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Investigation of Cytotoxic Effects and Antimicrobial Activities of Light-cured and Self-cured Universal Adhesive Systems

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Research Article

History

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ABSTRACT

Introduction: This study aimed to compare the cytotoxicity and antimicrobial activity of a light-cured adhesive system and a self-cured adhesive system from the same company.

Materials and Methods: A Tokuyama BOND force II (Light-cured) adhesive system (TF2B) and a Tokuyama Universal Bond (Self-cured) adhesive system (TUB) were selected for the study. The cytotoxicity evaluation of these two systems on cell cultures was performed using MTT assay and Agar Diffusion assay in L929 fibroblast cells. Disk diffusion method and broth microdilution (MIC) method were used to evaluate their antimicrobial activity. The experiments were performed on 6 pathogenic bacteria and 1 yeast fungus.

Results: According to MTT test results, both adhesive systems have no significant toxic effect on healthy cells (L929). However, when TUB and TF2B were compared with each other, it was found that TF2B had almost no toxic effect. In the agar diffusion test, when the two bonds were compared with each other, a weak color lightening was observed only around the first concentration of TUB. No visible melting was detected in other concentrations of TUB and TF2B. Both adhesive systems failed to reach MIC values effectively on the test microorganisms. Since the results were far above the MIC values of the reference antibiotics, it was determined that they did not have antimicrobial effects. Disk diffusion results similarly showed that both bonds did not form an inhibition zone on the test microorganisms.

Conclusions: In dentistry, cytotoxic effects of universal adhesive systems on living cells can be observed. Self-cured and Light-cured adhesive systems did not show toxic effects on L929 cells. In addition, antimicrobial effects on test microorganisms were not detected. The cytotoxicity of the materials can be tested on different cells.

Keywords: Universal Adhesive Systems, Cytotoxicity, Antimicrobial.

Light-cured ve Self-cured Üniversal Yapıştırıcı Sistemlerinin Sitotoksik Etkilerinin ve Antimikrobiyal Aktivitelerinin Araştırılması

Research Article

Süreç

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ÖZ

Amaç: Bu çalışmanın amacı, aynı firmaya ait light-cured adeziv sistem ile bir self-cured adeziv sistemin toksisitelerinin karşılaştırılması ve antimikrobiyal etkinliklerininin incelenmektir.

Gereç ve Yöntem: Çalışmada bir Tokuyama BOND force II (Light-cured) adeziv sistem (TF2B) ve bir Tokuyama Universal Bond (Self-cured) adeziv sistem (TUB) seçilmiştir. Bu iki sistemin hücre kültürleri üzerinde sitotoksisite değerlendirilmesi L929 fibroblast hücrelerinde MTT testi ve Agar Difüzyon testi kullanılarak gerçekleştirildi. Antimikrobiyal etkinliklerinin değerlendirilmesi amacıyla disk difüzyon yöntemi ile sıvı mikrodilüsyon (MIC) yöntemi kullanılmıştır. Deneyler 6 patojen bakteri ve 1 maya mantarı üzerinde gerçekleştirilmiştir.

Sonuçlar: MTT testi sonuçlarına göre sağlıklı hücreler üzerine (L929) her iki adeziv systemin de belirgin bir toksik etkisi yoktur. Ancak TUB ve TF2B birbirleri ile kıyaslandığında TF2B' nin hemen hemen hiç toksik etkisinin bulunmadığı tespit edilmiştir. Agar difüzyon testinde ise iki bond birbiriyle karşılaştırıldığında ise TUB'un sadece ilk konsantrasyonunun etrafında zayıf bir renk açıklığı görülmektedir. TUB ve TF2B'nin diğer konsantrasyonlarında ise gözle görülebilir bir erime tespit edilememiştir. Adeziv sistemlerin her ikisi de test mikroorganizmaları üzerinde etkili MIC değerlerine ulaşamamıştır. Referans antibiyotiklerin MIC değerlerinin çok üstünde sonuçlar elde edildiği için antimikrobiyal etkilerini bulunmadığı tespit edilmiştir. Disk difüzyon sonuçları da benzer şekilde her iki bondun da test mikroorganizmaları üzerinde inhibisyon zonu oluşturmadığını göstermiştir.

