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CERTIFICA:

Que los trabajos efectuados en la elaboración de la Tesis Doctoral titulada ": **BOND STRENGTH TO ROOT DENTIN AND FLUID FILTRATION TEST OF SEVERAL RESIN SEALERS AND MICRO-RAMAN SPECTROSCOPY STUDY OF DENTIN AFTER USING IRRIGATION SOLUTIONS**" presentada por Da. **Alaa S. Abdulmahdi Shoman** han sido realizados bajo mi dirección y supervisión, reuniendo las condiciones académicas necesarias para su presentación para optar al Grado de Doctor, si así lo considera el Tribunal designado por la Universidad de Granada.

Y para que así conste donde proceda, firmo la presente en Granada a 21 de octubre de 2013.

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"BOND STRENGTH TO ROOT DENTIN AND FLUID FILTRATION TEST OF SEVERAL RESIN SEALER AND MICRO-RAMAN SPECTROSCOPY STUDY OF DENTIN AFTER USING IRRIGATION SOLUTIONS"

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Part 1

RESUMEN

"Fuerza adhesiva a la dentina radicular, test de filtración de fluidos de varios selladores de resina y estudio mediante espectroscopia micro-Raman de la dentina tras la utilización de soluciones irrigantes"

Fuerza adhesiva a la dentina radicular and test de filtración de fluídos de varios selladores de resina

Introducción

Los objetivos fundamentales del tratamiento endodóncico son la limpieza y conformación del conducto radicular y la obtención de sellado apical hermético. De forma ideal, un sellador de conductos radiculares debe adherirse tanto a las paredes del conducto como al material de relleno en el interior del canal radicular. Por este motivo, se han desarrollado numerosos materiales de sellado radicular basados en los principios de la adhesión dentinaria; estos selladores, en ocasiones, no han logrado mejorar los resultados, en términos de fuerza adhesiva, obtenidos con la utilización del sellador no adhesivo AH Plus[231,230,212]

Aunque no se ha encontrado una relación significativa entre la fuerza de adhesión y la microfiltración apical, hemos de asumir que un sellado firme es, quizá, más importante que la fuerza adhesiva para evitar la filtración apical[72]. Lamentablemente, no existe un método aceptado universalmente para medir la filtración apical y se han obtenido en ocasiones resultados contradictorios que han llevado cuestionar la relevancia clínica de estos tests[99,226].

El test mecánico de expulsión del relleno radicular, habitualmente conocido por su nombre en inglés, Push-out bond strength test, se ha popularizado para la determinación de la eficacia adhesiva en endodoncia, aunque la relación entre test y la filtración apical permanece indeterminada.

Objetivos

Determinar la fuerza adhesiva a la dentina del conducto radicular y la capacidad de sellado de dos selladores de resina de metacrilato (RealSeal and EndoREZ) y comparlas con las del sellador no adhesivo AH Plus.

Para ello, se aplicaron los test de expulsión y de filtración de fluidos. La hipótesis nula a verificar es que no hay diferencias en la fuerza de adhesión y las propiedades de sellado de RealSeal, EndoREZ y AH Plus.

Material y métodos

Se utilizaron 60 dientes humanos unirradiculares extraídos por patología periodontal. Tras seccionar la corona de forma perpendicular al eje longitudinal del diente, las raíces se sometieron a preparación endodóncica manual, utilizando la técnica de paso atrás (telescópica). La longitud de trabajo se determinó sustrayendo 1 mm de la longitud de una lima k, de tamaño 15 (Dentsply Maillefer, Ballaigues, Switzerland) llevada hasta el foramen apical. La preparación biomecánica se llevó a cabo manualmente, con limas k de la misma marca, hasta el n° # 40. El irrigante endodóncico fue hipoclorito sódico al 2.5% durante 1 minuto, tras cada lima. En las raíces del grupo sellado con RealSeal, se usó EDTA al 17% (Colgate Oral Care Company, Waverly, Australia). En todos los grupos se practicó una irrigación final con agua destilada durante 1 minuto y el conducto se secó con puntas de papel.

Las raíces se asignaron al azar en grupos iguales (n=20) según el sellador a utilizar: Grupo 1: AH PlusTM (Dentsply De Trey, Konstanz, Germany), Grupo 2: EndoREZ® (Ultradent products, Inc. Utah, USA) y Grupo 3: RealSeal TM (SybronEndo. Glendora, CA, USA). Los selladores se utilizaron según las instrucciones y utilizando los aditamentos recomendados por cada fabricante.

En el grupo AH Plus se utilizó como relleno intracanal un cono maestro de gutapercha de conicidad 40.02 y conos accesorios de los números 25 y 20. En el grupo 2, EndoREZ se aplicó en la totalidad del conducto, obturándose con un cono de gutapercha impregnado en resina de conicidad 40.02 (EndoREZ point) y conos adicionales de tamaños #25, 20 de la misma marca. En el grupo 3, el imprimador RealSeal (Sybron Endo, Glendora, CA) se dejó actuar en el conducto durante 30 s, aplicando posteriormente el sellador en la totalidad del canal y obturando con un cono maestro RealSeal de conicidad 40.02 y puntas accesorias #25, 20 de la misma marca. En todos los casos se utilizó la técnica de condensación lateral en frío.

Las raíces se almacenaron en cámara de humedad a 37ºC durante 24 horas, para completar el proceso de polimerización, tras lo cual se asignaron al azar a los test de fuerza adhesiva (10 especímenes muéstrales) o de filtración de fluidos (n=10).

Los especímenes asignados al test de filtración de fluidos se seccionaron apicalmente, para conseguir una longitud radicular de 10 mm y se unieron a un sistema para la medición de fluidos, diseñado por Pashley et al.[199] Cada espécimen se insertó en posición corono-apical en un tubo de goma 2mm de diámetro interior conectado a una micropipeta de 10 ml unida, a su vez, a un reservorio de agua destilada de 250 ml situado a

una altura de 1 m sobre el plano de situación del espécimen. En el sistema se inyecta una burbuja de aire cuyo desplazamiento durante 24 horas indica la cantidad de fluido que ha permeado la raíz en ese tiempo. La conductancia hidráulica se expresó en µL/min/cm de H2O. Para verificar la hermeticidad del sistema se usaron dos especímenes adicionales en los que se había sellado el ápice con barniz.

Micro push-out test (µPBS)

Cada grupo compuesto por 10 especímenes muéstrales, se cortó en 4 láminas de, aproximadamente 1 mm de grosor, denominadas Cervical1 [C1], Cervical2 [C2], Medio1 [M1] y Medio2 [M2], en dirección corono-apical.

Se sometieron a presión en una máquina Instron 3345 (Instron Ltd, High Wycombe, UK) equipada con un vástago cilíndrico de 0.5 mm de diámetro que se situaba en contacto con el centro del conducto radicular, en el lado apical del conducto. La velocidad de carga fue de 0.5 mm/min. La máxima carga en el momento del fallo, cuando ocurría la extrusión del cono de relleno radicular, se registró en Newton (N) y se dividió entre la superficie adhesiva expresada en mm2, obteniéndose así la fuerza adhesiva expresada en Mega Pascales (MPa).

Una vez producido el fallo, los especímenes se recuperaron y se examinaron a 40 aumentos en un estéreo-microscopio (SZ60, Olympus, Tokio, Japón), clasificando el modo de fallo como adhesivo (entre la superficie de la dentina y el material de relleno), cohesivo (en el material de relleno radicular) o mixto.

Análisis estadístico:

Tras explorar la normalidad y homoscedasticidad de las distribuciones, el movimiento de fluido se comparó entre grupos mediante Análisis de la Varianza de una vía. El análisis de los resultados del test de fuerza adhesiva se realizó mediante contrastes no paramétricos (Kruskal-Wallis y test U de Mann Whitney, para la comparación entre grupos y tests de Friedman y de Wilcoxon para la comparación entre los niveles radiculares). La significación estadística se aceptó para un valor de $p < 0.05$.

RESULTADOS:

Test de permeabilidad

El sellador AH Plus/gutta percha obtuvo los mayores valores en el test de permeabilidad (10.45 ±6.45) seguido por el EndoREZ (8.49±5.92) y RealSeal (6.20±3.82), aunque no se obtuvieron diferencias estadísticamente significativas entre ellos.

Test de fuerza adhesiva

El sellador por sí mismo no influyó de forma significativa en los resultados del test de adhesión, aunque sí lo hizo el nivel radicular y la interacción entre el sellador y el nivel radicular.

En el conjunto de la muestra se obtuvieron diferencias significativas en función de la profundidad radicular, con valores crecientes en dirección corono-apical, aunque sin diferencias significativas entre los niveles M1 y M2.

Al considerar cada material aisladamente, AH Plus/gutapercha demostró una menor fuerza adhesiva en el nivel C1; para RealSeal, la fuerza adhesiva aumentó en sentido corono-apical; EndoREZ demostró un comportamiento similar en todos los niveles radiculares.

Cuando se compararon los valores del test μPush-out entre los sistemas de sellado radicular en cada nivel de profundidad radicular, no se observaron diferencias significativas para los niveles más superficiales C1 y C2; sin embargo, en el nivel M1 RealSeal demostró mayor fuerza adhesiva que EndoREZ, mientras que, en el nivel M2, EndoREZ desarrolló la menor fuerza adhesiva entre los tres sistemas de sellado radicular.

El modo de fallo fue similar entre los tres sistemas de sellado tanto en el conjunto de la muestra como en cada nivel de profundidad radicular. Con mayor frecuencia se detectaron patrones de fallo adhesivos, que representaron el 60% para RealSeal y el 90% para EndoREZ, seguidos por los fallos de tipo mixto.

Conclusiones

No hay diferencias significativas en la permeabilidad ni en la fuerza adhesiva a dentina radicular medida mediante el test de expulsión μPush-out, entre los sistemas AH Plus/gutapercha, EndoREZ y RealSeal.

ESPECTROSCOPIA MICRO-RAMAN DE LA DENTINA RADICULAR TRAS EL USO DE SOLUCIONES IRRIGADORAS.

Introducción

El éxito del tratamiento de conductos radiculares depende del método y la cali-dad de la instrumentación, irrigación, desinfección y de la obturación del canal radicular. La instrumentación endodoncia produce residuos de material calcificado y orgánico, que conocemos como barrillo y tapones dentinarios, que pueden interferir el pleno contacto y la adherencia de los materiales de obturación endodoncia al interior de los túbulos dentinarios. Además, esta capa de residuos que conocemos como barrillo dentinario, contiene bacterias y sus productos. La remoción del barrillo dentinario puede permitir la penetración de los medicamentos intra-conducto en el interior de los túbulos dentinarios para su mejor desinfección.

Estudios previos han demostrado que las soluciones de irrigación alteran el con-tenido mineral de la dentina radicular[245,247,248] en particular, las soluciones quelantes.

Objetivos

Caracterizar la estructura química y la cristalinidad mineral de la dentina del conducto radicular tras la instrumentación convencional, o su tratamiento adicional con NaOCl durante 5 minutos, EDTA, y un imprimador autograbador, mediante espectros-copia micro-Raman.

Específicamente, este estudio analiza las diferencias en la composición del con-tenido orgánico e inorgánico de la dentina del conducto radicular en función de:

1. La profundidad radicular, considerando cuatro niveles en la raíz en dirección corono-apical.
2. La aplicación de NaOCL durante 5 minute, con énfasis en las posibles dife-rencias respecto a su aplicación durante sólo 1 minuto y a la dentina no tra-tada.
3. El uso de tratamiento intra-canal de efectos principalmente desmineralizado-res (EDTA e imprimador de auto-grabado) en relación a la dentina del con-ducto no tratada y a la tratada con NaOCL, de efecto preferentemente des-proteinizador.

Material y métodos

En esta fase del estudio se utilizaron 20 dientes anteriores humanos extraídos. La corona se eliminó a nivel del límite amelo-cementario. Las raíces se asignaron aleatoriamente a uno de los cinco grupos del estudio (n=4), en función del producto irrigador utilizado durante la instrumentación y conformación del conducto. El Grupo I se irrigó con NaOCl al 2.5%, durante 1 minuto durante la instrumentación. El grupo II se instrumentó e irrigó igual que el grupo I, aplicando el NaOCl al 2.5% durante 5 min como irrigador final. El Grupo III se instrumentó e irrigó como el grupo I, con una irrigación final con EDTA (ácido etilen diamino tetra acético) al 17%, durante 1 minuto. En el Grupo IV, tras la instrumentación e irrigación como en el Grupo III, se aplicó el imprimador autograbador del sistema RealSeal, durante 30 segundos. El grupo V se instrumentó y se lavó solamente con agua destilada durante la instrumentación sin ningún irrigador adicional. Cada raíz se seccionó en cuatro láminas perpendicularmente al eje longitudinal de la raíz, correspondientes a los niveles Cervical 1 (C1), Cervical 2 (C2), Medio 1 (M1) y Medio 2 (M2).

De cada espécimen se obtuvieron 5 espectros Micro-Raman, en la dentina adyacente al conducto radicular, a una distancia máxima de 4 µm respecto a la luz del canal.

Los espectros se registraron con un Espectrómetro Micro-Raman Dispersivo JASCO NRS-5100, utilizando, como fuente de excitación un láser de Diodo Rojo de 785.11 nm y 500 mW (Torsana Starbright) y refrigeración por aire. Este espectroscopio está dotado de Microscopio confocal con apertura seleccionable desde software y objetivos OLYMPUS (x5, x20 y x100) y platina porta muestras automática controlable desde software y desplazable en los tres ejes del espacio. El Software Spectra Manager II permite el control del sistema, adquisición y análisis de los datos.

Los parámetros espectrales fueron: Raman shift de 800 a 1800 cm-1, 10 acumulaciones, 15 segundos por acumulación. En esta región espectral se pueden identificar las vibraciones de los componentes moleculares del colágeno y del componente mineral de la dentina. La identificación y asignación de los picos más importantes en esta región, se ha realizado según lo descrito en la literatura[258,398]: el pico más intenso a 961 cm^{-1} (ν_1 tensión simétrica) se asigna al fosfato mineral de la dentina. El pico a 1070 cm^{-1} (ν_1 tensión simétrica, CO_3^{2-}) corresponde al carbonato mineral. Los principales picos asociados con los componentes orgánicos de la matriz dentinaria aparecen a 1242 cm^{-1} (NH-, Amida III), a 1667 cm^{-1} (Amida I) y 1452 (CH2 deformación-vibración)

El análisis cuantitativo de la intensidad de los picos se realizó tras ajustar la línea base mediante la técnica multipuntos, utilizando el software SpectraManager II.

Las ratios analizadas en este estudio han sido:

Razón carbonato/fosfato. (1072/959 cm^{-1}). Puede proporcionar información valiosa sobre la composición química inorgánica de la dentina.

Razón carbonato/ CH2 wagging (1072/1450 cm^{-1}): informa sobre las diferencias relativas en el contenido en carbonato tipo B15.

Razón fosfato/ CH2 wagging (959/1450 cm^{-1}): informa sobre el contenido mineral, representado por la intensidad de la vibración en tension del fosfato respecto a la de las vibraciones de agitación de las cadenas laterales del colágeno, a 1450 cm^{-1}. Esta banda se elige como referencia por su baja sensibilidad a la orientación molecular, al contrario que la banda de Amida I, a 1,670 cm^{-1} [393]

Razón Amida III/ CH2 (1243/1450 cm^{-1}): Indica diferencias estructurales. Se ha obtenido un valor más alto de está razón en dentina intertubular respecto a dentina peritubular, reflejando un mayor contenido en colágeno[394].

Razón Amida I /CH2 (1665/1450 cm-1): Un aumento de la banda Amida I respecto a la vibración agitación CH2 indica una alteración en la calidad del colágeno, que se ha relacionado con el envejecimiento[395], hidratación/deshidratación[396] o daño radiológico[397].

Además, para el pico del fosfato se estimó la anchura total a la mitad de la intensidad máxima (FWHM, full width at half máximum) se estimó para el pico de la vibración en tensión del fosfato, $\nu_1 PO_4^{3-}$ a 961 cm^{-1}; un pico más estrecho indica una mayor cristalinidad.

Análisis estadístico

El análisis y comparación de los datos entre los grupos del estudio se ha realizado mediante tests no paramétricos. El test de Kruskal-Wallis y test U de Mann-Whitney, se utilizaron para la comparación entre grupos y los tests de Friedman y de los rangos con signo de Wilcoxon para la comparación entre los niveles radiculares. La significación estadística se aceptó para un valor de $p< 0.05$.

RESULTADOS

I. Efectos del hipoclorito sódico en los components orgánico y mineral de la dentina del conducto radicular.

La irrigación del conducto NaOCl al 2.5%, 1 minuto durante la instrumentación (Grupo I) no demostró diferencias significativas en el componente orgánico respecto al uso de agua destilada.

La irrigación final del conducto radicular con NaOCl al 2.5% durante 5 minutos (Grupo II) demostró una disminución de la razón Amida I/Amida III (1667/1242 cm^{-1}) debida al descenso de la intensidad de la banda de la Amida III, así como disminución de la relación Amida I y Amida III respecto a la banda 1450 cm^{-1} (vibración de -CH2 de la matriz dentinaria), en algunos niveles de profundidad radicular con respecto al grupo control irrigado sólo con agua destilada (Grupo V). Respecto al grupo I, la aplicación prolongada de NaOCl demostró un aumento de la razón AmidaI/AmidaIII, a todos los

niveles radiculares, junto con una disminución en algunos niveles de la relación Amida III/CH2. Como hecho destacable, no se detectaron alteraciones del pico a 1670 cm^{-1}, asignado a la Amida I.

En la composición mineral, la aplicación del NaOCl durante 1 minuto produjo, respecto al grupo control, como efecto más importante una disminución de la ratio carbonato/fosfato detectable en los tres niveles más coronales de la raíz (C1, C2 y M1).

El Grupo II, en el que se realizó una aplicación adicional de NaOCl durante 5 minutos, demostró un aumento de carbonato y fosfato a nivel superficial (C1) respecto al grupo control, aunque la relación carbonato/fosfato disminuyó en algunos niveles radiculares (C1 y M1). Las diferencias del grupo II respecto al grupo I (aplicación breve de NaOCl) fueron irregulares, demostrando el grupo II niveles más elevados de fosfato y carbonato, pero una relación entre ambos componentes mayor para el nivel C2 y menor en el nivel M1.

II. Effectos de la irrigación con EDTA al 17%irrigation (Grupo III) sobre los componentes orgánico y mineral de la dentina del conducto radicular.

La aplicación de EDTA como irrigante final, respecto al grupo control y al grupo I, (irrigación con NaOCl 1 minuto), no produjo alteraciones detectables de la banda asignada a Amida I ni de la relación Amida I/Amida III. Se detectó una disminución de la intensidad de la banda la Amida III, con un descenso de la ratio 1245/1450 cm^{-1} en los niveles más coronas y de 1268/1450 en los niveles C1 y M1.

En el componente mineral, las diferencias con el grupo control fueron irregulares entre los distintos niveles radiculares. Respecto al grupo I, se obtuvo un aumento del carbonato (1072/1450 cm^{-1}) en los niveles C1 y C2 y un disminución del fosfato mineral en el nivel M2. En conjunto, la ratio carbonato/fosfato fue mayor en los especímenes tratados con EDTA que la obtenida en el grupo I en tres de los cuatro niveles radiculares analizados.

III. Efectos del imprimador auto-grabador en los componentes orgánico y mineral de la dentina del conducto radicular.

En el componente orgánico, las principales diferencias respecto al grupo control consistieron en una disminución de las ratios Amida III/CH2, en los niveles C2 y M2 para la razón 1242/1450 y en el nivel M2 para la razón 1268/1450. Se observó, en consecuencia, una tendencia al aumento de la relación AmidaI/AmidaIII, que fue significativamente mayor que la del grupo control en el nivel M2. Similares resultados se obtuvieron al comparar el grupo IV con el grupo I (irrigación con NaOCl, 1 minuto), con un valor mayor de la razón AmidaI/Amida III en el nivel C2.

Respecto al grupo III, el grupo IV (con aplicación adicional de imprimador) alteró la intensidad de los picos de Amida III aunque de forma irregular y demostró en conjunto un valor mayor de la razón AmidaI/Amida III, significativa en los niveles C2 y M2.

En cuanto a los efectos sobre el componente mineral, el grupo IV presentó, como efecto más destacable, una disminución de la relación fosfato/matriz en los niveles inferiores M1 y M2, sin afectación del carbonato mineral, al ser comparado con el grupo control. Respecto al Grupo I, el grupo IV presentó como hallazgo más importante, una relación ligeramente mayor carbonato/fosfato, aunque solo fue significativa para el nivel C2.

La comparación del componente mineral en el Grupo IV respecto al Grupo III, sólo mostró niveles más elevados de carbonato y fosfato de forma ocasional.

Finalmente, el valor de la medida de la anchura del pico de fosfato a 960 cm^{-1}, no demostró diferencias significativas entre ninguno de los grupos del estudio, indicando una escasa afectación de la cristalinidad en función del tratamiento radicular.

Conclusión

- La irrigación con NaOCl al 2.5% durante 1 minuto durante la instrumentación (Grupo I) no altera los componentes orgánicos de la dentina (Amida I y Amida III). Pero, si se realiza una irrigación final con NaOCl al 2.5% durante 5 minutos, se produce un descenso de la Amida III en algunos niveles radiculares junto con efectos inconstantes sobre el componente mineral de la dentina que indican una disminución en la razón carbonato/fosfato.

- La irrigación final con EDTA durante 1 minuto tras la instrumentación con irrigación con NaOCl al 2.5% durante 1 minuto produce escasa o nula afectación de las bandas de carbonatos y fosfatos, aunque con un ligero incremento de la razón carbonato/fosfato. En lo que respecta al componente orgánico, no afecta a la Amida III ni a la razón Amida I/Amida III.

- La aplicación de imprimador auto-grabador durante 30 segundos, al final de la instrumentación e irrigación con EDTA, produce efectos inconstantes sobre el componente mineral, sobre todo un descenso en la concentración de fosfato. A nivel orgánico, produce un descenso irregular de la Amida III en algunos niveles radiculares, sin afectación de la Amida I, y un aumento ocasional de la razón Amida I/ Amida III.

- Las variaciones en la cristalinidad no difieren significativamente entre la dentina radicular no tratada y la tratada con irrigantes.

Part 2

BOND STRENGTH TO ROOT DENTIN AND FLUID FILTRATION TEST OF SEVERAL RESIN SEALERS

2.1 Abstract

Objectives

To investigate the bond strength and seal ability produced by AH Plus/gutta-percha, EndoREZ and RealSeal systems to root canal dentin.

Material and methods

Sixty extracted single-root human teeth, instrumented manually to size 40, were divided into three groups (n=20) according to the sealer used; G1: AH Plus, G2: EndoREZ, and G3: RealSeal sealers. After filling using the lateral condensation technique, each sealer group was randomly divided into two subgroups according to the tests applied (n=10 for μPush-out test and n=10 for fluid filtration test). A fluid filtration method was used for quantitative evaluation of apical leakage. Four 1-mm-thick slices (cervical and medium level) were obtained from each root sample and a μPush-out test was performed. Failure modes were examined under microscopy at 40X, and a one-way ANOVA was applied to analyze the permeability. Non-parametrical statistics for related (Friedman's and Wilcoxon's rank tests) or unrelated samples (Kruskal-Wallis and Mann-Whitney tests) allowed for comparisons of μPush-out strength values among materials at the different levels. Statistical significance was accepted for p values < 0.05.

Results

There are no significant differences among fluid filtration of the three sealers. The sealer/core material does not significantly influence the μPush-out bond strength values ($F=2.49$; $p=0.10$), although statistically significant differences were detected with regard to root level ($Chi^2=23.93$; $p<0.001$). AH Plus and RealSeal obtained higher bond strength to intraradicular dentin in the medium root slices.

Conclusions

There are no significant differences between the permeability and global μPush-out bond strength to root canal dentin achieved by AH Plus/gutta-percha, EndoREZ and RealSeal systems.

Keywords

AH Plus. Fluid filtration. Intraradicular dentin bonding. Resin sealer. Push-out bond strength.

2.2 Introduction

A.2.2. Permeability

A.2.2.1. Permeability definition

Permeability consists of the condition of being open to passage, especially fluids, ions, bacteria, and minute particles. In physics it is the rate of diffusion through a body or tissue under standard situations, including the movement of fluids, ions, molecules, particulate matter and bacteria into and through a substance or tissue under different and varying conditions.

A.2.2.2. Importance of permeability

The permeability of the dentin is crucial to support the physiology and reaction patterns of the pulp-dentin organ. The permeability of dentin has become a fundamental part of modern restorative dentistry, where adhesive technology plays a central role. Recent attention to dentin permeability surrounds the penetration of resin monomers into dentin. The penetration of resin monomer into dentin tubules and their branches, and its impregnation of the thin layer of demineralized intertubular collagen matrix exposed as a result of acid etching, are essential components in bonding resin based restorations to dentin. Measurements of changes in permeability or of fluid filtration through dentin are repeatedly used for testing the sealing ability of restorative adhesive[1-5]or non-adhesive[6] materials, the mobility of potentially toxic materials[7-9] the effectiveness of toothpastes[10] or desensitizing materials,[11-14] the uptake of substances,[15] or the effect of diverse clinical procedures[16-20].

A.2.2.3. Factors affecting fluid filtration

Many factors affect this passage, including the area exposed, the chemistry and structure of the tissue involved, the tissue thickness, and the pressure exerted on the process. The size of the particle is also important when testing dentin permeability as well as any chemical interaction between the dentin and the penetrating agent, the characteristics of the dentin (such as density), dentin calcification and topical application (dentin may have open tubules, as in newly erupted teeth, or it may have tubules that are partly or completely occluded by mineralized deposits), and the volume of dentinal tubules (some parts of root dentin have relatively few tubules). These differences will affect fluid filtration and the

penetration of sealers into the dentin tubules. The use of fluid filtration to assess the patency of dentinal tubules has the advantage of generating quantitative values, which are clearly more objective than a qualitative assessment.

Clinical conditions that reportedly affect or are associated with dentin permeability include aging, dentin hypersensitivity, different types of wear, biological reactions to restorative materials, dental caries and bonding to dentin.

A.2.2.4. Mechanism of fluid filtration

Fluid transport through dentinal tubules is transited by either the difference in concentration between the outer and inner dentin surfaces (diffusion) [21] or the presence of a pressure gradient, meaning the pressure on one side is higher than on the other (convection) [22]. Most dentine permeability studies use a pressure gradient to cause fluid movement.

A.2.2.5. Root canal treatment

A.2.2.5.1. Objectives

It has been found that apical periodontitis is caused by bacteria derived from the root canal[23-25]. Therefore, one chief goal of root canal treatment is the elimination from the root canal space of microorganisms which are the cause of pulpitis, apical periodontitis[23,26,27] and failure in endodontic treatment[28], and the prevention of reinfection. An additional goal is to seal the root canal system from the outside environment using an obturating material to stop leakage from the oral cavity and the periradicular tissues into the root canal system. Chemo-mechanical preparation is considered the most important step in the management of the infected root canal system, though it is difficult or even impossible to remove all organisms from the canal space[29]. Microorganisms present inside root canals may remain alive in the dentinal tubules even after vigorous chemical-mechanical preparation. Bacteria can continue in areas such as lateral canals and dentinal tubules, where they may be protected from the disinfecting actions of irrigant and medicaments[30]. These remaining bacteria can play a role in continued periapical disease[31].

A.2.2.5.2. Three dimensional filling

A number of studies have shown that most teeth with apical periodontitis will be cured despite having a positive bacterial culture at the time of root filling[32]. Filling may overcome some of the limitations of chemo-mechanical preparation, with the main objective being to eliminate all lines of leakage from the oral cavity and the periradicular tissues into the root canal system by producing a fluid tight seal[33]; and to remove space and seal within the root canal system any irritants that cannot be fully removed during cleaning and shaping procedures[32]. Thus, three dimensional obturation of the root canal system is widely accepted as a key to successful endodontic therapy. Schilder et al.[34] state, "The objective of root canal procedures should be the total three dimensional filling of the root canal and all accessory canals." A well-fitted three dimensional root canal filling stops percolation and microleakage of periapical exudates into the root canal space, prevents reinfection, and produces a favorable biological environment for curing.

A.2.2.5.3. The basic principles of root canal filling

The standard root filling is a combination of sealer cement with a central core material. The core acts like a piston upon the flowable sealer, causing it to spread, filling voids and wetting and attaching to the instrumented dentin wall. It follows that the sealer should possess many of the critical properties of the root filling.

A.2.2.5.4. Properties of root canal filling material

A root canal filling material must present suitable biological and physicochemical properties. First of all, it must be inert biocompatible material, not irritating the periradicular tissues, amenable to different obturation methods, radiopaque, antimicrobial, easy to manipulate and easily removed for post placement or retreatment. Ideally, it would be desirable that it stimulates reformation and biologic sealing by mineralized tissue deposition in the apical foramen. It should be noted that some desirable technical, practical, and even biological properties must be subordinated to the main functions of the root filling: filling and sealing.

A.2.2.5.5. Function of root filling

Sundqvist and Figdor[35] assigned three primary functions to the root filling: sealing against ingrowth of bacteria from the oral cavity; entombment of remaining micro-organisms; and complete obturation at a microscopic level to prevent stagnant fluid from accumulating and serving as nutrients for bacteria from any source. This notion of bacterial entombment suggests that bacteria staying within the root canal space are rendered harmless as they are deprived of crucial nutrients and space required for growth and multiplying.

A.2.2.5.6. Types and composition of endodontic filling materials

A.2.2.5.6.1. Gutta-percha

The most commonly used core filling material is gutta-percha. The introduction of thermoplastic gutta-percha to dentistry in the mid-19th century was a turning point in endodontic treatment. Plasticity combined with physical durability made it possible for the material to move into the recesses of the root canal system and to adapt to the canal walls. This material consists of coagulated exudates isolated from several species of the tropical tree palaquium (sapotaceae). It is a trans-isomer of natural rubber of caoutchouc, but is harder, more brittle and less elastic[36]. Crystalline gutta-percha may occur in α- or β-phase, and there are only minor differences in the chemical behavior and physical properties of the two. The α phase appears in nature; the β-phase occurs during refining and is dominant in the products used in endodontics. In their final form, gutta-percha points contain some 20% gutta-percha and up to 80% zinc oxide. A dye and metal salts are added for color and radiographic contrast. Some manufacturers add antimicrobials, for example calcium hydroxide[37], chlorhexidine[38] or iodoform[39], to impart some disinfectant properties to the materials. Gutta-percha is used as filling and impression material in dentistry and orthopedics, and as an insulator in electronics. It has also been used as a rubber substitute. It is compressible, allowing adaptation to the walls of the canal preparation during condensation, and it is dimensionally stable, undergoing little or no dimensional change despite temperature changes. It is very well tolerated in tissues, being considerably less reactive than gold or silver, and it is radiopaque, but does not spontaneously bond to the dentin wall.

A.2.2.5.6.2. Resin-based filling material

Recently resin-based material was introduced. Synthetic resins have been discussed and tested as endodontic filing materials for many decades[40]. This material is an alternative to gutta-percha in clinical and practice, first present with the introduction of Resilon. A new root canal filling material, RealSeal, is a thermoplastic synthetic polyester, difunctional methacrylate resin based root canal filling material that contains bioactive glass and radiopaque fillers based on Resilon. This material handles like traditional gutta-percha and is therefore called resin-percha[41]. It is obtainable in standardized points that fit endodontic instruments and in various tapers, as well as in accessory points for use with the lateral condensation technique, and pellets for use with the Obtura II delivery system. Different techniques can be used to place this material into the canal (single-cone method, cold lateral condensation and thermoplastic techniques), with the same instruments and devices that are used for gutta-percha condensation[42-43]. The main advantage of thermoplastic resin rather than gutta-percha as a core material resides in the extent to which it will bond to the sealer.

A.2.2.6. Sealer

Because of the lack of a chemical union between gutta-percha and the root canal dentin, regardless of the filling technique used[44], gutta-percha should be used with a sealer to achieve an optimal seal. The use of sealer cement together with a core filling material is suggested with most obturating techniques[34].

A.2.2.6.1. Function of sealer

The sealers are responsible for the principal functions of the final root filling: sealing off the root canal system, entombment of remaining bacteria, and filling irregularities in the prepared canal. Sealer cements produce a union between the core material and the canal wall by filling any residual spaces[45]. In addition, sealer cements often have the ability to penetrate areas such as lateral canals and dentinal tubules.

A.2.2.6.2. Requirements of sealer

Requirements for an ideal root filling cement, according to Grossman[40] are as follows. It should be easily introduced into the canal, should seal the canal laterally as well as apically, and should not shrink after being inserted. Furthermore, it should be impervious to moisture, bacteriostatic or at least not encourage bacterial growth, radiopaque, not stain tooth structure and not irritate periapical tissue; and it should be sterile, or quickly and easily sterilized before insertion, and easily removed from the root canal if necessary. A suitable endodontic sealer[46] should present good adhesion to the root canal wall and gutta-percha, and good sealing ability[47]. The quality of the filling relies largely on the sealing capacity offered by sealers[48-49], acting as a lubricant[50], while dimensionally stable to avert fluid circulation between canal compartment and the periapex. Neither shrinkage nor expansion is considered desirable for a root canal filling material. Shrinkage may cause slits and passageway for bacteria and their products; expansion may create forces leading to the fracture of dentin. An ideal root canal sealer should be nontoxic, hermetically seal the root canal system, and have good tissue compatibility and a lasting tightness, providing dimensional stability against shrinkage, expansion and solubility. In addition, it should be both insoluble in tissue fluids and able to fill all the unoccupied spaces, which is expected from a material with a suitable flow property [51-54].

