ORIGINAL ARTICLE

The effect of hyaluronic acid conditioned media on hDPSCs differentiation through CD44 and transforming growth factor-β1 expressions

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ABSTRACT

Hyaluronic acid (HA) has the capability to influence dentin niche which is important in regenerative process. The CD44 as a specific receptor of HA was found to be related to dentin mineralization process. Meanwhile, transforming growth factor β1 (TGF-β1) has a vital role in the transition from proliferation into the differentiation of human dental pulp stem cell human dental pulp stem cells (hDPSCs) to become odontoblast cells and dentin mineralization. This study aims to analyzed HA's effect on dentin mineralization through CD44 and TGF-β1 expressions. Stem cells were cultured in four different supplemented conditioned media (control, +10 μg/mL, +20 μg/mL, and + 30 μg/mL of HA). Evaluation of CD44 expression was analyzed using flow cytometry and TGF-β1 was analyzed using enzyme-linked immunosorbent assay reader. Qualitative result using Alizarin red test after 21 days was done to confirm the formation of mineralization nodules. It was shown that HA expression of CD44 and TGF-β1 on hDPSCs were higher in AH groups compared to the control group and 30 μg/mL HA induced the highest TGF-β1 expression on hDPSCs. Alizarin red test also showed the highest mineralization nodules in the same group. Therefore, from this study, we found that supplemented 30 μg/mL of HA was proved in initiating hDPSCs differentiation process and promote dentin mineralization.

Key words: Cells receptors, growth factor, mineralization, stem cells

INTRODUCTION

Dental pulp stem cells Human dental pulp stem cells (hDPSCs), are having a mesenchymal stem cells phenotype, which have therapeutic and have a high capacity to proliferate and differentiate into different cell types including odontoblast, adipocytes, osteoblast or chondroblast, neuron cells, and form dentin or pulp-like tissues, respectively, for repair process after injury.[1-3] This differentiation process regulates by the modulation of growth factors, transcriptional factors, extracellular matrix proteins, and receptor molecules.[4,5]

Extracellular proteins and growth factors induce stem cells differentiation depending on their niche biology.[4,6] Hyaluronic acid (HA) is a glycosaminoglycan and the main component of the extracellular matrix that can create an adequate microenvironment, accelerate cell proliferation and improve healing which is important in tissue regeneration.[7,8]

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In the human body, at homeostatic conditions, HA is in high molecular weight (HMW) but can degrade into low molecular weight when inflammation occurs.[2,9] HMW HA can regulate cell's adhesion, motility, proliferation, and migration to the injury area.[9,10] La Noce *et al*., studied the effect of HA on hDPSCs with different molecular weights. The result showed that HMW HA can induce osteogenic differentiation of hDPSCs, where this process begins with proliferation, followed by matrix production, and mineralization. It was also showed increasing mineralization nodules at 14 and 21 days. From this study, we concluded that HA's molecular weight substantially affects biological processes and tissue regeneration.[10]

HA regulates cells activity by binding with a specific-targeted cell surface receptor, CD44, which was found to be expressed in cells undergoing remineralization processes such as ameloblasts, odontoblasts, and osteoblasts.[7,9,11,12] HA-CD44 interaction allows a better cell adhesion, migration, proliferation rates, and stimulates transforming growth factor β (TGF-β)-mediated differentiation which is crucial in the healing process.[7,13]

Chen *et al*. conducted to see the effect of CD44 on mineralization by providing CD44 knockdown and the results showed a decrease in hDPSCs mineralization. This result concluded that CD44 plays a role in the mineralization process of hDPSCs.[14] Furthermore, Umemura *et al*. conducted a study of additional 10 μg/mL HMW-HA influence hDPSCs and proof a significant increase in CD44 expression of hDPSCs culture within 5 min incubation. This study also proved significantly increased alkaline phosphatase (ALP) values after 1-week cultures in HA.[11] Another study by Chen *et al*. used HMW HA, proving the same result of ALP value. These researches indicated that the higher the concentration of HA, the higher the ALP value, as a marker of differentiation of osteoblasts and odontoblasts expressed.[11,14,15]

The transition from proliferation to differentiation in the cell cycle depends on the inhibition of the proliferation process and activation of specific gen cells, which created different types of cells. One of the growth factors that play role in this transition is TGF-β1.[16,17] In addition, TGF-β1 also plays a role in cell growth, extracellular matrix production, apoptosis, angiogenesis, and immune response.[3] It was known that the differentiation of hDPSCs into odontoblast cells and dentin mineralization regulate by TGF-β1, but there was no study proving this related to the addition of HA as supplemented conditioned media in hDPSCs culture.[10,16,18,19]

The present study aimed to analyze the expression of CD44 and TGF-β1 on hDPSCs by adding different concentrations of HA in hDPSCs culture media. Therefore, by knowing the potency of additional HA in hDPSCs culture media will give a new perspective in terms of the future use of HA in regenerative endodontic therapy.

