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Effect of aging on the microstructure, hardness and chemical composition of dentin

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ABSTRACT

Objective: Understanding the effects of biological aging on human tissues has been a topic of extensive research. With the increase in healthy seniors and quality of life that topic is becoming increasingly important. In this investigation the effects of aging on the microstructure, chemical composition and hardness of human coronal dentin was studied from a comparison of teeth within "young" and "old" age groups.

Methods: The microstructure of dentin within three regions (i.e., inner, middle and outer) was analyzed using electron and optical microscopy. The mineral-to-collagen ratio in these three regions was estimated using Raman spectroscopy and the hardness was evaluated using microindentation.

Results: Results showed that there were significant differences in tubule density, tubule diameter and peritubular cuff diameter with depth. Although there was no difference in tubule density and diameter of the tubules between the age groups, there was a significant difference in the occlusion ratio. A significant increase in hardness between young and old patients was found for middle and outer dentin. An increase in mineral-to-collagen ratio from inner to outer dentin was also found for both groups. In old patients, an increase in mineral content was found in outer coronal dentin as a consequence of tubule occlusion.

Conclusions: An increase in occlusion ratio, hardness, and mineral content was found in the dentin of adult patients with age. This increase is most evident in the outer coronal dentin.

1. Introduction

The effect of aging on the microstructure and mechanical properties of bone has been studied extensively due to its importance to the elderly and their quality of life (e.g., (Currey, Brear, & Zioupos, 1996; Nalla, Kruzic, Kinney, & Ritchie, 2004; Ural & Vashishth, 2006; Ural & Vashishth, 2007; Wang & Puram, 2004; Wang, Shen, Li, & Agrawal, 2002; Zioupos & Currey, 1998; Zioupos, Currey, & Hamer, 1999)). However, the effect of aging on dental hard tissues (including dentin and enamel) has received rather limited attention. That is surprising when one considers the importance of human teeth to mastication and dietary intake.

Dentin is a hard tissue that occupies the majority of the human tooth. By volume it consists of approximately 45% mineral material, 33% organic material (collagen type I) and 22% water (Nanci, 2012). The thickness of dentin (i.e., from the pulp to the dentin enamel junction (DEJ)) is largely dependent on tooth type, but generally ranges from roughly 2 mm for mandibular incisors up to 3 mm in canines and molars. Furthermore, the thickness of dentin tends to increase with aging as a result of appositional growth (Gómez de Ferraris & Campos Munoz, 2009).

The microstructure of dentin is largely dominated by its tubules, which are responsible for housing the odontoblastic processes. The tubules extend from the pulp to the DEJ. A highly mineralized cuff of peritubular dentin surrounds the lumen of each tubule and contains mainly apatite crystals and a small proportion of organic proteins. The tissue located between the tubules is called intertubular dentin and contains a matrix of collagen fibers reinforced by apatite (Marshall, Marshall, Kinney, & Balooch, 1997). Based on its composition and structure, dentin is considered a hierarchical biological composite (Ziskind, Hasday, Cohen, & Wagner, 2011).

Dentinal tubules possess diameters ranging from approximately 1 to 3 μm, depending on patient age (Ingle, Bakland, & Baumgartner, 2008). Studies have shown that after the third decade of life there is a transition in the microstructure of dentin, in which the tubules become gradually filled with inorganic

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material (Kinney, Nalla, Pople, Breunig, & Ritchie, 2005). After a significant number of tubules have been filled, the tissue appears transparent, and is generally considered as "sclerotic". This process results in an increase in the mineral content of dentin, opposed to what occurs in bone where there is largely a decrease in mineral content with aging (Rosen, Glowacki, & Bilezikian, 1999). Furthermore, this increase in mineral content has been usually associated with increasing dentin fragility and, therefore, causes a variation in its mechanical properties (Koester, Ager, & Ritchie, 2008; Nazari, Bajaj, Zhang, Romberg, & Arola, 2009). Understanding the mechanical properties of dentin is important to comprehend the structural behavior of teeth with aging and in the development of new dental materials.

According to Kinney, Marshall, and Marshall (2003), the Young's Modulus of young dentin ranges from 20 GPa to 25 GPa, and the tensile strength ranges from 52 MPa to 105 MPa. The flexure strength ranges from roughly 130–180 MPa (Ryou et al., 2011). The results obtained for fracture toughness are similar to those found for bone. Values of roughly 1.7 MPa $m^{0.5}$ and 2.0 MPa $m^{0.5}$ have been obtained when tests were performed with crack extension parallel and perpendicular to the dentinal tubules, respectively (Arola et al., 2009). When measured by means of Vickers and microhardness tests, the average hardness of dentin is of about 0.5 GPa, with no significant dependence on indentation load or indentation time (Chuenarrom, Benjakul, & Daosodsai, 2009).

Within the field of dentistry, the importance of aging has become of greater interest in recent years due to its impact on the practice of restorative dentistry. Indeed, the tooth undergoes certain changes with age, including wear of enamel, the formation of transparent dentin, a decrease in the number of odontoblasts and an increase in dentin thickness as well as a production of reactionary dentin (Nanci, 2012). The changes in dentin microstructure produce variations in its mechanical properties, which are important for the introduction of restorative treatments and the greater potential for tooth fractures.

Several studies have been performed toward understanding the influence of aging on the mechanical behavior of dentin. For instance, Zheng, Nakajima, Higashi, Foxton, and Tagami (2005) analyzed the changes in hardness and Young's modulus of dentin with aging and reported that dentin does not undergo a significant change in hardness or Young's modulus with age in the middle and inner dentin. However, they found an increase of 16% in hardness and around 5% in Young's modulus within the outer dentin. Further studies have shown that the flexural strength of coronal dentin decreases almost 20 MPa per decade of life, beginning after adulthood. In addition, a reduction of 75% in the energy required to fracture dentin between young (age $\leq$ 30) and old patients (age > 55) was reported (Arola et al., 2009).

Changes in mechanical properties of dentin with aging have largely been attributed to the increase in mineralization due to filling of the dentinal tubules. However, it remains unclear whether these changes can be attributed to the mineral occupying the dentinal tubules, a complimentary change of the mineral of the intertubular dentin, or crosslinking of collagen by non-enzymatic processes (Miura et al., 2014). In fact, little information is available on the relationship between the changes in microstructure of dentin with age and spatial variations in chemical composition. Thus, the aim of this work was to identify the changes in microstructure, chemical composition and hardness of dentin with aging from selected age groups of Colombian patients.

