EUROPEAN ENDODONTIC JOURNAL

ORIGINAL ARTICLE

The Effect of Hydrogel Hyaluronic Acid on Dentine Sialophosphoprotein Expression of Human Dental Pulp Stem Cells

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ABSTRACT

Objective: Hyaluronic acid (HA) is glycosaminoglycan and one of important factors in extracellular matrix. In an inflamed pulp, when niche biology is conducive, the recruitment of human dental pulp stem cells (hDPSCs) will take place and differentiate into odontoblast like cell, creating reparative dentine and expressing dentine sialophosphoprotein (DSPP). Therefore, the purpose of this study was to analyze the potential of hydrogel HA in various concentration towards hDPSCs differentiation via DSPP expression at day 7 and 14.

Methods: After hDPSCs incubation reaching 80% confluence, cells were then starved for 24 hours. Then, culture media were supplemented with osteogenic media. hDPSCs planted into 96 well plate and HA 10 µg/mL, 20 µg/mL, and 30 µg/mL were added. DSPP expression was analysed using elisa reader at day 7 and 14, qualitative result was analysed using alizarin red at day 21. Data was analysed using one-way ANOVA.

Results: At day 7, there was a statistically significant different potential of HA conditioned media in various concentration ($p<0.05$) towards hDPSCs differentiation via expression of DSPP with HA 30 µg/mL being the most potential concentration to increase DSPP expression.

Conclusion: HA have the potential to increase odontoblast differentiation process via expression of DSPP, with HA 30 µg/mL being the optimum concentration for hDPSCs.

Keywords: Culture media, dental pulp, dentine sialophosphoprotein, hyaluronic acid, regenerative endodontics, stem cells, tissue regeneration

HIGHLIGHTS

- In addition to three well-known components of regenerative endodontics (stem cells, scaffolds, and growth factors), two other factors which is microbiological environment of the dentine and other survival factors play an important role in the success of treatment.
- Hyaluronic acid (HA) is a glycosaminoglycan and an important component of the extracellular matrix in the dentine niche.
- Hyaluronic acid has the potential to increase process of odontoblast differentiation, observed by a high DSPP expression.

Please cite this article as:
Nugraheni VI, Bagio DA, Margono A, Julianto I. The Effect of Hydrogel Hyaluronic Acid on Dentine Sialophosphoprotein Expression of Human Dental Pulp Stem Cells. Eur Endod J 2023; 8: 280-5

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Received January 23, 2023,
Revised May 08, 2023,
Accepted May 10, 2023

Published online: July 27, 2023
DOI 10.14744/eej.2023.59672

INTRODUCTION

In recent years, the field of regenerative endodontics has presented a promising potentials of pulp regeneration, therefore, treatment modality for inflamed pulp has been shifting towards regenerative endodontics (1). The concept of the dentine-pulp complex regeneration focuses mainly on the potential of human dental pulp stem cells (hDPSCs) to differentiate into odontoblast like cell which plays an important role in the formation of dentine pulp complex (2, 3).

Tissue engineering in regenerative endodontics requires three main components consisting of

stem cells, scaffolds, and growth factors (4). Materials that can induce odontoblast differentiation in regenerative endodontics range from synthetic materials such as MTA, bioceramics, to the emerging cements such as strontium incorporated tetracalcium phosphate cement (STTCP) to biological scaffold such as collagen and hyaluronic acid (5, 6).

The dental pulp is a low compliance tissue because it is placed in a cavity surrounded by hard tissue and only communicates with the outside environment through apical foramina (7). Because of this low compliance condition, compared with the wound healing process of skin or oral mucosa, the healing process of dentine-pulp complex is much more complicated and several factors contribute to the healing process (8). Regeneration of the dentine-pulp complex in mature teeth may be challenging compared to regeneration in immature teeth with abundant blood flow and cells (9). There are two types of stem cells, active stem cells that are inside the cell cycle and quiescent stem cells that are out of the cell cycle. hDPSCs are considered quiescent stem cells because they rarely proliferate in adult under physiological conditions (8, 10).

