



Biocompatibility and osteogenic potential of baghdadite, mineral trioxide aggregate, and their combination on human dental pulp stem cells: an in vitro study

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Abstract

INTRODUCTION. Dental pulp stem cells (DPSCs) are of interest in regenerative endodontics due to their multipotency. Mineral trioxide aggregate (MTA) is highly sought after due to its biocompatibility, but the limitations of long setting time and poor handling have created interest in newer products such as Baghdadite. To evaluate the biocompatibility and osteogenic potential of Baghdadite, MTA, and their combination on DPSCs using MTT and Alizarin Red assay.

MATERIALS AND METHODS. DPSCs were cultured and characterized by flow cytometry and CFU assays. Experimental groups (MTA, Baghdadite, MTA+Baghdadite) were exposed to cytotoxicity test (MTT assay) and mineralization test (Alizarin Red staining).

RESULTS. Cell viability of all the groups was higher than control. Combination group showed maximum viability (mean OD: 0.4066) than Baghdadite (0.3975) and MTA (0.3563). Alizarin Red staining showed the maximum mineralization in combination group (mean OD: 1.7069) than MTA (0.5788) and Baghdadite (0.4020).

CONCLUSIONS. The association of MTA and Baghdadite showed improved biocompatibility and osteogenic ability, which is promising for application as a pulp-capping agent in regenerative endodontics.

Keywords: dental pulp stem cells, mineral trioxide aggregate, baghdadite, biocompatibility, osteogenic differentiation

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Биосовместимость и остеогенный потенциал багдадита, минерального триоксидного агрегата и их комбинации в отношении стволовых клеток пульпы человека: in vitro исследование

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Резюме

ВВЕДЕНИЕ. Стволовые клетки зубной пульпы (DPSCs) представляют интерес в регенеративной эндодонтии благодаря своей мультипотентности. Минеральный триоксидный агрегат (MTA) высоко ценится за биосовместимость, однако длительное время схватывания и трудности в обращении стимулировали интерес к новым материалам, таким как багдадит. Целью исследования было оценить биосовместимость и остеогенный потенциал багдадита, MTA и их комбинации на DPSCs с использованием МТТ-теста и окрашивания аллизарином красным.

МАТЕРИАЛЫ И МЕТОДЫ. DPSCs культивировали и характеризовали с помощью проточной цитометрии и CFU-тестов. Экспериментальные группы (MTA, багдадит, MTA+багдадит) подвергались тесту цитотоксичности (МТТ) и тесту минерализации (окрашивание аллизарином красным).

РЕЗУЛЬТАТЫ. Жизнеспособность клеток во всех группах была выше, чем в контроле. Комбинированная группа показала максимальную жизнеспособность (средняя ОП: 0,4066) по сравнению с багдадитом (0,3975) и MTA (0,3563). Окрашивание аллизарином красным продемонстрировало наибольшую минерализацию в комбинированной группе (средняя ОП: 1,7069) по сравнению с MTA (0,5788) и багдадитом (0,4020).

ВЫВОДЫ. Ассоциация МТА и багдадита показала улучшенную биосовместимость и остеогенную активность, что делает ее перспективной для применения в качестве материала для покрытия пульпы в регенеративной эндодонтии.

Ключевые слова: стволовые клетки зубной пульпы, минеральный триоксидный агрегат, багдадит, биосовместимость, остеогенная дифференцировка

