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Effect of Biotitania and Titania Addition on Bioactivity and Antibacterial Properties of Calcium Silicate Cement

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ABSTRACT

Introduction: Nanoparticles are gaining more interest in dentistry for their antimicrobial, physical as well as other properties. This study aimed to evaluate the effect of adding two types of nanoparticles (NPs) on calcium silicate hydraulic cement's (CSHC) unique bioactivity and antibacterial properties. **Methods and Materials:** Biotitania/AgCl NPs were synthetized and characterized for its morphology, types of formed functional groups and crystalline AgCl using field emission scanning electron microscope (FE-SEM) equipped with energy-dispersive X-ray spectroscopy (EDS), X-ray diffractometer (XRD), Fourier transformation infrared spectroscopy (FT-IR) and thermo-gravimetric analysis (TGA). The former NPs and commercial titania (TiO2) NPs were added (0.5, 1.5 and 3-weight %) to commercial CSHS powder. A total of 140 disk-shaped specimens (10 mm×1 mm) were prepared (seven material groups per each test in addition to the eighth cell control group) to evaluate cell viability and alkaline phosphatase activity (ALP) after 3 and 12 days, respectively. All were incubated with mesenchymal stem cells. Antibacterial efficacy against Streptococcus mutans (S. mutans) was evaluated through the bacterial growth curve slopes while being in direct contact with the tested material groups for 18 h. One-way analysis of variance (ANOVA) and post hoc Tukey's tests were used to analyze the obtained data. **Results**: Addition of all NPs percentages had no significant effect ($P>0.05$) on cell viability in comparison to positive control CSHC. Commercial TiO2 NPs (0.5 weight %) had statistically significant lower values ($P\leq0.05$) for bacterial growth curve slope. However, addition of all NPs percentages had significantly improved ($P\leq0.05$) the ALP activity of CSHC with the most prominent effect to 3-weight% biotitania/AgCl NPs. **Conclusion**: Based on this in vitro study, addition of biotitania/AgCl NPs up to 3-weight% significantly improved the bioactivity of CSHC without having a significant negative impact on its antibacterial efficacy. Interestingly, the addition of commercial TiO2 even in small amounts can significantly improve CSHC antibacterial efficacy.

Keywords: Antibacterial Efficacy; Bioactivity; Biotitania; Calcium Silicate Cements; Silver Chloride; Titania

Introduction

Biodentine is a new bioactive calcium silicate hydraulic cement (CSHC). It has been lately introduced to the market in attempt to improve some physical properties of the commonly known mineral trioxide aggregate (MTA) and extend its applications [1]. The former sets in approximately 12 min and is effective in pulp therapy while being able to act as a coronal dentine replacement. It was also used as reparative material in perforations, internal/external resorptions, apexification, and retrograde fillings [1, 2] It was reported to have a significant antibacterial effect on against *Enterococcus faecalis (E. faecalis)* [3] high flexural [4], and compressive strengths. It had a good sealing ability with less microleakage [5], except when used for root-end filling [6].

There were several trials adding nanoparticle (NPs) to improve physicochemical and biological properties of hydraulic types of cement like MTA, modified Portland cement, and few on Biodentine [7-10]. However, there is still a need to improve the performance of such CSHC especially for its reported lower radiopacity values within both *in vivo* and *in vitro* studies [5, 11].

Furthermore, it had poor bond strength with composites in its early stage after setting [12]. Several trials were done evaluating the appropriate radiopacifier in addition to improving the physicochemical properties of calcium silicate-based cements. For example, silver NPs especially for their antibacterial effect [13], nano and micro-particles of niobium oxide with calcium silicate endodontic sealer and MTA where it promoted its cell biocompatibility [14]. It was recommended to study the impact of various NPs addition on the biocompatibility and antibacterial efficacy of CSHS [15]. Titania (TiO_2) was reported as a radiopaque agent and filler for endodontic sealers and cements [16]. TiO_2 NPs are chemically stable, biocompatible, and non-toxic [17] with reported antibacterial effect [18]. TiO_2 synthetized from biological source (biotitania) is more acceptable as an environment-friendly or green chemistry process. It is regarded as safe, cost-effective, biocompatible and non-toxic sustainable. A vast array of natural biological resources is available in nature like living plants, fungi and yeast [19]. Combination of both TiO_2 and synthetized biotitania in addition to silver NPs with CSHC may adversely affect its positive biological properties. As a result, the objective of the present study is to assess the cytotoxicity, osteogenic potential, and antibacterial efficacy of CSHC after the addition of both TiO_2 and synthetized biotitania combined with silver NPs.

Materials and Methods

A CSHC (Biodentine; Septodont, Saint-Maur-des-Fosses, France) was used in the present study. Two types of NPs powder were used, TiO_2 NPs (Batch number: MKBC-4174, Sigma Aldrich, Steinheim, Germany) with particle size≈21 nm) and synthetized (biotitania/AgCl) NPs (Table 1).

