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Original Article

Micro-CT evaluation of a novel periodontal ligament simulation technique for dental experimental models

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Abstract A reliable technique for simulation of the periodontal ligament (PDL) is required for *in vitro* studies. This paper proposes a simple technique to construct a tooth-PDL-bone experimental model with a reproducible PDL layer of a uniform width. In a preliminary study, two PDL simulation techniques were compared; transitional wax technique: wax layer was used to create a space for the PDL simulating material (light-body silicon) and direct rubber application technique: PDL simulating material (rubber die-spacer) was painted directly on the root surface. In both techniques, teeth were mounted in acrylic resin to simulate the supporting bone. The tooth-PDL-bone models were scanned using SkyScan 1076 micro-CT scanner and PDL-layer width was measured at selected sites on the roots using CTAn software. Based on the results of the preliminary study, 10 experimental models were constructed using the direct rubber application technique to confirm the reproducibility and consistency of the PDL layer width using micro-CT. Intra-class correlation coefficients (ICC) were calculated to assess the reproducibility. The transitional wax technique showed significantly greater variability in the PDL layer width when compared with the direct rubber application technique (F=66.0, p<0.001). The direct rubber application technique showed excellent reproducibility (ICC= 0.94; 95% confidence interval: 0.86, 0.98).

Keywords: Experimental model; micro-CT; periodontal ligament; rubber; simulation.

Introduction

Well-designed laboratory-based dental investigations permit a preliminary assessment of the likely clinical suitability of new dental products and procedures. *In vitro* investigations of teeth and dental restorative materials response to both static and dynamic occlusal loads have been extensively documented in the literature (Newman *et al*., 2003; Sahafi *et al*., 2005; Abdul Salam *et al*., 2006; Fokkinga *et al*., 2006a; Ambica *et al*., 2013; Adanir *et al*., 2015). Such investegations require construction of dental experimental models that simulate oral and clinical conditions. It has been recognized that mechanical behavior of the experimental models is influenced by parameters such as the physical and biomechanical properties of the tested material (Assif and Gorfil, 1994; Cheung, 2005), the nature of the applied load (Caplan *et al*., 2002; Naumann *et al*., 2005), the remaining tooth structure (Iqbal *et al*., 2003; Akkayan, 2004; Stankiewicz and Wilson, 2008), and the simulated supporting apparatus, namely PDL and bone (Soares *et al*., 2005; Brosh *et al*., 2011; Pérez-González *et al*., 2012; Marchionatti *et al*., 2014). Nevertheless, some, but not all, laboratory-based investigations simulate the PDL layer in the experimental models.

Evaluation of dental experimental model that comprise a simulated PDL layer in contrast to a model that lacks this feature has often appeared in the literature (Soares *et al*., 2005; Brosh *et al*., 2011; Pérez-González *et al*., 2012; Marchionatti *et al*., 2014). The results suggested that the presence of a PDL layer permits the realistic simulation of tooth movement with uniform stresses distribution within the artificial PDL material (Brosh *et al*., 2011). It has also been demonstrated that the presence of a PDL-simulating material modifies the mode of fracture when subjecting the models to occlusal loads (Soares *et al*., 2005). This has been supported by finite element modeling/analyses (FEM/FEA) that have evaluated different approaches to build numerical computed dental models (Cattaneo *et al*., 2005; Chen *et al*., 2005; Aversa *et al*., 2009). These studies have shown that the inclusion of a simulated PDL modifies the stress (Chen *et al*., 2005) and strain (Aversa *et al*., 2009) distribution in the computed models. It has been concluded that PDL simulation is a crucial step in FEA models construction (Cattaneo *et al*., 2005; Chen *et al*., 2005; Aversa *et al*., 2009) and recommended to include a simulated PDL in experimental models (Rosentritt *et al*., 2011; González-Lluch *et al*., 2016).

