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Osteogenic Differentiation of Stem Cells Treated with Fast Set NeoMTA Plus

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ABSTRACT

Aim: To evaluate the osteogenic activity of NeoMTA Plus using rat mesenchymal stem cells (MSCs). Materials & Methods: Pre-seeded rat MSCs were cocultured with either NeoMTA Plus or ProRoot-MTA and incubated for 1, 3 and 7 days (6 well for each) .The cell growth, viability and proliferation were evaluated with light and scanning electron microscope. The specimens were also subjected to quantitative real time-polymerase chain reaction for osteogenic gene expression. Results: After the treatment with either material, cell viability gradually increased by time, and the cells showed signs of differentiation into osteoblasts. ProRoot-MTA exhibited a significant higher mean value than NeoMTA plus after 7 days. At the third and seventh days, the cells treated with both materials exhibited deep alkaline phosphatase staining more than control untreated cells. Upregulation of osteogenic gene expression including bone morphogenetic-2, alkaline phosphatase, bone sialoprotein, osteopontin and osteocalcin was observed with both materials. Conclusion: Both NeoMTA Plus and ProRoot-MTA had osteogenic activity when used as pulp and endodontic repair material.

Key words: *Mesenchymal Stem Cells, Root Repair Materials, Alkaline Phosphatase, Mineral Trioxide Aggregates, Osteogenic Gene Expression, Calcium Silicate Cement, Fast-Set Neomta*

INTRODUCTION

Mineral trioxide aggregates (MTA) is the standard material used in endodontic treatment and pediatric dentistry as pulp capping, repair for root perforation, retrograde filling restoration, apexification and/or revascularization. Due to its handling difficulty and prolonged setting, an accelerator has been added for its improvement, although it had a negative impact on its biocompatible properties [1]. Accordingly, new fast setting NeoMTA Plus has been advocated in the market. It is a powder/gel system of tricalcium silicate-based bioactive cement. Like conventional MTA, it has nearly similar composition with varying amount of aluminum, sulfate and zirconium oxide with addition of tantalum oxide [2].

There was a scarcity of researches related to its biocompatibility and osteogenic potential. The current study aimed to analyze the effect of NeoMTA Plus (NuSmile Ltd, Houston, USA) on gene expression related to osteogenic activity in rat mesenchymal stem cells (MSCs) compared with ProRoot-MTA (Dentsply, De-Trey,

Germany). The expected null hypothesis had no difference between both materials when applied on Mesenchymal stem cells (MSCs) to induce gene expression related to osteogenic activity of stem cells.

MATERIALS AND METHODS

The institutional ethical approval was obtained from King Abdulaziz university ethical committee (# 057-15). The osteogenic potential of NeoMTA Plus was analyzed as compared with white ProRoot-MTA.

Materials procedure

Under sterile condition, both NeoMTA Plus and ProRoot-MTA were mixed, powder to liquid in ratio 3:1 according to manufacture recommendation until homogenous mix and backed into polyethylene ring (4 mm radius and 2 mm height) to prepare small discs of each material. For 24 hours at 37^0 C, the prepared discs were covered with moistened gauze, and incubated until complete setting and then sterilized by UV light for 1 hour.

Culture of Mesenchymal stem cells

In King Fahd Research center, King Abdulaziz University, the procedure of stem cells culture was performed. MSCs were derived from bone marrow of 6-8 weeks old, 120 gram weight albino rat femurs and tibias. The cells were then suspended in DMEM supplemented with 10% FBS and antibiotic of penicillin/streptomycin, incubated for 24 hours at 37ºC in 100% humidified 5% carbon dioxide (CO_2) incubator [3]. Using Flow cytometry (FACSAria III cell sorter, BD Bioscience, Belgium), the isolated cells were evaluated to be MSCs by labeling their monoclonal antibodies for $CD29^+/CD45^-$[4].

