Sensitivity of *Streptococcus mutans* to Selected Nanoparticles (in Vitro Study)

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ABSTRACT

Background: Nanoparticles are clusters of atoms in a size range from (1-100) nm. Nano dentistry creates amazing useful structures from individual atoms or molecules (nanoparticles), which provides a new alternative and a possibly superior strategy in prevention and treatment of dental caries through management of dental plaque biofilms. The aim of the study was to test the sensitivity of *Streptococcus mutans* to different concentrations of hydroxyapatite and iron oxide nanoparticles suspension solutions, in comparison to chlorhexidine, and de-ionized water, in vitro.

Materials and methods: Agar well technique was applied to test the sensitivity of *Streptococcus mutans* to different concentrations of hydroxyapatite and iron oxide nanoparticles compared with chlorhexidine 0.2% as a control positive and de-ionized water as control negative. Zone of inhibitions which is clear zone of no growth of the bacteria were measured across the diameter of each well, no zone indicated a complete resistance of bacteria to the agents.

Results: Values of mean of inhibition zone for all concentrations of hydroxyapatite nanoparticles were zero. While for iron oxide nanoparticles, they were zero until reaching the last three concentrations, in which there was a respective increase with a highly significant difference between groups ($p<0.01$). When making multiple comparisons of the inhibition zones of iron oxide nanoparticles between groups, findings showed that the inhibition zones of 17%, 20% and 22.5% of iron oxide nanoparticles were more than all other concentrations that had no inhibition zones with a significant difference ($p<0.05$). There was a highly significant difference between each concentration of hydroxyapatite and iron oxide nanoparticles with chlorhexidine and de-ionized water ($p<0.01$).

Conclusion: *Streptococcus mutans* were not sensitive to hydroxyapatite nanoparticles, as there was a complete resistance for the agent. While for iron oxide nanoparticles, *Streptococcus mutans* were sensitive to 17.5%, 20% and 22.5% and sensitivity increased with the increase in concentration with a statistically highly significant difference and this indicates an antibacterial activity of this material.

Keywords: Hydroxyapatite nanoparticles, Iron oxide nanoparticles, *streptococcus mutans*, Inhibition zone. **(J Bagh Coll Dentistry 2018; 30(1): 69-75)**

INTRODUCTION

Dental caries is an infectious microbiological disease of the teeth result in localized dissolution and destruction of the calcified tissue [1,2]. It is a multifactorial disease related to the interaction of the bacteria on the tooth surface, oral biofilm, diet specifically fermentable carbohydrate, which are fermented by the plaque microflora together over time [3]. *Streptococcus mutans* was found to be the predominant bacteria in caries process [3-5]. It is a gram-positive, facultative anaerobic bacterium commonly found in the human oral cavity. Many studies showed a positive association between *Streptococcus mutans* and initiation of a carious lesion, such studies demonstrated that these bacteria can be isolated from dental plaque adjacent to a carious tooth surface [5-8]. Nanomaterials are materials with components less than 100 nm. Increasing interest in future medical applications of nanotechnology is leading to the appearance of a new field called nanomedicine. It is the science and technology of diagnosing, treating and preventing disease and preserving and improving human health, through the use of nanoscale structured materials [9]. Nanomaterials due to their small size have a much increased surface area per unit mass compared to bigger particles, this allows a higher binding capacity and excellent dispersibility in solutions. It has also a strong ability to bind with proteins, as well as with fragments of plaque and bacteria due to their size, which increase the surface area, owing its antimicrobial properties [10,11]. Nano hydroxyl-apatite with chemical formula $Ca_{10}(PO_4)_6(OH)_2$ has long been among the most studied biomaterials in the medical field for both its proven biocompatibility and for being the main constituent of the mineral part of bone and teeth. It is also an important source of calcium and phosphate, that are very important for the remineralization of demineralized enamel areas [12]. This nanoparticle attracting interest as a biomaterial due to its outstanding properties: biocompatibility, bioactivity, osteo-conductivity, non-toxicity, non-inflammatory nature and similarity in size, crystallography and chemical composition with human hard tissue [13]. Mechanisms of the antibacterial activity of this material are not fully explained. However, there are three hypothetical mechanisms. According to the

