Effect of teriparatide on ligamentum flavum mesenchymal stem cells isolated from patients with ossification of the posterior longitudinal ligament

（テリパラチドによる脊柱靭帯骨化症由来 MSCs の骨分化促進に関する検討）

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ABSTRACT

Ossification of the posterior longitudinal ligament (OPLL) within the spinal canal sometimes leads to severe myelopathy. Teriparatide (TPD) is a recombinant human parathyroid hormone (PTH) (1–34), which promotes osteogenesis of mesenchymal stem cells (MSCs) via PTH 1 receptor (PTH1R). Although ligamentum flavum (LF)-MSCs from patients with OPLL have a high osteogenic potency, the effect of TPD on them remains unknown. In this study, we determined PTH1R expression in LF-MSCs from patients with OPLL and investigated whether TPD promotes osteogenic differentiation in them. First, LF-MSCs were isolated from patients with OPLL and cervical spondylotic myelopathy (CSM) (controls). Cultured LF-MSCs were treated with different concentrations of TPD on days 0, 7, and 14. On day 21, osteogenic gene expression was quantified. Mineralization was measured based on optical density after Alizarin Red S staining. LF-MSCs from both groups expressed PTH1R at the same level. TPD did not enhance osteogenic gene expression and mineralization in LF-MSCs from both groups. TPD did not promote the osteogenic differentiation of LF-MSCs from patients with OPLL. Thus, it may be safe for patients with OPLL. However, further confirmation of our results with *in vivo* studies is necessary.

Keywords: Ossification of the posterior longitudinal ligament; Ligamentum flavum; Mesenchymal stem cells; Teriparatide; Parathyroid hormone 1 receptor

1. Introduction

Ossification of the posterior longitudinal ligament (OPLL) slowly damages the cervical spinal cord, sometimes causing myelopathy.[1-3] Genetic, hormonal, environmental, and lifestyle factors promote the formation and progression of OPLL.[4] The pathophysiology of ossification involves the erroneous differentiation of mesenchymal stem cells (MSCs) within the spinal ligament. MSCs are localized in the perivascular area, collagenous matrix of posterior longitudinal ligament (PLL), and ligamentum flavum (LF).[5, 6] It has been reported that LF-MSCs isolated from patients with OPLL had a higher osteogenic differentiation potential than those from patients without OPLL,[7] suggesting that MSC differentiation may lead to spinal ligament ossification.

The reported prevalence rate of OPLL was between 1.9% and 4.3%,[8] and it increased with the age of the general Japanese population.[9] In the aging society, the number of patients with osteoporosis who experience fragility fractures is increasing worldwide, constituting a burden to the society.[10] Patients with OPLL are increasingly receiving treatment for osteoporosis. Anti-osteoporosis drugs, including bisphosphonates, estrogen, raloxifene, and parathyroid hormone (PTH), prevent bone density loss and osteoporotic fractures.[11] Among these drugs, teriparatide (TPD) has been used in approximately 15% of the initial treatments performed in patients with osteoporosis at a high risk of fractures.[12]

TPD is a recombinant human PTH (1–34), which is also used in the Ellsworth–Howard test. The Ellsworth–Howard test is performed to differentiate idiopathic hypoparathyroidism

from pseudohyperparathyroidism by assessing renal responsiveness to TPD.[13,14] TPD binds to parathyroid hormone 1 receptor (PTH1R) and promotes osteoblast maturation via the Wnt signaling pathway.[15] It is also approved as an osteoanabolic agent for treating severe osteoporosis.[16] Recent studies have demonstrated that TPD administration to patients with osteoporosis undergoing spine surgery improved the rate of spinal fusion and bone grafting,[17, 18] possibly by affecting MSCs and promoting bone differentiation.[19]

In our previous study, LF-MSCs isolated from patients with OPLL showed high osteogenic potency.[7] Therefore, despite the benefits of TPD for patients with osteoporosis, there is a concern over its safety for patients with OPLL. If LF-MSCs express PTH1R, TPD may act on the spinal ligaments and enlarge ossifying lesions. The enlarged ossifying lesions compress the spinal cord and lead to neurological deficiencies. A previous *in vivo* study using an OPLL mouse model indicated that TPD promoted the ossification of mice spinal ligaments.[20] However, to the best of our knowledge, there are no reports on TPD treatment of MSCs in patients with OPLL. The objective of this study was to determine whether PTH1R is expressed in LF-MSCs and investigate whether TPD promoted the osteogenic differentiation of LF-MSCs from patients with OPLL.