Öneriler: Diş hekimliğinde, üniversal adeziv sistemlerin canlı hücrelere sitotoksik etkileri gözlenebilir. Self-cured ve Light-cured adeziv sistemleri L929 hücreleri üzerine toksik etki göstermemiştir. Ayrıca test mikroorganizmaları üzerinde antimikrobiyal etkileri tespit edilmemiştir. Adeziv sistemlerin sitotoksisitesi farklı hücreler üzerinde de test edilebilir.

Anahtar Kelimeler: Üniversal Adeziv Sistemler, Sitotoksisite, Antimikrobiyal.

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Introduction

Today, the increasing importance given to aesthetics and the increasing awareness of human health provide physicians with a more sensitive restoration. In light of these developments, a new resource is added to the literature every day with the studies of both clinicians and academicians on aesthetic materials. In adhesive dentistry, the effects on the durability, aesthetic properties, and toxicity of materials developed with new technologies compared to existing dental materials are investigated. In various studies in the literature, it has been determined that residual monomer is released during and after polymerization of adhesive systems.[1]

Since 2010, universal adhesives have been produced rapidly and in various forms.[2] The basic principle of universal adhesives, which can be used in three different modes, is to make a simpler application by including etch-rinse and self-etch adhesive systems. In the literature, there are different studies on the bonding of universal adhesives to dentin. Although adhesive systems are similar to each other in terms of the monomers they contain, universal adhesive systems are different from other adhesive systems with their monomers that can form chemical and micromechanical bonds.[3] Universal adhesives are known to contain monomers such as HEMA, Bis-GMA, UDMA and PENTA as well as biphenyl dimethacrylate (BPDM) and polyalkenioc acid. HEMA and UDMA, which are hydrophilic monomers, provide better resin infiltration, increase bond strength, and ensure adequate polymerization of the monomers. It is known that residual monomers released from adhesive agents that are not sufficiently polymerized can cause toxic effects. It has been reported that this can be prevented by adding hydrophobic monomers such as Bis-GMA and PENTA into the adhesive systems.[4]

LED light sources, which provide light at a wavelength of 455-486 nm, contain electrons in two separate conductors. This wavelength range is sufficient for the activation of initiators in resin-containing systems.[5] Easy to use and long-lasting, LED light sources do not require filters, unlike halogen light sources.[6]

In addition to having mechanical, physical, functional, and aesthetic properties, it is also very important that the materials produced in adhesive dentistry are biocompatible. Biocompatibility is the cariogenic effect of a material on living tissue after its application to living tissue.[7] Non-biocompatible materials can alter the functioning of metabolism during contact with tissue and cause cell death.[8] Studies on the harmful effects of dental materials in the literature are increasing due to the application of resin-based materials and newly developed treatments.[9]

The first document on the examination of biocompatibility of materials used in dentistry was reported by the American Dental Association (ADA) in 1926. The ADA, the International Dental Association (FDI) and the International Organization for Standardization (ISO) classified biocompatibility tests in three different methods by a joint declaration in 1982.[10] These tests are in-vitro tests (primary or primary tests), in-vivo animal tests (secondary tests) and usage tests. A declaration has been published by both national (TSE 8227) and international (ISO 10993) organizations to standardize these tests. This standardization specifies how samples should be prepared, how materials should be applied and which tests are appropriate.[11]

These tests, which are performed in culture dishes outside of a living organism, are based on material culture contact. The biocompatibility of the material tested in in-vitro tests is determined by the number, growth rate, and metabolic activity of the cells in contact with the material.[12] In-vitro tests are rapid, easy to standardize, and low-cost tests.

