Tissue processing and optimal visualization of cerebral infarcts following sub-acute focal ischemia in rats

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\textbf{ABSTRACT}

Transient cerebral ischemia followed by reperfusion in an infarcted brain comes with predictable acute and chronic morphological alterations in neuronal and non-neuronal cells. An accurate delineation of the cerebral infarct is not a simple task due to the complex shapes and indistinct borders of the infarction. Thus, an exact macroscopic histological approach for infarct volume estimation can lead to faster and more reliable preclinical research results. This study investigated the effect(s) of confounding factors such as fixation and tissue embedding on the quality of macroscopic visualization of focal cerebral ischemia by anti-microtubule-associated-protein-2 antibody (MAP2) with conventional Hematoxylin and Eosin (HE) staining serving as the control. The aim was to specify the most reliable macroscopic infarct size estimation method after sub-acute focal cerebral ischemia based on the qualitative investigation. Our results showed that the ischemic area on the MAP2-stained sections could be identified macroscopically on both cryo-preserved and paraffin-embedded sections from both immersion- and perfusion-fixed brains. The HE staining did not clearly depict an infarct area for macroscopic visualization. Therefore both immersion-fixed and perfused-fixed MAP2 stained sections can be used reliably to quantify cerebral infarcts.

\begin{enumerate}
\item \textbf{Introduction}

Focal cerebral ischemia results from a sudden reduction of cerebral blood flow (CBF) in a specific brain region (Bacigaluppi et al., \textit{2010}). The middle cerebral artery occlusion (MCAO) model is a well-known rodent stroke model that produces a cerebral infarction (an area of necrotic tissue) in one hemisphere of the brain (Bacigaluppi et al., \textit{2010}). In studies using animal models of stroke, changes in the cerebral infarction size are often the primary endpoint, and it is an essential variable for translational stroke research (Overgaard et al., \textit{1992}).

In general, there are several comparative methods for visualizing cerebral ischemia, such as positron emission tomography (PET) and magnetic resonance imaging (MRI), which are widely used in clinical studies (de Leon et al., \textit{1996}; Venkataraman et al., \textit{2004}; Watson et al., \textit{1997}). Furthermore, the 2,3,5-triphenyltetrazolium hydrochloride (TTC) staining method gives the possibility of quick detection of ischemic tissues via a redox indicator reaction, but it is of limited use in fixed tissues due to the need for a reaction with mitochondria. This method has been reported to provide identical results to Hematoxylin and Eosin (HE) staining 24 h after reperfusion in a rat model of stroke (Bederson et al., \textit{1986}). However, TTC staining further limits quantitative histological investigations and leads to a lack of detailed information about important features of ischemic cell death. In preclinical research, the histological approaches are often preferred due to the straightforward, cost-efficient setup, which still provides a high resolution of the tissue structure.

Several routine histological staining methods have been used for visualizing ischemic brain regions. Nissl staining, using an aniline stain

\textbf{Abbreviations:} MAP2, microtubule-associated-protein-2; HE, hematoxylin and eosin; CBF, cerebral blood flow; MCAO, middle cerebral artery occlusion; PET, positron emission tomography; MRI, magnetic resonance imaging; TTC, triphenyltetrazolium hydrochloride; TBS, tris-buffered saline.

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to label extranuclear RNA granules which mainly localize the soma of neurons, is a preferable staining method for microscopic evaluation of ischemic tissue (triangular shaping, exhibiting dark staining due to condensation of cytoplasm and karyoplasm) (Chen et al., 2013; Garcia-Cabezas et al., 2016). HE is still the most widely used stain (Atchoin et al., 2017; Choi et al., 2017; Min et al., 2017; Pradillo et al., 2017), and the usage of HE-stained brain sections for microscopically assessing infarct volume is often considered the ‘golden standard’ (Sim et al., 2016), even though in some cases the appearance of the ‘dark neuron’ in HE-staining is representative of an artifact (Zille et al., 2012).

