Effect of tricalcium silicate cements in gene expression of COL1A1, MAPK’s, and NF-κB, and cell adhesion in primary teeth’ pulp fibroblasts

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Tricalcium silicate cements (TSCs) regulate gene expression and cell responses from dental tissues surrounding the repair site. The study aimed to evaluate the gene expression levels of Collagen Type I Alpha 1 Chain (COL1A1), Mitogen-Activated Protein Kinases (MAPK’s), Nuclear Factor-Kappa B (NF-κB), cell adhesion, and morphology of human dental pulp fibroblasts (hDPFs) from primary teeth treated with eluates obtained from Mineral Trioxide Aggregate (MTA) and Biodentine. hDPFs were treated with eluates from Biodentine and MTA (2.5 mg/mL in culture medium). The control group was a culture without the eluates. Gene expressions of COL1A1, MAPK’s, and NF-κB were evaluated using Polymerase Chain Reaction (PCR) and cell adhesion by immunocytochemistry for Vinculin and Integrin β1 expression. Gene expression of MAPK’s and NF-κB in hDPFs with the eluates from MTA and Biodentine showed no significant difference versus the control group (p > 0.05), but COL1A1 exhibited a significant difference (p < 0.05). The expression of COL1A1, MAPK’s, and NF-κB was lower in cultures with MTA and Biodentine eluates regarding the control group, with no significant difference between MTA and Biodentine (p > 0.05). After 72 h of incubation, the hDPFs cultured with MTA and Biodentine eluates showed an elongated morphology: after 7 d, a loss or/and reduction of the cytoplasmic processes, and smaller nuclei were observed. Vinculin and Integrin β1 were expressed in hDPFs treated with MTA and Biodentine eluates. MTA and Biodentine did not inhibit or generate a significant difference in the expression levels of COL1A1, MAPK’s, and NF-κB in hDPFs.

Keywords: Gene expression, Human dental pulp fibroblast, Mineral trioxide aggregate, Biodentine, Cell adhesion

INTRODUCTION

Vital pulp therapy in primary teeth has the main objective of maintaining the integrity of the pulp tissue through the promotion of mineralized tissue and for this purpose, it is necessary to place a biomaterial that provides an adequate seal of the compromised dental tissues. For many years, diverse materials have been used to protect the pulp tissue. Calcium hydroxide (Ca(OH)2) was the first choice material indicated for pulp capping, as well as glass ionomer cements and acetone-or-alcohol-based adhesive systems. Tricalcium silicate cements (TSCs), such as Mineral Trioxide Aggregate (MTA) and Biodentine, have replaced the use of (Ca(OH)2) in clinical applications in permanent teeth; for instance, pulpotomies, direct pulp capping, apexification, apicectomy, root perforation, and furcation repair. Because of TSCs are placed in direct contact with dental tissues, numerous studies have focused on and evaluated the response and behavior of various cell populations. It has been reported that the TSCs induces certain gene expressions, irrespective of
the cell type investigated\(^5-8\). The chemical composition and dose of TSCs are key factors in the regulation of behavior and cellular responses, including adhesion, differentiation, viability, proliferation, tissue formation, deposition, and regeneration\(^9,10\).

MTA is a biocompatible and bioactive material with the capacity to promote the formation of mineralized tissue. Its main components are calcium phosphate, tricalcium silicate, tricalcium aluminate, tricalcium oxide, and silicate oxide\(^11\). It has been reported that MTA promotes mRNA expression of Tumor Necrosis Factor, Interleukin-12, Interleukin-10, and Interleukin-6\(^12-16\). Biodentine is one of the most popular calcium silicate-based bioactive cements and considered as a substitute for the dentin. Several studies have stated that its molecular mechanism on the pulp tissue regeneration includes the induction of gene expression of markers involved in the cell differentiation processes, such as odontoblastic differentiation of human dental pulp stem cells (hDPSCs)\(^15,17\), induction of mRNA expression of alkaline phosphatase, osteocalcin, and bone sialoprotein in human mesenchymal stem cells\(^18\). It also stimulates the expression of angiogenic genes vascular endothelial growth factor and c-fos–induced growth factor\(^19\), induces the up-regulation of osteocalcin, dentin sialophosphoprotein, dentin matrix acidic phosphoprotein, and runt-related transcription factor\(^16,19\).