MATERIALS AND METHODS

This study was approved by the Ethical Committee Faculty of Dentistry, Universitas Indonesia (No. 82/ethical/approval/FKGUI/IX/2019; No. Protocol: 070940819) and conducted at Prodia Stem Cell (ProStem) Laboratory, Jakarta, Indonesia. (No.13/Ethical Approcal/FKGUI/III/2022 No. Protocol: 050160222).

Human dental pulp stem cells

hDPSCs biological stored raw material (from the previous study) at passages 3 and 4, was incubated in a humidified atmosphere of 5% CO_2 at 37°C until reaching 80% confluence. After 24 h incubation, culture media was supplemented using 10 mM β glycerophosphate, 50 μg/mL ascorbic acid, and 10–8 M dexamethasone to create an osteogenic condition. Cells were then starved by replacing the cell culture supplement with Dulbecco's Modified Eagle Medium (DMEM) (DMEM; Thermo Fisher Scientific Inc., MA, United States) with 1% fetal bovine serum for 24 h. Then, hDPSCs were cultured into four different supplemented conditioned media (control [DMEM], DMEM +10 μg/mL, DMEM +20 μg/mL, and DMEM +30 μg/mL of HA).

Hyaluronic acid

HA (Z fill deep®, New-Ulm, Germany) which consisted of HA 23 mg/ml. HA was sterilized and diluted with DMEM until reaching a concentration of 10 μg/mL, 20 μg/mL, and 30 μg/mL.

CD44 expression of the hDPSCs

hDPSCs cells were divided into 24 microtubes that contain 1×10^5 cells/microtube and then centrifugated for 5 min with a centrifuge machine (Thermo Fisher Scientific, Inc., Massachusetts, United States). The hDPSCs cells groups were incubated for 5 min before given 5 μl CD44 antibody (BD Biosciences, California, United States). Then, samples were analyzed by flow cytometry (Becton, Dickinson, and Company [BD], New Jersey, United States).

Transforming growth factor β1 expression of the hDPSCs

Human dental pulp stem cells (hDPSCs) were placed in 96 wellplate, each well containing 5×10^3 cells then given all groups of culture media. TGFβ-1 expression was measured after 7 and 14 days of incubation, enzymelinked immunosorbent assay (ELISA) test was following the manufacturer's protocol, on an ELISA microplate reader (Bio-Rad Laboratories, Inc., California, United States) under a wavelength of 405 nm, a detection range of 31.25–2000 pg/mL, and a sensitivity value of 18.75 pg/mL.

Statistical analysis

The statistical analysis was performed using IBM SPSS

Statistics Software, version 22.0 (IBM Corp., Armonk, NY, United States). All the data were analyzed using one-way ANOVA with a significance level of 95% ($P < 0.05$).

Alizarin red test

After 21 days, all groups were undergoing the alizarin red staining test (Sigma-Aldrich, Massachusetts, United States). This test was conducted as qualitative result of the formation mineralization nodules.

RESULT

CD44 expression of hDPSCs

Adding 10 µg/mL, 20 µg/mL, and dan 30 µg/mL of HA in hDPSCs culture media compared to control groups, showed an increase of CD44 expression after 5 min of incubation ($P < 0.05$) with the highest expression observed in 10 µg/mL group [Table 1], as seen in the flow cytometry chart [Figure 1].

Transforming growth factor β1 expression on hDPSCs

TGF-β1 expression in hDPSC was evaluated using the ELISA reader after incubation for 7 days and 14 days. Compared to control groups, all experimental groups showed increase in TGFβ-1 expression for both times ($P < 0.05$) with the highest expression observed in 30 µg/mL group after incubation for 7 days [Table 2]. When comparing each group, after incubation for 7 days, 20 µg/mL, and 30 µg/mL of HA significantly increased TGFβ-1 expression compared to the control group while 30 µg/mL significantly increased compared to 10 µg/mL and 20 µg/mL of HA. After incubation for 14 days, 10 µg/mL and 30 µg/ml significantly increased compared to the control group while 20 µg/mL was significantly different compared to 30 µg/mL of HA [Table 3].

Alizarin red test

After 21 days of incubation, qualitative result from alizarin red staining it was confirmed that there was an increased mineralization nodule, especially in 30 µg/mL HA conditioned media of the hDPSCs group [Figure 2].