2. Materials and methods

2.1. Patient tissue samples

From October 2010 to March 2019, LF samples were aseptically collected from 13 patients with OPLL (9 males and 4 females) and 13 patients with cervical spondylotic myelopathy (CSM) (1 male and 12 females) during spinal surgery. The diagnosis of OPLL and CSM was confirmed by plain radiography, computed tomography, and magnetic resonance imaging of the cervical spine. The age of patients with OPLL ranged between 50 and 77 years (mean 61.2 years) and that of patients with CSM ranged between 51 and 80 years (mean 66.7 years). There was no significant age and sex difference between the two groups of patients ($P = 0.170$, $P = 0.135$). Out of the thirteen samples per group, three were used to examine the expression of PHT1R and ten to investigate the effects of TPD on LF-MSCs. This study was approved by the committee of medical ethics of Hirosaki University Graduate School of Medicine. All subjects provided written informed consent before participating in the study.

2.2. LF-MSC isolation and culture

The collected LF samples were washed with phosphate-buffered saline (PBS) to remove blood and debris. To isolate MSCs, the tissues were minced and incubated in α-modified Eagle's medium (α-MEM; Invitrogen, Carlsbad, CA, USA) containing 3 mg/mL collagenase

type V (Wako Pure Chemical Industries, Osaka, Japan) at 37 °C for 3 h. After removing the debris using a 70 μm nylon filter (BD Biosciences, San Jose, CA, USA), nucleated cells were plated in 95 mm plastic dishes at a density of 5×10^5 cells. The cells were cultured in α-MEM Complete Culture Medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (JRH Bioscience, Lenexa , KS, USA), 100 U/mL penicillin G sodium, and 100 U/mL streptomycin sulfate (Invitrogen) for 14 days in a humidified atmosphere at 37 °C, composed of 95% air and 5% CO_2 (passage 0).[5]

2.3. Detection of PTH1R on LF-MSCs by western blotting

The expression of PTH1R in LF-MSCs was analyzed by western blotting. At passage 3, LF-MSCs from patients with OPLL (n = 3) and CSM (n = 3) were washed with PBS and incubated with RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) at 4 °C for 1 h for protein extraction. The extracted proteins were then incubated with anti-PTH1R antibody (1:850; Abcam, Cambridge, MA, USA), and PTH1R detection was performed using an automated capillary-based western system (Wes; Protein Simple, San Jose , CA, USA) with the 12– 230 kDa separation module (#SM-W004), according to the manufacturer's instruction.[21-23] β-Actin protein expression (1:2000; Abcam) was used as the internal control.

2.4. In vitro osteogenic induction of LF-MSCs by TPD administration

At passage 5, LF-MSCs from patients with OPLL and CSM (n = 10/group) were plated on 35 mm plates at 3 × 10^4 cells/well and cultured in α-MEM until subconfluency. The osteogenic differentiation medium (PromoCell, Heidelberg, Germany) supplemented with different concentrations of TPD (Asahi Kasei Pharma, Tokyo, Japan) (3, 10, and 30 nM) was then added to the cells at the beginning of culture (Day 0) and on days 7 and 14. Real-time quantitative PCR (qPCR) and Alizarin Red S staining were performed on day 21 (Fig. 1).

2.5. Evaluation of osteogenic gene expression in LF-MSCs by qPCR

The expression of osteogenic genes, including osteocalcin (*OCN*), runt-related transcription factor 2 (*RUNX2*), osteopontin (*OPN*), osterix (*OSX*), and bone morphogenetic protein 2 (*BMP2*), was quantified by qPCR using fluorescent dye SYBR Green Ⅰ (SYBR Green PCR Master Mix; Applied Biosystems, Foster City, CA, USA). The total RNA was extracted on day 21 using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol and retrotranscribed to cDNA. Amplification of cDNA with specific primers (Table 1) was monitored using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used to normalize the RNA expression level of the described osteogenic markers.

2.6. Quantification of calcium deposits

Mineral deposits on the cells were visualized by 1% Alizarin Red S staining (Sigma-Aldrich, Tokyo, Japan), and the calcium-bound dye was extracted using 100 mM hexadecylpyridinium chloride monohydrate (Wako Pure Chemical Industries) and quantified by optical density (OD) at 550 nm.