Up to date, there are some challenges in the regeneration of the dentine-pulp complex. One of the reasons is the tissue growth in the regenerative endodontics using scaffold has not been yet able to produce healthy functional tissue (11, 12). This result suggests that there might be some important factors that are not being considered in regenerative endodontics; hence, the identification of the most suitable combination of regenerative endodontic components still need to be continuously studied (13).

A recent study by Mari-Beffa et al. (13) suggested that the microbiological environment of the dentine and other survival factors play an important role in the success of regenerative endodontics, besides stem cells, scaffolds, and growth factors (13).

Hyaluronic acid (HA) is a glycosaminoglycan and an important component of the extracellular matrix in the dentine niche (14). Extracellular matrix proteins play an important role in regulating stem cell homeostasis and differentiation in the microbiological environment of the dentine niche (15). Hyaluronic acid, in various molecular weights, are known to have an important role in each stage of regeneration including the repair of the extracellular matrix, the process of migration, proliferation and differentiation of cells (16).

In an inflamed pulp, when the microbiological environment is conducive, there is a recruitment of pulp stem cells which will differentiate into odontoblast-like cells to form reparative dentine and there will be an expression of dentine sialophosphoprotein (DSPP) which is a specific regulatory protein in pulp stem cell differentiation (17).

Previous research by Chrepa et al. (18) has observed the effect of commercial hyaluronic acid (Restylane) on stem cells of the apical papillae (SCAP) and showed that on day 14 there was an increase in DSPP expression with a quantitative real-time reverse-transcription test polymerase chain reaction (qRT-PCR), while another study by Chen et al. (19) showed that the application of high molecule hyaluronic acid derived from *Streptococcus equi* diluted with saline with a concentration of 2 mg/mL on hDPSCs in osteogenic media showed an increased mineralization activity on days 3, 7 and 14 using the alkaline phosphatase (ALP) activity test. Osteogenic media was used in this study to ensure mineralization (18, 19).

Another study by Umemura et al. (20) which applied high molecule hyaluronic acid in powder preparations diluted with distilled water at a concentration of 1 g/mL, 5 g/mL, 10 g/mL, and 20 g/mL to hDPSCs showed an increase in mineralization after 7 days with ALP activity test and qRT-PCR test showed that there was DSPP expression on 10 g/mL hyaluronic acid after 24 hours (20).

However, until now, there has been no study about the effect of hyaluronic acid as culture media for hDPSCs with observation of odontoblast differentiation and dentine mineralization time up to 21 days. Therefore, the purpose of this study was to analyze the potential of hydrogel HA conditioned media in various concentration towards hDPSCs differentiation via DSPP expression at day 7 and 14 and observe the qualitative process at day 21.

MATERIALS AND METHODS

This study was approved by Ethics Committee at March 7th 2022 with approval number as followed 01/Ethical Exempted/FKGUI/III/2022 No. Protocol: 030180222. We were using hDPSCs from previous research (No.49/Ethical Approval/FKGUI/X/2020 (amendment); No. Protocol: 070260820) and conducted in accordance with The Declaration of Helsinki. This study was conducted at Prodia Stem Cell (ProStem) Laboratory, Jakarta, Indonesia. Two operators performed the experimental study, Each experimental group were in triplicates, and repeated two times. Figure 1 shows the flowchart of the experimental procedures of the study.

Hyaluronic Acid (HA)

Hyaluronic acid (Z fill deep®, New-Ulm, Germany) which consisted of hydrogel hyaluronic acid 23 mg/mL (molecular weight 3 Mio. Daltons) was used. HA was sterilized and diluted with DMEM until reaching concentration of 10 µg/mL, 20 µg/mL, and 30 µg/mL.

Dental Pulp Cell Culture

hDPSCs at passage 3 and 4 was incubated in a humidified atmosphere of 5% CO_2 at 37°C until reaching 80% confluence. Cells were then starved by replacing the cell culture supplement with Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific Inc., MA, United States) with 0% Fetal Bovine Serum (FBS) for 24 hours.

