Evaluation of Microvasculature at the Auditory Midbrain–The Benefits of Sectioning at a Tangential Angle

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KEY WORDS inferior colliculus; functional capillaries; histology sectioning

ABSTRACT Vascular remodeling in the brain occurs as a plastic change following neural over-activity. The auditory midbrain (or inferior colliculus, IC) is an ideal place to study sound-induced vascular changes because it is the brain's most vascularized structure and it is tonotopically organized. However, its micro-vascular pattern remains poorly understood. Since the IC is a sphere-like structure, the histological assessment of vasculature could depend on the angle of sectioning. Here, we studied the effects of cutting the IC at different angles on microvascular assessment, specifically: micro-vascular density and the shape of microvascular lumen. Photomicrographs were taken from 5 μm toluidine blue-stained histological sections obtained at two angles of sectioning: (a) the conventional coronal sectioning, and (b) a novel "tangential" sectioning (tangential to the dorso-medial surface of the IC). Results showed that the tangential sections, in comparison with the coronal sections, yielded (a) a higher count of micro-vascular density and (b) a higher proportion of round-shaped micro-vascular lumens. This discrepancy in results between two cut angles is likely related to the spatial pattern of blood vessels supplying the IC. We propose that the tangential sectioning should be adopted as standard for the accurate study of microvasculature in the IC. *Microsc. Res. Tech. 78:105–110, 2015*.

INTRODUCTION

Energy is supplied to brain cells through cerebral circulation which continues to develop after birth (Bar, 1980, 1983). Blood vessels are plastic structures which particularly at young ages can be remodeled after various physiological manipulations (Benjamin et al., 1998). For example, rearing rats in an enriched environment lead to plastic changes in the cortex like increased microvasculature in the visual corex (Black et al., 1987). In addition, exposing young rats to tones can enlarge neurons in the auditory cortex (Lu et al., 2009, 2014). For the study of stimulus-induced changes of cerebral vasculature, the auditory midbrain (inferior colliculus, IC) offers special advantages. First, it is the most vascularized structure in the whole brain (Bar, 1980; Gross et al., 1987). Second, it is tonopically organized (Cheung et al., 2012; Schreiner and Langner, 1997), so its frequency lamina can be selectively targeted with a tonal stimulus. Indices for microvasculature are obtained from images taken from thin histological sections cut typically according to conventional anlges (Andrew and Paterson, 1989). Because of the known anisotopy of vasculature in the brain, the angle of sectioning is likely critical. As far as the IC is concerned, which angle of cutting is better than the conventional ones remains unclear. Here, we determined if cutting the IC at two different angles may yield different results on microvasculature. Specifically, microvascular density and the shape of microvascular lumens were compared between (a) the coronal section and (b) a novel "tangential" section.

MATERIAL AND METHODS

Animal Preparation

Pregnant rats (Sprague–Dawley) were obtained from the Animal Center of National Cheng Kung University and kept in standard housing (food and water *ad libitum*) until the day of delivery. Litters were reduced to 10/litter, at equal numbers of male and female whenever possible (Smart and Dobbing, 1971) . Each litter and the nursing mother were kept in a

