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COMPARATIVE EVALUATION OF THE ANTIBACTERIAL ACTIVITY OF GLASS IONOMER RESTORATION INCORPORATED WITH DIFFERENT METAL OXIDES NANOPARTICLES ON ORAL STREPTOCOCCUS MUTANS

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ABSTRACT

Objective: The objective of this study is to compare and evaluate the antibacterial activity of modified conventional Glass ionomer restoration (GI) with zinc oxide (ZnO-NPs) and cerium oxide Nanoparticles (CeO2-NPs) on the oral Streptococcus mutans (SM).

Materials and Methods: one hundred and five disc-shaped specimens were divided into seven groups; GI, GI incorporated with three, five and seven percent ZnO-NPs and CeO2-NPs. Antibacterial activity was assessed by counting of colony forming unit (CFU) of SM after direct contact test and 96 hours biofilm evaluation. Fourier transform infrared spectroscopy (FTIR) was used to illustrate interaction between different concentrations of Nanoparticles incorporated with GI.

Results: Direct contact test showed a significant reduction of CFU of GI incorporated with seven percent ZnO-NPs in relation to GI (P value 0.007). Biofilm evaluation showed significant reduction of CFU of GI incorporated with three, five and seven percent ZnO-NPs and five and seven percent CeO2-NPs (P value 0.000). FTIR showed Peaks at 1405 and 1635 cm–1 related to symmetric and asymmetric vibrations of COO–, those peaks were broader and red shifted in GI incorporated with ZnO-NPs and CeO-NPs.

Conclusion: Incorporation of conventional GI with ZnO-NPs and CeO2-NPs enhance its antibacterial activity against oral SM.

KEY WORDS: Biofilm, Nanoparticles, Streptococcus mutans, Zinc oxide.

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INTRODUCTION

Secondary caries is defined as a carious lesion developed at the margins of an existing restoration. It is a one of the primary causes of failure of the restorative materials [1,2]. Enamel demineralization occurs by the adhesion of microorganism to the tooth surface and restorative materials to produce dental plaque [3]. Oral Streptococcus mutans (SM) is a main causative factor of dental plaque biofilm formation. Therefore, one of the most important methods for prevention of secondary caries is applying restorative materials to prevent bacterial growth and colonization, and it should be able to reduce acid production and dental plaque accumulation [4,5].

One of the most popular restorative dental materials is Glass ionomer restoration (GI). GI is set based on acid base reaction [6,7] . It is being used in many dental applications as restoration of primary teeth, class V restorations, cementation of crowns, restoration by sandwich technique and as restorative materials in atraumatic restorative therapy [8,9].

GI presented a chemical adhesion to the moist tooth structure with low coefficient of thermal expansion close to the tooth structure, in addition to fluoride release which is knowing for many years to have an antibacterial action [10,11]. In spite of these advantages, the internal cracks, air voids and high porosity of GI may allow the microleakage occurrence leading to secondary caries [12,13]. Also, some studies revealed no inhibition effect of fluoride after setting reaction as the low pH of GI during the setting reaction allows leaching of high amount of fluoride which relatively stops after setting [1].

In the light of the above mentioned disadvantages of GI restoration, the incorporation of Nanoparticles with antibacterial effect were introduced [14,15]. Nano dimension of the nanoparticles allow a considerable interaction with the microorganisms which increase the antibacterial action. This can prevent the secondary caries and prolong the effectiveness of the restorative material [1,16].

For many years, zinc was used as an antibacterial agent and incorporated in many dental products [17]. Zinc oxide Nanoparticles (ZnO-NPs) is a biocompatible material, non-toxic to human cells but more toxic to the bacteria. ZnO-NPs can inhibit the growth of oral SM and plaque formation [5,18,19]. Also, the antibacterial effect of ZnO-NPs increase by increase of the concentration and decrease in Nanoparticles size [13,20].

Among the metal oxides, the Nano particulate cerium oxide (CeO2-NPs) which belongs to lanthanide series and is a rare earth metal oxide, with antibacterial properties against many types of bacteria [21,22].

The aim of this study is to compare and evaluate the antibacterial activity of modified conventional GI restoration with ZnO-NPs and CeO2-NPs on the SM. The null hypothesis to be tested that there is no antibacterial effect of modified GI with ZnO-NPs and CeO2-NPs on the SM.

MATERIALS AND METHODS

Conventional Glass ionomer (GC Gold Label 9, Tokyo, Japan) powder and liquid was modified by addition of different concentration of ZnO-NPs and CeO_2-NPs.

