Research Paper

Tumor Necrosis Factor Superfamily 14 Promotes Odontogenic Differentiation of Human Dental Pulp Stem Cells

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This study aimed to evaluate the effect of tumor necrosis factor superfamily 14 on the viability and differentiation of human dental pulp stem cells. Human dental pulp stem cells were treated with 25 ng/ml and 50 ng/ml tumor necrosis factor superfamily 14. Cell viability, alkaline phosphatase activity and messenger ribonucleic acid of odontogenic markers were assessed *via* quantitative reverse transcription polymerase chain reaction. Mineral deposit formation was determined using alizarin red staining. Statistical analysis was conducted using a one-way analysis of variance parametric test. Results were considered statistically significant if the p value was <0.05. Tumor necrosis factor superfamily 14 at 50 ng/ml significantly enhanced human dental pulp stem cells viability on d 3 and alkaline phosphatase activity on d 7 and d 14 compared with the control group. Tumor necrosis factor superfamily 14 (50 ng/ml) significantly upregulated the messenger ribonucleic acid expression of odontogenic markers including alkaline phosphatase, osteocalcin, dentin sialophosphoprotein and dentin matrix protein-1. Runt-related transcription factor 2 expression was significantly increased by treatment with 25 ng/ml tumor necrosis factor superfamily 14. The treatment groups showed higher expression of osteopontin messenger ribonucleic acid, but the difference was not statistically significant. Furthermore, an increased formation of mineral deposits was observed in the tumor necrosis factor superfamily 14-treated groups after alizarin red staining. Tumor necrosis factor superfamily 14 promotes human dental pulp stem cells expression of odontogenic differentiation markers and formation of calcific deposits.

Key words: Calcific deposits, differentiation, human dental pulp stem cells, tumor necrosis factor superfamily 14

Dental pulp is located within a solid chamber consisting of dentin, enamel and cementum. This chamber offers firm mechanical support and protects the dental pulp from a microorganism-filled oral environment[1]. Pulp and dentin function physiologically as a unit called the pulp-dentin complex. This complex is a dynamic tissue that responds to mechanical, bacterial or chemical irritation to decrease its effects[2].

In cases where the dental pulp is exposed due to dental caries, root canal treatment has been considered as the treatment of choice; however, there has been increased interest in procedures that attempt to preserve pulp vitality using Vital Pulp Therapy (VPT) techniques. These procedures included direct pulp capping, partial pulpotomy and complete pulpotomy[3]. VPT has shown increased success in recent years[4-6]. VPT depends on recruiting undifferentiated stem cells for differentiation into cells involved in regenerating lost dentin pulp complex tissues[7].

Hard tissue formation on the site of pulp injury is considered a sign of successful management *in vivo*; therefore, *in vitro* studies use various markers to evaluate successful stem cells differentiation to odontoblast-like cells when exposed to different materials[8]. Several markers and transcription factors are involved in stem cell differentiation into odontoblasts and odontoblast-like cells. These markers include Dentin Matrix Protein 1 (DMP-1), Dentin Sialophosphoprotein (DSPP), Osteopontin (OP), Osteocalcin (OC), Alkaline Phosphatase (ALP) and Runt-related transcription factor 2 (RUNX 2) [9-11]. Interestingly, most markers of odontoblastic differentiation such as ALP, OC and RUNX2 are shared with osteoblastic differentiation[12].

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Multiple biomaterials have been used in VPT, ranging from calcium hydroxide to the more recent different forms of calcium silicates, to promote undifferentiated stem cell differentiation into odontoblast-like cells[6,13]. Although considerable success has been achieved, the quality of newly formed tissue differs from that of the original physiological structures. In fact, the newly formed dentin bridge in VPT is made of osteodentin with irregular tubular dentin and some tunnel defects[14].

Despite the availability of multiple biomaterials, the endodontic field still lacks a material that promotes high-quality regeneration of the pulp-dentin complex with reduction or complete resolution of the inflammatory process. Therefore, finding a material that promotes odontoblast differentiation while reducing the inflammatory response at the site of tissue regeneration could potentially improve the observed outcome.