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INTRODUCTION

Regenerative endodontics is a rapidly developing discipline that aims to restore the functional and biological vitality of the dentin–pulp complex with the aid of biomaterials and stem cell-mediated therapy [1]. The use of DPSCs, a group of mesenchymal stem cells (MSCs) of dental pulp with multilineage differentiation capacity, including to odontoblast-like and osteoblast-like cells [2; 3], is one of the pillars of this discipline. The cells have been found to be of interest in tissue engineering and regenerative medicine due to their self-renewal and immunomodulatory characteristics [4; 5]. To harness the regenerative potential of DPSCs, biocompatible and bioinductive materials must be used to guarantee cellular viability, growth, and differentiation. Of the most commonly used materials in regenerative endodontics, MTA has been used as a gold standard for direct pulp capping, pulpotomy, and apexification. It is valued for its best-in-class sealing, biocompatibility, and mineralized tissue induction potential [6; 7]. MTA has been shown to release calcium ions that increase the expression of osteogenic markers like alkaline phosphatase (ALP), osteocalcin, and bone sialoprotein, facilitating hard tissue regeneration [8]. However, despite its excellence, MTA has certain limitations, including a prolonged setting time, difficult handling, potential tooth structure discoloration, and early-stage cytotoxicity owing to its high alkalinity [9; 10]. These limitations have prompted efforts to develop new bioceramic materials that are capable of replacing or augment the clinical performance of MTA. A candidate material is Baghdadite – a calcium-zirconium-silicate ceramic of the silicate-based bioactive ceramics family [11]. Baghdadite was initially created for orthopedic use, but it has exhibited superior osteoconductive behavior, good mechanical behavior, and controlled ion release kinetics that promote cell differentiation [12]. Its ion release of biologically active ions like Ca^{2+} , Si^{4+} , and Zr^{4+} is critical in modulating the local environment and initiating signaling pathways conducive to bone and dentin regeneration [13]. Baghdadite is seen to promote the proliferation of mesenchymal stem cells, enhances ALP activity, and stimulates matrix mineralization and is thus a potential candidate in dental tissue engineering [11; 14].

Though their biocompatibility and osteoinductive properties have been studied individually for MTA and

Baghdadite, their combination in dental pulp regeneration has not been studied in depth. It is hypothesized that their combination would exhibit synergism by taking advantage of the bioactivity of MTA and the ionic delivery properties and mechanical strength of Baghdadite. The synergy could be translated as increased DPSC viability and mineralization and enhanced clinical performance of pulp-capping and regenerative materials.

Therefore, the present study aims to evaluate and compare the biocompatibility and osteogenic potential of MTA, Baghdadite, and their mixture on human DPSCs utilizing MTT assay for cytotoxicity evaluation and Alizarin Red S staining for mineralization investigation. The present study could shed some information on the development of future regeneration materials for endodontic therapies.

MATERIALS AND METHODS

Study Design and Ethical Considerations

This in vitro experimental study was conducted in the Dental Regenerative Laboratory at Dr. D.Y. Patil Dental College and Hospital. Ethical clearance was given by the Institutional Ethics Committee, and informed consent of all the participants was taken prior to tooth extraction.

Materials and Reagents Used

The following reagents were used for experimentation: 2% Alizarin Red S, Ascorbate-2-phosphate, Sodium Pyruvate, L-Proline, ITS (Insulin, Transferrin, Selenous acid), Transforming Growth Factor Beta3 (TGF- β 3), 0.1% Safranin O, 3-Isobutyl-5-methylxanthine (IBMX), Indomethacin, Insulin, 0.3% Oil Red O, MTT solution (5 mg/ml), DMSO (Dimethyl sulfoxide), TRIzol reagent, chloroform, chilled isopropanol, 75% ethanol, and 100% ethanol. Experimental biomaterials used were Mineral Trioxide Aggregate (Angelus) and Baghdadite (Nano Research Elements).

Extraction of Dental Pulp

Dental pulp was obtained from teeth extracted after informed consent under the institutional ethics approval. Aseptic technique was employed throughout. Teeth were extracted after pre-rinse with chlorhexidine and manipulated with sterilized instruments. The pulp was extracted immediately after tooth extraction and

stored in a sterile transport medium of double-strength antibiotic-antimycotic solution and phosphate-buffered saline (PBS). The samples were transported to the Dental Regenerative Laboratory.

Isolation and Culture of Human Dental Pulp Stem Cells (DPSCs)

Pieces of pulp tissue were seeded onto FBS culture plates. Following incubation at 37°C in 5% CO₂ for 24 hours, Dulbecco's Modified Eagle Medium (DMEM) supplemented with FBS and antibiotics was added. Outgrowth of cells was viewed using an inverted phase contrast microscope. The cells were dissociated using 0.25% Trypsin-EDTA at confluence, centrifuged, then re-seeded in fresh culture medium. The cells were sub-cultured at intervals in T75 flasks until passage 4.

Subculture and Cell Growth

After confluence, cells were trypsin-EDTA harvested, spun down and resuspended in complete media. Cells were seeded in T75 flasks and grown at 37°C in a humid atmosphere. Media were changed every 2–3 days. One flask was used at confluence for flow cytometry and the other for colony forming unit (CFU) assay.

