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Antibiofilm Evaluation of Two Different Denture Liners Incorporated with Zirconium Oxide Nanoparticles

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Research Article

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ABSTRACT

Objectives: This *in vitro* study was purposed to examine the antibiofilm activity, weight change, and surface properties including glucose sorption, and roughness of novel nano-ZrO_2 incorporated denture liners.
Materials and Methods: Modified nano-ZrO_2 were added to silicone-based and acrylic resin-based prosthetic lining materials at two different concentrations (0.5% and 1%). The antibiofilm potentials of test groups against Candida albicans (*C. albicans*), Staphylococcus aureus (*S. aureus*), and Streptococcus mutans (*S. mutans*) were determined using 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium (MTT) method. Surface roughness, weight change, and glucose sorption of denture liners were also evaluated after modifying them with nano-ZrO_2.
Results: According to the antibiofilm activity results, 1% nano-ZrO_2 addition to silicon-based and acrylic resin-based denture liners resulted in 16.48% and 13.39% of biofilm inhibition for *S. aureus*, respectively. 1% nano-ZrO_2 addition to silicon-based denture liners also inhibited the *S. mutans* biofilm formation at an 8.16% rate. Nano-ZrO_2 addition to the test groups had no inhibition effect on *C. albicans* biofilm formation. Surface roughness decreased significantly once nano-ZrO_2 was added in acrylic resin-based test groups; however, addition of 0.5% nano-ZrO_2 increased silicone-based test group significantly.
Conclusions: To mitigate microbial biofilm problems caused by the use of denture liners addition of nano-ZrO_2 might be a promising method owing to its antibiofilm capacities especially against biofilms of *S. aureus* and *S. mutans*.

Keywords: Denture Liners, Glucose, Nanoparticles, Surface Properties, Zirconium Oxide

Zirkonyum Oksit Nanopartikülleri İlave Edilmiş İki Farklı Protez Astarlarının Antibiyofilm Değerlendirmesi

Süreç

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ÖZ

Amaç: Bu *in vitro* çalışma, güncel nano-ZrO_2 ilave edilmiş protez astarlarının antibiyofilm aktivitesi, ağırlık değişimi, glukoz emilimi ve pürüzlülüğü dahil yüzey özelliklerini incelemek amaçlanmıştır.
Gereç ve Yöntemler: Modifiye nano-ZrO_2 silikon esaslı ve akrilik rezin esaslı protetik astar malzemelerine iki farklı konsantrasyonda (%0,5 ve %1) eklendi. Test gruplarının *Candida albicans (C. albicans)*, *Staphylococcus aureus (S. aureus)* ve *Streptococcus mutans (S. mutans)*'a karşı antibiyofilm potansiyelleri, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) yöntemi kullanılarak belirlendi. Nano-ZrO_2 ile modifiye edildikten sonra protez astarlarının yüzey pürüzlülüğü, ağırlık değişimi ve glikoz emilimi de değerlendirildi.
***Bulgular*:** Antibiyofilm aktivite sonuçlarına göre, silikon esaslı ve akrilik rezin esaslı protez astarlarına %1 nano-ZrO_2 ilavesi *S. aureus* için sırasıyla %16,48 ve %13,39 biyofilm inhibisyonu ile sonuçlandı. Silikon esaslı protez astarlarına %1 nano-ZrO_2 ilavesi de *S. mutans* biyofilm oluşumunu %8,16 oranında engelledi. Test gruplarına Nano-ZrO_2 ilavesinin *C. albicans* biyofilm oluşumuna inhibisyon etkisi olmadı. Akrilik esaslı test gruplarında nano-ZrO_2 eklendiğinde yüzey pürüzlülüğü önemli ölçüde azaldı, ancak %0,5 nano-ZrO_2 eklenmesi silikon esaslı test grubunu yüzey pürüzlülüğünü ise önemli ölçüde artırdı.
***Sonuçlar*:** Protez astar kullanımından kaynaklanan mikrobiyal biyofilm problemlerini azaltmak için nano-ZrO_2 ilavesi, özellikle *S. aureus* ve *S. mutans* biyofilmlerine karşı antibiyofilm kapasiteleri nedeniyle umut verici bir yöntem olabilir.

Anahtar Kelimeler: Protez Astarları, Glikoz, Nanopartiküller, Yüzey Özellikleri, Zirkonyum Oksit.

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Introduction

In spite of the increasing health awareness of patients, the requirement for the use of removable denture is growing steadily.[1] Removable complete denture aims to replace the patient's missing teeth and soft tissue with hard acrylic dentures. Tolerance of the patient depends on how well the denture is fit in the mouth.[2] The thickness of patients' lining mucosa decreases due to resorption in the cases with highly resorbed ridge[3], thus the overlying thin mucosa cannot tolerate high chewing forces distributed by hard base material.[2] Soft denture liners are suggested to provide functional load distribution equally on the denture foundation area and to reduce local point pressures.[4] In addition, these materials improve the fitting tissue surface of the denture base and retention of the prosthesis.[5]

Tissue conditioners (TCs) are short-term soft liners. They usually consist of polyethyl methacrylate (PEMA) polymer powder and liquid components.[6] TCs are widely used in; the treatment of damaged mucosal tissues underlying poorly fitting dentures, in making dynamic impressions[7], for provisional relining of poorly fitting dentures and immediate dentures, and for tissue conditioning during implant healing.[8]

Denture liners can be classified as provisional or permanent depend on their duration of use, and as silicone rubber or acrylic resin regarding their composition. They can also be categorized depending on their polymerization method for instance heat, chemical or light cure.[9,10] There are some problems related to these materials: loss of resilience[5,6,11], change in color[5,6,9,12], porosity[5,9], water sorption and solubility[6,12], rough surface character[9], microbial adhesion and colonization.[11,13,14] In addition, the use of these materials harbor biofilm growth of fungal or bacterial pathogens, especially in geriatric patients with poor oral hygiene.[15]