2. Materials and methods

Human third molars were obtained from selected patients after written consent and following all the protocols required by the Dental Clinic at Universidad Cooperativa de Colombia (UCC). Exclusion criteria included presence of caries and previous restorations. The teeth were obtained from donors residing in Medellín, Colombia, and were divided into two age groups, namely a "young" group with donors between 18 and 25 years of age (N = 12), and an "old" group with donors between 47 and 65 years of age (N = 8). There were an equal number of male and female samples in both groups. Immediately after extraction, all the specimens were kept in Hank's Balanced Salt Solution (HBSS) at 2 °C to avoid dehydration and loss of mineral (Habelitz, Marshall & Balooch, 2001). In addition, the specimens were tested within two weeks of extraction to limit the loss of mineral and organic materials.

Each molar was sectioned along its longitudinal axis (section A–A in Fig. 1a) using diamond abrasive slicing equipment with

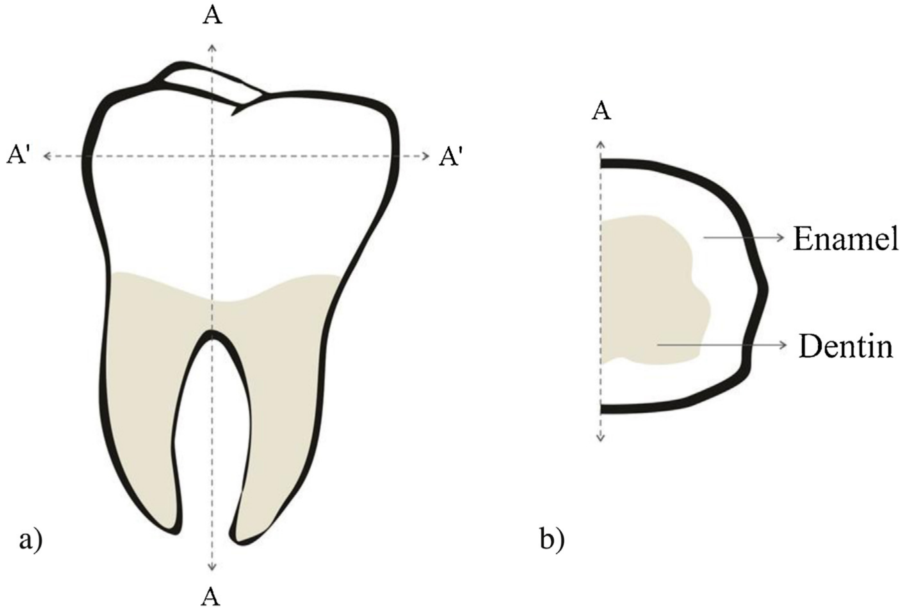

Fig. 1. Schematic diagram of a sectioned molar after (a) longitudinal (A–A), and (b) transverse (A′–A′) cutting. The specimen is then embedded in cold-cure epoxy resin with the sectioned surface facing outwards.

continuous water coolant. Secondary sections were cut transversely (section A′–A′) in order to expose the dentin as shown in Fig. 1. For indentation analysis and microscopic evaluations, the specimens were embedded in cold-cured epoxy resin, following similar procedures used by other researchers (Brauer, Hilton, Marshall, & Marshall, 2011; Park, Wang, Zhang, Romberg, & Arola, 2008; Rivera, Arola, & Ossa, 2013). The exposed dentin in the resin mount was polished using silicon carbide abrasive paper with successive smaller particle sizes until reaching 1200 grit. Further polishing by means of standard red felt polishing cloth wheels was then performed using diamond particle suspensions of 3 μm in size. After polishing, all samples were ultrasonically cleaned in an HBSS bath for 30 min before microscopic observation in order to eliminate particles of the diamond particle suspension or tissue resulting from the polishing process. The polished specimens were then kept in a HBSS bath solution prior to testing.

Vickers testing was used to study the variation in hardness as a function of dentin depth. Microindentation was performed using a micro-hardness tester (Wilson Instruments, Model 402 MVD, Norward, MA, USA) with a Vickers diamond indenter. Ten indentations were made on each surface, starting at the DEJ. Grinding and polishing was then performed to remove approximately 500 μm of material, after which another 10 indentations were performed. This procedure was repeated until the pulpal surface was reached. Indentations were made using an indentation load of 1.96 N and dwell times of 10 s. These testing conditions generate an indentation large enough so that the hardness corresponds to the overall dentin hardness, which includes hardness of intertubular and peritubular dentin. Indentations were carefully made with a distance of at least 10 diagonals in length from each other in order to avoid any deformation from neighboring indentations.

The Vickers hardness number (HV) was estimated following the ASTM C1327 (2008) standard according to:

$$HV = \frac{0.1891 \times F}{d^2}, \qquad (1)$$

where F is the indentation load and d is the indentation diagonal.

The same specimens were used to evaluate the dentin microstructure using an optical microscope (Axiovert 40 MAT, Carl Zeiss Microscopy, NY). Tubule density, diameter of the tubule lumens and diameter of the peritubular dentin were measured and calculated at each depth. A determination of such parameters was carried across the sample surface. Seven images, each with constant area, were randomly selected from every polished surface. In each image, the amount of tubules was calculated and expressed as tubules/mm^2. The tubule diameter and peritubular dentin diameter were also obtained. Values from the seven images were averaged to obtain information from each depth.

The results obtained for hardness and microstructure at each depth were normalized to the dentin thickness and then classified as outer (normalized depth between 0.76 and 1.00), middle (between 0.36 and 0.75) or inner (depth between 0.00 and 0.35) dentin. Differences in hardness between the outer, middle and inner dentin, as well as between young and old patients were evaluated using a two-way analysis of variance (ANOVA), defining significance of results by p-value ≤ 0.05.

For the analysis of chemical composition, selected molars from each of the two age groups were sectioned along the longitudinal axis (section A–A in Fig. 1a) and embedded in cold-cure epoxy resin with the sectioned surface facing outwards. The section surfaces were polished following the process previously explained. Chemical composition analysis of outer, middle and inner dentin was performed using Raman spectroscopy and the results were used to determine the mineral-to-collagen ratio for the three