Flow is the ability of sealer cement to infiltrate into irregularities and accessory canals of the root canal system, and it is held to be a very important property. The greater the flow, the greater the ability to penetrate into irregularities. Vice versa, if the flow is excessive, the danger of material extravasations to the periapex is increased, which could harm periodontal tissues[55].

Many authorities consider that, regardless of coronal seal, a complete seal of the root is needed to preserve long-term periapical health[56]. With this goal in mind, new endodontic sealers have arisen to improve the root canal seal beyond current possibilities using conventional materials.

A.2.2.6.3. Types of endodontic sealers

A great variety of endodontic sealers are available commercially. The hierarchy of sealers runs from zinc oxide and eugenol, calcium hydroxide, and glass ionomer, to epoxy resin and methacrylate resin-based sealers spanning some 80 years, and they are divided into groups according to their chemical composition.

Endodontic sealers based on zinc oxide and eugenol have been used clinically for several decades, and they have satisfactory physico-chemical properties[57]. The glass ionomer sealers were introduced into root canal treatment because of their adhesion to dental hard tissues[58]. Sealers based on resin were then introduced, including epoxy-resin sealers because of their adhesive ability, and methacrylate resin sealers due to the formation of a monoblock.

A.2.2.6.3.1. Epoxy resins

Epoxy resins are organic compounds containing an epoxide group. They are characterized by a reactive epoxy ring and are polymerized by the breaking of this ring[59]. Epoxy resins, with their strong thermosetting capacity, are often used as dental materials, affording very good physical properties and ensuring suitable biological performance. Excellent apical sealing has been observed with epoxy resin-based sealers[60].

A.2.2.6.3.1.1. AH Plus

AH Plus (De Trey-Dentsply, Konstanz, Germany) is an epoxy resin-based sealer shown to have low solubility and disintegration[61] and good adhesion[62]. It is placed in the canal without any dentin preparation or dentin adhesive, and can be used with any obturating technique. It contains no eugenol, which inhibits the polymerization of resins[63] and can interfere with bonding[64-65]. It can be used with gutta-percha in vertical or lateral compaction techniques. Previous studies showed that the epoxy resin-based root canal sealer AH Plus is cytocompatible[66], and has good tissue tolerance[67].

The composition of AH Plus sealer is AH Plus paste A, containing bisphenol-A epoxy resin, bisphenol-F epoxy resin, calcium tungstate, zirconium oxide, silica and iron oxide pigments; and AH Plus paste B contains dibenzydiamine, aminoadamantane, tricyclodecane-diamine, calcium tungstate, zirconium oxide, silica and silicone oil.

Tightness and insolubility of the polymerized material are pertinent for the function of a root canal sealer. These properties and the viscosity during application are directly reliant on the filler. Therefore, finely ground calcium tungstate with an average particle size of 8 µm and finely ground zirconium oxide of 1.5 µm average particle size are used. The mixed and polymerized AH Plus has a filler content of 76% in weight, the remainder of the constituents being polymers, Aerosil and the pigment.

In addition to the tube delivery, the proven and unchanged AH Plus sealer chemistry is now available as AH Plus Jet™ Mixing Syringe. The new double-barrel syringe significantly improves working ergonomics. AH Plus Jet comes with a mixing tip, which automatically mixes the sealer components in an ideal ratio. It features an intra-oral tip adjustable to individual anatomic conditions through rotation and angulations. Thus, AH Plus Jet allows direct application of the sealer into the root canal orifices. The sealer can be clinically applied with a single hand.

AH Plus has high radiopacity, owing to new fillers with a greater absorption capacity, thereby ensuring suitable visibility of the filling material even in thin layers. AH Plus is characterized by very low shrinkage, or high dimensional stability, decisive for the impermeability of the treated root canal. AH Plus has demonstrated good sealing properties[68].

A bacterial leakage study was carried out using *Enterococcus faecalis* as a microbial tracer to determine the length of time for bacteria to infiltrate through the obturated root canal to the root apex. The conclusion drawn from this comparative experiment was that the epoxy resin root canal sealer was more adaptable to the root canal wall and filling material than a calcium hydroxide sealer when bacterial coronal leakage was studied[69].

The antimicrobial effects of endodontic AH Plus, investigated[70] after 2, 20 and 40 days, showed slight inhibition of *Streptococcus mutans* at 20 days and on *Actinomyces israelii* at every time interval. No effect was found on *Candida albicans* and *Staphylococcus aureus*.

AH Plus has also been tested for possible interactions with living tissue. According to the present level of knowledge, AHPlus can be classified as harmless and safe.

Summary of AH Plus features:

1. Long-term sealing properties.
2. Manifesting dimensional stability.
3. Self-adhesive properties.
4. Very high radiopacity.
5. Excellent scientific documentation in many clinical and in-vitro studies.
6. Use as reference and standard in many investigations.
7. Extensive market history.
8. Fulfillment of requirements ISO 6876:2001 (E) for dental root canal filling materials.

Indication: Permanent obturation of root canals of teeth of the secondary dentition in conjunction with root canal points.

Contraindication: Hypersensitivity against epoxy resins, amines, or other components of the root canal filling material.

Removal of root canal filling: If AH plus is used in combination with gutta-percha points, the root canal fillings can be removed using conventional techniques for the removal of gutta-percha.

Working Time: The working time is at least 4 hours at 23°C[2].

Setting Time: The setting time is at least 8 hours at 37°C[2].

A.2.2.6.3.2. Methacrylate Resin Based Sealers

Methacrylate Resin Based Sealers (MRBS), based on polymer chemistry technology, appeared in the late 1990´s. The organic polymer matrix in most composite resins used is either aromatic or urethane diacrylate oligomer composite resins polymerized by the free radical-addition mechanism. Methacrylate resin based sealers enable obturation in a slightly moist root canal because they are hydrophilic. This hydrophilicity encourages the creation of deep resin tags stretching into the dentinal tubules from the root canals. Deep resin tags reinforce bonding and the clinical success of obturation. While several formulas have been introduced, two dominate the market, and were used in this study: EndoREZ (Ultradent Products Inc. Utah, USA) and RealSeal (Sybron Endo Glendora, CA, USA).

This genre of bondable root canal sealers has been encouraged in view of the highly desirable property of producing a monoblock within the root canal space[71]. The term monoblock refers to the adhesion of sealer to dentin and filling materials, the canal space becoming perfectly filled with a gap-free solid mass that stops or reduces microleakage, improving the fracture resistance of filled canals[72-73] and simplifying the clinical technique.

A.2.2.6.3.2.1. EndoREZ

(Ultradent Products Inc., South Jordan, Utah, USA) is a hydrophilic, two component, radiopaque, chemical or dual-curing sealer designed to bond to resin-coated gutta-percha[74] forming a monoblock. Its active ingredient is urethane dimethacrylate resin, which affords a hermetic seal deep into dentinal tubules and accessory canals. According to the manufacturer, EndoREZ has satisfactory sealing properties and an easy delivery system[75]. EndoREZ has the following properties:

- The first injectable, self-priming sealer —no mixing pads or primers necessary.
- Same radiopacity as gutta-percha.
- Superior flow and wetting for easy handling.
- Proven to reinforce roots and provide long-lasting obturation.
- Penetrates and adapts to intricate canals and dentinal tubules.
- Allows for easy post placement to facilitate simple post preps or retreatment.

EndoREZ is a second generation of bondable sealer[76-78] containing zinc oxide, barium sulphate, resins and pigments in a matrix of urethane dimethacrylate. It is non-etching, hydrophilic in nature, and does not need the adjunctive use of dentin adhesive.

It is designed to flow into accessory canals and dentinal tubules. The increased hydrophilicity of EndoREZ enhances its penetration, thereby aiding resin tag creation for retention and seal after smear layer removal[79]. In spite of this, however, gap formation occurs as a result of polymerization shrinkage[80]. EndoREZ is recommended for use with either traditional gutta-percha or resin-coated gutta-percha. Low bond strength to the dentinal wall was reported with conventional uncoated gutta-percha[81,82] because of a lack of chemical union between the polyisoprene component of gutta-percha and methacrylate-based resins. To overcome this problem, specific EndoREZ points are used; they are traditional gutta-percha cones with a resin coating that is a polybutadiene-diisocyanate-methacrylate adhesive[83]. This adhesive resin includes a hydrophobic portion that is chemically compatible with the hydrophobic polyisoprene substrate, and a hydrophilic portion that is chemically compatible with a hydrophilic methacrylate resin that creates a chemical bond between EndoREZ sealer and the gutta-percha cone, resulting in a durable, contiguous seal throughout the obturation infrastructure that works best with EndoREZ points. It has been recommended for a single gutta-percha cone technique, but can be used with other obturating methods. ENdoREZ is fully polymerized in 20-30 minutes.

In the early toxicology studies of EndoREZ by Pameijer, Zmener and Bangas, EndoREZ was determined to be biocompatible and was introduced to the dental profession. It was not found to have antimicrobial properties[84].

A.2.2.6.3.2.2. RealSeal endodontic obturation system

RealSeal (Sybron Endo, Glendora, CA, USA) is a synthetic polyester resin. This radiopaque endodontic obturation material contains bioactive and radiopaque fillers. RealSeal is based on Resilon (Pentron Clinical Technologies, Wallingford, CT) which is a thermoplastic synthetic polymer based root canal filling material containing bioactive glass and radiopaque fillers. RealSeal seems and handles like gutta-percha, though unlike gutta-percha, the RealSeal points bond to an associated sealer. RealSeal demonstrates all the advantages of gutta-percha (radiopacity, biocompatibility, retrievability, insolubility and thermo-plasticity), and there are master and accessory cones in ISO sizes. For retreatment purposes, it may be heat-softened or dissolved with solvents such as chloroform.

RealSeal sealer is a dual-cured dental composite resin sealer[42], containing a mixture of urethane dimethacrylate, polyethylene glycol dimethacrylate, ethoxylated bisphenol A dimethacrylate and Bis-GMA resins, silane-treated barium borosilicate glasses with a small amount of aluminum oxide, barium sulfate, calcium hydroxide, bismuth oxychloride with amines, peroxide, photo initiator, stabilizers and pigment. It can be used in conjunction with Resilon points.

RealSeal is a third generation methacrylate resin-based sealer that incorporates the use of self-etching primers, reduced from a 2-bottle system to a single-bottle system. The primer/adhesives mostly contain 2-acrylamido -2-methyl- propansulfonic acid (Amps) as the functional acidic monomer[85]. In the single-bottle type self-etching primer, the contents are functional acidic monomers, solvents, water that is necessary for ionization of the acidic monomers, and self-cured catalysts incorporated into a ‘‘one-component’’ (a single bottle). This is similar to the so-called all-in-one adhesives currently available in restorative dentistry.

The acidic primer, which is a resinous material incorporated in a volatile liquid carrier such as acetone or alcohol, is applied to the dentin surface, where it penetrates through the smear layer and demineralizes the superficial dentin. The acidic primer is air dried to remove the volatile carrier; then RealSeal sealer is applied and polymerized with the resin already in the matrix, locking it into the dentin surface. The primer forms a hybrid layer that bonds to the sealer, which in turns bond to the core. RealSeal Thinning Resin (SybronEndo), an ethoxylated bisphenol-A-dimethacrylate (EBPADMA) based resinous solvent, is also included in these systems to adjust the sealer viscosity. However, addition of the thinning solvent to the sealer without photo activation did not increase adhesion to dentin[86].

ADVANTAGES:

1. Designed like gutta percha for ease of use and reduced learning curve.
2. Highly radiopaque.
3. Potential reduction in microleakage.
4. May improve fracture resistance.
5. Eugenol-free.

6. Retrievable.
7. Immediate coronal seal when light cured.

DISADVANTAGES:

1. Small sealer syringe and potential waste with mixing tips.
2. Microbrushes may be too large.
3. Basic kits lack pellets for warm gutta-percha backfill.
4. Lacks larger cone sizes in larger taper systems.
5. Sealer may be more expensive than conventional ZOE systems.
6. More time-consuming, with additional steps.
7. Relatively sticky when heated.
8. No clinical studies.

Pending further investigation, it was found that the black material causing the darkening of the root canals was bismuth sulfide, which resulted from the reaction between the bismuth oxy chloride in the RealSeal root canal filler and a protein (from body fluid). The possible causes of the bismuth sulfide formation are primarily due to technique variations or not following the instructions of the RealSeal system perfectly, such as overheating and incomplete flushing of NaOCl from the root canal. Overheating can degrade the RealSeal root canal filler and may lead to improper sealing and leaking, which can result in the leaching of proteins into the root canal and subsequent bismuth sulfide formation. Failure to completely flush all NaOCl from the root canal can also result in the formation of bismuth sulfide.

A.2.2.7. Microleakage definition

Microleakage is the leak of fluids, debris and microorganisms between the walls of a prepared root canal and the restoration. Trowbridge[87] describes microleakage as the ingress of oral fluids into the space between the tooth structure and a restoration. These descriptions have been widely used by researchers[88,89,90]. Microleakage can occur at a micron level or at nanometer level. Microleakage is possibly more important for endodontic applications than bond strength, because even if materials have relatively low bond strength to dentin, they may be considered good obturating materials if effective in stopping microleakage [91,92,93].

A.2.2.7.1. Types of microleakage

Leakage at the micron level (bacterial microleakage) is the passage of bacteria to the root-filling interface. Leakage at the submicron level (nano leakage) would be the ingress of ions and molecules through root-restoration interface, while bacteria are not able to enter. It is agreed that fluid containing ions and molecules access dentinal tubules with ease when the dentin surface is treated with acid-etch or other conditioning agents. Investigation of microleakage is important in the assessment of restorative materials.

A.2.2.7.2. Location of microleakage

In practice, the use of a solid core with a sealer leaves two interfaces along which leakage could occur: the core sealer and dentin-sealer interfaces[46]. According to Timpawt[94], endodontic sealers are used to remove the interface between the gutta-percha and the dentinal walls. Leakage may occur at the interfaces between the sealer and dentin, or sealer and gutta-percha, and in spaces within the sealer itself.

A.2.2.7.3. Microleakage development

There are many factors that can contribute to microleakage. Polymerization shrinkage of materials is well documented, where the hardening phase causes contraction in volume, creating stress and forming gaps between the canal walls and filling[95]. Secondly, some materials have the property of thermal expansion and water absorption, making them susceptible to leakage formation[96]. Thirdly, long term effects of mechanical loading and thermal changes can cause elastic deformation and physical alteration of both the tooth substance and filling material, resulting in microleakage[87,91].

A.2.2.7.4. Adverse effect of microleakage

Microleakage, whether apical or coronal, may be clinically undetectable. It is a major factor influencing the longevity of dental restoration, as well as a clinical problem which may cause failure of endodontic therapy[97,98].

A.2.2.7.5. Factors affect microleakage

Microleakage is influenced by many variables such as different filling techniques, the physical and chemical properties of sealers, and the presence or absence of a smear layer[99,100]. Setting up a seal in the root canal may depend on the ability of the root canal sealer to penetrate into the dentinal tubules[101], and the adhesion of the sealer to gutta-percha and dentin, which, when complete, prevents apical leakage[102].

The penetration of sealer cements into dentinal tubules is believed to be a desirable result for a number of reasons. It will increase the interface between the material and dentin, thus improving the sealing ability, and retention of the material may be improved by mechanical locking. The other main advantage of penetration is the potential for these materials to exert antibacterial effects against bacteria that may reside within these areas. Sealers that display greater penetration will have a greater propensity to entomb viable bacteria within tubules, isolating them from potential nutrient sources. Penetration of sealer cements into dentinal tubules is influenced by factors that include smear layer removal, dentine permeability and filling technique[103,104,105,106]. Variations in the physical and chemical properties of sealer cements also influence the depth of penetration[104]. The ability of any one particular sealer cement to penetrate dentinal tubules consistently and effectively will be one factor influencing the choice of material for filling. The penetration of sealer cements into dentinal tubules may differ, given the physico-chemical properties of the sealer such as viscosity and particle size.

As described by McComb and Smith, the smear layer that affects microleakage, the penetration of a sealer and the bond between the sealer and the canal wall is a combination of organic and inorganic debris present on the root canal wall after instrumentation[107]. Its presence may act as a path for the ingress and growth of bacteria[108]. If filling materials leak out of the root canal and the smear layer is not removed, it can be eliminated by bacterial byproducts such as acids and enzymes, or it may slowly disintegrate and dissolve[109], or joined with saliva or any liquid present are forced or smeared onto the surface and often

into the tubules covering the prepared surface. The smear layer and the smear plugs in the opening of the tubules will reduce the permeability of the dentin, and interfere with the adhesion and penetration of root canal sealers into dentinal tubules. Many studies have reported reduction of apical leakage after removal of the layer[110].

A.2.2.7.6. Microleakage modification

Microleakage is an active process and thus varies over time. The progression of microleakage is due to long term biochemical reactions within the material itself and between the material and the surrounding environment, where the distance along the root filling may increase or decrease with time[87]. Moreover, dissolution of the sealer may increase leakage[111]. RealSeal is susceptible to alkaline[112] and enzymatic hydrolysis, meaning that biodegradation of RealSeal by bacterial/salivary enzymatic[113] and endodontically relevant bacteria might increase leakage. On the other hand, swelling of gutta-percha may decrease leakage[114] in the root canal. Physical and chemical properties of the sealer, such as the thickness of the sealer layer, may also play an important role in sealing the root canal[115] because 50% of the root canal surface is covered by sealer after lateral condensation of gutta-percha[116].

A.2.2.7.7. Microleakage studies

Microleakage studies used to comprehend the leakage pattern of filling materials can lead to an increased perception of the mechanism and etiology of microleakage. Establishment of the microleakage pattern is of considerable relevance for filling material selection in dental practice[89,91]. Different in vitro methodologies are used to estimate sealing quality, and laboratory-based experimental models are used to detect and assess leakage along root fillings. In the past, leakages of different root canal filling materials were measured by the penetration of dyes, glucose, bacteria, radioisotopes, microorganisms, or electro-chemical means, while dye leakage and bacterial penetration were the most repeatedly used in laboratory models to assess microleakage after root sealing. All of these techniques have been shown to have a variety of deficiencies. Some investigators harbor doubts about the relevance of these tests under clinical conditions[117] because the size and shape of the tracers are different from the bacteria and endotoxins that cause periapical disease and endodontic failure[118]. These tracer methods also have the shortcoming of being semi-quantitative. In dye-penetration studies trapped air has been shown to limit the penetration of dye[119,54]. Furthermore, if the specimen is divided into

sections, for leakage to be measured on the cut surface, the tooth must be destroyed, so longitudinal studies cannot be performed. It may be more pertinent to assess the amount of fluid crossing through a canal than measuring the length of a gap in a filled canal[120].

A.2.2.8. Fluid filtration system

For the reasons mentioned above, Wu[121] affirm that the fluid filtration technique may be more suitable for assessing endodontic sealing strategies. Derkson[122] developed a fluid filtration technique to measure microleakage around coronal restorations, a technique adapted by Wu[121] to measure microleakage of root end fillings. The measuring device consisted of a calibrated micropipette of appropriate volume, with an air bubble placed into it, connected via tubes to the specimen on one side and the liquid reservoir on the other. Displacement of the bubble along the micropipette, usually toward the specimen, measured the amount of fluid movements. They described this technique as being capable of quantitatively measuring volumetric microleakage. It has been used for 20 years to explore the physiology of dentin[123], and the effects of various restorative treatments on dentin permeability[124]. The introduction of the fluid filtration method in endodontics[125] has gained popularity in evaluating apical or coronal microleakage. Many researchers have thereby evaluated the sealing efficiency of root end filling materials[126-130], apical leakage of post and core restoration in endodontically treated teeth[6] and the microleakage of temporary restorative materials[131]. This method has also been used to compare the sealing efficiency of different root canal filling methods[115,132.133].

A.2.2.8.1. Advantages of fluid filtration system

This method presents several advantages: the samples are not destroyed, permitting the evaluation of sealing efficiency over time; no tracer is needed with the related problems of molecular size, or affinity for dentin; and no intricate materials are required as in bacterial penetration studies or radioactive tracer studies[134]. The results are generally expressed as µl/min/cm H2O. Fluid filtration is based on the principle that no fluid movement will be discovered if the root canal system is completely closed. Further, the fluid filtration technique calculates microleakage and allows for repeated measurements because it is non-destructive[135].

A.2.2.8.2. Properties of fluid filtration system

This system is a sensitive, reproducible and non-destructive method, making possible repeated observation of the same specimen over time[121]. In combination with other evaluation protocols such as SEM analysis[122], it can be used to assess and compare fluid movement at two observation periods. It provides some level of quantitative and qualitative analysis. It is fairly effective, cheap and straightforward.

It has some drawbacks, however. First, it does not allow for continuous recording of fluid flow, and because some changes are related to time, discontinuous recordings impede an accurate discernment of different parts of the recording. Secondly, if changes are to be introduced in the environment via simulation of stimuli to the specimen, recording of the precise timing is fundamental. Moreover, the exact location of leakage cannot be directly determined. Bearing in mind that in the micropipette method readings are made visually, this method is more susceptible to personal determinant errors[136].

A.2.2.7.7.1. Factors influencing microleakage studies

Factors that influence microleakage studies include, firstly, the substrate for microleakage studies, given the limited availability of human teeth and the concern about infection control effects. Secondly, storage factors such as time, media, and temperature for the storage of extracted teeth can play a role in microleakage studies. The duration of tooth storage after extraction can range from minutes to years[91]. Notwithstanding, a review by Rueggeberg[137] concluded that time after extraction has no impact on bonding result.

B.2.2. Micro Push-out bond strength

B.2.2.1. Bond strength definition

In recent years obturating materials and sealers have been developed based on dentin adhesion technologies. Adhesive materials are frequently compared using bond strength and microleakage tests. Bond strength is defined as the ability of two materials to adhere to each other[138], or it is the force per unit area required to break the bond between the adhesive material and dentin. It is usually limned in megapascals (MPa) that is, Newtons per square millimeters. According to Tagger[139] “bonding” is a better term than “adhesion”, because it suggests that the attachment between substances could have been initiated by other factors such as mechanical interlocking.

B.2.2.2. Importance of adhesion

Crossman postulated that an ideal endodontic sealer should adhere firmly to both dentin and gutta-percha[140]. Adhesion of the root canal filling to the dentinal walls is essential in both static and dynamic states, as it removes any space that permits penetration of fluid between the filling and the wall, and withstands dislodgment of fillings during future operation[46]. The adhesion of root canal sealers to gutta-percha seems to be an important property for preserving the integrity of the apical seal, thereby reducing apical microleakage. Differences in the adhesive properties of endodontic sealers may be expected, because their interaction with gutta-percha can vary along with their chemical composition.

B.2.2.3. Mechanism of adhesion of resin-based sealers

Hybridization is the primary process used today to bond restorative resin materials to dentin. Contrary to common belief, the dentinal tubules make only a minor contribution to dentin adhesion. Most retention is due to micromechanical retention from the collagen matrix in the intertubular dentin[141-143]. While micromechanical retention is believed to be the primary cause of retention overall, there is also a minor chemical interaction between dentin and some adhesive systems[144].

Another principle of adhesion of a resin-based material to root canal walls is based on forming a good adhesive interface to dentin as well as penetrating the adhesive agent within the tubules and the formation of tags[145,146]. Acid etching of dentin surface will remove the smear layer and the smear plugs, leaving a mesh of collagen on the prepared surface. The resin monomer gets into dentin tubules and their branches, and its impregnation of the thin layer of demineralized intertubular collagen matrix exposed as a result of acid etching[147] is an essential component of bonding resin based restorations to dentin. The thin layer of collagen exposed as a result of the demineralization, when infiltrated by resin monomer and polymerized, forms the so-called “hybrid layer”. This layer is an essential part of adhesive dentistry. With most products, the hybrid layer is between 2 and 5μm in thickness[148]. If failure occurs during the penetration of the monomer into dentin, the exposed collagen fibers are not filled. If the fibers are not filled, hydrolysis of this layer may occur due to the removal of hydroxyapatite crystals without replacement by the resin-based material. The result is a failed union between the dentin and resin-based material, formation of gaps and subsequent decrease in bond strength[149] when methacrylate-based cements etch the dentin through acidic primers; these cements are called self-etching system. These adhesive cements encourage the retention to dentin walls by chemical reaction and micromechanical retention1[150].

B.2.2.4. Modification of dentin bonding

Different modifications can affect the bonding of sealers to dentin over time. Plasticization is a process in which fluids are absorbed by resins, causing them to swell, resulting in degradation of their mechanical properties[151]. Hydrolysis due to water ingress between the hybrid layer and unaffected dentin can break the covalent bonds within collagen fibrils and the resin polymers[152]. This process is enhanced by enzymes released by bacteria[153] and from the dentin itself [154]. The breakdown products diffuse out of the interfacial area, weakening the bond, and allowing more fluid to ingress. Collagen degradation is thought to stem from host-derived matrix metalloproteinases (MMPs) that are present in dentin and released slowly over time[154]. MMPs are released by bacteria, along with other enzymes[153,154], but bacteria are not necessary for collagen degradation to occur[154]. Another potential modification is the deterioration of the resin bond over time, a process well documented in vitro[155,156,157,158,159] and in vivo[160,161]. Interfacial leakage increases as the bond degrades [162,163]. Functional forces have been shown to contribute to the degradation of the resin bond in restorative applications[164,165]. This is also true in the root canal system, where torsional and flexural forces stress the dentin/resin interface repeatedly during function. Repeated stress causes microfractures or cracks within the resin[151].

B.2.2.5. Adhesion Studies

Several studies have looked into the adhesion of different kinds of root canal sealers to root dentin and gutta-percha[148,62]. Although the American Dental Association[166] put out a series of rules and tests for the study of physical properties of root canal sealers, adhesion tests have not yet been standardized because no consensus on test parameters has been reached among researchers. Moreover, the divergent results obtained in studies and the difficulties in testing materials with great plasticity, such as gutta-percha and Resilon® (RealSeal), or materials with high modules of elasticity, such as radicular posts, have led to the development of different methodologies for determining the bond strength of endodontic sealers to coronal or root dentin[167-170].

B.2.2.6. Micro Push-out test

Bond strength can be determined by several techniques. An adequate method for evaluation of the adhesion of root canal filling materials would supply more reliable results, allowing comparison of the materials to validate their clinical choice. Numerous studies have looked into the bond between the sealer and the canal wall[171,172], including the effect of the smear layer on bond strength[173,174]. More recently, a "push-out" test was developed to measure the bond between sealer, canal wall and core material[81,175]. The test is intended to evaluate the extent to which the sealer and core material are bonded into a solid mass, as well as the strength of the bond to the canal wall. The push-out test permits an accurate standardization of the specimens[176], and may offer a better estimation of the actual bonding effectiveness. The push-out test would appear to be more reliable because of the absence of premature failures and the variability of data distribution[177].

B.2.2.7. Factors affecting the bond strength of cement to dentin

The adhesion of cements to root dentin has always represented a challenge for dental clinicians because of the unfavorable ovoid canal configuration[178,179]; the anatomical and histological characteristics of the root canal, including the orientation of the dentin tubules[180-184]; the influence of different root canal regions[185,186], since the number of tubules decreases from the crown to the apical root[187]; and the response to acid etching. Consequently, dentin bonding can vary among different areas of the same root canal[181]. Bond strength values are higher for the crown (above the cementum-enamel junction) than for the root dentin[188]. Further conditioning factors are the hydration degree of the root canal dentin; the type of resin sealer and adhesive used[189,190]; the conditioning agent and accompanying cement used; the filler content of the cement[191,192]; the thickness of cement[193-195]; the use of eugenol-containing sealers; the presence or absence of smear layer; and finally, differences in root dentin among specimens. Moreover, moisture control and the difficulty of viewing the root canal can complicate bonding procedures. If resin-based sealers undergo polymerization shrinkage, the quality of the bond to dentine and to core material is affected[79]. A pulling of resin sealer tags out of the tubules during polymerization shrinkage of the sealer produces gaps along the sealer dentin interface[75,196,197].

2.3 Objectives

1. Measure and compare the fluid filtration of roots treated endodontically with gutta-percha/AH Plus, EndoREZ point/EndoREZ sealer and RealSeal point/RealSeal sealers.
2. Measure and compare the μPush-out bond strength of the roots treated endodontically with gutta-percha/AH Plus, EndoREZ point/EndoREZ sealer, and RealSeal points/RealSeal sealer.
3. Study regional differences when comparing the μPush out bond strength among root thirds (1st coronal- 2nd coronal- 1st middle and 2nd middle).
4. Try to establish associations between fluid filtration and μPush out bond strength.

2.4 Materials and methods

2.4.1. Collection of teeth

Sixty extracted non-carious, unrestored intact crowns, from straight and single-rooted permanent human anterior teeth with fully formed apices, were collected. Any excess calculus and soft tissue was removed, and specimens were preserved in 0.01% thymol at 4ºC. The roots with an isthmus, lateral accessory canals or more than one canal, open apices, and resorptive defects were excluded from the sample.

2.4.2. Specimen preparation

2.4.2.1. Instrumentation procedure

Having obtained sixty single-root teeth, the crowns of the teeth were resected at the cement-enamel junction with a diamond coated disk at slow speed with constant water-cooling (figure 1). The cuts were made perpendicular to the long axis of the root (figure 2). Any residual pulpal tissue was removed carefully with a barbed broach. Root canals were instrumented using the step back technique to obtain a flared preparation. A size 10 k-file (Dentsply, Ballaigues, Switzerland) was inserted into the root canal until the tip was just visible beyond the apex; the file was measured, and working length was determined by subtracting 1mm from this length. The canal systems were instrumented to the working length until a size 40 k-file. After each step in the flare preparation, the canal was irrigated with 2.5% sodium hypochlorite (NaOCl) by virtue of its antimicrobial and tissue dissolving properties. It is considered an ideal endodontic irrigant, in that it removes debris and wets the environment to facilitate canal enlargement, it causes alteration the cellular metabolism of microorganisms and destruction of phospholipids, and it degrades lipids and fatty acids. Its oxidating actions provoke the deactivation of bacterial enzymes[198]. Then, 17% ethylene diamine tetra acetic acid (EDTA; Colgate Oral Care Company, Waverly, Australia) was used as a final irrigant for roots filled with RealSeal sealer. A final rinse of distilled water was used to remove any remnants of the irrigating solutions. The canals were dried using paper points.

Figure1: Cutting machine with a diamond disk

Figure 2: Crown resected at the cement-enamel junction

2.4.2.2. Obturation procedure

The teeth were obturated using a lateral condensation technique with the following materials: gutta-percha/AH Plus, EndoREZ point/EndoREZ sealer, and RealSeal point/Real Seal sealer. A size 40.2 taper master gutta-percha cone (Dentsply Maillefer) or 40.02 taper of RealSeal point (SybronEndo) was placed in the canal to the full working length.

2.4.2.2.1. AH Plus

AH Plus (DENTSPLY De Trey, Konstanz, Germany) sealer was prepared according to manufacturer's instructions. AH Plus was mixed using the AH Plus jet mixing system (Figure 3), then introduced into the root canal orifices with the intraoral tip. The apical part of the master gutta-percha cone with 40.02 taper was coated with sealer and placed in the canal. The master cone was vertically and laterally condensed by inserting a stainless steelfinger spreader (Moyock union broach) between it and the root canal wall. The spreader was inserted to a point 1mm short of the working length, and rotated to 180º several times before disengaging it from the canal. The void created by the spreader was filled by condensing an auxiliary gutta-percha point. The procedure was repeated until gutta-percha points could not be introduced more than 3 mm into the root canal. Excess gutta-percha was removed with a hot instrument and the remainder was condensed vertically with a plugger.

Figure 3: AH Plus jet mixing system and intraoral tip.

2.4.2.2.2. EndoREZ

After canal instrumentation and rinsing with distilled water, the canal was not dried with multiple paper points. The hydrophilic nature of EndoREZ allows the clinician to use just a few paper points, to leave a small amount of moisture inside the canal. A two-part chemical set material was mixed in an auto mix nozzle (Figure 4). According to the manufacturer's recommendations, the material was dispensed into a narrow diameter syringe (Skini^{TM} syringe; Figure 5) with a fine tipped cannula (NaviTip^{TM}; Figure 6). The NaviTip^{TM} was inserted into the canal 2 to 3 mm short of the working length and the sealer was dispensed while withdrawing the syringe (Figure 7). Sealer was placed into the canal until the level of the sealer reached just short of the canal orifice. Then, a resin coated gutta-percha cone with 40.02 taper previously fitted to the working length was placed in the canal with lateral condensation. Additional cones were put into the canal as necessary to fill the space. The excess gutta-percha was then removed with a hot instrument and the remainder was condensed vertically.

Figure 4: EndoREZ nozzle

Figure 5: Skini ™ syringe

Figure 6: Navi Tip ™.