Cell Characterization by Flow Cytometry

Passage 4 cells were trypsinized with 0.25% Trypsin-EDTA and washed with PBS. Cells were resuspended in PBS + FBS and centrifuged. Cells were incubated with the following antibodies: CD105-PE/CD34-FITC, CD73-PE/HLADR-FITC, CD90-PE/CD45-FITC, and one unstained control. The samples were incubated for 30 minutes in the dark and analyzed using FACS and Cell Quest Pro software.

Cell Characterization by Colony Forming Unit Assay

Trypsinized cells passage 4 were stained with trypan blue to assess for viability and counted. 100 viable cells were seeded per 60 mm dish and incubated in 5% CO₂. The colonies were fixed with 0.3% crystal violet after 12 days and examined microscopically for colony formation.

Preparation of Materials

Baghdadite (10 µg) was dissolved in distilled water. Sterile water was added to MTA and created 5 × 3 mm cylinders, incubated for 24 hours at 37°C with 5% CO₂, UV sterilized, and infused in α-MEM. Media was filtered (0.2 µm) and diluted to 1 mg/mL after 7 days. Fixed 10 µg concentrations of MTA and Baghdadite were combined together to create stock solutions in the combination group.

MTT Assay for Cytotoxicity

Passage 4 DPSCs were seeded at 5 × 10³ cells/well and grown to 70% confluence. Cells were exposed to MTA, Baghdadite, or their combination in DMEM + 10% FBS for 48 hours. MTT reagent (5 mg/mL) was added and incubated for 4 hours. Formazan crystals were dissolved in DMSO, and absorbance was read at 560 nm to quantify viability against controls (Fig. 1).

Osteogenic Induction and Mineralisation Assay

Osteogenic medium was prepared with DMEM, FBS, β-glycerophosphate (10 µM), ascorbic acid (2 mM), and dexamethasone (0.1 µM). DPSCs were seeded in 24-well plates and cultured for 24 hours. Three wells were cultured with osteogenic medium, and three control wells were cultured with complete DMEM. Plates were cultured for 13–21 days with bi-weekly replacement of media. Post-incubation, cells were stained with 2% Alizarin Red S (pH 4.1–4.3) and observed under an inverted microscope to determine mineralization (Fig. 2).

RESULTS

Fig. 3 (A–D) shows all events in the flow-cytometry. Surface markers expression for human dental pulp stem cells are positive for CD90-PE-H, CD73-PE-H, CD105-PE-H and negative for CD45-FITC-H, HLADR-FITC-H, CD34-FITC-H.

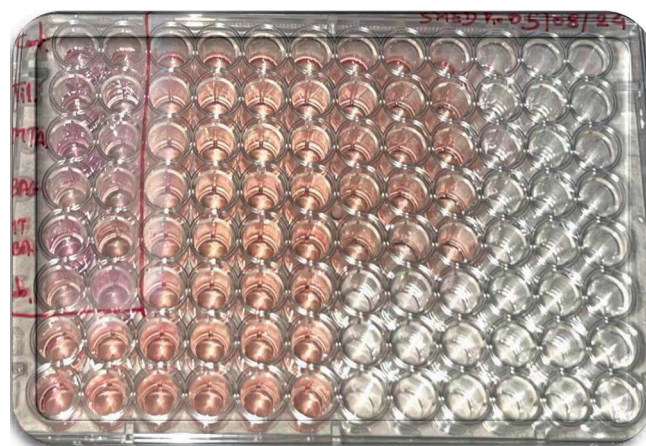


Fig. 1. MTT assay in 96-well plate evaluating the cytotoxicity of MTA, Baghdadite and the combination of MTA and Baghdadite

Рис. 1. МТТ-тест в 96-луночном планшете для оценки цитотоксичности МТА, багдадита и их комбинации