The decision on whether or not to include a PDL layer in the experimental models varies among researchers. PDL was simulated in the experimental models in several *in vitro* studies that applied both linear (Akkayan and Gülmez, 2002; Newman *et al*., 2003; Fokkinga *et al*., 2006b; Krastl *et al*., 2014) and dynamic compressive loads (Rosentritt *et al*., 2004; Sahafi *et al*., 2005; Naumann *et al*., 2006; Balkenhol *et al*., 2011; Pereira *et al*., 2014). On the other hand, Naumann *et al*. (2009) reviewed the study designs of sixty-nine dental laboratory studies and found that 50% of the studies that applied a dynamic testing protocol and 72% of the studies that applied a linear compressive loads have not simulated the PDL (Naumann *et al*., 2009). Some researchers exclude the PDL simulation step to avoid complicating the test design (Cormier *et al*., 2001; Hu *et al*., 2003; Marchi *et al*., 2008). Other researchers believe that the presence of a PDL layer will cause dislodgement of the tooth during testing, which interferes with the accurate assessment of the material being investigated (Martínez-González *et al*., 2001; Al-Omiri and Al-Wahadni, 2006).

Natural PDL has irreplaceable functional and structural roles. Healthy PDL consists of collagen fiber bundles that are arranged in different directions around the tooth to provide attachment and support during mastication (Lindhe *et al*., 2008). The cellular content of the PDL facilitates fiber and bone remodeling in response to pathological conditions as in periodontal diseases or in response to orthodontic tooth movement (Lindhe *et al*., 2008). The extracellular fluid motion in the PDL provides a hydrostatic and damping reaction to protect the tooth in response to physiological forces (Natali *et al*., 2004; Komatsu, 2010). PDL mobility is determined largely by the PDL width, height and structural quality. The healthy PDL is situated within the alveolar bone proper and continuously surrounds the root with a width that ranges between 0.2 to 0.4 mm (average of 0.25mm) (Lindhe *et al*., 2008).

To represent the structural and functional features of the PDL, different simulating techniques and materials with variable PDL thicknesses have been documented in the *in vitro* studies (Bortoluzzi *et al*., 2007; Nishimura *et al*., 2008; Büttel *et al*., 2009; McLaren *et al*., 2009; Ayad *et al*., 2011; Rosentritt *et al*., 2011; Sterzenbach *et al*., 2011; Barcellos *et al*., 2013; Palamidakis *et al*., 2013; Marchionatti *et al*., 2014). The tooth-PDL-bone dental experimental model consists of the tooth root surrounded by a resilient material (representing the PDL) and embedded in a rigid material (representing the bone). The most commonly used technique to build this model involves several steps (Akkayan, 2004; Bortoluzzi *et al*., 2007; Marchionatti *et al*., 2014). It starts with covering the root with a transitional isolating material before mounting in the bone representative material. The transitional material acts as a spacer that is discarded and replaced with the resilient, PDL simulating material. While, petroleum jelly (Brosh *et al*., 2011), wax (Bortoluzzi *et al*., 2007; Marchionatti *et al*., 2014) and aluminum foil (Akkayan, 2004) have been used as transitional isolating materials, a wide range of PDL simulating materials with different properties and consistencies have been used by researchers. These include; addition silicone (Nishimura *et al*., 2008; Ayad *et al*., 2011), condensation silicone (Barcellos *et al*., 2013; Palamidakis *et al*., 2013; Pereira *et al*., 2014), polyether (Rosentritt *et al*., 2004; Balkenhol *et al*., 2011), polyvinyl siloxane (Fokkinga *et al*., 2006a; Krastl *et al*., 2014), polysulfide (Soares *et al*., 2005), polyurethane (Soares *et al*., 2005; Sterzenbach *et al*., 2011) and industrial rubber materials (Naumann *et al*., 2006; McLaren *et al*., 2009).

