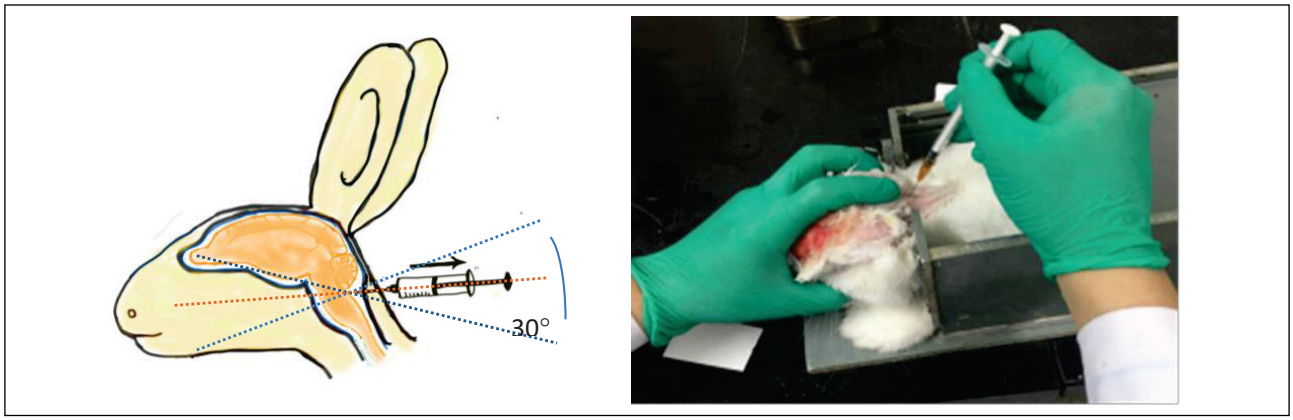

Fig. 1. Acquisition of cerebrospinal fluid (CSF) samples from awake rabbits. We would relax the neck muscles through appropriate stretching and gentle patting, which not only pacified the animal to prevent restlessness but also shortened the depth of percutaneous puncture. The puncture direction can be performed toward the 30º ranges between the lower edge of orbit and the tip of the mandible, but it was ideal toward the tip of the nose.

pH was monitored in each group. The non-foaming group was exposed at room temperature until the pH stabilized and foamed. The fully foaming group stopped foaming while stabilized and was exposed at room temperature until the pH stabilized again and then foamed. The continous foaming group kept foaming for two hours. The 38ºC foaming group stopped foaming while the pH stabilized in a 38ºC water bath, was exposed at 38ºC until the pH stabilized again, and then foamed.

The foamed artificial cerebrospinal fluid in sealed package

The sterile saline infusion soft package (100 ml, 250 ml) was used to save foamed ACSF. We randomly selected five samples of A and B in 50 ml each and mixed them evenly in a sterile beaker on an aseptic bench. They were sealed into the soft infusion bag after fully foaming at 25ºC with the pH marked. We then filled the bag with foaming gas. After 24 hours, the first stable pH in each was measured to compare with the initial pH.

Statistical analysis

All results were expressed as mean ± standard deviation (M ± SD). Graphpad prism 8.0 software was used for statistical processing and drawing. The one-way ANOVA analysis and Tukey multiple comparisons were used to compare the components' indifferent time. $P < 0.05$ was considered statistically significant.

RESULTS

Acquisition of cerebrospinal fluid samples from awake rabbits

We obtained 19 qualified CSF samples with a success rate of 79.17% (19/24). The majority of awake rabbits cooperated with the percutaneous suction of CSF from the cisterna magna. There were three cases where the cisterna magna could not be reached

Table 1. Comparison of main components among human cerebrospinal fluid (CSF), rabbit CSF, and common artificial cerebrospinal fluid (ACSF).

