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Original Article

The additive effect of iloprost on the biological properties of Mineral trioxide aggregate on mesenchymal stem cells

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Abstract *Background/purpose:* Iloprost has been proposed as a potential biomaterial owing to angiogenic and odontogenic properties. However, the liquid form can limit its use during clinical applications. Mineral trioxide aggregate (MTA) has been used for various dental applications in which cell–material interaction is essential. This study aimed to investigate additive effects of iloprost on the biological properties of MTA on the viability, attachment, migration and differentiation of human mesenchymal stem cells (hMSCs).

Materials and methods: Standardized human dentin disks were prepared. MTA was prepared by mixing distilled water or iloprost solution, and the lumen of the disks was filled with MTA or MTA-iloprost. hMSCs on disk alone and hMSCs on culture plates were used as controls. Cell viability and attachment were measured after 1, 7 and 14 days using AlamarBlue assay and scanning electron microscopy (SEM). Cell migration in MTA or MTA-iloprost extracts was determined using a wound-healing model.

Osteogenic differentiation was evaluated by real-time reverse transcriptase polymerase chain reaction for alkaline phosphatase (ALP), bone sialoprotein (BSP), osteocalcin (OCN), and osteopontin (OSP) gene expressions after 7 and 14 days of osteogenic induction.

Results: Cells on MTA-iloprost surface showed similar viability with MTA at 1 and 14 days but enhanced cellular viability and cell spreading compared to MTA at 7 days ($p < 0.05$). Cell

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migration was similar by MTA-iloprost and MTA extracts ($p > 0.05$). MTAiloprost significantly up-regulated BSP, OCN and OSP expressions compared to MTA ($p < 0.05$).
Conclusion: The addition of iloprost to MTA improved the initial cell viability and osteogenic potential of hMSCs.

Introduction

Calcium silicate-based materials (CSBMs) have been used in various endodontic applications, including vital pulp therapy, regenerative endodontics, perforation repair and root-end sealing.[1] These bioactive materials can induce the proliferation and odontogenic/osteogenic differentiation of stem cells.[2] Mineral trioxide aggregate (MTA) represents the first developed CSBM, and has been widely used during endodontic applications.[3]

For extracellular matrix synthesis and mineralization of stem cells, angiogenesis is essential for recruiting stem cells and providing oxygen and nutritional supplies.[4] Previous studies have shown that angiogenic signaling molecules can improve the initial proliferation and differentiation of stem cells.[5,6] Although MTA has shown high clinical success rates, a recent study showed a diminished proangiogenic effect of MTA that can affect the initial cell—material interaction and its regenerative properties.[7] To improve its angiogenic properties, the combined use of MTA with growth factors have been explored in the literature.[8,9] However, there are no standard combinations of these materials due to the limitations of clinical translations of the growth factors.

Prostacyclin, a member of the endogenous prostanoid family, is known for its potent angiogenic activity.[10] Iloprost is an exogenous long-acting prostacyclin that stimulates the dilation of blood vessels.[11] In medicine, iloprost is used in cases where a lack of blood flow damages the tissues.[12] Previous studies showed that iloprost can induce angiogenic differentiation by vascular endothelial growth factor (VEGF) expression in human dental pulp cells.[13] Iloprost was also reported to enhance angiogenesis in a growth factor-free tooth slice organ culture system,[14] and promote tertiary dentin formation when applied to exposed pulp tissue in a rat model.[15] Therefore, iloprost has been suggested as a potential endodontic biomaterial with angiogenic and odonto/osteogenic properties. However, the use of iloprost in a liquid form limits its functions and delivery at the targeted site and requires the investigation of potential carrier materials for its clinical use.[15]

Considering the limited proangiogenic activity of MTA and limitations with the clinical delivery of iloprost during regenerative applications, the combined use of MTA and iloprost is considered to induce a synergistic effect that improves their biological and functional properties. Therefore, the present study aimed to evaluate the additive effects of iloprost mixed with MTA on the viability, migration, and differentiation of mesenchymal stem cells.