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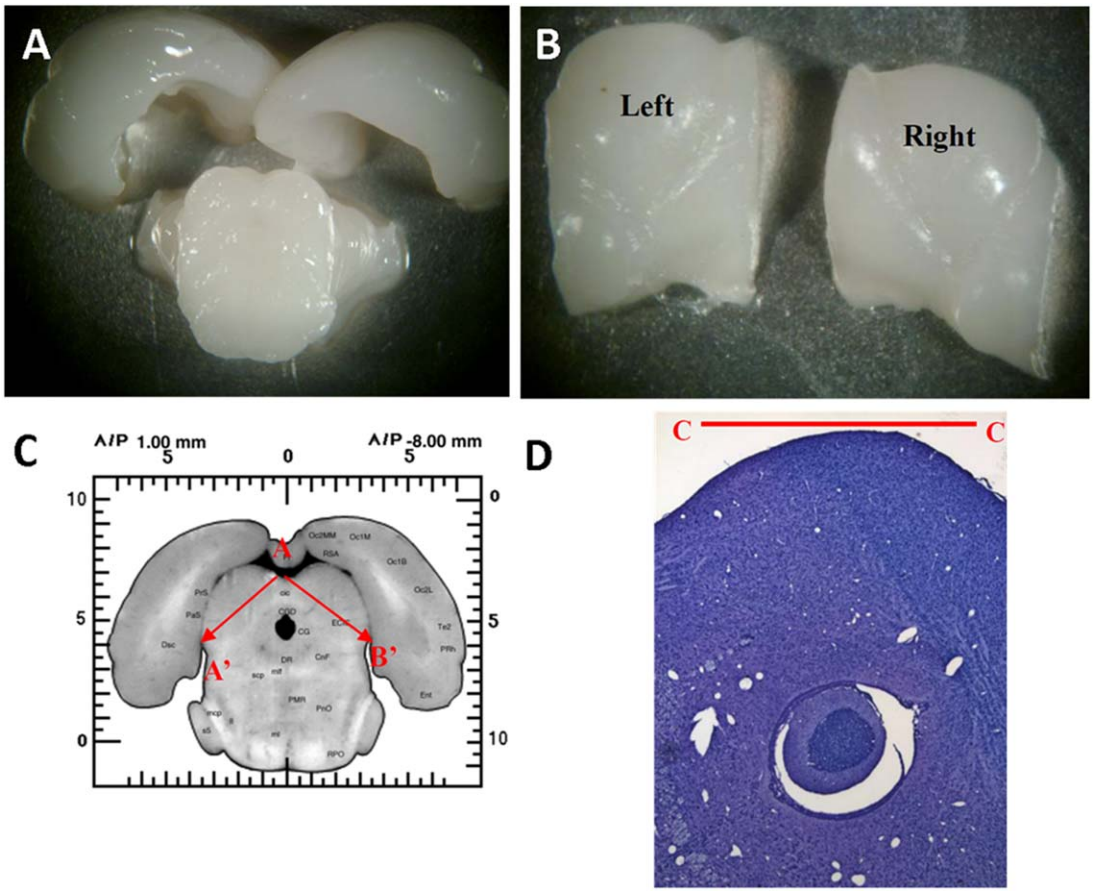

Fig. 1. Details on the tangential cut through the inferior colliculi (IC). (**A**) A caudal view of the block of brian tissure containing the brainstem and cerebral cortices; (**C**) the corresponding atlas view (http://www.loni.usc.edu/Research/Atlases/Data/rat/RatAtlasViewer.php ef) showing the two cuts (lines AA', AB') for removing the IC on each side using a specially designed cutter; (**B**) lateral view of the two IC after removal from the brainstem; (**D**) a coronal section of IC showing the angle of the tangential cut running in parallel to the line CC'. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

chamber (dimension W × L × H: 0.9 × 1.8 × 1.8 m) equipped with a quiet ventilation system. On postnatal day 14 (P14), rat pups were euthanized (Thiopental, 25 mg/kg, i.p.) and perfused transcardially with normal saline (37°C), followed by 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS, pH 7.4, 0°C). Two transverse cuts were made through the brainstem to expose the IC (Andrew and Paterson, 1989). After removing the overlying pia and connective tissues, the block (containing the IC and superior colliculus) was placed on a brain cutter specially designed for this experiment. The brain tissues were carefully trimmed under a dissection microscope to isolate the IC on each side for either the coronal or tangential sectioning that followed later (Fig. 1). Brain tissues were washed (normal saline, three times), dehydrated (alcohol gradient, 75–100%), and embedded in paraffin. The IC was cut serially at 5 μm thickness and stained with toluidine blue (1%, in 1% sodium borate) for light microphotography. For coronal or tangential sections, two regions of the IC were selected (Fig. 2, rectangles A, B) and imaged with a digital camera system (Nikon, Coolsnap) at a magnification of 100X. These regions represent roughly the low and high frequency regions of the IC as known from the literature (Schreiner and Langner, 1997). Images were processed by a commercial software Image ProPlus (and cross-checked with a tailor-made Matlab program we developed for quantifying microvascular lumens). We analyzed only micro-vessels in the stained sections (i.e., capillaries, connecting arterioles, and venules) or specifically lumens with diameters <20 μm. Statistical comparison were made using a commercial software (GraphPad Prism 4) using unpaired *t*-test with a significance level set at $P < 0.05$.