For the preparation of biotitania/AgCl (silver chloride) NPs; Titanium bis (ammonium lactato)-di-hydroxide (TiBALDH, Sigma-Aldrich, Steinheim, Germany) and lysozyme were used. Biotitania/AgCl NPs were prepared according to methods adapted according to previous literature [20]. Briefly, solution A was prepared by adding 2 mL of 100 mg/mL of lysozyme to 18 mL of 100 mM TiBALDH prepared in 0.1 M Tris-HCl buffer. In a separated beaker, solution B was prepared by adding 2 mL of 10 mM silver nitrate solution to 10 mL of 10 mg/mL lysozyme dissolved in Tris-HCl buffer; pH=8. Thereafter, few drops of 1M NaOH were added to solution B until the pH reached 12. To reduce ionized silver ions, 40 µL of 10 mM of ice-cold sodium borohydride was drop-wise added and then the solution was left stirring for 15 min. Then, the reduction reaction was stopped by adding 1 mL of 1 M acetic acid. Finally, solution B was added to solution A and left stirring for 1h, the final orange precipitates were washed 3 times with distilled water, centrifuged at 14,000 rpm, and lyophilized for 2 days. Characterization of the morphology of the synthetized NPs, types of formed functional groups and AgCl crystalline phase were done using field emission scanning electron microscope (FE-SEM, Zeiss, Oberkochen, Germany) equipped with energy-dispersive X-ray spectroscopy (EDS, OXFORD X-Max, UK), X-ray diffractometer (XRD; Rigaku, Tokyo, Japan) with the 2θ range from 20 to 60 degree, with a step size of 0.02 and a scanning speed of 8 degree/min), Fourier transformation infrared spectroscopy (FT/IR 4600, FT-IR; Jasco, Tokyo, Japan), and thermo-gravimetric analysis (TGA) (Q5000IR; TA instruments, Newcastle, DE, USA) under a stream of air from 50° C to 900° C with a heating rate of 10°C/min.

Table 1. Materials used in the present study

Material	Composition	Manufacturer
Biodentine	*Powder;* tricalcium silicate, calcium carbonate & zirconium oxide, iron. *Liquid;* water, calcium chloride (accelerator) and a hydrosoluble polymer (modified polycarboxylate) that serves as a water reducing agent.	Septodont, St. Maurdes Fossis, France. Batch No. B16479
Titania (TiO_2)	TiO_2 nanoparticles	Sigma-Aldrich, Germany
Biotitania ($BioTiO_2$)	Biotitania doped with silver chloride nanoparticles	Synthesized

Table 2. Mean and standard deviation (SD) of bacterial growth rate indicated by the slope values of linear portion in growth curves for Biodentine with $BioTiO_2$ TiO_2 NPs

Group	Mean value (slope of linear potion of curve)	Group	Mean value (slope of linear potion of curve)	*P*-Value
Positive Control	0.042 (0.00079) ^{A}	Positive Control	0.042 (0.00079) ^{A}	≤0.05
Biodentine	0.002 (0.0002) ^{B}	Biodentine	0.002 (0.0002) ^{B}	
Biodentine+0.5% $BioTiO_2$	0.005 (0.0003) ^{C}	Biodentine+0.5% TiO2	0.001 (0.00006) ^{C}	
Biodentine+1.5% $BioTiO_2$	0.0058 (00006) ^{C}	Biodentine+1.5% TiO2	0.0016 (0.00006) ^{BC}	
Biodentine+3% $BioTiO_2$	0.0083 (00000) ^{D}	Biodentine+3% TiO2	0.0025 (0.0002) ^{B}	

P≤0.05 means there is a significant difference and the letters are for Tukey's test

