Histologic Assessment of Neurons in Rat Models of Cerebral Ischemia

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We describe a method for typing neurons into four progressive stages of ischemic deterioration based on visual characterization of the nucleus in terms of its optical contrast, delineation along the nuclear–cytoplasmic interface, and its shape. Difficulty in assessing nuclear shape required the introduction of an angularity comparator chart to improve the investigator's accuracy. Three investigators typed neurons obtained from normal, ischemic, and ischemic-reperfused rat brains. Accuracy and reproducibility of the investigators' typing decisions with and without the angularity comparator charts were evaluated. The accuracy of subjective shape assessment was compared with objective digitizer measurements of the same. The angularity comparator charts reduced subjective shape classification error by two thirds, and group error (overall performance expressed by the coefficient of variance) decreased from 15.9% to 4.7% for Type I (normal cells), from 33.9% to 17.3% for Type II (cells with angular nuclei), from 15.5% to 14.1% for Type III (cells with smeared nuclei), and from 3.2% to 5.5% for Type IV (dead cells). Thus, Type I and IV neurons can be assessed at a higher reproducibility than the intermediate Types II and III. Our typing method can also be used to evaluate the effect of treatment regimes on ischemic neuronal damage. (Stroke 1990;21:299–304)

The last two or three decades have witnessed significant advances in our basic understanding of the pathophysiology of cerebrovascular ischemic disease. Fundamental mechanisms contributing to ischemic neuronal damage have been elucidated. The most secure strides in scientific progress have generally occurred in those fields insisting on strict quantification. Despite the advent of exciting technologic advances that permit in vivo imaging of metabolic derangements associated with brain ischemia, it is likely for the foreseeable future that light microscopy will remain the most practical, reliable, and definitive laboratory tool for assessing the degree of ischemic tissue damage. However, shortcomings do exist in the histologic assessment of ischemic brain damage. Experimental investigations of neuronal grading schemes to date, virtually without exception, have certain weaknesses in common. To our knowledge, no cell typing scheme has yet been examined critically or documented fully for user reliability, accuracy, and internal redundancy. Until such an evaluation is performed, any proposed taxonomy of ischemic neuronal injury must be viewed as suspect and arbitrary. It is the burden of each research group using an ischemic grading scale to establish its scientific rationale and to prove the discriminative capabilities of its choices.

A human observer perceives many distinct features of cellular pathology under oil-immersion light microscopy in 1-μm-thick plastic sections. Only a small fraction of these features are pertinent to ischemic cellular decay and can be detected at an acceptable level of confidence. These features are increased cytoplasmic density, smearing of the nuclear–cytoplasmic boundary, and total disintegration of the nucleus.1 We have also found that subtle changes in the shape of the nucleus are reliable early signs of ischemic cell pathology.

In an earlier study,1 it was demonstrated that the nuclear features show early and consistent ischemic changes that can be followed through to complete cell deterioration. Since the nucleus has not only a prominent, central location within the cell that favors visualization but also a central role in governing vital cell processes, it is reasonable to assume that monitoring the deterioration of the nucleus is a reliable way of assessing the degree of overall cellular injury during ischemia. Hence the results of our earlier study have been formalized into a few neuronal injury definitions based on coherent visual criteria so that investigators...
**Materials and Methods**

Samples of cortical brain tissue were obtained from Sprague-Dawley rats that had been subjected to either severe partial cerebral ischemia or cerebral ischemia followed by reperfusion in a combined occlusive and hemorrhagic model. Rats in the first group were maintained under severe partial ischemic conditions for 35 minutes; the rats were then killed by in vivo perfusion-fixation. Rats in the second group were subjected to equivalent severe partial ischemia for 35 minutes, followed by release of the carotid ligatures and reperfusion of the brain for 30 minutes before perfusion-fixation. Full experimental details and a description of the processing of histologic specimens for light microscopy is given elsewhere.¹

The nucleus of an ischemic neuron deteriorates in stages that can be readily assessed by a human observer at ×1,000 magnification in toluidine blue-stained 1-μm plastic sections.¹ These stages can be characterized in terms of optical contrast (high, low, none), delineation of the nuclear–cytoplasmic interface, and shape of the nuclear envelope. Figure 1 illustrates the visual characteristics of the nucleus that we used to designate the stages of ischemic decay. The four types correspond to four progressive stages of neuronal deterioration.

