# Microbiological evaluation for antifungal activity of some metal oxides nanofillers incorporated into cold cured soft lining materials: clinical based study

Avaliação microbiológica da atividade antifúngica de algumas nanopartículas de óxidos metálicos incorporadas em materiais de revestimento macio curados a frio: estudo clínico

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## ABSTRACT

**Objective:** The aim of the current study was to evaluate the efficacy of addition of zirconium oxide ($ZrO_2$), titanium oxide ($TiO_2$), and silica oxide ($SiO_2$) nanoparticles to cold-cured soft liner on adhesion of *Candida albicans* (CA). **Material and Methods:** Fifty-four patients had been selected and divided into three groups according to the modification of soft liner with $ZrO_2$, $TiO_2$, and $SiO_2$ nanoparticles (18 each of). Each patient received maxillary complete denture having three cavities, the cavities were lined using cold cured soft liner modified with different concentration (0%, 3%, and 7%) of metal oxide nanoparticles. On days 14 and 28, swaps were taken out from relining site and immediately cultured for fungal evaluation. The number of colonies were counted, data collected and explored for normality using Shapiro-Wilk test, logarithmic transformation of CA count was performed. Repeated and one-way ANOVA were used followed by Tukey HSD. Independent-t test used to compare between CA counts at different periods. **Results:** The CA adhesion was significantly decreased by the addition of $ZrO_2$, $TiO_2$ and $SiO_2$ nanoparticles in comparison with control group, also the antifungal coverage increased with nanoparticles concentration increased ($P<0.005$). The highest CA count was identified in group $SiO_2$ followed by $ZrO_2$, while $TiO_2$ showed the lowest CA count ($P<0.001$). **Conclusion:** Addition of different nanoparticles; $ZrO_2$, $TiO_2$ and $SiO_2$ to cold-cured soft liner is an effective method for reducing CA adhesion.

## KEYWORDS

Antifungal agent; *Candida albicans*; Denture liners; Nanoparticles.

## RESUMO

**Objetivo:** O objetivo do presente estudo foi avaliar a eficácia da adição de nanopartículas de óxido de zircônio (ZrO2), óxido de titânio ($TiO_2$) e óxido de sílica ($SiO_2$) a um material de revestimento macio curado a frio na adesão de *Candida albicans* (CA). **Material e Método:** Cinquenta e quatro pacientes foram selecionados e divididos em três grupos de acordo com a modificação do revestimento com nanopartículas de $ZrO_2$, $TiO_2$ e $SiO_2$ (18 cada). Cada paciente recebeu prótese total maxilar com três cavidades, as cavidades foram revestidas com forro macio curado a frio modificado com diferentes concentrações (0%, 3% e 7%) de nanopartículas de óxido metálico. Nos dias 14 e 28, as trocas foram retiradas do local de realinhamento e imediatamente cultivadas para avaliação fúngica. O número de colônias foi contado, os dados coletados e explorados para normalidade usando o teste de Shapiro-Wilk e a transformação logarítmica da contagem de CA foi realizada. ANOVA para medidas repetidas e de uma via (one-way) foram usados, seguidos por teste de Tukey (HSD). O teste t independente foi usado para comparar as contagens de CA em diferentes períodos. **Resultados:** A adesão do CA foi significativamente diminuída pela adição de nanopartículas de $ZrO_2$, $TiO_2$ e $SiO_2$ em comparação com o grupo controle, também a cobertura antifúngica aumentou com o aumento da concentração de nanopartículas (p <0,005). A maior contagem de CA foi identificada no grupo $SiO_2$ seguido por $ZrO_2$, enquanto $TiO_2$ apresentou a menor contagem de CA (p <0,001). **Conclusão:** Adição de diferentes nanopartículas; $ZrO_2$, $TiO_2$ e $SiO_2$ para revestimento macio curado a frio é um método eficaz para reduzir a adesão de CA.

## PALAVRAS-CHAVE

Antifúngico; *Candida albicans*; Reembasadores de dentadura; Nanopartículas.

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## INTRODUCTION

Increase the prevalence of denture stomatitis in association with Candida albicans (CA) has been reported to range between 11-67% in complete denture wearers that can be explained by the fact that dentures decrease the flow of saliva and oxygen to the underlying tissue producing local acidic and anaerobic microenvironment that favors yeast overgrowth[1].

Patients with oral candidiasis may display various symptoms including burning, painful sensation, swallowing difficulty and change of taste but most often are asymptomatic. It was showed that this oral inflammation may occur on the mandible and maxilla; however, it is more often associated with the maxilla[2].

Denture related stomatitis, even if asymptomatic, should be treated as it may act as a source of infection and encourage the alveolar bone resorption. This condition can be managed by improvement of denture and oral hygiene, correction of the adaptation of the old denture with a tissue conditioner, systemic antifungal and/or topical application of an antifungal agent when the presence of yeast has been confirmed[3].

The soft lining materials are widely used in the management of traumatized oral mucosa; they have many disadvantages including color instability, loss of resiliency, low abrasion resistance, inferior bond strength and porosity. One of the most serious problems is the surface colonization by CA and other microorganisms[4].

The systemic antifungal therapy as Amphotericin B is recommended for immunocompromised patients, but it may induce hepatotoxic and nephrotoxic furthermore, reinfection will occur within two weeks after treatment stopped [5]. The gradual release of antifungal agents through the soft liners provides an effective therapeutic concentration at the infected sites even under the diluent effects of saliva and tongue movements also, it does not require patient's cooperation. On the other hand, one of the major problems with adding antifungal to biomaterials is the negative impact on its mechanical properties, Garner et al. found that the incorporation of chlorhexidine, nystatin or miconazole into resilient materials have shown to cause significant increase in the material's hardness[6].

Many studies evaluated the effect of many disinfectants on the physical properties of temporary soft lining materials, and reported that their application significantly affects some of the physical properties of such materials as water solubility, water sorption and hardness[7,8].

