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In Vitro Cytotoxicity Assessment of Different Thermoplastic Aligner Materials

In vitro *procjena citotoksičnosti različitih termoplastičnih materijala za izradu ortodontskih alignera*

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Abstract

Objectives: This study evaluated the cytotoxicity of different thermoplastic materials and the impact of thermoforming on their cytotoxicity. **Materials and methods:** Four thermoplastic materials were tested before and after thermoforming: Zendura A (Bay Materials), Zendura FLX (Bay Materials), Erkoloc-pro (Erkodent), and CA Pro + (Scheu Dental). The samples were stored in artificial saliva and incubated at 37 °C for 14 days to simulate conditions in the oral cavity during one stage of clear aligner treatment. Then, the saliva was diluted with a complete culture medium at three concentrations: c1=10%, c2=20% and c3=30%. Cytotoxic activities of the materials were evaluated after incubation for 4h, 24h, 48h, and 72h by using the CCK-8 assay on human oral fibroblast cells (Innoprot, REF: P10868). **Results:** The mean measured values of metabolically active cells below 70% of the control were for c3 of non-thermoformed Zendura A and Erkoloc-pro, and thermoformed Zendura A, after 72-hour incubation. The analysis showed differences between non-thermoformed: Zendura A and Erkoloc-pro and Zendura FLX and CA Pro +, thermoformed: Zendura A and CA Pro +, and Zendura FLX and CA Pro + at certain conditions. Differences between concentrations (c2 vs. c3 and c1 vs. c3) in the non-thermoformed group were found. There were no statistically significant differences in cytotoxicity between non-thermoformed and thermoformed groups. **Conclusions:** Cytotoxicity was observed in non-thermoformed and thermoformed materials, with significant differences between concentrations of the same material. However, the thermoforming process did not impact the cytotoxicity of clear aligner materials.

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Introduction

Clear aligner orthodontic treatment is based on the application of a series of custom-made plastic trays that gradually move the teeth. Unlike traditional metal braces, they are transparent, comfortable, and removable, making them a more aesthetically pleasing choice for patients (1, 2).

Thermoforming is a manufacturing method in which heat and pressure are used to create specific forms from plastic materials (3). It is commonly used to produce removable orthodontic appliances, including clear aligners and retainers (1, 4-6). In terms of clear aligners, the thermoforming process must be carried out precisely to ensure that each custom-

Uvod

Ortodontska terapija prozirnim aparatićima za zube temelji se na primjeni niza individualnih prozirnih udlaga koje postupno pomiču zube. Za razliku od tradicionalnih metalnih bravica, oni su prozirni, udobni i mogu se skinuti, što ih čini estetski prikladnijim izborom za pacijente (1, 2).

Termoformiranje je proizvodna metoda u kojoj se toplinom i tlakom stvaraju specifični oblici od plastičnih materijala (3). Obično se koristi za proizvodnju mobilnih ortodontskih naprava, uključujući prozirne alagnere i retencijske naprave (1, 4 – 6). Postupak termoformiranja prozirnih alignera mora se obaviti precizno kako bi se osiguralo da svaka

made thermoplastic tray fits perfectly to the patient's dentition and moves his/her teeth into a new position. To achieve this, a detailed multi-step process must be performed (4, 7).

The first step involves taking dental 3D scans or impressions and creating a digital model of the patient's dentition. The next step is treatment planning, which uses specialized software programs and produces a series of models for each aligner tray in orthodontic treatment (8, 9). Thermoplastic sheets, often made from polyurethane (PU), polyethylene terephthalate glycol (PET-G), or polymer blends, are then heated and placed over each model for vacuum- or pressure forming (1, 8, 9). When the thermoforming process is complete, the excess material is removed, and aligners are polished and checked for fit (8, 9).

Due to its complex technical process, thermoforming might alter various properties of the aligners. According to the study by Bucci et al. (10), aligner thickness is reduced after thermoforming. Similar conclusions were made in another study, where the results showed that the thickness of the aligner after thermoforming varied depending on the measurement position on the tooth, with the edges of the aligner being thinner than the occlusal surfaces (11). The research results by Daniele et al. (12) showed that Invisalign was susceptible to staining, likely due to surface irregularities formed during the thermoforming process. In a study where one of the objectives was to investigate the impact of thermoforming on the mechanical properties of clear aligners, it was concluded that the tested materials exhibited reduced flexural strength after thermoforming (13). Tamburrino et al. (14) reported that thermoforming can lead to changes in tensile yield stress and elastic modulus, depending on the type of material tested. The results of a study that involved testing the morphological, mechanical, optical, and chemical properties of thermoformed materials showed that the manufacturing process reduces the thickness and molecular weight of the material, thereby affecting its mechanical properties. Thermoforming also led to an increase in surface roughness, which in turn impacted the optical properties of the material (15).

A study by Martina et al. (16) observed a slight cytotoxic effect of different thermoplastic materials, which was increased after thermoforming for PET-G materials. Another study reported that thermoplastic materials were slightly to moderately cytotoxic compared to the control group (17).

This study aims to evaluate the cytotoxicity of different clear aligner materials in a simulated intraoral environment and to assess the potential effect of the thermoforming process on the cytotoxicity of clear aligner materials. It is expected that there may be differences between materials regarding cytotoxicity, and that the thermoforming process may influence the cytotoxic effects of the tested materials.

Materials and methods

Four thermoplastic clear aligner materials were tested: Zendura A (*Bay Materials LLC, Fremont, California, USA)* 0.76 mm (material A), Zendura FLX (*Bay Materials LLC, Fremont, California, USA*) 0.76 mm (material B), Erkoloc

individualna udlaga savršeno odgovara pacijentovoj denticiji i pomiče zube u novi položaj. Da bi se to postiglo, mora se provesti detaljni postupak u više koraka (4, 7).