Tumor Necrosis Factor Superfamily 14 (TNFSF14) also known as Lymphotoxin-like Inducible proteins that competes with Glycoprotein D for binding to Herpes virus entry mediator on T cells (LIGHT), is an immunomodulatory cytokine that is a member of the tumor necrosis factor superfamily[15]. TNFSF14 modulates innate and adaptive immune responses by promoting the homeostasis of lymphoid organs, liver and bone[16]. When used on human Bone Marrow Mesenchymal Stem Cells (BM-MSCs) it enhances survival, proliferation, differentiation into osteocytes and osteogenesis[17]. Interestingly, when TNFSF14 was added to BM-MSCs, markers associated with both osteogenesis and odontogenesis were upregulated[12,17].

To date, no study has investigated the effect of TNFSF14 on the odontogenic differentiation of human Dental Pulp Stem Cells (hDPSCs). Therefore, this study aimed to evaluate the effect of TNFSF14 on the viability and odontogenic differentiation of hDPSCs. The null hypothesis that guided this work was TNFSF14 had no effect on odontoblastic differentiation of hDPSCs.

MATERIALS AND METHODS

Cell culture:

hDPSCs purchased from Lonza (Basel, Switzerland; Catalog No.: PT-5025) were expanded and cultured in alpha-modified Minimum Essential Medium (α-MEM) (Thermo Fisher Scientific, Waltham, United States of America) supplemented with 10 % Fetal Bovine Serum (FBS) (Thermo Fisher Scientific), 1 % penicillin/streptomycin and 1 % MEM Non-Essential Amino Acids (NEAA) solution, subsequently referred to as supplemented α-MEM. All cells were cultured at 37° at 5 % CO_2 and 95 % humidity. Cells were transferred to 96, 24 and 12-well plates with the supplemented α-MEM once they reached 90 % confluency in advance of the subsequent experiments. Passages 4-6 of hDPSCs were used in the experiments.

Reagents:

Recombinant human TNFSF14 (R&D Systems, Minneapolis, United State of America; Catalog No: 664-LI-025), was dissolved in 0.1 % Bovine Serum Albumin (BSA)-Phosphate-Buffered Saline (PBS) and stored at -20° until use according to the manufacturer's instructions.

Osteogenic medium preparation:

The Osteogenic differentiation Medium (OM) consisted of α-MEM supplemented with 10 % FBS, 1 % penicillin-streptomycin, 50 µg/ml *L*-ascorbic acid (Wako Chemicals, Neuss, Germany), 10 nmol/l calcitriol 1α,25-dihydroxy vitamin D3 (Sigma-Aldrich, Burlington, United State of America), 10 mmol/l β-glycerophosphate (Sigma-Aldrich) and 10 nmol/l dexamethasone (Sigma-Aldrich).

Cellular viability:

Alamar blue assay: The effect of TNFSF14 on the viability of hDPSCs was evaluated at 25 ng/ml and 50 ng/ml on d 1, d 3 and d 7 using an Alamar blue assay according to standard protocols[18]. Briefly, the cells were seeded in 96-well plates at a density of 5×10^3 cells/well. At the end of each time point, 10 % AlamarBlue™ reagent (Bio-Rad Inc. Hercules, United States of America) was added to each well, after 1 h of incubation, fluorescence was measured at an excitation and emission wavelength of 530 nm and 590 nm, respectively using the SpectraMax® M5/M5e Multimode Plate Reader (Molecular Devices, San Jose, United States of America). Data were collected using SoftMax® pro 6 microplate data acquisition and analysis software (Molecular Devices). Experiments were performed in duplicate.