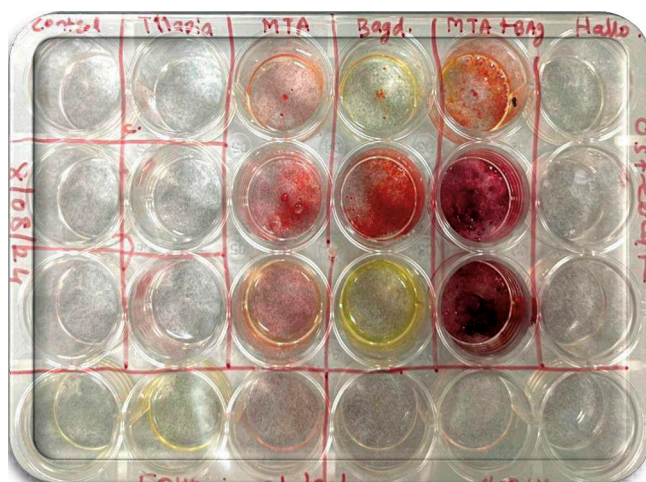


Fig. 2. Alizarin red staining

Рис. 2. Окрашивание аллизарином красным

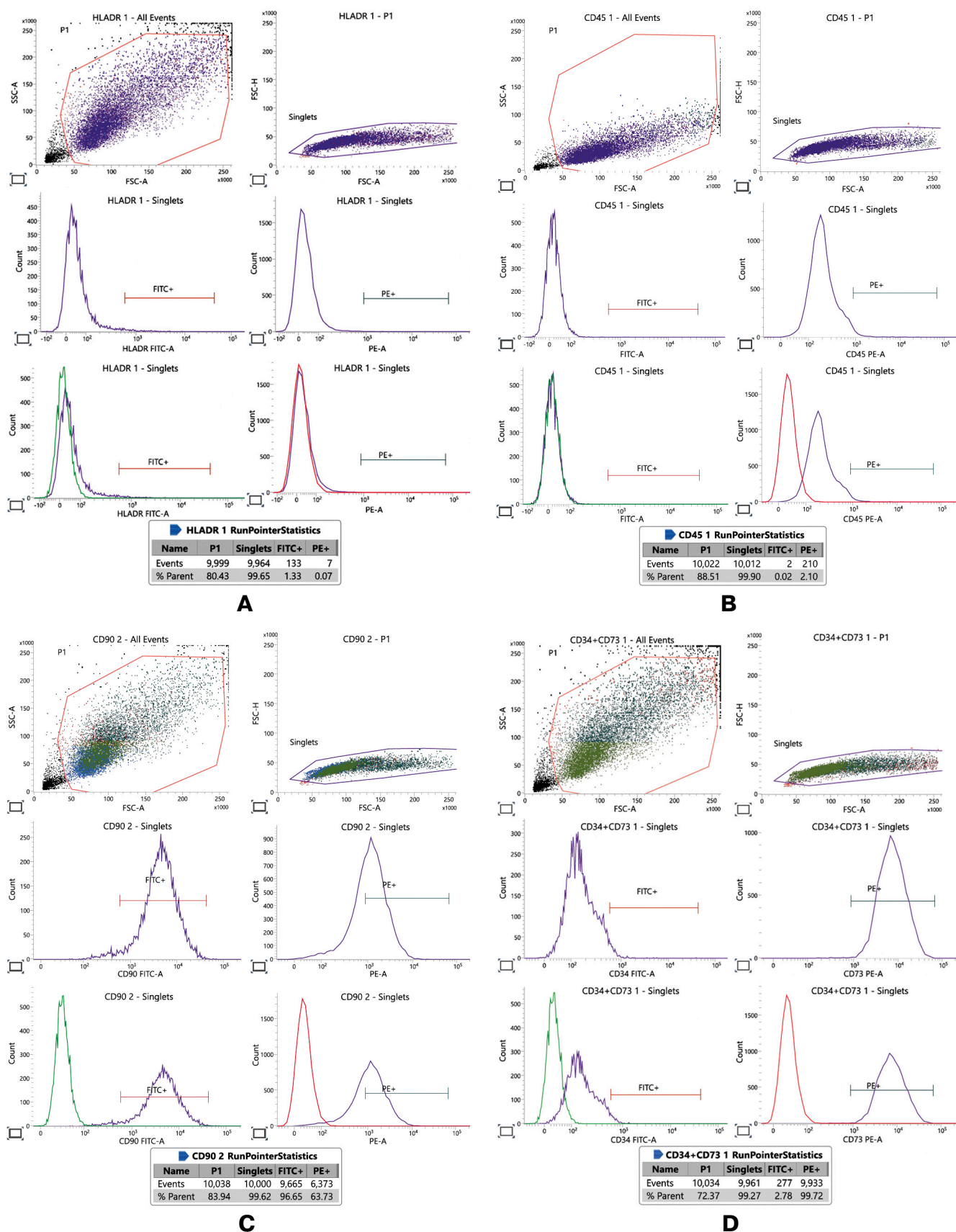


Fig. 3. A–D Showing all events in the flow-cytometry. surface markers expression for human dental pulp stem cells are positive for CD90-PE-H, CD73-PE-H, CD105-PE-H and negative for CD45-FITC-H, HLADR-FITC-H, CD34-FITC-H