Corrosion Cast of the IC Macro-Vessels

To verify the surface macrovascular branching pattern in the IC, we studied the corrosion cast of the cerebral vessels in some selected animals following the reported procedure (Krucker et al., 2006). The animal was first anesthetized (pentobarbital, 40 mg/kg, i.p. with heparin, 700 U/kg) and chest opened for transcardial perfusion with normal saline (60–80 mmHg, 37°C, 5 min) followed by 4% paraformaldehyde in PBS (20 mL, 0°C). The flow of perfusate was powered by a perfusion pump (25 mL/min) and further regulated with reference to a manometer reading of the perfusion system. Immediate after fluid perfusion, 10 mL of resin (Mercox/catalyst, 10/0.3, 20g/0.4g, Mercox/methyl-methacrylate 8/2/0.4; Ladd

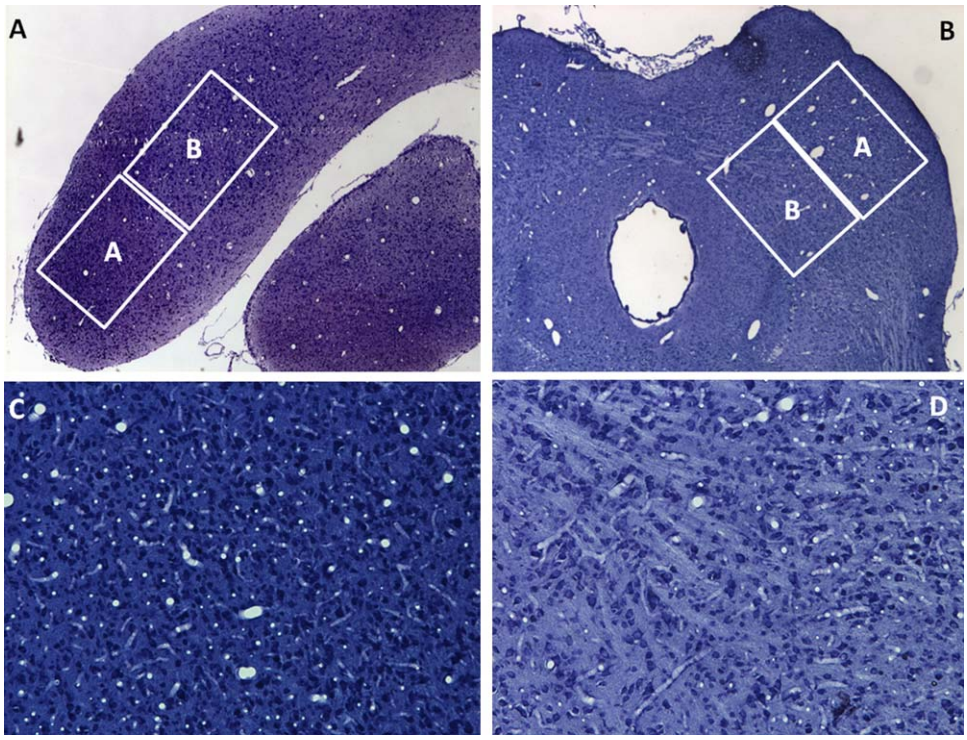

Fig. 2. Photomicrographs of sections of the IC cut at two different angles (stained with toulidine blue). (**A**) Areas sampled in the tangential sections. Two areas, A & B were taken from each IC section (x20). Vessels (<20 μm) was quantified and results averaged for the individual animal. (**B**) Areas sampled in the coronal sections, showing two areas, A and B (x20). Vessels (<20 μm) was quantified and results averaged to represent the animal. (**C**) Vessels in tangential sections (x100). (**D**) vessels in coronal sections (x100). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

