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# MORPHOMETRIC AND STEREOLOGIC ANALYSIS OF CEREBRAL CORTICAL MICROVESSELS USING OPTICAL SECTIONS AND THIN SLICES

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## ABSTRACT

Following single dose fractions of 25-50 Gy the brain is subject to a late delayed effect which progresses to necrosis 7-12 months after irradiation. Loss and dilatation of microvessels is observed accompanying and preceding this necrosis but the role of these changes as a cause of necrosis is not established. To study these responses of the microvessels a perfusion method has been developed to selectively stain and thus clearly visualize these vessels. This consists of a vascular perfusion fixation with mixed aldehydes followed by Schiff's reagent. Stereo-pair composite images of vertical vibratome slices (150 $\mu$m) consisting of 6 optical sections 5 $\mu$m apart were prepared using an image analysis system. Data obtained from optical sections were compared with those obtained from composite images using the method for vertical slices developed by Gokhale (1992). Morphometry, needed to assess microvessel size, and stereology, needed to estimate length density and volume fraction, were performed on the composite stereo images. Profiles of cut ends of vessels were counted at the same time their widths were measured. Intersections of cycloids and vertical line probes with the projected images of the vessel segments within the reconstructed slices were recorded while a ribbon probe applied to profiles of cut ends of vessels (McMillan, 1992) was used to estimate volume fraction. Correlations were found between the length densities computed from counts of cut capillaries, the estimate made from the vertical slices using Gokhale's method and that computed from volume fraction and average area of vessel cross sections. In spite of vascular changes and necrosis in the white matter no changes in capillary length density, volume fraction or diameters were found in layer VI of the parietal cortex.

Key words: cerebral microvessels, diameter, length density, proton radiation, vertical slices, volume fraction.

## INTRODUCTION

The late, delayed response of brain to irradiation is well described but the population and tissue architectural changes have not been quantified. White matter forming the fimbria of the hippocampus has been found to be especially sensitive to irradiation. In the rat demyelinization and necrosis occur 40-50 weeks after irradiation and are believed to be secondary to microvessel damage and loss (Calvo et al., 1988, Reinhold et al., 1990). The

relative resistance of the cortex to radiation damage, even though it has a more dense microvascular bed than does the white matter, has not been explained. In an effort to detect radiation induced changes in the microvessels of the deep part of the parietal cortex (layer VI), their 3-dimensional structure has been visualized and evaluated using stereologic and morphometric methods following proton irradiation. Conventional modes of irradiation cannot be used without undesirable effects either on the oral mucosa or the control brain hemisphere. Using the 100 Mev proton facility at Loma Linda University it is possible to irradiate only the left hemisphere without damaging the oral mucosa thus leaving the right hemisphere as a control.

## MATERIAL AND METHODS

Sixteen female Sprague-Dawley rats (200-350 g) had their left cerebral hemisphere irradiated with protons at doses of either 25 Gy (9 rats) or 40 Gy (6 rats). The animals were killed at 8 to 14 months following irradiation. Under deep anesthesia (Ketamine (87 mg/Kg)-Xylazine(13 mg/Kg)) they were perfused through the ascending aorta with about 10 ml of saline followed immediately by 2.5 % glutaraldehyde and 2% formaldehyde in 0.1 M phosphate buffer pH 7.3. Perfusion was initiated at about 200 Torr through a 14 gauge cannula for 2-3 min and then continued at less than 100 Torr for 10-15 min. One hundred ml of Schiff's reagent was then injected in boluses of 15-20 ml at a pressure of more than 200 Torr with a 2-4 min delay between injections. A vertical plane for the preparation of vibratome slices 150 $\mu$m thick was selected as illustrated in Fig. 1. Four 150 $\mu$m slices separated from each other by 300 $\mu$m were collected from each sample and then dehydrated, cleared and mounted for imaging and analysis.

