**Original Article**

# An In vitro Evaluation of Antimicrobial Efficacy of new Nano-zinc Oxide Eugenol (NZOE)

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***Received 12 June 2018 and Accepted 24 August 2018***

## Abstract

**Introduction:** This interventional in-vitro study aimed to evaluate the antimicrobial activity of a new Nano Zinc OxideEugenol (NZOE) sealer in comparison with AH26 and Pulpdent common root canal sealers against endodontic pathogens. **Methods:** The antimicrobial efficacy of three sealers(NZOE, AH26 and Pulpdent) against Enterococcus faecalis (ATCC 29212), Escherichia coli (ATCC 25922), Streptococcus mutans (ATCC700610), Candida albicans (ATCC 90028), and Staphylococcus aureus (ATCC 25923) were evaluated by Direct Contact Test (DCT) at five different time intervals 0, 2, 14, 48 hours and 7 days. The results were statistically analyzed using SPSS software and Kruskal–Wallis at 5% significance level. **Results:** All three tested sealers had antimicrobial activity against microorganisms involved in this study. Fresh NZOE sealer eliminated all microorganisms tested, except the strain of E. faecalis colony which was reached to zero after 2 hours. However, AH26 and Pulpdent failed to completely kill all of the E. faecalis colony during the entire observation period. Also, NZOE showed a significant antimicrobial action ($P<0.05$) in comparison with other two sealers by effectively eliminating the Candida albicans colonies at zero time and 7 days. **Conclusion:** Highest antimicrobial effect of NZOE sealer was shown followed by Pulpdent sealer and AH 26 against Candida albicans and Enterococcus faecalis.

**Key words:** AH26, Antimicrobial, Direct-contact test, Nano Zinc Oxide, Pulpdent.

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Zarei M, Javidi M, Joybari M, Tajzadeh P, Arefnejad M, Gharechahi M. An In vitro Evaluation of Antimicrobial Efficacy of new Nano-zinc Oxide Eugenol (NZOE). J Dent Mater Tech 2018; 7(4): 167-73.

## Introduction

Microorganisms and their byproducts are the main etiological factors of pulpits and apical periodontitis. The main goal in endodontic therapy is the elimination of microorganisms from the root canal system by instrumentation, irrigation, and intra-canal medication. Unfortunately, complete elimination of microorganisms and their by-products from the root canal system is impossible (1). So, antimicrobial activity of root canal filling materials is considered to be a beneficial property for reduction of the remaining microorganisms' number (2).

There are the numbers of root canal sealers currently available that possess quite different formula. Zinc-oxide-Eugenol based sealers are the most commonly used sealers in root canal treatment. Studies have shown that, nanotechnology is extensively used in manufacturing dental materials (3). Some advantages such as penetration into dentinal tubules, antibacterial activity and low micro-leakage property of nano-particles have drawn attention in endodontics (4,5).

A new endodontic sealer with Nano-sized ZOE powder particles (NZOE) has been developed in the Dental Material Research Center, Mashhad University of Medical Sciences, Mashhad, Iran. This sealer is similar to various ZOE-based sealers, but with different sizes of ZOE nanoparticles (3). This sealer exhibit cyto-compatibility (6, 7), similar tooth discoloration compared to resin sealers (3), and proper sealing ability (8).

The aim of the current *in vitro* study was to evaluate the antimicrobial activity of NZOE, compared with two common endodontic sealers AH26 (resin-based) and Pulpdent (ZOE-based) against *Enterococcus faecalis*, *Streptococcus mutans*, *Staphylococcus aureus*, *Escherichia coli* and Candida *albicans* at five different time intervals.

## Materials and Methods

Endodontic sealers used in this study were $AH_{26}$ (Dentsply Sirona, Germany), Pulpdent (Watertown, USA) and NZOE (Dental Material Research Center, Mashhad Iran). All the sealers were prepared in accordance with the manufacturer's recommendations. *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Streptococcus mutans* (ATCC700610), *Candida albicans* (ATCC 90028), and *Staphylococcus aureus* (ATCC 25923) strains were used. All the reference strains were obtained from the Department of Microbiology, Pasteur Institute of Iran, Tehran.