The documented artificial PDL width varies greatly between studies. While a very thin PDL layer of 0.1 mm width has been reported (Heydecke *et al*., 2001), a thick layer of 1.0 mm width has also been documented (Rosentritt *et al*., 2006). The majority of the documented simulated PDL layer widths have been in the range of 0.2- 0.3 mm (Soares *et al*., 2005; Büttel *et al*., 2009; Balkenhol *et al*., 2011; Krastl *et al*., 2014). However, there is some uncertainty regarding the achievement of this width in experimental models. Despite its popularity, a major limitation of the transitional isolating material technique is the absence of confirmation that the purported material width has been achieved accurately and uniformly around the root. Simulating the natural PDL width and continuity is assumed to be a fundamental requirement to achieve a realistic response of the experimental model to the applied load in *in vitro* studies (Hu *et al*., 2003; Al-Omiri and Al-Wahadni, 2006; Marchi *et al*., 2008). Failure to achieve this could negatively affect the accuracy of the mechanical tests results and therefore, the PDL is considered as an unintended variable in the study design. Also, and more importantly, the reproducibility and consistency of the simulated PDL among experimental models often have been overlooked by researchers.

Therefore, this paper aims to evaluate the uniformity and reproducibility of the frequently applied PDL simulation technique that involves utilization of wax as a transitional isolating material and to compare it with an alternative, novel and simple PDL-simulation technique.

Materials and methods

Approval of the study was obtained from the Faculty of Health Science at the University of Adelaide (No. H2014-237). A preliminary study was first conducted to evaluate the PDL layer uniformity and root coverage quality for the transitional wax technique and a proposed PDL simulation technique. The transitional wax technique followed previous studies protocols (Soares *et al*., 2005; Bortoluzzi *et al*., 2007; Marchionatti *et al*., 2014). The proposed PDL simulation technique involved a direct construction of the PDL layer around the root without a transitional isolating material step. Based on the results of the preliminary study, subsequent micro-CT verification was conducted on 10 experimental models to confirm the reproducibility of the PDL in the proposed simulation technique.

Single-rooted human teeth were used to construct tooth-PDL-bone experimental models for all investigatory procedures. All roots were cleaned from calculus, deposits and any attached soft tissue using an ultrasonic scaler before commencing the experimental models' construction. On all teeth, a line representing the simulated alveolar bone crest was placed on the root surface 1mm apical to the cemento-enamel junction (CEJ) as illustrated in figure 1. A second line was placed 2mm below the CEJ (1 mm below the first line) to mark the coronal limit of the PDL (Nugala *et al*., 2012). All experimental steps were performed by one operator.

Tooth-PDL-bone experimental model construction

Transitional wax technique

In this PDL simulation technique, wax was used as the isolating medium to create a space for the PDL around the root. The root was immersed for two seconds into a liquefied (60°C) base-plate wax (Kerr Dental, Orange, CA, USA) until the root is completely covered with the wax up to the lower line marked on the root. This creates a wax layer around the root that is approximately 0.3 mm thick (Marchionatti *et al*., 2014). A cylindrical brass mould was filled with autopolymerising acrylic resin (ProBase Cold, Ivoclar Vivadent, Schaan, Liechtenstein) to represent the alveolar bone surrounding the tooth. Using sticky wax, the tooth was attached to the vertical rod of the dental surveyor (J.M. Ney Company, Bloomfield, CT, USA) to maintain the tooth vertical orientation in the acrylic resin mould. The filled mould was placed on the surveyor-base directly below the tooth-vertical rod assembly. The tooth-rod assembly was lowered into the autopolymerising acrylic resin filled mould until the higher marking on the root is reached. The tooth was secured in this position until the acrylic resin is fully polymerized. A putty index of the mounted tooth in the acrylic resin mould was made to assess in tooth repositioning in the block at a later stage.

The tooth was removed from the fully polymerized acrylic resin and the wax spacer around the root was removed using hot water and hand instrument. A socket for the root and the artificial PDL layer was created inside the acrylic resin block. A light-body silicon impression material (Imprint 4, 3M ESPE, MN, USA) was injected into the resultant root socket in the acrylic block to simulate the PDL layer. The tooth was relocated immediately into the socket and secured in the original place using the putty index. After setting of the impression material, the excess impression material was removed using a scalpel blade.

