



# **Effect of rhBMP-2 and Chitosan in Differentiation of Periodontal Ligament Stem Cells into an Osteoblastic Lineage**

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## **Authors' contributions**

*This work was carried out in collaboration between all authors. Author MY designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author BA prepared chitosan sample. Author EWB managed the analyses of the study and managed the literature searches. All authors read and approved the final manuscript.*

## **Article Information**

DOI: 10.9734/ARRB/2018/38859

### Editor(s):

(1) George Perry, Dean and Professor of Biology, University of Texas at San Antonio, USA.

### Reviewers:

(1) Promila Verma, King George Medical University, India.

(2) Ömer Birkan Ağralı, Marmara University, Turkey.

Complete Peer review History: <http://www.sciencedomain.org/review-history/22866>

**Original Research Article**

**Received 17<sup>th</sup> November 2017**  
**Accepted 19<sup>th</sup> January 2018**  
**Published 26<sup>th</sup> January 2018**

## **ABSTRACT**

**Aims:** To analyze the effect of rhBMP-2 and Chitosan in differentiation of Periodontal Ligament Stem Cells (PDLC) into an osteoblastic lineage.

**Study Design:** This study was designed as *in vitro* study and osteogenic biomarkers were determined in the culture supernatant.

**Place and Duration of Study:** Laboratory of Oral Biology Faculty of Dentistry Universitas Indonesia. Jakarta 10430 Indonesia, January – September 2016.

**Methodology:** Human periodontal ligament stem cells (PDLC) were isolated from the root of vital teeth, followed by identification of stem cells by antibody anti STRO-1. Chitosan was used at the concentration of 0.15%. The culture cells were divided into four groups as follow, the control group (PDLC) and treatment groups with recombinant human Bone Morphogenic protein 2 (rhBMP-2), the

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combination chitosan-rhBMP-2 and chitosan only. The levels of alkaline phosphatase (ALP) was determined by colorimetry and osteocalcin and collagen type I were measured using ELISA. **Results:** The results showed that levels of ALP tended to increase in all groups. At day 14, the highest levels of ALP was in chitosan treated group. The concentration of collagen type I managed to raise in all groups on days 14, and the highest levels Collagen type I occurred in RH BMP-2 and chitosan treated cells, after that decrease in all groups until day 21 ( $p < 0.05$ ). Osteocalcin concentration tended to increase in all groups, and at days 21, the highest levels in with rhBMP-2 + chitosan. **Conclusion:** The rhBMP-2, chitosan, and its combination induce differentiation of periodontal ligament stem cells into the osteoblastic lineage.

**Keywords:** *Periodontal ligament stem cells (PDLC); alkaline phosphatase (ALP); recombinant human bone morphogenic protein 2 (rhBMP-2); chitosan; osteocalcin.*

## 1. INTRODUCTION

Stem cells from periodontal ligament (PDL) play a role in the process of osteogenesis or the formation of regenerative periodontal tissue. Huang, et al. (2009) suggested that the PDL may contain pluripotent stem cells that originate from the neural crest [1]. Stem cells in the PDL are important not only for formation and maintenance of the tissue but also for repair, remodeling, and regeneration of adjacent alveolar bone and cementum. The previous study has shown that progenitor cells isolated from PDL tissue by selection with cell surface markers STRO-1+ and CD146+ are capable of differentiating into chondrogenic, osteogenic, and adipogenic phenotypes under appropriate culture conditions [2]. To optimize the role of osteoblast in the process of osteogenesis will require trigger molecules (growth factors) which together with the extracellular matrix (scaffold) will induce proliferation and differentiation of stem cells into osteoblast-like cells. The scaffold provides a three-dimensional environment for cells to attach and grow, therefore mimicking the *in vivo* condition. Additionally, these synthetic matrices can be fabricated such that it may form any desired shape and carry needed growth factors to guide the process of cell differentiation and tissue formation [3,4]. The tissue-engineering technology involves generating tissue or organ constructs *in vitro* for subsequent implantation. The biodegradable material can be synthetic polymers, e.g., Chitosan. Chitosan is the deacetylated form of chitin. Fungi synthesize chitin and chitosan in their cell walls, while the shells of crabs and shrimps and the bone plates of squids and cuttlefish are composed of chitin only [5,6]. Chitosan has a biological advantage that can be used as a source of biomaterials in dental practice in Indonesia. Chitosan can be used either alone [7], or in combination with