Materials and methods

Specimen preparation

This study was approved by the Institutional Review Board at the College of Dentistry Research Center (PR 0094) of King Saud University (E-19-3908). Dentin disks were prepared from freshly extracted mandibular premolars without caries and resorption as described previously.[16] Briefly, the crowns were sectioned to obtain 2-mm-thick transverse slices, the diameter of the outer disks was standardized to 5—6 mm. The diameter of the central part was prepared to 2.5 mm for experimental groups and dentin disks without a central part preparation were used as a control (dentin disk group). Dentin disks were then sterilized using gamma irradiation at a dose of 25 kilo gray before use for experimental procedures.

Dentin disks were then assigned to the following groups; dentin disk, MTA group and MTA-iloprost group. For the MTA group, MTA powder (ProRoot, Dentsply, Tulsa, OK, USA) was mixed with distilled water in accordance with the manufacturer's instructions. For the MTA-iloprost group, the MTA powder was mixed with iloprost solution (10^{-6} mol/L) (MedChemExpress Inc., NJ, USA) using the same powder to liquid ratio. The concentration of iloprost was used according to a previous study showing the highest mineralization effect of iloprost at 10^{-6} mol/L compared to 10^{-7} and 10^{-8} mol/L.[17] The lumens of the dentin disks were filled with MTA or MTA-iloprost. The cells on dentin disks alone or on culture plates were used as the control groups. Each disk was placed in a well of 24-well plates and incubated in a cell culture incubator (HERAcell 150i, Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C and 5% CO_2 humidity for 4 h for the material setting.

Cell culture and viability assay

Immortalized human bone marrow-derived mesenchymal stem cells (hMSCs) cell line was created from normal bone marrow mesenchymal stem cells by the overexpression of human telomerase reverse transcriptase gene.[18] Cells were cultured according to the protocol described in the supplementary material. Human MSCs (5×10^4 cells/well) were seeded on each sample surface and incubated for 1, 7, and 14 days by changing the culture medium three times a week. After each time period, the culture medium was removed and the cells were incubated with AlamarBlue agent (AbD Serotec, Kidlington, UK) for 4 h. The

fluorescence signal from the samples was measured at an excitation wavelength of 530 nm and an emission wavelength of 590 nm using a SpectraMax M5/M5e Multimode Plate Reader (Molecular Devices/BioTek®, Winooski, VT, USA), and the data were obtained using SoftMax Pro Microplate Data Acquisition and Analysis Software (Molecular Devices/BioTek®).

Determination of cell morphology using scanning electron microscope

Cells were seeded on disks filled with MTA and MTA-iloprost and disks alone as described above in the cell viability assay. Cells seeded on glass coverslips were used as control. The specimens were fixed in 2.5% glutaraldehyde and dehydrated in a graded series of ethanol. All samples were dried using a critical point dryer and coated with gold. Specimens were analyzed by scanning electron microscopy (SEM, JSM-6360 LV, JEOL Corp., Peabody, MA, USA) at 1000× magnification.

Migration assay

The migration ability of hMSCs was evaluated using a wound-healing model. Cell migration was determined using material extracts. The extracts were prepared and artificial scratch was created according to the protocol described in supplementary material. The cells were incubated with culture medium (control) or conditioned medium containing MTA or MTA-iloprost extracts. The scratched area was captured at 0, 24, and 48 h using a time lapse imaging system (Evos FL Auto Imaging System,* Life Technologies, Carlsbad, CA, USA). Cell migration was determined according to the percentage of the scratched area closure using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Determination of the osteogenic differentiation ability of hMSCs using real-time reverse transcriptase polymerase chain reaction

The expression levels of the osteogenic markers, alkaline phosphatase (ALP), bone sialoprotein (BSP), osteocalcin (OCN), and osteopontin (OSP) were determined using reverse transcriptase polymerase chain reaction (RT-qPCR). Human MSCs were incubated in an odonto/osteogenic differentiation medium containing 50 μg/ml ascorbic acid, 10 nM vitamin D, 10 mM β-glycerophosphate, and 100 nM dexamethasone for 7 and 14 days. Cells were seeded as described above in the cell viability assay. Total RNA was extracted, and real-time PCR was performed as described in the supplementary material. Primer sequences for differentiation markers are detailed in Table 1.

