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CLINICAL STUDY

The evaluation of *Candida albicans* biofilms formation on silicone catheter, PVC and glass coated with titanium dioxide nanoparticles by XTT method and ATPase assay

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Abstract: Lots of *Candida albicans* infections involve in biofilm formation on medical devices. This kind of biofilm can impede antifungal therapy and complicates the treatment of infectious diseases particularly in field of chronic diseases associated with implanted devices. This study has investigated the influence of treating silicone catheter, PVC and glass coated with Titanium dioxide (TiO2) nanoparticles on attachment of *C. albicans*. In this study TiO2 nanoparticles were synthesized from precursor TiCl4 and characterized by scanning electron microscopy (SEM) and X-ray diffraction (XRD) which showed TiO2 nanoparticles are 70-100 nm in size. In the simplest model of biofilms formation, *C. albicans* isolates (ATCC10231) and (ATCC 76615) were grown on the surface of small disks of catheter, PVC and glass in a flat-bottomed 12-well plates and evaluated biofilm formation using ATP bioluminescence and tetrazolium salt (XTT) reduction assays. In addition, morphology of *C. albicans* biofilms after 48 h incubation was observed by SEM. Results indicated that there is a statistical difference between mean of coated samples especially catheter and glass before and after TiO2 nanoparticles coating ($p<0.05$). In SEM analysis, *C. albicans* biofilm was more aggregated on the surface of glass and catheter than PVC and control groups and after treatment by these nanoparticles, catheter and glass both showed most significant decrease of *C. albicans* attachment in comparison to the control groups *(Fig. 4, Ref. 23)*. Full Text in PDF *www.elis.sk*.
Key words: antifungal agent, biofilm, *Candida albicans*, coating, TiO2 nanoparticles.

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The incidence of mortality due to opportunistic microorganism has shown a marked increase in recent years. Candidiasis is one of the actually most ordinary fungal infections worlds widely (1). Currently *C. albicans* has more effective role among nosocomial pathogens due to proper potential for biofilm formation (2). Biofilms related medical device infections increase hospital patients and impose hospitalization costs. As the use and types of indwelling medical devices in modern healthcare are continuously expanding, especially with an aging population, the incidence of biofilms infections will be also rising. The main problem with microbial biofilms infections is their tendency to resist to the host immune system and all antimicrobial agents. In fact, counterparts of their free floating planktonic microbes within a biofilms are more resistant to antimicrobial agents (3, 4). Therefore, achieving a novel method to inhibit attachment of cells to the surface and eliminate of fungal mass over surfaces is valuable to control infections (5).

Nowadays great growing of nanotechnology in different branches of science such as engineering and medical is obviously noticeable. In recent years, nanomaterials have considerable acceptance to use as an antimicrobial effect due to different physical, chemical and electrical properties in ultra tiny form that unavailable in larger forms (6, 7).

In this study it was evaluated the influence of treatment of silicone catheter, PVC and glass coated using Titanium Dioxide (TiO2) nanoparticles on susceptible (ATCC 10231) and resistant (ATCC76615) standard strains of *C. albicans* strains.

These biofilms were analyzed through two viability cell techniques. First, XTT (Tetrazolium salt) assay as a colorimetric method and second one ATPase assay to assess biofilm biomass.

Material and methods

1) Preparation of TiO2 nanoparticles

TiO2 nanoparticles were synthesized through the hydrolysis of Titanium Tetrachloride (TiCl4) as precursor. In this step TiCl4 was slowly added into the 58 ml distilled water under constant string for 5 hour. The solution was aged for 24 h at ambient temperature. Then gel was dried in oven for 12 h at 60 °C and finally calcinated at the 550 °C.

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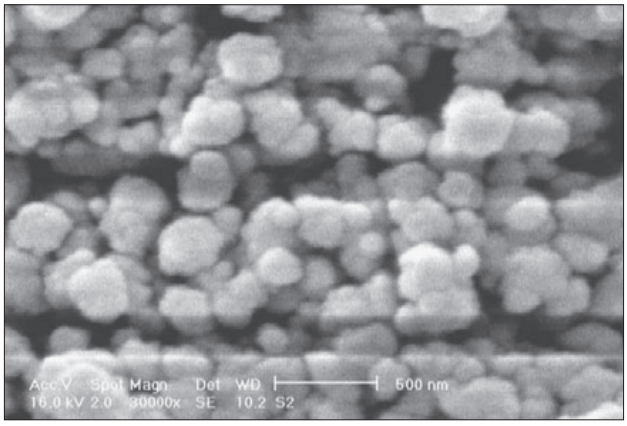

Fig. 1. SEM micrograph of TiO2 nanoparticles, Sizes of particles are 70-100 nm with rather uniform round shape.

