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Original Article

Ultrastructural changes in postcapillary-sized venule morphology in aged mouse periodontal ligament

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The aim of this study was to compare the postcapillary-sized venule (PCV) morphology of four young ALCA mice (35 days) and four colony-related aged mice (365 days) using the transmission electron microscope (TEM). Right and left mandibular first molar mesial roots with associated periodontal ligament (PDL) and bony socket, were used for TEM assessment. Five PCV profiles were selected at each 160μm interval, from the alveolar crest to the tooth apex. PCV profile dimensions were measured on standardised micrographs magnified x2900. Age affects were tested using multiple regression analysis. The number of PCV profiles in the tooth third of the PDL was higher in aged mice ($p < 0.01$) and comprised predominantly apericytic vessels ($p < 0.001$). The number of PCV profiles increased significantly ($p < 0.001$) in aged mice in the PDL middle circumferential third halfway down the molar root. Age had no significant affect on PCV diameter. Aged PDL permeability studies are needed to investigate whether the changes in aged PCV profile number are associated with functional modification of the PDL microvasculature.
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Introduction

In all industrialised countries, there is a trend towards an ageing population that has profound economic, political, and social significance.[1] Maintenance of optimal cellular function is dependent on the blood circulation, which is crucial for the provision of nutrients and the removal of metabolic waste products.[2] Alterations in structural or functional aspects of the peripheral vasculature have been implicated in the decreased functional capacity of tissues with ageing.[3] The accumulation of intracellular waste products including the production of damaging reactive oxygen metabolites that occur with age may be attributable to changes in vascular blood flow and permeability.[4]

Many pathways, which vary between different vascular beds depending on the physiological or pathophysiological conditions, have been described for the passage of different nutrients and metabolic waste.[5] Two water-filled pathways have been defined across endothelium for the exchange of water, gases, ions and small hydrophilic molecules:[5] 1. transmembranous (directly across the endothelium by diffusion and convection) and, 2. extramembranous routes (transcellular vesicles, channels, fenestrae and diaphragms, and intercellular endothelial junctions). Clearly, there are many factors that can affect microvascular permeability. It is noteworthy that capillary permeability to macromolecules, micromolecules and transvascular fluid movement has been shown to decrease with age.[6,7,8,9] However, an age-related increase in vascular permeability in rat cochlear microcirculation has been shown using intravital microscopy and, using infused fluorescently labelled dextran, by differences between recovery slopes of intraluminal and extraluminal light intensity.[4]

The periodontal ligament (PDL) is traditionally considered to be a dense, fibrous connective tissue that attaches tooth to alveolar bone. The PDL has an important role in several functions,[10] including support and protection during mastication, homeostatic, formative, resorptive, sensory and nutritive functions provided by the cell populations, connective tissue fibres, vascular systems and nerves. The vascular supply of the PDL provides for the physiological exchange of nutrients and waste products, and may reflect the high metabolic activity and rapid turnover of both cellular and extracellular elements.[11] A quantitative electron microscope study[12] found the PDL extracellular matrix did not change with age. However, using immunolocalisation, marked cytoskeletal changes with aged PDL have been demonstrated in rats.[13]

The periodontal ligament endothelial cells are dynamic structures important for regulating vascular permeability, leukocyte extravasation and vascular remodelling.[14] The clinical significance of any age-related alteration in permeability of the PDL microvasculature would be relevant to the function of the PDL during mastication, tooth eruption, orthodontic tooth movement in adult patients, and periodontal health and disease. It has been postulated that postcapillary-sized venule (PCV) profiles may be the most sensitive microvessel to experimental extrusive force, but that any changes in the PCV profiles are transitory.[15]

Periendothelial cells that characterise venous capillaries and postcapillary venules in the rabbit thigh fascia are pericytes and veil cells,[16] which are closely associated with an abluminal intercellular distance of 20 nm.[17] Luminal diameter and the number of pericytes distinguish a venous capillary (< 8 µm diameter) from a postcapillary venule (8 to 30 µm diameter), the latter having a gradual increase in the number of pericytes.[16] An increased number of pericytes surrounding endothelial cells is related to the increased degree of tightness of the interendothelial junction and, therefore, "the better the microvascular barrier".[18]

Ongoing investigations of the morphology of PCV of the aged PDL are needed to provide a more complete picture of the various factors involved in the determination of diffusion pathways and vascular efficiency. The purpose of this study was to obtain and evaluate morphometric data of the mandibular molar periodontal ligament microvasculature of young and aged mice.

