THE EFFECT OF NANOPARTICLE MINERAL TRIOXIDE (NMT) ON THE PROLIFERATION AND DIFFERENTIATION OF STEM CELLS HUMAN EXFOLIATED DECIDUOUS TO ODONTOBLASTS

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Abstract

To analysis the effect nano mineral trioxide (NMT) on the proliferation and differentiation of Stem Cells Human Exfoliated Deciduous (SHED) to odontoblast.

This research is a laboratoric experimental study. Mesenchymal stem cells (SHED) isolated from deciduous teeth and identified using immunochytochemistry were cultured until they reached confluent state. This culture was divided into 3 groups: (i) culture without any treatment (control group), (ii) Culture that treated with MTA dose 2 mg and (iii) Culture that treated with NMT on dose 2 mg. The observation of stem cells proliferation used MTT test and differentiation obserced by using ELISA method to detect alkaline phosphatase (ALP) and dentin sialophospho protein (DSPP) concentration.

NMT was not toxic and increased the proliferation of SHED, and did not impede SHED viability especially on the second day compared to MTA ($p < 0.05$). Meanwhile on the third day the viability of MTA was higher rather than NMT ($p < 0.05$). NMT could increase the activity of ALP and DSPP compared to MTA on the SHED ($p < 0.05$).

NMT could increase proliferation of SHED, increased ALP and DSPP activity in SHED and did not impede SHED viability.

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Introduction

The data from WHO Bank (2000) obtained from 6 WHO areas (AFRO, AMRO, EMRO, EURO, SEARO, WPRO) showed that the average of caries incidence (DMFT) in 12-year-old children is around 2.4 %. The current caries index for the same age group in Indonesia, one of SEARO (South East Asia Regional Offices) countries, is around 2.2. The age group serves as a critical indicator since around 76.97% of caries infection occur in the age group.[1] Dental caries in children may impair the function of chewing and food grinding, which, in turn, may impair their growth and development.[2]

The fundamental principle of restorative dentistry is keeping defective dental pulp healthy and functional in order to heal caries or trauma which related by exposed pulp. Pulp tissues are connective tissues with restorative potential. The characteristics of exposed pulp restoration include the reorganization of impaired smooth muscle tissue caused by the regeneration of *odontoblast- like cells* from sub-odontoblast cells and the restoration of open dentin marked by the formation of reparative dentinal bridge. One of the most influential factors in pulp restoration is bacterial penetration through thesurface of pulp-filling material.[3-6]The materials commonly used in treating vital pulp with reversible inflammation in children are formocresol, ferric sulfate, calcium hydroxideandmineral trioxide aggregate.[7]

Currently, formocresol (FC), commonly used in pulpotomy, is considered toxic and

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carcinogenic for human.[8] As an alternative to FC, now the MTA potential in pulpotomy of deciduous teeth is researched intensively. Mineral trioxide aggregate (MTA) is a material commonly used in endodontics.[7] Some research indicated that the use of MTA in vital pulpotomy of deciduous teeth was not as effective as FC and calcium hydroxide (CH). Previous research showed that MTA effectiveness was equal to FC[9] and MTA was not different from CH statistically.[10] Other clinical research demonstrated that the effectiveness of MTA in pulpotomy was just around 66.6% and significantly different from that of FC (76.9%) in treated deciduous molar.[11] Based on the background, it is assumed that the effectiveness of MTA can be increased by modifying the size of MTA particles to be nanoparticles, more specifically nanoparticle mineral trioxide (NMT). In nano (1-100 nm), the modified MTA could excite cellular system more effectively because nano-sized materials (nano particle) can through an organism cell membrane and interact with biological system easily.[12]

Mineral trioxide aggregate (MTA) belongs to metal oxide group (CaO, MgO, SiO, FeO and AlO). In nano size, MTA has higher antibacterial effect so that increase the proliferation, differentiation and maturation rate of pulp cells. Nano particle MTA called nano particle mineral trioxide (NMT). The research used stem cells human exfoliated deciduous (SHED) from remaining deciduous teeth with resorption. It has been found that dental pulp remaining in deciduous teeth contains multipotent stem cell population.[13] The research showed that the teeth, which would eventually fall out naturally, was similar to umbilical cord, since both of them contained mother cells which provided a unique and potential stem cell resource for clinical trials. The stem cells can be isolated and cultured ex vivo by providing a unique stem cell population and can be obtained from unexpected sources of tissues. Stem cells human exfoliated deciduous (SHED) seems to represent multipotent stem cells that may be more mature compared to the population of post natal stromal stem cells previously observed.[13]

