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Comparative analysis of cytotoxicity effects of two denture hard lining materials on human gingival fibroblasts: an in vitro study

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ABSTRACT

Background: The objective of this study was to compare the cytotoxicity of TDV and Rebase II denture hard liners on human gingival fibroblasts, aiming to address issues associated with incomplete polymerization and free monomers that affect material properties.
Methods: Seventy-two specimens (24 each of TDV, Rebase II, and controls) were prepared under aseptic conditions according to factory instructions. Cytotoxicity was determined using the MTT test with methyl tetrazolium salt added to the cell culture medium. A two-way ANOVA and a post-hoc Tukey test was used to evaluate the results of incubation before mitochondrial activity was measured using Multiscan spectrophotometry (570 nm).
Results: There were significant differences in cell viability between the groups after 24 hours ($P < 0.001$), with TDV having higher viability than Rebase II. The difference between Rebase II and TDV, however, was not significant at 48 and 96 hours ($P > 0.131$). At 24 hours, Rebase II exhibited significantly lower viability than TDV liner, with a significant difference between the two groups ($P = 0.001$).
Conclusion: Due to the maximum monomer release in the early hours of incubation, the amount of cytotoxicity decreased with increasing incubation time.

1. Introduction

The fit of dentures naturally decreases over time due to changes in the tissues that support them. The denture's adaptability to oral tissues can be improved by both hard and soft lining materials (Wicks et al., 2015). Linings for dentures are polymers applied to their interior surfaces to improve their ability to adapt to oral tissues. Hard liners were introduced in the 1950s and are composed of a powder and liquid combination containing a polymer polyethyl methacrylate (PEMA), a liquid monomer isobutyl methacrylate (IBMA), and a reaction initiator, benzoyl peroxide (Lefebvre et al., 2001). In the 1940s, polymethyl methacrylate resins became the most common denture base materials because of their aesthetic quality, repair capabilities, and physical properties (Kreve and Dos Reis, 2019).

Denture liners are not required to meet biological tests, but they must still meet safety requirements, such as being nontoxic, nonirritating, and preventing bacterial and fungal colonization (Jagdish et al., 2017). Due to leaching or absorption, direct autopolymerizing hard liners may undergo physical, mechanical, or biological changes, which may include volumetric changes, hardening, color changes, and changes in cytotoxicity (Atay et al., 2012; Muller-Borer et al., 2012; Song et al., 2014). Cytotoxicity can result from incomplete polymerization or free monomers, which can directly irritate the oral mucosa and cause long-term damage (Marigo et al., 2022). During polymerization, free monomers can be released at varying rates, based on factors including temperature and polymerization time, and too much unpolymerized monomer can adversely affect a material's mechanical and physical properties and may cause allergic reactions. In addition, resin properties and toxicity may differ based on the components, structure, purity, and monomer conversion rate (Ebrahimi Saravi et al., 2012; de Souza Costa et al., 2014; Makvandi et al., 2020).

Using liners can reduce direct contact between the polymerization and the oral mucosa, which can mitigate the toxicity of direct liners (Regis et al., 2012; Weaver and Goebel, 1980; Rokaya et al., 2018; Jontell et al., 1995). Chairside relining has gained popularity because it requires less time and effort than laboratory procedures. Removable or provisional denture bases can be directly relined with self-curing resins to achieve a reliable fit (Hong et al., 2010; Chaves et al., 2012; Wicks

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et al., 2015; Masetti et al., 2018). TDV and Rebase II have been commonly used in recent years to reduce the delivery time of removable dental prosthesis bases and improve their compatibility with removable dental prosthesis bases (Jontell et al., 1995; de A.L. Chaves et al., 2010). Liners with these properties are purported to produce less heat, less odor, and less chemical irritation as well as less complicated laboratory procedures in a shorter time. The porosity of these materials is minimal as well. However, the mechanisms by which these monomers cause toxicity and their interactions with human cells remain unclear (Lefebvre et al., 2001; Chaves et al., 2012; Regis et al., 2012; Caldas et al., 2016). Therefore, understanding the mechanisms of their toxicity is crucial for the future development of safe and biocompatible monomers. The simplicity, cost-effectiveness, and immediacy of relining will make it popular, particularly among elderly patients who require additional assistance with their oral hygiene needs (Kreve and Dos Reis, 2019).