The living stem cells were pre-plated in 96-well culture plate containing osteogenic culture DMEM and 10% FBS supplemented with pencilline/streptomycin antibiotic at a density of $1x\ 10^5$ cell/ well for cell proliferation assay [5], while seeded in 6-well culture plate at a density of $3x\ 10^5$ cell per well for gene expression procedures. The culture plates were incubated for 10 days at 37º C in 100% humidified 5% carbon dioxide (CO_2) incubator with regular changes of culture medium every 2-3 days until the appropriate cell/well density [5, 6].

Cell proliferation Assay

The discs of each material were applied on the pre-seeded cells, and incubated for 1, 3 and 7 days (6 well for each) at 37ºC in 100% humidified 5% carbon dioxide (CO_2) incubator [7]. Further 6 wells of culture cell were incubated without treatment (negative control). After each incubation period, 10 µl of cell proliferation reagent (WST-1 cell proliferation assay kit reagent, Sigma-Aldrich, Inc. Germany) was added for 4 hours, then the cells were dissolved by sodium Dodecyl sulfate for 10-15 minutes. Using ELISA absorbance reader (ELx 808, Bio Tek Instrument, Inc., USA), cell viability, growth and proliferation were then evaluated at 450 nm absorbance [8]. The procedures were repeated three times to confirm the results. The cell cultured wells exposed to the investigated materials as well as the untreated cells of different incubation periods, were also evaluated by scanning electron microscope (Field Emission SEM, 450 FEI, Amsterdam, Netherlands)

Staining procedure for Alkaline phosphatase

After each incubation period, the MSCs were extracted from culture media using 70% ethanol followed by deionized water rinsing 3 times. For alkaline phosphatase staining, the cells were treated with 300µL/well live stain (1-Step NBT/BCIP solution; Thermo Fisher Scientific Inc, Rockford IL. USA) for 15 minutes, then scanned and photographed under light microscope.

Quantitative Real-time Polymerase Chain Reaction (qPCR):

Pre-seeded MSCs, as previously described, were further incubated after applying the investigated materials for 1, 3 and 7 days (6 wells for each). 6 wells of untreated cells were used as control. After each incubation periods, RNA was extracted from co-cultured cells, and then complementary deoxyribonucleic acid (cDNA) was synthesized using cDNA reverse transcription kit (ImPromII™ reverse Transcription System cat #A3800, Promega, Madison, USA). The reaction was performed at 25 ºC for 5 minutes, 42ºC for 120 minutes and 70ºC for 15 minutes respectively. Then qPCR was done using QuantiFast® SYBR® Green PCR Kit Master Cat No 204054 (QIAGEN GmbH, QIAGEN Strasse, Hilden, Germany), in StepOne Plus Real-Time PCR system (AB Applied Biosystem, Life technology, Foster City, CA) by initial denaturation at 95ºC for 10 minutes followed by 34 cycles at 95^0C for 5 seconds, 65^0C for 10 seconds, and 72^0C for 15 seconds [9]. Different gene expression related to osteogenic activity was evaluated including Bone morphogenetic protein(BMP)-2, Alkaline phosphatase (ALP), Bone sialoprotein (BSP) and Osteocalcin (OC) were analyzed using specific primer sequence for bone morphogenetic protein-2 (BMP-2 reverse: 5'-TTCCCACTCATTTCTGAA, forward: 5'-

AACACCGTGCTCAGCTTCCA-3'AGT-3'), Osteocalcin (rOC-2 reverse:5'-CATAGATGCGCTTGTAGG-3', forward: 5'-TACCAGGGAGGTGTGTGA-3'), Alkaline phosphatase (rAlp-1 reverse: 5' -ACTGGTCAGAGTCACCTG-3', forward: 5'-GCTCATTTCCAACATCATGGTC-3) bone sialoprotein (rBsp-1reverse: 5'-TTACCCCTGAGAGTATGG-3', forward: 5'-GATAGTTCGGAGGAGGAG-3',)osteopontin (rOP reverse 5'-CAGGATTCCATACCCAAGAAGG-3', Forwad 5'-GAGAAGATCTGGCACACCT-3'and GAPDH (rGapdh-2 reverse: 5'-GCAGGGATGATGTTCTGG-3', forward: 5'-AGTCCATGCCATCATTGC-3') as endogenous control gene [10].The reactions were triplicated.