The laboratory aspect to be examined was whether the modification of MTA particle size to be nano (NMT) could increase and stimulate the rate of proliferation and differentiation, of mesenchymal dental pulp stem cells to odontoblasts by quantifying differentiation and maturation markers**.** The research covered: (1) observing the proliferation of mesenchymal dental pulp stem cells using cell viability test – if a material or a drug can stimulate cell proliferation, it means that material is non toxic[14]; (2) observing the differentiation of mesenchymal dental pulp stem cells using the activities of: (A) Alkaline phosphatase (ALP). The activity of the enzyme is related to the formation of dental hard tissues which are widely used as the marker of calcification tissue descendant cells like odontoblasts.[15] (B) Dentin sialophospho protein (DSPP). The activity of DSPP serves as differentiation marker of dentin-pulp complex. DSPP is secreted selectively by odontoblaststo stimulate collagen in secondary dentin formation.[16,17]

Materials and Method

The sample of this research was mesenchymal stem cells which isolated from deciduous teeth. Dental pulp stem cells from deciduous teeth would proliferate and reach the confluent stage after 21 days. Sample were distributed to 24 SHED *well culture plates*. The stages of research were as follows:

The Modification of MTA to NMT

The MTA modification process consisted of several stages: MTA particle size was modified using a high energy *millin*g machine (HEM). Before the milling process, the MTA particle size was measure using particle size analysis (PSA). The 4 gram of MTA was put inside the HEM tube and milled for 30 hour to obtain the nano-sized particles. To obtain particles smaller than 100 nm, the milling result was dissolved in 99.8% isopropanol which served as the medium of wave transmission that would break down the particles into smaller particles. The dissolved materials were placed inside a small tube and were treated with ultrasonic for 30 minutes to reached the maximum size of nano particles as expected. The resulting particles were measured using particle size analysis (PSA) and scanning electron microscopy (SEM) and referred as Nanoparticle Mineral Trioxide (NMT) powder and this particles were used as the materials of this research.

We used energy dispersive analysis with X-ray (EDAX) to know the elements inside of

MTA and NMT. Meanwhile, we used X-ray diffraction (XRD) to analyse the composition of MTA and NMT.

The Isolation and Identification of Stem Cells Using Immunochytochemistry Technique

Stem cells were isolated from deciduous teeth that taken from our dental clinics, and placed in a tube containing DMEM. The teeth were rinsed using Chlorhexydin (Minosep) and PBS and divided into two using mortar and pestle. The pulp tissues were taken from the broken teeth using extirpation needle. The tissues were chopped and put inside a tube containingof 5ml of PBS. The resulting cell suspension was centrifugated at 2000 rpm for 10 minutes. The result was sediment (pellet) which was dissolved in DMEM. Astrainer (70 µm) was used to obtain a single cell. The next stage, the stem cells selection were done using bead magnet coated with an antibody of anti human STRO-1 as a stem cell marker. As the cells attached to the bead magnet, cells were eluted by adding buffer to release STRO-1 positive stem cells from the bead magnet. The result of stem cellsisolation was inoculated in tissue culture plate filled with DMEM which was enriched with 10-20 % of fetal calf serum (FCS) or fetal bovine serum (FBS), 10 µM ascorbic acid 2-phophate, 2 mM L-glutamine, 100 Units/ml penicillin and 100 µg/ml streptomycin as proposed by Laino*et al* [18]. The cells were put into an incubator and incubated in a controlled atmosphere consisting of 95% O_2 and 5% CO_2 at 37^0C and the culture medium was replaced every 2-3 days.