Holguín conducted a systematic review and reported that MTA attained an efficacy of 97.9% over 86.9% for ferric sulfate. Regarding formocresol, MTA achieved 99% versus 98.3% efficacy\(^20\). MTA is one of the dental materials commonly used in pediatric patients to preserve the function and vitality of temporary teeth. It is also a successful material for apical filling, perforation repair, vital pulp therapy, and apical barrier formation of permanent teeth with necrotic pulps and open root apex\(^11\). Previous studies have reported that the Biodentine is considered a suitable treatment to achieve acceptable therapeutic results when applied on deeply carious primary teeth without degenerative symptoms; so, it is an effective and promising medicament for the pulpotomy procedure\(^22\). Therefore, MTA and Biodentine are two biomaterials that are under continuous clinical and scientific research because of their extensive clinical use\(^23,24\).

There are diverse genes that have essential participation in the physiological processes of repair and mineralization of dental tissues induced by TSCs. They include the Collagen Type I Alpha 1 Chain (COL1A1) that possesses a direct effect on the generation of hard dental tissue and mineralization\(^25\); the Mitogen-Activated Protein Kinases (MAPK’s) and Nuclear Factor-Kappa B (NF-κB), which are transcription factors that activate a cascade of intracellular signals in order to regulate some cellular activities, such as induction of cell growth, proliferation, differentiation, and apoptosis\(^26,27\).

During the repair process, the activation of signaling factors is an indispensable process to initiate a response in which the cellular capacity for repairing the damaged tissue is activated. The cellular response at the site to be repaired involves cell adhesion, guided by the integrin family, which are membrane receptor glycoproteins involved in cell adhesion and other physiological processes, including embryogenesis, hemostasis, and immune response. These proteins disappear when the cells migrate and reappear when they reach the point of destination, where they assemble with other cells to form or regenerate the damaged tissue\(^28\).

Studies have established the gene expression in osteoblasts, odontoblasts, fibroblasts, cementoblasts, osteoclasts, stem cells, and inflammatory cells in response to TSCs\(^5-8\). However, there are only a few studies that have reported the gene expression and cellular response in human dental pulp fibroblasts (hDFPs) when directly interacting with TSCs; hDFPs are the most abundant cells in the pulp tissue. They produce collagen fibers and are responsible for collagen turnover\(^29\).

The main aim of this in vitro study was to evaluate the gene expression of COL1A1, MAPK’s, and NF-κB, and to assess the cell adhesion through the presence of focal contacts of Integrin β1 and Vinculin in hDFPs treated with eluates from MTA and Biodentine. The null hypothesis of this study is that the eluates from MTA and Biodentine neither inhibit the gene expression of COL1A1, MAPK’s, and NF-κB nor the presence of focal contacts of Integrin β1 and Vinculin in hDFPs.

**MATERIALS AND METHOD**

**Cell cultures**

Human dental pulp fibroblasts (hDFPs) were isolated from extracted deciduous teeth of patients aged between 11 and 12 years, after signing a signed informed consent from their parents. The exclusion criteria were molars with deep caries, fractured, and/or with previous endodontic treatment. The study was approved by the Institutional Ethics Committee (CEI-FE-018-018). The whole culture process was based on a previous method by Escobar-Garcia et al.\(^30\). Briefly, the cells were obtained by the explant technique and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; D6046, Sigma-Aldrich, St. Louis, USA) supplemented with 10% fetal bovine serum (A5955, Sigma-Aldrich, St. Louis, USA) and 1% antibiotic solution (A5955, Sigma-Aldrich), at 37 °C in a humidified atmosphere of 95% and under 5% CO\(_2\). The culture medium was changed every 2 d. Once the cell cultures reached a confluence of >80%, the cells were harvested by classical trypsin (0.025%) and ethylenediaminetetraacetic acid (EDTA) (0.02%) treatment for 5 min; posteriorly, the pellet was resuspended in 4 mL DMEM.