Statistical analysis

The statistical analysis for cell growth results at different incubation periods were performed by ANOVA and Post-Hoch tests using SPSS software (Munich, Germany) at significance level of 0.05.

For qPCR, the data of five target genes and reference gene (GAPDH) results were statistically analyzed by Livak method ($\Delta\Delta C_T$) and comparative cycle threshold (ΔC_T) [11]. After normalization of ΔC_T of the target gene to the ΔC_T of the reference gene, $\Delta\Delta C_T$ was determined and statistically analyzed by StepOne software version 2.3 (Applied Biosystems). The Quantification of control target was normalized to be 1.

RESULTS

Cell viability

Figure 1 shows the variation in viability of MSCs during different experimental periods according to the effect of the material used. There was no significant difference in viable cell account among the materials and control group in the first and 3th days (F = 3.765 at P =.047 and F = .079 at P = .925 respectively). The significant difference among groups was only detected at the 7th day experimental period (F =9.044 at P = .003)

The ProRoot-MTA exhibited gradual increases in the MSCs account by time (0.281 ± 0.06, 0.222 ± 0.05 and 0.364 ± 0.05 at the 1st, 3rd and 7th day respectively) with statistically significant difference (F = 10.994at P = 0.001). The cell count in ProRoot-MTA group was greater than control and NeoMTA Plus groups in the first and seventh days (table1).

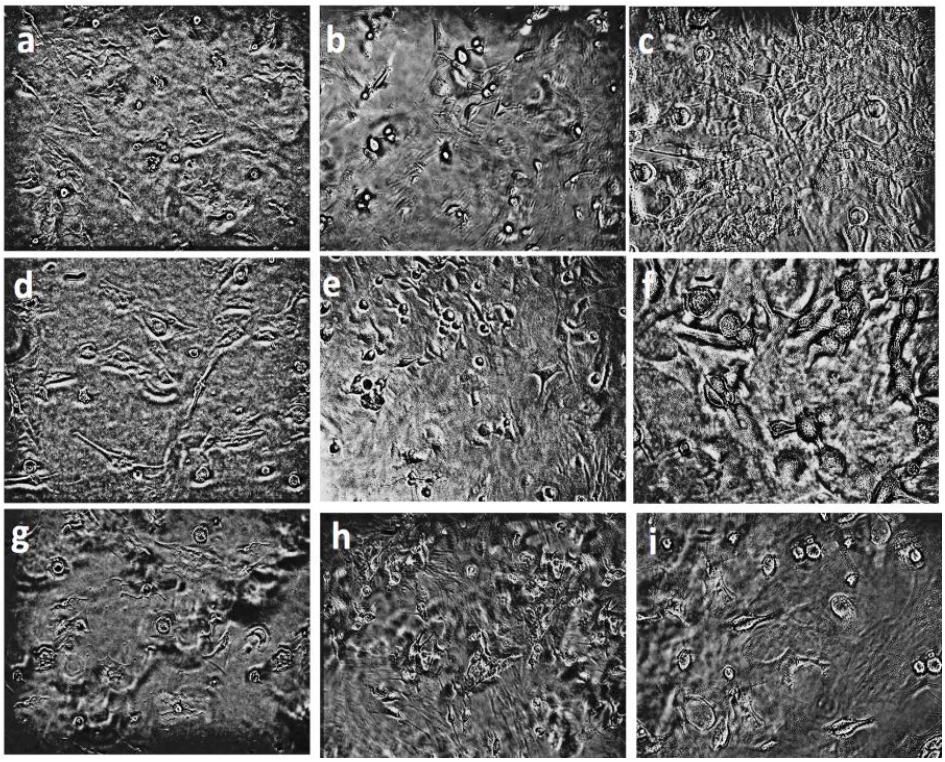

Figure 1: Photomicrographs of viable control untreated mesenchymal stem cells (a-c) and cells treated with either NeoMTA Plus (d-f) or ProRoot-MTA (g-i) and incubated for 1, 3 and 7 days respectively. Magnification X20