The stem cells of deciduous teeth (SHED) was identified using immunochytochemistry technique. The stem cells that had been cultured for14 days were confirmed using an antibody of antihuman STRO-1, the mark the mesenchymal stem cells were done by washing the cells three times using PBS and conducting blocking by adding blotto reagent consisting 5% skim milk and 0.03% Tween 20 to prevent nonspecific binding. The result was washed using PBS for three times. In this step showed that the stem cells were reacted with the antibody of anti STRO-1 labeled Fluorescent isothiocyanate (FITC) that had been diluted in 2 µl/ml blotto inside well and was incubated for 20 minutes at 37^0C and 5% CO_2. After 20 minutes, another process of three times washing using PBS was done and the result was observed using a microscope of 200 times magnification. STRO-1 positive cells appeared blue and were identified as mesenchymal dental pulp stem cells of permanent and deciduous teeth. Some of the dental pulp stems cells (SHED) were subject to proliferation test using cells viability test. The viability test used MTT method and trypan blue dye exclusion that had previously been exposed to MTA and NMT. Some other dental pulp stem cells were subject to differentiation test to odontoblasts.

The Observation of Proliferation and Differentiation of SHED to Odontoblasts After NMT and MTA Administration

Mesenchymal stem cells which had been in confluent state were distributed into 24 well culture plates containing around 10^4 or 2 X 10^5 cells per ml in culture medium (DMEM + 10% FBS/FCS + penisillin/streptomisin). In the cells culture plates dexamethason (10nM), ascorbic acid (50 µg/ul), and glycerophospate (10 mM) and bioactive molecule powder of NMT and MTA of 100 ng/ml concentration in every well culture plate were given. The proliferation ability of MTT in odontoblast forming were done using ELISA by detecting the concentration of ALP and DSPP. All processes in stage 2 were repeated 6 times in 3 different periods.

The Observation of the Proliferation of SHED Using MTT Test

Proliferation refers to the growth rate of dental pulp stem cell which indicates the cell viability after the MTA and NMT were given. The stages of viability test are as follows: The culture of SHED which used in viability test was the culture of DPSC in which the SHED had been harvested. The number of cells/ml in the culture was marked using trypan blue exclusion test and counted using hemocytometer under a light microscope. The amount of cells in 10^4 cells/well were cultured in 96 well plates and incubated at 37^0C and 5% CO_2 for 24 hours. After 24 hours, 50µl of MTA and NMT were given in the pulp cells cultures and observed from day 1 to day 3 and the control group was the medium of DMEM. The pulp cells cultures then were incubated at 37^0C and 5% CO_2 for 4 hours. In each well of the cultures of pulp were given 15 µl of MTT assay

and incubated at 37^0C and 5% CO_2 for 3 hours and 15µl acidified isopropanol was added to each well and the wells were put in a shaker for 1 hour. The result was read using microplate reader with a wave length of 405 nm. The percentage of NMT absorbance value was computed and then compared to the absorbance value of the control group.

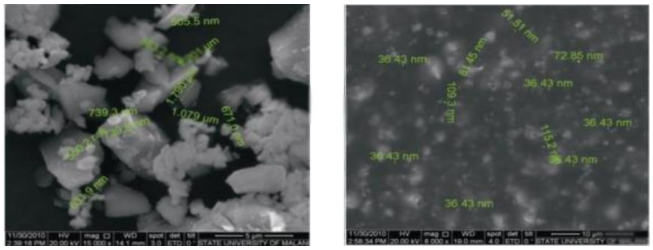
The Observation of the Differentiation The Observation of the Differentiation of SHED to Odontoblasts Using ELISA Test

The activity of ALP was determined using Colorimetri ALP reaction. This was reaction between supernatant MTA and NMT and fluorescent substrat-spesific ALP. The number of fluorescence indicated the number of ALP activity. The stages in the process of measuring ALP activity were: buffer reagent of 290 µl 1 M dietanolamine at pH 9.8 was placed in 300 µl well plate. 5 µl 0.65 M (247 mg/l ml H_2O) p-nitrophenyl phosphate was incubated at 37^OC for 5 minutes. 5 µl of the sample (MTA, NMT and blank) was added to each well and mixed inside microplate. The microplate was put inside microplate reader/ELISA and read at 405 nm wave length. The DSPP was determined using the human DSPP ELISA Kit. In general, the procedure of Human DSPP ELISA was as follows: 100 µl of standard human DSPP or sample was put in each ELISA plate which was previously coated with DSPP-specific monoclonal antibody. The amount of 100 µl of biotin-conjugated polyclonal antibody was added, followed by 100 µl of avidin-conjugated HRP. The presence of antigen-antibody bindings to DSPP was detected by adding 90 µl of TMB substrate. The bindings were indicated as shown of the color changing into blue color in each ELISA well plate. The reaction of enzyme-substrate was halted by adding 50 µl of stop solution that containing of sulfide acid. The absorbance value was read at 450nm±2 nm wave length. The absorbance value of each standard and sample group were equal to the number of antigen-antibody binding in a particular well. The curve of standard human DSPP was obtained by entering the absorbance log value and the log value of standard human DSPP which concentration value as determined (20 ng/ml; 10 ng/ml; 5 ng/ml; 2.5 ng/ml; 1.25 ng/ml; 0.625 ng/ml; 0.312 ng/ml and 0 ng/ml). The result is shown as a linear equation of curve. The concentration of each sample was obtained by entering absorbance log value of sample in the linear equation of standard human DSPP curve.