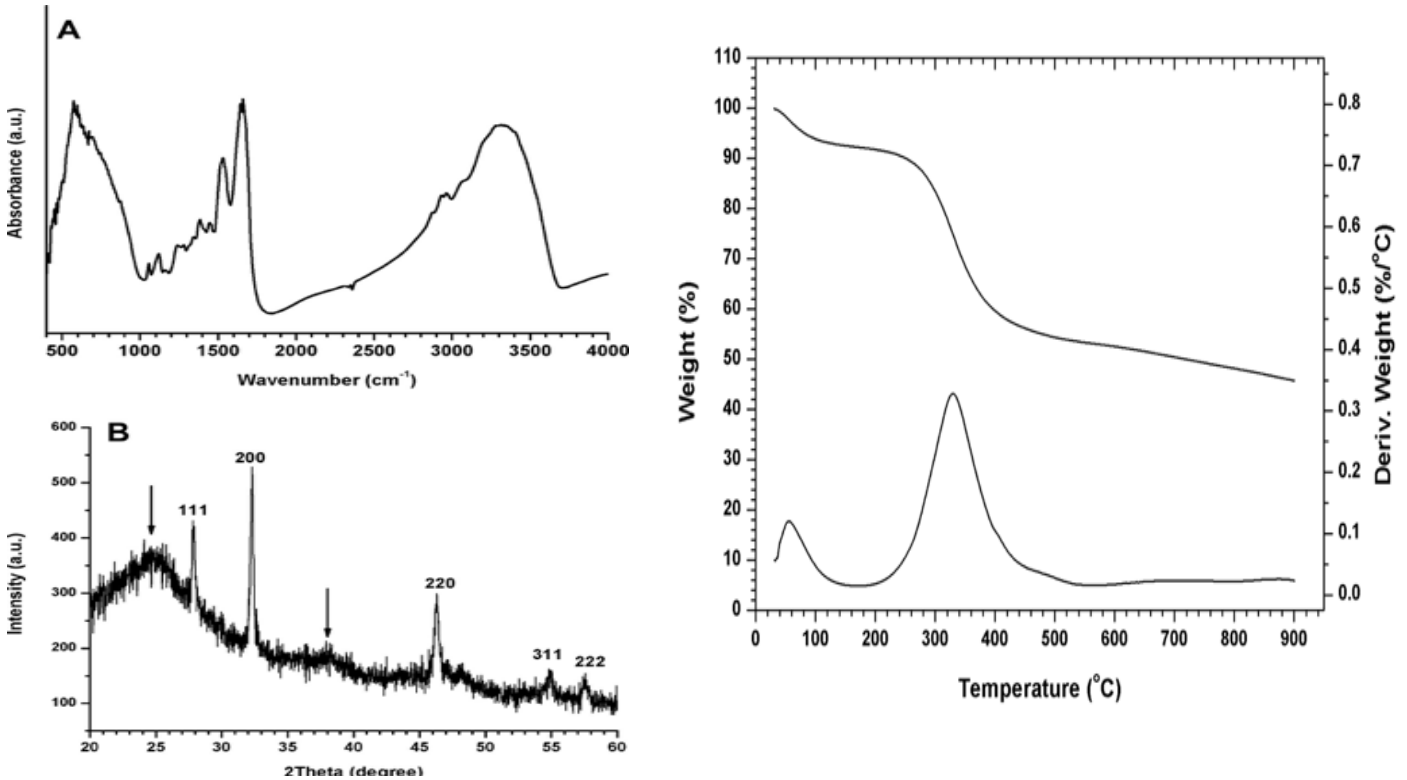

Figure 1. Characterization of synthetized biotitania/AgCl NPs. Left image shows FTIR spectrum. *A*) shows the appearance of amide I and II peaks of lysozymes at 1658 and 1530 cm^{-1}, respectively and XRD diffraction pattern; *B*) showing broad peaks (arrows indicated zones) that correspond to amorphous TiO_2 and sharp overlapped peaks at 2**θ** of 27.8°, 32.2° & 46.2° corresponding to cubic silver chloride crystalline phase. Right image, *C*) Therrmo-gravimetric analysis (TGA) showing two weight loss curves as the temperature increased to 900°C, first region (30 -150°C) that is related to water loss and second region (200- 450°C) that is related to weight loss of 35 % due to the decomposition of lysozyme

Specimens preparation and mesenchymal stem cells (MCs) isolation:

A split Teflon mold (10 mm diameter and 1 mm thickness) was used to prepare 140 disk-shaped specimens of CSHC. Six groups (for each type of NPs) were created according to the added percentages of TiO_2 and synthetized biotitania/AgCl NPs (0.5, 1.5 and 3%). A number of 60 (20 for each added % percentage to be used for testing MTT assay and osteogenic effect, *n*=20) disks were used for addition of each type of NPs with the mentioned percentages. The last twenty specimens were two control groups made of only CSHC for both MTT and bioactivity tests. The NPs of different percentages were added to CSHC powder capsules and mixed with its liquid according to the manufacturer's instructions.

The mesenchymal stem cells (MSCs) were prepared at Medical Experimental Research Centre (MERC), Mansoura University, Egypt. They were isolated from 6-8 weeks old Sprague-Dawley female rat bones marrow under the approval of Ethical Comity (number. 1480518). Afterwards, the bone was flushed with low glucose (1000 mg/L)-Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin, 1% streptomycin and 2 mM glutamine, all were purchased from Lonza, Basel, Switzerland. The cells were cultured in a controlled environment of 5 % CO_2 at 37 °C. The cells were sub-cultured when they reached 70-80% confluence, and cells of passages 3-4 were used for the experiment.

MTT assay

Seventy disks were used for the test (a total number of seven materials group, 10 per each group, 6 groups with added different percentages of added NPs and seventh material control group without additions. The eighth group was positive control cell group without using any of tested materials). The cytotoxicity of different groups was assessed by the MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium assay. A number of 0.1×10^6 MSCs sub-cultured was seeded on materials' disks of different groups within 48 well plates. Cells seeded on the culture plates without tested material was used as a control group. After three days of incubation at 37°C, 5% CO_2 and 100% humidity, cytotoxicity was measured using thiazolyl blue tetrazolium bromide or MTT (Bio Basic

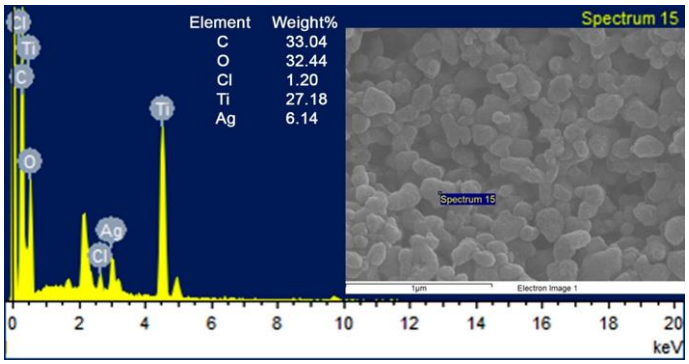

Figure 2. Spot scan energy-dispersive X-ray spectroscopy (EDS), spectra of biotitania/AgCl NPs showing the elemental compositions and their corresponding weight percentage values of each element with high elemental weight% of both Ti and O and lower weight% for Ag

Canada Inc. Biotechnology, Markham, Canada). MTT stock solution (5 mg/mL) was diluted 1:10 by adding 200 µL of MTT stock into 2 mL of cell culture medium with concentration and incubated for 4 h at 37° C in incubator. Re-suspend formazan crystals (MTT metabolic product) in 200 µL DMSO (dimethyl sulfoxide) was placed on a shaking table, 150 rpm for 5 min, to thoroughly dissolve formazan into the solvent. The optical density at 560 nm was detected using Elisa reader (Mindary Reader MR-96A, Biomedical Electronics Shenzhen, China) and subtracted from the background at 620 nm.