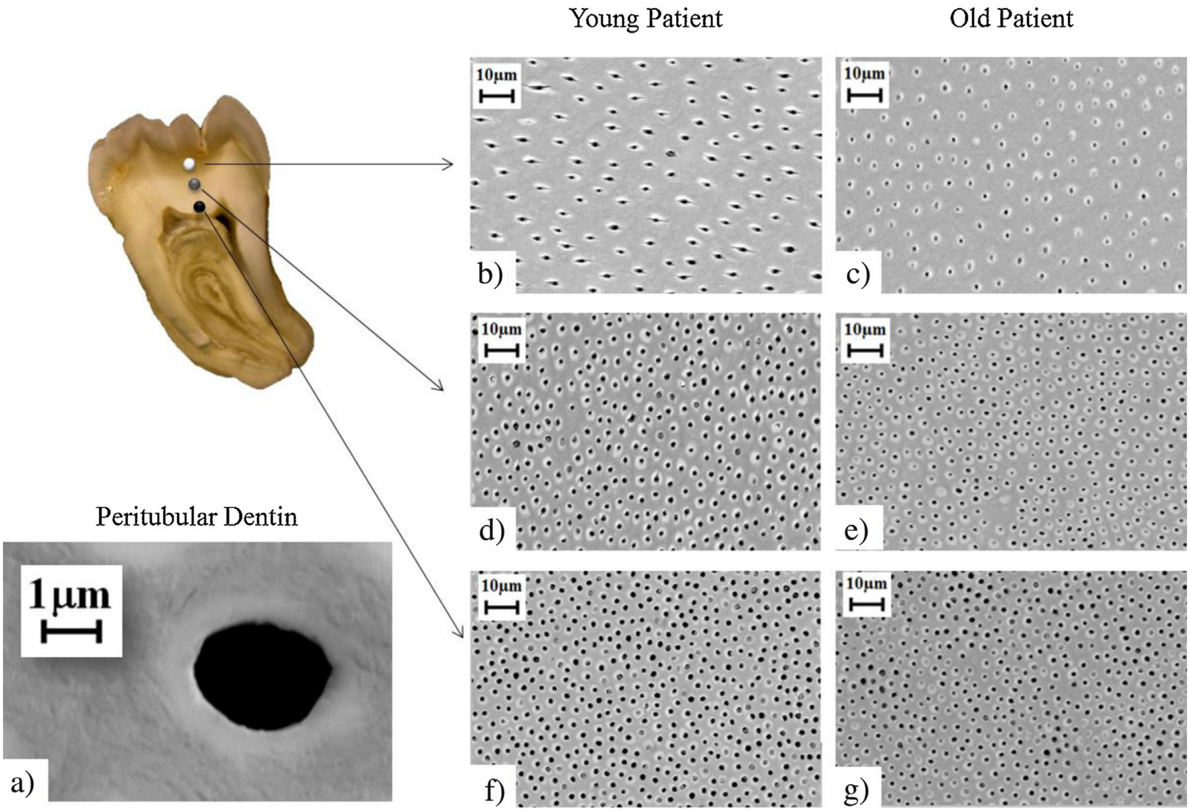

Fig. 2. Micrographs of the dentin microstructure. (a) Single tubule; (b and c) outer dentin; (d and e) middle dentin; (f and g) inner dentin. Note the obliterated dentinal tubules for the old donor teeth in the middle and outer regions (evident in (e) and (g)).

regions (i.e., inner, middle and outer). A confocal Raman spectrometer (Horiba Jobin Yvon LabRAM HR) was used and the spectrums were obtained over the spectral region of 400–1100 cm^{-1}. The Raman spectrometer had a laser diode with a wavelength of 785 nm and a spot diameter of approximately 1.1 μm. The mineral-to-collagen ratio was calculated from the ratio of area under the υ_4PO_4 peak at 589 cm^{-1}, which is associated with the phosphate bending of hydroxyapatite, and the area under the amide III peak at 1254 cm^{-1}, which is associated with movements of the peptide bond present in collagen (Goodyear, Gibson, Skakle, Wells, & Aspden, 2009; Kazanci, Roschger, Paschalis, Klaushofer, & Fratzl, 2006). These bands were selected for analysis as they are reportedly less susceptible to orientation effects and the polarization direction of the incident light (Kazanci et al., 2006).

Differences in the mineral-to-collagen ratio between the outer, middle and inner dentin, as well as between young and old patients were evaluated using a two-way analysis of variance (ANOVA), defining significance of results by p-value ≤ 0.05. Representative maps of hardness and mineral-to-collagen ratio were also obtained from selected teeth to convey the spatial variations present.

3. Results

3.1. Microstructure

The microstructure of dentin from selected young and old donor teeth is shown in Fig. 2. The microstructures shown for each group correspond to the three regions of coronal dentin evaluated, including the outer (Fig. 2b and c), middle (Fig. 2d and e) and inner (Fig. 2f and g) regions. As evident from the figures, there was an increase in tubule density and diameter of the tubules as the distance from the DEJ increased. Peritubular dentin can be seen surrounding each dentinal tubule (e.g., Fig. 2a). There was no differences in appearance between the peritubular dentin from young and old donor teeth. In comparing the two age groups, some of the tubules in the old donor group appeared to be obliterated, with greater number in the middle and outer dentin. Micrographs of tubules for young and old donor teeth are shown in Fig. 3.

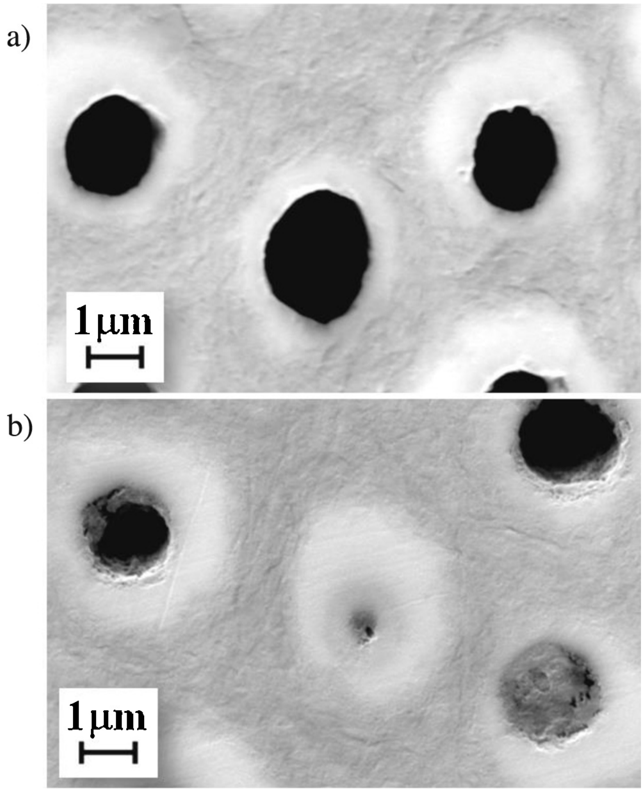

Fig. 3. Micrographs of dentinal tubules from outer dentin. (a) Young donor; (b) old donor.