Prvi je korak uzimanje dentalnih 3D skenova ili otisaka i izrada digitalnog modela pacijentove denticije. Slijedi planiranje terapije u specijaliziranim računalnim programima i proizvodnja niza modela za svaki aligner u ortodontskom tretmanu (8, 9). Termoplastične ploče, često izrađene od poliuretana (PU), polietilen-tereftalat-glikola (PET-G) ili mješavine polimera, zatim se zagrijavaju i stavljaju preko svakog modela za oblikovanje pod vakuumom ili tlakom (1, 8, 9). Kada je proces termoformiranja završen, višak materijala se uklanja, a udlage se poliraju i provjerava se njihovo prilijeganje na zubne lukove (8, 9).

Zbog složenoga tehničkog procesa termoformiranje može promijeniti svojstva alignera. Prema studiji Bucci i suradnika (10), debljina alignera smanjuje se nakon termoformiranja. Slični su zaključci i u jednoj drugoj studiji u kojoj su rezultati pokazali da debljina alignera nakon termoformiranja varira ovisno o poziciji mjerenja na zubu, pri čemu su rubovi alignera tanji od okluzalnih površina (11). Rezultati istraživanja Danielea i suradnika (12) pokazalo je da je Invisalign podložan obojenju, vjerojatno zbog površinskih nepravilnosti nastalih tijekom procesa termoformiranja. U studiji, čiji je jedan od ciljeva bio istražiti utjecaj termooblikovanja na mehanička svojstva prozirnih alignera, zaključeno je da ispitani materijali pokazuju smanjenu čvrstoću na savijanje nakon termooblikovanja (13). Tamburrino i suradnici (14) izvijestili su da termooblikovanje može rezultirati promjenom u vlačnoj granici popuštanja i modulu elastičnosti, ovisno o vrsti ispitivanog materijala. Rezultati istraživanja, koje je uključivalo ispitivanje morfoloških, mehaničkih, optičkih i kemijskih svojstava termoformiranih materijala, pokazali su da proizvodni proces smanjuje debljinu i molekularnu masu materijala, a time utječe na njegova mehanička svojstva. Termoformiranje je također povećalo hrapavost površine, što je zatim utjecalo na optička svojstva materijala (15).

Studija Martina i suradnika (16) pokazala je blagi citotoksični učinak različitih termoplastičnih materijala koji je povećan nakon termoformiranja za PET-G materijale. Autori druge studije izvijestili su da su termoplastični materijali blago do umjereno citotoksični u usporedbi s kontrolom (17).

Ova studija ima za cilj procijeniti citotoksičnost različitih prozirnih materijala za alignere u simuliranom intraoralnom okruženju i procijeniti potencijalni učinak procesa termoformiranja na citotoksičnost prozirnih materijala za izradu alignera. Očekuje se da bi mogle postojati razlike između materijala kad je riječ o citotoksičnosti te da proces termoformiranja može utjecati na citotoksične učinke ispitivanih materijala.

Materijal i metode

Ispitana su četiri termoplastična prozirna materijala za alignere: Zendura A (Bay Materials LLC, Fremont, Kalifornija, SAD) 0,76 mm (materijal A), Zendura FLX (Bay Materials LLC, Fremont, Kalifornija, SAD) 0,76 mm (materijal B), Er-

Pro (*Erkodent Erich Kopp GmbH, Pfanzgrafenweiler, Germany*) 1.0 mm (material C) and CA Pro + (*Scheu Dental GmbH, Iserlohn, Germany*) 0.75 mm (material D).

The materials were divided into non-thermoformed (as-supplied) and thermoformed groups. Thermoplastic sheets were heated and pressed over a flat plate using a Biostar® device (*Scheu Dental GmbH*) for the thermoformed group. The as-supplied thermoplastic sheets and thermoformed samples were cut into 2x2 cm squares. To simulate the intraoral environment during clear aligner therapy, the samples were stored in artificial saliva (*Artificial Saliva for Pharmaceutical Research, Sigma-Aldrich Chemie GmbH, Taufkierchen, Germany*) in an incubator at 37 °C for 14 days. Before storage, the samples were weighed on a precision scale to ensure an equal ratio of sample weight and liquid volume of 0.1 mg/ml according to the ISO (International Organization for Standardization) standard 10993-12:2012 (18).

After the incubation period, the saliva containing leachates from materials was diluted with complete culture medium at three concentrations: c1=10% of saliva with leachates and 90% of culture medium, c2=20% of saliva with leachates and 80% of culture medium, and c3=30% of saliva with leachate and 70% of culture medium. Higher dilutions were not performed to avoid possible cell death due to nutrient deficiency.

Human oral fibroblast cells (*Human Oral Fibroblasts REF: P10868, Innoprot, Derio, Spain*) were cultured in a complete culture medium containing DMEM (*Dulbeco's Modified Eagle's Medium, Sigma-Aldrich*) with 10% FBS (*Fetal Bovine Serum, Sigma-Aldrich*) and 1% antibiotic and antimytotic solution (*Antibiotic and Antimycotic Solution, Sigma-Aldrich*) at 37 °C in a humid atmosphere with 5% CO2.

Following serial subculturing, fibroblasts were plated on 96-well plates at a density of 5,000 cells per well and incubated overnight to allow attachment. The cells were subsequently exposed to three concentrations of various materials in triplicates and incubated for 4, 24, 48, and 72 hours at 37°C. A triplicate of complete culture medium was used as the control.

Cytotoxicity was evaluated by adding 10 µL of CCK-8 reagent (*Cell Counting Kit-8, MedChemExpress, New Jersey, USA*) in each well, after each incubation. The 96-well plates were then again incubated at 37 °C for 2h.

The metabolic activities of the cells were measured in a spectrophotometer (*HiPo MPP-96, Biosan, Riga, Latvia*) at 450 nm. The following formula was used to calculate cell viability: Cell viability = Optical density of the sample / Optical density of the control * 100 (ISO 10993-5:2009 (19)).