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**Figure 1.** Deterioration of neuronal nucleus through visually defined sequential stages of cerebral ischemia. Stages are defined by contrast loss, shape distortion, and membrane smearing. Type I neurons are normal; Type IV neurons are the most injured.

Type I and II neurons contain sharply delineated nuclei that can be readily differentiated from the surrounding cytoplasm. In toluidine blue-stained 1-μm sections, the nucleus stains lighter than the surrounding cytoplasm and stands out with high contrast. When the nucleus is sectioned through its equator, the lighter nuclear and darker cytoplasmic compartments are sharply separated (Figure 1, high-contrast Type Ia and Ib cells). Cuts tangential to the nucleus smear the nuclear membrane, resulting in neurons with less clearly delineated nuclear–cytoplasmic interfaces, while high-contrast discrimination between the nuclear and cytoplasmic compartments is maintained (Type Ia and Ib cells). In thinner sections (approximately 0.5 μm), nuclei do not stain with high contrast but do have well-delineated nuclear compartments (Type Ic and Id cells). The shape of the nucleus distinguishes normal Type I from abnormally angular Type II cells.

Type III neurons show low nuclear–cytoplasmic contrast and have a smeared nuclear–cytoplasmic border; their nuclei can be either nonangular (Type IIIa) or angular (Type IIIb). Type III encompasses a wide range of neuronal alterations and includes all neurons with a visible nucleus that cannot be classified as Type I or II.

Type IV neurons do not have a recognizable nucleus. These cells may be of either low- (Type IV, a or b) or high-density staining (Type IV, c or d) and
may contain nuclear remnants (Type IV, a or b), typically a surviving nucleolus.

An angularity comparator chart was also used to aid the investigator in subjectively distinguishing normal nonangular nuclear shapes (Type I, a, b, or c) from angular changes of the nuclei (Type II, a, b, or c). A comparator chart presents an array of images sorted by absolute measurements of a feature (here, the shape parameter) and its associated binary decision (here, angular/nonangular). The feature is assessed by an absolute method and examples are organized and quantified in sequence on a chart in which the target cut-off point (here, 0.89) is shown (Figure 2). A second (abstract) angularity comparator chart illustrating three geometric transformations of a circle—that is, the normal shape of a nucleus—with their corresponding shape parameters is given in Figure 3.

Digitized data from nuclear envelopes was processed to give objective measures of the shape parameter of nuclei as described elsewhere.1

Three investigators repeatedly evaluated 183 neurons from a projection slide database under the same viewing conditions. In study 1, pictures of two reference neurons with shape parameter values on either side of the cut-off point were provided to the three investigators. All investigators repeated the typing six times. In study 2, the typing was repeated five more times to determine the impact of the angularity comparator charts shown in Figures 2 and 3 on the reliability and accuracy of the investigators’ typing.

Percentages of neuronal types obtained in each typing session were determined. Mean and standard deviations were then calculated for each investigator for studies 1 and 2 (Figure 4).

One investigator’s performance in subjectively determining angularity of the neuronal nucleus (Type II cells) was compared with an objective measure of the nuclear shape parameter. The ratio of the number of this investigator’s Type II cells to the number of cells in each digitizer shape parameter class was calculated and plotted (Figure 5) to show his accuracy in shape discrimination. The difference between the investigator’s actual angularity cut-off point and the target cut-off point was evaluated by calculating the mean of his cut-off point classes weighted by the relative frequency of his angularity decisions across the shape parameter classes. Only the nonangular-to-angular transition was evaluated, starting with the last class typed as 100% nonangular and ending with the first class found to be 100% angular.