Materials and methods

Tissue specimens used in the present study were from previous studies[19,20] and consisted of mandibular right and left molar PDL from four young mice (35 days) and four aged (365 days) ALCA-strain mice that had been prepared for Transmission Electron Microscopy (TEM). Tissue blocks isolated from the mesial root PDL were sectioned ultra-thinly at 160 µm intervals, beginning at the crest of the alveolar bone representing the zero level, through to the root apex representing the end level.

An initial survey of the sample found that more than five PCV profiles were present at each level in the specimens studied. Venous capillaries and collecting venules numbered less than the five per level, and were excluded because of the lack of a statistically adequate sample size. Therefore, only PCV profiles were included in the investigation. However, PCV profiles contain 88 per cent of the mouse PDL vascular volume[20] and are the predominant vascular segment of the PDL microvascular bed. Thus, PDL tissue blocks were taken from the right and left sites of four young mice and four aged mice, representing 16 PDL tissue blocks (young: 8 blocks, aged: 8 blocks). From each PDL tissue block, five PCV profiles per level (coronal to apical) for 7 to 9 levels were selected, to provide a minimum sample size of 640 PCV profiles for each of the young (n = 320) and aged (n = 320) mice.

Each specimen was randomly positioned when placed in the TEM for examination. A polaroid photograph of the final quadrat (x100) was used to assist in identifying the PDL located at the mesial area of the tooth.[19] Each grid was examined, beginning at the top left hand corner of the grid and proceeding across the grid from left to right and in the reverse direction for the adjacent row below, until five PCV profiles were identified within the grid square (Figure 1). Electron micrographs of the PCV profiles were exposed at a magnification of x500 to cover all the area in that grid square. If there were fewer than five blood vessels in the first grid square, then the adjacent grid square was examined (Figure 1). The PCV profiles were recorded as being located in the tooth third, middle third, and bone third of the PDL. If any PCV profiles were present in both regions, they were allocated to the region of the PDL where the major segment of it was located. PCV profiles were excluded if more than one quarter of the vessel intersected with a grid bar, as this made assessment of the luminal diameter difficult.