Graphical analysis of cytotoxic tendency was carried out in GraphPad Prism version 10.4.1 (*GraphPad Software, Boston, Massachusetts, USA*). Statistical analysis was performed in the JASP program (*JASP, University of Amsterdam, Amsterdam, and the Netherlands*). For the comparison between different materials and concentrations, the Kruskal-Wallis test, along with Dunn's posthoc analysis with Bonferroni correction was used. The Mann-Whitney test was used to compare non-thermoformed and thermoformed groups.

koloc Pro (Erkodent Erich Kopp GmbH, Pfanzgrafenweiler, Njemačka) 1,0 mm (materijal C) i CA Pro + (Scheu Dental GmbH, Iserlohn, Njemačka) 0,75 mm (materijal D).

Materijali su podijeljeni u netermoformirane (kako su isporučeni) i termoformirane skupine. Termoplastične ploče zagrijavane su i prešane preko ravne ploče s pomoću uređaja Biostar® (Scheu Dental GmbH) za termoformiranu skupinu. Netermoformirane termoplastične ploče i termoformirani uzorci izrezani su na kvadrate 2 x 2 cm. Kako bi se simulirali uvjeti u usnoj šupljini tijekom terapije prozirnim alignerom, uzorci su bili pohranjeni 14 dana u umjetnoj slini (Artificial Saliva for Pharmaceutical Research, Sigma-Aldrich Chemie GmbH, Taufkierchen, Njemačka) u inkubatoru na 37 °C. Prije pohranjivanja izvagani su na preciznoj vagi kako bi se osigurao jednak omjer mase uzorka i volumena tekućine od 0,1 mg/mL prema ISO standardu (International Organisation for Standardization) 10993-12:2012 (18).

Nakon inkubacije slina koja je sadržavala eluat materijala razrijeđena je kompletnom podlogom kulture u trima koncentracijama: c1 = 10 % sline i 90 % medija kulture, c2 = 20 % sline i 80 % medija kulture te c3 = 30 % sline i 70 % medija kulture. Nisu primijenjena veća razrjeđenja kako bi se izbjegla moguća smrt stanica zbog nedostatka hranjivih tvari.

Humani oralni fibroblasti (Human Oral Fibroblasts REF: P10868, Innoprot, Derio, Španjolska) uzgajani su u punom mediju za kulture koji je sadržavao DMEM (Dulbeco's Modified Eagle's Medium, Sigma-Aldrich) s 10 % FBS-a (Fetal Bovine Serum, Sigma-Aldrich) i 1-postotnu otopinu antibiotika i antimikotika (antibiotik i antimikotik otopina, Sigma-Aldrich) na 37 °C u vlažnoj atmosferi s 5 % CO_2.

Nakon serijskog supkultiviranja, fibroblasti su postavljeni na ploče s 96 jažica u gustoći od 5000 stanica po jažici i inkubirani preko noći da bi se omogućilo pričvršćivanje. Stanice su zatim izložene trima koncentracijama različitih materijala u triplikatu i inkubirane tijekom 4, 24, 48 i 72 sata na 37 °C. Kao kontrola korišten je triplikat punog medija za kulture.

Citotoksičnost je procijenjena dodavanjem 10 µL reagensa CCK-8 (Cell Counting Kit-8, MedChemExpress, New Jersey, SAD) u svaku jažicu nakon svake inkubacije. Ploče s 96 jažica zatim su ponovno inkubirane 2 sata na 37 °C.

Metabolička aktivnost stanica mjerena je spektrofotometrom (HiPo MPP-96, Biosan, Riga, Latvija) na 450 nm. Za izračun vijabilnosti stanica korištena je sljedeća formula: vijabilnost stanica = optička gustoća uzorka / optička gustoća kontrole * 100 (ISO 10993-5:2009 (19)).

Grafička analiza citotoksične tendencije provedena je u GraphPad Prism verziji 10.4.1 (GraphPad Software, Boston, Massachusetts, SAD). Statistička analiza obavljena je u programu JASP (JASP, Sveučilište u Amsterdamu, Amsterdam, Nizozemska). Za usporedbu između različitih materijala i koncentracija korišten je Kruskal-Wallisov test, zajedno s Dunnovom posthoc analizom s Bonferronijevom korekcijom. Mann-Whitneyjev test korišten je za usporedbu netermoformiranih i termoformiranih skupina.

Results

The cytotoxic tendency of materials after 14 days in a simulated intraoral environment is shown in Figures 1 and 2. The graphs present the mean values of cellular metabolic activity, with error bars indicating the standard deviation. The values of cell viability that were below 70% of the control for the non-thermoformed group were: concentration 3 of material A after incubation for 72 hours (minimal, mean, and maximal measured value), concentration 3 of material B after incubation for 72 hours (minimal measured value), and concentration 3 of material C after incubation for 72 hours (minimal and mean measured value). All cell viability values for non-thermoformed material D were above 70% of the control (Figure 1).

The values of cell viability that were below 70% of the control for the thermoformed group were concentration 3 of material A after incubation for 72 hours (minimal, mean, and maximal measured values) and concentration 1 of material C after incubation for 4 hours (minimal measured value). All values of cell viability for thermoformed materials B and D were above 70% of the control (Figure 2).