Table 1 Reverse transcription polymerase chain reaction primers and conditions.

Gene symbol	Direction	Primer sequence
ALP	Forward	GACGGACCCTCGCCAGTGCT
	Reverse	AATCGACGTGGGTGGGAGGGG
BSP	Forward	CAGTTCAGAAGAGGAGG
	Reverse	TCAGCCTCAGAGTCTTCATC
OCN	Forward	GGCAGCGAGGTAGTGAAGAG
	Reverse	CTCACACACCTCCCTCCTG
OSP	Forward	CAGTTCAGAAGAGGAGG
	Reverse	TCAGCCTCAGAGTCTTCATC
GAPDH	Forward	CTGGTAAAGTGGATATTGTTGCCAT
	Reverse	TGGAATCATATTGGAACATGTAAACC

ALP, alkaline phosphatase; BSP, bone sialoprotein; OCN, osteocalcin; OSP, osteopontin.

Statistical analysis

All the experimental procedures were conducted in triplicate with three measurements in each group ($n = 9$/group). The homogeneity of the data was analyzed using the Shapiro–Wilk test, and the data were analyzed using one-way analysis of variance followed by Tukey's post hoc test for multiple comparisons using SPSS software (version 21.0, SPSS Inc., Chicago, IL, USA). Data are presented as the means ± standard deviations, and the differences were considered significant at $p < 0.05$.

Results

Cell viability and morphology

The cells in the control-disk group showed a significant reduction in the cell viability compared to the cells cultured on well plates on day 1 ($p < 0.05$). On days 7 and 14, cell viability was not altered by the presence of dentin. Therefore, the results in the experimental groups were compared with those of the control-disk group. On days 1 and 14, the experimental groups showed similar cell viability compared to the control-disk group ($p > 0.05$). The cell viability in the control-disk group and MTA-iloprost group showed higher cell viability than the MTA group on day 7 ($p < 0.05$) (Fig. 1A).

Representative scanning electron micrographs are shown in Fig. 1B. Human MSCs showed higher initial cell spreading in the control group but lower cell attachment and more contracted cell morphology in the control-disk group (Fig. 1B, cells are shown by yellow arrows). The cells showed rounder cell morphology on the MTA or MTA-iloprost surface on day 1. On day 7, hMSCs appeared to be flattened and the cells in the MTA-iloprost group showed higher spreading than those in the MTA group. On day 14, cells were fully spread and well attached to the dentin covering the material surfaces.

Cell migration

Representative images are shown in Fig. 2A. Quantitative results showed that the hMSCs incubated with extracts of MTA and MTA-iloprost decreased the migration ability compared to the control group ($p < 0.05$) on day 1 (Fig. 2B). However, the cells showed similar migration ability on day 2 in the experimental and the control groups ($p > 0.05$) (Fig. 2B).