2.7. Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using SPSS version 22.0 (SPSS, Chicago, IL, USA). Two-way ANOVA was conducted to compare the differences of each parameter between TPD concentrations and between the OPLL and CSM groups. The results with a *P* value of <0.05 were considered statistically significant.

3. Results

3.1. LF-MSCs from patients with OPLL and CSM expressed PTH1R

LF-MSCs from patients with OPLL and CSM expressed PTH1R (Fig. 2A). The relative expression of PTH1R in LF-MSCs from patients with OPLL and CSM was 1.67 ± 0.04 and 1.50 ± 0.24, respectively, with no significant difference between the two groups ($P = 0.51$) (Fig. 2B).

3.2. Gene expression levels of osteogenic markers did not increase after TPD administration

The mRNA levels of *OCN*, *RUNX2*, *OSX*, and *BMP2* were significantly higher in LF-MSCs from the OPLL group than in the CSM group (*OCN*, $P = 0.009$; *RUNX2*, $P = 0.018$; *OSX*, $P = 0.015$; *BMP2*, $P = 0.011$) (Fig. 3A, B, D, and E). The mRNA level of *OPN* was not significantly higher in the OPLL group than in the CSM group ($P = 0.167$) (Fig. 3C). No significant differences were observed among each concentration of TPD ($P > 0.05$) (Fig. 3A-E).

3.3. Mineral content was not increased by TPD administration

Following the incubation of LF-MSCs with the osteogenic induction medium, calcium deposits were visualized by Alizarin Red S staining in both OPLL and CSM groups (Fig. 4A). Visually, the cultured dishes of MSCs from the OPLL group were strongly stained compared

with the CSM group. The OD of samples from patients with OPLL was 2.16 without TPD, and it was 2.03, 2.10, and 1.94 after the administration of TPD at concentrations 3, 10, and 30 nM, respectively. On the contrary, the OD of samples from patients with CSM was 1.22 without TPD, and it was 1.22, 1.28, and 1.27 after the administration of TPD at concentrations of 3, 10, and 30 nM, respectively. The OD values in the OPLL group were significantly higher than those in the CSM group ($P < 0.01$) (Fig. 4B). However, there were no significant differences in the OD values among the different TPD concentrations ($P = 0.98$) (Fig. 4B).

4. Discussion

LF-MSCs from patients with OPLL expressed PTH1R, which is required for the osteoblastic differentiation of MSCs. The expression of osteogenic genes, such as *OCN*, *RUNX2*, *OSX*, and *BMP2*, and the degree of mineralization were significantly higher in the OPLL group than in the CSM group. These results indicated the high osteogenic potency of LF-MSCs from patients with OPLL. Although TPD administration induced the commitment of MSCs toward osteogenesis,[24] in this study, TPD did not promote the osteogenic differentiation of LF-MSCs.

TPD increased the commitment of mesenchymal precursor cells to the osteoblast lineage, promoted osteoblast maturation, and inhibited osteoblast apoptosis, thereby increasing the osteoblast numbers and functions.[24] Additionally, Kaback *et al.* reported that the expression of *OSX* and *RUNX2* was upregulated in mice bone marrow MSCs after systemically treating the mice with TPD.[19] Moreover, TPD had opposite effects on chondrogenesis depending on its concentration. TPD at a low concentration promoted the chondrogenic differentiation of murine bone marrow MSCs with the expression of Sox9, Col2a1, and PTH1R,[25] and the expression of OPN in human bone marrow MSCs was higher in the TPD group than in the control group.[26] These findings were not consistent with our result, that is, the administration of TPD to LF-MSCs did not promote ossification and mineralization. There are two possible reasons that could explain why TPD did not affect LF-

MSCs.

Osteogenic differentiation was not promoted by TPD probably because the characteristics of MSCs vary depending on the source of tissue. Several studies have evaluated source-dependent properties of MSCs isolated from bone marrow, synovium, periosteum, adipose, and muscle. Sakaguchi *et al.* reported that the osteogenic differentiation potential of human bone marrow, synovium, and periosteum MSCs was higher than that of adipose MSCs.[27] Another study showed that rat synovium MSCs had the highest proliferation and chondrogenesis potentials, whereas the osteogenic differentiation capacity of rat periosteum MSCs was the highest.[28] Our results demonstrated that TPD administration to human LF-MSCs did not promote osteogenic differentiation. Considering these results, human LF-MSCs may be less responsive to TPD administration and/or have a lower osteogenic differentiation potential than MSCs isolated from other tissues.

