Cytotoxicity Assessment of Metal and 3D Printed Resin Orthodontic Attachments

An *in vitro* cell culture behavior study

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Materials used in orthodontics are constantly changing and improving, but biocompatibility remains one of their most important characteristics.The purpose of this study is to evaluate the in vitro cellular behavior of dental MSC cells when put in contact with metal and 3D printed resin orthodontic attachments.Cell monolayers were seeded in wells and left to adhere. Polymer and metal orthodontic attachments were placed into the wells. The cells were analyzed on an inverted light microscope. The cellular viability was determined using Alamar Blue assay. Results showed that the morphology and the density of cells are not affected by the presence of either orthodontic material compared to the controls.The metabolic activity of the cells was not altered. Similar cellular reactions,were observed in contact with both materials used in our short term in vitro study.

Keywords : Biocompatibility, 3D printing, orthodontic materials, cytotoxicity

Materials used in orthodontics are constantly changing and improving. In the beginning of the century usage of gold, copper, zinc and vulcanite was widespread. In the present day, biomaterials used in orthodontics are developing at a great pace. After Eliades predicted advancements in elastomeric materials, fiber reinforced composites and plastic bracket manufacturing with new polymer formulation[1], in 2016, Krey et. al was able to prove the concept of implementing an orthodontic treatment plan with individual computer designed and 3D printed resin brackets in the dental clinic [2]. Customisation of an orthodontic resin attachment design and 3D printing provided a good tool for upper bicuspid impaction cases [3].

Biocompatibility is an important characteristic of all material used in orthdontics given that during treatment they come in contact with the patients oral tissues [1]. Research in the field of orthodontic materials cytotocity has been done using a variaty of methods. Grimsdottir et al. evaluated the antibacterial and cytotoxic effect of metal brackets using agar overlay [4]. Baby et al. did a similar study assesing orthodontic stainless steel brackets coated with different phases of photocatalytic titanium oxide [5]. Kloukos et al assessed the biological effects of water eluents from polycarbonate based esthetic orthodontic brackets [6]. Resin composites that set through polymerization are cytotoxic before and immediately after the process [7]. Jonke et al. compared cytotoxicity levels between chemically cured orthodontic adhesive systems and light-cured orthodontic adhesive systems and noticed a diminished cytotoxic effect in the latter [8].

Although physical, chemical and biological testing has been done on many of the orthodontic materials used in the present day, there is no unanimous opinion concerning the allergic reactions due to the use of orthodontic devices and materials [9].

Device fabrication has experienced a revolution when 3D printing technology began to produce high quality appliances. Providing materials suitable for both layered fabrication techniques and use in the dentistry field is a challenge for the dental materials research community [10]. The emergence of new innovative materials like biocompatible 3D printed resins, require further reaserch regarding their behavior when used during orthodontic treatment in contact with the oral tissue an saliva of the patients.

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Experimental part

Materials and methods

In the present study, two kinds of orthodontic lingual button attachments were compared. The metal attachments were standard stainless steel lingual buttons. The 3D printed resin attachments were generated using computer aided design (CAD) software Exocad (exocad GMBH, Germany) and printed using the Form2 (Formlabs Inc., USA) 3D printer. The resin used for producing the lingual button polymer attachments was NextDent C&B (Vertex-Dental, Netherlands), which is a biocompatible class IIa monomer based on acrylic esters. Rinsing of the printed attachments was done twice in an ultrasonic bath filled with an alcohol solution (96%) to remove any excess material. The next step was drying the printed orthodontic attachments. Final polymerization was achieved using a UV-light curing box. The support structures were removed and sharp edges were smoothened.

The metallic and 3D printed resin orthodontic attachments were decontaminated by immersion in 70% ethanol and then sterilized by exposing them for 30 min to UV light. To verify the efficiency of sterilization, the materials were incubated for 96 hours, in sterile, cell culture growth medium RPMI 1640 [Lonza] at 37°C, humidified atmosphere, with 5% CO_2. Every 24 h, the medium was checked under the microscope for possible microbial contamination.