A quantitative comparison of the microstructural characteristics of dentin from the young and old groups is shown in Fig. 4. Specifically, Fig. 4(a) shows the tubule density in different regions of coronal dentin. A reduction in the tubule density was observed with increasing proximity to the DEJ in both age groups. For the young donor group the average tubule density in the outer and inner regions was 25,000 $tubules/mm^2$ and 35,000 $tubules/mm^2$, respectively. A similar distribution was found for the old donor group (i.e., decrease in tubule density approaching the DEJ), but there was some difference in the tubule count. The most noticeable change was found in the outer dentin with lower tubule density (10% less) in comparison to that of young donors.

A comparison of the average tubule diameter within the different regions of dentin is shown in Fig. 4(b). In the young donor group the average lumen diameter in the outer and inner regions was 1.36 ± 0.12 μm and 1.84 ± 0.10 μm, respectively. For the old group these values were 1.24 ± 0.08 μm and 1.81 ± 0.35 μm, respectively. Results obtained for the peritubular cuff diameter are shown in Fig. 4(c). A decrease in the peritubular cuff diameter was found with increasing proximity to the DEJ for both age groups. In the young group the average diameters within the inner and outer regions were 3.51 ± 0.69 μm and 2.81 ± 0.40 μm, respectively. A similar result was found for old patients, where a larger diameter of tubules near the pulp was observed.

Overall, there was a significant decrease in tubule density, tubule diameter and peritubular cuff diameter with depth ($p \leq 0.05$) with proximity to the DEJ for both age groups. In comparing results for the microstructure between young and old donor teeth, the differences found for tubule density, diameter, and peritubular dentin diameter were not statistically significant ($p > 0.05$).

3.2. Hardness

The influence of indentation load on hardness was studied in order to identify the proper load for measuring the hardness of dentin. Results of that evaluation are shown in Fig. 5. The measured hardness values decrease from approximately 1.5 GPa at 0.23 N indentation load to 0.7 GPa at 1.96 N load. A plateau in hardness is observed for loads of 1.96 N and greater. Consequently, all further hardness measures were conducted with a load of 1.96 N. The average hardness for the three regions of dentin are shown in Fig. 6 for both the young and old donor teeth. These values correspond to hardness measured with load applied parallel to the dentinal tubules, as shown in Fig. 5b and c.

Representative indentations made within the outer dentin of young and old donor teeth are shown in Fig. 7a and b, respectively. Indentations within the outer dentin covered an average of 36 tubules within the body of the indentation; for the inner dentin that number was 170 tubules. For the old donor teeth an average of 27 and 156 tubules were included in the outer and inner dentin, respectively. It is worth noting that no cracks were observed emanating from the indentation corners for either group, even at the highest indentation load (9.8 N). According to the distribution in measurements, an increase in hardness was found with proximity to the DEJ for both age groups. Average hardness values of 0.65 ± 0.03 GPa and 0.68 ± 0.01 GPa were found for the young and old donor groups, respectively. When comparing hardness results with respect to the specific regions of dentin between young and old donors, significant differences were found for the

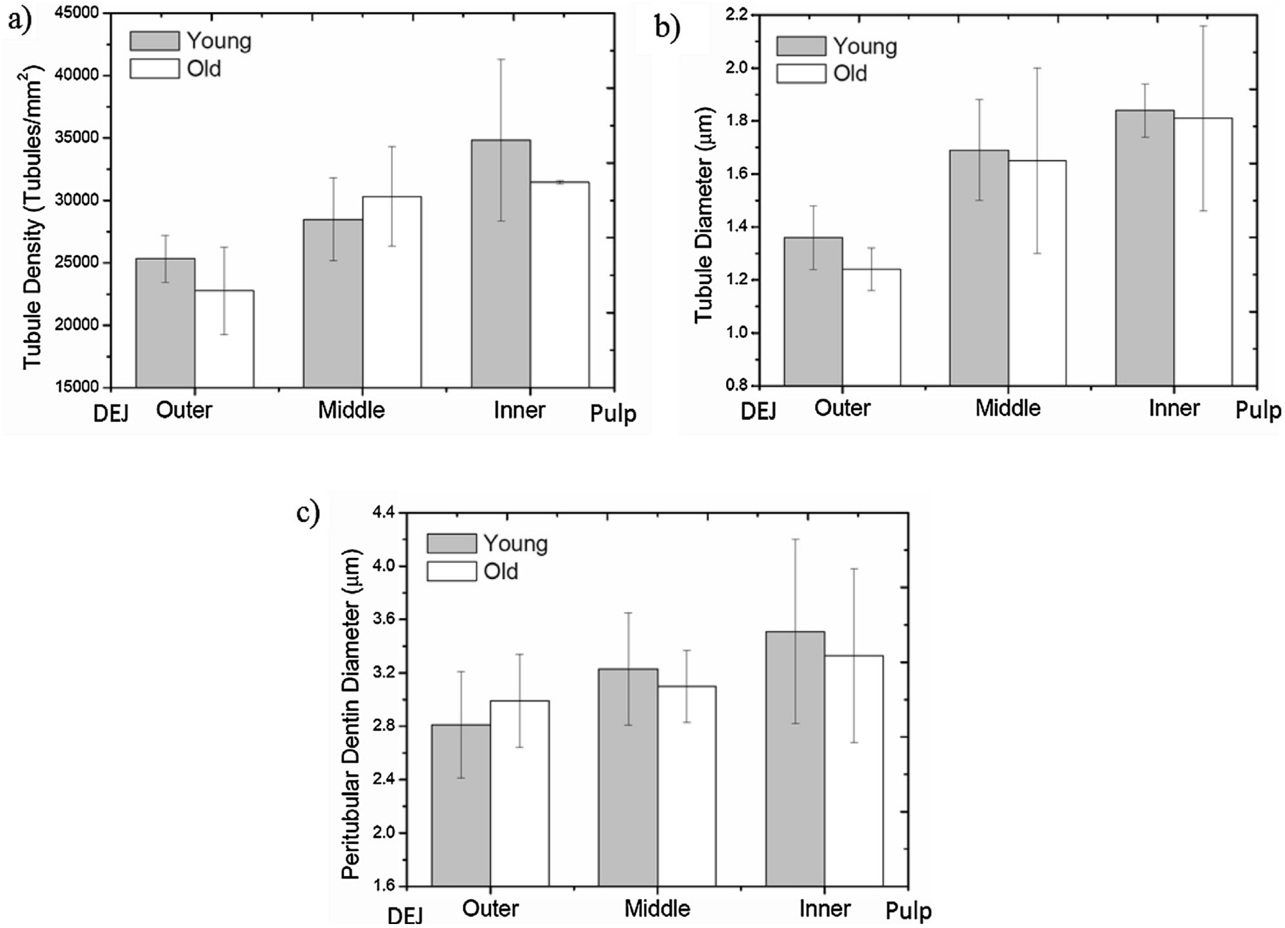

Fig. 4. Comparison of microstructure as a function of depth in the coronal dentin. (a) Tubule density; (b) tubule diameter; (c) peritubular dentin diameter.