Limited research has been conducted on the cytotoxicity of Rebase II and TDV hard liners, as biological safety must be confirmed before they can be used clinically. In this study, human gingival fibroblasts (HGFs) were used to analyze the cytotoxicity of these two denture lining materials. The null hypothesis is that there would be no difference in cytotoxicity between TDV and Rebase II liners.

2. Material and methods

The sample size for this in vitro study was calculated based on research conducted by Çakırbay et al. (2018), using a fixed-effect ANOVA power analysis conducted with PASS 15 software (NCSS). The parameters used for the calculation included a significance level of $\alpha = 0.05$, an effect size for the material factor of 1.95, and an effect size for the time factor of 5.4. Consequently, six specimens were chosen for each group. In this study, direct hard liners TDV (TDV, Brazil) and Rebase II (Tokuyama, Japan), which contain polyethyl methacrylate compounds, were used together with HGF cells (National Genetic Resource Center, Iran). A culture medium containing Dulbecco's Modified Eagles medium (Biosra, France) with fetal bovine serum, 45 microliters of DMEM (Biosra, France), 7.5 microliters of FBS (Biosra, France), and 500 microliters of antibiotics (penicillin/streptomycin) was used to culture the cells (total of 552.5 μL). The medium was changed every two days, and a passage was performed after the flask was filled. MTT was performed on the third passage of the cells. An incubator with 37°C, 95% humidity, and 5% CO_2 was used to maintain the cells in a 24-well plate (SPL, South Korea) for one day. Each well contained approximately 10^4 cells. For each of the three designated time points in the study, a total of 24 specimens- semicircles with 1 cm diameter- were prepared. This consisted of 8 specimens from each of the two liner material groups — TDV and Rebase II — and an additional 8 specimens from the control group, amounting to 72 specimens in total.

Mixing the materials was performed according to the manufacturer's instructions. Using a 24-well plate (SPL sterile cell culture plate), a paste-like liner was placed in the bottom. Immediately following completion of polymerization, the setting and final polymerization steps were carried out in the well. A 24-well plate containing the liner was filled with previously incubated and passaged cells (Kostić et al., 2012). The toxicity test was performed for all groups three times (at 24, 48, and 96 hours).

Three groups of specimens were randomly selected, with the control group (culture medium + fibroblast cells) receiving no substance. Each group was tested three times to avoid bias. The study groups consisted of Group 1: TDV liner after 24 hours, Group 2: Rebase II liner after 24 hours, Group 3: Negative control after 24 hours, Group 4: TDV liner after 48 hours, Group 5: Rebase II liner after 48 hours, Group 6: Negative control after 48 hours, Group 7: TDV liner after 96 hours, Group 8: Rebase II liner after 96 hours, and Group 9: Negative control after 96 hours..

Cytotoxicity was measured using the MTT test, which detected cell viability via mitochondrial activity. MTT is metabolized by succinic dehydrogenase in live cells, producing blue formazan crystals, with the intensity measured spectrophotometrically indicating cell quantity. After incubating the culture with MTT for 24 hours at 37°C, formazan is dissolved in 100 ml of dimethyl sulfoxide (DMSO) solvent solution (Biosra, France) was added to each plate. The mitochondrial activity was measured at 570 nm with a Multiscan spectrophotometer (T80-PG-UK).

The cells were evaluated under a microscope (Nikon XDS-1B), and when 80% of the flask was filled with cells, the cells were ready for the next step (37°C, using the Bain-Marie method). A diluted PBS1 (Biosra) solution was used to wash the cells and discard the supernatant. Trypsin (DENAzist Asia, Iran) was added to the PBS and incubated for 5 min at 37°C, in a Memmert (Memmert, Germany) after discarding the PBS. Separated cells were incubated in DMEM-Lg with 10% FBS for 1 min and then centrifuged at 1500 rpm for 10 min in a Hermle centrifuge (Hermle, Gosheim, Germany). After counting, the cells were divided and transferred to a new flask after discarding the supernatant and adding it to the sediment in the culture medium. A 25 mL flask was filled with about 3 mL of medium, while a 75 mL flask was filled with about 10 mL of medium.