Another possible limitation of HE-staining for infarct volume estimation is the necessity for microscopic delineation of the infarcted area, which is time-consuming and requires comprehensive morphological knowledge. Additionally, an accurate delineation of the cerebral infarct is not necessarily a simple task due to the complex shapes and indistinct borders of the infarction (Gavin et al., 2004). Thus, an accurate macroscopic histological approach for infarct volume estimation can lead to faster and more reliable preclinical research results.

Immunohistochemical staining, particularly the cytoskeletal protein microtubule-associated protein 2 (MAP2), provides a discernible parameter of the infarction (Kitagawa et al., 1989; Popp et al., 2009). A significant concern with histological assessment is that different factors such as brain swelling or shrinkage due to tissue processing may interfere with visualization of the infarcted tissue (Diemer, 1982). To the best of our knowledge, there are no previous studies that compare the effects of fixative methods (immersion- and perfusion-fixation) in combination with different tissue processing methods on the macroscopic visualization of infarcted brain areas for MAP2 immunostaining.

Recently, we showed that the size of infarcted brain area in MCAO rat model is roughly 31% larger in mean kurtosis tensor (MKT) than in the mean diffusivity (MD) using recent fast diffusion kurtosis imaging (DKI) methods. Subsequent histological analysis (hematoxylin) revealed similar lesion volumes to MD. When we compared our results with the size of infarction on MAP2 stained sections, larger MKT infarct lesion indicates that MKT do provide increased sensitivity to microstructural changes in the lesion area compared to MD (Bay et al., 2018).

Accordingly, this study’s primary purpose was to investigate the effect of confounding factors on the quality of macroscopic visualization of focal cerebral ischemia using both HE histology and MAP2 immunostaining. Many studies can only utilize immersion fixation since they require one fragment/hemisphere of the brain for molecular techniques, hindered by perfusion fixation. This study shows that the usage of immersion-fixed MAP2-stained sections and perfusion-fixed MAP2 sections were both reliable and could be used interchangeably depending on the individual experimental setup. Consequently, our study provides novel information on preparing brains for MAP2 immunostaining of sub-acute ischemia suitable for macroscopic infarct size estimation.

2. Methods

2.1. Animal experiment

This study was performed on five adult male Wistar rats (440 ± 70 g). The animals were kept on a 12:12 light-dark cycle with free access to food and water and were housed in pairs at a temperature of 20–22 °C. Rats were subjected to 1 h of focal cerebral ischemia using a transient filament middle cerebral artery occlusion (MCAO) model as previously described (Belayev et al., 1996). Rats were anesthetized with a hypnorm-midazolam mixture (1.8 mg/kg s.c.) and maintained on a 1/3-dose given every 30 min. After 24 h of reperfusion, the animals were divided into two groups based on fixation method: 1) Transectional perfusion fixation (adapted from Gage et al. (Gage et al., 2012)), immersion fixation in 4% formaldehyde which contained methanol as a stabilizer. Before fixation, both groups were euthanized with an overdose of pentobarbital (20%, 2 ml i.p.) under isoflurane anesthesia.

Animal care was carried out under the Danish committee’s guidelines on animal ethics (permission no. 2013–15–2934–00987).

2.2. Tissue processing

Brains were divided into two additional subgroups based on tissue processing methods: 1) cryo-sectioning and 2) paraffin-embedded sectioning. The brains processed for cryo-sectioning were placed in a cryoprotective solution containing 30% sucrose until they were fully submerged. Subsequently, the brains were surrounded by Tissue-Tek O.C.T. compound (Sakura® Finetek, USA) and frozen in isotopane at −40 °C. Parafin blocks were cut into 20 µm thick sections with a fraction of one out of 30 on a microtome (Leica RM2255 Rotary Microtome, Leica Biosystems, UK). The frozen brains were cut into 40 µm thick sections with a fraction of one out of 15 in a cryostat (Microm HM560, Thermo Scientific, Waltham, USA) and stored in De Olmers solution. One set of sections from each brain was HE-stained (as the standard histological approach), and the other one was MAP2-immunostained. In doing so, the difference in infarct visualization between sections suitable for either microscopic (HE) or macroscopic (MAP2) assessment could be evaluated directly.