CSF composition	**Healthy humans**	**Common ACSF**	**Healthy rabbit** (n = 19)	**95% CI** (n = 19)
Glu (mmol/l)	2.8 – 4.4	3.386※	4.151 ± 0.247	4.032 – 4.270
Pro (g/l)	0.1 – 0.4	–	0.254 ± 0.201	0.1467 – 0.3606
Na^+ (mmol/l)	135 – 150	145.5※	151.400 ± 2.104 Δ	150.4 – 152.5
K^+ (mmol/l)	2.6 – 3.0	2.8※	3.034 ± 0.056	3.007 – 3.061
Cl^- (mmol/l)	115 – 130	128.5※	125.400 ± 1.861 Δ	124.5 – 126.3
Ca^{2+} (mmol/l)	1.00 – 1.40	2.3※	1.354 ± 0.0419	1.334 – 1.374
Mg^{2+} (mmol/l)	1.2 – 1.5	2.2※	0.668 ± 0.071 Δ	0.6338 – 0.7020
Phosphates (mmol/l)	0.4 – 0.6	1.1※	0.367 ± 0.033 Δ	0.3512 – 0.3825
Cr (umol/l)	50 – 110	–	47.910 ± 4.838	45.57 – 50.24
Ur (mmol/l)	3.0 – 6.5	–	6.221 ± 1.396	5.548 – 6.894
Osmotic pressure (mosmol/kg·H_2O)	280 – 300	289.0※	301.000 ± 4.429 Δ	298.9 – 303.2
pH	7.28 – 7.32	7.3※	7.419 ± 0.041 Δ	7.399 – 7.439
PO_2 (mmHg)	40 – 44	–	127.600 ± 16.350 Δ	119.7 – 135.4
PCO_2 (mmHg)	44 – 50	–	41.16 ± 3.304	39.57 – 42.76
TCO_2 (mmol/l)	20 – 25	–	61.27 ± 2.816 Δ	59.92 – 62.63
lactate (mmol/l)	1.1 – 2.4	–	2.000 ± 0.325	1.843 – 2.157
buffer excess (mmol/l)	–	–	2.011 ± 1.577	1.251 – 2.770
HCO_3^- (mmol/l)	24 – 27	23.1※	26.030 ± 1.398	25.48 – 26.72

There were significant differences in the CSF between rabbits and humans, hinting at the physiological differences. The common ACSF that mimics the human CSF was more different from the CSF of rabbits. It served as a reminder that the ACSF for rabbits needs to be specifically prepared. Note: CI, Confidence interval. Δ The ingredients of CSF in rabbits were beyond the physiological range of humans; ※ The ingredients of common ACSF compared with the CSF of rabbits beyond 95% CI.