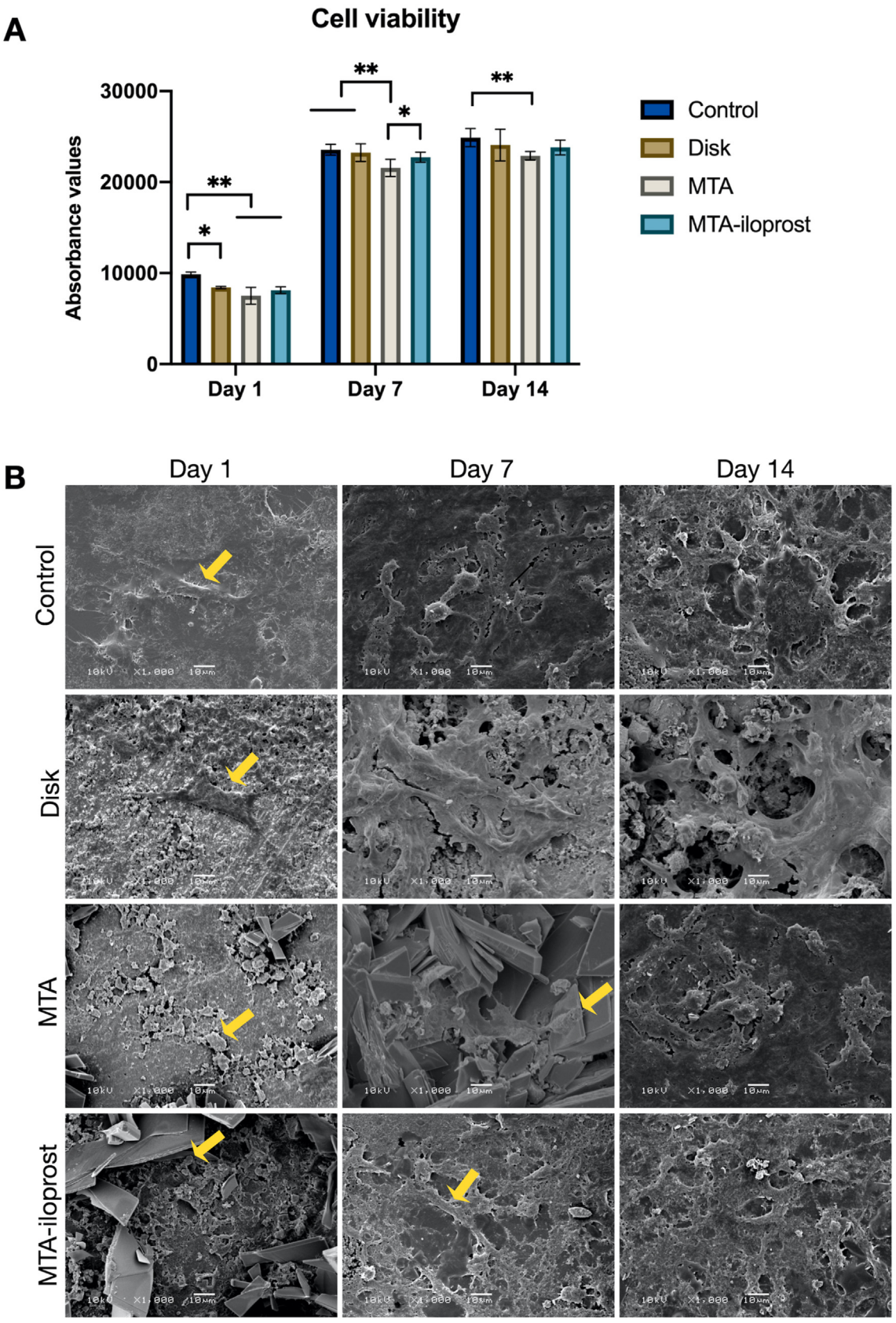

Figure 1 (A) Cell viability using an AlamarBlue assay (*$p < 0.05$; **$p < 0.001$). (B) Representative scanning electron micrographs of human mesenchymal stem cells grown on glass cover slips (control), dentin disks, MTA, and MTA-iloprost at 1, 7, and 14 days (scale bars: 10 μm, 1000× magnification).