After 24 hours, culture media was supplemented using 10 mM β glycerophosphate, 50 µg/mL ascorbic acid, and 100 Nm dexamethasone to create an osteogenic condition. Then, hDPSCs were cultured in four different groups consisting of control group (osteogenic culture media), osteogenic culture media+HA 10 µg/mL, osteogenic culture media+HA 20 µg/mL, and osteogenic culture media+HA30 µg/mL.

DSPP expression of the hDPSCs

hDPSC were placed in 96 well-plate, each well containing $5x10^3$ cells. These samples of control and DSPP experimental groups expression was measured after 7 and 14 days of incubation.

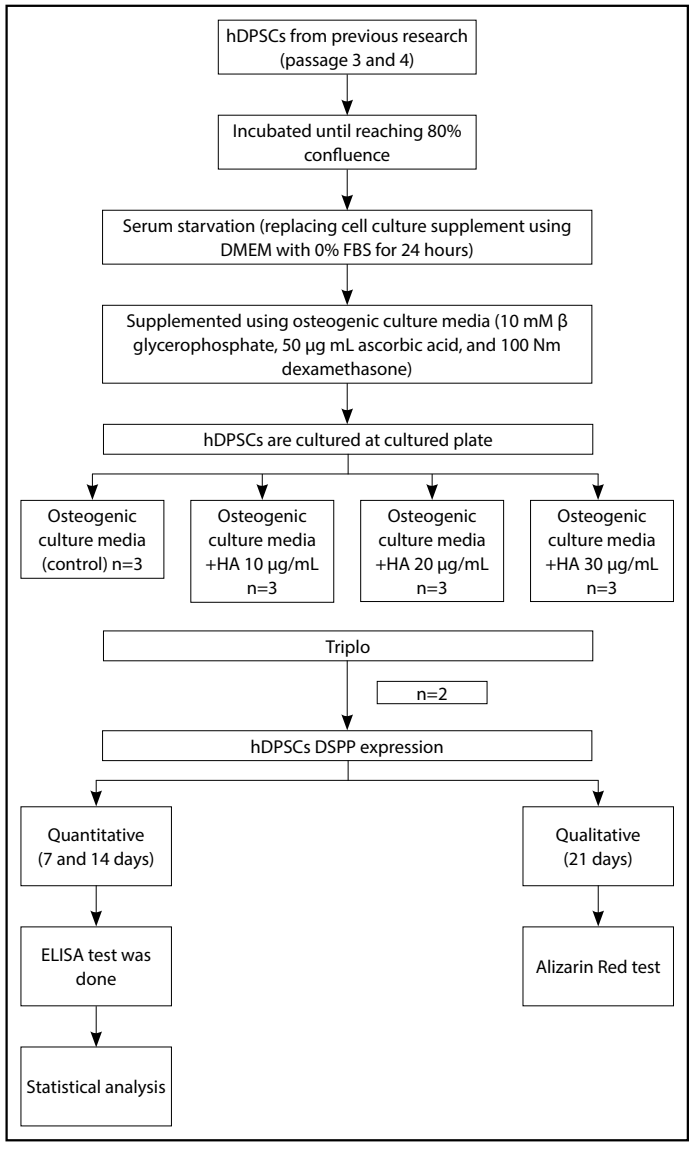

Figure 1. The experimental procedure flowchart

hDPSCs: Human dental pulp stem cells, DMEM: Dulbecco's Modified Eagle Medium, FBS: Fetal Bovine Serum, HA: Hyaluronic acid, DSPP: Dentine sialophosphoprotein, ELISA: Enzyme Linked Immunosorbent Assays

Enzyme Linked Immunosorbent Assays (ELISA) test was done following the manufacturer's protocol on a microplate reader under a wavelength of 405 nm (Bio-Rad Laboratories, Inc., California, United States).

Alizarin Red Test

Samples of control and experimental groups were incubated for 21 days to undergo Alizarin Red staining test (Sigma-Aldrich, Massachusetts, United States). This test was conducted as a qualitative analysis of the mineralization nodules formation.