Novel PDL-simulation technique (Direct rubber application technique)

In this technique, an artificial PDL material, latex rubber die-spacer (Rubber-Sep, Kerr Dental, Orange, CA, USA) was applied directly to the root surface using the accompanying brush. The material was applied up to the lower line marked on the root in successive coats until the desired width was reached. According to the manufacturer, each coat has a thickness of 12 µm, and to achieve a selected PDL width of approximately 0.25 mm, twenty coats of the rubber material were applied. Each coat required few seconds to dry before the application of the next coat. The tooth was then mounted in the acrylic resin block as described previously. After setting of the acrylic resin, tooth-PDL-bone experimental model was created (Fig. 1).

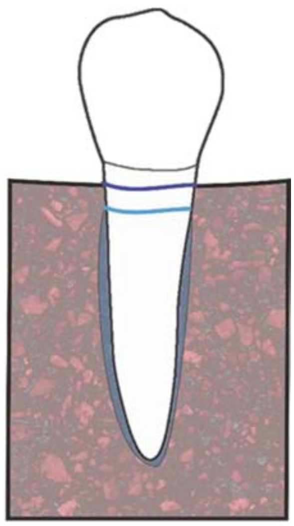

Fig. 1 A schematic drawing of the tooth-PDL-bone experimental model with two lines drown on the root; top line represents the simulated alveolar bone crest, and the bottom line represents the height of the simulated PDL.

Micro-CT scanning and analyses

Experimental models were scanned using a micro-CT SkyScan 1076 (Bruker Micro-CT, Kontich, Belgium) scanner. Before scanning, the fully polymerized acrylic resin blocks containing the teeth were removed from the brass cylinders to avoid radiographic artifacts. The tooth-PDL-bone model was secured to a mounting foam holder that facilitated proper positioning in the carbon-composite bed inside the micro-CT scanner chamber. The acquisition parameters were set and saved to ensure consistency of the scanning procedure of all teeth (voltage; 50 Kv, current; 160 MA, resolution; 9 µm, rotation step; 0.8°, rotation; 180°, frame averaging; 2). The projected raw images were saved as Tag Image File Format (TIFF) files.

NRecon software (SkyScan, version 1.6.9.4) was used to reconstruct and combine the projected images of the models. After importing to NRecon software, one cross-section was previewed to set the parameters to ensure maximum contrast and visualization of different parts of the viewed images. These parameters include smoothing, beam-hardening correction, malalignment compensation and thresholding. The same parameters were used for all cross-sections of all scanned models to create bitmap format (BMP) files.

The reconstructed BMP files of the scanned models were then visualized in three orthogonal views (coronal, sagittal and trans-axial) using Data-Viewer software (SkyScan, version 1.5.1.2). The dataset was saved for further analysis. CTvox software (SkyScan, version 3.0.0.0) was used for detailed three-dimensional reconstruction and assessment of the models.

Using the Data-Viewer software images dataset, CTAn software (SkyScan, version 1.14.4.1) was used to measure the width of the simulated PDL layer in both techniques. The measurements were performed at three predetermined sections on the root (coronal, middle and apical) in the sagittal plane images. The most coronal measurements were performed in the cross-section that was located three millimeters below the CEJ. The middle measurements were performed in the cross-section that was located three-millimeter below the previous one and the apical measurements were performed in the cross-section that was located three-millimeter below the middle one. In each section, the measurements of the PDL material width were performed in the mid-buccal (M-B), mid-lingual (M-L), mid-distal (M-D) and mid-mesial (M-M) in the trans-axial plane images. All measurements were recorded in millimeters.

CTvox and Data-Viewer software images were inspected visually to evaluate the simulated PDL material adhesion to the root, uniformity and overall coverage quality. Mean (± SD) for the PDL layer widths were calculated for all points on root sections for experimental models of both techniques.

Direct rubber application technique reproducibility verification

On the basis of the preliminary data on both techniques, the transitional wax technique was not pursued, and micro-CT scanning and data analysis were performed for 10 experimental models constructed using the direct rubber application technique to verify the technique reproducibility.

