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Manoeuvrability and biocompatibility of endodontic tricalcium silicate-based putties

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ABSTRACT

Objectives: The present study evaluated the indentation depth, storage modulus and biocompatibility of an experimental endodontic putty designed for endodontic perforation repair and direct pulp-capping (NeoPutty). The results were compared with the properties associated with the commercially available EndoSequence BC RRM Putty (ES Putty).

Methods: Indentation depth was measured by a profilometer following indentation with the 1/4 lb Gilmore needle. Elastic modulus was evaluated using a strain-controlled rheometer. The effects of eluents derived from these two putties were examined on the viability and proliferation of human dental pulp stem cells (hDPSCs) and human periodontal ligament fibroblasts (hPDLFs), before (1 st testing cycle) and after complete setting (2nd testing cycle).

Results: The ES Putty became more difficult to ident and acquired a larger storage modulus after exposure to atmospheric moisture. Biocompatibility results indicated that both putties were relatively more cytotoxic than the bioinert Teflon negative control, but much less cytotoxic than the zinc oxide–eugenol cement negative control. NeoPutty was less cytotoxic than ES putty in the 1st testing cycle, particularly with hDPSCs. Both putties exhibited more favourable cytotoxicity profiles after complete setting.

Conclusions: NeoPutty has a better window of maneuverability after exposure to atmospheric moisture. From an *in vitro* cytotoxicity perspective, the NeoPutty may be considered more biocompatible than ES putty.

Clinical significance: The experimental NeoPutty is biocompatible and is capable of reducing the frustration of shortened shelf life when jar-stored endodontic putties are exposed to atmospheric moisture during repeated opening of the lid for clinical retrieval.

1. Introduction

Biomaterials play an integral role in contemporary medicine and dentistry for restoring function and facilitating healing after injury or disease. They may be natural or synthetic and are used to support, enhance or replace damaged tissues or biological functions. As matters or constructs that are designed to interact specifically with living biological systems, biomaterials differ from materials used in other fields of science in that they should promote regeneration or repair without inducing undue inflammation, toxic reactions or allergenic responses in the human body, and at the same time, possess good handling properties [1]. As such, the degree of tolerance of a biomaterial by cellular components in the vicinity of the biomaterial-living tissue interface is as critical as its physicochemical properties [2].

Tricalcium silicate-based hydraulic (water-setting) cements are currently the material of choice for regeneration and repair procedures in endodontics because of their bioactivity. These materials are used clinically for pulp capping, pulpotomy, apexogenesis, apexification,

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perforation repair and root-end filling [3,4]. One of these cements have also been promoted as a dentine replacement "restorative" material [5,6]. The major components of these hydraulic cements are tricalcium silicate and dicalcium silicate [7]. They are biocompatible, bioactive, possess clinically-acceptable sealing properties and have the ability to induce reparative hard tissue formation [8,9]. The original tricalcium silicate-based products, denoted as "mineral trioxide aggregate", suffer from limitations such as suboptimal handling, long setting times (> 2 h), washout during setting, low adhesion to canal wall dentine, relatively high solubility before setting and discolouration of tooth structure over time [10–12]. To overcome the drawbacks of tricalcium silicate-based cements, a range of bioactive endodontic cements have been developed [13,14]. These bioactive cements release calcium, silicon and hydroxyl ions and produce an alkaline pH. The high pH induces precipitation of a layer of carbonated apatite between the cement and dentinal wall and pulp, when these cements are examined in synthetic calcium and phosphate-containing tissue fluid environments [15,16].

Pre-mixed tricalcium silicate-based cements have been introduced since 2010 to overcome the potential drawback of heterogeneous consistency during on-site mixing of conventional tricalcium silicate-based hydraulic cement powders with water [17,18]. These materials require long setting times *in vivo*, but the dental procedure can be finished quickly without washout issue and these cements are bioactive *in vivo*. One of the first commercialised materials was based on tricalcium silicate with monobasic calcium phosphate [19]. Pre-mixed hydraulic materials are available in two forms: a lower viscosity paste for endodontic sealing, and a higher viscosity putty version for direct pulpal contact and other endodontic restorative procedures [18,20–22]. Tricalcium silicate-based premixed putties are marketed in North America as iRoot SP from Innovative BioCeramix, Inc. (Vancouver, Canada). Brasseler USA (Savannah, GA, USA) as EndoSequence BC Root Repair Material Putty (RRM) and EndoSequence BC RRM-Fast Set Putty, and as EdgeSeal (Edge Endo, Albuquerque, New Mexico). These materials have also been marketed as TotalFill BC RPM Putty and Fast Putty by FKG Dentaire (La Chaux-de-Fonds, Switzerland). These sealers and putties do not set in their package but harden *in vivo* in the presence of moisture. The pre-mixed putty version contains zirconium oxide and tantalum oxide as radiopacifying agents. During retrieval from their containers, these materials are exposed to the moisture in the atmosphere [17,23]. Hence, the putty version often has a short shelf-life after opening. It has been speculated that the cause of premature aging of the putties involves reaction with atmospheric moisture after their removal from the sealed foil pouch, moisture permeability of the container or evaporation of the organic carrier. NeoPutty (NuSmile, Houston, TX, USA) is an experimental premixed, bioactive tricalcium silicate-based repair putty that was claimed by the manufacturer to have overcome this problem. The putty contains tantalum oxide as radiopacifying agent and is blended with a non-aqueous, water-miscible carrier to impart good handling properties. Nevertheless, the setting time of this premixed putty is unknown after exposing to atmospheric moisture.

For endodontic procedures such as vital pulp therapy, perforation repair and filling of root-end preparations, the cellular components of the dental pulp and/or periapical tissues are exposed to the capping or repair materials [24]. Tricalcium silicate-containing endodontic putties have also been recommended for direct pulp-capping materials [25,26]. During vital pulp therapy, a capping putty placed directly on, or in close proximity with pulpal tissues, must be non-toxic to pulpal stem cells to expedite reparative dentinogenesis [27]. Likewise, a putty used as a root-end filling material or for perforation repair should promote, or at least not impair the healing process and regeneration of the cementum, and should not cause severe inflammatory reaction in the periapical tissues [28]. Hence, there were two objectives of the present study. The first objective was to evaluate changes in the indention depth and storage modulus of the NeoPutty and a control tricalcium silicate-based putty to determine their window of manoeuvrability after exposure to atmospheric moisture. The second objective was to examine the effects of the experimental putty on the viability and proliferation of human dental pulp stem cells (hDPSCs) and human periodontal ligament fibroblasts (hPDLFs). The null hypotheses tested were: (1) there is no difference in the window of manoeuvrability between the experimental putty and the control putty; and (2) there are no differences in the various facets of cytotoxicity induced by the two putties when they are placed in close proximity with hDPSCs and hPDLFs.

2. Materials and methods

2.1. Indentation testing

Indentation tests are used for analysing the setting characteristics of hydraulic cements. These tests evaluate the point when the setting material has sufficient resistance to indentation from a standardised weight. The International Organisation for Standardisation methods currently employed for evaluating the setting time of tricalcium silicate cements, namely, ISO 6876 and 9917.1, are designed for testing root canal sealers and dental restorative materials, respectively [29,30].

Two tricalcium silicate-based endodontic putties were tested: NeoPutty and EndoSequence BC RRM Putty (ES Putty). The compositions of these putties are shown in Table 1. Indentation depths were measured by a non-contact optical profilometer (VR-5000, Nanovea, CA, USA) (Fig. 1A). Each putty was dispensed and placed in polytetrafluoroethylene (Teflon) moulds (5-mm diameter and 2-mm thick; Fig. 1B). Three specimens of each putty were prepared. The specimens were exposed to ambient air at 43% relative humidity. Each specimen was indented using a 1/4 lb Gilmore needle for determining the indentation depth. The Gilmore needle was coated with water-clear silicone fluid with a viscosity of 200 cP (Dow Corning 200 Fluid, Dow Corning Corp., Midland, MI, USA) to prevent the needle from sticking to the unset putty (Fig. 1C). The indentation depth was measured immediately with the optical profilometer. Indentations of two putties were made daily for 30 days. Data were expressed as percentage reduction of the original indentation depth. The experiment was conducted in triplicate.

