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TGF-β1 induces the proliferation, migration and differentiation of stem cells from human exfoliated deciduous teeth

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Aim: This study evaluated the effect of different concentrations of transforming growth factor beta 1 (TGF-β1) on stem cells from human exfoliated deciduous teeth (SHED) viability, proliferation, migration and differentiation into odontoblasts. **Methods:** SHED was treated with different concentrations of TGF-β1 (1.0, 5.0 and 10.0 ng/mL). Sulforhodamine B and MTT assays evaluated the cell proliferation and viability at 1, 3, 5, and 7 days and Migration assay at 24h. RT-PCR verified Dentin matrix protein1 (DMP-1) and Dentin Sialophosphoprotein (DSPP) mRNA expression for 1, 7 and 14 days. The data were analyzed by one-way and two-way ANOVA, followed by Tukey test ($p<0.05$). **Results:** All tested TGF-β1 concentrations increased SHED proliferation compared with the negative control (untreated), from day 3 of treatment ($p=0.000$), with no loss of cell viability. Cell migration was higher in media containing TGF-β1 (1.0-10.0 ng/mL) than in negative and positive control media, with 10 or 20% Fetal Bovine Serum, respectively ($p=0.000$). Treatment with TGF-β1 for up to 14 days induced the expression of the odontoblast markers DMP-1 and DSPP. DMP-1 expression was intense early after treatment with 10.0 ng/mL TGF-β1 and increased progressively from days 1-14 of treatment with 1.0 and 5.0 ng/mL TGF-β1. In contrast, DSPP expression was detected after 14 days of treatment with 10.0 ng/mL TGF-β1. **Conclusion:** Different concentrations of TGF-β1 on SHED promoted positive effect on proliferation and migration, with no loss of cell viability. The concentrations of 10.0 ng/mL TGF-β1 for 14 days induced the expression of the odontoblast markers DMP-1 and DSPP.

Keywords: Cell differentiation. Stem cells. Transforming growth factor beta. Extracellular matrix proteins.

Introduction

Tissue engineering is a multidisciplinary science focused on the development of new tissues and organs[1], to repair congenital defects and injured tissues[2-4]. Successful tissue engineering relies on the ability of undifferentiated cells to respond to specific signals that induce proliferation, migration, and differentiation into more specialized cell lines, when placed in a biocompatible matrix.

In clinical dentistry, tissue engineering has emerged as an exciting area of research. Although few pre-clinical experimental models are available for mechanistic and translational studies involving the regeneration of the human dental pulp, stem cells appear to play a major role in this field[5]. Deciduous teeth contain a population of post-natal stem cells, stem cells from human exfoliated deciduous teeth (SHED), which are capable of extensive proliferation and multipotential differentiation *in vitro*[6]. In dental tissues, SHED constitute an autogenous source for endodontic regeneration, due to their potential for pulp regeneration *in vivo*[7,8] which could be applied in pulp tissue engineering[9-11]. Also, SHED represent good candidates for stem cell sources in human regenerative therapies against systemic diseases[12-16].

SHED have higher proliferative potential than dental pulp stem cells (DPSCs) and bone marrow mesenchymal stem cells (BMMSCs), when plenty of extracellular matrix and growth factors are provided[4,12-14,17]. Moreover, current studies indicate that BMMSCs may contribute for cancer development, while SHED have immunologic properties similar to those of BMMSCs, being capable of suppressing the activation of human T lymphocytes *in vitro*[18].

In dental tissue, growth factors play an important role in mineralization, as well as their canonical roles in angiogenesis, progenitor cell recruitment, cell proliferation and differentiation[8,19]. Growth factors also signal the differentiation of stem cells into odontoblasts, and stimulate dentin matrix secretion[20,21]. Odontoblasts secrete growth factors on dentin, where they remain protected in an active form through the interaction with other components, such as latency associated peptide, betaglycan and decorin[22,23]. Upon dental tissue damage, dentin-pulp complex regeneration is likely to be triggered by the release of growth factors and other bioactive molecules[20,24,25]. Notably, bone morphogenetic protein-2 inhibition prevented the dentin-induced differentiation of SHED into odontoblasts, suggesting a critical role for dentin-derived BMP-2 in this process[26].

Among other growth factors, the multifunctional regulator transforming growth factor beta 1 (TGF-β1) is released by dentin and increases the proliferation/differentiation of human dental pulp stem cells *in vitro*[27-29]. TGF-β1 also is a key mediator of odontoblast differentiation and dentin mineralization[9,28-30]. It is noteworthy to examine the potential for use of this growth factor in dental tissue engineering and regenerative approaches using SHED. Thus, this study evaluated the effect of different concentrations of TGF-β1 on SHED viability, proliferation, migration, and differentiation into odontoblasts.

Materials and methods

Culture of stem cells from human exfoliated deciduous teeth (SHED)

SHED, gently provided by Dr. Bruno N. Cavalcanti (PhD, Institute of Science and Technology, São Paulo State University, São José dos Campos, SP, Brazil) were isolated by standard enzymatic digestion protocol and characterized according to Miura et al.[6] (2003), after Institutional Review Board approval (CAAE 02210312.1.0000.0077).