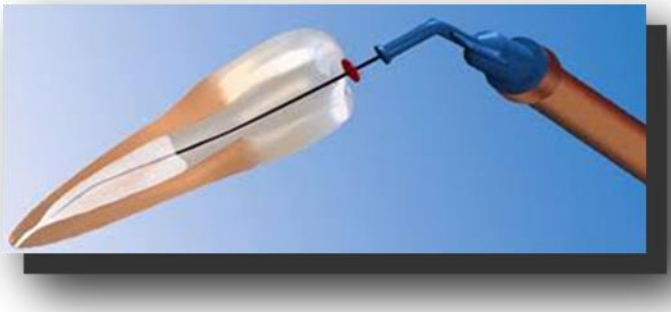


Figure 7: Insertion of EndoREZ sealer 2-3mm short of the working length

2.4.2.2.3. RealSeal sealer

After rinsing the canal with sodium hypochloride, 17% EDTA was used as the final irrigant. The canal was then rinsed with distilled water and dried. RealSeal Primer (Sybron Endo, Glendora, CA) was introduced into the root canal using a paper point (Roeko Langenau, Germany) soaked with the primer for the apical region. The primer was placed into the middle and coronal part using a micro brush (Micro brush, international, Grafton, WI, USA). After 30 seconds, excess primer was removed with paper points. An ISO standard 02 taper RealSeal master cone was probed in to within 1mm of working length. RealSeal root canal sealant was dispensed onto a mixing pad, after which the RealSeal thinning resin was applied to adjust the viscosity of the sealer, which was then placed into the root canal using a premeasured master point coated with the sealer and condensed with a finger spreader vertically and laterally. The rest of the canal was filled up with accessory points dipped in a small amount of sealer. This procedure was repeated until RealSeal points could not be introduced more than 3mm into the root canal (Figure 8). The RealSeal sealer sets in the canal in approximately 45 minutes, creating a monoblock that resists leakage.

Figure 8: RealSeal kit

In all specimens the length was kept identical: the roots were resected at the apex perpendicular to the long axis of the tooth until 10 mm of root length was obtained, so as to avoid anatomical variations and ensure standardization for the leakage measurements.

In the present study, the teeth were divided into three groups (n=20) according to the sealer used; Group1: AH Plus, Group2: EndoREZ, and Group3: RealSeal sealers. After filling using the lateral condensation technique, each sealer group was randomly divided into two subgroups in view of the tests applied (n=10 for µPush-out test and n=10 for fluid filtration test).

- Group I. AH Plus sealer and gutta-percha point for fluid filtration measurement.
- Group II. EndoREZ sealer and resin-coated gutta-percha point for fluid filtration measurement.
- Group III. RealSeal sealer and RealSeal points for fluid filtration measurement.
- Group IV. AH Plus sealer and gutta-percha cone for µPush-out test.
- Group V. EndoREZ sealer and resin-coated gutta-percha points for µPush-out test.
- Group VI. RealSeal sealer and RealSeal points for µPush-out test.

The teeth of all groups were kept moist at all times by wrapping them in a distilled water-soaked gauze at 37ºC for 24 hours to allow the sealer to set completely.

2.4.3. Leakage evaluation

Twenty-four hours after completion of the filling procedure, the roots of each group (I, II, and III) were resected at the apex perpendicular to the long axis of the tooth until 10 mm of root length was obtained.

A layer of nail varnish was then applied on the external root surface, leaving the coronal and apical orifices open for fluid exchange with the environment. Nail varnish was used to limit the movement of fluid across the dentinal tubules and ensure that any fluid flow assessed was caused by flow along the interface between the filling and the dentin. At that point, the specimen was attached to the apparatus to measure the fluid filtration.

2.4.4. Description of the fluid filtration apparatus

The apparatus used to assess dentin permeability was constructed by the West of Scotland Health Board's Department of Clinical Physics and Bio-engineering using the same principles as those described by Pashley[199]. The system involves the evaluation of fluid transport in specimens calculated from bubble movement. It is necessary to apply pressure so that the fluid moves through the specimen and displaces the bubbles. For this purpose, a reservoir of 250 ml of distilled water was used and placed 100 cm higher than the root, to produce a pressure of 100 cm H2O at room temperature. A specific rubber tube (2mm internal diameter) was attached to the reservoir of the system, as was a 10 ml insulin syringe (syringe A in Figure 9), connected to introduce an air bubble into the system, and then the rubber tube was closed with glue. The rubber tube was connected to a 10 ml micropipette with an inner diameter of 1 mm, which served to measure movement of the air bubble (Figure 10). The other end of the micropipette was connected to another rubber tube, to which a 10 ml plastic syringe was attached (syringe B). Syringe B was used to pull the air bubble into the micropipette and then adjust its position before the start of each measurement. The apparatus was flushed to ensure that there were no air bubbles trapped in the tubing. The diameter of the air bubble must not be smaller than the internal diameter of the micropipette so that its movement will be a precise indicator for fluid movement in the micropipette. At the end of the rubber tube, the root was inserted and placed in a plastic syringe filled with distilled water. All the parts were fixed onto a wooden board, which was kept horizontal using a spirit level.

System sensitivity can be adjusted by altering the pressure used or the diameter of the micropipette. Various parameters could modify the test results, such as diameter of the capillary containing the bubble, the length-measuring time, and the pressure applied[123]. This should be expressed as µl/min cm H20 instead of µl/min.

Figure 9: insulin syringe

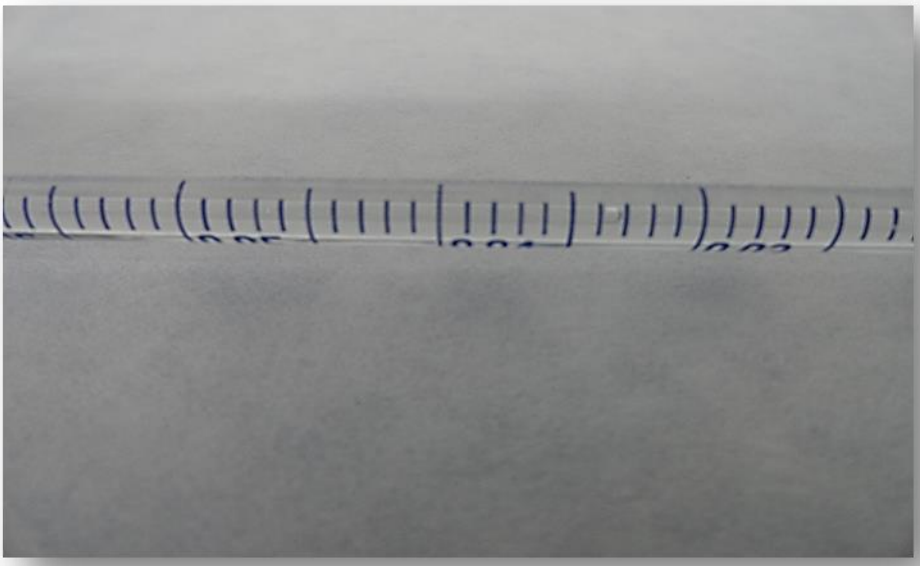


Figure 10: 10ml micropipette with air bubble inside

2.4.5. Measurement of fluid filtration

At the start of each day, the system was sealed and checked for 10 min to ensure there was no leakage. Throughout all measurements of fluid filtration, the root was covered with water and then covered with a plastic dish to prevent evaporation, which could have potentially altered dentin permeability artificially. Each specimen was attached to the apparatus, and the system was bled by opening the joint between the water reservoir and the specimen. This joint (between the specimen and the apparatus) was used to bleed the system after the connection of each specimen. In order that the solution could infiltrate into the root of the specimen, 10 min were allowed to elapse before measurement. The air bubble was then adjusted so that it was aligned with the zero point of the 10 ml scale of the micropipette (Figure 11). After a further 24 hours, any change in the position of the bubble was recorded by looking at the scale on the micropipette. This measurement showed the amount of fluid that had permeated through the root filling. The unit of fluid volume within the micropipette was micro-liters. Permeability was taken as the distance, in mm, that the bubble had moved by the end of the 24 hours measuring period. Accordingly, 1mm displacement of the bubble in the pipette is equivalent to a constant volume of fluid movement in the sample that means 1 mm of the scale corresponds to 1 µl fluid movement in the sample.

The measurements were done in 10 specimens of each group (sealant), the averages were calculated.

Figure 11: Air bubble at zero position.

2.4.6. Preparation of samples for µPush-out test

Twenty-four hours after the filling procedure, the roots of groups IV, V, and VI were divided into sections using a 300 µm thick sintered diamond wafering blade (Struers, 5010323, Denmark; Figure 12) perpendicular to the long axis of the root canal at low speed with constant water cooling. Four slices of 1mm thickness sections of root dentin were prepared (Figure 13). The thickness of each root slice was assessed by means of a digital caliper. The specimens were mounted on the machine to perform the µPush-out test.

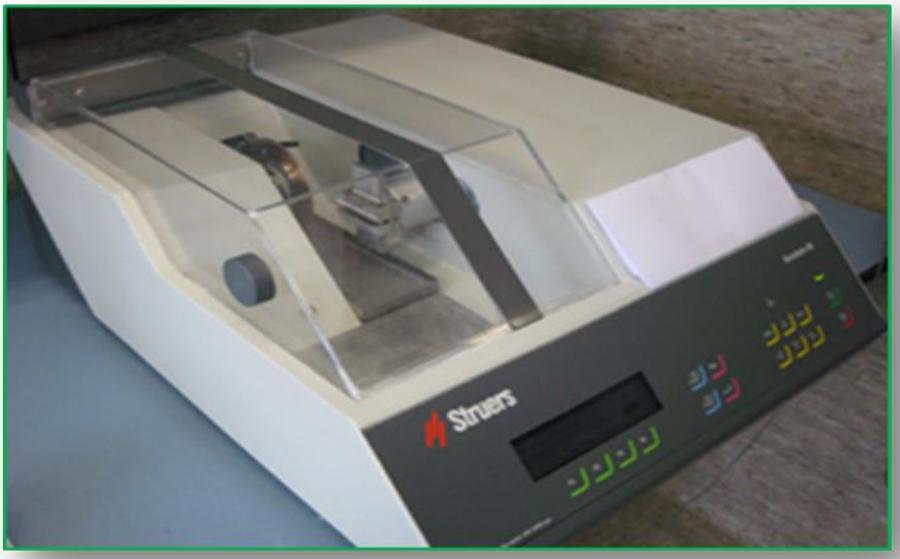


Figure 12: Struers machine for serial cutting.

Figure 13: Root slices for µPush-out test.

2.4.7. Micro Push-out test

The µPush-out test was used to evaluate the bond strength of the three types of resin-based sealer. After cutting the root to four slices of 1mm thickness, each slice was subjected to compressive loading via a universal testing machine (Instron 3345, Instron Ltd, High Wycombe, UK; Figure 14), equipped with a 1 mm-diameter cylindrical plunger. The plunger was positioned so that it only made contact with the root filling upon loading, without touching the canal wall, thereby introducing shear stresses along the interfaces. For the test, a stainless steel support was used to carry the specimen (metallic ring). Specimens were placed within a centralizing ring to ensure centered application of the load. The side with the smaller diameter of the root canal faced upwards and was aligned with the shaft that would exert pressure load on the filling (apical-coronally) until the filling was displaced. This method thus ensured the alignment of the specimen in a reproducible manner, and also avoided any restriction interface due to root canal taper during the push-out test. The machine was calibrated at a constant speed of 0.5mm/min; the maximum failure load was recorded in Newtons (N) and converted (into MPa) by dividing the applied load by the bonded area (A). Failure was indicated by the extrusion of the intact cone of root filling from the root slice (Figure 15) and confirmed by the appearance of a sharp drop along the load/time curve recorded by the testing machine (Figure 16). The highest value recorded was taken as the push-out bond strength. The computer and software attached to the universal testing machine calculated the push-out bond strength value for each specimen from the average of the perimeters (coronal-apical) and the thickness of the specimen, via the formula:

$$\text{Debond stress (MPa)} = \frac{\text{Debonding force (N)}}{\text{Area (mm)}}$$

Where the debonding force is the maximum force before debonding, and area (of the bonded interface) is the average value of the perimeter times the thickness.

Once the failure happens, the specimens were recovered and examined at 40 magnification on a stereo-microscope (SZ60, Olympus, Tokyo, Japan), classify the failure mode as an adhesive (between the surface of the dentin and filling material), cohesive (in root filling material) or mixed

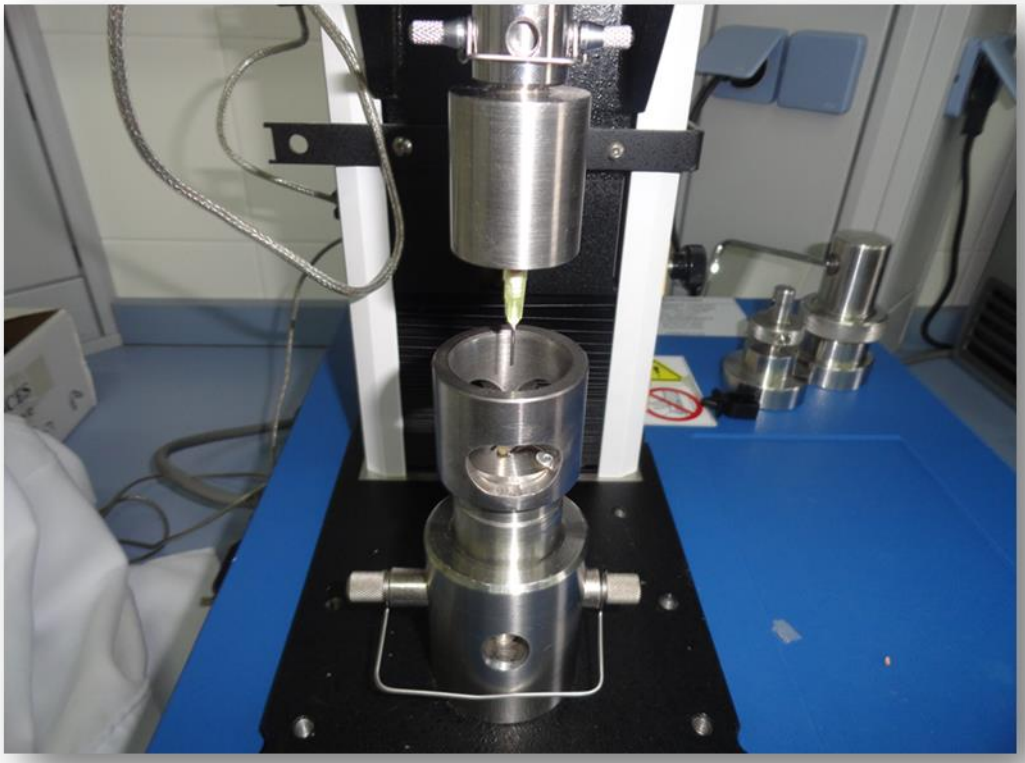


Figure 14: Instron universal testing machine for push-out test.

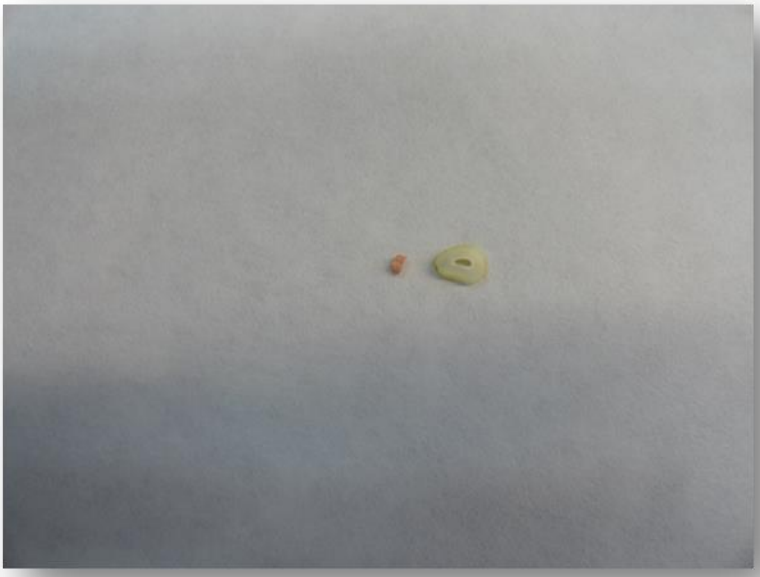


Figure 15: Extrusion of the root filling from the root slice.

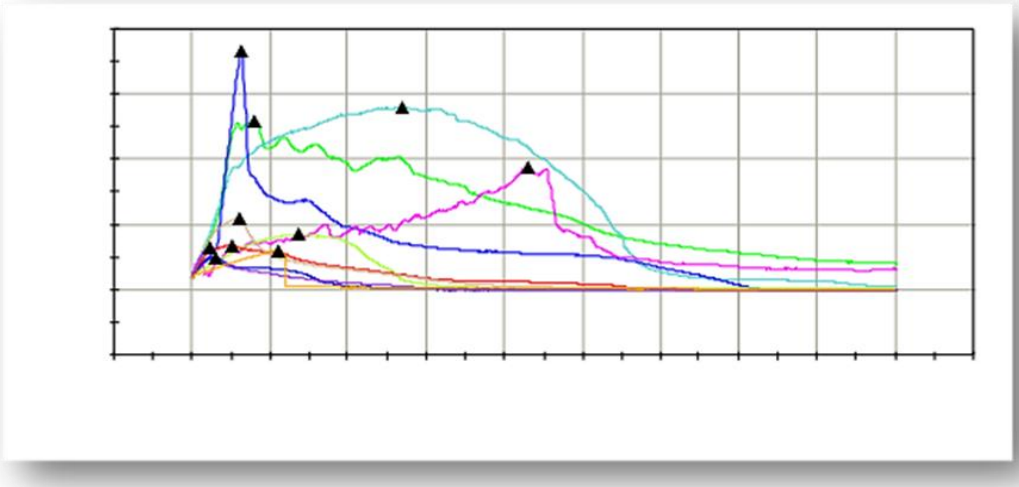


Figure 16: The graph from the instron machine illustrates the sharp drop due to extrusion of the intact cone of root filling.

2.5 Results

A. Permeability

A.2.5.1. Statistical analysis

Fluid movement values, recorded after 24 hours subjected to 100 cm water pressure, were compared among the three different sealers and filling materials by One-way ANOVA, after verifying the normality of distribution by means of the Shapiro-Wilk test and the homogeneity of variances by Levene's test. Values were considered significant for a p value <0.05.

A.2.5.2. Results

Shapiro-Wilk tests allowed for testing the normality of data distribution in all the study groups ($p<0.05$). Levene's tests revealed the homogeneity of variance among groups ($p=1.510$). Mean values (SD), maximum and minimum values are recorded in Table 1.

Table 1: Mean values of permeability (µL/24h) and standard deviations.

PERMEABILITY	N	MEAN(SD)
AH Plus	10	10.45 (6.45)
EndoREZ	10	8.49 (5.92)
RealSeal	10	6.20 (3.82)

As results, the numerical values showed the AH Plus sealer group to have the highest value of microleakage, followed by EndoREZ sealer; RealSeal sealer had the lowest value of fluid, meaning it provided better resistance to microleakage. But one-way ANOVA test revealed that the differences among the three sealers in micro leakage were not significant. This confirms that all of the materials used in this study allowed fluid to flow along the filled root canal twenty-four hours after filling.

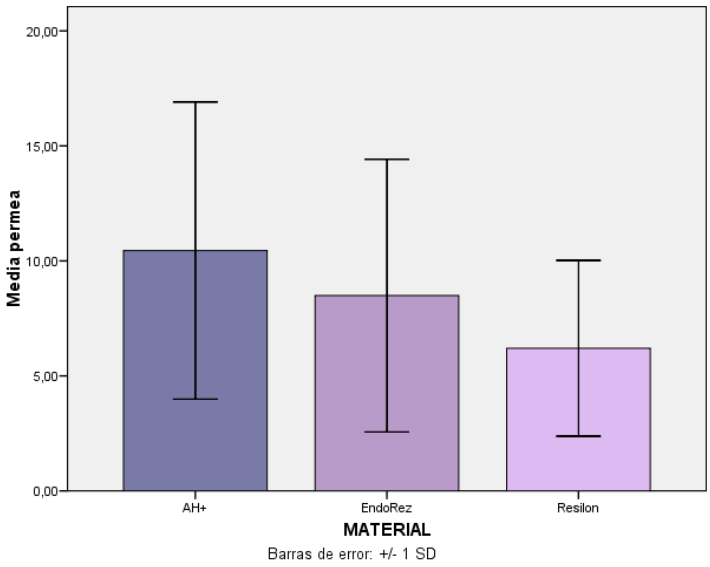


Figure 17: Mean permeability value of three types of sealer.

B. MICRO PUSH-OUT BOND STRENGTH

B.2.5.1. Statistical analysis

Descriptive statistical analysis was performed taking the µPush-out bond strength test values as a dependent variable. The filling material and root level were the independent variables.

The normality of the data distribution in all the study groups was verified by applying the Shapiro-Wilk test, and Levene's test was used to explore the homogeneity of variance among groups.

A factorial model of ANOVA for repeated measures was used to determine the influence of the root level (inter-subjects factor) and material (intra-subjects factor) on the bond strength to root walls. Tukey's test was used for post-hoc comparisons.

Non-parametrical statistics for related (Friedman's and Wilcoxon's rank tests) or unrelated samples (Kruskal-Wallis and Mann-Whitney tests) allowed for comparisons of µPush-out strength values among materials at the different depth levels. Statistical significance was accepted for p values < 0.05.

B.2.5.2. Results

The Shapiro-Wilk test revealed that data distribution for the µPush-out test was normal. Descriptive statistics of the µPush-out bond strength values are given in Table The linear model of ANOVA for repeated measures demonstrated that root level ($F=27.148$, $p<0.001$), and the interaction between the root thirds and the filling material ($F=8.66$, $p=0.001$), significantly affected the µPush-out bond strength to the walls of the root canal. The filling material in itself does not significantly influence the µPush-out bond strength values ($F=2.492$; $p=0.102$).

Despite the normality of the data, Levene's test showed that the variances were not homogenous across all the study groups.

Table 2: Adhesion values in the push-out test. Mean (standard deviation).

Group	C1	C2	M1	M2	Total N=30
AH Plus n=10	1.69(1,18)a	2.05(1,00)a,b	2.48(1,32)a,b	3.30(2,22)b 1	2.38(1.14)
EndoREZ n=10	0.95(1,08)	2.06(1,77)	1.59(0,98) 2	1.44(1,11) 2	1.51(0.94)
RealSeal n=10	0.77(0,46)a	0.94(0,68)a,b	3.41(1,88)b,c, 1	4.59(2,34)c 1	2.43(1.01)
Total N=30	1.14(1.01)A	1.69(1.31)B	2.49(1.58)C	3.11(2.31)C	

Different capital letters indicate significant differences in µPush-out strength values among root levels in total. Different lower case letters indicate significant differences among levels for each sealer.

Numbers indicate significant differences in columns (among sealers for each depth level).

B.2.5.2.1. Comparison among root levels for each material.

For the whole sample, statistically significant differences were detected in function of the root level (Friedman's test; Chi^2=23.93; p<0.001). The Wilcoxon test showed differences among all the levels, with an increased value from cervical to apical, excepting in M1 and M2 levels.

When each material was considered separately, AH Plus/gutta-percha also demonstrated significant differences in function of the root level, obtaining the lowest values in slice C1 (Chi^2=8.40; p<0.05) (Friedman's test).

EndoREZ showed a similar behavior at all the root levels (Chi^2=3.545; p=0.315).

For RealSeal, differences in µPush-out bond strength (Chi^2=18.758; p<0.001) were detected. The coronal level showed similar µPush-out bond strength to the level immediately below, but significantly lower than the intermediate levels. Intermediate levels were similar among themselves. The µPush-out bond strength at the 2nd middle level was significantly greater than the more superficial levels (1st and 2nd cervical), that is, C1≤C2≤M1=M2.

B.2.5.2.2. Comparisons among materials in each root level.

The Kruskal-Wallis test was used to compare the µPush-out bond strength among the three materials used in the study.

For the superficial half of the root (1st and 2nd cervical levels) no differences were found among the materials, but the µPush-out bond strength differed for the deeper levels, medium 1 (Chi^2= 6.225; p<0.05) and medium 2 Chi^2=9.264; p= 0.010).

Mann-Whitney's U tests allowed for comparison in pairs. At the Medium1 level, RealSeal demonstrated greater bond strength than EndoREZ (U=21.00; p=0.028). AH Plus showed an intermediate value for µPush-out bond strength, without significant differences with respect to the other two sealers. For the 2nd medium level, EndoREZ showed a significantly lesser bond strength than AH Plus (U=22.00; p= 0.034) and RealSeal (U=13.00; p=0.005). There was no difference in the µPush-out bond strength between RealSeal and AH Plus.

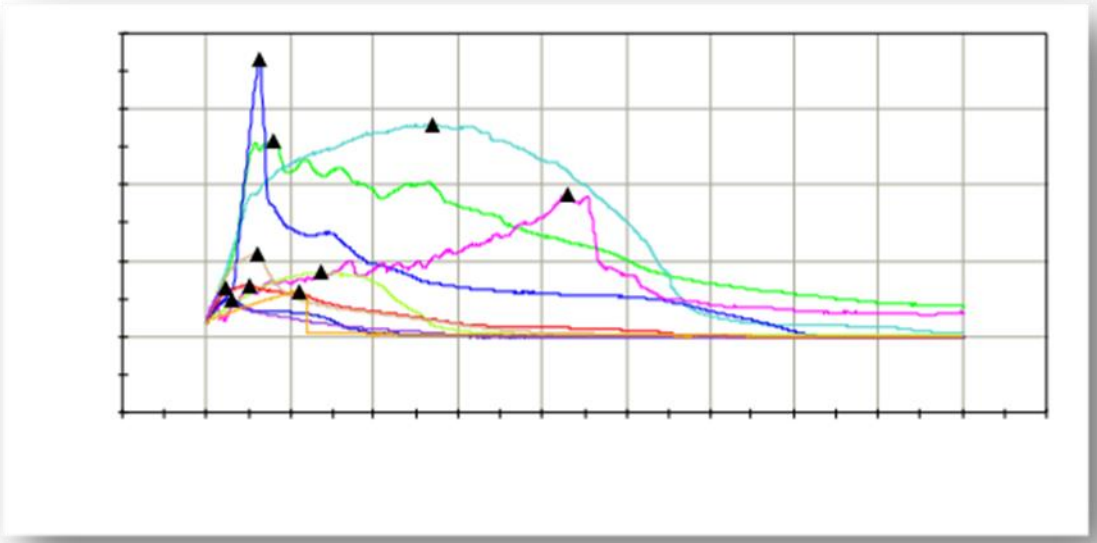


Figure 18: Graph of µPush-out test of AH Plus sealer

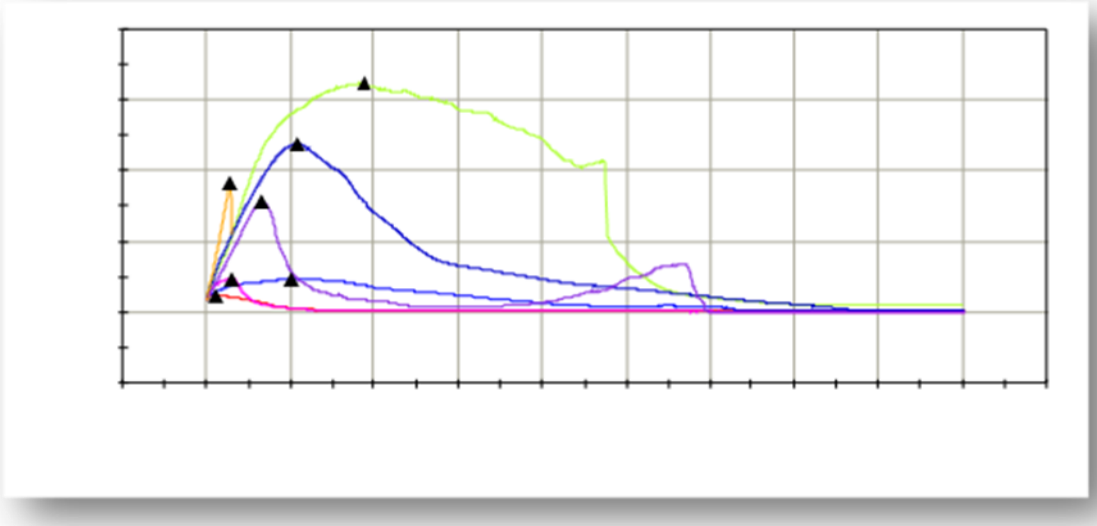


Figure 19: Graph of µPush-out test of EndoREZ sealer

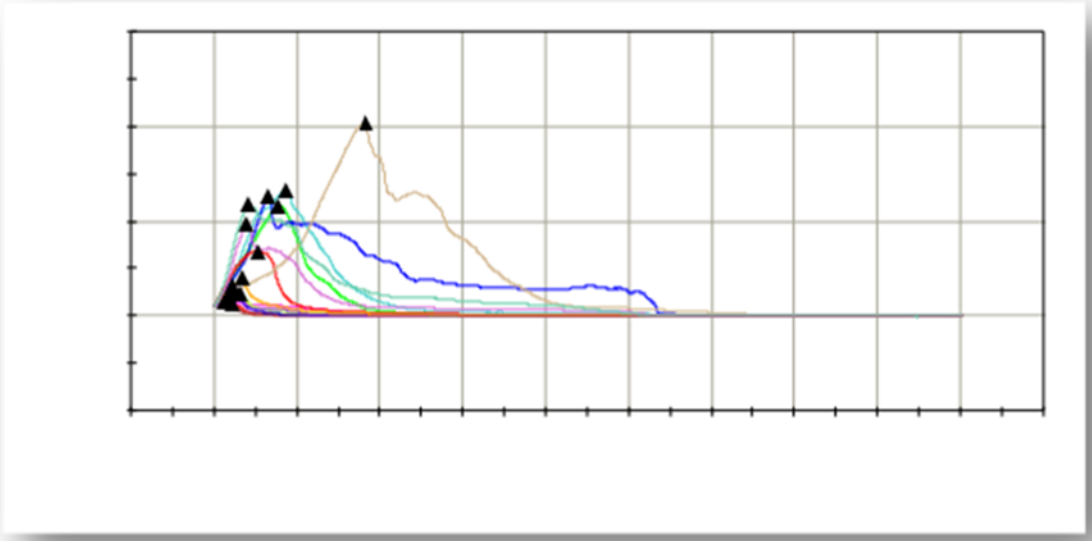


Figure 20: Graph of µPush-out test of RealSeal sealer

B.2.5.2.3. Mode of failure

To determine the mode of failure, µPush-out bond strength (MPa) was recorded. A total of 120 slices per group (approximately 4 slices each for 30 teeth) were tested. The predominating type of failure for all three sealer/core materials tested was adhesive failure, followed by mixed type failures. For the whole sample this distribution was similar among the three sealers used (Chi^2=2.45, p=0.65), and the four root levels (Chi^2=4.66, p=0.58) (Table 3).

Table (3): failure mode

Groups	failure	Cervical 1	Cervical2	medium1	Medium2	total
AH Plus	Adhesive	4 (13.30%)	5 (16.5%)	5 (16.7%)	7 (23.3%)	21 (52.5%)
	Mixed	5 (16.7%)	3 (10.0%)	3(10.0%)	3 (10.0%)	14 (35%)
	Cohesive	1(3.3%)	2 (6.7%)	2 (6.7%)	0 (0.0%)	5 (12.5%)
EndoREZ	Adhesive	6(20.0%)	4 (13.3%)	8 (26.7%)	9 (30.0%)	27 (67.5%)
	Mixed	3 (10.0%)	3 (10.0%)	2 (6.7%)	1(3.3%)	9 (22.5%)
	Cohesive	1 (3.3%)	3 (10.0%)	0 (0.0%)	0 (0.0%)	4 (10.0%)
RealSeal	Adhesive	7 (23.3%)	7 (23.3%)	6 (20.0%)	6 (20.0%)	26 (65.0%)
	Mixed	3 (10.0%)	3 (10.0%)	3 (10.0%)	2 (6.7%)	11 (27.5%)
	cohesive	0 (0.0%)	0 (0.0%)	1(3.3%)	2 (6.7%)	3 (7.5%)
total	Adhesive	17 (56.7%)	16(53.3%)	19 (63.3%)	22 (73.3%)	
	Mixed	11 (36.7%)	9 (30.0%)	8 (26.7%)	6 (20.0%)	
	cohesive	2 (6.7%)	5 (16,7%)	3 (10%)	2 (6.7%)	

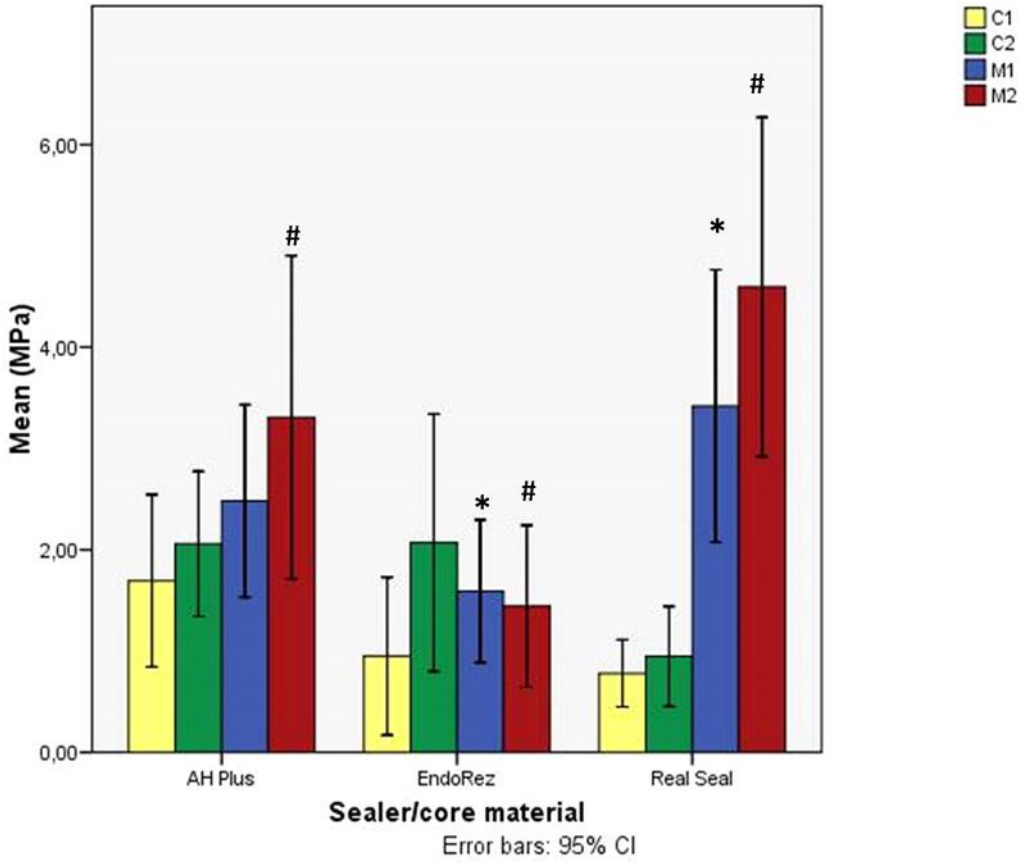


Figure 21. Bar chart showing µPush-out bond strength values, and comparisons among sealer/core materials in each root level (Mann-Whitney's U test).