Statistical Analysis

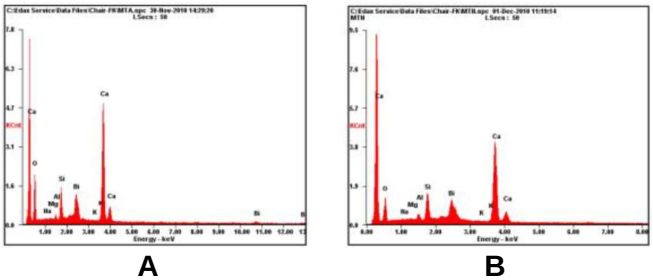
The data was tested using one way ANOVA when the data were normally distributed and had homogenous variance, to know the difference between three groups of research used Tukey test. If the data were not normally distributed and did not have a homogenous variance, they were made into normally distributed data with homogenous variance using transformation. If the data were not normally distributed and did not have homogeneous variance, even after the transformation, Kruskal Wallis.

Results



A B

Figure 1. The result of SEM of MTA and NMT (A) SEM result of MTA showed the average MTA particle size was 0.508 µm (15000x magnification). (B) SEM result of NMT showed the average NMT particle size was 75.8 nm. (8000x magnification).



A B

Figure 2. The result of EDAX of MTA and NMT (A) EDAX result of MTA showed that the quantity of calcium was the highest, followed by oxide, bismuth, and silicate. (B) EDAX result of NMT showed that the quantity of calcium was the highest, followed by silicate, oxide, and bismuth respectively.

The result showed that the particle size of MTA was between 330 nm – 1 µm (< 1 µm) or 0.508 µm on average and the particle size of NMT was < 115 nm or 75.8 nm (nano size) on average (Figure 1). Calcium and silicate were shown more dominant in the group of NMT than in MTA (Figure 2).

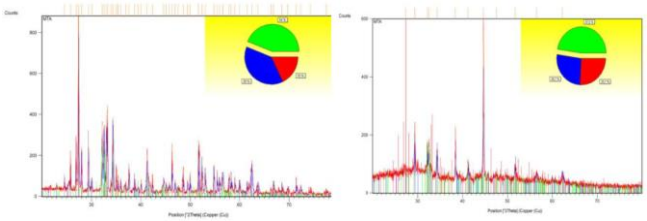
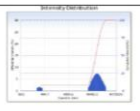


Figure 3. (A) XRD analysis of MTA showed that dicalcium silicate (44%) was the highest percentages among of MTA compositions. (B) XRD analysis of NMT showed that dicalcium silicate (47.5%) was the highest percentages among of NMT composition and was higher rather than MTA composition.

The result showed composition of MTA consisted of tricalcium silicate (38%), dicalcium silicate (44%) and bismuth oxide (18%). The composition of NMT consisted of tricalcium silicate (26.7%), dicalcium silicate (47.5%) and bismuth oxide (25.7%).

The research also involved particle size analysis (PSA) of MTA and NMT. The PSA result showed that MTA particle size was 7.4µm, while NMT particle size was 41.1nm. (Figure 4).

Sample	Number Distribution	Milling Time	Size (nm)
MTA		0	7397.8

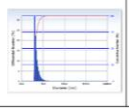
Sample	Number Distribution	Milling Time	Size (nm)
NMT		30 hours UltraSonicisop ropil 30minutes	41.1

Figure 4. (A)**.** PSA result of MTA with particle size of 7397.8 nm or 7.4 µm (B). PSA result of NMT with particle size of 41.1 nm (*nano size*)

The PSA technique for cells analysis was shown gave more accurate result than given by SEM and EDAX since PSA was the tool specifically designed to quantify particle size.

