Current Research in Dentistry

Research Articles

Biocompatibility of Orthodontic Resins: *In vitro* Evaluation of Monomer Leaching and Cytotoxic Effects

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Abstract: The aim of this study was to investigate the effect of orthodontic resins on cell survival and to evaluate monomer leaching both before and after resin polymerization. Materials and methods: 3T3 mouse fibroblasts were exposed to three cured and uncured orthodontic resins. Cellular viability was assessed by Alamar Blue assay after 24, 48 and 72 h. High Performance Liquid Chromatography was used to measure the amount of monomers released by the tested samples. Data were analyzed by means of ANOVA and Tukey's test ($p<0.05$). All tested materials exerted a cytotoxic response. Cytotoxicity tests showed that the uncured samples were more cytotoxic than the polymerized ones. A time-dependent reduction in cellular viability was found. Monomer release analyses indicated a higher elution of Triethylene Glycol Dimethacrylate (TEGDMA) compared to Bisphenol A Glycidyl Methacrylate. TEGDMA release was higher in the uncured samples and showed a time-dependent pattern. Our results showed the role of resin curing in determining the cytotoxic effect of orthodontic resins and suggested that the differences in the chemical composition of resin matrix appeared to be much more related to the decrease in cell viability than the amount of monomer leaching from orthodontic resins. Clinicians should pay greater attention to resin curing after bracket placement in order to reduce the potentially dangerous effect of monomer release.

Keywords: Biocompatibility, Composite Resins, Orthodontics, Monomer Elution

Introduction

The introduction of the acid etch technique by Buonocore (1955) and the direct bonding bracket by Newman (1992) revolutionized the orthodontic clinical practice using Resin-Based Adhesively bonded Materials (RBDM). Since then, their use has widely increased owing to numerous advantages for patients and clinicians, including shorter bonding sessions, aesthetics, reduction in discomfort and gingival irritation (Jonke *et al*., 2008).

Orthodontic composites and adhesives are made up of two main monomers: Bisphenol A diglycidyl dimethacrylate (Bis-GMA) and Triethylene Glycol Dimethacrylate (TEGDMA). The former is characterized by higher molecular weight, size and hydrophobicity compared to the latter (Geurtsen, 1998).

Incomplete RBDM conversion and/or their degradation in the oral environment causes the release of monomers which may affect adjacent tissues (Jagdish *et al*., 2009; Gioka *et al*., 2005). Light-cured or chemically cured dental composite resins leave a soft, sticky superficial layer upon polymerization, commonly referred to as an Oxygen-Inhibited Layer (OIL) because of its origin (Suh, 2004). In orthodontic clinical practice, this can concern the bonding agents around the bracket base.

The elution process depends on the size, the weight and the chemical composition of the leachable

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molecules. Unlike the bulky basic and large monomers such as Bis-GMA, TEGDMA molecules leach out faster into the aqueous oral environment (Tanaka *et al*., 1991).

Resin dental monomers were found to be cytotoxic and the tests all indicated damages to cell membrane integrity and alteration of cell functions, such as enzyme activities or synthesis of macromolecules (Schweikl *et al*., 1998). *In vitro* studies revealed that TEGDMA caused a dose-dependent mutagenic effect in mammalian cells inducing formation of micronuclei, cell cycle delay and apoptosis via Reactive Oxygen Species (ROS) production (Schweikl *et al*., 2001; Eckhardt *et al*., 2009a). Furthermore Bis-GMA and TEGDMA reduced the levels of the radical scavenger glutathione (GSH), which protects cell structures from damage caused by oxidative stress (Schweikl *et al*., 2006). Depletion of the intracellular GSH pool may increase ROS levels leading to cell death through necrosis or apoptosis (Spagnuolo *et al*., 2013; Heil *et al*., 1996).

TEGDMA monomers may influence specific cell responses of the innate immune system (Eckhardt *et al*., 2009b). Moreover, Bis-GMA resulted positive in the DNA synthesis inhibition test (Yano *et al*., 2011) and direct exposure to high concentrations induced apoptosis (Franz *et al*., 2003).

