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A COMPARATIVE ANALYSIS OF TRANSCARDIAL PERFUSION TECHNIQUES FOR BRAIN FIXATION IN RODENTS: A CALL FOR STANDARDIZATION OF TRANSCARDIAL PERFUSION

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ABSTRACT

Transcardial perfusion is a crucial technique in rodent research for tissue fixation and preservation. The process typically involves flushing the vascular system with saline to remove blood, followed by fixation with 4% paraformaldehyde (PFA) or formalin. However, differences in species, procedural techniques, and fluid volumes can influence perfusion efficiency and tissue quality. This literature review compares six transcardial perfusion protocols used in mice and rats, focusing on procedural variations and fluid volume requirements. Data were collected from published protocols detailing perfusion techniques, obtaining evidence that substantial variability is observed in the protocols, including differences in anesthesia regimens, surgical approaches, perfusion techniques, fluid compositions, and fixation outcomes. Future research should focus on standardizing perfusion protocols to improve consistency in fixation quality, minimize tissue degradation, and preserve structural and molecular integrity.

KEYWORDS

Transcardial Perfusion; Brain Fixation; Animal Model; Paraformaldehyde; Tissue Preservation

INTRODUCTION

The brain is a highly complex organ, highly susceptible to various neurological diseases, with its major ones still facing major challenges in developing therapies. The therapy and identification for each disease differ in various aspects (Abed, 2023; Cervellati *et al.*, 2020; Zehravi *et al.*, 2022). Although various therapies are being developed, many of them still face issues such as limited efficacy, significant side effects, or even fail in the clinical trial phase (Kim *et al.*, 2022). This can be seen in the phenomena of stroke therapies, where drugs that are promising in preclinical trials have failed in clinical trials. This might be caused by the mismatch of animal models used with the clinical scenario of the disease, which underlines the importance of efforts to continuously improve the animal models used in research and their relevance to the realistic clinical situation (Campbell *et al.*, 2019; Trotman-Lucas and Gibson, 2021).

Before interventions are tested in clinical trials, preclinical testing in animal models is done to evaluate the safety and initial efficacy of the therapy (Trotman-Lucas and Gibson, 2021). Thus, animal models must have high validity, both in mimicking the pathophysiology of the disease studied and in the preservation of brain tissue, allowing accurate histological and molecular analysis of the effects of the therapy being tested (Domínguez-Oliva *et al.*, 2023; Ruggeri, Camp and Miknyoczki, 2014). In preclinical testing, the brains from animal models are taken for study, which begins with fixation methods. However, all fixation methods have the potential to alter neural tissue structure. Therefore, it is necessary to study the potential for artifacts and distortions in fixation methods, even for established techniques. Several fixation methods for brain fixation are often used, one of which is transcardial perfusion (Kasukurthi *et al.*, 2009).

Transcardial perfusion is a standard method in fixation of experimental animal tissues, especially in neuroscience and histopathology research (Kasukurthi *et al.*, 2009). This technique is carried out by flowing buffer and fixative solutions through the cardiovascular system to ensure that the tissue is maintained in optimal condition before histological or immunohistochemical analysis (Wu et al., 2021). Good perfusion is essential to prevent autolysis, maintain tissue structure, and ensure even distribution

of fixative in target organs, such as the brain. In experimental models, perfusion is necessary to preserve tissue morphology, allowing for further analysis of histopathological changes, protein expression, and molecular changes (McFadden *et al.*, 2019). Although TP protocols for brain perfusion have been widely used, there is still variability in the procedure, including differences in perfusion pressure, solution volume, type of fixative, and duration of perfusion. Variations in these parameters can affect the tissue quality obtained regarding histological clarity and cellular integrity (Kasukurthi *et al.*, 2009). Therefore, protocol evaluation and optimization are needed to determine the most effective perfusion parameters in maintaining tissue quality. This study explores variations in transcardial perfusion techniques in hopes of identifying the most effective and efficient method.

MATERIALS AND METHODS

This study was conducted as a literature review, utilizing scholarly databases such as PubMed, ScienceDirect, and Google Scholar using the keyword ("transcardial perfusion" AND brain) AND ("rodent" OR "rat" OR "mouse" OR "animal model"). The inclusion criteria included established protocols or renewed experimental methods of transcardial perfusion involving fixation liquids for fixating brains of rodent models, and the exclusion criteria included studies without specific perfusion details, without a focus on brain fixation, or those focusing on non-rodent species.