Рис. 3. А–Д Демонстрация всех событий проточной цитометрии. Экспрессия поверхностных маркеров для стволовых клеток пульпы человека: положительная для CD90-PE-H, CD73-PE-H, CD105-PE-H и отрицательная для CD45-FITC-H, HLA-DR-FITC-H, CD34-FITC-H

As evident from Table 1, the MTT assay values represent the biocompatibility of the test materials with the hDPSCs. The maximum mean absorbance value of 0.4066 ± 0.0236 was found in the combination group of MTA + Baghdadite, representing the maximum cell viability compared to the application of the two materials separately. The next highest mean absorbance of 0.3975 ± 0.0351 was found in the Baghdadite group, followed by the MTA group (0.3563 ± 0.0451). All three test groups were significantly superior to the control group (0.1552 ± 0.0086) as seen in Fig. 4, confirming the cell viability-improving effect of the biomaterials. The combination group among the three test groups not only possessed the maximum cell viability but also the least variation, representing a consistent cellular response.

Table 1. MTT Assay Results – Cell Viability

Таблица 1. Результаты МТТ-теста – жизнеспособность клеток

Group	1	2	3	Average	Std Dev
MTA	0.4084	0.2984	0.3620	0.3563	0.04509
Baghdadite	0.4402	0.3543	0.3980	0.3975	0.03507
MTA + Baghdadite	0.3771	0.4348	0.4080	0.4066	0.02358
Control	0.1638	0.1466	0.1560	0.1552	0.00860

Table 2. Alizarin Red S Absorbance Values (Raw Data)

Таблица 2. Значения оптической плотности аллизарина красного S (исходные данные)

Group	1	2	3
Control	0.5924	0.5987	0.5245
MTA	0.5595	0.5807	0.5962
Baghdadite	0.4010	0.3993	0.4056
MTA + Bag	1.6780	1.7595	1.6832

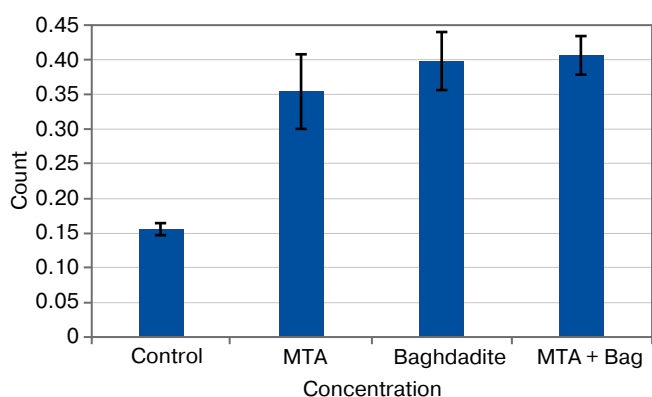


Fig. 4. MTT Assay of Baghdadite, Mineral trioxide aggregate and its combination

Рис. 4. МТТ-тест багдадита, минерального триоксидного агрегата и их комбинации

Osteogenicity of the materials was assessed by Alizarin Red S staining and is given in Tables 2 and 3. Unprocessed absorbance readings of triplicates in Table 2 showed that MTA + Baghdadite had the greatest calcium deposition with readings of 1.6780–1.7595. Baghdadite alone exhibited the lowest mineralization ability (mean: 0.4020), whereas MTA and control groups exhibited either greater or lesser similar absorbance readings of 0.5245–0.5987.

Table 3 shows mean absorbance and standard deviation of groups. Mean absorbance (1.7069 ± 0.0373) of the combination group (MTA + Baghdadite) was significantly higher than MTA (0.5788 ± 0.0150) and Baghdadite (0.4020 ± 0.0027), to the benefit of the improved mineralization ability of the combination group. Mean of the control group was 0.5719 ± 0.0336 , nearly equal to MTA but significantly lower than the combination group as seen in Fig. 5. Fig. 6 shows the microscopic images of mineralization of all the groups done using Alizarin red stain.