Due to normal distribution of data, the ANOVA-2 test was used to compare cell viability among all groups at the three time points. To examine the differences between groups, Levene's test was first performed to check for data homogeneity, and subsequently, Tukey's HSD test was applied for pairwise group comparisons. Applying ISO10993-5:2009 standards, the substances were categorized according to their cytotoxicity effect: non-cytotoxic (cell viability at least 75% that of the control group), slightly cytotoxic (cell survival 50–75% that of the control group), moderate cytotoxicity (cell survival 25–50% that of the control group), and strongly cytotoxic (cell survival less than 25% that of the control group).

3. Results

The MTT was performed on 72 samples—24 samples (8 from each of the TDV and Rebase II liner material groups and 8 samples as a control group) at 3-time points. Results of the MTT test for each acrylic group were obtained in the form of optical density values, which were converted into cell viability based on the values of the control group (Tables 1–4).

In the first 24-hour follow-up, the highest biocompatibility was observed in the control group, followed by the TDV hard liners (65.85 ± 3.03), and the lowest was in the Rebase II hard liners (22.36 ± 1.75). After 48 hours, the highest biocompatibility besides the control group was still the same as that measured at 24 hours—TDV hard liners (71.39 ± 1.05), and the lowest was in Rebase II hard liners (63.11 ± 1.74); however, the differences were not statistically significant ($P > 0.05$). After 96 hours, the highest biocompatibility was observed in the control group, followed by the TDV hard liners (73.38 ± 1.97), and the lowest value was observed in the Rebase II hard liners (67.10 ± 2.26). No statistically significant differences were observed between the 2 liners ($P > 0.05$). There was a statistically significant difference between the two liners and the control groups. However, in the first 24 hours of the experiment, the two liner groups reflected a statistically significant difference ($P < 0.001$).

Table 1
Mean and standard deviation (STD) of cell viability after 24, 48, 96 hours for all groups.

Groups	Mean±STD after 24 hours	Mean±STD after 48 hours	Mean±STD after 96 hours
Control N1=24	100±3.59	99.9±4.7	99.98±2.32
TDV N2=24	65.8±3.03	71.39±1.05	73.38±1.97
RebaseII N3=24	22.36±1.75	63.11±1.74	67.10±2.26
P Value	*P*<0.001	*P*>0.05	*P*>0.05

Table 2
Comparison of cell viability at different times based on ANOVA test. Re: Rebase II; TD: TDV.

ANOVA		Sum of Squares	df	Mean Square	F	Sig.
Cell viability after 24h	Between Groups	24223.762	2	12111.881	180.143	0.000
	Within Groups	1411.934	21	67.235		
	Total	25635.696	23	-		
Cell viability after 48h	Between Groups	5989.281	2	2994.640	42.746	0.000
	Within Groups	1471.199	21	70.057		
	Total	7460.480	23	-		
Cell viability after 96h	Between Groups	4876.865	2	2438.433	63.418	0.000
	Within Groups	807.457	21	38.450		
	Total	5684.322	23	-		

To check the difference between the groups, since the dispersion of the data was the same according to Levene's test, the Tukey HSD test was used to compare the two groups. According to the results of Tukey's test, on the first day of the experiment (first 24 hours), the average values of cell viability in the three groups of TDV and Rebase II hard liners and in the control group had a statistically significant difference ($P < 0.001$) (TDV > Rebase II < control).

4. Discussion

The present study evaluated and compared the cytotoxicity of a Rebase II direct denture liner and TDV, used to improve denture fit, in vitro on HGFs. The few studies available did not make it clear about cytotoxicity of hard liners, and just limited studies used HGF. (Çakırbay Tanış et al., 2018). One of the novelties in our study arises from the utilization of HGFs Fibroblasts, the predominant cells of gingival connective tissue, are the first cells to encounter dental materials when a mucosal injury occurs in the oral cavity (López-García et al., 2021; López-García et al., 2021). Therefore, understanding how denture resin materials might affect these cells is crucial. Several previous articles have shown that the MTT test is a standard method for checking the toxicity and metabolism of cells (Kostić et al., 2012), Mar 15; (Çakırbay Tanış et al., 2018).

