

In Vitro Cytotoxic Evaluation Of Conventional Denture Base Material And Soft Lining Material Using Colorimetric Mtt Assay

Kolorimetrik Mtt Testi Kullanarak Geleneksel Protez Kaide Materyali İle Yumuşak Astar Materyalinin İn Vitro Sitotoksik Özelliklerinin Değerlendirilmesi

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Öz

Amaç: Bu çalışmada protez yapımında kullanılan geleneksel kaide materyali ile yumuşak astar materyalinin fare fibroblast hücreleri üzerinde zamanla meydana gelen sitotoksik etkilerinin değerlendirilmesi amaçlandı. **Materyal ve Metod:** Protez kaide materyali (Rodeks) ve yumuşak astar materyalinin (Dentusil) disk şekilli test numuneleri üreticinin talimatlarına göre aseptik şartlar altında hazırlandı.. Örnekler, ağız ortamını taklit etmek için 5.000 termal döngüye tabi tutuldu. Yaşlandırma prosedürlerini takiben, materyallerin sitotoksik etkisi, 24 saat, 48 saat ve 72 saatlik hücre inkübasyon döneminden sonra L929 fare fibroblast hücreleri kullanılarak [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] testi ile değerlendirildi. Her grup için hücre canlılığı değerleri hesaplandı. Verilerin istatistiksel analizi, iki yönlü tekrarlanan bir ölçüm yöntemi kullanılarak gerçekleştirildi. (P <0.001) **Bulgular:** 24 saat ve 48 saat inkübasyon periyodunda yumuşak astar materyali, 72 saat inkübasyon periyodunda ise kaide materyali daha fazla hücre canlılığı göstermiştir. İstatistiksel olarak iki materyal arasında anlamlı fark bulunmuştur. İnkübasyon periyotları arasında ise 24 saat inkübe edilen grup 48 saat ve 72 saatden istatistiksel olarak farklıdır. 72 saat ve 48 saat arasında anlamlı fark bulunmamıştır. **Sonuç:** Kaide materyallerinin altına kullanılmış olduğumuz yumuşak astar materyali kaide materyaline göre daha biyouyumludur.

Anahtar Kelimeler: Geleneksel kaide materyali, yumuşak astar, sitotoksosite, termal siklus, MTT

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Abstract:

Purpose: In this study, it was aimed to evaluate the time course of cytotoxic effects of the conventional base material and soft lining material on the mouse fibroblast cells. **Material and Method:** Disc-shaped test samples of denture base material (Rodex) and soft lining material (Dentusil) were fabricated according to manufacturers' instructions under aseptic conditions. The samples were subjected to 5,000 thermal cycling to mimic the oral environment. Following aging procedures, the cytotoxic effect of the materials was assessed by [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay using L929 mouse fibroblast cells after 24 h, 48 h and 72 h cell incubation period. Cell viability values were calculated for each group. Statistical analysis of the data was performed using a two-way repeated measurement method. ($p < 0,001$) **Results:** For 24 hours and 48 hours incubation period soft lining material, and for 72 hour incubation period the base material showed higher cell viability. Statistically, there was a significant difference between the two materials. The group incubated 24 hours is statistically different from 48 hours and 72 hours. No significant difference was found between 72 hours and 48 hours. **Conclusion:** The soft lining material we used under the base materials is more biocompatible than the base material.

Keywords: Conventional denture base material, soft liners, cytotoxicity, thermal cycles, MTT

INTRODUCTION

Verifying the biological and toxicological safety of a dentist is a prerequisite for clinical use, as the absorption of certain substances released from a patient's body's substances may be toxic at high concentrations.¹

Allergic reactions caused by denture base mate-

rials and local chemical irritation have been reported in the literature. The main clinical symptoms are redness, swelling, painful vesicles, ulceration and labial edema in the oral mucosa. The most common complaints of patients are the palatal mucosa, which is often in direct contact with the maxillary prostheses, in the form of burning in the mouth. Occasional oropharynx and oral mucosal diseases are seen in these disorders.^{2,3,4,5}

