BMC Oral Health

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Cytotoxicity and proliferation effects of cold ceramic on stem cells from human exfoliated deciduous teeth compared to MTA: an in vitro study

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Abstract

Background This study aimed to assess the cytotoxicity and proliferation effects of cold ceramic (CC) on stem cells from human exfoliated deciduous teeth(SHEDs) compared to mineral trioxide aggregate(MTA).

Methods In this in vitro study, the cytotoxicity of fresh and set MTA and CC for SHEDs was assessed after 24 and 72 h using the methyl thiazolyl tetrazolium(MTT) assay. The scratch test was used to evaluate cell migration, while cell morphology and adhesion were assessed by scanning electron microscopy (SEM). Data were analyzed by one-way ANOVA and Tukey test (alpha = 0.05).

Results At 24 h, the cell viability percentage was higher in fresh MTA than fresh CC ($P < 0.0001$), and in set CC than set MTA ($P = 0.0003$). At 72 h, cell viability in the presence of both fresh and set MTA was similar to the control group ($P = 0.871$). Set CC showed significantly higher cell viability ($P < 0.0001$) while fresh CC decreased cell viability. The scratch was completely healed in the control group. Cell density was lower in the MTA group, and the lowest in the CC group. SHEDs preserved their natural morphology and had optimal cytoplasmic attachment to MTA and CC surfaces after 24 and 48 h.

Conclusion Cell viability and migration in CC were comparable to those in MTA and even superior in set form after 72 h. CC caused cell proliferation in addition to migration. Cells had a normal morphology and optimal adhesion in both groups. CC may be suitable for use as an alternative to MTA in pulpotomy of primary teeth.

Clinical trial number Not applicable.

Keywords Adult stem cells, Cell adhesion, Cell migration, Cell survival, Dental cements, Mineral trioxide aggregate

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Introduction

Vital pulp therapy (VPT) has been recommended to preserve pulp vitality in immature primary or permanent teeth with pulp exposure due to caries in absence of radicular pathologies [1]. Conservative VPT of primary teeth is performed to preserve the tooth integrity and supporting structures, decrease the need for pulpotomy, and retain the primary teeth until their physiological exfoliation [2].

There are three treatment options for the management of deep caries in primary teeth: indirect pulp capping, direct pulp capping, and pulpotomy. Stem cells play a pivotal role in such treatments. Stem cells from human exfoliated deciduous teeth (SHEDs) are a population of cells with a high proliferation rate capable of differentiation [3]. Since minimally invasive dentistry supports VPT, knowledge about the interactions between the pulp cells and capping agents is highly important, aiding in selection of the best materials for clinical use [4–6].

An ideal pulp capping agent should be antibacterial and non-toxic, capable of creating a hermetic seal, reinforcing the radicular pulp healing, and not interfering with the process of physiological exfoliation of primary teeth [7]. In search for more biocompatible materials for pulpotomy, mineral trioxide aggregate (MTA) was introduced by Torabinejad in the mid-1990s as a new biomaterial for pulpotomy of primary and permanent teeth. To date, a number of studies have reported its high success rate in VPT [8–10]. MTA is an alkaline agent that induces dentinal bridge formation and has properties such as excellent biocompatibility, induction of tissue regeneration, and provision of optimal seal without microleakage. Nonetheless, it has some drawbacks, such as difficult application, long setting time, short half-life, tooth discoloration potential, low compressive strength, and high cost, encouraging further research to find an equally effective alternative [11, 12].

Cold ceramic (CC) is a bioceramic material similar to MTA, which is used as a root-filling material in endodontic treatment. CC, with calcium hydroxide as its main constituent, can also be used as a pulp-capping agent in VPT [13]. The optimal properties of CC include shorter setting time than MTA, providing greater seal than glass ionomer, optimal biocompatibility and non-toxicity, and favorable radiopacity [14–18]. The available studies on the biocompatibility of CC have shown that its cytotoxicity is comparable to that of MTA [16, 17, 19, 20], and the clinical and radiographic success of pulpotomy with CC is equal to that of pulpotomy with MTA [21]. Considering the optimal properties of CC, such as shorter setting time and lower cost than MTA, it may be considered as a pulpotomy agent for primary teeth. However, CC has not been compared with other materials such as Biodentine.

Nonetheless, to the best of the authors' knowledge, no previous study is available on the effects of CC on SHEDs. Thus, this study aimed to assess the cytotoxicity and proliferation effects of CC on SHEDs compared to MTA (as the gold standard).

Methods

In this in vitro experimental study, which was conducted on SHEDs, the cytotoxicity of different concentrations of set and fresh forms of MTA and CC was evaluated by indirect exposure of cells to the extracts and evaluation of their viability, proliferation, and migration. The morphology and adhesion of cells were assessed directly by their seeding on MTA and CC discs and inspection under a scanning electron microscope (SEM). The study protocol was approved by the ethics committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.DRC.REC.1402.105). Three to five repetitions were considered for each test.

