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Age-Related Blood Flow and Capillary Changes in the Rat Utricular Macula: A Quantitative Stereological and Microsphere Study

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ABSTRACT

Vascular change may contribute to age-related vestibular dysfunction. Previously, we reported a significant age-related decrease in blood flow (BF) and mean capillary diameter (D_{cap}) in the rat posterior canal crista. The purpose of this study was to examine an otolith organ, the utricle, for similar changes. Old male Fischer 344 rats (O; 28–31 mos) were anesthetized, and the left cardiac ventricle was transcutaneously injected with radioactive microspheres to determine BF. The temporal bones were removed, fixed, and decalcified. The utricles were dissected free and placed into a gamma counter with the reference samples. The specimens were then plastic embedded and serially sectioned at 1 μm according to the vertical section technique. Microsphere surface counts were made and neuroepithelial BF calculated. A systematic random set of sections was sampled and analyzed using stereological techniques for estimates of D_{cap}, capillary surface area/unit volume ($S_{v,cap}$), capillary length/unit volume ($L_{v,cap}$), and volume of utricular neuroepithelium (V_{ut}). Using these data, total capillary surface (S_{cap}) and total length (L_{cap}) were calculated. Statistical comparisons were made with data from our previous study of young animals (Y; 3–6 mos). Results indicate a significant age-related decrease in BF (Y = 0.125 μL/min, O = 0.062 μL/min; $P = 0.003$), D_{cap} (Y = 5.95 μ, O = 4.57 μm; $P = 0.0002$), $S_{v,cap}$ (Y = 12.33 mm^2/mm^3, O = 9.87 mm^2/mm^3, $P = 0.016$), S_{cap} (Y = 0.178 mm^2, O = 0.129 mm^2; $P = 0.01$), and V_{ut} (Y = 0.014 mm^3, O = 0.013 mm^3; $P = 0.04$) with no significant change in $L_{v,cap}$ (Y = 655 mm/mm^3, O = 686 mm/mm^3, $P = 0.41$) or L_{cap} (Y = 9.47 mm, O = 8.96 mm; $P = 0.49$). These age-related vascular changes are likely to have a significant impact on utricular physiological and thus, dysequilibrium.

Keywords: microcirculation, stereology, aging, vestibular

INTRODUCTION

Falling is a serious health problem among the elderly because of the frequency and morbidity of the injuries and resulting health care costs. Approximately 30% of people living at home over 65 fall each year and the frequency becomes much higher among the elderly in nursing homes (Rubenstein and Josephson 1992). The etiology of this complaint is often multifactorial and can include defects in sensory function, central nervous system integration, and neuromuscular and skeletal function (Houx and Jolles 1993; Lord et al. 1991; Woollacott et al. 1986). Clearly, a decline in vestibular function, either peripheral or central, would be a major contributing factor in age-related dysequilibrium. Morphological studies of the human vestibular system have reported losses of Type I and Type II hair cells in all of the end organs (Merchant et al. 2000),

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loss of Scarpa's ganglion cells (Park et al. 2001), and loss of neurons from the vestibular nuclei (Lopez et al. 1997). These age-related changes have been shown to impact vestibular physiology. For example, the dynamic response properties of the vestibulo-ocular reflex and optokinetic reflex decline (Peterka et al. 1990), sway increases (Peterka and Black 1990), and, in general, there is a decline in vestibular control of eye movements (Paige 1994).

A factor that has been postulated to contribute to age-related inner ear changes is a decline in blood flow (BF; Johnsson and Hawkins 1972). Animal studies have shown that there is a decreased cochlear BF (Prazma et al. 1990) and endocochlear potential associated with age-related vasculature changes (Gratton and Schmiedt 1996). While there are a number of reports on the vasculature of the aging auditory system, there has been only one quantitative study of the vestibular periphery. Lyon and Wanamaker (1993), using microsphere and stereological techniques, reported a significant decrease in BF and mean capillary diameter of the posterior canal crista in old rats. Impaired circulation affects many aspects of cellular homeostasis and would likely result in a decline of end organ function, and thus imbalance, as well as contributing to a general physiological decline of vestibular function.