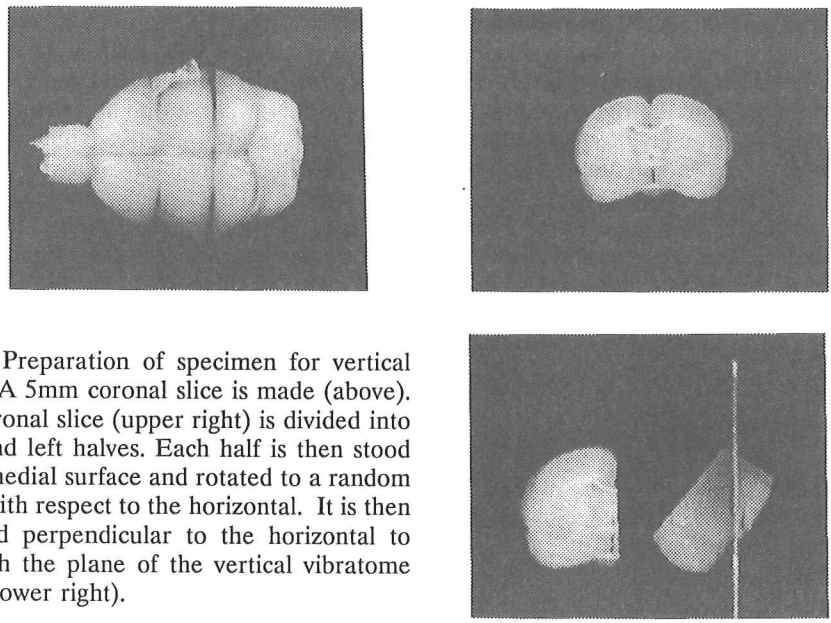

**Fig. 1.** Preparation of specimen for vertical slices. A 5mm coronal slice is made (above). The coronal slice (upper right) is divided into right and left halves. Each half is then stood on its medial surface and rotated to a random angle with respect to the horizontal. It is then trimmed perpendicular to the horizontal to establish the plane of the vertical vibratome slices (lower right).

Layer VI of the parietal cortex, located next to the fibers of the corpus callosum, was oriented in the microscopic field so that the cortical surface was up and therefore the vertical axis was always directed up and down in the digitized images. These images were collected using a SUN SPARCstation2 and videopix frame grabber using the 20X objective. Six focal plane images 5 $\mu$m apart were used to form the left and right stereo-pair images in the following manner. The first digitized image was copied into the developing right and left composite images. Then as each new image was digitized it was offset to the left or right by one pixel and compared, pixel by pixel, with its corresponding left and right composite image. A new composite image was formed consisting of the minimum pixel in each case. In this way 6 images were combined to form each of the stereo-pair images. These images were then printed on a TEAM (Toyo) video printer at a final magnification of 619. When viewed through a stereopticon device depth is clearly perceived and it is relatively easy to identify where vessels enter and leave the slice through the near and far surfaces.

The microvessels of 8 rats were analyzed. They were killed 12-14 months after irradiation with either 25 Gy (4 rats) or 40Gy (4 rats). The profiles of the "cut" vessels were outlined, the near ones on one image and the far ones on the other. Analysis of stereo-pairs was accomplished by placing on the image an overlay (Fig. 2.) consisting of a set of systematically placed and arranged cycloids with their minor axis perpendicular to the vertical axis and a set of lines parallel with it (according to Gokhale's method, 1992). Next to each of the vertical lines a second line was drawn that was equal, at the

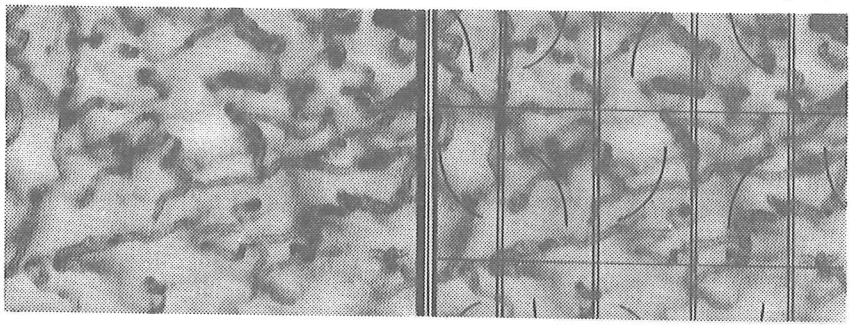

**Figure 2.** Stereopair image of parietal cortex layer VI microvessels with the overlay used for morphometric and stereologic analysis. 230 X