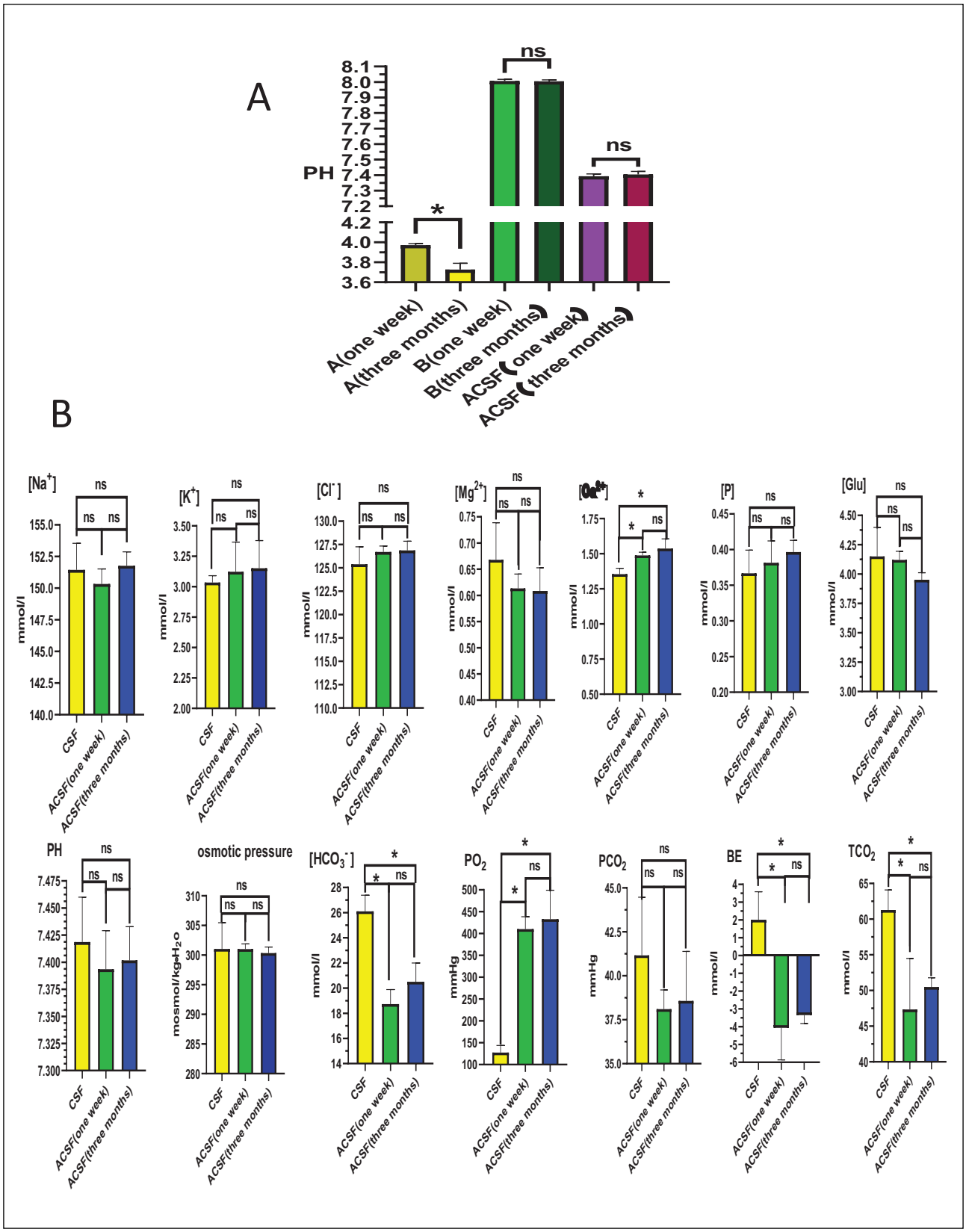

Fig. 2. The chemical stability of artificial cerebrospinal fluid (ACSF) in three months. In general, there were no significant differences in the final component concentrations of ACSF between three months and one week ($P < 0.05$). It showed the chemical stability of ACSF for long-term storage in part A/B at room temperature. It was stable for Part B with airtight packaging but relatively unstable for part A containing glucose. There was some inevitable degradation in glucose but no significance in statistics. Although there was a significant change in pH of part A, the pH of ACSF still stabilized around 7.4 due to the carbonate buffer system forming after the foaming mixture B/A. In addition, there were significant deviations in gas indexes and [Ca^{2+}] compared with the cerebrospinal fluid (CSF) of rabbits. They were attributed to reagent weighing and foaming gas, which could easily be improved. Note: CSF (n = 19), ACSF (n = 5), Part A (n = 5), Part B (n = 5). * meant $P < 0.05$, ns meant $P > 0.05$. BE, buffer excess.

