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Micrometric measurement of the density of stained odontoblast processes

ALICIA KOHLI[1], STELLA M. PEZZOTTO[2], GRACIELA GARCIA[3], LEONOR C. POLETTO[2].

1. Histology and Embryology Department, School of Dentistry, National University of Rosario. Argentina.
2. Research Council, National University of Rosario. Argentina.
3. Biochemistry School. National University of Rosario. Argentina.

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ABSTRACT: The embryological, structural and functional unit of the dentine-pulp complex shares the odontoblast, located in the border of the dentine pulp, with basal nuclei and organelles. The odontoblast process emerges from its apical pole. It is formed by microtubules, microfilaments and vesicles covered by membranes penetrating the dentinal tubules, isolated from the inter-tubular matrix, along the extent of the dentine. The objective of this study was to evaluate the efficacy of three staining techniques: hematoxylin-eosin, periodic acid-Schiff and Schmorl, by staining the process, from beginning to end, and compare the results with the erosion technique. Thirty human teeth were employed in the trial; after their extraction the pulp was fixated, the pieces demineralized in nitric acid at 8%, the collagen filaments eliminated with Type II Collagenase, the tissue was stained, and the measurements were made. The portions with no pulp were prepared with the erosion technique.
Results: Comparing the best results obtained by staining with the values obtained with the erosion technique, the former showed lower values. Conclusion: Staining techniques show lower density of the staining processes compared with the dentinal tubules in the erosion technique.

Introduction

The dentine-pulp complex is a structural (Kikuchi *et al*., 1994) and functional embryological unit (Nakashima, 1994) that shares a specialized cell called odontoblast or dentinoblast, which has a body peripherally located in the dental pulp, with the same basal core and cell organoids involved in protein synthesis (Akama, 1991). From its apical pole emerges an odontoblast process formed by microtubules, microfilaments, membrane-covered vesicles and bodies that resemble lysosomes (Couble *et al.*, 2000; Holland, 1985). Some researchers have located this process by combining decalcification, partial dissolution with type II collagenase and observing it with scanning electron microscopy. The processes appeared larger in volume when they emerged from the odontoblasts, then became thinner in the middle portion of the dentine due to the emission of collaterals, and ended in very thin ramifications in the dentine enamel junction (Yamada *et al*., 1983). Using the same preparation plus immunofluorescence technology, it was verified that the processes extended to the limit of the dentine enamel junction both in mice and in human beings (Sigal *et al.*, 1984a; Sigal *et al*., 1984b; Goracci *et al*., 1999; Byers, 1984; Brannstrom and Astrom, 1972).

*Address correspondence to: Alicia Kohli.
Pasaje Rizzuto 3473, (2000) Rosario, Argentina.
E-mail: *aliciakohli2009@hotmail.com*
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In a previous work (Kohli *et al*., 2004) dental pieces extracted for orthodontic reasons were demineralized, their collagen fibers were eliminated with the Yamada method (Yamada *et al*.,1983) and the resulting material was stained by three techniques in order to observe them under an optical microscope, given that this School of Dentistry has no electronic microscopes. In that piece of research we used: -the already traditional method of hematoxylin-eosin to stain the core and cellular cytoplasm; - periodic acid-Schiff to identify the plasmatic membrane of the odontoblast and its process, and - Schmorl's staining, also used to identify the odontoblast process in other cells (Kohli *et al*., 2010). The odontoblast process appeared pink-violet with the first technique, greenish violet with the second, and rosy violet covered with brownish or light blue granules with the third one (Kohli *et al*., 2004). The aim of this study was to apply a micrometric measurement technique to evaluate which of the stainings employed would be more effective in identifying the process from beginning to end, observing it with an optical microscope, and comparing it with the erosion technique.

