Determining Efficacy and Minimum Inhibitory Concentrations of a Denture Adhesive Containing Particles and Nanoparticles of Zirconium against *Candida albicans*

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ABSTRACT

BACKGROUND

Candidiasis as a common complication in patients who wear dentures. Zirconia particles and nanoparticles have antimicrobial properties. This study aimed to investigate the effect of adding zirconia particles and nanoparticles, to denture adhesive on candidiasis.

METHODS

A total of 46 wells in 6 columns and 6 rows were selected from a cell culture plate. Equal amounts of *Candida albicans* and denture adhesive were added to all wells. Zirconia was then added to the first three rows at different concentrations of particles and the second three rows received different concentrations of nanoparticles, so that the concentrations were half the previous column. After 24 hours, by adding one type of Thiazolyl Blue Tetrazolium Bromide (MTT) to all the wells, the growth or lack of growth of *C. albicans* was investigated. Finally, the death or survival (merely lack of growth) of *C. albicans* was studied by sampling the wells with no growth.

RESULTS

C. albicans growth was seen in all concentrations of zirconia particles. However, it did not grow in all three rows of nanoparticles up to 31 µgr/mL concentration. Further, all wells with no growth continued to grow after removal of nanoparticles from the medium. Zirconium particles may have anti-candida properties at concentrations above 250 µgr/mL. However, nanoparticles with a minimum concentration of 31 µgr/mL inhibit the growth of *C. albicans* but do not cause death of these microorganisms.

CONCLUSIONS

Nano-zirconia can be added to the denture adhesive to reduce the possible occurrence of *C. albicans* in the denture users.

KEY WORDS

Particle, Nanoparticle, Zirconium, Denture Adhesive, *Candida albicans*

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BACKGROUND

Patients with dentures may experience conditions that affect the gear and stability of denture in addition to altering the soft and hard tissues. Some of these conditions are deficient neuromuscular control and altered quantity and quality of saliva.[1] Various methods such as relining, rebasing, denture adhesive, and implant are used to increase denture gear. The most important method is denture adhesive, which is considered a coating material for denture, improves the gear, chewing function, and patient's comfort, and reduces accumulation of food under the denture.[2,3]

Candidiasis is the most prevalent oral infection in humans. The percentage of people with candidiasis has increased with age. It is seen in the mouth of 60% of people aged over 60 years without any symptoms.[4] The lesions created in the oral mucosa associated with the use of denture are due to the reactions of oral tissues to microbial plaque, denture trauma, or the material used in denture manufacturing. One of the most common types of these lesions is denture stomatitis that involves specific inflammation in the mucosa under denture along with chronic erythema and oedema of all or parts of the oral mucosa. This inflammation occurs in both maxilla and mandible.[5]

Nanotechnology includes manufacture of nanomaterials that can be used for economic and therapeutic purposes depending on their properties. Nanoparticles are defined as particles with a diameter less than 100 nm, the size of which is smaller than bacteria and eukaryotic cells.[6] The properties of nanoparticles are different from those of their main type.[7,8] Some of these nanoparticles such as titanium, zinc oxide, aluminium, and zirconium oxide exert antibacterial and antifungal effects on microorganisms like *C. albicans*.[2,9,10,11] However, the cytotoxicity of various bacteria and fungi varies depending on the type of nanoparticle.[12,13] Since *C. albicans* growth in oral cavity brings about unfavourable complications in the patients, which may discourage the patient to use the denture, the present study was carried out to investigate the effect of zirconia particles and nanoparticles on *C. albicans* growth.

METHODS

Study Design

Groups and subgroups of this study is designated according to Gerald. R Chase et al.'s study. According to result of this study the ideal repetition of each examination in serial dilution method is three times. The first three rows in table 2 (A, B and C) indicates zirconia particles and the second three rows (D, E and F) indicates zirconia nanoparticles. More repetition can increase the probability of different outcomes and less repetition can increase the chance of error.(14)

Culture Medium Preparation

Sabouraud Dextrose Agar (SDA) and Muller Hinton Broth (MHB) provided from HiMedia®. According to manufacturer's instructions for MHB media preparation 21 g of media powder should be solved in 1000 cc distilled water. In this research 125 cc of media is sufficient for experiments. due to a simple proportion ($\frac{21}{1000} = \frac{x}{125} \rightarrow x = 2.63$) 2.63 gr of powder solved in 125 cc of distilled water so a clear solution is obtained and heated for one minute to start boiling. Also, according to manufacturer's instructions for SDA media preparation 65 g of media powder should be solved in 1000 cc distilled water. So, for providing the required volume of medium 8.125 g of raw medium power solved in 125 cc of distilled water ($\frac{65}{1000} = \frac{x}{125} \rightarrow x = 8.125g$).