Several studies investigated the cytotoxic effects of various RDBM used in operative dentistry (Schweikl *et al*., 2005), while there are few data regarding orthodontic resin materials and their biological properties. Recently, the cytotoxicity of orthodontic primers (D'Antò *et al*., 2009) and composites (Malkoc *et al*., 2010) has been demonstrated, but no research focus on the relationship between toxicity and monomers elution rate. Therefore, the purpose of our study was to assess the cytotoxicity of three conventional orthodontic composites, correlating their cytotoxic effects to the analysis of monomer elution before and after polymerization.

Materials and Methods

Cell Cultures

3T3 mouse fibroblasts were grown in a 5% CO_2 atmosphere at 37°C, in Dulbecco's Modified Eagle Medium (DMEM) with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 10 mM), glucose (1.0 g L^{-1}), $NaHCO_3$ (3.7 g L^{-1}), penicillin (100 units mL^{-1}), streptomycin (100 mg mL^{-1}) and 10% fetal calf serum. Reagents and cells were purchased from Lonza (Treviglio, Italy).

Test Materials and Samples Preparation

The orthodontic composites tested in this study were Transbond XT (3M Unitek, Monrovia, USA), Eagle Spectrum (American Orthodontics, Sheboygan, USA) and Greengloo (Ormco Europe, Glendora, USA) (Table 1).

Resin specimens were stratified in uniform-size samples (6 mm in diameter and 2 mm in height) into cell culture inserts (Fig. 1), with 8 μm size pore membrane filters (Corning Costar Transwell cell culture inserts, Corning Incorporated, NY, USA), under aseptic conditions to limit the influence of biologic contamination on the cell culture tests. All composites were analyzed as both cured and uncured specimens. Specimens that required light curing were polymerized with the maximum time recommended by the manufacturer (Table 1) by a LED light curing unit (wavelength range: 430-480 nm; light intensity: Approx. 1000 mW/cm^2; Elipar FreeLight 2, 3M ESPE Dental Products, St Paul, Minn).

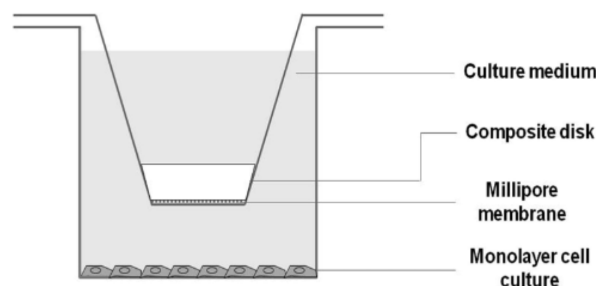

Fig. 1. Schematic illustration of Transwell assay used. Resin specimens were stratified into cell culture inserts, with 8 μm size pore membrane filters, under aseptic conditions to limit the influence of biologic contamination on the cell culture test. Cell monolayers were seeded in the lower chambers of the tissue culture plates. Cell culture with inserts without test materials were used as a negative control

Table 1. Tested orthodontic composites

Material	Manufacturer	Batch number	Suggested curing time (seconds)	Composition
Eagle Spectrum	American Orthodontics, Sheboygan, WI, USA	001-940/001-945	5-10	Bis-GMA, TEGDMA, Ethoxylated bisphenol A dimethacrylate, inorganic filler, camphoroquinone
Greengloo	Ormco Corporation, Orange, CA, USA	740-0320/740-0322	10-30	TEGDMA, Ethyloxylated trimethylopropane triacrylate, Ethoxylated bisphenol A dimethacrylate, inorganic filler, camphoroquinone
Transbond XT	3M Unitek Ortho Prod, Monrovia, CA, USA	712-030/712-036	10-20	Bis-GMA, silane, n-dimethylbenzocaine, phosphorus hexafluoride, inorganic filler, camphoroquinone

Tested materials: Composition of materials (information obtained from the manufacturers). Suggested curing times for each resin is reported according to the manufacturer's recommendation

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Cell Viability

Cytotoxicity of cured and uncured orthodontic composites were evaluated by Alamar Blue (AB) staining (Biosource Internation, Camarillo, CA) in order to measure the influence of tested materials on cell viability (Borra *et al*., 2009). Cells were seeded in the lower chambers of a 24 trans well tissue culture plate (Costar, Corning Incorporated, NY, USA) at 20×10^3 cells/well density in 2 mL of complete medium. Cell culture with inserts without test materials were used as a negative control.