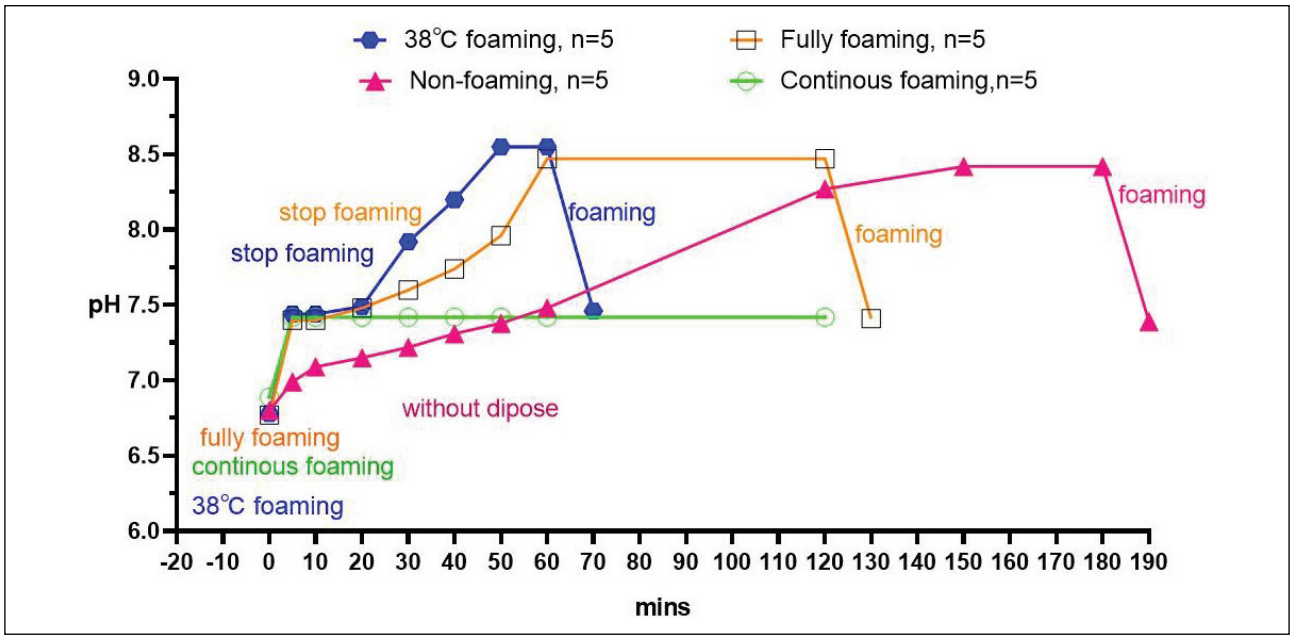

Fig. 3. The pH changes in foaming artificial cerebrospinal fluid (ACSF). The non-foaming group reflected that ACSF was unstable in pH and needed to be foamed before use. Next, the fully and continous foaming groups reflected that ACSF must keep foaming during use. Further, the 38ºC foaming and fully foaming groups reflected that ACSF had a similar pH at room temperature (25ºC) and body temperature (38ºC).

due to thickening of the neck muscles with tension, and two unqualified samples were excluded because of animal restlessness.

The main cerebrospinal fluid components in normal rabbits

The components in healthy humans' CSF and common ACSF came from the previous literature (13, 17, 18). There were some differences between rabbits and humans in the CSF at [Na^+], [Cl^-], [Mg^{2+}], [P], [TCO_2], PO_2, osmotic pressure, and pH. Further, when comparing common ACSF to the rabbits' CSF, there were more differences at [Glu], [Na^+], [K^+], [Cl^-], [Ca^{2+}], [Mg^{2+}], [P], [HCO_3^-], osmotic pressure, and pH. Although lacking the gas indicators of common ACSF, the difference in gas status must be great, given that it is usually used directly without being foamed (*Table 1*).

The chemical stability of artificial cerebrospinal fluid

Part B, containing bicarbonate, was stable in airtight packaging during the three months, with the pH of 8.007 ± 0.010 versus 8.005 ± 0.008 (P = 0.9995).

Part A, containing glucose, was relatively unstable during the three months, with the pH as 3.972 ± 0.015 versus 3.727 ± 0.064 (P < 0.001). However, the pH of ACSF still stabilized around 7.4 as 7.393 ± 0.015 versus 7.405 ± 0.019 (P = 0.8667). Some degradation in glucose inevitably occurred, but it was not statistically significant in [Glu] of ACSF as 4.120 ± 0.075 versus 3.952 ± 0.061 (P = 0.3334) (*Fig. 2A* and *2B*).

The other components and indexes in ACSF showed stable status in three months (*Fig. 2B*). They were as follows: [Na^+] (150.3 ± 1.189 versus 151.8 ± 1.102 mmol/l, P = 0.3733), [K^+] (3.123 ± 0.243 versus 3.152 ± 0.228 mmol/l, P = 0.9412), [Cl^-] (126.7 ± 0.636 versus 126.9 ± 0.228 mmol/l, P = 0.9816), [Ca^{2+}] (1.488 ± 0.022 versus 1.537 ± 0.069 mmol/l, P = 0.1758), [Mg^{2+}] (0.590 ± 0.014 versus 0.608 ± 0.044 mmol/l, P = 0.9889), [P] (0.405 ± 0.012 versus 0.397 ± 0.016 mmol/l, P = 0.6639), [BE] (–4.07 ± 1.800 versus –3.35 ± 0.047, P = 0.6855 mmol/l), [HCO_3^-] (18.73 ± 1.152 versus 20.50 ± 1.501 mmol/l, P = 0.0657), [TCO_2] (47.33 ± 7.155 versus 50.48 ± 1.295 mmol/l, P = 0.3391), PO_2 (410 ± 28.45 versus 432.8 ± 66.32 mmHg, P = 0.4680), PCO_2 (37.22 ± 2.243 versus 38.57 ± 2.825 mmHg, P = 0.9593), and osmotic pressure (301.0 ± 0.89 versus 300.3 ± 1.03 mosmol/kg·H_2O, P = 0.9449).

