Histological quantification of brain tissue inflammatory cell infiltration after focal cerebral infarction: a systematic review

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Acquired brain injury such as ischemic stroke causes intense inflammatory cell infiltration in and around the injured brain tissue [1]. This infiltration starts within hours, lasts days to weeks, and appears to contribute to cell loss during both early and late phases [1]. Cerebral ischemia activates various cell types, including microglia, macrophages, neutrophils, lymphocytes, and endothelial cells, which secrete many inflammatory factors, including interleukin-1β and tumor necrosis factor-α, which promote the inflammatory response in the brain [1]. Reliable quantification of infiltration of these inflammatory cells after cerebral infarction is needed to determine if experimental treatments affect this important tissue outcome. Although flow cytometry is a common and reliable method for identifying infiltrated leukocytes, we chose to study histological methods. Histological methods allow tissue to remain intact and the location of inflammatory cells in relation to the infarct to be analyzed. We therefore sought to determine the available evidence comparing histological methods for quantification of brain tissue inflammatory cell infiltration after cerebral infarction.

Materials and methods

We searched PubMed (NLM) and World of Knowledge (Science Citation Index) in November 2012 with the search terms: histolog* AND (stroke OR cerebral ischemi* OR cerebral infarct*) AND (inflammat* OR leukocyte OR microglia OR macrophage OR neutrophil OR lymphocyte). We chose these search terms after conducting preliminary searches to explore the literature. We focused on the most prominent inflammatory cell types on which our prior studies have focused. We included full-text articles in English published prior to November 2012 of unique experimental data that described a histological method to quantify inflammatory cells in the brain tissue after cerebral infarction. We excluded abstracts and articles that cited methods described in previous publications if no modifications were described. Titles, abstracts, or full articles were reviewed to determine if each search result matched our selection criteria.
Results

The search returned 686 results, of which 10 matched our selection criteria (Table 1). All of the included studies evaluated brain sections of rodent models of focal cerebral infarction by multiple methods, including the use of endothelin-1 (ET1) and middle cerebral artery occlusion (MCAO). The studies used hematoxylin and eosin staining or immunohistochemistry for markers of cells known to be involved in brain tissue inflammation.

We found variability in the species evaluated, the timing of histology following cerebral infarction, the reported section thickness used, the section interval evaluated, the number of sections evaluated, the exact tissue location evaluated relative to the ischemic area or anatomic landmarks, the magnification and number of areas evaluated per brain section, and other specifics of the quantification methods used. We found no studies directly comparing any of the described methods.

Table 1. Summary of review results comparing the histological preparation and quantification methods for brain tissue inflammatory cell infiltration after focal cerebral infarction.

| Study Details | Histological Preparation | Quantification Methods |
|---------------|---------------------------|------------------------|
| Chen et al. 1994 | Brains sectioned into seven coronal sections of 2 mm | Polymorphonuclear (PMN) leukocytes counted in bregma 0.8 mm section, light microscopy |
| Histology 46 h after focal cerebral infarction by MCAO in rats [2] | Unstated number and interval of 6 μm thick brain sections cut from each block | PMNs identified by nuclear morphology, counted, averaged in six fields at 100× magnification in “lesion area...selected randomly in the cortex and the basal ganglion” |
| Zhang et al. 1994 | 6 μm thick coronal brain section at approximately bregma 0.8 mm, centered in ischemic lesion | Neutrophils identified by nuclear features |
| Histology at 6, 12, 24, 48, 72, 96, 168 h after focal cerebral infarction by MCAO in rats [3] | H&E staining | Counted and averaged in 12 fields at 100× magnification “chosen randomly” in cortical, striatal, and preoptic areas of the lesion |
| Weston et al. 2007 | “Approximately 10” 16 μm sections prepared from six 2 mm coronal slices | Immunoreactive (IR) cells counted with software tool in 20× fields |
| Histology at 0, 1, 2, 3, 7, 15 days after ET1-induced focal cerebral infarction in rats [4] | Number and interval of sections unstated, but noted six distances from bregma | Data presented for both hemispheres, cortex, and striatum |
| | Immunostaining for macrophages (anti-ED1 antibody) and neutrophils (PMN neutrophil antisera) | Not stated if all areas were analyzed on each section or if varied by section |
| Veldhuis et al. 2003 | Unstated number and interval of 8 μm sections at center (bregma 0.7 mm) and caudal part (bregma - 1.8 mm) of infarct | Neutrophil number and PMN IR cells colocalized with ED1 IR each calculated by integrating PMN IR cells over six fixed stereotaxic levels |
| Histology at 24 h after focal cerebral infarction by MCAO in rats [5] | Immunostaining for leukocytes (CD45), neutrophils (HIS48), macrophages (ED1) | Leukocyte and monocyte data collected from unstated areas of center and caudal parts of MCA territory with AnalySIS software |
| Nakagawa et al. 2006 | Prepared unstated number and interval of 10 μm coronal sections | Neutrophil data collected from cortical and striatal regions in center and caudal sections of both hemispheres, indicated via illustration, reported as IR cells |
| Histology at 48 h after focal cerebral infarction by MCAO in rats [6] | Immunostaining for microglia/macrophages (CD11b) | Pixels in photographs divided into hue, saturation, and intensity, and thresholds set only for IHC-positive areas |