This study aimed to compare the toxicity and antimicrobial activity of a light-cured adhesive system and a self-cured adhesive system from the same company. Accordingly, cytotoxicity evaluation was performed on L929 mouse fibroblast cells and antimicrobial activities were performed on pathogenic gram positive and gram negative bacteria and yeast fungi.

Materials and Methods

One light-cured adhesive system and one self-cured adhesive system were selected for cytotoxicity evaluation on cell cultures (Table 1). In addition to these two different experimental groups, a positive control group containing only the L929 fibroblast cell line without any material, which was kept in the same cell culture medium as the groups, was added. The contents and manufacturers of the adhesive systems used are shown in Table 1.

Table 1. Chemical contents and manufacturers of the adhesive systems used

Adhesive systems	Components	Manufacturer	pH
Tokuyama Universal Bond (Self-cured)	Phosphoric acid monomer (3D-SR monomer), MTU-6 HEMA Bis-GEMA, TEGDMA, Acetone-MPTES Borate Peroxide Acetone, Isopropyl alcohol, water	Tokuyama Dental Corp (Kaliforniya, ABD)	2.2
Tokuyama BOND force II (Light-cured)	Self-reinforcing 10-MDP (10-methacryloyloxydecyl dihydrogen phosphate), Bis-GMA, TEGDMA, HEMA, alcohol, water	Tokuyama Dental Corp (Kaliforniya, ABD)	2.8

Preparation of Samples

In order to obtain the cytotoxic values of the adhesive systems used; preparation of test samples, sterilization, preparation of cell culture and evaluation by MTT and Agar diffusion method were performed respectively. All procedures were evaluated using solid disc samples in accordance with ISO 10993-5 protocol to ensure standardization. Light-cured adhesive system (Tokuyama BOND force II) (TF2B) was applied to the surface with TF2B using a single applicator according to the manufacturer's protocol and waited for 10 seconds. Afterwards, it was polymerized with LED (Valo Led, Ultradent) light device for 10 seconds. Self-cured adhesive system (Tokuyama Universal Bond) (TUB) was poured into the mixing chamber with one drop each of TUB ingredients A and B in accordance with the manufacturer's instructions. It was mixed thoroughly with a disposable applicator. TUB was applied to the surface using a disposable applicator. It was dried with gentle air for 30 seconds to ensure homogeneous film thickness. Using an oil-free air/water syringe, weak air was continuously applied to the TUB surface until the flowing TUB remained in the same position without any movement. The surface was finished with soft air.

The norm ISO 10993 (2018 edition) requires the evaluation of the chemical and physical properties of a medical device, including endodontic materials. This name summarizes a series of standards published mostly by ISO and the European Committee for Standardization (CEN). This series includes guidelines for selecting appropriate test methods to assess various aspects of biocompatibility. Nine biological tests are listed in ISO 10993 for biological evaluation and risk assessment of implanted materials. Based on this standardization system, comprehensive implantation assessments support systemic toxicity assessments (acute, subacute and chronic).[13]

ISO 7405 is a biocompatibility standard specifically related to ISO 10993-1 for dental materials, including endodontic materials. The various tests in these two standards are similar. Furthermore, ISO 7405 defines dental bioactive endodontic materials as materials capable of stimulating apical hard tissue formation applied by various methods (retrograde or orthograde treatments).[14]