Results

Study 1 (Figure 4) showed that although individual investigators performed consistently, there was poor agreement between them. Only Type IV neurons were determined with satisfactory interobserver agreement. The study was then repeated using the
angularity comparator charts. In study 2, two investigators showed increased reliability, but investigator 3 showed increased variability (greater standard deviation) in determining Type I and II cells. Analysis of the investigators' angularity cut-off points revealed that the deterioration of investigator 3's performance was a result of a learning process that occurred during five consecutive runs (Figure 5).

The improvement in group performance resulting from the use of the angularity comparator charts is demonstrated by marked decreases (60% and 50%) in the coefficient of variation for Type I and II neurons, respectively. There was no change in the evaluation of Type III and some deterioration in the evaluation of Type IV cells. The coefficient of variation for the group changed from 15.9% to 4.7% for Type I cells, from 33.9% to 17.3% for Type II cells, from 15.5% to 14.1% for Type III cells, and from 3.2% to 5.5% for Type IV cells.

In the first session of Study 2, the angularity cut-off point of investigator 3 was 0.91 (Figure 5), quite similar to the average angularity cut-off point of 0.92 found in Study 1. However, as study 2 progressed, this investigator's familiarity with the use of the angularity comparator charts apparently produced readjustment of his cut-off points until by the fifth session an experimental cut-off point of 0.886 was achieved.

The use of angularity comparator charts markedly reduced the difference between the investigator's mean cut-off point and the target cut-off point for two investigators (Figure 6); there was a modest reduction in the coefficient of variation of the cut-off point for all three investigators. The combined effect of these two factors increased the precision of the angularity assessment for all investigators threefold.

Discussion

A reasonably detailed yet dependable description of the sequential structural changes in injured or dying neurons is essential to understanding the basic pathophysiology of cerebrovascular ischemia, as well as to evaluating the effectiveness of therapy. Several laboratories have advanced qualitative schemes of varying complexity to characterize the basic types of ischemic and ischemic-anoxic neuronal injury.2-6 Neuronal damage has been quantified by three distinct methods. Irreversibly damaged neurons have been quantified as a single class,7-9 neuronal densities have been determined,10 and distributions of neuronal types have been evaluated.2,5,11,12

We have confirmed the reliability of counting irreversibly damaged neurons by our low individual and group variance found for Type IV neurons in studies 1 and 2 (Figure 4). It is reasonable to assume that determining the densities of surviving neurons is also a valid grading tool in a region of known neuronal density.10 However, the quantification of multiple neuronal types has been shown to be error-
prone even when type definitions are quite simple and explicit (see Figure 5). Therefore, it seems imperative that multityping methods be validated.

Traditional typing methods seem to use terms that provide a very textured, pictorial description of the panoramic view of a histologic section or a cell, but when used as the building blocks for complex typing schemes, the terms produce ambiguity in classification. These typing methods are more often than not multifactorial, that is, incapable of resolving the decay path of a single feature. Instead, these methods attempt to and are very effective in following, documenting, and describing a collage of cellular features. The very completeness of the definitions excludes the possibility that all neurons can be unambiguously assigned to types within the method.

These problems can be minimized if the multifactorial, panoramic approach is abandoned; in other words, when out of the abundance of features only one is selected that is 1) representative of the overall decay process, 2) readily visualizable, and 3) evaluated using simple visual descriptors such as contrast, delineation, and shape. This produces a simplistic but verifiable view of the ischemic decay process that can be readily evaluated for typing consistency, accuracy, and reliability.

Oil-immersion light microscopy permits rapid visualization of cellular features (cytoplasmic shape, cytoplasmic density, cytoplasmic microvacuolation, nuclear shape, nuclear delineation, and nuclear contrast) that may have relevance in characterizing ischemic neuronal injury. Features that show transient reversible changes during reperfusion, such as cytoplasmic microvacuolation and chromat clumping seem inappropriate for inclusion in a sequential typing scheme.