Table 1: MSCs viability after using the investigated root repair materials (Means ± standard deviation)

Duration / Materials	One day	3 days	7 days	One Way ANOVA
Control	0.212 ± 0.08	0.243 ± 0.09	0.217 ± 0.06	F = 0.268 at P = 0.768
NeoMTA Plus	0.193 ± 0.02	0.234 ± 0.12	0.296 ± 0.07	F = 2.474 at P = 0.118
ProRoot-MTA	0.281 ± 0.06	0.222 ± 0.05	* 0.364 ± 0.05	F = 10.994at P = 0.001
	F = 3.765 at P =.047	F = .079 at P = .925	F = 9.044 at P = .003	

Scanning Electron Microscopy (SEM)

Upon scanning electron microscopic examination, there was no marked difference in the appearance of control untreated MSCs than that cells treated with either NeoMTA Plus and ProRoot-MTA (figure 2 a, b and c respectively) at the first day. Some of MSCs treated with ProRoot-MTA, showed an evidence of cell mitosis starting from day 1.

At day three, no changes were observed in control untreated MSCs except little cell elongation of some of them (figure 2 d). The cells treated with either NeoMTA Plus or ProRoot-MTA showed differentiation to osteoblasts- and fibroblasts-like cells (figure 2 e and f) isolated or collected in group (clump). The cells appeared ready for the proliferation stage.

At day seven, some of control untreated MSCs underwent apoptotic changes (figure 2 g). MSCs exposed to NeoMTA Plus or ProRoot-MTA become more elongated with evidence of mitotic activity (figure 2 hand i).