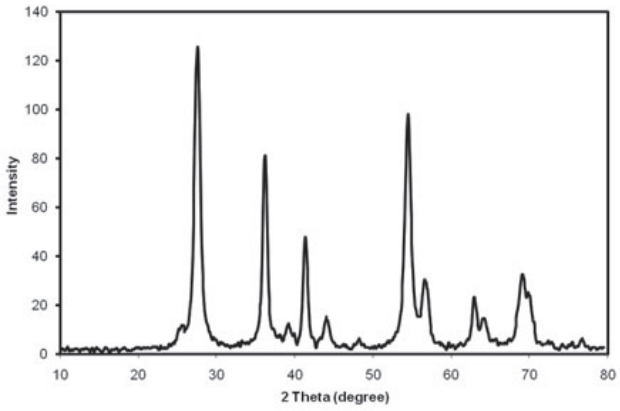

Fig. 2. XRD pattern of TiO2 nanoparticles with Cu Ka radiation at wavelength λ = 1.54178 Å.

2) Characterising of TiO2 nanoparticles:

a) Scanning Electron Microscopy (SEM) method,

b) To access information about shape and estimating size of nanoparticles, SEM (Philips) micrograph was taken Figure 1. After synthesize of TiO2 nanoparticles,

c) X-Ray Diffraction (XRD) method,

d) To identifying type of TiO2 nanoparticles, the synthesized TiO2 powder was characterised by XRD technique (XPERT: model 95) with kα radiation, wavelength (λ=1.54178 Å) (Fig. 2),

3) Coating of catheter, PVC and glass by TiO2 nanoparticles.

Small pieces of silicone catheter, PVC and glass were cut in size of 1 cm^2. Then surfaces of samples were washed with NaOH and they were finally coated with TiO2 nanoparticles by deep coating method. In this process, samples coated with continuous immersing and ejecting to the TiO2 nanoparticles solution.

4) Preparation of standard fungal cell suspension

Susceptible *C. albicans* (ATCC 10231) and resistant (ATCC 76615) standard strains of *C. albicans* was used to form biofilm and tested the antifungal activity of samples which was coated by TiO2 nanoparticles. At first, two strains were grown on sabouraud dextrose agar medium (SDA Merck, Germany) at 37 °C for 18 h. Then, freshly colonies inoculated into yeast nitrogen base medium (YNB medium; Himedia Co) containing 50 mM glucose and incubated at 37 °C for 24 hours. After that, little amounts of the yeasts were transferred into a test tube containing sterilized PBS with pH: 7.2 (difco). Next the mixture was centrifuged (10000 g, 10 min) and turbidity of suspension of cells was compared to 0.5 McFarland standard tubes to estimate cell density. Finally yeast cells were counted and adjusted at 1×10^6 cells/ml by using Neubauer slide**.**

5) Biofilm formation

According to Chandra et al (33) artificial candidal biofilm formation were carried out on 12-well tissue culture plates. Samples were placed in each well of 12-well tissue culture plates. The yeast cells were incubated for 2 h at 37 °C in 1 ml of 1 % bovine serum albumin (BSA). After this pretreatment, 80 µl of *C. albicans* cell suspension was inoculated onto the surfaces. The yeast cells were allowed to adhere to the surface at 37 °C for 90 min. After this