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first mechanism, the ions penetrate into bacterial cell and, by affecting the production of intracellular Adenosine Triphosphate, they disrupt the process of DNA replication. The second mechanism is associated with the accumulation of ions in cell membranes of bacteria, due to their large surface area to volume ratios, enabling greater presence of atoms on the surface, provides maximum contact with the environment, makes penetration through cell membranes easier and this will lead to changes in their permeability (the gradual release of proteins and lipopolysaccharides). The transportation of protons through the cell membrane is prevented, and consequently it leads to the destruction of the cell membrane and the death of the bacterial cell. The third mechanism is based on the ion induction of reactive oxygen species. Oxygen radicals are able to react with the components of the membrane and cell wall of bacteria, as well as other cell components (e.g., mitochondria), causing irreversible changes in their structure and thus death of the bacterial cell [14,15]. Magnetic nanoparticles are iron oxide Fe_3O_4 (magnetite), are one of the most versatile and safe material used in medicine with unique magnetic properties and superior biocompatibility. In the area of antibacterial activity, metal nanoparticles are of a particular interest because they could be synthesized with high surface area and with highly potential active sites [16,17]. The antibacterial activity of Iron oxide nanoparticles could be due to several mechanisms. The first mechanism suggested is the oxidative stress generated by reactive oxygen species including superoxide and hydroxyl radicals, hydrogen peroxide, and singlet oxygen, which may cause chemical damage to proteins and DNA in bacteria. Secondly, electrostatic interactions between nanoparticles and bacterial cell membranes or cell membrane proteins can result in physical damage, which ultimately leads to bacterial cell death [18]. Other studies demonstrated that the high surface-to-volume ratio of nanoparticles allow a close interact with microbial membranes, damaging their structure and inactivate bacteria [17,19-21]. As far as, there was no Iraqi study conducted to investigate the effect hydroxyapatite and iron oxide nano particles on salivary *Streptococcus mutans*. For this reason, this study was conducted.

MATERIALS AND METHODS

An ethical consent was achieved from dental departments, private clinics and specialist governmental dental health centers in Baghdad city for teeth collection. An approval was achieved from the followings:

- University of Technology, Department of Laser and Optoelectronic Engineering, for using laser application laboratory.
- Baghdad University, College of Education for Pure Science Ibn-Al-Haitham, Central Service Laboratory for using scanning electron microscope and energy dispersive spectometrym devices.
- Ministry of Industry and Minerals, Ibn albetar research center, section of plant extraction researches, unit of microbiology for using the microbiological lab.

Agar well technique was applied to test the sensitivity of *streptococcus mutans* to different concentrations of hydroxyapatite and iron oxide nanoparticles suspension solutions compared with chlorhexidine 0.2% as a control positive and de-ionized water as control negative. Mueller Hinton Agar media was prepared according to the instructions of Hi-Media[22]. Different concentrate-ions of nanoparticles suspension solutions were prepared according to previous studies starting from 0.5%, 1%, 2.5%, 5%, 7.5%, 10%, 20%, 30%, 40%, 50% for hydroxyapatite nanoparticles; and 0.5%, 1%, 1.5%, 2%, 2.5%, 5%, 7.5%, 10%, 12.5%, 15%, 17.5%, 20%, 22.5% for iron oxide nanoparticles [23,24]. Hydroxyapatite nanoparticle used in this study (mk nano, Mississauga, Canada) was 99% in purity, white in color, 20 nm and insoluble in water [25]. To prepare hydroxyapatite suspension solution, Hydrochloric acid (HCl) was used to dissolve the nanomaterial and then de-ionized water was added. Sodium hydroxide (NaOH) was added to adjust pH to 7. Suspension solution which holds salts that were pulled out by separating funnel was obtained [26]. Iron oxide nanoparticle used in this study (Sigma Aldrich, Malaysia) was 98% in purity, reddish black in color, 20-30 nm and water soluble. It has a high dispersity and stability in water. To prepare iron oxide nanoparticles suspension solution, dissolve powder in distilled water; then ultrasonicated for 20 minutes using an ultrasonic device to result in a uniform suspension as ultrasonication will promote an effective and fast dissolution of samples, based on the mechanical agitation caused by the ultrasound waves into liquids [27].

Procedures:

1) A volume of 25 ml of Muller Hinton Agar (MHA) (pH 7) was poured into sterile Petri dishes, left at room temperature for 24 hour.
2) To each plate 0.1 ml of activated *streptococcus mutans* inoculums was spread, left at room temperature for 20 minutes.
3) Four wells of equal size (6 mm in diameter) and depth were prepared in each agar plate; each well was filled with 0.1 ml of the tested agent.