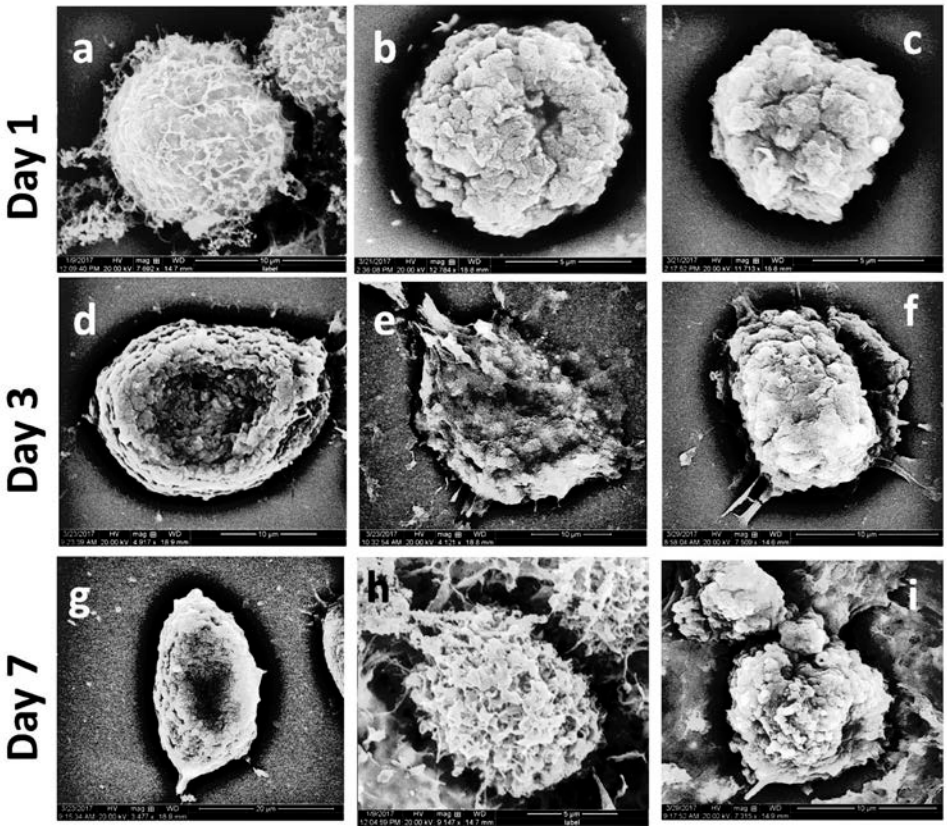

Figure 2: SEM photographs of control MSCs (a) and cells treated with either NeoMTA Plus (b) and ProRoot-MTA (c) showed no marked difference at day one. At the third and seventh days, compared with the control untreated MSCs (d and g), the cells treated with NeoMTA Plus (e and h) or ProRoot-MTA (f and i) showed differentiation into osteoblast-like cells with an active process.

Alkaline phosphatase

In the first day, there was no obvious staining difference between the investigated materials (Figure 3). The staining was greater in cells exposed to either investigated materials than that of control untreated cells. At the third and seventh day, the cells treated with NeoMTA Plus and ProRoot-MTA exhibited staining more than control untreated cells.

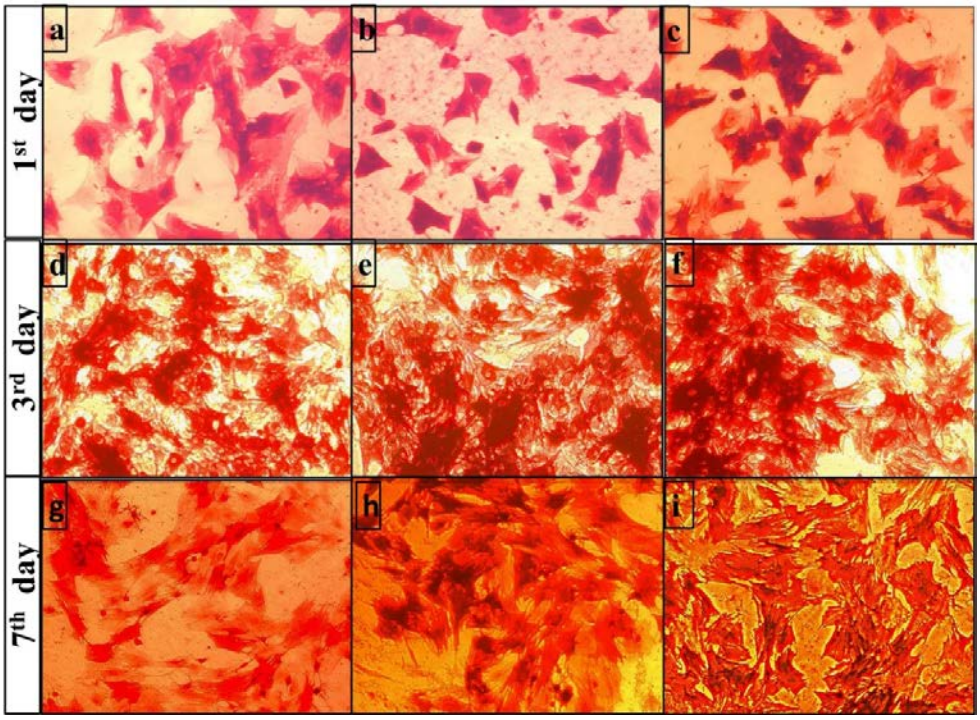

Figure 3: The Alkaline phosphatase staining of control MSCs at day 1 (a) appeared nearly similar to that treated with NeoMTA Plus (b) and ProRoot-MTA (c). At day 3 and 7, the control untreated MSCs (d and g) showed lighter staining density than that treated with NeoMTA Plus (e and h) and ProRoot-MTA (f and i) ; respectively.

Reverse-transcription Polymerase Chain Reaction and Quantitative Real-time Polymerase Chain Reaction

Bone morphogenetic protein-2

Both NeoMTA PlusNeo and ProRoot-MTA exhibited the upregulation of BMP-2 gene expression that was increased by time up to the seventh day. The greatest expression values were obtained by ProRoot-MTA all over the experimental periods (figure 4).