Alkaline phosphatase (ALP) activity and Alizarin red staining

Before seeding the MSCs, 70 prepared CSHC disks (for seven material testing groups) of the tested materials were placed in six-well culture plates and treated for 2 h with 1% antibiotic/antimycotic solution. After disinfection, they were washed twice with phosphate-buffered saline (PBS) and finally pre-wetted with culture media. Afterwards, specimens received a number of 3×10^4 MSCs in each 1 mL of osteogenic media with composition of 10 % FBS, 1% penicillin, 1% streptomycin and 2 mM glutamine (Lonza, Basel, Switzerland), 50 µg/mL ascorbic acid, 10 mM β-glycerophosphate, and 10 nM dexamethasone (Sigma-Aldrich, Steinheim, Germany). The cells were allowed to be in direct contact with the material and incubated for 8 h prior to observation. The culture medium and the osteogenic supplements were refreshed every 2 days. After culturing for 12 days, osteogenesis was assessed by Alizarin Red staining and evaluation of ALP activity. An eighth control group of MSCs without tested material was added.

For Alizarin red staining, MSCs were first washed with PBS and fixed in 10% (v/v) formaldehyde (Sigma, Chicago, USA). After 15 min, 2% Alizarin Red solution at pH 4.1 was added to each flask. The cells were incubated at room temperature for 20 min and then they were washed four times with distilled water for 5 min per each washing time. Digital pictures of the proliferated

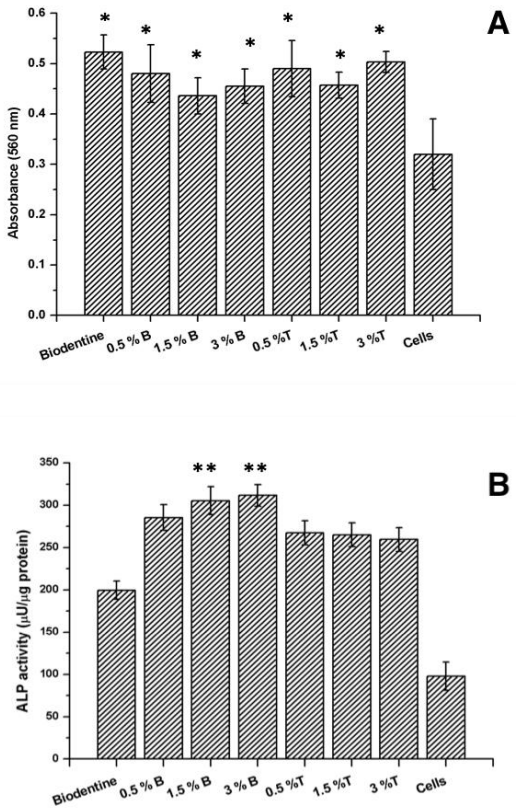

Figure 3. Bar charts showing cytotoxicity of all groups; *A*) Through MTT assay, after three days of incubation with mesenchymal stem cells (MSCs), there was no statistical significant difference within all groups (*) except for the control cell group; *B*) ALP activity of all groups after 12 days of incubation with MSCs. ** means they have the highest statistically significant and mean values. The 0.5, 1.5 and 3% B are for CSHC (Biodentine)+different percentages of biotitania. 0.5, 1.5 % and 3 % T are for CSHC (Biodentine)+different percentages of Titania

cells after osteogenic differentiation at the surrounding area of the specimens were taken using an inverted light microscope (Olympus America Inc., Pennsylvania, USA). The pictures were taken with 4× and 10× magnifications of the lens.

For ALP, specimens were washed with PBS, and the cells were trypsinized and the cell pellets were re-suspended in 100-200 µL of the lysis buffer, and then centrifuged for 15 min at 2000×g. The ALP activity was assayed utilizing the conversion of a colorless p-nitro-phenyl phosphate to a colored p-nitro-phenol according to the manufacturer's protocol (Sigma, St. Louis, MO, USA). The color change was measured spectrophotometrically at 405 nm (Spectra III, TECAN, Salzburg, Austria), and the alkaline phosphatase levels were normalized to the total protein content of cells measured by BCA assay Kit (Sigma, St. Louis, MO, USA) according to the company's guidelines. The results were expressed in µU (micro-unit) of yellow p-nitro-phenol per microgram (µg) of protein. A similar protocol was followed in a previous work [21].

