BJOS
Brazilian Journal of Oral Sciences

Original Research

Volume 23
2024
e243733

Assessment the bioactivity of zinc oxid eugenol sealer after the addition of different concentrations of nano hydroxyapatite-tyrosine amino acid - an *in vitro* study

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Editor: Dr. Altair A. Del Bel Cury

Received: June 14, 2023
Accepted: April 19, 2024

Aim: Zinc oxide eugenol sealer has been used till now in endodontic obturation. However, despite many improvements in its formula, it still does not have, the essential root canal sealer's properties which is the apatite forming ability. The aim of the present study is to assess the effect of the incorporation of nano Hydroxyapatite- tyrosine amino acid at different concentrations in the zinc oxide eugenol sealer formula in terms of bioactivity analysis. **Methods:** The nano hydroxy apatite-tyrosine amino acid was incorporated into the original zinc oxide eugenol (endosell) at different concentrations starting from (10 – 20)%. The chemical changes in zinc oxide eugenol before and after addition were characterized using FTIR and XRD. The setting time test was done according to ADA specification no. 57. The bioactivity analysis for the zinc oxide eugenol before and after the addition was evaluated according to ISO/FDIS 23317:2007(E) by using 28 days of storage in phosphate buffer saline, and then the hydroxyapatite precipitation and Ca/P ratio was evaluated using FESEM/EDX. **Results:** The FTIR and XRD confirmed the setting reaction occurrence among the (original ZOE, nHAP, and Tyr). The XRD and FESEM/EDX analyses confirmed the HAP precipitation on the ZOE sample surfaces after the addition of (nHAP-Tyr a.a) and this precipitation was increased with increased concentrations of additions. **Conclusion:** Incorporated (20) % of equal amounts of "nHA-Tyr a.a" can convert the ZOE to bioactive sealer as confirmed by XRD and FESEM/EDX. However, other characteristic analyses like Nuclear magnetic resonance, atomic force microscopy, and in vivo animal study were needed to further confirm the results.

Keywords: Root canal filling materials. Tyrosine. Nanocomposites. zinc oxide-eugenol cement.

Introduction

The main goal of root canal obturation is to provide an appropriate filling of anatomical irregularities with minimal voids. To achieve this objective, gutta-percha cones are associated with endodontic sealers[1,2].

The sealers are mainly grouped according to their main chemical components into (zinc oxide eugenol, glass ionomer, silicone, resin, and bioceramics-based root canal sealers[3,4].

The zinc oxide eugenol (ZOE) sealers are the early root canal sealers based on Grossman or Rickerts's formula that was commonly used amongst clinicians. It has good antimicrobial activity. However, ZOE has a weak mechanical characteristic, its sealing property was inferior in comparison to other sealers due to its relatively high solubility in addition to the recorded cytotoxic effect of these types of sealers and it lacks the ability of bone formation[5,6].

Biomaterials are materials that possess some novel properties that make them appropriate to come in immediate contact with the living tissue without eliciting any adverse immune rejection reactions. There are many types of biomaterials: Bioinert material (material that once placed in the human body has minimal interaction with its surrounding tissue, examples of these are metals, ceramics, and zirconia); Bioactive material (a material that elicits a specific biological response at the interface of the material, which results in the formation of a bond between the tissue and that material, these materials are either non-resorbable like bioactive glasses, hydroxyapatite, and ceramic) or Bioresorbable materials (material that upon placement within the human body starts to dissolve, slowly replaced by advancing tissue (bone) for example tricalcium phosphate[7].

A bioactive material is defined as a material that has an effect on or induces a response from living tissue, organisms, or cells to form nano-hydroxyapatite (nHAP). Bioactive endodontic sealers have been developed to improve the quality of root canal obturation. However, the bioactivity, and remineralization potential, may provide additional benefits for root canal obturation materials by strengthening root dentin and promoting the formation of hard tissue[8].

The Hydroxyapatite with a chemical formula $Ca_{10}(PO_4)_6(OH)_2$ is a calcium phosphate bioceramic regarded as the main inorganic biomineral constituent in enamel and dentine with a Calcium/Phosphate (Ca/P) ratio of 1.67 which is a major component of hard tissues such as bone and dentin and Ca/P ratio of 2.17 in Enamel[9,10].