Dental pulp-derived mesenchymal stem cells (dMSC) cells used in this study were kindly provided by the

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laboratory of Prof. Paunescu (OncoGen Center of Cell and Gene Therapy in Cancer,, Timisoara). The cells were cultured in RPMI 1640 medium [Lonza], supplemented with 10% FCS [Sigma] and 1% penicillin-streptomycin solution [Sigma] and maintained at 37°C, in a humidified atmosphere of 5% CO_2. The medium was changed every 3 days. Cells grown to confluence were harvested by trypsinization with Tryple Express [Gibco]. All experiments were performed using cells at passages 7-12.

In order to investigate the effect of the orthodontic attachments on the morphology and proliferation of dental MSCs, cell monolayers were seeded on 24 well-plates at 2 different densities ($1.4x10^4$ and $3x10^4$ cells per well) and left to adhere for 24 hours. Afterwards, the resin and metallic orthodontic attachments were placed into the wells in duplicates. After 24-and 48h incubation intervals, the cells were analyzed under an inverted light microscope [Zeiss Axio Observer]. Additionally, to better discern any changes in the growth patterns of dMSC, the orthodontic attachments were removed and the cells were stained with lipophilic tracer dye SP-$DiOC_{18}$(3) [ThermoFisher], at a final concetration of 5μM, for 5 min at 37°C. The medium was then removed and the cells were washed 2 times with PBS [Sigma]. After the washing procedure, 1 mL of fresh culture medium was added to the wells. Cell morphology was observed under the microscope, using the green fluorescent light filter, at 519 nm wavelength.

The viability of dental MSCs in the presence of polymer and metal orthodontic material was determined using an Alamar Blue assay, in which the metabolic activity of viable cells correlates directly with the reduction of resazurin (blue) dye to resorufin (red). For this assay, orthodontic materials were affixed to the individual wells of a 24-well plate using a 0.2% gelatin solution [Sigma]. All orthodontic materials were assessed in triplicates. The plate was kept for 20 minutes at room temperature, in the sterile cell culture hood, and then for 1 h in the incubator, at 37°C. After the gelatin polymerised, the excess was removed and the plate was dried for 2 h before adding the cells. Confluent flasks of dental MSCs were harvested by trypsinization with TrypleExpress [Gibco], counted with Trypan Blue [Sigma] and seeded in the coated 24-well plate, at a density of $3x10^4$ cells/well, in 1 mL complete growth medium. After 24 h from seeding, the growth medium was replaced with 0.9 mL fresh medium and 0.1 mL Deep Blue Viability Cell dye [Biologend]. For absorbance measurements, 100μL aliquots from each well were transferred to a 96 well plate. Absorbance of the reduced dye was determined at 570 nm and 600 nm wavelengths with TECAN Infinite m200 Pro [Männedorf, Switzerland] microplate reader, after 1 h, 24 hand 48 h of incubation at 37°C.

Statistical Analysis

One-way analysis of variance (ANOVA) test was used to compare the mean differences between the orthodontics materials and the control groups, at different time points. Statistical analysis was performed using GraphPad Prism 7.0 [GraphPad Software, San Diego, USA], with statistical significance set at $P<0.05$.

Results and discussions

Mesenchymal stem cells of dental origin were allowed to adhere for 24 h in the presence of orthodontic materials and then cultivated for an additional 24 h, in order to observe possible changes in the cell morphology and behaviour. Figure 1 shows morphological aspects of the cell culture at 24 h from seeding. We used two different densities of cells ($1.4x10^4$ and $3x10^4$ cells per well) in order to exclude any effect of cell culture overgrowth on the assessment of morphology.

In the presence of metal orthodontic material (Fig. 1B), as well as in the presence of polymer orthodontic material (Fig. 1C), dental MSCs grown at the lower density show good adherence and are well spread on the plate, even in the close proximity of the orthodontic materials. Compared to the control group (fig.1A), the cells have the same fibroblast morphology and similar density. Similarly, in the case of cells grown at the higher confluence, observation of the morphology and the density of cells showed that the cultures were not affected by the presence of the metal orthodontic materials (fig.1E) or the resin material (fig.1F).