It is not known whether aging affects the otolith organs in a manner similar to that demonstrated for the posterior semicircular canal. The goal of the research presented here was to determine if age-related changes in BF and vascular morphology occur within the rat utricle. Data show significant decreases in mean capillary diameter and BF as well as neuroepithelial volume.

METHODS AND MATERIALS

Male Fisher 344 rats (28–31 mos, $N = 7$) were anesthetized using 3% chloral hydrate (250 mg/kg) in saline and placed in the supine position. Both femoral arteries and one femoral vein were cannulated using PE 50 tubing. One arterial catheter was used for constant blood pressure monitoring (for instrumentation details, see Payman and Lyon 1993) and the other for obtaining an arterial blood sample for blood-gas analysis (0.3 mL, replaced with 0.3 mL saline, analyzed in a Radiometer Model ABL2 blood-gas analyzer, Copenhagen, Denmark) and microsphere reference sample. A rectal temperature probe was inserted and body temperature maintained at 37°C using a heat lamp. A transcutaneous injection of ^{141}Ce microspheres (7.8×10^6; 9.21 ± 0.53 μm in diameter, specific activity of 7.24 mCi/g; 3M, St. Paul, MN; suspended in 1.0 mL saline at 37°C, by sonication), lasting 20 s, was made into the left cardiac ventricle using a 25-gauge needle. Simultaneously with the beginning of the injection, a 1.0 mL reference sample was taken at a rate of 1.0 mL/min using a syringe pump (Harvard Apparatus Model 2200). After two minutes, the animals were killed with an intracardiac injection of saturated KCl, decapitated, and the temporal bones removed. The bulla, oval, and round windows were opened. The inner ear was perfused with 3% glutaraldehyde in 0.1M phosphate buffer, pH 7.2, and the temporal bones post-fixed for 24 h.

The temporal bones were decalcified in 10% ethylenediaminetetra-acetic acid and the utricles dissected free and post-fixed in 1% osmium tetroxide for 1 h. The specimens and reference samples were placed into a Beckman gamma counter (Beckman Instruments, Fullerton, CA) and counted, 3×100 min. The results were used to calculate total end organ BF in μL/min by using a ratio of the reference sample counts to tissues sample counts. The kidneys were also removed and bisected. The superior halves were fixed in glutaraldehyde, weighed, and gamma counted to document uniform left–right microsphere distribution.

Once the gamma counting was completed, the end organs were dehydrated and embedded in soft Spurr's embedding medium. Serial "vertical sections" were cut at 1 μm, collected onto coverslips, and counterstained with 1% sodium borate, 1% methylene blue. To avoid bias due to orientation of the section plane, isotrophic uniformly random (IUR) sections would ordinarily be used, giving all surfaces an equal chance of being sampled. However, with IUR sections it frequently becomes impossible to identify tissue compartments, which was essential in our study. Therefore, the vertical section technique was used (Baddeley et al. 1986). Briefly, a vertical section is a plane perpendicular to a given "horizontal" plane, which is an arbitrary plane of reference used to define the orientation of the sectioning. This results in vertical uniformly random sections (VUR). Using a cycloid arc test probe in conjunction with VUR sections allows them to become equivalent to IUR for the purpose of surface estimation. In this study, the fixed but arbitrary axial direction designated as the horizontal plane was the side on which the nerve enters the end organ. All sections were cut perpendicular to this axis. A random starting point of sectioning was obtained for the first specimen using a random number table. Each subsequent specimen was rotated 10° from the previous sample to insure that each end organ would be sectioned in a different orientation, thus increasing efficiency (Fig. 1).

