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Cytotoxicity evaluation of different clear aligner materials using MTT analysis

Esra Cifci Ozkan,[*] Gulay Dumanli Gok,[*] Nazli Ece Ordueri[†] and Tugba Elgun[‡]
Biruni University, Faculty of Dentistry, Department of Orthodontics, Istanbul, Turkey[*]
Biruni University, Faculty of Medicine, Department of Histology and Embriology, Istanbul, Turkey[†]
Biruni University, Faculty of Medicine, Department of Medical Biology, Istanbul, Turkey[‡]

Abstract

Backround: The in vitro cytotoxic effects of six different clear aligner materials were evaluated using the MTT analysis.
Methods: The clear aligner material samples [Duran (ScheuDental GmbH, Iserlohn, Germany), Zendura-Flx (Bay Materials LLC, Fremont, CA, USA), Taglus (Laxmi Dental Export Pvt. Ltd, Mumbai, India), Smart Track (Align Technology, San Jose, CA, USA), Zendura (Bay Materials LLC, Fremont, CA, USA), Essix C + (Essix® (Raintree Essix, Inc., 4001 Division St, Metairie, LA-USA)] were initially kept in a saline solution in airtight test tubes for 8 weeks at 37°C. According to the recommended ISO standards, the weights of the samples were divided by the volumes of the dilutions in the ratio of 0.1 g/ml. To evaluate the cytotoxicity of the samples, an MTT analysis was performed using a human gingival fibroblast cell line (HGF). To analyse the data, the Kruskal–Wallis test was applied (α=0.05).
Results: Zendura was the most cytotoxic material resulting in 67.3 ± 16.20% cell viability, followed by Smart Track with 87.6 ± 5.53% cell viability. While Duran, Essix C + had 92.6 ± 26.34% and 94.9 ± 8.54% cell viability, Zendura-Flx, Taglus had 106.9 ± 12.76% and 113.183 ± 7.45% cell viability, respectively.
Conclusion: While Zendura and Smart Track showed mild cytotoxicity, other materials showed greater cell viabilities. According to the ISO standards, the clinical use of each brand of aligners, except Zendura, may be considered reliable. Taking into account standard deviation, Zendura and Duran should be used with caution. The suppliers of aligners should adhere to the manufacturer's recommendations since an increase in ion release might arise from material wear.
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Esra Cifci Ozkan: cifcie@gmail.com; Gulay Dumanli Gok: drgulaydumanli@gmail.com; Nazli Ece Ordueri: nordueri@biruni.edu.tr; Tugba Elgun: telgun@biruni.edu.tr

Backround

Clear aligner treatment is a recent development in orthodontics that has gained worldwide popularity, partly due to company advertising and to patient's perceptions of 'invisible' orthodontics. Clear aligners are not only aesthetic but also comfortable compared to conventional brackets and wires. Although clear aligner treatment is accepted, safety related to biocompatibility remains a controversial issue. Experiments have demonstrated that the cytotoxicity of clear aligners is identical or lower than that of several dental materials including bands and brackets, mini-implants, and bonding agents.[1–3]

The clear aligner material from which an appliance is fabricated plays a crucial role in the treatment outcome. The most-used transparent materials are polymers such as polyethylene (PE), polyethylene terephalate (PET), polyethylene terephalate glycol (PETG), polyurethane (PU) and polypropylene (PP).[4] Changes in the oral environment may modify these polymeric components, causing the release of potentially hazardous chemicals that may affect intraoral cells. To counter the drawbacks of

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clear aligner treatment and upgrade the quality of treatment provided, new materials are being developed and former materials are being improved. It is therefore critical to determine the cytotoxicity of the modern materials against those previously used. Although prior studies have assessed the past material biocompatibility, there is limited information regarding the cytotoxicity of clear aligners used in orthodontics.[5] Therefore, the purpose of this study was to determine the cytocompatibility of currently used and up-to-date brands of clear aligner materials.

Materials and methods

The providers of six different clear aligner materials used in active orthodontic treatment [Duran (ScheuDental GmbH, Iserlohn, Germany), Zendura-Flx (Bay Materials LLC, Fremont, CA, USA), Taglus (Laxmi Dental Export Pvt. Ltd, Mumbai, India) and Smart Track (Align Technology, San Jose, CA, USA), Zendura (Bay Materials LLC, Fremont, CA, USA), Essix C + (Essix® (Raintree Essix, Inc., 4001 Division St, Metairie, LA-USA)] were tested. The aligner brands and their components are indicated in Table I.