4) Plates were left at room temperature for 1 hour then incubated aerobically for 24 hours at 37°C. Zone of inhibition of bacteria were measured across the diameter of each well by using a ruler, no zone indicated a complete resistance of bacteria to the agents.

Statistical analysis was carried out using SPSS 21 version (Statistical Package for Social Sciences) and R.3.3.2. Shapiro Wlilk test was used for testing the normality of data. For the normally distributed data; mean, standard deviation and ANOVA test were calculated. Levene's test was used to test the homogeneity of variance and Dunnet T3 test was used to make comparisons among groups. For the not normally distributed data; mean, standard deviation, relative effective size and BDM (Brunner, Dette, Munk) rank based ANOVA test were calculated and Cliff's method was used to make comparisons among groups.

RESULTS

The values of mean for all concentrations of hydroxyapatite nanoparticles were zero as there were no inhibition zones. While for iron oxide nanoparticles, values of mean and standard deviation were zero until reaching the last three concentrations (17.5%, 20% and 22.5%), in which there was a respective increase in mean values as seen in table 1 and figures 1 and 2. De-ionized water showed no zone of inhibition. While chlorhexidine showed an inhibition zone. ANOVA test showed a statistically highly significant difference between groups ($p<0.01$). When making multiple comparisons of the inhibition zones of iron oxide nanoparticles between groups, findings showed that the inhibition zones of 17%, 20% and 22.5% were more than all other concentrations that had no inhibition zones with a significant difference ($p<0.05$). Inhibition zones of 22.5% were more than 17.5% and 20% and the inhibition zones of 20% were more than 17.5% by percentage of 100% with a statistically significant difference between them ($p<0.05$). Table 2 revealed that there was a statistically highly significant difference between hydroxyapatite and iron oxide nanoparticles with chlorhexidine and de-ionized water ($p<0.01$). Making multiple comparisons of the inhibition zones in table 3 between chlorhexidine with each concentration of hydroxyapatite and iron oxide nanoparticles revealed a highly significant difference ($p<0.01$) except with 20% iron oxide nanoparticles, the result was not significant. when comparing the inhibition zone of 17.5%, 20% and 22.5% iron oxide nanoparticles with de-ionized water, a highly significant difference was found ($p<0.01$).

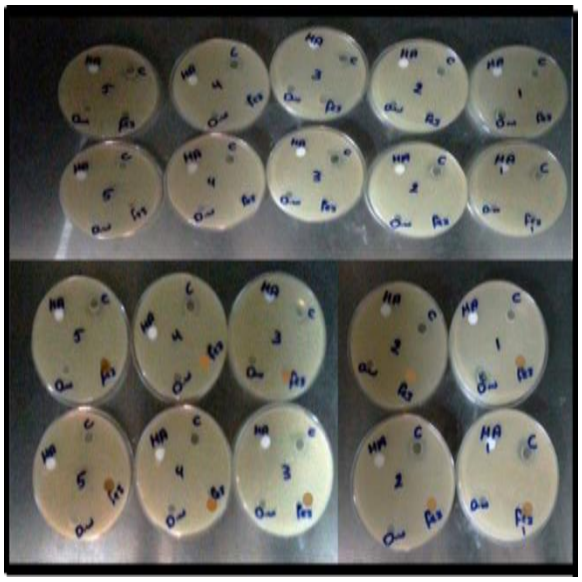

Figure 1: Agar well diffusion method for different concentrations of hydroxyapatite and iron oxide nanoparticles

Figure 2: Agar well diffusion method for (1) 17.5%, (2) 20%, and (3) 22.5%) of iron oxide nanoparticles

Table 1: Inhibition zone (mean and standard deviation) of iron oxide nanoparticles groups by concentrations with a statistical test

Groups	Mean ± SD	Relative EZ
0.5%IONPs	0.00 ± 0.00	0.385
1% IONPs	0.00 ± 0.00	0.385
1.5% IONPs	0.00 ± 0.00	0.385
2% IONPs	0.00 ± 0.00	0.385
2.5% IONPs	0.00 ± 0.00	0.385
5% IONPs	0.00 ± 0.00	0.385
7.5% IONPs	0.00 ± 0.00	0.385
10% IONPs	0.00 ± 0.00	0.385
12.5% IONPs	0.00 ± 0.00	0.385
15% IONPs	0.00 ± 0.00	0.385
17.5% IONPs	11.40 ± 0.55	0.808
20% IONPs	13.40 ± 0.55	0.885
22.5% IONPs	15.40 ± 0.55	0.962