The antimicrobial activity of the endodontic sealers was performed by means of the modified Direct-Contact Test (9) against above reference strains, freshly mixed at five different time intervals 0, 2, 14, 48 hours and 7 days. The tested sealers were prepared according to the manufacturer's instructions. . Each sealer was placed on the side wall of the Micro-tubes (Eppendorf 2.0 mL) by using a cavity liner applicator. The sealers immediately after preparation was considered as $T_0$; the others were allowed to set for 2 hours ($T_2$), 14 hours ($T_{14}$), 48 hours ($T_{48}$) and 7 days ($D_7$). Afterwards, the Micro-tubes were incubated at 37°C in humidity for five different time intervals 0, 2, 14, 48 hours up to 7 days.

The microorganisms inoculated in Brain Heart Infusion (Condo, Spain) and incubated for 24 h at 37 °C. After incubation, the broth cultures were adjusted to 0.5 McFarland ($1.5 \times 10^8$ CFU; colony-forming units) by using McFarland standard. Ten µL of bacterial suspension was placed on the surface of each fresh aged and sealers in Micro-tubes and exposed one hour in an incubator shaker at 37ºC (available humidity). All interval time Micro-tubes was done as well.

Consequently, 240 µL of BHI broth was added to each interval time Micro-tubes, mixing well for one minute and transfer 10 µL of each diluted samples are then spread onto BHI agar.

The BHI agar plate incubated at 37°C for 24 hours. The microbial culture was prepared for each interval time Micro-tubes on BHI agar. After incubation, the CFU/mL was calculated from bacteria dilution.

All experiments were performed in triplicate. Microbial suspension without cements used as positive control and sealer in addition to BHI without microbial suspension, considered as negative control. The normality was checked by shapiro-wilk test and the data distribution was not normal. Kruskal–Wallis comparison tests were used for the statistical analysis. The data were processed using SPSS software and the level of significance was set at 5% ($P < 0.05$).

## Result

The results of the antimicrobial effects of three endodontic sealers are presented in Tables (1-5). Fresh sealers and sealers set for 2, 14, 48 hours, and 7 days showed differences in their antimicrobial effects. In fresh

sealers, significant difference in antimicrobial activity had present only against *E.coli* and *Candida albicans* (Table3, 4). Fresh NZOE eradicated all bacteria except E. *faecalis.* But after two hours of contact, NZOE eliminated all *E. faecalis* strains.

NZOE, AH-26, and Pulpdent killed all *S. mutans* in 0, 2, and 14 hrs. Of contact, and in 48 hrs. no microbial colony was performed in Pulpdent and NZOE.

After 7 days, the significant difference were only present against S. *aureus* and *candida*. Against *s. aureus*, AH26 showed the most activity and Pulpdent was the worst. Against candida, NZOE presented the best antimicrobial activity and Pulpdent was the worse.

However, after 7 days most sealers had lost much of their antimicrobial effect.

**Table 1.** Mean and standard deviation of colony count for sealers in different intervals in E.faecalis

| Time | Materials | Min max | Mean ± SD | Min | Max | P-Value |
|---|---|---|---|---|---|---|
| T0 | Pulpdent | 10000 | 40000±51962 | 10000 | 100000 | P=0.304 |
| | NanoZno | 10000 | 40000±51962 | 10000 | 100000 | |
| | AH26 | 10000 | 7000±5196 | 1000 | 10000 | |
| 2h | Pulpdent | 100000 | 70000±51962 | 10000 | 100000 | P=0.304 |
| | NanoZno | 0 | 0 | 0 | 0 | |
| | AH26 | 0 | 17±29 | 0 | 50 | |
| 14h | Pulpdent | 100000 | 70000±51962 | 10000 | 100000 | P=0.044 |
| | NanoZno | 1000 | 1000 | 1000 | 1000 | |
| | AH26 | 1000 | 4000±5196 | 1000 | 10000 | |
| 48h | Pulpdent | 400 | 367±58 | 300 | 400 | P=0.025 |
| | NanoZno | 1000 | 4000±5196 | 1000 | 10000 | |
| | AH26 | 1000000 | 7000000±5196152 | 1000000 | 10000000 | |
| 7d | Pulpdent | 10000 | 40000±51962 | 10000 | 100000 | P=0.050 |
| | NanoZno | 10000 | 40000±51962 | 10000 | 100000 | |
| | AH26 | 100000000 | 70000000±51961524 | 10000000 | 100000000 | |