Cell culture

The cells were cultured in cell culture flasks containing Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) with high glucose, 10% fetal bovine serum (FBS; Gibco, USA), and 1% penicillin-streptomycin, and then incubated at 37 °C and 95% humidity with 5% CO2 (Binder, Germany).

Preparation of the discs and extracts

MTA (Angelus, Brazil) and CC (SJM Co., Iran) were prepared as instructed by the manufacturers. Discs with 1 cm diameter and 2 mm thickness were fabricated from MTA and CC ($n = 5$ from each) under aseptic conditions and UV-sterilized for 30 min (each side). Some plates were assessed after 24 h to evaluate acute cytotoxicity while some others were assessed after 72 h to evaluate chronic cytotoxicity. To obtain the extract of fresh MTA and CC, DMEM containing 10% FBS was added to some of the discs according to ISO-10993-12 [22].

To obtain the extract of set MTA and CC, the discs were incubated at 37 °C and 95% humidity in a CO_2 incubator for 24 h to ensure their complete setting. Next, DMEM containing 10% FBS was added to the discs according to ISO-10993-12 (1 mL of the culture medium per 3 cm^2 surface area of the discs) and the discs were incubated at 37 °C and 95% humidity in a CO_2 incubator for 24 h. Next, 50% and 25% concentrations [23, 24] were prepared from the pure (100%) extract.

The obtained extracts were refrigerated until use.

Cytotoxicity assessment

The methyl thiazolyl tetrazolium (MTT) assay was used for quantitative evaluation of the effects of MTA and CC on the viability and proliferation of SHEDs. For this

purpose, SHEDs were seeded in 96-well plates (SPL, Korea) containing 100 µL of DMEM, 10% FBS, and 1% antibiotic at a density of 5000 cells/well and incubated at 37 °C and 95% humidity to reach 70% confluence in the logarithmic growth phase. The overlaying medium was removed and replaced with the extracts. Extracts were tested in undiluted (100%) and diluted (25% and 50%) forms. Cells treated with the extracts served as the test groups, and those treated with the culture medium alone served as the control group. The plates were then incubated at 37 °C and 95% humidity in a CO2 incubator. The MTT assay was performed according to ISO-10993-5 [22]. The percentage of cell viability was calculated using the following formula:

$$\text{Cell viability percentage} = \frac{Mean\ optical\ density\ of\ treatment\ group}{Mean\ optical\ density\ of\ the\ control\ group} \times 100$$

According to ISO-10993-5, a material causing over 30% reduction in cell viability compared with the control group (100% viability) and lowering the viability percentage below 70% would be considered cytotoxic [25].

Migration of sheds

The migration of cultured SHEDs adjacent to MTA and CC extracts was assessed by the wound healing assay, also known as the scratch assay, which is a semi-quantitative test. Accordingly, SHEDs in the logarithmic growth phase were seeded in wells of a 24-well plate (SPL, Korea) at a density of 100,000 cells/well. On day 2 (24 h after incubation), the cells reached 100% confluence. A sterile #10 sampler was used to create a quick vertical scratch in each well (time 0). Each well was rinsed with complete cell culture medium twice to eliminate the detached cells. Next, each well was treated with either the culture medium alone (control group) or with the completely set MTA and CC extracts. The cells were stained at times 0 and 24 and 48 h after scratching. For this purpose, the overlaying medium was removed from each well, and the cells were rinsed with cold (4 °C) positive (containing calcium and magnesium) phosphate-buffered saline. To fix the cells, 500 µL of precooled (-20 °C) 100% methanol (Merck, Germany) was added to each well, followed by incubation at room temperature for 10 min. For staining, methanol was removed, and replaced with 0.5% crystal violet (Merck, Germany) solution. The plate was placed on a shaker at room temperature for 10 min. The dye was removed, and the cells were rinsed with deionized water three times. The cells at the scratch site were digitally photographed under an inverted microscope (Nikon, Japan) at x4 magnification, and the distance between the scratch borders was measured by ImageJ software (National Institute of Health, USA) and reported in inches.