Denture liners are easily colonized by pathogenic microorganisms.[13] The surface topography and the composition of material are the parameters that are directly associated with microbial adhesion to dental materials.[16] Denture liners are prone to microbial colonization due to degeneration and further degradation over time.[17,18] A rough surface supports microbial growth caused by the adhesion of microbial cells. This microbial growth occurs with the adhesive interactions between Candida species and oral bacteria (mostly *C. albicans* and *oral streptococci*).[19] Surface roughness is a significant surface property affects the adhesion of microorganisms on denture liners. As the surface roughness of denture liners increases, the accumulation of biofilm will also increase.[16] Biofilm adherence with its enzymatic activity leads to the degradation of liner materials and irritate the denture foundation area.[17]

Nanotechnology has an effective and fundamental role in developing the properties of dental materials.[20] The size, distribution, and morphology of nanoparticles are specific properties. Thanks to these properties, they show either completely new or improved features. Recently, new applications of nanoparticles are increasing rapidly.[21,22] Metallic nanoparticles exhibit improved reactivity and increased surface area. Therefore, the antimicrobial properties of these nanoparticles have been of interest.[20]

Nano-ZrO_2 has properties as high strength, high fracture toughness, excellent abrasion resistance, and superior chemical resistance. Due to these properties, it is added as a filler to the denture bases.[23] The addition of nano-ZrO_2 is recommended to improve the mechanical properties of PMMA.[24,25] Nano-ZrO_2 possesses excellent mechanical and chemical properties as well as antimicrobial properties.[26] Several studies have reported that it has antibacterial and antifungal effects on *C. albicans* and *S. aureus*.[26-28] It was reported that the antibacterial effect of nano-ZrO_2 on *C. albicans* may result from the active oxygen species produced by nano-ZrO_2. These produced active oxygen species disrupt the cell membrane of microorganisms.[27]

Numerous *in vitro* studies have been conducted focusing on different aspects of soft lining materials related to microbial and fungal colonization.[29-31] Among these studies include the use of denture cleaners[14,32], the addition of antimicrobial or antifungal agents[30,31], and the addition of fillers at low concentrations.[19,33] Although there are studies about the addition of nano-ZrO_2 to PMMA denture base[22,25,26], there is not enough information regarding the incorporating of nano-ZrO_2 into denture liners in the literature.[34]

The purpose of this *in vitro* research was to examine the antibiofilm activity of an acrylic-based tissue conditioner and a silicone-based soft denture liner combined with nano-ZrO_2. Additionally, the objective was to evaluate the impact of adding nano-ZrO_2 on glucose sorption, weight change, and surface roughness of two types of different denture liners. The null hypothesis of this study was that the addition of nano-ZrO_2 into denture liners would not affect the evaluated parameters.

Materials and Methods

The test materials used in this research study are presented in Table 1. ZrO_2 nano-powder (St. Louis, MO, USA average particle size of ≤100 nm) and silane coupling agent [3-aminopropyltriethoxysilane, (APTES, St. Louis, MO, USA)] were supplied by Sigma-Aldrich.

Surface functionalization of Nano-ZrO_2 Using a Silane Coupling Agent

The introduction of reactive groups to the surface of the filler was achieved by reaction of the silane coupling agent with nano-ZrO_2. Since the silane agent was cross-linked with the surface hydroxyl groups of nano-ZrO_2, the homogeneous distribution of nano-ZrO_2 fillers was ensured without agglomeration in the denture liners.

250 mg of nano-ZrO_2 and 5 mL pure toluene (≥99.7%, Sigma-Aldrich) were placed into a flask and then ultrasonicated at ambient temperature for 20 min. After that, this solution was placed into a flask equipped with a

magnetic stirrer (WiseStir MSH-20A, Germany) at speed 0-1500 rpm. The silane in the amount of 13.21 µL was added dropwise using a sterile syringe under a rapid stirrer. After the flask was covered by parafilm, the slurry was left for two days. The solvent (toluene) was removed from the slurry using a rotary evaporator (Büchi rotavapor R-210, Switzerland) under vacuum at 60°C rotary 150 rpm for 30 min. The modified nano-ZrO_2 particles were dried in a vacuum oven (BINDER vacuum drying Model VD 53, Tuttlingen, Germany) at 60°C for 20 hours and, then the nanoparticles were stored at room temperature before use.

The Fourier Transform Infrared Spectroscopy (FT-IR) measurements

Binding analysis showing before and after the modification step with the silane binding agent of nano-ZnO_2 was performed using FTIR (Nicolet iS5, Thermo Scientific, Madison, WI, USA) within wavelength the range of 450–4500 cm^{-1} obtaining by 40 scans. The spectrum peaks were recorded using OMNIC Spectra Software and then analyzed.

Preparation of test specimens

The two commercial denture liners materials were examined in this study. These materials were one silicone-based soft denture liner (Ufi Gel P; VOCO GmbH, Cuxhaven, Germany) and one acrylic-based tissue conditioner (Visco-gel, Dentsply DeTrey GmbH, Konstanz, Germany). Concentrations of nano-ZrO_2 added to the test materials are presented in Table 2. Three sub-groups were formed in each group including the control group [Ufi Gel P (UGP) / Visco-gel (VG)]. Two weight percentage of modified nano-ZrO_2 (0.5% and 1%) were used. These groups were tissue conditioner (Visco-gel) and soft lining material (Ufigel P) containing 0.5% and 1% modified ZrO_2 (respectively VG 1, VG 2, UGP 1, and UGP 2).

Modified nano-ZrO_2 and hexane solvent (Sigma Aldrich, St. Louis, MO, USA) was placed into a glass beaker, and then this solution was ultrasonicated (20 min) to provide nano-ZrO_2 particles to disperse well. After the incorporation of the catalyst of UGP test material into the solution, this mixture subjected to ultrasonication for 20 min. The hexane was removed from the mixture using an evaporator at the room temperature. The base of UGP was mixed with the composite including the catalyst and modified nano-ZrO_2. Before adding nano-ZrO_2 fillers to the VG test materials, nano-ZrO_2 particles were modified aforementioned method. The liquid of VG test material and modified nano-ZrO_2 was mixed with the powder of VG. According to the manufacturer's instructions, for UGP test materials, the obtained mixture was prepared in a weight ratio of 1:1 base to a catalyst, while for VG test materials, at a 3: 2.2 powder to liquid ratio.