2.3. Histochemistry and immunohistochemistry

For HE staining, the sections were mounted on gelatin-coated glass slides (Superfrost, 76 × 26 mm, cut edges, Thermo Scientific, Waltham, USA) and stained with hematoxylin (0.1%, Hematoxylin Mayer Sur obtained from the pharmacy of the Capital Regional Hospital, Denmark) for 8 min and eosin for 2 min followed by a dehydration procedure.

For the MAP2 immunostaining, the sections were first rinsed in Tris-buffered saline (TBS-T) (1% BSA, 0.3% Triton-X and TBS) (2 × 10 min) before antigen-retrieval and then were incubated in DAKO buffer (1:9 DAKO to distilled deionized water) for 40 min at 85–90 °C for cryosections and boiled 10 min for paraffin sections. Slides were then rinsed twice (TBS-T 10 min; TBS 5 min) followed by a 15 min block of endogenous peroxidase (8 ml TBS, 1 ml methanol, and 1 ml 30% H2O2) and a 30 min block using milk-solution (0.2% non-fat dry milk) with TBS (10 min) and TBS-T (2 × 10 min) rinses in between. Afterward, sections were incubated overnight in polyclonal rabbit anti-MAP2 (Abcam, Ref# ab32454, Denmark) as a primary antibody (1:500). The next day, sections were rinsed with TBS-T (2 × 15 min), and secondary goat anti-rabbit IgG antibody/HRP (1:200; Dako, Ref# P044801) was added to the sections for 2 h. Subsequently, sections were rinsed in TBS-T (2 × 15 min) followed by immunobellabeing for 2 min using 3,3’-diaminobenzidine (DAB) solution (Kem-En-Tech Diagnostics, Denmark, Ref#4170) and dehydration.

2.4. Delineation of ischemic brain area

The identification and delineation of the ischemic lesion was performed on the MAP2 and corresponding HE-stained sections’ images. The images were captured using the newCAST software (Visiopharm, Horsholm, Denmark) and a Leica light microscope (Leica DM6000 B, Germany) modified for stereology with a digital camera (Leica DFC 295, Germany) and a motorized microscope stage (Ludl MAC 5000, US) with 4 × and 20 × objective lenses. The ischemic area was categorized on three groups based on the clarity of the ischemic area borders. Each group got one score: 1) macroscopic ischemic area with no obvious border 2) ischemic area with unclear border 3) ischemic area with a clear border. Three independent blinded researchers performed macroscopic delineation of the ischemic area based on the image scoring system. The highest score (3) belongs to the section with the clear border of the ischemic area, and the lowest score (1) belongs to the section with no clear border of the ischemic area. A summary of the scoring analysis
Table 1: Macroscopic delineation of the infarcted area by three independent blinded researchers.

| Researcher | Perfusion-fixed | Immersion-fixed |
|------------|----------------|-----------------|
|            | Paraffin | Cryopreserved | Paraffin | Cryopreserved |
|            | HE | MAP2 | HE | MAP2 | HE | MAP2 |
| Researcher 1 | | | | | | | |
| 1 | x | x | x | x | x | x |
| 2 | x | x | x | x | x | x |
| 3 | x | x | x | x | x | x |
| Researcher 2 | | | | | | | |
| 1 | | | | | | | |
| 2 | x | x | x | x | x | x |
| 3 | x | x | x | x | x | x |
| Researcher 3 | | | | | | | |
| 1 | | | | | | | |
| 2 | x | x | x | x | x | x |
| 3 | x | x | x | x | x | x |