Cytotoxicity assay

L929 (Mouse healthy fibroblast) cell line was used in this study. L929 cell line was grown in 25 cm^2 and 75 cm^2 flasks in a carbon dioxide incubator containing 5% CO_2 and 95% humidity. "Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum (FBS), and 1% antibiotic (100 IU/mL penicillin-streptomycin-PS) were used as a medium for the cells. The cells were passaged twice a week until the cell density required for the cytotoxicity test was obtained and the cells were kept in a humid atmosphere at 37 ºC. The cells were detached from the flask with 0.05% trypsin solution. Samples prepared according to ISO 10993.5 and ISO 10993.12 (Standardization IOF, 2009, 2012) were fully immersed in DMEM+FBS+PS medium under sterile conditions at 37°C for 24 hours. The samples in the medium were diluted in culture medium at twofold serial concentrations (100, 50, 25, 12.5, 6.25, 3.125 µg/ml). Cytotoxicity was evaluated using an MTT assay. L929 cells were seeded in 96-well plates and incubated for 24 hours. Samples of different concentrations dipped in the medium of both materials were added to the wells. After 24 hours of incubation, MTT solution (20 µl, 5 mg/ml) was added to each well and incubated for another 4 hours in a 37°C incubator. Then, 150 µl DMSO was added to dissolve formazan crystals. Absorbance was measured with a microplate reader at 570 nm.[15-17]

Agar diffusion test

According to ISO 10993-5 and ISO 7045 (Standardization IOF, 2009, 2011), agar diffusion test as a barrier test method for cytotoxicity was performed for non-specific cytotoxicity of its components that can leach from the samples after agar diffusion.

Neutral red dye (powder), a vital dye, was dissolved in PBS at 4 mg/ml and a stock solution was prepared. Cells were seeded in 6-well plates at a density of 1x 10^5 and incubated for 24 hours. At the end of the period, the medium was removed and the medium containing DMEM and 5% agar prepared for agar diffusion analysis was added to the 6 plates. After 60 minutes of solidification time, the samples prepared for cytotoxicity analysis were placed in the center of the wells. Blank disks and DMSO-impregnated disks were placed in the wells for positive and negative controls. Cells were stained with neutral red stock solution (0.01% in phosphate-buffered saline) diluted 1:100 with culture medium. After 24 hours of incubation, the plate was examined under an inverted microscope (Olympus, JAPAN). Neutral red stain, which stains viable cell nuclei, was used to determine the viability of the cells surrounding the material.[16,18,19]

Antimicrobial assay

The antimicrobial activity of two different bond samples against some bacteria and one yeast was determined by the Kirby-Bauer disk diffusion test and broth microdilution method.[20,21] *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* 29213, *Bacillus cereus* ATCC 11778, *Enterococcus faecalis* ATCC 29212, *Streptococcus mutans* ATCC 25175, *Escherichia coli* ATCC 25922, *Candida albicans* ATCC 10231 were used as test microorganisms.

Disk diffusion assay

Mueller-Hinton Agar (MHA; Difco, Detroit, MI, USA) medium was used in this method, which was performed by Kirby-Bauer disk diffusion test.[20] After adjusting 0.5 McFarland turbidity suspensions for each bacterium and yeast in 0.85% sterile saline solution, Mueller-Hinton Agar was inoculated onto the surface of the plates using a sterile swab. After the medium surface dried, two different bond samples were placed on the agar plates

and incubated at 35 ± 1 °C for 18 ± 2 hours. After incubation, zone diameters were measured and evaluated according to EUCAST recommendations.[21] Amoxicillin-clavulanic acid (30µg, Oxoid), meropenem (10µg, Oxoid), amikacin (30µg, Oxoid), levofloxacin (5µg, Oxoid), vancomycin, (5µg, Oxoid) imipenem (10µg, Oxoid) were used as reference antibiotics and fluconazole (25µg, Oxoid) was used as reference antifungal. Our experiments were performed in three replicates.