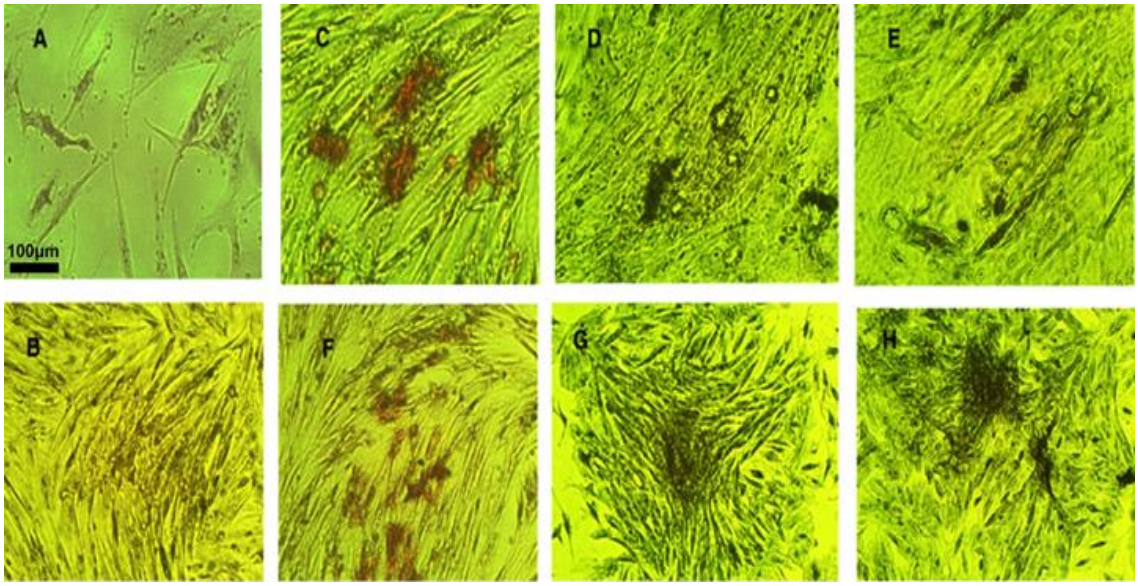

Figure 4. Inverted light microscope pictures (x10 magnifications) after 12 days of incubation within osteogenic media. They show appearance of calcium deposits, irregular and flatter cell appearance for calcium silicate hydraulic cement's (CSHC) (B), titania and biotitania with small weight% (0.5) in *C* and *F*), respectively, than control cell group (A). Other groups with titania (D and E, 1.5 & 3 weight%, respectively) and with biotitania (G and H, 1.5 & 3 weight%, respectively) showed flat cell appearance with starting of cellular colonization, especially for higher biotitania percentages

Antibacterial activity with Direct Contact Test (DCT)

This test was done for assessing the impact of both types of NPs addition on the antibacterial effect of such CSHC. The DCT relays on the evaluation of turbidity related to bacterial growth in 96-well microtiter plates [22]. *Streptococcus mutans* (*S. mutans*) (ATCC 25175) was cultured in 5 mL of brain-heart infusion broth (BHI) (Oxoid, Basingstoke, England) and incubated overnight at 37° C. Prior to testing, bacterial culture was centrifuged at 3000 rpm for 10 min and cells were re-suspended in fresh media to an optical density at 650 nm (OD650) of 0.5. Afterwards, this suspension was 10 folds serially diluted and plated on BHI agar to determine colony-forming units (CFU). In a 96 well microtiter plate (Nunc, MaxiSorp, Denmark), each row represented one testing group. Within each row, three sets of four adjacent wells (first, middle and last) were created. When testing procedures started, CSHC capsules, either control or with added NPs different percentages, were mixed according to manufacturer's instructions. The sidewalls of the first well sets within seven rows were coated evenly with an equal amount of tested materials while holding the plate vertically. After complete CSHC setting according to manufacturer's recommendations, 10 µL of the bacterial suspension (10^6 CFU) was placed on each well of the first set in each row and incubated for one h at 37°C while the plate is still in a vertical position to ensure the direct contact between the bacteria and the tested materials. Afterwards, 245 µL of BHI broth was added to each well of the first set and mixed well. Then, 15 µL were transferred from the wells of the first set to the adjacent middle set of four wells containing 215 µL of fresh BHI broth, respectively. The last set received 230 µL of un-inoculated BHI broth and served as a negative control. Positive control was included in a separate eighth row in the same microtiter plate, which consisted of 2 sets of uncoated wells, that were treated the same as the first and middle experimental sets. The bacterial outgrowth was estimated, after DCT for all tested groups, depending on the changes in the readings of optical density (OD) at 650 nm. The OD readings were evaluated using spectrophotometer (ELISA micro-titer plate reader model 608, Bio-Rad) every 2 h for 18 h at 37°C. The negative control wells' values were considered as the baseline as they were subtracted from the respective experimental data. A regression line was calculated using the ascending linear portion of the curves for each well [18].

Statistical analysis

Statistical analysis was performed using SPSS 16.0 (SPSS, Chicago, IL, USA) for Windows. One-way analysis of variance (ANOVA) was used to analyze the results of MTT assay, ALP enzyme activity and growth curve slopes of *S. mutans* for all tested groups. Before that, data were evaluated for normal distribution using Shapiro and Wilk test. Post hoc Tukey's test was used afterwards. Significance was determined at P-value<0.05. Results were expressed as mean±standard deviation (SD).