* Significant differences at M1 level between EndoREZ and RealSeal

Significant differences at M2 level: EndoREZ µPush-out was significantly lesser than the rest of sealer/core materials.

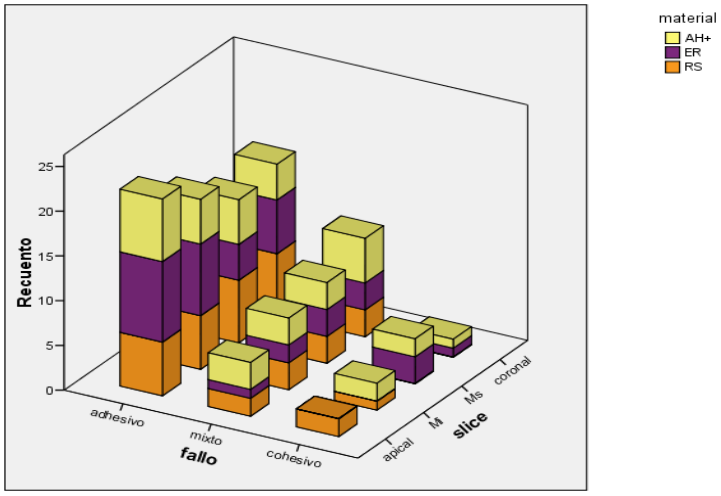


Figure 22: Mode of failure of the three materials in each root level

2.6 Conclusion

In this study, permeability and μPush-out bond strength was compared among roots treated endodontically with three different types of sealers. Root canals were filled using the lateral condensation technique with AH Plus, EndoREZ, and RealSeal sealers.

Microleakage was recorded for each type of sealer and none of the investigated materials achieved a tight seal at the filling-cement-root interface at 24 hours.

Regarding the μPush-out bond strength, no significant difference was found among the three sealers/filling materials.

The root third significantly affects the value of adhesion to the walls of the canal. There were no significant differences between the different filling materials in any of the four levels of the canal considered, except in the 2nd medium third. Analyzing separately each of the materials according to root level, it appears that only the 2nd medium third manifested significant differences in the behavior of the materials in terms of interfacial strength achieved with RealSeal and AH Plus to intraradicular dentin —in the 2nd medium third it was significantly higher than that achieved with EndoREZ.

This study revealed that μPush-out bond strength and permeability were not affected by the kind of sealer. There was no relation found between the permeability and μPush-out bond strength.

2.7 Discussion

The three dimensional obturation of the root is a main objective of endodontic treatment and proves essential for preventing apical or coronal leakage in the root canal. Gutta-percha, used for many years as a solid material in root fillings associated with different types of sealers[200], has suboptimal short and long term apical sealing properties and does not provide chemical bonding to the root canal wall. Recent advance in obturation materials have centered on the introduction of resins into the filling material in the cones, the sealer or both. Resin-filling materials have gained popularity and are now accepted as a root canal filling material[201]. Epoxy resin AH Plus is a two component paste root canal sealer, placed in the canal without any dentin preparation or dentin adhesive, and used with gutta-percha and the lateral condensation technique. The dentin surface is cleaned and then treated with a weak acid[202,203]. This acid removes debris from the dentin surface, removes the smear layer, and exposes hydroxyapatite crystals. Micromechanical retention occurs between AH Plus sealer, based on the polymerization reaction of epoxy resin amines and with the collagen matrix in the intertubular dentin.

Recently, new methacrylate resin-based endodontic sealers were introduced. EndoREZ is a non-etching root canal sealer and does not require the adjunctive use of a dentin adhesive. It is designed to flow into accessory canals and dentinal tubules. Because it is hydrophilic in nature, it is applied in a moist environment to facilitate tag formation and close adaptation to the canal walls.

Another type is RealSeal, a dual cured resin composite sealer. One of the claimed advantages of using adhesive endodontic sealers with bondable polymeric root canal filling materials is that they bond throughout the length of the root canal, producing micromechanical retention through the formation of a thin hybrid layer that forms when the self-etching primer demineralizes the superficial dentin and exposes the thin layer of collagen. The resin composite sealer is then applied and polymerized.

A chemical coupling of the urethane dimethacrylate- containing RealSeal root-filling material to the methacrylate based sealer; a continuum is anticipated by the manufacturer that results in the creation of a monoblock between the root filling and the intraradicular dentin[204]. These new materials need to be carefully evaluated.

Leakage studies of resin-based endodontic materials are still relevant[205]. Different methods have been used to evaluate the sealing of endodontic cements, including bacterial penetration, dye penetration, radio isotopes, light microscopy or SEM. These methods have some disadvantages, the most important one being their qualitative, rather than quantitative information —they can reveal the presence or absence of leakage, but not the amount[206]. This study involved the most popular method used for microleakage assessment, the fluid

filtration method, which was originally described by Derkson and has been extensively used to measure dentin permeability, comparing the sealing properties of restorative materials. More recently, Wu, applied this technique in root canals, by means of air bubble movement inside a capillary tube. This method presents several advantages over the traditional microleakage studies[207]:

- The samples are not destroyed, allowing repeated measurements over a period of time.
- No tracer is needed, thereby avoiding the related problem of molecular size and affinity for dentin, and no intricate materials are required as in bacterial penetration studies or radioactive tracer studies.
- There is no modification of seal with this technique, because the measurements are made directly after filling without dipping the roots in acids, alcohol or methyl salicylate.
- This technique also avoids problems caused by entrapped air or fluid, not using the high pressures employed in vacuum studies.

The results are very accurate because every small volume can be recorded. Thus, a better standardization of the methodology is achieved. Reliability, reproducibility and compatibility are enhanced by using the fluid filtration system[206].

This technique is simple, less time-consuming and makes it possible to assess microleakage in individual samples in different observation periods.

Meanwhile, obstacles associated with this method include the sealing of the space between root and plastic tube, which is important for bias prevention; in this study super glue was used to seal this area.

To obtain accurate results one must check:

- Presence of leaky tube connection
- The stability of the apparatus is important, and the pipette must be perfectly straight to allow the air bubble to flow along its entire length
- The dentin must be sealed by using nail varnish to seal the external root surface.

- The length of specimens should ideally be kept identical, as in this study, to avoid anatomical variations and ensure standardization for the leakage measurements.

The sensitivity of the fluid transport method system can be adjusted by altering the pressure used and altering the diameter of the micropipette[208].

Microleakage is also affected by the type of filling technique. Here, the lateral condensation technique was used. Its most widely accepted due to advantages such as long term use, controlled placement of materials and relative ease of use. This technique does not reproduce canal irregularities, but it does produce many irregularities in the final mass of gutta-percha, leading to an inadequate dispersion of the sealer and the formation of voids in and around the gutta-percha points. Therefore this drawback, may contribute to leakage.

In the context of bond strength, the concept of bonding resin material to enamel was first introduced by Bounocore[209]. Because of the distinction in composition and morphology between enamel and dentin, the need for wet bonding arose. Polymer-based sealers have been introduced as root canal sealers to obtain dentinal tubular penetration, bond to the collagen matrix, and consequently provide adhesive strength to dentin[207].

Adhesion is defined as a process in which two surfaces of different molecular compositions are bonded by chemical, physical or mechanical attraction. Adhesion of an endodontic sealer is its capacity to adhere to the root canal walls and promote the union of gutta-percha cones to each other and to the dentin[62,210] by either mechanical attraction, which occurs by the entrapment of material into another body, within natural or artificial cavities. In contrast, chemical adhesion may result from primary valence forces, such as covalent and metallic bonds. The micromechanical retention to a tooth structure occurs when resin completely infiltrates dentinal surfaces and creates a hybrid or resin reinforced layer[211]. The materials to be adhered must be sufficiently close to each other. Therefore, a primary condition is the wettability of the liquid in a solid material[212], which will provide the required proximity between the materials, facilitating molecular attraction and promoting adhesion[213].

Various methods have been used to evaluate the bond strength of endodontic sealer in root dentin; including pull-out[214], micro tension and push-out[215,188,168,216] techniques. The thin slice µPush-out test is an important experimental instrument[81]. It can evaluate the bond strength in a high c-factor with high stress generation directed toward the bonding area. In addition, the confidence of the µPush-out test may be confirmed by the low variability of the data when the results show low standard deviations. It offers some advantages over the microtension technique for the study of canal adhesion, in that specimens are rarely lost;

advantages over tensile and shear strength test are that it is less sensitive to small variations among specimens and to variations in stress distribution during load application, and it is easy to align samples for testing[217] µPush-out force reportedly increases linearly with greater thickness of the dentin disc[218]. The optimal thickness of dentin for the performance of this technique is controversial, however, with different authors recommending 4mm[218], 3mm[219], 2.5mm[64], and 1mm[168]. A thin slice (1mm) was used in the present study because it has proven reliable in the evaluation of bond strength in 1mm thick samples[220], and it simplifies calculations of the bond area. Moreover, the use of thicker discs increases the area of friction and may lead to an over-estimation of the bond strength[81].

In this study, permeability and µPush-out bond strength were compared among roots treated endodontically with AH Plus, EndoREZ and RealSeal sealers and root canals filled using lateral condensation technique at 24 hours. The results showed that the permeability and global µPush-out bond strength were not affected by the kind of sealer/core material.

We used different samples for fluid filtration and µPush-out bond strength, to be performed at exactly 24 hours´ time, thus avoiding different degrees of conversion for the sealers. We did not light-cure the coronal portion of the RealSeal sealer as recommended by the manufacturer, because fast-setting light-cured resin sealers produce restriction in the flow and consequent defects or weakening of bond strength[221]. Moreover, the slow chemical reaction of methacrylate-based self-etching sealers may reduce shrinkage stress by means of prolonged plastic flow during the setting time of the material[222]. We held that such behavior would allow for complete resin infiltration into the demineralized dentin.

Our results showed that there were no statistically significant differences in the values of fluid filtration among the three sealers used in the study. It is noteworthy, however, that none of the investigated sealers/core materials achieved a tight seal at the filling-cement-root interface, a point also brought out by Vasconcelos et al.[223] allowing fluid to flow along the filled root canal twenty-four hours after filling, whereas the control showed no filtration. The authors speculated that AH Plus did not adapt well to dentin walls because of the hydrophobic properties of epoxy resin[224]. Also, the leakage of AHplus may have resulted from inadequate bonding between the sealer and the gutta-percha points, allowing fluid to flow at the interface[225]. In turn, for RealSeal sealer leakage may be due to

- Uneven application of the self-etching primer to the root canal.
- Inadequate evaporation of the solvent from the primer, promoting the formation of hydrogel, which is leaky.
- Uneven application of the root canal sealer to the root canal.

- Inadvertent stripping of the sealer off the canal wall during the placement of cones.
- Disruption of the maturing resin-root dentin bond during cold lateral condensation.
- The amount of dimethacrylate in the RealSeal points might not be optimal for effective chemical coupling to a methacrylate-based sealer.

Currently, the clinical relevance of leakage tests in vitro is questioned[226] because it is difficult to interpret results when, in our opinion, only zero filtration can be considered a good result. Moreover, there is no correlation among various methods to evaluate microleakage, and outcome of the tests depends on the evaluation method[226,99]. In the absence of correlation between the ex vivo sealing ability of root filled teeth and "clinical success"[227], we believe there is a threshold for microleakage values that would prove clinically relevant [228].

In this study we used three totally different kinds of sealers: a conventional non-bonding epoxy resin-based sealer, AH Plus; a first generation non-acidic diurethane dimethacrylate and triethyleneglycol dimethacrylate, EndoREZ; and a second generation sealer, RealSeal, based on adhesive technology with a self-etching primer and in association with a thermoplastic synthetic polyester polymer-based root canal filling material. It should be noted that the conventional nonbonding AH Plus/gutta-percha root filling was equal to RealSeal and EndoREZ. These discouraging results are in line with those of Ungor et al.[217] who found that the Epiphany/Resilon combination was not superior to the AH Plus/gutta-percha combination. Still, most studies report that AH Plus sealer presents greater adhesion to dentin than RealSeal, regardless of root canal wall treatment[229,230,231]. Clinicians need to be aware that methacrylate resin based sealers did not meet expectations regarding adhesion to root dentin, and at this point in their development there are no clear benefits in their use[72].

The µPush-out bond strength results were significantly influenced by dentin location. AH Plus and RealSeal obtained higher values in the deeper slices, owing to the fact that in the deepest third there is more intertubular dentin and higher collagen fiber density available for hybridization[181], and both materials (AH Plus and RealSeal) depend mainly on intertubular dentin for micromechanical retention, whereas EndoREZ showed the lowest values, regardless of location, because:

- EndoREZ differs from RealSeal in that it contains only TEGMA, diurethane, di-methacrylate and bisglycol dimethacrylate phosphate[232], which probably leads to long but discontinuous tags, the resin tags making only a minor contribution to bond strength[141,143,233]. Adhesion is a complex process and depends on several physical and chemical aspects of molecules and the interaction between these materials and the dentin wall[104], so the quality of dentin hybridization is what most important[146], not the quantity of tags is.

- The interface between the gutta-percha resin coating and the resin sealer is the real bondable interface in this system, but this interface is a weak link that failed during polymerization shrinkage of the sealer.

- The removal of the oxygen inhibition layer[234] from the surface of resin coated gutta-percha cones during packaging has been hypothesized for their weak adhesion to the methacrylate resin-based root canal sealer, resulting in their frequent delamination from the sealer after root canal obturation.

All sealers exhibited their lowest μPush-out bond strength values in two superficial slices. This can be attributed to the presence of oxygen, inhibiting sealer setting and producing a layer with low polymerization.

The values of the μPush-out test are very low, consistent with results of other studies using the same methodology, indicating that the bonding of two methacrylate resin-based sealers and root dentin is much weaker than with resin-dentin bonds, 25-30 MPa[71]. Furthermore, the adhesive failures between sealer/dentin interface clearly suggest an inadequate level of adhesion between sealer and dentin in terms of bond strength[235,236,237]. This may be due to the difficulties in testing materials with great plasticity, such as gutta-percha, RealSeal and EndoREZ points. In contrast, when the test was done with sealer and no core material, values were higher[238,235], suggesting failure may be traced to the sealer/material interface. In a root canal there is a highly unfavorable configuration factor (ratio of bonded to unbounded resin surfaces)[239] that contributes to maximizing the polymerization stress of resin-based materials along the root canal walls; this may even exceed the bond strength of dentin adhesives to dentin, resulting in gap formation along the surfaces[240,239]. Along these lines Souza et al.[241] report that there is a correspondence between the presence of gaps and microleakage.

Despite a material´s relatively low bond strength to root dentin, it may be effective in preventing microleakage[242]. The main problem is that this low μPush-out bond strength is accompanied by fluid filtration. The adhesion of resin sealer to gutta-percha or core seems

to play an important role in microleakage prevention, since it does not have to be at the sealer-dentin interface. Microleakage can affect the bonding of sealers to dentin by plasticization (fluids are absorbed by resins)[240] and hydrolysis due to water entry in the interface. In addition, collagen degradation may occur due to host-derived matrix metalloproteinases (MMPs) in dentin that are slowly released over time when self-etching adhesives are used[154]. RealSeal may therefore adversely affect the longevity of bonded root canal fillings by accelerating degradation of the bond through the movement of fluid between the hybrid layer and unaffected dentin[240,154].

This study reveals that global μPush-out bond strength and permeability were not affected by different sealer/core materials. This leads us to partially accept the null hypothesis, because in the two deeper slices, RealSeal and AH Plus achieved higher μPush-out bond strength than EndoREZ. We cannot confirm any clear relationship between permeability and μPush-out bond strength.

Part 3

MICRO-RAMAN SPECTROSCOPY OF ROOT DENTIN AFTER USING IRRIGATION SOLUTIONS

3.1. Abstract

Objective

The purpose of this study was to evaluate and compare the effect of different chemical agents used in endodontics on the root dentin composition.

Materials and methods

The crown was removed at the cement-enamel junction from 20 human anterior teeth. The roots were divided into five groups, each having four roots. Group I was irrigated with 2.5% NaOCl for 1 minute during instrumentation. Group II was irrigated with 2.5% NaOCl for 5 minutes as a final rinse after instrumentation like group I. Group III was treated with 17% EDTA as a final rinse for 1 minute after instrumentation like group I and irrigation with 2.5% NaOCl for 5 minute as group II. In Group IV, RealSeal self-etching primer was applied for 30 seconds after instrumentation, followed by irrigation as in groups I, II and III. Group V served as the control group, treated with distilled water during instrumentation. Each root was sectioned into 4 slices (n=80). Micro-Raman spectroscopy was performed on these root thirds.

Results

Final irrigation of the canal with 2.5%NaOCl solution for 5 minutes (Group II) showed a decrease in the amide I/amide III (1667/1242) ratio corresponding to the amides III, while a lower value of the two amide III/matrix ratios, significant in some root levels, was observed in relation with the control group (V). Regarding Group I, irrigated with 2.5% NaOCl for 1 minute during instrumentation, there was a significant increase of 1667/1242 ratio at all levels. In addition, there was a significant decrease in the 1072/960 ratio with respect to the control group at the first three levels (C1, C2 and M1). The 2.5% NaOCl solution for 1 minute decreased the carbonate/phosphate ratio.

EDTA showed little or no affectation of the bands of carbonates and phosphates, just slightly increasing the carbonate/phosphate ratio. Application of primer for 30 seconds produced an inconsistent effect on the mineral component, mainly originating a decrease in the phosphate concentration, with no affectation of amide I and non-constant affectation of amide III. There was no significant difference in the crystallinity between the treated dentin and the control group.

Conclusion

In short, 2.5% NaOCl irrigation for 1 minute during instrumentation had no effect on organic components, while increasing time to 5 minutes led to an irregular decrease in amide III and an inconsistent effect on the mineral component. Final irrigation with EDTA for 1 minute after instrumentation, with 2.5% NaOCl irrigation for 1 minute during instrumentation, produces little or no affectation of bands of carbonates and phosphates. The application of a primer produces an inconsistent decrease in the phosphate concentration, without affectation of amide I.

3.2 Introduction

During root canal preparation, root dentin is exposed to different mechanical and chemical factors. An understanding of the chemical composition of dentin is the first step toward predicting the behavior of the dentin after treatment.

The success of root canal therapy depends on the method and the quality of instrumentation, irrigation, disinfection, and three-dimensional obturation of the root canal. Endodontic instrumentation using either manual or mechanized techniques produces a smear layer and plugs of organic and inorganic particles of calcified tissue. The smear layer contains additional organic elements such as pulp tissue debris, odontoblastic processes, microorganisms, and blood cells in the dentinal tubules[109]. A smear layer can create a space between the inner wall of the root canal and the obturating materials, thus preventing the complete locking and adherence of the root canal filling materials into the dentinal tubules[107]. It also contains bacteria and bacterial by-products and thus must be completely removed from the root canal system[243]. In addition, removal of the smear layer can allow intra-canal medicaments to penetrate the dentinal tubules for better disinfection[244].

Among the different root canal irrigants for removal of smear layer, sodium hypochlorite (NaOCl) and ethylene diamine tetra-acetic acid (EDTA) solution are commonly used. It has been reported that some chemicals used for endodontic irrigation are capable of causing alterations in the chemical composition of dentin[245,246,247]. and change the Ca/P ratio of the dentin surface[246]. Any change in the Ca/P ratio may alter the original proportion of organic and inorganic components, which in turn changes the microhardness, permeability, and solubility characteristics of dentin[248], which may also affect the quality of its adhesion to root canal sealers[249]. This results in compromised sealer penetration and significant apical microleakage[243]. Indeed, studies have shown that different concentrations of EDTA are capable of decreasing the microhardness of root canal dentin[250,251]. Changes in the mineral content of superficial dentin may also adversely affect the sealing ability and adhesion of dental materials such as resin-based cements and root sealers to dentin[252,253].

Although NaOCl is not a chelating agent, it can significantly decrease the Ca/P ratio of superficial root dentin[245,247], and its microhardness[254,255], depending on the concentration of the solution.

Dentin is a complex, hydrated, dynamic bonding substrate[149,256]. It has been speculated that regional differences in density and orientation of dentin tubules, mineral/collagen matrix content, presence of various form of dentin, varying smear layer thickness, and differences in dentin hydration state as a function of intratooth location would result in complex, non-uniform acid etching of dentin[257]. The complexity and non-uniformity of this demineralized dentin layer will directly affect subsequent

penetration/infiltration of bonding agents and, ultimately, adhesive bond formation with the dentin substrate[258,259]

Measuring the structure, composition, or suitability for bonding of this complex, etched dentin substrate, especially in its natural, hydrated state, is a formidable problem. To date the most popular techniques for studying the etched dentin layer have relied on morphologic characterization of this layer after fixation by scanning electron microscopy (SEM)[260,261]. Numerous morphological and chemical studies have been done in an effort to obtain a clearer picture of the dentin-resin interface. Bonding systems can be characterized by how they treat the smear layer to obtain micro-mechanical retention by infiltration of resin into the demineralized dentin and exposed collagen fibers[262]. Because of the very small thickness of the hybrid layer, particularly with self-etching primer systems[263, 264], methods to analyze this layer must have a very high resolution similar to that of electron microscopy. The major drawback of electron microscopy is that information regarding the depth of demineralization and morphology could be modified during the specimen preparation procedure, which involves fixation, dehydration and drying.

Raman micro-spectroscopy is a useful analytical technique for studying the composition and structure of bonding of a sample[265]. It can potentially be used to obtain a longitudinal view of the demineralized dentin layer in its wet condition. There is no need for specimen preparation/metallization as for ESEM [266,267]. In contrast to other microscopic techniques, Raman microscopy can be used to detect and quantify the molecular chemistry of microscopic specimens. By combining spectroscopy with microscopy, the laser beam can be focused on a very small spot size (0.6-0.8 μm) with the CCD camera, and molecular information can be obtained with great spatial resolution at the microscopic level. Specimens can be analyzed directly in water at room temperature and pressure without destroying the sample when using a water immersion lens. The capability of performing spatially resolved chemical analyses of microscopic regions of specimens in situ has been applied to materials science and biological sciences. Raman micro-spectroscopy is an exceptional tool for investigating the chemistry of interfaces or surface profiles because it does not rely on homogenization, but rather each structure is analyzed in situ.

The aim of the present in vitro study was to assess the effect of instrumentation with 2.5%NaOCl during 1 minute, 2.5% sodium hypochlorite during 5 minute, 17% EDTA and self-etching primer on the dentin component using micro-Raman spectroscopy.

3.2.1 Dentin

Dentine is the most voluminous avascular mineralized connective tissue of the tooth, supporting enamel and enclosing the central pulp chamber, characterized by a mineralized matrix that is mainly hydroxyapatite with an organic matrix of collagenous proteins. Macroscopically, dentin —along with enamel, cementum, and pulp— is one of the four major components of teeth. In the crown it is covered by enamel, in the root it is covered by cementum. Dentin, which is less mineralized and less brittle than enamel, is necessary for the support of enamel[268]. Dentin is the calcified tissue that forms in the internal bulk of the tooth and is a hydrated biological composite made up largely of intertubular dentin that is collagen type I reinforced with apatite. Mineralized dentin together with the pulp tissue forms the dentin-pulp complex, which is responsible for the formation and maintenance of the tooth mass.[269,270]

3.2.1.1. Physical properties

- Color: Its color is pale yellow.
- Hardness: It is the second hardest surface of the body, harder than cementum yet softer than enamel, so it decays more rapidly and is subject to severe cavities if not properly treated; but it still acts as a support substance for enamel, which is extremely brittle. Dentin with enamel forms a composite structure that can withstand high loads.
- Elasticity: Being highly elastic, it provides flexibility and prevents fracture of the overlying more brittle enamel. It also provides a chamber and protective barrier for the vital pulp tissues.
- Permeability: Semipermeable in both directions (to and from the pulp).
- X-ray: More radiolucent than enamel and more radiopaque than cementum.
- Thickness varies from 3-10mm.
- Dentin is living tissue. The odontoblast residue in the pulp continues to be active throughout the life of the tooth and can be initiated in response to stimuli.

3.2.1.2. Chemical characteristics of dentin

In terms of weight, mature dentin is approximately 70% inorganic material (mineral phase), 20% organic material, and 10% water (adsorbed on the surface of the mineral or in interstices between crystals). On a volume basis, these proportions are 45%, 33% and 22%, respectively[271].

The mineralized substance is hydroxyapatite in the form of small plates. Hydroxyl apatite, also called hydroxyapatite (HA), is a naturally occurring mineral form of calcium apatite with the formula Ca5(PO4)3(OH), but it is usually written Ca10(PO4)6(OH)2 to specify that the crystal unit cell comprises two entities. Hydroxyl apatite is the hydroxyl end member of the complex apatite group. The OH-ion can be replaced by fluoride, chloride or carbonate, producing fluorapatite or chlorapatite. It crystallizes in the hexagonal crystal system. It is reported that the growth of apatite crystals occurs with the long axis running parallel to the long axis of collagen fibrils[272], and their nucleation is associated with non-collagenous proteins, e.g., phosphoryn[273]. The organic phase is about 90% fibrous proteins, mainly type I collagen, while the rest of the organic phase is composed of lipids, non-collagenous matrix proteins[271]and ground substance, inclusions of insoluble protein and glycoprotein. Non-collagenous matrix proteins mainly consist of osteopontin, bone sialoprotein, dentin sialophosphoprotein (DSPP), matrix extracellular phosphoglycoprotein and dentin matrix protein 1 (DMP1)[274,275].

DMP1 is a bone- and tooth-specific protein initially identified from mineralized dentin matrix[576], but also expressed in non-mineralized tissues[277], while DPP (dentin phosphophoryn) is a dentin (circumpulpal) specific, highly phosphorylated protein (phosphoprotein), It is the most abundant non-collagenous protein in dentin[278,279,280] accounting for 50% of dentin. It plays a role in the mineralization of circumpulpal dentin, and is present in root dentin to a lesser extent. DSP is dentin sialo protein, accounting for 5%-8% of dentin non-collagenous protein.

Other elements of dentin include:

Carbon makes up over 45% of human tooth and is present in both the enamel and dentin.

Calcium is very important in the formation of teeth, making up 13% of the tooth, and it is present in both enamel and dentin.

Phosphorus makes up 8% of the enamel and dentin. Phosphorus and calcium work closely to build strong teeth.

Oxygen forms 33% of human teeth, present in enamel and dentin.

Other elements, including sodium, magnesium and chlorine, make up the rest of the tooth.

3.2.2. Dentinogenesis

The formation of dentin, known as dentinogenesis, is a highly regulated and controlled course of actions in which several constituents of both cellular and extracellular nature play a role. It begins prior to the formation of enamel. Dentin is secreted by odontoblasts, which are a special type of biological cell on the outside of dental pulp, and it begins at the late bell stage of a developing tooth. Odontoblasts differentiate from cells of the dental papilla. The main task of the odontoblasts is to synthesize and secrete collagens and several non-collagenous proteins of which the dentin organic matrix is formed. In addition, odontoblasts secrete signaling molecules, mainly of the TGF-β superfamily[281], which are significant for cellular functionality[282]. Odontoblasts control dentin matrix mineralization at least by determining the nature of the extracellular matrix and by controlling the influx of mineral ions[283]. The odontoblasts begin to move toward the center of the tooth, forming an extension called the odontoblast process. Thus, dentin formation proceeds toward the inside of the tooth. Collagen is the main organic component of the dentin, comprising about 93% of the organic materials. A complex compound of type I collagen fibers and a carbonate-rich apatite mineral phase (Ca10 (PO4)6(OH) 2) gives a quality of rigidity and strength to the dentin matrix[284]. Citric acid is present and additional minor organic components include glycosaminoglycan, lipids, and various protein components.

3.2.2. Organic matrix

The organic matrix of dentin primarily consists of fibrous collagens and other proteins such as proteoglycans, phosphoproteins and phospholipids, etc. The matrix provides a framework for mineralization. Collagens comprise 90% of the dentin matrix, and are principally type I[285,286]. Type I collagen is composed of two identical α1 (I) chains and one α2 (I) chain, and a glycine in every third amino acid position in an individual chain is needed for the formation of a triple helix structure[287,288].

Type I collagen is predominant in bone and dentin, the most abundant collagenous protein in the body, although there is some type III detectable in most dentin preparations[289]. Type I collagen fibers in dentin form a network with which the mineral crystallites are aligned in a tightly packed manner[290]. Electron microscopic studies of dentin suggest that the bulk of the mineral is on the surface of the dentin collagen, with the crystals densely packed and not reflecting the arrangement of the collagen[291].

Type I collagen is synthesized as a larger procollagen, which contains extensions at both the N- and C-terminal ends, called the amino terminal and carboxy terminal propeptides, which prevent premature collagen aggregation into fibrils. After procollagen secretion from cells, extracellular modification takes place, and propeptides are removed by specific proteinases and mature collagen molecules aggregate into a fibrous matrix[288], which then serves as a support for mineral deposition. Thus, the collagen provides a template, which requires the association of specific matrix proteins, upon which the mineral can deposit in an organized fashion.

Type III collagen, a homopolymer of three α1 (III) chains, is a prominent constituent of soft connective tissues, such as pulp tissue, where it comprises approximately half of the collagen matrix[292,293]. In addition, there is strong evidence that calcified tissues are also able to express type III collagen. Karjalainen et al.[294] has shown that mature and intact human odontoblasts produce type III collagen after tooth development. The role of type III collagen in normal physiological dentin mineralization is unknown.

Proteins other than collagens comprise the remaining 10% of the dentin organic matrix. In addition, a minor part of the dentin matrix is composed of lipids, which possibly participate in mineral formation[272]. The non-collagenous proteins —dentin phos-phoprotein (DPP; phosphophoryn) and dentin sialoprotein (DSP)— represent the most abundant dentin-specific acid proteins in the dental matrix.[295]

DPP is a highly phosphorylated protein, hydrophilic in character. It is capable of binding a large amount of calcium, facilitating the initial mineralization of the organic collagen frame. DPP is secreted by odontoblasts just ahead of the mineralization front[296,297]. The function of DSP is not yet known, but it may also have a role in the matrix mineralization reaction[295]. Proteoglycans, such as decorin, bigclycan, fibromodulin and lumican, which carry glycosaminoglycan (GAG) carbohydrate side chains within their structures, comprise another sizeable portion of the non-collagenous proteins[298]. Since proteoglycans are also able to bind calcium[299], they may play a part in mineralization of the organic matrix of dentin, together with acid phosphoproteins.

3.2.4. Mineralization

Dentin mineralization sharply occurs at a specific mineralization front[300,272,270]. At the onset of dentin formation, odontoblasts synthesize and secrete type I collagen and proteoglycans, and other significant constituents to the predentin layer. In predentin, collagen molecule fibers aggregate with their long axes in parallel into fibrils, which further arrange to bundles, possibly with the help of proteoglycans[301,302].

Formation of hydroxyapatite and the other collagen-based tissues (cementum, calcified cartilage, tendon, and bone) in dentin occurs through cell-mediated events. The cells produce the extracellular matrix upon which the mineral is deposited, regulate the flux of calcium and phosphate ions into this matrix, and produce the enzymes and growth factors that regulate both their own function and the composition of the matrix. The extracellular matrix of dentin consists of mineral (hydroxyapatite), collagen, non-collagenous matrix proteins, extracellular organelles, and water[303]. The hydroxyapatite mineral crystals in dentin, as in bone, and cementum, are arranged in an oriented fashion, with their long axis parallel to the fibril axis of the predominant matrix protein, collagen. Mineralization occurs during dentinogenesis: the odontoblast, a highly polarized cell[301], secretes its extracellular matrix from its basal laminar portion. The distal secretory pole of the odontoblast also has a high concentration of calcium, which appears to be transported to the extracellular matrix, where mineralization commences[301]. Calcium ions (Ca2+) are transported to the mineralization front by a transcellular route. This route includes a Ca2+-activated ATPase, which in concert with Na+/Ca2+ -exchangers, calcium channels and intracellular calcium binding proteins, maintain delicate calcium ion homeostasis in odontoblasts[304,305]. Phosphophoryn, initially released from the odontoblast in its calcium-

dependent conformation, is one of the factors facilitating initial crystal deposition, presumably because it stabilizes hydroxyapatite nuclei. With dentin maturation, the phosphophoryn that is secreted into the extracellular matrix may bind to the preformed hydroxyapatite crystals, leading to phosphophoryn accumulation at the mineralization front and to the regulation of crystal growth[306]. Association of phosphophoryn with collagen fibrils[307] would help orient the mineral crystals relative to the collagen. Concurrently, highly phosphorylated dentin phosphoprotein and dentin sialoprotein, phospholipids, and possibly another pool of proteoglycans, are added to the mineralization front, where they act as mineral nucleators and induce apatite formation. They bind to the collagen fiber surface and enhance the ability of the fibers to bind calcium ions and, thus, mineral deposition[308]. The binding of calcium appears to be dependent on the presence of the phosphate groups, since the dephosphorylated protein can no longer bind cations[309]. Upon binding Ca ions, the protein undergoes a conformational change, forming a structure with abundant, ß-pleated sheets[310,311]. It is this structure that has a high affinity for hydroxyapatite. Primary dentin matrix is synthesized at a rapid rate during tooth development[272,270]. Thus, the tooth mass consists principally of primary dentin, which outlines the pulp chamber, and therefore it may be referred to as circumpulpal dentin. Following primary dentinogenesis, odontoblasts continue to deposit a physiological, secondary dentin around the pulp at a slow rate, leading eventually to the reduced size of the pulp chamber. Structurally, secondary dentin resembles primary dentin, having a similar tubular pattern, which is, however, less regular than that of the primary dentin[272,270].

