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Photocatalytic antifungal activity against
Candida albicans by TiO_2 coated
acrylic resin denture base

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TiO₂를 코팅한 아크릴릭 레진 의치상의
캔디다에 대한 광촉매적 항진균 활성화에 관한 연구

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이 논문을 치의학 석사학위신청 논문으로 제출함.

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양지연의 석사학위논문을 인준함.

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국 문 초 록

TiO₂를 코팅한 아크릴릭 레진 의치상의 켄디다에 대한 광촉매적 항진균 활성에 관한 연구

양 지 연

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본 연구에서는, 의치의 세척 및 소독법으로서 TiO₂를 코팅한 의치상 레진 표면의 광촉매적 산화작용을 제안하였다. 물과 산소가 존재할 때, 높은 반응성을 지닌 OH-라디칼이 TiO₂ 광촉매와 자외선 A (UVA) 로부터 발생되며, 이 라디칼들이 세균을 파괴하고, 전 면적에 걸친 항진균 작용을 가능케한다.

시편은 열중합형 의치상 레진 (Vertex RS; Dentimex, Netherlands) 을 이용하여 제조사의 지시에 따라 통상적인 방법으로 온성하였다. Ti-alkoxide를 진구체로 상온에서 코팅 가능한 TiO₂ 광촉매 졸 (LT)을 제조하고 XRD패턴, TEM사진, 질소흡착 실험을 통해 상용 TiO₂ 광촉매인 P-25 (Degussa, Germany)와 특성을 비교하였다. 제조한 TiO₂ 광촉매 졸(LT)을 바인더와 혼합한 후 딥코터를 이용하여 의치상 레진 시편에 코팅하고, 코팅하지 않은 시편은 대조군으로 분류하였다. 완성된 시편은 XRD를 사용하여 TiO₂의 결정화도를 검사하였으며 FE-SEM/EDS를 통해 입자크기 및 모양을 확인하고 화학적 성분을 분석하였다. 시편의 광촉매 활성 정도

는 접촉각 측정과 메틸렌블루 분해 반응을 통해 조사하였으며, 끝으로 항진균 활성도를 실험하였다. 초기 농도 10^5 cells/ml의 *Candida albicans* KCTC 7629를 실험군과 대조군 시편 위에 1ml 씩 떨어뜨린 후, 10W의 최대출력을 가진 352nm 파장의 UVA 광원 (Blacklight Blue; Sankyo Denki, Japan) 두 개를 15cm 상방에서 조사하였다. 120분 동안 10분 간격으로 $2\mu\text{l}$ 씩 채취하여 적당히 희석한 후 영양 한천 배지(Bacto™ Brain Heart Infusion; BD, USA)에 도말하였다. UVA-광촉매 반응 후 살아남은 균체는 24시간 동안 37°C에서 배양 후 계수하였다.

LT 광촉매와 상용 P-25의 특성을 비교한 결과, LT가 보다 높은 광촉매 활성을 가진 것으로 나타났다. LT 광촉매로 코팅한 실험군 시편에서, 코팅면은 anatase 결정형을 보이고, 20nm 이하의 입자 크기와 넓은 비표면적을 나타냈다. 시편의 광촉매 활성을 메틸렌 블루 분해 반응으로 조사한 결과 2시간 후 약 5%의 분해율을 보였다. 접촉각은 평균 20° 이하로, 친수성을 지님을 알 수 있었다. *Candida albicans*에 대한 항진균 실험 결과, UVA 광원 조사 후 30분 이내에 0%의 생존율을 나타냈다.

이상의 결과에서, UVA-광촉매 반응을 이용하여 의치 표면에 존재하는 *Candida albicans*에 대한 항진균 작용이 가능함을 확인하였으며, 임상에서 활용할 수 있으리라 사료된다.