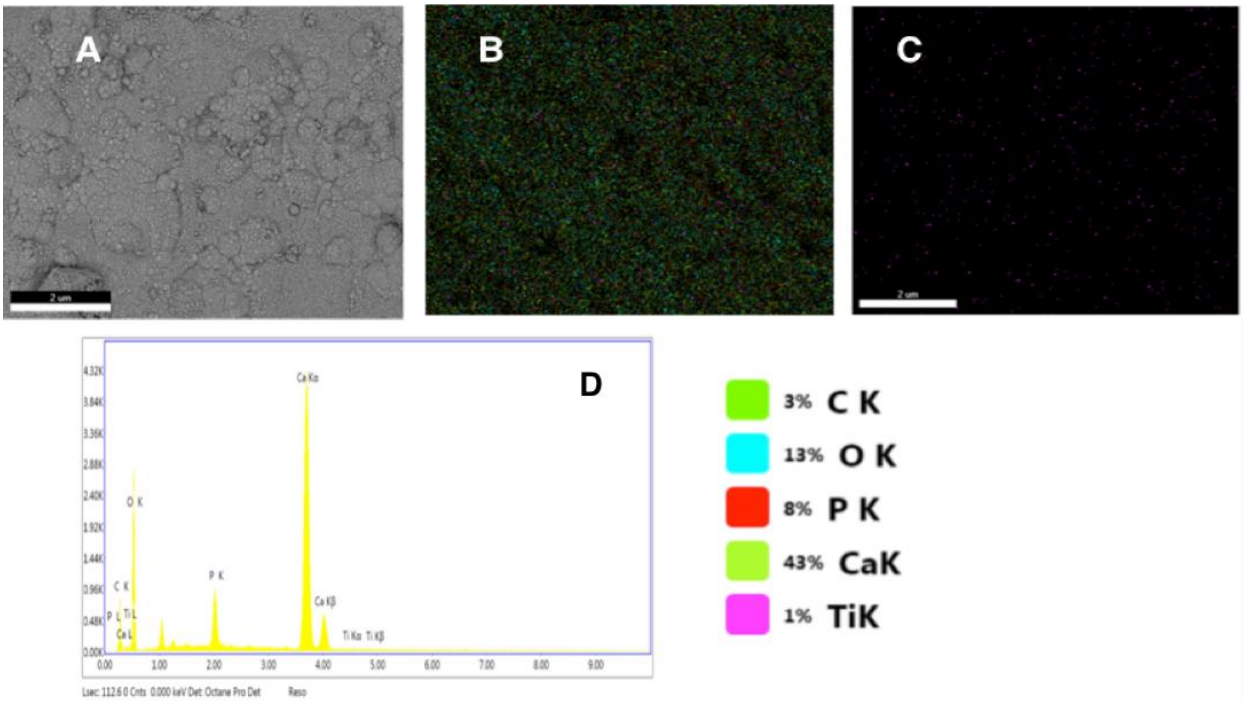

Figure 5. Surface mapping of calcium silicate hydraulic cement (CSHC) with 3-weight% biotitania NPs addition, *A*) showing high elemental concentrations of Ca and P; *B*), in addition to appearance of small weight% Ti on the surface ;*C*) after 12 days of incubation in osteogenic media; *D*) Represents surface elemental peaks by energy-dispersive X-ray spectroscopy (EDS). Surface elemental weight% are color coded

Results

Characterization of synthetized NPs

The presence of lysozyme attaching both amorphous titanium and AgCl NPs together was confirmed by Fourier transformation infrared spectroscopy (FTIR) analysis in figure (1-A). The appearance of intense amide I and II peaks at 1658 and 1530 cm^{-1}, respectively, confirms the presence of lysozyme on the surface of the particles [23]. The X-ray diffractometer (XRD) analysis (Figure 1B) shows broad peaks, indicated by arrows that correspond to amorphous TiO_2. On the other hand, sharp overlapped peaks at 2**θ** of 27.8º, 32.2º & 46.2º are indexed to be cubic silver chloride crystalline phase [24]. Thermo-gravimetric analysis (TGA) and its corresponding derivatives weight loss curves showed two mean regions for weight loss as the temperature increased to 900º C. The first region was between 30 and 150º C that is related to the loss of adsorbed water. However, the second region was detected between 200 and 450º C with a total weight loss of 35 % due to the decomposition of lysozyme. After 500º C, about 8 % of weight loss was detected, probably due to the transformation of anatase to rutile phase. TiO_2 NPs is found in three phases including anatase, rutile and brookite. Anatase is the predominant commercial phase of TiO_2 which is also widely used for photocatalysis [25]. Rutile is a stable phase while anatase is a metastable one which means that anatase can transform to rutile by heating [26]. Thus, during characterization of the formed biotitania NPs phases by TGA, this weight loss above 500º C was attributed to the latter phase transformation (Figure 1C). There was high weight % of both Ti and O (27.18 & 32.44, respectively) on elemental examination using energy-dispersive X-ray spectroscopy (EDS), while the weight % of silver was recorded to be only 6.14 % (Figure 2).

Cytotoxicity and ALP activity

CSHC control group showed the highest mean value of cell viability (OD.56±0.034) followed by group of 0.5 % addition of TiO_2 NPs (OD=0.55±0.056). There was no statistically significant difference (P>0.05) within all groups except for the control cell group (Figure 3A). CSHC with the addition of 1.5 and 3 % biotitania NPs showed the highest statistically significant (P≤0.05) ALP activity values (305.49±16.4 and 311.8 ±12.8 µU/µg protein, respectively). The groups with biotitania/AgCl NPs addition showed the highest statistically significant increase (P≤0.05) in ALP activity than other groups (Figure 3B). Figure 4 shows the flatter and irregular appearance of MCSs with appearance of Ca deposits especially in TiO_2 and biotitania with small weight% (0.5) (Figure 4C and 4F) than control cell group (Figure 4A). Higher weight% (1.5 and 3) of