The pathologic consequences of nuclear shape changes are difficult to interpret. If a cell is swollen, then it may at least be inferred that the cellular membranes are intact. Although shrinkage and deformation are obvious signs of cellular pathology, these changes provide no obvious direct information about the state of the nuclear or cytoplasmic membranes.

Separation of cellular compartments implies that each has intact membranes; this separation can be seen at either high or low contrast. Diminished contrast between compartments can result from either technical or pathologic factors (i.e., extremely thin sections, mixing of compartment contents as a result of leakage through disrupted membranes, and chemical changes). The nucleus of Type 1a and 1b neurons is highly contrasted with the cytoplasm, indicating the presence of an intact nuclear membrane in spite of its smeared appearance as a result of tangential sectioning. However, 0.5-μm tangential cuts of Type lc or Iic neurons can be mistaken for Type IIIa or IIIb neurons. Absence of contrast and delineation represents the most degenerative change seen because this implies destruction of the nuclear membrane and dissolution of the nucleus.

Because the number of cells in each type was adequate for statistical characterization, the following conclusions can be drawn concerning the accuracy of our typing method. 1) The use of angularity comparator charts markedly improved the reproducibility of determining Type I and II neurons from 15.9% to 4.7% and from 33.9% to 17.3%, respectively. 2) As expected, the use of angularity comparator charts had no significant effect on the reproducibility of determining Type III and IV neurons. 3) When type distributions are compared, one can anticipate finding significant differences between Types I, II, III, and IV neurons only when differences exceed 5%, 17%, 14%, and 6%, respectively. 4) The two extremes of our typing method (Types I and IV) can be assessed at much higher reproducibility than the intermediate Types II and III. This finding is in accord with the high acceptance of counting dead neurons (equivalent to our Type IV) as a method of quantifying the extent of ischemic injury in tissue. It should be noted, however, that our method makes it possible to assess the fraction of normal neurons in a population at equally high accuracy, that is, approximately 5%. It should also be recognized that identifying normal neurons is more challenging and potentially more rewarding than counting blobs of high-density debris from dying neurons.

We used controlled and standardized tissue processing and staining techniques. One can rightfully postulate that reliable typing requires tissue processing and staining free of artifacts that interfere with visual evaluation. Our method effectively compen-
sates for the reductions in contrast associated with thin sections by interpreting the morphologic integrity of Type Ic and IIC cells as equivalent to that of Type I, a or b and Type II, a or b cells. However, we recommend that sections be cut at 1.0 μm, which excludes a type Ic to IIIa or a Type IIC to IIIb transition in tangential sections.

All facets of the analytic process leading to a typing decision are lost with the completion of a global typing method. If the details of this analytic process are needed for a subsequent study, then we recommend that the method of neuronal bit feature analysis and computer typing be used instead of global typing.1 Global typing is quite complex, and to ensure its consistency all investigator cut-off points in the typing scheme must be stable.

While arguing and demonstrating that it is possible to rigorously characterize the consistency of traditional grading instruments (i.e., investigator analysis and typing), this article also underscores the subtle assessment difficulties and optimization failures of these methods.

The instrumentational requirements of our technique do not exceed those of standard oil-immersion light microscopy. Our method requires angularity comparator charts, which are provided. If a different typing scheme is to be developed based on a different but cohesive separation logic, new comparators ought to be developed, too. The methods of cellular bit feature analysis1 and investigator cut-off point analysis described in this article can be used in conjunction with standard morphometric procedures to develop these new tools. We describe a method that has been validated by detailed scrutiny of all its basic components, namely 1) feature reliability assessment by bit analysis,1 2) matching the scheme to features of decay dynamics using experimental data rather than a priori hypotheses,1 and 3) evaluation of investigator performance in terms of typing accuracy (with and without grading tools) against an absolute morphometric reference technique. We are not aware of any other neuronal typing schemes the performance of which have been subjected to equivalent scrutiny.

References


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