2.2. Rheological testing

A method for measuring the maneuverability of hydraulic putties exposed to the atmosphere during material retrieval is to utilise rheometric measurement methods that are used commercially for characterization of creams, pastes and semi-solids. Dynamic viscoelasticity parameters such as storage modulus, loss modulus and tangent delta are of particular interest, because they measure the resistance of a viscoelastic material such as hydraulic putty or sealer to elastic (temporary) deformation under an oscillating applied force (Fig. 2B). By tracking the changes in these properties over time, one can document the change in a material from a gel-like state (with less resistance) to a solid-like state (with greater resistance). The elastic modulus asymptotically approaches a plateau at long time periods when the material is fully set.

A rheometer (MCR 302, Anton-Paar Graz, Austria) was used for controlled strain rate rheology testing in the dynamic mode. The storage modulus and loss modulus of the hydraulic putties were recorded at controlled temperature (25 °C) and ambient air at 43% relative humidity. Rheological measurements were conducted using 4 cycles. A

Table 1
Composition of the putties examined in the present study.

Material	Manufacturer	Components
EndoSequence BC RRM	Brasseler USA Dental Ltd.	Tricalcium and dicalcium silicate, calcium sulfate, tantalite, zirconia and proprietary organic liquid
NeoPutty	NuSmile Ltd.	Tricalcium and dicalcium silicate and aluminate, tantalite, proprietary organic liquid and stabilizers

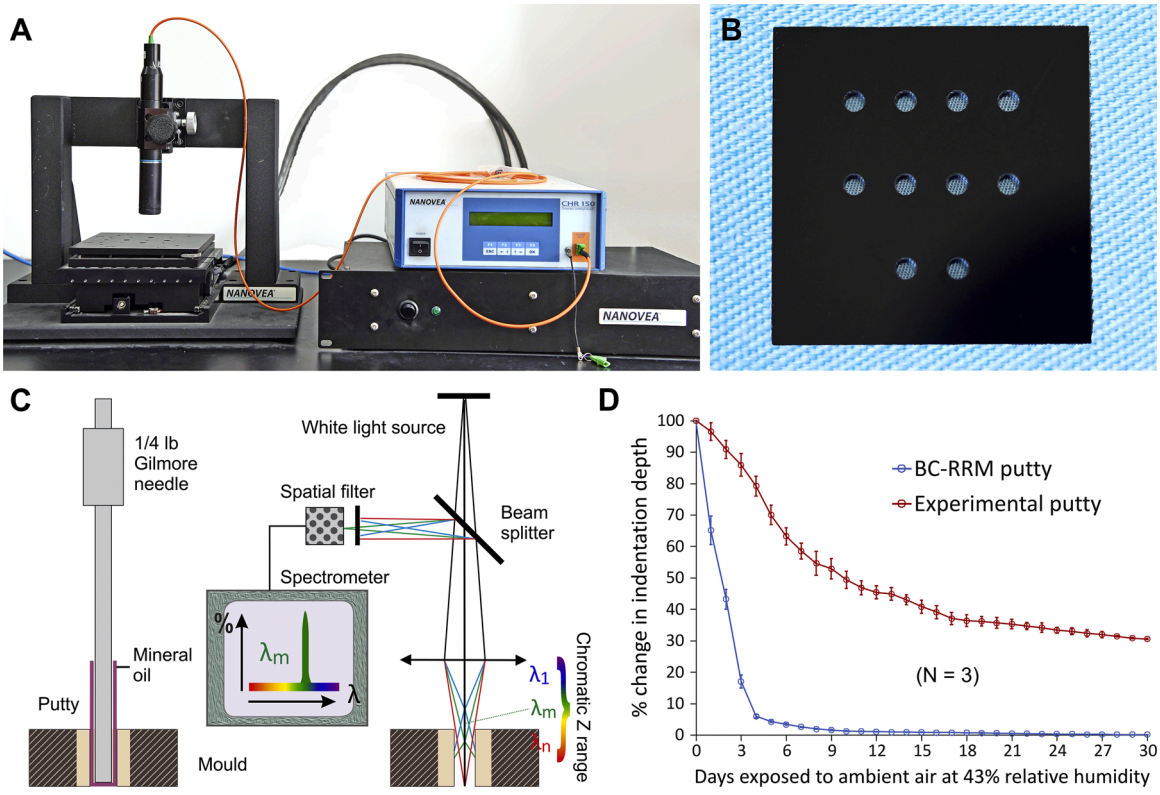

Fig. 1. Indentation testing of endodontic putties. **A**. Nanovea profilometer. **B**: Teflon mould used for confinement of putties for indentation testing. **C**. Schematic of the set-up of the 1/4 lb Gilmore needle and measurement of indentation depth by the optical profilometer. **D**: Changes in indentation depth of the EndoSequence BC-RRM putty and the experimental NeoPutty. Both putties were exposed to ambient air at 43% relative humidity for 30 days. The profilometer measured the indentation of the Gilmore needle. Data are means and standard deviations (N = 3).

freshly opened jar of each putty was used initially. Each cycle consisted of exposing the putty from the jar to ambient air at 37 °C for 2 min. A small amount of putty was retrieved from each jar and the lid of the jar was closed immediately and re-opened only at the next cycle. The retrieved putty was divided into 3 portions for rheological testing (N = 3). Each putty portion was placed between two parallel plates of the rheometer. The lower plate was fixed and maintained at a temperature of 25 °C. The rheometer was operated in an oscillatory mode with an oscillation frequency of 0.159 Hz and an applied strain of 0.01% (determined from a pilot experiment). Changes in the maximum storage modulus of the putties after 1800 s of dynamic oscillatory shearing in the four cycles were used for comparison.

2.3. Specimen preparation for biocompatibility testing

NeoPutty and ES Putty were dispensed and placed in pre-sterilised polytetrafluoroethylene (Teflon) moulds (5-mm diameter and 2-mm thick, milled from 10 mm x 10 mm Teflon blocks). A sterilised Mylar strip was placed at the bottom of each mould. Teflon moulds of the same dimensions were used as the negative control. Other Teflon moulds were filled with a zinc oxide–eugenol cement (Intermediate Restorative Material (IRM); Dentsply Sirona, York, PA, USA) as the positive control. The specimens were placed in hDPSC or hPDLF culture medium to collect eluents using a cyclic aging protocol that took into consideration elution of toxic components from the set cements.

2.4. Cell culture

Cryopreserved hDPSCs (Poietics™, Lonza, Walkersville, MD, USA) and hPDLFs (Clonetics™, Lonza) were used for cytotoxicity evaluation. The hDPSCs are reported by the supplier to express Cluster of Differentiation (CD) cell-surface molecular markers CD105, CD166, CD29, CD90 and CD73, but not CD34, CD45 and CD133. The hDPSCs were cultured in DPSC Dental Pulp Stem Cell BulletKit™ Medium (PT-3005; Lonza) in 5% CO_2 at 37 °C. The hPDLFs were cultured in SCGM™ BulletKit™ medium (CC-3205; Lonza) in 5% CO_2 at 37 °C. The hDPSCs and hPDLFs from the second to third passages were used for subsequent experiments.

2.5. Cyclic testing

Cytotoxic components can diffuse out of set hydraulic cements. Accordingly, a cyclic protocol was used to evaluate the effects of putties on the viability and proliferation of the hDPSCs and hPDLFs. After placing each putty into the Teflon mould, the assembly was added to the respective culture medium (1 disk/2 mL) and incubated at 37 °C for 24 h. This testing condition simulated elution of toxic components from the putty after its immediate placement into a root-end cavity or pulpotomy prior to setting. After incubation, the eluent-containing culture medium was filtered through cellulose acetate filters with 0.22 μm diameter pore size (MilliporeSigma, St. Louis, MO, USA). The filtered culture medium was sterilised with ultraviolet light for 4 h and used for the cytotoxicity testing described in the subsequent sections.

After the first testing cycle, the Teflon moulds with set putties were retrieved and re-immersed in hDPSC or hPDLF culture medium (1 disk/2 mL) for 4 days to enable potentially toxic components to leach out of the materials. This testing condition simulated continued diffusion of materials from the corresponding putty after setting. Filtered eluent-containing culture media derived from the set putties were used for testing during the 2nd cycle.