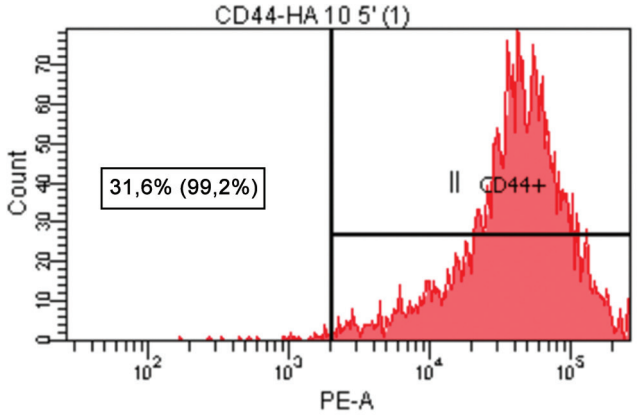

Figure 1: Flowcytometry result of 10 µg/mL HA after 5 min incubation. HA: Hyaluronic acid

DISCUSSION

Umemura *et al*. proved that 10 µg/mL HA conditioned media can increase CD44 expression on hDPSCs.[11] Based on that study, we used an HMW HA with different concentration; 10 µg/mL, 20 µg/mL, and 30 µg/mL. This study showed that

Table 1: Analysis of CD44 expression of human dental pulp stem cell in three different concentration of hyaluronic acid cultured media

Treatment	*n*	Mean (SD)	*P*
Control	3	23 (1)	0.023*
10 µg/mL HA	3	30 (2.5)	
20 µg/mL HA	3	27 (3)	
30 µg/mL HA	3	28 (1)	

*One-way ANOVA, $P<0.05$. HA: Hyaluronic acid, SD: Standard deviation

Table 2: Analysis of expression transforming growth factor β-1 of human dental pulp stem cell in different concentration of hyaluronic acid

Conditioned media groups	TGF-β1 expression on hDPSC after incubation for 7 days and 14 days, mean (SD)	
	7 days	14 days
Control	424.3 (15.7)	673.2 (28.6)
HA 10 µg/mL	535.6 (26.8)	569.5 (50.5)
HA 20 µg/mL	677.1 (71.6)	641.2 (43.8)
HA 30 µg/mL	1142.3 (150.1)	504 (23.6)
P	0.028*	0.003*

*One-way ANOVA $P<0.05$. TGF-β1: Transforming growth factor β-1, hDPSC: Human dental pulp stem cell, HA: Hyaluronic acid, SD: Standard deviation

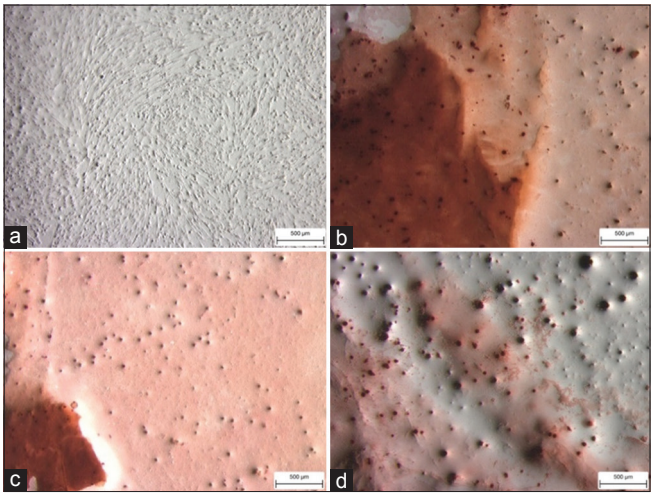

Figure 2: Qualitative result seen under Microscope (ZEISS, Primovert Inverted Cell Culture Microscope) using Alizarin red staining on hDPSC culture media supplemented with HA, then incubated for 21 days. (a) Control group (b) 10 µg/mL HA, (c) 20 µµg/mL HA, (d) 30µg/mL of HA. HA: Hyaluronic acid

Table 3: Comparison of transforming growth factor-β1 expression on human dental pulp stem cell in 10 μg/mL, 20 μg/mL, and 30 μg/mL hyaluronic acid culture media

Conditioned media groups comparison	*P*	
	7 days	14 days
Control versus HA 10 μg/mL	0.146	0.010*
Control versus HA 20 μg/mL	0.006*	0.335
Control versus HA 30 μg/mL	0.000*	0.001*
HA 10 μg/mL versus HA 20 μg/mL	0.075	0.051
HA 10 μg/mL versus HA 30 μg/mL	0.000*	0.069
HA 20 μg/mL versus HA 30 μg/mL	0.000*	0.002*