Assessment of cell morphology and adhesion

The UV-sterilized set specimens (after 24 h of incubation) were placed in wells of a 24-well plate and sealed with agar. SHEDs were seeded on the surface of each specimen at a density of 50,000 cells/surface and incubated. The plate was removed from the incubator after 24 and 48 h, the overlaying medium was carefully removed, and the specimens were transferred to a new well plate. They were rinsed with phosphate-buffered saline once, and then 500 µL of 2.5% glutaraldehyde in phosphate-buffered saline was added to each well. The plate was refrigerated for 24 h to fix the cells on the specimen surface. The fixator was removed, and the specimens were rinsed and dehydrated using graded concentrations of alcohol, as follows:

Deionized water (10 min), 30% alcohol (10 min), 40% alcohol (10 min), 50% alcohol (10 min), 60% alcohol (10 min), 70% alcohol (10 min), 80% alcohol (10 min), 90% alcohol (10 min), 100% alcohol (30 min), and 100% alcohol (30 min). Finally, the alcohol was removed, and the samples were air-dried under a chemical hood for 48 h. Next, they were gold sputter-coated, and inspected under a scanning electron microscope (SEM; TESCAN MIRA3 Aria Electron Optic, Iran). Cell adhesion was assessed qualitatively by evaluation of the presence/absence of cell adhesion and pseudopods.

Statistical analysis

Data were analyzed using GraphPad Prism version 9 (La Jolla, CA, USA). Normal distribution of data was confirmed by the Kolmogorov-Smirnov and Shapiro-Wilk tests ($P > 0.05$), and the homogeneity of the variances was confirmed by the Levene's test ($P > 0.05$). Thus, comparisons were made by one-way ANOVA and Tukey test at 0.05 level of significance.

Results

Cytotoxicity

After 24 h: No significant difference was noted in the cell viability percentage in the presence of different concentrations of set MTA extract with each other or with the control group ($P > 0.05$), and all showed approximately 100% viability. However, a significant reduction occurred in the cell viability percentage by approximately 20% in the groups treated with different concentrations of fresh MTA extract (with no significant difference with each other) compared with the control group ($P < 0.05$). In general, the percentage of cell viability in set specimens was significantly higher than that in fresh specimens ($P < 0.05$).

The viability percentage of cells treated with different concentrations of set CC extract (with no significant difference with each other) was significantly higher than that in the control group by 20–30% ($P<0.05$). A reduction in the cell viability percentage occurred in the presence of undiluted and ½ diluted concentrations of the fresh extract by 80% and 60%, respectively ($P<0.0001$). However, ¼ diluted extract had no significant difference in cell viability with the control group ($P>0.05$). In total, the percentage of cell viability was significantly higher in cells in the set groups compared with the fresh groups.

In undiluted and ½ diluted extracts, the percentage of reduction in cell viability was significantly greater in CC than in MTA ($P<0.05$). However, in ¼ diluted extracts, the percentage of cell viability was significantly higher in CC than in MTA ($P<0.05$). In undiluted and diluted extracts, the percentage of cell viability in CC was significantly higher than MTA ($P<0.05$).

After 72 h: No significant difference was found in the cell viability percentage of different concentrations of set MTA extract with each other or with the control group ($P>0.05$) except for the undiluted form that showed approximately 20% lower cell viability than the control group ($P<0.05$). No significant difference existed between ½ and ¼ concentrations of fresh MTA ($P>0.05$) except for undiluted form which caused a significant (80%) reduction compared with the control group ($P<0.05$).

The viability percentage of cells treated with different concentrations of set CC (with no significant difference with each) was significantly higher than the control group by approximately 40% ($P<0.05$). Undiluted and ½ diluted fresh extracts caused a significant reduction in cell viability by 90% and 40%, respectively ($P<0.05$). However, ¼ diluted extract had no significant difference with the control group in cell viability percentage ($P>0.05$). In general, the percentage of cell viability of set extracts was significantly higher than fresh extracts ($P<0.05$).

In ½ diluted extracts, the percentage of reduction in cell viability was significantly greater in CC than MTA ($P<0.05$). However, in undiluted ($P>0.05$) and 1/4 diluted ($P>0.05$) extracts, CC and MTA were not significantly different. In comparison to undiluted extracts, the percentage of cell viability of CC was significantly higher than that of MTA ($P<0.05$).

Figures 1 and 2 compare the cytotoxicity of different concentrations of MTA and CC extracts in undiluted and set forms with each other and with the control group after 24 and 72 h of exposure, respectively.

Migration of sheds

As shown in Fig. 3, cell migration was not significantly different between the two groups at 24 h. However, at 48 h, the scratch was completely healed in the control group, almost healed in the MTA group with lower cell density than the control group, and partially healed in the CC group with lower cell density than the MTA and control groups. Cell density in other areas was greater in the CC group than in the MTA and control groups, pointing to a higher cell proliferation rate in the CC group.

Morphology and adhesion of sheds

As demonstrated in Fig. 4, in the MTA group, cells had completely normal morphology and optimal cytoplasmic adhesion to the surface at 24 and 48 h. The number of cells was higher after 48 h, compared to 24 h, pointing to cell proliferation. The same pattern was observed for the CC with the difference that the number of cells was much higher than the MTA group at both 24 and 48 h.

