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Investigating best practice for specimen preparation for biological testing of root canal sealers

Sweta Surana Bhandari [a,1], William Palin [a,2], Sarah A. Kuehne [b,3], Josette Camilleri [a,*,4]

[a] School of Dentistry, Institute of Clinical Sciences, College of Medical and Dental Sciences, University of Birmingham, Birmingham, United Kingdom
[b] Nottingham Trent University, School of Science and Technology, Nottingham, United Kingdom

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ABSTRACT

Introduction: Biological characterization of root canal sealers is important as it assesses the ability of the root canal sealer to exert antimicrobial properties thus avoiding treatment failures caused by microbial challenge and also assess the cytotoxic effect on the periapical tissues. Assessment of the biological testing of root canal sealers necessitates the sterilisation of the materials prior to evaluation. This study aims to analyse the influence of various sterilisation techniques conducted prior to biological testing on the microstructure and surface properties of endodontic sealers. Assessment of the initial microbial contamination on the material was also undertaken.
Methods: Four commercial sealers were investigated. The sealers were either prepared in a laminar flow cabinet or on a laboratory bench top under ambient conditions. Each group was further divided into 5 groups (n = 3) based on the sterilization technique:1) ethanol-10 mins, 2) ultraviolet-1 h, 3) ethanol-10 mins + ultraviolet-1 h, 4) autoclave, and 5) no sterilisation (control). Microbial levels in the materials were assessed by plate streaking technique. The materials were characterized by scanning electron microscopy and energy dispersive spectroscopy, and Fourier transform infrared spectroscopy, before and after sterilisation, to assess any changes in microstructure and chemical composition.
Results: All the materials did not exhibit contamination when prepared in laminar flow chamber in sterile conditions compared with sealers prepared on the bench top. Three of the commercial materials showed changes in microstructure while one (TotalFill) was not affected by the sterilisation. AH Plus and BioRoot RCS exhibited alterations in water and alcohol peaks in FT-IR while the single syringe sealers (TotalFill and BioRoot Flow) showed no changes.
Conclusions: Sterilisation methods cause physical and chemical alterations to sealers. Material preparation should be performed in a laminar flow cabinet and a test for sterility should be performed prior to any biological testing being undertaken. If the materials are not sterile, assessment of the effects of the sterilization methods is recommended.

1. Introduction

Mechanical debridement, irrigation, and intra-canal medication in root canal treatment are performed for reduction of bacterial load. However, even after these procedures, it may be difficult to eliminate microorganisms from dentinal tubules, lateral canals, and apical ramifications [1,2]. These spaces have the potential to be infected or reinfected resulting in the failure of endodontic treatment. Thus, antimicrobial and antibiofilm properties of sealers used during obturation play a critical role in preventing the growth of residual microorganisms [3]. To further undertake biological characterization, the effect of sealers on the periodontal ligament cells is assessed. A review of the studies that have assessed antimicrobial and also biological investigation of sealers has been undertaken [4].

Prior to antimicrobial and biological testing, materials should be disinfected or sterilised as microbial contamination will affect the test

* Corresponding author.
E-mail address: J.Camilleri@bham.ac.uk (J. Camilleri).
[1] https://orcid.org/0000–0002-5886–8035
[2] https://orcid.org/0000–0003-2225–7635
[3] https://orcid.org/0000–0001-6790–8433
[4] https://orcid.org/0000–0003-3556–6365

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being undertaken. This indication is noted in the standards for biological and microbial testing of dental materials [5–10]. All standards and guidance recommend methods of sterilization or decontamination that do not interfere with the material properties.

The disinfection or sterilisation procedures of resin restorative materials using steam sterilisation, ethylene oxide, and ethanol has been assessed and ethylene oxide was shown to modify the chemistry of glass ionomers and resin-based materials [11]. Ethanol disinfection also affected the physical properties of resins thus steam sterilization was shown to be a more suitable technique with no resultant degradation of the material [12]. The use of ultraviolet light for disinfection was shown not to affect the properties of composite resins [13]. Even some biomaterials used as scaffolds have been shown to be difficult to sterilize due to their particular chemistry [14]. Microstructural changes caused by the sterilisation will result in limited clinical translation of the biological testing as the material tested will be modified during the in vitro study and the changes recorded will not be related to the unmodified material used in clinical practice.