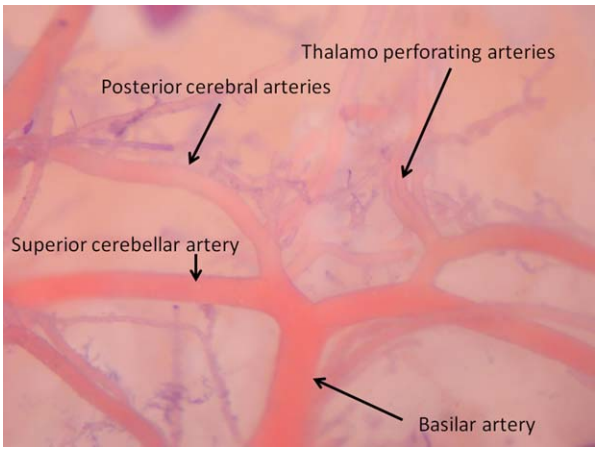

Fig. 3. Picture showing the origin of posterior cerebral artery from the basilar artery. (Mercox resin corrosion casting). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

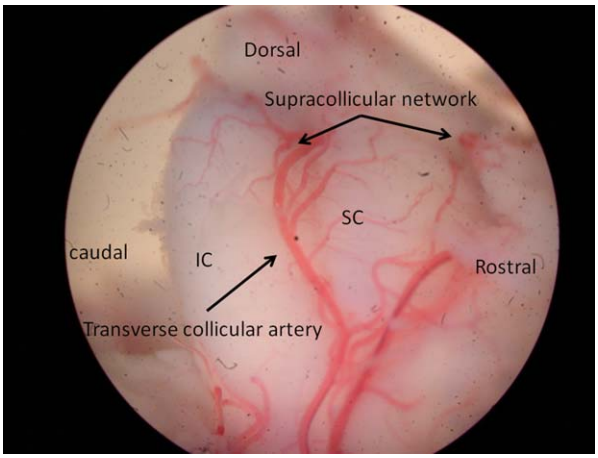

Fig. 4. A close look at vessels supplying the inferior colliculus (IC). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Research, Burlington, VT) was infused at a rate of 5 mL/min for 5 min. The head of the resin-filled animal was then immersed in hot water (50°C, 60 min), and kept at room temperature overnight for curing. Soft tissue from the head was removed by maceration in 20% NaOH at room temperature for 24 h, followed by decalcification in 5% formic acid for 24 h. The casts were then thoroughly cleaned with and stored in distilled water before drying by lyophilization.

RESULTS

Corrosion Cast of the IC

Corrosion cast of the IC confirmed the presence of two major feeding arteries from its surface, namely the transverse collicular artery and supracollicular network. The transverse collicular artery was a branch from the posterior cerebral artery which itself was a