Regarding differences between materials, post hoc analysis showed differences between non-thermoformed materials: A versus C at concentration 3, and after incubation for 48 hours (p=0.030), and B versus D at concentration 1 and after incubation for 72 hours (p=0.019) (Table 1). Significant

Rezultati

Citotoksična tendencija materijala poslije 14 dana u simuliranom intraoralnom okruženju prikazana je na slikama 1. i 2. Grafikoni pokazuju srednje vrijednosti stanične metaboličke aktivnosti, sa stupcima pogrešaka koji pokazuju standardnu devijaciju. Vrijednosti vijabilnosti stanica koje su bile ispod 70 % kontrole za skupinu koja nije termoformirana bile su: koncentracija 3 materijala A nakon inkubacije od 72 sata (minimalna, srednja i maksimalna izmjerena vrijednost), koncentracija 3 materijala B nakon inkubacije od 72 sata (minimalna izmjerena vrijednost) i koncentracija 3 materijala C nakon inkubacije od 72 sata (minimalna i srednja izmjerena vrijednost). Sve vrijednosti vijabilnosti stanica za materijal D koji nije termoformiran bile su iznad 70 % kontrole (slika 1.).

Vrijednosti vijabilnosti stanica koje su bile ispod 70 % kontrole za termoformiranu skupinu bile su koncentracija 3 materijala A nakon inkubacije od 72 sata (minimalne, srednje i maksimalne izmjerene vrijednosti) i koncentracija 1 materijala C nakon inkubacije od 4 sata (minimalna izmjerena vrijednost). Sve vrijednosti vijabilnosti stanica za termoformirane materijale B i D bile su iznad 70 % kontrole (slika 2.).

Kad je riječ o razlikama između materijala, *post hoc* analiza pokazala je razlike između materijala koji nisu bili termoformirani: A u odnosu na C pri koncentraciji 3 i nakon inkubacije od 48 sati (p = 0,030), te B u odnosu na D pri

Table 1 Post-hoc analysis of the difference in cell viability between all materials with the same concentration and incubation.
Tablica 1. Post-hoc analiza razlike u vijabilnosti stanica između svih materijala s istom koncentracijom i inkubacijom

Group	c (%)	Incubation (h)											
		4			24			48			72		
		p#	†	p_{bonf}	p#	†	p_{bonf}	p#	†	p_{bonf}	p#	†	p_{bonf}
NT	10	0.248	-	-	0.183	-	-	0.176	-	-	0.032	B vs D	0.019
	20	0.132	-	-	0.347	-	-	0.074	-	-	0.100	-	-
	30	0.392	-	-	0.025	-	ns	0.040	A vs C	0.030	0.100	-	-
T	10	0.197	-	-	0.204	-	-	0.082	-	-	0.053	B vs D	0.039
	20	0.347	-	-	0.727	-	-	0.038	A vs D	0.039	0.467	-	-
	30	0.347	-	-	0.040	-	ns	0.079	-	-	0.116	-	-

NT - non-thermoformed; T - thermoformed; c – concentration; # Kruskal Wallis test, significance p<0.05; † Comparisons; Dunn post hoc test with Bonferroni correction, significance p_{bonf}<0.05; ns - not significant; A - Zendura A; B - Zendura FLX; C - Erkoloc-pro; D - CA Pro +

Table 2 Post-hoc analysis of the difference in cell viability between the three concentrations of the same material and incubation.
Tablica 2. Post-hoc analiza razlike u vijabilnosti stanica između triju koncentracija istog materijala i inkubacije

Version	Material	Incubation (h)											
		4			24			48			72		
		p#	†	p_{bonf}	p#	†	p_{bonf}	p#	†	p_{bonf}	p#	†	p_{bonf}
NT	A	0.148	-	-	0.113	-	-	0.027	c2 vs c3	0.021	0.180	-	-
	B	0.670	-	-	0.066	-	-	0.039	c1 vs c3	0.034	0.051	-	-
	C	0.491	-	-	0.061	-	-	0.069	-	-	0.044	c2 vs c3	0.042
	D	1.000	-	-	0.113	-	-	0.061	-	-	0.119	-	-
T	A	0.177	-	-	0.133	-	-	0.051	-	-	0.069	-	-
	B	0.670	-	-	0.058	-	-	0.119	-	-	0.062	-	-
	C	0.291	-	-	0.051	-	-	0.051	-	-	0.062	-	-
	D	0.430	-	-	0.079	-	-	0.113	-	-	0.062	-	-

NT - non-thermoformed; T - thermoformed; A - Zendura A; B - Zendura FLX; C - Erkoloc-pro; D - CA Pro +; # Kruskal Wallis test, significance p<0.05; † Comparisons; Dunn post hoc test with Bonferroni correction, significance p_{bonf}<0.05; c1 = 10%; c2 = 20%; c3 = 30%