The search results that are free from duplicates will be filtered in the following order: (1) Title and abstract if available; (2) Availability; (3) Full paper. The final six studies included in the screening will be extracted and analysed.

RESULTS

This study reviews methods of transcardial perfusion used by the included studies from various aspects such as surgical methods, perfusion techniques, and brain collection.

Table 1. Characteristics of the included studies

Author, Year	Article type	Sample	Intervention
Wu *et al*., 2021	Protocol	Mouse	Transcardial perfusion
Gage, Kipke, & Shain, 2012	Protocol	Mouse	Transcardial perfusion
Kwan *et al*., 2022	Protocol	Mouse	Transcardial perfusion
Paul, Beltz, & Berger-Sweeney, 2008	Protocol	Rat	Transcardial perfusion
Arévalo *et al*., 2022	Experimental protocol	Neonatal mice	Transcardial perfusion
Rana, Massa, & Jie Chen, 2022	Experimental protocol	Mice	Gravity fed transcardial perfusion

Table 1 presented the characteristics of the included protocols and studies, with rodents as the sample and transcardial perfusion as the standard intervention.

Table 2. Surgical procedures of included studies

Author, Year	Anesthesia	Surgical Approach	Needle/Cannula Insertion	Atrium Incision
Wu *et al*., 2021	1.5% sodium pentobarbital with a dose of 0.06 ml per 10 g of body weight	Thoracotomy with sternum reflection	Inserted into the left ventricle and advanced into the aorta	Right atrium incision for blood drainage
Gage, Kipke, & Shain, 2012	Ketamine (80 mg/kg) & Xylazine (10 mg/kg) via intraperitoneal	Thoracotomy with diaphragm incision and sternum reflection	15G blunt or olive-tipped needle inserted into the ascending aorta	Right atrium cut to maximize outflow

Kwan *et al*., 2022	Sodium pentobarbitone (100 mg/kg, lethally overdose via intraperitoneal)	Thoracotomy with sternum reflection	Gavage needle (attached to pump tubing) inserted into the apex of the heart, 2-3mm into the tissue	Right atrium incision for blood drainage
Paul, Beltz, & Berger-Sweeney, 2008	Sodium pentobarbital (60 mg/kg) via intraperitoneal	Thoracotomy with sternum reflection	Cannula inserted through the left ventricle into the aorta	Right atrium incision for blood drainage
Arévalo *et al*., 2022	Ketamine (520 mg/kg) & Xylazine (78 mg/kg) intraperitoneal	Small thoracic incision exposing organs	27G needle inserted into the left ventricle	Right atrium cut to allow fixative outflow
Rana, Massa, & Jie Chen, 2022	Isoflurane exposure until respiration stops	Thoracotomy with Y-shaped incision to expose the heart	Butterfly needle inserted into the left ventricle (~0.5 cm depth)	Right atrium or inferior vena cava cut for drainage

The surgical aspects include anesthesia usage, thorax opening, needle insertion, and atrium incision, which are evaluated in Table 2.

Table 3. Perfusion techniques of included studies

Author, Year	**Perfusion Technique**	**Perfusion Fluids & Completion**	**Perfusion Rate**	**Fixation signs**
Wu *et al*., 2021	Manual or peristaltic pump	Saline (~20 mL) followed by 4% PFA (~15-20 mL)	~1 mL/5 s	Stiff body, slight brownish liver color
Gage, Kipke, & Shain, 2012	Pressure-controlled pump system (80–130 mmHg)	Pressure-controlled pump system (80–130 mmHg)	200 mL PBS, then pressure up to 130 mmHg	Fixation tremors, liver turns pale
Kwan *et al*., 2022	Peristaltic pump	1x PBS (5 min perfusion at 5 mL/min, ~25 mL total) followed by ice-cold 4% PFA in 1x PBS (8 min perfusion at 5 mL/min, ~40 mL total)	5 mL/min	Fixation tremors observed after 1-2 min
Paul, Beltz, & Berger-Sweeney, 2008	Aspirator jar (2-3 ft above work area)	Saline (~150 mL) followed by 4% formalin (~400 mL)	Pumped through perfusion system	Stiff and rigid body, liver changes to light brown
Arévalo *et al*., 2022	Pump-based system, controlled flow	10 mL PBS, then 10 mL 4% PFA	1 mL/min (controlled pump)	Liver turning pale, full-body rigidity
Rana, Massa, & Jie Chen, 2022	Gravity-fed system (no pump)	1/3 bottle PBS (~20–30 mL), then 4% PFA (~20–30 mL)	Passive gravity flow (~3 min saline, ~50 min PFA)	Tail curling, stiffened body, liver color change

Table 3 lists the whole perfusion process of every protocol from the tool, fluids used, perfusion rate, and fixation signs.