A systematic random sampling design (Gundersen and Jensen 1987) was used to select a set of sections (25–30) for analysis. Sections were placed on the light microscope under oil immersion at 100X. Systematic random selection of sample fields was achieved using a computer-controlled microscope stage. The field was

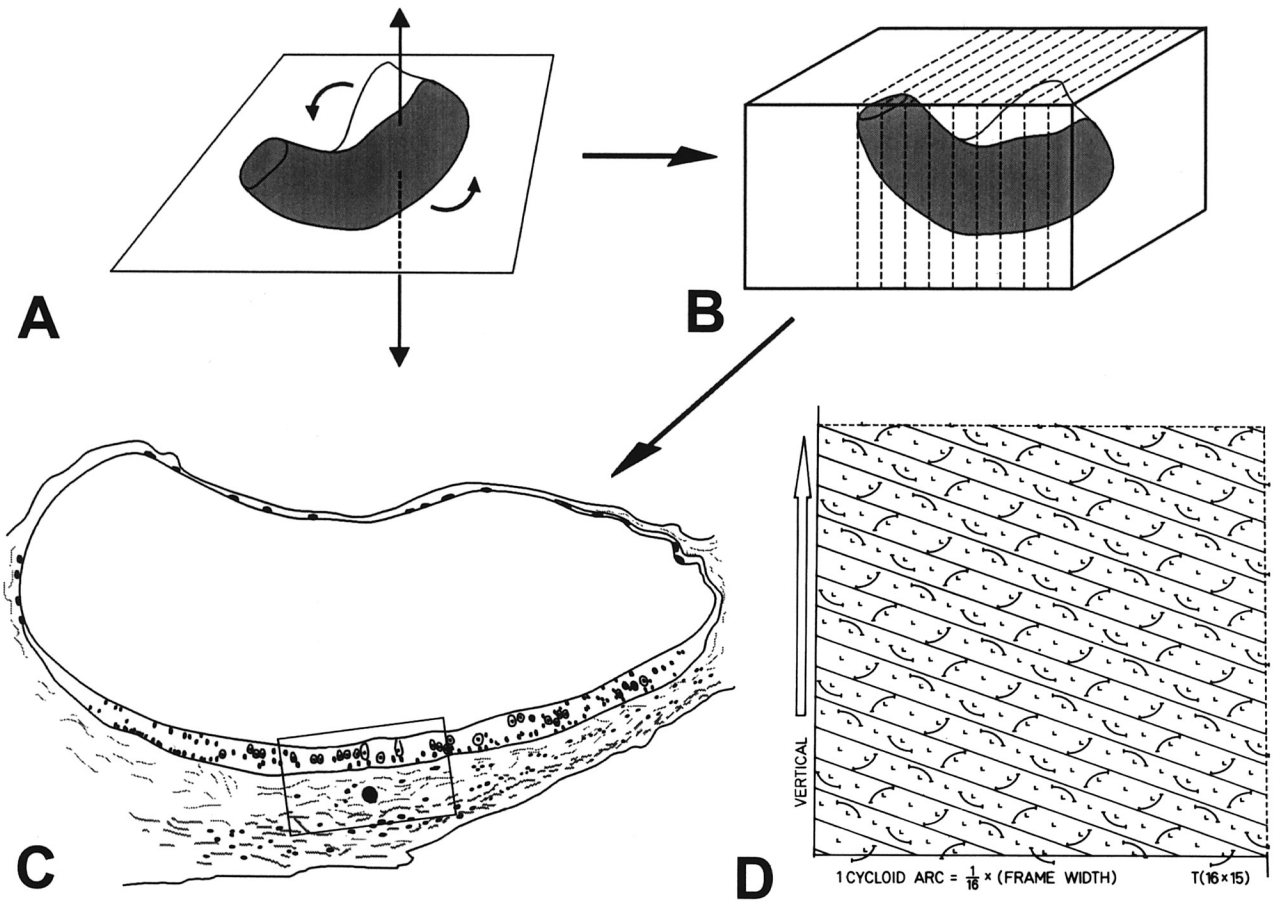

FIG. 1. In this study, the sections used were "vertical sections," perpendicular to a fixed but arbitrary horizontal axis whose direction was designated as the side on which the nerve enters the utricle. One degree of freedom around the vertical axis (**A**) is allowed so that a random starting point for the sectioning was obtained. All sections were cut perpendicular to this axis (**B**). Each subsequent specimen was systematically rotated 10° from the previous sample, insuring that each specimen would be sectioned in a different orientation. Microspheres within the neuroepithelium (depicted by the black dot in **C**) were identified and counted by serially examining all sections. A set of these sections was then microscopically examined and $S_{v,cap}$ determined using the unbiased test system shown in **D** (see text for details), which was placed into the ocular. Boxed area in **C** is shown in Fig. 2 (modified from Payman and Lyon 1993).