Preparation of Nano particles:

Zinc oxide Nanoparticles (ZnO-NPs):

A zinc acetate salt (Alfa Chemika, Mumbai, India) was dissolved in methanol and stirred until the entire amount was completely dissolved, then sodium hydroxide solution (pH 10) (Loba Chemie PVT.LTD, Mumbai, India) was added drop by drop. A white precipitate was formed indicating the formation of ZnO-NPs. The precipitate was washed several times using distilled water, then dried at 100 ^{o}C [23].

Cerium oxide Nanoparticles (CeO_2-NPs):

Cerium IV oxide, nanoparticle size is < 25 (Sigma Aldrich Company, St. Louis, USA)

Characterization of ZnO-NPs:

The morphology of ZnO-NPs was observed by transmission electron microscopy (HR-TEM, JOEL JEM-2010, Peabody, MA, USA) operating at an accelerating voltage of 200 kV, with Gatan digital camera (Erlangshen ES500, Abingdon, UK).

Preparation of tested specimens:

One hundred and five disc-shaped specimens were prepared by using four mm diameter and two ml thickness Teflon mold. The specimens were divided into seven groups, control group (G1) GI without modifications. G2, G3 and G4, GI modified by 3, 5 and 7% ZnO-NPs, G5, G6 and G7, GI modified by 3, 5 and 7% CeO2-NPs, respectively.

The different concentrations of the Nanoparticles were weighed by using electronic balance with four digits precision (Sartorius AG, Goettingen, Germany). The weighed nanoparticles were freshly mixed with GI powder by using plastic spatula. The recommended powder/liquid ratio was 3.6/1.

GI was mixed according to the manufacturer instructions and placed in the Teflon mold. The specimens were covered with Mylar strip and glass plate were pressed over the strip to squeeze the excess material and to obtain smooth and standardized surface. Specimens were left to dry for 20 minute at room temperature, then removed from the mold and sterilized by UV light for 30 minutes.

Antibacterial tests:

Bacterial strain and growth condition:

Streptococcus mutans (SM) ATCC 25175 were cultured aerobically in brain heart infusion (BHI) (Lab M, Lancashire, UK) for 24 hour at 37 ^{o}C. The bacterial suspension prepared at optical density of DO_{600} = (0.6-0.7) measured by using Spectrophotometer (TECAN Nanoquant Infinite 200 Pro, Männedorf, Switzerland)

Agar diffusion test:

200 μl of SM suspension was spread on BHI agar plates and left for 30 minute at room temperature. Thirty five set discs (five discs from each group) were placed in direct contact with the BHI agar. The plates were incubated aerobically for 24 hours at 37^{o}C. The inhibition zones were measured in millimeters [1].

Direct contact test:

Thirty five set discs were placed in 96-well microtitre plates (Cellstar, Greiner, Germany). 10 μl of SM suspension was spread on the surface of the discs. Five empty wells were considered as a positive control and (G1) were considered as a negative control. The plates were incubated for one hour to evaporate the liquid leaving a thin layer of bacteria in contact with the discs. Then 500 μl of BHI broth were added to each well. After 24 hour a 10 μl of the mixture (bacterial suspension + BHI broth) were diluted to 10^{-6}, then spread on BHI agar plates and incubated aerobically for 24 hours at 37^{o}C. The SM colonies were counted to determine the colony forming unit (CFU/ml) [16].

Evaluation of biofilm:

Thirty five set discs were placed in 96-well microtitre plates. One ml of bacterial suspension were added to each well, then the plates were incubated for 96 h at 37^{o}C.After 48 hours the growth media (BHI+1% sucrose) were renewed. After incubation the discs were drained and transferred to sterile tube with 1 ml BHI. The biofilm attached to the disc surface was collected by using a vortex mixer (Stuart, Staffordshire, UK) for one min at 2500 rpm. The collected bacteria was serially diluted to 10^{-6} and inoculated to BHI agar plates and incubated for 24 h at 37^{o}C. Finally the SM colonies were counted to determine (CFU/ml) [15].

Fourier transform infrared spectroscopy (FTIR):

FTIR spectrometer (IR Affinity-1S, Shimadzu Co, Kyoto, Japan) was used within scanning range from 400-4000 cm^{-1} using KBr as reference to demonstrate the structural composition and illustrate interaction between different concentrations of ZnO-NPs and CeO_2-NPs incorporated with GI.

