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Research article

The role of NMT induction on odontogenic proliferation and differentiation of dental pulp stem cells

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ABSTRACT

This study was conducted to investigate the odontogenic proliferation and differentiation of dental pulp stem cells (DPSCs) after induction by nanoparticle mineral trioxide (NMT). DPSCs were isolated from permanent teeth and placed in tubes containing Dulbecco's modified Eagle's medium, followed by immunocytochemistry analysis. The viability of DPSCs exposed to NMT was measured using MTT assay with trypan blue dye exclusion. Alkaline phosphatase (ALP) activity was evaluated using ALP colorimetric reactions by reacting NMT supernatants with fluorescent-specific ALP substrates. The concentration of osteocalcin was determined using an instant human osteocalcin enzyme-linked immunosorbent assay (ELISA) kit. A human dentin sialophosphoprotein (DSPP) ELISA kit coated with anti-human DSPP antibody was employed to measure DSPP levels. There was a significant difference between ALP activity after exposing the cells to NMT and trioxide mineral aggregate on days 3, 7, and 21. Osteocalcin activity showed a significant difference on days 3, 7, 14, and 21. There was a significant difference in DSPP levels on days 7 and 21. DPSCs exposed to NMT and to trioxide mineral aggregate showed extracellular matrix formation on day 7 and 14, respectively. Furthermore, NMT may effectively increase the proliferation and differentiation of DPSCs as well as their maturation toward odontoblasts.

1. Introduction

Until recently, dental caries has become a common health concern in Indonesia. Based on the results of National Basic Health Research, the decay missing filling teeth index in Indonesia is quite high at 4.6, which means that the tooth decay average reaches 460 pieces of teeth per 100 people [1]. Dental caries in children can affect mastication, which may disturb the growth, development, and quality of children's life [2]. On this issue, a proper dental diagnosis and treatment to keep the teeth healthy, known as restorative dentistry, is required. The basic principle of restorative dentistry is to maintain the health of damaged dentin-pulp and achieve functional and successful healing of open pulp formed by caries or trauma [3]. Open pulp healing is characterized by regeneration of odontoblast-like cells from subodontoblast cells and repair of the open dentine via dentinal bridge formation. One factor affecting pulp recovery is the penetration of bacteria through the surface of the pulp filler material [4]. Trioxide mineral aggregate (MTA) [5], a pulp filler commonly used in endodontic treatment [6], is often used to treat vital pulps with reversible inflammation in children.

According to previous works, MTA is only effective in 66.67% of pulpal healing treatments [6, 7]. MTA has an extended setting time [8] due to its large particle size that prevents cell membrane penetration. The MTA particle size has important effects on the success of pulp healing. Nano-sized MTA particles can easily pass through the cell membrane and interact with biological molecules [9]. Nanoparticle mineral trioxide (NMT) can function effectively in existing cell systems because of its larger nanoparticle surface area, which increases the bioactivity of the material to stimulate the growth of cells, including odontoblasts. According to Effendi et al., NMT intensifies the proliferation of human exfoliated deciduous stem cells [10].

Odontoblasts can form a single layer and synthesize a matrix to further produce dentin. The dentine matrix formation during the mineralization process can be identified and quantified by the protein or gene expression level [11]. The important odontoblasts secreted proteins

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are alkaline phosphatase (ALP), an enzyme that stimulates calcification, osteocalcin (OC), and dentin sialophosphoproteins (DSPPs), which stimulate secondary dentin formation [12]. Current research has focused mostly on stem cells having the ability or potential to proliferate and differentiate into specific cell types which make up various tissues of the body. In addition, dental pulp stem cells (DPSCs) are a heterogeneous population of cells isolated from the human permanent third molar pulp. Stem cells derived from pulp tissue can differentiate into either to odontoblast or fibroblast strains [13].

Cell maturation is a biological process involving the formation of calcium deposits and the extracellular matrix. The presence of calcium deposits indicates the occurrence of one regeneration [14]. The effect of NMT on stem cell differentiation towards odontoblasts is observed as dentin matrix formation during the mineralization process, which can be identified and quantified at the protein level by analyzing odontoblast phenotype markers, such as ALP, OC, and DSPP [11]. Therefore, in the present study, we investigated the ability of NMT to increase the growth and proliferation of dental pulp cells and their differentiation into odontoblasts.

2. Materials and methods

Mesenchymal stem cells were isolated from permanent teeth (DPSCs). The materials used in this research were MTA powder (Dentsply, Charlotte, USA), NMT powder, 99.8% isopropanol, Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS) or fetal bovine serum (FBS), 10 μM ascorbic acid 2-phosphate, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, anti-human antibody STRO-1, and a human DSPP ELISA Kit. NMT was modified from MTA using the milling technique [15]. The tools used in this research included High Energy Milling (HEM) tube, culture plate, and incubator. The experiment of DPSCs was approved by Institutional Review Board Bogor Agriculture University, Indonesia, with ethics certificate number 05-2015-IPB. In this work, all regulations and confirmation that informed consent was obtained.