Statistical Analysis

This data was analyzed using one-way ANOVA, followed by Tamhane post-hoc to compare between groups. Analysis using Wilcoxon non-parametric test was also used to indentify differences between the study groups. All the tests were conducted at a significance level of 95% ($p<0.05$). All of the data were analyzed using IBM SPSS Statistics Software, version 23.0 (IBM Corp., Armonk, NY, United States).

TABLE 1. DSPP Expression in hDPSCs culture media with hyaluronic acid (HA) 10 µg/mL, 20 µg/mL, 30 µg/mL at day 7 and 14 intervals

Culture media	DSPP Expression in hDPSCs culture media (ng/mL)	
	7 days mean (SD)	14 days mean (SD)
Positive control	12.9 (8.02)	6.9 (0.30)
HA 10 µg/mL	9.3 (0.60)	5.33 (1.87)
HA 20 µg/mL	7.8 (0.64)	7.10 (0.60)
HA 30 µg/mL	19.83 (0.40)	8.86 (0.56)
p	0.027*	0.021*

*: *One-way ANOVA*, $p<0.05$. hDPSCs: Human dental pulp stem cells, DMEM: Dulbecco's Modified Eagle Medium

TABLE 2. Different potential of hDPSCs DSPP expression in various hyaluronic acid concentration 10 µg/mL, 20 µg/mL, 30 µg/mL at day 7 and 14 intervals

Culture media group	p	
	7 days	14 days
Control vs HA 10 µg/mL	0.988	0.865
Control vs HA 20 µg/mL	0.949	0.998
Control vs HA 30 µg/mL	0.852	0.074
HA 10 µg/mL vs HA 20 µg/mL	0.259	0.807
HA 10 µg/mL vs. HA 30 µg/mL	0.000*	0.361
HA 20 µg/mL vs HA 30 µg/mL	0.000*	0.119

*: *Post Hoc Tamhane*, $p<0.05$. hDPSCs: Human dental pulp stem cells, DSPP: Dentine sialophosphoprotein, HA: Hyaluronic acid

TABLE 3. Different potential of hDPSCs DSPP expression in various hyaluronic acid concentration 10 µg/mL, 20 µg/mL, 30 µg/mL between day 7 and day 14 intervals

DSPP expression	Median (min-max)	p
Day 7	9.65 (5.8-21.6)	0.004*
Day 14	7.15 (4.2-9.5)	

*: *Wilcoxon*, $p<0.05$. hDPSCs: Human dental pulp stem cells, DMEM: Dulbecco's Modified Eagle Medium

RESULTS

The effect of HA on the expression of DSPP (ng/mL) in hDSPCs was evaluated using ELISA reader after incubation for 7 and 14 days. Based on the one-way ANOVA test in Table 1, there were significant differences between the treatment groups HA 10 µg/mL, HA 20 µg/mL, and HA 30 µg/mL in the expression of DSPP hDPSCs at 7 days and 14 days ($p<0.05$).

Based on the post hoc Tamhane test in Table 2, at day 7 interval, there was a significant difference in DSPP expression ($p<0.05$) between treatment group HA 10 µg/mL and HA 30 µg/mL, also between treatment group HA 20 µg/mL and HA 30 µg/mL. At day 14, there was no significant difference of DSPP expression ($p<0.05$) in hDPSCs culture media between all groups.

Based on the Wilcoxon test in Table 3, there was a significant difference between the treatment groups HA 10 µg/mL, HA

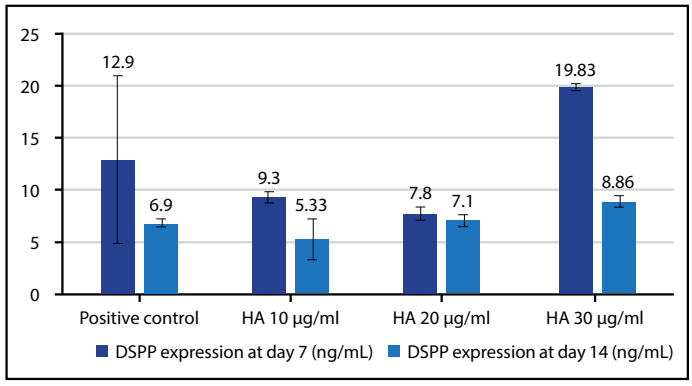

Figure 2. Means comparison of DSPP expression at day 7 and 14

HA: Hyaluronic acid, DSPP: Dentine sialophosphoprotein

20 µg/mL and HA 30 µg/mL on the expression of DSPP between days 7 and 14.