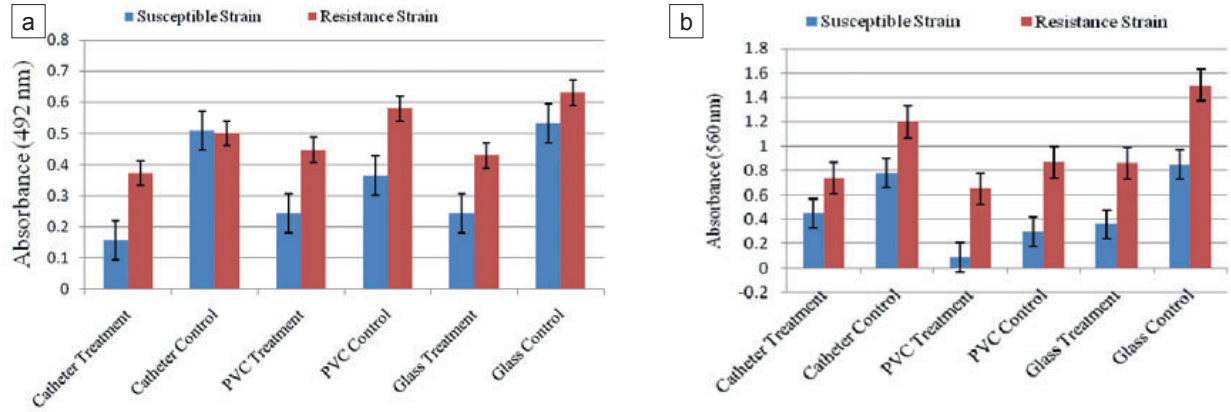

Fig. 3. Absorbance of *C. albicans* biofilms that formed at the surfaces of silicone catheter, PVC and glass disks and coated with TiO2 nanoparticles in comparison to control groups for both standard susceptible and resistance strains. The methods was used for biofilm quantification were XTT reduction assay (a), ATP bioluminescence assay (b). Treatment of Catheter, PVC and Glass also their control groups are represented in this figure. Data are means ± standard deviations of three independent experiments performed in triplicate ($p < 0.05$).