Test methods

Glucose sorption and weight change

The glucose sorption test method was performed according to Muttagi and Subramanya.[35] Artificial saliva (AS) was prepared by mixing 0.400g NaCl, 0.400g KCl, 0.795 g $CaCl_2H_2O$, 0.69g NaH_2PO_4, 0.005g $Na_2S.9H_2O$, 1.0g urea, and 1000mL distilled water[36] was prepared. The pH of AS was adjusted to 7.00 with NaOH or HCl, and then the volume was increased to 1 L. Artificial saliva with glucose (G-AS) was obtained by adding 150 g of glucose to the AS with the same composition.

Table 1. Test materials evaluated in the study

Materials (Code)	Material Type	Main Composition	Lot No.	Manufacturer
Visco-gel (VG)	auto-polymerized acrylic-based tissue conditioner	Powder: Polyethyl methacrylate Liquid: Ethanol, Butyl phthalyl butly glycolate, Dibutyl phthalate	1610000172	Dentsply DeTrey GmbH, Konstanz, Germany
Ufi Gel P (UGP)	auto-polymerized silicone-based soft denture liner	Modified polydimethylsiloxane and platinum catalyst	1645226	VOCO Gmbh, Cuxhaven, Germany

Table 2. Classification of test materials used in the study and percentages and amounts of powder, liquid and nano-ZrO_2 powder of these materials

Groups	nano-ZrO_2 conc. percentages	amounts of nano-ZrO_2 (mg)	amounts of powder or base (g)	amounts of liquid or catalyst (g or µl)
Group UGP 0 (Ufigel UGP 0)	0%	0 mg	0.1 g	0.1 g
Group UGP 1 (Ufigel UGP+nano-ZrO_2)	0.5%	1 mg	0.1 g	0.1 g
Group UGP 2 (Ufigel UGP+ nano-ZrO_2)	1%	2 mg	0.1 g	0.1 g
Group VG 0 (Viscogel)	0 %	0 mg	0.2 g	146.6 µl
Group VG 1 (Viscogel+nano-ZrO_2)	0.5%	1 mg	0.2 g	146.6 µl
Group VG 2 (Viscogel+nano-ZrO_2)	1%	2 mg	0.2 g	146.6 µl

Each of the 3 test specimens of the 6 groups (Group UGP0, UGP1, UGP2, and VG0, VG1, VG2) were submerged in 50 mL of G-AS and were shaken at 75 rpm for 20 minutes. Test specimens were removed from G-AS and excess saliva was wiped dry. After the test specimens were rinsed for 2 minutes with 20 mL of distilled water, they immersed in 50 mL AS for 6 hours. While the control group was kept in AS throughout the experiment, the other test specimens were taken from AS after 6 hours and then immersed in G-AS. The described procedure was repeated for all test specimens 3 times a day throughout 3 days. Both G-AS and AS were changed for each reading and every 24 hours, respectively.

The amount of glucose in the distilled water was determined with the Phenol sulfuric acid method.[35] The standard calibration curve was used to calculate the amount of glucose in the distilled water for all specimens. After every 24 hours, the amount of glucose in AS was calculated similarly. At the end of the third day, the test specimens which were divided into small pieces were placed in 100 mL of ethyl acetate. The specimens in the flasks were kept on a shaker for 4 hours to completely dissolve. The ethyl acetate solution was washed using 20 mL of distilled water every 30 minutes and this process repeated 5 times. The separating funnel separated the washed distilled water. This distilled water collected in the flask and then the glucose amount was calculated.

Antibiofilm Activity

Microorganisms and Culture Medias

Candida albicans (C. albicans) (ATCC 10239), Staphylococcus aureus (S. aureus) (ATCC 6538P), and Streptococcus mutans (S. mutans) (ATCC 25175) were purchased from the American Type Culture Collection (ATCC) and used as three standard strain organisms. S. aureus was cultivated in Nutrient Broth (NB) culture at 37°C; S. mutans was cultured in Brain Heart Infusion Broth (BHIB) under a humidified atmosphere of 5% CO_2 at 37°C and C. albicans strain was grown in Sabouraud Dextrose Broth (SDB) at 30ºC.

Disc specimens (5x1.5 mm dimensions; n=10) from per group were prepared to evaluate their antibiofilm activities. They were assigned into 3 subgroups concerning the added amounts of nano-ZrO_2 (Group 0: control, Group 1: 0.5% nano-ZrO_2 added, Group 2: 1% nano-ZrO_2 added). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining method was used to measure the biofilm inhibition rates of the denture liners incorporated with nano-ZrO_2. The disc-shaped specimens were incubated with microbial inoculum in Tryptic Soy Broth (TSB) supplemented with 5% D-Glucose for 72 h. Following the end of the incubation period, the specimens were washed twice with PBS. Then, these specimens were immersed in 5 mL PBS and sonicated for 5 min to remove the attached microbial biofilm layer. The sonicated solution was centrifugated. The pellet was suspended with 150 µL PBS in microplate wells. 50 µL MTT solution added to the wells for staining the live microorganisms and incubated for 2 h at 37°C. The media was removed and 150 µL of dimethyl sulfoxide (DMSO) and 25 µL glycine buffer were added. The experiment was done in triplicate. The absorbances were read at 550 nm in a microplate reader (Multiskan GO UV/Vis Microplate Spectrophotometer, Thermo-Fisher Scientific, Rockford, IL, USA). Results compared with the control discs that were not containing nano-ZrO_2 and percentage of biofilm inhibition was calculated.[38]

Surface Roughness Test

For the surface roughness test, specimens with a diameter of 10 mm and a thickness of 1.5 mm were produced using a stainless-steel mould (n = 10). As the specimens were produced, the glass plate which approximately 0.009 µm, was placed under and above the stainless-steel mould and kept under vertical force. The surface roughness test of all specimens was performed with the profilometer device (SJ-210, Mitutoyo, Kanagawa, Japan) (cut-off wavelength of 0.8 µm and a speed of 0.5 mm/s). 3 different areas in the same direction for each specimen were measured. Average surface roughness (Ra) calculated.

Scanning Electron Microscope (SEM) Examination

Two test specimens from each group were randomly chosen for surface analysis under Scanning Electron Microscope (SEM) (Zeiss, SUPRA-55, Carl Zeiss NTS GmbH, Oberkochen, Germany). The selected specimens were made conductive under the vacuum of 10^{-1} mbar/Pa and a current of 10 mA in the gold-palladium coating unit (Quorum Q 150 R ES DC Sputter, Kent, UK) by coating with Au-Pd for 180 seconds. After coating, the SEM micrographs of specimens were taken at a magnification of x50, x100, x200. The surface morphological features for each subgroup's tissue conditioner and soft denture liner were visualized and then analyzed.