At day 7 and 14 intervals, it was found that the application of hyaluronic acid on hDPSCs culture media resulted in the mean expression of DSPP with the highest expression in the HA 30 µg/mL. During the day 14 interval, the level of DSPP expression decreased in all control and treatment groups compared to the 7 days observation period (Fig. 2).

Figure 3 shows the results of Alizarin Red staining at day 21 of observation using a microscope (Zeiss Primovert) with red nodules showing mineral deposits originating from differentiated hDPSCs cells. The nodules were more evident at the concentration of HA 30 µg/mL.

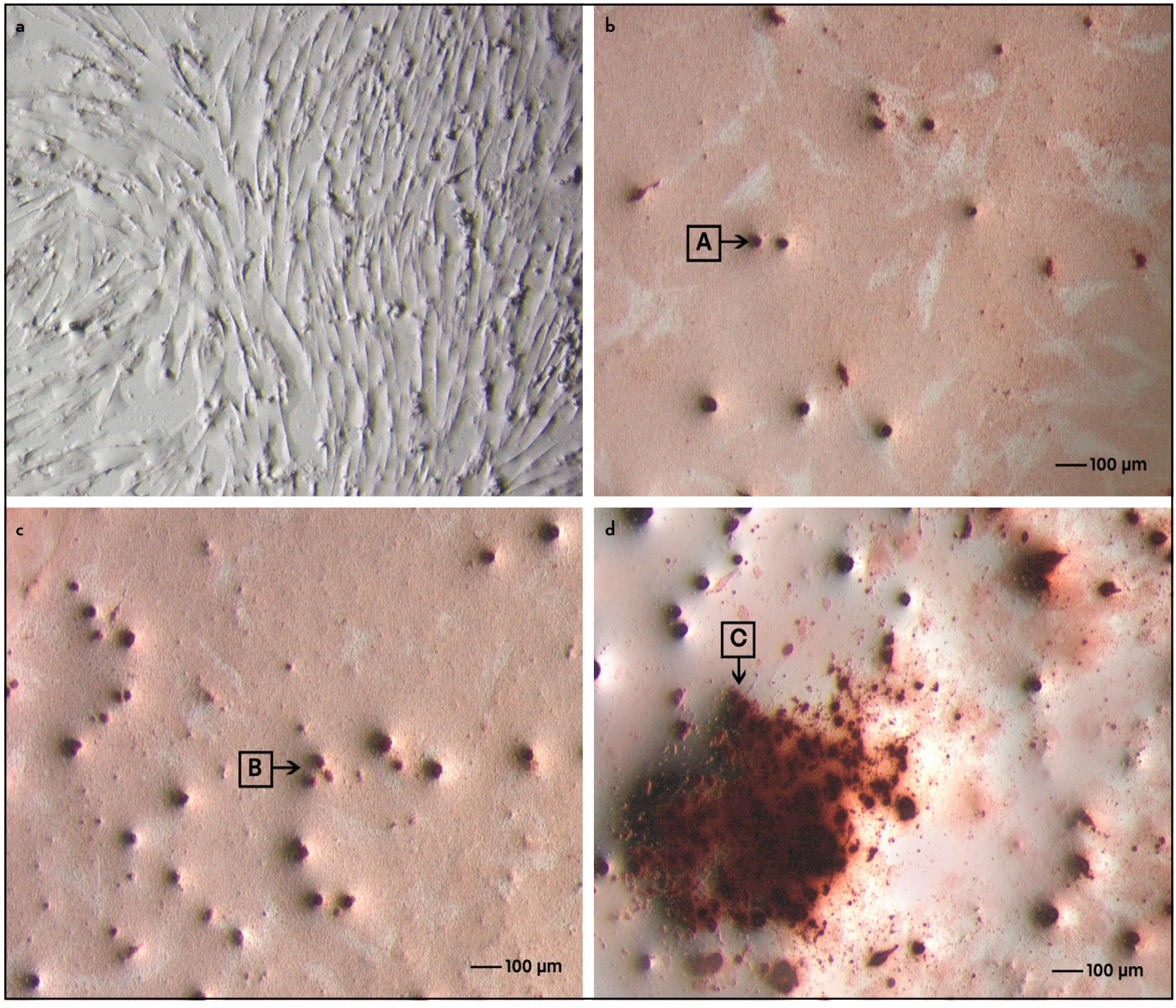

Figure 3. Alizarin red staining result. Positive control at day 21 **(a)**, HA 10 µg/mL at day 21: black arrow (A) shows mineralized deposit from differentiated hDPSCs that can be visualized by several red nodules **(b)**, HA 20 µg/mL at day 21: black arrow (B) shows more nodules but still scattered and present in less than 50% of the area **(c)**, and HA 30 µg/mL at day 21: black arrow (C) shows more than 50% of histological area consist of red nodules **(d)**

HA: Hyaluronic acid, hDPSCs: Human dental pulp stem cells

DISCUSSION

Hyaluronic acid (HA) added to the hDPSCs culture media in this study had a high molecular weight [called high molecular weight hyaluronic acid (HMW HA)]. HA is divided into low molecular weight hyaluronic acid (LMW HA) with a molecular weight of about 100 KDa and HMW HA with a molecular weight of 1 MDa or more (21). LMW HA has proinflammatory and proangiogenic effects, and plays an important role in cancer development. Based on study by Price et al. (22) a molecular weight range of 200-500 kDA appears to be important in promoting cancer cell. While in this study, HMW HA was used because it can bind to CD44 and cause upregulation of TGF-β1 so that cell migration and differentiation can occur (21, 23, 24).