Evaluation of odontogenesis:

ALP assay: hDPSCs were seeded into a 24-well plate in α-MEM with a density of 5×10^4 cells/well for ALP activity. After cells reached a confluency of 90 %, TNFSF14 was added to each group with α-MEM for

24 h. The following day, the cells were cultured in OM supplemented with 25 ng/ml or 50 ng/ml TNFSF14. ALP activity was evaluated on d 7 and d 14[19]. ALP Diethanolamine Activity Kit (Thermo Fisher Scientific) was used to quantify ALP activity by absorbance level measured at 405 nm by the SpectraMax® M5/M5e Multimode Plate Reader (Molecular Devices). The ALP assay was conducted in two independent experiments with three samples per group.

Calcified deposits evaluation:

Alizarin red staining: Alizarin red staining was conducted on d 14 and d 21 according to standard protocols[17]. Briefly, hDPSCs were exposed to 25 ng/ml and 50 ng/ml TNFSF14 in 24-well plates at a density of 1×10^5 cells/well. Wells were rinsed and then fixed with 4 % paraformaldehyde followed *via* incubation with 2 % Alizarin red stain (ScienCell Research Laboratories, Carlsbad, United States; Catalog No.: 0223) for 30 min at room temperature and then washed with distilled water three times for 5 min each time. Light microscopy images were recorded and assessed by two blinded examiners in each group. Alizarin red staining was performed in duplicate.

Quantitative reverse transcription polymerase chain reaction:

The hDPSCs were seeded in 12-well plates in OM supplemented with 25 ng/ml and 50 ng/ml TNFSF14 at a density of 2×10^5 cells/well. On d 7, the cells were collected and Ribonucleic Acid (RNA) lysis buffer was added to the cell pellet. RNA was isolated using the RNeasy Mini Kit (RNeasy; Qiagen, Hilden, Germany) and quantified using a Nanodrop spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific)[20]. The extracted RNA was reverse-transcribed using a High-Capacity complementary Deoxyribonucleic Acid (cDNA) Reverse Transcription kit (Thermo Fisher Scientific) and cDNA was synthesized using a Multigene thermocycler (Labnet International, Inc., Edison, United States of America). Messenger RNA (mRNA) expression was analyzed using fast SYBR™ Green polymerase chain reaction master mix (Thermo Fisher Scientific) under the following thermal conditions according to the manufacturer; 95° for 12 min followed by 40 cycles of 95° for 15 s, 65° for 30 s, and 72° for 30 s. The primer sequences used for ALP, RUNX-2, OC, OP, DSPP and DMP-1 (OligoTM, Seoul, South Korea) are listed in Table 1. Experiments were conducted in duplicate. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as an endogenous control. The data was normalized using the comparative delta-delta Cycle threshold (Ct) method ($2^{-\Delta\Delta}$ CT). This normalization is represented by the relative fold change in percentage compared to the values obtained from the control group[21].

TABLE 1: PRIMER SEQUENCES FOR ALP, RUNX-2, OC, OP, DMP-1 AND DSPP

Gene	Sequence (5´-3´)
GAPDH	Sense (forward primer)
	5`-CTGGTAAAGTGGATATTGTTGCCAT-3`
	Antisense (reverse primer)
	5`-TGGAATCATATTGGAACATGTAAACC-3`
ALP	Sense (forward primer)
	5`-GACGGACCCTCGCCAGTGCT-3`
	Antisense (reverse primer)
	5`-AATCGACGTGGGTGGGAGGGG-3`
RUNX-2	Sense (forward primer)
	5`-ACGIGGCTAAGAATGTCATC-3`
	Antisense (reverse primer)
	5`-CTGGTAGGCGATGTCCTTA-3`
OC	Sense (forward primer)
	5`-GGCAGCGAGGTAGTGAAGAG-3`
	Antisense (reverse primer)
	5`-CTCACACACCTCCCTCCTG-3`
OP	Sense (forward primer)
	5'-CAGTTCAGAAGAGGAGG-3'
	Antisense (reverse primer)
	5'-TCAGCCTCAGAGTCTTCATC- 3'
DMP-1	Sense (forward primer)
	5`-CAGGAGCACAGGAAAAGGAG-3`
	Antisense (reverse primer)
	5`-CTGGTGGTATCTTGGGCACT-3`
DSPP	Sense (forward primer)
	5`-AATGGGACTAAGGAAGCTG-3`
	Antisense (reverse primer)
	5`-AAGAAGCATCTCCTCGGC-3`