Material and Method

We used 30 human teeth from patients of both sexes, between the ages of 6-18, whose pieces had to be extracted for orthodontic reasons. Immediately after the extraction the dental pulps were fixated in situ and divided in halves with the standard procedure. The halves with no adhered dental pulp were prepared with the erosion technique. The other halves that preserved the adhered pulp were demineralized with nitric acid at 8%, renewed daily for the needed period for each dental piece according to their volume and tissue maturity in order to obtain material soft enough to be embedded in paraffin, and the pH was restored with calcium carbonate. The collagen fibers were eliminated with type II collagenase (MG Sigma Cat C-6885), following Yamada's method, and treated with 5 N HCl at 60°C for ten minutes. The pieces were drained and submerged in type II collagenase for 8 hours and then drained again and placed in a phosphate buffer solution to restore the pH. Proceeding with the routine technique for optical microscopy, the pieces were embedded in paraffin and each tooth was cut in three 5 mm thick portions and then stained with Schmorl's and with Biopur kits for hematoxylin-eosin and periodic acid Morphological measurements.

A five-division grid was employed, placed in a Kyowa-Unilux 12 light-microscope with 10 X oculars. The grid surface was measured with a micrometer objective (Swift, 0.01 mm/div) using a 40 X objective. A total of 13 divisions correspond to each side (0.13 mm) with a total area of 0.0169 mm″, that is, 16900 μm″. This value represents the unit of density of the area, with which the density of the stained processes was calculated by dividing the number of processes we counted in each square by itself.

The grid was first placed in contact with the apical pole of the odontoblasts, where the processes were starting from, and this field became our internal reference. The total number of processes within the grid was then divided by the area. Since the grid was applied 7 consecutive times, 7 density areas of stained processes were obtained and the results were averaged. Then the grid was applied to the external border of the dentine, where the same measurement was made to obtain the average density of the stained processes. The crown and root of 90 stained histological sections were measured. The average density of the dentinal canaliculi was calculated in the same way in 30 erosions. The measurements of density of the stained processes and dentinal canaliculi in the erosion were expressed in μm^{-2}.

Statistical analysis: Average measurements were shown as means ± standard deviations. Comparisons were done applying the Friedman analysis of variance and the Wilcoxon Signed-Rank Test.

Results

In the 30 histological sections obtained for each staining, we focused on the areas where the dental pulp remained neatly adhered to the remnant dentine, which allowed us to localize it in the three stainings. Therefore, it was possible to compare erosion results with the three measurement techniques. Eighteen histological sections (60%) showed the pulp adhered to the pulp chamber, 6 (20%) showed it joined to the radicular canal, and another 6 (20%) showed it joined to the pulp chamber and the radicular canal.

Table 1 shows the results of the measurements for the areas of 30 teeth stained with hematoxylin-eosin, PAS, and Schmorl. The average density of stained processes obtained with the hematoxylin-eosin technique in the internal area of the crown was 0.008 ± 0.002, slightly lower than the same area in the root: 0.008 ± 0.001. But considering the average of the external areas in the crown and root, they were higher (In crown: 0.001 ± 0.002; in root: 0.001 ± 0.002).

Employing periodic acid-Schiff staining, the aver-

age density of the stained processes was high for the internal areas of the crown (0.007 ± 0.002) and root (0.007 ± 0.001), but they were lower than the values obtained with hematoxylin-eosin staining. The averages of the external areas of the crown (0.001 ± 0.002), and root (0.001 ± 0.001) were lower than the values obtained with the previous staining. With the Schmorl technique, the average densities of the stained processes were lower for the internal areas of the crown (0.008± 0.001) than the values obtained with the hematoxylin-eosin staining in the same areas. For the external areas of the crown (0.002±0.001) and root (0.003 ± 0.002) they were higher than the values obtained by the previous stainings. With the Schmorl technique the internal area was covered with a microgranulated brownish color that later disappeared.

TABLE 1.