Dentine extracellular matrix is composed of proteoglycans and non-collagenous proteins. Non-collagen proteins consist of small integrin binding ligands, n-linked glycoprotein (SIBLING) consisting of dentine sialophosphoprotein (DSPP), dentine matrix protein 1 (DMP1), bone sialoprotein (BSP), osteopontin (OPN), and matrix extracellular phosphoglycoprotein (MEPE) (25). SIBLING plays a role in the mineralization of collagen fibrils and crystal growth when predentine is converted to dentine. In this study, the potential of odontoblast differentiation was analysed through the expression of DSPP (25, 26).

DMP1 is secreted prior to DSPP expression. DMP1 is also found in bone in greater numbers than teeth, so it is not a specific protein coding gene for odontoblast differentiation, while DSPP is a protein coding gene or precursor protein which is a specific factor for odontoblast differentiation (26). DSPP will then be cleaved by proteinases into dentine sialoprotein (DSP) and dentine phosphoprotein (DPP) (25). DPP is a non-collagenous protein that is most commonly found in dentine extracellular matrix as an initiator and modulator of the formation and growth of hydroxyapatite crystals, while the mechanism of DSP is still unclear in dentineogenesis (25, 26).

The results of this study showed that there were potential of hyaluronic acid in various concentrations (10 µg/mL, 20 µg/mL and 30 µg/mL) in hDPSCs culture media towards DSPP expression with an observation time of 7 days and 14 days. The highest DSPP expression appeared to be in the HA 30 µg/mL treatment group. There was a significant difference between the treatment groups HA 10 µg/mL, HA 20 µg/mL and HA 30 µg/mL towards DSPP expression between 7 days and 14 days of observation, with DSPP expression at 14 days of observation decreasing in all group. This has never been proven in previous studies and is in line with the process of odontoblast differentiation.