Alkaline phosphatase

Both investigated materials exhibited upregulation of alkaline phosphatase gene expression at day one with the greatest mean CT value obtained by ProRoot-MTA (64.19) compared with NeoMTA Plus (24.28) and control untreated MSCs. The expression markedly decreased at the third day, then re-increased at the seventh day (figure 4).

Bone sialoprotein

At day one, both investigated root repair materials exhibited downregulation of bone sialoprotein gene expression. However, at the third day, they showed marked upregulation of gene expression, This expression was maintained by NeoMTA Plus (mean CT value= 2.3) while down-regulation was obtained by ProRoot-MTA (mean CT value= 0.75) (figure 4).

Osteopontin

At day one, ProRoot_MTA exhibited upregulation of osteopontin gene expression (2.19) more than that obtained by NeoMTA Plus (1.11) as compared with control untreated MSCs. Whereas, both materials exhibited down regulation of this gene expression at the third day. At the seventh day, ProRoot-MTA was the only one to induce upregulation (1.19) (figure 4).

Osteocalcin

At day one, both investigated materials induced upregulation of osteocalcin gene expression with greater mean CT value exhibited by ProRoot (3.95). This expression decreased at day three with down-regulation obtained by NeoMTA Plus (0.85) then re-increased at day seven with no marked difference in their gene expression (figure 4).