Deciduous tooth used in this experiment was the anterior tooth in which some part of its root, at maximum 2/3 of the root is resorped. In the day 21, the pulp cells were reached its confluent state (Figure 5). After culturing the dental pulp cells, the next step was isolating mesenchymal stem cells using magnetic bead and anti STRO-1 antibody to find the mesenchymal stem cells marker. The isolated stem cells were identified using immunochytochemistry and anti STRO-1antibody labeled FITC as the marker (Figure 6). The culture of dental pulp cells succeeded after 2 months of experimentation with the repetitive cultures of different samples. Some of the failure found in this experiment was caused by bacterial contamination.

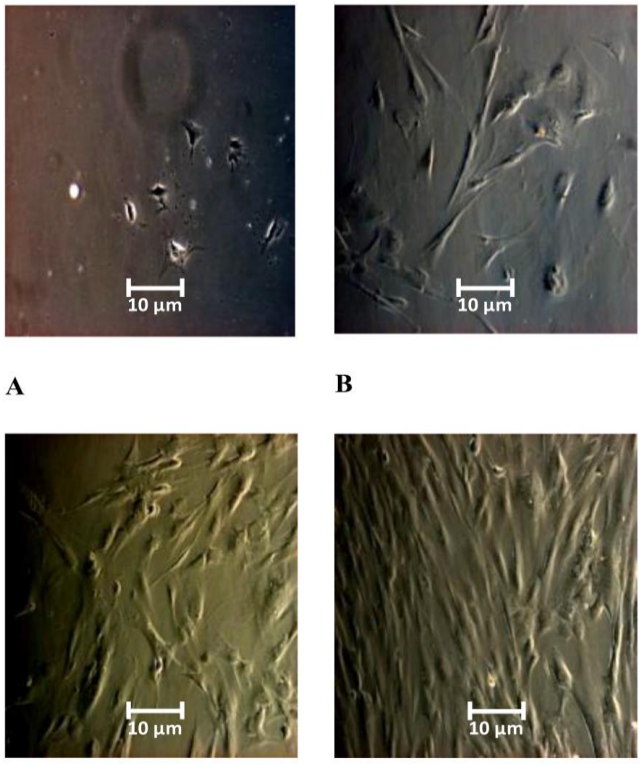


Figure 5. The microscopic picture of pulp cell of deciduous teeth. (A) on day 1. (B) on day 7. (C) on day 14. (D) on day 21. (100x magnification)

The result of stem cells isolation showed that all of stem cells were STRO-1 positive (100%). The culture of STRO-1 positive stem cells reached confluent state in the day of 17. Some stem cells sample (SHED) then included for viability test. The other samples were used to measure the activity of ALP, DSPP (Figure 7).

The result of Tukey test showed that in the day 1 there is no significant difference between SHED exposed NMT with MTA p = 1.00. On the second day there was a significant difference between SHED exposed NMT and SHED exposed MTA p = 0.012. Meanwhile, on

the third day there was a significant differences between SHED exposed MTA with SHED exposed NMT p = 0.043 (Figure 8).

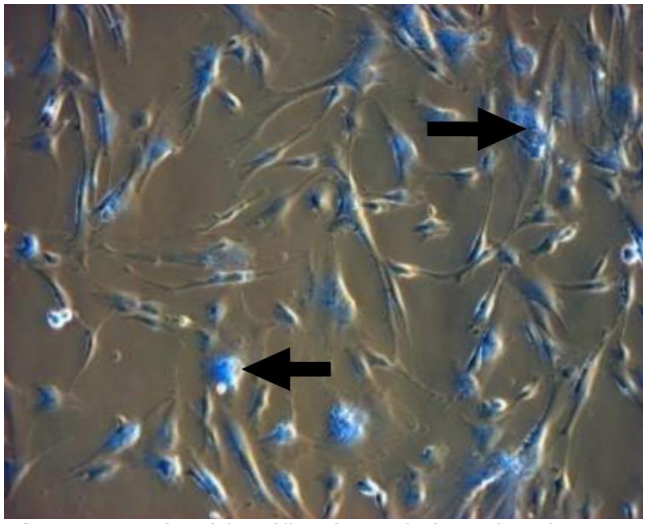


Figure 6. The identification of dental pulp stem cells using immunochytochemistry. STRO-1 positive SHED in fluorescent blue. The arrows indicate SHED (200x magnification)