The aim of this study was to determine whether sterilising procedures used before biological testing could alter the physical, chemical and antimicrobial characteristics of root canal sealers.

2. Materials and methods

The following commercial root canal sealers were investigated:

AH Plus Jet (AH; Dentsply DeTrey GmbH, Konstanz, Germany) (batch no. 1904000728).

- BioRoot RCS (BR; Septodont, Saint-Maur-des-Fossés, France) (batch no. B27015)
- BioRoot Flow (BRF; Septodont, Saint-Maur-des- Fossés, France) (batch no. B24447BB)
- TotalFill BC Sealer (TF; FKG Dentaire, La Chaux-de-Fonds, Switzerland) (batch no. 21001SP)

The composition of various sealers used in this study is provided in the Table 1a.

2.1. Sample preparation

Two sets of samples were prepared. One set was prepared in a laminar flow cabinet (Guardian MSC T1200, Monmouth Scientific, Bridgwater, UK) under aseptic conditions with autoclaved (121°C, 15 psi for 15 mins) instruments (tweezer, mixing spatula) and materials (glass slab, rubber moulds). Sealer discs with dimensions of 10 mm diameter and 1 mm thickness were prepared in rubber moulds in triplicates for four sealer groups. A second set of sealer discs was prepared in nonsterile conditions on the laboratory bench to be used as control.

For BioRoot RCS (Septodont), 5 droplets of the liquid provided by the manufacturer were mixed with the powder and the material was manipulated according to the manufacturer's instructions with a sterile metal spatula upon a sterile glass slab. The two pastes of AH Plus (Dentsply) Jet were also mixed on the sterile glass slab using a sterile metal spatula to avoid using the plastic tips provided that could not be sterilised. TotalFill (FKG Dentaire) and BioRoot Flow (Septodont) are premixed pastes, and these sealers were therefore directly applied in the sterile rubber moulds using a sterile metal spatula since the provided plastic tips also could not be sterilised. These specimens were placed in closed petri dishes wrapped with cling film inside an incubator (Thermo Scientific, Langenselbold, Germany) at 37 °C and 100% relative humidity till their complete setting. Once set, each disc (n = 3), was sterilised using one of five sterilisation methods (Table 2). These methods included the use of 70% ethanol, ultraviolet (UV) light-254 nm wavelength (UV irradiation systems BIO-LINK, BLX-254 - BDH Laboratory and Scientific Equipment, Merck Life Science UK Ltd) and steam sterilization using an autoclave (Astell Scientific, Kent, United Kingdom). One set of discs was used as control with no sterilisation.

2.2. Sterility test

Each disc was placed in 5 ml of Brain Heart Infusion Broth (BHI) overnight in a universal tube in a shaking incubator (N-Biotek, INC, 110 rpm) at 37°C. After 24 h, 100 μL of this BHI suspension was spread with a spreader on a BHI agar plate. The streaked agar plates were placed in an incubator (Thermos Scientific, Langenselbold, Germany) overnight at 37°C. The agar plates were checked for bacterial growth after 24 h. Triplicates of each material were assessed.

2.3. Material characterization

2.3.1. Scanning electron microscopy and energy-dispersive X-ray spectroscopy

One disc from each of the 5 groups was mounted on aluminium stubs, held in place with carbon tape, and sputter coated 60 mm diameter by 0.1 mm thick/ 20 nm with gold (K550X Sputter Coater, Quorum Technologies Ltd., Kent, UK). The specimen was then viewed under the scanning electron microscope (EVO MA 10, Carl Zeiss Ltd., Cambridge, UK). The sealer disc was assessed in secondary electron mode to obtain elemental contrast at 500x magnification, operated at accelerating voltage of 20 kV with a working distance of around 8.5 mm. Energy dispersive spectroscopy of selected areas was performed to assess the elemental distribution.

2.3.2. Fourier transform infrared spectroscopy

The sealers discs from all 5 groups were ground using an agate mortar and pestle to a fine powder. 5 mg of each crushed sample of the sealers were mixed with 500 mg potassium bromide in an agate mortar and pestle, pressed into a pellet (13 mm diameter) using a pellet die (Specac, Orpington, UK) and a manual laboratory compactor (Clarke CSA10bb 10 Tonne Hydraulic Bench Press, UK). This KBr pellet was then analysed in the infrared spectrometer (Thermo Nicolet Nexus 4700 FT-IR Spectrometer) using transmitted infrared spectroscopy. All spectra were collected within the spectral range of 4000–400 cm^{-1} wavenumbers. Background spectra of KBr were also collected. Each spectrum was scanned three times to improve the signal-to-noise ratio.