**Preparation of eluates from TSCs**

Both cements, MTA (Angelus, Londrina, PR, Brasil) y Biodentine (Septodont, Saint-Maur-des-Fosses, France), were prepared according to the manufacturers’ instructions and were placed under ultraviolet light for 15 min to prevent bacterial contamination. The TSCs were mixed with a supplemented medium (2.5 mg/mL), agitated overnight, and stored at 4 °C for future use. Three mL of DMEM medium supplemented with MTA or Biodentine were added to the hDFPs cultures and maintained under incubation for 7 d at 37 °C, in a humidified atmosphere of 95%, under 5% CO\(_2\). Cell cultures with culture medium but without the TSCs served as the control group. The medium was twice-weekly changed, and the cells were then cultured at 37 °C, under an atmosphere of 95%
oxygen (O₂), and 5% CO₂ until assessment. Cell morphology was evaluated with an optical microscope (Leica, Germany) according to international organization for standardization (ISO) Standard 10993-5 20.

RNA isolation and cDNA synthesis
RNA was isolated from hDPFs cultures exposed to MTA and Biodentine using the Tri Reagent technique (Sigma-Aldrich), according to the manufacturer’s instructions. The concentration of the genetic material was quantified using a NanoDrop (Thermo Scientific FC Multiskan; Thermo Scientific, Vantaa, Finland). Complementary DNA synthesis (cDNA) from 1 mg/mL of RNA was performed using the reverse transcription technique with a long-range reverse transcriptase reagent (QIAGEN GmbH; D-40724 Hilden, Germany) with 2 subsequent incubation periods of 60 min at 37 °C, and then 5 min at 85 °C, the temperature at which transcriptase becomes inactive.

Relative gene expression: Polymerase Chain Reaction (PCR)
Specific amplification of genes involved in the cell cycle was performed separately: Mitogen-Activated Protein Kinases (MAPK’s), Collagen Type I Alpha 1 Chain (COL1A1), and Nuclear Factor-Kappa B (NF-κB). This process employed primers and specific alignment conditions for each gene, as described in Table 1. The PCR reaction was performed on 25 µL of the final volume, which contained: green GoTaq (Promega Co; Madison, WI, USA) Master Mix, 250 ng cDNA, and PRIMERS 0.5 µM. For the design of the primers, the Ampliflex V1.5.4 (Institute of NeuroPhysiopathology UMR 7051, Faculty of medical and Paramedical Sciences, 27 Boulevard Jean Moulin 13385 Marseille Cedex, France) software was used. PCR products were exposed to electrophoresis in 1% agarose gels, 90 volts for 1 h. Finally, these gels were analyzed in a Quantity One BioRad (Bio-Rad Laboratories; Hercules, CA, USA) Photo Register, to obtain the relative values of genetic expression, and compared against the control group; the glycerol 3-phosphate dehydrogenase (GAPDH) gene was used as the housekeeping gene. All the tests were performed in triplicate. Statistical analysis was performed using the Sigma Plot software (v. 11.0) (2479E, Bayashore Rd, Suitr195 Palo Alto, CA 94303 United States). Means were compared by using the one-way analysis of variance (ANOVA), followed by post-hoc Tukey’s comparisons between group means. Alpha value was set at 0.05, as statistically significant.

Immunocytochemical assay
A culture medium was added without the TSCs to allow for cell adhesion; the samples were incubated for 24 h, at 37 °C, 5% CO₂, and 95% humidity. The culture medium was replaced with a new culture medium prepared with the TSCs and cultured for 24 h. The samples were then fixed with 10% neutral formalin and blocked-in phosphate-buffered saline (PBS) with 1% albumin and 0.025% Tween, before contacting with the primary antibody (Integrin β1 mouse monoclonal IgG 2a and vinculin mouse monoclonal IgG1). This incubation procedure was performed overnight. The samples were washed with PBS and placed in contact with the secondary antibody (normal mouse IgG Alexa Fluor 488). Finally, the samples were observed and assessed under the confocal laser scanning microscope (CLSM, Leica, Germany).