3.2.5. Root formation

The root structure promotes the exchange of nutrient from the blood into the dentin, which is responsible for the major biomechanical changes occurring throughout the lifetime of a tooth and its underlying components. Radicular dentin forms at a slightly later stage than coronal dentin. It is formed during tooth eruption and is uniformly calcified in the root. It is secreted by the odontoblast of the dental pulp[312].

The dentin in the root of a tooth forms only after the presence of Hertwig's epithelial root sheath (HERS), near the cervical loop of the enamel organ. Root dentin is considered different from dentin found in the crown of the tooth (known as coronal dentin) because of the different orientation of collagen fibers (parallel to the basement membrane), the decrease of phosphoryn protein levels, lesser mineralization, and a slower rate of deposition of dentin.

3.2.6. Dentin microstructure

Although the primary function of dentin is mechanical, it is only in the last few years that its mechanical properties have begun to be understood in terms of its hierarchical microstructure[313,314]. Prominent features in the microstructure of dentin are tubules that radiate outward from the pulp to the dentin-enamel junction in coronal dentin, and from the pulp canal to the cementum in the root[312]. Dentinal tubules contain dentinal fluid and the cytoplasmic processes of the cells that have formed the dentin, the odontoblasts, are located[270]. In addition, the tubule contains a complex mixture of proteins such as albumin, transferrin, tenascin and proteoglycans[272,270]. As a result, dentin has a degree of permeability which can increase the sensation of pain and the rate of tooth decay. The tubules´ numerical density varies with intra-tooth location, a lower number being in the outer portions and increased density near the pulp chamber. In normal human dentin, the tubules are lined with a highly mineralized cuff of peritubular dentin[315,316] which is much more mineralized than the surrounding intertubular dentin. The intertubular dentin matrix is formed of mineralized collagen fibrils arranged in a felt-like structure, oriented perpendicular to the tubules. Pashley et al.[315] reported that the number of tubules and peritubular dentin area decrease with distance from the pulp, whereas the intertubular area increases with distance from the pulp.

The collagen fibrils are randomly oriented in a plane perpendicular to the direction of dentin formation[301]. The mineral occupies two sites within this collagen scaffold: intrafibrillar (inside the periodically spaced gap zones in the collagen fibril) and extrafibrillar (in the interstices between the fibrils). The partitioning between these two sites is uncertain, although it is believed that between 70% and 75% of the mineral may be extrafibrillar[317,318].

3.3. Mechanical instrumentation

The general purposes of root canal instrumentation are cleaning, shaping and smoothing the root canal. Schlider[319] considered that root canal cleaning and formation were the basis of endodontic treatment success. Cleaning of the root canal involves the removal of all contents of the root canal systems before and during shaping, and removal of vital and/ or necrotic pulp tissue, infected dentin and dentin in order to eliminate most of the microorganisms from the root canal system[320]. In turn, shaping entails the enlargement of the canal system to facilitate the placement of a root filling[321], developing a continuous tapering conical form of the canal and maintaining the original shape and position of the apical foramen[319]. Buchanan[322] suggested avoiding an excessive enlargement on the apical site by making minimum root canal narrowing. Walton and Torabinejad[323] affirm that the root canal should be shaped into a continuous cone shape from the apex to the coronal part. Ram[324] suggested that the root canal needed to be enlarged to a number 40 file, so the maximal irrigation could contact apical debris and better eliminate the smear layer[325].

Mechanical instrumentation and irrigation stand as endodontic treatment principles and an essential component supporting treatment success. Recent advances in the field of endodontics have given rise to the use of nickel-titanium rotary instruments in dental practice. It believed that they may improve the efficacy of root canal preparation. The introduction of nickel-titanium rotary files to endodontics has changed the way root canal preparations are performed, enabling more complicated root canal systems to be shaped with fewer procedural errors. Its application would reduce the number of instruments used for root canal cleaning and formation. Grossman et al.[326] hold that mechanical cleaning is an important measure in root canal treatment. The original canal shape is maintained better when using rotary nickel-titanium instruments as opposed to a hand-preparation technique with stainless steel k- flexo file[327]. Another advantage is that a more conical preparation of the root canals permits the placement of a master gutta-percha point of grater taper (non-standardized). Musikant et al.[328] concluded that the greater the taper, the more resistant the gutta-percha cones are to apical displacement by condensing forces.

Engine-driven nickel-titanium instruments require less chair time to prepare the root canals as compared to stainless steel hand k-files[329]. In addition, it appears to produce more centered canal preparations than stainless steel hand files[330,331,332]. These devices are moreover easy to use, which contributed to their popularity.

All endodontic files and reamers were manufactured from stainless steel and had standard 0.02 tapers. According to some theories, the irrigation that a 0.02 tapered spaceprovides may be insufficient for maximum dissolution of organic debris by sodium hypochlorite. This can alter the treatment outcome, since the cleaning of all spaces is a very important phase during endodontic therapy[319,333]. The use of greater tapers (0.04, 0.06, 0.08, and 0.10) should allow for a more apical placement of the irrigant.

The golden roles for nickel-titanium rotary preparation:

- Assess case difficulty
- Provide adequate access
- Prepare with hand files up to size #20 prior to rotary use
- Use light touch and low rpm
- Proceed with crown-down sequence
- Replace rotary instruments frequently

3.3.1. Nickel titanium elasticity

Stainless steel files have a high stiffness that increases along with the instrument size, and causes high lateral forces in curved canals[334]. These restoring forces attempt to return the instrument to its original shape and act on the canal wall during preparation, influencing the amount of dentin removed, also leading to straightening in the apical, middle and coronal thirds[335,321]. The resulting transportation and canal aberrations (including ledges, zipping and perforations) leave a significant portion of the canal wall uninstrumented, and may create an irregular cross-sectional shape that is harder to obturate[336,337]. According to some reports, less transportation is caused with nickel-titanium than with hand instrumentation[329,330].

Nickel-titanium files, which are two to three times more flexible than stainless steel files, result in more uniform preparation with regard to taper, and they enhance obturation by providing resistance. The high flexibility of nickel-titanium is critical for endodontic files for two reasons. With highly elastic instruments, forces between the file and the canal wall during instrumentation are reduced. This results in the file remaining centered in the root canal space, and in a lower propensity towards canal straightening or other preparation errors. Secondly, rotational movement in curved canals will bend rotary files once per

revolution. This leads to work hardening and brittle fracture, known as cyclic fatigue.

Given their super elasticity, one might surmise that Ni-Ti instruments are less efficient, since little force is applied to the dentin as the file is deflected or bent away from the surface. However, studies demonstrate that nickel-titanium instruments are as effective or better than comparable stainless steel instruments in machining dentin[338].

3.3.2. Crown-down technique

In this technique, larger instruments are used first in the coronal aspect of the canal, followed sequentially by smaller instruments in the apical aspect of the canal. Using standardized gutta-percha points and a lateral condensation technique, a more conservative taper is advisable. Nevertheless, the additional use of orifice openers and 0.06 tapers in the Profile series facilitates spreader placement after canal preparation[339]. This improved deep shaping could compensate the less close fit of the 0.02 tapered master point and improve the lateral obturation with additional cones.

The advantages of this technique are that:

- The elimination of debris and microorganisms from the more coronal parts of the root canal system prevents inoculation of apical tissues with contaminated debris.
- Elimination of coronally placed interferences that might adversely influence instrumentation.
- Early movement of large volumes of irrigant and lubricant to the apical part of the canal.
- Facilitation of accurate working length determination, as coronal curvature is eliminated early on in the preparation.
- Reduction in postoperative pain that may occur with apical extrusion of debris.
- Better dissolution of tissue with increased irrigant penetration.
- Easier smear layer removal because of better contact with chelating agents.
- Enhanced disinfection of canal irregularities due to irrigant penetration.

In a study by Blum[340] in which the root canals of mandibular incisors were prepared with Ni-Ti rotary instruments using either a step-back or crown-down technique, the researchers found that less vertical force and torque were created with the crown down

technique and that instrument tips had less contact with dentin and less stress during the early phases of instrumentation.

There are two types of preparation. The first is mechanical, done by instrument; but instrumentation alone has a limited ability to debride and clean the canal[341,342]. The second one is chemical. Regardless of the instrumentation technique or system used, the use of irrigant is essential for debridement of the canal system[343,344]. Wu and Wesseelink[345] reported an uninstrumented area in 65% of instrumented oval canal because of the morphology of the root canals. Furthermore, because the instrumentation technique does not sterilize the canal, debriding the remaining 40% of the canal surface involves irrigation, which has an important role during endodontic treatment.

Chemo-mechanical action is key to optimally clean the root canal system from debris (defined as dentin chips and residual or necrotic pulp tissue attached to the root canal wall, which in most cases is infected[346]). The smear layer is a surface film of a thickness of approximately 1-2mm[347]. A thick and non-homogenous smear layer can prevent the efficient elimination of intra-canal microorganisms, and compromise the complete sealing of the root canal[348]. Irrigation is necessary for its removal.

During each instrumentation procedure, the canals are simultaneously washed out or irrigated with a solution capable of disinfecting them and dissolving organic matter[349]. It is recommended to use antibacterial irrigant in combination with chelating agents in order to remove debris as well as the inorganic/organic smear layer[350].

Irrigation with appropriate solutions contributes to the cleaning of the canal system in several ways including:

- Cleaning of debris.
- Lubrication of the canal system to avoid instrument separation.
- Dissolution of organic and inorganic remnants of the pulp system.
- Antibacterial properties; destruction of bacteria and deactivation of antigenic products.
- Softening and eliminating the smear layer.
- Penetrating into areas unreachable to instruments, thereby extending the cleaning processes.

There are three main categories of irrigant:

Lubricants: they decrease the friction between the endodontic instrument and the wall of canal and make stress-free instrumentation. Instrument lubrication can be done by using either sterile water, sodium hypochlorite (NaOCl), or 17% EDTA (ethylene diamine tetra acetic acid).

Disinfectants: they include the most frequently used irrigant, NaOCl, which dissolves necrotic tissue and kills bacteria effectively.

Chelating agents: these compounds have a central metal ion surrounded by covalently bonded atoms which possess additional bonds for chemical reaction. Chelating agents were introduced into endodontics as an aid for the preparation of narrow and calcified root canals in 1957. They remove the inorganic components by binding with calcium and carrying it out of the canal. The most commonly used endodontic chelating agent is EDTA.

The characteristics of an ideal irrigating system would be: biocompatibility, bactericidal agent, low toxicity, low superficial tension, physical flushing of debris, smear layer removal, capacity to dissolve tissue or organic residue, be lubricant, disinfected, and not affect physical properties of dentin.

Other factors related to the utility of the irrigating agent include:

a) Should be readily available

b) Reasonable price

c) Suitability

d) Satisfactory shelf life

e) Simple storing

Irrigants cause changes in the mechanical properties of dentin[351,352]. Studies on modes of action and efficiency of various chemical irrigating solutions have shown their effect on both organic and inorganic components of root canal dentin. In turn, the mechanical, chemical, and physical properties of the dentin structure changes. Thus, irrigating solutions cause alterations on dentin and enamel surfaces and effect interactions with materials used for obturation and coronal restorations[352]. Widely used irrigation solutions are sodium hypochlorite, EDTA, and chlorhexidine. This study used sodium hypochlorite.

3.3.3. Sodium hypochlorite

It is a chemical compound with the formula NaOCl. It is a vital and effective endodontic irrigant, with excellent efficacy as a primary irrigant on account of its ability to dissolve both vital and necrotic remnant pulp tissues[353]. It is both an oxidizing and hydrolyzing agent[354], bactericidal, virucidal[355], and features deodorizing action[356]. Sodium hypochlorite has been used as a wound irrigant since at least 1915[357], and as an endodontic irrigant since 1920[358]. It has a long history of successful usage in endodontics because of its antimicrobial activity[359]. Studies show that sodium hypochlorite decreases microorganism numbers during the treatment of teeth with apical periodontitis[343,360], dissolves soft tissue[361,362]removes the smear layer, lubricates and flushes away loose debris. It moreover has the capacity of dissolving proteins, saponification of fats and neutralization of toxic products, it has low viscosity and a reasonable shelf life. A concentration between 0.5% and 5% is recommended for clinical use to degrade necrotic tissue[363].

Three important factors are behind its widespread use among clinicians: 1) non-specific antimicrobial effect and ability to dissolve biofilms[364,365], as its mechanism of action causes biosynthetic alterations in cellular metabolism and the destruction of phospholipids, chloramine formation that restricts cellular metabolism, oxidative action with irreversible inactivation of enzyme in bacteria, and lipid and fatty acid degradation; 2) its unique capacity to solubilize necrotic tissue[366]; and 3) its reasonable price and availability from many commercial sources[367].

Disadvantage

Sodium hypochlorite is toxic to living tissue, except keratinized epithelia[368]. Another drawback is its high surface tension, which limits its penetration into the depth of dentinal tubules. It causes corrosion to ultrasonic instruments, has an undesirable taste for patients, and causes irritation of the eye[369].

3.3.4. EDTA

Among the different root canal irrigants for removal of smear layer, ethylene diamine tetra acetic acid solution, or EDTA, is generally recognized as the most effective chelating agent in endodontics. Almost all manufacturers of nickel-titanium instruments recommend use of EDTA as a lubricant during rotary root canal instrumentation. A liquid solution of EDTA can chemically soften the root canal dentin and dissolve the smear layer, as well as increase dentine permeability and prepare the dentinal walls for better adhesion of filling materials[109]. Serper and Calt [370] found that 17% EDTA had a good demineralizing effect by measuring the amount of phosphorus liberated. EDTA demineralize the inorganic component of dentin via calcium chelation[371,372]. The 17% concentration and neutral pH of disodium EDTA is widely chosen for root canal treatment. An adverse effect of EDTA is that it may cause dentinal erosion.

Dentin is a molecular complex with calcium ions in its composition. The chelating agent is applied over dentin; this facilitates dentin disintegration for the EDTA[373].

EDTA provides the following benefits:

1) Helps to clean and disinfect the wall of the root dentin, since it eliminates dentinal debris resulting from instrumentation usage in the process of forming the canal.

2) Facilitates the action of medication used inside the canal by increasing dentinal tubule diameter as well as dentin permeability.

3) Conditions the root canal dentin wall, providing for an increased degree of adhesion of the obturation material[374]. EDTA used for one minute inside the root canal effectively removes dentinal debris[375]. Nevertheless, a 10 minute application erodes dentin around and inside the canals.

3.3.5. Bonding agent

This material of low viscosity, when applied on the tooth surface, forms a thin film after setting. This film strongly bonds to the tooth surface upon which the viscous composite restorative resin is applied. When it sets, it forms an integrated resin restoration.

Components: 1) Etchants/conditioners, 2) Primers, 3) adhesives.

It has been reported that the quality of resin dentin adhesion is greatly influenced by the duration of the etching process, and by the amount of dentin surface humidity following rinsing of the acid and prior to resin infiltration[376,377]. Therefore, much present research and development in dentin adhesion aims to simplify the bonding procedures and to eliminate all possible technical sensitivities by reducing the number of bonding steps.

A relatively new formulation comprises both a self-etching and self-priming adhesive system with a pH low enough (1 to 2) to etch through smear layers into underlying dentin when the concentration of acidic monomers is increased and dissolved in 30%-40% (by weight): hydroxyl ethyl methacrylate, or HEMA, a very water soluble priming monomer. The smear layers are either incorporated into the hybrid layer if the solution cannot be well agitated[378,379], or they are completely dissolved if the surface can be scrubbed with the product. Importantly the self-etched, self-primed surfaces are not to be rinsed with water. The surfaces are scrubbed for 30 seconds and then gently air dried to evaporate the small amount of water that is in the product, in order to ionize the acidic monomers and facilitate solubilization of calcium and phosphate ions from the etched smear layer and underlying intact tooth structure.

The hydrophilic primer is applied to increase dentin surface energy and to facilitate the penetration of the bonding resin monomer, generating a mixed zone of resin entangled (intertwined) collagen fibrils, known as the hybrid layer[211]or the resin-dentin inter diffusion zone[262]. Self-etching primer systems have been developed to simplify and shorten bonding procedures[380]. A great advantage is that self-etching primers are designed to be used on dry dentin. Since no rinsing step is required, the clinician does not need to be concerned about the level of dentin wetness. Another advantage is that a separate etching step is no longer required. They do not etch very far into the dentin beneath the smear layer, and dentin is simultaneously demineralized and infiltrated by the same resin component[381]. This makes removal of smear plugs in the tubules unnecessary.

Self-etching systems may potentially reduce post-operative sensitivity by providing simultaneous infiltration of the adhesive to the depth of demineralization and dissolving the smear layer without exposing dentinal tubules. However, in general, laboratory tests have

shown a reduction in bond strengths and only limited clinical data is available to date[148]. These self-etching primer adhesives can be used to etch both ground enamel and dentin simultaneously[382]. They bond equally well to superficial and deep dentin[382].

In the case of RealSeal (SybronEndo, Orange, CA), the self-etching primers are further reduced from a 2-bottle system to a single bottle system, consisting of hydroxyethyl meth-acrylate, sulfonic acid and water.

3.4. Confocal micro-Raman spectroscopy

Micro Raman spectrometery is used in lieu of a standard Raman spectrometer. It is a spectroscopic technique involving a specialized Raman spectrometer integrated with an optical microscope. A Raman microscope begins with a standard optical microscope, with its lenses and mirrors, and adds an excitation laser, a monochromator, and a sensitivity detector (such as a charge-coupled device, CCD, or photomultiplier tube, PMT). In many cases CCD is the detector of choice for Raman spectroscopy.

Raman spectroscopy has become an important analytical and research tool. This light scattering technique can be applied to study vibrational, rotational, and other low frequency modes in material. It is based on inelastic scattering, or Raman scattering of monochromatic light, usually from a laser in the visible, near infrared or near ultraviolet range. The sample is illuminated through the objective by a laser of a very narrow wave length range. Confocal Raman microscopy refers to the ability to spatially filter the analysis volume of the sample, in the XY (lateral) and Z (depth) axes. The use of a confocal microscope greatly improves Raman micro spectroscopy. The confocal microscope has a pinhole (25 to 100 μm in diameter) that rejects out-of-focus signals. The smaller the pinhole size, the better the rejection. As a result, the background signals can be reduced significantly. Furthermore, it is possible to measure Raman spectra of the sample at different depths by moving the focal plane in the axial direction. Confocal Raman microscopy has very high spatial resolution, and stands as a powerful non-destructive and non-contact method of sample analysis. Since the objective lenses of the microscope can focus the laser beam to several and/or sub-micrometers in diameter, the resulting photon flux is much higher than that achieved in conventional Raman setups. In the past, this technique was used to a very limited extent, to extract information about the chemical structure of mineralized tissue mainly due to the influence of fluorescence[383]. The confocal Raman microscope used in this study has the added benefit of enhanced fluorescence quenching.

3.4.1. The theory of micro Raman spectroscopy

Spectroscopy was originally the study of the interaction between radiation and matter as a function of wavelength (ƛ). When monochromatic radiation is incident upon a sample, this light will interact with the sample in some fashion. It may be reflected, absorbed or scattered. It is precisely the scattering of radiation that gives Raman spectroscopy information about a sample´s molecular structure.

The laser light interacts with molecular vibrations, photons or other excitations in the system, causing the energy of the laser photons to be shifted up or down. The shift in energy reveals information about the vibrational modes in the system. Spontaneous Raman scattering is typically very weak, and as a result the main difficulty of Raman spectroscopy lies in separating the weak inelastically scattered light from the intense Rayleigh scattered laser light. Scattered light is collected with a lens and sent through an interference filter or spectrometer to separate desired Raman modes or obtain the Raman spectrum of a sample. Raman spectrometry relies on holographic gratings and multiple dispersion stages to achieve a high degree of laser rejection.

Raman spectra were recorded using the NRS series of benchtop, singly dispersive micro Raman spectrometers based on JASCOS proven technology, emphasizing sensitivity, reliability, and ease of operation through a computer-controlled optical system. With super stability, effortless software controlled optics and no time consuming realignment, complete operator safety is maintained by the fully enclosed automated sample chamber door, which provides a 120-degree opening to allow full microscope access.

3.4.2. Raman scattering phenomenon

The phenomenon of inelastic light scattering is known as Raman radiation. It was first documented in 1928 by Raman[384], who won the Nobel Prize for his work. Light is elastically scattered when the frequency of photons in monochromatic light changes upon interaction with a sample. The photons of the laser light are absorbed by the sample, so that the photon excites the molecule from the ground state to a virtual energy state. When the molecule relaxes, the photon is reemitted and the molecule returns to a different rotational or vibrational state. The difference in energy between the original state and this new state is the reason why the frequency of the reemitted photons is shifted lower or higher in comparison with the original monochromatic frequency; this is known as the Raman effect. The Raman shift provides information about vibrational, rotational and other molecular modes.

Scattered light may take the form of two types: the first, called Rayleigh scattering, is strong, having the same frequency as the incident beam. This type of signal is useless for practical purposes of molecular characterization. The other, called Raman scattering, is very weak. The light hits the molecule, the incident photon excites one of the electrons into a virtual state, and, depending on the state of the electron, Stokes Raman scattering or anti-Stokes Raman scattering can be generated. If the final vibrational state of the molecule is more energetic than the initial state, then the emitted photon will be shifted to a lower frequency in order for the total energy of the system to remain balanced. This shift in frequency is designated as a Stokes shift. If the final vibrational state is less energetic than the initial state, then the emitted photon will be shifted to a higher frequency, and this is denoted as an anti-Stokes shift.

3.4.3. The components of the Raman system

The Raman system consists of four major components:

- Excitation source (laser)
- Sample illumination system and light collection optics.
- Wave length selector (filter or spectrophotometer).
- Detector (photo diode array, CCD, or PMT).

Raman is both qualitative and quantitative. Quantitative analysis is performed by measuring the relative intensities of bands that are directly proportional to the relative concentrations of the compounds.

Raman is usually not destructive, but if too much laser power is used, or if the power is focused on a small point (in a microscope), it can be destructive, burning the sample. Using a large sample spot or lowering the laser power significantly would reduce the risk of sample damaging.

3.4.4. Micro Raman spectrometer

Optical spectrometers often said to be the most useful device used in science overall. They serve to scatter light into its various component wavelengths and to determine the wavelength of each resolved component. They provide much reliable information concerning the composition, structure, and dynamics of matter. The Raman spectrometer portion of the micro Raman is an optical instrument for measuring the intensity of light relative to its stock shift from the wavelength of the exciting laser light. This shift is given in wave numbers. A beam of light collected from the sample enters the device and is separated into its stoke shifted frequencies by a diffraction grating.

The Raman spectrometer is built into the microscope, along with a digital imaging system, so that a maximum amount of light can be collected from the smallest samples. In this sense, micro Raman spectrometers are very flexible instruments, able to measure the Raman spectra of microscopic areas. Raman spectra provide unique molecular fingerprints which can be used to classify a material, and distinguish it from others.

3.4.5. Micro Raman spectroscopy applications

Raman spectroscopy is very information rich, capable of identifying different molecules and even functional groups within larger molecules, chemical identification and effects of bond. The bonds formed between atoms have specific vibrational frequencies that correspond to atom masses and the strength of the bond between them. Complex molecules therefore exhibit many peaks and can be readily identified by the pattern or fingerprint of those peaks. There are many uses for micro Raman spectrometers as they can non-destructively identify microscopic samples or microscopic areas of larger samples. Indeed, they are appropriate for any application where non-destructive, microscopic, chemical analysis or imaging is required, whether the goal is to derive qualitative or quantitative data. Raman analysis makes it possible to determine the chemical structure of a sample whether solid, liquid, gas, gel, slurry or powder, and identify the compounds present by measuring molecular vibrations. It yields better spatial resolution and can be used for the analysis of smaller samples. Raman spectroscopy is moreover suitable for the microscopic examination of minerals and materials such as polymers and ceramics, cells and proteins. The chemical profile of the demineralized dentin layer can be obtained using Raman microscopy.

The micro Raman technique has been shown to be powerful tool for detecting and quantifying the molecular structure of hybrid layers at a spatial resolution comparable to optical microscopy, degree of conversion of resin-based composite[385,386] , of dental resins in different mixtures[387,388], and of adhesive systems[389].

For biological and medical specimens, Raman micro-spectroscopy generally uses near-infrared (NIR) lasers (785 nm and 1064 nm Nd: YAG are especially common). This reduces the risk of damaging the specimen by applying higher energy wavelengths.

General uses of Raman:

- To identify organic molecules, polymers, biomolecules, and inorganic compounds both in the bulk and in individual particles.
- Raman imaging and depth profiling is used to chart the distribution of components in mixtures.
- To determine the presence of different carbon types (diamond, graphic, amorphous carbon, etc.) and their relative proportions.
- To determine inorganic oxides and their valence state.
- To measure the stress and crystalline structure in semiconductors and other materials.

3.4.6. Raman advantages

Raman spectroscopy offers several advantages for microscopic analysis. Since it is a scattering technique, specimens do not need to be fixed or sectioned (no special treatment is required). Analysis can be performed at room temperature. Raman spectra can be collected from a very small volume (<1 μm in diameter); these spectra allow the identification of species present in that volume. Water does not generally interfere with Raman spectral analysis, as it can use a water immersion lens; they are less sensitive to water content than FTIR techniques[390]. The techniques require minimal specimen preparation[265], and can be conducted in a normal atmosphere[391]. It affords good reproducibility, and the presence of a molecule to which a spectrum is assigned can be appraised by calculating the spectrum peak.

3.3. Objectives

The aim of this study was to characterize the surface chemistry & mineral crystallinity of root canal dentin after conventional instrumentation, or its treatment with NaOCl for 5 min, EDTA, and a self-etching primer, by means of micro-Raman spectroscopy.

Specifically, this study analyzes the compositional differences in the organic and inorganic contents of the root canal dentin as a function of:

1. The depth into the root, by considering four root levels in a crown-apex direction (mean, coronal to apical)

2. The application of NaOCl during 5 minutes, with emphasis on the differences with respect to a shorter application of 1 minute and no-treatment.

3. The use of canal treatments with a main demineralizing effect (EDTA, self-etching primer), as opposed to no-treatment and to mainly deproteinizing treatment with NaOCl.

3.4. Materials and methods

3.4.1. Sample selection

Twenty extracted intact permanent maxillary and mandibular incisor teeth with straight single roots, fully developed with closed apices, free from cracks, caries and restoration in the root region, and not having undergone root canal treatment were selected from anonymous subjects after their signed consent under a protocol approved by the Ethics Committee of the School of Dentistry, University of Granada. Following extraction, the teeth were stored in 0.1% thymol at 4ºC for a maximum of six months.

3.4.2. Preparation of teeth

3.4.2.1. Instrumentation

The crowns of teeth were sectioned at the cement-enamel junction (CEJ), perpendicular to the long axis of the teeth using a water cooled low-speed diamond disc (Struers, 44310019, Copenhagen, Denmark). After cutting, the crown instrumentation was done using Profile nickel-titanium rotary instruments (Dentsply Maillefer) by means of the crown-down technique. The canal orifices were identified and the canals were negotiated with stainless steel K files, sizes 8 to 15, until the tip was visible at the apical foramen. This led to three things: the establishment of the working length; canal patency; and the creation of a pathway or glide path for rotary instruments. The working length was established as 1 mm short of the apical foramen. All roots were instrumented mechanically using an ENDO-MATE DT apparatus (Figure 23). This ultra-slim & compact hand piece featuring Torque Control & Auto Reverse is smart enough to memorize exact speeds and torque settings for up to 9 nickel-titanium files from all major suppliers.

The basic steps common to all ‘crown to apical’ techniques imply early coronal and mid-root flaring and enlargement before proceeding to the apical part of the canal. The initial coronal flaring can be completed most efficiently with Profile orifice shapers No.3 (0.06/40) as the first instrument used. The rotating instrument was inserted, after which slight push/pull motions were executed for about 5-10 seconds. When progression became difficult it was stopped, and the next instrument was chosen (profile No.2 (0.06/30). As the instrument initially moves into the coronal third of the canal, the pathway is enlarged. The approach permits rapid irrigant penetration and facilitates further movement of small instruments deeper into the root canal. The same movements were performed till there was resistance, and repeated sequentially with 0.06/25, 0.06/20 and 0.04/25 to 3mm less from the working length. This allowed an unimpeded placement of instruments to the middle and apical parts of the canal system. Apical preparation up to the exact working length was

done using 0.04/20 followed by 0.04/25 (smaller to larger). Final flaring was done using 0.06/20 with a slight in-and-out motion. Irrigation during cleaning and shaping was accomplished using 2.5% NaOCl solution for 1 min.

Figure 23: ENDO-MATE DT apparatus.

3.4.2.2. Preparation of control group

The tooth structure in the control group was used to analyze the dentin before any treatment. Thus, crystallinity, carbonate substitution, amide I and amide III were compared between normal and treated dentin. Four teeth served as the control. Each tooth was cut at the cement-enamel junction to remove the crown using a low speed diamond disc under constant water coolant. The tooth was instrumented mechanically using distilled water as an irrigant during instrumentation for 1 minute, and the root was prepared for micro-Raman analysis.

After instrumentation of all roots, 20 of them were divided into five groups according to the treatment agent used. Each group contained four teeth:

3.4.3. Grouping

- Group I: Roots instrumented mechanically using 2.5% NaOCl with pH 12; irrigation for 1 minute during instrumentation.
- Group II: Roots exposed to 2 ml of 2.5% NaOCl with pH 12 for 5 minutes as a final rinse, after mechanical instrumentation including the use of 2.5% NaOCl irrigation for 1 minute during instrumentation.
- Group III: Roots exposed to 2 ml of 17% EDTA pH 7.5 as a final rinse for 1 minute after mechanical instrumentation, using 2.5% NaOCl irrigation for 1 minute during instrumentation. A final flush of 10 ml distilled water was included to prevent the prolonged effect of EDTA.
- Group IV: Application of self-etching primer to dry dentin for 30 seconds after mechanical instrumentation, with the use of 2.5% NaOCl irrigation for 1 minute during instrumentation, then 2 ml of 17% EDTA as a final rinse for 1 minute. Excess primer was removed with paper points.
- Group V: control group roots instrumented mechanically using only distilled water during instrumentation.

3.4.4. Preparation of samples for micro-Raman

Following cleaning, shaping and treatment, each root was sectioned horizontally perpendicular to the long axis of the root, into four slices —coronal 1, coronal 2, middle1, middle 2— Figure 24; using a water cooled low speed diamond disc (Struers, 5010323, Copenhagen, Denmark).

Figure 24: Four root slices with 1mm thickness

In this way, 80 root slices were obtained, having thickness 1mm. The dentin surface of each slice was then abraded to smoothness with a series of silicon carbide paper (500, 800, 1200 and 2500) successively increasing in grit size, and polished with a fine silicon carbide paper (4000 grit) under water coolant to obtain a mirror-like surface (Figure 25). The final thickness of each slice was 0.3mm of flat dentin surface. The samples were cleaned using an ultrasonic bath (with distilled water) for 30 minutes to remove excess debris. The slices were then ready for micro-Raman spectroscopy characterization (Figure 26).

All 80 specimens were kept in a wet environment at 37°C for 24 hours until micro-Raman analysis.

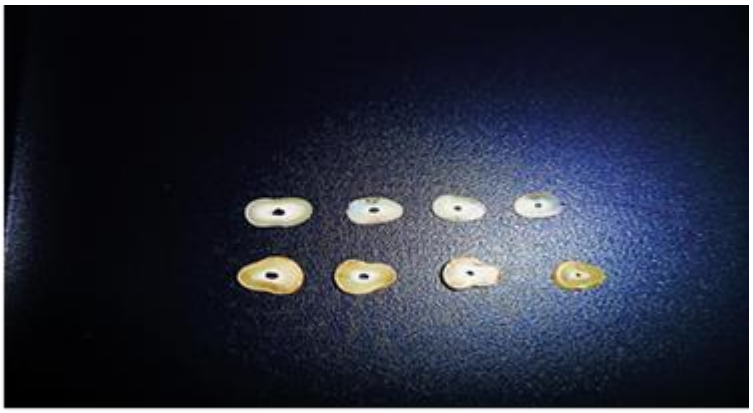


Figure 25: Root slices before and after smoothing

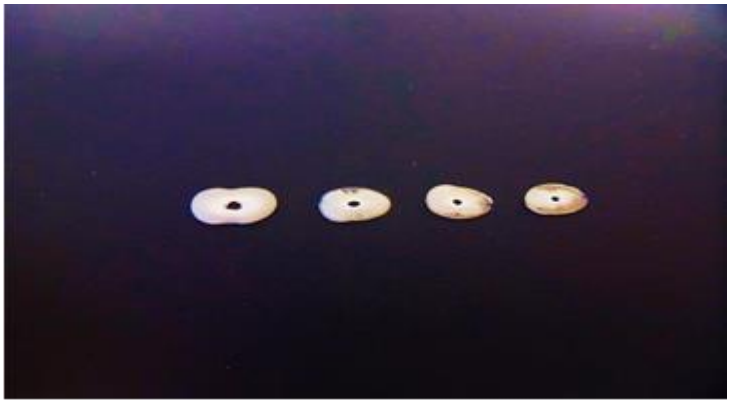


Figure 26: Root slices after smoothing.