Table 1
Sealer composition as provided by the manufacturers.

Sealer	Presentation	Composition
AH Plus	Auto mix syringe	(a) Epoxide paste: Diepoxide, Calcium tungstate, zirconium Oxide, aerosil, pigment. (b) Amine Paste:1-adamantane-amine, N,N′-dibenyl-5-oxa-nonandiamine-1-9,TCD-diamine, calcium tungstate, Aerosil, silicone oil
BioRoot RCS	Powder and liquid	(a) Powder: Tricalcium silicate, zirconium oxide, povidone. (b) Liquid: Aqueous solution of calcium chloride
BioRoot Flow	Preloaded syringe	Zirconium Oxide, tricalcium silicate, calcium carbonate, propylene glycol, povidone, aerosil (silica), acrylamide / sodium acryloyldimethyltaurate copolymer, isohexadecane and polysorbate
TotalFill	Preloaded syringe	Zirconium Oxide, tricalcium silicate, dicalcium silicate, calcium hydroxide

Table 2
Table showing different sterilisation methods used for sterilisation of sealer discs.

1. Discs prepared in microbiology hood	
Group A	70% ethanol 10 mins
Group B	UV Light 1 h (each side ½ hour)
Group C	70% ethanol 10 mins + UV light 1 h
Group D	Autoclave 121°C for 15 mins
Group E	Control (No sterilisation)
2. Discs prepared outside microbiology hood (in open lab)	

2.3.3. Macroscopic imaging of sealer discs

After sterilisation, photographs of the discs were imaged to assess for any visible changes.

3. Results

3.1. Sterility test

Microbial levels in all samples were below the bacterial detection limit when prepared in the laminar flow cabinet (Fig. 1B) compared to the ones prepared on the laboratory bench top that showed contamination (Fig. 1A).

3.2. Characterization of set materials before and after sterilisation

3.2.1. Scanning electron microscopy

The SEM of the material surface subjected to different sterilisation protocols are shown in Fig. 2. All sterilisation methods used in the current study appeared to have an impact on the material surface composition. The steam and ethanol caused deterioration to the material surface of AH Plus with areas devoid of radiopacifier present. Some surface deposits were seen when BioRoot RCS was steam sterilized. Ultraviolet irradiation seemed to have increased the porosity of the material. The ethanol plus ultraviolet irradiation was more destructive with areas showing deposits interspersed with areas that are darker and without deposits. In the case of BioRoot Flow, few changes were seen except some cracking and increased porosity in ethanol plus ultraviolet. For TotalFill, no microstructural changes were shown other than some surface deposits in the ethanol plus ultraviolet irradiation group.

3.2.2. Energy-dispersive X-ray spectroscopy

EDS analysis of the materials subjected to different sterilisation protocols is shown in Table 3 The mean Ca/Si ratio and Ca/Zr ratio increased in BioRoot RCS after autoclaving while it was elevated after ultraviolet sterilisation in BioRoot Flow as compared to the control group (no sterilisation). TotalFill depicted opposite effects, where the Ca/Si and Ca/Zr ratios showed a drastic decline in their values with all sterilisation techniques specifically with the ethanol + ultraviolet group.

3.2.3. Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy analysis in Fig. 3 depicted changes in water and alcohol peaks at 3421 cm^{-1} for AH Plus and BioRoot RCS, whereas TotalFill and BioRoot Flow seemed unaffected by different sterilisation techniques.

3.2.4. Macroscopic imaging of sealer discs

No significant changes were seen under visible inspection as shown in Fig. 4.

4. Discussion

Four sealer types were tested in the current research. The hypothesis that hydraulic cement sealers will be affected by sterilization processes undertaken prior to biological testing is justified by the fact that dental and biomaterials exhibited changes in microstructure and physical characteristics after they were sterilized [11–14]. Furthermore, hydraulic cements are susceptible to environmental changes due to their specific chemistry [15]. The methods used to assess the changes to the materials were similar to previous research [11].