DISCUSSION

The present in vitro research was aimed at evaluating and comparing the cytocompatibility and osteogenic differentiation potential of MTA, Baghdadite, and their combination on human dental pulp stem cells (hDPSCs). The results evidently indicate that the combination of MTA and Baghdadite is superior in terms of being more biocompatible and mineralization capable compared to their individual forms.

Table 3. Alizarin Red S Quantification – Averages and SD

Таблица 3. Количественная оценка аллизарина красного S – средние значения и стандартное отклонение

Metric	Control	MTA	Baghdadite	MTA + Bag
Average	0.5719	0.5788	0.4020	1.7069
Std Dev	0.0336	0.0150	0.0027	0.0373

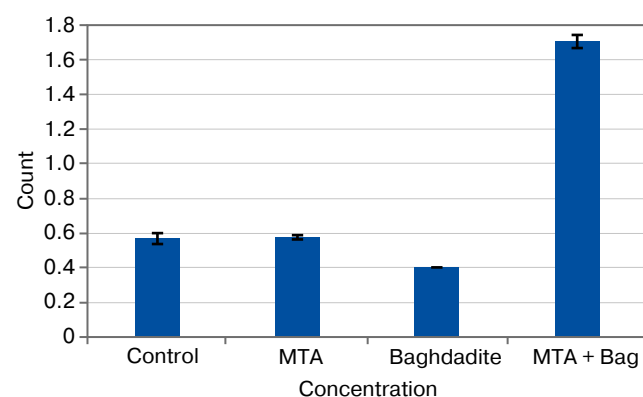


Fig. 5. Osteogenic potential of Baghdadite, Mineral trioxide aggregate and its combination

Рис. 5. Остеогенный потенциал багдадита, минерального триоксидного агрегата и их комбинации

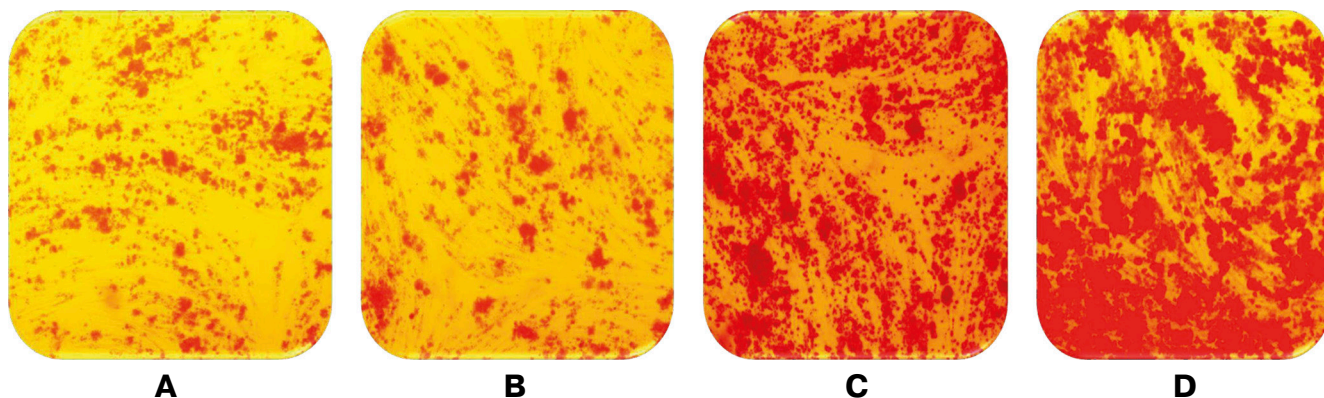


Fig. 6. Microscopic images of mineralization using alizarin red stain: A – Control, B – MTA, C – Baghdadite, D – MTA + Baghdadite

Рис. 6. Микроскопические изображения минерализации с использованием окраски аллизарином красным: A – контроль, B – МТА, C – Багдадит, D – МТА + Багдадит

MTA has been the gold standard material in endodontic vital pulp therapy and regenerative endodontics because of its sealing capacity, biocompatibility, and ability to promote hard tissue formation [15; 16]. In this study, MTA has exhibited strong biocompatibility (mean OD: 0.3563) and moderate mineralization potential (mean OD: 0.5788) as observed in earlier reports [17; 18]. For example, Reyes-Carmona et al. reported that MTA induced the expression of osteogenic markers like ALP and osteocalcin in human osteoblast-like cells and induced deposition of mineralized matrix [17]. Similarly, Gomes-Filho et al. reported that MTA induced mild inflammatory response and induced mineralized tissue formation in subcutaneous connective tissue in vivo [18].