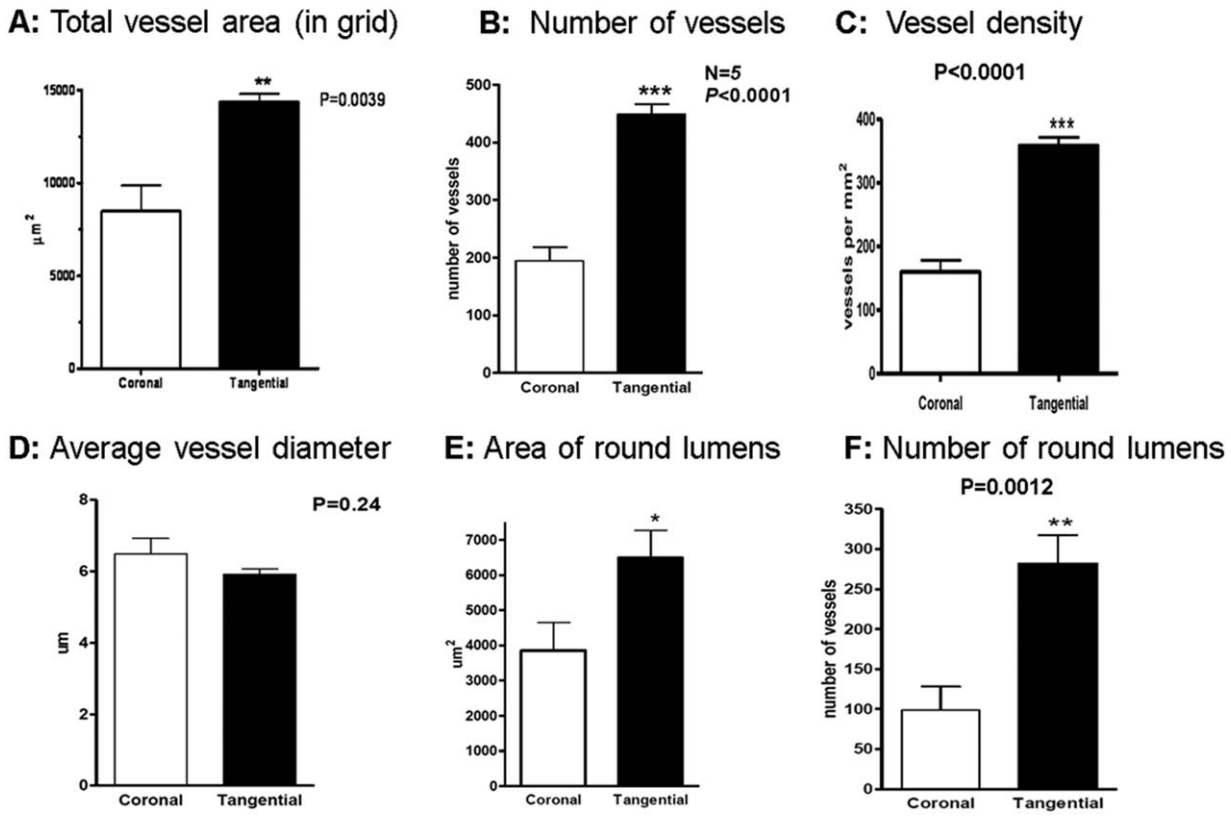

Fig. 5. Results from vessel profile studies.

terminal branch of the basilar artery (Fig. 3). Arteries supplying the IC stemmed from the collateral vessels of both the trasverse collicular artery and the supracollicular and passed perpendicuarly into the IC from its dorso-lateral surface (Fig. 4). Therefore, our coronal sectioning basically was cut in parallel to the major arteries descending from the dorso-lateral surface of the IC; whereas, the tangential sectioning was cut grossly perpendicular to the longitudinal axis of the major arteries penetrating the IC.

Micro-Vascular Cross-Sectional area

The total microvascular cross-sectional area (summed area of lumens) for the tangential sections was 14.360 ± 996 μm^2 (mean $\pm$ SD) within the sample grid ($1{,}300 \times 970$ μm^2) which was greater than the corresponding values (8.481 ± 3.121 μm^2) for the coronal sections ($P < 0.005$, $n = 5$, Fig. 5A).

Total Surface Area of Micro-Vessels

The total number of microvascular surface area vesels for the tangential sections was 448 ± 38.9 μm^2 (mean $\pm$ SD) within the sample grid ($1{,}300 \times 970$ μm^2) which was greater than the corresponding values (195 ± 52.8 μm^2) for the coronal sections ($P < 0.0001$, $n = 5$, Fig. 5B).

Micro-Vascular Density

The micro-vascular density for the tangential sections was $358 \pm 13.8/mm^2$ (mean $\pm$ SD), which was greater than the corresponding values ($160 \pm 18.4/mm^2$) for the coronal sections ($P < 0.0001$, $n = 5$, Fig. 5C).

Micro-Vascular Size

The average microvascular lumen size for the tangential sections was 5.9 ± 0.4 μm (mean $\pm$ SD), which was not significantly different from the coronal secions (6.5 ± 0.9 μm; $P = 0.24$, $n = 5$, Fig. 5D).