Statistical Analysis

Data were analyzed using IBM SPSS Statistics version 17.0 software (IBM Corporation, Armonk, NY, USA). The evaluation of continuous variables was first performed for homogeneity and normality by Levene and Shapiro-Wilk tests, respectively. Mann Whitney U test was used to determine the differences in continuous variables between test groups. However, in comparisons between more than two independent groups, the data were subjected to the Kruskal Wallis test. When the p-values were statistically significant according to the Kruskal Wallis test, the differences among all the combination groups were determined by Conover's multiple comparison test.

Friedman's test was used to evaluate whether the differences among measurement times in terms of both glucose concentrations in distilled water and weight of specimen were statistically significant or not. $p<0.05$ was considered statistically significant. However, the Bonferroni correction was performed for all possible multiple comparisons to check Type I errors.

Results

FT-IR Analysis

FTIR spectra taken to confirm the covalent binding between the nano-ZrO_2 particles and the silane are shown in Figure 1.

In the transmittance spectrum of the silane, the two peaks at 1072 and 952 cm^{-1} were attributed to the asymmetric and symmetric stretching vibrations of Si-O-C bond, respectively. And the peak at 764 cm^{-1} correspond to the bending of Si-C bond. The absorption bands at 2884 and 2974 cm^{-1} arose from CH_3 symmetric and asymmetric stretching vibrations, respectively. The peak at 2927 cm^{-1} is due to the CH_2 asymmetric stretching vibration. In the transmittance spectrum of the silane modified nano-ZrO_2 particles, the strong peaks resulting from vibrations of Si-O-C bond were disappeared due to breaking of these bonds in the silane molecule. And two new strong peaks appeared at 1154 and 1225 cm^{-1}, which could be assigned to the stretching vibration of Si-O-Si and twisting vibration of CH_2, respectively. On the other hand, the peaks originating from the methyl group in silane molecule disappeared due to the removal of the methyl group as a result of the reaction. In addition, absorption of Zr-O-Si bond seemed to be overlapped with the strong absorption of Zr-O-Zr bond below 800 cm^{-1}. According to the peaks of these covalent bonds, we can say that the surface of the nano-ZrO_2 particles with the silane coupling agent has been successfully modified.

Glucose sorption and weight change

In all VG test groups for the first day, there was no statistically significant difference between the 6th hour, the 12th hour, and the 24th hour in terms of median glucose concentrations in the distilled water (VG0: p = 0.050, VG1: p=0.097, VG3: p=0.050). Likewise, as far as the comparison between the 6th hour, the 12th hour, and the 24th hour regarding median glucose concentrations in the distilled water for the first day, no statistically differences were found in all UGP test specimens (UGP0 and UGP1 p = 0.050, UGP2: p = 0.097) (Table 3). Besides, the glucose sorption of test groups was not statistically significant among all subgroups for all three days in artificial saliva (p>0.05) (Figure 2).

Among the VG0, VG1 and VG2 groups, respectively; there was no statistically significant difference according to the Bonferroni correction in terms of the median sample weight of 0 h, 24 h, 48 h and 72 h (p>0.00625). Similarly, no statistically significant difference could be determined among the sample weights of the UGP0, UGP1 and UGP2 groups that measured every 24 hours (p>0.00625) (Table 4).

Antibiofilm Activity

Biofilm inhibition rates of the nano-ZrO_2-incorporated denture liners are given in Table 5. In the pilot study, a 0.25% added nano-ZrO_2 group was excluded from the study because this group did not show antibiofilm activity against all microorganisms tested. The highest antibiofilm activities were observed against *S. aureus* for UGP2 and VG2 with 16.48% and 13.39% inhibition, respectively. The biofilm formation of *S. mutans* was only inhibited by UGP2 with an 8.16% inhibition rate. *C. albicans*' biofilm formation was not inhibited by any mixture of the nano-ZrO_2-denture soft liner.

Table 3. The concentration of glucose in distilled water according to follow-up time, hours and groups

	Day 1			Day 2			Day 3		
	6th h	12th h	24th h	6th h	12th h	24th h	6th h	12th h	24th h
Group 0 VG 0	116.47 (4.50)	131.47 (3.76)	143.67 (1.46)	132.17 (4.85)	126.37 (3.98)	114.39 (1.02)	154.38 (2.72)	144.55 (3.06)	132.74 (2.19)
UGP 0	97.89 (1.10)	102.68 (1.04)	116.58 (3.91)	122.37 (3.11)	115.67 (3.27)	108.67 (4.02)	131.22 (2.80)	122.35 (3.01)	115.08 (2.09)
Group 1 VG 1	107.69 (1.88)	111.48 (7.09)	126.47 (7.60)	128.17 (6.11)	119.87 (1.72)	117.59 (2.36)	165.74 (6.68)	141.32 (2.48)	123.57 (4.20)
UGP 1	103.68 (2.20)	107.59 (2.99)	119.54 (4.46)	113.67 (1.55)	114.59 (2.33)	103.28 (4.28)	135.48 (2.67)	127.49 (3.03)	125.67 (1.62)
Group 2 VG 2	109.36 (2.25)	117.49 (4.89)	131.27 (4.11)	145.36 (7.20)	137.86 (3.12)	120.03 (2.02)	171.28 (2.67)	156.31 (5.07)	141.28 (2.40)
UGP 2	103.68 (2.45)	103.58 (1.02)	124.01 (6.19)	129.98 (2.48)	130.25 (2.50)	119.32 (5.99)	139.67 (3.09)	128.74 (6.71)	124.69 (4.31)

Values are shown in the form of the median (distribution between quarters).