Statistical analysis:

The normality of the distribution was assessed using the Shapiro-Wilk test. A parametric test (one-way Analysis of Variance (ANOVA)) was performed when data followed a normal distribution. The Levene test was performed to confirm the equality of variances

and Welch ANOVA with Games-Howell post-hoc tests were applied if the assumption was not observed. Results were considered statistically significant if the p value was <0.05. The analysis was conducted using the International Business Machines (IBM) statistical package for the social sciences statistics software version 29. GraphPad Prism 10 (GraphPad Software, San Diego, United States of America) was used to design the graphs.

RESULTS AND DISCUSSION

On d 1, no statistically significant differences were observed between the control and treatment groups. On d 3, the group treated with 50 ng/ml showed a statistically significant increase in viability compared to the groups treated with 25 ng/ml and the control group ($p<0.001$). The 50 ng/ml group showed higher viability on d 7, but the difference between the groups was not statistically significant (fig. 1).

On d 7, the activity of ALP on hDPSCs significantly increased in the treatment groups compared with the control group 25 ng/ml ($p=0.006$), 50 ng/ml ($p\leq0.001$). Furthermore, significantly higher ALP activity was observed at 50 ng/ml compared to 25 ng/ml ($p=0.008$). On d 14, the 50 ng/ml group showed a significant increase in activity compared to the control group ($p=0.013$). Similarly, the 50 ng/ml groups showed an increase in ALP activity compared to the 25 ng/ml group; however, the difference was not statistically significant (fig. 2).

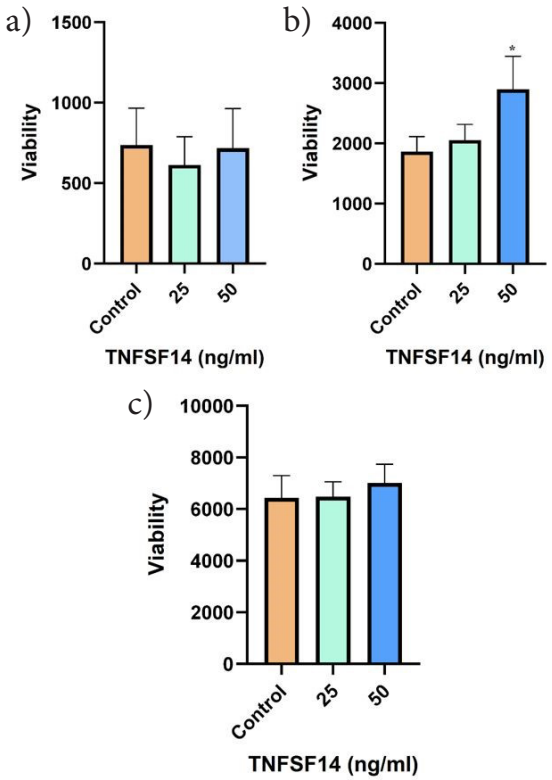

Fig. 1: hDPSC viability on (a): d 1, (b): d 3 and (c): d 7
Note: Data are shown as mean±standard deviation and *p<0.05 is statistically significant compared to the control group

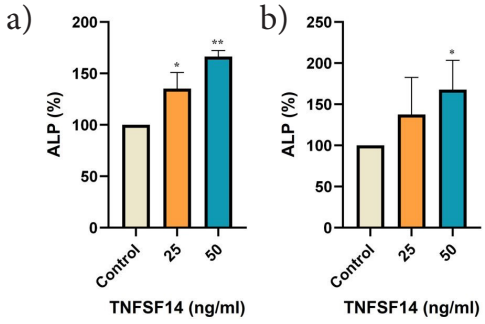

Fig. 2: ALP assay results are shown as the percentage change compared to the control group on (a): d 7 and (b): d 14
Note: *p<0.05 is statistically significant compared to the control group and **p<0.05 statistically significant compared to 25 ng/ml and the control

hDPSCs stained with alizarin red on d 14 showed that none of the groups exhibited calcified nodules. On d 21, the quantity, color and size of the calcified nodules in the TNFSF14-treated groups were noticeably higher than those in the control group. The 50 ng/ml concentration group had more calcified nodules than the 25 ng/ml group (fig. 3).