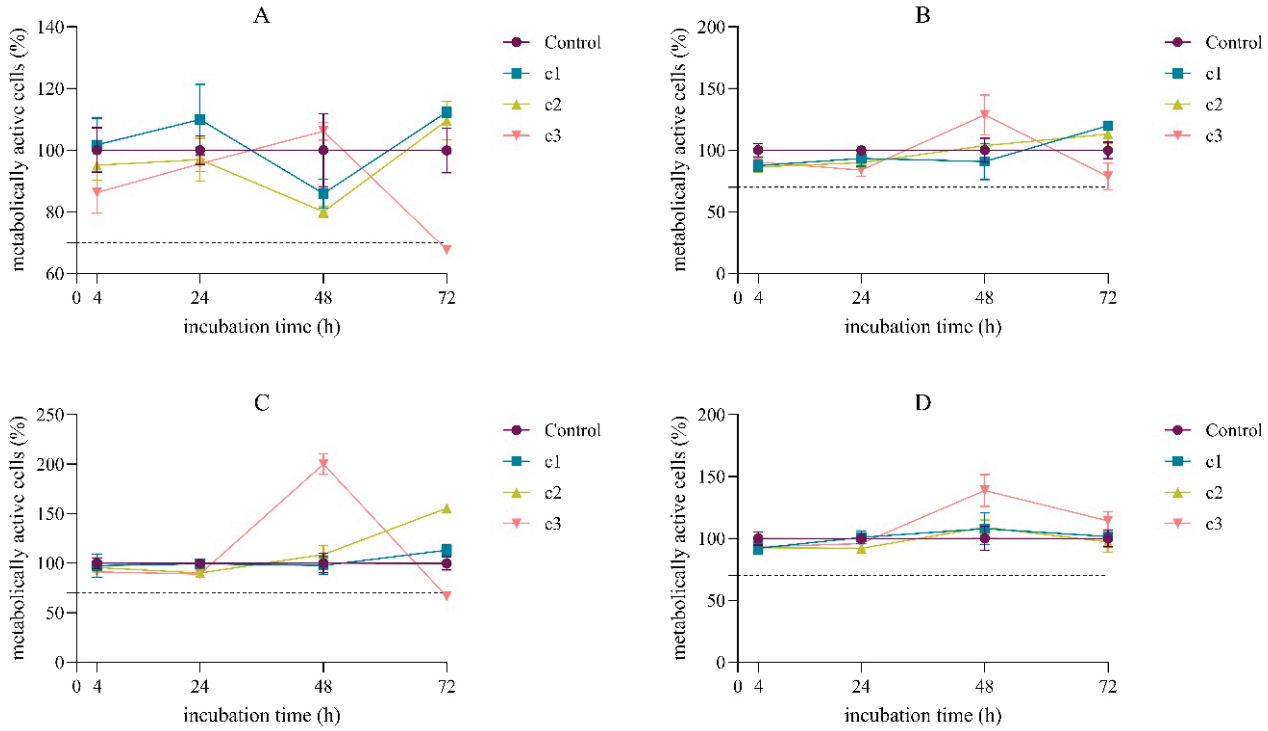

Figure 1 Cytotoxicity tendencies of non-thermoformed materials. A-Zendura A; B-Zendura FLX; C- Erkoloc-pro; D-CA Pro. The graphs show the mean values, with error bars representing standard deviation (SD). The dashed line represents 70% of the control, and according to the ISO standard (ISO-10993-5:2009), all values below this threshold are considered cytotoxic.

Slika 1. Tendencije citotoksičnosti netermoformiranih materijala: A-Zendura A; B-Zendura FLX; C- Erkoloc-pro; D-CA Pro; grafikoni pokazuju srednje vrijednosti, sa stupcima pogrešaka koji predstavljaju standardnu devijaciju (SD); isprekidana linija pokazuje 70 % kontrole, a prema ISO standardu (ISO-10993-5:2009) sve vrijednosti manje od toga praga smatraju se citotoksičnima

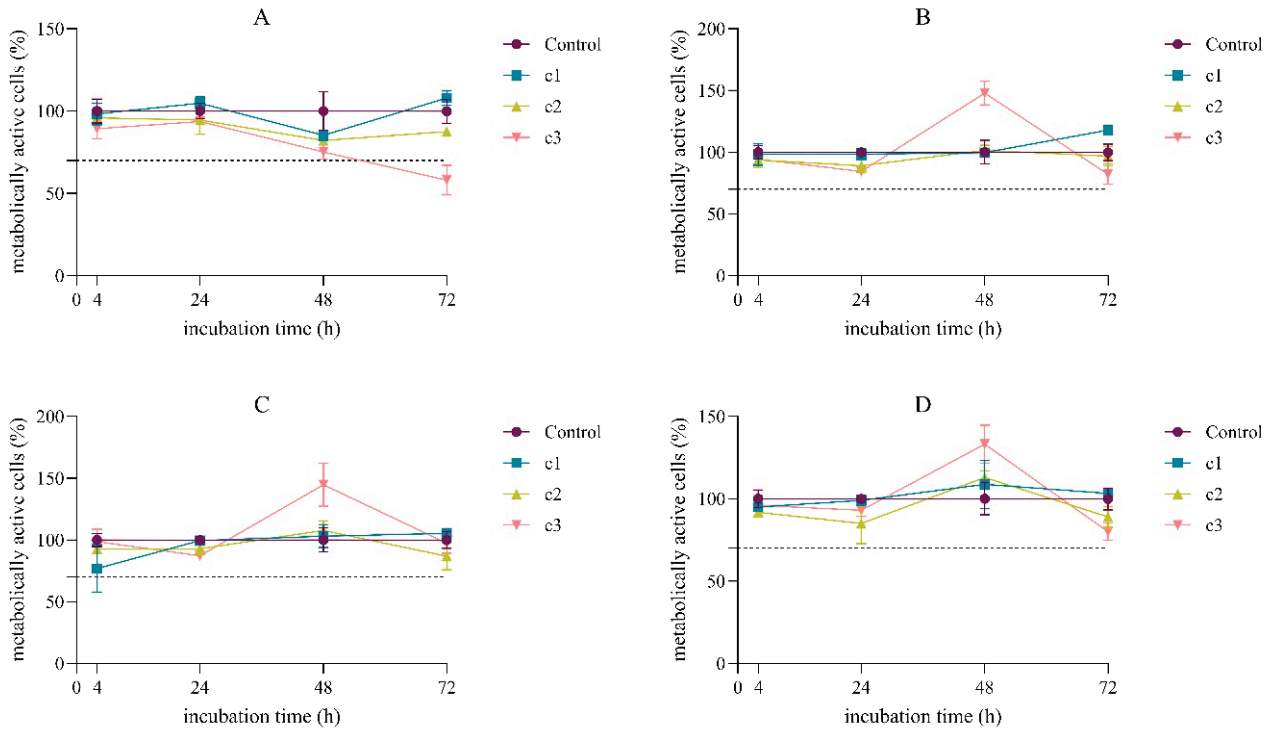

Figure 2 Cytotoxicity tendencies of thermoformed materials. A-Zendura A; B-Zendura FLX; C- Erkoloc-pro; D-CA Pro. The graphs show the mean values, with error bars representing standard deviation (SD). The dashed line represents 70% of the control, and according to the ISO standard (ISO-10993-5:2009), all values below this threshold are considered cytotoxic.