Discussion

The results showed that cell viability and migration in CC were comparable to those in MTA and even superior in set form after 72 h. Materials in freshly mixed form constantly release elements that might be toxic. Moreover, cytotoxicity may be related to the release of hydroxyl ions and the resultant alkaline pH, which is intense at first and is attenuated over time.

According to the latest guideline of the American Academy of Pediatric Dentistry for VPT, application of calcium silicate cements increases the success rate of pulpotomy of vital primary teeth with deep carious lesions more than other materials and techniques [26]. Calcium silicate-based cements have favorable physical and biological features such as interactivity (Ca and OH ions release, and production of higher alkalinity), apatite forming stimulation, biocompatibility and a bactericidal effect. The release of Ca and OH ions promotes cell differentiation and proliferation, wound healing, tissue repair, and hard tissue mineralization (dentinal bridge formation); thus, mantling the pulp tissue vitality [27].

Several studies have shown favorable biocompatibility of MTA [4, 23, 28–31]. Da Silveira et al. [24] assessed the cytotoxic effects of 1:1 and 1:2 dilutions of bioceramic materials on SHEDs and found that MTA had the lowest cytotoxicity even in 1:1 concentration. In the present study, by a reduction in the concentration of CC to 1:4, its cytotoxicity decreased, indicating its dose-dependent cytotoxicity pattern. Unlike MTA, studies on the biocompatibility of CC are scarce. Mozayeni et al. [17] compared the cytotoxicity of MTT and CC in fresh and set forms using the MTT assay. They reported comparable biocompatibility and cell viability in the CC and MTA groups. However, cell viability was lower in the presence of freshly mixed CC compared to freshly mixed MTA. MTA in freshly mixed form showed higher cell viability compared with the set form in their study, which was in contrast to the present results. Khedmat et al. [19] evaluated