positioned to the section's lower left and the microscope stage was then offset in the x and y axes by an amount randomly generated by the computer and the first sample field selected. Subsequent sample fields were obtained by moving the stage in uniform increments, thus eliminating operator bias and any overlap between sample fields. The test system, consisting of cycloid arcs (Fig. 1C) of known length and number of points, was used to estimate capillary surface/unit volume of utricular neuroepithelium ($S_{v,cap}$). The system was superimposed on the tissue image by placing it in a microscope ocular. The $S_{v,cap}$ was estimated by counting the number of capillary lumen intercepts made by the cycloid arcs and points falling on the reference space using the following equation:

$$\hat{S}_{v,cap} = 2(p/l)\frac{\sum_{i=1}^{n} I_i}{\sum_{i=1}^{n} P_i} \quad (1)$$

where I is the number of intersections between the cycloid arc and the capillary luminal surface and P is the number of test points hitting the reference space. The ratio of test points [Eq. (2)] to test curve length in real units (μm) is represented by p/l.

Estimates of the volume fraction of capillary lumen ($V_{v,cap}$) was made using point-counting techniques (Howard and Reed 1998) and the following relationship:

$$\hat{V}_{v,x} = \frac{\sum_{i=1}^{m} P_x}{\sum_{i=1}^{m} P_{ref}} \quad (2)$$

where m is the number of sample fields, P_x is the number of points falling on the structure of interest, and P_{ref} is the number of points falling on the reference space. Once $V_{v,cap}$ had been determined, it was possible

to estimate mean capillary diameter (D_{cap}) using Eq. (3):

$$\hat{D}_{cap} = \frac{4}{S_{v,cap}/V_{v,cap}} \quad (3)$$

The capillary length per unit volume ($L_{v,cap}$) was estimated based on the assumption that a capillary is a cylinder and using the estimates of $S_{v,cap}$ and D_{cap} in the following relationship:

$$\hat{L}_{v,cap} = \frac{S_{v,cap}}{\pi \cdot D_{cap}} \quad (4)$$

where r represents the mean capillary radius.

Using the same section set, the volume of the utricular neuroepithelium (V_{ut}), which excluded the main portion of the utricular nerve, was estimated using Cavalieri's principle (Gundersen et al. 1988). A point-counting grid was placed into the microscope ocular, superimposed over the tissue at 20X, and each point falling on the utricular neuroepithelium counted. The V_{ut} can then be estimated using the following equation:

$$\hat{V}_{ut} = T \cdot \frac{a}{p} \cdot \sum_{i=1}^{m} P_i \quad (5)$$

where T is the average section thickness, a/p is the area associated with each point (μm^2), and P is the sum of points taken over all sections. Once V_{ut} had been determined, the total values for capillary surface area and length for each end organ were calculated.

The total number of microspheres contained within the neuroepithelium, as well as in the rest of the specimen was ascertained by surface counting (Angelborg et al. 1985) using all sections. Since the sections were considerably thinner than the microspheres, the possibility of superposition effect was eliminated (Gundersen 1986). Neuroepithelial BF was then calculated by multiplying the total BF, as determined by the gamma counting, by the percentage of microspheres within the neuroepithelium.