3.4.5. Micro Raman spectroscopy

Raman spectra were recorded with a JASCO NRS 5100 spectrometer (Figure 27). The optical microscope allowed for visual identification of the position at which the Raman spectrum was obtained (Figure28). The Raman spectrometer itself consisted of red Diode laser beam with a wavelength of 785.11nm, and the 500 mW laser power incided upon the sample used to induce the Raman scattering effect. The spectral coverage of this model ranges from 800 to 1800 cm^{-1}, therefore spanning the finger print region associated with collagen and mineral. The calibration of the wavelength and intensity was performed according to the manufacturer's specifications using a silicon standard (520cm^{-1}). Samples were placed on a glass slide and focused under an X100 microscopic objective lens (Figure 29). The spectra were acquired in the dentine adjacent to the root canal at a maximum distance of about 4 microns from the dentin internal border. Samples were analyzed using the following micro Raman parameters: spatial resolution was ~0.5µm, spectral resolution 1.7cm-1, accumulation time 15 seconds, with 10 accumulations obtained from each point per cycle. Measurements were taken at 5 points on the same level inside the first 4µm from the internal canal border. The entire dentin surface was examined on the x and y axes. Five spectra were taken from each sample, giving 20 spectra from each of the three teeth in each group (300 spectra for all teeth); the mean ± standard deviation (SD) values were calculated. Software was used to analyze the acquired Raman spectra. To favor precise optical focusing at X100 magnification, prior to acquiring spectra, excess water was removed from the samples. Subsequently, spectra were acquired. At all times the samples were kept hydrated by wrapping them in humid gauze.

Quantitative analysis of the peak height (intensity) was carried out using a standard base line method, the baselines having been adjusted by means of a multipoint technique. The baseline was set as a line joining the lowest point in the upper and lower halves of each region under consideration, and the maximum distance from the baseline was understood to be the peak height. Bands associated with inorganic components (minerals), appearing in the spectral range between approximately 959 and 1.070cm-1, included bands v1phosphate (PO43-) and B type mineral carbonate (CO32-); the organic bands associated with collagen appeared in the spectral range near 1.240 cm-1 for (NH) amide III and 1,665 cm-1 for (NH) amide I.

Raman spectra divided the viewed sample into three areas. The first spectral area, from 800 to 1100 cm-1, contains the CO32- and PO43- bands; the second spectral area, from 1100 to 1400 cm-1 primarily entails type III bands; and 1400 to 1800 cm-1 corresponds to CH2 and amide I (Figure 30).

Figure27: Micro-Raman spectrometer

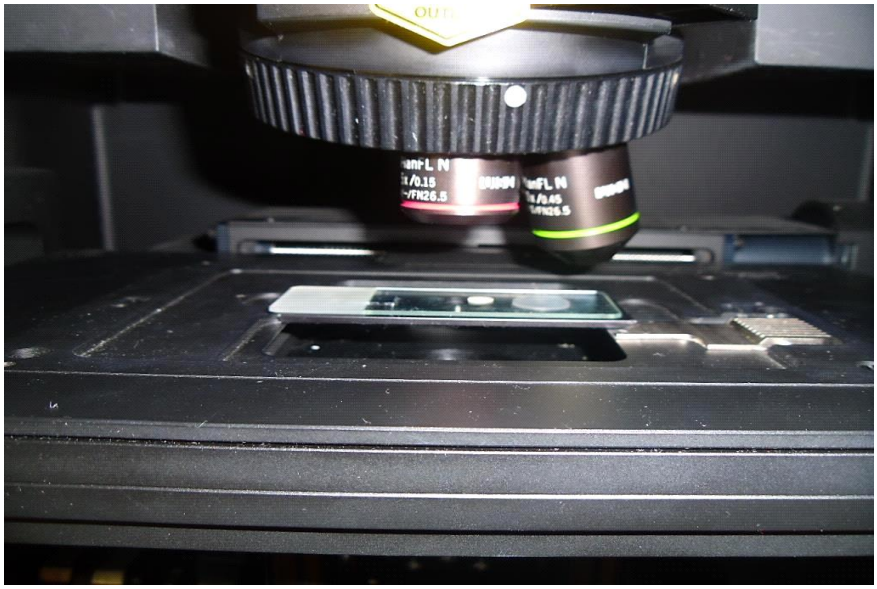


Figure 28: the optical micro-Raman microscope

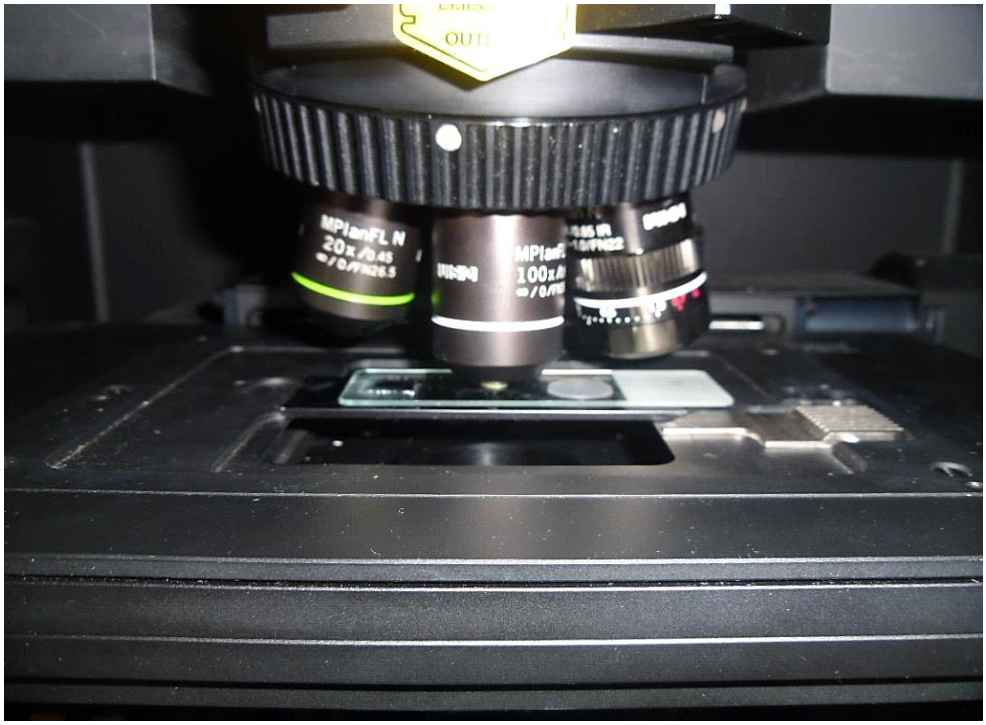


Figure 29: The sample was placed on a glass slide and focused under an X100 microscopic objective lens

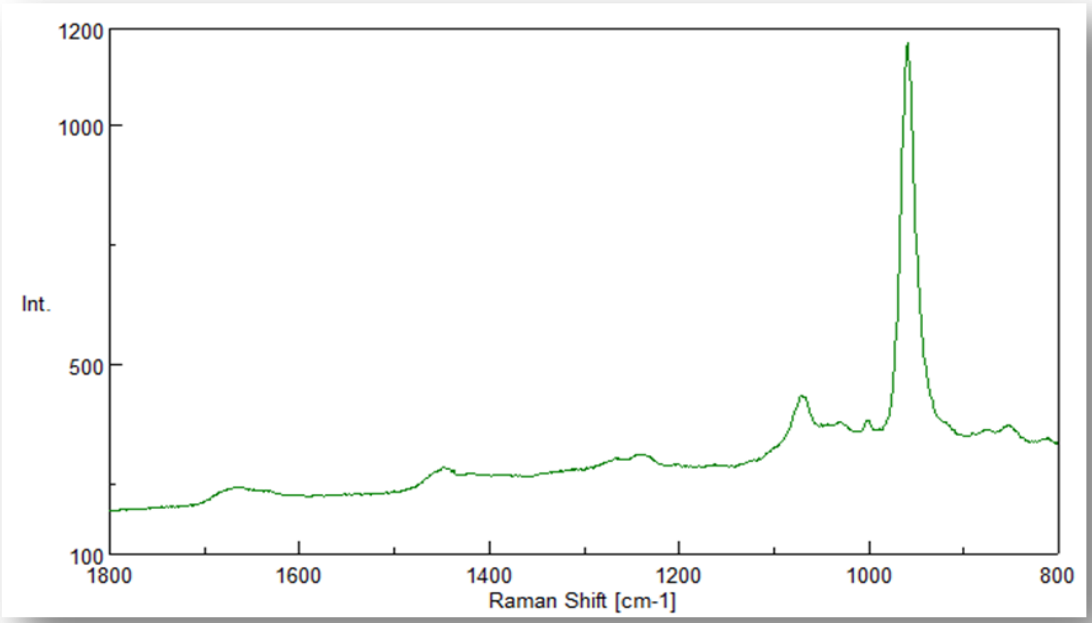


Figure 30: Raman spectrum of normal dentin. The strong peak at 960 cm^{-1} is associated with the P-O group of the dentin mineral component, while peaks at 1245 (C-N), 1450 (CH2), and 1667 cm^{-1} (C=O) represent the dentin organic components.

Comparisons of the intensity ratios among the different dentin specimens are given below.

Carbonate to phosphate ratio: It can provide valuable insight into the inorganic chemical composition of dentine.

Carbonate to CH2 ratio (1,072–1,450 cm^{-1}): The intensity ratios of CO_3^{2-} to the CH2 wagging mode were calculated to measure the relative differences in carbonate[392].

Phosphate to CH2 ratio (959–1,450 cm^{-1}): The mineral to organic ratio was calculated as the ratio of the phosphate intensity to the intensity of vibrations resulting from the CH2 flapping mode of side-chains of collagen molecules. This 1,450 cm^{-1} band was chosen by virtue of its low sensitivity to molecular orientation compared with amide I (at 1,670 cm^{-1}) [393].

Amide III to CH2 ratio (1,243–1,450 cm^{-1}): It indicates structural differences. A higher ratio was noted in intertubular dentine than in peritubular dentine, which indicates a higher content in collagen[394].

Amide I to CH2 ratio (1,665–1,450 cm-1): An increase of the amide I band at 1,655 cm^{-1} relative to the CH2 band indicates altered collagen quality induced by aging[395], hydration/dehydration[396], or radiologic damage[397].

3.5. Results

The spectra covering the region of Raman peaks within the range of 800 – 1800 cm^{-1} reveal molecular vibrations of dentin mineral and collagen. The identification and assignment of Raman sensitive peaks associated with organic and inorganic components of dentin is well documented. Some relevant assignments in this region are as follows: the most intense peak at 961 cm^{-1} (ν_1 symmetric stretch) is assigned to the dentin mineral phosphate. The peak at 1070 cm^{-1} (ν_1 symmetric stretch, CO_3^{2-}) is assigned to the mineral carbonate. The dentin collagen matrix features presenting the major peaks associated with the organic component appear at 1242 cm^{-1} (amide III) and at 1667 cm^{-1} (backbone amide I) and 1452 (CH2 deformation vibration). [258, 398]

Additionally, 960 cm^{-1} arose from ν_1 (PO_4^{3-}), and the band at 1030 cm^{-1} was assigned to pyridine ring, and 1070 cm^{-1} to $\nu_1(CO_3^{2-})$.

Detailed information about assignments and positions of these peaks is listed in Table 4.

Table (4): Wave numbers (in cm^{-1}) and peak assignments of micro-Raman spectra from the intertubular and peritubular dentin

ITD (cm-1)	PTD(cm-1)	Assignment
961vs	961vs	$\nu_1(Po_4^{3-})$
1031m	1031m	pyridine ring
1071ms	1071ms	$\nu_1(CO3)$[405,407,408] type B carbonate
1245m	1241m	s(NH) amide III[405,406,408]
1268m	1268m	s(NH) Amide III[271,272]
1450m	1450m	s(CH) deformation[405,406,409]
1670m	1668w	v(c=o) stretch/amide I[405,406,408]

Changes in the organic and inorganic components of the dentin samples were calculated by taking ratios of intensities of amide I, amide III, CH2 Wagging, & carbonate to phosphate peak intensity, as shown in Table 5.

A prime interest of this study was to evaluate any compositional and structural alterations associated with the dentin treated by different chemical agents (that is, 2.5% NaOCl for 1 minute during mechanical instrumentation in group I and for 5 minutes as a final irrigant in the group II, 17% EDTA for 1 minute as a final irrigant in group III & RealSeal self-etching primer for 30 seconds in group IV applied after mechanical instrumentation as in Group I). These findings were viewed in comparison with the control group (V), in which dentin was irrigated with distilled water during instrumentation.

To determine whether application of these agents caused any denaturation of dentin collagen, the Raman spectra containing amide I and III (1100-1670 cm^{-1}) were compared for treated dentin and sound dentin. The similarities of the two spectra indicate that these procedures did not denature dentin collagen.

Amide peaks are thought to be representative of protein conformation. The position and intensity of these peaks (amide I and III) are typical of collagen fibril with a triple helical structure.

In this study, each endodontic agent had been applied onto 4 teeth in each group, and then each tooth was divided into four sections. Raman spectra were recorded from each specimen at 5 points in the same plane inside the first 4μm from the internal canal border.

Table (5): the ratios of various peaks

Ratios	
1031/960	Pyridine ring /Phosphate
1072/960	Carbonate/phosphate
961/1450	Phosphate/matrix
1072/1450	Carbonate/matrix
1667/1242	amide I (NH) /amideIII
1670/1450	amide I /CH2
1245/1450	amide III(NH)/CH2
1268/1450	Amide III(NH)/CH2

3.5.1. Statistical analysis

Descriptive statistics and normality plots were obtained for each treatment group and root level. Shapiro-Wilk's test was applied to explore the data distribution. Afterwards, non-parametrical tests were used.

To compare among treatment groups, the Kruskal-Wallis and Mann-Whitney U tests were applied. Analysis of the effect of chemical agents among root levels was done using Friedman's test for multiple comparisons and Wilcoxon's rank tests, for pairwise comparisons. Significance was established for a p value lesser than 0.05.

3.5.2. Effect of sodium hypochlorite on the organic and mineral component of root canal dentin (table 6 and 7)

Irrigation of the canal with 2.5%NaOCl solution during instrumentation for 1 minute —Group I— showed no significant difference in amide I, amide III and amide I/amide III ratios with respect to the control group, irrigated with distilled water during instrumentation.

After final irrigation, the canal with 2.5%NaOCl solution for 5 minutes —Group II— showed a decreased amide I/amide III (1667/1242) ratio corresponding to the amides III, in relation with control group (V) at all levels, but only significant in the lower medium levels (M1 & M2). and there was a decrease in the amide I and amide III band about 1450 cm-1 (-CH2 vibration dentin matrix), in some rooting depth levels compared to the control group irrigated only with distilled water (Group V. In relation with Group (I), irrigated with 2.5% NaOCl for 1 minute during instrumentation, there was a significant increase of 1667/1242 ratio at all levels, yet a lower value for the two amide III/matrix ratios that was significant in the 1st cervical level of 1245/1450 and in the upper levels (cervical and middle) of 1268/1450 ratio.

It should be noted that the final irrigation for 5 minutes did not cause change in the bands 1670/1450 associated with amide I in relation to the instrumentation and control groups.

There was significant difference in the bands corresponding to amide I (1670/1450), among the levels of control group (V) due to regional variability.

Table 6: Means and standard deviations of organic ratios of groups (I, II and V) (N=20)

Group	third	1667/1242	1670/1450		1245/1450	1268/1450
Group(I) Instrumentation With 2.5%NaOCl	C1	0,704(0,135)B	0,717(0,044)		1,060(0,241)A	0,979(0,325)A
	C2	0,711(0,123)B	0,726(0,053)		1,049(0,194)A/C	0,987(0,311)
	M1	0,669(0,122)B	0,683(0,059)		1,060(0,241)	1,012(0,270)A
	M2	0,733(0,047)B/C	0,702(0,079)		0,963(0,147)A/B	0,899(0,216)
Group(II) 2.5%NaOCl as final irrigant	C1	0,817(0,093)A	0,709(0,056)		0,873(0,079)B	0,751(0,115)B
	C2	0,771(0,056)A	0,699(0,051)		0,908(0,045)A/B	0,789(0,054)
	M1	0,811(0,068)A	0,713(0,075)		0,882(0,079)	0,761(0,090)B
	M2	0,804(0,063)A	0,714(0,047)		0,890(0,050)A	0,784(0,073)
Group (V) Control (instrumentation with distilled water)	C1	0,753(0,133)A/B	0,703(0,104)	a/b	0,942(0,079)A	0,797(0,084)A
	C2	0,714(0,104)A/B	0,737(0,048)	a	1,053(0,172)C	0,960(0,248)
	M1	0,687(0,124)B	0,661(0,107)	b	0,974(0,147)	0,905(0,215)A
	M2	0,686(0,122)B	0,657(0,118)	b	0,958(0,137)B	0,834(0,165)

Different capital letters indicate significant differences among the groups in each root level.

Different small letters indicate significant differences among the levels in each treatment group.

Regarding mineral component, group (I), involving irrigation with 2.5% sodium hypochlorite for 1 minute during instrumentation, showed a decreased carbonate/matrix (1072/1450) ratio in relation with the control group at all levels that was significant at the 2nd cervical level. It also showed significant decrease in the phosphate/matrix (960/1450) ratio at the 2nd cervical level only, with no significant differences at other levels. It also showed a significant decrease 1072/960 ratio than control group at the first three levels (C1, C2 and M1) except at the 2nd middle level was not significant.

Final irrigation with sodium hypochlorite for 5 minutes showed a significant increase of 1072/1450 and 960/1450 ratios in relation with the control group at the 1st cervical level (figure31). while showed a decreased 1072/960 ratio that was significant in the upper levels (cervical and middle). In relation with instrumentation group (I), 2.5% NaOCl for 5 minutes showed an increase of 1072/1450 and 961/1450 ratios that was significant at the 1st and 2nd cervical levels, and a significant increase of 1072/960 ratio at

the 2nd cervical level, but a significant decrease at the 1st middle level.

It is clear that sodium hypochlorite for 5 minutes did not cause change of the bands 1031/960 associated with phosphate in relation with instrumentation and control group.

There was a significant difference in the band corresponding to phosphate (960/1450) among the levels of instrumentation group I (2.5%NaOCl). There was likewise a significant difference in the band corresponding to carbonate (1072/1450) among the levels of the 2.5%NaOCl group (II). Significant differences were found among the levels of control group (V) in all mineral ratios, which can be attributed to regional variability.

Table 7: Means and standard deviations of minerals ratios of groups (I, II and V) (N=20)

Group		1031/960	1072/960	961/1450	1072/1450
Group(I) Instrumentation With 2.5%NaOCl	C1	0,116(0,09) a	0,165(0,014) A	14,241(1,730)A a	2,366(0,322)A
	C2	0,110(0,019)	0,161(0,013) A	15,057(2,250)A a	2,426(0,347)A
	M1	0,110(0,014)B	0,168(0,017) A/B	14,817(1,665)A a	2,485(0,235)
	M2	0,104(0,017) b	0,164(0,008) A	16,255(0,957)A b	2,633(0,162)
Group(II) 2.5%NaOCl as final irrigant	C1	0,113(0,005)	0,162(0,010) A	16,532(1,631)B	2,698(0,356) a/b
	C2	0,113(0,009)	0,164(0,004) B	17,086(1,337)B	2,817(0,188)B a
	M1	0,114(0,013)A/B	0,162(0,014) A	15,478(1,734)A	2,502(0,321) b
	M2	0,109(0,008)	0,164(0,006) A	17,134(3,381)A	2,811(0,510) a
Group (V) Control (instrumentation with distilled water)	C1	0,125(0,022) a	0,178(0,016) B a	13,653(3,642)A a	2,378(0,504)A a
	C2	0,107(0,007) b	0,164(0,006) B b	17,469(1,481)B c	2,880(0,281)B b
	M1	0,107(0,010)B b	0,175(0,007) C a	15,401(2,511)A a/b	2,693(0,397) a/b
	M2	0,108(0,015) b	0,167(0,007)A/B b	16,018(1,741)A b	2,689(0,278) b

Different capital letters indicate significant differences among the groups in each root level.

Different small letters indicate significant differences among the levels in each treatment group.

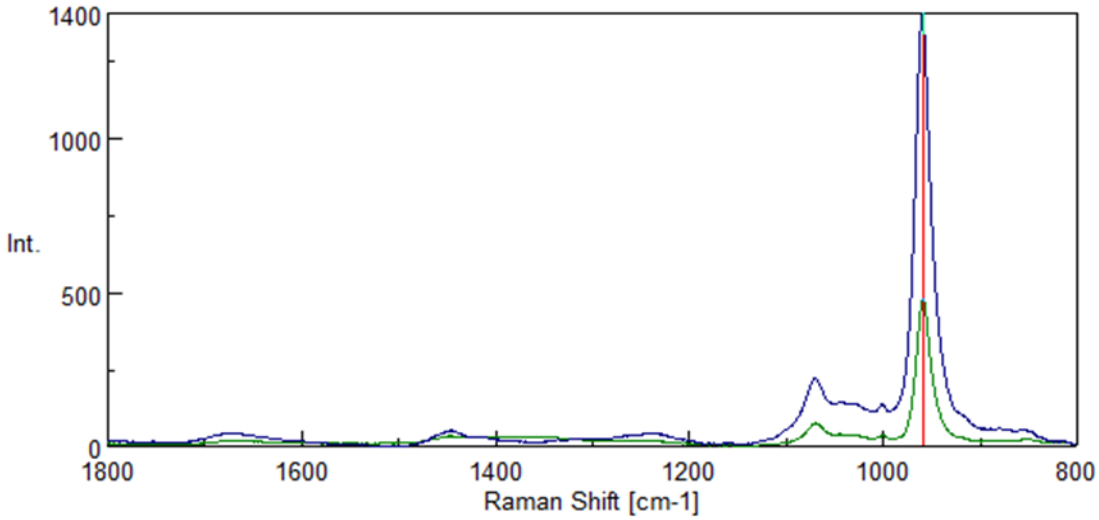


Figure 31: Representative micro-Raman peak intensity differences between NaOCl (blue) and distilled water (green) at 1st cervical level.

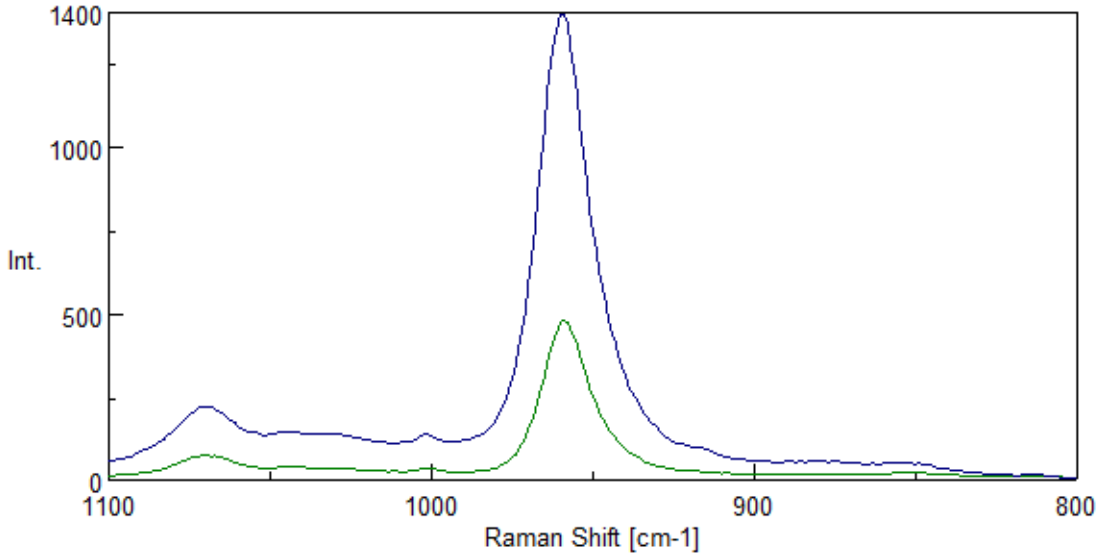


Figure (31 A): The differences between the mineral features of the spectra between NaOCl (blue) and distilled (green) water are clearly noticed in the region of 800–1100 cm^{-1}. NaOCl showed higher carbonate and phosphate than distilled water at 1st cervical level.

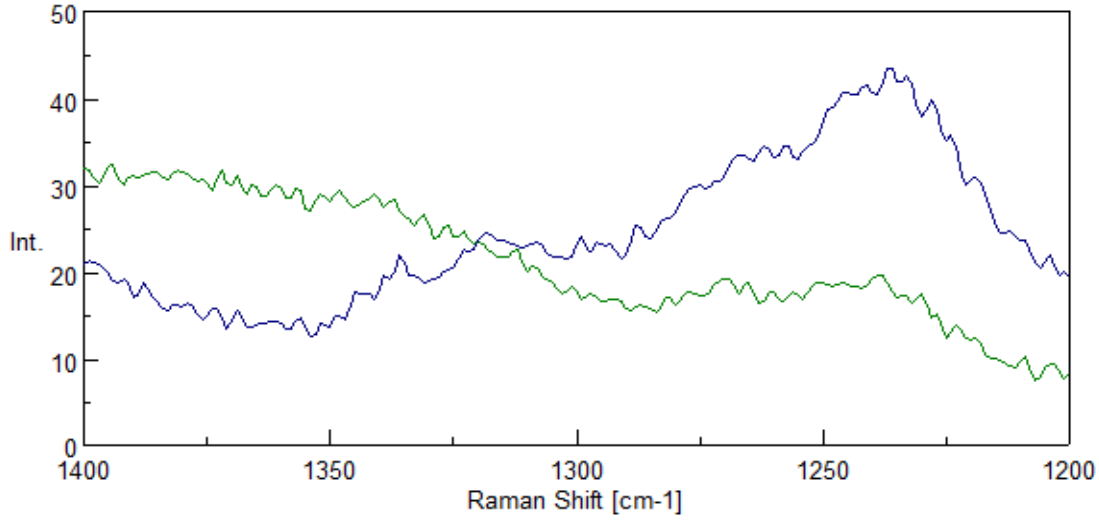


Figure (31 B): The differences between the collagen features of the spectra between NaOCl (blue) and distilled (green) water are clearly noticed in the region of 1200–1400 and from 1400-1800 cm^{-1}.

3.5.3. Effect of 17% EDTA on the organic and mineral component of root canal dentin (table 8 and 9)

After final irrigation of the canal with 17% EDTA solution for 1 minute, Group (III) showed no significant difference of the amide I/amide III ratio (1667/1242) in relation with control (group V) and group I, only irrigated with NaOCl between file steps. Group (III) showed decreased 1245/1450 at all levels, which proved significant in the coronal levels (1st and 2nd cervical level), while also showing a significant decrease 1268/1450 in the 1st (cervical and middle) levels (figure 32).

It should be noted that 17% EDTA did not cause change of the bands 1670/1450 associated with amide I in relation with the instrumentation using 2.5%NaOCl and the instrumentation with distilled water (control group).

There was a significant difference in the band corresponding to amide III (1268/1450) among the levels of the 17% EDTA group (III).

Table 8: Means and standard deviations of organic ratios of groups (I, III and V) N=20

Group		1667/1242	1670/1450	1245/1450	1268/1450
Group(I) Instrumentation With 2.5%NaOCl	C1	0,704(0,135)B	0,717(0,044)	1,060(0,241)A	0,979(0,325)A
	C2	0,711(0,123)B	0,726(0,053)	1,049(0,194)A/C	0,987(0,311)
	M1	0,669(0,122)B	0,683(0,059)	1,060(0,241)	1,012(0,270)A
	M2	0,733(0,047)B/C	0,702(0,079)	0,963(0,147)A/B	0,899(0,216)
Group(III) Final irrigation with 17% EDTA	C1	0,739(0,067)B	0,657(0,032)	0,894(0,065)B	0,754(0,088)B a
	C2	0,716(0,065)B	0,643(0,040)	0,902(0,064)B	0,785(0,074) ab
	M1	0,745(0,049)B	0,676(0,060)	0,910(0,085)	0,789(0,077)B ab
	M2	0,715(0,051)B	0,676(0,051)	0,950(0,065)B	0,836(0,104) b
Group (V) Control (instrumentation with distilled water)	C1	0,753(0,133)A/B	0,703(0,104) a/b	0,942(0,079)A	0,797(0,084)A
	C2	0,714(0,104)A/B	0,737(0,048) a	1,053(0,172)C	0,960(0,248)
	M1	0,687(0,124)B	0,661(0,107) b	0,974(0,147)	0,905(0,215)A
	M2	0,686(0,122)B	0,657(0,118) b	0,958(0,137)B	0,834(0,165)

Different capital letters indicate significant differences among the groups in each root level.

Different small letters indicate significant differences among the levels in each treatment group.

In terms of mineral component, final irrigation of the canal with 17% EDTA, Group III, as opposed to the control group irrigated only with distilled water, showed a significant increase 1072/1450 (carbonate/matrix) ratio at 1st cervical level while showed significant decrease at 2nd cervical level. For the 960/1450 (phosphate/matrix) ratio, group III, showed a significant decrease at 2nd (cervical and middle) levels while the 1072/960 (carbonate/phosphate) ratio showed a significant increase in 2nd cervical yet a significant decrease in 1st middle levels.

Final irrigation with 17% EDTA (Group III), in relation with instrumentation with 2.5% NaOCl (Group I) showed a significant increase 1072/1450 (carbonate/matrix) ratio at 1st and 2nd cervical levels while showing a significant decrease of 960/1450 (phosphate/matrix) ratio at 2nd middle. In the 1072/960 ratio, Group III showed a significant increase at upper cervical levels and the 2nd middle level.

Meanwhile, 17% EDTA did not cause changes in the bands 1031/960 associated with phosphate in relation to instrumentation with 2.5% NaOCl and the distilled water control group.

Table 9: Means and standard deviations of mineral ratios of groups (I, III and V)

Group		1031/960	1072/960	961/1450	1072/1450
Group(I) Instrumentation With 2.5%NaOCl	C1	0,116(0,09) a	0,165(0,014) A	14,241(1,730)A a	2,366(0,322)A
	C2	0,110(0,019)	0,161(0,013)A	15,057(2,250)A a	2,426(0,347)A
	M1	0,110(0,014)B	0,168(0,017)A/B	14,817(1,665)A a	2,485(0,235)
	M2	0,104(0,017) b	0,164(0,008)A	16,255(0,957)A b	2,633(0,162)
Group(III) Final irrigation with 17% EDTA	C1	0,110(0,007)	0,173(0,011)B	15,272(1,865)A	2,634(0,358)B
	C2	0,112(0,016)	0,175(0,017)C	15,349(2,553)A	2,670(0,294)C
	M1	0,106(0,015)B	0,170(0,014)A/B	16,069(2,455)A	2,718(0,356)
	M2	0,110(0,010)	0,171(0,006)B	14,505(1,146)B	2,486(0,162)
Group (V) Control (instrumentation with distilled water)	C1	0,125(0,022) a	0,178(0,016)B a	13,653(3,642)A a	2,378(0,504)A a
	C2	0,107(0,007) b	0,164(0,006)B b	17,469(1,481)B c	2,880(0,281)B b
	M1	0,107(0,010)B b	0,175(0,007)C a	15,401(2,511)A a/b	2,693(0,397) a/b
	M2	0,108(0,015) b	0,167(0,007)A/B b	16,018(1,741)A b	2,689(0,278) b

Different capital letters indicate significant differences among the groups in each root level. Different small letters indicate significant differences among the levels in each treatment group.

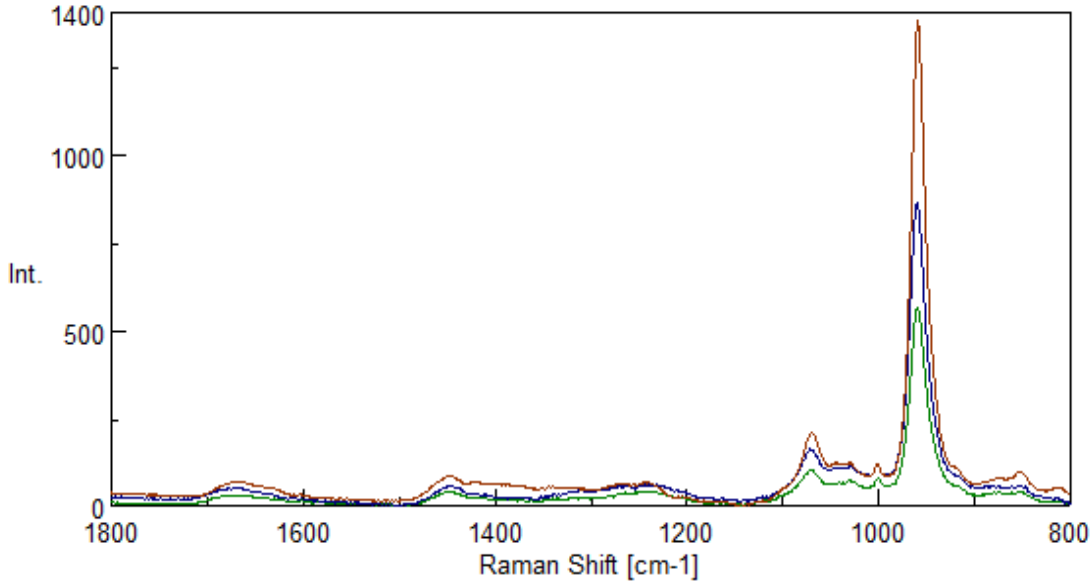


Figure (32): representative micro-Raman peak intensity difference among EDTA (red), 2.5% NaOCl during instrumentation (blue) and distilled water (green) at 1st cervical level.