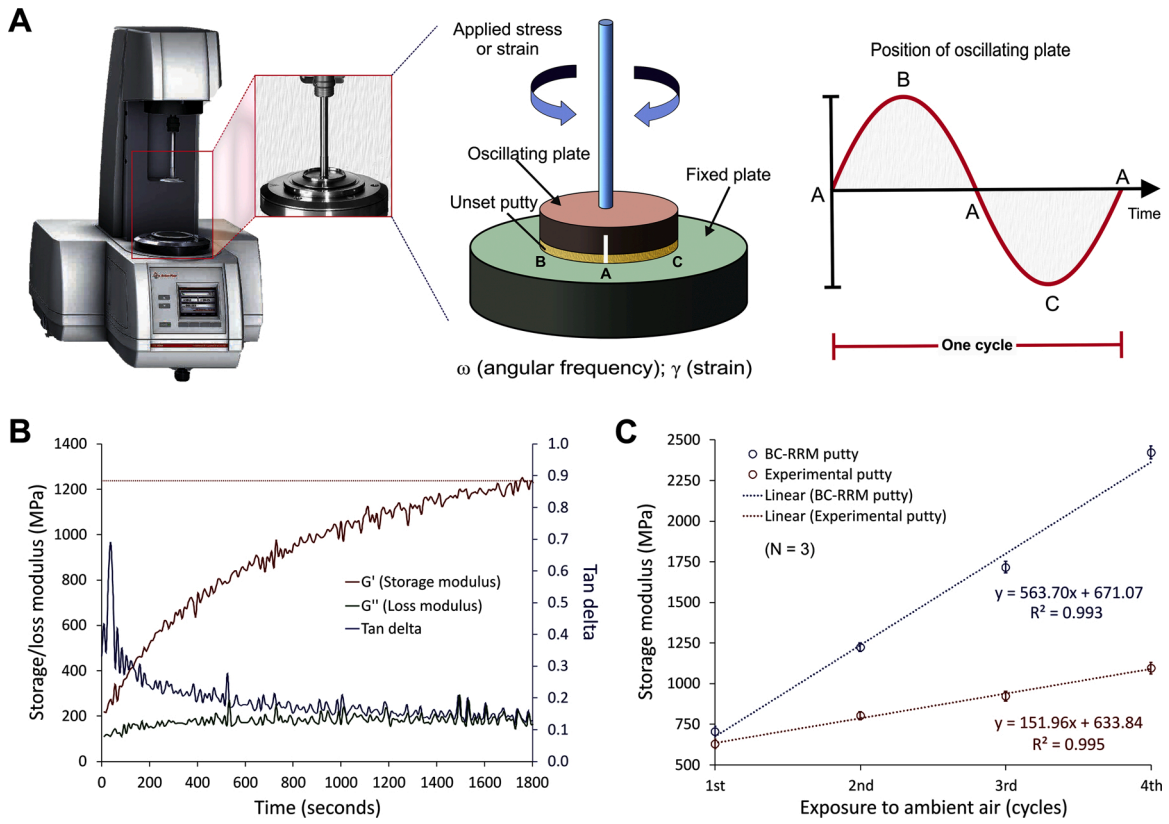

Fig. 2. Rheology testing. **A**. *Left*: Example of a rotational rheometer. *Middle*: Schematic of the rheometry setup, with the specimen to be tested placed between two parallel plates. Right: schematic of the position of upper oscillating plate. **B**. A representative example of the actual dynamic mechanical properties (storage modulus (G'), viscous modulus (G'') and their ratio (tan delta) recorded for one of the putties during the 1800-sec rotational period. The dotted line on the storage modulus curve represents the maximum storage modulus used for comparison. **C**. Maximum storage modulus of the EndoSequence BC-RRM putty and the experimental NeoPutty recorded at each testing cycle. Data are means and standard deviations (N = 3).

2.6. Cell viability

2.6.1. Membrane integrity

Plasma membrane permeabilisation is a late process in the cascade of events that commences from the delivery of a lethal stimulus to cell death [31]. Flow cytometry was used in conjunction with a differential staining technique to evaluate changes in plasma membrane permeability caused by cytotoxic components eluted from the set putties. This procedure sorted and counted the individual cells within a cohort of hDPSCs or hPDLFs. For each cycle, the hDPSCs and hPDLFs were plated in 6-well plates at 10^5 cells/cm^2. The cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere until 70–80% confluency was achieved. The eluent-containing culture medium derived from the 1st or 2nd cycle was used to replace the original culture medium. The established hDPSCs or hPDLFs were exposed to these elution media for 3 days. The exposure cells were centrifuged to discard the supernatant and re-suspended at 2×10^6 cells/mL in the binding buffer included in the Apoptosis and Necrosis Quantification Kit (Biotium Inc., Hayward, CA, USA). The cells were stained with FITC-Annexin V (AnV; $\lambda_{abs}/\lambda_{em} = 492/514$ nm) and ethidium homodimer III (Etd-III; $\lambda_{abs}/\lambda_{em} = 528/617$ nm) and incubated for 15 min at room temperature in the dark. During apoptosis, phosphatidylserine is translocated from the inner to the outer surface of the cell membrane for phagocytic cell recognition [32]. Human anti-coagulant Annexin V is a 35 kDa, Ca^{2+}-dependent phospholipid-binding protein with a high affinity for phosphatidylserine. Annexin V labelled with fluorescein (FITC) identifies apoptotic cells by binding to phosphatidylserine exposed on the outer leaflet of the cytoplasmic membrane, which results in the expression of green fluorescence within the cytoplasm. Necrosis is usually caused by a severe cellular insult, resulting in the loss of nuclear membrane integrity. Ethidium homodimer III is a highly positive-charged nucleic acid probe. Live or apoptotic cells are impermeable to ethidium homodimer III, but necrotic cells are stained with red fluorescence.

The stained hDPSCs and hPDLFs were subjected to fluorescence-activated cell sorting using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). This process determines the percent distribution of healthy (AnV/Etd-III negative), early apoptotic (AnV positive, Etd-III negative), late apoptotic (AnV/Etd-III positive) and necrotic cells (AnV negative, Etd-III positive). Experiments were performed in triplicate.

The hDPSCs and hPDLFs were plated on glass cover slips in 24-well plates, at a density of 400 cells/cm^2 and allowed to establish for 24 h. After exposure to the respective eluent-containing culture medium for 3 days, the hDPSCs or hPDLFs were triple-stained with AnV (green-fluorescence), Etd-III (red-fluorescence) and 4,6-diamidino-2-phenylindole (DAPI; MilliporeSigma), a blue-fluorescent nucleic acid stain that preferentially stains double-stranded DNA. The stained hDPSCs and hPDLFs were examined by a fluorescent microscope (Axioplan 2, Carl Zeiss, Oberkochen, Germany).

2.6.2. Leakage of cytosolic enzyme

Lactate dehydrogenase (LDH) is a cytosolic enzyme present in many cell types. The enzyme catalyses the conversion of lactate to pyruvate via reduction of nicotinamide adenine dinucleotide (NAD) to NADH. It is also a biomarker for irreversible cell membrane damage induced by the toxicity of materials [33]. Damage to plasma membrane releases LDH into the cell culture medium. These extracellular LDH may be quantified by a coupled enzymatic reaction. The Pierce™ LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) was performed to

quantify LDH activity. The hDPSCs and hPDLFs were separately plated in 96-well plates at 10^5 cells/well and cultured in the respective eluent-containing culture medium derived from each cycle for 3 days, without further change of the medium. At the designated time, the culture medium was transferred to a new 96-well plate, mixed with Reaction Mixture and subsequently, Stop Solution. Absorbance at 490 nm and 680 nm were measured using a microplate reader (Synergy HT, BioTek Instruments, Winooski, VT, USA). The LDH activity was determined by subtracting the 680 nm background absorbance from the 490 nm absorbance. For spontaneous LDH activity control, the hDPSCs or hPDLFs were cultured in sterile ultrapure water. To test the maximum LDH activity, the hDPSCs and hPDLFs were cultured by adding 10X Lysis Buffer. Experiments were performed in sextuplicate. The following formula was used for calculating the percent cytotoxicity: [(Material-mediated LDH activity – Spontaneous LDH activity) / (Maximum LDH activity – Spontaneous LDH activity)] x100.

2.6.3. Caspase-3 activity

Caspase 3 is a member of the CED-3 subfamily of caspases. It is one of the critical enzymes of apoptosis and plays a central role in mediating nuclear apoptosis that includes chromatin condensation, DNA fragmentation and cell blebbing [34]. After exposure to the eluent-containing culture media, the caspase-3 activity of hDPSCs and hPDLFs was determined using a Caspase-3 Colorimetric Assay Kit (MilliporeSigma). The cells were lysed for 15 min and the cell lysates were exposed to p-nitroanilide (pNA) for 90 min. Hydrolysis of the peptide substrate by the caspase-3 present in the cell lysate releases pNA, the absorbance of which was recorded at 405 nm. The concentration of the pNA released from the substrate was calculated from a calibration curve prepared with different concentrations of a p-NA standard. Experiments were conducted in sextuplicate.