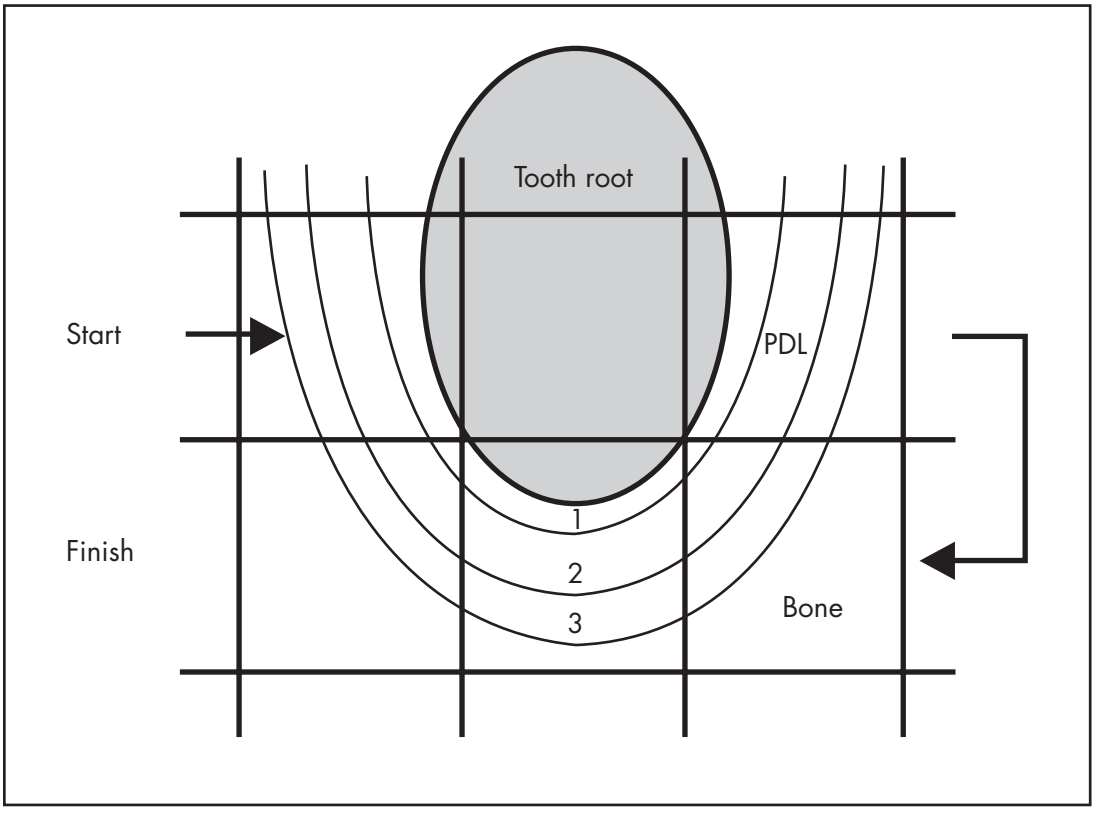

Figure 1. Representation of the preferred quadrant sampling procedure (arrows) and the periodontal ligament (PDL) divided into three regions, tooth (1), middle (2), and bone (3) circumferential thirds.

A micrograph of a replicating graticule* (2160 lines/mm) was exposed at both the low and high magnifications every TEM photographing session to ensure magnification standardisation. Each micrograph was enlarged and printed at x 2900. Each micrograph was assigned a number from a random-numbers table[21] and placed in sequence, blind to the observer, to eliminate observer bias during measurement procedures.

The PCV profiles classification, location and measurement were based on:

1. Apericytic PCV profiles (aPCV) or pericytic PCV profiles (pPCV) classifications were based on published criteria.[16,22]

2. PCV profiles location was recorded as being in the tooth, middle or bone circumferential thirds of the PDL.

3. Luminal diameter was measured with a Max-Cal electronic digital calliper with an accuracy of ± 0.03 mm and a resolution of 0.01 mm.

Error analysis

Every tenth specimen was selected from the randomly assigned specimens resulting in thirteen specimens being used for measurement error. Two randomly assigned PCV profiles from each of these specimens were measured blind under the same conditions as previously. The data were tested for normality and constant variance. Provided these assumptions were satisfied, a paired *t*-test was used for data analysis. Otherwise, a Wilcoxon signed-rank nonparametric test was used. The significance level was set at $p < 0.05$. No significant differences were found between the first and second measurements. Cohen's kappa coefficient[23] yielded a measure of 1.00, indicating that no significant differences were found between the first and second classifications.

Statistical analysis

A statistical analysis was completed using the Genstat™5 Release 3† Initially, data were tabulated to give arithmetic means with standard error

* Arithmetic means with standard error values in parentheses.

† Mean values calculated using analysis of covariance. They were adjusted because of different numbers of values at each periodontal ligament level.

Table I. Total number of apericytic and pericytic postcapillary-sized venule profiles in each periodontal ligament level (μm) for young (Y, *n*=4) and aged (O, *n*=4) mice.

	Apericytic				Pericytic			
Level (μm)	Y	Range	O	Range	Y	Range	O	Range
0	26	5–8	28	5–10	14	2–5	12	0–5
160	24	4–8	26	5–8	16	2–6	12	2–5
320	35	8–9	32	6–9	5	1–2	7	1–3
480	31	3–11	30	6–9	10	0–7	7	1–3
600–675	32	6–9	26	4–8	8	1–4	9	1–4
750–860	38	7–10	32	4–10	4	0–3	6	0–6
900–960	33	6–11	29	3–10	12	1–4	4	0–2
Apex	52*	8–18	54*	10–17	8	0–2	10	0–4
Total	271		257		77		67	

* Significant increase ($p < 0.05$) in postcapillary-sized venule profile number located in the bone 1/3 at the apex. However, this result may not be significant due to the inclusion of more levels at the apex.

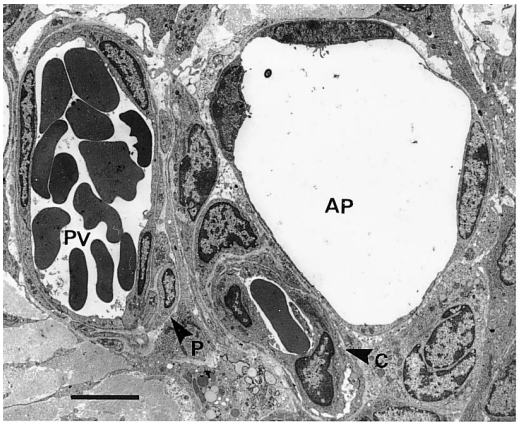

Figure 2. An arterial capillary (C) in close proximity to an apericytic postcapillary-sized venule profile (AP), a pericytic postcapillary-sized venule profile (PV) and a pericyte (P). Uranyl acetate and Reynold's lead stain. Mag. = x2,900 Bar = 5 μm.

values, and classified by mouse group, PDL level and side (left and right). Arithmetic means did not account for the variation in the numbers of measurements, indicating that the data sets were unbalanced. The means for each PDL level, therefore, were estimated using an analysis of covariance,[21] with the effect of mice being viewed as covariants.