The material selected to make partial or complete denture bases are acrylic resins because of some advantages, such as satisfactory dimensional stability and fracture resistance, easy handling and repair, better thermal conductivity, and significant color stability that allows simulating of natural gum.^{6,7} Since polymethyl methacrylate (PMMA) was first introduced in dentistry in the 1930's, it has been successfully used to make removable prostheses due to its low cost, adequate aesthetic properties and ease of manipulation.⁸

Acrylic resins are the preferred prosthetic base material. However, acrylic resins used as prosthetic base material do not have shock absorption properties on the prosthetic incoming loads. Consequently, the biting forces from the occlusal are transmitted directly to the underlying tissues via the denture. If the occlusal force distribution on the thin mucosal tissue covering the alveolar bone is distributed in an inhomogeneous manner, the biting force creates a sensation of pain. Over time, alveolar bone loss may occur. Soft lining materials are often used when patients suffer from symptoms indicated in full denture patients. Clinical trials have shown that soft lining materials develop chewing function compared to traditional denture base materials.^{9,10,11,12}

The aim of this in vitro study was to evaluate biocompatibility properties of denture base material and soft lining material using cell culture method with L 929 mouse fibroblasts by MTT (tetrazolium salt 3- [4,5-dimethylthiazol-2-yl] -

2,5-diphenyltetrazolium bromide) cell viability test.

MATERIALS-METHODS

Materials used in this study are listed in Table1.

Preparation of the test specimens of denture base and soft lining materials and measurement were performed according to International Organization of Standardization (ISO) 10993-512.13

36 samples per each material were prepared in dimensions of 5 mm diameter and 2 mm height. Mixing proportions were applied according to instructions of the manufacturers.

Group I: Heat-cured acrylic base resin (Rodex (Rodont, Milano, Italy)) samples were prepared by investing plastic patterns in dental stone to form molds in dental flasks as routine denture processing. Then the patterns were removed from the molds. The resin mixture of powder and liquid was placed in the molds and then polymerization was employed as following; the molds were placed in cool water and raised temperature to 100 °C over 45 minutes and then continued boiling for 15 minutes.

Group II: Self-cure vinyl polysiloxane soft lining (Dentusil (Bosworth(USA), Skokie,IL, USA)) samples were prepared in stainless steel mould.

The samples were subjected to 5,000 thermal cycling to mimic the oral environment. The samples were placed in a wire basket and changed in water between 5 and 55 °C. The waiting period lasted 40 seconds in each water tank with a transfer time of 5 seconds.

Prior to cytotoxicity tests, the samples were ultrasonically cleaned in distilled water for 20 minutes and to prevent bacterial contamination is kept for 20 minutes under UV light.

The samples of each material were divided into three groups for 24 h, 48h and 72 h incubation periods.

Evaluation of cell viability (MTT assay)

During the experimental work, 10% fetal bovine serum (FBS), 1% Penicillin-Streptomycin and 1% L-glutamine (Biochrom, Germany) cell culture medium supplemented with Dulbecco's Modified Eagle's Medium (DMEM / F12) as chemicals and reagents were used. MTT assay for cell viability test was obtained from Sigma-Aldrich (M5655, Sigma-Aldrich, Germany). The test materials for 30 minutes before eluting preparations were exposed to ultraviolet light to prevent bacterial contamination. The materials in the cell culture medium was incubated at 37 °C for 24 hours.