Recently, nanoparticles are representing a promising antifungal agent; the mechanism of their action is completely different from traditional antibiotics and can target microorganisms compromising the development of resistant strains[9]. Furthermore, inorganic antimicrobial materials can achieve appropriate disinfection without forming harmful byproducts and are more stable than organic antimicrobial agents[10]. As a consequence, different nanoparticles have been incorporated into dental biomaterials to improve their antibacterial activity, as Ag [11], $TiO_2$ [11-13], $SiO_2$ [14-16] and $ZrO_2$ [17-19].

$ZrO_2$ nanoparticles possess remarkable antibacterial properties; Gad et al. investigated the effect of $ZrO_2$ nanoparticles on CA adhesion of cold-cured acrylic resin, and reported that CA adhesion was decreased in $ZrO_2$ specimens [17]. Also, Alaa et al. reported that the addition of $ZrO_2$ nanoparticles into acrylic-based resilient lining material could provide antifungal properties, so reducing the incidence of denture-related stomatitis[20]. $TiO_2$ nanoparticles had a wide spectrum of antimicrobial activity against a wide range of microorganisms including bacteria, fungi, and viruses due to its photocatalytic properties. The reactive oxygen (ROS) generated by $TiO_2$ can oxidize the components of the cell membrane of the bacterial cell leading to its destruction[21]. $SiO_2$ nanoparticles can offer antimicrobial action through interruption of cell functions as cell differentiation, adhesion and spreading of bacteria. Moreover, it could inhibit the adhesion of microorganisms through the modification of surface characteristics of denture materials[14,16]. Many studies reported that incorporation of silane-$SiO_2$ nanocomposite into PMMA acrylic resin resulted in a statistically significant reduction in CA adhesion [22,23].

The exact mechanism of antimicrobial activity of different nanoparticles is not fully understood, it is suggested that the inhibitory activity of nanoparticles generally can be along multiple pathways that are related to each other and in most cases occur simultaneously; reaction with peptidoglycan cell membrane;

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inhibition of protein synthesis, obstructing DNA replication and generation of reactive oxygen species (ROS) leading to cell death [13,19]. Others, suggested that there is an electromagnetic attraction between the microorganism which has a negative charge on its surface and the metal oxide nanoparticle which has a positive charge and by this attraction and surface contact, the microbe will die due to its oxidation[24].

It was reported that silanization of the nanoparticles resulting in greater surface area exposed for interaction with CA leading to enhancing their antibacterial activity, however, few studies found antibacterial activity of nanoparticles without surface treatment[20,23,25,26].

Although the *in vitro* antifungal activity of regular metal oxides have been investigated, there are no or little *in vivo* studies are available that concerning the antifungal activity of $ZrO_2$, $TiO_2$, $SiO_2$ nanoparticles, so the aim of the current in vivo study was to evaluate the efficacy of $ZrO_2$, $TiO_2$ and $SiO_2$ nanoparticles added to cold cured soft liner on CA adhesion. The null hypothesis was that the effect of metal oxide nanoparticles ($ZrO_2$, $TiO_2$ and $SiO_2$) incorporated into cold cured soft liner on CA adhesion would be insignificant.

## MATERIALS AND METHODS

Fifty-four completely edentulous patients had been selected from the clinic of Removable Prosthodontic Department, Faculty of Dental Medicine, Boys, Al-Azhar University. All selected patients were with normal ridge relationship, firm and healthy mucosa, healthy temporomandibular joint and good oral hygiene. Patients with any systemic disease that may affect the salivary flow or soft tissue health or any infectious disease were excluded. Also, patients with history of antibiotic, topical or systemic antifungal or anti-inflammatory drugs for the last 6 months were also excluded. Heavy smoker patients, patients with history of radiotherapy or chemotherapy in jaws within the last 12 months or patients with para-functional habits were excluded from the study.

Ethical approval was obtained from the institutional ethical review committee (Proposal ID; 497-11-16). Signed written consent forms were obtained from all subjects before conducting any procedures.

The materials used in the present study and their manufacture's specifications are listed in Tables I, II and III, while grouping and coding of different variables used in the study are listed in Table IV.

Medical and dental history were taken from each patient to confirm the selection criteria then intra oral, extra oral and radiographic examination were done to detect any problems or pathological changes which should be treated before denture construction.

The selected patients were assigned and distributed into three groups according the type of metal oxide nanoparticles that added to the soft liners (18 subjects of each). 1st group included patients received maxillary complete denture that were lined with cold cured soft liner modified with $ZrO_2$ nanoparticles (Z), 2nd group included patients received maxillary complete denture that were lined with cold cured soft liner modified with $TiO_2$ nanoparticles (T), and 3rd group included patients received maxillary complete denture that were lined with cold cured soft liner modified with $SiO_2$ nanoparticles (S). Each patient of each group received upper and lower complete denture processed by the same prosthodontist and technician performing standardized finishing and polishing procedures. The maxillary complete denture having three empty spaces (Figure 1)

**Table I -** Materials used in the study

| No. | Trade name | Manufacture | Exp. date |
|---|---|---|---|
| 1 | Soft lining material | Acrostone, Anglo-Egyptian Co. | 12/2021 |
| 2 | Zirconium oxide nanoparticles | NanoGATE, Egypt | 2/2022 |
| 3 | Titanium oxide nanoparticles | NanoGATE, Egypt | 7/2022 |
| 4 | Silica oxide nanoparticles | NanoGATE, Egypt | 7/2021 |
| 5 | Heat cured acrylic resin | Acrostone, Anglo-Egyptian Co. | 10/2021 |
| 6 | TMSPM silane coupling agent | Shanghai Richem International Co., Ltd., Shanghai, China | 5/2022 |

**Table II -** The manufacture's specifications of soft lining material

| Categories | Composition | Color |
|---|---|---|
| Acrylic based, | Powder: Polymethylmethacrylate (50 gm). | Pink |
| Temporary soft liner | Liquid: Aromatic ester, ethyl alcohol (25ml). | |