I . Introduction

Denture-related stomatitis is an inflammatory process that mainly involved the palatal mucosa when it is covered by complete or partial dentures and presents as an intensely red, glistening, and slightly swollen palatal epithelium^{1,18)}. The prevalence has been reported as varying ranges from 9% to 97%^{4,23,34)}. It has been suggested that denture-related stomatitis has a multi-factorial cause. Nonsystemic factors are bacteria³⁸⁾, yeast³⁾, ill-fitting dentures, poor denture hygiene¹⁾, consumption of carbohydrate-rich diet³²⁾, and effects arising from denture materials¹⁾. However, generally the etiologic factor is microbial, and the presence of the opportunistic pathogen *Candida albicans* in denture plaque is considered as an important factor in the development of this inflammation^{3,6)}. This dimorphic fungus exists in both yeast and mycelial (hyphal) forms. During tissue invasion, the mycelial form predominates³⁶⁾. Because of its large size, host phagocytes are unable to completely destroy it. Therefore, extracellular killing mechanisms are required to eliminate the candida. The usual treatment of denture-related stomatitis involves elimination of the source of the infectious agent (denture cleansing and disinfection) and elimination of the oral tissues (antifungal therapy)^{7,22)}. To minimize reinfection, dentures should continue to be soaked in solutions that destroy or remove the organism. Reduced use of denture or the removal of it during the antifungal therapy can be helpful in eliminating the source of the infectious agent. Generally, tissue treatment involves topical application of Nystatin (Sandoz

Pharmaceuticals, East Hanover, NJ), but patient complication is often poor because the unpleasant taste causes nausea and vomiting. These side effects somehow decrease its therapeutic value²²⁾.

Proliferation of *Candida albicans* is primarily within the plaque on the fitting surface of the denture rather than on the inflamed mucosa. Consequently, the treatment of the denture is equally important as treatment of the tissue^{7,22)}. With regard to the treatment of dentures, cleansing and disinfection should be efficiently carried-out as the organisms can penetrate into the voids of the acrylic resin and grow in them, from which they can continue to infect and reinfect bearing tissues²²⁾. Often, approximately 1mm of internal denture base acrylic is removed, and a soft liner is placed. This liner must be changed frequently as it also becomes penetrated by the organism.

Most commercially available denture cleansers aim to remove hard and soft deposits and bacterial plaque either by chemical or mechanical ways¹⁷⁾. There are a large number of solutions, pastes and powders with a variety of claims for their relative efficacies. It was reported, however, that some of them caused problem such as discoloration, the generation of porosity and surface roughness of the denture base resin surface²⁸⁾.

A potential alternative may be provided by substrates made of light-guiding materials, coated with specific semiconductors and stimulated by indirect mild ultraviolet A (UVA) light (320-400nm). This method shows oxidative and disinfectant activity. The semiconducting materials about which most information is available is titanium dioxide (TiO₂). It is well known that the TiO₂ in anatase is capable of oxidizing and the decomposing

various kinds of compounds^{33,35}). The recent review article provides a comprehensive report of the mechanisms involved and the potential fields of application²⁶). There is now a wide agreement regarding the mechanism: in TiO₂, an electron is transferred from the valance band to the conduction band by absorption of a photon, and the resulting electron hole pair reacts with molecules on the surface of the semiconductor. Various reactive oxygen radicals caused by reactions of the hole have been identified in aqueous solution, mainly the OH radical^{12,15,16}). The free electron simultaneously created reacts with dissolved oxygen, to produce among other things hydrogen peroxide^{9,10}). These reactive species can oxidize organic material up to complete mineralization, depending on the experimental conditions²⁶). Overall, the organic molecules react with dissolved oxygen to produce CO₂ and H₂O.

Photocatalytic materials such as titanium dioxide(TiO₂) have been studied for more than 20 years on their capacity to degrade organic contaminants from air and water. Because of its unique photoinduced characteristics, it has been widely studied for applications such as auto cleaning agent, deodorant, antibacterial, and for purification of water and air^{8,29}).

In 1985, Matsunaga et al. first reported the antibacterial effect of TiO₂ photocatalytic action: bacteria cultures in contact with UV-irradiated TiO₂-Pt thin film, during 60 to 120 min, had a significant reduction in number of cultivable cells²⁴). Since this report, the photocatalytic property has been widely studied in variety of microorganisms such as viruses, bacteria, fungi, algae, and also in cancer cells^{2,39}).