After a 24 h exposure for uncured samples and 24, 48 and 72 h for cured samples, the AB assay was performed according to the manufacturer's protocol. The optical density was measured at wavelength between 540 and 590 nm by using a plate reader spectrophotometer (Sunrise, TECAN, Männedorf, Zurich, Switzerland) and the percent of AB reduction was calculated using the manufacturer formula.

Monomer Leaching Evaluation

High Performance Liquid Chromatography (HPLC) technique was used in our study in order to determine the amount of Bis-GMA and TEGDMA leached from cured and uncured samples.

The same types of cured and uncured specimens used for cell cultures were incubated for 24, 48 and 72 h in 2 mL of DMEM. The media were then centrifuged and filtered through a 0.45 μm syringe filter (Whatman, Maidstone Kent, UK), the residue precipitated in each tube was suspended again with 300 μL of acetonitrile (CH_3CN), then 300 μL of filtered media were added. Lastly, samples were heated to 37°C for 5 min, centrifuged at 15000 g for 5 min and diluted 1:10 in CH_3CN. Samples were then analyzed using a JASCO HPLC system (2 PU-980 pumps, UV-970 UV/VIS detector and AS-1555 auto sampler). The analyses were performed at a wavelength of 214 nm with a C-18 (5 μm) Supelco reversed phase column (250×4.6 mm) using an elution gradient of water (A) and methanol (B) starting from 50 to 30% of A (10 min) and to 15% of A (5 min), 0.7 mL min^{-1} flow, 50 μL injected volume.

The concentration of TEGDMA and Bis-GMA in each sample was quantified using a calibration line performed with standard solutions (Sigma Aldrich, Milan, Italy) before and after each analysis.

Statistical Analysis

Individual values were summarized as means ± SD from independent experiments (n = 4) and the data were analyzed by one-way Analysis Of Variance (ANOVA) followed by Turkey's test for multiple comparisons.

The level of significance was set at $p<0.05$.

Results

Cytotoxicity of Tested Materials

Cytotoxicity of cured and uncured orthodontic composites, evaluated by AB, showed that the percentages of cell viability at 24 h after exposure were lower than in controls: Uncured composites were significantly more cytotoxic than cured (Fig. 2). Greengloo showed the highest percentages of reduction in cell viability, both before and after polymerization, between the tested materials (Fig. 2).

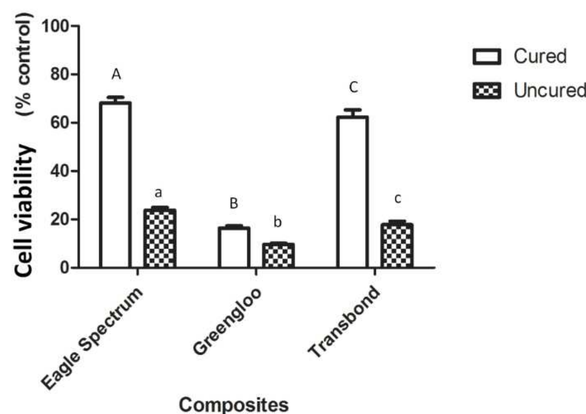

Fig. 2. Cell survival rates after 24 h exposure to cured and uncured orthodontic resins. Results are reported as a percentage of negative controls (mean ± SD). Different case of the same letter indicates significant difference among cured and uncured sample of the same composite. Different capital or small letters indicate difference among cured or uncured composites respectively ($p\leq0.05$).

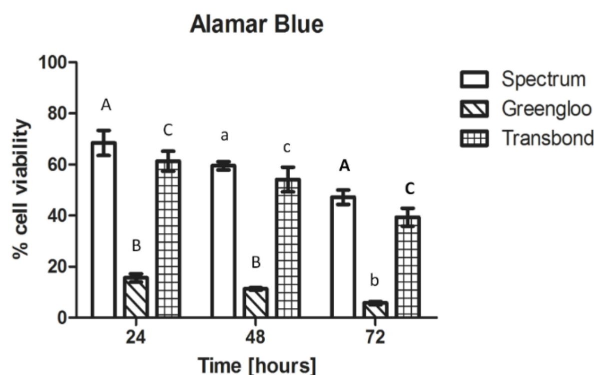

Fig. 3. Cell viability, evaluated after 24, 48 and 72 h exposure to cured orthodontic composites. Results represent means ± SD. Different letters indicate statistically significant difference among resins within the same timepoint. Different style (upper case, lower case, upper bold case) of the same letter indicates statistical significant difference of samples of the same resin between different timepoints

