Original Article

Cytotoxicity of bracket identification dyes

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ABSTRACT

Objectives: To investigate the potential cytotoxicity of the bracket identification dyes commonly used in orthodontic fixed appliances.

Materials and Methods: Six bracket brands representing the market in various aspects were selected. Ten sets of each bracket brand were acquired, and the identification dyes on them were scraped. They were tested for cytotoxicity at three different levels of concentration (2.5 mg/mL, 5 mg/mL, and 10 mg/mL), with the aid of a real-time cell analysis system. The results were compared within and between the groups. One-way analysis of variance and Tukey's post hoc test were used for statistical analysis.

Results: None of the six investigated dyes displayed cytotoxicity at the 2.5 mg/mL concentration. Of the investigated brands, three at 5 mg/mL and four at 10 mg/mL displayed cytotoxicity.

Conclusions: Some of the identification dyes in this study did display cytotoxicity at the higher concentrations tested. Alternative methods for bracket identification should be considered. (*Angle Orthod.* 2019;89:426–431.)

KEY WORDS: Bracket identification dye; Cytotoxicity; Real-time cell analysis

INTRODUCTION

Identification of the brackets is necessary for their placement on the correct teeth. A marking at the disto-gingival tie wings of the brackets is a part of this identification, helping to signal the correct placement orientation, which in turn supplies accurate torque values. Especially in brackets that have nonzero degree torque, this placement is crucial; if the brackets that have positive values are placed backward, the torque will be expressed negatively, and vice versa. In the market, colored dotting is the commonly preferred method for identification. By using different colors for different dental quadrants or teeth, the clinician can determine the required position of the disto-gingival wings.

The dyes used for coloring the dots are not specified by the manufacturers. Particularly because of expanding market conditions, serious questions are being raised about the quality of the dyes. None of the bracket manufacturers recommend removing the color codes after bonding. This presents a logical cause for concern, as the dyes cannot be seen by clinicians in subsequent appointments after bracket placement; they disappear, likely because they have either been swallowed by the patient or spit out during brushing.

In the literature, many different orthodontic materials that could be considered innocent on first assessment, perhaps even more innocent than the dye, have been examined for their toxicity. Orthodontic brackets,[1] bands,[2] elastomeric ligatures,[3] mini-implants,[4] composites,[5] and aligners[6] have been evaluated by researchers, and information has been presented previously. In those papers, the biocompatibility of different orthodontic materials was investigated to determine whether they release ingredients that can be harmful to cells.

Previous studies have stated concerns about the biocompatibility of metallic brackets. In particular, the release of metal ions and the consequent biological reactions that occur have received attention in the literature.[7] Concerns have been especially focused on metal brackets that contain high amounts of nickel. In addition, alternative bracket types, such as nickel-free

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Table 1. Tested Brands and Their Manufacturers

Brand	Manufacturer
Mini Master	American Orthodontics, Sheboygan, Wis
Damon Q	Ormco Corporation, Orange, Calif
MIM	HangzhouXingchen, 3B Dental Instrument & Materials Co Ltd, Hangzhou, China
Mini-Taurus	Rocky Mountain Orthodontics, Denver, Colo
Mini 2000	Ormco Corporation, Orange, Calif
Kirium	3M/Abzil, SãoJosé do Rio Preto, SP, Brazil

or esthetic brackets, have also been studied.[1,8] Ceramic, which is manufactured from alumina, was found to be chemically inert in the oral cavity.[8,9] Another esthetic alternative, polycarbonate brackets, spurred debate because of their bisphenol A content. Bisphenol A is an estrogenic substance, causing early puberty in female subjects.[10] Retamoso et al.[1] showed that polycarbonate brackets were highly cytotoxic to mouse fibroblast cells.

Although orthodontic materials and especially bracket types have been investigated for cytotoxicity for years, bracket identification dyes, which might be presumed to be more toxic than most of the other studied materials, have not been previously examined. The present study, therefore, tested whether the identification dyes used on brackets are toxic to living cells.

MATERIALS AND METHODS

The identification dyes were acquired from six different bracket brands. The tested brands and their manufacturers are listed in Table 1. The dyes of 10 sets of brackets belonging to each brand were scraped, and the total amount of dye collected from each brand was nearly 10 mg. The dye was sterilized using ethylene oxide. Real-time cell analysis tests were performed in cell cultures (L929 fibroblast cell line) with the aid of xCELLigence real-time cell analysis dual purpose (RTCA DP; ACEA Biosciences, Inc, San Diego, CA, USA). The present study was approved by the Ethical Committee of Cumhuriyet University in Sivas, Turkey.