F=2542.037, d.f=12, p=0.000
**** Highly Significant, EZ= Effect size

The relative effect size of chlorhexidine in table 4 is more than the relative effect size of each concentration of hydroxyapatite and iron oxide nanoparticles with a statistically highly significant

difference ($p<0.01$). Upon making comparisons in table 5 it was found that the inhibition zone of chlorhexidine was more than de-ionized water and each concentration of hydroxyapatite and iron oxide nanoparticles by a percentage of 100% with a significant difference ($p<0.05$), while the comparisons between the inhibition zone of de-ionized water and each concentration of hydroxyapatite and iron oxide nanoparticles was zero with a nonsignificant difference ($p>0.05$).

Table 2: Descriptive (mean and standard deviation) of inhibition zone according to concentrations of hydroxyapatite and iron oxide nanoparticles by groups with chlorhexidine and de-ionized water with a statistical test

		0.5% HANPs	2.5% HANPs	5% HANPs	7.5% HANPs	0.5% IONPs	1% IONPs	1.5% IONPs	2.5% IONPs	10% IONPs	17.5% IONPs	20% IONPs	22.5% IONPs
Group		0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	11.40 ±0.55	13.40 ±0.55	15.40 ±0.55
CHX		13.80 ±1.10	14.00 ±1.00	13.60 ±0.55	14.40 ±0.55	13.80 ±1.10	13.60 ±0.55	13.60 ±0.55	13.80 ±0.84	13.00 ±0.00	13.20 ±0.45	13.00 ±0.00	13.20 ±0.45
DW		0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00
ANOVA	**F**	793.500	980.000	3082.667	3456.000	793.500	3082.667	3082.667	1360.286	-	1537.200	2906.000	2081.200
	df	2											
	p-value	0.000 **	0.000 **	0.000 **	0.000 **	0.000 **	0.000 **	0.000 **	0.000 **	-	0.000 **	0.000 **	0.000 **

** Highly Significant

Table 3: Multiple comparisons of inhibition zone between groups of hydroxyapatite and iron oxide nanoparticles with chlorhexidine and de-ionized water

Groups Concentration	**CHX**				**DW**	
	Group		**DW**		**Group**	
	MD	**p-value**	**MD**	**p-value**	**MD**	**p-value**
0.5%HANPs	-13.80	0.000**	13.80	0.000**	0.00	.
2.5% HANPs	-14.00	0.000**	14.00	0.000**	0.00	.
5%HANPs	-13.60	0.000**	13.60	0.000**	0.00	.
7.5% HANPs	-14.40	0.000**	14.40	0.000**	0.00	.
0.5%IONPs	-13.80	0.000**	13.80	0.000**	0.00	.
1%IONPs	-13.60	0.000**	13.60	0.000**	0.00	.
1.5%IONPs	-13.60	0.000**	13.60	0.000**	0.00	.
2.5%IONPs	-13.80	0.000**	13.80	0.000**	0.00	.
17.5%IONPs	-1.80	0.002**	13.20	0.000**	11.40	0.000**
20%IONPs	0.40	0.391	13.00	0.000**	13.40	0.000**
22.5%IONPs	2.20	0.000**	13.20	0.000**	15.40	0.000**

**Highly significant, MD= Mean Difference

Table 4: Descriptive (mean, standard deviation and relative effect size) of inhibition zone according to concentrations of hydroxyapatite and hron oxide nanoparticles with chlorhexidine and de-ionized water with a statistical test