Not all dental materials are supplied sterile [11]. The oral cavity is naturally contaminated; thus, the sterility is only mandatory for implantable materials. For biological and antimicrobial testing, specimen sterility is important as contamination will lead to cell death during cytocompatibility studies and interference with microbial assessment in microbiology assays. The standards [7–10] and guidance documents [5,6], suggest specimen sterilization that does not effect the material properties.

In the current study, the sealers tested proved to be contaminated when prepared on the laboratory bench top thus requiring sterilization. Preparation of materials in a laminar flow cabinet is always recommended, however it is not always possible due to the need of equipment

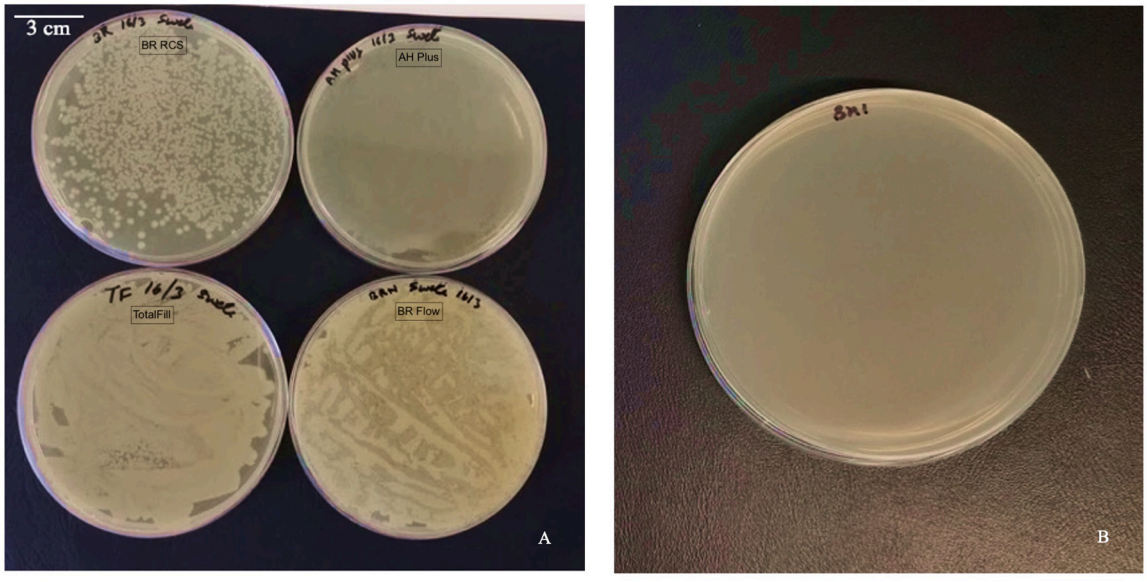

Fig. 1. Agar plates showing microbial growth when disks of AH Plus, BioRoot RCS, BioRoot Flow and TotalFill were prepared in the open lab.