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### Table 1. Summary of review results comparing the histological preparation and quantification methods for brain tissue inflammatory cell infiltration after focal cerebral infarction. (Continued)

| Study Details                                      | Histological Preparation | Quantification Methods |
|----------------------------------------------------|---------------------------|------------------------|
| - Bizzoco et al. 2007                              | Unstated number of 30 \( \mu m \) sections prepared between bregma \(-0.10 \) and \(-0.60 \) mm | Analyzed two adjacent slices per brain |
| - Histology at 72 and 144 h. after MCAO focal cerebral infarction in rats [7] | Immunostaining for microglia (CD11b) | Cells counted from the somatosensory cortex to the border of the agranular insular cortex |
|                                                    |                           | Labeled cells counted in \( 20 \times \) fields of 0.3 mm\(^2\) with AxioCision 4 software |
|                                                    |                           | Mean labeled cell numbers reported for striatum and fronto-parietal cortex in ipsilateral hemisphere |
|                                                    |                           | Striatal microglia counted in 10 fields next to corpus callosum and capsule externa and 3 medial striatal areas |
|                                                    |                           | Counted IR microglia in six parietal cortex fields, nine in frontoparietal cortex, three in frontal cortex; areas illustrated |
|                                                    |                           | Counted Iba-1-positive cells |
|                                                    |                           | Cells counted in ipsilateral and contralateral cortex and striatum |
|                                                    |                           | No details provided on counting method or magnification |
|                                                    |                           | Average Iba-1-positive cell count “in randomly selected four sections” presented as cells per 0.02 mm\(^2\) |
|                                                    |                           | Labeled cells counted, but did not state location relative to ischemic area or anatomic landmarks, magnification, number of areas evaluated per section, or counting method |
|                                                    |                           | Iba-1 optical densitometry measured with Image J software |
|                                                    |                           | Images taken in unspecified regions in ipsilateral striatum and somatosensory cortex at an unstated magnification |
|                                                    |                           | Labeled cells counted in cortex “between the lines crossing the center of the anterior commissures and the top of the striatum,” area traced with Image J, cell data shown as cells/mm\(^2\) |
|                                                    |                           | Density of CD68- and Iba-1-cells measured in “3 coronal sections from each rat” between 0.9 and 0.2 mm anterior to bregma” in striatum and cortex |
|                                                    |                           | Labeled cells counted in same three 1 mm\(^2\) areas of ipsilateral striatum (shown by illustration) on each section using a counting grid and Image J software |
|                                                    |                           | Labeled cells counted in cortex “between the lines crossing the center of the anterior commissures and the top of the striatum,” area traced with Image J, cell data shown as cells/mm\(^2\) |

In 1994, Chen et al. reported histology at 46 h after focal cerebral infarction by MCAO in rats. Each brain was sectioned into seven coronal sections of 2 mm [2]. An unstated number and interval of 6 \( \mu m \) thick brain sections were cut from each block and stained with hematoxylin and eosin. One section (bregma 0.8 mm) was used to count polymorphonuclear (PMN) leukocytes using a light microscope. PMNs were identified by nuclear morphology and counted and averaged in six fields at 100\( \times \) magnification “within the lesion.
area...selected randomly in the cortex and the basal ganglion”.

A 1994 study by Zhang et al. reported histology data at 6, 12, 24, 48, 72, 96, and 168 h after focal cerebral infarction by MCAO in rats [3]. A single 6 μm thick coronal brain section at approximately bregma 0.8 mm, which the authors stated was centered in the ischemic lesion, was stained with hematoxylin and eosin. Neutrophils, identified by their nuclear features, were counted and averaged in 12 fields at 100× magnification “chosen randomly” in the cortical, striatal, and preoptic areas of the lesion.