Groups			**CHX**		**DW**		**BDM**		
Concentration	**Mean ± SD**	**EZ**	**Mean ± SD**	**EZ**	**Mean ± SD**	**EZ**	**F**	**Df**	**P-value**
1% HANPs	0.00 ± 0.00	0.33	15.20 ± 0.45	0.83	0.00 ± 0.00	0.33	225.000	4	0.000**
10%HANPs	0.00 ± 0.00	0.33	15.20 ± 0.45	0.83	0.00 ± 0.00	0.33	150.000	4	0.000**
20%HANPs	0.00 ± 0.00	0.33	15.00 ± 1.22	0.83	0.00 ± 0.00	0.33	125.000	4	0.000**
30% HANPs	0.00 ± 0.00	0.33	14.20 ± 1.10	0.83	0.00 ± 0.00	0.33	140.625	4	0.000**
40% HANPs	0.00 ± 0.00	0.33	15.00 ± 0.71	0.83	0.00 ± 0.00	0.33	140.625	4	0.000**
50% HANPs	0.00 ± 0.00	0.33	14.20 ± 1.10	0.83	0.00 ± 0.00	0.33	140.625	4	0.000**
2%IONPs	0.00 ± 0.00	0.33	14.00 ± 1.41	0.83	0.00 ± 0.00	0.33	140.625	4	0.000**
5%IONPs	0.00 ± 0.00	0.33	14.00 ± 1.41	0.83	0.00 ± 0.00	0.33	140.625	4	0.000**
7.5%IONPs	0.00 ± 0.00	0.33	14.00 ± 0.71	0.83	0.00 ± 0.00	0.33	140.625	4	0.000**
12.5%IONPs	0.00 ± 0.00	0.33	13.20 ± 0.45	0.83	0.00 ± 0.00	0.33	225.000	4	0.000**
15%IONPs	0.00 ± 0.00	0.33	13.60 ± 0.89	0.83	0.00 ± 0.00	0.33	140.625	4	0.000**

**Highly significant, EZ= Effect size, BDM= Brunner, Dette, Munk rank-based ANOVA

Table 5: Multiple comparisons of inhibition zone among groups of hydroxyapatite and iron oxide nanoparticles with chlorhexidine and de-ionized water

Groups Concentration	Groups				CHX	
	CHX		DW		DW	
	P(x<y)	p-value	P(x<y)	p-value	P(x<y)	p-value
1% HANPs	1	0.04*	0	0.99	0	0.04*
10%HANPs	1	0.04*	0	0.99	0	0.04*
20%HANPs	1	0.04*	0	0.99	0	0.04*
30% HANPs	1	0.04*	0	0.99	0	0.04*
40% HANPs	1	0.04*	0	0.99	0	0.04*
50% HANPs	1	0.04*	0	0.99	0	0.04*
2%IONPs	1	0.04*	0	0.99	0	0.04*
5%IONPs	1	0.04*	0	0.99	0	0.04*
7.5%IONPs	1	0.04*	0	0.99	0	0.04*
12.5%IONPs	1	0.04*	0	0.99	0	0.04*
15%IONPs	1	0.04*	0	0.99	0	0.04*

* Significant

DISCUSSION

Result showed that the mean values of inhibition zones for all concentrations of hydroxyapatite nanoparticles used in the study were zero, this indicates a complete resistance of the bacteria to the agent. This may be attributed to the dissolution property of the hydroxyapatite nanoparticles in solution, in which these nanoparticles have high crystallinity, so lower solubility and lower rate of dissolution, hence no antimicrobial activity [26]. For iron oxide nanoparticles, the inhibition zones started to appear when reaching the concentrations of 17.5%, 20% and 22.5% and their mean values were increased with the increase in the concentration with a highly significant difference and the maximum value was at 22.5% followed by 20% then 17.5% with a percentage of 100% and this could be attributed to the antibacterial activity of iron oxide nanoparticles that is poorly understood, but the most accepted mechanisms may be: oxidative stress and metal ion release. Reactive oxygen species induced oxidative stress is an important antibacterial mechanism for nanoparticles, it is a generic term for molecules and reactive intermediates that have strong positive redox potential, and different types of nanoparticles produce different types of reactive oxygen species by reducing oxygen molecules and whenever the concentration of nanoparticles increase, the amount of reactive oxygen species increased so increasing antimicrobial activity. The four reactive oxygen species types are the superoxide radical (O^{2-}), the hydroxyl radical (-OH), hydrogen peroxide (H_2O_2), and singlet oxygen (O^2), which exhibit different levels of dynamics and activity. Under normal circumstances, the production and clearance of reactive oxygen species in bacterial cells are balanced. In contrast, with excessive production of reactive oxygen species, the redox balance of the cell favors oxidation. This unbalanced state produces oxidative stress, which damages the individual components of bacterial cells. Oxidative stress has been confirmed as a key contributor to the change in the permeability of the cell membrane, which can result in bacterial cell membrane damage. Moreover, reactive oxygen species are beneficial to increase the gene expression levels of oxidative proteins, which is a key mechanism in bacterial cell apoptosis. Furthermore, they can attack proteins and depress the activity of certain periplasmic enzymes that are essential to maintaining normal morphology and physiological processes in bacterial cells. The generation of these species degrades the active components that are responsible for maintaining the normal morphological and physiological functions of the microorganism. Metal ions are slowly released from metal oxide and are absorbed through the cell membrane, followed by direct interaction with the functional groups of proteins and nucleic acids, such as mercapto (–SH), amino (–NH), and carboxyl (–COOH) groups, damaging enzyme activity, changing the cell structure, affecting the normal physiological processes, and ultimately inhibiting the microorganism [21,27-29].