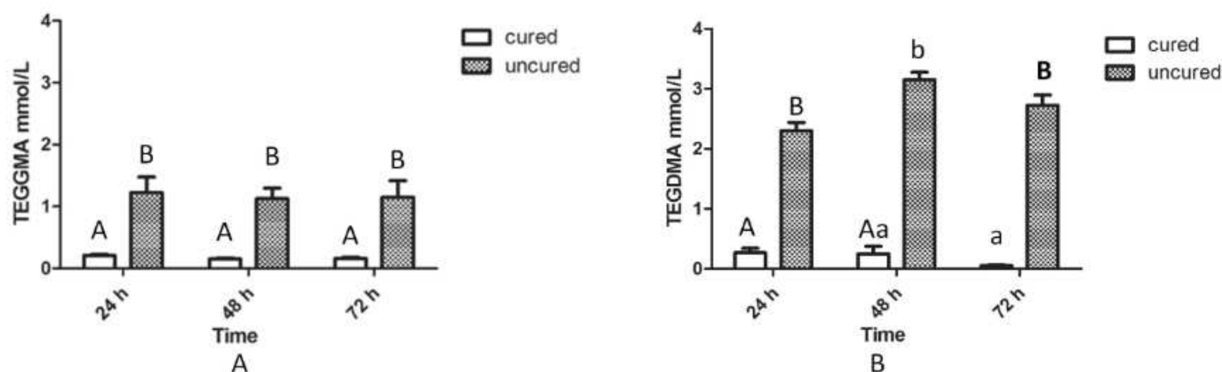

Fig. 4. Cumulative TEGDMA monomer leaching from Eagle Spectrum (A) and Greengloo (B) eluates after 24, 48 and 72 h. Letters shared in common between or among the columns would indicate no significant difference

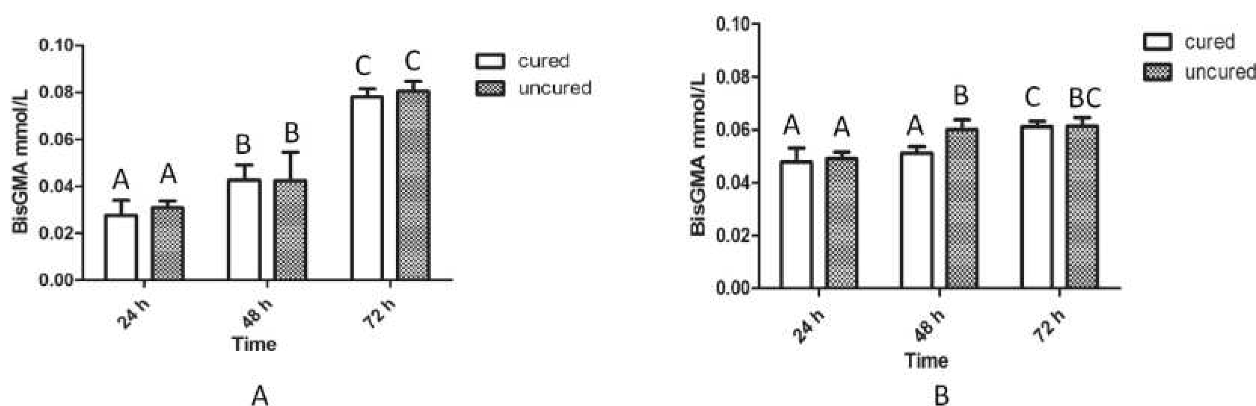

Fig. 5. Cumulative Bis-GMA monomer leaching from Eagle Spectrum (A) and Transbond XT (B) eluates after 24, 48 and 72 h (A) Different letters indicate statistically significant difference in Bis-GMA release between timepoints for the cured and uncured sample respectively. No difference was found between the release in cured and uncured specimen within the same timepoint ($p \leq 0.05$). (B) Letters shared in common between or among the columns would indicate no significant difference ($p \leq 0.05$).