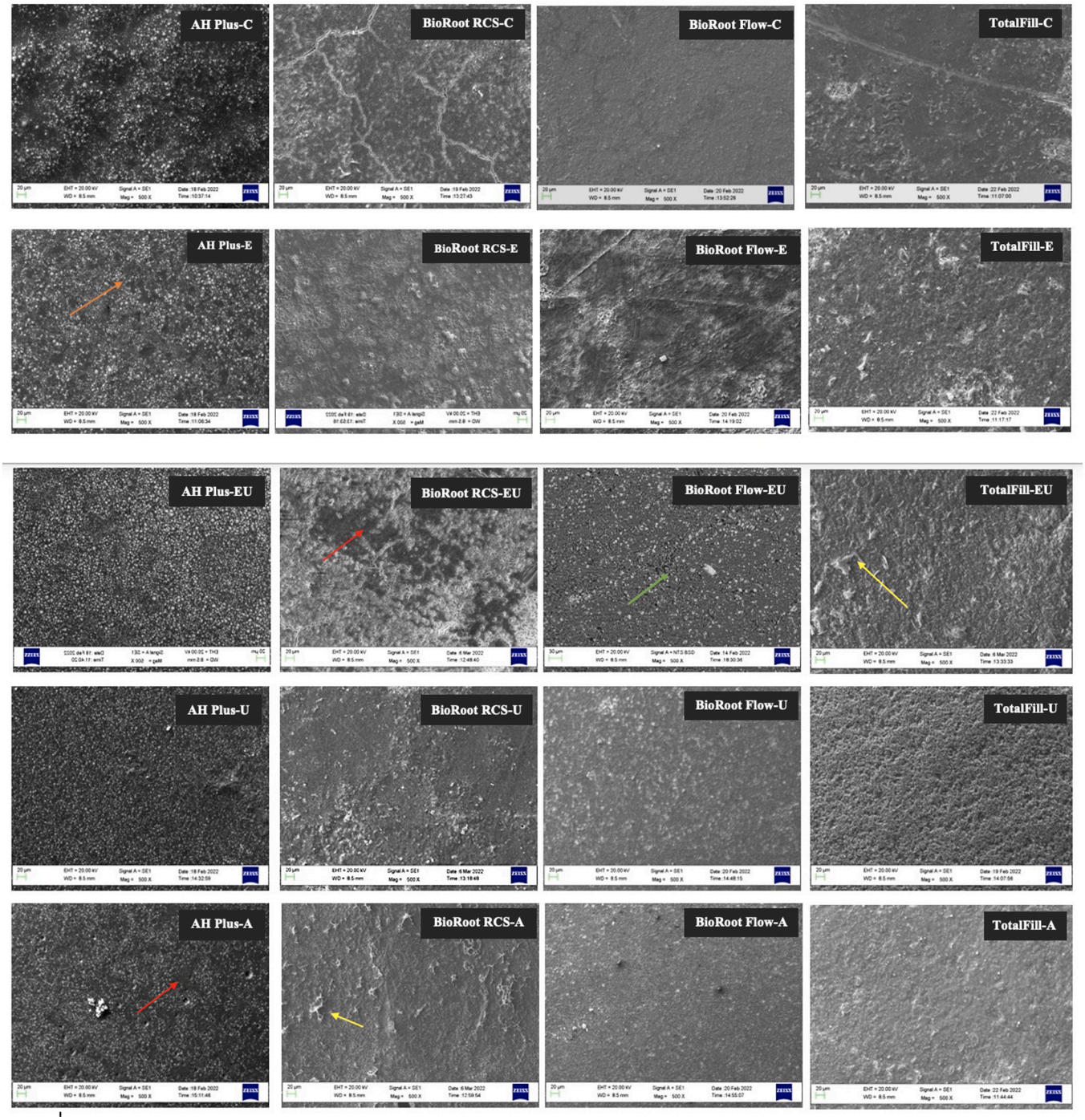

Fig. 2. Secondary electron Scanning electronic micrographs of AH Plus, BioRoot RCS, BioRoot Flow and TotalFill sealers after sterilisation with Ethanol & Ultraviolet (EU), Ultraviolet alone (U) and Autoclave(A) at 500k magnification. In ethanol plus ultraviolet group, red arrow indicates areas with deposits interspersed with areas that are darker and without deposits in BioRoot RCS, green arrow indicates cracking and increased porosity in BioRoot Flow, blue arrow indicates some surface deposits in TotalFill. In autoclave group red arrow indicates deterioration to the material surface of AH Plus with channels devoid of radiopacifier. Yellow arrow indicates some surface deposits in BioRoot RCS.

that cannot be placed in the cabinet. In this case the materials are prepared on the bench top and then decontaminated. Testing of sterility prior to commencement of testing is thus always recommended.

The assessment of sterility and the detection of the viable microorganisms could be performed by using macroscopic and microscopic examinations, measurement of the turbidity, pH measurement and direct streaking [16]. The most common analytical method for monitoring the growth of pure bacterial cultures is the turbidity measurement of liquid cultures, or optical density (OD) via spectrophotometer [17]. Sometimes the spectrophotometer may not be able to identify low viable count; in such instance, an agar plating method could provide a qualitative value (presence/absence) of the result [17]. In this study, agar plating method was used for confirmation of bacterial growth. The properties of the sterilized samples were compared with untreated controls. The absence of colonies on the plate could be explained by the inhibition of the microorganism at high pH.

In the absence of plating and specimen preparation and sterilization, the effects of steam, ethanol, ultraviolet, and ethanol plus ultraviolet

Table 3
Energy-dispersive X-ray spectroscopy (EDS) mean values with standard deviation for sterilisation of sealers. Ratio of calcium with silicon and ratio of calcium with zirconium of distinct groups of sterilisation methods on four sealers AH Plus, BioRoot RCS, BioRoot Flow and TotalFill; no sterilisation as control.