Nanotechnology is also used to produce a large number of dental materials. The advantages of nanoparticles, that have attracted attention in endodontics, are their better penetration into the dental tubules, profound antibacterial properties, and decreased microleakage. Because of these valuable properties, the utilization of nanoparticles in producing endodontic sealers has become favorable for many researchers[11,12].

Calcified human tissues consist of the collagen matrix as the organic phase and the apatite as the inorganic phase; deposition of the latter is regulated by noncollagenous proteins (NCPs), which have highly anionic nature that enable them to cap-

ture calcium ions from the body solution and to provide bounded ions to collagen fibrils during the formation of bone and dentin. Thus, it has been assumed that binding of NCPs/ collagen fibrils could be a means of promoting intrafibrillar nucleation of hydroxyapatite[13,14].

Tyrosine (Tyr) is a type of amino acid, which serves as the building block of proteins. It is a non-essential amino acid and is also classified as an aromatic amino acid, derived from phenylalanine[15,16].

Tyrosine (Tyr) has an aromatic ring that consists of a six-link carbon-hydrogen ring with three conjugated double bonds. This ring's substituents regulate whether the side chain of amino acids employs polar or hydrophobic interactions. In the case of Tyr, a hydroxyl group on the phenyl ring participates in hydrogen bonds; that is, the side chain of Tyr is more polar and hydrophilic. In other words, Tyr is less hydrophobic and more reactive due to its aromatic ring with a hydroxyl group[17,18].

The purpose of the research was developed as a trying to induce the bioactivity of ZOE sealer by the addition of (nHAP – Tyr.) a.a. materials.

Materials and Methods

Addition of Materials

The addition of (nHAp) - tyr powders was done equally (50% nHA, 50% tyr). The addition starts from 10% before this concentration. There was apatite precipitation that could be seen on the surface of sealer specimens after 28 days of storage in PBS as confirmed by FESEM/EDX data. The selective concentrations used in the current study were (10, 12, 16, 20) %. However, the addition ended at 20 % since no further setting reaction for the original ZOE sealer had occurred.

The addition of the equal parts of each (nHA and tyr) powder was done by adding the same amount of powder that would be withdrawn from the original ZOE sealer, as the following:

Dispense one spoon of the original ZnO on the sensitive balance of 0.0001 g accuracy which weighs about (0.150) g, then remove from this mass what is equal to the percent of (nHAP and Tyr) powder that will be added.

The additions occur as the following:

- 0.150 / 100 × 2 = 0.003 g [amount of original ZOE powder that will be withdrawn].
- 0.003/ 2 = 0.0015 g [for each nHAP and Tyr] powder.
- Final spoon mass = 0.150g (after addition).

Chemical Setting Reaction

The chemical setting reactions for nHAP alone, Tyr. a.a alone, original ZOE, and ZOE after addition at (10 and 20) % were done using FTIR and XRD analyses.

Setting Time Test

Setting time Test for the ZOE after addition at (10-12-16-20) % was done according to ADA specification no. 57 using a Gilmore needle with a mass of (100 ± 0.5) g and a flat end with a diameter of (2.0 ± 0.1) mm positioned vertically at right angles against the sealer sample.

Five samples for each freshly mixed original ZOE sealer and the experimental sealer at different concentrations were prepared using a stainless-steel ring mold with a diameter of (10 mm and height of 2 mm). The time from the end of mixing until the set of the material was recorded as the setting time.

Bioactivity (Assessment of apatite forming ability)

The bioactivity of the sealer was assessed according to ISO 23317 (Implants for surgery - *In vitro* evaluation for apatite-forming ability of implant materials) using the FESEM/EDX analysis to confirm the apatite deposits on the sealers' surface after immersion in (PBS) for 28 days.

Phosphate-buffered saline is a buffer solution (pH ~ 7.4) commonly used in biological research. One of the characteristics of a bioactive material is its ability to form an apatite-like layer on its surface when it comes in contact with physiological fluids in vivo or with simulated body fluids such as (PBS) in vitro[16,17].

Three samples for each of the original ZOE (control positive group) and selective concentrations of experimental sealer (10%, 12%, 16%, and 20%) were prepared measuring (2.0 ± 0.1) mm in thickness and (20 ± 0.1) mm in diameter, and used for the assessment of apatite forming ability. Then all the prepared samples were placed in an incubator at 37 °C and 95% relative humidity for 1 week to ensure the complete setting of sealer. Then in such a way, the sealer samples were soaked in (10) ml PBS-containing container, which was refreshed every 3 days and then incubated at 37 °C and 95% relative humidity for 28 days.