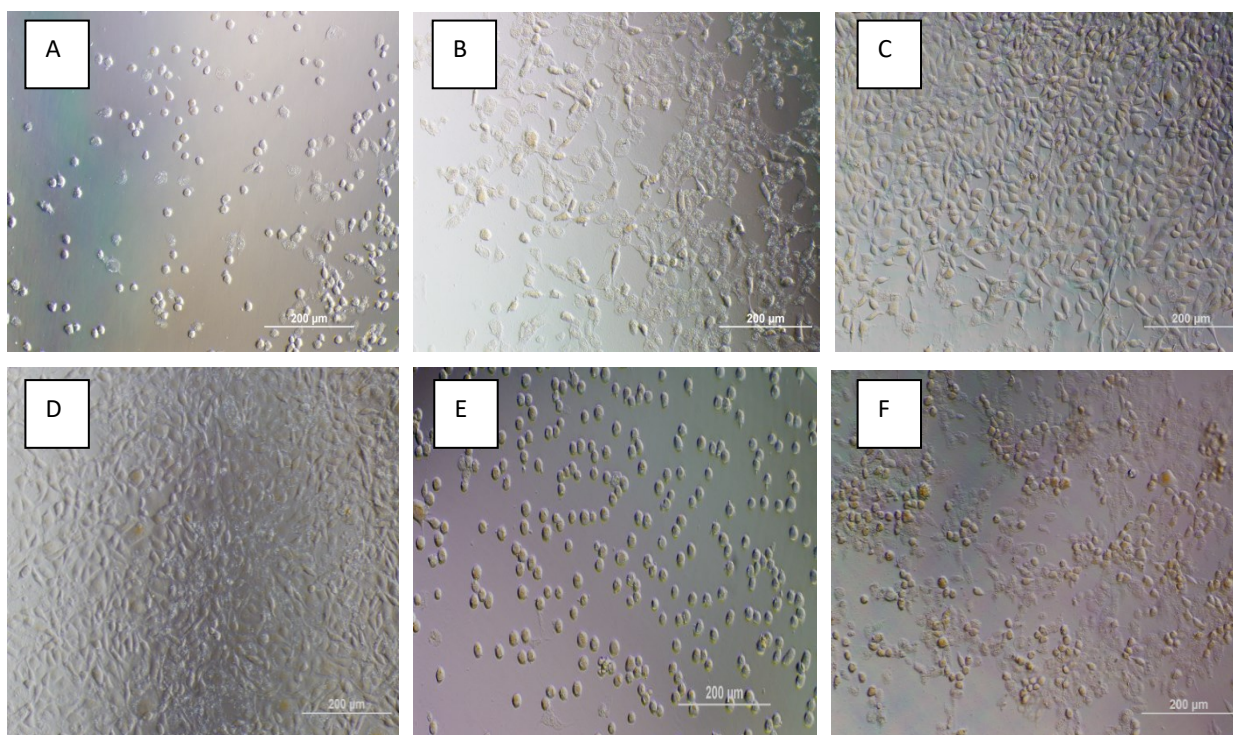
L929 mouse fibroblast cells were used to establish the soft liner and denture base materials cytotoxicity. (Şap Institute Ankara, Turkey) 4X10⁴ cells / ml with 96-well plates (100 µL per well) and 5% CO₂ in air at 37 °C, L 929 mouse fibroblasts were incubated.

After the 24 hour incubation period, following removal of the cell culture medium, fresh media containing elutes of the test materials were added to each well. For the control group, cells incubated in DMEM / F12 were used.

The cells were incubated for 3 days. MTT test was performed daily, and cell morphology was visualized by inverted microscopy (IX70 Olympus, Japan). : L929 mouse fibroblast cells exposed to denture base material and soft lining material are shown in figure 1.(Figure1)

After 24, 48, 72 hours of incubation, all media were removed from the wells and MTT-containing culture medium was added to each well and incubated for 4 hours at 37 °C. To protect from light, the culture plates were

Figure 1: L929 mouse fibroblast cells exposed to denture base material and soft lining material. (A) 24 h incubation period dentusil, (B) 48h incubation period dentusil, (C) 72 h incubation period dentusil, (D) 24 h incubation period rodex, (E) 48 h incubation period rodex, (F) 72 h incubation period rodex



covered with aluminum foil and the cells were incubated in a dark environment. Following 4 hours of incubation, the medium was aspirated, 100 µL of acid isopropanol (0.05 N HCl in absolute isopropanol) was added to each well. It was then measured using a UV-irradiated spectrophotometer with absorbance at 570 nm. (EZ Read 400 Microplate reader, Biochrom, UK).