ANALYSIS

The coefficient of error was calculated for the individual estimates using the methods of Gundersen and Jensen (1987) for systematic sample design. Data were statistically compared with those obtained in a previous study of young animals (3–6 mos, $N = 7$; Payman and Lyon 1993) using a one-way analysis of variance (BMDP Statistical Software, Statistical Software, Los Angeles, CA). The equality of variance was also tested using Levene's test, and when the null hypothesis was rejected, the Welch and the Brown and Forsythe tests were used. A probability of $P < 0.05$ was set as the level of all hypothesis testing.

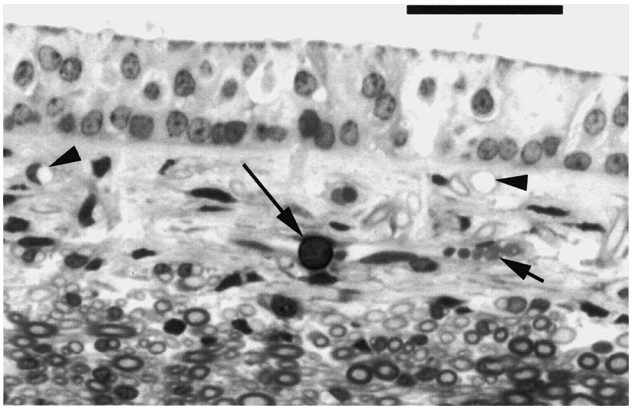

FIG. 2. A capillary containing a microsphere (long arrow) and red blood cells (short arrow) as well as several empty capillaries (arrowheads) can be clearly seen within the utricular neuroepithelium in this section from an old rat. Calibration bar = 40 μm.

RESULTS

There were no significant differences in blood chemistries between the young (Y; all values are mean ± standard error; MAP = 80.5 ± 4.1 mm Hg, pH 7.33 ± 0.01, pO_2 = 89.6 ± 1.5 mm Hg, pCO_2 = 43.1 ± 1.2 mm Hg, Hb = 16.3 ± 0.62; $N = 7$) and old animals (O; MAP = 78.3 ± 1.7 mm Hg, pH 7.34 ± 0.01, pO_2 = 84.6 ± 3.3 mm Hg, pCO_2 = 42.0 ± 2.9 mm Hg, Hb 15.9 ± 1.2; $N = 7$) and are all within normal limits for chloral hydrate-anesthetized animals. There was no right versus left difference in kidney BF, demonstrating the uniform distribution of the microspheres (Y: 4.4 ± 0.3 μL/mg/min, left; 4.3 ± 0.3 μL/mg/min, right; O: 1.5 ± 0.3 μL/mg/min, left; 1.5 ± 0.3 μL/mg/min, right).

Microspheres were identified within the utricular neuroepithelium (Fig. 2) and followed for several serial sections, which provided confirmation of a single microsphere. The number of microspheres trapped in the young specimens was 16.9 ± 2.1 and 6.3 ± 1.7 in the old. Results indicate that there is a significant age-related decrease of utricular BF (Y = 0.125 μL/min, O = 0.062 μL/min; $P = 0.003$), D_{cap} (Y = 5.95 μm, O = 4.57 μm; $P = 0.0002$), $S_{v,cap}$ (Y = 12.33 mm^2/mm^3, O = 9.87 mm^2/mm^3; $P = 0.016$) as well as total surface (S_{cap}: Y = 0.178 mm^2, O = 0.129 mm^2; P = 0.01) and volume (V_{ut}: Y = 0.014 mm^3, O = 0.013 mm^3; $P = 0.04$). There were no significant changes in either $L_{v,cap}$ (Y = 655 mm/mm^3, O = 686 mm/mm; $P = 0.41$) or total length (L_{cap}: Y = 9.47 mm, O =

TABLE 1

Summary of blood flow and stereological data for the different age groups

	D_{cap}[a] (μm)	$S_{v,cap}$[b] (mm^2/mm^3)	$L_{v,cap}$[c] (mm/mm^3)	$V_{v,cap}$[d]	BF[e] ($\mu L/min$)	V_{ut}[f] (mm^3)	S_{cap}[g] (mm^2)	L_{cap}[h] (mm)	$BF_{v,ut}$[i] ($\mu L/min^3/min$)
Young									
Mean	5.95	12.33	655	0.0185	0.125	0.0144	0.178	9.47	8.91
SEM	±0.22	±0.73	±30.2	±0.002	±0.014	±0.001	±0.015	±0.63	±0.001
Old									
Mean	4.57*	9.87*	686	0.0115*	0.062*	0.0130*	0.129*	8.96	4.72*
SEM	±0.14	±0.5	±20.9	±0.001	±0.011	±0.0001	±0.007	±0.35	±0.001