Table 4. Weight change of test specimens according to groups at certain time intervals

	0. hour	12. hour	48. hour	72. hour
Group 0				
VG	0.472 (0.007)	0.488 (0.010)	0.507 (0.009)	0.510 (0.007)
UGP	0.547 (0.012)	0.542 (0.007)	0.541 (0.004)	0.537 (0.008)
Group 1				
VG	0.490 (0.010)	0.501 (0.014)	0.500 (0.014)	0.497 (0.012)
UGP	0.549 (0.001)	0.557 (0.011)	0.559 (0.007)	0.560 (0.006)
Group 2				
VG	0.498 (0.015)	0.502 (0.014)	0.504 (0.012)	0.510 (0.011)
UGP	0.570 (0.005)	0.579 (0.019)	0.580 (0.005)	0.576 (0.013)

Values are shown in the form of the median (distribution between quarters)

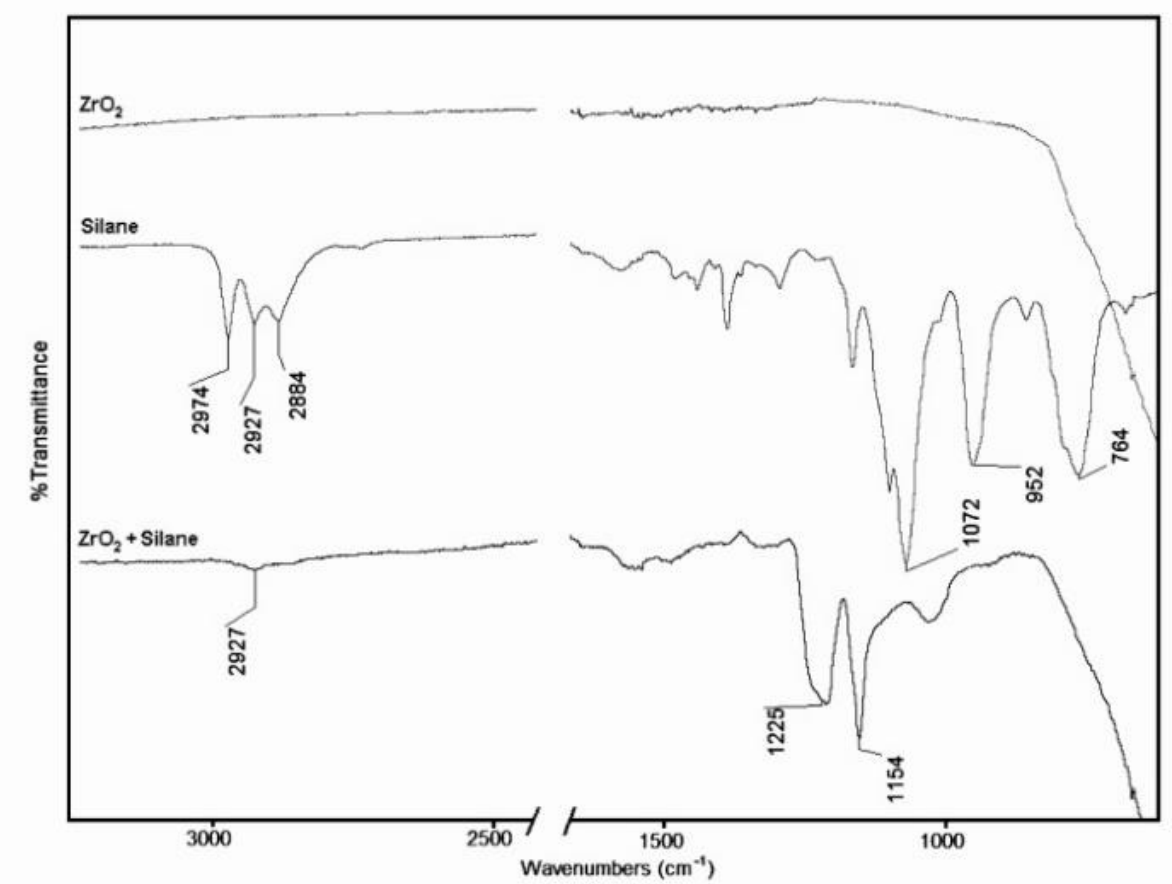

Figure 1. FT-IR spectrum of nano-ZrO_2 and modified nano-ZrO_2 with the silane coupling agent

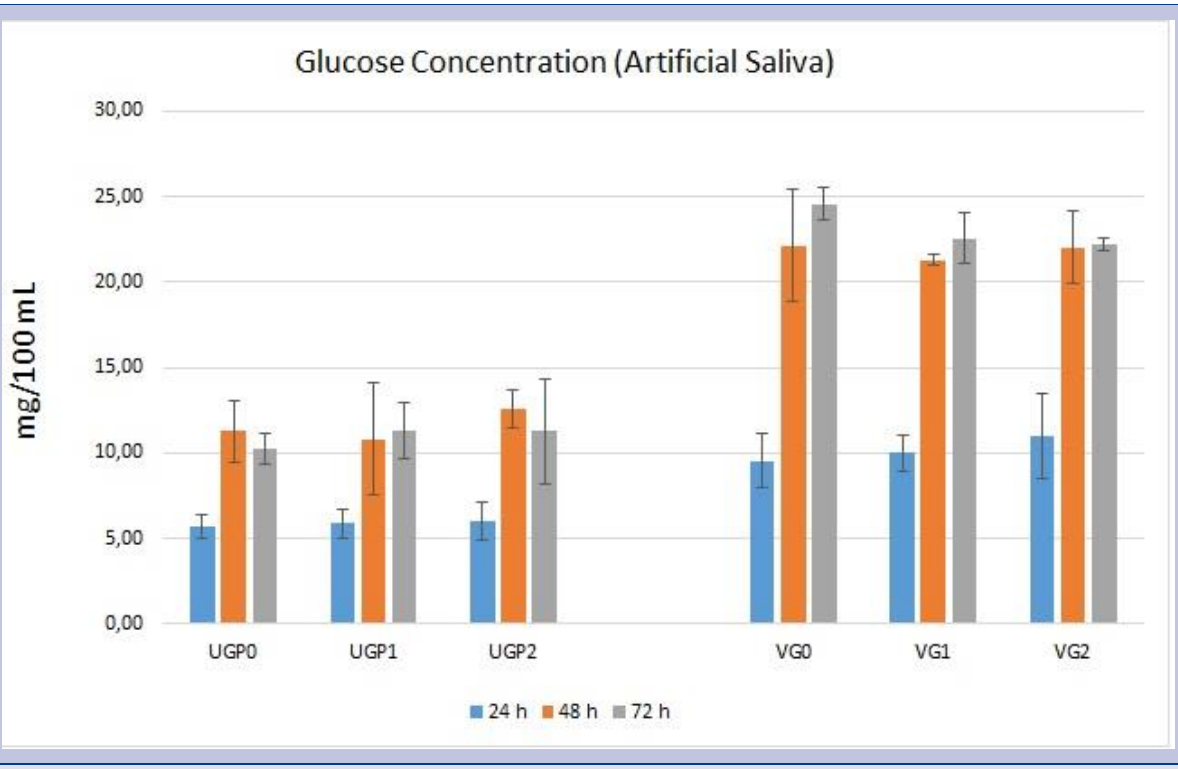

Figure 2. Glucose concentrations calculated in artificial saliva after certain time intervals