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**Table III -** The manufacture's specifications of metal oxide nanoparticles

|  | Zirconium oxide ($ZrO_2$) | Titanium oxide ($TiO_2$) | Silica oxide ($SiO_2$) |
|---|---|---|---|
| Color | White | White | white |
| Form | Powder | Powder | Powder |
| Average size (TEM) | $12 \pm 3$ nm | $15 \pm 3$ nm | $21 \pm 3$ nm |
| Purity | Purity >99% | Purity >99% | Purity >99% |
| Shape (TEM) | Spherical | Spherical | Spherical |
| Crystal system | Tetragonal | Anatase 95-97%, Brookite 5-3% | Amorphous |

**Table IV -** Grouping and coding of different variables used in the study

| Group | Subgroups and codes | Description |
|---|---|---|
| Z | Z0 | Resilient soft liner without $ZrO_2$ nanoparticles |
|  | Z3 | Resilient soft liner with 3wt.% of $ZrO_2$ nanoparticles |
|  | Z7 | Resilient soft liner with 7wt.% of $ZrO_2$ nanoparticles |
| T | T0 | Resilient soft liner without $TiO_2$ nanoparticles |
|  | T3 | Resilient soft liner with 3wt.% of $TiO_2$ nanoparticles |
|  | T7 | Resilient soft liner with 7wt.% of $TiO_2$ nanoparticles |
| S | S0 | Resilient soft liner without $SiO_2$ nanoparticles |
|  | S3 | Resilient soft liner with 3wt.% of $SiO_2$ nanoparticles |
|  | S7 | Resilient soft liner with 7wt.% of $SiO_2$ nanoparticles |

created by placing three brass discs with 15mm and 1.5mm diameter and depth respectively; two on the left and one on the right side placed on the hard palate before packing the acrylic resin denture base material at the laboratory stage [27].

Each group subdivided into three subgroups according to the concentration of nanoparticles (0%, 3% and 7%) [12,17,28,29] as follow, in the first group (Z) the right empty space was filled with soft liner without $ZrO_2$ nanoparticles (Z0), the superior left empty space was filled with soft liner with 3% $ZrO_2$ (Z3) and the inferior left empty space was filled with soft liner with 7% $ZrO_2$ (Z7). The same steps were carried out for the $TiO_2$ (T) and $SiO_2$ (S) groups.

$ZrO_2$, $TiO_2$ and $SiO_2$ nanoparticles were treated separately by using silane coupling agent [3-Trimethoxysilyl propyl methacrylate (TMSPM), Shanghai Richem International Co., Ltd., Shanghai, China] creating reactive groups on its surface to allow for better adhesion between nanoparticles and resin matrix. The amount of silane coupling agent (X) required for efficient and uniform coverage of nanoparticles was calculated by the following equation [30]:

$$X = (A/\omega) f \qquad (1)$$

where A is the surface area of the nanoparticles $ZrO_2$, $TiO_2$ and $SiO_2$ (m$^2$/g), $\omega$ is the surface



**Figure 1 -** Three empty spaces on the fitting surfaces of the maxillary denture denoting the relining sites.

coverage per gram of silane MPS ($\omega = 2525$ m$^2$/g) and $f$ is the amount of nanoparticles (g).

TMSPM was dissolved in acetone to ensure that it would evenly coat the surfaces of the nanoparticles, then nanoparticles were added to the TMSPM/acetone solution and stirred with a magnetic stirrer (HS-350C, China) for 60min. Then the solvent was eliminated using a rotary evaporator (Rotavapor® R-300, Buchi AG, Flawil, Switzerland) under vacuum for 30min at

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60°C and 150 rpm. When the sample was dried, it was heated at 120°C for 2 hours then bench cooled at room temperature to get the surface-treated nanoparticles[31,32].

Treated nanoparticles and cold cured soft-liner powder were weighted using electronic balance of 0.0001gm accuracy (Denver instrument, Göttingen, Germany) as shown in Table V, then mixed together using a mortar and pestle followed by mechanical mixing for 30 minutes to ensure the homogenous mix with uniform color. The mixture was mixed with the monomer according to the manufacturer's instructions in a mixing jar and mechanically spatulated for 30 seconds with a wooden tongue depressor and left until smooth creamy mix was obtained. A separating medium (Cold-Mold Seal, Pyrax polymers, Switzerland) was applied to all areas that were not included in the lining process (around the borders of the created cavities) then a thin layer of soft-liner mix was applied to the created cavities and inserted in the patient's mouth, while the lower denture in place and the patient was guided to close in centric occlusion and maintained light occlusal pressure for 7-8 minutes. A scalpel or bur were utilized to detach the excess material avoiding its distortion, polishing discs (3M ESPE, USA) were used for finishing the soft liner. Finally, the tissue surface of the denture is examined for any defect or air bubbles. The denture was reinserted in the patient's mouth and checked for proper fit and occlusion. Furthermore, application of soft liner and sampling procedures were done by one clinician.

All participated patients were given written and verbal post-insertion instructions and a standardized oral and denture hygiene protocol along the whole study with avoidance of any factors affecting the soft-liner roughness.

**Microbiological evaluation**

For each patient three microbiological evaluations were made as following; The 1st one was done immediately after relining procedures (day 0) to be ensure that all participated patients are free from CA at the relining procedures. The 2nd was done after 2 weeks from the time of relining procedures. The 3rd evaluation was done after 4 weeks from the time of relining procedures.