2.1. Isolation of DPSCs

Stem cells isolated from permanent teeth were placed in tubes containing DMEM. The teeth were cleaned with chlorhexidine (minosep) and phosphate-buffered saline (PBS) and broken into two using a mortar and pestle. The pulp tissue was collected with an extirpate needle chopped with a scalpel in a culture plate containing 1 mL PBS and was added to a tube containing 5 mL PBS. The produced cell suspension was centrifuged at 2000 rpm for 10 min to obtain a precipitate (pellet) which was then dissolved in DMEM. A strainer was used to obtain single cells (70 μm). The stem cell selection was performed using magnetic beads coated with anti-human antibody STRO-1 stem cell marker. Cells bound to the beads were eluted with washing buffer to release STRO-1-positive stem cells. The isolation method of DPSCs was adapted from the previous work [10]. The isolated stem cells were then inoculated onto a tissue culture plate containing DMEM enriched with 10–20% FCS or FBS, 10 μM ascorbic acid 2-phosphate, 2 mM L-glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. The cells were incubated in an atmosphere of 95% O_2 and 5% CO_2 at 37 °C. The culture medium was changed every 2–3 days [11].

2.2. Identification of DPSCs

DPSCs were identified by immunocytochemistry analysis. Briefly, stem cells were cultured for 14 days and the expression of STRO-1 was confirmed using a human anti-STRO-1 antibody. The stem cells were washed with PBS three times and blocked with blotto solution (consisting of 5% skim milk and Tween 20.03%) to prevent nonspecific binding, and finally washed three times with PBS. The stem cells were labeled with fluorescent isothiocyanate-tagged anti-STRO-1 antibodies dissolved in 2 μL/mL blotto and incubated in a 5% CO_2 atmosphere at 37 °C for 20 min. After 20 min, the cells were washed three times with PBS and observed under a microscope (Zeiss Axio Vert 40CFL, Jena, Germany) at 200x magnification. STRO-1-positive cells fluoresced as blue and were identified as mesenchymal stem cells.

2.3. DPSC proliferation

The viability of DPSCs exposed to MTA and NMT was examined in an MTT assay with trypan blue dye exclusion. Briefly, the DPSCs were propagated until they reached confluence. The cells (approximately 10^4 or 2×10^5 cells per mL) were plated in a 24-well culture plate containing culture medium (DMEM +10% FBS/FCS + penicillin/streptomycin). The cells in the culture plate were exposed to dexamethasone (10 nM), ascorbic acid (50 μg/μL), glycerophosphate (10 mM), and bioactive powdered NMT and MTA at a concentration of 100 ng/mL per well. While the DPSCs were incubated, the number of cells/mL was counted using trypan blue and a hemocytometer. Next, 10^4 DPSCs/well were cultured in 96-well plates and incubated at 37 °C and 5% CO_2 for 24 h. The DPSCs were then exposed to 50 μL of MTA and NMT, incubated for 1–3 days, and incubated at 37 °C and 5% CO_2 for 4 h. In this study, DMEM was used as a control. Next, pulp cells in each well were exposed to 15 μL of MTT reagent and incubated at 37 °C and 5% CO_2 for 3 h. Finally, acidified isopropanol (15 μL) was added and the plate was incubated while shaking for 1 h. The results were measured with a microplate reader (Bio-Rad, California, USA) at 405 nm. NMT absorbance was calculated compared to the control group. This proliferation method of DPSCs was adapted from the previous work [10].

2.4. Differentiation of DPSCs

Differentiation of DPSCs into odontoblasts was observed by measuring the concentrations of ALP, OC, and DSPP. ALP activity was measured using ALP colorimetric reactions in the MTA and NMT supernatants with fluorescent ALP substrates. The level of fluorescence indicated the amount of ALP activity. Buffer (290 μL) and diethanolamine (1 M, pH 9.8) were added to a 96-well plate, followed by the addition of 5 μL of *p*-nitrophenyl phosphate (0.65 M, 247 mg/mL H_2O), and the plate was incubated at 37 °C for 5 min. Subsequently, 5 μL of the cell sample was added to each well (MTA, NMT, and blank) and mixed. The absorbance was determined at 405 nm with a microplate reader.

The concentration of OC was determined using an instant human OC ELISA kit. Briefly, 100 μL H_2O was added to a 96-well plate; wells for the standards and blank were filled with 150 μL H_2O. The supernatant (25 μL) supernatant from the cultured cells was added to each well and the plate was shaken at 100 rpm for 2 h. After washing three times with 400 μL wash buffer, the plate was turned onto a water-absorbent paper for 5 min to dry. Next, 100 μL of tetramethyl-benzidine substrate was added to each well in the dark and the plate was shaken at 100 rpm for 15 min. After that, 50 μL of stop buffer was added to each well and the results were read using an ELISA reader at a wavelength of 450 nm. A human DSPP ELISA kit using plates coated with anti-human DSPP antibody was used to measure the DSPP concentration. A total of 100 μL of human DSPP was added to each pre-coated well, followed by 100 μL biotin-conjugated polyclonal antibody and 100 μL of avidin-conjugated horseradish peroxidase. Finally, 90 μL of tetramethyl-benzidine substrate was added to each well. The reaction was stopped by adding 50 μL stop solution containing sulfuric acid, and the absorbance was immediately read at 450 ± 2 nm.

2.5. Maturation of odontoblasts

To measure calcium deposits, we performed Von Kossa staining after the stem cell cultures were exposed to NMT and MTA. The DPSC culture was exposed to NMT and MTA in the culture plates and the culture medium was removed. The plate was washed with PBS and added with

1% silver nitrate prior to exposure to UV light for 20 min. The plate was then cleaned with distilled water and incubated with sodium thiosulfate for 5 min. The next plate cleaning was done using aquadest for being stained with Nuclei Fast Red for 5 min. A careful test of the plate under a microscope was finally after the plate was cleaned using aquadest. We quantified the calcium deposits by calculating the cell percentage containing the black spots, which indicate the presence of calcium ions. In this scenario, cell without black spots indicated a lack of calcium ions.