[a] D_{cap} = mean capillary diameter.
[b] $S_{v,cap}$ = capillary surface density/unit volume.
[c] $L_{v,cap}$ = capillary length density/unit volume.
[d] $V_{v,cap}$ = capillary lumen volume fraction.
[e] BF = neuroepithelial blood flow.
[f] V_{ut} = volume utricular neuroepithelium.
[g] S_{cap} = total neuroepithelial capillary surface area.
[h] L_{cap} = total neuroepithelial capillary length.
[i] $BF_{v,ut}$ = blood flow/unit volume utricular neuroepithelium.
SEM = standard error of the mean.
* = significantly different from young animals, $P < 0.05$.

8.96 mm; $P = 0.49$). Summary data for all variables are presented in Table 1.

DISCUSSION

Data from the present study show that there is a significant age-related decrease in utricular BF. These findings are similar to those reported for the posterior canal crista (Lyon and Wanamaker 1993) as well as for the cochlea of the rat (Seidman et al. 1996) and the gerbil (Prazma et al. 1990). The BF measurements from our study may represent a minimum value since the mean capillary diameter was smaller (4.57 μm) than that of the microspheres (9.21 μm). These would tend to lodge more proximally within the vasculature and not reach the terminal capillaries in the utricular neuroepithelium, thus leading to a lower BF measurement. On the other hand, cochlear BF measurements show no difference when the diameter of the microspheres used varied by 50% (Hultcrantz and Angelborg 1978; Rodgers et al. 1986). While these data may indicate that the distribution of capillaries of different diameter is similar within the regions of the cochlea that were examined, they also suggest that the passage of microspheres is not limited by capillary diameter alone. Other factors such as endothelial cell dilatation due to shear stress, perfusion pressure, neural influences, and/or other local mechanisms may also be contributing factors. Even if our quantitative BF data represent a minimum value, the decreased capillary diameter found in the present study could account for a significant reduction in BF. For example, Zweifach et al. (1981) reported that BF diminished by 64% with a capillary diameter reduction of 28%, decreases that are similar to those reported here (BF 50%, D_{cap} 22%).

A possible factor that may contribute error to the BF findings is the low number of microspheres that were trapped in the neuroepithelium. Despite the low number, we argue that the blood flow measurements are valid given the results of the above-mentioned studies and since the standard error for both the young and old animals is within what would be expected for normal biological variation. In addition, in an evaluation of the microsphere method for measuring cochlear BF in the rat, Hillerdal et al. (1987) showed that biological variation contributed more error than if the microsphere number varied from 23 to 400 μL^{-1} min^{-1}.

Our data also show a significant decrease in V_{ut}. This is likely due to the loss of hair cells and/or supporting cells. While there are no quantitative data available for the rat utricle, in a qualitative study by Iurato (1967) reported a reduction in hair cell volume. More recently, in a quantitative analysis of mice, Park et al. (1987) demonstrated an age-related 14% decease of hair cells in the utricle. Whether there is also nerve fiber loss is not certain. Studies of Scarpa's ganglion show that there is no age-related cell loss in rats (Alidina and Lyon 1990) or mice (Cohen et al. 1990). Recently it was demonstrated that an age-related decline in cochlear BF occurs prior to a significant change in auditory brainstem response and an increase in mitochondrial DNA deletions (Seidman et al. 1996). This strongly suggests that hypoperfusion and subsequent free radical production was a major mechanism for the decline in auditory sensitivity. No similar data are available for the vestibular system. However, data from Lyon and King (1997) show a

significant decrease in mitochondrial volume fraction, with a corresponding increase in lipofuscin granules, which have been shown to contain mutated mitochondrial DNA likely representing the phagocytosis of damaged mitochondria (Hirai et al. 2001).