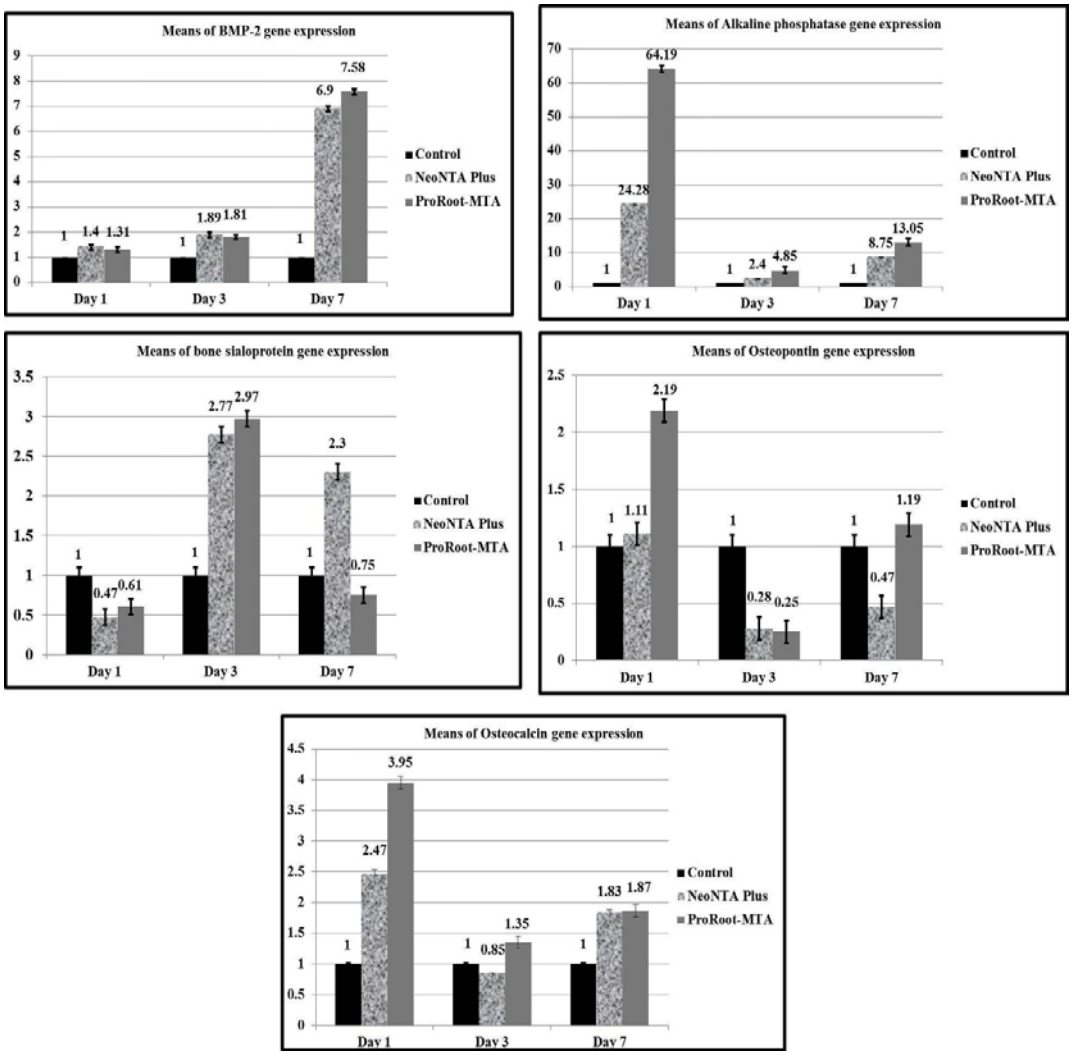

Figure 4: Gene expression of bone and osteogenesis genes (including BMP-2, alkaline phosphatase, bone sialoprotein, osteopontin and osteocalcin) induced by NeoMTA Plus and ProRoot-MTA compared with control untreated MSCs.

DISCUSSION

Based on the prolonged setting time and handling difficulty of conventional MTA (ProRoot-MTA), fast setting NeoMTA Plus was developed to offer its bioactive and osteogenic potential. It was reported that there was some variation of their constituents as NeoMTA Plus contained higher aluminum and sulfur than conventional MTA that might interfere with its biological behavior [2]. There has been scarcity of studies evaluating their biocompatibility and bioactivity. In a previous in-vitro study, the evidence of bioactivity of NeoMTA Plus was reported as the calcium phosphate crystals precipitated on the material surface [2]. The current study was designed to evaluate their bioactivity on stem cells, and their ability to induce osteogenic differentiation when used as a root repair material.

Because MSCs of mice are multipotent cells, they are capable of in vitro differentiation into various non mesenchymal lineages such as calcified-forming cells including osteoblasts-, cementoblasts- and/or odontoblasts-like cells [9, 12]. This is why they were used in the current in vitro study. MSCs were proved to have high rate of proliferation with possible growth regulation [13], and to effectively evaluate the osteogenic potential of dental repair materials [14].

The mechanisms of osteogenesis related to NeoMTA Plus repair material have not been fully understood. To elucidate its effects, the viability, morphology, growth, proliferation and differentiation of MSCs were evaluated. The viability of cells with progressive growth and proliferation has been indicative of the favorable therapeutic effect of repair material used [8].

In the present study, ProRoot-MTA exhibited higher cell viability/proliferation along during the whole experiment than that obtained by NeoMTA Plus with significant difference at day seven. This finding might be related to higher calcium hydroxide (CH) content of the material. CH has been the main byproduct of both NeoMTA Plus and ProRoot-MTA, with extra amount within the original composition of ProRoot-MTA [2]. As CH dissociated to calcium and hydroxyl ions in the culture medium, the extracellular calcium might stimulate the cell viability [6].

Bone formation or bone repair has been a complex process based on the differentiation of MSCs present in pulp, periapical and/or periodontal tissues into osteoblast-like cells in association with the expression of different staged osteogenic-related factors and several genes [15] under suitable environment and/or therapeutic effect of repair material to generate calcified deposits, and repair the root defects [9, 16, 17].

In the current study, both investigated repair materials enhanced the gene expression of BMP-2 that gradually increased by time up to the seventh day, so the null hypothesis of this point was accepted. The calcium silicate-based material including MTA was reported to induce significant upregulation of BMP-2 gene expression in the rat dental pulp cells [18] and human periodontal cells [9]. It was suggested that BMP-2 is the most osteo-inductive factor [19], regulating cell differentiation and proliferation [10]. This fact has been supported by the scanning electron microscopic finding as the cell proliferation and differentiation increased gradually synchronized with the upregulation of BMP-2 gene expression. Maeda et al 2010 determined that cocultures of MTA with human periodontal ligament cells induced early upregulation of BMP-2 gene expression through calcium sensing receptor stimulation. This stimulation might attribute to increase the extracellular calcium ion that released from MTA into culture media [9].