3. Results

Figure 1 shows microscope images of DPSCs on days 1, 5, 14, and 17. Meanwhile, Figure 2 shows the isolation of 100% STRO-1-positive cells. STRO-1-positive stem cells were grown to confluence until day 17 and then observed for DPSC proliferation, differentiation, and maturation. In addition, Figure 3 shows the viability of DPSCs exposed to MTA and NMT. The results of variance analysis indicated a significant difference ($p < 0.01$) in the viability of DPSCs exposed to NMT compared to controls and between cells exposed to NMT and MTA ($p < 0.05$). The ALP activity in dental pulp stem cells on different days is depicted in Figure 4. On day 3, we observed a significant difference between ALP activity in DPSCs exposed to MTA or NMT ($p < 0.05$) and between those exposed to NMT and the control ($p < 0.01$). On day 7, there was a significant difference between ALP activity in cells exposed to MTA or NMT ($p < 0.01$). In contrast, on day 14, there was no significant difference. However, on day 21, there was a significant difference in the ALP activity of cells exposed to NMT or MTA ($p < 0.01$), between those exposed to NMT and the control ($p < 0.05$), and between those exposed to MTA and the control ($p < 0.05$).

Based on the results of statistical analysis, the OC concentration in DPSCs exposed to NMT on day 3 was significantly different from that in cells exposed to MTA ($p < 0.01$), and both were significantly different from the control ($p < 0.05$). On days 7 and 14, there was a significant difference between the OC concentration in DPSCs exposed to NMT or MTA ($p < 0.05$). On day 21, the OC concentration in DPSCs exposed to NMT was similar to that of the control group ($p > 0.05$). However, the OC concentration in DPSCs exposed to NMT was significantly higher than that of cells exposed to MTA (Figure 5). On days 3 and 14, the DSPP concentration in DPSCs exposed to NMT or MTA did not significantly differ, nor was there a significance between DSPP in DPSCs exposed to NMT and the control ($p > 0.05$). However, on days 7 and 21, the DSPP concentration in DPSCs exposed to NMT was significantly higher than that of cells exposed to MTA ($p < 0.05$), whereas there was no significant difference between NMT-treated cells and controls (Figure 6).

In the observation of calcium deposits, DPSCs exposed to NMT showed extracellular matrix formation on day 7, whereas in DPSCs exposed to MTA, extracellular matrix formation was observed on day 14. The control group showed no extracellular matrix formation on day 21. Although, the number of cells was decreased on day 21, calcium deposits were detected in almost 100% of DPSCs exposed to NMT or MTA; the control group showed fewer calcium deposits. The calcium deposits were observed on day 7 in both DPSCs exposed to NMT or MTA, but not in the control group. Furthermore, an extracellular matrix was formed on day 7 in DPSCs exposed to NMT. In more detail, calcium deposit of dental pulp stem cells exposed to NMT or MTA and the control group can be seen in Figure 7. The statistical analysis, as summarized in Figure 8, revealed significant differences between calcium deposits in DPSCs exposed to NMT or MTA on days 7, 14, and 21 ($p < 0.05$). We found no significant relationship between ALP activity and calcium deposits in DPSCs exposed to MTA between days 7 and 21 (Pearson's correlation analysis, $p > 0.05$) with a very weak correlation strength (0.00–0.199). The weak correlation was observed on day 7 (0.20–0.399). On days 7 and 14, a negative correlation was observed, indicating that lower ALP activity was correlated with fewer calcium deposits. In contrast, on day 21, a positive correlation was observed, indicating that higher ALP activity was correlated with a larger number of calcium deposits, although the strength of the correlation was very weak. The correlation between the OC concentration and calcium deposits in DPSCs exposed to MTA was also not significantly different between days 7 and 21 ($p > 0.05$). In contrast to the correlation between ALP activity and calcium deposits in DPSCs, the OC concentration and calcium deposits in DPSCs exposed to MTA on days 7 and 14 showed a moderately positive correlation (0.400–0.599), suggesting that a higher OC concentration is associated with a larger number of calcium deposits. On day 21, the correlation power decreased (0.00–0.199) and became negative. The correlation between the DSPP concentration and calcium deposits in DPSCs exposed to MTA was not significant between days 7 and 21 ($p > 0.05$) and showed a negative correlation, with a greater DSPP concentration associated with

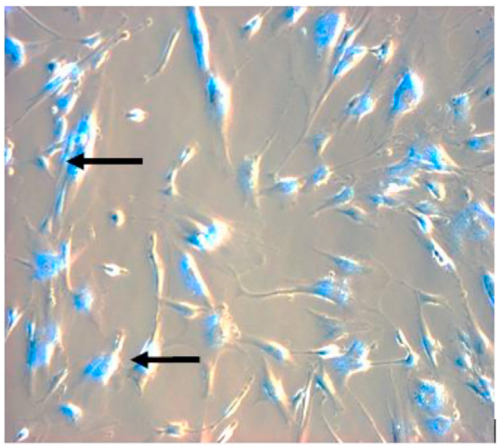


Figure 2. Identification of dental pulp stem cells (DPSCs) by immunocytochemistry. Blue colored DPSCs were identified as STRO-1 (200× magnification).