1. Ischemic lesion with no obvious border; 2. Ischemic lesion with unclear border; 3. Ischemic lesion with clear border.

Fig. 1. Left: Top: Perfusion-fixed paraffin brain sections from sub-acute rat stroke (MCAO) model. A. HE-stained brain section captured on the light microscope with a 4 × objective lens. Scale bar = 4.5 mm. B. Selected region of the HE-stained brain section with microscopic illustration of the border between infarcted and non-infarcted tissue captured on the light microscope with a 20 × objective lens. Scale bar = 100 µm. C. MAP2-stained brain section captured on the light microscope with a 4 × objective lens. Scale bar = 4.5 mm. D. Selected region of the MAP2-stained brain section with microscopic illustration of the border between infarcted and non-infarcted tissue captured on the light microscope with a 20 × objective lens. Scale bar = 100 µm. Right: Top: Perfusion-fixed cryopreserved brain sections from sub-acute rat stroke (MCAO) model. A. HE-stained brain section captured on the light microscope with a 4 × objective lens. Scale bar = 4.5 mm. B. Selected region of the HE-stained brain section with microscopic illustration of the border between infarcted and non-infarcted tissue captured on the light microscope with a 20 × objective lens. Scale bar = 100 µm. C. MAP2-stained brain section captured on the light microscope with a 4 × objective lens. Scale bar = 4.5 mm. D. Selected region of the MAP2-stained brain section with microscopic illustration of the border between infarcted and non-infarcted tissue captured on the light microscope with a 20 × objective lens. Scale bar = 100 µm. Right: Bottom: Immersion-fixed paraffin brain sections from the sub-acute rat stroke (MCAO) model. A. HE-stained brain section captured on the light microscope with a 4 × objective lens. Scale bar = 4.5 mm. D. Selected region of the HE-stained brain section with microscopic illustration of the border between infarcted and non-infarcted tissue captured on the light microscope with a 20 × objective lens. Scale bar = 100 µm. Right: Bottom: Immersion-fixed cryopreserved brain sections from the sub-acute rat stroke (MCAO) model. A. HE-stained brain section captured on the light microscope with a 4 × objective lens. Scale bar = 4.5 mm. D. Selected region of the HE-stained brain section with microscopic illustration of the border between infarcted and non-infarcted tissue captured on the light microscope with a 20 × objective lens. Scale bar = 100 µm. C. MAP2-stained brain section captured on the light microscope with a 4 × objective lens. Scale bar = 4.5 mm. D. Selected region of the MAP2-stained brain section with microscopic illustration of the border between infarcted and non-infarcted tissue captured on the light microscope with a 20 × objective lens. Scale bar = 100 µm.
of one image per condition is included in Table 1.

3. Results

3.1. Perfusion-fixed brains

In the current study, results from the perfusion-fixed brains depicted defined ischemic areas on the MAP2-stained sections for cryo-preserved and paraffin-embedded tissue. However, the cryo-preserved tissue was superior to the paraffin-embedded tissue (Fig. 1). Comparing the results of macroscopic visualization of cerebral ischemia between HE- and MAP2-stained cryo-sections suggested that MAP2 immunostaining provides superior infarct visualization with a clear border compared to the HE staining, which depicted either no obvious or an unclear border of the infarcted cerebral area (Fig. 1 & Table 1).

3.2. Immersion-fixed brains

The macroscopic investigation of focal cerebral ischemia on both MAP2-stained paraffin-embedded and cryo-preserved sections revealed an ischemic area with a reduction in MAP2 immunostaining and a distinct border (Fig. 1). The HE-stained sections also depicted visible ischemic areas on both paraffin-embedded and cryo-preserved sections. However, these had a less distinct and more blurred border of the ischemic area than MAP2 stained sections (Fig. 1 Table 1).

3.3. Image scoring system

The results by three independent blinded researchers showed that MAP2-stained perfused-fixed cryo-sections and immersion-fixed cryo-preserved brain sections were the most reliable stained sections for the macroscopic delineation of the infarcted brain regions by getting the highest score (9 scores in total). Conversely, the HE-stained paraffin section from the perfused-fixed brain got the lowest score (4 scores in total) (Table 1). Importantly, to test internal consistency (“reliability”) this measurement, we calculated Cronbach’s alpha which was 0.521, which indicates a high level of internal consistency for our scale with this specific staining.