Table 4. Brain collection of included studies

Author, Year	**Brain collection**
Wu *et al*., 2021	The brain is placed in PBS with 0.01% sodium azide for long-term storage at 4°C. Alternatively, it can be submerged in a 30% sucrose solution until it settles at the bottom and then stored at −20°C to −80°C.
Gage, Kipke, & Shain, 2012	Brain dissected post-perfusion and stored in fixative

Kwan *et al*., 2022	The excised brain is immediately transferred to a 50 mL sample container filled with ice-cold 4% PFA in 1x PBS and placed on a vertical rocker at 4°C for up to 24 hours.
Paul, Beltz, & Berger-Sweeney, 2008	N/A
Arévalo *et al*., 2022	Whole pup post-fixed, then cryosectioned
Rana, Massa, & Jie Chen, 2022	Whole mouse placed in PFA overnight, then brain excised

Articles with information on the collection of fixated brain after perfusion are evaluated in Table 4 (N/A: not announced).

DISCUSSION

Fixation is done to preserve tissue in its life-like state in a rapid and uniform way. Unlike immersion fixation, which works more optimally on smaller tissues, where fixative liquid can reach all regions of the tissue, the use of transcardial perfusion can directly perfuse fixative through the circulation to reach all parts of the organism (Gage, Kipke, and Shain, 2012). The included studies utilized various protocols of transcardial perfusion, each differing in anesthesia type, surgical approach, perfusion technique, and fixation parameters. These methodological differences may influence tissue preservation quality, reproducibility, and suitability for downstream histological and molecular analyses (Kasukurthi *et al.*, 2009).

The Choice of Anesthesia and Surgical Approach Across Protocols

Rodents, such as the laboratory mouse (*Mus musculus*), are widely used as animal models because of their relative ease of care, size, and availability (Navarro *et al.*, 2021). Rats (*Rattus norvegicus*) are also used immensely, as over 2 million of it are used in biomedical research in the European Union and Canada every year. Owing to the widespread use of rodents in research and the necessity of euthanasia, it is crucial to continuously refine humane methods to ensure minimal handling, simple administering technique, and painless death (Zatroch *et al.*, 2016).

Euthanasia can be done physically, involving methods such as exsanguination and chemicals, one of which is the use of anesthesia. A commonly used one is sodium pentobarbital, a fast-acting depressant of the central nervous system acting via the GABA receptors, causing loss of consciousness (Mohamed *et al.*, 2020). Another one frequently used in experimental models is the combination of ketamine and xylazine (McFadden *et al.*, 2019).

The usage of anesthesia varied among the protocols—some utilized sodium pentobarbital while others combined ketamine and xylazine. Pentobarbital, as seen in Wu et al. (2021) and Kwan *et al.* (2022), ensures deep anesthesia but may affect cardiovascular dynamics, potentially influencing perfusion efficiency (Matsubara and Da Silva-Santos, 2024). In contrast, the combination of ketamine-xylazine (Gage, Kipke and Shain, 2012; Pérez Arévalo *et al.*, 2022) provides sufficient anesthesia while maintaining more stable cardiovascular function (Matsubara and Da Silva-Santos, 2024), which may lead to more consistent perfusion results.

Contrary to aspects of anesthesia, the surgical approach was consistent across protocols as seen in Table 2, most commonly employing thoracotomy and sternum reflection. However, Pérez Arévalo *et al.* (2022) employed a small thoracic incision, which may reduce tissue damage and blood loss, which could be relevant when preserving peripheral nervous system structures are considered.