Broth Microdilution assay

Broth Microdilution Method was used to determine the MIC (Minimum Inhibitory Concentration) values of the bond structures against the bacteria and yeast used in our experiments. Bond structures were dissolved in 10% DMSO (Dimethyl Sulfoxide) at 20.48 mg/ml. Cation-adjusted Mueller Hinton liquid medium (CAMHB, Becton Dickenson BBL, Sparks, MD, USA) was used for the preparation of dilutions and 0.5 McFarland turbidity of microorganisms. Our experiments were performed in 96-well "U" bottom microplates. After two-fold serial dilutions, concentrations of $5x10^5$ cfu/ml for bacteria and $0.5\text{-}2.5\text{-}2.5x10^3$ cfu/ml for yeast were obtained in each well by the addition of bacteria and yeast suspensions adjusted to 0.5 McFarland turbidity. With the addition of bacteria, the concentrations of bond structures ranged from 1024 ug/ml to 2 ug/ml. After dilutions, the microplates were incubated at 35 ± 1 °C for 18 ± 2 hours and the well containing the lowest antibiotic concentration in which no growth was observed was considered as the MIC value.[21] Wells containing only CAMHB medium were considered as sterilized control and wells containing medium and bacteria were considered as growth control. Our experiments were performed in three replicates. Linezolid, levofloxacin, and amikacin were used as reference antibiotics and fluconazole was used as reference antifungal.

Statistical analysis

One Way Anova test as well as as Tukey test were used for statistical analysis of the findings. For this purpose, the SPSS 16.0 (SPSS, Chicago, IL, USA) statistical program was used and a $p<0.05$ value at a 95% confidence interval was considered significant between groups.

Results

The cytotoxic effect of the materials on healthy cells (L929 cell line) was evaluated using MTT and Agar Diffusion test. Medium was used as positive control and DMSO was used as negative control.

MTT Cytotoxicity assay

The cytotoxic activity of Tokuyama Bond irradiated and non-irradiated materials and the percentage viability of cells after 24 hours of incubation were determined according to the MTT assay results based on the formula in reference sources (Figure 1).[22]

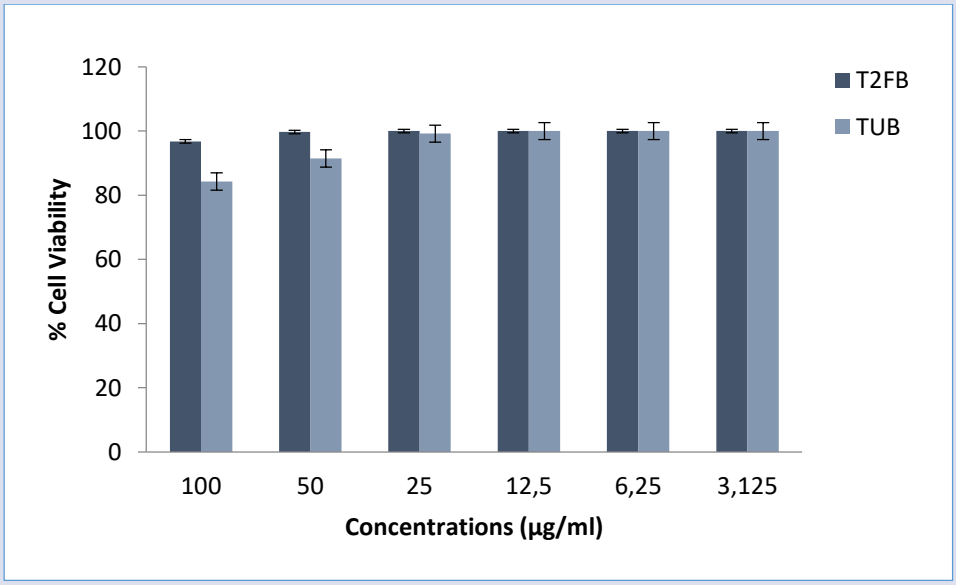

Figure 1: Cell viability of Tokuyama Universal BOND (self-cured) – (TUB) and Tokuyama BOND force II (light-cured) – (TF2B)

At the first concentration, cell viability of TF2B was 96.8%, while that of TUB was 84.3%. Both materials reached 100% cell viability by the third concentration. Both adhesive systems have no significant toxic effect on healthy cells. However, when TUB and TF2B were compared with each other, it was found that TF2B had almost no toxic effect.