On the day of taking the samples, each patient was instructed not to rinse their mouth, not to brush their denture and a fasting period of at least 3 hours before taking the sample. The samples were taken in the morning at 10-12 o'clock. Three samples were taken from the fitting surface of the maxillary denture at the site of relining (relined three cavities), each relined area was swabbed using gamma sterilized disposable cotton swab stick (CITOSWAB®, Labware manufacturing Co. Ltd, China), the swab stick was held against the relined area with a rotating motion to ensure proper adherence of the microorganisms to the swab for 30 seconds immediately after the prosthesis was removed from the oral cavity then emulsified in 1ml sterile nutrient broth (OXOID LTD, Hampshire England) transporting media and stored in a cold place (icebox) to avoid dehydration, overgrowth or death of microorganisms with misleading results and transported within two hours to the microbiology laboratory. The swabs were cultured using Sabouraud Dextrose Agar (SDA) (OXOID LTD, Hampshire England) supplemented with chloramphenicol (0.05g/L) to suppress the growth of other bacteria and increase its selectivity to CA, the vial containing the swab was vortexed for one minute to suspend the microorganisms from the swab into the broth then; $20\mu L$ from the nutrient broth was taken by pre-adjustable micropipette and applied to the surface of SDA plate by sterile glass rods to facilitate spreading procedure. The Petri dishes were labeled and incubated aerobically in an electric incubator (Shanghai, Boxun Medical Biological Instrument Corp., China) at 37⁰C for 48 hours [33,34]. CA colonies were identified by its morphology, Gram staining and Germ tube test. CA colonies appeared as distinct, creamy white, smooth, convex and raised with yeasty

**Table V -** Weight of the nanoparticles and soft lining material

| Nanoparticles % | Powder | Liquid | Amount of the NPs |
|---|---|---|---|
| 0% (control) | 10gm | 4.4ml | 0 gm |
| 3% | 9.7gm | 4.4ml | 0.3gm |
| 7% | 9.3gm | 4.4ml | 0.7gm |

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odor, (Figures 2-7). By direct microscope, CA appear violet, Gram-positive large budding yeast cell (oval in shape). The evaluation was done by counting the number of colonies that appeared on the Petri dish (CFU/sample) using a digital counter (Koicaxy, China)[33,35].

The data were collected, tabulated and prepared for statistical analysis. Statistical analysis was performed with IBM® SPSS® (SPSS Inc., IBM Corporation, Armonk, NY, USA) Statistics Version 26 for Windows. Data were presented as mean and standard deviation (SD). Data were explored for normality using Shapiro-Wilk test. CA count showed a non-normal (non-parametric) distribution so logarithmic transformation of CA count was performed and showed normal distribution. Repeated ANOVA was used to show the effect of different nanoparticle types ($ZrO_2$, $TiO_2$ and $SiO_2$) and concentrations (0%, 3% and 7%) and time (2 weeks and 4 weeks) on the difference of CA concentration (Log CA count).
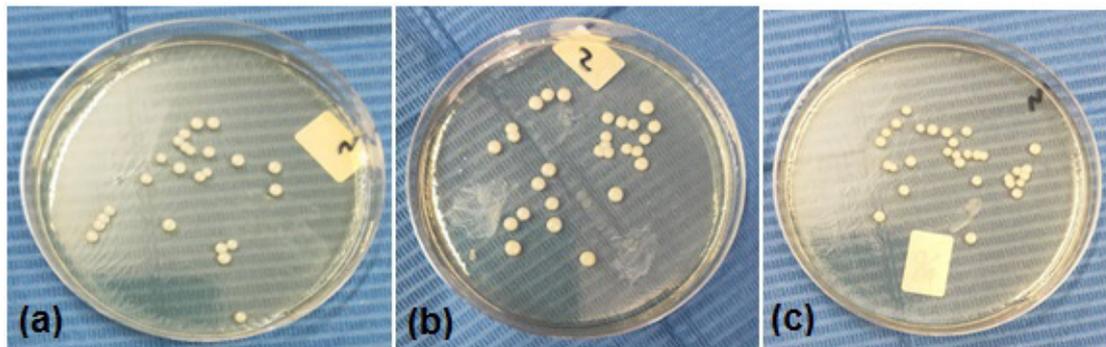


**Figure 2 -** The CA colonies count on SDA of different NPs at 0% concentration of NPs (unmodified soft liner) after 2 weeks (control), (a) Z0, (b) T0, and (c) S0.
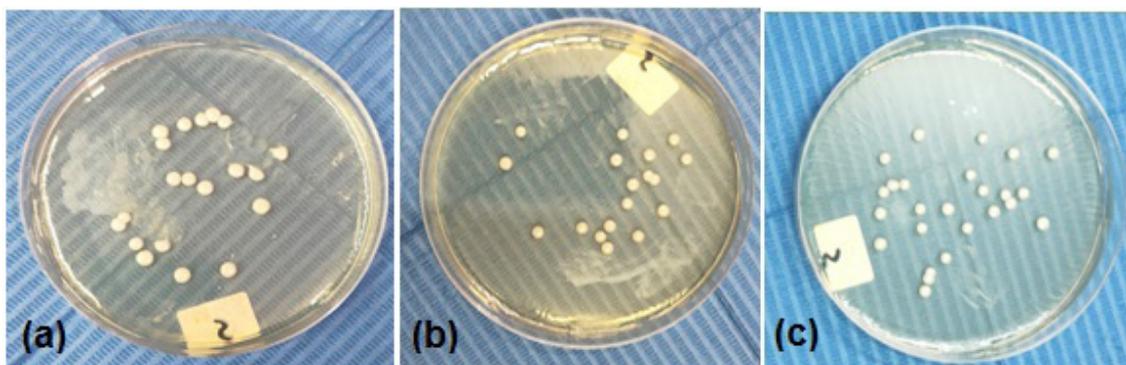