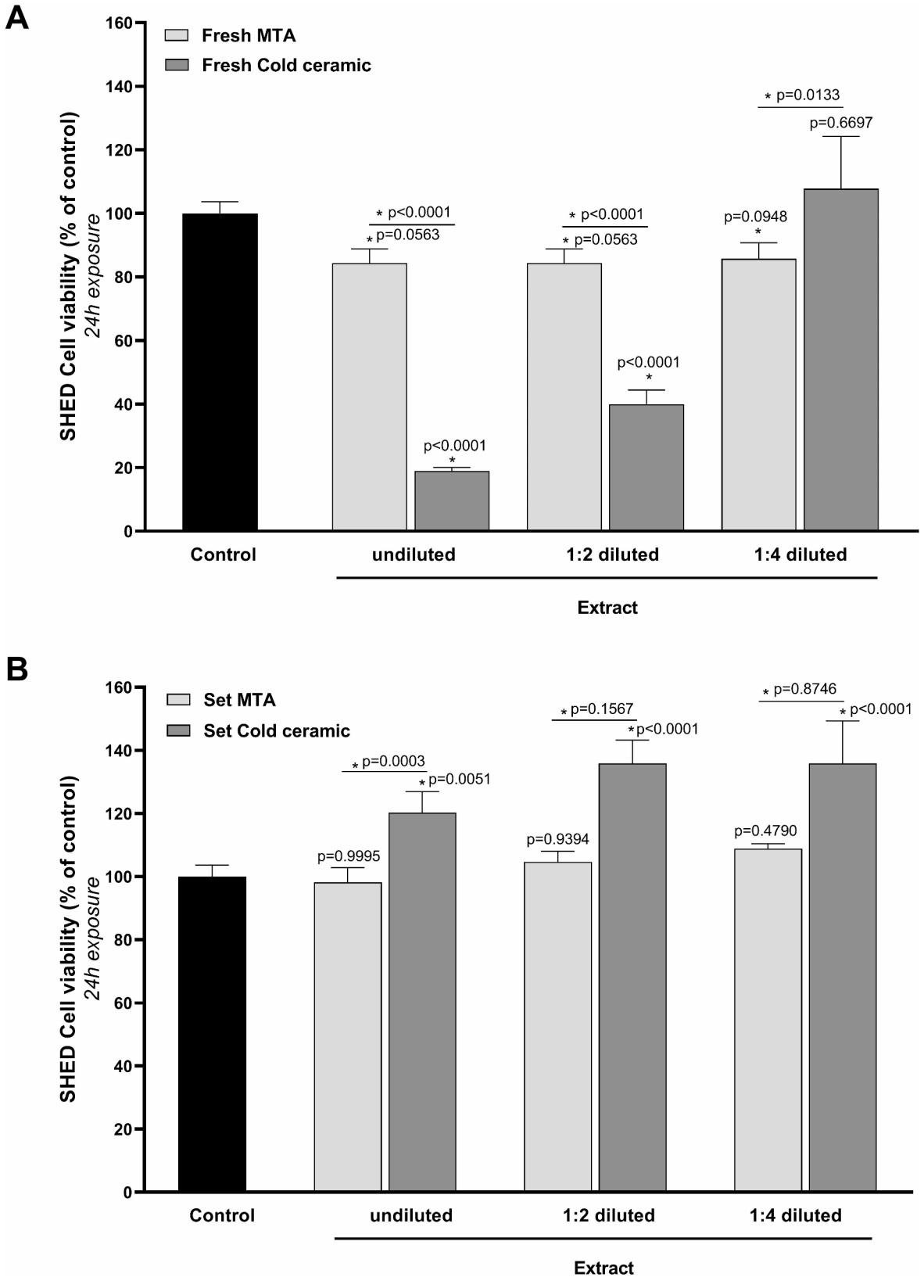

Fig. 1 Cytotoxicity of fresh (**A**) and set (**B**) MTA and CC extracts in undiluted and diluted forms for SHEDs after 24 h of exposure in comparison with each other and with the control group. Starts on top of the columns indicate presence of a significant difference with the control group, and starts on the lines indicate presence of a significant difference between the two groups ($P < 0.05$)

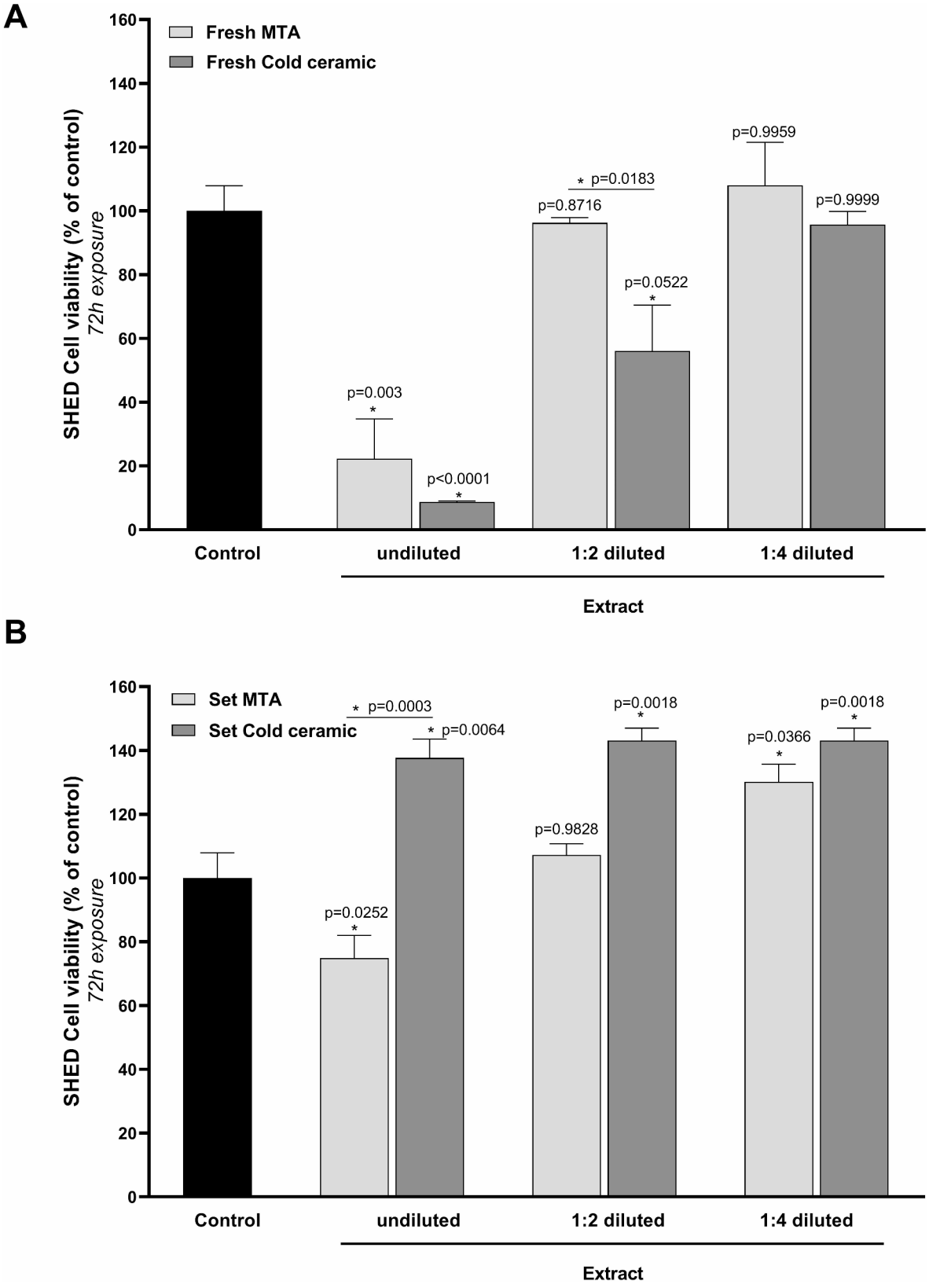

Fig. 2 Cytotoxicity of fresh (**A**) and set (**B**) MTA and CC extracts in undiluted and diluted forms for SHEDs after 72 h of exposure in comparison with each other and with the control group. Starts on top of the columns indicate presence of a significant difference with the control group, and starts on the lines indicate presence of a significant difference between the two groups ($P < 0.05$)