other biodegradable polymers, such as aliphatic polyesters [8,9], other natural polymers with ceramics such as hydroxyapatite (HA) [10]. Chitosan as a scaffold is biocompatible, bioresorbable, and bioactive polymer so that it can be used as biomaterials for medical applications, such as regenerative periodontal tissue engineering and implant fixation. The osteogenic medium can also be supplemented with growth factors that naturally occur in bone, such as bone morphogenic proteins (BMPs), fibroblast growth factors, platelet-derived growth factor (PDGF), transforming growth factor beta, and insulin growth factors [11]. Under normal growth conditions, BMP-2 is localized in bone tissue as a complex with high molecular glycosaminoglycans, and is released in response to bone damage, resulting in the stimulation of differentiation of mesenchymal cells into osteoblasts and induction of cell proliferation [12]. Human recombinant Bone Morphogenetic Protein-2 (RH BMP -2) is one of growth factor, able to stimulate the differentiation and proliferation of Periodontal Stem Cells into osteoblast-like cells, which play a role in the process of osteogenesis or the formation of regenerative periodontal tissue. The purpose of this study was to evaluate whether the administration of rhBMP-2, Chitosan, as well as a combination of both, can stimulate Periodontal Stem Cells – derived cells into an osteoblastic lineage.

## 2. MATERIALS AND METHODS

This study has been approved by Ethical committee Faculty of Dentistry Universitas Indonesia. Human periodontal ligament stem cells (PDLC) were isolated from human third molars of impacted teeth. The PDLC was taken by surgical scalpel and chopped by scalpel under sterile condition. Collagenase type I and dispase

were added to obtain a single cells suspension. The tissues were digested with for an hour at 37° C incubator in a humidified atmosphere containing 5% CO<sub>2</sub>. Serial pipetting were conducted every 15-20 minutes to separate the cells. PDLSC were seeded in culture flask containing Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA, USA) added with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 IU/ml penicillin, 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA, USA), 250 µg fungizone (Invitrogen, Carlsbad, CA, USA), 10 nM dexamethasone (Invitrogen, Carlsbad, CA, USA), 100 µg ascorbic acid, and 100 mM β-glycerophosphate (Sigma, St. Louis, MO, USA). They were cultured in an atmosphere of 5% CO<sub>2</sub> at 37°C incubator until 90% confluency was achieved. Purification of PDLSC was conducted as previously described by Bachtiar et al. [13]. Briefly, cells were transferred in 5 mL tube, washed with PBS containing 2% of inactivated FBS and resuspended in PBS/2% FBS with a concentration of 10<sup>6</sup> per 100 µL. The antibody of CD 10<sup>6</sup> and STRO-1 are used (1:40) to select and isolate stem cells and added to the cell mix, incubated for 20 minutes, washed in PBS/2% followed by second antibody incubation for 20 minutes. Cells are washed, resuspended in PBS/2%FBS containing 4',6-diamidino-2-phenylindole (DAPI; final concentration 1 µg/mL). Identification of stem cells from Periodontal

ligament with STRO-1 immunostaining. The PDLSC culture was divided into four groups, the control group, and treatment groups with rhBMP-2, Chitosan, combination of rhBMP- 2 + 0.15% of Chitosan. To evaluate Alkaline Phosphatase (ALP), osteocalcin and collagen type 1 were determined by ELISA.

### 3. RESULTS

#### 3.1 Alkaline Phosphatase Activity

In this study, the alkaline phosphatase activity of PDLSCs treated by rhBMP-2, Chitosan, and the combination of both was measured for up to 18 days. The results show that the ALP activity of the cells treated with rhBMP-2 and rhBMP-2 + chitosan were in highest concentration at day 18 (Fig. 1). ALP on chitosan only treated group showed lower than those rhBMP-2 and rhBMP-2+ chitosan on days 18 (P < 0.05).

#### 3.2 Concentration of Collagen Type 1

The level of collagen type 1 tended to increase is in all groups on days 14, and on day 18 the highest levels in the treatment group are presented with rhBMP-2 and chitosan, and then decrease in all groups until 21 days.