**Figure 3 -** The CA colonies count on SDA of different NPs at 3% concentration of NPs after 2 weeks (a) Z3, (b) T3, and (c) S3. Showing, least number of colonies observed in T group then Z group and S group.
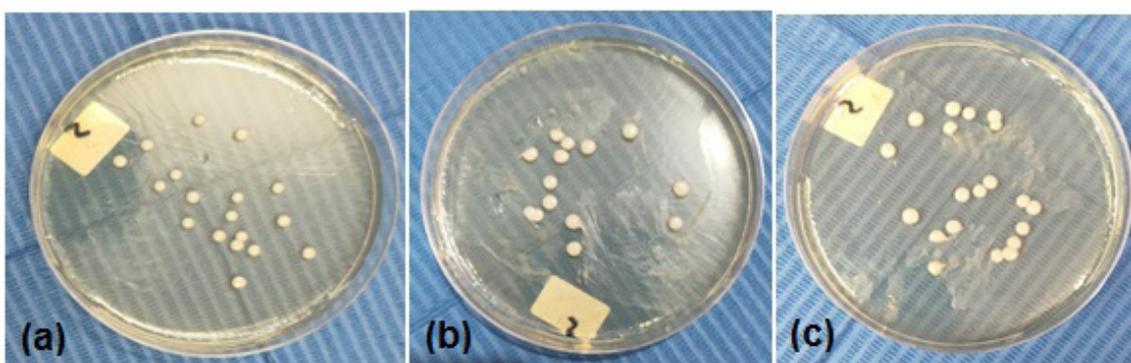


**Figure 4 -** The CA colonies count on SDA of different NPs at 7% concentration of NPs after 2 weeks, (a) Z7, (b) T7, and (c) S7. Showing, the number of colonies was reduced with increasing concentration.

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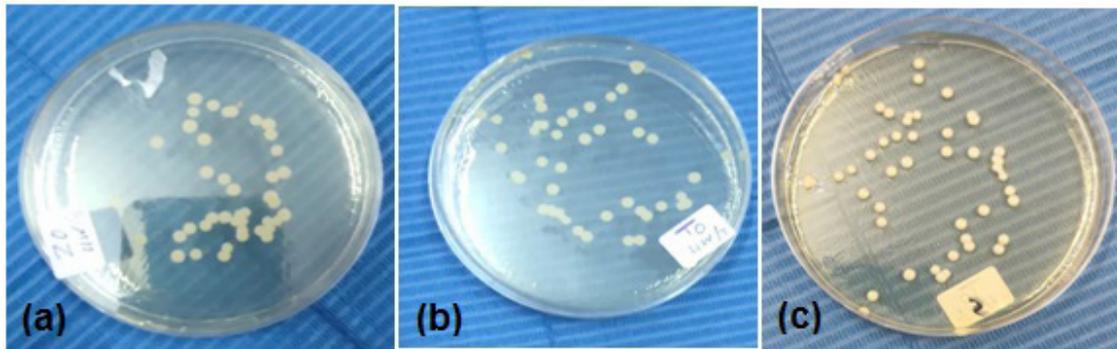
Figure 5 - The CA colonies count on SDA of different NPs at 0% concentration of NPs (unmodified soft liner) after 4 weeks (control), (a) Z0, (b) T0, and (c) S0.
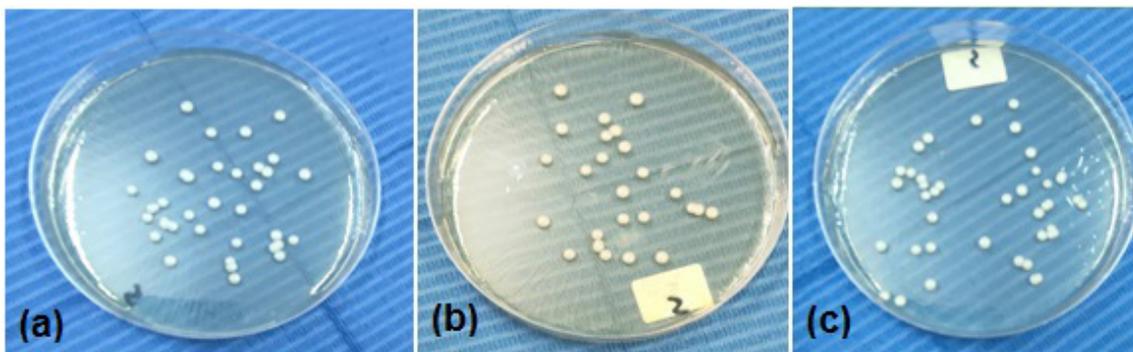


Figure 6 - The CA colonies count on SDA of different NPs at 3% concentration of NPs after 4 weeks (a) Z3, (b) T3, and (c) S3. Showing, the number of colonies was increased with increasing time, and least count was observed for T group.
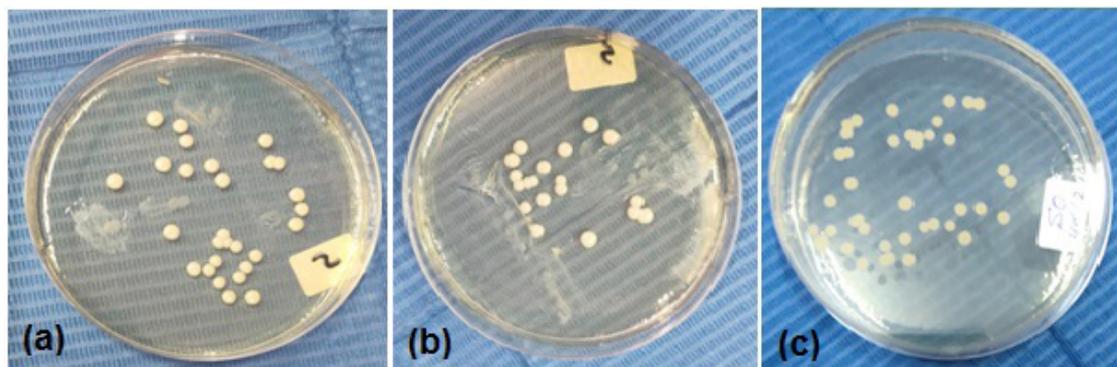


Figure 7 - The CA colonies count on SDA of different NPs at 7% concentration of NPs after 4 weeks, (a) Z7, (b) T7, and (c) S7. Showing, the number of colonies was reduced with increasing concentration.

Furthermore, one-way ANOVA was used to compare between all groups followed by Tukey HSD for multiple comparisons. Independent-t test used to compare between log CA counts at different periods. The significance level was set at $p < 0.05$.