**Table 2.** Mean and standard deviation of colony count for sealers in different intervals in S.aureus

| Time | Materials | Min max | Mean ± SD | Min | Max | P-Value |
|---|---|---|---|---|---|---|
| T0 | Pulpdent | 10000 | 40000±51962 | 10000 | 100000 | P=0.304 |
| | NanoZno | 10000 | 40000±51962 | 10000 | 100000 | |
| | AH26 | 10000 | 7000±5196 | 1000 | 10000 | |
| 2h | Pulpdent | 100000 | 70000±51962 | 10000 | 100000 | P=0.304 |
| | NanoZno | 0 | 0 | 0 | 0 | |
| | AH26 | 0 | 17±29 | 0 | 50 | |
| 14h | Pulpdent | 100000 | 70000±51962 | 10000 | 100000 | P=0.044 |
| | NanoZno | 1000 | 1000 | 1000 | 1000 | |
| | AH26 | 1000 | 4000±5196 | 1000 | 10000 | |
| 48h | Pulpdent | 400 | 367±58 | 300 | 400 | P=0.025 |
| | NanoZno | 1000 | 4000±5196 | 1000 | 10000 | |
| | AH26 | 1000000 | 7000000±5196152 | 1000000 | 10000000 | |
| 7d | Pulpdent | 10000 | 40000±51962 | 10000 | 100000 | P=0.050 |
| | NanoZno | 10000 | 40000±51962 | 10000 | 100000 | |
| | AH26 | 100000000 | 70000000±51961524 | 10000000 | 100000000 | |

**Table 3.** Mean and standard deviation of colony count for sealers in different intervals in E.*coli*

| Time | Materials | Min max | Mean ± SD | Min | Max | P-Value |
|---|---|---|---|---|---|---|
| T0 | Pulp dent | 0 | 42±72 | 0 | 125 | P=0.560 |
| | NanoZno | 0 | 7±12 | 0 | 20 | |
| | AH26 | 100 | 75±66 | 0 | 125 | |
| 2h | Pulp dent | 300 | 267±153 | 100 | 400 | P=0.044 |
| | NanoZno | 0 | 0 | 0 | 0 | |
| | AH26 | 400 | 467±208 | 300 | 700 | |
| 14h | Pulp dent | 10000 | 7000±5196 | 1000 | 10000 | P=0.0610 |
| | NanoZno | 60 | 60±20 | 40 | 80 | |
| | AH26 | 60 | 70±26 | 50 | 100 | |
| 48h | Pulp dent | 100000 | 67000±57158 | 1000 | 100000 | P=0.044 |
| | NanoZno | 20 | 17±6 | 10 | 20 | |
| | AH26 | 0 | 7±12 | 0 | 20 | |
| 7d | Pulp dent | 100000000 | 70000000±51961524 | 10000000 | 100000000 | P=0.027 |
| | NanoZno | 3000 | 11667±15885 | 2000 | 30000 | |
| | AH26 | 500 | 523±166 | 370 | 700 | |