Perfusion Techniques and the Composition of Perfusion Fluids

The studies included manual, peristaltic, pressure-controlled pump-based, and gravity-fed perfusion techniques as mentioned in Table 3. Manual and peristaltic pumps, as used by Wu et al. (2021) and Kwan *et al.* (2022), need precision of the operators, allowing room for variability in perfusion pressure and rate. In contrast, pressure-controlled pumps as used by (Gage, Kipke and Shain (2012) and Pérez Arévalo *et al.* (2022) allows a more standardized pressure application ranging from 80 to 130 mmHg, allowing enhanced consistency in fixation outcomes. The usage of pump optimizes controlled delivery and reduces the possibility of tissue damage with a steady flow of buffer (PBS) followed by fixative (PFA) through the circulatory system (Pérez Arévalo *et al.*, 2022).

The fluid composition also varied, with some protocols using PBS pre-wash followed by 4% PFA (Wu et al., 2021; Paul, Beltz, & Berger-Sweeney, 2008), while others employed ice-cold 4% PFA in PBS (Kwan *et al.* 2022; Rana, Massa and Chen, 2022). The use of pre-wash steps ensures the removal of blood components, which is crucial in minimizing autolysis and post-fixation artifacts. Notably, Paul, Beltz, & Berger-Sweeney (2008) utilized an aspirator jar for perfusion, a less common method that may result in variable perfusion efficiency due to pressure fluctuations.

Perfusion Rate and Fixation Quality

The perfusion rates ranged from 1 to 5 mL/s, with some studies, such as one from Kwan *et al.* (2022), reporting fixation tremors within 1-2 minutes as an indication of adequate perfusion. Lower perfusion rates at 1 mL/min, as seen from Pérez Arévalo *et al.* (2022) may allow for more gradual and uniform fixative penetration, preventing structural damage, but could be less effective in large animal models. Conversely, higher rates at 5 mL/min (Rain Kwan *et al.*, 2022) may rapidly displace blood but risk inducing pressure-related tissue distortions. Fixation signs, including body rigidity, liver discoloration, and tremors, were observed across studies (Table 3). Liver discoloration (light brown or pale) was commonly reported and may indicate successful vascular clearance, while tremors suggest active fixative distribution throughout the vasculature.

Brain Collection, Post-Fixation Handling and Quality

Differences in post-fixation handling may influence histological and molecular downstream applications, significantly impacting the fixated outcome (Bauer, Otter and Chafin, 2018). The methods of brain extraction and handling varied widely across studies, as seen in Table 4. For instance, the difference of fixation usage can be examined, where Wu et al. (2021) maintained the brain in PBS containing 0.01% sodium azide for long term storage at 4°C, while Kwan *et al.* (2022) fixed the brain in a 50 mL sample container filled with ice-cold 4% PFA in 1x PBS followed by placement of the container on vertical rocker at 4°C for up to 24 hours. Other protocols from Gage, Kipke and Shain (2012) and Pérez Arévalo *et al.* (2022) did not specify the fixation liquid used.

Two studies from Rana et al. and Arévalo et al. provide substantial evidence supporting the superiority of transcardial perfusion over other methods of fixation, such as immersion. Rana et al. found that perfused brains exhibit better structural integrity with firmer consistency, preventing possibilities of mechanical distortion during handling. Arévalo et al. demonstrated that perfusion ensures complete blood clearance, to prevent the presence of residual hemoglobin that can interfere with downstream immunohistochemical staining. Immersion fixation results in uneven tissue preservation, while perfusion preserves fine anatomical details and prevents degradation. Both studies provided evidence that perfusion leads to better immunofluorescence reading by ensuring antigen preservation and reducing background staining.

CONCLUSIONS

Transcardial perfusion allows maximal fixation within the fixated tissues. However, the reviewed protocols demonstrate substantial variability, with differences observed in anesthesia regimens, surgical approaches, perfusion techniques, fluid compositions, and fixation outcomes. These variations highlight the lack of a standardized method that ensures optimal brain tissue preservation across different experimental settings. A significant limitation in comparing these protocols is the lack of standardized outcome assessments for brain tissue quality. While some studies report gross fixation signs, only a few provide quantitative or histological validation of fixation effectiveness. Additionally, the difference in species used introduces variability in perfusion dynamics due to vascular architecture and metabolic rate differences. Given these discrepancies, future research should focus on standardizing or optimizing perfusion fixation protocols to identify the most effective approach for ensuring quality, minimal tissue degradation, and maximal structural and molecular integrity preservation. Establishing consensus guidelines through comparative studies would improve inter-study reproducibility and enhance the reliability of neuroscientific and histopathological research utilizing rodent brain models.

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Conflict of Interest

The authors declare that no competing interests exist.

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