The aligner materials were sterilised using the methods recommended by the manufacturer and defined by the International Standards Organization (ISO) 10993-5 norm.[6] The samples were incubated in saline for 8 weeks and stored at 37°C under constant conditions. The weights of the samples were divided by the volumes of the dilutions in a ratio of 0.1 g/ml, as recommended by ISO standards. As a negative control, saline without any clear aligner material was cultured under the same conditions. Samples were stored at –20°C until analysis. A human gingival fibroblast (HGF) cell line (PCS-201-018, ATTC; Manassas, Virginia, USA) was used to evaluate the cytotoxicity of the clear aligner material. The cell line was obtained from the American Cell Culture Collection (ATCC) cell bank.

Fibroblast cells were grown in a T25 cm^2 cell culture flask containing Minimal Essential Medium (DMEM) (Biochrom KG, Berlin, Germany) supplemented with 10% fetal bovine serum (Biochrom AG, Germany), 2 mL glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in a 5% carbon dioxide (CO_2) incubator. The solutions were refreshed every 2 days. At the end of the seventh day, cell growth and cell distribution at the flask base were evaluated using an inverted light microscope. When the primary cultures became viable and the cells completely covered the bottom of the flask, the medium in the culture dish was aspirated. Cellular debris and serum residues in the culture medium were removed by washing the cells with phosphate buffer solution (Invitrogen, California, USA) (pH = 7.0). The flask was shaken after adding 1 ml of trypsin/ethylenediamine tetraacetic acid (EDTA) (0.05% trypsin + 0.02% EDTA, Thermo, Germany) solution. The solution was allowed to permeate each cell and incubated at 37°C (NuAire, Inc. Plymouth, USA) for 5–10 min. Subsequently, the cells were separated from the flask base and new medium containing 10% saline was added into the flask. After mixing, the solution was centrifuged at 15,000 rpm for three minutes (Hettich Zentrifugen, Rotofix 32A, Germany). After the supernatant was aspirated, the cells in the remaining pellet were homogenised in 10% saline and DMEM medium at 37°C. Subsequently, the fibroblasts were seeded into 96-well plates following which the

Table I. Clear aligner brands and their contents.

Brand	Material content	Supplier
Duran	Polyethylene terephalate glycol (PETG)	Scheu-Dental GmbH
Essix C+	Polypropylene/ ethylene copolymer (>95%), stabilizers (<5%)	Raintree Essix, Inc.
Smart Track	Polyurethane from methylene diphenyl diisocyanate and 1,6-hexanedial, Additives	Align Technology, Inc.
Zendura	Polyurethane resin	Bay Materials LLC
Taglus	Polyethylene terephthalate glycol (PETG)	Laxmi Dental Export
Zendura Flx	Copolyester and Polyurethane resin	Bay Materials LLC

culture vessels were checked daily and the media were renewed every 2 days. The passage process was repeated regularly and multiplied in a single layer until a density of 80% was reached. All procedures were carried out in a sterile cabinet (Microtest, Class II-A2, Turkey) to avoid the risk of contamination.

A ratio of 0.1 g/mL between the weight of the clear aligner samples and the volume of the saline dilution was arranged and incubated at 37°C for 48 hr in a 5% CO_2 environment. Control cells were prepared in saline without a plaque eluate. After an incubation period of 72 hr, the MTT method [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma Chemical Co., Milan, Italy) was applied. Each well received 10 µl of MTT solution and incubated for 4 hr at 37°C in a CO_2 oven. In order to dissolve the formazan crystals formed by living cells, 100 µl of sodium dodecyl sulphate solution was added to each well, and left in the CO_2 incubator for a further 16 to 18 hr. At the end of this period, the optical densities of the cells were read in an ELISA device (SPECTROstar® Nano, Germany) at a wavelength of 540 nm. The optical density of the cells cultured in the DMEM medium without any clear aligner material was used as a negative control. Negative control media were attributed to 100% cell viability and used as a reference for determining the level of cytotoxicity. This test was repeated six times for each group.