The purpose of this study was to evaluate the applicability of

photocatalytic reaction to eliminate *Candida albicans* from acrylic resin denture base, and to investigate the antifungal effect with various UVA illumination time.

II. Materials and Methods

1. Photocatalyst preparation and characterization

LT (Low Temperature) photocatalyst used in this study was made from alkoxide (titanium isopropoxide, Aldrich Co., 99.9%) and nitric acid. Titanium precursor was dropped to distilled water little by little and agitated with inorganic acid at room temperature and pressure. Final solution was made by hydrothermal method for 6 hours. It was reported that anatase crystalline phase could be made by hydrothermal method from hydro titanium²⁰.

The crystalline structure, particle size, and specific surface area of the LT were tested. Powder acquired from photocatalyst sol after drying at 10 0°C was tested with X-Ray diffraction (XRD, Rigaku D/MAX-1200, Japan), transmission electron microscopy (TEM, JEOL, JEM-2000FXII, Korea Basic Science Institute/ Gwangju Branch), and N₂ adsorption method (ASAP 2010, Micromeritics, USA). Characteristics of the LT photocatalyst used in this study were compared with those of the commercial P-25 photocatalyst (Degussa, Germany).

2. Preparation of acrylic resin plates

A mold for acrylic resin plates, which was 35×35×3mm, was prepared. A 3mm layer of base plate wax (Plate wax rose; Degussa Dental, Vienna,

Austria) was placed between 2 heat-resistant glass plates measuring 35×35×5mm and flasked with dental stone (New Plastone; GC, Tokyo, Japan) according to the conventional procedures. As soon as the stone was set, flasks were separated, then the wax was removed.

Polymerization of acrylic resin materials (Vertex RS; Dentimex, Netherlands) was performed in strict compliance with the manufacturer's instructions. The clamped flask with heat-polymerizing resin was immersed in boiling water for 20 minutes. Then the flask was bench cooled before deflasking.

After a completion of polymerization, each plate was retrieved from the mold and all six sides of the plates were mechanically polished with #320, #600, and 1000 grit SiC papers on the polishing machine (OMNILAP 2000 SBT Inc). The plates then were cleaned with compressed steam.

3. TiO₂ thin film coating and characterization

One of the resin plates was coated with the LT photocatalyst. The specimen was coated with TiO₂ (LT) and binder material (silane) using a dip-coater (1mm/min speed) and was dried for 2 hours at 80°C.

The specimens divided into two classes according as whether they were coated or not ; 1) polished type, 2) TiO₂ coated type. Prior to testing, the polished specimens were stored in tap water at 22°C for 48 hours.

For the characterization of TiO₂ film, field emission scanning electron microscope/ energy dispersive x-ray spectrometer (FE-SEM, XL 30 SFEG,

Phillips Co., Netherlands / EDS, Link Analytical LTD. AN ISIS 310, England), UV spectrophotometer (UV-1601), XRD and contact angle analyzer (Phoenix 450) were used to confirm nano-coating of TiO₂, chemical composition on particle surface, photocatalytic activity with methylene blue, the crystallinity and hydrophilicity of TiO₂ film.

Specimens were observed under SEM at low magnification($\times 2,000$) and high magnification($\times 100,000$) each. XRD patterns were obtained at the angle of diffraction, $2\theta=10-70^\circ$. The operating conditions of the X-ray source were 30kV, 15mA, target=Cu, and a scan speed of $2^\circ/\text{min}$. 35mℓ of methylene blue solution at 10 ppm of initial concentration was dropped on Petri dish, then the specimens of the experimental and control groups were put on the Petri dish and adsorbed the methylene blue for 30min. The amount of methylene blue degradation was measured for 2 hours with the UVA light on. Right and left contact angle was measured 5 times and the average was taken.