Mean and the standard deviation for the CTAn software recorded widths were calculated for each point of every predetermined root section. To confirm the reproducibility of the PDL layer width, an Intra-class Correlation Coefficient (ICC) was calculated using Stata Statistical Software (Release 14. College Station, TX: StataCorp LP). This estimates correlations between individual and average PDL layer widths made by the same experimental model, “raters”. In this sense, “raters” are the four-point CTAn measurements recorded for the three root sections, in each experimental model (total of 12 “raters” /experimental model). The ICC measures the strength of inter-“raters” reliability. Cicchetti (1994) interpreted the ICC values less than 0.4 as “poor” inter-rater reliability, between 0.40 and 0.59 as “fair”, between 0.60 and 0.74 as “good” and between 0.75 and 1.00 as “excellent”.

Results

Preliminary results

The PDL layer simulated in the preliminary study was first visually assessed on micro-CT images. Figures 2a, 2b and 2c show root cross-sections of the transitional wax technique experimental model. Figures 3a, 3b and 3c show root cross-sections of the direct rubber application technique experimental model. The PDL layer in the direct rubber application technique is closely adherent to the root with a uniform and continuous coating, as displayed in the images. In the images of the transitional wax technique, the PDL layer is unevenly distributed on the root with loss of attachment between the PDL layer and the root, which appeared as spaces between the surfaces.

The results of the CTAn software measurements for the transitional wax technique and the direct rubber application techniques are presented in Table 1. The data shows that the PDL layer width was inconsistent in the transitional wax technique with PDL width ranging from 0.00-0.42 mm. On the other hand, the PDL layer simulated by direct rubber application technique shows less variation ranging from 0.20-0.27 mm. Interestingly the mean width for both groups across all sites was 0.24 mm but the transitional wax technique widths were significantly more variable ($F=66.0$, $p<0.001$).

Direct rubber application technique reproducibility verification results

Mean (±SD) of the PDL layer widths simulated by the direct rubber application technique are presented in Table 2. The mean of the PDL layer width was between 0.24±0.006 and 0.25±0.004 in the coronal section, 0.25± 0.003 and 0.25±0.005 in the middle section and 0.24±0.006 and 0.25±0.002 in the apical section. The calculated ICC value for the average width measurements was found to be 0.94 (95% confidence interval: 0.86-0.98). According to Cicchetti (1994) interpretation, 0.94 indicates an excellent inter-“raters” reliability.