4. Discussion

This study’s findings show well-defined MAP2-immunostained infarcts for macroscopic visualization of sub-acute focal cerebral ischemia following 24 h of reperfusion regardless of the fixation method.

The experimental findings on cryo-embedded sections from perfused brains showed a well-defined focal ischemic area with clear borders using MAP2 immunostaining. In contrast, the HE-stained infarct either had no obvious border or unclear borders, making it unreliable for macroscopic assessment. Indeed, the MAP2 immunostaining facilitated macroscopic infarct visualization across several tissue processing varieties and will therefore not be restricted by different preferences or restrictions in laboratory procedures. Macroscopic evaluation enables the use of several other volume estimation methods, giving each researcher the ability to use their preferred method.

Lesion size in rodent models of focal cerebral ischemia may reach its maximum level within hours. Accordingly, it is necessary to perform staining methods to visualize and delineate the ischemic brain region at correspondingly early time points. Thus, the main point of selecting MAP2 immunostaining was to introduce a reliable marker of infarcted area with post-occlusion survival periods of 24 h or earlier time points by considering different fixation and tissue processing methods. MAP2 has previously been suggested as an indicator of acute focal cerebral ischemia with lower intensity of staining 1 h following MCAO with the precisely well-defined border of the lesion (Dawson and Hallenbeck, 1996; Popp et al., 2009). However, here we confirm that MAP2 can indeed be used as an indicator for macroscopic visualization of the ischemic area on both perfusion- and immersion-fixed sub-acute focal cerebral ischemia brains 24 h after occlusion.

Even though several different tissue-processing methods have been utilized for HE and MAP2 immunostaining, only one occlusion and reperfusion time has been investigated. Thus, we cannot for sure conclude that MAP2 immunostaining is suitable across all experimental stroke settings since the lengths of occlusion and reperfusion will significantly impact the tissue morphology. However, Popp et al. (Popp et al., 2009) investigated the MAP2 staining using permanent, 30 and 120 min of occlusion with 2 h, 1 day, 7 and 30 days of reperfusion, whereas Dawson and Hallenbeck (Dawson and Hallenbeck, 1996) investigated the usability of MAP2 in permanent MCAOs of 1, 2 and 4 h of duration. Both studies found that MAP2 was a reliable staining method for all investigated occlusion and reperfusion time points. So overall, the studies point to MAP2 stains as being reliable at different occlusion and reperfusion times.

It is well-known that HE stains all types of cells, including neurons and glial cells, without distinguishing between them. However, the water content of these cells is not equal (Tshii et al., 1999). Therefore, possible explanations for the apparent ambiguity of focal cerebral ischemia areas on HE sections could be the decline in tissue water content following perfusion, especially the unpredictable tissue deformation in the x-, y-, and z-axes in paraffin-embedded sections (Dorph-Petersen et al., 2001). Accordingly, if the infarct is small, HE staining could lead to indistinct infarcts on paraffin-embedded sections at the macroscopic level, thereby requiring a thorough and time-consuming investigation at a microscopic level. Further, it is suggested that a large amount of neuronal death occurs acutely following the ischemic intervention, while the alteration in glial plasticity happens gradually (Kroemer et al., 2009).

In contrast, MAP2 immunostaining targets mainly neurons and neuronal dendrites (Dawson and Hallenbeck, 1996), resulting in fewer confounds and a correspondingly more well-defined infarct border. Additionally, the usage of histological methods compared to, e.g., TTC staining provides the opportunity for both in vivo and, more interestingly, ex vivo MRI scanning. By ex vivo scanning, the fixed brains can be compared directly between methods since the tissue morphology remains similar and is not affected by structural changes due to post-fixation after in vivo scanning (Bay et al., 2018).