**Post hoc* LSD, $P<0.05$. HA: Hyaluronic acid, LSD: Least significant difference

all experimental groups had a higher CD44 concentration compared to the control group. This was in accordance with Umemura *et al*. and La Noce *et al*. which found that HMW of HA can induce CD44 expression.[10,11] Other previous studies by Chen *et al*. showed that CD44 as a marker of hDPSCs differentiation, therefore, to be able to prove the initial process of hDPSCs differentiation, in this study will also observe TGF-β1 expression as one of the regulator in hDPSCs differentiation.[14]

TGF-β1 will form odontoblast-like cells, and result in the expression of specific odontogenic proteins such as dentin matrix protein 1 (DMP-1) and dentin sialophosphoprotein (DSPP).[20] Growth factors will activate the proliferation signaling network to initiate the process of cell differentiation or apoptosis (cell death), senescence (cell aging), or stop proliferation and begin to differentiate.[16] The proliferative phase in the wound healing process, begin around day 3–5, which can be continued by simultaneous differentiation or transition from proliferation starting from day 3 to 7, then deposition of extracellular matrix formation of hard tissue can last up to day 21.[21] Therefore, if proliferation occurs continuously, tumorigenesis will occur, so an increase in TGF-β1 expression is expected to regulate the cell cycle process by inhibiting proliferation and starting the differentiation process by reducing inhibitor protein binding DNA (Id).[4,17,20]

This study considered that TGF-β1 has an important role in regulating this process. The observation time used was 7 days and 14 days, based on research by La Noce *et al*. that used HA on hDPSCs culture media with an observation time of 7 days, 14 days, and 21 days. That research observed the expression of differentiation osteoblast hDPSCs with markers of osteocalcin, osteopontin, and bone sialoprotein. It was shown that HA increased osteocalcin expression on day 14 and there was a significant increase in osteopontin expression on day 7 and day 14.[10]

The result of this study showed that there were various potential concentrations of HA in hDPSCs culture media on TGF-β1 expression within 7 days of observation. This is in line with a previous study by Umemura *et al*., which showed HA can increase the induction of odontoblast differentiation of hDPSCs in culture media with DSPP and DMP-1 markers through CD44 signaling with the addition of HA concentration of 10 μg/mL in hDPSCs culture media. In 7-day observation, compared to other groups and the control group, HA 30 μg/mL was the most potent concentration. Other studies proved that HA can induce osteoblast differentiation and hard tissue mineralization.[11]

However, the results of this study have not been carried out by previous studies that showed the relationship between increased TGF-β1 expression and the addition of HA on hDPSCs. Nevertheless, this study was in line with the previous study that reported ALP differentiation markers increased on hDPSCs cultured with AH 1 μg/mL, AH 5 μg/mL, AH 10 μg/mL, and 20 μg/mL for 7 days, and increased ALP values compared to controls (highest ALP at 20 μg/mL).[10]

In accordance with another study by Chen *et al*.,[15] the administration of HA 20 μg/mL on hDPSCs can increase mineralization at 3 and 7 days of observations. It was also proven that there was an increase in ALP value, mineral deposition in hDPSCs, and an increase in the activity of bone morphogenetic protein 1 (BMP-1), which is a family of TGF-β1, on 3 days and 7 days of observation. The study by Suzuki *et al*. also showed that an increase in BMP will increase odontoblast differentiation and the formation of mineralized nodules in hDPSCs.[14,15,22]

A study by Sabbagh *et al*. showed the process of odontoblast differentiation of hDPSCs began on day 3–day 7. This also explains the results of this study which showed an increase in HA concentration was directly proportional to TGF-β1 expression on day 7 and a decrease in TGF-β1 hDPSCs expression at 14 days of observation. This phenomenon explains that the transition from proliferation to differentiation of hDPSCs peaks on day 7 and decreases on day 14.[23]

In, accordance with the previous study results of the [Figure 2] qualitative picture in this study also showed mineralized nodules that were increasing at 21 days of observation of 30 μg/mL HA group.[10,11]

However, the result of this study was limited to CD44 and TGF-β1 expressions as a regulator for hDPSCs differentiation using specific HA.