4. Microorganisms

Candida albicans KCTC 7629 was inoculated in a BHI (Bacto™ Brain Heart Infusion; BD, USA) agar medium and grown aerobically at 37°C for 24h. One colony of the culture strain was taken and was inoculated into the 3mℓ of BHI broth.

After overnight culture, an accurate measurement of optical density of the cells was done by enzyme-linked immunosorbent assay (ELISA) at 450nm.

Then, the yeast was centrifuged, washed twice with phosphate-buffer saline (PBS, pH 7.2), and resuspended to required final concentration (10^5 cfu/ml) by serial dilution with $1\times$ PBS.

5. Antifungal activity test

Each specimen was placed in a Petri dish. 1ml of the fungal solution was pipetted onto the coated and polished specimens and the specimens were illuminated from 15cm above with two of UVA light ($2\times 10\text{W}$, 352nm peak emission, Blacklight Blue; Sankyo Denki, Japan). Each $2\mu\text{l}$ of sample was plated on the BHI agar plate in duplicates at 10 min intervals for 2 h. The temperature increase of the samples was 8°C (from 27°C to 35°C) at most after 120 min of irradiation. All plates were incubated for 24 h at 37°C , and the colony-forming units (CFUs) were then counted.

controls: The effect of UVA light alone on the microorganisms was determined on uncoated (polished) specimen.

III. Results

1. Characterization of TiO₂ photocatalysts

LT photocatalyst used in this study is composed of TiO₂ which has photocatalytic activity under room temperature. Fig.1 shows XRD patterns of the LT particles dried under 100°C and the commercial P-25 photocatalyst. LT showed the diffraction peak of anatase phase at 25° but showed very little rutile phase. On the contrary, P-25 showed not only the peak of anatase phase but also that of rutile phase at 27, 36, 41°. The rutile content of the P-25 measured from the peak area was 20–25%, which seemed somewhat high.

Fig.2 is TEM photographs which show the size and morphology of TiO₂ particles. P-25 particles were 20–50nm in diameter and had irregular shapes. The shape of LT particle was like an embryo bud of rice which was 30nm in long axis and 0–7nm in short axis.

Fig.3 shows N₂ adsorption isotherms to measure the surface areas of LT powder dried at 100°C and P-25 photocatalysts. Surface area of LT was about 200m²/g, which was four times larger than that of P-25 (about 50m²/g).

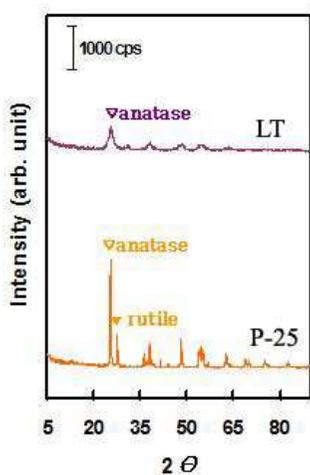


Fig.1. XRD patterns of LT and P-25 photocatalysts.

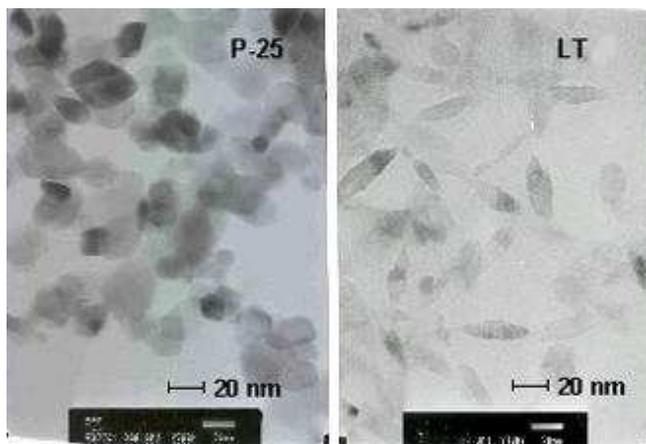


Fig.2. TEM images of LT and P-25 photocatalysts.