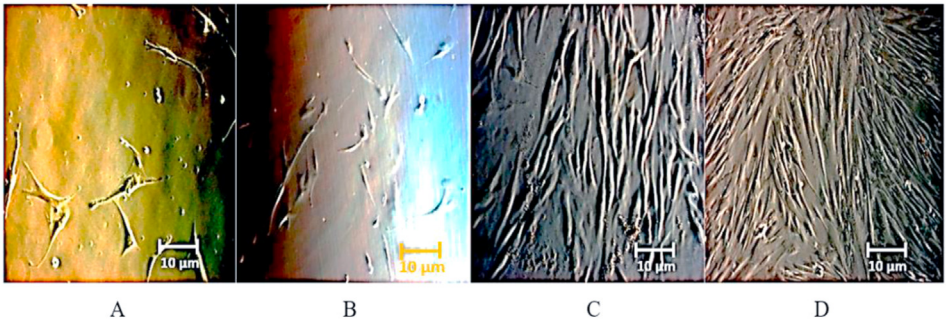
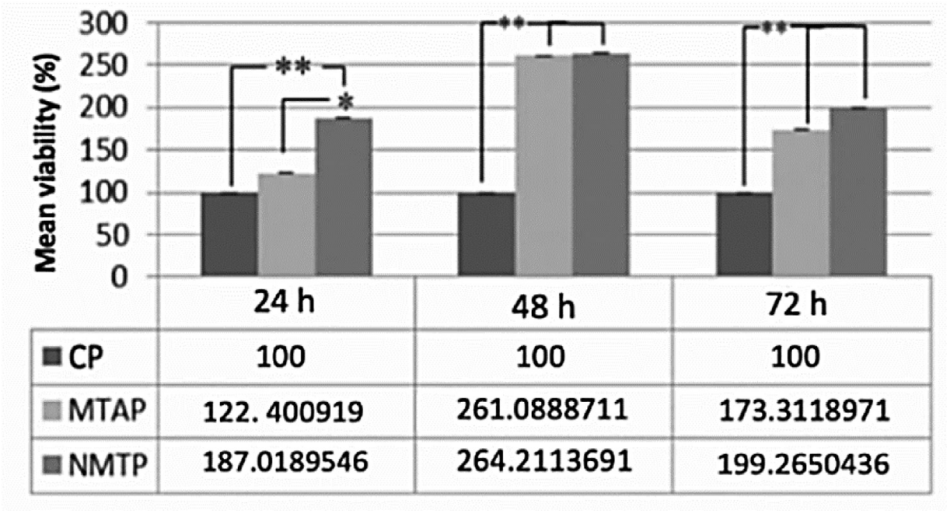
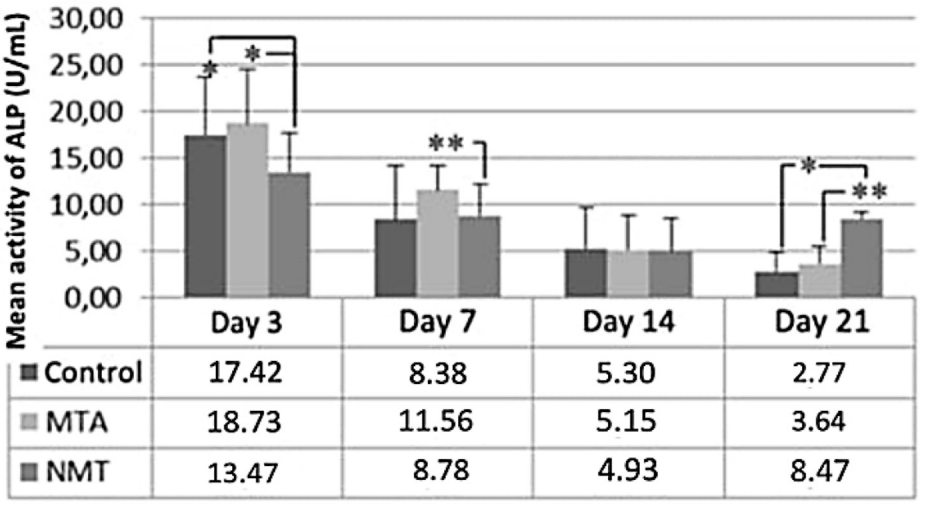


Figure 1. Microscopic images of dental pulp stem cells (DPSCs): (A) DPSCs on day 1. (B) DPSCs on day 5. (C) DPSCs on day 14. (D) DPSCs on day 17. (100× magnification). Scale bar 10 µm.



	24 h	48 h	72 h
CP	100	100	100
MTAP	122. 400919	261.0888711	173.3118971
NMTP	187.0189546	264.2113691	199.2650436

Figure 3. Viability of dental pulp stem cells on days 1, 2, and 3 **) $p < 0.01$; *) $p < 0.05$; Error bar = Standard deviation.