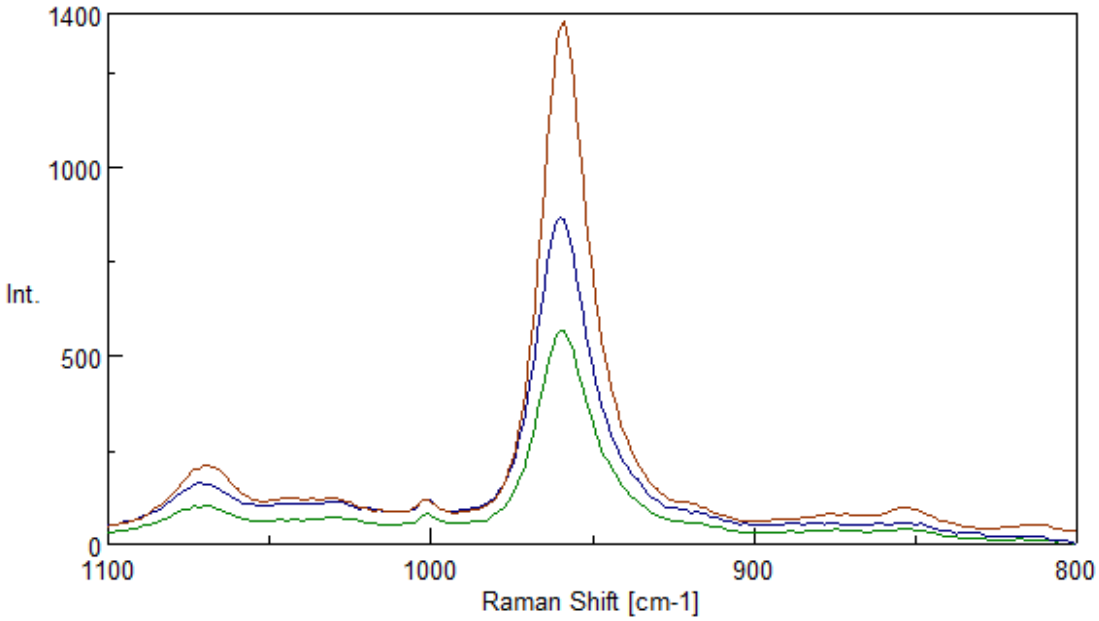
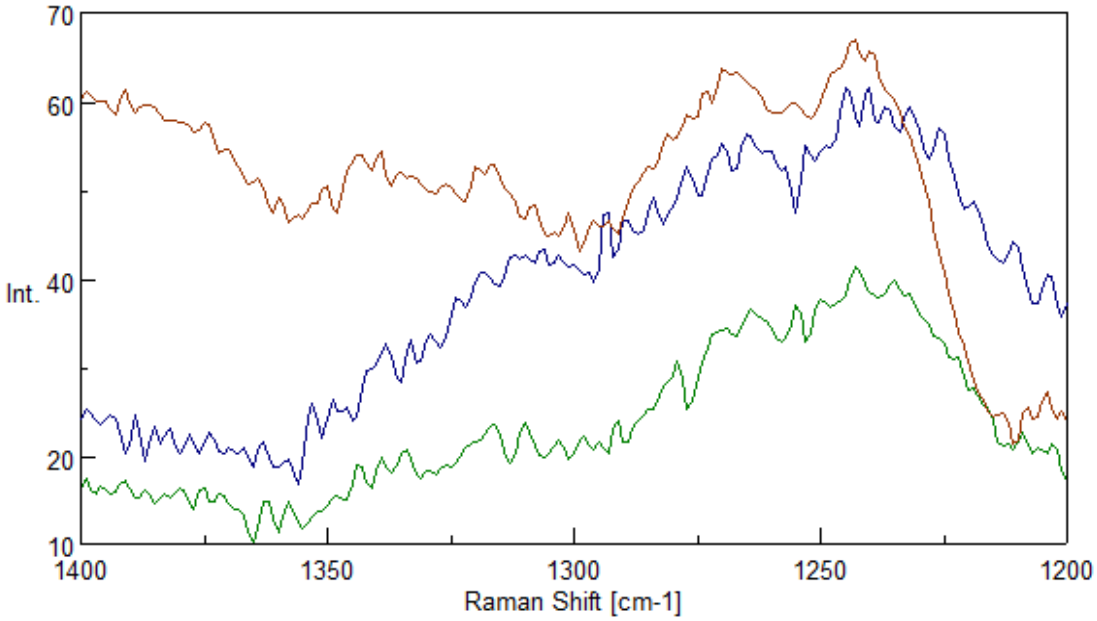


Figure (32 A): Differences in the mineral features of the spectra between EDTA (red), 2.5% NaOCl during instrumentation (blue) and distilled (green) water are clearly noticed in the region of 800–1100 cm^{-1}; EDTA showed higher value of carbonate at 1st cervical level.



Fi**gure (32 B):** Differences in the collagen features of the spectra between EDTA (red), 2.5% NaOCl during instrumentation (blue) and distilled (green) water are clearly noticed in the region of 1200–1400 and from 1400-1800 cm^{-1}.

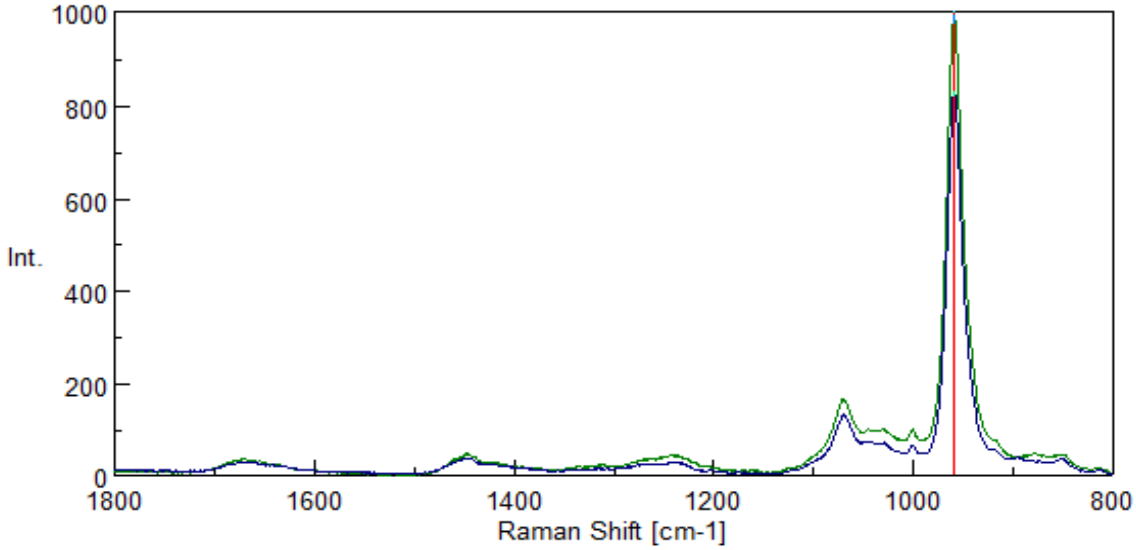


Figure (33): Representative micro-Raman peak intensity difference among EDTA (blue color) and distilled water (green) 2nd cervical level.

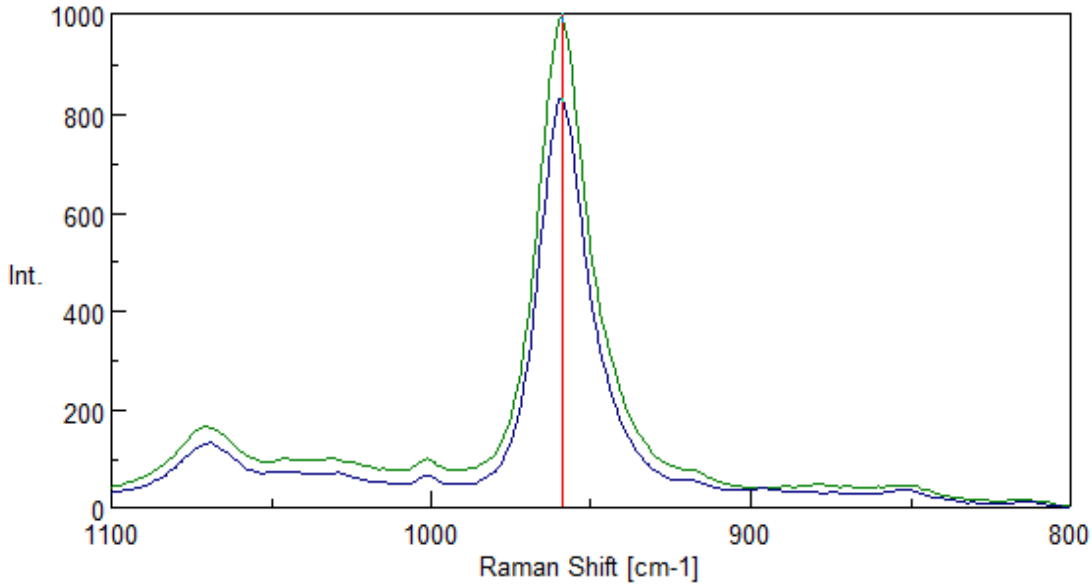


Figure (33 A): EDTA (blue color) showed lower 960 than distilled water (green) in the 2nd cervical level.

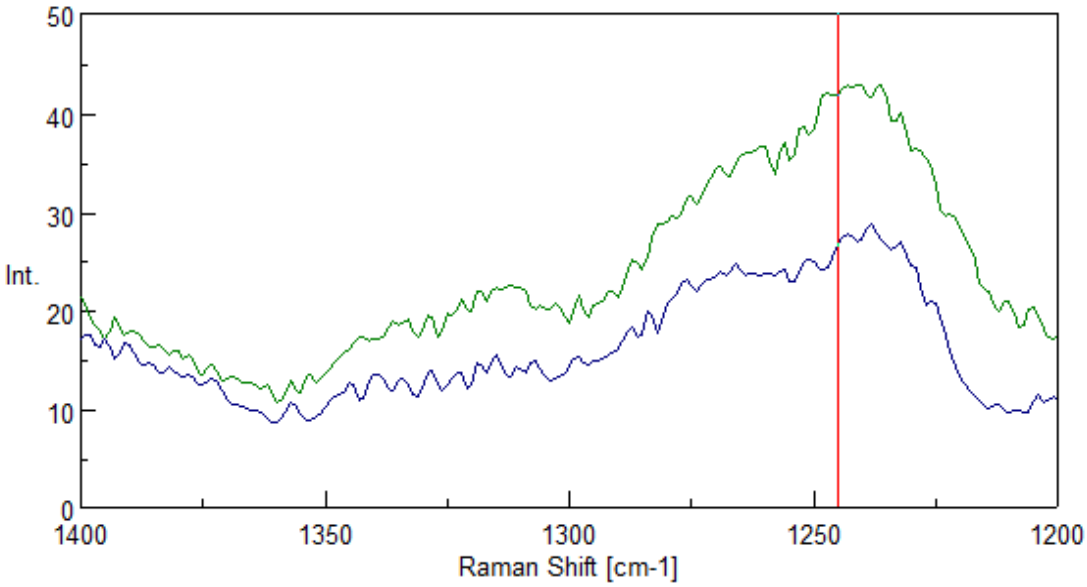


Figure (33 B): EDTA (blue color) showed lower 1245 than distilled water in 2nd cervical level.

3.5.4. Effect of self-etching primer on the organic and mineral component of root canal dentin (table 10 and 11)

After application of the self-etching primer of RealSeal sealer for 30 seconds on teeth irrigated with 2.5% NaOCl during instrumentation, and 17% EDTA as a final irrigant for 1 minute, a decrease in the two amide III/matrix ratio was observed in relation with the control group; it was significant in 2nd levels (cervical and middle) of 1245/1450 and in 1st middle of 1268/1450 ratio, an increased 1667/1242 (amid I/amide III) was significant in the 2nd middle level. Application of primer after instrumentation with 2.5% NaOCl and final irrigation with EDTA in relation with Group I instrumentation with 2.5% NaOCl showed a decreased two amide III/matrix ratio that was significant in the 1st middle of 1268/1450 ratio, along with an increased 1667/1242 that was significant for the 2nd cervical level. In turn, Group III, with EDTA as final irrigant for 1 minute, showed significanly lower 1245/1450 at the 2nd middle level and significant lower 1268/1450 at the 1st middle level, yet significantly higher 1268/1450 at 1st cervical level, while giving significantly higher 1667/1242 at the 2nd cervical and middle levels. The primer did not cause change of the bands 1670/1450 associated to amide I in relation with instrumentation and control group.

There was significant difference in the bands corresponding to amide I (1670/1450), among the levels of primer Group IV.

Table 10: Means and standard deviations of organic ratios for Groups I, IV and V.

Group		1667/1242	1670/1450	1245/1450	1268/1450
Group(I) Instrumentation With 2.5%NaOCl	C1	0,704(0,135)B	0,717(0,044)	1,060(0,241)A	0,979(0,325)A
	C2	0,711(0,123)B	0,726(0,053)	1,049(0,194)A/C	0,987(0,311)
	M1	0,669(0,122)B	0,683(0,059)	1,060(0,241)	1,012(0,270)A
	M2	0,733(0,047)B/C	0,702(0,079)	0,963(0,147)A/B	0,899(0,216)
Group(IV) primer	C1	0,755(0,088)A/B	0,680(0,047) a	0,907(0,071)A/B	0,776(0,084)A
	C2	0,787(0,074)A	0,729(0,080) b	0,925(0,042)A/B	0,803(0,048)
	M1	0,722(0,074)B	0,643(0,058) c	0,893(0,048)	0,817(0,080)C
	M2	0,772(0,069)A/C	0,677(0,048 a/b/c	0,880(0,063)A	0,805(0,042)
Group(III) Final irrigation with 17% EDTA	C1	0,739(0,067)B	0,657(0,032)	0,894(0,065)B	0,754(0,088)B a
	C2	0,716(0,065)B	0,643(0,040)	0,902(0,064)B	0,785(0,074) ab
	M1	0,745(0,049)B	0,676(0,060)	0,910(0,085)	0,789(0,077)B ab
	M2	0,715(0,051)B	0,676(0,051)	0,950(0,065)B	0,836(0,104) b
Group (V) Control (instrumentation with distilled water)	C1	0,753(0,133)A/B	0,703(0,104) a/b	0,942(0,079)A	0,797(0,084)A
	C2	0,714(0,104)A/B	0,737(0,048) a	1,053(0,172)C	0,960(0,248)
	M1	0,687(0,124)B	0,661(0,107) b	0,974(0,147)	0,905(0,215)A
	M2	0,686(0,122)B	0,657(0,118) b	0,958(0,137)B	0,834(0,165)

Different capital letters indicate significant differences among the groups in each root level. Different small letters indicate significant differences among the levels in each treatment group.

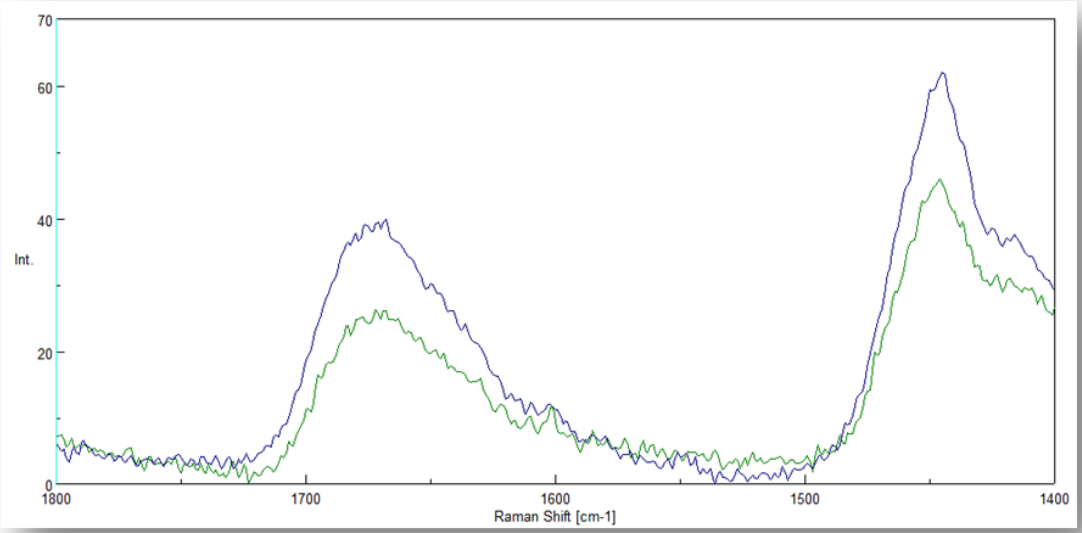


Figure 34. Represent differences between the collagen features of the spectra between primer (blue) and distilled water (green) are clearly noticed in the region from 1400-1800 cm^{-1}. The primer showed an increased amide I/amide III at 2nd middle level.

Regarding the mineral component, application of primer after instrumentation with 2.5% NaOCl (Group IV) showed a significant decrease of 960/1450 (phosphate/matrix) ratio in relation with the control group irrigated with distilled water at lower medium levels, but no significant difference of 1072/1450 and 1072/960 at any level. In relation with Group I, instrumentation with 2.5%NaOCl, primer, there was a significant increase 1072/1450 (carbonate/matrix) ratio at 2nd cervical level; in addition, the 960/1450 ratio showed a significant increase at 2nd cervical level, and a significant decrease at lower medium levels. Meanwhile, there was a higher 1072/960 ratio at all levels that was significant at the 2nd cervical level. In relation with Group III, using EDTA as final irrigant for 1 minute, significantly higher 1072/1450 was found at the 2nd cervical level and significantly higher 961/1450 at the 2nd cervical and 1st middle levels. The primer caused a significant increase of 1031/960 ratio associated with phosphate in relation with instrumentation and control group.

There were significant differences among the levels of primer Group IV in all mineral ratios.

Table 11: Means and standard deviations of mineral ratios of Groups I, IV and V; N=20

Groups	Level	1031/960	1072/960	961/1450	1072/1450
Group(I) Instrumentation With 2.5%NaOCl	C1	0,116(0,09) a	0,165(0,014) A	14,241(1,730)A a	2,366(0,322)A
	C2	0,110(0,019)	0,161(0,013)A	15,057(2,250)A a	2,426(0,347)A
	M1	0,110(0,014)B	0,168(0,017)A/B	14,817(1,665)A a	2,485(0,235)
	M2	0,104(0,017) b	0,164(0,008)A	16,255(0,957)A b	2,633(0,162)
Group(IV) primer	C1	0,121(0,024) a	0,176(0,028)A/B a/b	14,437(4,408)A/Ba/c	2,436(0,457)AB a
	C2	0,109(0,022) b	0,167(0,016)B/C a	17,319(1,907)B b	2,881(0,157) B b
	M1	0,126(0,015)A a	0,174(0,013)B/C b	13,621(2,117)B a	2,338(0,277) a
	M2	0,109(0,010) b	0,165(0,006)A a	14,404(2,366)B c	2,383(0,350) a
Group(III) Final irrigation with 17% EDTA	C1	0,110(0,007)	0,173(0,011)B	15,272(1,865)A	2,634(0,358)B
	C2	0,112(0,016)	0,175(0,017)C	15,349(2,553)A	2,670(0,294)C
	M1	0,106(0,015)B	0,170(0,014)A/B	16,069(2,455)A	2,718(0,356)
	M2	0,110(0,010)	0,171(0,006)B	14,505(1,146)B	2,486(0,162)
Group (V) Control (instrumentation with distilled water)	C1	0,125(0,022) a	0,178(0,016)B a	13,653(3,642)A a	2,378(0,504)A a
	C2	0,107(0,007) b	0,164(0,006)B b	17,469(1,481)B c	2,880(0,281)B b
	M1	0,107(0,010)B b	0,175(0,007)C a	15,401(2,511)A a/b	2,693(0,397) a/b
	M2	0,108(0,015) b	0,167(0,007)A/B b	16,018(1,741)A b	2,689(0,278) b

Different capital letters indicate significant differences among the groups in each root level. Different small letters indicate significant differences among the levels in each treatment group.

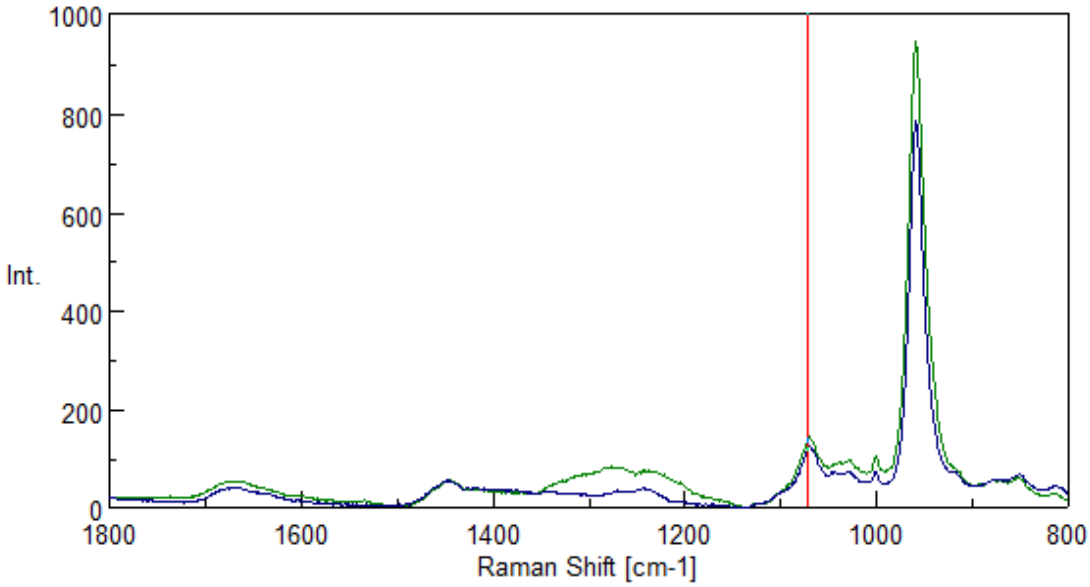


Figure 35. Representative micro-Raman peak intensity difference between Primer (blue color) and 2.5% NaOCl during instrumentation (green) at 1st middle level.

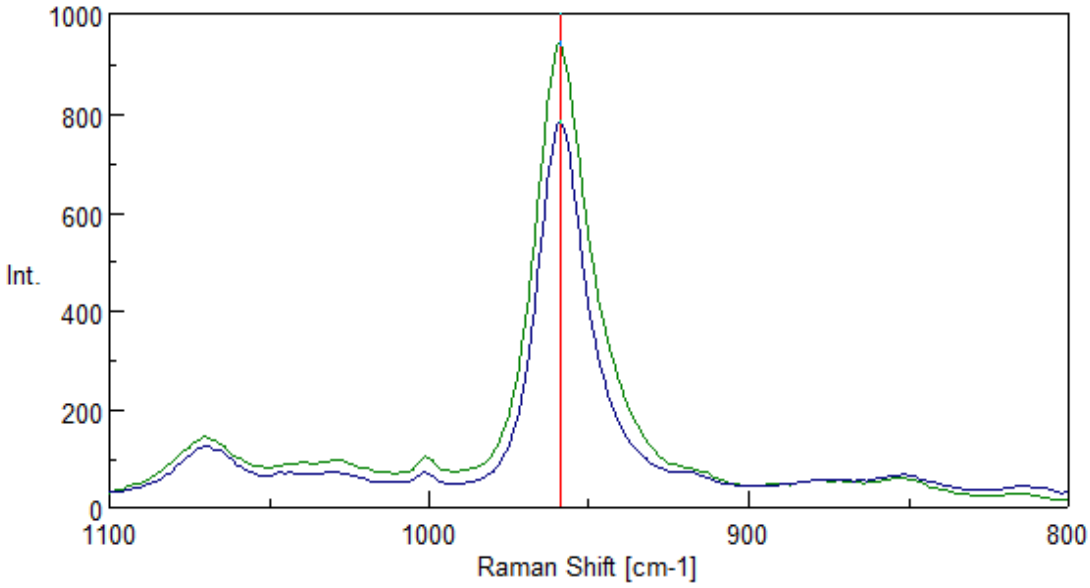


Figure 35 A. Primer (blue color) lower phosphate band than instrumentation (green color) at 1st middle level

In order to detect changes of the mineral matrix, the full width at half maximum (FWHM) of the strongest peak at 961 cm^{-1} arosing from $\nu_1PO_4^{3-}$ has been estimated. The area under the Raman curve is governed by its width, which corrects for changes in crystallinity. The width of these peaks can provide information on the quality of the mineral, whereas narrowing of the peak indicates crystalline in the material. The full peak width was measured at half of the maximum height of that particular peak. The FWHM of phosphate at 959.9 cm^{-1} was measured to obtained crystallinity of the mineral (phosphate).

Table 12: Mean values and standard deviation of FWHM

Level	instrumentation	NaOCl	EDTA	Primer	Distilled water
1stcervical	18,164(0,940)	18,318(0,495)	17,907(0,654)	17,762(0,853)	18,030(0,615)
2ndcervical	17,870(1,331)	18,034(0,626)	17,041(0,863)	18,764(0,674)	17,547(0,252)
1st middle	17,642(0,981)	18,135(0,827)	17,813(0,810)	17,794(0,904)	17,725(0,898)
2nd middle	17,451(1,153)	17,694(0,360)	17,888(0,673)	17,292(0,735)	17,613(0,519)

Compared to normal dentin, the variations in crystallinity did not differ significantly between normal and treated dentin. This finding indicating that, poor affectation of crystallinity as a function of root canal treatment.

3.6. Conclusion

The following important results have come out of this study:

- 2.5% NaOCl irrigation for 1 minute during instrumentation (Group I) showed no effect on organic component (amide I, amide III); but a subsequent final irrigation with 2.5% NaOCl solution for 5 minutes produces an irregular decrease of amide III in some root levels, and inconsistent effect on the mineral component —most notably a decrease in the carbonate/phosphate ratio.

- Final irrigation with EDTA for 1 minute after instrumentation, with 2.5% NaOCl irrigation for 1 minute during instrumentation, produces little or no affectation of the bands of carbonates and phosphates, and just a slight increase of carbonate/phosphate ratio. In terms of the organic component, it does not affect the amide III ratio or amide I/amide III.

- Application of primer for 30 seconds after the end of instrumentation and irrigation with EDTA produces a consistent effect on the mineral component. It mainly leads to a decrease in the phosphate concentration. At the organic component level, an irregular decrease of amide III was seen in some levels, without affectation of amide I, along with an inconsistent increase of the amide I/amide III ratio.

- The variations in crystallinity did not differ significantly between normal and treated dentin.

3.7. Discussion

The success of root canal therapy depends on the method and quality of instrumentation, irrigation, disinfection, and three-dimensional obturation of the root canal. In the endodontic literature, techniques for cleaning and shaping of root canals differ, and no single-best procedure for all conditions has been established[404,405,406]. Nickel-titanium rotary systems solve most of the deficiencies of traditional stainless steel instruments. Original canal shape can be better maintained when using rotary nickel-titanium instruments as compared to a hand-preparation technique with stainless steel K-Flexofiles[327]. The major retention is provided by micromechanical interactions of the bonding agent with the collagen matrix and the underlying mineralized zone in the intertubular dentin. Chemicals should be applied on instrumented root canal surfaces in order to remove the smear layer[407,408]. Such procedures may induce considerable changes in the surface morphology of dentin, which may also exert changes in its mechanical and physical properties[245,247,409,410], in turn modifying its permeability and solubility characteristics[411,412]. Moreover, alteration of the inorganic phase of dentin surfaces by acidic pretreatments modifies their surface properties, and undoubtedly, their hardness[413]. It is important to test the effect of irrigation solutions on all dentin tissues, because contact might occur during irrigation, meaning that radicular and coronal dentin is exposed to irrigating solution deposited in the pulp chamber; this may cause an alteration of dentin and effect its interaction with material used for obturation[414]. Earlier studies showed that irrigating solutions significantly change the mineral content of root dentin[247,245,215]. In particular, chelating solutions could play a part in influencing the mineral content of dentin. In the current study, we evaluated the mineral and organic contents of root canal dentin treated with different chemical agents, using micro-Raman spectroscopy. It has been conclusively shown that the organic element of dentin (collagenous component) is depleted by soaking in NaOCl[416,417], while the mineral component is left relatively intact. Hence, the recommendation of combining chelating agents with NaOCl solution, because no single irrigation solution has been shown capable of dissolving organic remnants or demineralizing the inorganic calcified portion of the root canal wall. If irrigation with NaOCl is alternated with EDTA, the hydroxyapatite is also degraded and consequently leads to greater dentin strain and a change in visco-elastic properties[417].

Root canal preparation and the use of irrigating solutions such as sodium hypochlorite would be responsible for eliminating the majority of microorganisms in an infected root canal system[360,418]. Sodium hypochlorite is recommended and used by the majority of dentists because this solution presents several important properties: antimicrobial effect,[360,359] tissue dissolution capacity[419,420]and acceptable biologic compatibility in less concentrated solutions[421,422]. All the NaOCl solutions that are available to the clinician are alkaline. The reason for this is two-fold: unaltered sodium hypochlorite

solutions are alkaline, and neutralized or acidified solutions are unstable and thus cannot be stored or marketed that way[423]. NaOCl with PH of 7.4-11.5 caused 70% protein description from the hydroxyapatite surfaces[362]. Dentin contained 22% organic material, mainly collagen type I, which played a major mechanical role. Depletion of the organic phase after NaOCl treatment caused mechanical changes.

Many studies have investigated the influence of different concentrations of sodium hypochlorite on the mechanical properties of dentin[410,424]. Cameron has shown that concentrations greater than 2% remove protein remnants from root canals when combined with ultrasound, whereas lower concentrations do not[425]. A 2.5% concentration of sodium hypochlorite, as used clinically, was chosen to establish the possible effect of such a concentration on root dentin.

The interaction between sodium hypochlorite and organic material of the pulp tissue results in a reduction in the pH of sodium hypochlorite[420]. Understanding the mechanism of action of sodium hypochlorite is necessary to appreciate its tissue dissolving ability. The mechanism of its action on organic tissues was described to be mediated via dissociation of sodium hypochlorite in water into sodium hydroxide and hypochlorous acid[198]. Sodium hydroxide (NaOH) interacts with fatty acids to form soap and glycerol (alcohol) and with amino acids to form salt and water. Hypochlorous acid (HOCl-) interacts with amino acids to form chloramines and water. Both of these ions were found to be essential for the dissolution of organic tissues[420].

The tissue-dissolving ability of NaOCl has been found to be related to the duration of exposure[426] and its concentration and temperature[427]. It is also dependent on the amount of organic tissue present, the frequency and intensity of the irrigant fluid flow, and the available surface area for interaction[428].

EDTA, which is the most widely used chelating irrigant, was first used in root canal therapy by Nygaard–Ostby[429] in 1957. EDTA is an organic acid which eliminates the mineral part of pulp tissue[430]. EDTA solution mainly contains disodium salts, which appear to give the best chelating action. It also plays an important role in the reduction of inflammatory reaction by inhibiting the affinity of macrophages to the vasoactive peptides of the pulpal tissue[431]. The use of EDTA is common during mechanical preparation of root canals. Goldberg[432]explains that EDTA action is not selective for dentin debris. This demineralizing effect also acts upon the root canal walls, leaving them almost devoid of mineralized surface, which is soft and permeable. On the contrary, it can affect the sealing ability of the filling material[433]. Chemical solutions that have softening effects on the dentinal walls could be beneficial in the clinical setting, permitting rapid preparation and

negotiation of narrow/calcified root canals. However, the degree of softening and demineralization may bear an influence on the physical and chemical properties of the root canal dentin.

The basic mechanism of bonding is based on micro-mechanical interlocking, derived from an exchange process that involves replacement of minerals from the hard dental tissue by resin monomers[434]. During this exchange process, the smear layer that covers the cut tooth tissues acts as a barrier to resin infiltration. There are two approaches for treating the smear layer: (1) by complete removal of the smear layer through rinsing after application of a mineral acidic etchant, such as 30-40% phosphoric acid; or (2) by demineralization of the smear layer through the use of a self-etching primer, which is a mixture of a hydrophilic resin and acidic monomers. When the self-etching primer is applied to the dentin surface, its acidic component either completely dissolves the smear layer, or it creates diffusion channels through the smear layer where the resin can diffuse a short distance into the underlying dentin[435]. The extent of adhesive penetration with self-etch systems was almost identical to the extent of dentin demineralization. Such knowledge surrounding the depth and chemical profile of the etched dentin surface is important for improving the reliability and durability of these systems.