Although TPD was administered to LF-MSCs from patients with OPLL once a week, as performed in clinic settings when treating patients with osteoporosis, TPD did not promote osteogenic differentiation, which could be due to the metabolic differences of TPD between *in vivo* and *in vitro* systems. *In vivo*, TPD is released into the bloodstream following subcutaneous or intravenous administration and metabolized by the liver and kidneys and finally eliminated mainly by the kidneys.[29] A previous *in vivo* study showed that the half-life elimination time of TPD was 28 min following intravenous administration.[30] In contrast, when

TPD was incubated *in vitro* with osteoblastic cells, TPD concentration gradually decreased after 6 h.[31] During *in vivo* studies, TPD initially stimulated bone formation and then promoted bone remodeling, in a sequence of events during an “anabolic window,” a period when the TPD actions are maximally anabolic.[32] The fact that this *in vivo* mechanism cannot be replicated *in vitro* could explain the discrepancy in the results between our study and previous study.

There are some limitations in our study. First, we used MSCs isolated from LF, and not from PLL. Because numerous ligaments of patients with OPLL are ossified, it is likely that even MSCs isolated from non-ossified ligaments would exhibit the features of this pathological condition. Second, we did not extract the membrane fraction of MSCs. Therefore, we could not assess PTH1R expression on the surface of MSCs. However, we showed that LF-MSCs from patients with OPLL and CSM expressed PTH1R at the same level. Third, we did not categorize the types of cervical OPLL ossification. A previous study revealed genetic differences in osteogenic differentiation potential between the continuous and segmental groups.[33] Fourth, this study included only a small number of tissue samples (OPLL: n = 13, CSM: n = 13) to detect a statistically significant difference between the groups.

5. Conclusions

In conclusion, we investigated the effects of TPD on the osteogenic differentiation of LF-MSCs from patients with OPLL. In our *in vitro* study, TPD did not promote the osteogenic differentiation of LF-MSCs from patients with OPLL. These results suggest that TPD may be safe to OPLL patients with osteoporosis, because it did not affect the MSCs from the ligamentum flavum. However, further confirmation of the findings with *in vivo* studies is necessary.

Conflicts of interest

The authors indicated no potential conflicts of interest

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Table 1. List of primers used for real-time quantitative PCR (qPCR)

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
OCN	GACCCTTTCCCTCCATCTGA	ATTTAGTTTGGCTTGGGGGC
RUNX2	GGCAGTTCCCAAGCATTTCA	ACTGGCGGGGTGTAAGTAAA
OPN	GCAGTGATTTGCTTTTGCCTC	AGATGGGTCAGGGTTTAGCC
OSX	GGACTCAACAACTCTGGGCA	GTAAAGGGGGCTGGATAAGC
BMP2	GGGCATCCTCTCCACAAAAG	ATCCAGTCATTCCACCCCAC
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG

OCN: osteocalcin, RUNX2: runt-related transcription factor 2, OPN: osteopontin, OSX: osterix, BMP2: bone morphogenetic protein 2, GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Fig. 1

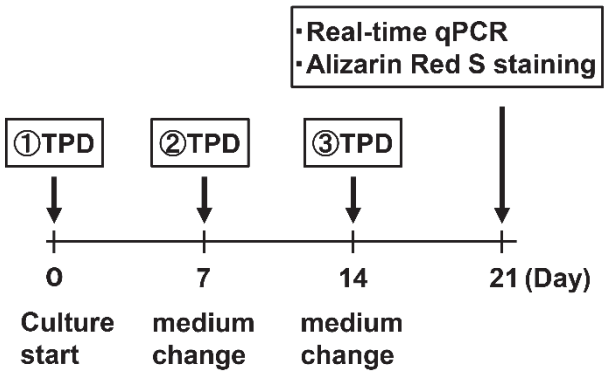

Fig. 1. Experimental schedule. TPD was administered at different concentrations (3, 10, and 30 nM) to LF-MSCs from patients with OPLL and CSM on days 0, 7, and 14 after *in vitro* culture. Real-time quantitative PCR and Alizarin Red S staining were performed with cells cultured until 21 days.