	Day 3	Day 7	Day 14	Day 21
Control	17.42	8.38	5.30	2.77
MTA	18.73	11.56	5.15	3.64
NMT	13.47	8.78	4.93	8.47

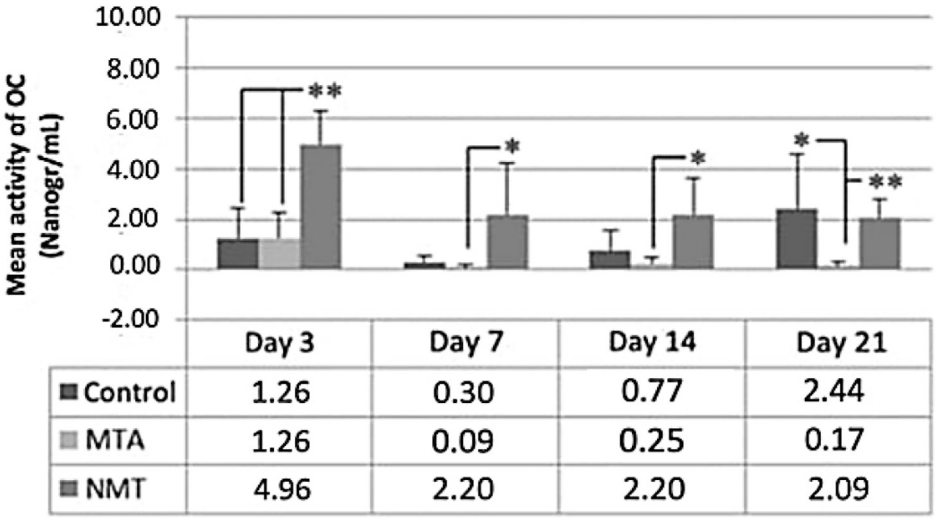
Figure 4. Alkaline phosphatase activity in dental pulp stem cells on days 3, 7, 14, and 21 evaluated by analysis of variance. **) $p < 0.01$; *) $p < 0.05$; Error bar = standard deviation.

a smaller number of calcium deposits. Day 7 showed the strongest correlation (0.600–0.799), after which the correlation power continuously decreased. On day 21, the correlation strength was very weak (0.00–0.199). Table 1 shows the correlation between odontoblast markers and calcium deposits in DPSCs exposed to MTA.

The correlation between ALP activity and calcium deposit in DPSCs exposed to NMT did not differ from that of those exposed to MTA, indicating no significant difference between days 7 and 21 ($p > 0.05$) (see Table 2). On day 7, the correlation was very weak (0.00–0.199) and negative, demonstrating that higher ALP activity indicated fewer calcium deposits. Additionally, on day 21, a strong negative correlation was observed (0.400–0.599). On day 14, the correlation was positive, suggesting that higher ALP activity is associated with a larger number of calcium deposits, but the correlation power was weak (0.200–0.399). The correlation between the OC concentration and calcium deposits in DPSCs exposed to NMT was not significantly different ($p > 0.05$) and was very weak, except on day 21 when a moderately negative correlation strength was observed (0.400–0.599). This indicates that higher ALP activity is associated with a smaller number of calcium deposits. The correlation between the DSPP concentration and calcium deposits in DPSCs exposed to NMT was very weak and insignificant from days 7–21. Similar results were observed on days 7 and 21, with a negative and weak correlation (0.00–0.199). However, the correlation was positive after day 21, indicating that a higher DSPP concentration is associated with a larger number of calcium deposits. On day 14, a negative and very weak correlation was observed (0.00–0.199).

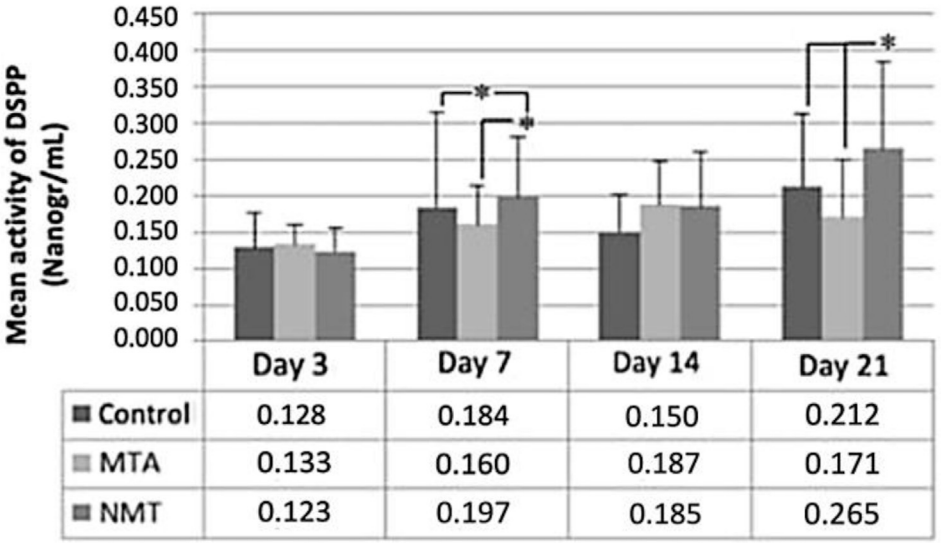
4. Discussion

Oral-derived MSCs are captivating to be employed in research and clinical purposes. The immune responses associated to tissues and organs transplants using oral-derived MSCs have been investigated through several studies. Oral-derived MSCs have been shown to prevent alloreactivity, to induce immunologic tolerance by suppressing T-cell proliferation, and to efficiently reduce autoimmune disorders by promoting the recovery of the ratio between T-reg and T-helper [16]. Beside their propitious roles as immunomodulators, oral-derived MSCs are easier to harvest than other anatomical sites [16, 17]. Several studies reported that MSCs therapy is related to bone tissue regeneration. Different stem cell types and genes have been explored in terms of their osteogenic ability [18]. The most investigated MSCs from dental tissues are dental pulp stem cells (DPSCs). DPSCs have a main role in regenerative dentistry due



	Day 3	Day 7	Day 14	Day 21
Control	1.26	0.30	0.77	2.44
MTA	1.26	0.09	0.25	0.17
NMT	4.96	2.20	2.20	2.09

Figure 5. Osteocalcin concentration in dental pulp stem cells on days 3, 7, 14, and 21. **) $p < 0.01$; *) $p < 0.05$; Error bar = standard deviation.