Using the values obtained from the optical reader and the formula presented by Vande Vannet et al.,[7] the cell proliferation percentages of each test material were calculated by the following formula:

Cell viability (%) = (Optical density of test group/ Optical density of cellular control group)×100

Subsequently, the classification of Ahrari et al.[8] was applied to determine cell viability by defining:

More than 90% cell viability: no cytotoxicity

60 to 90% cell viability: mild cytotoxicity

30% to 59% cell viability: moderate cytotoxicity

Cell viability less than 30%: severe cytotoxicity

Statistical analysis

Descriptive statistics were used to summarise the characteristics of the data set for each material. Differences between the groups were analysed using the Kruskal Wallis test. When statistical significance was found, the post-hoc Dunn–Bonferroni test was applied for pairwise group comparisons. A value of $p<0.05$ was considered statistically significant. Analyses were performed using the IBM SPSS Statistics program for Windows, Version 20.0 (Armonk, NY: IBM Corp).

Results

The cell proliferation values obtained from the MTT analysis for each clear aligner material is shown in Table II and Figure 1. While Duran, Essix C+, Smart Track and Zendura groups had lower than 100% cell proliferation value, Taglus and Zendura-Flx groups' values were higher than 100%.

The highest cytotoxicity was associated with the Zendura material at 67.31% ± 16.20% cell viability and followed by Smart Track with 87.66 ± 5.53% cell viability. While the Zendura and Smart Track material cytotoxicity was labelled as mild, the other materials showed no cytotoxicity.

Discussion

Clear aligner therapy is changing orthodontic practice and continues to grow rapidly due to public demand for a more comfortable and aesthetic method of orthodontic treatment. Although, over the last years, clear aligner materials and technology have advanced to increase the range of achievable tooth movements, there is a scarcity of clinical research to support the biological safety of these devices. Therefore, the present paper assessed the cytotoxicity of the common clear aligner materials.

The cytotoxicity of a material may be evaluated by in vitro and in vivo tests. An in vitro test is provided by cell culture which may be performed by colorimetric, luminimetric or enzymatic assays.[9] MTT analysis is a sensitive, quantitative, calorometric assay working on the basis of a reduction of yellow tetrazolium MTT salt to dark purple formazone facilitated by the succinate dehydrogenase enzyme found in mitochondria. The reduction of MTT to formazone occurs only in active cells in a process used to measure cell viability. It provides fast results which are highly sensitive and detect a very low level of toxicity.[10]

In this study, the cytotoxicity of six different brands of clear aligner material containing media were evaluated on HGFs. HGFs are preferred because

Table II. Cell proliferation obtained from MTT analysis.

Clear aligner	*n*	Mean±SD (Min-Max)	Kruskal Wallis Test Statistic	$**p^a$	$**p^b$	$***p^c$	$**p^d$	$**p^e$
Duran	6	92.625±26.345 (53.15–124.59)	p=0.001*					
Essix C+	6	94.947±8.543 (81.29–103.82)		1.000				
Smart Track	6	87.662±5.535 (78.01–93.03)		1.000	1.000			
Zendura	6	67.31±16.209 (38.13–81.01)		0.397	0.456	1.000		
Taglus	6	113.183±7.459 (104.51–123.77)		0.937	0.827	0.085	0.001	
Zendura Flx	6	106.922±12.765 (83.75–120.35)		1.000	1.000	0.598	0.011	1.000

*p indicates the differences between groups.

**Post-hoc binary group comparisons according to Dunn-Bonferroni test: a—Differences between Duran, b—Differences between Essix C+, c—Differences between Smart Track, d—Differences between Zendura, e—Differences between Taglus.

they constitute the main cell line in oral tissues and are clinically exposed to the potential toxic effects of aligner materials through gingival contact. In addition, HGFs are the most commonly used cell line to assess the biocompatibility of dental materials.[6]

More recently, literature has emerged that offers contradictory findings regarding the cytotoxicity of preferred clear aligner materials. The absence of a cytotoxic effect by Smart Track Invisalign aligner material asserted by Eliades[11] was challenged by Martina et al.[5] who determined a mild cytotoxic effect of Smart Track material by claiming the thermoplastic polyurethane was changed from that assessed by Eliades et al. in 2012.