Preparation of the Cell Cultures

The L929 fibroblast cells used for preparing the cultures were provided from The Culture Collection of Animal Cells, Foot and Mouth Disease Institute in Ankara, Turkey. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich Chemie, Germany), supplemented with 1% penicillin/streptomycin (Biochrom, Germany) and 10% fetal bovine serum (Biochrom). The cells were incubated at 37°C in a carbon dioxide (CO_2) incubator equipped with an atmosphere composed of a minimum relative humidity of 95%, and 5% CO_2. When 70% confluence was obtained, the cells were passaged at least twice a week with 0.05% trypsin and ethylenediamine tetraacetic acid. To neutralize the effect of the trypsin, DMEM was added to the cell suspension, and the suspension was shared to the flasks. The operation was carried out in a laminar flow cabinet equipped with ultraviolet light sterilization. After passaging, centrifugation and pipetting were performed until the target concentration of 2.5×10^6 cell/mL was reached. The cells were counted using a hemocytometer.

The xCELLigence RTCA DP system was developed to detect biological analysis processes electronically. It consists of three main parts: (1) the RTCA DP, (2) the RTCA computer and integrated software, and (3) the E-Plate 16 or CIM-Plate 16. According to the manufacturer's statement, the system uses noninvasive electrical impedance monitoring to quantify cell proliferation, morphology change, and attachment quality in a label-free, real-time manner.[11] The system is based on the measurement of the changes in the properties of cells or molecules through sensors. When changes occur in the biological status of the cells or proteins, the system measures these as analog electronic signals and then converts them to digital signals for processing and analysis.[12]

The test and control specimens were prepared according to the manufacturers' instructions. In this study, an E-Plate that had 16 wells with a volume of 250 μL per well was used. Cells were seeded to these wells at a volume of 100 μL. After seeding, the plate was placed in the incubator, and growth was observed at 1-hour intervals with the aid of the electronic impedance of the proliferating cells. Thus, growth rate indices of the cultures were established.

For the same dye, three different concentrations of test specimens (2.5 mg/mL, 5 mg/mL, and 10 mg/mL) were prepared with the aid of artificial saliva as the extraction solution. Then, in the log phase of growth, the cultures determined as the test groups were exposed to bracket identification dyes contained in 10 μL of extraction solution.

In addition to these groups, control groups consisting of the culture medium with the cells were constituted. After the process, the specimens were placed in the incubator again, and measurement continued at 1-hour intervals. Growth proliferation rates (cell indices) were calculated through the data obtained from the xCELLigence RTCA DP system. In addition, for dyes found toxic at the 10 mg/mL concentration, the IC_{50} values, which showed the half maximal inhibitory concentration of a substance in inhibiting biological or biochemical function, were calculated and presented.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Science (SPSS for Windows, version 16.0, SPSS Inc, Chicago, Ill). For all data, descriptive statistics including mean and standard deviation were calculated. Intergroup comparisons were analyzed using one-way analysis of variance. In the event of a difference being detected, Tukey's post hoc test was used to determine in which group the difference occurred. *P* values of less than .05 were considered statistically significant.

RESULTS

Some of the identification dyes obtained from the brackets had toxic effects at different levels on the L929 fibroblast cells. When the cytotoxicity findings of different concentrations of the test materials were compared with the control groups, none of the materials displayed cytotoxicity at the 2.5 mg/mL concentration. However, three of the tested dyes at 5 mg/mL (Kirium, Mini-Taurus, and MIM) and four of the tested dyes at 10 mg/mL (Kirium, Mini-Taurus, MIM, and Mini Master) displayed cytotoxicity (Figure 1A through F).

In the study, the IC_{50} values of the groups that were toxic at the 10 mg/mL concentration were compared. Through this comparison, it was observed that the lowest IC_{50} value, which showed the highest cytotoxicity, was found in the Kirium group, and the highest IC_{50} value, which showed the lowest cytotoxicity, was found in the Mini Master group (Figure 2). There was no statistically significant difference between the Kirium and MIM groups, but statistically significant differences were found between all the other groups ($P < .05$).

DISCUSSION

In the straight-wire technique, brackets are manufactured with different slot geometries for each tooth to bring each of them into the correct spatial position. These attachments, which are specific to the related teeth, are identified both on the box and on the bracket by the manufacturer using various methods. Although there are different methods for identification, colored dots are the most commonly used method among the manufacturers.