tissue level, to 3 $\mu$m away from the first. These two lines formed a ribbon probe (McMillan, 1992) for the analysis of volume fraction. The parallel lines forming the ribbon probe are used to estimate area fraction. The area of each sectioned profile encountered by the ribbon is estimated and an equivalent area is marked off on the ribbon. Then the ratio of the total length of marked ribbon segments to the total length of ribbon applied is equal to the area and volume fractions. Three counts are needed to apply Gokhale's method; A) intersections per unit length of vertical lines with projections of vessel profiles, B) intersections per unit length of cycloids (minor axis perpendicular to the vertical) with those same projected profiles and C) the number per unit area of cut ends of vessels. The estimate of length density is then given by the relationship $Lv = (2 \cdot B \cdot C)/A$ and section thickness is equal to A/C. While counting the cut ends the width of every third one was measured. Arterioles and venules were excluded from the analysis. The counts and measurements made on each of the 5-6 frames from

a single hemisphere were collected into a single data file using DataVoice™ (McMillan and Harris, 1990) from which length densities, volume fractions, vessel diameter histograms, mean vessel diameters and areas and section thickness were computed. Three methods were used to compute length density; 1) from profile counts of cut ends, 2) by Gokhale's (1992) method for vertical slices of unknown thickness and 3) from the volume fraction (Vv) and average area of vessel cross sections (Acs) (Lv=Vv/Acs). Means and standard deviations were computed for each group of 4 animals.

## RESULTS

The tissue slices were first examined for evidence of pathology; necrosis, cyst formation or enlarged vessels. Such lesions were uniformly found in white matter in the region of the lateral ventricle, especially the fimbria of the hippocampus (Table 1.)

Table 1. Histologic evaluations of the left hemisphere of each rat.

| Months post-irradiation | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|
| Necrosis and cyst | | 0 X | | | X X | 0 0 | X |
| Enlarged vessels in fimbria | | | 0 | | 0 | | 0 0 |
| No lesion found | 0 X | | X | 0 | | | X |

0 = 25 Gy X = 40 Gy

Only 33% of those receiving 25 Gy had cerebral necrosis or cyst formation while 57% of those receiving 40 Gy developed these conditions. Vascular changes without necrosis were seen in 44% of rats exposed to 25 Gy. On the other hand no lesions were found in 43% of the 40 Gy rats but only 22% of those irradiated with 25 Gy had none. No effect of radiation on the microvessels of layer VI of the parietal cortex was seen.

Table 2. Microvessel length densities ($mm/mm^3$) computed using different methods.

| | Irradiated 25 Gy | | | Not Irradiated | | |
|---|---|---|---|---|---|---|
| Rat I.D. | Profile Counts | Vertical Slices | Vv/ Area | Profile Counts | Vertical Slices | Vv/ Area |
| 393A | 664 | 759 | 515 | 863 | 714 | 635 |
| 393B | 597 | 329 | 432 | 527 | 414 | 352 |
| 413B | 599 | 705 | 694 | 617 | 651 | 581 |
| 493B | 583 | 686 | 519 | 638 | 822 | 436 |
| Mean | 611 | 620 | 540 | 661 | 650 | 501 |
| S.D. | 36 | 196 | 110 | 143 | 173 | 130 |

Table 2 reports the length density estimates for each of the 4 animals which received 25 Gy of radiation. Table 3 gives the same data for the 4 rats receiving 40 Gy radiation. Correlations between the different methods of estimating capillary length density were

found (profile counts vs. vertical slices - r=0.65, slope (m)=0.86, y intercept = 100; profile counts vs. Vv - r=0.54, m=0.59, y=127; vertical slice vs. Vv - r=0.63, m= 0.52, y=168). Mean section thickness from Gokhale's method was 50 $\mu$m(S.D. 15). For Gokhale's method the number of intersections of vertical lines on projected profiles of capillaries ranged from 71 to 140 for each hemisphere; intersections with cycloids ranged

Table 3. Microvessel length densities (mm/mm$^3$) computed using different methods.

| | Irradiated 40 Gy | | | Not Irradiated | | |
|---|---|---|---|---|---|---|
| Rat I.D. | Profile Counts | Vertical Slices | Vv/ Area | Profile Counts | Vertical Slices | Vv/ Area |
| 493A | 588 | 587 | 365 | 588 | 737 | 609 |
| 493C | 668 | 750 | 647 | 451 | 476 | 409 |
| 2193 | 838 | 845 | 670 | 561 | 529 | 328 |
| 293B | 685 | 670 | 374 | 820 | 817 | 559 |
| Mean | 695 | 713 | 514 | 605 | 640 | 476 |
| S.D. | 104 | 110 | 167 | 155 | 163 | 130 |

Table 4. Capillary diameters ($\mu$m) and volume fractions (%).

| | Irradiated 25 Gy | | Not Irradiated | |
|---|---|---|---|---|
| | Diameter | Vv | Diameter | Vv |
| Mean | 6.6 | 1.8 | 6.5 | 1.7 |
| S.D. | 1.1 | 0.5 | 0.4 | 0.4 |
| | Irradiated 40 Gy | | Not Irradiated | |
| Mean | 6.1 | 1.7 | 5.9 | 1.5 |
| S.D. | 0.5 | .6 | 0.4 | 0.4 |

from 31 to 97; cut end counts ranged from 167 to 383 per hemisphere. Histograms of vessel diameters (0.5$\mu$m steps) from which mean areas of vessel cross sections were calculated were symmetrical and based on 54 to 155 measurements per hemisphere.