Cross-Sectional Area of Round Micro-Vessels

When focused on vessels with a round lumen, the total microvascular surface area for the tangential sections was 6.503 ± 1.711 μm^2 (mean $\pm$ SD) within the sample grid ($1{,}300 \times 970$ μm^2) which was greater than the corresponding values (3.853 ± 1.784 μm^2) for the coronal sections ($P < 0.05$, $n = 5$, Fig. 5E).

Number of Round Micro-Vesssels

The total number of round micro-vessels for the tangential sections was 282 ± 78 within the sample grid ($1{,}300 \times 970 \mu m^2$) which was greater than the corresponding values (99 ± 29) for the corronals sections ($P < 0.005$, $n = 5$, Fig. 5F).

Proportions of Micro-Vascular Area Based on Shapes (Round, Oval, or Irregular)

Micro-vessels were first classified according to the shape of their lumens. Their respective proportions in total area (round : oval : irregular) were 51% : 42% : 7% in the tangential sections compared to 44% : 46% :

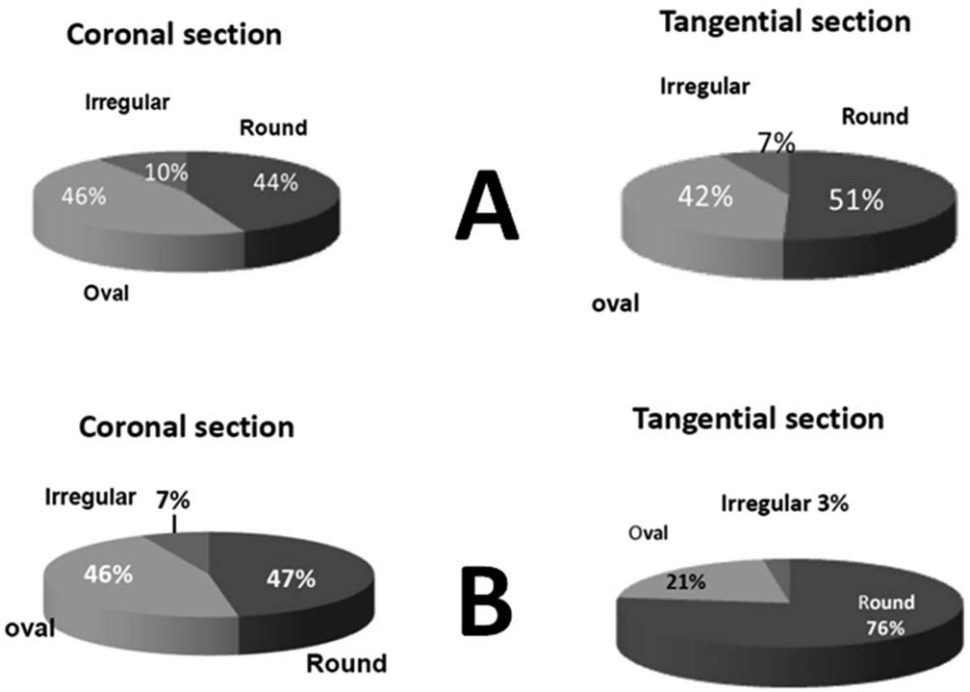

Fig. 6. (**A**) Comparison of vessel area (%) to vessel shapes on different orientations. Round vessels in tangential sections increase 7% in area when compared with coronal sections. (**B**) Comparison of vessel numbers (%) to vessel shapes on different orientations. Round vessels in tangential sections increase 29% in numbers when compared with coronal sections.

10% in the coronal sections (Fig. 6A). The difference in proportions was statistically significant for round lumens ($P < 0.05$, $n = 5$, Chi square test).

Proportions of Micro-Vessel Numbers Based on Shapes (Round, Oval, or Irregular)

Again, the proportions in micro-vessel number (round : oval : irregular) were 76% : 21% : 3% in the tangential sections, compared with 47% : 46% : 7% in the coronal sections (Fig. 6B). The difference in proportions was statistically significant for round lumens ($P < 0.05$, $n = 5$, Chi square test).