The biocompatibility of the marking dyes, which come into contact with tissue fluids and oral mucosa, has been neglected by manufacturers, clinicians, and researchers. During the appointments following after the brackets are attached, dyes are often not visible on the brackets, and it is likely that they are swallowed. Therefore, it is possible that not only the oral tissues but also the entire gastrointestinal tract and whole body through the circulatory system may be exposed to any harmful effects of the dyes. Previously, many studies have examined the tissue biocompatibility of orthodontic materials.[1–10] However, there has been no trial of the dyes used in the colored identification dots.

In previous studies, various testing methods were used to investigate the biocompatibility of orthodontic materials. Kloukos et al.[13] investigated the cytotoxic effects of polycarbonate-based orthodontic brackets by the activation of mitochondrial apoptotic mechanisms. They preferred the cell staining system. Jacoby et al.,[2] who examined orthodontic bands with and without silver solder, used a spectrophotometer to measure cytotoxic outcomes in their study. In a different study that researched the cytotoxicity of the components of orthodontic acrylic materials, real-time cell analysis was used as the evaluation method.[14] These are only a few examples of the many studies that have been conducted in relation to the cytotoxicity of orthodontic materials.

In this study, L929 fibroblast cells were used for preparing the cultures, and the xCELLigence RTCA DP system was used as a real-time cell-monitoring system that allowed for the label-free and dynamic measurement of cell response to cytotoxicants. Cell cultures include cells derived from an animal or plant and a favorable artificial environment for subsequent growth. Relatively well-controlled variables and quantitative results in short time periods make them attractive as a test medium for researchers. The L929 fibroblast cell line is one of the most commonly used cell cultures for biocompatibility essays. The cultivation and maintenance of these cells is simple, and they have a high correlation with specific animal analysis.[15] This model has also been used in many previous orthodontic studies.[3,4,7] Real-time cell-monitoring systems can be used conveniently for a wide variety of cell-based assays in scientific trials.[16] Although it is an in vitro method, it has considerable advantages over conventional animal testing methods and over other in vitro methods. In these methods, subjects are exposed to the material being investigated and are then monitored over a period of time. In addition, cell labeling can be necessary for histological examination. With a real-time cell-analyzing system, the dynamic response of cells to the materials can be evaluated. Therefore, instead of endpoint information, longitudinal data sets, including data from the desired time points, can be obtained by this method. In addition, microelectronic sensors are used and no labeling is required.[16] These major advantages were the basis of the choice to employ this system in this study.

Six different bracket brands were investigated in the present study. In the selection, brackets were chosen