2.6.4. Oxidative stress

Release of reactive oxygen species (ROS) is inevitable for aerobic organisms and occurs at a controlled rate in healthy cells. Under oxidative stress, ROS production is dramatically increased, resulting in alteration of membrane lipids, proteins and nucleic acids [35]. Oxidative stress reflects an imbalance between ROS production and the cell's ability to detoxify the reactive intermediates and repair damage. Evaluation of intracellular ROS formation reflects the severity of cytotoxicity caused by elution of toxic components from the unset and set premixed putties.

Intracellular ROS in hDPSCs and hPDLFs was detected by CellROX® Orange Oxidative Stress Reagents (Life Technologies, Thermo Fisher Scientific). After exposing the hDPSCs and hPDLFs to the respective eluent-containing culture medium for 3 days, the cells were harvested and re-suspended in complete growth medium. CellROX® Orange (a fluorescent redox cytoplasmic stain; $\lambda_{abs}/\lambda_{em} = 545/565$ nm) was added to the cells at a concentration of 5 μM and incubated at 37 °C for 60 min. SYTOX® Red Dead Cell stain dissolved in dimethyl sulfoxide (DMSO) was added to each sample. The percentage of ROS-positive cells for each material/aging period was detected using the FACSCalibur flow cytometer. Experiments were conducted in triplicate.

Additional hDPSCs and hPDLFs were plated on coverslips in 24-well plates. After culturing in the respective eluent-containing medium, the cells were double-stained with DAPI and CellROX® Orange to evaluate intracellular ROS distribution using confocal laser scanning microscopy (CLSM).

2.7. Cell proliferation

2.7.1. Cell viability

Cell viability was evaluated by incubating 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) with hDPSCs or hPDLFs that had been exposed to the respective eluent-containing growth medium for 3 days. The hDPSCs or hPDLFs were incubated with MTT-succinate solution for 60 min and then fixed with Tris-formalin. The purple intracellular MTT formazan was dissolved *in-situ* using DMSO-NaOH. The optical density of the formazan was measured at 562 nm. The optical density of blank DMSO-NaOH was subtracted from all wells. The formazan content of each well was computed as a percentage of the mean of the Teflon controls, which was taken to represent 100% biocompatibility. The experiments were performed in sextuplicate.

2.7.2. Cellular DNA content

A CyQUANT Cell Proliferation Assay Kit (Thermo Fisher Scientific) was used to detect the effect of test materials on the proliferation of hDPSCs and hPDLFs. Established hDPSCs and hPDLFs were cultured using the respective eluent-containing culture medium derived from the two cycles, for 3 days each. At the designated time, the cultured cells were lysed with CyQUANT GR dye/cell lysis buffer. The absorbance of the cell lysate was determined by a fluorescence microplate reader (FL600, BioTek Instruments, Winooski, VT, USA) at $\lambda_{abs}/\lambda_{em} = 480/520$ nm. The concentration of DNA (in ng/μL) was calculated using a pre-established standard curve that correlates fluorescence intensity with known DNA concentrations. The experiments were performed in sextuplicate.

2.8. Statistical analyses

For each analysis, the data sets were analysed for their normality (Shapiro-Wilk test) and equal variance (modified Levene test) assumptions prior to the use of parametric statistical methods. When those assumptions were violated, the respective data set was non-linearly transformed to satisfy those assumptions prior to the use of parametric statistical procedures. For the indentation depth test and storage modulus, data sets with two groups were analysed with the Student *t*-test. For the cytotoxicity experiments, data obtained from each assay were analysed separately to examine the effects of "material" and "aging cycle", and the interaction of these two factors on the parameter investigated, using two-way analysis of variance. Because the IRM positive control was only included for identifying the discriminatory potential of each assay, data from this group were excluded to increase the robustness of the statistical analyses. Post-hoc comparisons were performed using Holm-Šidák procedures. Statistical significance for all tests was set at $\alpha = 0.05$.

3. Results

3.1. Indentation depth

The percentage reduction indentation depth over the 30-day testing period is summarised in Fig. 1D. The EndoSequence BC-RRM putty hardened quickly after exposure to ambient air at 43% relative humidity. There was a radical decrease in indentation depth during the first four days, with 95% reduction of the indentation depth by the 4th day. The 1/4 lb Gilmore needle was unable to make further indentation after the 10th day, with no change in indentation depth thereafter.

Changes in indentation depths were milder for the experimental NeoPutty. There was gradual decrease in indentation depth and indentation of the partially-set putty was still possible (~65% decrease in indentation depth) at the end of the 30-day period.

3.2. Storage modulus

A representative plot of the changes in storage modulus, loss modulus and tan delta (i.e. lost modulus/storage modulus) of a putty over a period of 1800 s of rheological testing is shown in Fig. 2B. The data indicates that the putties are viscoelastic materials. During the 3 h of rheological testing, the putty absorbed moisture from ambient air and became progressively thicker and with less ability to flow. Changes in the maximum storage modulus obtained over the four designated cycles

of ambient air exposure are summarised in Fig. 2C. For both putties, the maximum storage modulus increased linearly with the number of cycles. The slope of the regression line is much higher for the EndoSequence BC-RRM putty (563.7) compared with that of the experimental Neoputty (152.0), confirming the indentation testing results that the Endo-Sequence BC-RRM putty was more prone to harden after exposing to ambient air.

3.3. Cell viability

Effects of the putties on plasma membrane integrity are summarized in Fig. 3. Representative fluorescent microscopy images of hDPSCs and hPDLFs that had been exposed to eluents derived from the 2 testing cycles are shown in Fig. 3A and C, respectively. Cells exposed to the medium obtained from Teflon, the negative control, were healthy and exhibited blue-fluorescent nuclei with minimal signs of apoptosis (green fluorescence) or necrosis (red fluorescence that appeared pink after merging of the channels). In contrast, cells that were cultured with the elution medium derived from the IRM positive control were mostly apoptotic or necrotic. Cells that were exposed to NeoPutty or ES Putty elution medium from the two cycles exhibited variable degrees of apoptosis or necrosis.

Flow cytometry quantification of vital, AnV/Etd-III negative hDPSCs and hPDLFs in 1st and 2nd testing cycle are represented in Fig. 3B and D, respectively. For the hDPSCs, there were significant differences among the materials (two putties and the Teflon negative control; $P < 0.001$) and testing cycles ($P < 0.001$) on cell viability. The interaction of these two factors was statistically significant ($P < 0.001$); that is, the effect of different materials was dependent on the cycle level. For hDPSCs, cell viability was in the order: Teflon > NeoPutty > EndoSequence BC-RRM putty (ES Putty) for the 1st cycle and Teflon > NeoPutty = ES Putty for the 2nd cycle ($P < 0.05$). While there was no significant difference in the number of vital hDPSCs in the 1st and 2nd cycle for Teflon, significantly more vital cells were present in the 2nd cycle for both putties ($P < 0.05$).

For the hPDLFs, significant differences were also noted among the materials ($P < 0.001$) and testing cycles ($P < 0.001$) on cell viability. The interaction of these two factors was statistically significant ($P < 0.001$). Cell viability was in the order: Teflon > NeoPutty > ES Putty for both cycles ($P < 0.05$). Except for the negative control, significantly more viable hPDLFs were present in the 2nd cycle for both putties ($P < 0.05$).

The flow cytometry results were reinforced by assays specific for identification of necrosis and apoptosis. Release of lactate dehydrogenase, an indicator of irreversible membrane disruption in necrotic or apoptotic cells, is quantitatively depicted in Fig. 4A for hDPSCs and Fig. 4B for hPDLFss. For the hDPSC, there were significant differences among the materials ($P < 0.001$) and testing cycles ($P < 0.001$) on material-induced toxicity that led to the extracellular leakage of the mitochondrial enzyme. The interaction of these two factors was statistically significant ($P < 0.011$). Percentage cytotoxicity based on the leakage of lactate dehydrogenase was in the order: Teflon < NeoPutty < ES Putty for both the 1st and 2nd cycles ($P < 0.05$). For the hPDLFs, leakage of lactate dehydrogenase was significantly affected by the type of material ($P < 0.001$) but not by the testing cycle ($P = 0.30$). The interaction of those two factors was not statistically significant ($P = 0.251$). Although the two putties were relatively more cytotoxic than Teflon in the 1st and 2nd testing cycles ($P < 0.05$), they were not significantly different from each other. For all materials that were analysed statistically, no significant difference was determined in the percentage cytotoxicity between the 1st and the 2nd cycle.