Statistical Analysis

All data were evaluated the Statistical Package for the Social Sciences, version 21.0 (SPSS Inc., Chicago, IL, USA). Statistical analysis of the

data was performed using a two-way repeated measurement method. ($p < 0,001$) All pairwise multiple comparison procedures were performed by Bonferroni t-test method.

RESULTS

Statistically there is a difference between the acrylic base material and the soft lining material. When the periods of incubation are evaluated; the 24 hour incubation period is statistically different from 48 hours and 72 hours. There is no statistical difference between the 72 hour incubation period and 48 hours. The soft lining ma-

terial (Dentusil) is incubated for 24 hours and 48 hours and the base material (Rodex) is more biocompatible for 72 hours incubation period. When we evaluate materials within themselves; for soft lining material (Dentusil), 48 hours and 24 hours incubation periods statistically different, 48 hour and 72 hour incubation periods

are statistically different from each other, there is no statistical difference between 72 hour and 24 hour incubation period. When the base material (Rodex) is evaluated; all incubation periods, 24 hours, 48 hours, 72 hours, are statistically different from each other. (Table 2,3)

Table 1: Materials used in the study

Materials	Manufacturer	Composition	Material Type
Rodex	Rodont, Milano, Italy	Methyl methacrylate	Heat-cured
Dentusil	Bosworth(USA), Skokie,IL, USA	Self-cure vinyl polysiloxane	Autopolymerized Soft relining

Table 2

Groups	N	Mini- mum	Maxi- mum	Mean		Std. Deviation
				Statistic	Std Er- ror	
Dentusil 5000 cycles	12	,61	,89	,7097	,02722	,09430
24 h	12	,42	1,08	,9315	,05020	,17389
48h	12	,62	,90	,7928	,02522	,08738
72h						
Rodex 5000 cycles	12	,14	,58	,3380	,03728	,12915
24h	12	,38	,84	,5793	,03876	,13427
48h	12	,59	1,20	,8358	0,5543	,19203
72h						

Table 3: The paired multiple comparison procedures (Bonferroni t-test) results are also included in the table.

Comparisons for factor: Group				
Comparison	Diff of Means	t	P	P<0,050
dentusil , rodex mufla	0,227	5,901	<0,001	yes
Comparisons for factor: Incubation period				
Comparison	Diff of Means	t	P	P<0,050
72h , 24 h	0,290	7,887	<0,001	yes
72 h, 48h	0,0589	1,600	0,350	no
48h, 24h	0,232	6,287	<0,001	yes
Comparisons for factor: Incubation period within rodex				
Comparison	Diff of Means	t	P	P<0,050
72h , 24 h	0,498	9,558	<0,001	yes
72 h, 48h	0,257	4,926	<0,001	yes
48h, 24h	0,241	4,632	<0,001	yes
Comparisons for factor: Incubation period within Dentusil				
Comparison	Diff of Means	t	P	P<0,050
48h, 24h	0,222	4,259	<0,001	yes
48h, 72h	0,139	2,664	0,032	yes
72h, 24h	0,0831	1,595	0,353	no
Comparisons for factor: Group within 24				
Comparison	Diff of Means	t	P	P<0,05
dentusil , rodex mufla	0,372	6,482	<0,001	yes
Comparisons for factor: Group within 48				
Comparison	Diff of Means	t	P	P<0,05
dentusil , rodex mufla	0,352	6,144	<0,001	yes
Comparisons for factor: Group within 72				
Comparison	Diff of Means	t	P	P<0,05
Rodex, dentusil	0,0431	0,751	0,455	no

DISCUSSION

This study evaluated the time course of cytotoxic effects of the conventional base material and soft lining material. Based on the results, for 24 hours and 48 hours incubation period soft lining material, and for 72 hour incubation period the base material showed higher cell viability. Statistically, there was a significant difference between two materials.