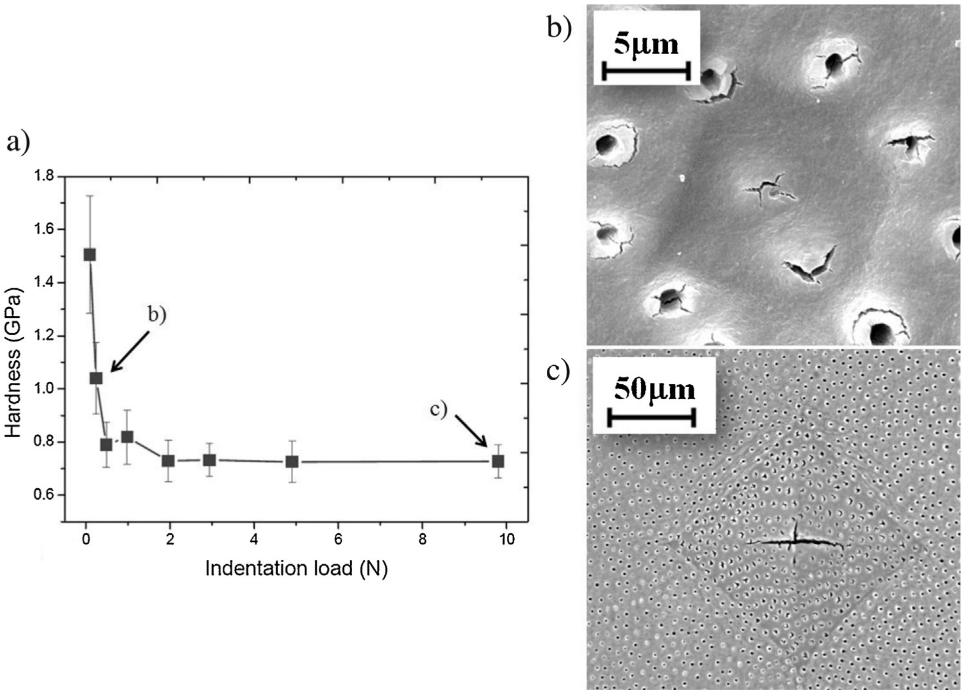

Fig. 5. Effect of indentation load on the Vickers hardness of dentin from a young donor tooth. (a) Change in hardness with indentation load; (b) indentation at 0.23 N; (c) indentation at 9.80 N.

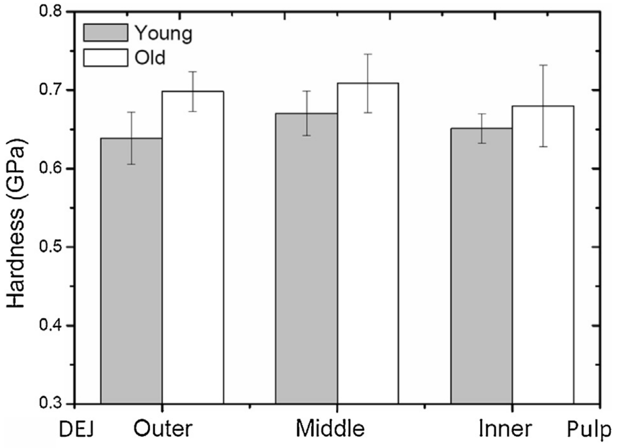

Fig. 6. Vickers hardness obtained for dentin from young and old donor teeth according to depth. The direction of applied load is parallel to the dentinal tubules.

outer and inner dentin between young and old dentin (p-value ≤ 0.05).

3.3. Chemical composition

The distribution of mineral-to-collagen ratio as a function of distance across the coronal dentin for young and old donor teeth is shown in Fig. 8. The dentin in both age groups showed a similar behavior, with increasing mineral-to-collagen ratio approaching the DEJ. A higher ratio indicates a lower proportion of organic material. The differences found among the areas evaluated in the young donors group were not statistically significant ($p > 0.05$), as opposed to the old donors group where they were statistically significant ($p \leq 0.05$).

When comparing the mineral-to-collagen ratios between the young and old patients, nearly a 4% difference was found nearest the pulp within the inner dentin. However, the difference in mineral-to-collagen ratio was approximately 40% in the middle dentin and 70% in the outer dentin. In comparing results for the mineral-to-collagen ratio between young and old donor teeth, the differences were statistically significant ($p \leq 0.05$).

Comparisons of hardness and mineral-to-collagen ratio distributions in representative teeth from each age group after longitudinal sectioning can be seen in Fig. 9. The results correspond to a young donor (18 years of age) and an old donor

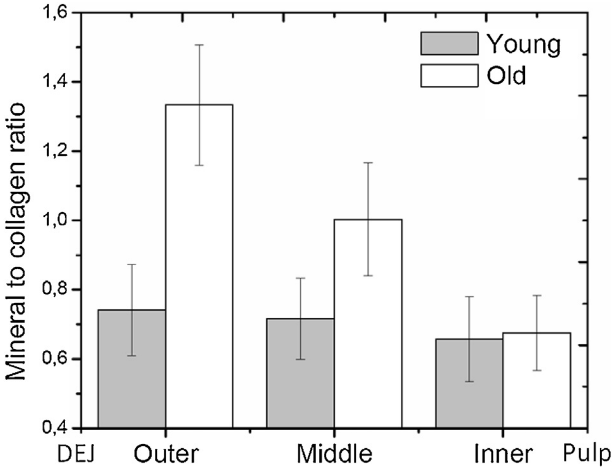

Fig. 8. Distribution of mineral-to-collagen ratio of dentin from young and old donor teeth according to depth.

(65 years of age). For the young donor tooth there was an increase in hardness from the pulp up to the DEJ (Fig. 9a). As for the chemical composition of the young dentin (Fig. 9b), there was an increase in the mineral-to-collagen ratio with increasing proximity to the DEJ. In comparing Fig. 9a and b it is seen that lower hardness values were obtained in regions of lower mineral-to-collagen ratio (inner dentin). Conversely, where a higher proportion of mineral was present, the hardness was greater.

Results of the hardness and mineral-to-collagen ratio measurements for an old donor tooth are shown in Fig. 9c and d. The chemical composition map for the old donor tooth shows a distorted pulp chamber due to deposition of secondary dentin within. This process can lead to complete pulp obliteration over sufficient time (Tronstad, 2008).

4. Discussion

4.1. Microstructure

Consistent with the findings of earlier studies, there were differences in tubule density and dentinal tubule diameter as conveyed from the measures within the three regions of dentin evaluated (Fig. 2). The results obtained for tubule density in the young patients group are consistent with those of Marshall et al.