**Table 4.** Mean and standard deviation of colony count for sealers in different intervals in Candida albicans

| Time | Materials | Min max | Mean ± SD | Min | Max | P-Value |
|---|---|---|---|---|---|---|
| T0 | Pulpdent | 0 | 13±23 | 0 | 40 | P=0.046 |
| | NanoZno | 0 | 0 | 0 | 0 | |
| | AH26 | 80 | 73±31 | 40 | 100 | |
| 2h | Pulpdent | 100 | 400±520 | 100 | 1000 | P=0.123 |
| | NanoZno | 700 | 667±153 | 500 | 800 | |
| | AH26 | 1000 | 1000 | 1000 | 1000 | |
| 14h | Pulpdent | 0 | 7±12 | 0 | 20 | P=0.035 |
| | NanoZno | 0 | 0 | 0 | 0 | |
| | AH26 | 150 | 167±126 | 50 | 300 | |
| 48h | Pulpdent | 200 | 300±173 | 200 | 500 | P=0.139 |
| | NanoZno | 300 | 300±100 | 200 | 400 | |
| | AH26 | 1000 | 800±346 | 400 | 1000 | |
| 7d | Pulpdent | 100 | 400±520 | 100 | 1000 | P=0.289 |
| | NanoZno | 800 | 833±153 | 700 | 1000 | |
| | AH26 | 600 | 600±100 | 500 | 700 | |

**Table 5.** Mean and standard deviation of colony count for sealers in different intervals in S.*mutans*

| Time | Materials | Min max | Mean ± SD | Min | Max | P-Value |
|---|---|---|---|---|---|---|
| T0 | Pulpdent | 500 | 467±351 | 100 | 800 | P=0.022 |
| | NanoZno | 0 | 0 | 0 | 0 | |
| | AH26 | 0 | 0 | 0 | 0 | |
| 2h | Pulpdent | 100 | 83±76 | 0 | 150 | P=0.105 |
| | NanoZno | 0 | 0 | 0 | 0 | |
| | AH26 | 0 | 0 | 0 | 0 | |
| 14h | Pulpdent | 1000 | 700±520 | 100 | 1000 | P=0.051 |
| | NanoZno | 1000 | 700±520 | 100 | 1000 | |
| | AH26 | 100000 | 70000±51962 | 10000 | 100000 | |
| 48h | Pulpdent | 100000 | 70000±51962 | 10000 | 100000 | P=0.670 |
| | NanoZno | 10000 | 40000±51962 | 10000 | 100000 | |
| | AH26 | 100000 | 70000±51962 | 10000 | 100000 | |
| 7d | Pulpdent | 1000000 | 700000±519615 | 100000 | 1000000 | P=0.040 |
| | NanoZno | 1000 | 4000±5196 | 1000 | 10000 | |
| | AH26 | 10000 | 40000±51962 | 10000 | 100000 | |

## Discussion

Residual microorganisms are the most important cause of failure in endodontic treatment. Thus, in addition to biocompatibility, sealing ability, and dimensional stability, antimicrobial effect of sealers is one of the critical properties (2).

In this study, we investigated the antimicrobial properties of AH26, pulpdent, and NZOE against facultative bacteria and yeast, which are predominant in persistent or refractory periapical lesions. E. faecalis is robust microorganism that may infect root canal (9) and are more likely to be found in cases of failed endodontic therapy than in cases of primary infection (10). E.coli is sometimes recovered from root canals and represent a standard organisms used in antimicrobial testing (11). C. albicans has the ability to form biofilms on different surface, and may be involved in cases of persistent and secondary infection (12). S. mutans may have a major influence on both the initial pulpal lesion and subsequent pulpal pathology (13).

It should be noted that in this study, the samples were evaluated at 0, 2, 14, 48 hrs. and 7days by the Direct-Contact Test (DCT) method. The aim for selecting these intervals was that the antibacterial effects of sealers were evaluated in fresh and set manner. The setting time for pulpdent, AH26, and Nano-Zno is 2, 14, and 48 hours, respectively. And finally, we choose 7days for evaluating aging effect on antibacterial effect of sealers. The DCT is a quantitative and reproducible method that simulates the contact of the test microorganism with endodontic sealers inside the root canal (14). The effect of sealers at various stages of the setting reaction on microbial viability can be evaluated (15). The method also allows for better control of possible confounding factors than ADT. In DCT, the turbidimetric method allows detecting the prevention of growth (bacteriostatic effect). Also, in cases in which carryover effect is controlled, turbidimetric measurements in DCT can show whether all (100%) bacteria have been killed (16).