## DISCUSSION

The lesions seen in the white matter were similar to those described by Calvo, et al. (1988) and Reinhold, et al. (1990) and the data can be interpreted to indicate a dose and time dependency for the development of lesions. The lesions limited to the vasculature in the 25 Gy animals may be interpreted to be precursors to necrosis. This study also confirms the relative resistance of the cerebral cortex to the late delayed necrosis. No changes in the microvessels of the deep cortex have been seen in this study.

The facts that a correlation was seen in the length densities computed from section profile counts and from vertical slices and that the mean values were nearly the same is

due to the fact that both methods are based upon counts of cut ends of the vessels and that the cortical microvessels are isotropically oriented (Eins and Bar 1978). Standard deviations ranged from 5 to 32% of the means although the number of cut ends counted ranged from 167 to 383 per hemisphere. This variability reflects both biological variability and some ambiguity in the images (the investigator had to distinguish between out of focus and in focus components of the images). This ambiguity may also account for the lower values computed from the Vv and areas. Even so the mean values obtained in this study correspond to those reported earlier (Bar, 1980, Craigie, 1920).

The data with the least variability in this study was that for capillary diameter. Standard deviations ranged from 6 to 17% of the means. Still this measure of capillary integrity gave no evidence of radiation damage. This does not mean that the cortex is immune to such damage for in other studies (unpublished) we have infrequently observed focal regions of dramatic loss of microvessels with an accompanying irregular dilatation.

The apparent discrepancy between the nominal section thickness, 25 $\mu$m, and that computed from Gokhale's method, 50 $\mu$m, is a consequence of the fact that objects appear at least 67% closer than actual when immersed in a medium with a refractive index of 1.5. Also depth of focus adds at least 3 $\mu$m to the nominal thickness of 25 $\mu$m.

In conclusion it has been shown that when microvessels are clearly delineated, relatively simple image processing can be used to create stereopair images from which their 3-dimensional configuration can be assessed and that can be analyzed by Gokhale's (1992) method for the estimation of length density from vertical slices. While this is not necessary to estimate length density of isotropically oriented cortical microvessels (counts of cut ends could suffice) it would be necessary in a study of the microvessels of the white matter where they tend to be oriented parallel with the nerve fibers. On the other hand since perfusion fixed capillaries are cylindrical the model based estimation of length density from volume fraction and average area of cross sections is unbiased irrespective of isotropy of sections or vessels providing section profiles can be clearly visualized.

## REFERENCES

Bar T. The vascular system of the cerebral cortex. Advances in Anatomy, Embryology and Cell Biology 1980; 59:1-61.

Calvo W, Hopewell JW, Reinhold, HS, Yeung TK. Time- and Dose-related changes in the white matter of the rat brain after single doses of X rays. Br J Radiol 1988; 61:1043-1052.

Craigie EH. On the relative vascularity of various parts of the central nervous system of the albino rat. J Comp Neurol 1920; 31:429-464.

Eins S, Bar T. Orientation distribution of blood vessels in central nervous tissue. in Quantitative analysis of microstructures. Sonderbande der Praktischen Metallographie 1978; 8:381-388.

Gokhale AM. Estimation of length density $L_v$ from vertical slices of unknown thickness. J Microscopy 1992; 167:1-8.

McMillan PJ. Areal analysis revisited: Precision of the ribbon probe. Acta Stereol 1992; 11/Suppl 1:387-390.

McMillan PJ, Harris JG. DataVoice™: A microcomputer- based general purpose voice-controlled data-collection system. Comp Biol Med 1990; 20:415-419.

Reinhold HS, Calvo W, Hopewell JW, Van Den Berg AP. Development of blood vessel-related radiation damage in the fimbria of the central nervous system. Int J Rad Oncol Biol Phys 1990; 18:37-42.