## RESULTS

As summarized in Table VI, repeated ANOVA showed that different nanoparticle types,

concentrations and time had a significant effect on the mean CA concentration ($p<0.001$), while the interaction between all variables as well as between time and NPs type showed insignificant effect on mean CA concentration ($p=0.520$) and ($p=0.138$), respectively.

### Effect of nanoparticle concentrations

As shown in Table VII, regarding group Z; there was a statistically significant difference between log CA count after 2 weeks with

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**Table VI -** Repeated ANOVA used to show the effect of different nanoparticle types, concentrations and time on the difference of CA concentration (log CA count)

| Source | Type III Sum of Squares | df | Mean Square | F | p-value | Partial Eta Squared |
|---|---|---|---|---|---|---|
| Concentration | 4919.420 | 2 | 2459.710 | 362.743 | <0.001* | 0.910 |
| Type | 593.975 | 2 | 296.988 | 43.798 | <0.001* | 0.549 |
| Time | 9552.691 | 1 | 9552.691 | 962.997 | <0.001* | 0.930 |
| Concentration* Type | 197.432 | 4 | 49.358 | 7.279 | <0.001* | 0.288 |
| Time*Concentration | 1233.420 | 2 | 616.710 | 62.170 | <0.001* | 0.633 |
| Time* Type | 40.346 | 2 | 20.173 | 2.034 | 0.138 | 0.053 |
| Time*Concentration*Type | 32.321 | 4 | 8.080 | 0.815 | 0.520 | 0.043 |

*Significant.

**Table VII -** Descriptive statistics and results of one-way ANOVA test for comparison between log CA counts with different NPs concentrations

| | | 0% | | 3% | | 7% | | p-value | effect size |
|---|---|---|---|---|---|---|---|---|---|
| Mean | | Mean | SD | Mean | SD | Mean | SD | | |
| Z | 2W | 1.38[a] | 0.07 | 1.31[ab] | 0.06 | 1.26[b] | 0.06 | 0.005* | 0.360 |
| | 4W | 1.67[a] | 0.03 | 1.53[b] | 0.04 | 1.46[c] | 0.04 | <0.001* | 0.860 |
| T | 2W | 1.39[a] | 0.08 | 1.27[b] | 0.06 | 1.21[b] | 0.06 | <0.001* | 0.619 |
| | 4W | 1.68[a] | 0.03 | 1.48[b] | 0.03 | 1.37[c] | 0.05 | <0.001* | 0.931 |
| S | 2W | 1.42[a] | 0.04 | 1.36[b] | 0.03 | 1.32[b] | 0.06 | <0.001* | 0.486 |
| | 4W | 1.69[a] | 0.03 | 1.57[b] | 0.02 | 1.52[c] | 0.04 | <0.001* | 0.884 |

*Significant. Different superscript letter within each row indicates significant difference at p<0.05 (Tukey HSD).

different concentrations (p-value <0.005, Effect size = 0.360). Pair-wise comparison between concentrations revealed that there was no statistically significant difference between Z0 and Z3 as well as between Z3 and Z7 subgroups, while there was a statistically significant difference between Z0 and Z7. After 4 weeks, there was a statistically significant difference between log CA count with different concentrations (p-value <0.001, Effect size = 0.860) where Z0 showed the statistically significant highest log CA counts, followed by Z3 while Z7 showed the statistically significant lowest log CA count.

As regards group T, there was a statistically significant difference between log CA counts after 2 weeks with different concentrations (p-value <<0.001, Effect size = 0.619). There was no statistically significant difference between T3 and T7 subgroups while both of them showed statistically significant lower log CA counts than T0. After 4 weeks, there was a statistically significant difference between log CA counts with different concentrations (p-value <<0.001, Effect size = 0.931) where T0 showed statistically significant highest log CA counts followed by T3 while T7 showed statistically significant lowest log CA count.

As regards group S, there was a statistically significant difference between log CA counts after 2 weeks with different concentrations (p-value <0.001, Effect size = 0.486). There was no statistically significant difference between S3 and S7 subgroups while both of them showed statistically significant lower log CA counts than S0. After 4 weeks, there was a statistically significant difference between log CA counts with different concentrations (p-value <0.001, Effect size = 0.884) where S0 showed statistically significant highest log CA counts followed by S3 while S7 showed statistically significant lowest log CA count.

**Effect of nanoparticle types**

As shown in Table VIII, regarding concentration 0%; there was no statistically significant difference between log CA counts with different fillers at 2 weeks as well as 4 weeks (p-value = 0.318, Effect size = 0.091) and (p-value = 0.526, Effect size = 0.052), respectively.

With concentration 3%, there was a statistically significant difference between log CA counts with different fillers at 2 weeks (p-value = 0.007, Effect size = 0.340). There was no statistically significant

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**Table VIII -** Descriptive statistics and results of one-way ANOVA test for comparison between log CA counts with different nanoparticle types

| Mean | | Z | | T | | S | | p-value | effect size |
|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SD | Mean | SD | Mean | SD | | |
| 0% | 2W | 1.38[a] | 0.07 | 1.39[a] | 0.08 | 1.42[a] | 0.04 | 0.318 | 0.091 |
| | 4W | 1.67[a] | 0.03 | 1.68[a] | 0.03 | 1.69[a] | 0.03 | 0.526 | 0.052 |
| 3% | 2W | 1.31[ab] | 0.06 | 1.27[a] | 0.06 | 1.36[b] | 0.03 | 0.007* | 0.340 |
| | 4W | 1.53[b] | 0.04 | 1.48[a] | 0.03 | 1.57[c] | 0.02 | <0.001* | 0.647 |
| 7% | 2W | 1.26[ab] | 0.06 | 1.21[a] | 0.06 | 1.32[b] | 0.06 | 0.003* | 0.385 |
| | 4W | 1.46[b] | 0.04 | 1.37[a] | 0.05 | 1.52[c] | 0.04 | <0.001* | 0.704 |

*Significant. Different superscript letter within each row indicates significant difference at p<0.05 (Tukey HSD).