A 2007 study by Weston et al. reported histology at days 0, 1, 2, 3, 7, and 15 days after ET1-induced focal cerebral infarction in rats [4]. Six coronal slices 2 mm in thickness were prepared. “Approximately 10” sections of 16 μm thickness were then immunostained from each of the 2 mm slices. The number of sections and the interval between sections used for analysis was not stated, although data were presented for six distances from bregma. Sections were immunostained with an anti-ED1 antibody for macrophages and a polymorphonuclear neutrophil (PMN) antisera for neutrophils. Immunoreactive (IR) cells were counted with a software tool in “a 20× field for each brain section,” with data presented for both hemispheres and both the cortex and striatum. It was not stated if all these areas were analyzed on each section or if that varied by section. “The total number of neutrophils in each brain was calculated by integrating the PMN IR cells counted in the six predetermined stereotaxic levels and the distance between each of the levels according to the method of Osborne et al. (1987). The total number of PMN IR cells that were also colocalized with ED1 IR cells were counted in a similar manner.”

In 2003, Veldhuis et al. reported histology at 24 h after focal cerebral infarction by MCAO in rats [5]. An unstated number and interval of 8 μm thick sections were prepared at the center (bregma 0.7 mm) and caudal part (bregma 1.8 mm) of the infarct. Sections were immunostained for leukocytes (CD45), neutrophils (HIS48), and macrophages (ED1). Sections were analyzed with AnalySIS software. Leukocyte and monocyte data were collected from unstated areas of the center and caudal parts of the MCA territory. Neutrophil data were collected from cortical and striatal regions in the center and caudal sections from both hemispheres that were not specified in the text but were indicated on an illustration. The authors stated “Each pixel in the photographs was divided into three color components (hue, saturation, and intensity). [A constant] threshold for these three color components was defined in such a manner that only immunohistochemical-positive areas were selected for analysis...The number of pixels in the immunopositive area was used and expressed in arbitrary units.” Leukocyte and monocyte data were presented as “nr of cells,” though the authors provided no explicit description of which data was used for quantification. Neutrophil data was presented as number of IR cells.

In a 2006 study by Nakagawa et al., histology data was reported at 48 h after focal cerebral infarction by MCAO in rats [6]. An unstated number and interval of coronal sections 10 μm thick were immunostaining for microglia/macrophages (CD11b). Labeled cells were counted “in the penumbra, the contralateral area, and the striatum (0.06 mm², respectively)...in three areas randomly selected in three sections per animal.” The authors did not further describe the location or magnification used for cell counting.

A study by Bizzoco et al. in 2007 reported histology data 72 and 144 h after MCAO focal cerebral infarction in rats [7]. An unstated number of sections 30 μm thick between bregma −0.10 and −0.60 mm were immunostained for microglia (CD11b). The authors stated that quantitative “analysis was performed on two adjacent sections taken from each rat brain,” and “The region of the cortical mantle examined comprised the infarct area extending from the somatosensory cortex to the border of the agranular insular cortex.” Labeled cells were counted in 20× fields of 0.3 mm² using AxioCision 4 software. Mean labeled cell numbers were reported for the striatum and fronto-parietal cortex in the ipsilateral hemisphere. Striatal microglia were counted in 13 fields, 10 adjacent to the corpus callosum, and capsula externa and 3 medial striatal areas. For data collection in the fronto-parietal cortex, IR microglia were counted in nine fields, three in the frontal cortex, and six in the parietal cortex; the counting areas were illustrated.

A 2009 study by Baba et al. reported histology data at 1 week after focal cerebral infarction by MCAO in rats [8]. An unstated interval and number of 14 μm thick coronal sections were prepared. The locations of the prepared sections relative to the infarct or other anatomical landmarks were not stated. Sections were immunostained for microglia (Iba-1). The authors stated that “The number of Iba-1+...positive cells was counted to evaluate glial reaction” but did not say how or where or at what magnification. “The average of cell numbers in randomly selected four sections” for Iba-1-positive cells was presented as cells per 0.02 mm² in the ipsilateral and contralateral cortex and striatum.