Table 5. Antibiofilm activity of denture liners incorporated with nano-ZrO_2

Tested Materials							
	UGP + nano-ZrO_2				VG + nano-ZrO_2		
Group	Antibiofilm Activity (%) C. albicans	Antibiofilm Activity (%) S. aureus	Antibiofilm Activity (%) S. mutans	Group	Antibiofilm Activity (%) C. albicans	Antibiofilm Activity (%) S. aureus	Antibiofilm Activity (%) S. mutans
UGP0	Control (100% Biofilm) (0% Activity)	Control (100% Biofilm) (0% Activity)	Control (100% Biofilm) (0% Activity)	VG0	Control (100% Biofilm) (0% Activity)	Control (100% Biofilm) (0% Activity)	Control (100% Biofilm) (0% Activity)
UGP1	0.00	3.46	0.00	VG1	0.00	2.28	0.00
UGP2	0.00	16.48	8.16	VG2	0.00	13.39	0.00

Table 6. Mean values of surface roughness for the test materials

	VG	UGP	*p*-value †
Group 0	3.97 (1.29)[a,b]	0.29 (0.37)[a]	**< 0.001**
Group 1	1.74 (2.01)[a]	0.66 (0.76)[a,c]	**0.004**
Group 2	2.38 (2.50)[b]	0.44 (0.26)[c]	**< 0.001**
p-value ‡	0.012	0.020	

†A comparison between VG and UGP groups showed the results to be statistically significant according to the Mann Whitney U test and Bonferroni Correction ($p<0.0167$), ‡ Likewise, a comparison within VG and UGP groups showed the results were of statistical significance for $p<0.025$ according to the Kruskal Wallis test and Bonferroni Correction, a: The difference between group 0 and group 1 was statistically significant ($p<0.001$), b: The difference between group 0 and group 2 was statistically significant ($p=0.002$), c: The difference between group 1 and group 2 was statistically significant ($p=0.002$).

Surface Roughness Test

The results of surface roughness are shown in Table 6. Among the UGP and VG groups, there was a statistically significant difference according to the results of surface roughness ($p<0.001$). The UGP groups (incorporating with 0.5% nano-ZrO_2 and 1% nano-ZrO_2) had statistically lower values than those of the VG groups (respectively $p=0.004$; $p<0.001$).

In VG groups, the addition of nano-ZrO_2 (0.5% and 1%) significantly reduced the surface roughness of the test specimens ($p<0.001$ and $p=0.002$). Among the VG 1 and VG 2 test groups, no difference of statistical significance could be determined according to Bonferroni correction ($p=0.050$).

Among the UGP test specimens, there was a statistically significant difference in terms of surface roughness ($p=0.020$). UGP 1 group had statistically higher values than those of UGP 0 and UGP 2 groups ($p<0.001$ and $p=0.002$). There was no statistically significant difference between UGP 0 and UGP 2 test groups ($p=0.098$).

SEM Observation

SEM analysis indicated a rougher surface with the pits and fissures in Group VG 0 (Figure 3A), while the incorporation of nano-ZrO_2 for tissue conditioning test materials displayed relatively smooth surface due to the existence of nanoparticles (Figure 3B and 3C).

The surfaces of Group UGP 0 and UGP 2 were relatively smoother than UGP 1 (Figure 3D, 3E and 3F). In both test groups with zirconium oxide added, nano-ZrO_2 was observed in a globular-shaped throughout the liner surfaces (Figure 3B, 3C, 3E and 3F). In UGP 1 and UGP 2 groups nano-ZrO_2 well distributed without agglomeration or clusters within the soft denture liner (Figure 3E and 3F). However, in VG1 and VG2 test groups, nano-ZrO_2 slightly agglomeration in the tissue conditioner matrix was observed (Figure 3B and 3C). The SEM images showed that the nanoparticles were successfully embedded without disrupting the matrix integrity.

Discussion

In this present study, nano-ZrO_2 were added into denture liners in an attempt to improve the antibiofilm properties of the liner against *C. albicans*, *S. aureus*, and *S. mutans*. The influence of nano-ZrO_2 addition on surface roughness, weight change, and glucose sorption of denture liners were also evaluated. The null hypothesis of our study, 'that the addition of nano-ZrO_2 into denture liners would not affect the evaluated parameters' has been partially rejected. Tissue conditioner and soft denture liner containing 1% nano-ZrO_2 were found to exhibit antibiofilm activity for *S. aureus*. The addition of nano-ZrO_2 did not affect *C. albican's* biofilm. Surface roughness (Ra) decreased significantly once nano-ZrO_2 was added in the VG group, but Ra increased in the UGP group.

Microbial colonization on the surfaces of denture liners is a clinically significant problem for dental applications.[15,38] There are studies about the incorporation of antimicrobial compounds to denture liners indicated that the antimicrobial effect was occurring in a dose-dependent matter.[39,40] Within the present study, nano-ZrO_2 addition into denture liners showed an antibiofilm effect against *S. aureus* biofilm formation for both silicone and acrylic-based resin denture liners. However, there was no inhibition effect on *C. albicans* biofilm formation. There was a difference between microorganisms to inhibit biofilm formation. This may also be related to the type of microorganism. The bacteria

have a thin and slack cell wall. On the flip side, *C. albicans* is a eukaryotic organism. The cell wall of *C. albicans* is thick and complex.[18] The lack of antibiofilm activity against *C. albicans* in tested materials might be explained by the complexity and density of the cell wall structure of these microorganisms.