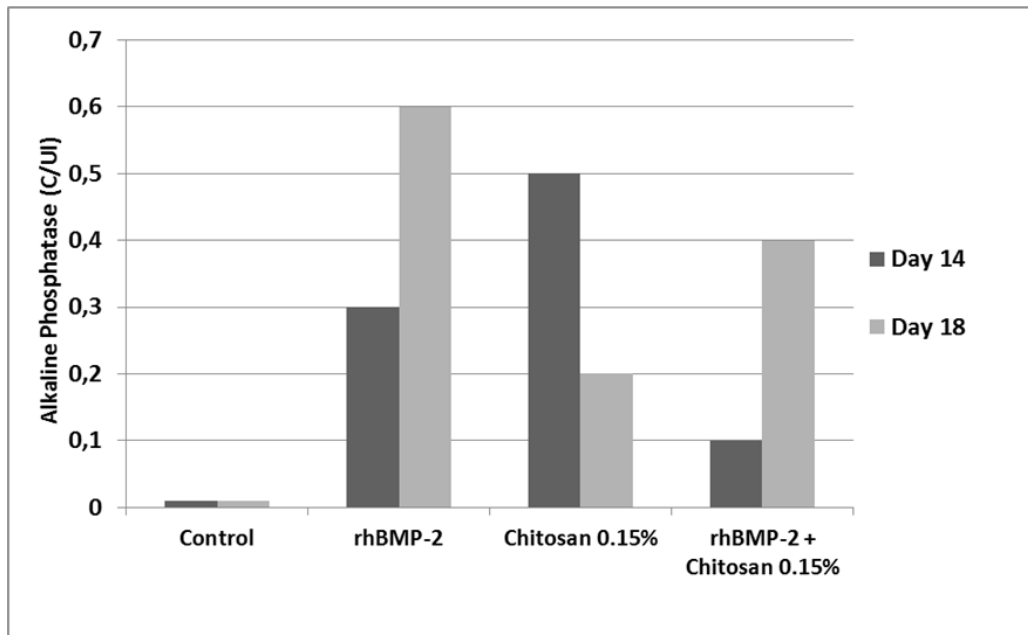


Fig. 1. Alkaline phosphatase activity PDLSC on day 14 and day 18 of experiments

### 3.3 Osteocalcin Concentration

This study found that the osteocalcin activity of PDLSC treated by rhBMP-2, chitosan and rhBMP-2+chitosan were in highest concentration at day 18 of culture (Fig. 3). On day 18,

osteocalcin activity of the cells treated by rhBMP-2+chitosan showed highest osteocalcin activity than the other groups. Compared to day 18, osteocalcin concentration tended to decrease in all groups on days 21.

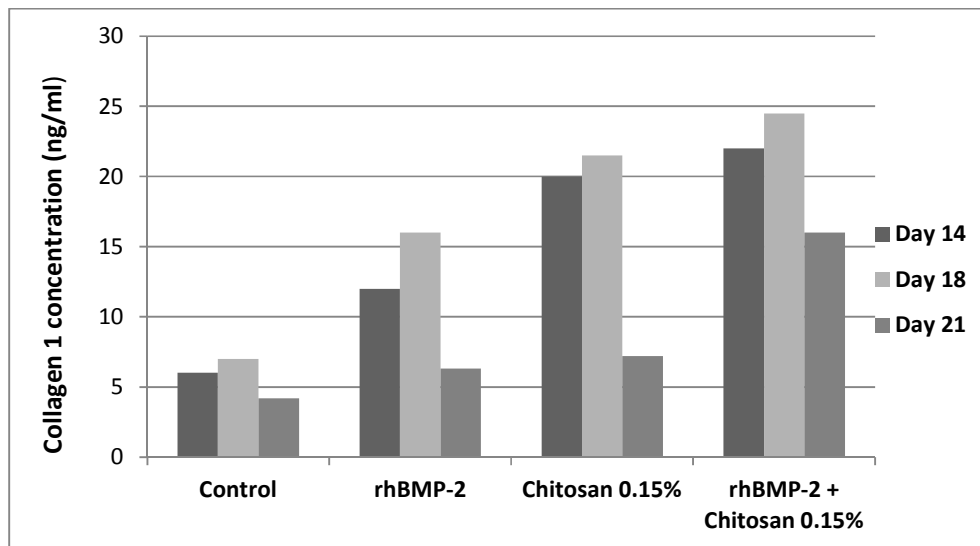


Fig. 2. Concentration of collagen 1 of PDLSC culture medium on day 14, day 18 and day 21 of experiments