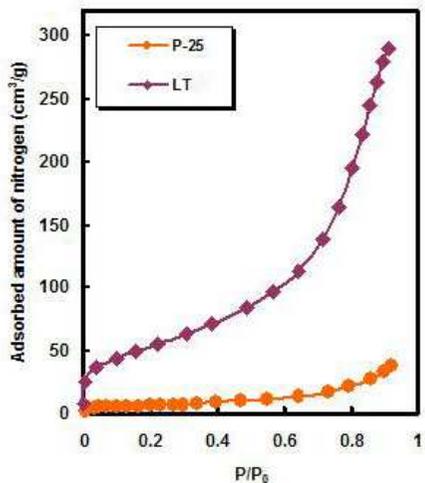


Fig.3. Nitrogen adsorption isotherms of the LT powders dried at 100°C and P-25 photocatalysts.

2. Characterization of TiO₂ thin film supported on acrylic resin

XRD was used to detect the anatase and rutile crystallite phases of TiO₂ coated surface. XRD patterns of the polished and TiO₂-coated specimens are shown (Fig.4). The diffraction peaks of polished type had a very low intensity indicating that the material was amorphous. In TiO₂-coated type, intensities of diffraction peaks were too weak to confirm their crystalline structure; Despite this, the observed peaks corresponded to the anatase phase. And these patterns showed a high dispersion of TiO₂.

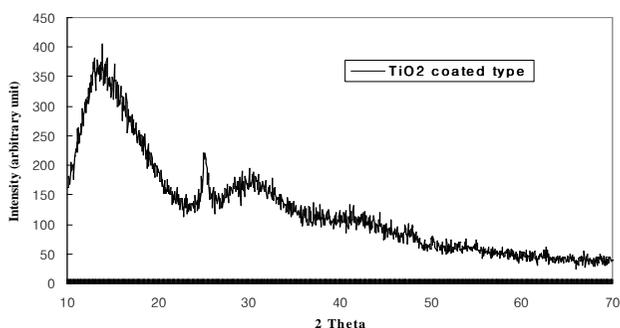
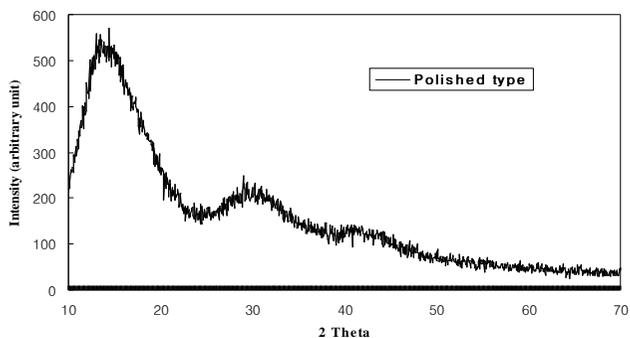


Fig.4. XRD patterns of polished and TiO₂(LT)-coated acrylic resin specimens.

To investigate the shape and size of the TiO₂ particles, the author examined the TiO₂-coated specimen with scanning electron microscopy. And general surface texture of the specimen was compared with the polished specimen. Fig.5 shows SEM images of the specimens. TiO₂ thin film consisted of heterogeneous spherical particles with a very small size (below 20nm). At low magnification, polished specimen had a rough surface and scratch due to polishing procedure. On the contrary, TiO₂-coated specimen had a relatively smooth surface.