Agar diffusion test (Neutral Red assay)

The viability of L929 cells around the BONDs was determined as Zone index (ZI) or Lysis index (LI) with a neutral red stain, which stains live cell nuclei (Table 2). Materials above 40% or No:3 melt index are considered toxic.[22,23]

Table 2. Description of zone index (ZI) and lysis index (LI) in the agar diffusion assay

ZI or LI	Decolorization zone diameter	Cell lysis
0	No detectable	No detectable
1	There is lightening within the borders.	Less than 20%
2	There is lightening around 5 mm.	20–40%
3	There is a lightening around 10 mm.	40–60%
4	There is lightening more than 10 mm in the surrounding area	60–80%
5	The total culture is decolorized	More than 80%

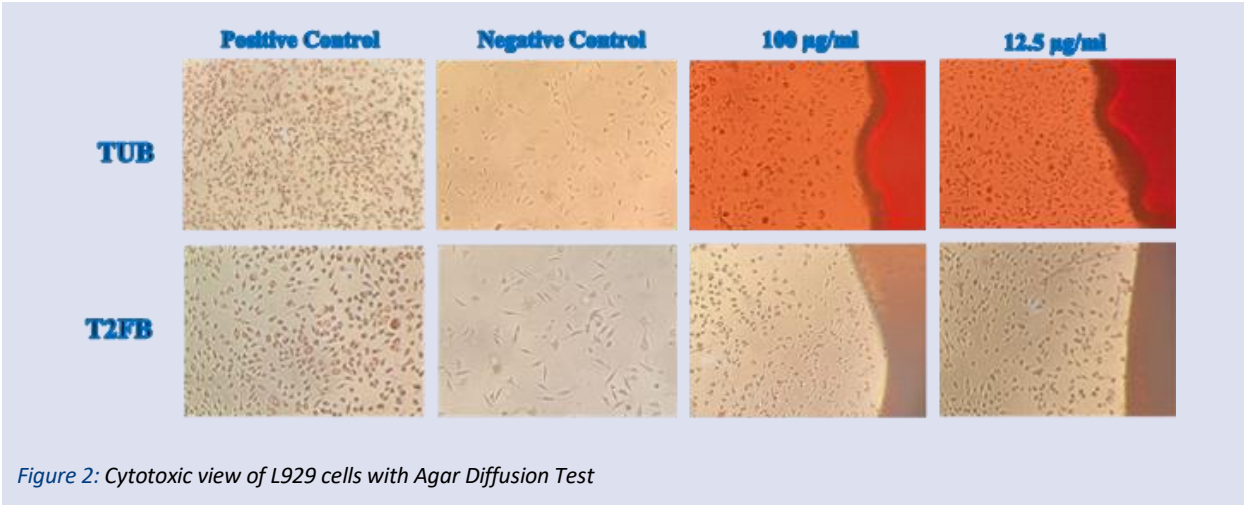

Figure 2: Cytotoxic view of L929 cells with Agar Diffusion Test

Table 3. Bonds of zone index (ZI) and lysis index (LI) in the agar diffusion assay

Material	Scala	Cell Lysis Index
TUB – 100 µg/ml	1	Less than 20% cell lysis
TUB – 12.5 µg/ml	0	No cell lysis detectable
TF2B – 100 µg/ml	0	No cell lysis detectable
TF2B – 12.5 µg/ml	0	No cell lysis detectable
Control - DMSO	4	60-80% cell lysis
	0	No cell lysis detectable

TUB = Tokuyama Universal BOND (self-cured)
TF2B = Tokuyama BOND force II (light-cured)

A melting index with a scale of 3 or higher, i.e. a toxic area, was not observed around the bonds placed on the cells. When the two bonds are compared with each other, only the first concentration of TUB shows a weak discoloration around it. No visible melting was detected in other concentrations of TUB and TF2B (Table 3, Figure 2). Agar diffusion test results and cytotoxicity results were found to be compatible with each other.