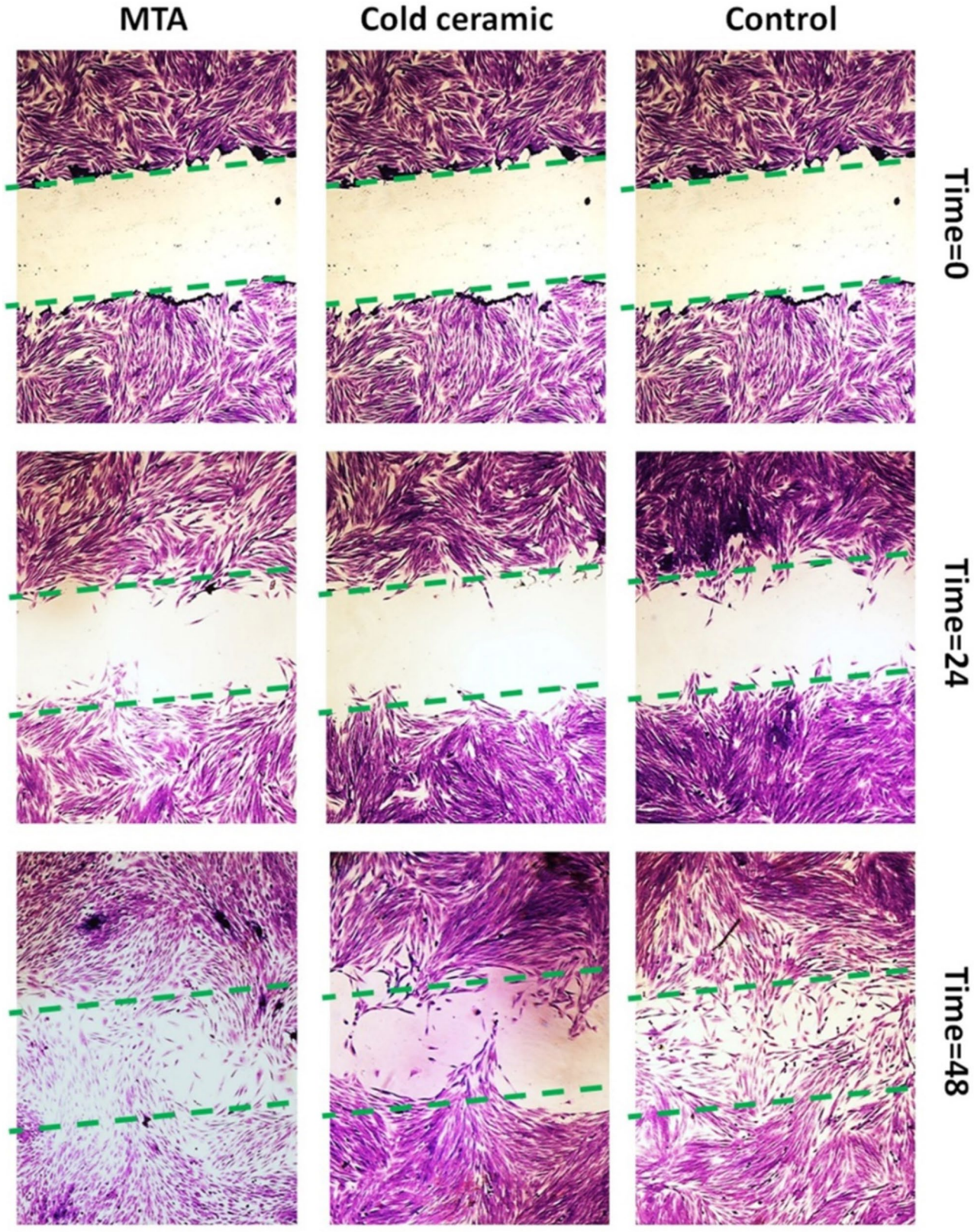

Fig. 3 Micrographs of SHEDs at 0, 24, and 72 h after scratching in the presence of set undiluted CC and MTA extracts in comparison with the control group at x40 magnification