Statistical analysis:

Data were statistically described in terms of mean ±, standard deviation (± SD), and range. Comparison between the study groups was done using one way analysis of variance (ANOVA) test with Bonferroni Post Hoc multiple two group comparisons. P values less than 0.05 was considered statistically significant. All statistical calculations were done using computer program IBM SPSS (Statistical Package for the Social Science; IBM Corp, Armonk, NY, USA) release 22 for Microsoft Windows.

RESULTS

Characterization of ZnO-NPs

TEM image of ZnO-NPs was shown with a mean diameter about 20 nm in a uniform hollow spheres. (Figure 1)

Antibacterial tests

Agar diffusion test:

There was no inhibition zone around the specimens of different groups.

Direct contact test (DCT):

The results of CFU of SM regarding to the DCT are represented in figure 2. G3, G4 and G7 showed a significant CFU reduction in relation to the positive control (P value 0.000, 0.013, 0.037) respectively, while only G4 showed a significant CFU reduction in relation to G1 (P value 0.007).

Evaluation of biofilm:

CFU of SM after 96h biofilm formation are depicted in figure 3. All tested groups showed significant reduction of SM CFU in relation to G1 (P value 0.000) except G5.

Fourier transform infrared spectroscopy (FTIR):

The characteristic bands observed in all FTIR charts are tabulated in table (1), whoever. The FTIR of ZnO-NPs, G1, G2, G3 and G4 were presented in figure 4**.** Figure 5 showed FTIR of the CeO_2-NPs, G1, G5, G6 and G7.

TABLE (1) FTIR spectral bands assignment of ZnO-NPs, CeO2-NPs, GI and GI incorporated with diffrent concentrations of ZnO-NPs and CeO2-NPs

Peak assignment	G1	ZnO-NPs	CeO_2-NPs	G2	G3	G4	G5	G6	G7
υO-H*	3460	3433	3454	3430	3437	3425	3454	3443	2448
Zn-O	-----	562	-----	451	431	429	-----	-----	-----
υC-H*	2363	------	2314	2372	2372	2372	2371	2365	2355
Symmetric COO–	1405	------		1420	1420	1420			
Asymmetric COO–	1635	------	1635	1623	1623	1623	1635	1635	1635
Ce-O	-----	-----	474	-----	-----	-----	-----	-----	-----

*: ***υ = stretching***

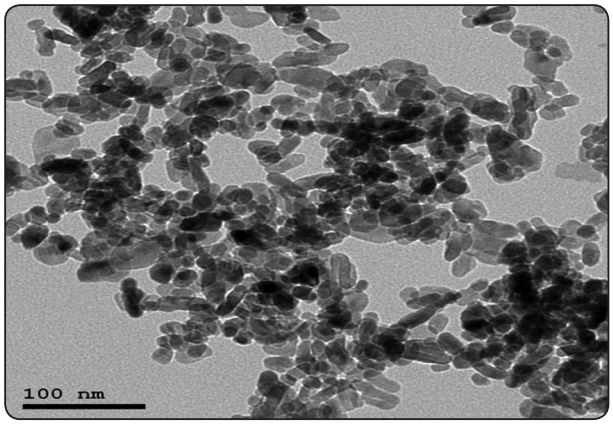

Fig. (1): TEM image represent the morphology of zinc oxide Nanoparticles

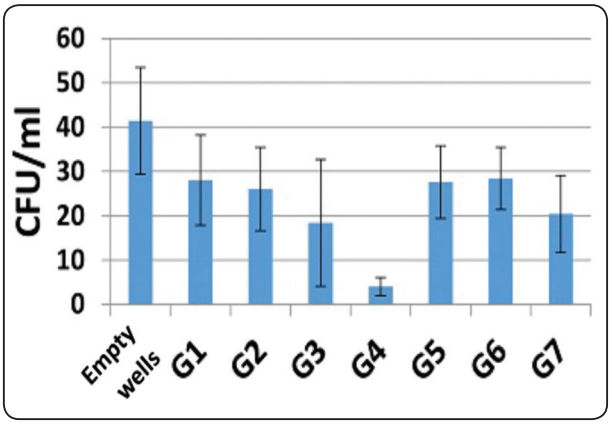

Fig. (2): Mean and SD of colony forming unit of Streptococcus mutans after direct contact test