After incubation, the apatite precipitate on the surface of the sealer specimens was confirmed using an X-ray diffractometer, with 2θ = (10˚-80˚) and radiation source with Cu Kα (λ = 1.5406 ˚A) at 30 kV and 30 mA. The surface morphology of the specimens was analyzed using FESEM adjusted at 20 kV accelerating voltage and 10 mA and the Ca/P ratio was assessed using EDX analysis.

Results

Chemical-Setting Reaction Using Fourier Transform Infra Reds (FTIR)

The FTIR was used to detect the functional groups changed before and after the addition to identify the chemical bonds and to produce the sample profile[19,20].

The FTIR Spectra of Tyrosine Amino Acid

The functional groups of Tyr a.a were illustrated in Figure 1 as:

At (3399.52) which represented the (OH) phenolic part of Tyr.

At (3198.52) is represented the amine part (NH_2).

At (1266.47-1213.22) are represented the phenolic, C–O "aromatic"

At (1014.91-876.95) are represented the carboxylic acid part (COOH)

The FTIR spectra of nano-hydroxyapatite

The two major functional groups of nHAP are illustrated in Figure 1:

At (1024.84) which represented the (P=O) functional part of nHAP.

At (599.99) which represented (C–Ca) functional part of nHAP.

The FTIR spectra of the original ZOE sealer

The functional groups of ZOE are illustrated in Figure 1:

At (636.89) is represented (C–H) bend of the eugenol group.

At (1637.87) is represented (C=O) of the zinc oxide.

At (2928.73-2866.63) are represented the (C–H) "aliphatic"

At (3076.74) is represented the (C–H) "aromatic".

The FTIR of experimental sealer (10% and 20 %) nHA+Tyr

The FTIR spectrum confirmed the occurrence of chemical reactions among the components of ZOE sealer and nHAP and Tyr. a.a. and this was confirmed as the functional groups of (amine and phenol) of Tyr. disappeared at (3500-3000). However, the following representative functional groups were identified, in Figure 1:

At (2926.65-2866.27) are represented the aliphatic (C–H), from Tyr.

At (1719.77) is represented the carbonyl group (C=O), from Tyr.

At (1359.95-1260.93) are represented the (P=O), from nHAP.

At (1030.33-982.68) are represented the aliphatic phosphates (P-O-C) from nHAP.

At (1226.81- 1180.87) are represented the aromatic phosphates (P-O-C) from nHAP.

The possibility of the formation of the (COO–Ca) complex that results from the acid-base reaction between the calcium oxide (base) from nHAP and Tyr a.a. (acid) in the presence of water, in which the carboxylic groups of Tyr act as a proton donator (H^+) that replaced by the Ca^{++} ions to form (COO–Ca) complex with its characteristic peaks at (1610-1550/1420-1300).

The FTIR spectral pattern of 10% and 20% experimental sealer confirmed the disappearance of some functional groups that are present in the original ZOE sealer, these are (1655.54, 1578.89, 1560.60, 1450.10, 1430.43, 1388.31, 993.87, 795.97, 722.36) and (2928.73, 2866.63, 1655.54, 1578.89, 1450.10, 1430.43, 1388.31, 722.36, 993.87) respectively

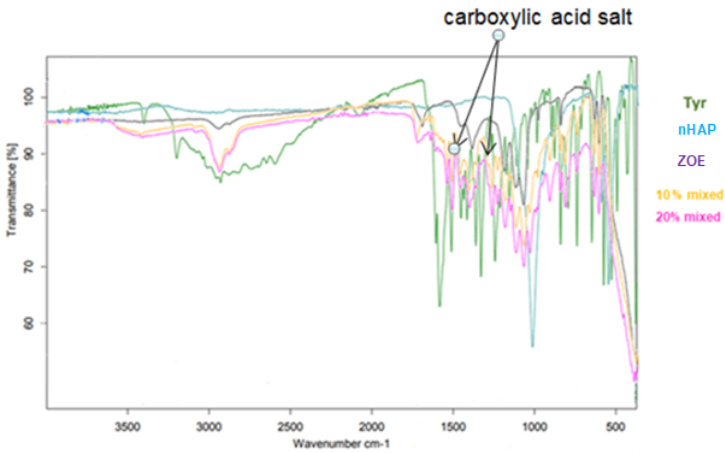

Figure 1. Show FTIR spectra for tyrosine amino acid (Tyr), nano hydroxyapatite (nHAP), original ZOE, 10% ZOE, and 20% ZOE.