In the next step, the provided medium (SDA) heated to obtain a homogenous and clear solution. Then each of two media (MHB and SDA) poured into two 50 cc falcon tube (four tubes in total). As the aim of this work was just media isolation for maintenance and autoclaving; there was no need of volumetric measurement. Each of four falcon tubes was autoclaved at 121°C and 1.5 atm for 15 minutes. Finally, the SDA medium poured into two plate and transferred to refrigerator to become solid and suitable for microbial culture.

Denture Adhesive Dissolution

The maximum concentration of the adhesive dissolved in MHB was 1.25 mgr/mL; on the other hand, for avoidance of confounding bias Corega denture adhesive was used which has no zinc oxide as an antifungal agent. Hence, 1.25 mgr/mL of Corega denture adhesive was dissolved by heating in the MHB culture medium used in this experiment.

Fungal Sample Preparation

The *C. albicans* yeast prepared from the Iranian national center for genetic and biological resources was incubated at 37 °C for 24 h in MHB and SDA. in order to detect any bacterial contaminations a colony of microorganisms was obtained and stained through gram's method and for fungal visualization lactophenol cotton blue was applied then the final sample was studied through optical microscope. Finally, a 0.5 McFarland standard was prepared from the *C. albicans* in MHB medium after detecting no contamination.

Preparation of Zirconia

Zirconium particles (5 µm) and nanoparticles (20 nm) were prepared from PubChem and Iranian nanomaterials companies, respectively. Through trial and error, the maximum concentration of zirconia that remains suspension in the culture medium and do not sediment is 250 µgr/mL—determined by heating during zirconia dissolution and sonication at 50 °C for 30 min— from which the serially diluted concentrations were prepared. The concentrations, prepared based on serial dilution method,(14) (see table 1). To this purpose the concentration of active material was considered as a highest primary concentration and poured into a falcon tubes named A1 for particles and B1 for nano particles. Then the contents of each tube diluted to the half of their concentration and was named A2 and B2. The diluting process continued till the microorganism's growth was observed in the presence of active material and minimum inhibitory concentration (MIC) reported for the least diluted concentration inhibited microorganism's growth.

Tube	Zirconia Nanoparticle Concentration µgr/mL	Tube	Zirconia Particle Concentration µgr/mL
B1	250	A1	250
B2	125	A2	125
B3	62.5	A3	62.5
B4	31.2	A4	31.2
B5	15.6	A5	15.6
B6	7.8	A6	7.8

Table 1. Different Concentrations of Zirconia Particles and Nanoparticles

Cell Culture Plate Preparation

For the main experiment 56-well cell culture (Sorfa Life Science Co) provided. The wells of this plate are arranged in 7 rows and 8 columns. The contents of A1 to A6 falcon tubes was poured into A1 to A6 wells and the contents of B1 to B6 falcon tubes poured into D1 to D6 wells.

The diluted concentrations of the Zirconia were repeated three times which is showed by three rows in table 2; the rows A, B and C are assigned to the particles, and the rows D, E and F are for the nanoparticles. As well, negative and positive control indicated respectively in column 7 and 8 of table 2, were considered to increase the accuracy of each row's experiments. Also, for detecting culture medium contamination and conforming microbial growth ability G1 and G2 groups was designed respectively. On the other hand, G3, G5 and G6 groups was assigned to represent any colour change after denture adhesive, particles and nanoparticles dissolution respectively and G4 group could illustrate any interference of denture adhesive with *C. albicans* growth. G7 and G8 tubes also was prepared to show any interference of denture adhesive with the function of zirconia particles and nanoparticles. Table 2 are summarized all the contents, gradients and concentrations used in the present study.