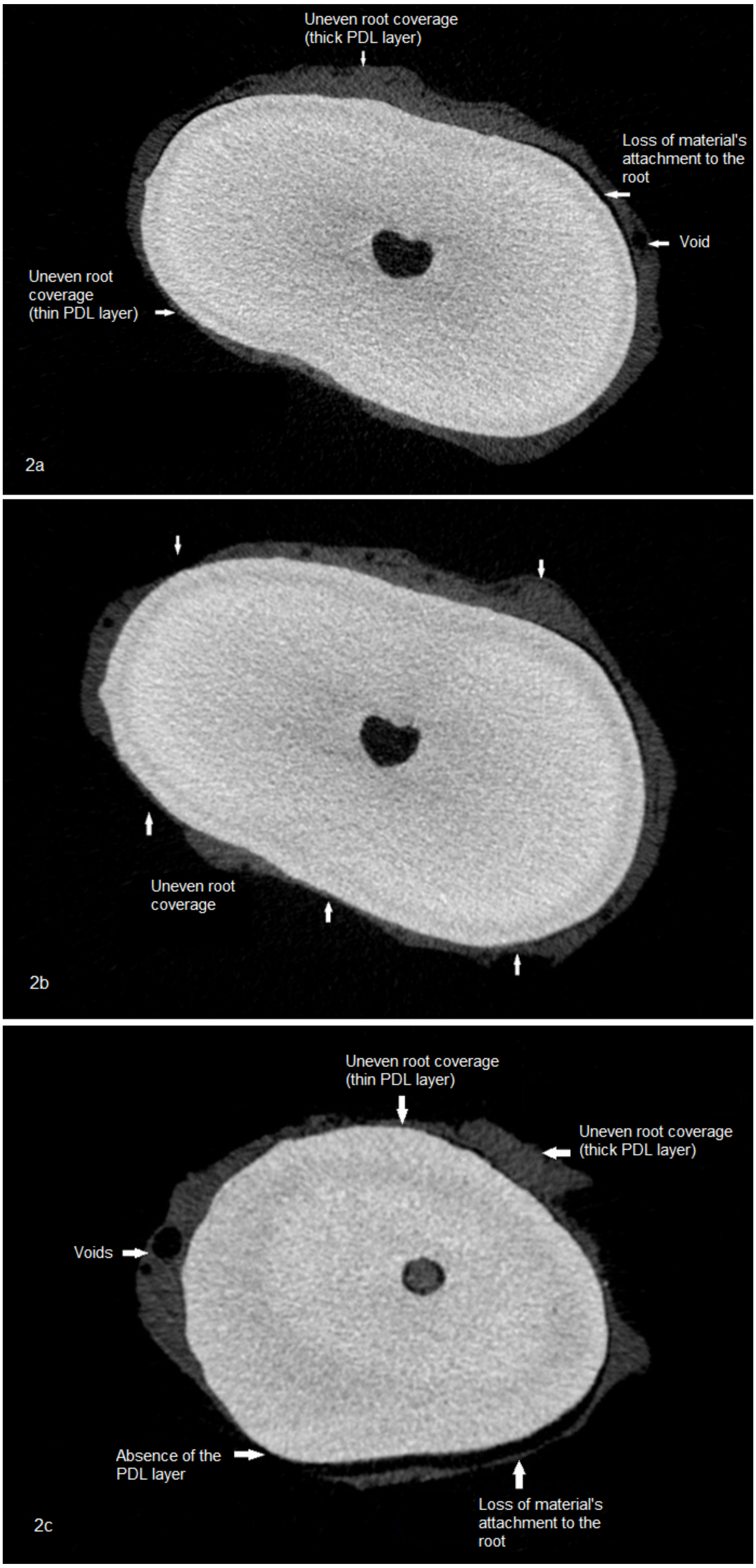

Figure. 2a, 2b and 2c Micro-CT images of PDL simulated with transitional wax technique at different trans-axial sections. Note the loss of material's attachment to the root surface in some areas (presented as a space between the PDL material and the root in all sections), voids and uneven root coverage.