The X-Ray Diffraction Analysis (XRD)

The XRD was done for nHAP alone, Tyr. a.a alone, original ZOE, and ZOE after addition at (10 and 20) % as shown in Figure 2.

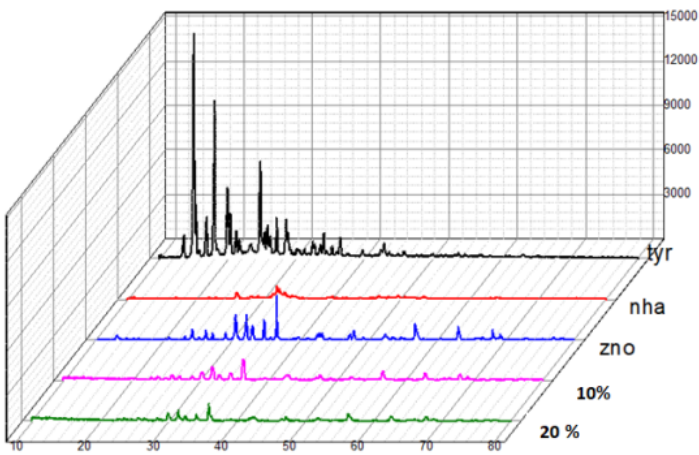

Figure 2. Show the XRD spectra pattern for tyrosine amino acid (Tyr), nano hydroxyapatite (nHAP), original ZOE, 10% ZOE, and 20% ZOE.

The XRD of Tyrosine amino acid

Tyrosine amino acid has significant characteristics peaks at the following 2 theta values (15.18°, 18.00°, 20.30°, 25.70°), these peaks disappeared or decreased in XRD spectra results in data of the experimental ZOE sealer (after the addition of 10 and 20 % of nHA + Tyr), Figure 2.

The characteristic peaks of Tyr were in good agreement with the ICDD data (00-031-1970) that represented the high purity of Tyr that was used in this study[21,22].

The XRD of nHA

The XRD pattern represents the significant characteristics peaks at the following 2 theta values (25.93°, 33.10°, 34.12°, 34.63°) that were in good agreement with the ICDD data (01-082-1429) indicating that the nHAP was in hexagonal phase[20]. However, these specific peaks were decreased after mixing (10 and 20)% of (Tyr and nHAP), indicating the occurrence of a chemical reaction, Figure 2.

The XRD of the original ZOE

The original ZOE has significant characteristics peaks at the following 2 theta values (24.10°, 26.04°, 31.96°, 32.96°, 36.43°, 35.49°, 36.50°,43.15°, 47.13°, 56.73°, 62.98°, 68.05°) according to JCPDS card No. 79-22053 3a, and ICDD data (01-079-0205)[23,24].

These peaks either increased or decreased after mixing (10 and 20) % of (Tyr and nHAP), indicating the occurrence of setting chemical reactions among the compositions of the original ZOE root canal sealer with the nHAP and Tyr, Figure 2.

The Setting Time

Table 1 demonstrates the descriptive statistics of setting a time that includes the mean and standard deviation (SD) of the control (original ZOE) and the experimental groups, at different concentrations (10, 12, 16, and 20) %. However, with more than 20 % (Tyr + nHAP) addition; the original ZOE no further underwent setting.

Table 1. Descriptive statistics (mean, standard deviation) for the experimental groups of four different concentrations and original ZOE.

Sealer types/ Concentration	N	Control (ZOE) M ± SD	Experimental (ZOE) M ± SD
Control	10	0.310±0.000	
10%	10		0.5902±0.0016
12%	10		1.0200±0.01581
16%	10		1.0340±0.01140
20%	10		1.0700±0.01000
Total			0.8048±0.30972

N = Number of specimens, M = Mean, SD = Standard deviation
ZOE = zinc oxide eugenol.

One-way analysis of variance "ANOVA test", (Table 2) was performed to compare the four experimental concentrations. The results revealed that there was a significant difference at ($P \leq 0.01$) between four concentrations of setting time.