Expression of caspase-3, a crucial mediator of programmed cell death

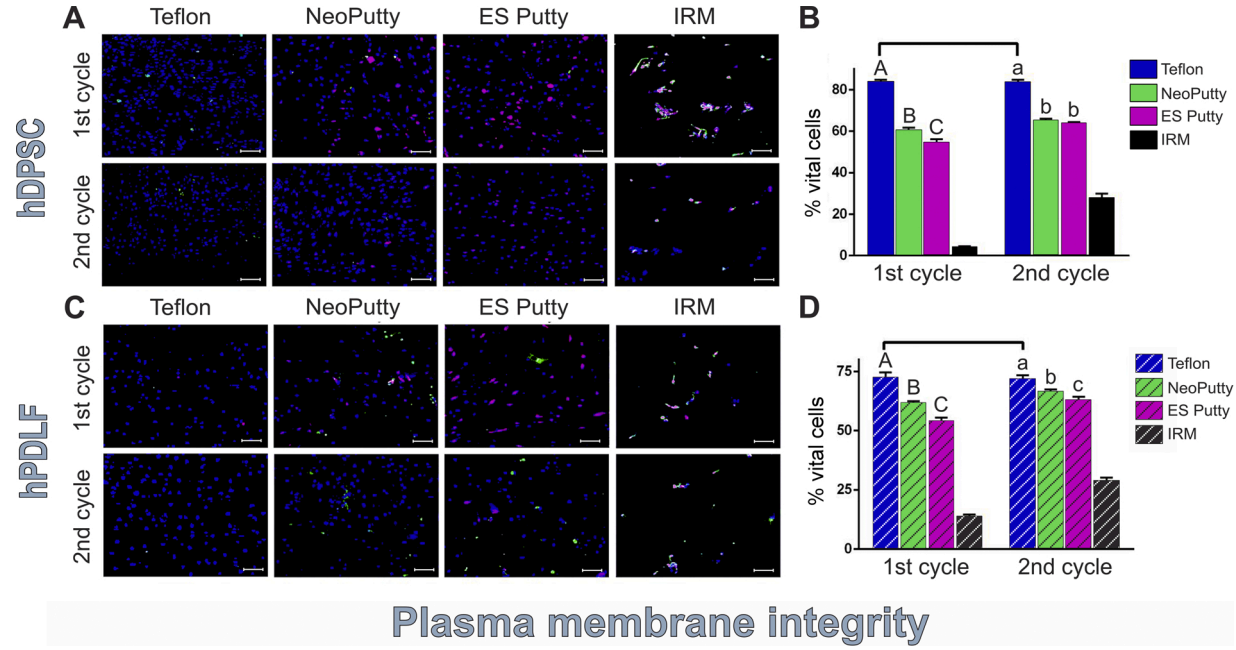

Fig. 3. Effects of endodontic putties on plasma membrane integrity. Representative fluorescent microscopy images of **A**. human dental pulp stem cells (hDPSC) and **C**. human periodontal ligament fibroblasts (hPDLF) that had been exposed for three days to eluents derived from the 2 aging cycles. Bars =25 μm. Teflon was used as the negative control and Intermediate Restorative Material (IRM) was used as the positive control. Both cell types were triple-stained with DAPI (blue-fluorescent nuclear counterstain), FITC-Annexin V (green-fluorescent phosphatidylserine-binding cytoplasmic dye) and ethidium homodimer III (red-fluorescent non-vital DNA dye). Healthy cell nuclei were stained blue. Apoptotic cells showed green cytoplasm and blue nuclei. Necrotic cells showed red or pink nuclei. Dead cells progressing from the apoptotic cell population were stained green, red and blue. Flow cytometry data of **B**. hDPSC and **D**. hPDLF that were cultured for 3 days in eluent-containing culture medium with the eluents derived from the 1st or 2nd cycle. Cells were stained with FITC-Annexin V (AnV) and ethidium homodimer III (Etd-III) for cytoplasmic membrane phospholipids and nucleic acids, respectively. The charts represent the percentage of healthy vital cells (AnV/Etd-III negative). Data are means and standard deviations (N = 3). For each chart, columns in the 1st cycle that are labelled with the same upper case letters are not significantly different ($P > 0.05$). Columns in the 2nd cycle that are labeled with the same low case letters are not significantly different ($P > 0.05$). For comparison between the 1st and 2nd cycle, columns linked with a horizontal bar are not significantly different ($P > 0.05$) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

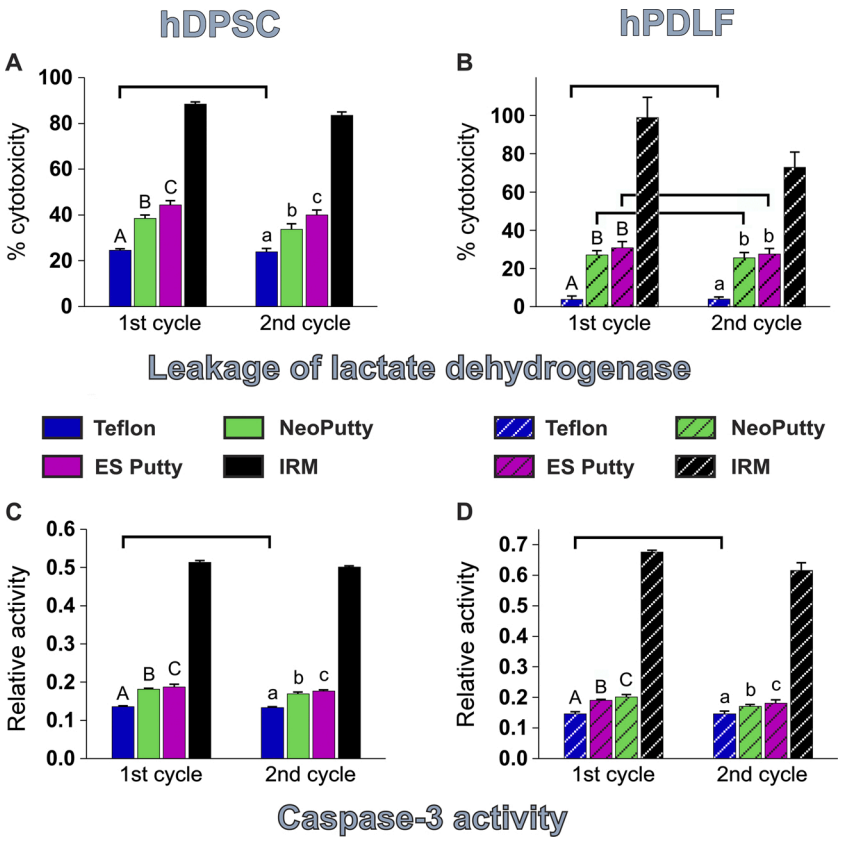

Fig. 4. Effects of endodontic putties on apoptotic and necrotic cell death. Leakage of lactate dehydrogenase from **A**. necrotic hDPSC and **B**. necrotic hPDLF with disrupted plasma membranes. The cells were exposed for three days to eluents derived from the 2 aging cycles. Lactate dehydrogenase catalyses the interconversion of pyruvate and lactate with concomitant interconversion of reduced nicotinamide adenine dinucleotide (NADH) and oxidized NAD. Expression of caspase-3 by **C**. apoptotic hDPSC and **D**. apoptotic hPDLF that had been exposed to elution medium derived from the two aging cycles for 3 days. Caspase-3, one of the effector caspases involved in apoptosis, is induced by the corresponding initiator caspases from the respective extrinsic and intrinsic pathways during apoptosis. Data are means and standard deviations (N = 6). For each chart, columns in the 1st cycle that are labelled with the same upper case letters are not significantly different ($P > 0.05$). Columns in the 2nd cycle that are labelled with the same low case letters are not significantly different ($P > 0.05$). For comparison between the 1st and 2nd cycle, columns linked with a horizontal bar are not significantly different ($P > 0.05$).