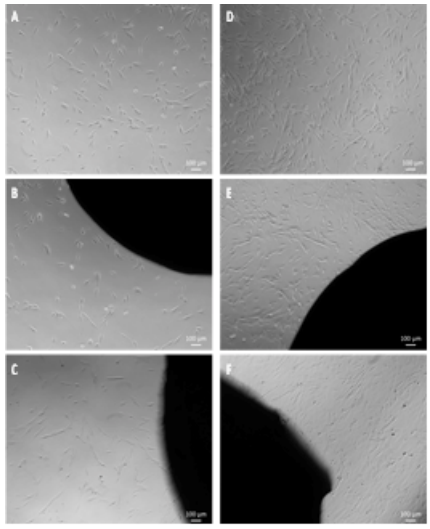

Fig. 1 Dental pulp-derived mesenchymal stem cells after 24 hours from seeding at a density of $1.4x10^4$ cells/well: A control cells, B cells grown in presence of metal orthodontic material, C cells grown in presence of polymer orthodontic material, or at a density of $3x10^4$ cells/well: D control cells, E cells grown in presence of metal orthodontic material, F cells grown in presence of polymer orthodontic material. Optical microscopy, magnification 50x.

Serial microscopic observation of the cell cultures during the 48 h of culture demonstrated that the proliferation and the morphology of cells were not impaired by the presence of the orthodontic materials in both metal and resin groups (fig.2). Close to the orthodontic attachments and also further away from the materials, the aspect of the MSCs was normal and the cell density was similar compared to the control groups. After two days of cultivation, almost no toxicity and good compatibility of the 3D printed resin material was observed (fig. 2F).

To obtain a better image and more details of cell structure, we labeled dental pulp-derived MSCs with lipophilic tracer SP-$DiOC_{18}$. Carbocyanine dyes are weakly fluorescent in aqueous solutions, but highly fluorescent and photostable when incorporated into lipohilic biomolecules, making them ideal for staining the cytoplasmic membranes of cells. Furthermore, labeling does not affect cell viability or basic physiological functions. The sulphonated derivative of DiO, SP-$DiOC_{18}$ is more soluble in culture medium than other lipohilic tracers and emits green fluorescent light when excited. Cell staining facilitated observation of the cytoplasmic membrane and liphophilic molecules important for cell structure and function (fig.3). In all groups, control, metal and polymer orthodontic attachments, cell membranes appear to be intact, with no obvious cell damage or apoptosis.

The Alamar Blue results of cell viability are presented in figure 4. The metabolic activity of cells was determined

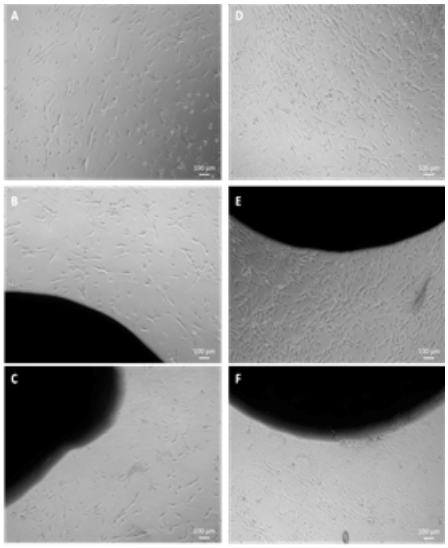

Fig. 2 Dental pulp-derived mesenchymal stem cells after 48 h from seeding at a density of $1.4x10^4$ cells/well: A control cells, B cells grown in presence of metal orthodontic material, C cells grown in presence of polymer orthodontic material, or at a density of $3x10^4$ cells/well: D control cells, E cells grown in presence of metal orthodontic material, F cells grown in presence of polymer orthodontic material. Optical microscopy, magnification 50x