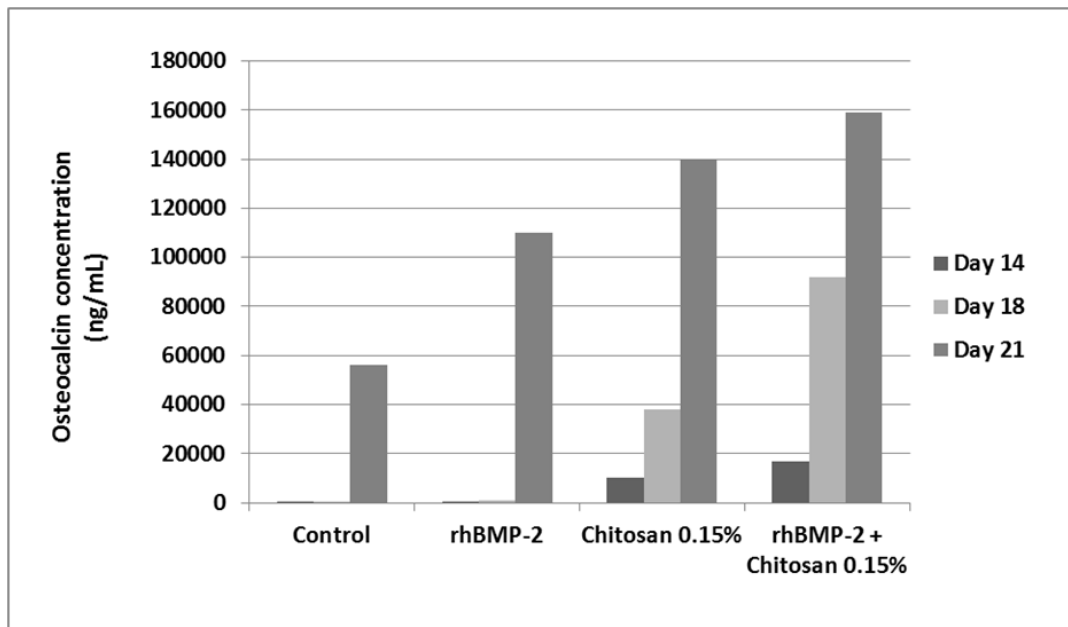


Fig. 3. Concentration of osteocalcin of PDLSC culture medium on day 14, day 18 and day 21 of experiments

#### 4. DISCUSSION

The previous study reported that DPSCs, isolated from human third molars was able to induce differentiation into cementoblast-like cells, adipocytes and collagen forming cells with the material similar to periodontal tissue cement in immunodeficient mice and rats [14]. In the periodontal regeneration process, cells involved in regeneration have shown to express STRO-1 [15]. The result of our study showed that the addition of RhBMP2 and Chitosan had induced osteogenic differentiation of stem cells from periodontal ligament. Similar studies have been reported by Saito et al. [16].

Alkaline phosphatase activity is a well-defined biomarker of osteogenesis and is assumed to reflect the degree of differentiation [17]. Alkaline phosphatase levels are indicative of osteoblast activity and the early stage of osteoblast differentiation [17]. In this study, compared to the control Alkaline phosphatase levels were tend to increase in all groups. These results indicate that in an early stage of PDLSC differentiation RhBMP2 and RhBMP2 +Chitosan were capable of promoting differentiation of PDLSC into osteoblast lineage. Similar outcomes regarding induction of higher alkaline phosphatase activity in proliferation and differentiation of Periodontal Ligament Stem Cells on chitosan have already been reported by our group and by other researchers [17,18].

The concentration of collagen type 1 tended to increase in all groups on days 4, and the highest level is in the treatment group presented with RH BMP-2 + Chitosan, and then decrease in all group until days 21. Based on the results of the study, this suggests the role of rhBMP-2 in enhancing differentiation Peridontal Stem Cells. And also showed the role of Chitosan and as a scaffold quite good because it can provide an environment suitable to trigger adhesion, proliferation, and differentiation of cells. Osteocalcin is usually used as a marker for the late stage of osteoblast differentiation [19]. Osteocalcin concentration tended to increase in all groups, and days 21 the higher expression level osteocalcin are in the group presented with rhBMP-2 + Chitosan followed by the group presented with chitosan and rhBMP-2. High levels of osteocalcin showed in advanced stages of differentiation might be due to the chitosan has a calcium content of mineral apatite which is a natural constituent of bone and teeth, then becomes conductive to PDL-SC into osteoblast-

like cells. This result was supported by Tang XJ et al. in vivo experimental, the NHC composite has good hard tissue biocompatibility and an excellent osteoconductivity [20,21]. Further study needed to evaluate in vivo effect of PDLSC compared to dental pulp-derived stem cells (DPSCs) on bone defect regeneration. The study conducted by Bachtiar et al. showed that transplantation DPSCs was effective to induce bone regeneration [22].

Cells and scaffolds strategies can promote the ingrowth of new bone as these are designed to provide mechanical stability of the implanted material, while promoting osteogenesis, osteoconduction, and osteoinduction, these scaffolds were resorbable and thus be gradually degraded and replaced by new bone [23]. Biomaterials can incorporate bioactive factors to attract host stem cells, bone ingrowth cytokine to promote osteoformation and pro-angiogenesis factors [24].

#### 5. CONCLUSION

In conclusion, our study demonstrates that rhBMP-2, Chitosan, and combination of both could stimulate Periodontal Stem Cells differentiation into the osteoblastic lineage.

#### ACKNOWLEDGEMENT

Part of this study was supported by Universitas Indonesia PUPT Grant 2016 from the Ministry of Research and Higher Education Republic of Indonesia.

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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