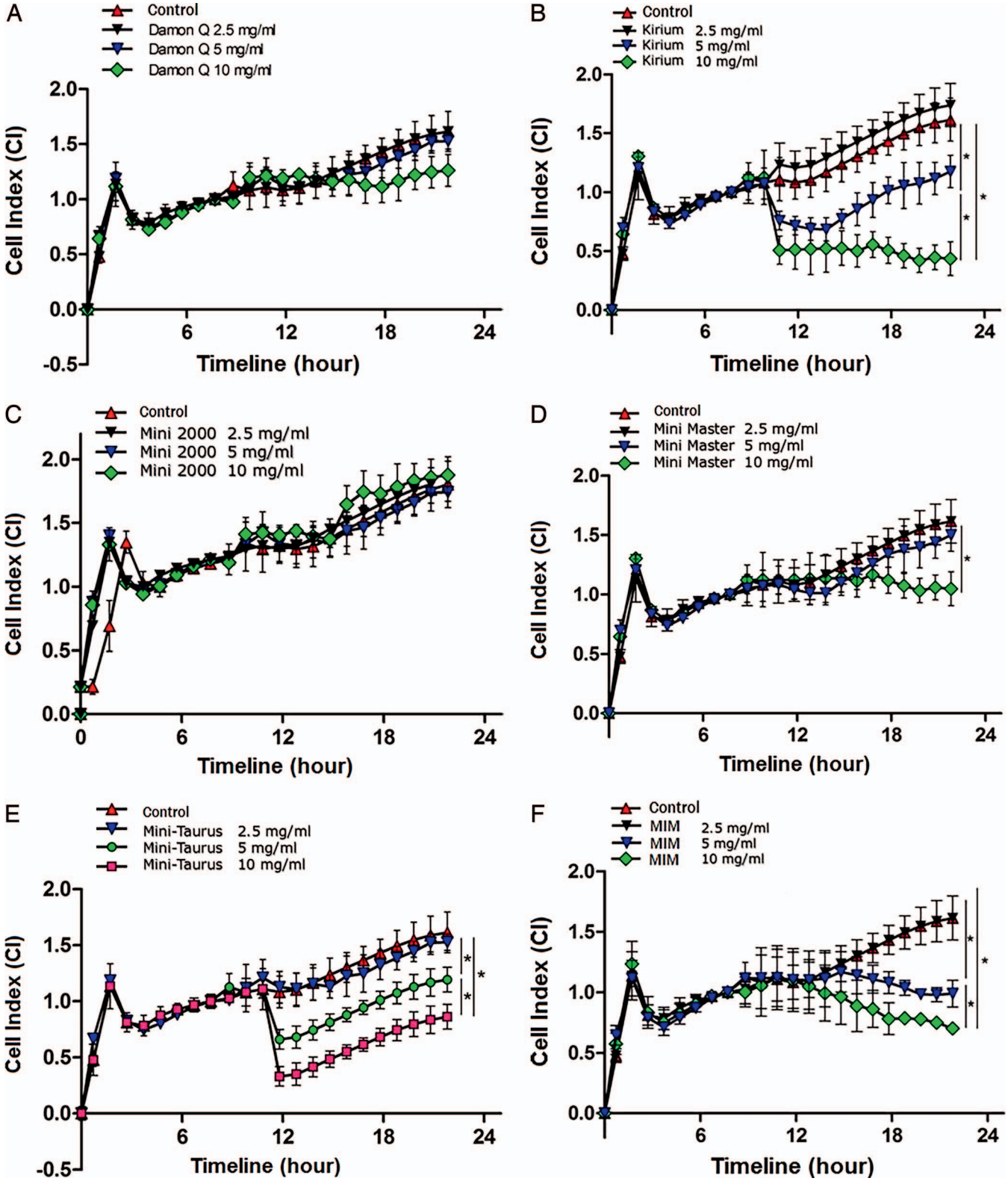

Figure 1. (A) Dynamic monitoring of cell life courses subjected to Damon Q dye. (B) Dynamic monitoring of cell life courses subjected to Kirium dye. (C) Dynamic monitoring of cell life courses subjected to Mini 2000 dye. (D) Dynamic monitoring of cell life courses subjected to Mini Master dye. (E) Dynamic monitoring of cell life courses subjected to Mini-Taurus dye. (F) Dynamic monitoring of cell life courses subjected to MIM dye.

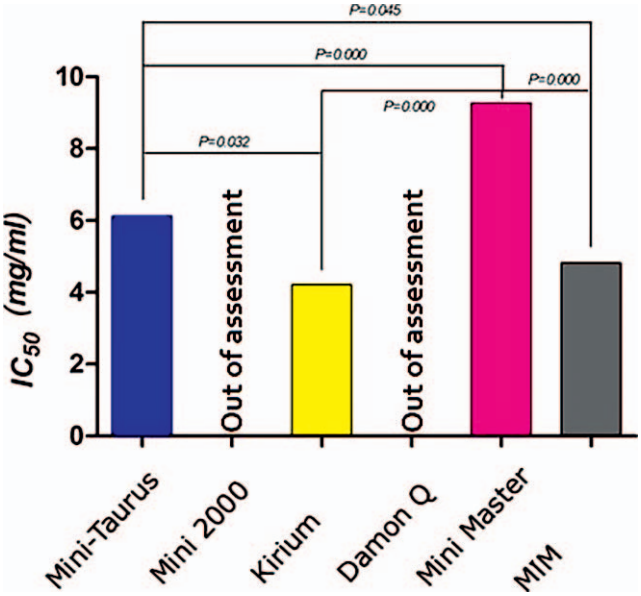

Figure 2. Intergroup comparisons of the dyes that were cytotoxic at the 10 mg/mL concentration.

to reflect the general market conditions. This enabled the evaluation of whether the dyes used in expensive brackets differed from the dyes used in cheaper brackets in terms of cytotoxicity. There was also a self-ligating bracket in the test samples, which is a bracket type of interest among some clinicians.[17] Thus, taking into account both commercial values and popularity, an attempt was made to create a sample group representative of the brackets existing in the market.