DISCUSSION

The rat IC contains the largest micro-vascular blood volume (Cremer et al., 1983) and the highest rate of blood flow (Landau et al., 1955). It is located underneath the junctional area of the sagittal and transverse sinuses and appeared to be an oval body in shape. Macroscopically, the long axis of the IC runs from ventro-lateral to dorso-medial at an angle of about 45° with the sagittal plane, and 15° with the frontal plane (Faye-Lund, 1985). In order to visualize the IC, the overlying cranium together with sagittal and transverse sinuses had to be surgically removed. Collateral vessels and the supracollicular network were destroyed during the removal of these sinus and tissues. We suspect this might be the reason why arterial and venous supply in the IC area was seldom discussed.

By applying Mercox resin, we had successfully produced castings of the arterial and venous supply of the rat brain after removal of tissues by 20% NaOH. With careful tracing of the vessels, we were able to reconstruct the vascular supply of IC. IC is basically nourished by three main arterial systems: namely, posterior cerebral artery, superior cerebellar artery, and anterior inferior cerebellar artery. The posterior cerebral artery originates from the initial portion of the superior cerebellar artery. The first branch anatomizes with contralateral homologous vessels, thalamoperforating arteries which course rostrally and dorsally to reach the ventral posterior region of the thalamus. Around the junction with the posterior communicating artery is the transverse collicular artery (0.15 mm in diameter). The transverse collicular artery courses over the surface of the brachium of the IC and its external cortex, with some of its anastomosis forming a part of the supracollicular network. The posterior cerebral artery ends in an anastomotic network, spreading over the dorsal surface of the superior and inferior colliculi. This supracollicular network perforating vessels nurture all the colliculi (Dorr et al., 2007). At the rear end, this network anatomizes with the cortical pial network over the occipital cortex. Anteromedially, it anatomizes with the terminal branches of the azygos pericallosal artery. Corrosion casting study of rat cerebral vessels showed a perpendicular penetration of vessels through the IC cortex into the central nucleus (So, 2007).

Previous studies on the vascular supply of the rat IC depended on coronal sections to produce pictures for vessel studies (Andrew and Paterson, 1989, Boero et al., 1999, Iwagaki et al., 2000). The study by Andrew and Paterson (1989) showed that blood vessels with

diameter >20 μm were rarely observed in the central nucleus of the rat IC. Our observation concurred with their findings. For vessel profiles, we adopted the method used by Andrew and Paterson (1989). Instead of using cross, oblique, and longitudinal to name the shapes of the vessels, we opt for round, oval, and irregular to describe the shapes we observed during our study. Our findings supported our hypothesis that more round vessels were observed in a tangential rather than a coronal cutting. And number of round vessels in tangential sections increase 29% in numbers when compared with coronal sections. This increase in round vessels profiles probably would be due to more transverse sections made along a longitudinal running vessels in the tangential sections.

For vessel surface area, again a significant increase was found in tangential sections compared with the coronal sections. This is due to a greater number of vessels were cut. The number of vessels in tangential sections increased significantly over coronal sections, since we were cutting the vessels along its own distribution like following a river down its tributaries. Coronal sections would produce more irregular or oval vessels while cutting longitudinally on these vessels, and thus, less surface area was encountered.

When we compare average vessel diameters between the two different profiles, vessels in coronal sections had greater mean diameter than tangential sections but the difference was not significant. This finding was likely due to more longitudinal parts of the vessels in coronal sections, leading to a larger mean diameter of the vessels.

This idea of producing sections by tangential cuts originated from our observations on the vessel distribution of the IC. We wanted a picture that allows us to see the tributary system of the supplying vessels similar to a geologist studying a river. We are the first to show that by tangential cut of the IC, a new perspective of looking at the same area of interest. We found significant differences in terms of vessel numbers, surface area, densities, and vessel profiles. A tangential cut produced a more representative data (additional valuable data) for the future vascularization studies.

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