Age effects were tested using a multiple regression technique that compared the effect of age with the residual between mouse variance. By assuming that the occurrence of PCV profiles across the PDL from tooth root to alveolar bone was independent for each mouse group, a test of significance could be made. In this case, a generalised linear model with a poisson distribution[21] was used to test for the effect of age. A value of $p < 0.05$ was taken as significant.

Results

PCV profiles type

The blood vessels assessed were aPCV profiles and pPCV profiles (Figure 2). Some fenestrated PCV profiles also were identified. The total number of aPCV profiles and pPCV profiles counted for young mice (348) was comparable to the number for aged mice (324; Table I). aPCV profiles were more common in both groups at all PDL levels. The number of aPCV profiles increased significantly with increasing depth of the PDL from the alveolar crest to the apex ($p < 0.05$) in each age group; however, this result may not be significant due to the inclusion of more levels at the apex (Table I).

The type of PCV profiles also differed across the PDL, as shown in Table II. Although there were few PCV profiles in the tooth third in both young and aged mice, they were with a single exception aPCV profiles. A significantly larger number of aPCV profiles ($p < 0.001$) in the tooth third of the aged mice was found when compared with the young mice.

Table II. Total number and ratios of pericytic and apericytic postcapillary-sized venule (PCV) profiles for young (Y) and aged (O) mice in each location horizontally across the periodontal ligament.

PCV profiles type	Region Tooth Y	1/3 O	Middle Y	1/3 O	Bone Y	1/3 O	Total PCV
Pericytic	0	1	22	23	55	43	Y = 77, O = 67
Apericytic	1	14***	54	47	216	196	Y = 271, O = 257
Pericytic:Apericytic	0:1	1:14	1:2.5	1:2.0	1:3.9	1:4.6	
Pericytic:Total PCV	0:1	1:16	1:3.5	1:3.0	1:4.9	1:5.6	

*** Significant increase (p< 0.001) in the number of apericytic postcapillary-sized venule profiles in the tooth 1/3 in aged mice.

Table III. Total number of postcapillary-sized venule profiles (apericytic and pericytic) in each of 8 mice classified by location across the periodontal ligament (Region) from the root to the alveolar bone.

Mouse age	Region Tooth 1/3	Middle 1/3	Bone 1/3	Total
Young (Y)	0	16	65	81
Y	0	20	66	86
Y	0	19	73	92
Y	1	21	67	89
Total Y	1	76	271	348
Aged (O)	3**	11	60	74
O	4**	13	66	83
O	8**	29	46	83
O	0	17	67	84
Total O	15	70	239	324

** Significant increase (p <0.01) in aged mice postcapillary-sized venule profile number located in the tooth circumferential 1/3 of the periodontal ligament.

PCV profile location

The sampling of PCV profiles in each of the three circumferential regions of the PDL was examined in a random order. Therefore, a difference between ages could be investigated, but not quantified stereologically. The numbers of PCV profiles sampled in each mouse and in each of the three circumferential regions (tooth third, middle third and bone third) across the PDL are summarised in Table III. PCV profiles were significantly (p < 0.01) more common in the bone third of the PDL, and declining in the middle third, with few found in the tooth third. Using a generalised linear model with a poisson distribution, a significant increase in the number of PCV profiles in the tooth third in the aged mice (p < 0.01) was found, as shown in Tables II, III and IV. As noted above, these PCV profiles were predominantly apericytic (p < 0.001). There was animal variability, such that one mouse in the aged group did not have any PCV profiles in the tooth third of the PDL in the tissue samples examined. Except for one mouse, no PCV profiles were found located in the tooth third in young mice.

The numbers of PCV profiles in the PDL middle circumferential third of the aged mice were more uniformly found from coronal to apical levels (Table IV), and significantly greater numbers of PCV profiles (p < 0.001) were observed halfway down the PDL; whereas, in the middle PDL circumferential third of the young mice, there were significantly fewer (p < 0.001) PCV profiles, and a steady reduction in PCV profile numbers from the alveolar crest to a minimum at the mid-point of the PDL, followed by a steady increase in numbers at the apex (Table IV). A significantly (p < 0.05) greater number of PCV profiles was found in the apical bone third in aged mice (Table IV). However, these data may be skewed due to the inclusion of additional PDL levels at the apex.