The biologic and toxicological properties of the material are important for the acceptance of the prosthesis by the patients. Cytotoxicity is used to describe the steps of molecular events that interfere with macromolecular synthesis, causing cellular functions and impairment of structural damage. It is the first step of cell culture tests in evaluating the biocompatibility of materials. In vitro cytotoxicity tests are essential to test the new materials used in humans because it is a scanning stage reduces the effect of confounding variables makes a simplified system toxicity study.¹⁴

Testing of dental materials by cell culture methods is easy and repeatable according to other test methods and it is low cost.¹⁵

The MTT test, originally developed by Mosmann in 1980, is now regarded as the gold standard in determining cell viability and proliferation.¹⁶ This test measures cell viability in terms of reducing activity as an enzymatic conversion of the tetrazolium compound to water-insoluble formazan crystals, when dehydrogenases occur in the mitochondria of living organisms, including reducing agents and enzymes present in other organelles such as the endoplasmic reticulum.^{17,18}

As a highly efficient, miniaturized test, the assay improves cell counting technology by replacing the increased sensitivity of the assay with the potent, radioactive isotope-based ³H-thymidine uptake assay. At first, this test does not include washing steps, but requires resolution of formazan crystals in acid-

isopropanol, a time-consuming process. However, various changes, including the solubilization of formazan in aqueous media or the addition of DMF (Dimethylformamide) to remove excessive amounts of dye, followed by washing with PBS to dissolve the formazan crystals in DMSO (Dimethyl sulfoxide) have enhanced the simplicity and susceptibility of this test. A variety of tetrazolium-based tests have been developed, such as XTT, MTS and WST assays, in which water-soluble formazan products are removed from the washing and solvent solubility steps, but the well established MTT assay.¹⁹

Different cell types can be used to quantitatively assess the biocompatibility of dental materials and to evaluate different biological endpoints. Immortalized cell lines L929, HaCat, Raw 264.7 are susceptible to dental monomers and plasticizers obtainable from polymeric materials. HaCat cells are suitable substitutes for oral keratinocytes and can be easily cultivated and passaged indefinitely.^{20,21}

Raw 264.7 cells represent the majority of the functions of primary cultured macrophages, the first line for reestablishing potentially harmful substances.²²

The gingival tissue is in constant contact with the denture base material and soft lining materials, it is important to clarify the cytotoxic effects of these materials on fibroblast cells.²³ The fibroblast cells were selected because they are the predominant tissue types on the body and because of the easy sowing and 24-hour positive folding times. These cells are recommended by many standards institution.²⁴ In addition L929 mouse fibroblasts are recommended by ISO 10993-5 for cytotoxicity tests.¹³ For this reason we preferred mouse fibroblast cells in this study.

There are various methods for polymerizing acrylic resins, such as water bath

polymerization, microwave polymerization and chemical polymerization.²⁵ Polymerization with water bath is one of the common methods for prosthetic production because it produces prostheses which are cheaper than microwave energy polymerisation and have the appropriate mechanical properties.²⁶ However, an increase in surface porosity occurs due to the exothermic polymerization reaction.²⁷ The chemically polymerized resin is often used to repair prostheses because it polymerizes rapidly at room temperature.²⁸ It has been reported in the literature that acrylic resins affect the cytotoxicity of the material in the form of polymerization. It has been reported that polymerisation with water bath is more suitable for biocompatibility than microwave polymerization.²⁵

It is believed that the filtered residual monomer and plasticizers, as well as ethane, may contribute to the cytotoxicity of acrylic-based dental materials. The remaining monomer is often the irritating component of prosthetic-based resins. A properly processed prosthesis now has monomer content <1%.²⁹

In our study, 5,000 cycles of thermal cycling were applied before the cytotoxic properties of the samples were determined. This process may now be effective for removing the monomer. Because many researchers recommend reducing the cytotoxic properties of acrylic-based dental materials and keeping them in the hot water for 24 hours before use.³⁰

It is difficult to imitate and evaluate the in vivo situation.³¹ Base materials and soft lining materials are likely to come into contact with the oral mucosa and therefore the choice of cell line in our study may not be optimal for mimicking the oral condition. There is a need for clinical trials to support the accuracy of future in vitro studies.

Conflict of Interests

The authors declare that there are no conflicts

of interest regarding the publication of this paper.

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