When comparing the mean values and the relative effect size of inhibition zones between chlorhexidine with each concentration of hydroxyapatite and iron oxide nanoparticles except for 20% and 22.5% of iron oxide nanoparticles, it revealed a statistically highly significant difference, and this could be attributed to a better antibacterial activity of chlorhexidine. For 20% iron oxide nanoparticles, the result was not significant and this could be explained as both agents have the same antimicrobial activity. While for 22.5%, the increase in nanoparticles concentration, could explain the result.

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الخلاصة

المقدمة: الجسيمات النانوية هي مجموعات من الذرات في نطاق حجم من (١-١٠٠) نانومتر. استخدام الجسيمات النانوية في طب الأسنان سيخلق و بشكل مذهل هياكل مفيدة من الذرات الفردية أو الجزيئات (الجسيمات النانوية)، و الذي سيوفر بديلا جديدا وربما استراتيجيات متفوقة في الوقاية والعلاج من تسوس الأسنان من خلال إدارة الأغشية الحيوية لطب الأسنان. اجريت هذه الدراسة لاختبار

حساسية بكتريا المكورات العقَدية الميوتانز لتراكيز مختلفة من الهيدروكسي اباتيت النانوي و أوكسيد الحديد النانوي ، بالمقارنة مع الكلورهكسيدين (٠,٢%) و الماء غير الايوني.
المواد والطرق: تم اختبار حساسية الميوتانز للتراكيز المختلفة من الهيدروكسي اباتيت النانوي و أوكسيد الحديد النانوي مختبريا (خارج الفم), بطريقة الانتشار من الحفر في الوسط البكتيري وتم استخدام مادة الكلورهيكسدين في هذه الدراسة كمجموعة ضابطة موجبة بينما الماء الغير الايوني استخدم كمجموعة ضابطة سالبة. تم قياس منطقة المثبطات والتي هي منطقة عدم نمو البكتيريا عبر قطر كل حفرة، عدم وجود منطقة بكتريا مثبطة يشير الى مقاومة البكتريا بشكل كامل للعوامل.
النتائج: قيم منطقة التثبيط لجميع تراكيز الهيدروكسي اباتيت النانوية صفر. أما بالنسبة لأوكسيد الحديد النانوي ، فقد كانت القيم صفر حتى وصلت إلى التراكيز الثلاثة الأخيرة، حيث كان هناك زيادة في قطر منطقة التثبيط مع وجود فرق احصائي عالي. عند إجراء المقارنات لمناطق التثبيط بين المجموعات، أظهرت النتائج أن مناطق تثبيط ١٧٪ و ٢٠٪ و ٢٢,٥٪ من أوكسيد الحديد النانوي كانت أكثر من جميع التراكيز الأخرى التي لم يكن لها مناطق تثبيط مع فرق احصائي. كان هناك فرق احصائي عالي بين كل تراكيز الهيدروكسي اباتيت وأوكسيد الحديد النانوي مع الكلورهيكسيدين والماء الغير الايوني.
الاستنتاج: لم تكن المكورات العقدية حساسة للهيدروكسي اباتيت النانوي ، حيث كانت هناك مقاومة كاملة للعامل. أما بالنسبة لأوكسيد الحديد النانوي ، فقد كانت المكورات العقدية حساسة للتراكيز ١٧,٥٪ و ٢٠٪ و ٢٢,٥٪ وزادت الحساسية بزيادة التراكيز مع وجود فرق إحصائي عالي وهذا يشير إلى ان لهذه المادة نشاط مضاد للجراثيم.