Antimicrobial Assay

MIC and Disk diffusion test were used for antimicrobial activity determination. The antibiotics selected as the control group for 6 bacteria and 1 yeast fungus used in the test are given in Table 4 together with the MIC results.

Table 4. MIC results of Tokuyama bonds; TUB and TF2B.

Microorganisms (Bacteria and Yeasts)	TUB MIC (µg/mL)	TF2B MIC (µg/mL)	Antibiotics MIC (µg/mL)	Antibiotics used
Escherichia coli	>1024	>1024	2	Amikacin
Pseudomonas aeruginosa	>1024	>1024	2	Amikacin
Staphylococcus aureus	>1024	>1024	2	Linezolid
Enterococcus faecalis	>1024	>1024	2	Levofloxacin
Bacillus cereus	>1024	>1024	2	Linezolid
Streptococcus mutans	>1024	>1024	2	Levofloxacin
Candida albicans	>1024	>1024	0.25	Fluconazole

TUB = Tokuyama Universal BOND (self-cured)
TF2B = Tokuyama BOND force II (light-cured)

Both Tokuyama bonds did not reach effective MIC values on the test microorganisms. Since the results were far above the MIC values of reference antibiotics, it was determined that they did not have antimicrobial effects. Disk diffusion results are shown in Figure 3. Accordingly, it was observed that both bonds did not form an inhibition zone on the test microorganisms.

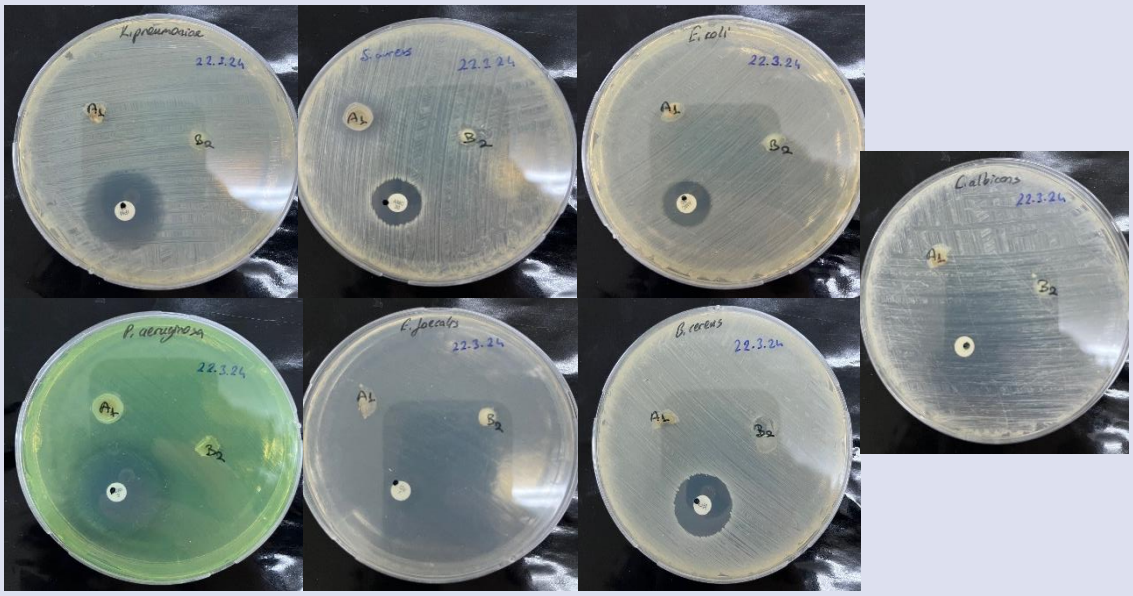

Figure 3: Disk diffusion results of Tokuyama bonds; TUB and TF2B.