Average area densities of 90 histological sections of stained processes, expressed in μm^{-2}

Hist Sec Nº	HEMATOXYLIN-EOSIN				PAS				SCHMORL			
	Crown		Root		Crown		Root		Crown		Root	
	Int	Ext	Int	Ext	Int	Ext	Int	Ext	Int	Ext	Int	Ext
1	0.009		0.008		0.007		0.007		0.008		0.007	0.003
2	0.008				0.011				0.008	0.002		
4	0.008				0.008							0.004
5			0.009				0.006	0.003	0.008	0.004	0.005	0.001
6	0.007		0.006		0.008		0.007		0.005	0.002		
7	0.003				0.002				0.007	0.003		
8	0.005			0.002	0.006				0.007	0.002	0.007	
9	0.008		0.008		0.008		0.007				0.008	0.001
10			0.008				0.008		0.006	0.002	0.007	0.006
11	0.005		0.007		0.005		0.008	0.002	0.008	0.002		
12	0.005				0.002				0.008	0.006		
13	0.008	0.004			0.008	0.003			0.008	0.004	0.009	0.002
14	0.010		0.009		0.008		0.008		0.007	0.001		
15	0.008				0.006						0.009	0.003
16			0.005				0.006	0.002	0.006	0.005		
17	0.008	0.007			0.008	0.008					0.007	
18			0.008				0.006		0.007	0.001	0.008	0.002
19	0.009		0.007		0.010		0.007		0.007	0.004		
20	0.008			0.008	0.008						0.008	0.007
21			0.009				0.007	0.005			0.008	
22			0.008				0.007		0.008	0.005		
23	0.009	0.002			0.008	0.005			0.010	0.002		
24	0.008				0.009						0.009	0.003
25			0.009				0.009		0.009	0.001		
26	0.009				0.009				0.010	0.002		
27	0.010				0.010				0.006	0.002		
28	0.008	0.004			0.009				0.008			
29	0.010	0.001			0.009				0.009			
30	0.009		0.008	0.003	0.006		0.007				0.007	0.002

* Hist Sec Nº: Histological Section Number.

The results of the internal and external measurements of each one of the 30 teeth, applying the erosion technique, and the average density of the canaliculi are shown in Table 2. The density of the internal area of the dentinal canaliculi was 0.014 ± 0.023 in the crown, and 0.009 ± 0.002 in the root. They were higher than the values obtained with stainings. In the external areas of the crown (0.007 ± 0.003), and in the root (0.006 ± 0.002) the density of the dentinal canaliculi was higher than the observed with the stainings.

In the internal and external areas of the crown, the density of the stained processes obtained with each staining and with erosion was compared (Table 3). In the internal surface of the crown, there were no significant statistical differences between the hematoxylin-eosin, periodic acid-Schiff and Schmorl techniques, applying the Friedman's test ($p \leq 0.554$). The test was applied to the four values, revealing that the significant difference ($p \leq 0.010$) corresponded to the erosion value, which was higher than the rest. Observing the external surface of the crown, applying the Friedman's test, the high value resulting from erosion showed a significant difference ($p \leq 0.0001$). By comparing two techniques -with the Wilcoxon Signed-Rank Test - significant differences appeared between hematoxylin-eosin and Schmorl ($p \leq 0.03$); hematoxylin-eosin and erosion ($p \leq 0.0001$); Schmorl and periodic acid-Schiff ($p \leq 0.001$); periodic acid-Schiff and erosion ($p \leq 0.0001$); Schmorl and erosion ($p \leq 0.0001$). The density of stained processes of the internal and external areas of the root obtained with the staining and the erosion techniques are shown in Table 4. The statistical difference between the four averages in the internal surface was significant (Friedman's test: $p \leq 0.005$). Comparing two techniques, significant differences were found between: hematoxylin-eosin and periodic acid-Schiff ($p \leq 0.041$); hematoxylin-eosin and Schmorl ($p \leq 0.064$); periodic acid-Schiff and erosion ($p \leq 0.013$); Schmorl and erosion ($p \leq 0.013$). Regarding the external surface, the difference between the four averages was statistically significant (Friedman Test: $p \leq 0.0001$). The significant differences comparing two techniques were between: hematoxylin-eosin and Schmorl ($p \leq 0.005$); hematoxylin-eosin and erosion ($p \leq 0.001$); periodic acid-Schiff and Schmorl ($p \leq 0.001$); periodic acid-Schiff and erosion ($p \leq 0.001$); Schmorl and erosion ($p \leq 0.001$).

