SHORT COMMUNICATION

Cytotoxicity and estrogenicity of Invisalign appliances

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Introduction: Our purpose was to study the in-vitro cytotoxic and estrogenic properties of Invisalign appliances (Align Technology, Santa Clara, Calif). **Methods:** Three sets, each consisting of a maxillary and a mandibular appliance, of as-received aligners were immersed in normal saline solution for 2 months. Samples of eluents were diluted to 3 concentrations (5%, 10%, and 20% vol/vol) and tested for cytotoxicity on human gingival fibroblasts and estrogenicity by measuring their effect on the proliferation of the estrogen-responsive MCF-7 breast cancer cells. All assays were repeated 4 times for each maxillary and mandibular set, and the results were analyzed with 2-way analysis of variance (ANOVA) with appliance and concentration serving as predictors at the .05 level of significance; differences among groups were investigated with the Tukey test. **Results:** There was no evidence of cytotoxicity on human gingival fibroblasts and no stimulation of proliferation of the MCF-7 cell line at any concentration, indicating no estrogenicity of aligner eluents. **Conclusions:** The use of Invisalign appliances did not seem to induce estrogenic effects under the conditions of this experiment. (Am J Orthod Dentofacial Orthop 2009;136:100-3)

The release of bisphenol-A (BPA) from dental polymeric applications has attracted the interest of many investigations over the past decade. Many articles have dealt with the potential estrogenicity of adhesives, composite resins, and polycarbonate products during the last 5 years.[1] The importance of identifying such incidents associated with dental resins is derived from the various effects assigned to BPA.[2-5] Starting in the late 1980s, the search for effects of BPA on the human organism has become a national concern after several publications demonstrated activity at doses lower than the reference dose of 50 μg per weight set by the U.S. Environmental Protection Agency.[6] This figure was calculated by dividing the lowest observed adverse effect level reported in the National Toxicology Program carcinogenesis bioassay (50 mg per kilogram) by an uncertainty factor of 1000, presumably to secure safety for the human organism.

Nonetheless, in the late 1990s, studies reported increased prostate weights and other effects on the male reproductive system in mice exposed to levels of BPA below the safety standard (2 and 20 μg/kg).[7,8] These articles were followed by many studies that found various effects, such as increased mammary gland tumors,[9] precancerous lesions in prostates of neonatally exposed animals,[10] development of hyperglycemia and insulin tolerance,[11] elevation of reactive oxygen species,[12] and oxidative stress.

The resultant turmoil on the hormonal endocrinologic disruptors provoked the investigation of estrogenic action of the full spectrum of polymeric materials used in everyday activities including plastic utensils and biomaterials for medical and dental applications. As a general rule, estrogenic action is confined to molecules with a double benzoic ring and that release BPA, which mimics the action of the female hormone estradiol.

In orthodontics, potential candidates for BPA release include plastic materials and auxiliaries such as adhesives and polycarbonate brackets and aligners. Although no BPA release and no estrogenicity have been reported for light-cured and chemically cured orthodontic adhesives,[13,14] there is no documentation for Invisalign appliances (Align Technology, Santa Clara, Calif). These aligners are placed in the oral cavity for 22 hours per day for approximately 2 weeks to achieve gradual tooth movement.[15,16] Whereas the in-vivo alterations of these appliances[17] and treatment variables[18] have been presented, the potential release of BPA has not been investigated.

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The purpose of this study was to investigate the estrogenicity of aligners by using an in-vitro aging protocol and assay.

MATERIAL AND METHODS

Three as-received sets of aligners, each consisting of a maxillary and a mandibular appliance, obtained from the manufacturer blindly, for the treatment of a malocclusion, were used in this study. Each set of appliances was immersed in normal saline solution in a glass container for 2 months at 37°C. Samples of the eluents were then diluted to 5%, 10%, and 20% vol/vol and processed for testing.

Cytotoxicity assay

Human gingival fibroblast strains were developed from healthy young donors. For the gingival cultures, clot-free tissue pieces were finely minced and placed into culture dishes in Dulbecco's Minimal Essential Medium (Biochrom KG, Berlin, Germany) (DMEM) supplemented with antibiotics, nonessential amino acids, and 15% fetal bovine serum (FBS) (Biochrom KG). After incubation for several days in an environment of 5% carbon dioxide, 85% humidity, and 37°C, fibroblasts migrated out of the tissue pieces and started proliferating. When the primary cultures became confluent, they were designated as in passage 1, and they were subcultured by using trypsin-citrate (0.25%-0.3%) solution at a 1:2 split ratio.[19] From this point on, serial passaging was performed once a week in DMEM with 10% FBS (Biochrom KG). Cells were tested and found to be free of mycoplasma. All cell culture media were from Gibco-BRL (Paisley, United Kingdom).