CONCLUSION

HA proven in inducing CD44 and TGF β-1 expressions on hDPSCs. Further study still needs to conduct, to determine the mechanism of HA on the dental pulp differentiation.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. da Rosa WL, Piva E, da Silva AF. Disclosing the physiology of pulp tissue for vital pulp therapy. Int Endod J 2018;51:829-46.
2. Schmidt J, Pilbauerova N, Soukup T, Suchankova-Kleplova T, Suchanek J. Low molecular weight hyaluronic acid effect on dental pulp stem cells *in vitro*. Biomolecules 2020;11:22.
3. Salkin H, Acar MB, Korkmaz S, Gunaydin Z, Gonen ZB, Basaran KE, *et al.* Transforming growth factor β1-enriched secretome up-regulate osteogenic differentiation of dental pulp stem cells, and a potential therapeutic for gingival wound healing: A comparative proteomics study. J Dent 2022;124:104224.
4. Marí-Beffa M, Segura-Egea JJ, Díaz-Cuenca A. Regenerative endodontic procedures: A perspective from stem cell niche biology. J Endod 2017;43:52-62.
5. Potdar PD, Jethmalani YD. Human dental pulp stem cells: Applications in future regenerative medicine. World J Stem Cells 2015;7:839-51.
6. Yu J, Jamal M, Garcia-Godoy F, Huang GT. Dental pulp stem cell niche. In: Turksen K, editor. Tissue-Specific Stem Cell Niche. Stem Cell Biology and Regenerative Medicine. Cham: Springer; 2015. p. 163-89.
7. Saravanakumar K, Park S, Santosh SS, Ganeshalingam A, Thiripuranathar G, Sathiyaseelan A, *et al.* Application of hyaluronic acid in tissue engineering, regenerative medicine, and nanomedicine: A review. Int J Biol Macromol 2022;222:2744-60.
8. Zhai P, Peng X, Li B, Liu Y, Sun H, Li X. The application of hyaluronic acid in bone regeneration. Int J Biol Macromol 2020;151:1224-39.
9. Misra S, Hascall VC, Markwald RR, Ghatak S. Interactions between hyaluronan and its receptors (CD44, RHAMM) regulate the activities of inflammation and cancer. Front Immunol 2015;6:201.
10. La Noce M, Stellavato A, Vassallo V, Cammarota M, Laino L, Desiderio V, *et al.* Hyaluronan-based gel promotes human dental pulp stem cells bone differentiation by activating YAP/TAZ pathway. Cells 2021;10:2899.
11. Umemura N, Ohkoshi E, Tajima M, Kikuchi H, Katayama T, Sakagami H. Hyaluronan induces odontoblastic differentiation of dental pulp stem cells via CD44. Stem Cell Res Ther 2016;7:135.
12. Zheng Z, Patel M, Patel R. Hyaluronic acid-based materials for bone regeneration: A review. React Funct Polym 2022;171:105151.
13. Hwang J, Kiick KL, Sullivan MO. Modified hyaluronic acid-collagen matrices trigger efficient gene transfer and prohealing behavior in fibroblasts for improved wound repair. Acta Biomater 2022;150:138-53.
14. Chen KL, Huang YY, Lung J, Yeh YY, Yuan K. CD44 is involved in mineralization of dental pulp cells. J Endod 2013;39:351-6.
15. Chen KL, Yeh YY, Lung J, Yang YC, Yuan K. Mineralization effect of hyaluronan on dental pulp cells via CD44. J Endod 2016;42:711-6.
16. Berridge MJ. Module 8: Development. In: Cell Signalling Biology. London: Portland Press Ltd; 2014. p. 1-6.
17. Berridge MJ. Module 9: Cell cycle and proliferation. In: Cell Signalling Biology. London: Portland Press Ltd; Biochemical Journal 2014. p. 1-45. .
18. Xia K, Xue H, Dong D, Zhu S, Wang J, Zhang Q, *et al.* Identification of the proliferation/differentiation switch in the cellular network of multicellular organisms. PLoS Comput Biol 2006;2:e145.
19. Keshet Y, Seger R. The MAP kinase signaling cascades: A system of hundreds of components regulates a diverse array of physiological functions. Methods Mol Biol 2010;661:3-38.
20. Liang C, Liao L, Tian W. Stem cell-based dental pulp regeneration: Insights from signaling pathways. Stem Cell Rev Rep 2021;17:1251-63.
21. Broughton G 2nd, Janis JE, Attinger CE. The basic science of wound healing. Plast Reconstr Surg 2006;117:12S-34S.
22. Suzuki T, Lee CH, Chen M, Zhao W, Fu SY, Qi JJ, *et al.* Induced migration of dental pulp stem cells for *in vivo* pulp regeneration. J Dent Res 2011;90:1013-8.
23. Sabbagh J, Ghassibe-Sabbagh M, Fayyad-Kazan M, Al-Nemer F, Fahed JC, Berberi A, *et al.* Differences in osteogenic and odontogenic differentiation potential of DPSCs and SHED. J Dent 2020;101:103413.