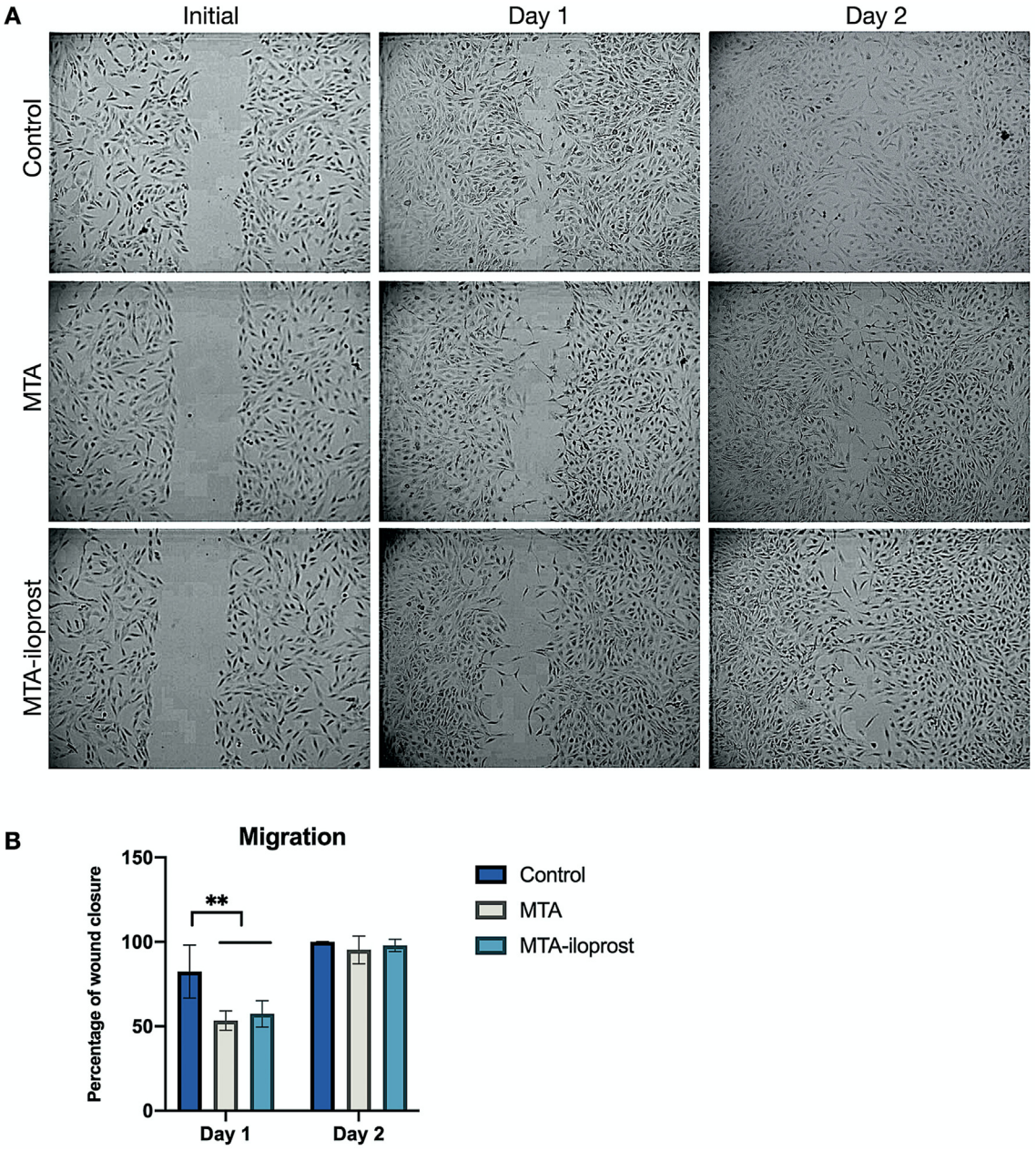

Figure 2 (A) Cell migration in the presence of culture medium, extracts of MTA and extracts of MTA-iloprost using an in vitro scratch wound healing assay. (B) Cell migration is represented as the percentage of the closure of the scratched area (*$p < 0.05$; **$p < 0.001$).

Osteogenic marker expressions

The expression levels of differentiation markers were similar between the control and disk groups ($p > 0.05$). Therefore, the results of the experimental groups were compared with those of the control-disk group and results are collectively presented in Fig. 3. On day 7, the control and experimental groups showed similar ALP expression ($p > 0.05$). On day 14, the ALP expression levels in the MTA and MTA-iloprost groups resulted in a 2.2- and 1.6-fold increase than those in the disk group, respectively ($p < 0.05$). The MTA group also showed a significant increase compared to the MTA-iloprost group on day 14 ($p < 0.05$).