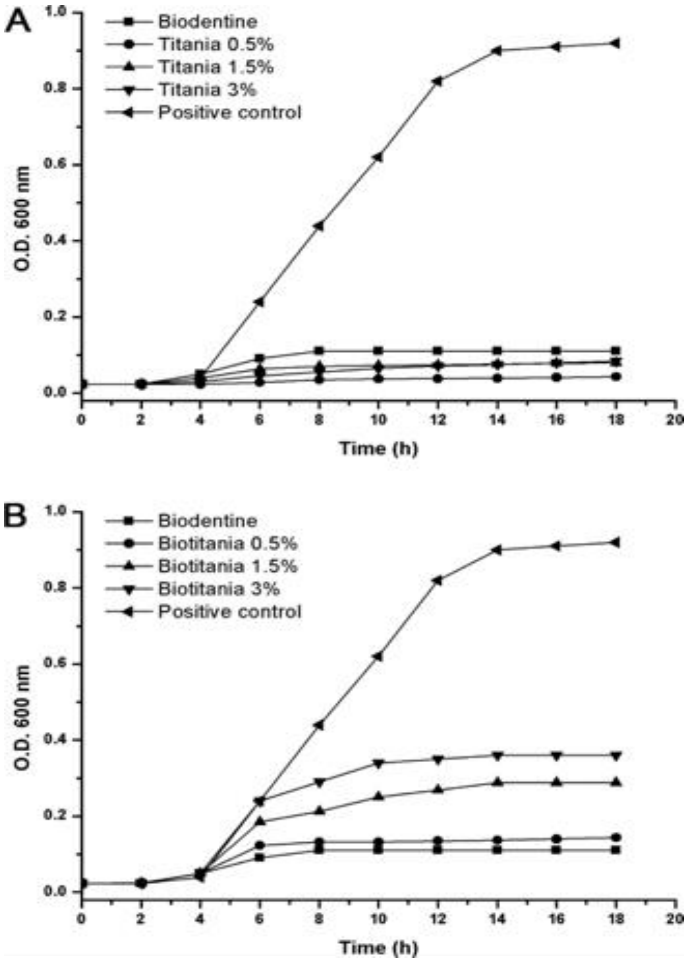

Figure 6. Bacterial growth curves of Direct contact antibacterial activity (DCT) for control groups (*S.mutans* and calcium silicate hydraulic cement's (CSHC)) and CSHC after addition of biotitania NPs. A curve shows almost flat bacterial growth curves especially for lower weight% of titania. B curve represents bacterial growth curves for CSHC mixed with titania NPs showing higher slopes of bacterial growth curves especially for higher weight% of Biotitania, all are after 18 h of bacterial incubation

TiO_2 and biotitania (Figure 4D-G) show flat cell appearance with starting of cellular colonization, especially for higher biotitania percentages. After 12 days in osteogenic medium, the morphology of CSHC surface incorporated with 3 weight% biotitania/AgCl NPs is shown in SEM micrograph (Figure 5A), indicating partial erosion of the surfaces of CSHC disk which lead to the detection of titanium element as indicated by EDX mapping in Figures 5B-D. Titanium appeared on the surface with low weight percentage and random distribution.

Direct contact antibacterial activity

CSHC with addition of 0.5 % TiO_2 NPs showed the lowest statistically significant ($P \leq 0.05$) mean curve slope value ($0.001 \pm 6 \times 10^{-5}$) for bacterial growth. Addition of TiO_2 NPs with 1.5 % showed the second-lowest curve slope value ($0.0016 \pm 6 \times 10^{-5}$) with no statistical significant difference ($P > 0.05$) than CSHC and 0.5 TiO_2 NPs addition groups. Table 2 shows the slopes of the linear portion for all drawn growth curves of the same test group were measured using excel software (they resemble the specimens within this group). Afterwards, the mean values, standard deviations using one way ANOVA for all tested groups slopes values as well as post-hoc *Tukey's* test were calculated using SPSS software. All tested groups showed statistically significant ($P \leq 0.05$) bacterial inhibition activity than the control bacterial growth curve of *S. mutans* (Figure 6A and 6B).

Discussion

Metallic NPs were used to enhance the physicochemical, mechanical and antibacterial properties of dental materials e.g. silver NPs [15], titanium/TiO_2 [27] and zirconium oxide [28]. This study tried to apply the nanotechnology approach to Biodentine™ using two types of NPs. It monitored the impact of adding NPs by 0.5, 1.5 & 3-weight % on unique properties of Biodentine like bioactivity and antibacterial action. The current results showed the superiority of TiO_2 NPs in the antibacterial efficacy. According to previous protocols [18, 22] evaluating the antibacterial effect, testing materials while in direct with micro-organisms introduces more realistic relation to the situation intra-orally than using the disk-diffusion method in agar plates. As previously discussed in the methodology section, the s-shaped curve for bacterial growth was drawn from measured turbidity by optical density of each well, every two hours, containing the tested material (on the side wall) with bacteria in direct contact to it, and all in the growth medium for bacteria. The more turbid the medium is indicating higher bacterial growth and vice versa.

The addition of 0.5-weight % of TiO_2 showed the least mean value for the slope of *S. mutans* growth curve hence the highest antibacterial effect among tested material groups. On the other hand, increase the fraction of synthetized biotitania/AgCl NPs negatively affected the antibacterial efficacy of CSHC, especially its highest fraction (3 -weight %). This is in accordance with previous findings where TiO_2 NPs have improved antibacterial efficacy of tested dental cement [29]. On the other hand, the addition of 3-weight % of biotitania/AgCl NPs showed the highest slope value for the bacterial curve hence the least antibacterial effect of tested material groups. Despite of that, 3-weight % of biotitania/AgCl NPs still has a highly significant antibacterial efficacy on *S. mutans*. This goes hand in hand with previous XRD analysis of the commercially used TiO_2 NPs showing it as a mixture of anatase and rutile phases with predominant anatase peak intensity [30]. Crystalline TiO_2

mixture, with major anatase phase and minor rutile phase percentage, had a profound antibacterial efficacy. However, current XRD results of biotitania/AgCl NPs showed TiO_2 as an amorphous structure, not in crystalline anatase/rutile mixture, similar to commercial TiO_2. Furthermore, the source of Ag ions within the biotitania molecule was AgCl instead of metallic Ag. The former has lower solubility to ensure long life and slow release of Ag ions [31]. Therefore, this study recommends further evaluation of biotitania/AgCl NPs for long-term antibacterial efficacy reaching one week and even more. Although increasing the TiO_2 concentrations within the present study did not significantly improve the CSHC antibacterial efficacy, it did not affect it negatively either.

TiO_2 NPs type has recently gained attraction in the dental materials field. It improved the physical properties of glass-ionomer and acrylic resin besides improving the antibacterial efficacy of mouthwashes against *S. mutans* and *S. sanguis* [9]. On the other hand, biotitania/AgCl NPs was synthetized using a biological substance like antibacterial enzyme (lysozyme) and AgCl [20]. The latter is a colorless solution and regarded as a low solubility product to ensure long life and a slow release of silver ions for the antibacterial property [32]. The TGA and FTIR of the synthetized NPs confirmed the decomposition of lysozyme and its presence on the NPs surface. Previously, the synthesis of such bio-nanoparticles has led to the improvement and progress in biomimetic approaches and advancement in nanomaterials. The interaction of bio-nanoparticles with living cells and microorganisms is an expanding field of research [33].