Slika 2. Tendencije citotoksičnosti termoformiranih materijala: A-Zendura A; B-Zendura FLX; C- Erkoloc-pro; D-CA Pro; grafikoni pokazuju srednje vrijednosti, sa stupcima pogrešaka koji predstavljaju standardnu devijaciju (SD); isprekidana linija pokazuje 70 % kontrole, a prema ISO standardu (ISO-10993-5:2009) sve vrijednosti manje od toga praga smatraju se citotoksičnima

Table 3 Differences between non-thermoformed and thermoformed groups at the same concentrations and incubation conditions.
Tablica 3. Razlike između netermoformiranih i termoformiranih skupina pri istim koncentracijama i uvjetima inkubacije

Material	c (%)	Incubation (h)			
		4	24	48	72
		p#	p#	p#	p#
A	10	0.700	1.000	1.000	0.400
	20	0.700	0.700	0.376	0.333
	30	0.400	0.400	0.100	0.333
B	10	0.100	0.268	0.700	0.400
	20	0.200	0.700	0.700	0.200
	30	0.200	0.700	0.400	1.000
C	10	0.400	0.700	0.700	0.100
	20	0.400	0.400	1.000	0.200
	30	0.700	1.000	0.200	0.200
D	10	0.700	0.700	1.000	0.400
	20	1.000	0.700	0.700	0.400
	30	0.700	0.400	0.700	0.333

A - Zendura A; B - Zendura FLX; C - Erkoloc-pro; D - CA Pro +; c1 = 10%; c2 = 20%; c3 = 30%; # Mann- Whitney U test, significance p<0.05

differences were also observed between thermoformed materials: A versus D at concentration c2 and after incubation for 48 hours (p=0.039) and B versus D at concentration c1 and after incubation for 72 hours (p=0.039) (Table 1).

Statistical analysis revealed differences between concentrations only for the non-thermoformed group: c2 versus c3 for material A after 48 hours of incubation (p=0.021), c1 versus c3 for material B after 48 hours of incubation (p=0.034), and c2 versus c3 for material C after 72 hours of incubation (p=0.042) (Table 2).

The results obtained in this research showed no statistically significant differences in cytotoxicity between non-thermoformed and thermoformed groups (P>0.05) (Table 3).

Discussion

Clear aligners are a transparent and comfortable alternative to traditional fixed appliances (20, 21). Thermoplastic materials must effectively move the teeth and not harm the patient. This study aims to assess the cytotoxicity of four clear aligner materials in a simulated intraoral environment and to evaluate the potential impact of the thermoforming process on their cytotoxicity.

Figure 1 and Figure 2 show the cytotoxic tendency of materials after 14 days in a simulated intraoral environment. In this study, the cytotoxicity threshold for the materials was based on the ISO standard (19), where a material is considered cytotoxic if the cell viability is less than 70% of the control. According to this, the cytotoxic potential was found for some materials in the non-thermoformed group: concentration 3 of material A after incubation for 72 hours (minimal, mean, and maximal measured value), concentration 3 of material B after incubation for 72 hours (minimal measured value), and concentration 3 of material C after incubation for 72 hours (minimal and mean measured value), as well as for some materials in the thermoformed group: concentration 3 of material A after incubation for 72 hours (minimal, mean

koncentraciji 1 i nakon inkubacije od 72 sata (p = 0,019) (tablica 1.). Značajne razlike uočene su i između termoformiranih materijala: A u odnosu na D pri koncentraciji c2 i nakon inkubacije od 48 sati (p = 0,039) i B u odnosu na D pri koncentraciji c1 i nakon inkubacije od 72 sata (p = 0,039) (tablica 1.).

Statistička analiza otkrila je razlike između koncentracija samo za skupinu koja nije termoformirana: c2 prema c3 za materijal A nakon 48 sati inkubacije (p = 0,021), c1 prema c3 za materijal B nakon 48 sati inkubacije (p = 0,034) i c2 prema c3 za materijal C nakon 72 sata inkubacije (p = 0,042) (tablica 2.).

Rezultati ovog istraživanja nisu pokazali statistički značajne razlike u citotoksičnosti između netermoformiranih i termoformiranih skupina (P > 0,05) (tablica 3.).

Rasprava

Prozirni aligneri transparentna su i za pacijenta ugodna alternativa tradicionalnim fiksnim aparatima (20, 21). Termoplastični materijali moraju učinkovito pomicati zube i ne štetiti pacijentu. Ovoj je studiji cilj procijeniti citotoksičnost četiriju prozirnih materijala za alignere u simuliranom intraoralnom okruženju i potencijalni utjecaj procesa termoformiranja na njihovu citotoksičnost.

Slike 1. i 2. prikazuju citotoksičnu tendenciju materijala poslije 14 dana u simuliranom intraoralnom okruženju. U ovoj se studiji prag citotoksičnosti za materijale temeljio na ISO standardu (19), pa se materijal smatrao citotoksičnim ako je vijabilnost stanica bila manja od 70 % kontrole.

U skladu s tim utvrđen je citotoksični potencijal za neke materijale u skupini koja nije termoformirana: koncentracija triju materijala A nakon inkubacije od 72 sata (minimalna, srednja i maksimalna izmjerena vrijednost), koncentracija 3 materijala B nakon inkubacije od 72 sata (minimalna izmjerena vrijednost) i koncentracija 3 materijala C nakon inkubacije od 72 sata (minimalna i srednja izmjerena vrijednost), te za neke materijale u termoformiranoj skupini: koncentracija 3 materijala A nakon inkubacije od 72 sata (minimalne,

and maximal measured values), and concentration 1 of material C after incubation for 4 hours (minimal measured value). It is noticeable that the curve for the C3 concentration shows the same trend for almost all materials in both groups (non-thermoformed and thermoformed), with the percentage of metabolically active cells significantly increasing above the control after incubation for 48 hours, then suddenly dropping during more prolonged incubation, even falling below the cytotoxicity threshold in some cases. This is likely a cellular defense mechanism against potential toxins, with prolonged exposure leading to decreased cell viability. The other concentrations do not follow any specific trend, and all values of metabolically active cells are above the cytotoxicity threshold, except for the minimum value of c1 of material C after incubation for 4 hours. However, since this was a short incubation period with the lowest concentration, this probably occurred due to a technical error.