MTA-iloprost upregulated the BSP expression on day 7 (3.9-fold increase) and day 14 (1.6-fold increase) compared to the control-disk and MTA groups ($p < 0.05$). BSP expression in the MTA group was significantly increased compared to the control-disk group on day 7 ($p < 0.05$, 1.8-fold increase) with similar BSP expression on day 14 ($p > 0.05$). OCN expression in the MTA-iloprost group on day 7 and day 14 was significantly upregulated compared to the control-disk and MTA groups ($p < 0.05$). The MTA and MTA-iloprost groups showed significant increase in OSP expression on day 7 (1.7- and 2.1-fold change, respectively) and on day 14 (1.6- and 2.2-fold change, respectively) compared to the control-disk group ($p < 0.05$). In addition, OSP expression

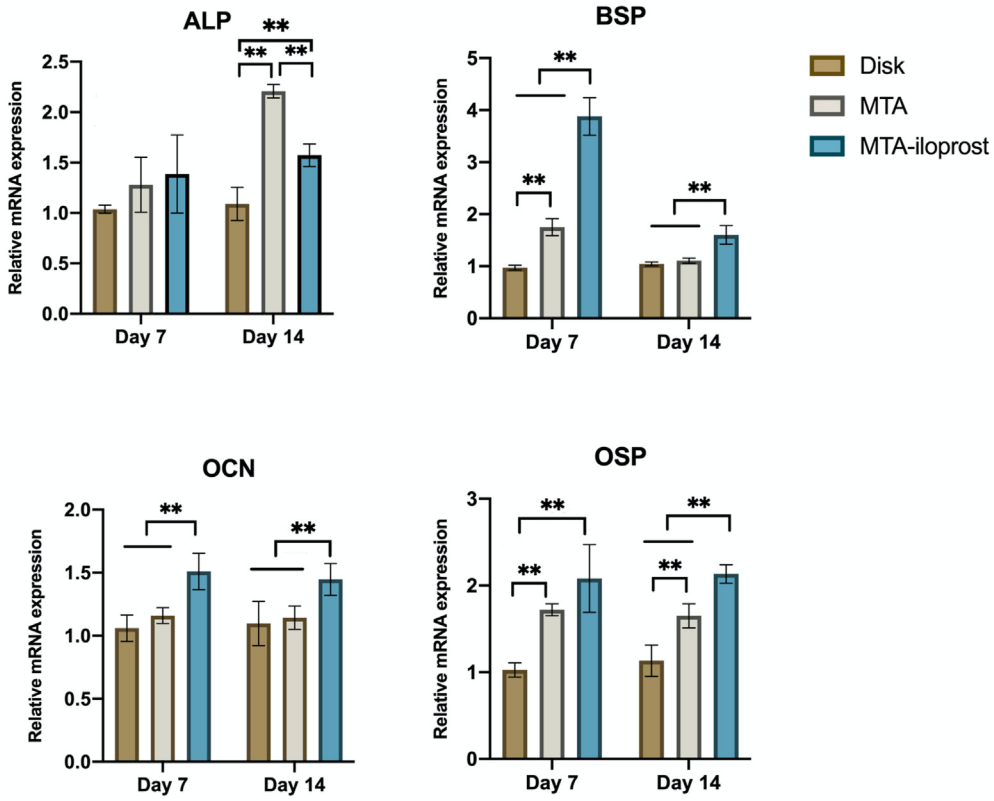

Figure 3 The mRNA expression levels of the osteogenic marker of ALP, BSP, OCN and OSP from human mesenchymal stem cells cultured on dentin disks, and dentin disks filled with MTA or MTA-iloprost (*$p < 0.05$; **$p < 0.001$).

was significantly increased in the MTA-iloprost group compared to the MTA group on day 14 ($p < 0.05$).