**Table IX -** Descriptive statistics and results of independent-t test for comparison between log CA counts at different period

| Mean | | 2W | | 4W | | p-value | effect size |
|---|---|---|---|---|---|---|---|
| | | Mean | SD | Mean | SD | | |
| Z | 0% | 1.38 | 0.07 | 1.67 | 0.03 | <0.001* | 0.893 |
| | 3% | 1.31 | 0.06 | 1.53 | 0.04 | <0.001* | 0.825 |
| | 7% | 1.26 | 0.06 | 1.46 | 0.04 | <0.001* | 0.801 |
| T | 0% | 1.39 | 0.08 | 1.68 | 0.03 | <0.001* | 0.876 |
| | 3% | 1.27 | 0.06 | 1.48 | 0.03 | <0.001* | 0.847 |
| | 7% | 1.21 | 0.06 | 1.37 | 0.05 | <0.001* | 0.732 |
| S | 0% | 1.42 | 0.04 | 1.69 | 0.03 | <0.001* | 0.951 |
| | 3% | 1.36 | 0.03 | 1.57 | 0.02 | <0.001* | 0.945 |
| | 7% | 1.32 | 0.06 | 1.52 | 0.04 | <0.001* | 0.810 |

*Significant.

difference between Z and T as well as between Z and S groups while there was a statistically significant difference between T and S groups. After 4 weeks; there was a statistically significant difference between log CA counts with different fillers (*p-value* <0.001, Effect size = 0.647). S group showed statistically significant highest log CA counts followed by Z while T group showed statistically significant lowest log CA count.

With concentration 7%, there was a statistically significant difference between log CA counts with different fillers after 2 weeks (*p-value* = 0.003, Effect size = 0.385). There was no statistically significant difference between Z and T as well as between Z and S groups, however, there was a statistically significant difference between T and S group.

After 4 weeks, there was a statistically significant difference between log CA counts between different fillers (*p-value* <0.001, Effect size = 0.704). The results showed that the significantly highest log CA counts was identified in group S followed by group Z, while group T showed the statistically significant lowest log CA counts.

**Effect of time periods**

As shown in Table IX, regarding group Z, there was a statistically significant change in log CA counts between Z0, Z3 and Z7 subgroups by time (*p-value* <0.001, Effect size = 0.893), (*p-value* <0.001, Effect size = 0.825) and (*p-value* <0.001, Effect size = 0.801), respectively.

As regards group T group, there was a statistically significant change in log CA counts between T0, T3 and T7 subgroups by time (*p-value* <0.001, Effect size = 0.876), (*p-value* <0.001, Effect size = 0.847) and (*p-value* <0.001, Effect size = 0.732), respectively.

As regards group S group, there was a statistically significant change in log CA counts between S0, S3 and S7 subgroups by time (*p-value* <0.001, Effect size = 0.951), (*p-value* <0.001, Effect size = 0.945) and (*p-value* <0.001, Effect size = 0.810), respectively. Pair-wise comparison between time periods for all groups revealed that there was a statistically significant increase in log CA counts from two to four weeks.

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## DISCUSSION

The present clinical trial was carried out on fifty-four patient to study the efficacy of $ZrO_2$, $TiO_2$ and $SiO_2$ nanoparticles added to cold-cured soft liner on CA. These three metal oxides NPs were picked out for their best antibacterial activity and they have attracted increased interest due to their unique biological, physical, chemical properties and inertness compared to their macro molecules. The concentration of NPs incorporated into soft lining and acrylic resin denture base materials to enhancement their biological and mechanical properties were very broad, the concentrations of NPs (3wt.% and 7wt.%) were chosen in the present study based on available evidences and reports and they were most commonly used concentrations[12,17,28,29].

Previous studies demonstrated that CA can find protection from mechanical cleansing measures in the surface irregularities of denture base materials so these methods are inadequate to completely diminish microorganisms on the denture surface and may showed an adverse effect on the soft lining materials[36]. Many approaches have been made to inhibit or at least reduce CA adherence on the denture base or resilient materials as surface modification using different coatings or incorporating a variety of antifungal additive. The usage of antifungal agents had proven to be a short-term therapy and not always effective due to the development of resistant strains[9,17].

Nanoparticles possess enhanced and unique physicochemical properties as large surface-area-to-mass ratio, ultra-small sizes and increased chemical reactivity which enable them to interact with the negatively charged surface of microbial cells resulting in enhanced antimicrobial activity so, different nanoparticles have been applied in several areas of dentistry because of their broad-spectrum bactericidal properties[25].

The findings of this study revealed that cold cured soft liner reinforced with different types of nanoparticles ($ZrO_2$, $TiO_2$ and $SiO_2$) significantly reduced the CA counts and the best result obtained at 7% concentration, so the null hypothesis of this study was rejected.

At day zero, the results of different groups confirmed that there was no adhesion of CA, this may be attributed to the strict selection criteria of patients who were healthy, have firm mucosa and of good oral hygiene.

The $ZrO_2$ nanoparticles improved the antimicrobial behavior of cold cured soft liner by significantly reducing CA adherence as $ZrO_2$ concentration increased. These results were in accord with Gad et al. who found that adding of $ZrO_2$ nanoparticles to cold-cured acrylic resin could be an effective method to decrease the adhesion of CA on the surfaces of PMMA[17].

Also, the findings of the present study were in agreement with Gowri et al. who explained that the improved antibacterial activity of $ZrO_2$ nanoparticles may be attributed to active oxygen species produced from the $ZrO_2$ nanoparticles, which subsequent causes a disruption of bacterial cell membrane. This disruption lead to an increase in the permeability of the cell membrane which result in $ZrO_2$ accumulation in the membrane and cytoplasmic regions of the bacterial cells[18,20]. Furthermore, $ZrO_2$ nanoparticles may actively inhibit the growth of CA and many other fungal strains by interfering in cell function, causing deformation in fungal hyphae[37].