	1	2	3	4	5	6	7	8
A	* MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + particle (250 µgr/mL)	* MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + particle (125 µgr/mL)	* MHB mixed with denture adhesive (100 λ)+fungus (50 λ) + particle (62 µgr/mL)	* MHB mixed with denture adhesive (100 λ)+ fungus (50 λ) +particle (31 µgr/mL)	* MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + particle (16 µgr/mL)	* MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + particle (8 µgr/mL)	* MHB mixed with denture adhesive (100 λ) + fungus (50 λ)	** MHB mixed with denture adhesive (100 λ)
B	* MHB mixed with denture adhesive (100 λ) +fungus (50 λ) + particle (250 µgr/mL)	* MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + particle (125 µgr/mL)	* MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + particle (62 µgr/mL)	* MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + particle (31 µgr/mL)	* MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + particle (16 µgr/mL)	* MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + particle (8 µgr/mL)	* MHB mixed with denture adhesive (100 λ) + fungus (50 λ)	** MHB mixed with denture adhesive (100 λ)
C	* MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + particle (250 µgr/mL)	* MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + particle (125 µgr/mL)	* MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + particle (32 µgr/mL)	* MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + particle (31 µgr/mL)	* MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + particle (16 µgr/mL)	* MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + particle (8 µgr/mL)	* MHB mixed with denture adhesive (100 λ) + fungus (50 λ)	** MHB mixed with denture adhesive (100 λ)
D	** MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + nanoparticle (250 µgr/mL)	** MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + nanoparticle (125 µgr/mL)	** MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + nanoparticle (62 µgr/mL)	** MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + nanoparticle (31 µgr/mL)	* MHB mixed with denture adhesive (100 λ)+fungus (50 λ) + nanoparticle (16 µgr/mL)	* MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + nanoparticle (8 µgr/mL)	* MHB mixed with denture adhesive (100 λ) + fungus (50 λ)	** MHB mixed with denture adhesive (100 λ)
E	** MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + nanoparticle (250 µgr/mL)	** MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + nanoparticle (125 µgr/mL)	** MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + nanoparticle (62 µgr/mL)	** MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + nanoparticle (31 µgr/mL)	* MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + nanoparticle (16 µgr/mL)	* MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + nanoparticle (8 µgr/mL)	* MHB mixed with denture adhesive (100 λ) + fungus (50 λ)	** MHB mixed with denture adhesive (100 λ)
F	** MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + nanoparticle (250 µgr/mL)	** MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + nanoparticle (125 µgr/mL)	** MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + nanoparticle (62 µgr/mL)	** MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + nanoparticle (31 µgr/mL)	* MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + nanoparticle (16 µgr/mL)	* MHB mixed with denture adhesive (100 λ) + fungus (50 λ) + nanoparticle (8 µgr/mL)	* MHB mixed with denture adhesive (100 λ) + fungus (50 λ)	** MHB mixed with denture adhesive (100 λ)
G	** MHB (100 λ)	* MHB (100 λ)+ fungus(50 λ)	** MHB mixed with denture adhesive (100 λ)	* MHB mixed with denture(100 λ)+ fungus (50 λ)	** MHB (100 λ) + particle(250 µgr/mL)	** MHB (100 λ) +nanoparticle (250 µgr/mL)	* MHB (100 λ)+ fungus (50 λ) + particle (250 µgr/mL)	** MHB (100 λ) + fungus(50 λ) +nanoparticle (250 µgr/mL)

Table 2. Cell Culture Plate Content and Colour of Wells after Adding MTT; * Wells Which Showed Colour Change to Dark Colour, ** Wells Which Did Not Show Any Colour Change

Detecting Fungicidal Activity

To analyse the growth ability of the microorganisms in each well, Thiazolyl Blue Tetrazolium Bromide (MTT) (Aldrich, Sigma co) was added to the wells. MTT was added to the wells of A and B rows for particles and rows D and E for nanoparticles. Change of the initial yellow colour to dark blue after 30 minutes indicates the metabolism and growth of the microorganisms and wells with no change of colour indicates lack of fungal growth. As adding MTT causes the death of microorganisms, for detecting inhibitory or cytotoxicity effect of particles and nanoparticle MTT was not added to wells of C and F rows at first. Samples were taken by a sampler from the wells of these rows which did not show any colour change in their equivalent wells in the rows A, B, D and E and were passaged on SDA. In that case microbial growth on SDA shows the inhibitory activities (not fungicidal) of each zirconia particles and nano particles, but absence of microbial growth, demonstrates adding zirconia causes the death of *C. albicans*. Finally, MTT was added to the wells of D and F row.