5. Conclusion

Macroscopic visualization and delineation of sub-acute cerebral ischemia to measure ischemic tissue size depends on proper fixation, embedding, and staining methods. In the current study, we have showed that MAP2 immunostaining using a combination of tissue processing methods can be used for accurate macroscopic estimation of an ischemic area. Our results provide evidence for MAP2 immunostaining as a gold standard for macroscopic visualization of sub-acute focal cerebral ischemia. Thus, it is a reliable technique for macroscopic estimation of ischemic region size.

Ethics approval and consent to participate

Animal care was carried out under the Danish committee’s guidelines on animal ethics (permission no. 2013-15–2934–00987).

Conflict of Interest

none declared.

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References

Atochin, D.N., Chernysheva, G.A., Alliev, O.I., Smolynkova, V.I., Otsipenko, A.N., Logvinov, S.V., Zhdanikina, A.A., Plonnikova, T.M., Plonnikov, M.B., 2017. An improved three- vessel occlusion model of global cerebral ischemia in rats. Brain Res. Bull 132, 213–221.

Bay, V., Kjolby, B.F., Iversen, N.K., Mikkelsen, I.K., Ardalan, M., Nyengaard, J.R., Jespersen, S.N., Dransbek, K.R., Ostergaard, L., Hansen, B., 2018. Stroke infarct volume estimation in fixed tissue: comparison of diffusion kurtosis imaging to diffusion weighted imaging and histology in a rodent MCAO model. PLoS One 13, e0196161.

Bederson, J.B., Pitts, L.H., Germaino, S.M., Nishimura, M.C., Davis, R.L., Bartkowiak, H.M., 1986. Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. Stroke J. Cereb. Circ. 17, 1304–1308.

Belavy, L., Alonso, O.F., Busto, R., Zhao, W., Ginsberg, M.D., 1996. Middle cerebral artery occlusion in the rat by intraluminal suture. Neurological and pathological evaluation of an improved model. Stroke J. Cereb. Circ. 27, 1616–1622.

Chen, C., Cui, H., Li, Z., Wang, R., Zhou, C., 2013. Normobaric oxygen for cerebral ischemic injury. Neural Regen. Res. 8, 2885–2894.

Choi, C.H., Yi, K.S., Lee, S.R., Lee, Y., Jeon, C.Y., Hwang, J., Lee, C., Choi, S.S., Lee, H.J., Cha, S.H., 2017. A novel voxel-wise lesion segmentation technique on 3.0-T diffusion MRI of hyperacute focal cerebral ischemia at 1h after permanent MCAO in rats. J. Cereb. Blood Flow Metab. 38, 1371–1384.

Dawson, D.A., Hallenbeck, J.M., 1996. Acute focal ischemia-induced alterations in MAP2 immunostaining: description of temporal changes and utilization as a marker for volumetric assessment of acute brain injury. J. Cereb. Blood Flow Metab.: Official J. Intern. Soc. Cereb. Blood Flow Metab. 16, 170–174.

de Leon, M.J., Conrve, A., George, A.E., Golomb, J., de Santi, S., Tarshish, C., Rusiniek, H., Bobinski, M., Incce, C., Miller, D., Winniewski, H., 1996. In vivo structural studies of the hippocampus in normal aging and in incipient Alzheimer’s disease. Ann. N. Y. Acad. Sci. 777, 1–13.

Diemer, N.H., 1982. Quantitative morphological studies of neuropathological changes. Part I. Crit. Rev. Toxicol. 10, 215–263.

Dorph-Petersen, K.A., Nyengaard, J.R., Gundersen, H.J., 2001. Tissue shrinkage and unbiased stereological estimation of particle number and size. J. Microsc. 204, 232–246.

Gage, G.J., Kipke, D.R., Shain, W., 2012. Whole animal perfusion fixation for rodents. J. Visualized Exp.: JoVE.