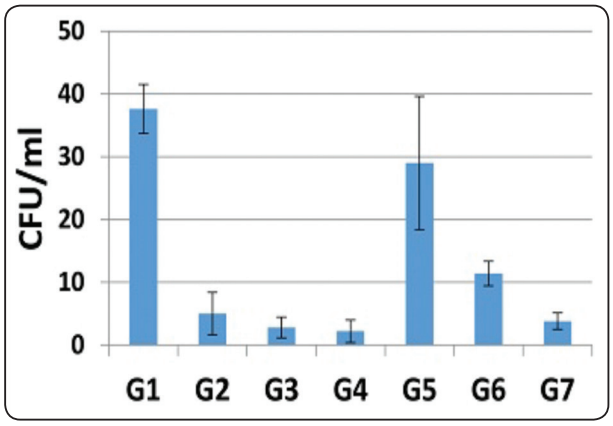

Fig. (3): Mean and SD of colony forming unit counting of Streptococcus mutans after 96 hours biofilm formation

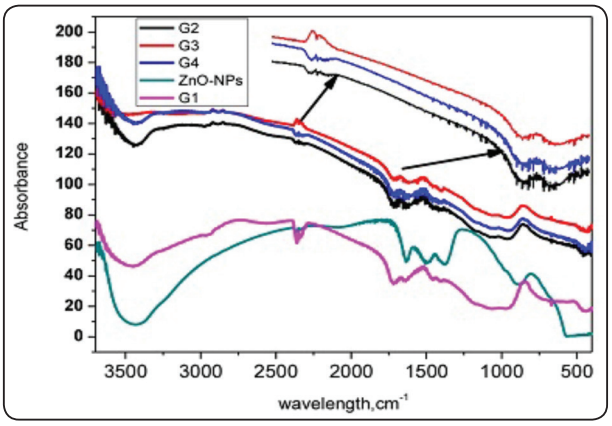

Fig. (4): FTIR spectra of the ZnO-NPs, GI and GI incorporated with different concentrations of ZnO-NPs

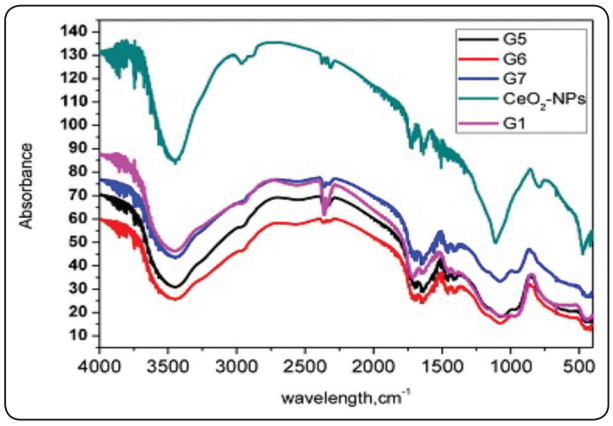

Fig. (5): FTIR spectra of the CeO2-NPs, GI and GI incorporated with different concentrations of CeO2-NPs

DISCUSSION

In the present study, the agar diffusion test didn't show any inhibition zone around all specimens. These findings were in agreement with Hojati et al. and Sungurtekin-ekci et al. [1,24]. The possible explanation for this result is the insolubility of high viscous conventional GI, ZnO-NPs and CeO2-NPs as they couldn't leash to the surrounding area to initiate an antibacterial action. The agar diffusion test is considered a traditional method to evaluate a soluble materials as antibiotics [24–26].

However one of the most important requirement of dental restoration is a low solubility in oral

cavity. DCT is considered a suitable test for evaluating materials with low solubility, as in DCT the bacteria was contact directly on the surface of tested materials [1]. DCT revealed that there was no significant antibacterial activity of unmodified GI in relation to the empty wells. This result was consistent with Elsaka et al. [27]. The possible explanation of this finding is that the manually mixed GI release less fluoride than the encapsulated GI due to the fact that the trituration of encapsulated GI enhances the reaction between glass particles and the cement liquid. This reaction increases the volume fraction of the GI matrix and decreases the unreacted particles. Fluoride tends to be released from the cement matrix, therefore the mechanically triturated GI would increase the fluoride release[28]. Moreover, studies revealed that the low pH of the freshly mixed GI could provide antibacterial, activity more than the set GI [29].

Also, there was a significant antibacterial activity of GI incorporated with 5 and 7% concentrations of ZnO-NPs in comparison to the empty wells and GI incorporated with 7% ZnO-NPs in relation to GI. This result is in agreement with Hojati et al. as they revealed that by increasing the Nanoparticles concentration the antibacterial activity increase [1].

The mechanism of antibacterial action of ZnO-NPs is releasing an active oxygen species like H2O2 and interacting with the bacteria with electrostatic forces, leading to the alteration of the bacterial cell wall and loss of extracellular content and bacterial cell death [15].