Table 2. Show One Way ANOVA tests among The Experimental Groups.

ANOVA					
	Sum of Squares	**df**	**Mean Square**	**Fc**	**(Sig.)**
Between Groups	2.300	4	0.575	5.990	0.000
Within Groups	0.002	20	.000		
Total	2.302	24			

Post Hoc Tests (Duncan's Multiple Range tests) were performed to evaluate the system that gives the best value of experimental concentrations (Table 3)

Table 3. Post Hoc Duncan 's Multiple Range tests of experimental groups at P<0.01.

Duncan's Group	**N**	**Subset for alpha = .05**				
		Control	**10%**	**12%**	**16%**	**20%**
Control	10	0.3100				
10%	10		0.5902			
12%	10			1.0200	1.0340	1.0700
16%	10					
20%	10					

Bioactivity Analysis

After immersion of the experimental sealer in (PBS) for about 28 days, HAP precipitation on the surface of the experimental groups was shown visibly. However, the HAP precipitation was confirmed using FESEM/EDX analyses.

The EDX spectra for the control (original) and experimental ZOE sealer groups were represented in Figure 3.A. The spectra do not reveal the presence of phosphorus and calcium elements in the control ZOE group. While the EDX for experimental groups at (10, 12, 16, and 20) % revealed the presence of phosphorus and calcium elements, Figure 3 (B-E).

The Ca/P ratio of the natural HAP is (1.67), the ratio for the experimental groups was calculated from EDX analysis and it indicates the formation of HAP. It was (1.60), (1.68), (1.77), and (1.83) for (10, 12, 16, 20) % respectively.