García-Quezada, M.A., John, Y.J., Barbas, H., Zikopoulos, B., 2016. Distinction of neurons, glia and endothelial cells in the cerebral cortex: an algorithm based on cytological features. Front Neuroanat. 10, 107.

Gavin, C.M., Smith, C.J., Emsley, H.C., Hughes, D.G., Turnbull, I.W., Vail, A., Tyrrell, P.J., 2004. Reliability of a semi-automated technique of cerebral infarct volume measurement with CT. Cerebrovasc. Dis. 18, 220–226.

Ishii, H., Arai, T., Morikawa, S., Inubushi, T., Toyama, I., Kimura, H., Mori, K., 1998. Evaluation of focal cerebral ischemia in rats by magnetic resonance imaging and immunohistochemical analyses. J. Cereb. Blood Flow Metab.: Official J. Intern. Soc. Cereb. Blood Flow Metab. 18, 931–934.

Kitagawa, K., Matsumoto, M., Niinobe, M., Mikoshiba, K., Hara, R., Ueda, H., HANDa, N., Fukunaga, R., Isaka, Y., kimura, K., 1989. Microtubule-associated protein 2 as a sensitive marker for cerebral ischemic damage–immunohistochemical investigation of dendritic damage. Neuroscience 31, 401–411.

Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E.S., Barberee, E.H., Blagosklonny, M.V., El-Deiry, W.S., Golstein, P., Green, D.R., Hengartner, M., Knight, R.A., Kumar, S., Lipton, S.A., Maloni, W., Nunez, G., Peter, M.E., Tschopp, J., Yuan, J., Piacentini, M., Zhivotovsky, B., Melino, G., 2009. Classification of cell death: recommendations of the nomenclature committee on cell death 2009. Cell Death Differ. 16, 3–11.

Lin, J.W., Li, L., Freeling, J.J., Martin, D.S., Wang, H., 2017. USP14 inhibitor attenuates cerebral ischemia/reperfusion-induced neuronal injury in mice. J. Neurochem. 140, 826–833.

Mikkelsen, I.K., Diemmer, N.H., 1992. Reduction of infarct volume and mortality by thrombolysis in a rat embolic stroke model. Stroke; A J. Cereb. Circ. 23, 1167–1173.

Popp, A., Jamiesch, N., Witte, O.W., Frahm, C., 2009. Identification of ischemic regions in a rat model of stroke. PLoS One 4, e4764.

Pradillo, J.M., Murray, K.N., Coutts, G.A., Moraga, A., Oroz-Conjar, F., Bourn, H., MORo, M., Lizasoain, I., Rothwell, N.J., Allan, S.M., 2017. Reparative effects of interleukin-1 receptor antagonist in young and aged/co-morbid rodents after cerebral ischemia. Brain Behav. Immun. 61, 117–126.

Sim, J., Jo, A., Kang, B.M., Lee, S., Bang, O.Y., Heo, C., Jhon, G.J., Lee, Y., Suh, M., 2016. Cerebral hemodynamics and vascular reactivity in mild and severe ischemic rodent middle cerebral artery occlusion stroke models. Exp. Neurol. 253, 130–138.

Venkataraman, A., Kingsley, P.B., Kalina, P., Pavlakis, S.G., Buckwald, S., Spinazzola, R., Harper, R.G., 2004. newborn brain infarction: clinical aspects and magnetic resonance imaging. CNS Spectr. 9, 436–444.

Watson, C., Jack Jr., C.R., Genders, F., 1997. Volumetric magnetic resonance imaging. Clinical applications and contributions to the understanding of temporal lobe epilepsy. Arch. Neuro. 54, 1521–1531.

Zille, M., Fart, T.D., Przedszczig, I., Muller, J., Sommer, C., Dirmagl, U., Wunder, A., 2012. Visualizing cell death in experimental focal cerebral ischemia: promises, problems, and perspectives. J. Cereb. Blood Flow Metab.: Official J. Intern. Soc. Cereb. Blood Flow Metab. 32, 213–231.