	AH Plus		BioRoot RCS		BioRoot Flow		Total Fill	
Mean	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr
Control	0.47 ± 0.8	0.10 ± 0.01	5.12 ± 0.1	1.63 ± 0.16	8.1 ± 0.41	3.11 ± 0.1	57.61 ± 5.23	3.18 ± 0.29
Ethanol	1.47 ± 0.49	0.12 ± 0.03	5.43 ± 0.43	1.40 ± 0.11	11.29 ± 0.61	5.52 ± 0.45	13.20 ± 1.67	1.50 ± 0.09
Ultraviolet	2.43 ± 0.15	0.15 ± 0.03	4.64 ± 0.14	1.29 ± 0.03	12.68 ± 1.56	6.01 ± 1.46	26.30 ± 6.30	1.53 ± 0.11
Ethanol+UV	1.57 ± 0.6	0.09 ± 0.003	4.27 ± 0.25	1.14 ± 0.1	7.72 ± 0.26	2.81 ± 0.10	9.1 ± 0.29	1.14 ± 0.05
Autoclave	2.24 ± 0.27	0.11 ± 0.34	8.45 ± 1.72	3.67 ± 5.94	7.96 ± 0.24	3.03 ± 0.13	14.44 ± 1.18	1.25 ± 0.19

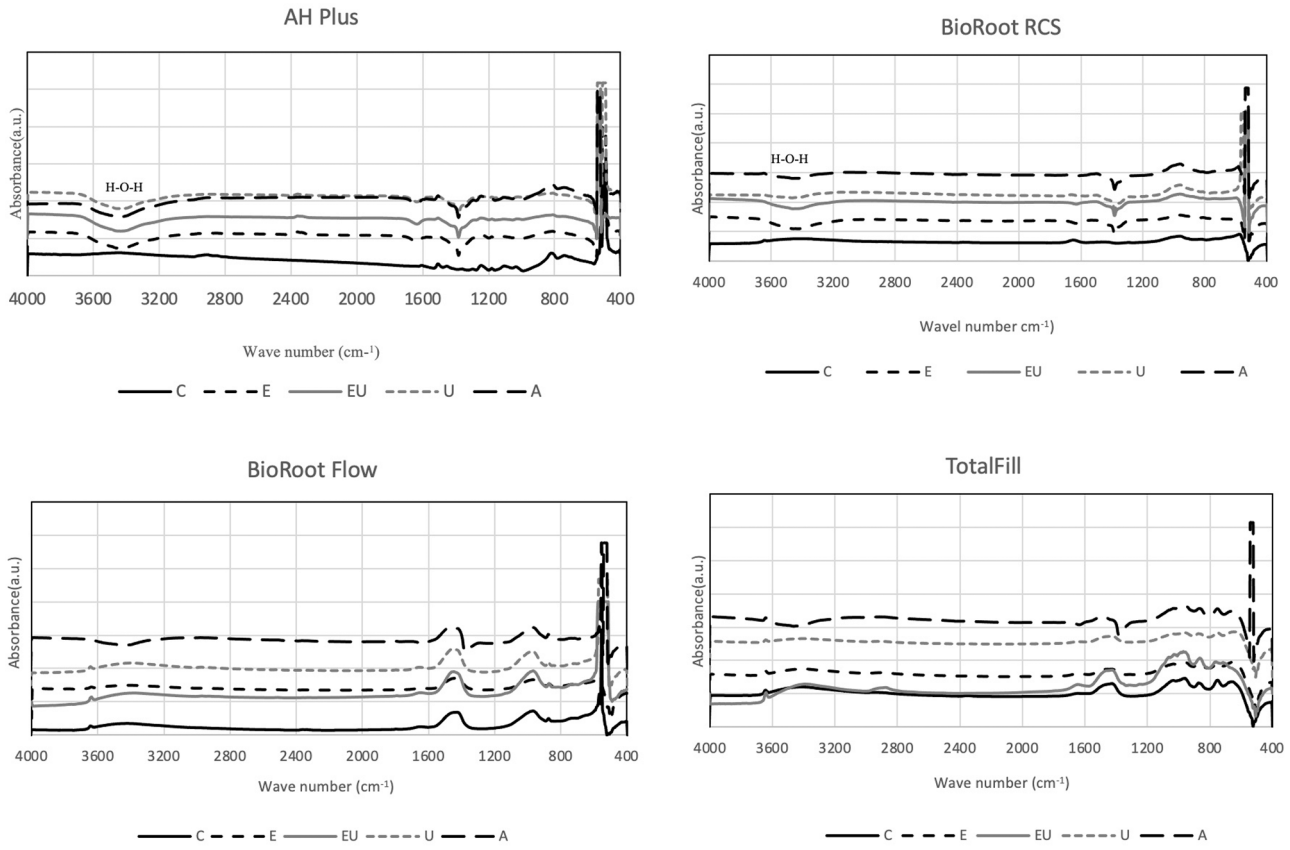

Fig. 3. Fourier transform infrared spectroscopy of AH Plus, BioRoot RCS, BioRoot Flow and TotalFill after different techniques of sterilisation: No sterilisation as control (C), Ethanol (E), Ethanol + Ultraviolet (EU), Ultraviolet (U) alone and Autoclave(A). Changes in water (H-O-H) and alcohol peaks could be seen at 3421 cm^{-1} in AH Plus and BioRoot RCS specimens.

sterilisation techniques on a range of hydraulic cement sealers were investigated. BioRoot RCS, BioRoot Flow and TotalFill are hydraulic tricalcium silicate sealers which have now been widely used as root canal sealers due to their notable antibacterial effect and high cytocompatibility [18,19]. The use of steam sterilization has been shown to be effective in some reports [11,13], while others reported that polymers exhibited hydrolysis, softening, melting, or material degradation due to elevated temperature, pressure, and aqueous environment [12]. Ethylene oxide has been recommended as a sterilising agent for products that are sensitive to heat, moisture, and radiation. While being ineffectual at sterilising composites, ethylene oxide also has mutagenic and carcinogenic effects and has shown chronic toxicity or reproductive effects in laboratory animals [20]. Ethanol works well as an antiseptic against most bacteria, fungi, and viruses but fails to kill bacterial spores [21]. Because of its fixative and dehydrating properties, it serves as a solvent in cleaning solutions and used as a disinfectant. This antimicrobial property of ethanol is due to protein coagulation and denaturation of cell membrane of microbes [22]. Gamma radiation is often used to sterilise medical devices, but this high energy radiation can lead to decreased mechanical properties via depolymerisation, oxidation, crosslinking, and chain scission. It has long been known that using ultraviolet light to kill microorganisms without the use of chemicals or heat is an efficient way for sterilisation of materials [23,24]. The ability of microorganisms to replicate is damaged and inactivation happens more quickly when exposed to UV irradiation at a specific wavelength range (200 - 280 nm).