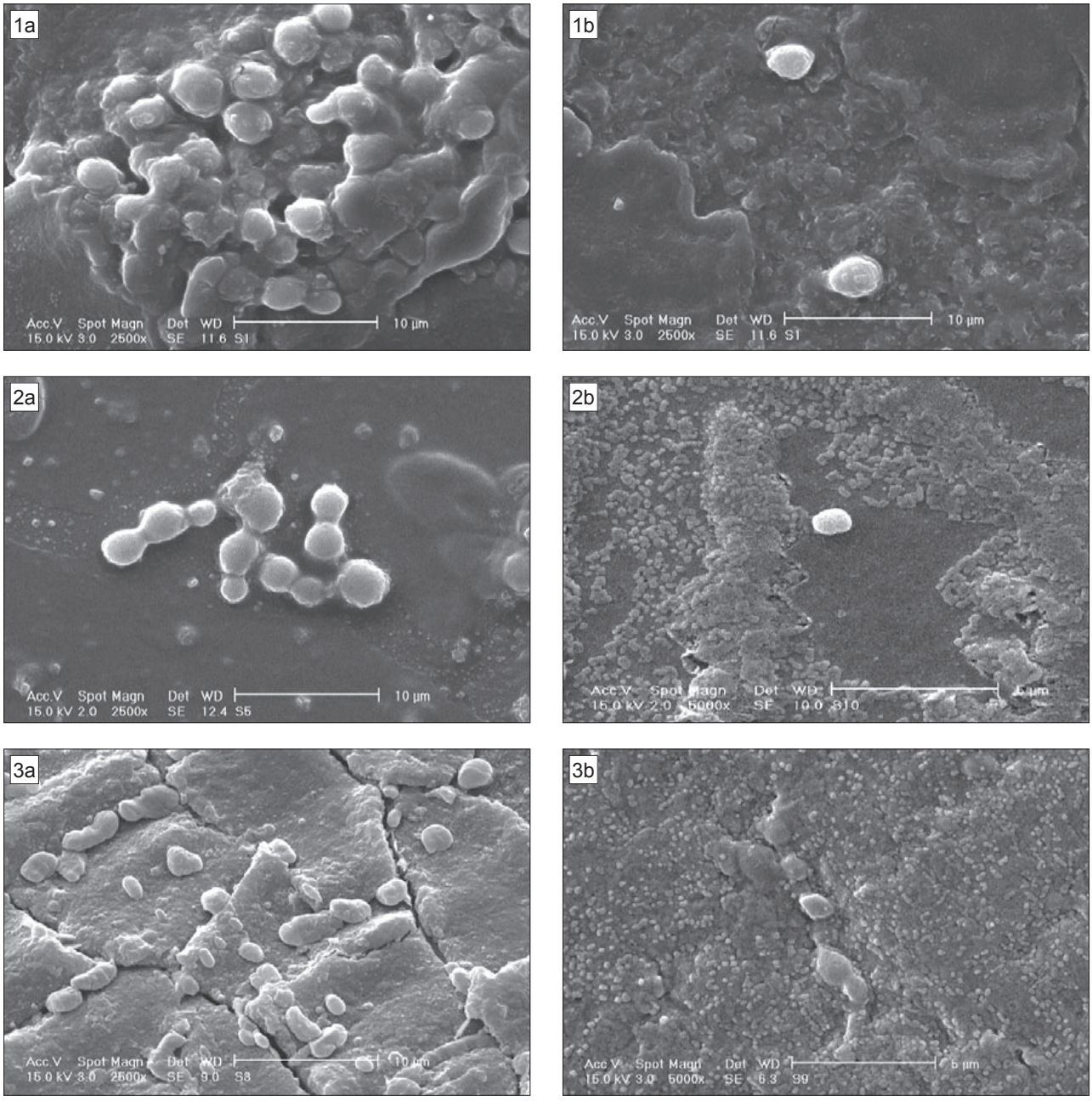

Fig. 4. SEM micrograph of biofilm formation on the surface of 1 cm^2 pieces of glass (1), PVC (2), silicone catheter (3) before and after treatment with TiO2 nanoparticles, respectively (a and b). The best biofilm were formed at the glass and catheter surfaces.

time the slice of samples were gently washed with PBS and then added 4 ml of YNB medium including 50 mM glucose. The plates were incubated at 37 °C for 48 h, and the biofilms were formed on the surfaces. Afterward, the YNB medium was removed and washed with 4 ml of PBS.

6) XTT assay

XTT [2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5- (phenyl amino) carbonyl)-2H-tetrazolium hydroxide] as colorimetric assay was carried out to evaluate antifungal effect of nanoparticles. This method is based on determining the viability of collected cells.

Antifungal assay was carried out by measuring the optical absorbance of incubated 96-wells plates containing 50 µl of the collected cell suspension per well. Plates were incubated with 50 µl of the YNB medium containing 50 mM glucose and 100 µl XTT (Sigma) and 50 µl coenzyme Q0 (Sigma) at 37 °C for 3 h. Optical absorbance (Fig. 3) was measured at the wavelength of 492 nm using an ELISA reader (Memmert, Germany).

7) ATPase assay

After biofilm formation and treatment of cells by nanoparticles, ATPase assay was used to determine the viability. For this reason,

biofilm was removed by scrapping method, 10 µl of susceptible and resistant of removed *C. albicans*, 10 µl complex reagent containing luciferin and luciferase which was contained Mg^{+2} ,mixed in each well of Luminescence microplates. Then optical density was measured by luminometer (Berthold Co) at 560 nm. ATP is a marker for cell viability. Because it is present in all metabolically active cells and its concentration declines very rapidly when the cells treated with the biocides. The ATPase assay system is based on production of light at 560 nm through the reaction of ATP with added luciferase and D-luciferin. All Data were analyzed by using statistical t-test method and SPSS software.

Results

Common morphology of the TiO2 nanoparticles is shown in Figure 1. It shows TiO2 nanoparticles which has regular shape.

The type of crystalline structure of the synthesized TiO2 nanoparticles has been considered by means of XRD with Cu Ka radiation at wavelength $\lambda = 1.54178$ Å, and measurements were performed using a θ–2θ goniometry (8). Figure 2 shows XRD pattern TiO_2 nanoparticles. All the diffraction peaks of samples show that synthesized TiO2 is the desired material. Crystal size of TiO2 nanoparticles are measured using Debye-Scherrer relation [$D=0.94\lambda/\beta\cos(\theta)$] (8). It was observed that the diameter of the TiO2 nanoparticles is on average 70-100 nm (Fig. 2).

In this study, small pieces of glass and catheter that coated with 7.03 µg/ml of TiO2 nanoparticles showed reduction in cell density after coating of the samples with TiO2 nanoparticles. This reduction was more for susceptible standard strain of *C. albicans* when it was used XTT technique to assay.