However, MTA's extended setting time and primary cytotoxicity have been of concern due to its increased pH [19]. In this study, while MTA favored cell viability, absorbance was lower when compared to Baghdadite and the blended group, a sign that could be indicative of early-stage cytotoxic effects previously reported. Gancedo-Caravia and Garcia-Barbero wrote that MTA's primary alkalinity might lead to small reductions in the viability of fibroblasts at the first 48 hours [20]. Therefore, the results of this study affirm the benefits and drawbacks of MTA in a regenerative environment.

Baghdadite is a calcium-zirconium-silicate ceramic and has been identified as a potential biomaterial due to its osteoconductivity, mechanical properties, and desirable degradation pattern [21]. Baghdadite alone in this research showed greater cell viability (mean OD: 0.3975) than MTA and caused moderate mineralization (mean OD: 0.4020). Schumacher et al. found that Baghdadite scaffolds promoted osteoblast proliferation and calcium phosphate deposition in vitro [22].

Notably, the current study is one of the few evaluations of Baghdadite in a dental pulp context. While most of the earlier research has focused on orthopedic and bone regeneration models, our finding extends its application to dental stem cells for the first time. Previous studies proved that Baghdadite ceramics support mes-

enchymal stem cell (MSC) adhesion, proliferation, and osteogenic differentiation primarily through controlled release of Ca^{2+} , Si^{4+} , and Zr^{4+} ions, reported to initiate osteogenic pathways [11; 23]. As an example, Roohani-Esfahani et al. found that nanostructured Baghdadite scaffolds promoted osteoblast proliferation and calcium phosphate deposition in vitro, confirming its osteoconductive nature [14]. While the lack of mineralization in the current study with Baghdadite alone is a testament to its baseline bioactivity, the comparatively lower response in the current study compared to the MTA+Baghdadite group could be a function of variability in cell type and osteoinductive sensitivity. DPSCs have been shown to have disparate signaling thresholds compared to bone marrow-derived MSCs and to be more demanding or require longer-duration inductive stimuli in order to realize maximum osteogenic potential [24].

The strongest outcome of our study was the enhanced performance of the combination group (mean viability OD: 0.4066; mineralization OD: 1.7069), demonstrating a synergistic interaction. To our knowledge, this is the first investigation on the direct study of the effect of co-use of Baghdadite and MTA on hDPSCs. The enhanced results can be ascribed to various factors, including the additive release of bioactive ions (Ca^{2+} , Si^{4+} , Zr^{4+}), increased surface microtopography, and a better pH balance.

Literature has proven that silicate and calcium ions are influential in triggering osteogenic signaling pathways such as Wnt/ β -catenin and MAPK that are crucial in DPSC differentiation [25; 26]. For instance, Zhou et al. proved that silicate ions of calcium silicate scaffolds triggered Wnt signaling and enhanced RUNX2 and ALP expression in DPSCs [27]. Furthermore, Zr^{4+} ions of Baghdadite have been associated with improved mechanical properties and cell-matrix interactions, in evidence of this, research by Lu et al. established that Baghdadite scaffolds caused much greater increases in the expression of RUNX2, osteopontin, bone sialoprotein, and osteocalcin genes in human osteoblasts than HA/TCP scaffolds. Fur-

thermore, Baghdadite scaffolds significantly induced RUNX2 and osteopontin gene expression in human adipose-derived stem cells (ASCs). In co-culture models, the use of Baghdadite scaffolds enhanced the osteogenic gene expression in ASCs and osteoblasts, signifying a modulatory influence on cell-cell interaction relevant to bone regeneration. The findings above prove that Baghdadite scaffolds not only improve the osteogenic differentiation of osteoblasts and ASCs but also modulate their cross talk, thereby enhancing their osteogenic potential [28].

Interestingly, similar synergistic effects have also been reported in other composite material systems. Gandolfi et al. experimented with the combination of MTA and calcium phosphate compounds and found much higher cell proliferation and ALP activity than MTA alone [29]. Similarly, Kim et al. in the present study observed that MTA combined with bioactive glass exhibited higher mineralization potential than plain MTA in DPSCs, as shown by higher ALP activity, denser Alizarin Red S staining, and higher expression of osteogenic markers such as DSPP, ALP, and bone morphogenetic protein-2 (BMP-2) [30]. These results validate the hypothesis that judicious combinations of bioactive materials can deliver synergistic biological effects more than their additive effect.