In comparison to the control group, the TNFSF14 concentration of 50 ng/ml exhibited the highest mean value for the messenger RNA (mRNA) of ALP ($p<0.001$), OC ($p<0.001$), DSPP ($p=0.023$), and DMP-1 ($p<0.001$). Additionally, OC levels were significantly higher in the 25 ng/ml group than in the control group ($p=0.028$). ALP, DSPP and DMP-1 levels were higher in the 25 ng/ml group than in the control group, however these differences were not statistically significant. RUNX-2 demonstrated a significant increase at a concentration of 25 ng/ml compared to the control and 50 ng/ml groups. Conversely, OP gene expression was higher in the experimental groups than that in the control group, however the difference was not statistically significant (fig. 4).

hDPSCs have the capacity to differentiate into multipotent mesenchymal stem cells. These cells can then further differentiate into osteoblast-like or odontoblast-like cells, which are responsible for the process of mineralization[22]. In this article, it has been demonstrated that adding TNFSF14 to hDPSC has the ability to induce upregulation of specific odontogenic marker mRNAs, such as ALP, RUNX-2, OC, DMP-1 and DSPP. In addition, it promotes increased ALP activity. Furthermore, an increased calcific deposit formation was observed in response to TNFSF14 application. Moreover, hDPSC viability increased when cells were grown in the presence of TNFSF14, similar to the effect of TNFSF14 on BM-MSCs[23].

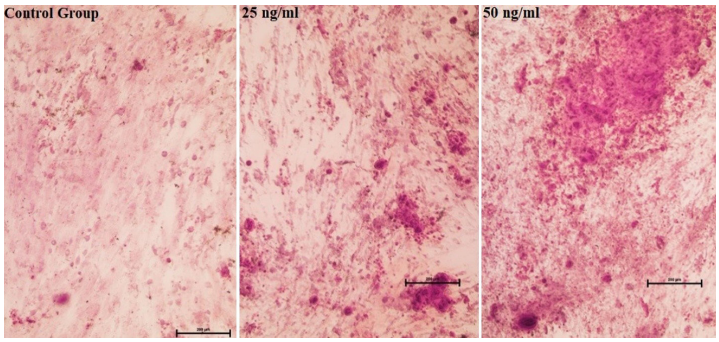

Fig. 3: Alizarin red stain photomicrograph on d 21
Note: Scale bar 200 µm and magnification 10X

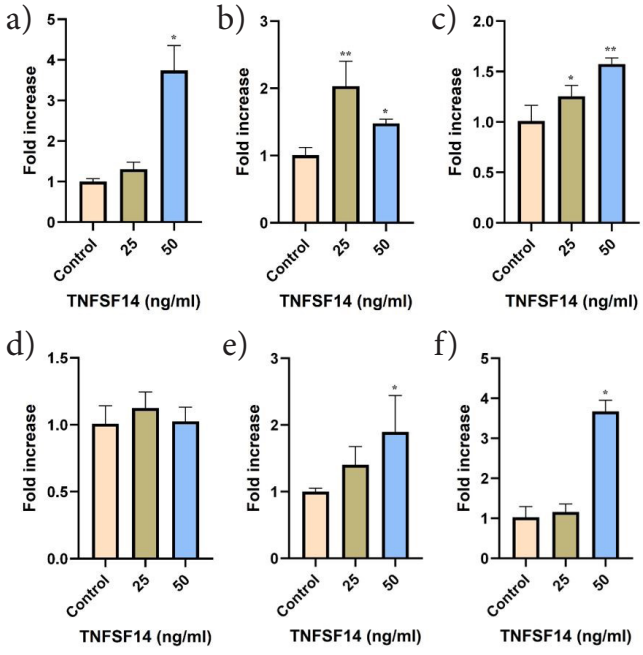

Fig. 4: Reverse transcription quantitative polymerase chain reaction of 25 ng/ml and 50 ng/ml on d 7 of (a): ALP; (b): RUNX-2; (c): OC; (d): OP; (e): DSPP and (f): DMP-1