ATPase assay results were according to XTT assay although; in comparison to control group differences was more than XTT method. All experiments were performed in triplicate and three independent experiments ($p < 0.05$).

Results of treated samples using scanning electron microscope showed that attached *C. albicans* to treated glass and catheter surfaces significantly decreased in comparison to control groups.

Discussion

Human tissues frequently have large and compound microbial communities that are growing as biofilm and according to the condition cause a variety of infections. As a result of greater use of implanted medical devices than before, microbes in biofilm display increased resistance towards biocides; antibiotic chemotherapy as well decreased susceptibility to the host defense mechanisms (9, 10). Recent evidences have revealed that in more than 65 % microbial infection, biofilms have critical role in infections and diseases associated biofilm and are becoming increasingly more difficult to treat (11). *Candida* is an important human fungal flora causing nosocomial infection. *Candida* species have capability to adhere to many surfaces and form biofilms. The surfaces of medical devices supply a substrate for colonization of microbes. The evidence linking *Candida* biofilms of medical devices associated with infections is growing as more consistent way for evaluating *Candida* biofilms (12).

Many studies have been investigated about eradicating the biofilm populations using variety of conventional drugs and antimicrobial agents to eliminate fungi in order to control unfavorable biofilm surfaces and in aqua environments by using antifungal biocides or UV radiation (13). The function of electronic microscopy and molecular methods has developed our understanding about biofilm structure and attachment of this structure to achieve important information about the prevention and treatment of biofilm formation on a variety of surfaces, important to the medical practices till industries which are useful to develop preventive strategies and methods for biofilm control (14). According to expensive and consuming treatment of infection disease and difficulty in treatment as well genetic variation and increasing drug resistance, introducing agents that possess antifungal properties and inhibitory biofilm formation could be effective role in three stages of controlling, prevention and treatment of disease associated biofilms (15).

Because of the great ability of nanoparticles in coating surfaces and regard to antimicrobial activity of these nanoparticles, we used TiO2 nanoparticles to coating silicone catheter, PVC and glass for analyzing *C. albicans* biofilm formation. According to Akiba (16) and Chen (17) studies, nanoparticles such as TiO2 have antimicrobial efficacy which could be considered as a self-cleaning agent.

According to the antifungal properties of TiO2 nanoparticles in amount of 4 µg/ml for susceptible and 4.55 µg/ml for resistance strain (Haghighi and colleagues in 2010) (18) in this study it was tried to use three different surfaces, silicone catheter, PVC and glass as a substratum for biofilm formation.

The results of XTT method and ATPase assay on biofilm formation showed glass and catheter coated with TiO2 nanoparticles had better ability to inhibit attachment, and formed biofilm was more for PVC surface (Fig. 4).

Studies by many laboratories have showed that microbial attachment to the treated PVC were stronger than latex and silicone surfaces because of some surface properties such as texture, charge and hydrophobicity. The catheters with rough, irregularities surface show considerably more adherent compared to catheters with smoother surfaces (19, 20).

It was proved by scientists that TiO2 nanoparticles, producing intracellular reactive oxygen species (ROS), induced destructive effects inside the microbial cells and oxidation of intra cellular Coenzyme A (CoA) and peroxidation of plenty lipids which is resulted decreased respiratory activity and consequently cell death (21, 22).

We evaluated the amount of viability of sessile cells of biofilms by using XTT assay. Furthermore, results of XTT assay have accordance with ATPase analyses. The major advantages of ATPase assay compared to conventional methods for viability detection are high sensitivity, excellent linearity, simplicity and fast to do and with lack of cell harvesting or separation steps without any need to incubate the cells (23).

This study showed that TiO2 nanoparticles able to inhibit *C. albicans* biofilms formation. Consequently in field of controlling and elimination of *C. albicans* biofilms, TiO2 nanoparticles can be considered as an alternative antifungal agent although further investigation related to optimizing TiO2 nanoparticles synthesize and procedure of coating surfaces must be done.

Conclusion

In this study, it was shown that TiO2 nanoparticles inhibit the growth of *candida* biofilms. And this inhibitory effect was more for the glass and then catheter. According to this results ATPase assay can achieve fast and more reliable results thus consuming less time.

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