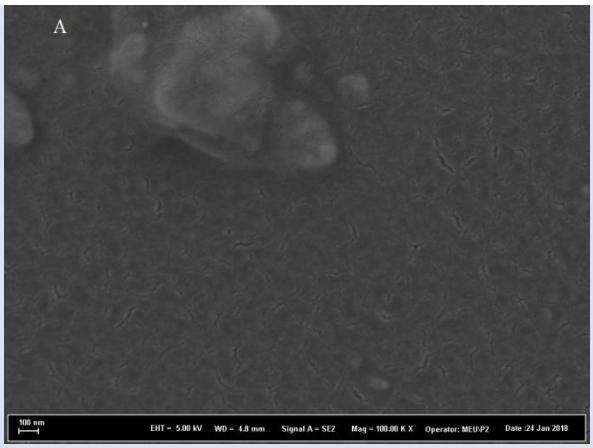

Figure 3(A). SEM image of specimen surface from Group VG 0 (control group)

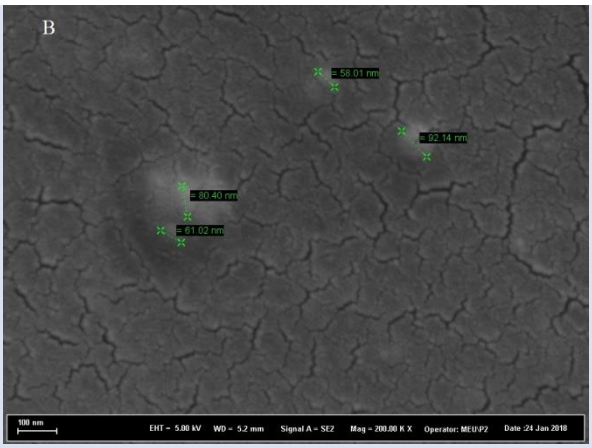

Figure 3(B). SEM image of specimen surface from Group VG 1 (0.5% nano-ZrO_2 added)

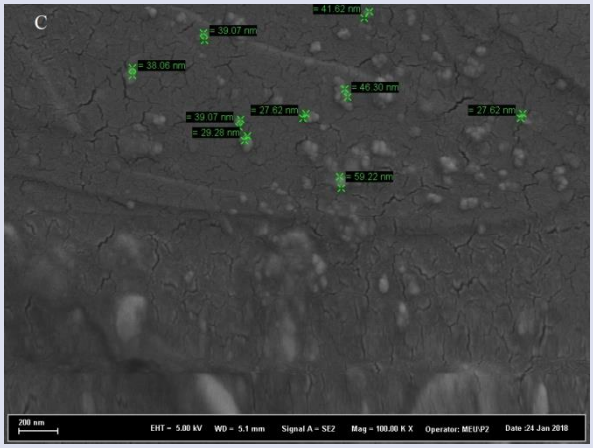

Figure 3(C). SEM image of specimen surface from Group VG 2 (1% nano-ZrO_2 added)

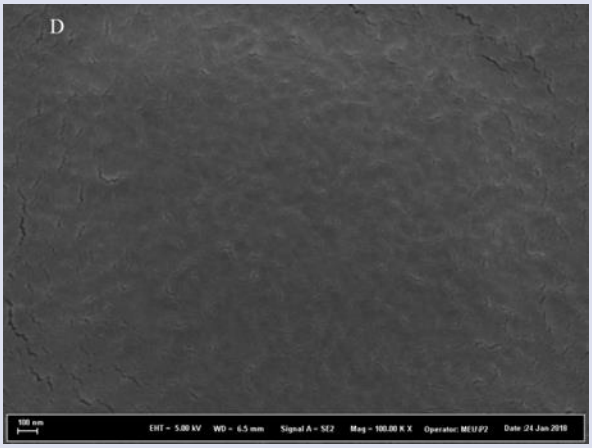

Figure 3(D). SEM image of specimen surface from Group UGP 0 (control group)

Figure 3(E). SEM image of specimen surface from Group UGP 1 (0.5% nano-ZrO_2 added)

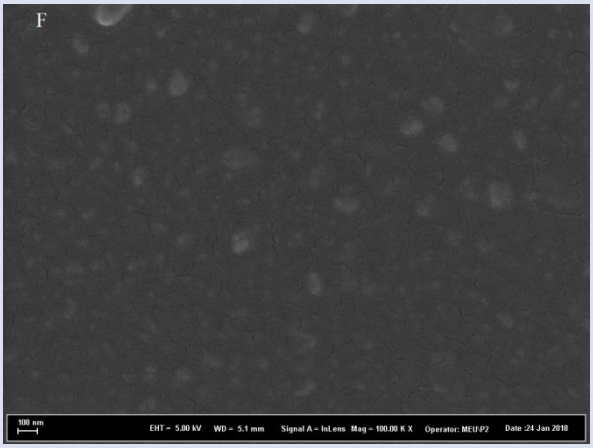

Figure 3(F). SEM image of specimen surface from Group UGP 2 (1% nano-ZrO_2 added)

Figure 3. SEM images of denture liner's surfaces

Our results infer that the antibiofilm activity of nano-ZrO_2 containing specimens against *S. aureus* was more effective than *S. mutans*. Both bacteria are Gram-positive cells.[18] However, the time for these microorganisms to appear in the biofilm of denture wearers is different. *S. aureus* is associated with various infections containing

systemic diseases like septicemia, endocarditis, pneumonia.[38] ZrO_2 nanoparticles produce active oxygen species. They accumulate on the surface of *S. aureus* cells, actively inhibiting the growth of *S. aureus* cells.[27] On the other hand, *S. mutans* is a precursor of biofilm formation, so it might lead to denture stomatitis with *C. albicans*.[38] From this information point of view, it is important to found that 8.16% rate antibiofilm activity against *S. mutans* in UGP2 test specimens in our study.

Gad *et al.*[26] reported that the addition of 7.5% nano-ZrO_2 into cold-cured resin significantly caused a reduction in the amount number of *C. albicans*. In the present study, the addition of nano-ZrO_2 to denture soft liners was not found to be beneficial to decrease the *C. albicans* biofilm formation. This might be due to the applied dose of the nano-ZrO_2. Candida accumulation on denture materials may also vary depending on the type of material.[41] Jangra *et al.* [28] evaluated the antimicrobial effect of zirconia nanoparticles against bacterial strains (*E. coli* and *S. aureus*) and fungal strain (*Aspergillus niger*) and found it to be effective only against the *E. coli*. Gowri *et al*.[27] performed the synthesis of nano-ZrO_2 using Aleo vera gel extract via a biological method. They found that nano-ZrO_2 inhibits the growth of fungal strains and also show superior antibacterial activity against *E. coli* than *S. aureus*. Findings different from our study might be due to surface charge. In our study, the antibiofilm activity was examined and in these mentioned studies, antibacterial and antifungal activity was investigated which are different test methods.