When erosion technique was applied, the average density of the canaliculi was lower in the external than in the internal area by 52.5% in the crown and 26% in the root.

The best results obtained with each staining, in internal and external areas, were compared with the erosion values, considered 100% of the measurement because they were the highest.

The hematoxylin-eosin average density of the stained processes in the internal area of the crown compared to the erosion was 44.7% lower, and when the Schmorl technique was applied in the external area the

TABLE 2.

Average area densities of 30 histological sections by erosion, expressed in μm^{-2}

Hist Sec Nº	Crown		Root	
	Int	Ext	Int	Ext
1	0.014	0.012	0.010	0.008
2	0.010	0.009		
3	0.009	0.006		
4			0.005	0.005
5	0.009	0.006	0.009	0.008
6	0.013	0.007		
7	0.012	0.005		
8	0.010	0.009	0.008	0.006
9			0.010	0.007
10	0.013	0.011	0.009	0.006
11	0.005	0.004		
12	0.007	0.005		
13	0.013	0.015	0.012	0.013
14	0.008	0.004		
15			0.012	0.009
16	0.006	0.005		
17			0.007	0.004
18	0.008	0.004	0.006	0.003
19	0.005	0.006		
20			0.008	0.007
21			0.008	0.005
22	0.009	0.007		
23	0.009	0.007		
24			0.008	0.005
25	0.009	0.005		
26	0.115	0.005		
27	0.009	0.006		
28	0.010	0.006		
29	0.010	0.004		
30			0.008	0.005

* Hist Sec Nº: Histological Section Number.

result was 63.4% lower. In the internal area of the root with hematoxylin-eosin staining, the value obtained was 9.3% lower compared to the average of the canaliculi with erosion. Comparing Schmorl and erosion in the external area of the root, the result was 58.4% lower.

Discussion

Locating the odontoblast process was an arduous task for the researchers, mainly because the processes are located inside the dentinal canaliculi, a mineralized matrix. One of the problems was fixating and maintaining the odontoblasts and processes in good condition inside the matrix (Evan *et al*., 1976).

With Transmission Electron Microscopy (TEM) and Scanning (SEM) it was possible to locate the processes, study their constitution and observe their behavior in healthy and decayed teeth. By employing SEM it was verified that the processes were in the entire dentine and that thinner ramifications emerged from them, joined together like bridges (Holland, 1985; La Fleche *et al*., 1985). Using a demineralization technique with EDTA and eliminating the collagen fibers of the dentine with type II collagenase and observing them with SEM, Yamada *et al*. (1983) were able to describe the emerging odontoblast processes, the originated collaterals and their end in the dentine enamel junctions (Sigal *et al*., 1984a, b).

In a previous work (Kohli *et al*., 2004) we were able to observe the processes with a combination of technologies available in this Faculty. We proceeded to the demineralization with nitric acid at 8%, applied type II collagenase, and later stained the resulting tissue with three different stainings, which allowed us to visualize the processes under an optical microscope. Based on these results we measured the density of the stained odontoblast processes and compared them with

TABLE 3.

Average area densities of 88 histological sections in 22 dental pieces, crowns, expressed in μm^{-2}

Area	Staining	Mean	Std Dev	Median	Minimum	Maximum
Internal[1]	**HE**		0.002	0.008	0.003	0.010
	PAS	0.008	0.002	0.008	0.002	0.011
	Sch	0.008	0.008	0.008	0.005	0.012
	E	0.014	0.023	0.009	0.005	0.115
External[2]	**HE**	0.001	0.002	0.000	0.000	0.007
	PAS	0.000	0.002	0.000	0.000	0.008
	Sch	0.002	0.001	0.002	0.000	0.006
	E	0.007	0.003	0.006	0.004	0.015