RESULTS

Zirconia Particles (Wells A1 to A6, B1 to B6, and C1 to C6)

All dark wells (in table 2) indicated *C. albicans* growth. None of the concentrations of zirconia particles inhibited *C. albicans* growth.

Zirconia Nanoparticles (Wells D1 to D6, E1 to E6, and F1 to F6)

Zirconia nanoparticle was found to be effective in inhibiting *C. albicans* growth in the first four concentrations. Therefore, before adding MTT to row F, it was necessary to take samples from the first four wells, F1, F2, F3, and F4, and culture them on SDA for 24 h at 37 °C in order to determine the type of effect of nanoparticle. All of samples of the wells grew indicating that the zirconia nanoparticle had merely inhibitory effect on *C. albicans* growth and didn't have cytotoxicity effect. Therefore, the nanoparticles inhibit *C. albicans* growth at concentrations higher than 31 µgr/mL, but the particles have no effect on *C. albicans* growth even at 250 µgr/mL concentration.

Control Groups

Positive and Negative Controls of Particle and Nanoparticle

The positive controls were wells contained fungus and culture medium with adhesive, all of which turned into dark colour as expected (wells A7, B7, and C7 for particle serial dilution, and wells D7, E7, and F7 for nanoparticle serial dilution,). The negative controls were wells contained the culture medium with adhesive to analyse the presence or absence of contamination during work after preparation of each serial dilution. None of these wells turned dark in colour (wells A8, B8, and C8 for the particles, wells D8, E8, and F8 for the nanoparticle).

Other Control Groups

- G1: It remained yellow in colour due to lack of *C. albicans* confirming the sterility of the culture medium and ELISA plate.
- G2: the dark colour in this well confirmed the ability of the microbial sample to grow.
- G3: The yellow colour in this well indicated that the denture adhesive did not change the MTT colour.
- G4: Its dark colour showed that the denture adhesive did not interfere with *C. albicans* growth.
- G5 and G6: the yellow colour of this these wells confirmed that the particles and nanoparticles did not change the MTT colour. Thus, the dark colour of all wells of particle serial dilutions can be only due to the metabolism of the fungus itself.
- G7 and G8: they turned to dark and yellow colours, respectively indicating that the denture adhesive did not interfere with the function of zirconia particles and nanoparticles.

DISCUSSION

An external object like removable denture may provide a favourable ground for microbial growth. As well, it may be an agent involved in oral lesions. On the other hand, de Oliveira et al. reported that use of denture adhesive enhanced the accumulation and adhesion of *C. albicans*-containing biofilms.[15] Higher accumulation of microbial factors in acrylic porosities of a removable prosthesis increases the need for an anti-microbial factor in the denture adhesive used for removable prosthesis. Trivial concentration of nanoparticles can be proposed as a positive factor both economically and practically because the lower is the amount of zirconia used, the lower is the cost, the possible change of properties, and interference with the denture adhesive function. Metal oxides have been seriously taken into account in the treatment of microbial diseases owing to high stability and low complications.[16] However, different types of nanoparticles may occasionally impair their host DNA.[17,18] Yet, unlike other therapies noted for candidiasis, use of zirconia might be more preferable relative to other metal oxides[4,19,20-22] as Yoshiakiikarashii reported that zirconia had no toxic effects on the body cells and organs.[21] Gouda (2012) reported that zirconia ameliorates the ulcer and has antimicrobial and disinfecting properties.

In a comparative study between zirconia particle and nanoparticle Doskoc Z, concluded that zirconia nanoparticles had a higher inhibitory effect on Pseudomonas putida growth.[6] The minimum effective concentration of zirconia nanoparticle was reported to be 27.2 mg/l and the most effective concentration of zirconia was 200 mg/l. However, both the particles and nanoparticles showed negative effects on the tested bacterial growth in their study. The difference may be the fact that they measured the decrease of bacterial growth and reported that the highest concentration of zirconia could inhibit bacterial growth by 25%.[6] But the present study considers the metabolism as fungal growth even if the fungal growth had reduced microscopically.