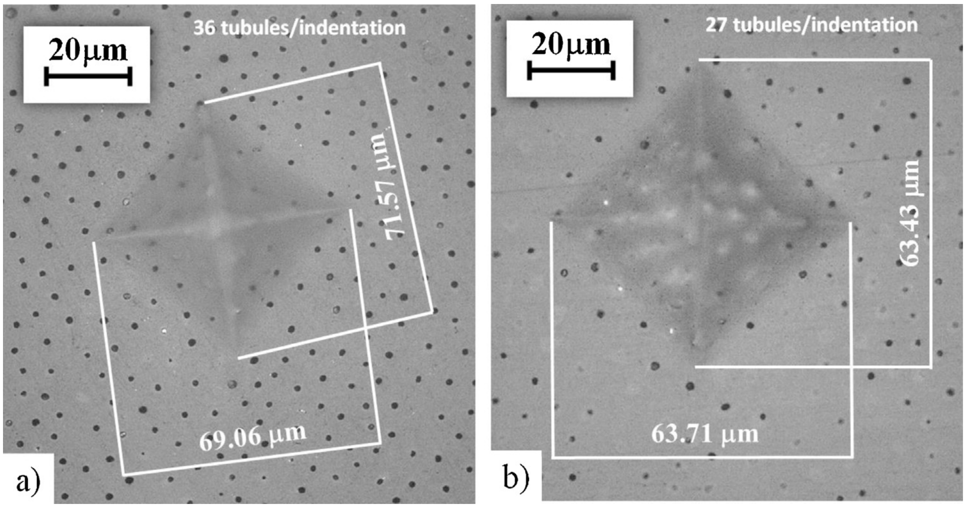

Fig. 7. Indentation of dentin for determination of Vickers hardness. The indentation locations are within the outer dentin of a young (a) and old (b) donor tooth.

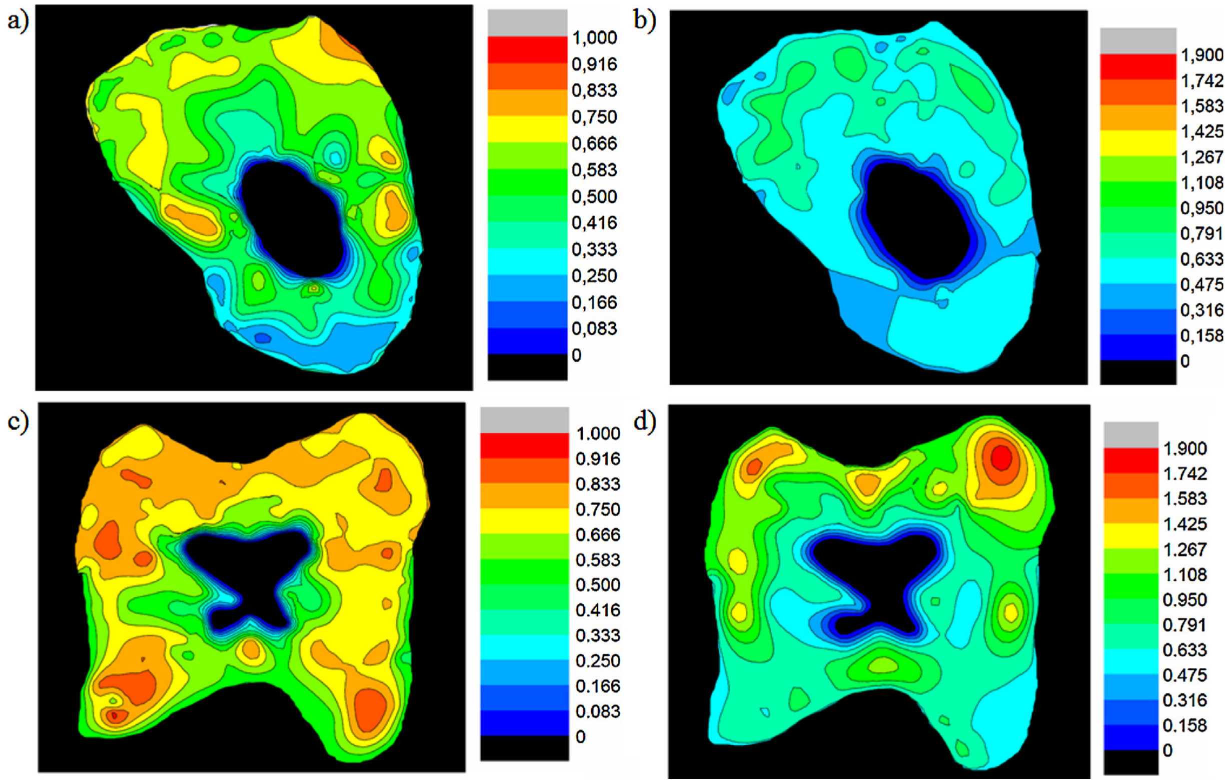

Fig. 9. Comparison of the hardness and chemical composition distributions in a tooth as evident from longitudinal sectioning. (a) Hardness and (b) chemical composition (mineral to collagen ratio) from the tooth of a 18 year old donor, (c) hardness and (d) chemical composition from the tooth of a 65 year old donor.

(1997), who reported values of 20,000 tubules/mm^2 (outer) to 43,000 tubules/mm^2 (inner).

For the dentin of old donor teeth only full or partially opened tubules were considered in the tubule density measurements. Obliterated tubules were excluded from the measurements to assess how dentinal tubule density changes with aging as caused by the filling process. Clearly a reduction in the number of open tubules contributes to changes in dentin permeability and potentially other aspects of its physical behavior. For example, the changes with aging could be important to dentinal sensitivity and the resistance to tooth fracture.

There was a decrease in tubule diameter from the pulp to the DEJ for both age groups evaluated. The measures of tubule diameters are consistent with those reported by Ivancik et al. (2014) for the dentin from young U.S. patients. Interestingly, larger tubule diameters have been reported for the dentin of Brazilian donor teeth (diameters of 2.99 μm and 2.42 μm for inner and outer dentin, respectively) and no significant differences with depth (Lopes, Sinhoreti, Gonini Júnior, Consani, & Mccabe, 2009). When comparing the measured values from young and old donor teeth of the present study, similar results were obtained for the two groups in the inner and middle regions. There was some difference in the measures for diameter in the outer dentin, with 9% lower values in the old dentin. Undoubtedly this difference is attributed to the onset of sclerosis.