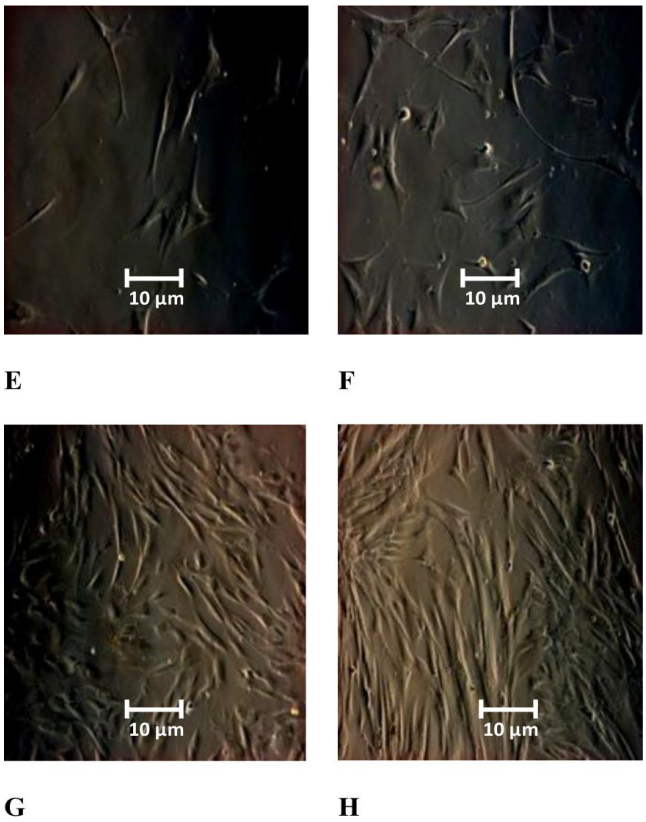
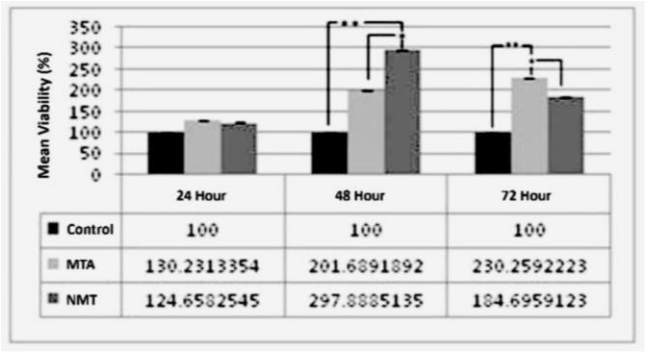
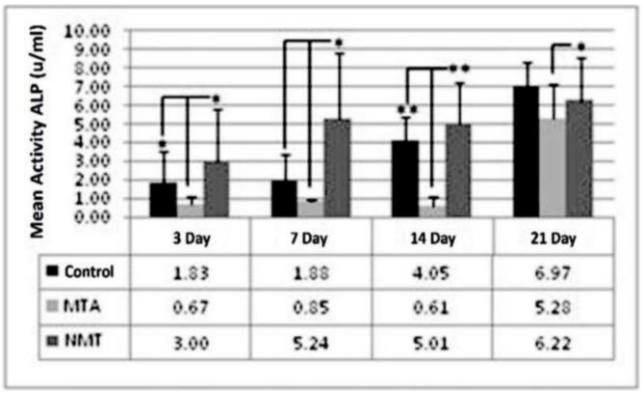


Figure 7. Microscopic picture of the stem cells of deciduous teeth (SHED): (E) SHED on day 1. (F) SHED on day 5. (G) SHED on day 14. (H) SHED on day 17. (100x magnification)



**) p < 0.01; *) p < 0.05

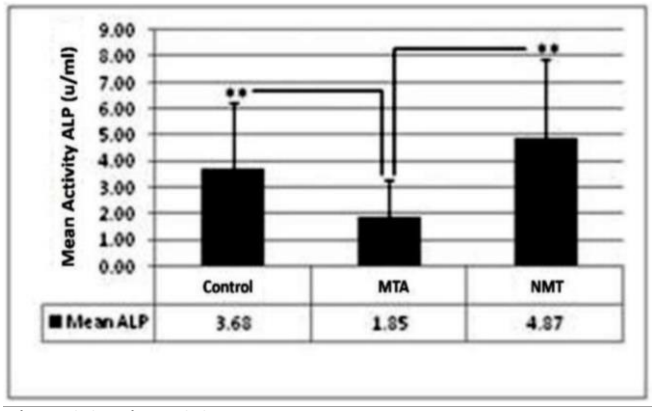
Figure 8. The mean of viability of SHED given NMT and MTA from day 1 to day 3.