RESULTS
Cell morphology
hDPFs of the control group, cultured without eluates from MTA and Biodentine, showed an elongated morphology, typical of a heterogeneous fibroblast with cytoplasmic projections, after 72 h (Fig. 1a) and 7 d (Fig. 1d) of incubation. In the experimental group, hDPFs exhibited some alterations in the cellular morphology (more elongated shape), an increase in cytoplasmic granules, and fewer cytoplasmic projections and cell swelling, considered a typical indicator of cell injury; these findings can be observed in the Figs. 1b (72 h post-incubation) and 1e (7 d post-incubation). hDPFs incubated with MTA eluates after 72 h (Fig. 1c) and 7 d (Fig. 1f) of incubation showed a more elongated spindle-shaped morphology regarding the control group, with fewer shortened cytoplasmic projections and smaller cell nuclei.

COL1A1, NF-κB, and, MAPK’s expression
The expression of COL1A1 and NF-κB was higher in hDPFs of the control group. COL1A1 expression was lower in hDPFs cultures of the experimental group, but with no significant statistical difference between them (p > 0.05). Both eluates (Biodentine and MTA) exhibited a significant difference in the control group (p < 0.05) (Fig. 2a). MAPK’s was higher expressed in the cell cultures with eluates from MTA than in the control group; however, in the medium supplemented with eluates from Biodentine, there was no significant difference between the TSCs and the control group (p > 0.05) (Fig. 2c).

Cell adhesion
Integrin β1 expression in hDPFs was expressed in focal contacts. hDPFs cultured without TSC’s (control group) (Fig. 3a) and those cultured with eluates from Biodentine (Fig. 3b) exhibited a similar expression; focal contacts were present, without causing major alterations. In cell cultures with eluates from MTA, decreased areas of focal contacts could be observed (Fig. 3c). In the control group, the expression of Vinculin was noted along with focal contacts (Fig. 4a), in hDPFs treated with eluates from Biodentine were observed a similar cell response (Fig. 4b) regarding the control group; in hDPFs treated with eluates from MTA, the focal contacts showed higher intensity (Fig. 4c).

DISCUSSION
MTA and Biodentine are frequently used as TSCs in vital pulp therapy of primary teeth; in permanent teeth, they are
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Table 1: Primers and conditions used to evaluate gene expression.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Primer sequence (5´-3´)</th>
<th>Size of PCR product (bp)</th>
<th>Annealing temperature (ºC)</th>
</tr>
</thead>
</table>
| COL1A1      | Fw: GATTCCCTGGACCTAAAGGTGC  
Rv: AGCCTTCCTCATCTTTGCCAGCA | 207                      | 58.1                      |
| MAPK’s      | Fw: TCGCCGAAGCACCATTCAAGTT  
Rv: CGGCTCAAAGGAGTCAAGTGGAT | 539                      | 58.9                      |
| NF-κB       | Fw: CCTTGCACTTGGCAGTGATCACTA  
Rv: ACTTCTGCTCTGACATGGACG | 294                      | 57.8                      |
| GAPDH       | Fw: CCATCAATGACCCCTTCATTGACC  
Rv: TGGTCATGAGTCCTTCCACGAT | 435                      | 62.1                      |

COL1A1: Collagen Type I Alpha 1 Chain, MAPK’s: Mitogen-Activated Protein Kinases, NF-κB: Nuclear factor kappa B, GAPDH: glyceraldehyde 3-phosphate dehydrogenase, PCR: Polymerase Chain Reaction.

Figure 1: Micrographs obtained from optical microscopy (40X) on the morphology of hDPFs after 72 h of incubation. control group (a), with Biodentine eluates (b) and MTA (c). After 7 d of incubation: control group (d), with Biodentine eluates (e) and with MTA eluates (f).

employed to seal communications between root canals and periradicular tissues. When TSCs are in contact with dental tissues, they induce a cellular response involving some physiological processes such as cell proliferation, migration, adhesion, and differentiation; so, it is necessary to evaluate their effects on the behavior of different pulp cell populations to determine more clearly their biological mechanism of action.