In general, the measures of peritubular cuff diameter were approximately twice the diameter of the tubule lumens. The formation of peritubular dentin occurs after the mineralization of intertubular dentin has completed (Gómez de Ferraris & Campos Munoz, 2009). Deposition of dentin is continued by odontoblasts following a circular pattern surrounding the dentinal tubules. During this process, and as the odontoblasts produce several layers of dentin, the pulpal chamber reduces its size, odontoblasts move to the interior of the pulp, and odontoblastic processes remain inside the dentinal tubules (Gómez de Ferraris & Campos Munoz, 2009).

To identify the change in number of obliterated dentinal tubules relative to the total number of open tubules for each region of dentin, the occlusion ratio was calculated according to:

$$\text{Occlusion ratio} = \frac{\text{Nr. Obliterated dentinal tubules}}{\text{Nr. Open tubules}} \tag{2}$$

A higher occlusion ratio indicates a higher fraction of obliterated dentinal tubules. Measured estimates for the occlusion ratio in the three regions of dentin evaluated are shown in Fig. 10. As expected, the occlusion ratio in young donor teeth is approximately zero in all three regions. Indeed, the visible changes in microstructure of dentin with aging begin in the third decade of life (Curtis & Watson, 2008; Gómez de Ferraris & Campos Munoz,

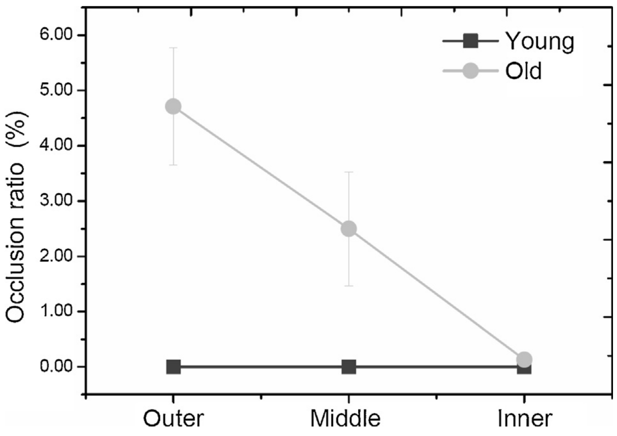

Fig. 10. Occlusion ratio for the three different regions of coronal dentin and a comparison of results for the young and old donor teeth.

2009 Nazari et al., 2009). On the other hand, an increase in occlusion ratio was noted as one approaches the DEJ. Hence, the highest proportion of obliterated tubules in the tooth crown is located in the outer dentin and decreases with depth. The occlusion ratio measurements correspond to an average of 1170 obliterated tubules/mm^2 in outer dentin and 120 obliterated tubules/mm^2 in inner dentin. Thus, in the tooth crown filling of the lumens begins at the ends of the tubules.

In comparing the results obtained for microstructure between young and old patients (e.g., tubule density, diameter, and peritubular dentin diameter) there was no significant difference ($p > 0.05$). However, there was a significant difference in the occlusion ratio between the young and old groups, and between the outer and inner dentin ($p \leq 0.05$) as shown in Table 1.

4.2. Hardness

There was load-dependency in the measured hardness of dentin as shown in Fig. 5a. This behavior is at least partly explained by the number of tubules involved within the indentation area. The difference in number of tubules involved in the indentation response is evident from a comparison of Fig. 5b and c. Load-dependent behavior has been previously reported in the indentation response of dental enamel (Park, Quinn, Romberg, & Arola, 2008; Rivera et al., 2013). In enamel, which is absent of tubules, the load dependence was described in terms of the change in mechanisms of deformation with increasing load. That could also play a role in the response of dentin through the relative viscous behavior and degree of water movement.

Results of the hardness measurements show that there are limited differences in the hardness between the three regions of dentin evaluated. An overall average hardness of 0.65 ± 0.03 GPa was obtained for the young dentin, which is in agreement with results from previous studies (Fuentes, Toledano, Osorio, & Carvalho, 2003). Fuentes et al. (2003) found that the hardness of dentin ranged between 0.60 GPa and 0.61 GPa, with only little variation along the tooth. Nevertheless, the spatial variations in hardness identified in the present study are not as significant as those found by other authors, where values between 0.25 GPa and 0.8 GPa have been reported (Pashley, Okabe, & Parham, 1985). It is important to note that previous studies have generally involved data derived from the teeth of patients living in North America. Differences in the microstructure and mechanical behavior of dentin have been noted in a previous comparison of donor groups from North and South America. That may be relevant here.

An increase in hardness occurred with increasing proximity of the DEJ for both age groups. The lower hardness of inner dentin is at least partly associated with the higher number (and area) of dentinal tubules (Pashley et al., 1985). The area occupied by dentinal tubules in the inner dentin is approximately 20% lower than the outer dentin (Ivancik & Arola, 2013; Pashley et al., 1985). This behavior might be attributed to differences in chemical composition and variations between the amount of organic and inorganic material within dentin. However, according to the results obtained for the mineral-to-collagen ratio, these differences were not statistically significant in the young patients group.

An analysis of the hardness results for the young and old donor groups showed that there were significant differences for outer and inner dentin between young and old groups ($p \leq 0.05$). These results are in agreement with those found for the occlusion ratio and obliteration of dentinal tubules. Nevertheless, the results are not consistent with some previous results concerning the hardness of dentin and age. For instance, Senawongse, Otsuki, Tagami, & Mjor (2006) used nano-indentation techniques to determine changes in hardness of different types of dentin with age and did identify significant differences. However, the secondary and mantle dentin did show an increase in hardness (Senawongse et al., 2006). Zheng et al. (2005), used nano-indentation to investigate the changes in hardness of dentin with age. In that investigation there were no significant differences in dentin hardness between age and location. However, the transparent regions of dentin from old donor teeth did exhibit significantly greater hardness. The discrepancy between the earlier studies and present results may be explained by the use of nano-indentation techniques, and differences in the representation of peritubular or intertubular dentin with single indents. A load-dependency may also contribute to unique responses for the intertubular and peritubular components. More work is needed in this area.

Table 1
Results from the ANOVA (p-values) in comparing the microstructure from young and old dentin donor teeth. Note statistically significant differences for the occlusion ratio for the middle and outer dentin.