All cured materials showed a significant decrease in cell survival rates compared to the control group and a time dependent cytotoxic effect resulted for all the tested materials (Fig. 3). Greengloo exerted the highest cytotoxic effect at 24, 48 and 72 h between tested resins even if no statistical difference was found between eluates at 24 and 48 h (Fig. 3).

HPLC Results

Bis-GMA signals were found in Transbond XT and Eagle Spectrum while TEGDMA was found in Eagle Spectrum and Greengloo.

TEGDMA elution from uncured samples was largely higher compared to cured ones (Fig. 4A and 4B) while there was no statistically significant difference in Bis-GMA elution between cured and uncured specimens (Fig. 5A and 5B).

TEGDMA release did not result to be time-dependent (Fig. 4A and 4B).

On the other hand, Bis-GMA elution resulted to be time-dependent for Eagle Spectrum (Fig. 5A) but not for Transbond XT (Fig. 5B). The highest Bis-GMA concentration was seen after 72 h for Eagle Spectrum (0.08 mmol L^{-1}) (Fig. 5A).

Discussion

The introduction of RBDM in the orthodontic clinical practice is well established due to their easy handling. To date many studies focused on their physical properties, such as shear bond strength (Finnema *et al*., 2010), with less emphasis on RBDM biocompatibility, despite the proximity of their application site to the periodontal tissues which may cause inflammation of gingiva, oral mucosa and alveolar bone (Borra *et al*., 2009).

Different studies, dealing with RBDM biocompatibility, are based on *in vitro* tests because of the ability to control the environment and cellular

responses (Jagdish *et al*., 2009; Franz *et al*., 2003; Malkoc *et al*., 2010). According to the strategies for material testing, presented by Wataha (2012), the purpose of our study, was to determine *in vitro* material liability, the dynamics of any component release and the potential of released monomers to alter cell metabolism and function. Polymerization and monomer conversion of the RBDM organic matrix is rarely fully completed and, this event seems to be responsible for most of the reported undesirable effects such as cytotoxicity, allergic and inflammatory potential (Goldberg, 2008; Borelli *et al*., 2017).

The effectiveness of a light-curing unit to cure a composite resin material efficiently depends on several factors, such as the wavelength of emitted light, type of photoinitiator, bulb intensity, exposure time, distance and angulation of light tip from the composite surface, type of RBC and shade of the resin composite (Jiménez-Planas *et al*., 2008; Marigo *et al*., 2015).

The light curing unit tip should ideally be in direct contact with the resin composite; however this is rarely clinically possible (Marigo *et al*., 2015; Caldas *et al*., 2003). In our study protocol, the tip was positioned as close as possible to the composite disk and a high intensity LED curing unit was used. The chosen curing time was the longest time suggested by the manufacturer (Table 1) in order to evaluate monomer release in the best curing conditions.

Monomer elution from dental resins mainly occurs through diffusion of the resin matrix and is, consequently, dependent on the size and the chemical composition of the leachable molecules (Gioka *et al*., 2005).

In our study, monomer leaching from cured and uncured orthodontic composites was evaluated through HPLC. The concentration of TEGDMA and Bis-GMA was measured in extracts of the resins, in order to evaluate the monomer release in eluates prepared in the same condition used for cell testing.

TEGDMA monomer was released significantly more from the uncured samples than from the cured ones. Moreover, among the tested materials which contain TEGDMA, Greegloo resulted to have the highest TEGDMA values that might explain its higher toxicity compared to Eagle Spectrum. On the other hand, Bis-GMA monomers, present in the Eagle Spectrum and Transbond XT resins, but not in Greegloo, was in an equally smaller amount from both cured and uncured samples. These results could be explained focusing on the chemical differences between the two mentioned monomers. TEGDMA is a hydrophilic monomer with a low molecular weight, therefore the unbound part of the monomer may be released in high amounts into the oral cavity or in any other aqueous environment (Geurtsen *et al*., 1999), whereas, Bis-GMA is a hydrophobic monomer with an higher molecular weight and, thus, only small amounts are released in the aqueous solution (Tanaka *et al*., 1999). Our results were in line with previous studies that demonstrated that TEGDMA was one of the most eluted monomer from various kind of resin materials (Geurtsen, 1998; Geurtsen *et al*., 1998). Moreover, Bis-GMA showed a time dependent release pattern from Eagle Spectrum but not from Transbond, but, in both cases, the amounts released were so small that they should not be able to affect cytotoxicity.