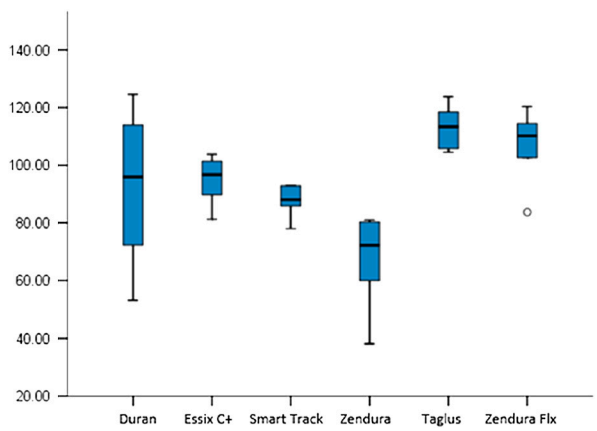


Figure 1. Percentile score distribution of cell proliferation of primary gingival fibroblasts obtained from MTT analysis in clear aligner groups.

Martina et al.[5] evaluated the cytotoxic effects of four different clear aligner materials using MTT analysis and showed a mild cytotoxic effect after 14 days. The highest cytotoxicity was seen in Biolon, followed by Zendura, Smart Track, and Duran materials producing 64.6, 74.4, 78.8, 84.6% cell viability, respectively.[5] Premaraj et al. provided support for the findings and found that exposure to the Invisalign aligner material changed the viability, membrane permeability, and adhesion of epithelial cells in a saline solution environment. In addition, saliva was determined a possible protective agent against cytotoxic activity.[12]

The search for materials with enhanced mechanical and biological properties is a considerable challenge in clear aligner technology. In 1999, Align Technology's first aligners were produced

as a single-layer polyurethane (PU) made from methylene diphenyl diisocyanate and 1,6-hexanediol. Accordingly, other companies have developed further thermoplastic materials for clear aligners in response to increased market demand.[5] In 2004, Zendura produced a new material that was composed of PU.[13] Basically, PU is synthesised by the reaction of polyols with isocyanates at a specific controlled temperature. Biomedical PU elastomers were synthesised by a reaction of 1,6-hexamethylene diisocyanate (HDI) or isophorone diisocyanate (IPDI) and poly diols.[14] While the HDI component is named as a hard segment, the poly diol component is named as a soft segment and the PU shows toxicity against human fibroblast cells because of the presence of aromatic and cycloaliphatic segments in the hard segment structure.[14,15] The cytotoxicity of PU can be changed by the synthesis of new polyurethanes via altering the ratio of the diol monomers.[16] In 2012, Smart Track (Align Technology, San Jose, CA, USA), a novel multi-layer thermoplastic PU, was developed to improve an aligner's flexibility, fracture resistance and transparency.[17,18] Although Zendura and the novel Smart Track materials are basically made of PU, a modification has increased the cytotoxicity of the earlier Smart Track material. This inference is consistent with the present result. While only Zendura and Smart Track showed mild cytotoxic effects, the other materials failed to influence the cells. In addition, Zendura's cytotoxicity was greater than Smart Track. This result was consistent with the study of Martine et al.[5]

In 2014 and in 2018, Duran and Taglus materials containing polyethylene terephthalate glycol (PETG) were introduced.[19,20] PETG is mostly preferred to optimise the mechanical properties of aligners.[21,22] PETG is a transparent thermoplastic that is known to possess the greatest abrasion-resistance, and is the least hygoscopic of most materials commercially available.[23,24] The hygroscopic nature of the polymers may increase the cytotoxic property by causing ion release through material degradation.[25] In this study, PETG-based Duran and Taglus aligners did not show cytotoxic properties which may be due to their reduced hygroscopic properties.

In 2018, Zendura-Flx was formulated and presented in a dual-shell, three layered structure. In this study, while Zendura-Flx showed 106.922 ± 12.76% cell viability, Zendura showed mild cytotoxicity with 67.31 ± 16.20% cell viability. The difference in the structural copolyester between Zendura and Zendura-Flx, has likely positively changed its cytotoxic properties.

