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Appendix E-1: Immunostaining and Stereology

Immunostaining

For histologic analysis, two whole-brain coronal sections of 1-cm thickness were taken in the midsagittal region just posterior to the foramen of Monro. One of the slabs was sectioned serially at a thickness of 50 μm to create a total of twenty brain slices, including left and right hemispheres (gray matter) and midbrain (white matter).

The first five sections included three controls and two sections that underwent AP staining and counterstaining with cresyl violet acetate plus light green and Gill hematoxylin. For sections 6 through 20 (the remainder of the block), the following staining pattern was used, repeating every three sections: immunohistochemistry with an antibody to (1) HIF-1α (1:250; NB100-131; Novus Biologicals); (2) HSC70/HSP73 (1B5) (1:4000; ADI-SPA-815-F; Enzo Life Sciences); and (3) AP histochemistry (i.e., this pattern corresponded to sections 6, 7, and 8, respectively; repeated again for 9, 10, and 11, respectively; and so forth, with each group of three considered a "set").

Floating sections were incubated overnight in the primary antibody, followed by incubation in biotinylated antimouse or antirat immunoglobulin G (IgG; KPL). Antibodies were visualized using streptavidin horseradish peroxidase and diaminobenzidine/hydrogen peroxide (DAB kit; Vector Laboratories).

Stereology

Every third section, 150 μm apart, was taken from the brain within the centrum semiovale in each dog. Immunolabeled slides were evaluated with brightfield microscopy using an Eclipse 90i microscope (Nikon) equipped with a motorized stage controller (MAC 5000; Ludl Electronic Products) and a high-speed video camera (MBF Bioscience) and interfaced with a PC workstation (Precision 390; Dell) and Stereo Investigator software (MBF Bioscience). Four brain regions of interest (the superior, middle, and inferior cortices, and the thalamus) were delineated by contours in each section prior to unbiased stereology of labeled cells or vessels. If areas of microinfarction were evident, these were also outlined for volume estimation.

A counting frame size of 300 × 300 μm and a grid size of 1300 × 1300 μm were used in both Optical Fractionator and Spaceballs workflows. Placement of the counting frame was randomly chosen by the software within the outlined structure.