Cytotoxicity was estimated by a modification of the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma, St Louis, Mo) assay.[15] Briefly, cells were plated in 96-well, flat-bottomed microplates at a density of approximately of 7000 cells per well (25,000 cells/cm^2), in medium containing 10% FBS. Eighteen hours after the plating, the aligner immersion media were added. The maximum immersion media concentration was 20% vol/vol to prevent further culture medium dilution and thus adverse effects on cell physiology. After 48 hours of incubation, the medium was replaced with MTT dissolved at a final concentration of 1 mg per milliliter in serum-free, phenol-red-free DMEM, for a further 4-hour incubation. Then, the MTT formazan was solubilized in isopropanol, and the optical density was measured at a wavelength of 550 nm and a reference wavelength of 690 nm.

Estrogenicity assay

These assays involved 2 cell lines: an estrogen-sensitive (MCF-7) and an estrogen-insensitive (MDA-MB-231 human breast adenocarcinoma). The reason for selecting these was to exclude the possibility that the decreased proliferation of cells induced by the aligner eluent would mask a potential induction of proliferation because of the estrogenic effect.

Cells were cultured in DMEM supplemented with 10% FBS, at 37°C, in 5% carbon dioxide, in a humidified incubator. The cells were regularly subcultured by using trypsin-citrate solution. To evaluate the estrogenicity of the tested materials, the cells were plated in 96-well, flat-bottomed microwells (5000 cells per well) in DMEM and 10% fetal calf serum. Twenty-four hours later, the medium was changed to phenol-free DMEM supplemented with 1% dextran-coated fetal calf serum pretreated with charcoal. After another 24 hours, new medium was added along with the solutions to be tested. Estradiol and BPA (5%, 10%, and 20% vol/vol) were used as positive controls, and normal saline solution was used as the negative control. After 6 days of incubation, with medium renewal at day 3, the medium was changed with a fresh one containing 10% FBS along with [methyl-^{3}H] thymidine (0.2 μ Ci/mL, 25 Ci/mmol) (Amersham, Buckinghamshire, United Kingdom). After incubation for a further 18 hours, the radioactivity incorporated into the DNA was estimated by scintillation counting, after fixation with trichloroacetic acid (10% wt/vol) and solubilization by 0.3 N sodium hydroxide and 1% sodium dodecyl sulfate. All assays were performed in quadruplicate, and the results were averaged.

The statistical analysis of data was performed with 2-way analysis of variance (ANOVA) with appliance and concentration as predictors. Differences were further investigated with the Tukey multiple comparison test at the .05 level of significance.

RESULTS

Figure 1 depicts the results of the cytotoxicity assay, with no effect indicated from the aligner eluents on human gingival fibroblasts at any concentration.

Figure 2 shows the results of the estrogenicity assay; the eluents from the aligners at all concentrations could not stimulate cell proliferation, in contrast to the positive controls BPA and estradiol. In comparison, in the estrogen receptor α-negative MDA-MB-231 cell line, no proliferative or cytotoxic effect was indicated that would mask the potential proliferative action of eluents on MCF-7 cell line. Collectively, the data from both assays indicated no cytotoxicity or estrogenicity in the eluents from these aligners.

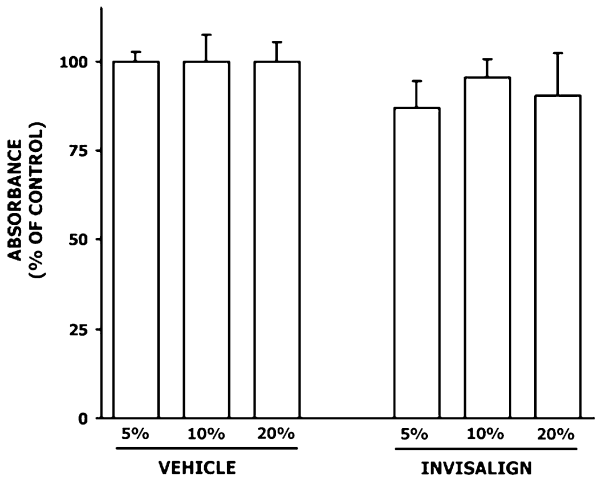

Fig 1. Effect of the Invisalign appliance eluents on the proliferation of human gingival fibroblasts; note absence of effect for aligners at any concentration (no difference from the control vehicle).