On the other hand, TEGDMA release was not influenced by time.

Several *in vitro* studies demonstrated that Bis-GMA and TEGDMA caused a dose-dependent mutagenic effect inducing formation of micronuclei, cell cycle delay and apoptosis (Schweikl *et al*., 1998; 2001; Heil *et al*., 1996; Yano *et al*., 2011) and may influence specific cell responses of the innate immune system (Eckhardt *et al*., 2009a; 2009b). Moreover TEGDMA, as well as Bis-GMA, is reported to affect GSH concentration (Spagnuolo *et al*., 2013), a natural radical scavenger, which protects cell structures from damage caused by oxidative stress (Schweikl *et al*., 2006). Depletion of the intracellular GSH pool may increase the intracellular ROS levels leading to cell death through oxidative damage (Spagnuolo *et al*., 2013; Heil *et al*., 1996).

In the present study, we used permanent mouse 3T3 fibroblasts in order to assess the cytotoxicity of orthodontic composites, because they can be easily amplified and are available in large numbers for testing (Koliniotou-Koubia *et al*., 2001), furthermore this cell line is recommended for the *in vitro* evaluation of materials by the International Standards Organization (ISO 7405).

Cell viability was evaluated by Alamar Blue after three different exposure periods (24, 48 and 72 h) because it is a non-toxic dye and this allows it to be used for continuous monitoring of cell viability (Borra *et al*., 2009). Our results showed that all the materials were cytotoxic and that uncured samples caused a major decrease in cell survival compared to cured ones. Tested materials caused different decrease in cell viability and this might be justified by the differences in chemical composition (Table 1). The highest reduction in cell viability was caused by the Greengloo, which was found to release a major amount of TEGDMA compared to Eagle Spectrum, in particular when uncured. Taken together, these observations indicated that TEGDMA might be the main causative factor of the severe cytotoxic effects determined by Greengloo eluates, but our results cannot exclude that different molecules, not included in our investigation, could contribute to the cytotoxic response exerted by this composite. TEGDMA toxicity is mainly related to the highest monomer release in the oral cavity; many studies

demonstrated that Bis-GMA molecules, tasted alone, showed a greater intrinsic toxicity compared to TEGDMA monomers (Schweikl *et al.*, 1998), but it show less toxicity when combined with others molecules and this could be justify the differences in our toxicity result between Grengloo (TEGDMA) and Eagle Spectrum (TEGDMA and Bis-GMA). The causes of this agonistic or antagonistic behavior remain unknown (Schuster *et al.*, 2000). However, the composite tested in which the major monomers was Bis-GMA (Transbond XT and Eagle Spectrum) exerted less toxicity in both experimental conditions (cured and uncured) and this could be related to the small amount of monomer present and released.

Conclusion

- This study illustrated that cured and uncured orthodontic composites can exert a cytotoxic effect. Not all the composites affected cell viability to the same extent
- Resin curing was shown to play a key role in determining cell toxicity: Our result suggested that the differences in chemical composition of resin matrix much more than the amount of monomer leaching from orthodontic resins appeared to be related to the decrease in cell viability in this *in vitro* study
- Practitioners should pay crucial attention to resin polymerization after bracket placement, because extensive monomer release due to inaccurate curing could play a synergic role in affecting RBDM biocompatibility

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Author’s Contributions

All authors read and approved the final manuscript.

Roberto Uomo: Performed cytotoxicity experiments, collected and analyzed data, drafted the manuscript and prepared it for submission.

Gianrico Spagnuolo: Designed the study, analyzed and interpreted data, wrote the paper and critically revised it.

Giuseppina Nocca: Designed and performed HPLC experiments, wrote the paper.

Rosa Valletta, Ambrosina Michelotti and Angela Galeotti: Supervised the project and revised the manuscript critically.

Vincenzo D’Antò: Designed the study, interpreted the data, wrote and critically revised the paper.

Conflict of Interest

The authors disclose any financial, personal or other associations that may influence or be perceived to influence, their work.

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