The TDV hard liner had higher cell viability (70–75%) and lower cytotoxicity than the Rebase II side liner (25–70%). Various factors are known to cause cytotoxicity of a substance, such as the type of elements released, the composition of saliva and mediator solution, and the incubation time (Elshahawy and Watanabe, 2014). Based on raw material ratios, contact time with tissue, and the host's systemic environment, the released elements have differing biological effects (López-García et al., 2019; Rodríguez-Lozano et al., 2021). Incubation time can influence cytotoxicity, since monomers and other side substances, such as isobutyl methacrylate (IBMA), are released at maximum levels with increasing incubation time.

Study results by Yokoyama et al. (2022) showed that 6 types of denture liners caused similar toxicity in mouse BALB/c cells, whether the materials were mixed immediately or after 24 hours. The toxicity of Mild Rebaron acrylic liners (photopolymers) was at its peak when mixed. In Rebaron liners, the maximum toxicity was seen at 24 hours, but it dropped drastically after 48 hours. According to Cakirbay et al. (2018), denture base resin materials should be tested for toxicity against L929 mouse fibroblast cells by MTT. In contrast with our study, the amount of cytotoxicity increased significantly after 15 days of immersion in water. Material types, cell types, methods, and experiment duration may be responsible for this discrepancy.

Jagdish et al. (2017))investigated the effect of a hard liner on human mucosal fibroblasts and discovered that differences in the substances released may cause disparities in toxicity results for varying materials. According to this study, materials released from the liners must reach a certain concentration to enter the near-toxic phase. There was always a greater toxicity of methacrylic acid (MA) than MA 72-144 mol/L and IBMA mol/L 40–80, which means that it had both 20–26% and 40–48% cell viability.

The high amount of MA released from the liner likely caused the high toxicity found in the Rebase II material during the first day of the present study. Caldas et al. (2016) tested two soft-liners at three different times (after 1 day, 1 week, and 1 month) and found that they were generally less than 20% toxic, a level not considered harmful. However, their study also showed that the toxicity could last for more than a week after

Table 3
Comparison of cell viability at different times based on Tukey's test. Re: Rebase II; TD: TDV.

Multiple Comparisons Tukey HSD Dependent Variable			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
CV24h	Control	Re	77.63279*	4.09985	0.001	67.2988	87.9667
		TD	34.14550*	4.09985	0.001	23.8115	44.4794
	Re	Control	-77.63279*	4.09985	0.001	-87.9667	-67.2988
		TD	-43.48730*	4.09985	0.001	-53.8212	-33.1533
	TD	Control	-34.14550*	4.09985	0.001	-44.4794	-23.8115
		Re	43.48730*	4.09985	0.001	33.1533	53.8212
CV48h	Control	Re	36.87477*	4.18501	0.001	26.3262	47.4234
		TD	28.59507*	4.18501	0.001	18.0465	39.1437
	Re	Control	-36.87477*	4.18501	0.001	-47.4234	-26.3262
		TD	-8.27970	4.18501	0.142	-18.8283	2.2689
	TD	Control	-28.59507*	4.18501	0.001	-39.1437	-18.0465
		Re	8.27970	4.18501	0.142	-2.2689	18.8283
CV96h	Control	Re	32.88634*	3.10042	0.001	25.0715	40.7012
		TD	26.60578*	3.10042	0.001	18.7910	34.4206
	Re	Control	-32.88634*	3.10042	0.001	-40.7012	-25.0715
		TD	-6.28055	3.10042	0.131	-14.0954	1.5343
	TD	Control	-26.60578*	3.10042	0.001	-34.4206	-18.7910
		Re	6.28055	3.10042	0.131	-1.5343	14.0954

Table 4
Comparison of pairwise cell viability at different time points based on the Tukey test.

Multiple Comparisons							
Tukey HSD							
Dependent Variable			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
CV24h	Control	Re	77.63279*	4.09985	0.001	67.2988	87.9667
		TD	34.14550*	4.09985	0.001	23.8115	44.4794
	Re	Control	-77.63279*	4.09985	0.001	-87.9667	-67.2988
		TD	-43.48730*	4.09985	0.001	-53.8212	-33.1533
	TD	Control	-34.14550*	4.09985	0.001	-44.4794	-23.8115
		Re	43.48730*	4.09985	0.001	33.1533	53.8212
CV48h	Control	Re	36.87477*	4.18501	0.001	26.3262	47.4234
		TD	28.59507*	4.18501	0.001	18.0465	39.1437
	Re	Control	-36.87477*	4.18501	0.001	-47.4234	-26.3262
		TD	-8.27970	4.18501	0.142	-18.8283	2.2689
	TD	Control	-28.59507*	4.18501	0.001	-39.1437	-18.0465
		Re	8.27970	4.18501	0.142	-2.2689	18.8283
CV96h	Control	Re	32.88634*	3.10042	0.001	25.0715	40.7012
		TD	26.60578*	3.10042	0.001	18.7910	34.4206
	Re	Control	-32.88634*	3.10042	0.001	-40.7012	-25.0715
		TD	-6.28055	3.10042	0.131	-14.0954	1.5343
	TD	Control	-26.60578*	3.10042	0.001	-34.4206	-18.7910
		Re	6.28055	3.10042	0.131	-1.5343	14.0954