The cultures were treated at different concentrations, including 2.5 mg/mL, 5 mg/mL, and 10 mg/mL of dye extract, in a volume of 10 μL of extraction solution. One set of brackets had nearly 1.5 mg of identification paint. Lagerlöf and Dawes[18] stated that the existing amount of saliva, depending on factors such as sex and frequency of swallowing, ranged from 0.60 to 1.19 mL. From this point of view, the tested groups, especially 2.5 mg/mL, simulated actual human exposure. However, in vitro study structures cannot exactly simulate actual in vivo conditions. Creating different concentration groups allowed for a survey of how different dosages affected toxicity for, in the words of Paracelsus, "all things are poison and nothing is without poison. Solely, the dose determines that a thing is not a poison."[19]

Evaluation of the Test Results

Some of the identification dyes obtained from the brackets exhibited toxicity at different levels on L929 fibroblast cells. By comparing the experimental groups formed at different concentrations with the control group, it was determined that cytotoxicity was not observed in any group at a concentration of 2.5 mg/mL. However, different levels of cytotoxicity were observed in some groups at the concentrations of 5 mg/mL and 10 mg/mL. In all toxic groups at 5 mg/mL, cytotoxicity was detected at 10 mg/mL as well. There was no fluctuation related to concentration. The trend was consistently from nontoxic to toxic. In this way, the results were consistent with the concept of "the dose makes the poison."[19]

MIM, Mini-Taurus, and Kirium displayed cytotoxicity at 5 mg/mL and 10 mg/mL; Mini Master was found toxic only at 10 mg/mL. Damon Q and Mini 2000 did not display cytotoxicity at any concentration. The bracket dyes that were not toxic at any concentration, Damon Q and Mini 2000, were produced by the same manufacturer, and it is likely that the same chemical agents were used in the identification dots of the brackets of both brands.

Even though the global economy sometimes shrinks due to recession, it generally experiences consistent growth over time. Production increases every year, and countries are in serious competition to market their products. In this competitive environment, products made in Western countries often get the forefront because of their quality. With the advantage of having access to cheap labor, rapidly developing Asian countries such as China and India often offer products that are lower in terms of quality but cheaper than their Western equivalents. In addition to having access to cheap labor, producers in these countries are trying to compete with their Western competitors by further reducing production costs. This situation, especially in the field of medical supplies, can become a threat to human health through the lowering of the quality of the products.

Although partially consistent with this trend, the present study does present a somewhat different outcome. The dyes scraped from brackets produced by an Asian manufacturer were found to be cytotoxic at concentrations of 5 mg/mL and 10 mg/mL. Similarly, the dyes scraped from the Mini-Taurus and Kirium brackets, produced by Western manufacturers, also showed toxic effects at concentrations of 5 mg/mL and 10 mg/mL. However, the dyes scraped from Damon Q and Mini 2000, produced by another Western manufacturer, did not show cytotoxicity at any concentration. In this regard, evaluations based on the origin of the manufacturer did not give us consistent results. It is believed that these results were caused by the fact that the biocompatibility of identification dyes was simply neglected by manufacturers, much as it has been of little interest to researchers. The results of the present study should be beneficial to manufacturers in terms of attracting attention to this issue.

While using dye is a common method of identification, it is not an innocent technique, as can be seen in the results of this study. Even if the dyes were shown to cause no harm, the dye is still being swallowed by patients without their knowledge or consent. As such, it is clear that alternative methods for identification should be developed, such as identification by laser marker and structural markers, such as an arrow or notch. Structural markers, however, can complicate bracket morphology. Many patients already have difficulty in maintaining oral hygiene because of the presence of the bracket on the teeth; complicating the bracket morphology could increase its volume and potentially worsen the situation, as the increase in retentive area may cause plaque to more easily accumulate over the teeth, and patients could have more difficulty removing it.

It is believed that laser marking is a more appropriate identification method to employ. Since color coding is not possible in this method, it may be difficult to determine which brackets belong to which jaw and quadrant, but laser marking could instead use symbols to indicate jaws and quadrants rather than dots. Thus, brackets can be identified without using dyes that can be harmful to patients and without adding structures to the brackets that could complicate the bracket morphology.

CONCLUSIONS

- Within the limitations of the in vitro study design employed, some of the investigated materials in this study did display cytotoxicity at the higher concentrations tested, making it clear that alternative methods should be considered.
- Among the alternatives, the laser marking method seems most appropriate.

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