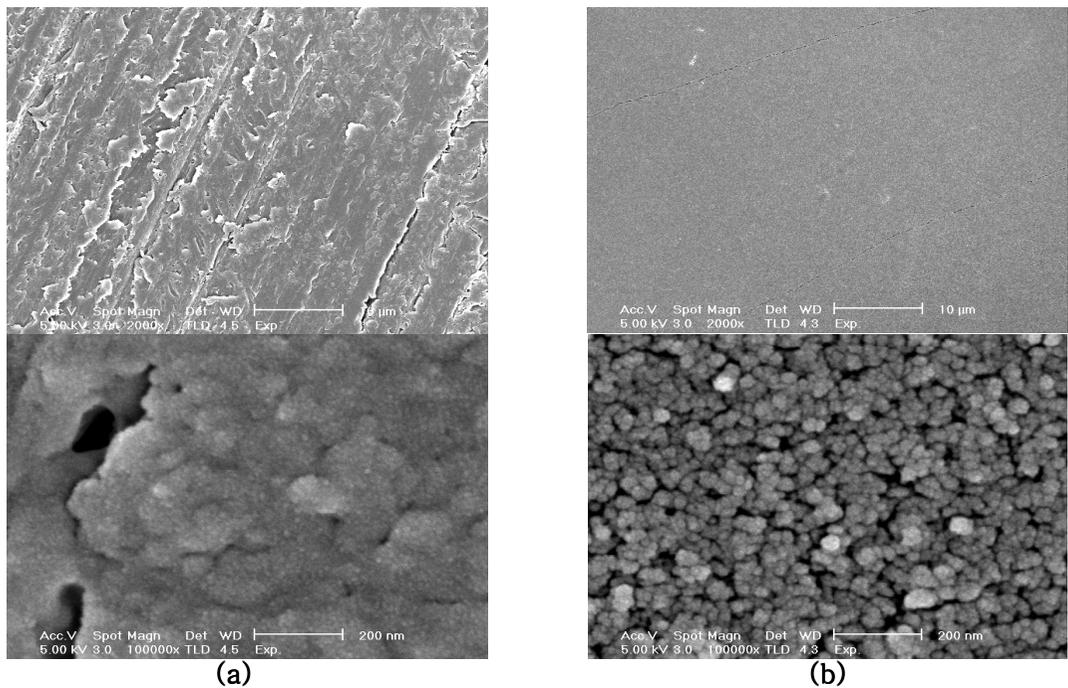


Fig.5. SEM images of polished(a) and TiO₂(LT)-coated(b) acrylic resin specimens. (Upper:×2000, Lower:×100000)

Table 1. showed the results of EDS which measured the chemical components of micro area (<1μm) of the surface. The polished specimen was composed with C and O. In LT-coated specimen, Ti and O were predominant. A small quantity of Na and Si was duo to the binder materials.

Table 1. EDS data of specimens.

<polished specimen>			<TiO ₂ (LT)-coated specimen>		
Element	Wt%	At%	Element	Wt%	At%
C	7.55	14.75	C	76.61	81.35
O	39.70	58.25	O	23.39	18.65
Na	1.70	1.73			
Si	0.69	0.58			
Ti	50.36	24.68			
Total	100.00	100.00	Total	100.00	100.00

Fig.6 shows the photocatalytic degradation efficiency of methylene blue on polished and LT-coated acrylic resin specimens. Methylene blue is useful to evaluate photocatalytic activity since it is easy to detect the color change in accordance with the degree of degradation. More than 5% of methylene blue was degraded after 2 hours in LT-coated specimen. However, uncoated specimen showed almost no change after UV irradiation.

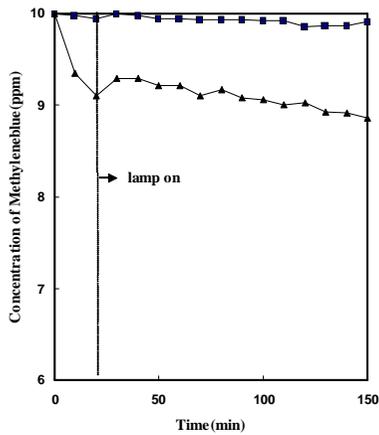


Fig.6. Photocatalytic degradation efficiency of methylene blue on polished(■) and TiO₂(LT)-coated(▲) acrylic resin specimens.

The hydrophilic property of photocatalyst is the phenomenon where H₂O molecule adsorb to the surface of a material to develop the highly hydrophilic membrane, therefore, water drops to disperse without drop formation. In this study, the uncoated specimen showed well-formed water drop. In LT-coated specimen, however, water dispersed to form aqueous membrane with its hydrophilic property (Fig.7). It means that it's easy to get rid of contaminated materials.