	Day 3	Day 7	Day 14	Day 21
Control	0.128	0.184	0.150	0.212
MTA	0.133	0.160	0.187	0.171
NMT	0.123	0.197	0.185	0.265

Figure 6. Dentin sialophosphoprotein concentration in dental pulp stem cells on days 3, 7, 14, and 21, evaluated by analysis of variance. **) $p < 0.01$; *) $p < 0.05$; Error bar = standard deviation.

to their abilities to differentiate into osteoblasts and into odontoblasts [16, 19]. Stem cells require ideal scaffolds to provide a microenvironment in facilitating their adhesion, proliferation, and differentiation processes [20] The ideal scaffold should exhibit excellent bioactivity and biocompatibility to support cell adhesion and proliferation. It should also meet specific requirements, such as odontoinductive and odontoconductive activity. Both natural and synthetic scaffolds have been used for dentin tissue engineering, such as ceramic bovine bone (CBB), demineralized dentin matrix (DDM), poly-L-lactate-co-glycolate (PLGA), small intestinal submucosa (SIS), B-tricalcium phosphate (β-TCP), and composite scaffold crossed-linked with collagen-chondroitin [21]. Innovative combinations of experimental scaffold colonized with autologous stem cells have offered a promising strategy for regenerative healing of bone [22]. In this study, we evaluated the effect of NMT on the proliferation and differentiation of DPSCs. NMT was modified from MTA using milling technique in accordance with the previous work [10]. NMT of 1–100 nm can function effectively in the cell system because of its increased nanoparticle surface area, which enhances bioactivity to stimulate cell growth. The viability of DPSCs exposed to NMT was higher than that of MTA exposure and cells in the control group. This high viability resulted from the larger amounts of calcium and silicate in NMT compared to in MTA, which affects the biocompatibility of a material. A high calcium content increases alkaline properties, which in turn increases cell activity [23]. Increased calcium was observed on the second day and tended to decline on day 3. This occurred because of the completion of the tricalcium silicate reaction in NMT, which causes calcium production to decrease [24]. Furthermore, the behavior of DPSCs in this study can also be affected by mechanical stresses transmitter to the pulp tissue [25, 26]. Odontoblast-like cell formation and differentiation from DPSCs rely on signal transduction from the environment [25, 27]. The proliferation and differentiation of DPSCs can effectively be promoted by appropriate mechanical stresses, such as low-intensity pulsed ultrasound (LIPUS), which is able to enhance the viability, proliferation and differentiation of several MSCs [25, 28].

ALP is a hydrophilic enzyme that functions without the organic phosphate of the phosphate ester. ALP activity is related to the formation

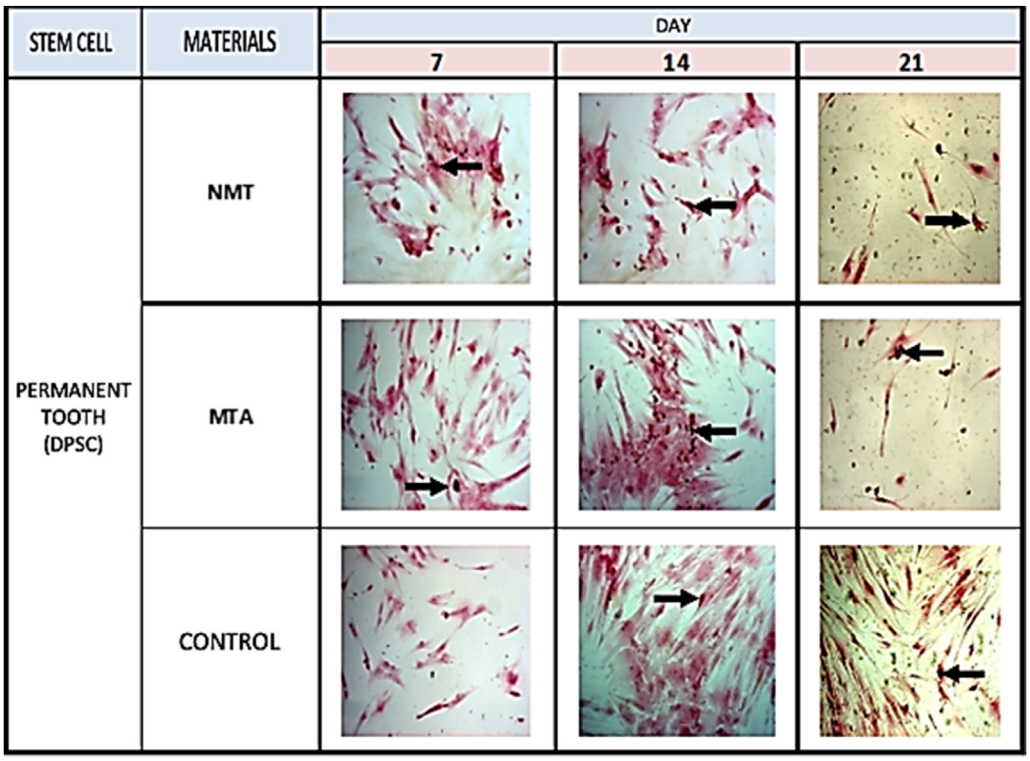


Figure 7. Calcium deposit of dental pulp stem cells exposed to nanoparticle mineral trioxide or trioxide mineral aggregate and the control group. (1) Calcium deposits on day 7. (2) Calcium deposits on day 14. (3) Calcium deposits on day 21. (100× magnification).