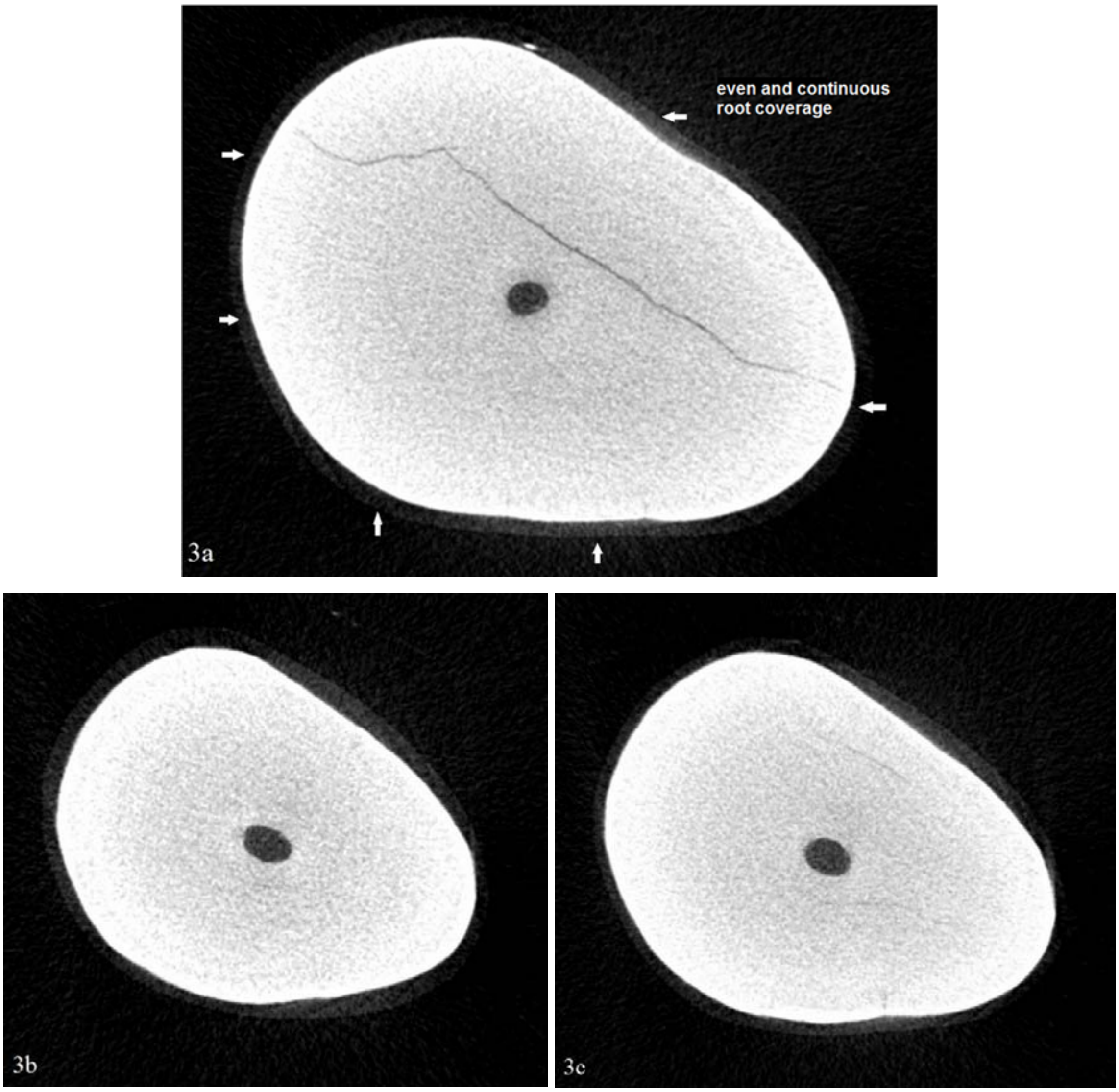

Figure. 3a, 3b and 3c Micro-CT images of PDL simulated with direct rubber application technique at different trans-axial sections. Note even root coverage in all sections.

Table 1 Width of PDL layer measured for both techniques in the preliminary study

Measurement levels		Coronal section				Middle section				Apical section				Mean ± SD
		M-B	M-L	M-M	M-D	M-B	M-L	M-M	M-D	M-B	M-L	M-M	M-D	
Transitional wax technique	model 1	0.35	0.41	0.02	0.38	0.20	0.28	0.00	0.35	0.22	0.40	0.35	0.16	0.24 ± 0.14*
	model 2	0.42	0.24	0.00	0.2	0.36	0.28	0.26	0.00	0.42	0.23	0.09	0.24	
Direct rubber application technique	model 1	0.20	0.22	0.25	0.25	0.25	0.25	0.25	0.24	0.25	0.25	0.26	0.25	0.24 ± 0.02
	model 2	0.27	0.25	0.25	0.24	0.21	0.24	0.24	0.23	0.26	0.26	0.26	0.24	

* Variation differs significantly; F=66.0, $p<0.001$. M-B: mid-buccal, M-L: mid-lingual, M-D: mid-distal and M-M: mid-mesial

Table 2 Mean and standard deviations of PDL layer width in direct rubber application technique

Sections	Coronal Section				Middle Section				Apical Section			
	M-B	M-L	M-M	M-D	M-B	M-L	M-M	M-D	M-B	M-L	M-M	M-D
Mean	0.25	0.24	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.20	0.24	0.25
SD	0.004	0.006	0.004	0.003	0.004	0.003	0.005	0.006	0.003	0.002	0.006	0.002

Discussion

When compared to clinical studies, laboratory-based dental studies permit standardization of specimens and testing parameters as well as control of experimental variables (Naumann *et al*., 2005; 2009). Additionally, laboratory studies can be conducted at a reasonable cost and over a shorter duration. However, creating an experimental protocol and model that simulates dental structures and oral cavity conditions is crucial to laboratory-based studies. The inclusion of a PDL layer in experimental models has been endorsed in the literature despite the acknowledged variability in techniques and materials (Soares *et al*., 2005; Brosh *et al*., 2011; Rosentritt *et al*., 2011; González-Lluch *et al*., 2016).