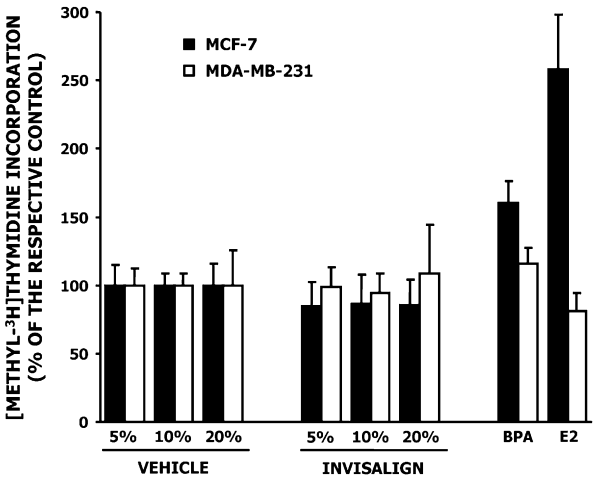

Fig 2. Results of the Invisalign appliance eluents on the proliferation of the estrogen-sensitive MCF-7 and estrogen-insensitive MDA-MB-231 cell lines. No effect is shown to be induced by the aligner eluents. The vehicle is normal saline solution; positive controls are estradiol (E2) and bisphenol-A (BPA). There is no significant difference between the vehicle and the aligner media at any concentration; however, the responsiveness of the MCF-7 cells is depicted in the intense proliferated effect caused by the E2 and BPA. Note also the lack of cytotoxic effect on the MDA-MB-231 cell line, which shows no evidence that could have masked the expression of a proliferative effect on the MCF-7 cells.

DISCUSSION

The results failed to demonstrate measurable biologic effects from aligners. Two reasons might contribute to this effect: the short time frame (although it was longer than in actual clinical conditions) and the stability of the aligners, which are basically polyurethane-derived products. In contrast to the aromatic rings in the configuration of BisGMA, polyether urethanes used as raw materials for aligner manufacturing have short rigid portions (the aromatic rings and the ureas) joined by short flexible "hinges" (the diamine linker and the methylene group between the aromatic ring) and long flexible portions (the polyether).[20]

The estrogenicity in the tested eluents was measured by an established assay, the estimation of the proliferation of the estrogen-responsive cell line. These cells are known to express estrogen receptor-α, which is of primary importance for the proliferative effect of estrogens. The classic method for measuring estrogenic action is the increase of mitotic indexes of rodent epithelia, but this approach might have limited relevance to human organisms.[4]

The basic differences between the study of common toxicants or other hazardous materials and BPA are that natural hormones, such as 17ß-estradiol, induce effects at concentrations much lower than the levels at which all hormone receptors become bound. Once all receptors are occupied, a further increase in natural hormone levels does not cause an increased response. Conventional testing of substances for toxicologic impact involves exposure to levels many times higher than those required for complete receptor binding. Thus, the lack of response to excessively high concentrations of effectors might be misinterpreted as lack of effect.[21] Therefore, in this study, a 20% vol/vol concentration of aligner eluents in the media of cells was considered adequate, since the induction of effects at higher concentrations was excluded.

Previous studies have shown that adhesive extracts can have a mild cytostatic effect.[22] This effect has been described in relevant studies in which a higher concentration of eluent was administered to cells probably because of deprivation of nutrients in the media. Thus, one could hypothesize that the absence of a potential estrogenic action of the eluent in this study could be the result of an antagonistic pattern of action between the proliferative effect of adhesive eluent because of the estrogenic activity and the inhibitory effect from the proven cytostatic effect. To assess this hypothesis, an estrogen-insensitive cell line, MDA-MB-231, was selected as the sham control, and the results excluded the possibility of interaction between these 2 modes of action.

Although aligners are exchanged every 2 weeks, this study included a longer time frame because the aging of the material in the oral cavity is substantially different

from that of in-vitro conditions. The release of BPA has been shown to be increased in alkaline environments and at high temperatures; intraoral conditions might expose the aligners to transient heat shock during consumption of hot liquids. Nonetheless, the identification of BPA release in vivo is not feasible for obvious reasons, where additional potent factors to assist in maximizing the severity of laboratory aging are inappropriate because these media should be then placed in cell cultures. Previous research involving exposure of aligners to aggressive environments such as ethanol showed no measurable leaching.[17]

CONCLUSIONS

No cytotoxic or estrogenic activity of Invisalign appliances was documented in this in-vitro assay, which used a standard model for the assessment of estrogenicity of materials.

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