PCV profile diameter

Age had no effect on PCV profile diameter (average 20.9 µm). The mean luminal diameters of the PCV profiles at each PDL level (coronal to apical) for each group are shown in Table V.

Table IV. Total number of postcapillary-sized venule profiles for young and aged mice classified by the location across the periodontal ligament (Region) from the tooth to the alveolar bone, and from coronal to apical levels (μm).

Level (μm)	Region Tooth 1/3 Young	Aged	Middle 1/3 Young***	Aged	Bone 1/3 Young	Aged
0	1	2	19	12	20	26
160	0	1	6	6	34	31
320	0	1	4	12	36	26
480	0	3	3	8	38	26
600-675	0	3	0	12***	40	20
750-860	0	4	9	8	33	26
900-960	0	1	11	6	34	26
Apex	0	0	24	6	36	58*
Total	1	15**	76	70	271	239

* Significant increase ($p< 0.05$) in aged mice postcapillary-sized venule profile number located in the bone 1/3 at the apex. However, this result may not be significant due to the inclusion of more levels at the apex.

** Significant increase ($p< 0.01$) in aged mice postcapillary-sized venule profile number located in the tooth circumferential 1/3 of the periodontal ligament.

*** Significant decrease ($p< 0.001$) in young mice postcapillary-sized venule profile number from the alveolar crest to halfway down the middle 1/3 of the periodontal ligament, and increasing towards the apex.

*** Significant increase ($p< 0.001$) in aged mice postcapillary-sized venule profile number halfway down the middle 1/3 of the periodontal ligament.

Table V. Luminal diameter (μm) of all postcapillary-sized venule profiles (apericytic and pericytic) for young and aged mice at each periodontal ligament level (μm).

Level (μm)	Young		Aged	
0	13.6†	14.3‡	13.1	12.1
	(1.8)		(1.0)	
160	15.3	13.5	12.6	12.8
	(1.1)		(1.3)	
320	13.3	16.8	13.3	12.4
	(1.1)		(1.3)	
480	15.1	15.5	14.5	12.0
	(0.9)		(1.5)	
600–675	14.9	14.8	13.8	9.4
	(1.3)		(0.7)	
750–860	14.9	14.6	13.2	12.3
	(0.8)		(0.9)	
900–960	15.0	16.5	12.3	12.8
	(1.0)		(1.0)	
Apex	14.4	13.6	13.8	14.3
	(1.2)		(1.2)	

† Arithmetic means with standard error values in parentheses.

‡ Mean values calculated using analysis of covariance. They were adjusted because of different numbers of values at each periodontal ligament level.

Discussion

The number of PCV profiles sampled in young mice in the current study was found to coincide with the volume distribution of PCV profiles (that is, number of PCV profiles per unit area) previously reported by Freezer and Sims[20] to be 0 per cent in the tooth third, 25 per cent in the middle third and 75 per cent in the bone third. The sampling techniques differed between the two studies. Namely, the current study sequentially sampled five PCV profiles per PDL level, while Freezer and Sims[20] utilised a stereological sampling method for all microvessels at each PDL level. The similar findings of the two studies indicate that the sample size used in the current study adequately represented the PCV profiles population in the PDL. Furthermore, because of variability in TEM section orientation and the large size of the total sample, it is argued that the sampling bias was sufficiently minimised.

The current study found significant age effects on PCV profiles type and location. In the aged mice, an increased number ($p < 0.01$) of PCV profiles was found in the tooth third of the PDL, which were predominantly aPCV profiles ($p < 0.001$). Furthermore, a significantly ($p < 0.001$) higher number of PCV profiles was found in the aged mice PDL middle circumferential third halfway down the molar root.

The aPCV profiles were more common overall for young and aged mice in the current study, which is consistent with the study by Freezer and Sims,[20] who found 70 per cent of PCV profiles were apericytic and 30 per cent of PCV profiles had associated pericytes. The PCV profile types differed between the lateral zones of the PDL for young and aged mice. The few PCV profiles in the tooth third for young and aged mice were, with a single exception, of the apericytic type. Freezer and Sims[20] reported an absence of any pPCV profiles in the tooth third of their tissue sample.