The pH change in foaming artificial cerebrospinal fluid

The pH stabilized at 7.39 ± 0.021 in five minutes after fully foaming or continous foaming at room temperature (25ºC). It promptly increased after foaming stopped and stabilized at 8.442 ± 0.033 after 60 minutes. It was reduced to 7.414 ± 0.0025 after foaming again. It showed the same process while foaming at 38ºC. The pH stabilized at 7.448 ± 0.023 after fully foaming, rose to 8.630 ± 0.152 when it stopped, and came back after foaming again. Additionally, the pH rose slowly, from 6.806 ± 0.048 to 8.452 ± 0.026 in three hours, while the ACSF exposed at 25ºC without disposal, and then stabilized at 7.406 ± 0.024 after foaming (*Fig. 3*).

The foamed artificial cerebrospinal fluid with stable pH in sealed package

The pH of ACSF with fully foaming compared with the later sealing for 24 hours was 7.376 ± 0.015 versus 7.382 ± 0.016 (P = 0.0705).

DISCUSSION

Previous CSF data in New Zealand rabbits were obtained from animals under general anesthesia (19). It is evident that the inhibition of anesthesia on respiratory, circulatory, and neural activations resulted in the changes in CSF, especially the gas indexes. It is necessary to obtain the composition data of CSF in non-anesthetized rabbits to provide more accurate data for ACSF preparation. It is a challenge to extract CSF from the cisterna

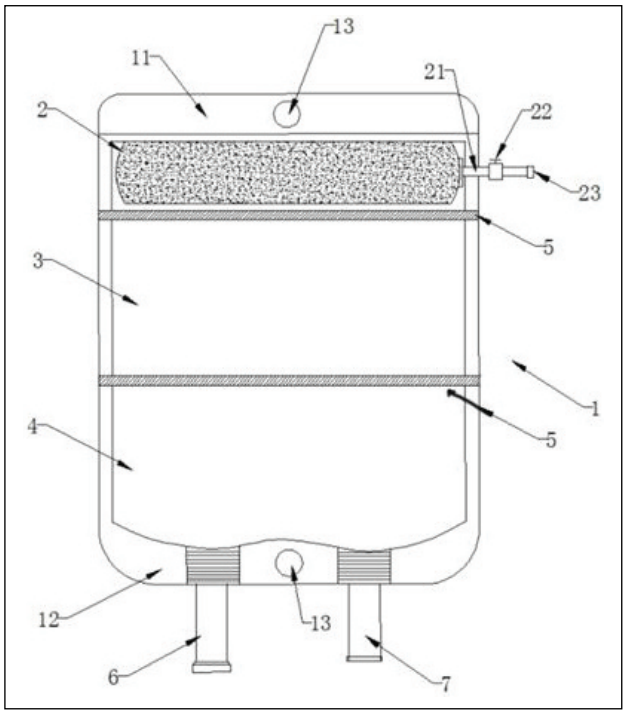

Fig. 4. A designed artificial cerebrospinal fluid (ACSF) packaging. Solution A and B are separately packed into chambers A (3) and B (4) during the production process for long-term storage. Before use, the separation (5) between A and B chambers is opened to evenly mix AB. Then, open the separation (5) of the foaming chamber and hang it upside down (12) to immerse the foaming stick. Connect the gas pipe (23) with sterile mixture gas containing 5% CO_2. Meanwhile, open the exhaust port (6) during fully foaming. Close the exhaust port (6) first and then the gas pipe (23) so that the package is saturated by gas. While using, hang it upside down (11) and connect the output interface (7) to release ACSF for treatment.

magna by a percutaneous puncture in awake rabbits. Our success was due to the proper animal immobilization, the relaxation of neck muscles, and the proper angle and depth in the puncture.