**) p < 0.01; *) p < 0.05

Figure 9. The mean of ALP activity in SHED starting from day 3 to day 21

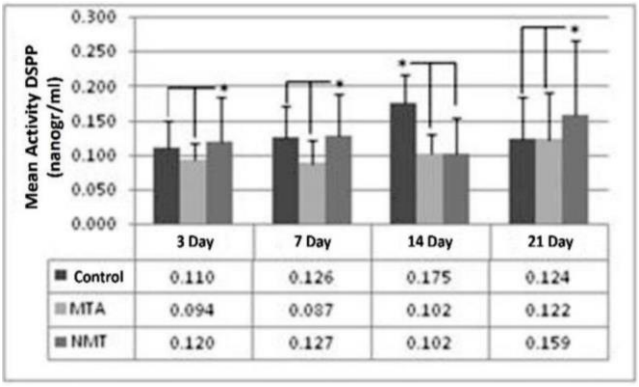
The result of Kruskal Wallis test on ALP activity in the day 3 there was a significant differences between SHED exposed NMT with SHED exposed MTA p = 0.028, On the 7th day p = 0.046, on the fourteenth day with Tukey test p = 0.000 and on the 21th with Kruskal Wallis test p = 0.000 (Figure 9).



**) p < 0.01; *) p < 0.05

Figure 10. The mean of ALP activity in SHED for 21 days

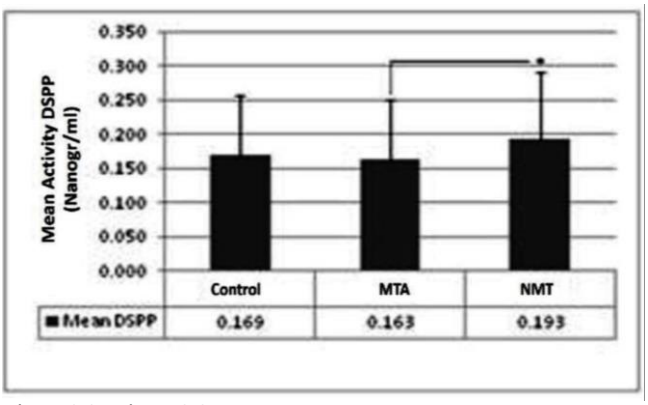
The result of Kruskal Wallis showed that in a complete 21 days there was a significant difference between ALP activity in SHED exposed with NMT and SHED exposed with MTA p = 0.000 and between control group p = 0.024 (Figure 10).



**) p < 0.01; *) p < 0.05

Figure 11. The mean of DSPP activity in SHED starting from day 3 to day 21.

The result of Tukey test on DSPP activity in the day 3 were significantly difference between SHED exposed with NMT and exposed with MTA p = 0.026, on the 7th day p = 0.046 and on the day 21st p = 0.042. Meanwhile there was no a significant differences on the day 14th between SHED exposed NMT with SHED exposed MTA p = 0.950 (Figure 11)



**) p < 0.01; *) p < 0.05

Figure 12. The mean of DSPP activity in SHED for 21 days.

The result of Kruskal Wallis test showed that in a complete 21 days there was a significant difference between DSPP activity in SHED exposed NMT and SHED exposed MTA p = 0.027 meanwhile the result of Tukey test showed that there was no significant differences between DSPP activity in SHED exposed NMT and control group p = 0.978 (Figure 12)

Discussion

The result of cells size that were observed using SEM and EDAX on MTA and NMT indicated that the particle size of MTA was found between 330 nm - 1 µm (< 1 µm) or 0.508 µm on average, while the particle size of NMT was < 115 nm or 75.8 nm in average. The result of this research is different from the research result done by Camilleri *et al* [19]. Camilleri reported the result of SEM and EDAX of MTA particle surface was < 1 µm - 30 µm for the biggest size and showed that the MTA used was not purely contained of tricalsium silicate and dicalsium silicate [20].The PSA result indicated that MTA particle size was 7.4 µm, while NMT particle size was 41.1 nm. The PSA technique for cells analysis was shown gave more accurate result than given by SEM and EDAX since PSA was the tool specifically designed to quantify particle size, while SEM and EDAX were designed to obtain the description of particle micro structure, particle size, and particle surface composition. The result of particle size measurement results using the PSA technique can be seen in figure 4 A dan B.