Fig. 2

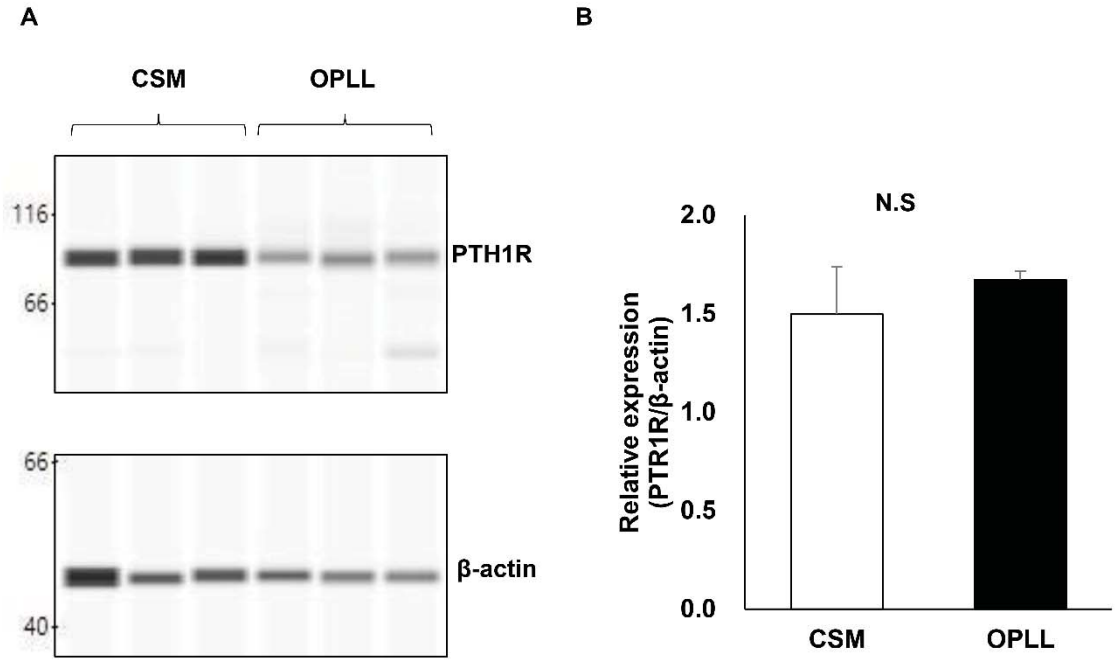

Fig. 2. Detection of expression of parathyroid hormone 1 receptor (PTH1R) protein using western blotting. (A) LF-MSCs from the CSM and OPLL groups expressed PTH1R protein. (B) Relative expression level of PTH1R is shown as mean ± SEM of three samples. There were no significant differences in the relative expression level of PTH1R between the two groups (*P* = 0.51).