Other studies reported that $ZrO_2$ nanoparticles can fills polymeric chain spaces and spaces on the polymer surface, as its proper bonding to the polymer matrix resulted in smooth surfaces, which leading to prevention of CA adhesion. Owing to this double effect of $ZrO_2$ nanoparticles; antimicrobial and surface texture changes might have an impact in minimizing the adhesion of CA[38].

On the other hand, these findings were in contradiction with Marwa et al. (2019) who reported that addition of $ZrO_2$ nanoparticles in PMMA results in an increased microbial colonization and surface roughness of the denture[39]. This may be due to the differences in the methodology or concentrations, size and shape of the nanoparticles used.

$TiO_2$ nanoparticles have a large spectrum of activity against microorganisms including Gram-negative and Gram-positive bacteria and fungi. As the concentration of the $TiO_2$ nanoparticles increased, the number of adherent CA was reported to decrease, this is in line with previous data[12,28].

In accordance with our results, Uchimaru et al. reported that $TiO_2$ combined with a tissue conditioner exhibited antimicrobial activity against *E. coli*, *S. mutans*, *S. aureus*, and antifungal activity

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against CA. The improvement in antifungal/ antimicrobial effects was concomitant with the increase of the mixing ratio of $TiO_2$ and the UV-irradiation time[40]. Also, Akiba et al. stated that $TiO_2$ nanoparticles exhibits strong oxidizing power under ultraviolet (UV) radiation from sunlight or an illuminated light source so they suggest that coating agents with photocatalyst as $TiO_2$ nanoparticles can be effective when the dentures are removed during sleep[41]. In addition, previous studies showed that $TiO_2$ also have antimicrobial activity in the absence of light indicating that (apart from the photocatalytic activity) direct contact and adsorption of $TiO_2$ nanoparticles on cell membrane may cause a loss of membrane integrity, loss of intracellular substances and suppression of the natural budding process[10,42].

Rongrong et al. reported that the 3 wt% additions of $TiO_2$ had significantly antibacterial activity compared to the control and blank groups, the antibacterial rate are 30.26% against *S. mutans* and 21.63% against CA respectively[28].

The results of the present study suggested that incorporation of $TiO_2$ nanoparticles into cold cured soft liner produce greater antifungal activity compared to $ZrO_2$ and $SiO_2$. These findings are in agreement with the work of Mazen et al. (2016) who attributed the poor antifungal activity of $ZrO_2$ and $SiO_2$ to the lack of ability to produce free reactive radicals that can attack CA cell walls and essential enzymes[43]. The difference between antifungal activity of different nanoparticles may be attributed to the individual properties of each nanoparticle such as size, morphology and electrical charge[11].

The antifungal effect of $SiO_2$ may be due to the modifications of surface characteristics of the organic resin, as hydrophobicity, roughness and surface free energy this is in line with the previous study which reported that coating of a denture base material with $SiO_2$ nanoparticles leading to increase the surface hydrophilicity which leading to decreased CA adherence to the denture base and resilient material surfaces[15,44].

Moreover, Rossano et al. found that the application of $SiO_2$ coating on the acrylic resin can help in reducing the adhesion of CA in removable prostheses which is essential to the long-term services and success of removable acrylic resin dentures and the treatment with resilient materials[16].

According to the outcome of this study, different nanoparticle types and concentrations had a significant effect on their antimicrobial activity when incorporated into cold cured soft lining materials, these results come in agreement with previous studies who found that the antimicrobial effectiveness increased with the increasing the concentration of NPs[17,40,45]. In addition, Ahmad et al. concluded that most NPs have an antimicrobial effect, but their actions are largely affected by their types, concentration, shapes, form and other factors; this will explain the difference in antibacterial action between different types of investigated NPs[46].

The results of the present study showed that there was a statistically significant increase in CA counts from two to four weeks in the tested groups, this may be attributed to, by time the material started to lose its plasticizers and subsequent its resiliency so become hardened and rough favoring adhesion of the microorganisms. This finding was in accord with Okita et al. who found that microbial adhesion increases with time[47].

The addition of NPs to soft lining materials, should not have adverse effects on their mechanical and physical properties, Amal et al. reported a significant decrease in the hardness of soft liner specimens and insignificant effect on the shear bond strength with highly significant decrease in the number of adhered CA cells which increased with increasing nano concentration[29]. In addition, Alaa et al. found that the addition of $ZrO_2$ NPs into soft lining material has antifungal properties and there is an increase in the shear bond strength of the soft lining material[20].

Limitations of this study include investigating one type of soft liner and short follow up period, so it suggests that long term in-vivo study to evaluate the efficacy of the antifungal effect of different nanoparticles incorporated into different resilient materials on the CA and other microorganisms.

Also, this report recommends to study the effect of addition of different nanoparticles and concentrations on the physical, optical and mechanical properties of soft lining materials for further investigations.

## CONCLUSIONS

Within the limitations of this in vivo study, the following conclusions can be derived:

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- $ZrO_2$, $TiO_2$ and $SiO_2$ nanoparticles have the capability of reducing CA counts so they can be used as antimicrobial agent in soft-lining materials to prevent denture stomatitis.

- $TiO_2$ nanoparticles having significant effect in reducing CA counts more than $ZrO_2$ and $SiO_2$ nanoparticles.

- Increase $ZrO_2$, $TiO_2$ and $SiO_2$ nanoparticles concentration leads to decrease the number of adherent CA.

## Clinical significance

Based on the results of the current study, $ZrO_2$, $TiO_2$ and $SiO_2$ nanoparticles have an antifungal effect, which could be incorporated into the soft lining material for removable prosthesis relining as a possible approach for denture stomatitis prevention.

## Author Contributions

Emad Azmy: performed the clinical steps, wrote the manuscript, collected the materials and papers related to this research, and financially supported the research. Mohamed Reda Zaki Alkholy: performed the clinical steps, revised the manuscript and financially supported the research. Mohamed Ahmed Helal: selected the idea, designed the research, supervised all the clinical steps, revised the manuscript and financially supported the research.

## Conflict of Interest

The authors report that no conflicts of interest.

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Supplementary data associated with this article are available on request.

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