The result of XRD as shown in figure 3 A and B showed that there was no compound change and new compound formation. However, there was a change in the number of the NMT composition, in which the dicalcium silicate and bismuth oxide were higher than those in found in the MTA group and the tricalcium silicate was lower than that found in the MTA group. The change of percentage in NMT composition most probably might be due to the overheated procedure during the MTA milling process. The result of XRD also indicated that there was no change in composition. However, there was a change in the number of each composition and a decrease in crystal size after the MTA became the NMT. The decreased of crystal size indicate that the regularity of the atom arrangement also decreased, which mean that the material became amorphous.

The Viability test using MTT on SHED showed that SHED exposed with MTA and NMT showed with higher means value than given in

the control group in the day 1, 2, and 3. The highest means value was found reached in the day 2. This indicates that both NMT and MTA are non toxic to SHED. The NMT means value was found higher than shown in the MTA group in the day 2 only, while MTA means value was found higher than NMT in the day 1 and 3 (Figure 8). The viability of SHED that given with NMT showed decreased in the day 3 and became lower than what seen in the MTA. This might be the NMT contained less tricalcium silicate compared to MTA. This situation related with the reaction of tricalcium silicate in the cells that already finished in the day 2 which caused the decrease in calcium production [19]. The result of this research proved that the present of the NMT in the SHED rose the proliferation process of the SHED significantly as shown in the day 2.

Starting from the day 3 to 21, the ALP activity in SHED that exposed with NMT was shown higher than those exposed with MTA and the control group (Figure 9). The result of this experiment showed that the NMT is better than what shown in the MTA in the process of reparative dentin in deciduous teeth shown by the increase of higher ALP activity compared to the MTA. The increases of ALP activity may also due to the fact that deciduous teeth contain less *Hydroxyapatite* (HA) than in the permanent teeth [21]. This increases of ALP activity showed in the SHED can be explain that the NMT contained of rich calcium ions that able to compensate the lack of HA in deciduous teeth. The ALP activity in SHED given with NMT showed higher than what given by the MTA for 21 days (Figure 10). This result represented that the NMT found to be more effective than MTA in stimulating the process of reparative dentin since the used of NMT showed with increases of the calcification process in SHED that taken from the deciduous tooth.

DSPP activity in SHED given with NMT showed with significantly different in day of 3, 7 and 21 than DSPP given with MTA and the control group. However, in day of 14, the DSPP activity of SHED given with NMT and SHED given with MTA showed in the same result, but shown with lower activity than in the control group (Figure 11). The DSPP activity in SHED given with NMT showed higher than what given by the MTA for 21 days as shown in Figure 12. This results indicates that the use of NMT found more effectively increases the formation of SHED secondary dentin than what given by the used of MTA.

The result of research showed that NMT gave more effective result in increasing the formation of secondary dentin and dentin calcification in SHED compared to what given by the MTA as the NMT has ability to increase the ALP and DSPP activities as shown in figure 8-11. The result of this research can be assumed that the NMT might give a better result comparing to MTA when it use for *pulpcapping* treatment, although further *in vivo* study is needed.

Conclusion

The result of this researchshowed that modification of the MTA particle size into smaller size in nano size was achieved by the milling the particles for 30 hours and followed with the used of the ultrasonic machine for 30 minutes.

The NMT can increase SHED proliferation, and was found non toxic to SHED, did not impede SHED viability, and increase ALP and DSPP activities, both functioning as reparative dentin materials.

The result of this research showed that the use of NMT stimulated higher level of calcium than given by MTA. This can be assumed that the use of NMT probably has a potential effect in stimulating the increase of calcium in deciduous teeth, which can be predicted its use to increases the dentin regenerative process in deciduous teeth, Schulze *et al*[22] which asserts that calcium absorption is positively correlated with calcium deposit.

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Declaration of Interest

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