Fig. 3

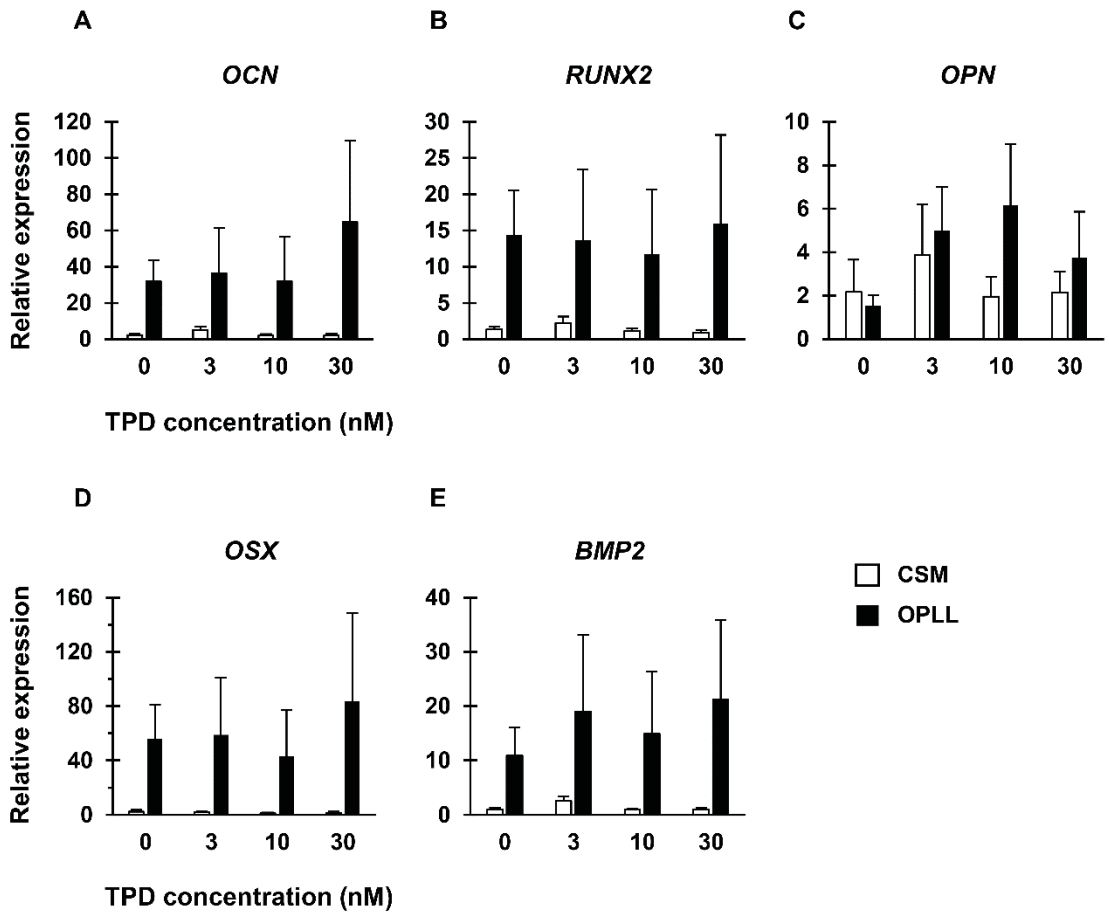

Fig. 3. Expression of osteogenic marker genes. (A) *OCN*, (B) *RUNX2*, (C) *OPN*, (D) *OSX*, and (E) *BMP2*. The graphs show the relative expression levels of these markers in LF-MCs between the two groups and among different TPD concentrations. The relative expression level of each mRNA is shown as mean ± SEM of 10 samples. The expression of *OCN*, *RUNX2*, *OSX*, and *BMP2* was significantly higher in LF-MSCs from the OPLL group than in the CSM group (*OCN*, $P = 0.009$; *RUNX2*, $P = 0.018$; *OSX*, $P = 0.015$; *BMP2*, $P = 0.011$). No significant differences were observed among different concentrations of TPD ($P > 0.05$).

Fig.4

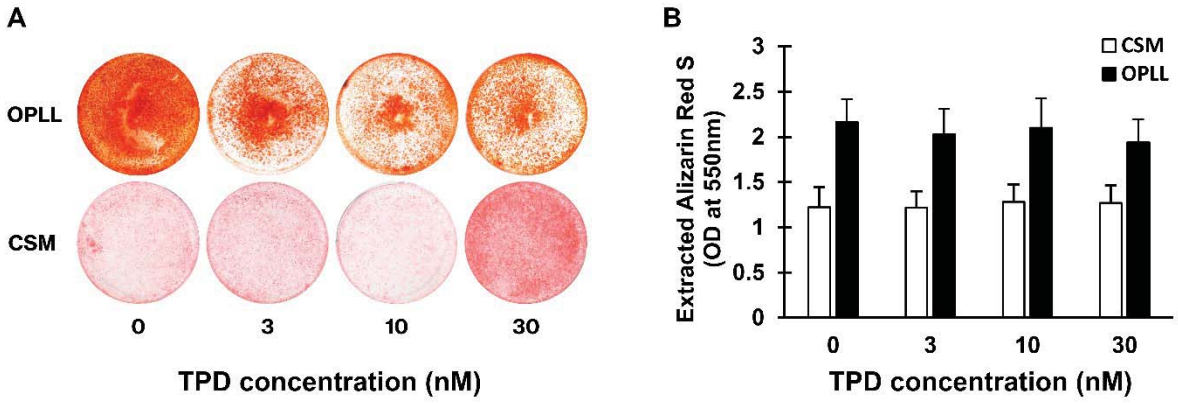

Fig. 4. Quantification of calcium deposits. (A) Calcium deposits were observed in both OPLL and CSM groups, by Alizarin Red S staining. Visually, MSCs from the OPLL group were strongly stained compared with those from the CSM group. (B) The OD values are shown as mean ± SEM of 10 samples. The OD values of the OPLL group were significantly higher than those of the CSM group ($P < 0.01$). However, there were no significant differences in the OD values among different TPD concentrations ($P = 0.98$).