Fig.7. Mean contact angle of polished(a) and TiO₂(LT)-coated(b) acrylic resin specimens.

3. Photocatalytic antifungal activity

Inactivation of *Candida albicans* by TiO₂ is shown in Fig.8. The time course of viable cells when cell suspensions were irradiated with TiO₂ under Black Light fluorescent lamp was determined. The number of viable cells decreased rapidly and complete inactivation was achieved after 30 min. However, UV light only without TiO₂ had a little effect on cell survival and about 50% of the cells lost viability. In addition, complete killing was

not achieved after 120min of illumination. On the contrary, the increase of the viable cell number was observed at the final stage. It was due to the use of young cultures being in a state of two about to divide into four.

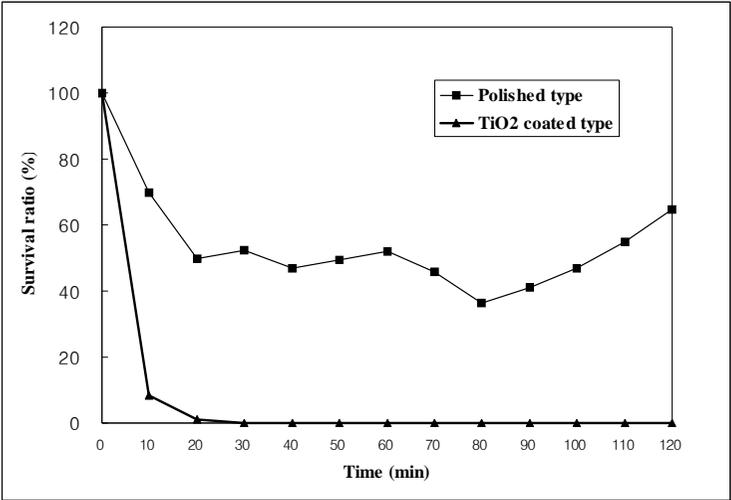


Fig.8. Antifungal activity against *Candida albicans*.

IV. Discussion

Bacterial and yeast plaque on dentures is thought to be an important etiologic factor in the pathogenesis of denture stomatitis. Since these fungi have been reported to colonize easily and penetrate denture materials, particularly tissue conditioners, and mechanical cleaning per se is insufficient to remove harbored *Candida* and harmful to soft-lining materials, chemical cleansing is suggested to be indispensable to denture plaque control. Therefore, many denture cleansers have been marketed for the removal or reduction of denture plaque. However, it has been pointed out that the chemical denture cleaning and sterilization often induced some problems such as color change, porosity and surface roughness on the denture base resin²⁸⁾ as well as porosity and surface roughness on the tissue conditioner. Moreover, in the preliminary study, fungicidal effects of some cleansers tested were less effective with a soaking period of 30 minutes⁵⁾.

The ability to reduce the colonized yeasts and/or fungal biofilm from acrylic resin may be particularly important in clinical term, since it is widely accepted that the biofilm organisms in vitro have a substantially reduced sensitivity to chemical agents such as antibiotics compared with the same organism in the dispersed form¹³⁾.

The use of the photocatalytic materials for denture cleaner seems to be able to solve those problems. In this study, *C. albicans* with 10^5 cfu/ml deposited on TiO₂-coated acrylic resin specimen was illuminated for 2 h.

The results of UVA-TiO₂ reaction showed that complete inactivation was achieved after 30 min.

However, the lethal mechanism of TiO₂ is not well understood yet. Some authors have proposed that the cell wall is the target of this effect. Sunada et al. showed endotoxin degradation in *E. coli*³⁷⁾, and Pinching et al. suggested the peroxidation of lipids as the initial effect³⁰⁾. Some papers deal with the structural damage on the bacterial cell; Satio et al. reported by TEM analysis a complete destruction of *Streptococcus sobrinus* AHT cells, after 60–120 min of photocatalytic action. They suggested a change in cell membrane permeability³¹⁾. *C. albicans* has a thick eukaryotic cell wall. So, *C. albicans* appears to be more resistant to cleansers than the bacteria¹¹⁾. It is assumed that primary step in photocatalytic decomposition is an attack by OH radicals on the cell wall, leading to punctures.