This study results showed that synthetized biotitania/AgCl had the most positive effect on the bioactivity of CSHC. The 3-weight % of biotitania/AgCl had a statistically significant increase in ALP activity. Both types of NPs, up to 3-weight %, had no statistical significant difference in cytotoxic effect from CSHC control group. This is not in agreement with previous work [34] where the cell viability was dependent on dose and exposure time of tested NPs including TiO_2 as cell death increased with higher NPs concentration and time. That could be attributed to the difference in the form of NPs that was being tested. As this study, another study [34] used suspension form of prepared NPs only while the current study used solid disks of CSHC with added fractions of tested NPs. That may give much more role to CSHC to have an impact on the cytotoxic effect. Based on the fact that Biodentine and MTA have the same basic constituents (Tri-calcium and di-calcium silicate), set through hydration reaction in presence of water [35] and there is no previous biological evaluation for TiO_2 additions to Biodentine, the results of the same previous studies on MTA were used as references. Former combination of up to 1 weight% TiO_2 with MTA did not significantly decrease the human gingival fibroblasts cell viability which is in accordance with the present study cytotoxicity results [9]. Furthermore, accelerating the setting time of modified Portland cement with TiO_2 NPs had improved its cell viability, which may also be applicable in Biodentine too, which has a short setting time (up to 12 min) [1, 35]. In addition to the latter findings, former strategies to functionalize TiO_2 NPs with biomolecules have proved to increase their bioactivitywhich goes hand in hand with the FTIR results of synthetized biotitania/AgCl NPs. The latter results confirmed the appearance of intense amide I and II peaks at 1658 and 1530 cm^{-1}, respectively, which confirms the functionalization of the NPs surface with lysozyme [23].

Although previous *in vivo* results focused on tissue reaction to MTA and Portland cement modified with TiO_2, they did not show the role of TiO_2 NPs (up to 1.5-weight %) in calcification or hard tissue deposition by these hydraulic cements [36]. However, the significant improvement of the bioactivity with both NPs types used in the current study may be related to the following factors. The fact that during osteogenic differentiation, cells express a sequence of osteogenesis-related proteins, including ALP, type I collagen (COLI), bone sialoprotein (BSP), osteocalcin (OCA), and osteopontin (OPN) [37]. These proteins build bone matrix, which sets a basis for the upcoming mineralization process. At the last stage of osteogenesis, the matrix is mineralized by the deposition of hydroxyapatite [38]. The ALP activity results showed 2 folds increase for CSHC sample as compared to stem cells cultured in osteogenic medium for 12 days, which is consistent with another published study [39]. The addition of different weight percentages of commercial titanium dioxide nanoparticles to CSHC resulted in a further increase in ALP activity, probably due to the ability of titanium dioxide nanoparticles to support stem cell adhesion and differentiation as has been reported earlier [40]. In our study, the staining of bone nodules is obvious in case of 0.5 % TiO_2 and biotitania; however, if we look carefully to biotitania (1.5 and 3-weight% groups, respectively) we can find that the cells are starting to form colonies which will, in turn, lead to large and dense bone nodule formation. Given that the duration of our culture is 12 days and the required period for the bone like nodules to completely form is not less than 21 days, we can assume that the addition of biotitania/AgCl NPs to CSHC stimulates stem cells differentiation and mineralization when compared to TiO_2 NPs [41, 42].

There are two factors that might contribute to the increase in ALP activity upon the interaction of stem cells with CSHC disks containing biotitania/AgCl nanoparticles. First, the amorphous nature of biotitania might be favorable to reduce the oxidative

stress in stem cells surrounding environment, which was reported to impair osteogenic differentiation of adult stem cells [41]. The second possible reason is the presence of AgCl NPs linked to the surface of biotitania by lysozyme which may have a stimulatory effect on stem cell differentiation. It was reported that Ag NPs promote the osteogenic differentiation of stem cells by upregulating the expression of RUNX 2, ALP, BMP-2, COLI, OCN, and OPN genes and enhancing ECM mineralization [42]. Moreover, Ag NPs improved bone fracture healing by stimulating the proliferation and osteogenic differentiation of mouse MSCs and driving the migration of MSCs and fibroblasts to the fracture sited. The low dissolution rate of silver chloride in comparison with metallic silver may cause minimum cytotoxicity to stem cells and stimulate their differentiation to osteoblast lineage [43].

MTT assay was used to evaluate cell viability in the current study, as it is simple, precise, and accessible. Furthermore, the blue formazan crystals produced by mitochondrial dehydrogenase enzymes in living cells are insoluble in tissue culture media and therefore, there is no need for the dissolving procedure [44]. The maximum percentage added of NPs types was 3 %, which could not be exceeded in order to stick to the manufacturer's instruction (5 liquid drops to each capsule powder). This 3 -weight % NPs amount did not need additional liquid drop thus had to be insignificant on setting time. Further future studies are recommended on *in vivo* evaluation of modified CSHC with both NPs within various capping and endodontic applications and on assessing their radiopacity.

Conclusion

The addition of TiO_2 NPs in a small percentage improved the antibacterial effect while higher percentages did not have additional improvement. Synthetized biotitania/AgCl NPs have significantly improved the bioactivity of CSHC, especially in their highest percentages. Both NPs did not negatively affect the cell viability and antibacterial properties of CSHC commercial products.

Conflict of Interest: 'None declared'.

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