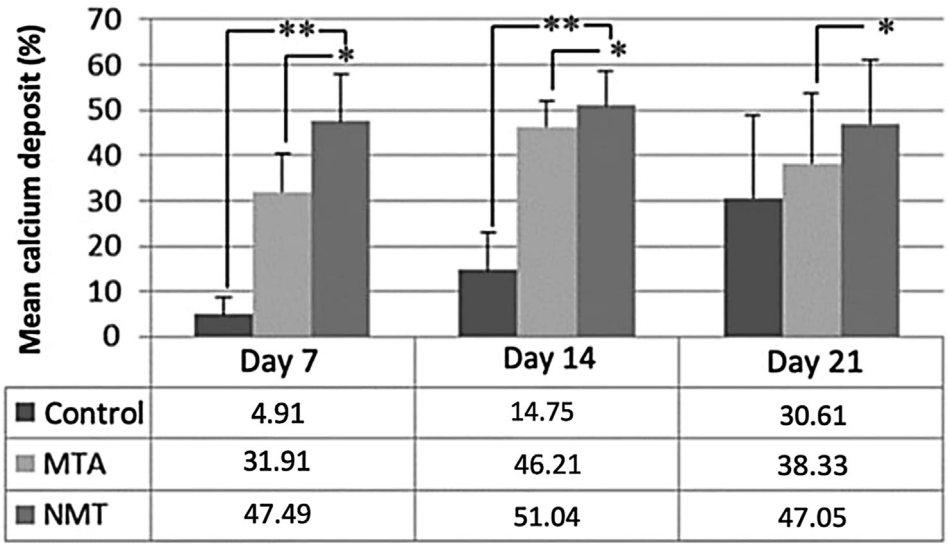


Figure 8. Calcium deposit in dental pulp stem cells on days 7, 14, and 21.**) $p < 0.01$; *) $p < 0.05$; Error bar = standard deviation.

Table 1. Correlation of odontoblast markers with calcium deposits in DPSCs exposed to MTA.

Odontoblast marker	Calcium deposit		
	Day 7	Day 14	Day 21
ALP (r)	-0.303 (low)	-0.155 (very low)	0.158 (very low)
OC (r)	0.442 (moderate)	0.565 (moderate)	-0.168 (very low)
DSPP (r)	-0.714 (strong)	-0.361 (low)	-0.154 (very low)

of hard-tissue and bone. This enzyme is therefore widely used as a marker of derived cells of calcified tissue such as odontoblasts [29]. Based on our results, the ALP activity in DPSCs exposed to NMT was more stable than that in cells exposed to MTA and control group cells. This is likely

Table 2. Correlation of odontoblast markers and calcium deposits in DPSCs exposed to NMT.

Odontoblast marker	Calcium deposit		
	Day 7	Day 14	Day 21
ALP (r)	-0.128 (very low)	0.206 (low)	-0.450 (moderate)
OC (r)	0.189 (very low)	0.141 (very low)	-0.405 (moderate)
DSPP (r)	-0.086 (very low)	-0.155 (very low)	0.086 (very low)

because of the high calcium ion content during the NMT exposure, which affected the ALP activity for 21 days. Calcium ion is produced when in contact with the tissue fluid, yielding amorphous calcium phosphate (ACP). Materials containing ACP and hydroxyapatite (HA) are recognized as preferable sources for converting materials in hard-tissue engineering because they are lightweight and have stable chemical properties [7]. NMT contains nanoamorphous calcium phosphates (nano ACPs) and nanocrystalline HA with a size of 41.1 nm each, leading to calcification that accelerates reparative dentine formation. The effects of ACP and HA on bone marrow stem cell proliferation, adsorption, and differentiation have been demonstrated at a nanoparticle size of 20 nm [30]. The results show that NMT and MTA have similar effects on the dentine calcification process in permanent teeth.

OC is a protein secreted by osteoblasts. The pattern of gene expression during odontoblast differentiation is similar in osteoblasts. In the lesion region, osteocalcin is secreted rapidly from odontoblasts to form predentine from reactionary dentine. OC is thought to be an odontoblast differentiation marker. Excessive osteocalcin appears in the rapid development of the Golgi body and vesicle of the columnar cell on the surface of the dentin-pulp below the cavity [31]. In this work, the OC concentration as an odontoblast marker of DPSCs exposed to NMT was higher than that of those exposed to MTA. The 21-day OC concentration in DPSCs exposed to NMT was significantly higher than that of those exposed to MTA. The high OC concentration in DPSCs exposed to NMT showed that NMT was more effective than MTA in the dentine repair process. DSPP is a marker of the differentiation of dentin-pulp complexes and is selectively secreted by odontoblasts [32]. DSPP expression by odontoblasts is initiated by predentin formation, and DSPP expression by pulp cells are active at dentinogenesis [33]. The DSPP concentration as an odontoblast marker in DPSC exposed to NMT or MTA on days 3 and 14 was almost the same but higher than in the control group. On days 7 and 21, the DSPP activity of DPSCs exposed to NMT was higher than that of those exposed to MTA and the control. The DSPP concentration in DPSCs exposed to NMT continued to increase from days 3–21. In contrast, MTA treatment increased the DSPP concentration until day 14, which was then decreased on day 21. Over 21 days, NMT treatment resulted in significantly higher total DSPP concentrations compared to after MTA treatment. The formation of dentine, known as dentinogenesis, begins before enamel formation initiated by odontoblasts [34, 35]. DSPP plays an important role in forming secondary dentine, particularly during pulp capping (reparative dentin) treatment.