HE: Hematoxylin-Eosin; Sch: Schmorl; E: Erosion
[1]Friedman's test considering four averages: $p \leq 0.010$; Friedman's test considering HE, PAS and SCH: $p \leq 0.554$;
[2]Friedman's test: $p \leq 0.0001$; Wilcoxon Signed-Rank Test: HE vs SCH ($p \leq 0.03$); HE vs ($p \leq 0.0001$); SCH vs PAS ($p \leq 0.001$); PAS vs E ($p \leq 0.0001$); SCH vs E ($p \leq 0.0001$).

the corresponding erosions. Our aim was to contribute more evidence to this controversial topic, and we agreed with the authors who described the processes in depth along the dentine, showing them with TEM in a transversal cut below the dentine enamel junction (Sigal and Chernecky, 1988). We also agreed with the work done with SEM applying a combined technique of demineralization, elimination of collagen fibers and immunohistochemical preparation (Frank and Steur, 1988), and with the study done with TEM in the thirds of children's radicular dentine (Isokawa *et al*., 1970). Like Izokawa we observed they were flattened and adhered to one of the walls of the dentinal canaliculi (Thomas and Carella, 1984), and we also visualized the lateral ramifications already described (Holland, 1985). In addition, we verified that the processes were thicker when they emerged from the odontoblasts and became smaller as they went through the dentine tissue (Yamada *et al*., 1983).

In the histological section stained with Schmorl, we observed that brownish microgranules covered the odontoblast when the process emerges from it, making its visualization difficult. These granules disappear as the process approaches the external border. In a MET and MEB correlation study the microgranules were seen covering the processes, which was attributed to the presence of glycosaminoglycans and proteoglycans. In our study the collagen was partially dissolved by the enzyme. This part of the technique should be carefully considered.

It is possible to conclude that hematoxylin-eosin staining was the best of the three methods used to study the histological sections. The Schmorl method proved more useful in the visualization of the external area of the processes, both in the crown and root, which was corroborated by the density of the stained processes obtained with the micrometric measurement technique. But these densities were lower than those obtained for

TABLE 4.

Root density comparison in 14 dental pieces expressed in μm^{-2}

Area	Staining	Mean	Std Dev	Median	Minimum	Maximum
Internal[1]	HE	0.008	0.001	0.008	0.005	0.009
	PAS	0.007	0.000	0.007	0.006	0.009
	Sch	0.007	0.002	0.007	0.000	0.009
	E	0.009	0.002	0.008	0.005	0.012
External[2]	HE	0.000	0.002	0.000	0.000	0.008
	PAS	0.000	0.001	0.000	0.000	0.005
	Sch	0.003	0.002	0.002	0.000	0.007
	E	0.006	0.002	0.006	0.003	0.013

HE: Hematoxylin-Eosin; Sch: Schmorl; E: Erosion
[1]Friedman's test: p≤0.005; Wilcoxon Signed-Rank Test: HE vs PAS (p≤0.041); HE vs SCH (p≤0.064); PAS vs E (p≤0.013); SCH vs E (p≤0.013). [2]Friedman's test: p≤0.0001; Wilcoxon Signed-Rank Test: HE vs SCH (p≤0.005); HE vs E (p≤0.001); PAS vs SCH (p≤0.001); PAS vs E (p≤0.001); SCH vs E (p≤0.001).

dentinal canaliculi with the erosion technique. The average density of the canaliculi, applying the erosion technique, was higher in the internal than in the external area, with a decrease of 47.5% in the crown, and 74% in the root. These results are discordant with other morphological studies (Komabayashi *et al*., 2008; Schellenberg *et al*., 1992).

We compared the values obtained for the internal and external areas by the erosion technique, considered as 100% because they were the highest, with the best results obtained with the other stainings. The average density of the internal area of the crown, applying the hematoxylin-eosin technique, was 44.7% lower than the value obtained with the erosion technique. The average density of the external area of the crown, applying the Schmorl technique, was 63.4% lower. In the internal area of the root, using hematoxylin-eosin staining, the value was 9.3% of that obtained by erosion. In the external area of the root, the value obtained with the Schmorl technique was 58.4% lower than that obtained with the erosion technique. We have no explanation for these differences, and there is no bibliography available to compare our findings.

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