In 2011, Patkar et al. presented histology data at 28 days after focal cerebral infarction by MCAO in mice [9]. An unstated number of 20 μm thick coronal sections were immunostained for microglia (Iba-1) from three randomly chosen brains. Labeled cells were counted, but the exact tissue location evaluated relative to the ischemic area or anatomic landmarks, the magnification and number of areas evaluated per brain...
section, and other specifics of the counting method was not stated. Optical densitometry was also performed for Iba-1, “Average intensity in the region of interest per area of field of view for each marker was also measured...using Image J software.” Images were taken at an unstated magnification in unspecified regions of interest in the ipsilateral striatum and somatosensory cortex.

In 2012, Bates et al. reported histology at 8 days after focal cerebral infarction by MCAO in rats [10]. An unspecified number and interval of 20 μm thick sections were immunostained for macrophages (ED1) and microglia (Iba1). Optical densitometry was performed with Photoshop, “Three to four sections were analyzed from each animal and a polygonal outline was drawn around the core region of the infarct area.” No information was provided describing the location of the sections. The mean pixel intensity within each region for that marker was averaged per animal.

A 2012 study by De Geyter et al. reported histology data at 24 and 72 h following ET1-induced focal cerebral infarction in rats [11]. An unspecified number and interval of 50 μm sections were prepared for immunohistochemistry. Sections were immunostained for microglia and macrophages (CD68 and Iba-1). The authors stated “The density of CD68- or Iba-1-stained cells in the striatum and cortex was calculated in three coronal sections from each rat, located between 0.9 mm and 0.2 mm anterior to bregma.” Labeled cells were counted in the same three areas, 1 mm² in size, of the ipsilateral striatum (shown by illustration) on each section using a counting grid and Image J software. Labeled cells were also counted in the cortex “between the lines crossing the center of the anterior commissures and the top of the striatum.” The area of these regions was traced with Image J, and labeled cell counts were presented as cells/mm².

We found no studies directly comparing any of the described methods.

Conclusion

Accurate histological quantification of brain tissue inflammatory cell infiltration after focal cerebral infarction is necessary for a reliable assessment of the interaction of this tissue response to neuroprotective and neurorestorative treatments. Our review found reports of multiple histological techniques to quantify post-infarction inflammation, but wide variability in the methods used, and no direct comparison studies. Most of the articles matching our selection criteria contained a method description that presented insufficient details for replication. Most of the described methods also contained one or more manual steps requiring evaluator judgement, such as deciding the location to count cells based on visual inspection or freely tracing infarct area. These steps most likely increase variability of results, decreasing the power of the study to find statistically significant differences, and trustworthy data.

Systematic and automatic quantification of inflammatory cell infiltration appears to be most effective in removing evaluator bias and producing reproducible results. These automatic methods commonly described use of imaging software which created a threshold level for identifying cell labeling, as in optical densitometry. Furthermore, developing a detailed image acquisition process to compare inflammatory cell activation seems to be integral to providing reliable data. “Random sampling” techniques should be replaced by outlined and diagrammed instructions indicating analyzed sections, number of sections, and number and locations of sampling areas.

To our knowledge, this is the first systematic review on this question, but our review has limitations. These methods are usually discussed in the context of larger overall studies, and therefore many studies using novel methods would not be indexed in a manner amenable to database searching. Additional applicable methods likely exist outside of the applied search terms as detailed in the methods. However, our search strategy is replicable and emphasizes the need for quantifiable histological methods for inflammation. This systematic review succeeds in clearly outlining search terms and methods to create a reproducible study with minimized bias. Our study can be further expanded upon in the future by exploring studies with additional inflammatory cell types and histological methods to incorporate newly described methods. Further systematic reviews may also include analysis on the most effective stereotactic methods and imaging software, information lacking in all of the described studies.

We conclude that the reported methods all seem reasonable, the technique description is often insufficiently detailed for replication, the lack of comparative data makes statements about the superiority of any particular method impossible, and further research is needed to optimize the analysis of this important experimental outcome. Further research should be conducted in which the aforementioned methods are conducted simultaneously. The methods could then be directly compared to determine which produces the most accurate, clear, efficient, and easily reproducible results.

Acknowledgements

We appreciate the editorial advice of Laura Cohen, Lindsey Jager, Rajeev Krishnaney-Davison, and Wai Yin Leung.
Declaration of Interest

We have no conflicts of interest to report. The authors alone are responsible for the content and writing of this paper.

Funding: NIH grant UL1TR000427.

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