Young vs. old				
Region	Tubule density	Occlusion ratio	Tubule diameter	Peritubular diameter
Outer	0.1425	0.0002	0.2123	0.6466
Middle	0.4333	0.0001	0.7358	0.4379
Inner	0.9703	0.3739	0.8497	0.6694

4.3. Chemical composition

An increase in mineral-to-collagen ratio was found with increasing proximity to the DEJ for both young and old donors, but no significant differences were found along the tooth. These results contradict the findings by Ryou et al. (2011), who found a reduction in the mineral-to-collagen ratio from the pulp to the DEJ in an evaluation of coronal dentin from US donors using FTIR. Ryou et al. (2011) used the bands associated with the υ_3PO4 peak and the Amide I peak to find the mineral-to-collagen ratio of dentin, contrary to the bands used in this study. Authors like Kazanci et al. (2006) have argued that it is important to consider the effect of orientation and polarization of different bands for Raman Spectroscopy analysis. They have also found that Amide III band (used in this study) is less susceptible to polarization effects than other amide bands. However, Märten, Fratzl, Paris, and Zaslansky (2010) measured the volume fraction of mineral within the coronal dentin using Small-angle X-ray scattering (SAXS) and found that the mineral volume fraction in dentin is uniform and only found statistically significant differences near the DEJ.

A lower mineral content within the inner dentin could be attributed to differences in the mineralization of the intertubular dentin at different regions. For example, Kinney, Balooch, Marshall, and Weihs (1996) measured the hardness of intertubular and peritubular dentin using AFM; the results obtained established a lower hardness in intertubular dentin near the pulp and an increase toward the DEJ. A correlation made by Pashley et al. (1985) between the change in hardness and tubule density demonstrated that the decrease in hardness of dentin toward the pulp can be accounted for by the decreased hardness of the intertubular dentin and that correlation with tubule density may be coincidental.

It is important to note that most of the results found in the literature were obtained from studies using teeth of mostly anglo-saxons living in the USA. Therefore, the differences in dentin features from Colombian donors (i.e., the variation in the mineral-to-collagen ratio along the tooth) might be attributed to individual characteristics, such as oral health and nutritional status, among others. In addition, several authors have suggested that a relation between ethnicity and some dental features, such as tooth size (Merz, Isaacson, Germane, & Rubenstein, 1991; Bishara, Jakobsen, Abdallah, & Fernandez Garcia, 1989), enamel thickness (Hall,

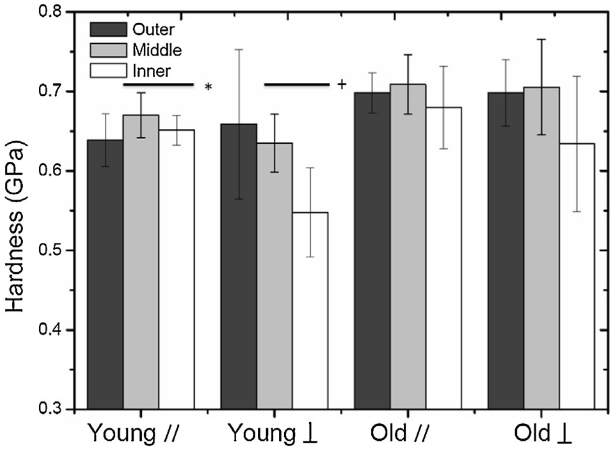

Fig. 11. Vickers hardness obtained for dentin of young and old donor teeth according to depth. The applied load is parallel (//) and perpendicular (⊥) to the dentinal tubules. Columns with significant differences ($p \leq 0.05$) are grouped in a line and marked with a cross (+) and a asterisk (*).

Lindauer, Tüfekçi, & Shroff, 2007) and tooth formation rate (Olze et al., 2007), might exist. Merz et al. (1991) found that dental arches in black patients are significantly wider and deeper than the ones in white patients; while Hall et al. (2007) found thicker enamel on the distal aspect of the black donor tooth. Differences between tooth dimensions of permanent teeth in three populations: Egyptians, Mexicans, and the North Americans were found by Bishara et al. (1989). However, only a few studies on dentin characteristics and how they might be determined by ethnicity have been carried out (Bajaj, Ivancik, & Arola, 2008).

The results obtained for the mineral-to-collagen ratio are in agreement with those found for hardness distribution, where higher hardness values were associated with areas where there was a higher amount of mineral.

The observed distribution in hardness and chemical composition of the old donors group corresponds to the progression of aging. It has been well established that aging of dentin starts from root dentin and continues in the coronal direction (Drusini, Calliari, & Volpe, 1991). It has also been reported that the total obliteration of tubules for coronal dentin might occur near the age of 70 (Tronstad, 2008).

It is important to note that the results shown in the hardness maps (Fig. 9a and c) were obtained after longitudinal sectioning of the tooth (section A–A in Fig. 1a). Consequently, the indentation load was applied perpendicular to the dentinal tubules. A comparison of hardness measurements from the parallel and perpendicular loading orientation is shown in Fig. 11. In comparing the results for these two directions, significant differences in hardness were found between the middle and inner dentin ($p \leq 0.05$) of young donor teeth. For the old dentin there were no significant differences ($p > 0.05$). The unique behavior of the inner and middle dentin may be attributed to a greater number of tubules in these regions, which yields a higher proportion of voids and lower hardness. For old dentin there is a consistent increase in hardness approaching the DEJ with both orientations of indentation. In this case, the increase is more significant than the one discussed earlier (load applied parallel to dentinal tubules) due to the presence of large areas of peritubular dentin within the indentation as a result of obliteration of the dentinal tubules.

5. Conclusions

According to the results obtained, the following conclusions were drawn:

1. The tubule density in the dentin of young and old donor teeth ranged from approximately 22,000 to 35,000 tubules/mm^2. The tubule diameter ranged from approximately 1.2 μm to 1.8 μm. There was a significant decrease in tubule density from the pulp to the DEJ in both age groups studied ($p \leq 0.05$).
2. There was a significant difference ($p \leq 0.05$) in the measures of occlusion ratio between the outer (4.71%) and middle dentin (2.50%) of the old donor teeth. The largest proportion of obliterated dentinal tubules was near the DEJ and decreased toward the pulp.
3. A significant decrease in hardness was found with increasing distance from the DEJ for both young and old donor teeth ($p \leq 0.05$). A larger hardness was found in the old dentin when compared to young dentin. There were significant differences ($p \leq 0.05$) in the hardness in the outer and middle dentin between the young and old donor teeth.
4. The mineral-to-collagen ratio was found to increase with proximity to the DEJ for both young and old patients. This behavior is opposed to previous results reported in the literature for dentin from US donors, which in turn raises questions whether the chemical composition of dentin and its percentages of organic material and collagen might be affected by ethnicity.
5. The outer dentin exhibited the highest mineral-to-collagen ratio, while lower values were found in the areas near the pulp. A greater mineral-to-collagen ratio was found for the old dentin, with the highest values found in the outer dentin.
6. A comparison of the hardness and mineral-to-collagen ratio distributions showed that the regions with the highest mineral-to-collagen ratio also exhibited the highest hardness.

Conflict of Interest

No conflict of interest

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