The direct PDL simulation technique proposed in this paper employs a latex rubber material that is originally designated as a die spacer for laboratory-constructed dental restorations. This material is supplied in a liquid form that allows direct application of controlled uniform thin coatings. According to the manufacturer, applying a thin coat using the accompanying brush results in approximately 12 µm thickness layer. This facilitates simulation of a planned PDL width and height on the root and ensures consistency and reproducibility among the experimental models.

The function of the wax layer in the transitional wax technique is to create a space between the root and the simulated alveolar bone that is to be filled with the PDL simulating material at a later stage. Therefore, the accuracy of wax layer thickness in this technique is a prerequisite for the accuracy of the PDL simulation. However, the consistency, thickness, and uniformity of the wax layer were difficult to control and verify. Furthermore, the technique is complicated and involves many steps and materials, which increases the chance of variation between the created experimental models.

Although the light-body impression material was the material of choice to simulate the PDL in most documented study designs (Akkayan, 2004; Soares *et al*., 2005; Brosh *et al*., 2011; Marchionatti *et al*., 2014), there was no guarantee of inter-specimens and intra-specimen uniformity in the PDL layer thickness. Due to the viscosity of the light-body impression material, shear thinning can occur when the root is placed in the impression material-filled socket (Pang and Chai, 1994). Therefore, the material's precise flow and height on the root surface are unknown. This could explain the uneven spread of the impression material on the root surface that was observed in this technique. This is particularly true when considered within the context of the natural unevenness of the root surface and the variation of the created wax thickness. In contrast, direct control of the material thickness was observed when the rubber die-spacer material was applied directly to the root. The paintable nature of the die-spacer permits application of a closely adherent layer on all root irregularities. In addition, the number of coats can be easily decreased or increased to establish a predetermined PDL width.

To date, PDL simulation lack standardization in terms of materials, techniques and dimensions. Most reports have focused on confirming the significance of incorporating a viscoelastic material to simulate the PDL (Natali *et al*., 2004; Cattaneo *et al*., 2005; Chen *et al*., 2005; Soares *et al*., 2005; Marchionatti *et al*., 2014). However, evidence of attaining a reproducible PDL layer in *in vitro* study designs has never been reported.

Micro-CT scanning and analyses were used in this paper to assess the width uniformity of the simulated PDL layer and quality of root coverage. Micro-CT is an advanced, modern and non-destructive investigation tool that has been utilized recently in various contexts in dental research (Swain and Xue, 2009). It requires minimal specimen preparation but allows high- resolution qualitative and quantitative specimen assessments through the commercially available software. The CTAn software data indicated that the desired artificial PDL material width in the direct rubber application technique is achieved uniformly with insignificant variability. This can be explained by the simplicity of the simulation technique, which does not involve multiple steps, and materials. On the other hand, the transitional wax technique showed significantly variable PDL width along the root surface, and most importantly, absence of the material in some areas around the root. This was confirmed by the trans-axial micro-CT images of the experimental models of both techniques (Figures 2a, 2b, 2c, 3a, 3b and 3c).

ICC is an inferential statistical method, which evaluates how strongly experimental models resemble each other in terms of quantitative measurements. In other words, ICC allows the assessment of reproducibility of a procedure and/or consistency of measurements. The experimental models constructed following the direct rubber application technique indicated excellent resemblance to each other with regard to the PDL width around the root. This confirms the reproducibility of the PDL layer simulated by the direct rubber application technique.

Within the limitations of this study, it has been concluded that the technique and material used for PDL simulation for *in vitro* experimental models affect the accuracy and reproducibility of the simulated PDL layer. In addition to technique simplicity, the paintable rubber material that is coated directly on the root surface can produce a continuous, uniform and reproducible artificial PDL layer for dental experimental models. However, further research is recommended to evaluate the viscoelasticity of the die-spacer rubber material and its effect on the mechanical behavior of the experimental models.

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Declaration

The authors declare no conflicts of interest.

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