The statistical analysis revealed a significant difference between calcium deposits in DPSCs exposed to NMT and MTA on days 7, 14, and 21. The role of nano-sized NMT supported that this nanomaterial is more reactive, as it stimulated catalytic cells and calcium content. Nano HA in NMT increased the calcium deposits in DPSCs. Calcium deposits were present on day 7 reaching their optimum content on day 14 and began decreasing on day 21. This is likely because by day 21, the cell growth and the ability to form calcium ions begin to decline. In addition, calcium in NMT and MTA also decreased because of the tricalcium silicate reaction. The calcium deposits in DPSCs exposed to NMT on day 7 showed the formation of an extracellular matrix, whereas in DPSCs exposed to MTA, extracellular matrix formation occurred on day 14. On the other side, these effects occurred on day 21 in the control cells.

The observations of calcium deposits above are consistent with the results reported by Schulze et al. [36], showing that calcium absorption is positively correlated with cutaneous deposits. Calcium levels in NMT exposed cells were higher than those in MTA-exposed cells, leading to increased numbers of calcium deposits and greater regeneration of permanent dentin. The modification of MTA to NMT showed a better impact on cell viability, demonstrating that NMT induces increased proliferation in DPSCs. This verifies that NMT contains more calcium ions which stably affect ALP activity for 21 days. Calcium ions are produced upon contact with tissue fluid, which in turn produces ACP, leading to calcification [37].

This work demonstrates that modification of MTA to NMT increased ALP activity and the concentrations of OC and DSPP, which have dentin reparative functions, whereas the calcium deposits indicate the regenerative process of dentin [38]. Increasing dentine repair and regeneration can maintain vital fixed tooth pulp, prevent the necrosis of pulp and formation of abscesses, and fight infection in the human body. The correlation between ALP activity and calcium deposits in DPSCs exposed to MTA and NMT was weak and likely to be negative, except for the correlation with calcium deposits in DPSCs exposed to NMT on day 14, which showed a weak positive correlation. This result is in line with the correlation of ALP activity and calcium deposits in DPSCs exposed to MTA, which had a weakly positive correlation. This is likely to occur due to the fact that NMT contains more calcium and nano HA than MTA, and thus it had a slightly better correlation power than MTA. The direction of the positive correlation indicated that greater ALP activity leads to larger numbers of calcium deposits.

The correlation between the OC concentration and calcium deposit in DPSCs exposed to MTA showed a moderately positive correlation on days 7 and 14, but the correlation strength became very weak and negative on day 21. These results are consistent with those of Schulze et al. [36], who reported that calcium deposits are associated with calcium absorption, osteocalcin, and leptin, particularly on days 7 and 14. The correlation of the OC concentration with calcium deposit in DPSCs was very weak and positive on days 7 and 14. The nanotubes of ACP and nano HA content are not directly related to the OC concentration and calcium absorption. OC is required for predentine formation after dentine injury. ALP is responsible for stimulating calcification in dentin. The results of the correlation of OC concentration with calcium deposits in DPSCs exposed to NMT differed from those of previous research, which showed that the OC concentration in DPSCs exposed to NMT was significantly higher than that in cells exposed to MTA from days 3–21. The correlation between the DSPP concentration with calcium deposits in DPSCs exposed to MTA was not significantly correlated from days 7–21, with a strong correlation on day 7 and very weak and negative correlation on day 21. This evidence confirms that there was no relationship between the formation of secondary dentin with calcium deposits in DPSCs exposed to MTA. In contrast, the correlation between DSPP concentration in DPSCs exposed to NMT was very weak and negative. The correlation between the DSPP concentration and calcium deposits in DPSCs exposed to NMT or MTA was not significant, indicating that the DSPP concentration following exposure to MTA or NMT did not affect calcium deposits. Therefore, secondary dentin formation was not associated with calcium deposits on permanent teeth.

5. Conclusion

In can be concluded that NMT increased the proliferation and differentiation of DPSCs, as well as their maturation toward odontoblasts. NMT showed the potential to effectively increase cell proliferation, differentiation, and maturation toward odontoblasts in dental pulp stem cells (DPSC) compared to MTA. It is because NMT contains more calcium and HA than MTA, so it has a slightly better correlation power than MTA. Furthermore, the direction of positive correlation indicates that the greater the value of ALP the greater the value of calcium deposits. The limitation of this study is that DPSCs have not been characterized by flow cytometry. Prior to the conduction of further experiments, human DPSCs, ideally, should be characterized by flow cytometry. By using flow

cytometry, it is essential to establish a more specific antigenic profile with more antigen expression. Furthermore, differentiation of DPSCs in this research focused on osteoblast differentiation potential. For further study, odontogenic, adipogenic, and chondrogenic differentiation potentials can be examined depending on the research objective.

Declarations

Author contribution statement

Muhammad Chair Effendi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Ahmad Taufiq: Analyzed and interpreted the data; Wrote the paper.

Boy Muchlis Bachtiar: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Endang Winiati Bachtiar: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ellyza Herda: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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