The stereological data presented here indicate that there is an age-related decrease in D_{cap} again similar to what has been reported for the posterior canal crista (Lyon and Wanamaker 1993). Other data for inner ear vascular change come from two-dimensional studies of the cochlea where stereological techniques were not used. Those data suggest that the area of capillaries decreases with age in the rat (Keithley et al. 1992) as well as in the gerbil (Gratton et al. 1997). Seidman et al. (1996) reported a trend toward a decreased D_{cap} in the rat. It is not known if decreased BF precedes this decrease in D_{cap}. In any case, as stated above, it has a significant impact on BF and the corresponding decrease of S_{cap} would affect the exchange of nutrients and waste products. Additionally, there is evidence that the basement membrane of strial vasculature has thickened due to age (Thomopoulos et al. 1997), which would also tend to decrease blood–tissue exchange.

Recently, the ability of the aging inner ear vasculature to respond to different challenges was examined using laser Doppler flowmetry. Brown et al. (1995) found a reduction in the ability of the vasculature to respond to vasodilating drugs as well as increased postdrug recovery times. In another study, the response of cochlear BF to occlusion of the anterior inferior cerebellar artery (Suzuki et al. 1998) demonstrated a decreased capacity of the cochlea to maintain stable BF. Both of these studies indicate a decreased ability of the inner ear vasculature to meet various challenges and suggest an increased susceptibility to damage due to altered BF. While these types of studies have not been performed on the vestibular vasculature, given the similar age-related decreases in BF and vascular morphological change, it is likely that this reduced responsiveness would also be present in the vestibular system. In addition, data from a recent quantitative study (Lyon and Jensen 2001) indicated that there is likely an overall coordination of inner ear BF; that is, when cochlear BF changes, vestibular BF changes by a relatively equal amount and in a similar direction.

The finding of an age-related BF decrease to the utricular macula raises the question of whether or not this change translates into clinically apparent vestibular dysfunction. While age-related changes in any one portion of the vestibular system may be insufficient to produce clinically significant symptoms, the summation of the deterioration of central and peripheral components would be significant. For example, in elderly humans it has been demonstrated that the ability to control eye movements at normal head velocities is diminished, that the adaptive plastic changes occurring to restore the vestibulo-ocular reflex to normal after a challenge are less effective, and that there is a more pronounced progression of vestibulo-ocular reflex phase change that can be accounted for by cell loss alone (Paige 1992). In the rat, recent data have shown that there is an age-related loss of glycine receptors within the vestibular nuclei (Nakayama et al. 1999). This, in conjunction with the loss of vestibular nuclear neurons (Lopez et al. 1997), Scarpa's ganglion cells (Park et al. 2001), and degeneration of the peripheral vestibular apparatus due to hypoperfusion, would have a clear impact on vestibular function.

Using antioxidant and free radical scavengers has produced significant decreases in mitochondrial lipid peroxides (Chung et al. 1992; Packer and Landvik 1990). In gerbils, treatment with a radical spin trap was shown to reverse temporal and spatial memory deficits by decreasing the production of oxidized proteins (Carney et al. 1991). Caloric intake can also influence the progression and severity of age-related degeneration caused by free radicals. Recently, Seidman et al. (2000) demonstrated in aged rats that a 30% caloric restriction resulted in a decrease of mitochondrial DNA deletions within the cochlea and less loss of auditory brainstem responses. Previously, Park et al. (1990) reported that dietary restriction could significantly decrease the number of spiral ganglion cells lost during aging in mice. While the mechanisms responsible for these changes remain unclear, these data indicate that pharmacological therapy with antioxidants, coupled with a dietary regime, may be a feasible approach in overcoming some of the manifestations of old age. However, further work must be done to elucidate the mechanisms responsible for these changes before a rational strategy for clinical therapy can be designed.

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