Since the effectiveness of heterogeneous catalysis also depends on the adsorption of reaction partners to the TiO₂²¹⁾, it would be interesting to measure the surface charge and the hydrophobicity of the bacteria and surfaces, and to correlate these findings with the sensitiveness to photocatalytic oxidation.

LT photocatalyst used in this study is specific synthesis which is able to coated to the acrylic resin at room temperature. In XRD patterns (Fig.2), LT photocatalyst which has few rutile structure is expected to have higher activity than P-25, because TiO₂ in anatase phase generally has been proved to have higher efficiency than in rutile phase^{19,27)}. Since degradation activity by the photocatalyst is shown on the surface of the TiO₂ particles, we can figure out that photocatalytic activity of LT with surface area four

times larger than p-25 is maximized.

To date, little emphasis has been placed on the possibility of preventing plaque formation and accumulation on dentures. LT photocatalyst-coated acrylic resin specimen used in this study had a hydrophilicity (Fig.7). We expect the self cleaning effect due to this hydrophilicity which suppresses biofilm formation on the denture base. The larger the contact angle is, the more irregular the surface has as the uncoated specimen. Irregularities and porosities present on the denture surface play a major role in reducing the activity of denture cleaning agents and hence increased stain and plaque retention.

It is confirmed by the results of SEM images (Fig.5). Uncoated specimen had irregularities, microcrack, and scratch made by polishing procedure. But, LT photocatalyst-coated specimen had a smooth surface, because the irregularities could be filled up with TiO_2 particles with a small size below 20nm.

UVA light source with 352nm peak emission was used in this study. The light used for photocatalyst is high energy-ultraviolet light whose mean wavelength is shorter than 400nm, and it is included in sunlight or lighting in the residential space. In comparison to the whole intensity of light, however, usable amount of ultraviolet light is very small. In case of sunlight, ultraviolet light intensity during day when the light is the most intensive is about $1\text{mW}/\text{cm}^2$, which is just 1% of the whole sunlight. So, in the room with less light intensity, the technology to maximize the amount of usable ultraviolet light is necessary.

The photochemical reaction of TiO_2 comes from the reaction of hole and

electron generated from the resorption of the photo-energy which corresponds to TiO_2 band gap energy (3.4 eV). The measures to lower the band gap energy to utilize long waved photo-energy should be investigated by slowing down the reunion rate of these electron pairs and doping method etc.

Generally, as in this study, photocatalyst adheres to the substrate by using condensing polymer of silicon alkoxide as a binder, or the photocatalyst nano-particles are fixed to the substrate by using fluorine resin as a binder which is relatively stable against photocatalytic reaction. A little amount of the silane binder can make fixed membrane with high hardness under high hardening temperature. The types and amount of the binder should be chosen in accordance with the substrate. The amount of the binder should be controlled according to the properties of matter that the operator is willing to use, because the increase of the membrane strength from the increased amount of the binder and the photocatalyst activity are in inverse proportion to each other. This study didn't consider binding strength of the binder and TiO_2 sol to the specimen, and so additional investigations about this subject are required.

V. Conclusions

Within the parameters of the study design and materials tested, it was possible to disinfect acrylic resin denture surfaces coated with a specific semiconductor(TiO_2) and stimulated by UVA. The results reported above suggest that TiO_2 photocatalysis may be a viable process for inactivation of *C. albicans* and could be applicable to the clinical use as a cleansing method.

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논문제목	국문: TiO ₂ 를 코팅한 아크릴릭 레진 의치상의 캔디다에 대한 광촉매적 항진균 활성에 관한 연구 영문: Photocatalytic antifungal activity against <i>Candida albicans</i> by TiO ₂ coated acrylic resin denture base				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DM구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함.
다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간 종료 3개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보 통신망을 이용한 저작물의 전송·출력을 허락함.

2005년 월 일

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