(apoptosis), was additionally used to supplement the flow cytometry results. Cell expression of caspase-3 after exposure to the corresponding eluent-containing culture medium are shown in Fig. 4C for the hDPSCs and Fig. 4D for the hPDLFs. For both hDPSCs and hPDLFs, significant differences were detected for the factors "materials" ($P < 0.001$) and "testing cycles" ($P < 0.001$). The interaction of those two factors was

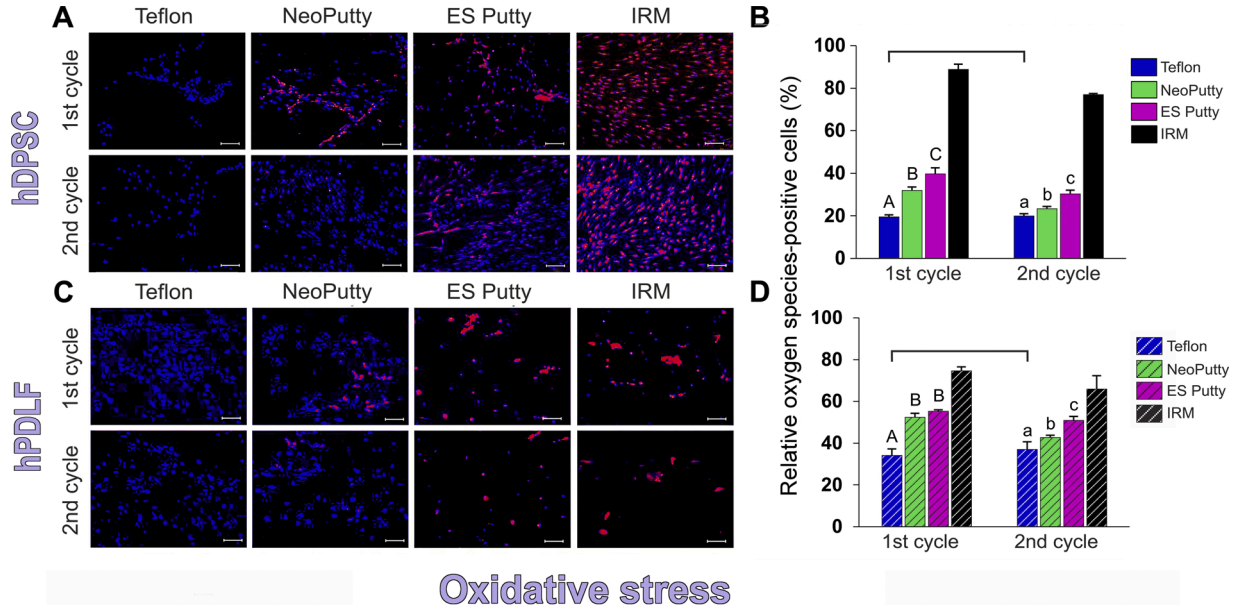

Fig. 5. Effects of endodontic putties on cellular oxidative stress. Representative fluorescence microscopy images of **A**. hDPSC and **C**. hPDLF that had been exposed for three days to eluents derived from the 2 aging cycles. Bars =25 μm. Both cell types were double-stained with DAPI and CellROX® Orange. Cells containing reactive oxygen species exhibited orange fluorescence upon oxidation of the fluorogenic probe used for identification of intracellular oxidative stress. Flow cytometry results of the percentages of CellROX® Orange-stained **B**. hDPSC and **D**. hPDLF that had been exposed for three days to eluents derived from the 2 aging cycles. Data are means and standard deviations (N = 3). For each chart, columns in the 1st cycle that are labelled with the same upper case letters are not significantly different ($P > 0.05$). Columns in the 2nd cycle that are labelled with the same low case letters are not significantly different ($P > 0.05$). For comparison between the 1st and 2nd cycle, columns linked with a horizontal bar are not significantly different ($P > 0.05$).

also statistically significant ($P < 0.001$ for hDPSCs; $P = 0.023$ for hPDLFs). For both the 1st cycle and the 2nd cycle, caspase-3 expression of the hDPSCs and hPDLFs were in the order: Teflon < NeoPutty < ES Putty ($P < 0.05$).

Fig. 5A and C are representative CLSM images showing ROS expression by hDPSCs and hPDLFs, respectively, in response to their exposure to different materials during the two testing cycles. Excess intracellular oxygen-based radicals generated during mitochondrial electron transport that the cells were incapable of degrading were seen as orange fluorescence within the cytoplasm, upon oxidation of the fluorogenic probe used for identification of intracellular oxidative stress. The orange fluorescence was most notable in the IRM positive control.

Quantification of the percentages of ROS-positive hDPSCs and hPDLFs by flow cytometry is depicted in Fig. 5B and D, respectively. For both cell types, the percentages of ROS-positive cells were significantly affected by the type of material they were exposed to ($P < 0.001$ for both cell types), as well as the cycle sequence ($P < 0.001$ for hDPSCs; $P = 0.004$). The interaction of those two factors were statistically significant ($P < 0.001$ for hDPSCs; $P = 0.001$ for hPDLFs). For pairwise comparisons, the percentages of ROS-positive hDPSCs were in the order: Teflon < NeoPutty < ES Putty for both cycles ($P < 0.05$); the percentages of ROS-positive hPDLFs were in the order: Telfon < NeoPutty = ES Putty for the 1st cycle ($P = 0.156$ for NeoPutty *vs* ES Putty) and Teflon < NeoPutty < ES Putty for the 2nd cycle ($P < 0.05$ for all comparisons). For both cell types, there were significantly more ROS-positive cells in the 1st cycle, compared with the 2nd cycle, for NeoPutty and ES Putty ($P < 0.05$).

3.4. Cell proliferation

Results of cell proliferation assessment based on analysis of cell metabolism are shown in Fig. 6A for hDPSCs and Fig. 6B for hDPLFs. For both cell types, increase in mitochondrial oxidoreductase enzyme activities, as indicated by the reduction of the tetrazolium salt to water-insoluble MTT-formazan, was significantly affected by the type of material that the cells were exposed to ($P < 0.001$ for both cell types) and the cycle sequence ($P < 0.001$ for both cell types). The interaction of those two factors were statistically significant ($P < 0.001$ for hDPSCs; $P = 0.001$ for hPDLFs). For the hDPSC, relative dehydrogenase activity was in the order: Teflon > NeoPutty > ES Putty for both the 1st and 2nd testing cycles ($p < 0.05$). For the hPDLFs, relative dehydrogenase activity was in the order: Teflon > NeoPutty > ES Putty for the 1st cycle ($P < 0.05$). Unlike the hDPSCs, the hPDLFs were more tolerant to eluents present in the 2nd testing cycle; there was no significant difference in cell numbers when the eluents derived from the three materials were used for culturing the fibroblasts. For both putties, significantly higher cell numbers were detected in the 2nd cycle, compared with the 1st cycle ($P < 0.05$).

Trends identified with the MTT assay were recapitulated when CyQYANT GR, a highly-sensitive nucleic acid binding cyanine dye, was used for determining cellular DNA content. For both cell types, cell numbers were significantly affected by the type of material ($P < 0.001$ for both cell types) and the cycle sequence ($P < 0.001$ for both cell types). The interaction of those two factors were statistically significant ($P < 0.001$ for hDPSCs; $P = 0.044$ for hPDLFs). Pairwise comparisons for the factor "materials" and "testing cycles" followed the same order described for the MTT assay.