The formation of calcific bridges is an important indicator of successful pulp recovery and regeneration[8]. In this study, the upregulation of odontogenic marker genes, including ALP, OC, DMP-1 and DSPP in response to TNFSF14 treatment, combined with increased ALP activity, suggests that TNFSF14 stimulates odontogenic differentiation pathways in hDPSCs, which is an essential foundation for the formation of mineralized tissues[12].

TNFSF14 has been previously shown to promote the osteogenic differentiation of BM-MSCs by upregulating osteogenic/odontogenic markers such as ALP, RUNX-2 and OC, which is in agreement with the current findings[17]. OC is a protein that is found in both bones and teeth. It has a role in the later stages of mineralization of the extracellular matrix[24]. The up-regulation of OC, along with the activation of ALP activity, significantly promotes the differentiation of hDPSCs into odontoblast-like cells[11].

OP is a multifunctional protein involved in various cellular processes including mineralization[25]. It is possible that the timing of our analysis did not capture peak OP expression. It is plausible that OP expression may have been more pronounced at later time points beyond the duration of our study[26]. Thus, assessing OP expression at additional time points may provide further insight into its role in odontogenic differentiation.

RUNX-2 together with ALP is key marker of early odontogenic differentiation[27,28]. The findings of the current study suggest that a concentration of 50 ng/ml TNFSF14 may have exceeded the necessary threshold to achieve RUNX-2 peak expression. Consequently, the observed effect on the expression of RUNX-2 may have reached a plateau or decreased in comparison with the lower concentration of 25 ng/ml. This saturation effect may explain why lower concentrations have a more significant impact on RUNX-2 expression[29]. Conversely, the mRNA levels were higher at 50 ng/ml TNFSF14 compared to 25 ng/ml in BM-MSCs, possibly due to the difference in cell types used in the two studies[17].

The differentiation markers examined, DSPP and DMP-1, have been identified as being primarily expressed in odontoblasts and are the primary indicators of odontoblast differentiation. The continuous presence of DMP-1 and DSPP during development indicates that these proteins play an important role in maintaining the balance of the dentin matrix[28].

Alizarin red staining is an effective method for examining the development of calcified nodules[30]. TNFSF14 induced calcific nodule production serves as an indicator of the odontogenic differentiation of hDPSC. These findings are consistent with the previous report on BM-MSCs[17].

Although *in vitro* studies, like this offer valuable insights into the cellular responses to stimuli, they fail to accurately recreate the complex microenvironment and physiological conditions of the living body. Therefore, further studies using *in vivo* models and clinical trials are required to verify the findings of the present study.

Our study provides novel insights into the ability of TNFSF14 to promote the odontogenic differentiation of hDPSCs. TNFSF14 has the potential to be a therapeutic agent for the management of pulp conditions requiring calcific bridge formation.

Ethical approval:

The research was approved by the Institutional Review Board (IRB) at King Saud University Medical City (KSUMC) and the College of Dentistry Research Centre (CDRC) at King Saud University (KSU) with IRB Project No. E-23-7902 and CDRC No PR 0160.

Acknowledgements:

The authors would like to thank College of Dentistry Research Center and Deanship of Scientific Research at King Saud University, Saudi Arabia for funding this research project.

Conflict of interests:

The authors declared no conflict of interests.

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This article was originally published in a special issue, "Integrative Approaches in Biomedical Sciences for Drug Discovery and Development" Indian J Pharm Sci 2024:86(6) Spl Issue "85-91"