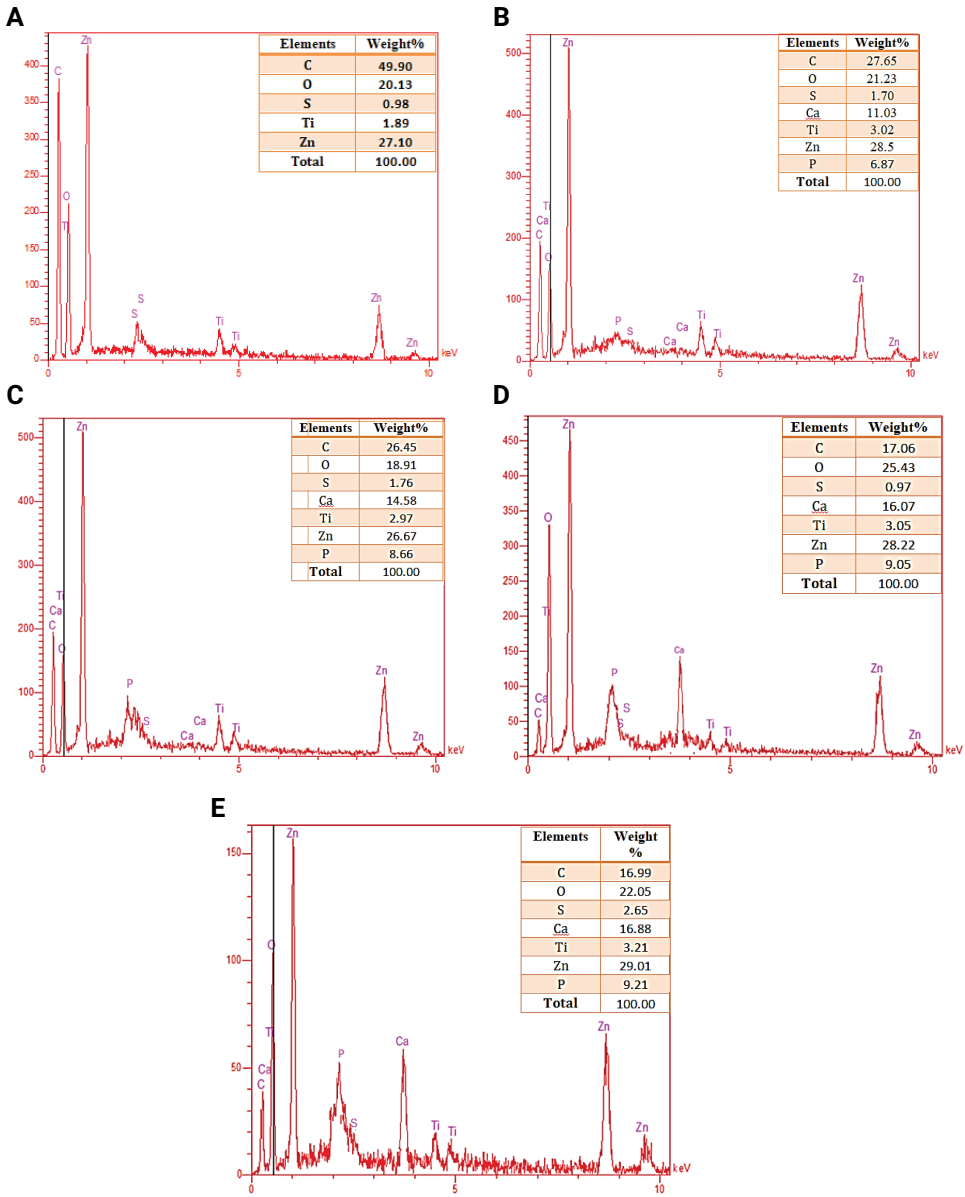

Figure 3. EDX data of the control group after immersion in PBS for 28 days (A). Compositional changes in the experimental group after immersion in PBS for 28 days showed the Ca/P ratio at 10% (B), at 12% (C), at 16% (D), and at 20% concentrations respectively (E).

The FESEM images showed that there was no HAP precipitate on the outer surface of the sample in the control group, Figure 4 (A).

The FESEM images of the experimental sealer groups revealed an irregular and amorphous HAP precipitate on the outer surfaces figure 4 (B-E) in comparison with the control group (original ZOE) which showed no HAP precipitation. However, the precipitation has appeared to be increased with increasing the percentage of addition with the higher at 20 %.

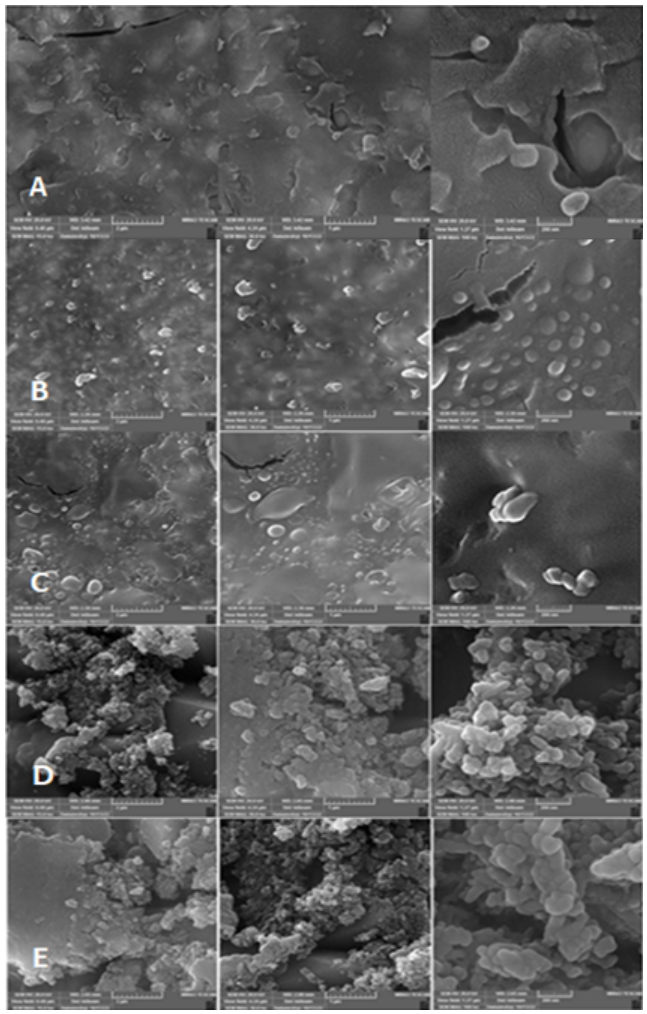

Figure 4. Show FESEM image of experimental ZOE group after 28 days of immersion in PBS, at different degree of magnification (2 µm, 1 µm and 200 nm) that registered HAP precipitate on the outer surface of the sample. At 200nm degree of magnification, the deposits represented the HAP precipitation which is usually taken plate-like morphology due to the presence of the amino acid, (A: Original ZOE, B:at 10 %, C: At 12%, D: At 16% and E:At 20%)

Discussion

Endodontic root canal sealer is used to achieve a fluid-tight or hermetic seal throughout the canal including the canal irregularities, the apical foramen, and minor discrepancies between the dentinal wall of the root and the core filling material. That is why, sealers help to prevent leakage, decrease the possibility of bacterial entrance from the canal to invade the periapical tissues, and resolve the lesion[25].