GI incorporated with 7% CeO2-NPs showed a significant antibacterial activity in relation to the empty wells but was non-significant in relation to the GI. The antibacterial action of CeO2-NPs has a wide range effect , not inhibiting bacterial growth but it interferes with the mitochondrial respiration function , DNA replication and cell division leading to the change of the oxidative stress induced by reactive oxygen species (ROS) and eventually cell death [30].

To our knowledge, there is a limited number of studies that have been evaluated the antibacterial activity of CeO2-NPs in dental field. Christiano et al. revealed that CeO2-NPs showed an antibacterial effect on SM when incorporated in 10% concentration with dental composite [31]. Also, CeO2-NPs revealed less antibacterial activity against gram positive bacteria as SM, which difficult to be penetrated with CeO2-NPs [22]. This fact could explain the lower antibacterial activity of CeO2-NPs in relation to ZnO-NPs, which have a higher antibacterial effect against gram positive bacteria [32,33].

In addition to the role of Nanoparticles concentration in antibacterial activity against SM. The present study revealed that the antibacterial activity of the GI incorporated with ZnO-NPs and CeO2-NPs have been enhanced after 96 h biofilm maturation, these results comply with Hojati et al. [1], who revealed that the antibacterial activity of the dental restorations incorporated with Nanoparticles increased by time.

The role of Zinc oxide in biofilm inhibition could be explained by its ability to inhibit sugar transportation and metabolism. As well as distribution of enzymes systems of dental biofilms by displacing magnesium ions which are essential for enzymatic activity of the dental plaque. Also, Zinc ions is able to reduce acid production by inhibition of glucosyltransferase activity [11].

The null hypnosis of this study was rejected, as the incorporation of conventional GI with ZnO-NPs and CeO2-NPs enhances its antibacterial activity against oral SM.

In the present study, the functional groups of ZnONPs, CeO2-NPs, unmodified GI and GI incorporated with various concentrations of Nanoparticles were measured using FTIR. ZnONPs FTIR spectrum was established a broad peak around 3433 cm^{-1} which was expressed to the O-H stretching mode of hydroxyl group of residual organic species or adsorbed water persisted during ZnONPs

preparation. This peak showed in unmodified GI at 3460 cm^{-1}, and at 3430, 3437 and 3425 cm^{-1} after GI incorporation with 3, 5, 7% ZnONPs, respectively. Moreover, ZnONPs revealed a Zn-O peak at 562 cm^{-1}, which was in accordance to the previously obtained by Sowribabu et al.[34]. This peak has been red shifted after GI modification with ZnONPs and appeared at 451, 431 and 429 cm^{-1} for 3, 5, and 7%, respectively.

The FTIR spectrum of unmodified GI showed two fork like small peaks centered at 2363 cm^{-1} attributed to C-H vibration mode. These peaks were appeared at 2372 cm^{-1} after incorporation with ZnONPs. Also, GI was showed emerged peaks at 1405 and 1635 cm^{-1} related to symmetric and asymmetric tensile vibrations of COO– (in carboxylic acid salt compounds) [35]. These peaks were looked broader and shifted to 1420 and 1623 cm^{-1} after GI modification with ZnONPs. It may be attributed to the incorporation of ZnO to the active -COO group in GI. This suggestion is confirmed by the decrease in the intensity observed in the C=O stretch of polyacrylic acid at 1720 cm^{-1}, which could be explained by the formation of polyacrylate salts [36].

Incorporation of GI with CeO_2-NPs influnced the absorption of some peaks. For example, the peak located around 1720 cm^{-1} in unmodified GI which was corresponding to C=O stretch of polyacrylic acid showed a small decreas in its intensity. On the other hand, the peak at 1635 cm^{-1} assigned to asymmetric tensile vibrations of COO– seemed sharper. However, peaks in the region from 2300-600 cm^{-1} attributed to organic species did not show any change.

In FTIR spectrum of CeO_2-NPs, the peak was developed at 474 cm^{-1} which could be explained by the presence of metallic Ce ion [37]. This peak was disappeared after incorporation of GI with CeO_2-NPs, which was indicated the linkage between CeO_2-NPs and GI. These results are consistent with that obtained by Sowribabu et al.[38].

CONCLUSION

Incorporation of conventional GI with ZnO-NPs and CeO2-NPs enhances its antibacterial activity against oral SM. The FTIR confirmed that, the interaction between the Nanoparticles and GI was occurred through the COO- functional group of polyacrylic acid in GI.

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