Steam sterilisation was shown to cause deterioration to the material surface of AH Plus and BioRoot RCS due to its elevated temperature. This is in agreement to a previous study assessing steam sterilization with resins [13]. Ethanol has shown no effect on resin-based materials and biomaterials in previous studies [12,13], but affected the mechanical properties of glass ionomer components in one of the studies [11]. This is similar to the present work where ethanol did not affect the physical and chemical properties of BioRoot RCS Flow and TotalFill and this was due to fact that the materials do not have available OH^- in the early stages of the reaction unlike BioRoot RCS. Therefore, ethanol may be

Fig. 4. Macroscopic view of all four sealer discs (BRF-BioRoot Flow, TF-TotalFill, AH-AH Plus, BR-BioRoot RCS) before and after sterilisation with different techniques (C-control, E-ethanol, EV-ethanol +ultraviolet, U- ultraviolet, A-autoclave).

used for sterilisation of few of the sealers.

The UV light can affect light curable materials [11] and in the current study it was shown that it even affected the surface of the BioRoot RCS sealer and changed the mean Ca/Si and Ca/Zr ratio in BioRoot Flow and TotalFill. Therefore, UV sterilisation should be avoided when sterilizing sealers. The combination of ethanol plus ultraviolet irradiation has not been used in prior studies testing sterilisation of dental materials. The combination of sterilization techniques altered the surface microstructure, the Ca/Si and Ca/Zr ratio as well as the water and alcohol peaks in all the sealers under investigation.

5. Conclusions

Sterilisation methods cause physical and chemical alterations to sealers. Material preparation should be performed in a laminar flow cabinet and a test for sterility should be performed prior to any antimicrobial testing being undertaken. If the materials are not sterile, assessment of the effects of the sterilization methods is recommended.

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Declaration of Competing Interest

The authors declare no conflict of interest related to this study.

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