the first day, as indicated by one of the tests they used, known as the XTT test.

In Wicks et al.'s (Wicks et al., 2015) study, the cytotoxic effects of three varieties of acrylic denture base materials were assessed on human gingival epithelial and fibroblast cells. Notably, some of the acrylic discs were left unpolished. The results revealed that the unpolished discs exhibited a significantly higher level of toxicity compared to their polished counterparts and that epithelial cells were less susceptible to toxicity than fibroblasts. Similarly, Saravi et al. (2012) explored the cytotoxic impact of three different acrylic resins, among them autopolymerized hard liners, on mouse fibroblast cells (L929). This examination was carried out at three intervals—1 hour, 24 hours, and 7 days—employing MTT and ELISA assays. The findings highlighted that both the type of acrylic resin and the duration of exposure considerably influenced cytotoxicity levels. Notably, there was a marked reduction in cytotoxicity at the 24-hour mark when juxtaposed with the initial hour, where toxicity peaked. However, no notable change in toxicity was observed over the subsequent seven days, suggesting a stabilization in toxicity levels. Variability in cytotoxicity can be attributed to the type of material, as well as the methodological approach, including the number of cell culture passages and the choice of cytotoxicity assessment technique (Kreve and Dos Reis, 2019).

Limitations of this study include its short-term in vitro design, the lack of evaluation of cytotoxicity in the first hour, the use of only one cytotoxicity test (MTT), and the inability to follow ISO recommendations due to a lack of equipment and access. Short-term in vitro studies cannot fully replicate the complex physiological environment of the body, and therefore may not be predictive of long-term in vivo effects. Additionally, cytotoxicity may occur rapidly within the first hour of exposure to a toxic substance. Therefore, the lack of evaluation of cytotoxicity in the first hour is a limitation of this study. The MTT test is a reliable and widely used cytotoxicity test, but it is not the only one available. Using other standard cytotoxicity tests, such as the lactate dehydrogenase (LDH) assay or the trypan blue exclusion test, would have strengthened the findings of this study. ISO recommendations provide standardized guidelines for conducting cytotoxicity testing. Following ISO recommendations would have ensured that the testing in this study was conducted in a rigorous and reproducible manner. However, the authors were unable to follow ISO recommendations due to a lack of equipment and access.

5. Conclusion

It was observed that the Rebase II hard liner group had the highest toxicity and the lowest cell viability at all time points, which exhibited a significant difference with the TDV liner only in the first 24 hours, which could be due to the difference in the ratio of powder to liquid in these two materials (Rebase II, 3:5 and TDV, 1:1). Moreover, with increasing time, cytotoxicity decreased in all groups with liners.

Ethical statement

This original review was ethically conducted in all facets. It is free from any form of plagiarism, with all pertinent articles and sources reviewed appropriately cited.

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This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Clinical relevance

Using direct autopolymerizing hard liners can cause cytotoxicity due to incomplete polymerization or free monomers. Increasing incubation time reduces cytotoxicity, since most monomers are released during the first few hours.

Ethical approval

This study received ethical approval from the Ethics Committee of Islamic Azad University, Tehran Branch, Dental School (Code: IR.IAU.DENTAL.REC.1401.010).

CRediT authorship contribution statement

Somayeh Hashemi: Conceptualization, Methodology, Software, Writing – review & editing. **Reza Nahidi:** Methodology, Supervision, Writing – original draft. **Homeyra Ansari:** Methodology, Supervision, Writing – review & editing. **Kiarash Firoozi:** Software, Writing – review & editing. **Rata Rokhshad:** Software, Writing – original draft, Writing – review & editing.

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