In order to explore the influences of MTA and Biodentine on hDPFs, different series of genes and proteins related to cell adhesion were used in this study. Hence, we hypothesized that MTA and Biodentine can also induce the expression of COL1A1, MAPK’s, and NF-κB in hDPFs. The results of this study demonstrated the expression of these genes after 7 d in hDPFs incubated with eluates from MTA and Biodentine. In hDPFs cultures without the eluates from the TSCs (control group), the expression of COL1A1, MAPK’s, and NF-κB was higher. However, a statistically significant difference was only reported in the expression of COL1A1 in the cultures treated with the eluates from both TSCs evaluated in this work regarding the control group. No significant difference could be found in the relative expression of MAPK’s and NF-κB, which means that even in conditions where there is no significant damage by an external agent, hDPFs cultures with the MTA and Biodentine eluates at a concentration of 2.5 mg/mL induce the relative expression of COL1A1, MAPK’s, and NF-κB genes.

The expression of COL1A1, MAPK’s, and NF-κB observed in the present in vitro study is a promising finding because the TSCs evaluated did not inhibit their expression, which means that an affected dental tissue repaired with MTA and Biodentine cements could be able to maintain the expression of these
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Figure 2: Relative gene expression. COL1A1 (a), MAPK’s (b), and NF-κB (c) in hDPFs cell cultures after 7 d of incubation in the culture media with MTA and Biodentine eluates. COL1A1: Collagen Type I Alpha 1 Chain, MAPK’s: Mitogen-Activated Protein Kinases, NF-κB: Nuclear factor kappa B, MTA: Mineral Trioxide Aggregate.

Figure 3: Integrin β1 expression in hDPFs cell cultures. control group (a), and with Biodentine’s eluates from (b) and with MTA’s (c).

Figure 4: Vinculin expression in hDPFs. control group (a), and with Biodentine’s eluates (b) and with MTA’s (c). *p < 0.05 vs. control.

genes. These genes are involved in the maintenance of the pulp vitality for the proper functioning of a primary tooth after invasive pulp therapy. There is a considerable number of studies that have evaluated the biocompatibility, cytotoxicity, and bioactivity of TSCs employing other cell lines; however, only a few works have evaluated their effects and responses on hDPFs. The most predominant cells in the pulp connective tissue are fibroblasts, which give rise to collagen fibers, de-
grade collagen, and are also responsible for collagen changes. Likewise, fibroblasts should be considered as central cells representing the real target in the strategies designed to induce the dentin-pulp regeneration process.

The results of this study demonstrated the expression of COL1A1 in hDPFs cultures treated with eluates from Biodentine and MTA. COL1A1 gene expression provides instructions to hDPFs for becoming part of a large molecule called type I collagen; the collagen secretion by this molecule is a crucial step during the development of new dentin and their synthesis is essential inside the extracellular matrix. COL1A1 is also implicated in the development of enamel, and it increased drastically at 72 h when MTA was applied in hDPFs. This means that MTA causes genetic positive changes in the pulp cells. Diverse studies have demonstrated that Biodentine stimulates similar mineralization markers as well as MTA, and promote the response of mesenchymal stem cells and umbilical vein endothelial cells; however, the osteogenic and angiogenic stimuli were slightly lower than MTA.

The expression of MAPK’s was also studied here. It has been reported that this gene is activated over some cellular functions, such as mitogenesis, proliferation, and differentiation. Different studies have reported that Biodentine is capable of inducing odontoblast differentiation of hDPSCs and involves MAPK’s and the calcium/calmodulin-dependent protein kinase II pathways. In the present in vitro study, Biodentine stimulated the odontoblastic differentiation and the mineralization of created nodules by activating the MAPK’s pathway, as did MTA. Calcium ions released from TSCs activate the MAPK’s signaling pathway and induce the messenger RNA expression of genes associated with mineralization in hDPSCs.

NF-κB is a transcription factor that plays a key role in the regulation of numerous genes with important roles in the cellular responses. Previous studies have proved that MTA can activate the NF-κB pathway in hDPSCs via the up-regulation of inflammatory cytokines. MTA can promote the odontogenic differentiation of stem cells of apical papilla via the NF-κB pathway in vitro. In this study, the qualitative cytotoxicity was evaluated through the detailed visualization of any changes in the cell morphology. Cultures of hDPFs incubated with the eluates from Biodentine and MTA after 72 h showed no significant changes in their morphology; however, after 7 d of incubation, the morphology of hDPFs in the experimental group showed shorter cytoplasmic prolongations with small nuclei. About, the assessment of qualitative cytotoxicity is a highly useful tool since numerous invasive treatments cause cytotoxicity in the cell as measured by enzymatic means. For instance, MTS uses a tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium) for detecting morphological alterations that can alert on possible damages. These damages are identified qualitatively by alterations in the shape, size, or presence of granules and vacuoles in the cytoplasm. The findings are signals of possible negative effects of a biomaterial in contact with pulp cells.