The differentiation process can be enhanced not only by BMP-2 but also by increasing the expression of various pro-mineralization genes like alkaline phosphatase concentration and non-collagenous matrix proteins like bone sialoprotein, osteopontin and osteocalcin [16] that are responsible for bone deposition and maturation process [20]. Each gene has a specific role in different stages during bone repair. Alkaline phosphatase, osteopontin and bone sialoprotein genes accompany osteoblast differentiation [15, 20, 21].

The present result showed the upregulation of alkaline phosphatase by both repair materials during the whole experiment with the significant increase after the first day. ProRoot-MTA induced higher expression than NeoMTA Plus. Its expression was decreased at day three, then re-increased at day seven. This finding might be attributed to the role of alkaline phosphatase in cell differentiation at the first day of using material [20]. On the other hand, the increase of alkaline phosphatase production at day seven might be attributed to its ability to initiate mineralization by supplying phosphate during cyto-differentiation stage [10] as it has been encoded by differentiated osteoblasts and enhanced bone turnover [17].

The present finding reported the higher expression of alkaline phosphatase, osteopontin and osteocalcin by both investigated materials in day one than that of third and seventh days. The same finding was previously reported that might be attributed to pH-changes during setting reaction [17]. Furthermore, the release of calcium ions from MTA upregulated the biologic marker including BMP-2, alkaline phosphatase, bone sialoprotein and octeocalcin [9, 17].

Bone sialoprotein, or osteopontin and osteocalcin have been mineralized tissue- proteins having important roles in regulation and differentiation of mineral-cells like osteoblasts, odontoblasts and cementoblasts [22]. In the current study, bone sialoprotein (BSP) was highly expressed by both investigated materials compared with control at day 3, whereas, at day 7, its expression was down-regulated by ProRoot-MTA, and up-regulated by NeoMTA Plus. Bone sialoprotein has been a noncollagenous protein, essential for the organization of collagen matrix of new bone and nucleation as well as the growth of hydroxyapatite crystals [21]. It has been expressed by fully differentiated osteoblasts during the mineralization phase responsible for nucleation and growth of hydroxyapatite crystals to form highly specific mineralized tissues in the presence of high affinity of calcium ions [17, 21] enhancing the mineral (hydroxyapatite) crystal formation [13, 20]. Therefore, it has been expected to be expressed at later stages after the differentiation and maturation. The null hypothesis related to the expression of alkaline phosphatase and bone sialoprotein was rejected as both investigated materials had different behavior during the incubation periods.

Osteopontin is phosphoprotein associated with cell survival, adhesion, proliferation and mineralization as it possesses several calcium binding domains [13, 14] that encoded within 9-24 hours after the activation [13]. This fact supported the present results as the expression of this gene was detected at the first day by both

investigated materials. While osteocalcin was formed by the mature osteoblasts during the bone mineralization [20].

Osteocalcin is an osteoblast-specific gene encoding a secreted protein essential for regulating the bone mineralization process. This fact was confirmed in the current study as the investigated materials showed their therapeutic effect on the undifferentiated mesenchymal stem cells that differentiated to osteoblast-like cells by encoding the bone morphogenetic protein-2 [21, 23]. Osteocalcin is the terminal differentiation of osteoblastic gene [21].

CONCLUSION

The relevance of the present result was that the upregulation of several bone-related genes expression including bone morphogenetic protein-2, alkaline phosphatase, bone sialoprotein, osteopontin and osteocalcin by MSCs exposed to either NeoMTA Plus or ProRoot-MTA indicated their ability to induce osteogenic activity. ProRoot-MTA had a greater osteogenic potential as it induced higher gene expression than that obtained by NeoMTA Plus. Furthermore, rat bone marrow derived MSCs were effectively used to evaluate the osteogenic gene expression of dental materials.

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