The Micro-Raman technique appeared to offer a powerful new method for direct measurement of the mineral distribution after acid etching. Raman studies are known to be very versatile and allow for easy characterization with little or no requirement for sample preparation[436]. The combination of Raman spectroscopy with microscopy makes it easier to characterize specimens with high resolution, giving better qualitative analysis as well as a comprehensive representation of the degree and depth of mineralization. Micro-Raman spectrometry allows a thorough molecular analysis of mineralized dental tissues. The information provided is in the form of curves representing the intensity of the signal according to the frequency, and mathematical analyses allow for any type of comparative and quantitative analysis[437]. Micro-Raman spectroscopy was used in this study to analyze the chemical composition of dentine, given that it is a useful, cost-effective analytical technique affording high specificity. Here, the acquired spectra are attributed to molecules rather than to single elements, and the laser beam can be focused on a very small spot size. A high spatial resolution at the sample surface can be achieved without dehydration of the sample. Measurements can be taken at room temperature and pressure, wet or dry, without destroying the sample. In relevant studies, vibrational spectroscopy provided information on the changes in the mineral and matrix composition as demineralization occurs. The spectroscopic parameters used here —mineral-matrix and crystallinity of the mineral— were selected for dentin analysis because they are independent of sectioning artifacts (being

ratios) and they represent important properties for any mineralized tissue.

The Raman technique can be used to measure the mineral loss during demineralization both qualitatively and quantitatively. For example, the phosphate peak in the range of 952-962 cm^{-1} can be used for identifying the presence of mineral in the demineralized layer, while ratios of 961/1454 can be analyzed for quantitative information such as depth and degree of dentin demineralization.

There is much debate regarding the ideal time of application for each chelating agent. Despite the huge amount of research on the topic, no clearly defined irrigation protocol has been established. That is, there is a current lack of consensus about how long a decalcifying agent must be in contact with the root canal walls to adequately remove the smear layer[250]. The optimal amount of time, according to diverse studies, varies from 1 to 15 minutes[247,438,439]. Other researchers suggest extending the application time to 10 to 15 minutes to obtain optimal results [432,440].

EDTA has been reported to remove the smear layer in less than one minute if the fluid is able to reach the root canal wall surfaces[441]. Serper reports that after just one minute of exposition on dentin, EDTA begins to affect dentinal structure[370]. Studies by Ari et al.[255], De-Deus et al.[250], Eldeniz et al.[442], and Sayin et al.[412] indicated that irrigation of root canals with 17 % EDTA solution reduced the microhardness of root canal dentin as the irrigation time was increased. The significant alteration in dentin hardness after the irrigation treatment indicates the potent direct effect of chemical solutions on the component of dentin structure. The decalcifying efficacy of these acids and chelating agents depends on the root length, duration of application time, diffusion into the dentin, the pH of the solution[443] and the concentration.

In the present study, all specimens were subjected to 1-minute contact with the 2.5%NaOCl solution during instrumentation (Group I), which represents the working solution. This duration is more accurate in terms of clinical practice. Then, 2.5%NaOCl was used for 5 minutes in Group II to compare it with the 1 minute group (I). A final rinse 17% EDTA for 1 minute was used in Group III; and self-etching primer RealSeal was applied in Group IV. Because it exerts no chemical changes on dentin[444], distilled water was used as the control. The objective of our investigation was to evaluate and compare the effect of these chemical agents on root dentin mineral and organic compositions.

In terms of organic matrix, the spectral region of amides I and III were thought to be the best possible region for studying the protein structural changes[445]. The position and intensity of these amide bands are sensitive to the molecular conformation/structure of polypeptide chains[446] and/or the orientation of collagen/proteins[447].

This indicated that the spectral differences were mainly due to molecular/structural changes. An increase of the amide I bands indicated altered collagen quality[397]. Our results showed that amide I did not increase in groups treated with 2.5% NaOCl solution, 17% EDTA solution and the self-etching primer of RealSeal sealer, meaning there was no alteration of collagen quality.

Comparing the collagen spectra collected from the treated dentin and the mineralized dentin (control), it was shown that 2.5% NaOCl solution for 1 minute during instrumentation did not cause any effect on organic, though increasing time to 5 minutes had an effect on the organic component (amide III) of dentin. The spectral region of amide I was believed to be the best possible region for investigating the protein structural changes[70]. Final irrigation with 2.5% NaOCl for 5 minutes gave a significant increase of 1667/1242 ratio when compared to 2.5% NaOCl for 1 minute during instrumentation. The increase in the 1667/1242 ratios ratio means that NaOCl causes a selective decrease of amide III.

The band at 1071 cm^{-1} (CO_3^{2-}) which could be explained by substitution of the phosphate ion with carbonate (B-type substitution). The $\nu_1\ CO_3^{2-}/\nu_1PO_4^{3-}$ represents the relative carbonate/phosphate content in mineral. The differences in carbonate content are important because the higher the carbonate level, the greater the susceptibility to mineral dissolution[448]. In our study, the intensity of carbonate/phosphate ratio reduced after exposure to 2.5% NaOCl solution for 1 minute during instrumentation in the first three levels, indicating a decrease of carbonate content of the mineral phase in these regions. Contrary to what may be commonly accepted, the treatment of dentin with NaOCl might not only remove the organic matrix but also some of the inorganic content, ultimately rendering dentin much weaker than normal[449]. The precise mechanism of this phenomenon is unknown, leaving room for speculation. For instance, it may be due to the high solubility of carbonate-apatite. Carbonate reportedly interferes with proper apatite crystallization and has a weakening effect on the bonds in the structure, as it increases the dissolution rate and solubility; it would therefore contribute to the susceptibility to caries[450] of dental apatite containing carbonate. Authors (Slutzky-Goldberg et al.[254], Ari et al.[255]and Oliveira et al.[451] conclude that sodium hypochlorite significantly reduces the microhardness of root canal dentin[254,451]. The significant alteration in dentin hardness after irrigation treatment indicates the potent direct effect of the chemical solutions on the component of dentin structure.

The fact that 17% EDTA solution as a final irrigant showed an inconsistent effect on mineral phosphate and carbonate may be due to factors inside the canal beyond our control:

- Thick and non-homogenous smear layer
- Short exposure time
- Length of canal, for which reason the irrigant did not reach the all root canal wall surfaces.
- The surface tension did not allow for a better contact with the dentin, hence a higher efficiency of the product. Defined as "the force between molecules that produces a tendency for the surface area of a liquid to decrease"[452], surface tension tends to limit the ability of a liquid to penetrate a capillary tube. Endodontic irrigants should have very low surface tension.
- The fact that with both instrumentation techniques, partially uninstrumented areas with remaining debris were found in all canal sections. This finding has been described by previous authors[453].

The application of RealSeal self-etching primer for 30 seconds also showed an inconsisten effect on mineral, which may be because of weak acidity of the self-etching type in comparison with total etch types.

As the mineral ratio of dentin may vary considerably within the same tooth[454], five points were taken in the 1st cervical, 2nd cervical, 1st middle, and 2nd middle root canal wall, and the means for each sample were calculated.

Biological materials such as dentin are far less homogenous, with dentin tubule density increasing from cervical to apical dentin[187], resulting in an inverse correlation between dentin microhardness and tubule density[455]. This may lead to deviations in the results owing to differences in adjacent regions of the dentin tissue[250].

This study showed there was irregular significant difference among root levels of treated groups and control group in mineral and organic ratios. It must be noted that studies have shown that dentin is not a homogenous tissue, and that its protein components change with age as the root matures.[456]

The study of Tesch et al.[457]also showed variations in the structural and mechanical properties of the mineral as a function of location in the mature human tooth by various methods, including FTIR microspectroscopy. This variation is in part due to the decrease of the dentinal tubule density and a respective decrease in the peritubular dentin density in areas farther away from the mineralization front[315]. As the mineral concentration and most likely the nature of the organic matrix in peritubular dentin differ from that in intertubular dentin[458], some spatial variation is anticipated.

In order to detect changes of the mineral matrix caused by treatment with chemical agents, the full width at half maximum (FWHM) of the strongest band (at 961 cm^{-1}) assigned to phosphate-ion was estimated for all groups. The peak width reflects the degree of mineral crystallinity[459]. In general, the narrower the spectral peak width is, the higher the degree of the mineral crystallinity. Our study showed no significant differences between the experimental groups and the control group.

It must be kept in mind that the idea of this study was to measure the effect of chemical agents on the surface of the root canal. In this study, the root was cut perpendicular to the long axis into four slices of 1mm thickness, polished with silicone carbide to obtain a 0.3mm thick flat dentin surface. Then, measurement was done at a depth of 4 µm in each slice of five points around the canal. However, Raman analysis required a polished surface that could not be performed on the internal canal surface, because the canal preparation was done as in clinic. This may be the reason why we did not find significant changes in the molecular structure.

It should be underlined that in the present study, the mineral contents in the same samples before treatment were not determined. Because the teeth were collected from patients at random, mineral contents would have varied from one tooth to the next, so it is unlikely that all groups of teeth were equivalent before treatment. Therefore, variation of results among groups may depend partly on the original content of dentin. For this reason, teeth similar to the experimental groups were used as control samples. Likewise, as no other methods (x-ray fluorescence spectrometry analysis, scanning electron microscopy, and energy dispersive spectrometry) were used to establish the mineral content of root dentin before treatment, the control group served as reference[245,415].

Part 4

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Part 5

Article

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Bond strength to root dentin and fluid filtration test of AH Plus/gutta-percha, EndoREZ and RealSeal systems

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ABSTRACT

Objectives: To investigate the bond strength and seal ability produced by AH Plus/gutta-percha, EndoREZ and RealSeal systems to root canal dentin. Material and Methods: Sixty extracted single-root human teeth, instrumented manually to size 40, were divided into three groups (n=20) according to the sealer used; G1: AH Plus, G2: EndoREZ, and G3: RealSeal sealers. After filling using the lateral condensation technique, each sealer group was randomly divided into two subgroups according to the tests applied (n=10 for μPush-out test and n=10 for fluid filtration test). A fluid filtration method was used for quantitative evaluation of apical leakage. Four 1-mm-thick slices (cervical and medium level) were obtained from each root sample and a μPush-out test was performed. Failure modes were examined under microscopy at 40x, and a one-way ANOVA was applied to analyze the permeability. Non-parametrical statistics for related (Friedman's and Wilcoxon's rank tests) or unrelated samples (Kruskal-Wallis' and Mann-Whitney's tests) allowed for comparisons of μPush-out strength values among materials at the different levels. Statistical significance was accepted for p values <.05. Results: There are no significant differences among fluid filtration of the three sealers. The sealer/core material does not significantly influence the μPush-out bond strength values (F=2.49; p=0.10), although statistically significant differences were detected with regard to root level (Chi^2=23.93; p<0.001). AH Plus and RealSeal obtained higher bond strength to intraradicular dentin in the medium root slices. Conclusions: There are no significant differences between the permeability and global μPush-out bond strength to root canal dentin achieved by AH Plus/gutta-percha, EndoREZ and RealSeal systems.

Key words: Resin cements. Root canal filling material. Compressive strength. Leakage.

INTRODUCTION

The fundamental goals of endodontic therapy are to achieve successful cleaning and shaping of root canals and a hermetic apical seal[25]. An ideal root canal sealer should adhere to both dentin and the core filling material. Methacrylate resin-based sealers, based on dentin adhesion technologies, have been developed in an attempt to seal the root canal system more effectively, improving bonding to radicular dentin[26], but their utilization requires removal of the smear layer and collagen exposure, because retention is largely achieved by micromechanical interlocking between collagen matrix and resin[29]. Ethylenediaminetetraacetic acid (EDTA) has been widely used for this purpose as the final irrigant before applying methacrylate resin-based sealers[5,13]; however, the results were poor regarding its bond strength adhesion to root dentin, whereas the non-bonding AH Plus sealer presented greater adhesion[7,10,17,18].

No correlation has been found between apical microleakage and sealer bond strengths to intraradicular dentin *in vitro*[1,2]. While we assume

that preventing effective microleakage is perhaps more important for endodontic application than bond strength[26], there is no universally accepted method for the evaluation of leakage[33]. Each has its limitations, being governed by electrical, filtration and/or diffusion laws[24]. Comparisons between different methods point to contradictory results, and, therefore, questionable clinical relevance[12,24].

Push-out bond strength testing has become a common method for determining the effectiveness of adhesion between endodontic materials and intraradicular dentin. Zicari, et al.[34] (2008) have correlated the push-out bond strength and sealing ability with the fluid filtration method of adhesive cements routinely used for fiber-post bonding, but to date there is no documented correlation using methacrylate resin-based sealers. We believe that this aspect is very important, because any filtration can hydrolytically degrade the adhesive interface. Hence, the purpose of this *in vitro* study was to quantitatively assess the bond strength to dentin root canal and the sealing properties of two methacrylate resin-based sealers and their corresponding core points (RealSeal and EndoREZ), then compare them with the gold standard conventional nonbonding AH Plus/gutta-percha. The μPush-out test and fluid filtration method were used to test the null hypothesis that there is no difference in the bond strength and sealing properties of RealSeal, EndoREZ systems and AH Plus/gutta-percha.

MATERIAL AND METHODS

We used 60 human anterior teeth, recently extracted for periodontal reasons from anonymous subjects under a protocol (nº 11/2011) approved by the Ethics Committee of the School of Dentistry, University of Granada. All teeth had single straight root canals and closed apices and none showed caries lesions or had received restorative or root canal treatment. After extraction, any calculus and soft tissue was removed and they were stored in 0.1% thymol diluted in distilled water at 4°C for use in this study within 3 months following extraction.

The crowns of the teeth were cut perpendicular to the long axis of the root at the cement-enamel junction with a diamond coated disk at slow speed with constant water-cooling. Root canals were instrumented using the step back technique to obtain a flared preparation. In all teeth, the working length was determined visually by subtracting 1mm from the length of a #10 K-file (Dentsply Maillefer, Ballaigues, Switzerland) at the apical foramen. Biomechanical preparation was performed manually with K-files until size #40. After each step in the flare preparation, the canal was irrigated with 2.5% sodium hypochlorite during 1 min. Then, 17% EDTA was used as a final irrigant for 1 min, after a rinse of distilled water (1 min) to remove any remnants of the irrigating solutions. The canals were dried using paper points.

The roots were randomly divided into three equal groups (n=20) according to the root canal sealer used; G1: AH Plus (Dentsply Maillefer, Ballaigues, Switzerland), G2: EndoREZ (Ultradent Products, Inc., South Jordan, Utah, USA) and G3: RealSeal (Sybron Endo, Glendora, CA, USA).

For the group filled with AH Plus, the sealer was mixed using AH Plus jet mixing system, and then introduced into the root canal orifices with the intraoral tip. A 40.02 taper master gutta-percha cone was placed into the canal and then the canal was filled up using the cold lateral condensation technique and accessory gutta-percha points size #25 and #20 dipped in a small amount of sealer.

EndoREZ was mixed in an ultra-mixer and dispensed using a narrow diameter syringe (Skini Syringe) (Ultradent Products Inc., South Jordan, Utah, USA) with a fine-tipped cannula Navi Tip (Ultradent Products, Inc., South Jordan, Utah, USA). Roots were filled totally with EndoREZ sealer. Then a 40.02 resin coated gutta-percha cone (EndoREZ point) (Ultradent Products, Inc., South Jordan, Utah, USA) was placed inside the canal, and additional EndoREZ points size #25 and #20 were placed into the canal using the cold lateral condensation technique.

For the group filled with RealSeal, RealSeal Primer (SybronEndo, Glendora, CA, USA) was introduced into the root canal with a paper point (Roeko, Langenau, Germany) soaked with the primer for the apical region. The primer was placed into the middle and coronal part using a micro brush (Microbrush International, Grafton, WI, USA). After 30 seconds, excess primer was removed with paper points. The sealer was dispensed directly from the tip of its auto mix dual-chamber syringe, according to the manufacturer's instructions. A 40.02 taper RealSeal master point was placed into the canal and then additional RealSeal accessory points size #25 and #20 were placed into the canal using the cold lateral condensation technique.

In all specimens, the excess of points was removed with a hot instrument and the remainder was condensed vertically. They were resected at the apex perpendicular to the long axis of the tooth until 10 mm of root length was obtained. Each root was transferred to a test tube (Eppendorf Ibérica, Madrid, Spain) and stored in a humidifier at 37°C for 24 hours to complete sealer setting. All preparation and obturation procedures were done by one operator.

Finally, each sealer group (n=20) was randomly divided into two subgroups according to tests applied (n=10 for μPush-out test and n=10 for fluid

filtration test).

Leakage evaluation

The external root surfaces of subgroups for the fluid filtration test were covered with a layer of nail varnish leaving the coronal and apical orifices open. The specimens were attached to a device to measure the permeability by fluid transport described by Pashley, et al.[22] (1988). The roots were inserted into a silicon tube (2 mm internal diameter), and attached with cyanoacrylate glue on the outer surface of the tube. A pressure tank of 250 ml of distilled water was placed 100 cm higher than the root to create a pressure of 100 cm H_2O. A 10 ml micropipette was inserted and attached with cyanoacrylate glue to measure movement of the air bubble introduced into the system with a micro syringe. Fluid pressure was applied from the coronal area in apical direction. The volume of fluid transport was measured by observing the movement of air bubbles within a 10 ml micropipette at 24 hours.

Previously, the system was sealed and checked for 10 min to ensure there was no leakage. The root was covered with water and then covered with a plastic dish to prevent evaporation. Each specimen was attached to the device, and then the system was bled by opening the joint between the water reservoir and the specimen. The air bubble was then aligned with the zero point of the 10 ml scale. Permeability was measured as the distance that the bubble had moved by the end of 24 hours. This measurement showed the amount of fluid which had permeated through the root filling in µl. A test was done with two additional roots, covering the apex with two layers of nail varnish, to ensure that there was no leakage or fluid movement anywhere within the device.

Micro Push-out test

Ten roots for each group were cut into four slices 1 mm thick, from cervical to apical direction (Cervical1 [C1], Cervical2 [C2], Middle1 [M1], Middle2 [M2]), using a 300 µm thick sintered diamond wafering blade perpendicular to the long axis of the root canal at low speed with constant water cooling. The thickness of each root slice was assessed by means of a digital caliper. Each slice was subjected to compressive loading via a universal testing machine (Instron 3345, Instron Ltd, High Wycombe, UK) equipped with a 0.5 mm-diameter cylindrical plunger. The plunger was positioned so that it only touched the root filling on loading, without touching the canal wall or introducing shear stress along the interfaces. Thus, the side with the smaller diameter of the root canal faced upwards and was aligned with the shaft that would exert pressure load on the filling (apical-coronally) until the filling was displaced. The machine was calibrated at a constant speed of 0.5 mm/min, and the maximum failure load was recorded in Newton (N). Failure was shown by the extrusion of the intact cone of root filling from the root slice and affirmed by the appearance of a sharp drop along the load/time curve recorded by the testing machine. The computer and software attached to the universal testing machine calculated the µPush-out bond strength value for each specimen, and converted them into MPa by dividing the applied load by the bonded area.

The slices were examined under a stereomicroscope at 40x magnification to determine modes of failure: adhesive at filling material with dentin interface, cohesive within filling material, or mixed failure.

Statistical analysis

The normality of the data distribution in all the study groups was verified by applying the Shapiro-Wilk test, and Levene's test was used to explore the homogeneity of variance among groups.

Fluid movement values at 24 hours were compared among the three different sealers and filling materials by means of one-way ANOVA and Tukey's tests.

To analyze µPush-out bond strength values, an ANOVA for repeated measures was first applied to explore the influence of the root level (within-subject factor) and sealers/filling material (between-subjects factor) on the µPush-out bond strength to root walls. As the sample groups did not fulfill homoscedasticity requirements, non-parametrical tests were used to compare µPush-out bond strength values among materials (Kruskal-Wallis' and Mann-Whitney's U test) and root levels (Friedman's tests for multiple comparisons and Wilcoxon's rank tests, for pairwise comparison).

The Chi^2 test was used to analyze the mode of failure distribution among groups. Differences were considered significant for $p<0.05$.

RESULTS

Permeability test

Mean fluid filtration values (SD) are recorded in Table 1. The AH Plus/gutta-percha group showed the highest value and the RealSeal group the lowest value, though there were no significant differences among the three sealer/core materials. This study makes manifest that all the materials used allowed fluid to flow along the root canal twenty four hours after filling.

Micro Push-out bond strength

Descriptive statistics of the µPush-out bond strength are given in Table 2.

ANOVA for repeated measures demonstrated that root level (F=27.148, p<0.001), and the interaction between the root level and sealer/core material (F=8.66, p=0.001), significantly affect the μPush-out bond strength to the walls of the root canal. The sealer/core material in itself does not significantly influence the μPush-out bond strength values (F=2.492; p=0.102).

For the whole sample, statistically significant differences were detected with respect to the root level (Friedman's test, Chi^2=23.93; p<0.001). Pairwise comparisons gave differences among all levels, with increased μPush-out values from cervical to apical except between M1 and M2 levels.

When each material was considered separately, AH Plus/gutta-percha also demonstrated significant differences according to root level, obtaining the lowest values in slice C1. For RealSeal system, the μPush-out bond strength increases apically, with C1≤C2≤M1=M2. EndoREZ system showed a similar behavior at all root levels (Chi^2=3.54; p=0.315).

μPush-out values of the three sealing systems were also compared in each level. Levels C1 and C2 showed no differences in terms of sealer/core materials, but there were differences between sealers at deeper levels: M1 and M2. RealSeal demonstrated greater bond strength than EndoREZ at M1 level (U=21.00; p=0.028). At M2 level, EndoREZ showed a significantly lesser bond strength than AH Plus (U=22.00; p=0.034) and RealSeal (U=13.00; p=0.005) (Figure 1).

Table 1- Mean values and standard deviation of permeability (μL/24 h)

Groups	Sealer	n	Mean permeability (sd)
I	AH Plus	10	10.34(6.45)
II	EndoREZ	10	8.49(5.92)
III	RealSeal	10	6.20(3.82)

Mode of failure

The predominating type of failure for all three sealer/core materials tested was adhesive failure, followed by mixed type failures. For the whole sample, this distribution was similar among the three sealers used (Chi^2=2.45, p=0.65), and the four root levels (Chi^2=4.66, p=0.58) (Table 3).

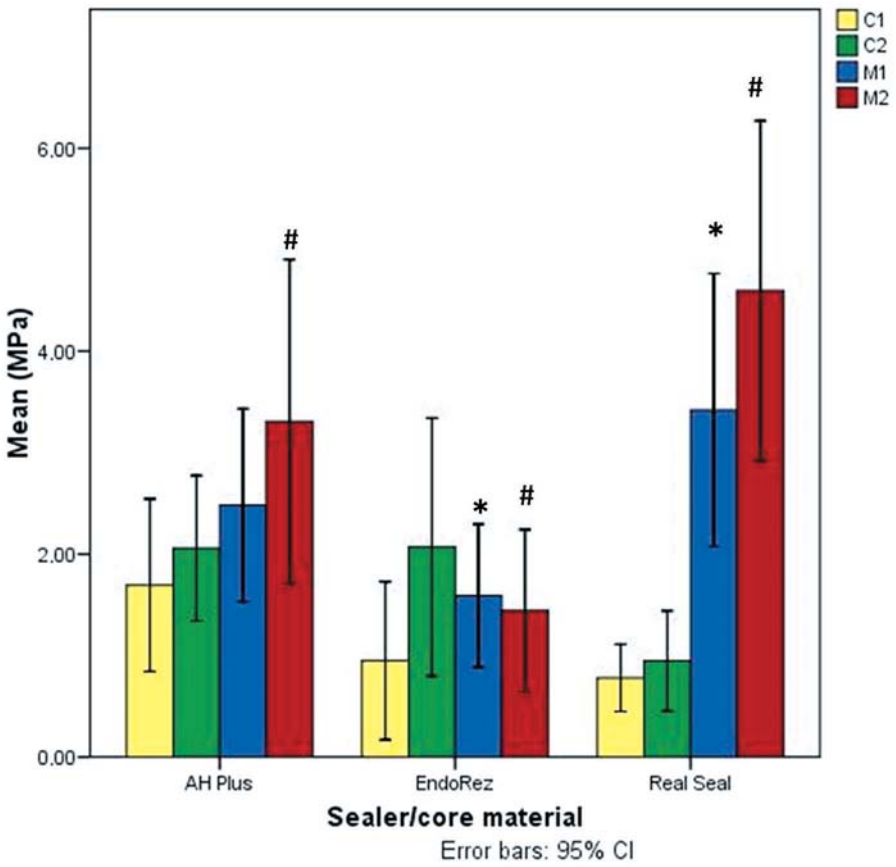
Table 2- Means and standard (MPa) deviations of μPush-out bond strength test. Different letters indicate significant differences in μPush-out strength values among root levels, for each material and for the whole sample

Groups	Cervical1	Cervical2	Medium1	Medium2	Mean(sd)
AH Plus	1.69(1.18)[a]	2.05(1.00)[a,b]	2.48(1.32)[a,b]	3.30(2.22)[b1]	2.38(1.14)
EndoREZ	0.95(1.08)	2.06(1.77)	1.59(0.98)	1.44(1.11)	1.51(0.94)
RealSeal	0.77(0.46)[a]	0.94(0.68)[a,b]	3.41(1.88)[b,c]	4.59(2.34)[c]	2.43(1.01)
Mean (sd)	1.14(1.01)[a]	1.69(1.31)[b]	2.49(1.58)[c]	3.11(2.31)[c]	

Different letters indicate significant differences in μPush-out strength values among root levels, for each material and for the whole sample

Table 3- Failure mode. n (%). Different letters indicate significant differences in μPush-out strength values among root levels, for each material and for the whole sample

Groups	Failure	Cervical1	Cervical2	Medium1	Medium2	Total
AH Plus	Adhesive	4 (13.30%)	5 (16.5%)	5 (16.7%)	7 (23.3%)	21 (52.5%)
	Mixed	5 (16.7%)	3 (10.0%)	3 (10.0%)	3 (10.0%)	14 (35%)
	Cohesive	1 (3.3%)	2 (6.7%)	2 (6.7%)	0 (0.0%)	5 (12.5%)
EndoRez	Adhesive	6 (20.0%)	4 (13.3%)	8 (26.7%)	9 (30.0%)	27 (67.5%)
	Mixed	3 (10.0%)	3 (10.0%)	2 (6.7%)	1 (3.3%)	9 (22.5%)
	Cohesive	1 (3.3%)	3 (10.0%)	0 (0.0%)	0 (0.0%)	4 (10.0%)
RealSeal	Adhesive	7 (23.3%)	7 (23.3%)	6 (20.0%)	6 (20.0%)	26 (65.0%)
	Mixed	3 (10.0%)	3 (10.0%)	3 (10.0%)	2 (6.7%)	11 (27.5%)
	Cohesive	0 (0.0%)	0 (0.0%)	1 (3.3%)	2 (6.7%)	3 (7.5%)
Total	Adhesive	17 (56.7%)	16 (53.3%)	19 (63.3%)	22 (73.3%)	
	Mixed	11 (36.7%)	9 (30.0%)	8 (26.7%)	6 (20.0%)	
	Cohesive	2 (6.7%)	5 (16,7%)	3 (10%)	2 (6.7%)	



*Significant differences among EndoREZ and Real Seal at M1 level
#Significant differences at M2 level: EndoREZ µPush-out was significantly lesser than the rest of sealer/core materials

Figure 1- Bar chart showing µPush-out bond strength values and comparisons among sealer/core materials in each root level (Mann-Whitney's U test)

DISCUSSION

In this study, permeability and µPush-out bond strength were compared among roots treated endodontically with AH Plus, EndoREZ and RealSeal sealers and root canals filled using lateral condensation technique at 24 hours. The results showed that the permeability and global µPush-out bond strength is not affected by the kind of sealer/core material.

Microleakage studies are most commonly used to measure the *ex vivo* sealing ability of a root canal filling, though various methods have been proposed or recommended for evaluation of leakage[33]. In this study, an endodontic fluid transport model was chosen to evaluate the sealing ability of three different root canal sealers, in view of its advantages: the samples are not destroyed, it provides quantitative measurement, and the results are precise, as small volumes can be recorded[15,19,24]. We used different samples for fluid filtration and µPush-out bond strength, to be performed at exactly 24 hours´ time, thus avoiding different degrees of conversion for the sealers. We did not light-cure the coronal portion of the RealSeal sealer as recommend by manufacturer's instruction, because fast-setting light-cured resin sealers produce restriction in the flow and consequent defects or weakening of bond strength[9]. Moreover, the slow chemical reaction of methacrylate-based self-etching sealers may reduce shrinkage stress by means of prolonged plastic flow during the setting time of the material[14]. We held that such behavior would allow for complete resin infiltration into the demineralized dentin.

Our results showed that there were no statistically significant differences in the values of fluid filtration among the three sealers used in the study. It is noteworthy, however, that none of the investigated sealers/core materials achieved a tight seal at the filling-cement-root interface, a point also brought out by Vasconcelos, et al.[32] (2011), allowing fluid to flow along the filled root canal twenty-four hours after filling, whereas the control showed no filtration. Currently, the clinical relevance of leakage tests *in vitro* has been questioned[12], because it is difficult to interpret results when, in our opinion, only zero filtration can be considered a good result. Moreover, there is no correlation among various methods to evaluate microleakage, and

the outcome of the tests depends on the evaluation method[12,24]. In the absence of correlation between the *ex vivo* sealing ability of root filled teeth and "clinical success"[20], we believe there is a threshold for microleakage values that would prove clinically relevant[21].

The adhesion tests of methacrylate bond sealer to dentin have not yet been standardized. The μPush-out test seems to be more reliable because it allows for adequate standardization of the specimens, the absence of premature failures and the variability of data distribution, while supplying a better estimation of the actual bonding effectiveness[11]. In all of the studies reviewed, biomechanical preparation is done with mechanical instrumentation because it is easier to perform and provides a more appropriate standardization of root canals. We used the step back technique and lateral condensation because it is still widely used throughout the world; moreover, there is no study of endodontic sealer adhesion and μPush-out, because the small final diameter of the canal makes the μPush-out test more difficult to perform.

In this study, we used three totally different kinds of sealers: a conventional nonbonding epoxy resin-based sealer, AH Plus; a first generation non-acidic diurethane dimethacrylate and triethyleneglycol dimethacrylate, EndoREZ; and a second generation sealer, RealSeal, based on adhesive technology with a self-etching primer and in association with a thermoplastic synthetic polyester polymer-based root canal filling material. It should be noted that the conventional nonbonding AH Plus/gutta-percha root filling was equal to RealSeal and EndoREZ. These discouraging results are in line with those of Ungor, et al.[31] (2006), who found that the Epiphany/Resilon combination was not superior to the AH Plus/gutta-percha combination. Still, most studies report that AH Plus sealer presents greater adhesion to dentin than RealSeal, regardless of root canal wall treatment[3,7,18]. Clinicians need to be aware that methacrylate resin-based sealers did not meet expectations regarding adhesion to root dentin, and, at this point in their development, there are no clear benefits in their use[26].

The μPush-out bond strength results were significantly influenced by dentin location. AH Plus and RealSeal obtained higher values in the deeper slices, while EndoREZ showed the lowest values, regardless of location. All sealers exhibited their low μPush-out bond strength values in two superficial slices. This can be explained by the presence of oxygen, inhibiting sealer setting and producing a layer with low polymerization.

Because we adopted a clinical approach, we were not able to ascertain whether an increase of the adhesive strength to the apex will remain in the most apical sections. Manual instrumentation until 40 ISO width produce very limited widening of the canal in the apical section, making it impossible to perform μPush-out tests without values having a frictional component with the canal walls. Previous authors have tested apices, but no realistic enlargements were made regarding canal preparation with drills for a post[2] or using a tapered diamond bur[18].

The values of the μPush-out test are very low, consistent with results of other studies using the same methodology, indicating that the bonding of two methacrylate resin-based sealers and root dentin is much weaker than with resin-dentin bonds, 25-30 MPa[30]. Furthermore, the adhesive failures between sealer/dentin interface clearly suggest an inadequate level of adhesion between sealer and dentin in terms of bond strength[2,17,27]. This may be due to the difficulties in testing materials with great plasticity, such as gutta-percha, RealSeal and EndoREZ points; in contrast, when the test was done with sealer and no core material, values were higher[1,2], suggesting failure may be traced to the sealer/material interface. In a root canal there is a highly unfavorable configuration factor (ratio of bonded to unbounded resin surfaces)[6] that contributes to maximizing the polymerization stress of resin-based materials along the root canal walls; this may even exceed the bond strength of dentin adhesives to dentin, resulting in gap formation along the surfaces[4,6,8]. Along these lines, Souza, et al.[28] (2012) report that there is a correspondence between the presence of gaps and microleakage.

Despite the material´s relatively low bond strength to root dentin, it may be effective in preventing microleakage[16]. The main problem is that this low μPush-out bond strength is accompanied by fluid filtration. The adhesion of resin sealer to gutta-percha or core seems to play an important role in microleakage prevention, since it does not have to be at the sealer-dentin interface. Microleakage can affect the bonding of sealers to dentin by plasticization (fluids are absorbed by resins)[8] and hydrolysis due to water entry in the interface. In addition, collagen degradation may occur due to host-derived matrix metalloproteinases (MMPs) in dentin that are slowly released over time when self-etching adhesives are used[23]. RealSeal may therefore adversely affect the longevity of bonded root canal fillings by accelerating degradation of the bond through the movement of fluid between the hybrid layer and unaffected dentin[8,23].

This study reveals that global μPush-out bond strength and permeability were not affected by different sealer/core materials: this leads us to partially accept the null hypothesis, because in the two deeper slices, RealSeal and AH Plus achieved higher μPush-out bond strength than EndoREZ. We cannot confirm any clear relationship between

permeability and μPush-out bond strength.

CONCLUSIONS

This study makes manifest that AH Plus/gutta-percha, EndoREZ and RealSeal systems allowed fluid to flow along the filled root canal in the twenty-four hours after filling. No significant differences were found among global μPush-out bond strengths of the three sealers/filling materials; but there does exist a regional influence, by which the μPush-out strength achieved with RealSeal and AH Plus to intraradicular dentin was found to be greater in the medium root slices.

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