In our study, the colony count of Candida was zero by Nano-Zno and AH26 sealers in 0 and 2 hours that showed these two sealers had similar and also strong antifungal properties. But fresh pulpdent sealer could not eradicate all microorganisms. Zhang et al. (15) showed that nano sealers were effective against E.coli, Candida, and S. *aureus*.

Fresh Nano-Zno sealer killed all microorganisms except for E. *faecalis*. In addition, Nano-Zno sealer eradicated all E. *faecalis* within 2 hours of contact that was better than AH26. AH26 and pulpdent could not eliminate all E. *faecalis* in any of intervals. Beyth et al. (17) in their microscopic evaluation reported that direct contact of nano particle with E. *faecalis* caused cellular lysis and immediate bacterial killing, while in contact to the other sealers, ability of biofilm formation still would present.

Also, significantly more colony reduction of S. *aureus* was observed by Nano-Zno in comparison to AH26 during the first 2 hours. Nano-Zno in fresh manner and also within 14 hours of contact killed all E.coli while average colonies were significantly higher for AH26.

Antimicrobial effect of ZOE refer to free eugenol that is Phenolic Substrata that can effect on myocyte in vegetative form (6). Nano-sized ZO powder particles (NZO) are biocompatible material that was broadly used in medicine industry and also have profound antibacterial effect that because of these favorable properties, utilization of nanoparticles in production of endodontic sealers has become the center of interest (7).

Cobankara et al. (18) showed that ZOE-based sealer in combination with Nano-Zno particle could penetrate deeply in dentinal tubules in addition to releasing antibacterial particles that sealer without nano particle did not have this properties.

Nair et al. (19) reported that particle size reduction and increase of sealer concentration could increase the antimicrobial effect of sealers against E.coli and Staphylococous ureous. In Rad Ms et al. study (20), similar to the present study, Nano sealer had better antimicrobial properties against Enterococous in comparison to pulp-dent sealer.

Cobankara et al. (18) showed that Nano sealers had antimicrobial effect after 90 days that associated to nano –particle concentration. Shrestha et al. (21) reported that Nano-Zno particles still had antimicrobial properties against E. *faecalis.* Samadi et al. (16) demonstrated that resin sealer had antimicrobial effect against E. *faecalis* immediately after mixing, but after 48 hours the bacterial colony was increased. The present study showed that antimicrobial effect of sealers were strong within 2 hours of contact and then until 7 days reduced gradually that was in accordance with the findings of Ehsani et al. (22) and Heling et al. (23). Altogether, antimicrobial effect of sealers against E. *faecalis* was lower than the other organisms. Nano-sealer in immediate contact to microorganism showed profound antimicrobial

properties that this characteristic was reduced by passing the time. NPs offer improved properties to classical organic antibacterial agents. One reason lies in their high surface area to volume ratio, resulting in appearance of new mechanical, chemical, electrical, optical, magnetic, electro-optical, and magneto-optical properties of the NPs that are different from their bulk properties (24).

The electrostatic properties of both NPs and biofilms influence how they interact (25). Moreover, Versiani et al. (4) have demonstrated that glass surfaces coated with ZnO nanoparticles are able to produce reactive oxygen species (ROS) that interfere with E. coli and S. aureus biofilm formation. Nanoparticles are able to attach to the membrane of bacteria by electrostatic interaction and disrupt the integrity of the bacterial membrane (26). Nanotoxicity is generally triggered by the induction of oxidative stress by free radical formation, that is, the ROS, following the administration of NPs (2).

## Conclusion

Based on the results of the present study, it can be concluded that Nano-ZNO sealer had higher antimicrobial properties compared to AH26 and Pulpdent sealers. Thus, in addition to another satisfactory characterize of Nano-ZNO, can be recommended to substitute the conventional sealer.

## Acknowledgment

This study was supported by a grant from the Vice Chancellor of Research Council of Mashhad University of Medical Sciences, Iran. The results presented in this study have been taken from a student thesis (no. 582) in Mashhad University of Medical Sciences (MUMS). The authors deny any conflicts of interest related to this study.

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