Discussion

Today, with the development of technology, many different adhesive materials have been introduced to the market. Biocompatibility studies have become increasingly important in newly developed materials.[24] Studies in the literature have shown that different monomers are released from resin-based dental materials before or after polymerization.[25] The monomers released from materials with different compositions determine biocompatibility.[26] In our study, the cytotoxic effects of adhesive systems on L929 mouse fibroblast cell lines were examined. The cytotoxic effects of adhesive systems widely used in restorative dentistry vary.[27]

It is known that different universal adhesives available in the market have different ingredients such as biphenyl dimethacrylate (BPDM), CQ, MDP, polyalkenioc acid, acetone and ethanol as well as monomers such as HEMA, Bis-GMA, UDMA and PENTA, different pH and polymerization methods.[28] Studies have shown that all these parameters have an effect on the cytotoxicity of the agent used.[29] Among the adhesives, Tokuyama Universal Bond is the only system with two different forms that polymerize chemically and with LED.

Animal experiments and cell culture tests are commonly used to evaluate the cytotoxicity of dental materials. However, animal experiments are controversial, long-lasting and expensive methods.[30] Cell culture tests have become an alternative to animal experiments due to their advantages such as low cost, controllability, and ease of construction.[31] Cell culture is widely used for dental materials whose biocompatibility will be evaluated, especially in in-vitro tests in restorative dentistry.[32] Direct contact of the medical materials with the cell culture or indirect application of the extract liquids obtained by soaking the material in a suitable liquid (>24 hours) to the cell culture is recommended in the International Organization for Standardization (ISO 10993-5) (10993 1999), which regulates the test methods to be used in the cytotoxicity evaluation of materials.[15]

Adhesive systems do not have direct contact with the pulp under in-vivo conditions. However, acute toxic effects due to residual monomer release before or after polymerization can reach the pulp through dentinal tubules, which increase in diameter as they approach the pulp.[33] Adhesive systems applied directly to dentin, in particular, may cause pulpal inflammation and necrosis of pulpal cells due to insufficiently polymerized monomers.[34] The cytotoxic effects of inadequately polymerized adhesive systems placed directly into culture media are determined by morphological changes in cells, changes in viability, disruption of cell membrane integrity, and changes in enzyme activity. The cytotoxic effects of many adhesive systems have been investigated and it has been reported that the monomer diversity of adhesive systems causes different cytotoxicities.[35] The high rate of polymerization of adhesive systems reduces possible biological risks. Therefore, the light devices used during polymerization are important. In a study by Ergün et al. the cytotoxic effects of LED and halogen light sources on three different adhesive systems were investigated.[36] In another study, the cytotoxicity of an adhesive system was evaluated on L929 mouse fibroblast cells by both LED light devices and chemical polymerization.[34] In both studies in the literature, the number of cells remaining in the experimental group polymerized with an LED light device was higher, i.e. the cytotoxic effects were found to be lower.[34, 36] Self-cured and light-cured polymerized adhesive systems were included in our study to compare the effect of different polymerization techniques on cytotoxicity value.

According to the results of our study, the toxic effect of Tokuyama Bond light-cured adhesive system (TF2B) on L929 healthy fibroblast cells was found to be very low. The self-cured adhesive system (TUB) had almost no toxic effect on the cells.

Conclusions

In our study to compare the biocompatibility and antimicrobial effects of self-cured and light-cured adhesive systems, both systems showed no toxic effect on healthy fibroblast (L929) cells. In MTT and agar diffusion test results, it can be said that self-cured (TUB) material has a negligible toxic effect compared to light-cured (T2FB) material. In addition, it was determined that both adhesive systems did not have antimicrobial effects on the test microorganisms according to MIC and disk diffusion results. The cytotoxicity of adhesive systems can be tested on different cells. Biocompatibility studies can be increased with different adhesive systems.

Conflict of Interest

The authors declare that they have no conflicts of interest in the publication.

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