4. Discussion

Tricalcium silicate-based cements are increasingly accepted by clinicians because of their bioactivity and well-reported clinical outcomes. Premixed tricalcium silicate-based repair putty materials are favoured because being premixed and hydrophilic, they are easy to use and handles well. The radiopacifying agent and proprietary organic liquid are premixed with a water-miscible carrier to improve handling

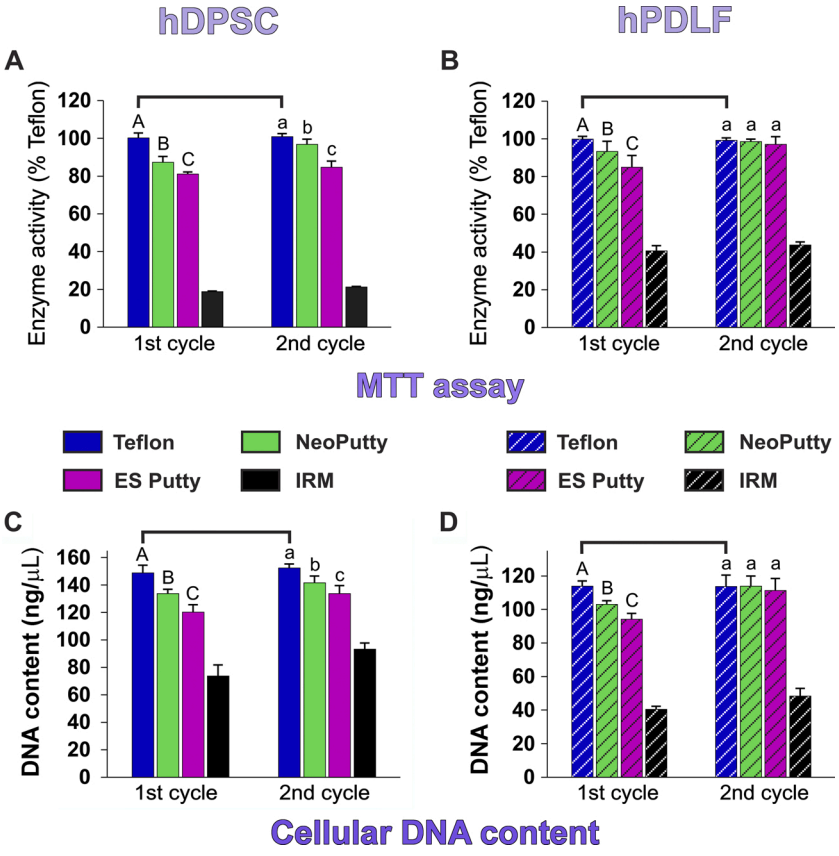

Fig. 6. Effects of endodontic putties on cell proliferation. MTT assay of metabolically-active, proliferating **A**. hDPSC and **B**. hPDLF that had been exposed for three days to eluents derived from the 2 aging cycles. Data are expressed as percentages of the negative control. Cellular DNA contents of proliferating **C**. hDPSC and **D**. hPDLF that had been exposed for three days to eluents derived from the 2 aging cycles. Cellular DNA content was determined from binding of the CyQUANT GR dye to cellular nucleic acids in RNase-digested cell lysates. Data are means and standard deviations ($N = 6$). For each chart, columns in the 1st cycle that are labelled with the same upper case letters are not significantly different ($P > 0.05$). Columns in the 2nd cycle that are labelled with the same low case letters are not significantly different ($P > 0.05$). For comparison between the 1st and 2nd cycle, columns linked with a horizontal bar are not significantly different ($P > 0.05$).

properties. Exposure of the putties to atmospheric moisture initiates the hydraulic setting reaction. Thus, opening the container repeatedly will shorten the shelf-life of the material. The manufacturer of NeoPutty suggests that the experimental putty has overcome this problem. The results of the present study indicate that there are significant differences between the indentation depth and storage modulus of NeoPutty and EndoSequence BC-RRM putty. Based on the results, it may be concluded that the shelf-life for NeoPutty is longer than BC-RRM putty when both putties exposing to the ambient air. Thus, the first null hypothesis that "there is no difference in the window of manoeuvrability between the experimental putty and the control putty" has to be rejected.

The present study utilised two potential methods (indentation depth and storage modulus) for determining the window of manoeuvrability of premixed putties. Indentation testing is commonly used for determining the setting times of dental cements, while rheology testing that is often employed for characterising the flow properties of endodontic sealers through narrow capillaries as well as the setting of glass ionomer cements [36]. Hydraulic cements are examples of viscoelastic cementitious materials, particularly during the unset stage [37]. As such, their viscoelastic properties are best characterized using dynamic mechanical analysis [38,39]. In dynamic mechanical analysis, the storage modulus measures the stored energy, representing the elastic portion of viscoelasticity. The loss modulus measures the energy dissipated as heat, representing the viscous portion of viscoelasticity. The data indicated the EndoSequence BC-RRM putty had a faster decrease in indentation depth and an earlier establishment of high storage modulus when compared with the experimental NeoPutty.

Tricalcium silicate-based materials placed in contact with body fluids are considered as permanent-contact implantable devices [40]. Accordingly, these products have to demonstrate biocompatibility before they can be recommended for clinical use [41]. It has been more than half a century since the 3R principle of replacement (of animals with non-living models), reduction (in the use of animals) and refinement (*of animal use practices*) was proposed by Russell and Burch to foster the development of alternative methods to animal testing [42]. The consensus report "Toxicity testing in the 21 st Century: A vision and a strategy" published by the U.S. National Academy of Sciences proposed a paradigm shift in toxicology from current animal-based testing towards the application of emerging technologies [43]. These strategies included assays based on human cells or non-mammalian models, high throughput testing, "omics" approaches, systems biology and computational modelling [44]. Although it is dubious if animal testing can be completely eliminated in contemporary medicine [45], the reproducibility of animal testing results of systemic toxicity has recently been challenged [46]. The use of fluidic microphysiological systems (organs-on-chips) that recapitulate the functions of the human organs are gaining momentum as the future tools for incorporation into the drug development pipeline [47]. Nevertheless, these tissue chips are more relevant for screening of systemic, organ or tissue-specific toxicity [48]. Tissue chips for the testing of locally-implanted materials in the dental arena are not yet commercially available. Hence cell-based evaluation assays are still a significant part of biomaterials testing and clinical research [49].

The International Standards Organization (ISO) 10993-5 standard distinguishes between the use of liquid extracts of material, direct contact and indirect contact of materials for evaluation of *in vitro* cytotoxicity [50]. Theoretically, direct cell-contact is best suited for simulating the placement of an endodontic putty in contact with an exposed dental pulp or in a root-end preparation within the bony crypt, wherein the crypt is filled with blood and cells derived therefrom prior to the setting of the material. In contrast, the use of liquid extracts is best utilized for evaluation gradual diffusion of toxic components from the set putties. However, the slow setting of the putty may adversely affect the reproducibility of assays evaluated using the direct contact method, as the cells are more susceptible to potential mechanical damage by the overlying test substrate [51]. In addition, indirect contact testing necessitates placement of a layer of agar over the unset putty. Because agar takes time to gel, this does not simulate immediate toxicity to the exposed cells. Hence, the unset putties were immersed in culture media to extract diffusible eluents for exposure to cells during the 1st testing cycle. Although the ISO 10993-5 standard does not specify the use of human cells, it was construed that the use of human cells would be more clinically relevant for testing of materials that are destined for implantation in the human body [52]. Human DPSCs and PDLFs were used because the putties are designed for placement over exposed human dental pulps or in close contact with periodontal ligament fibroblasts in vital pulp therapy or root-end fillings. The two types of tooth-associated human cells exhibit different sensitivities to the eluents derived from the putties. The hPDLFs appeared to be more tolerant to the eluents as some of their responses to the eluents derived from the 2nd testing cycle were not significantly different from the bioinert Teflon negative control.

Toxic chemical substances and their metabolites damage living cells by compromising structural and functional elements that are crucial for cellular housekeeping, beginning with initial injury that is followed by mitochondrial dysfunction and ultimately cell death [53]. Initial injury may be precipitated by disturbance of plasma membrane integrity, decompartmentalization of subcellular architectures or disruption of cell energy supplies. Mitochondrial dysfunction is attributed to increase in membrane permeability (i.e. mitochondrial permeability transition) that eventually results in apoptosis or necrosis [53]. A plethora of cell viability assays [54] and proliferation assays [55] are available for screening cell responses against a toxic reagent or material. These methods capitalize on the use of reagents to identify disruptions of cellular architecture or functions, including membrane permeability, dye uptake, metabolic activities, enzyme release, cell adherence, adenosine triphosphate (ATP) production, co-enzyme production, DNA synthesis and nucleotide uptake activity. For *in vitro* cytotoxicity testing, at least two cytotoxic assays are required to examine two of the three critical events involved in the chemical insult (i.e. initial injury, mitochondrial dysfunction and cell death) [53].