The post hoc analysis revealed differences between materials in both groups (non-thermoformed and thermoformed). Cytotoxicity of non-thermoformed material A significantly differed from that of non-thermoformed material C at concentration 3, and after incubation for 48 hours (p=0.030). A statistically significant difference was found between materials B and D in both tested groups (non-thermoformed, thermoformed) at concentration 1 and after incubation for 72 hours (p=0.019 and p=0.039, respectively). In the group of thermoformed materials, a significant difference was also observed between materials A and D at concentration c2 and after incubation for 48 hours (p=0.039). Differences between materials may arise due to the composition of each material. Material A is monolayer thermoplastic polyurethane (TPU) (22), while material C is a double-layered material composed of co-polyester (PETG) and polyurethane (TPU) (23). Both materials B and D are three-layered materials with two layers of copolyester and an elastomeric layer in between (24, 25). Other components of the materials may be toxic, and their proportions in the composition are proprietary, complicating the interpretation of results. It is known that the composition and conformation affect the mechanical properties of thermoplastic materials (26), while fewer studies have focused on their cytotoxicity.

This study found a statistically significant difference between different concentrations within the group of non-thermoformed materials. Concentration 3 differed from concentration 1 for material B after incubation for 48 hours (p=0.034), while it differed from concentration 2 for material A after incubation for 48 hours (p=0.021) and for material C after 72 hours (p=0.042). These differences occurred due to the previously described cytotoxicity tendency for concentration 3, where cell viability after 48 hours was significantly higher than the control, while after 72 hours, it dropped below 70%. In this case, c3 (30% volume/volume) can be considered the cut-off value in cytotoxicity tests. Higher dilutions than this would produce unclear results due to the possibility that the cells may die from insufficient nutrients rather than from potential toxins in the tested materials. Study by Alhendi et al. (17) has investigated the cytotoxicity of similar thermoplastic materials, also revealing significant

srednje i maksimalne izmjerene vrijednosti), te koncentracija 1 materijala C nakon inkubacije od 4 sata (minimalna izmjerena vrijednost). Može se uočiti da krivulja za koncentraciju C3 pokazuje isti trend za gotovo sve materijale u objema skupinama (netermoformirane i termoformirane), pri čemu se postotak metabolički aktivnih stanica značajno povećao u odnosu prema kontroli nakon inkubacije od 48 sati, a zatim naglo opadao tijekom dulje inkubacije te je čak u nekim slučajevima pao ispod praga citotoksičnosti. To je vjerojatno obrambeni stanični mehanizam protiv potencijalnih toksina, pri čemu produljena izloženost smanjuje vijabilnost stanica. Ostale koncentracije ne prate nikakav specifični trend, a sve vrijednosti metabolički aktivnih stanica iznad su praga citotoksičnosti, osim minimalne vrijednosti c1 materijala C nakon inkubacije od 4 sata. No kako se radilo o kratkom razdoblju inkubacije s najnižom koncentracijom, vjerojatno je riječ o tehničkoj pogreški.

Post hoc analiza otkrila je razlike između materijala u objema skupinama (netermoformirani i termoformirani). Citotoksičnost netermoformiranog materijala A značajno se razlikovala od one netermoformiranog materijala C pri koncentraciji 3 i nakon inkubacije od 48 sati (p = 0,030). Utvrđena je statistički značajna razlika između materijala B i D u objema ispitivanim skupinama (netermoformirani, termoformirani) pri koncentraciji 1 i nakon inkubacije od 72 sata (p = 0,019 odnosno p = 0,039). U skupini termoformiranih materijala također je uočena značajna razlika između materijala A i D pri koncentraciji c2 i nakon inkubacije od 48 sati (p = 0,039). Razlike između materijala mogu nastati zbog sastava svakog materijala. Materijal A jednoslojni je termoplastični poliuretan (TPU) (22), a materijal C dvoslojni je materijal sastavljen od kopoliestera (PETG) i poliuretana (TPU) (23). Oba materijala B i D troslojni su s dvama slojevima kopoliestera i elastomernim slojem između (24, 25). Ostale komponente materijala mogu biti toksične, a njihovi udjeli u sastavu su zaštićeni, što komplicira tumačenje rezultata. Poznato je da sastav i konformacija utječu na mehanička svojstva termoplastičnih materijala (26), no manji je broj studija usmjeren na njihovu citotoksičnost.

U ovom istraživanjem utvrđena je statistički značajna razlika između različitih koncentracija unutar skupine netermoformiranih materijala. Koncentracija 3 razlikovala se od koncentracije 1 za materijal B nakon inkubacije od 48 sati (p = 0,034), a razlikovala se od koncentracije 2 za materijal A nakon inkubacije od 48 sati (p = 0,021) i za materijal C nakon 72 sata (p = 0,042). Te razlike nastale su zbog prethodno opisane tendencije citotoksičnosti za koncentraciju 3, gdje je vijabilnost stanica nakon 48 sati bila značajno veća od kontrole, a nakon 72 sata pala je ispod 70 %. U ovom slučaju se c3 (30 % volumen/volumen) može smatrati graničnom vrijednošću u testovima citotoksičnosti. Veća razrjeđenja od ovoga dala bi nejasne rezultate zbog mogućnosti da stanice umru zbog nedovoljne količine hranjivih tvari, a ne zbog potencijalnih toksina u testiranim materijalima. Alhendi i suradnici ispitivali su u svojem istraživanju (17) citotoksičnost sličnih termoplastičnih materijala i također otkrili značajne razlike između različitih koncentracija istog materijala. Nađene su razlike između 5 % i 10 % te između 5 % i 20 %

differences between varying concentrations of the same material. Differences were found between 5% and 10% and between 5% and 20% concentrations. There was no difference between 10% and 20% concentration. In their study, the highest dilution (volume/volume) was 20%, while in ours, it was 30%, and they concluded that lower concentrations were less toxic.