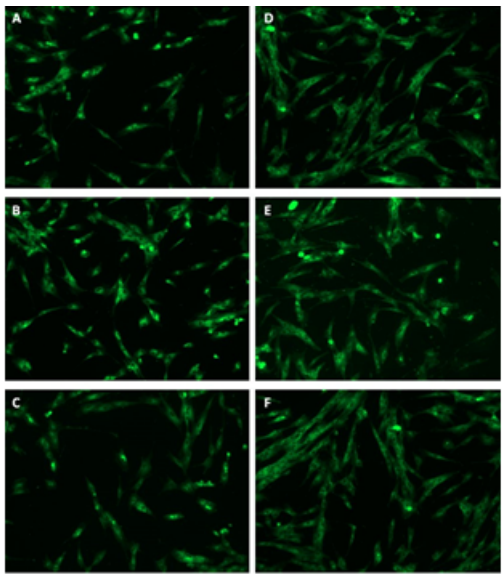

Fig. 3 Dental pulp-derived mesenchymal stem cells labeled with lipophilic tracer SP-$DiOC_{18}$ after 24 hours of contact with orthodontic materials: A control cells, B cells grown in presence of metal orthodontic material, C cells grown in presence of polymer orthodontic material, or at a density of $3x10^4$ cells/well: D control cells, E cells grown in presence of metal orthodontic material, F cells grown in presence of polymer orthodontic material. Fluorescence microscopy, magnification 100x.

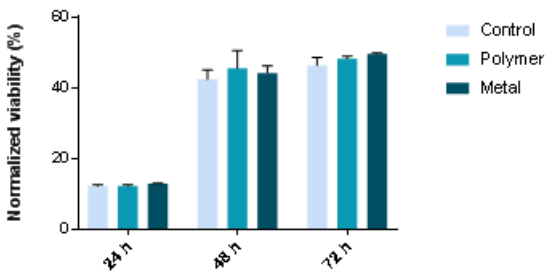

Fig. 4 Viability of dental MSCs cultured alone (control) or in the presence of resin and metal orthodontic materials at 24 h, 48 h or 72 h, as determined using an Alamar Blue reduction assay

after 24 h from seeding the dental MSCs in the presence of orthodontic materials attached to gelatin-coated 24-well plates. After 1 h of incubation with Alamar Blue, the MSC cells culture together with the polymer and metal attachments reduced the dye to resazurin with no significant difference when compared to the control. The cells were not only attached, but had similar metabolic activity with untreated cells. At 48 h and 72 h from co-culture with the orthodontic materials, comparable reduction of the dye showed that treated dental MSC still proliferate at the same rate as the control.

The purpose of this study was to evaluate the initial toxicity of a 3D printed resin material in comparison with standard metal orthodontic attachments routinely used in the orthodontic practice. Orthodontic brackets and archwires have been shown to have a slight citotoxicity in both in vitro and in vivo studies [11,12]. The main reason for this is the ion release from the alloys used in the production of these devices. It has been reported by Huang et al. that ions released from orthodontic archwires immersed in artificial saliva increase with longer immersion periods [13]. Costa et al used corrosion products of two types of stainless steel alloys immersed in artificial saliva to test in vitro effect on cell morphology and metabolism and showed that low nickel stainless steel alloys have a diminished cytotoxic effect [14].Comparison between the metallic and non-metallic materials effect on cell cultures showed results that were either similar in terms of cytotoxicity [15] or showed increased cytotoxicity for non-metallic materials [16].

Our study showed no cytotoxicty for dental pulp mesenchymal cells when the dental pulp MSC cells were grown for 72 hours in the presence of the metal and polymer orthodontic materials . In the Alamar Blue assay the treated cells had the same metabolism rate as the control cells indicating no cytotoxic effect from the materials.

Orthodontic adhesive resin composites have also been of interest when questioning the biocompatibility of orthodontic systems. Cell culture tests are simple, accurate, reliable, and rapid, they detect the agent's effect on isolated cells derived from animal or human tissues [17]. Previous studies used animal models to study monomer release [18]. Unreacted methacrylate monomers are dissolved in the lipid bilayers of cell membranes and can diffuse easily in the cell and cause damage to the cells [19]. We observed the effect of orthodontic materials at the membrane level by staining with a liphophilic tracer. The cells were well adherent with no damage to the cytoplasmic membrane and with an elongated shape like the control cells. The behavior of cells can indicate a proper medium to grow and proliferate. Damage to the cells could have been followed by DNA fragmentation and cell death [20].

Our results show similar behavior of MSC cells in the presence of both metal and resin orthodontic attachments for the fore mentioned time periods, thus providing some validation that, in the future, 3D printable resins could be an alternative to biomaterials used in orthodontics today.

Conclusions

Having similar cellular reactions in our short term *in vitro* study, we could conclude that in the future resins used in 3D printing can be an alternative to materials used in the present for orthodontic components, from a bio-combatibility stand point, but more research is needed regarding both biological and mechanical behavior of these materials .

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