Zirconia has significant effects on microorganisms and can decrease the complications of diseases when combined with other factors and treatments better than when it is used individually. For example, Jangara et al. carried out a study in 2012 and attached serine to zirconia nanoparticles and observed a wider range of bacteria and fungi that were affected.[23] Hence, similar to what we obtained in our study, adding other materials to nanoparticles not only does not reduce its efficacy, but also increases its efficacy occasionally.

Numerous studies have investigated the antimicrobial effects of other nanoparticles such as zinc, titanium, silver, etc. and have specifically documented the anti-candida properties of these materials.[2,9,10,11] Therefore, combining several other nanoparticles with zirconia may enhance their effect on pathogenic microorganisms.

The natural flora of the oral cavity protects the host against many pathogens. This protective barrier may be lost by changes in their quantity or quality and the host body becomes exposed to opportunistic pathogens.[24] *C. albicans* is a component of this microflora.[4] Inhibitory Static property of zirconia nanoparticle, which does not disturb the normal oral flora, can be considered as a positive point. Therefore, due to ineffectiveness of zirconia particles and nanoparticles on the viability of human cells[21] as well as invariability of the natural flora it maybe suggested to use it as an antifungal component to mix with denture adhesive.

By measuring the diameter of inhibition zone, Banerjee et al. reported that zirconia nanoparticles had no cytotoxic and inhibitory effects on the following normal bacterial flora of human skin, including Bacillus subtilis, Staphylococcus aureus, Proteus mirabilis, and Pseudomonas.[25] If we want to practically use zirconia in combination with denture adhesive, conditions similar to the diameter of inhibition zone will be created so that zirconia nanoparticles prevent *C.*

albicans growth at mucosal surface by dissemination into their surroundings. Gowri et al. used *C. albicans* and three other microorganisms through such measurement; various degrees of antimicrobial effects of zirconia against all four microorganisms were reported.[26]

Studies have shown that zirconia particles and nanoparticles, making a large surface, e.g. in the implant surfaces of heart, can prevent the growth of some microorganisms. On the other hand, zirconia surfaces can inhibit the growth of microorganisms. For example, Villard et al. showed that on the zirconia-coated implants, zirconia significantly exerted inhibitory effects on the formation of *C. albicans* colonies.[27] Moreover, Nascimento et al. conducted a study on the adhesion of 38 oral bacterial species, especially the bacteria related to periodontal diseases to the zirconia abutment surfaces. They found that Zirconia abutment surfaces showed fewer colonized bacteria.[13]

Silva et al. reported that zirconium surfaces prevented the biofilm formation more than titanium did.[28] These findings and those of our study indicate that the area of zirconia exposed to microorganisms is of great importance. The higher efficacy of nanoparticles than particles is due to their higher surface/volume ratio. Hence, nanoparticles have a better efficacy in comparison with particles.[29]

Given this, if zirconia nanoparticles are used in the acrylic resin for the production of full prosthesis, they may also show antimicrobial properties; this hypothesis needs further analysis. Further, by proving the efficacy of zirconia, it can be used topically for the treatment or control of *C. albicans* growth in other parts of the body such as vaginal candidiasis.

Zirconia nanoparticles exert inhibitory effects on *C. albicans* growth by interfering with DNA transcription and preventing the formation of proteins required for metabolism. For example, Dhanalekshmi and Meena found that silver surfaces reinforced with zirconia nanoparticles not only inhibited the accumulation of Candida microorganisms but also showed more antimicrobial and interfering effect on the normal activity of DNA than silver nanoparticles alone.[30]

In general, the present study showed that adding nano-zirconia to denture adhesive maintained its anti-candida properties, and nano-zirconia had a higher inhibitory effect against *C. albicans* than its common type. Therefore, nano-zirconia can be added to the denture adhesive to reduce the possible occurrence of *C. albicans* in the denture users and reduce the incidence of *C. albicans*. Moreover, nano-zirconia adhesives can be used as a surface coating in dentures in patients with candidiasis during the recovery period.

CONCLUSIONS

Zirconia nanoparticles have higher antifungal effects against *C. albicans* than zirconia particles, and denture adhesive does not interfere with the efficacy of zirconia. Furthermore, the nanoparticles inhibit *C. albicans* growth at concentrations higher than 31 µgr/mL, but the particles have no effect on *C. albicans* growth even at 250 µgr/mL concentration. Nano-zirconia merely inhibits the *C. albicans* growth but cannot cause *C. albicans* death.

Data Availability

There is no additional data about this manuscript and context contains all essential data.

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