It is suggested that the increased aPCV profiles in aged mice may be a compensatory measure to increase vessel permeability. Therefore, more aPCV profiles in the aged mice may balance the increased number of tight regions found in aged mice endothelial junctions.[24] In other words, in the aged mice PCV profiles there are more junctional regions with fewer pericytic cells. However, it should be noted that an increase in the number of tight regions simply may be due to an increase in the number of endothelial cells per unit area of PCV profiles. Investigation of the number of endothelial cells per unit area of PCV profiles in young and aged mice is necessary to determine whether changes in numbers of endothelial junctions are correlated with endothelial cell numbers.

It may be postulated that alterations in the proportions of pPCV profiles to aPCV profiles may indicate that the vessel wall becomes more permeable from the alveolar crest to the apex. The mean number of perivascular interstitial tissue channels per μm^2 (MNTC/μm^2) has been shown to increase in number with increasing fluid flow.[25,26] If there is increased permeability of PCV profiles in the apical regions, then there may be an increase in MNTC/μm^2. However, Tang and Sims[26] did not demonstrate any significant difference in the MNTC with increasing depth of normal rat PDL. This contrast may be due to other factors important in controlling permeability, such as the distribution of junction type and/or other PDL microvessels that maintain a consistent amount of fluid flow, with no corresponding change in the MNTC with an increasing depth of PDL.

It may also be postulated that the PCV profile vessel walls may have heterogenous permeability for the lateral zones of the PDL, taking into account the distribution of PCV profiles across the PDL.[20] In the current study, the numbers of PCV profiles were shown to increase from the tooth third to the bone third. Furthermore, the majority of PCV profiles in the bone third are apericytic, and, therefore, presumably more permeable. This is consistent with the reported steady increase in the MNTC/μm^2 from tooth third to the bone third of the rat PDL.[26]

Alterations in microvascular permeability can occur with increasing age.[9] Sims *et al.*[27] assessed ageing changes in mouse blood vessel luminal volumes stereologically, using TEM, and demonstrated that redistribution of blood vessels across and down the length of the mouse PDL was evident with a reduction in diffusion distances. These same researchers[27] examined PDL blood vessel luminal diameter, volume density and distribution; however, these parameters may not be directly related to permeability and further investigation is required to functionally assess microvessel permeability in young and aged mice in conjunction with assessment of tissue cleft per unit area. This would be valuable in providing further morphological data on all the blood vessel categories in the PDL, as it has been found that there is a decrease in volume, length and surface density of pPCV profiles and aPCV profiles in aged mice when compared with young mice.[27] It is necessary to investigate these aspects to determine whether changes in

volume, length and surface density of these vessels are associated with concomitant changes with age in endothelial junction type and widths[24] and proportion of pPCV profiles and aPCV profiles.

There was no significant effect of age on PCV profile luminal diameter in the current study. The PCV profile diameters measured in the current study were smaller in comparison with those reported by Sims *et al.*[27] and fall within the quoted ranges for PCV profile diameters.[5,16]

The number of PCV profiles differed between the lateral areas of the PDL with a significant increase in the number of PCV profiles located in the tooth third in the aged mice. Another significant variation in PCV profile numbers involved the middle circumferential third in the young mice: there was a steady reduction in PCV profile numbers in the young mice from the alveolar bone to a minimum at the mid-point of the PDL, followed by a steady increase in numbers to the apex. The implications of this numerical variation in relation to masticatory function need to be investigated. The absence of this trend in the aged mice, where the numbers of PCV profiles did not reduce in the middle circumferential third down the length of the PDL, was consistent with an increase in the vascularity of the PDL with age.[27] This raises the possibility that the PCV profile numbers become more uniform with age.

Conclusions

The findings of the current study may indicate that, with age, a variable degree of permeability in the microvascular barrier exists across and down the mouse PDL and that it is due to: 1. the larger number of aPCV profiles in the tooth third in the aged mice; 2. the larger number of PCV profiles halfway down the middle circumferential third of the PDL in aged mice, and 3. the decreased proportion of pPCV profiles to aPCV profiles in the tooth third in comparison with the middle and bone third in aged mice.

The alterations of PCV profile numbers in the PDL of mice found in the current study may represent functional modification of PDL microvasculature during ageing. However, microvascular permeability cannot be confirmed by ultrastructural findings alone. Studies assessing permeability in aged PDL in conjunction with other factors that contribute to permeability of the microvasculature are needed to confirm these concepts. Further assessments of the clinical significance of these aging changes is required.

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