Cell viability refers to the number of healthy cells in a sample. For the evaluation of cell viability in the present study, initial injury was examined by identifying the events and outcomes caused by alteration in plasma membrane integrity. Fluorescent microscopy and flow cytometry were indirectly used to detect this injurious event via the observation of the two modes of physiological and pathological cell demise. Lactate dehydrogenase release was subsequently used for direct quantification of the outcome of cell death. Lactate dehydrogenase is an enzyme found inside every living cell. After membrane integrity is compromised, this enzyme is release into the culture medium which may be used as an indicator of cell death [33]. Unlike complex living systems, genuine apoptosis does not occur in cell culture, particularly with the use of cell lines [56]; cultured eukaryotic cells undergoing apoptosis eventually die by secondary necrosis. This is because scavenger cells that are destined for removing cells that have undergone programmed cell death are lacking in a culture plate. "Find-me" or "eat-me" signals generated via cell-cell communication between scavenger cells and cells undergoing apoptosis are missing, and there is no information signifying how many cells are redundant and require elimination. In addition, part of the apoptosis event occurs within scavenger cells that generate enzymes to dispose their targets. This coordination between the predators and their prey is missing in culture plates with only one cell type.

Mitochondrial dysfunction represents the transitional link between initial chemical injury and cell death [53]. Ideally, assays on mitochondrial permeability transition pore opening should be used to examine mitochondrial dysfunction [57]. In the present work caspase-3 expression and oxidative stress assays were used as indirect indicators of those hDPSCs and hPDLFs with dysfunctioned mitochondria that are committed to the apoptotic death cascade. Caspase-3 is a key mediator in the mitochondrial events of apoptosis [58]. Caspase-3/7 detection is often utilised in conjunction with other analytical methods in

multiplexed and high-throughput cell viability assays to obtain multiple, correlated viability data [59,60]. Mitochondria is one of the main sources of oxidative stress as these organelles utilise oxygen for ATP production [61]. Oxidative stress occurs when the intracellular anti-oxidant mechanisms are incapable of balancing the homeostasis of reactive oxygen species, reactive nitrogen species and lipid peroxidation products [62,63]. Hence, intracellular oxidative stress may be viewed upon as an indicator of mitochondrial dysfunction [64,65]. The nexus between oxidative stress and the biocompatibility of implantable materials is well recognised [66].

Cell proliferation is the process that results in an increase in cell number within a population and is defined by the balance between cell division and cell loss through cell death or differentiation. Different assays are available for detecting cell proliferation. These methods may be classified as: i) metabolic assays which based on the reduction of tetrazolium salts or alamar blue, ii) DNA synthesis cell proliferation assays, iii) nucleoside-analogue incorporation assays, iv) cell cycle-associated protein detection [67]. Metabolic assays used to determine viability are used repeatedly over a period of time to investigate cell proliferation within a population. In the present study, MTT cell viability assay and quantification of DNA content were employed for examining the effects of the two putties on cell proliferation. The CyQUANT cell proliferation assay does not recognise DNA synthesis. However, measuring cell numbers with a DNA-binding fluorescent dye represents an indirect indicator of cell proliferation, which is a reasonable alternative for comparing the cytotoxic effects in different groups that have the same initial cell numbers.

Considering all data derived from the various cell viability and proliferation assays, the second null hypothesis that "there are no differences in the various facets of cytotoxicity induced by the two putties when they are placed in close proximity with hDPSCs and hPDLFs" has to be rejected. The statistical results indicate that NeoPutty is significantly more biocompatible to both periodontal fibroblasts and dental pulp stem cells, compared to the EndoSequence BC-RRM putty.

The differences in manoeuvring time and biocompatibility between the two putties may be attributed to the materials' compositions. EndoSequence BC-RRM putty and NeoPutty contain tricalcium silicate and dicalcium silicate blended with water-free proprietary organic liquids. The radiopacifiers employed in these pre-mixed putties consist of tantalite powder, although the EndoSequence BC-RRM putty also contains zirconia and calcium sulfate powders. Apart from tricalcium and dicalcium silicates, NeoPutty also contains calcium aluminate, although the quantity and type of calcium aluminate (e.g. mono-, di- or tricalcium aluminate) have not been disclosed by the manufacturer. These hydraulic putties are similar in that eluents derived from the unset or set putties are alkaline because of the release of calcium hydroxide on hydration with water derived from the culture medium or body fluids [68]. They differ in their pH, which may contribute to difference in cytotoxicity. For instance, the pH of calcium aluminate compounds varies depending on the medium, water or synthetic body fluids, but is about 11 [69]. In comparison, the pH of the tricalcium silicate cements is higher, between 12 to 13 [70].

Bone cements based on dicalcium silicate, calcium monoaluminate and combinations thereof were compared for their properties, including attachment and proliferation of mouse C57BL/6 calvaria (MC3T3) cells [71]. Although variations in cell proliferation were demonstrated among the compositions, the biocompatibility of all combinations was generally good. In subcutaneous implantation studies, calcium silicate and calcium aluminate cements were biocompatible, but the calcium aluminate cement created less inflammatory reaction than the tricalcium silicate-based Grey MTA Angelus cement [72]. Another study of a calcium aluminosilicate cement *vs* a tricalcium silicate cement with hDPSCs showed similar lower cytotoxicity for the former cement [27].

The liquid components of the two pre-mixed putties have not been undisclosed. Because the hydraulic cements have to set *in vivo*, one can surmise that the organic liquids are water-miscible. Examples of synthetic water-soluble liquids/polymers that are commonly used in medical devices are glycerol, polyacrylic acid, polyethylene glycol, polyvinyl pyrrolidone, polyvinyl alcohol, polyacrylamide, *N*-(2-hydroypropyl) methacrylamide, divinyl ether-maleic anhydride, polyoxazoline, polyphosphates and polyphosphazens. Other natural polymers such as xanthan gum, pectin, chitosan derivatives, dextran, carrageenan, guar gum, cellulose ethers, hyaluronic acid, albumin have also been used as carriers for biomedical applications [73]. Differences in the cytotoxicity of these liquid vehicles may also contribute to the variations in cytocompatibility between the two pre-mixed putties.

The use of cell culture models has significantly streamlined cytotoxicity testing for medical devices because these models have clearer definitions and greater agreement for what constitutes *in vitro* cytotoxicity: no cellular attachment, dramatic morphological changes, reduction in overall viability or adverse effects on cell proliferation [51]. These models are advantageous in that they shorten the time for data collection, enable rapid screening of materials for potential adverse eff ; ects, and possess the ability to test a small quantity of material, when compared with *in vivo* studies in which body weights or surface areas have to be considered. Undeniably, limitations exist for cell culture models. Exposure concentrations used in *in vitro* cytotoxicity evaluations have little relevance to the maximum concentrations achieved *in vivo* [74]. One needs not to be reminded of the renowned toxicological adage that all substances can be poisonous depending upon dosage [75]; toxicological risks only exist in relation to the conditions under which the cells are exposed to a substance. In addition, cell culture conditions are far from homeostatic and elimination of toxic substances such as what occurs *in vivo* by lymphatics and the immunological defence system does not exist [76]. Any extrapolation from the *in vitro* culture conditions should be used with caution. Nevertheless, data obtained from cell culture studies provide critical information prior to implementation of more labour-intensive *in vivo* studies.

Because hDPSCs are neural crest-derived mesenchymal stem cells isolated from adult teeth, they have the capacity to differentiate into osteoblastic, adipogenic, chondrogenic and odontoblastic lineages [77, 78]. Hence it is a logical extension of the present research, to examine whether the differentiation and osteogenic potential of these stem cells are affected by the hydraulic pre-mixed putties. Investigations of these responses are in order to justify the use of the experimental pre-mixed putty as a repair material in endodontics.

5. Conclusion

Within the limitation of the present study, it may be concluded that both NeoPutty and EndoSequence BC RRM putty have shortened shelf-lives after opening, although NeoPutty is much less susceptible to atmospheric moisture intake than EndoSequence BC RRM putty. In terms of biocompatibility, both putties are mildly cytotoxic to hDPSCs and hPDLFs when compared with a bioinert material such as polytetrafluoroethene (Teflon). From an *in vitro* cytotoxicity perspective, NeoPutty may be considered more biocompatible than EndoSequence BC RRM putty, particularly when the putty is exposed to the more sensitive human dental pulp stem cells. Future *in vivo* tissue responses are required to validate these *in vitro* results.

CRediT authorship contribution statement

Qin Sun: Investigation, Data curation, Writing - original draft. **Meng Meng:** Methodology, Investigation. **Jeffrey N. Steed:** Investigation. **Stephanie J. Sidow:** Investigation, Visualization. **Brian E. Bergeron:** Formal analysis, Visualization. **Li-na Niu:** Resources, Supervision, Funding acquisition. **Jing-zhi Ma:** Resources, Supervision, Funding acquisition. **Franklin R. Tay:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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