Morphological changes in the cells are indicators of inflammatory processes and apoptotic mechanisms. Cell enlargement usually occurs after the detection of intracellular signals of damage, as well as fragmentation of the genetic material, formation of pores, and rupture of the plasmatic membrane, which release the cytoplasmic content into the extracellular environment. The numerous cytoplasmic granules can be indicative of some degree of cytotoxicity; these granules may be derived from the formation of new vesicles from intracellular organelle debris and exhibit a high immuno-stimulatory potential. However, it is necessary to perform cellular ultrastructural tests to determine more clearly the cellular morphology.

Cell adhesion was determined through focal adhesions (FA), which are dynamically assembled and disassembled by the cells. Vinculin is one of the essential and best characterized FA proteins; it is a membrane-cytoskeleton protein located on FA, involved in the anchoring of integrin molecules to the actin cytoskeleton. Vinculin is a major regulator of cell adhesion; it contributes to the stability of focal adhesion under high forces by regulating the contractile stress generation. Intregin β1 constitutes a group of transmembrane proteins belonging to the family of adhesion molecules. The outcomes in this study demonstrated the expression of Vinculin and Intregin β1 through FAs in hDPFs cultures treated with MTA and Biodentine eluates. The biological effect on cell response of TSCs has been reported as dose-dependent; this means that the cellular behavior is also dose-dependent. In the present study, the cytotoxic effect of Biodentine on hDPSCs was demonstrated to be time- and concentration-dependent. In this regard, it has been reported that the cytotoxicity effect of Biodentine can be attributed to the resultant high pH values, and that the direct contact of Biodentine with the pulp tissue can increase the proliferation, migration, and adhesion of hDPSCs.

Some studies have evaluated the biological properties of TSCs with their corresponding eluates or direct contact in culture media, and the concentrations of the TSCs can vary; so, there is not a standardized protocol for evaluating the effect of TSCs in cell cultures and, therefore, results may vary between studies. This study was based on ISO 10993-5, which is a guide to standardizing the concentrations at which eluates must be prepared. The biological properties of MTA and Biodentine have been discussed; in terms of clinical use, the scientific literature has reported that both materials have proven to be successful in the pulpotomy treatment. Biodentine has also demonstrated both clinical and radiographic success rates of 100% in pulpotomies performed on primary molars with root resorption at 6 and 12 months. According to the outcomes from their systematic review and meta-analysis, Stringhini et al. reported that there is no superiority of MTA versus Biodentine when used as capping materials during the pulpotomy technique in primary teeth. This finding is in accordance with the results from Bani et al., who concluded that there is no significant difference in the clinical and radiographic successes between Biodentine and MTA after 24 months of follow-up.

In this study, only three genes were evaluated. Nevertheless, these genes are key to understanding the physiological
process of repair and mineralization of the dental tissues. Increasing the cell culture time could determine the expression of these genes evaluated in hDPFs cultures when exposed to the TSCs for prolonged periods. Further, in vivo studies would be necessary to provide a better and more accurate understanding of the cell response to the TSCs. The results obtained in this study support that MTA and Biodentine stimulate the expression of diverse functional genes to ensure pulp vitality and induce a regenerative process in damaged dental tissues.

CONCLUSIONS

Eluates from Biodentine and MTA at a concentration of 2.5 mg/mL did not inhibit the expression of \textit{COL1A1, MAPK,} and \textit{NF-κB} genes in hDPFs cultures, after 7 d of incubation, and no significant difference was found in the observed expression of these genes between the eluates from both Biodentine and MTA. Shortened cytoplasmic projections were observed in hDPFs incubated with eluates from Biodentine and MTA after 7 d of incubation, and expression of Integrin/β1 and Vinculin was observed through the FAs.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

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