In the previous experimental ACSF perfusion, the common ACSF prepared according to human CSF was used, regardless of the animal species. Our experimental data suggest that there are significant differences in the concentration of components and physicochemical characteristics between human CSF and rabbit CSF. There are further differences between common ACSF and rabbit CSF. The common ACSF was conspicuously lower in [Na^+], [HCO_3^-], [K^+], pH, and osmotic pressure and conspicuously higher in [Cl^-], [Ca^{2+}], [Mg^{2+}] compared with rabbit CSF. The experimental perfusion in rabbits with common ACSF may result in edema and brain injury. Therefore, the specialized ACSF prepared according to the CSF indexes of rabbits, which was used in the perfusion of rabbits' brains, can avoid side effects and deviation results.

The preparation of ACSF should consider not only the simulation of CSF but also the stability of its physical and chemical properties, which is convenient for long-term storage. Given the complex composition of CSF, ACSF simulates the critical indexes relating to physiological activity, especially [Glu], [Na^+], [HCO_3^-], [K^+], [Cl^-], [Ca^{2+}], [Mg^{2+}], pH, and osmotic pressure. It is well-known that glucose solution is relatively stable at pH 3.0 – 4.0, while the bicarbonate solution is relatively stable at pH > 7.4 (20, 21). Therefore, it is difficult to maintain the chemical stability of ACSF containing both glucose and bicarbonate for a long time. Commercially available ACSFs choose to discard the glucose component and pre-regulate pH at 7.4 to achieve chemical stability and avoid the foaming process. They not only lack the energy substance for brain cells but also quickly change the pH due to CO_2 escape at room temperature or 38ºC. For this reason, our protocol separates the electrolyte solution containing glucose (part A) or bicarbonate (part B), solving the issue of long-term storage. Our experiment showed that despite some glucose degradation with declining pH within three months, the [Glu] did not significantly decrease in ACSF as the final product. In addition, the pH stabilized around 7.4 under foaming.

ACSF is essentially a bicarbonate buffer system solution whose pH is affected by CO_2 solubility. It is affected by temperature, air pressure, and CO_2 concentration. The solubility of CO_2 decreases as temperature increases.

$$CO_3^{2-} + 2H+ \rightleftharpoons HCO_3^- + H+ \rightleftharpoons CO_2\uparrow + H_2O$$

Previous literature revealed that ACSF could stabilize the pH at 7.4, improving the CO_2 solubility by fully foaming with a mixture gas (5% CO_2 and 95% O_2) at room temperature (22). Our results are consistent with the reported literature. However, continous foaming must be maintained to stabilize the pH in the whole experimental process. Otherwise, a short time after foaming stops, pH will deviate from the physiological range, resulting in brain injuries (23). Moreover, a set of special foaming devices, much foaming gas, and an aseptic environment are required, which is difficult for many laboratories to provide.

We aimed to resolve these problems by replacing the special foaming device with a sandy pillar that was used for oxygenation in water. The ACSF was sealed into a soft infusion bag after being fully foamed in a sterile beaker and then filled with gas to keep CO_2 solubility, which can freely be used in 24 hours.

Inspired by this study, our team designed and developed ACSF packaging that integrates the functions of long-term storage, gas foaming, and safe use for ACSF. It avoids mistakes and pollution caused by the tedious operation in the A/B mixing and foaming process (*Fig. 4*).

There are four limitations in this study:

1). The addition of sodium acetate resulted in a decrease in [BE] and [HCO_3^-].

2). There was a higher [Ca^{2+}].

3). The higher PO_2 in ACSF may lead to cerebral oxygen poisoning, which could be avoided by replacing one part of oxygen with nitrogen in the foaming gas (17).

4). The stability of ACSF for more than three months was not observed.

Summarizing: the protocol of ACSF preparation and application that is separately prepared into A/B parts, balanced mixed, and foamed for use greatly improves the efficiency and neural safety of ACSF experiments. It is worthy of promotion.

Authors' contribution: Wen-He Zheng and Chao Yan contributed equally to this study.

Availability of data: The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Conflict of interest: None declared.

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