Biomimetic dentistry is the newest branch of minimally invasive dentistry that aids in mimicking the natural tooth structure utilizing bioactive materials that resemble the materials that teeth are composed of one of the most important materials that

biomimetic dentistry concerns is HAP. HAP is a type of mineral that is naturally a constituent of teeth and bones and can be used to repair and rebuild tooth structure[26].

The HAP is used in a variety of fields with many advantages including biocompatibility, bioactivity, and the absence of cytotoxicity effect, enhances the osteoconductive properties since it permits the attachment, proliferation, and migration of bone cells, thus actively promoting the growth and regeneration of new bone[26,27].

The presence of amino acid in the sealer formula has been supposed to form a chemical bond with calcium and phosphate ions of dentin hydroxyapatite through both carboxyl and amino functional groups in addition to the chemical bond that might occur between amino functional groups of amino acid and the collagen fibers of dentin. Therefore, this will aid in the improvement of the bond between the sealer and tooth dentin[26,28].

For the interaction of Tyr a.a. with uncharged polar side groups, It has been suggested that their adsorption on the surface of HAP according to the number of the adsorption sites. HAP surface has centers of positively and negatively charged ions and therefore the solute molecules may be accommodated on the surface of the crystals. It appears that these molecules adsorb onto specific sites of the surface where adsorption could take place through hydrogen bonding. Such interaction is expected in the case of Tyr and nHAP which all have a free hydroxyl side group. It has been proposed that the carboxyl and amino groups of the amino acid have a minor contribution to the adsorption process on the surface of HAP[12].

The finding of current study outcomes showed that the incorporation of (20 % nHAP-Tyr a.a.) to the original ZOE sealer produced high clinical results *i:e* better apatite formation on the surface of the original material among all other experimental concentrations, these results because of the use of bioactive remineralization can offer several advantages over traditional treatments in conservative dentistry such as: 1)Preservation of healthy tooth structure: the utilization bioactive materials can promote the remineralization of the defective tooth structure. 2) Decrease the need for invasive procedures such as drilling, filling, or crowns.3) Decrease sensitivity: through obstruction of the defect present in tooth tissue.4) Improved esthetics: Biomaterials remineralization can enhance the quality and appearance of the mineralized tooth tissues[18,22,29,30].

Also, the methodology of the current study included that the incorporation of synthetic nano Hydroxyapatite particles (nHAp) - tyrosine amino acid at different concentrations to the original composition of zinc oxide eugenol-containing sealer will have a positive effect on the original ZOE sealer. However, the ZOE sealer is used now in many private dental clinics although many sealers developed. However, it is not considered as a bioactive sealer. Therefore, the idea of the research was developed as an trying to induce the bioactivity and improve the biocompatibility of ZOE sealer by the addition of nano-hydroxyapatite - tyrosine amino acid materials.

In conclusion and according to the outcomes of FTIR, XRD, FESEM/EDX analyses in the current study, the addition of 20% nHAP-Tyr a.a equally (50% nHA, 50% tyr) to the original ZOE could be induced a reaction that produced the bioactive type of ZOE.

However, other characteristic analyses like NMR and AFM analyses were needed to further confirm the results.

Acknowledgments

The authors acknowledge the University of Mosul/College of Dentistry. Special thanks to the University of Mosul/College of Chemistry.

Data availability statement

The findings of the manuscript were discussed in-depth by all authors, who also contributed to its revision and gave their approval to the final edition.

Author Contribution

Rasha Mozahem Al-Shamaa: Study conception and design, data collection, analysis and interpretation of results and author; draft manuscript preparation. **Raghad Adnan Rashid:** Study conception and design, analysis and interpretation of results and author; draft manuscript preparation. Both authors actively participated in the discussion of the manuscript's findings, and the **Ethical policy and institutional review board statement**.

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