This study noted the cytotoxicity of material as Zendura >Smart Track >Duran >Essix C + >Zendura Flx >Taglus. This result is consistent with the study of Martine et al. in reference to Zendura, Smart Track and Duran[5]. The cell proliferation induced by of Zendura-Flx (106.922 ± 12.76%) and Taglus (113.183 ± 7.45%) materials was found to be above 100%. If the control medium had not been changed, these high percentages, which were similar to the control sample, could have been interpreted as mild retardation in control cell growth. However, all of the media were changed at the same time. As a result, cell proliferation was likely linked to the production of chemicals that promoted cell growth. In vitro cytotoxicity experiments for non-cytotoxic alloys or metals have already revealed changes in cell multiplication, cell adhesion, and cell migration.[26] In addition, a note of caution is required since the results were based upon data derived from mean values, and the standard deviation was not considered. If the standard deviations are evaluated in positive and negative directions, while a Zendura appliance can be labelled as a moderately cytotoxic material, Smart Track may be classified as non toxic, and non-toxic Duran and Essix C + may be identified as mildly cytotoxic materials according to the Ahrari classification.[8]

It is noteworthy that FDA regulates medical device safety using the terms "approved" and "cleared", and classifies medical devices into one of three categories. Accordingly, the risk is lowest for class I, moderate for class II, and high for class III devices. For Class III medical devices to be legally marketed, they must undergo a rigorous review and approval process and FDA-approval means that the benefits of the product outweigh the known risks and manufacturers must submit a pre-market approval application. For Class II and Class I materials, the FDA does not give "approval", but provides "clearance".

FDA-cleared means that the manufacturer is able to demonstrate that the product is "substantially equivalent" to another similar legally-marketed device that has previous approval, i.e. a new device is accepted as safe and effective as its predecessor.[27] In addition, FDA recognises ISO-10993-5 Consensus

Standards for biomedical devices and certifies according to ISO biocompatibility tests.[28] ISO 10993-5 states that cell viability of exposed cells should be > 70% to have non-cytotoxic potential by MTT analysis. A 50% extract of the test sample should have at least the same or a higher viability than the 100% extract.

Clear aligner materials, cleared by FDA are labelled as Class II materials.[27,29] According to this study mean value result, all of the materials, except Zendura, provided FDA and ISO standards for clinical usage. However, considering the standard deviation, a Zendura appliance may be classified as safe and a non-toxic Duran appliance may be labelled as cytotoxic according to ISO standard.

A biocompatibility evaluation is a multivariate process that involves in vitro and in vivo tests. In vitro tests assist in understanding the biological reaction to a substance. However, 100% biocompatibility is not determined as a result of validity. Because there is no foolproof way to estimate a biological reaction to a material, agreement regarding clinical usage ultimately must weigh the biological risks against potential benefits. In addition, it should be noted that intraoral aging alters the characteristics of thermoplastic aligner materials which may potentially affect biocompatibility.[30]

Conclusion

In the interpretation of the study results, the standard deviation was taken into account along with the mean and minimum-maximum values. The highest mean/standard deviation ratio (coefficient of variation) was found to be 4.15 and 3.5 in Zendura and Duran group, respectively. These ratios confirm that the distribution of values highly scattered around mean in Duran group and skewed in Zendura group.

This study concluded that Zendura and Smart Track aligner materials showed mild cytotoxicity. When the standard deviations were evaluated in positive and negative directions, a Zendura appliance could be labelled as moderately cytotoxic, Smart Track could be classified as non-toxic, and Duran and Essix C+ could be identitified as mildly cytotoxic materials according to the Ahrari et al. classification. The clinical use of clear aligners with mild cytotoxicity has been accepted and mild cytotoxicity has been reported to be clinically irrelevant.[5] In this case, Zendura should be used with caution.

According to the ISO standard indicating that cell viability of more than 70% is considered safe, the clinical use of each brand of aligners, except Zendura, may be considered acceptable. If the standard deviation is taken into account, Zendura and Duran will cross classification categories. In this case, Zendura could be identified as less cytotoxic or safe and Duran could be classified as slightly cytotoxic.

Consequently, while Zendura-Flx, Smart Track, Essix C + and Taglus may be used safely, Zendura and Duran should be used with caution. As a possible result of the increase in ion release that may occur due to material wear[31], the providers of aligners should follow the manufacturer's instructions.

Conflict of Interest

The authors declare that there is no conflict of interest.

Corresponding author

Esra Esra Cifci Ozkan, Ph.D
Biruni Universitesi, İstanbul, İstanbul TURKEY
Email: cifcie@gmail.com

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