Yasser and Abdul Fatah[34] investigated the antifungal effect of acrylic base soft denture liner material (Vertex™ Soft) with combined nano-ZrO_2. They concluded that this antifungal activity was enhanced with an increased duration of incubation in artificial saliva. The reason for the different antifungal activity in our study may be due to the denture liner polymerization method used. In addition, their study determined antifungal activity by disc diffusion test, while the antibiofilm activity of test specimens was examined by the MTT method in our study.

Although nano-ZrO_2 has the same surface geometry, different nanoparticle shapes may also affect antimicrobial activities.[26,28] Similarly, triangular silver nanoparticles exhibited superior biocidal activity against *E.coli* than rod- or spherically-shaped nanoparticles.[42] In our study, the SEM images of test materials were examined and the globular-shaped nanoparticle was observed. Also, future studies should be made by taking the TEM image of nano-ZrO_2 to better understand antibiofilm and antimicrobial efficiency.

Glucose is preferred by most cells as a source of carbon and energy, so the sensing and response of glucose are highly developed in most organisms.[43] *C. albicans* possesses glucose sensors.[35] The high-affinity glucose sensors in *C. albicans* respond even at very low levels of glucose (0.01%). *C. albicans'* sensing of sugar is essential to its colonization and show virulence optimally.[44] For this reason, in our study glucose absorption of the test specimens was evaluated by adding glucose to the prepared AS. The SEM showed a relatively smooth surface due to the existence of nanoparticles for nano-ZrO_2 added VG test materials (Figure 3B and 3C). Nano-ZrO_2 might fill spaces of the surface layer, causing a smoother surface. These findings are in agreement with previous studies.[25,26] In addition, the SEM images showed that the nanoparticles were successfully impregnated with denture liner without disrupting the surface integrity (Figure 3B, 3C, 3E and 3F). The comparisons among all the tested subgroups in the current study displayed that glucose sorption levels were close to each other (Figure 2 and Table 3). This might be related to the surface texture of the test specimens. Likewise, based on the weight change results of test groups evaluated, no difference was determined (Table 4). This outcome could be explained by molecular weight or similar glucose uptake into test materials.

Analyzing the results of our study, that although no statistically significant difference was observed compared between test groups ($p>0.05$), glucose sorption values for all VG groups slightly more than those of UGP test groups. This may be related to the surface properties or roughness of the test materials.

The evaluation of surface roughness was performed according to the Ra value. This value shows the average of the peaks and depressions on the surface for the evaluated test material. In this study, surface roughness values of test materials were obtained numerical data ranging from 1.74±2.01 to 3.97±1.29 µm for VG groups and from 0.29±0.37 to 0.66±0.76 for UGP groups. In all subgroups, surface roughness values of UGP groups were lower than VG groups. These results may be related to the natural characteristics and composition of test materials. VG comprises acrylic polymers (polyethyl methacrylate) and an ester-based plasticizer (ethanol, dibutyl phthalate, phthalyl butyl glycolate).[45] UGP consists of modified polydimethylsiloxane and platinum catalyst, but no plasticizer.[6]

0.5% and %1 nano-ZrO_2 that are incorporated into tissue conditioner (VG) decreased the surface roughness of test materials. The addition of nano-ZrO_2 may have reduced the porosity of the VG group. This will also reduce void formation and microbial adherence. As a result, nano-ZrO_2 incorporated VG group might cause obtaining a denser less porous mix. Incorporation of this nano-ZrO_2 into tissue conditioner will also improve the surface structure of the material. However, within UGP groups, surface roughness values increased in the experimental group containing 0.5% nano-ZrO_2. Since UGP has cross-linking agents in its chemical structure, the nanoparticles may not be able to penetrate completely into the polymer. Silicone-based denture liner materials are chemically more stable than acrylic-based ones.[46]

The differences in surface roughness observed between silicone and acrylic-based materials may be related to the consistency of the materials.[47] The acrylic-based tissue conditioner (Visco-gel) used in our study has more flowing consistency than the silicone-based (Ufigel P). On the other hand, the application of acrylic-based

material (Visco-gel) is easier. The material is already more consistent until polymerized between the glass plate. Acrylic-based material (Visco-gel) is less able to reproducing the surface details between a smooth glass plate during specimen fabrication. This may result in higher roughness values. The increased surface roughness of the resins may promote the biofilm deposition and colonization of *C. albicans*, resulting in prosthetic stomatitis.[41,47] Furthermore, the characteristic features of denture liners such as absorption, irregularities and porosity have considerable effects on microbial adherence.[48] The result of surface roughness in our study allows us to infer, that the UGP 0 and VG 1 test groups may cause less microbial adhesion and microorganism-related prosthetic stomatitis.

The limitations of this research contain that the fact the oral environment simulating the clinical situation such as the aging process, thermocycling, the occlusal force was not imitated. Extremely smooth glass plates were used in the production of test specimens. This test condition does not reflect the clinical environment. On the other hand, the oral cavity includes multiple-strain biofilms but such mixed biofilm was not used in the present study. The adherence of microorganisms to the tested denture liners was not examined by microscopy. Modified test liners showed superior antibiofilm and surface properties compared to their commercial counterparts although a single polymerization method of test materials was investigated in this study with an insufficient dispersion of ZrO_2 nanoparticles. Further studies on the influence of adding various amounts of nano-ZrO_2 to denture liners in which different polymerization methods on antimicrobial, physical, and biocompatibility properties are essential for clinical use.

Conclusions

It was concluded, that to mitigate microbial biofilm problems caused by the use of denture liners addition of nano-ZrO_2 might be a promising method owing to its antibiofilm capacities especially against biofilms of *S. aureus* and *S. mutans*.

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Conflicts of Interest Statement

The authors did not have any conflicts of interest in regards to this study.

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