Although thermoforming affects several aligner properties (10-13, 15), the results of our study revealed no statistically significant differences in cytotoxicity between non-thermoformed and thermoformed groups (P>0.05). On the other hand, the study by Martina et al. (16) concluded that the cytotoxicity of PET-G materials was higher after thermoforming. In their study, materials were stored in DMEM at 37 °C for 14 days, while we aimed to closely simulate the oral conditions by storing them in artificial saliva under the same conditions. Another difference is that we tested the cytotoxicity of the materials on human oral fibroblast cells using the CCK-8 test. In contrast, their research used the MTT assay and HGF (Human Gingival Fibroblast) cells. It seems that these discrepancies occurred due to differences in materials, testing methods, and experimental conditions used in the two studies.

In addition to the lack of data on the material composition, one limitation of our study is the possibility of potential technical errors during the execution of highly sensitive *in vitro* tests. Furthermore, under laboratory conditions, we could not analyze the impact of digestive enzymes and the chewing process on releasing compounds that may be toxic to oral mucosal cells. *In vivo* tests are needed to overcome these limitations.

Conclusions

Cytotoxicity was assessed for both non-thermoformed and thermoformed materials, with significant differences observed between different concentrations of the same material.

The thermoforming process did not affect the cytotoxicity of different clear aligner materials, used in this study.

koncentracija. Nije bilo razlike između koncentracije od 10 % i 20 %. U njihovoj studiji najveće razrjeđenje (volumen/volumen) bilo je 20 %, a u našemu je bilo 30 %, pa su zaključili da su niže koncentracije manje toksične.

Iako termoformiranje utječe na nekoliko svojstava alignera (10 – 13, 15), rezultati našeg istraživanja nisu otkrili statistički značajne razlike u citotoksičnosti između netermoformiranih i termoformiranih skupina (P > 0,05). S druge strane, u studiji Martine i suradnika (16) zaključili su da je citotoksičnost PET-G materijala veća nakon termoformiranja. U njihovoj studiji materijali su bili pohranjeni u DMEM-u na 37 °C tijekom 14 dana, a naš je cilj bio približe simulirati oralne uvjete pohranjivanjem u umjetnu slinu pod istim uvjetima. Druga je razlika u tomu što smo testirali citotoksičnost materijala na stanicama humanih oralnih fibroblasta s pomoću testa CCK-8. Suprotno tomu, oni su se u svojem istraživanju koristili MTT testom i HGF stanicama (humani gingivalni fibroblasti). Ta odstupanja vjerojatno su posljedica razlika u materijalima, metodama ispitivanja i eksperimentalnim uvjetima korištenima u dvjema studijama.

Uz premalo podataka o sastavu materijala, jedno od ograničenja naše studije jest mogućnost tehničkih pogrešaka tijekom obavljanja visoko osjetljivih testova *in vitro*. Nadalje, u laboratorijskim uvjetima nismo mogli analizirati utjecaj probavnih enzima i procesa žvakanja na otpuštanje spojeva koji mogu biti toksični za stanice oralne sluznice. Da bi se prevladala ta ograničenja, potrebni su testovi *in vivo*.

Zaključci

Citotoksičnost je procijenjena i za netermoformirane i za termoformirane materijale, uz značajne razlike uočene između različitih koncentracija istog materijala.

Proces termoformiranja nije utjecao na citotoksičnost različitih prozirnih materijala za alignere korištenih u ovoj studiji.

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Sažetak

Ciljevi: Procijeniti citotoksičnost različitih termoplastičnih materijala i utjecaj termoformiranja na njihovu citotoksičnost **Materijal i metode:** Četiri termoplastična materijala ispitana su prije i poslije termoformiranja: Zendura A (Bay Materials), Zendura FLX (Bay Materials), Erkoloc-pro (Erkodent) i CA Pro + (Scheu Dental). Uzorci su pohranjeni u umjetnoj slini i 14 dana inkubirani na 37 °C kako bi se simulirali uvjeti u usnoj šupljini tijekom jedne faze tretmana prozirnim alignerom. Zatim je slina razrijeđena punim medijem za kulture u trima koncentracijama: c1 = 10 %, c2 = 20 % i c3 = 30 %. Citotoksična aktivnosta materijala procijenjena je nakon inkubacije od 4, 24, 48 i 72 sata korištenjem CCK-8 testa na stanicama humanih oralnih fibroblasta (Innoprot, REF: P10868). **Rezultati:** Srednje izmjerene vrijednosti metabolički aktivnih stanica ispod 70 % od kontrole bile su za c3 netermoformirane Zendure A i Erkoloc-pro, te termoformirane Zendure A poslije 72-satne inkubacije. Analiza je pokazala razlike između termoformiranih: Zendure A i Erkoloc-pro i Zendure FLX te CA Pro +, termoformiranih: Zendure A i CA Pro +, te Zendure FLX i CA Pro + pod određenim uvjetima.Pronađene su razlike između koncentracija (c2 prema c3 i c1 prema c3) u skupini koja nije termoformirana. Nije bilo statistički značajnih razlika u citotoksičnosti između netermoformiranih i termoformiranih skupina. **Zaključci:** Citotoksičnost je uočena u netermoformiranim i termoformiranim materijalima, sa značajnim razlikama između koncentracija istog materijala. No proces termoformiranja nije utjecao na citotoksičnost prozirnih materijala za alignere.

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