Discussion

Enhancing cell—material interactions is essential for designing new biomaterials with improved functional properties, such as biocompatibility and bioactivity. Owing to angiogenic and odontogenic potential of iloprost, the additional effect of iloprost on the biological properties of MTA was investigated. MSCs are adult stem cells that differentiate into odontoblast-like cells under the stimulus, and they are capable of regenerating pulp-like tissue.[19,20] Compared to dental pulp stem cells, bone marrow MSCs (BMMSCs) have similar characteristics in their perivascular niche, gene expression profiles, and differentiation abilities.[21] Considering the use of MTA in various endodontic applications that requires cellular mineralization and odonto/osteogenic differentiation of the stem cells from the surrounding tissues, human BMMSCs were used in the present study. To mimic the dental micro-environmental niche, the materials were used in the presence of dentin disks. The findings showed an initial decrease in cell viability in the presence of dentin disks compared to cells on culture plates, which can be considered due to the 3D surface properties of the dentin compared to 2D flat surfaces.[22]

The present results showed that MTA decreased cell viability compared to the control group on day 7. A similar finding was reported in a previous study in which fibroblasts were exposed to undiluted extracts of MTA.[23] However, contradictory results have been also reported showing similar cell viability in the MTA and control groups.[24,25] The differences might be related to methodological differences, such as using different cell lines and the setting status of the material. The cell viability of hMSCs was not affected by exposure to MTA-iloprost, and after 7 days of exposure, the MTA-iloprost showed superior biocompatibility compared to the MTA group. This finding can be attributed to the ability of iloprost to upregulate proangiogenic factors and the initial cellular proliferation.[13] Furthermore, the long setting time of MTA might allow for a longer and controlled release of iloprost during the setting of the material. Further studies are required to assess the additive effect of iloprost on various physical properties of MTA. Morphological changes of the cells on the material surfaces were also investigated using SEM evaluation. The findings showed that hMSCs had reduced cellular spreading on the dentin disk compared to the control group aligned with the findings from the cell viability assay. Cells on the material surface appeared round, which might be caused by the leaching of substances from dental materials that affected cell—material interactions.[26] However, on days 7 and 14, the cells were flattened and covered the surface of the substrate, indicating the biocompatibility of the materials is consistent with a previous finding.[27]

Cell migration is essential step in dental pulp regeneration.[28] In the first 24 h, MTA extracts negatively affected hMSC migration and led to an almost 50% reduction in cell migration, which is consistent with a previous study.[23]

Iloprost has a role in cell migration by upregulating matrix metalloproteinase 9, which is responsible for extracellular matrix degradation.[29] In the present study, the addition of iloprost did not improve cell migration compared to MTA alone that can be attributed to the 2D environment of the cultured cells and needs to be explored via in vivo experiments or 3D culture conditions.

To understand the additive effect of iloprost and MTA on hMSC osteogenic differentiation potential, the expression of osteogenic differentiation markers was assessed. ALP is an early marker of osteogenic differentiation,[30] and OCN and BSP are major noncollagenous proteins expressed in the middle and later stages of differentiation, respectively.[31] OSP is an extracellular matrix acidic glycoprotein associated with the repair of mineralized tissues.[32] In the current study, dentin disks did not affect cell differentiation compared to the cells on culture plates, which might be explained by the absence of dentin conditioning with ethylenediamine acetic acid, which releases odontogenic-related growth factors before material placement.[16] The present findings showed that osteogenic marker expression was significantly upregulated in the cells on MTA-iloprost compared with MTA, suggesting that the material properties were enhanced with the addition of iloprost. This finding can be attributed to the upregulation of VEGF caused by iloprost, which induces osteogenic/odontogenic marker expression.[33] However, as a limitation of the current study, only the osteo/odontogenic activity of MTA-iloprost was evaluated. Angiogenic activity of stem cells and endothelial cells has been expected to increase but further studies are required to validate this assumption. The higher expression of ALP observed on day 14 in the MTA group compared to the MTA-iloprost group suggests that MTA induced early cell differentiation while the cells in the MTA-iloprost group showed mature cellular mineralization.

In conclusion, our study showed that the addition of iloprost to MTA significantly improved the initial cell viability and osteogenic differentiation potential of mesenchymal stem cells and can be potential biomaterial for future clinical applications. Further in vivo research is necessary to assess the associated cellular functions in vivo.

Declaration of competing interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jds.2021.03.018.

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