The total time for a cell to complete its cycle until it finally divides into two cells is 24 hours (27). Then after 48 hours, the termination process leading to cell differentiation occurs, where TGF-β1 plays a specific role in turning the proliferation process into cell differentiation by decreasing the expression of inhibitors of DNA binding (id) proteins (18, 19). Differentiation then occurs on day 3 to day 7 where in the study from Sabbagh et al. (28), it was found that on day 7, there had been odontoblast differentiation forming a columnar shaped cell (27, 29, 30). DSPP expression decreased on day 14 because as soon as DSPP was released in the tissue, DSPP was proteolytically cleaved into DSP and DPP (25, 26). The cleavage process from DSPP to DSP and DPP is an activation event that converts inactive precursors to active fragments such as zymogen activation (25). DPP then binds to calcium and hydroxyapatite ions, adheres to collagen and induces intrafibrillar mineralization (26). On day 21, the occurrence of mineralization in this study was observed through the results of Alizarin Red staining. It is obvious that the nodules were more evident at a concentration of HA 30 µg/mL. This result is in line with previous research although HA used in those studies have different type and sources. Study by Lu et al. (31) showed that in the Alizarin Red test the mineralization of hDPSCs appeared to be significantly increased on day 21. In addition, the study by La Noce et al. (14) also showed that the administration of HA in hDPSCs showed an increase in mineral nodules compared to the control group at 14 and 21 days of observation.

Although previous study by Palma et al. (32) showed that the addition of sodium hyaluronate: chitosan scaffolds in dogs did not improve the formation of new mineralized tissues along the root canals walls, the use of hyaluronic acid as a natural component to revitalize dentine niche might still be beneficial in regenerative endodontics (19, 32, 33). The use of chitosan as scaffold in the study by Palma et al. (32) showed that there is an initial inflammatory reaction associated with chitosan application to hard and soft tissues that impairs its application as a suitable scaffold for clinical application (33). While in this study, HA is used without scaffold to revitalize the dentine niche which plays an important role in regenerative endodontics. Some studies show that interaction between HA with hDPSCs could induce the formation of reparative dentine and HA have an important role in odontoblast differentiation in pulp capping and regenerative endodontics (19, 33). Future *in vivo* studies are still needed to determine the right dosage of HA as medicament used in regenerative endodontic procedures to revitalize dentine niche in order to achieve a successful odontoblast differentiation.

There are some limitations to this study. The result of this study could only be applied to the same type of HA and stem cell. Also, in this study, the effect of HA on odontoblast differentiation process only assessed through DSPP expression, which is an early marker of odontoblast differentiation. While as soon as DSPP was released, it was proteolytically cleaved into DSP and DPP. Therefore, further research is needed to analyze the expression of more specific odontoblast differentiation markers and mineralization potential such as dentine phosphoprotein (DPP). In addition, further research to determine HA signaling pathway in inducing odontoblast differentiation is also needed.

CONCLUSION

Hyaluronic acid 30 µg/mL has the most potential to increase the process of odontoblast differentiation through DSPP expression at 7 up to 21 days of observation. Hyaluronic acid could be a promising additive material in regenerative endodontic to achieve dentine pulp complex regeneration.

Disclosures

Acknowledgment: We are pleased to acknowledge Ms. Angliana Chouw, S.Si., M.Farm, Ms. Riska Agustina, S.Si. and Ms. Annisa, S.Si from PT Prodia Stem Cell Indonesia for the support.

Conflict of interest: The authors deny any conflict of interest.

Ethics Committee Approval: This study was approved by The Faculty of Dentistry Universitas Indonesia Ethics Committee (Date: 07/03/2022, Number: 030180222).

Peer-review: Externally peer-reviewed.

Financial Disclosure: This study was supported by a grant from the Directorate of Research and Community Engagement at Universitas Indonesia HIBAH PUTI 2022 with number No.: NKB-227/UN2.RST/HKP.05.00/2022.

Authorship contributions: Concept – V.I.N., D.A.B., A.M., I.J.; Design – V.I.N., D.A.B., A.M.; Supervision – D.A.B., A.M., I.J.; Funding - D.A.B.; Materials - V.I.N.; Data collection and/or processing – D.A.B., V.I.N.; Analysis and/or interpretation – D.A.B., V.I.N.; Literature search – V.I.N., D.A.B., A.M.; Writing – V.I.N., D.A.B., A.M., I.J.; Critical Review – V.I.N., D.A.B., A.M., I.J.

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