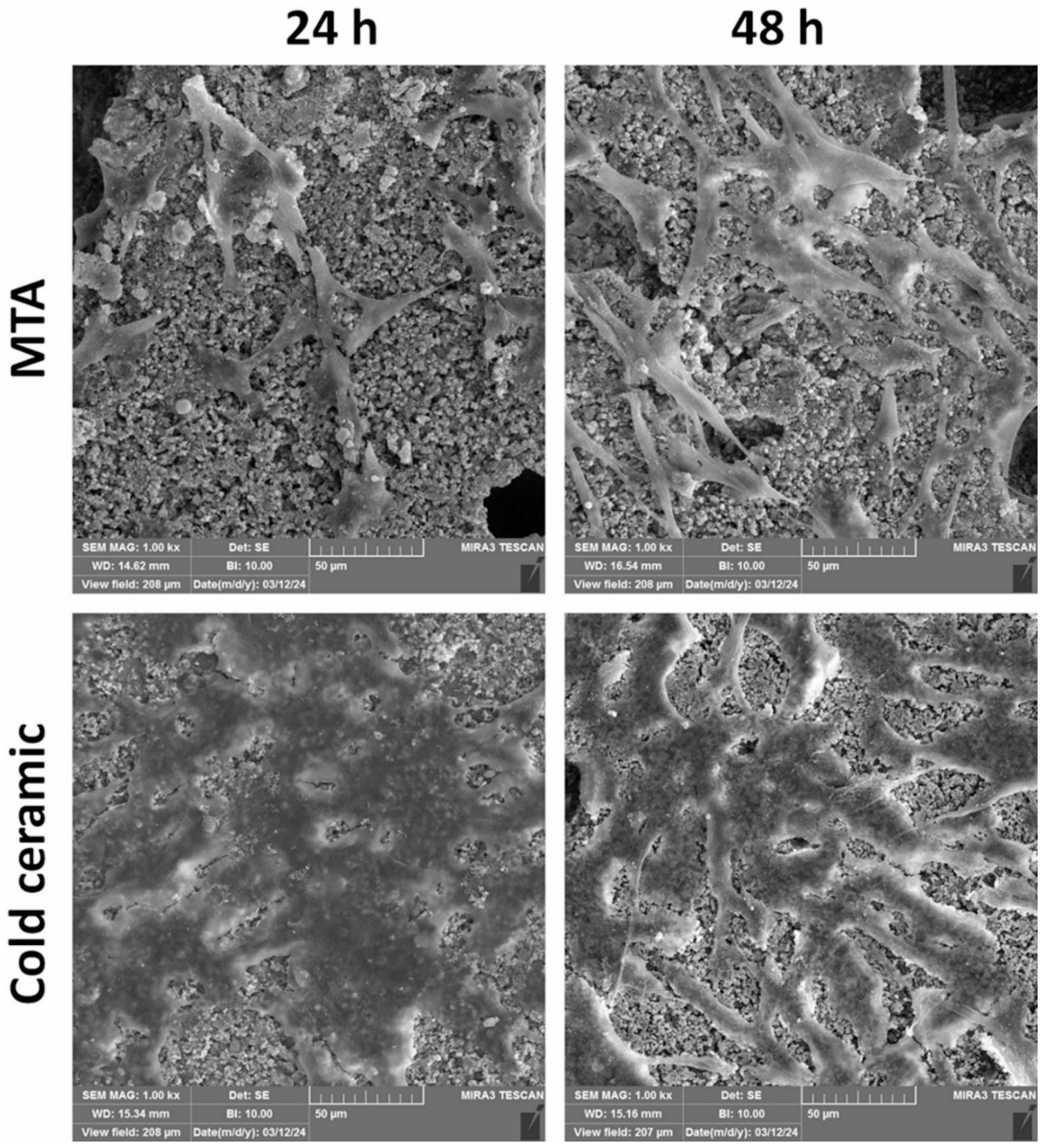

Fig. 4 SEM micrographs of SHEDs on the surface of MTA and CC specimens after 24 and 48 h

the effects of MTA and CC on the viability, adhesion, and differentiation of dental pulp stem cells and periodontal fibroblasts. To the best of the authors' knowledge, their study was the only one similar to the present study that assessed the cell viability in freshly mixed and set forms after 1, 3, 5, and 7 days. They showed that the biocompatibility of CC was similar to that of MTA in both fresh and set forms and at all time points and similar to the positive control group, except for the CC and MTA in fresh form following exposure to PDL cells, which yielded a cell viability lower than that of the control group. However, the present study indicated that CC in set form led to significantly higher cell viability than MTA. Although the results were highly favorable for CC in set form, it

showed a lower cell viability than MTA in freshly mixed form. But cell viability in the presence of its ¼ diluted form was comparable to that of MTA with no significant difference. It should be noted that SHEDs were used in the present study, while Khedmat et al. [19] evaluated PDL cells and dental pulp stem cells, which may partly explain the difference in the results. The initial and final setting times of CC are shorter than those of MTA, which is an advantage because viable cells are exposed to the freshly mixed and more toxic form of biomaterial for a shorter period of time in the clinical setting, decreasing the adverse effects.