The mechanisms involved in the enhanced mineralization in the combination group are likely to involve complex ionic and molecular interactions. Calcium ions released during MTA have been shown to enhance nucleation and as second messengers in intracellular osteogenic signaling cascades [31]. Conversely, silicate and zirconium ions in Baghdadite would most likely enhance cell adhesion and osteoblastic gene expression, as shown by Roohani-Esfahani et al. [14].

Additionally, the buffering action of Baghdadite would also reduce the surplus alkalinity of newly mixed MTA, reducing early cytotoxicity and creating a more stable environment for cell attachment. The initial setting of MTA results in a pH level of more than 12, which has been associated with early cytotoxicity [9]. By modifying this environment, Baghdadite would be able to preserve DPSC viability in the early hours of exposure, and this could be the reason for the higher and homogeneous viability values of our study's combination group (with the lowest standard deviation of all groups).

Few studies have directly evaluated the osteogenic potential of combinations of bioceramics on dental stem cells. Bottino et al. described a nanofibrous scaffold with incorporated bioactive glass nanoparticles and provided evidence of enhanced odontogenic differentiation of DPSCs with enhanced DSPP and DMP1 expression [32]. While their scaffold was of a different nature from ours, focusing on ionic release and surface bioactivity as two of the key differentiation inducers is consistent with our findings.

Similarly, calcium silicate-contained materials such as Biodentine have been studied for their effect on dental pulp stem cells (DPSCs), with favorable results comparable to or even better than MTA. For instance, Phang et al. demonstrated that Biodentine eluents in-

duced significantly high expression of the osteogenic markers RUNX2 and ALP in DPSCs and induced in vitro mineral deposition [33]. Küden et al. also reported that Biodentine and MTA exhibited high bioactivity and cytocompatibility, with the former exhibiting slightly greater cell viability and calcium nodule formation [34]. These findings attest to the capability of silicate-based cements to support odontogenic differentiation of DPSCs. Nevertheless, in our study, the combination of MTA with Baghdadite exhibited an even higher degree of mineralization than has previously been associated with MTA or Biodentine alone. This suggests that the incorporation of zirconium-based silicates – such as Baghdadite – can increase the bioactivity profile of conventional tricalcium silicate material. Incorporation of other bioactive ions such as Zr^{4+} and Si^{4+} into the composite might increase cell-matrix interaction, induce osteogenic gene expression, and offer a better ionic microenvironment for regenerative endodontics.

Although with encouraging outcomes, this research is not without limitations. Being an in vitro model, it can't replicate the intricate interaction of immune cells, blood flow, and mechanical stress in vivo. Moreover, we did not analyze gene expression markers or cytokine profiles, which would give more mechanistic insight into the differentiation process. Future research should incorporate quantitative PCR for markers such as ALP, RUNX2, DSPP, and osteocalcin and long-term in vivo analysis of biodegradability and inflammatory response.

The clinical interest of this hybrid material is its enhanced biological behavior and purported enhanced handling characteristics. If confirmed in vivo, the MTA + Baghdadite mixture would be an excellent pulp-capping material with the potential for dentin bridge formation and pulp vitality maintenance. The mixture also has the potential to show additional radiopacity and mechanical properties based on the zirconium content, which are clinically useful properties.

The novelty of this study lies in demonstrating, for the first time, that the synergy of MTA and Baghdadite yields superior outcomes in DPSC models against the use of either material in isolation. This not only adds to the sparse literature of Baghdadite in dentistry but also introduces a new paradigm in the optimization of regenerative materials through biomaterial synergy.

CONCLUSION

This in vitro study indicates the increased osteogenic potential and biocompatibility of a novel mixture of MTA and Baghdadite in human dental pulp stem cells. Compared to the use of the individual materials, the mixture not only supported increased cell viability but also significantly improved mineralized nodule formation. The enhanced effect is probably attributed to improved ionic release kinetics, favorable surface characteristics, and pH control. These findings suggest that the MTA–Baghdadite mixture might be a superior pulp-capping or regenerative endodontic material. Additional studies incorporating molecular analysis and in vivo models are required, however, to confirm its clinical application.

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