Migration of dental pulp stem cells to the site of injury and their differentiation to odontoblast-like cells occur in deep carious lesions or pulp exposure. Thus, ideal dental materials should induce cell migration to enhance the healing or protection of dental pulp. Accordingly, the present study also assessed the migration of SHEDs in the presence of MTA and CC extracts by the scratch assay at 0, 24, and 48 h. It should be noted that cells migrate in the first 24 h and then start to proliferate. In other words, they show migration in the first 24 h and proliferation in the second 24 h. The results showed no significant difference between the two groups in cell migration in the first 24 h. However, at 48 h, the scratch was completely healed in the control group. Cell density was lower in the MTA group and the lowest in the CC group. This study appears to be the first to assess the effects of MTA and CC on SHED migration and wound healing. Collado-González et al. [23] evaluated the migration of SHEDs in the presence of MTA and Biodentine using the scratch test and showed excellent cell migration and wound healing in both groups, especially in 1:4 concentration compared to the control group. Biodentine was superior to MTA. Their results were later confirmed by Araújo et al., [4] reporting the highest cell migration next to Biodentine and MTA. Cell migration was the lowest in the calcium hydroxide group despite the highest proliferation rate. The same results were reported by Maru et al. [30]. Thus, both CC and MTA appear to have optimal efficacy for wound healing.

Cell adhesion plays a pivotal role in the cascade of cell-biomaterial interactions and is imperative for cell proliferation and differentiation [32]. SEM assessment of cell morphology in the present study showed normal morphology and optimal cytoplasmic attachment of SHEDs in both the MTA and CC groups at 24 and 48 h. Also, the cell count increased in both groups after 48 h, indicating cell proliferation; however, this increase was much greater in the CC group. Collado-Gonzalez et al. [23] reported poor and limited adhesion of SHEDs to IRM and TheraCal LC, while MTA and Biodentine led to distinct cellular colonization, forming a cell layer covering the entire surface. Khedmat et al. [19] showed optimal adhesion of PDL fibroblasts to the surface of MTA and CC; nonetheless, cell spreading was greater on the MTA surface than CC, which was in contrast to the present results since CC caused greater cell adhesion and proliferation than MTA. Several parameters may affect cell adhesion, such as surface roughness and chemical properties, and cell spreading, proliferation, and differentiation [33], which may explain the difference in the results [34].

This study had an in vitro design. Thus, the results cannot be directly generalized to the clinical setting. Future animal studies and then clinical trials are required to obtain more reliable results. Lack of a similar in vitro study regarding the cytotoxic effects of CC on primary teeth was another limitation of this study. Future studies are required to obtain more reliable results. Also, the effects of CC on differentiation (expression of osteogenic/odontogenic markers) and bioactivity (extracellular mineralization) of SHEDs and dental pulp stem cells should be investigated. Antibacterial and apoptotic effects of CC, and its size, porosity, and discoloration potential should be assessed as well. Finally, clinical trials are required to obtain more generalizable results.

Conclusion

CC showed comparable cell viability and migration to MTA and was even superior to MTA in completely set form after 72 h. CC induced cell proliferation in addition to migration, and SHEDs had normal morphology and optimal adhesion in both groups. In total, both MTA and CC have acceptable biological effects. Considering the high cost of MTA, CC may be able to serve as a biocompatible and low cost alternative for pulpotomy of primary teeth due to its optimal biological properties, given that its other characteristics are confirmed in vitro, and also in clinical trials.

Abbreviations

MTA	Mineral Trioxide Aggregate
CC	Cold Ceramic
MTT	Methyl thiazolyl tetrazolium
SEM	Scanning electron microscopy
VPT	Vital pulp therapy
SHEDs	Stem cells from human exfoliated deciduous teeth

Acknowledgements

None.

Author contributions

A.A.S contributed to the study concept and design, and study supervision; M.B contributed to the drafting of the manuscript and critical revision of the manuscript for important intellectual content. M.T contributed to the statistical analysis and administrative, technical, and material support. N.M contributed to the acquisition of data and analysis and interpretation of data. All authors reviewed the final manuscript. The authors read and approved the final manuscript.

Funding

This research received no funding.

Data availability
The data used to support the findings of this study were supplied by corresponding author under license and data will be available on request. Requests for access to these data should be made to corresponding author.

Declarations

Ethics approval and contest to participate
The study protocol was approved by the ethics committee of the university (IR.SBMU.DRC.REC.1402.105).
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Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 2 November 2024 / Accepted: 5 March 2025
Published online: 13 March 2025

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