SHEDs were kept in MEMα culture medium supplemented with 10% fetal bovine serum (FBS; certified and heat-inactivated) and 1% penicillin-streptomycin, at 37°C and 5% CO_2 (all tissue culture reagents were from Gibco, Invitrogen). The culture medium was changed every two days. When monolayers reached 80% confluence, cells were passaged at a 1:3 ratio of cell suspension to fresh medium and SHED at passage numbers between 5 and 10 were used in all experiments.

Transforming Growth Factor-β1 (TGF-β1)

Lyophilized TGF-β1 (Life Technologies – Life Technologies do Brasil Com. Ind. Prod. Biotec Ltd.) was reconstituted in 0.1 mg/mL citric acid and diluted to 1 µg/mL into 0.1% bovine serum albumin (TGF-β1 stock solution, kept at 4ºC). Immediately v before use, TGF-β1 stock solution was diluted in MEMα, for final concentrations of 1.0, 5.0, and 10.0 ng/mL.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Assay

SHEDs were seeded in 96-well plates (2.5×10^3 cells/well), with 200 µL/well of MEMα supplemented with 10% FBS. Cells were stimulated with 1.0, 5.0 or 10.0 ng/mL TGF-β1 for 1, 3, 5, and 7 days. The culture medium was changed every two days. After stimulation, the medium was removed, and 200 µL of MTT solution (0.5 mg/mL, in MEMα supplemented with 1% FBS) were added to each well. Plates were covered with aluminum foil, and incubated at 37°C and 5% CO_2, for 4 hours. Next, plates were centrifuged at 200 x g, for 7 minutes (at 18°C), the MTT solution was removed, and 200 µL of DMSO (Fisher Scientific, Hampton, NH, USA) were added per well. The absorbance was determined in a spectrophotometer at 560 nm. Negative and positive controls represent cells kept in MEMα medium supplemented with 10% and 20% FBS, respectively. All absorbance measurements were blank corrected. Data represent results from three independent experiments, performed in triplicates.

Sulforhodamine B (SRB) Assay

SHEDs were plated in 96-well plates (1×10^4 cells/well), in 200 µL/well of MEMα medium supplemented with 10% FBS. Cells were stimulated with 1.0, 5.0 or 10.0 ng/mL TGF-β1, for 1, 3, 5, and 7 days. During stimulation, the culture medium was changed every two days. After stimulation, cells were fixed by the addition of 10% trichloroacetic acid and incubated for 1 hour at 4°C. Plates were washed in tap water 5 times and allowed to dry. Cellular protein was stained using 4% SRB in 1% acetic acid and incubated at room temperature for 30 minutes. The excess SRB was

removed by washing with 1% acetic acid and the remaining SRB was solubilized in 10 mM Tris-base (unbuffered). Absorbance was determined in a spectrophotometer at 565 nm. Negative and positive controls represented cells kept in MEMα medium supplemented with 10% and 20% FBS, respectively. All absorbance measurements were blank corrected. Data represent results from three independent experiments, performed in triplicates.

Migration Assay

For migration analysis, 24-well companion plates (Fisher Scientific, Pittsburgh, NA, USA) were loaded with 500 mL/well of MEMα with or without 10% FBS and TGF-β1 (1.0, 5.0 or 10.0 ng/mL). SHED that had been serum-starved for 24h were seeded onto 8-mm-pore-size inserts (5 x 10^4 cells/well) in 200 mL/well of culture medium and allowed to migrate overnight. Then inserts were removed, and migrated cells were trypsinized and collected in microcentrifuge tubes and centrifuged at 3.835 x g for 10 minutes, at 4°C. Pellets were washed with PBS and centrifuged at 3.835 x g for 5 minutes, at 4°C. The supernatant was removed, and pellets were transferred to new 24-well plates with 500 mL/well of 20 mM Cell Tracker™ Green CMFDA (Invitrogen, Carlsbad, CA, USA) in PBS. Plates were covered with aluminum foil, incubated for 30 minutes at 37°C, and fluorescence was read at 485 nm, in a spectrophotometer. Negative and positive controls represented migration assays with cells kept in MEMα medium supplemented with 10% and 20% FBS, respectively. All absorbance measurements were blank corrected. Data were obtained from three wells per condition and they are representative of three independent experiments.

RT-PCR Assay

To evaluate SHED differentiation into odontoblasts, cells were plated in 6-well plates (2 x 10^5/well) in 2 mL/well of MEMα culture medium and allowed to adhere overnight. Then, cells were stimulated with TGF-β1 (1.0, 5.0 or 10.0 ng/mL) for 1, 7 and 14 days. The medium was changed every two days. After stimulation, cells were collected, total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instruction, and total RNA concentrations were measured in a Nanodrop 2000C spectrophotometer (Wilmington, DE 19810, USA).

RT-PCR assays for the odontoblast markers DSPP and DMP-1 were performed using 1 µg of total RNA/reaction and the SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The following primers were used for RT-PCR: for DMP-1, 5'-CAGGAGCACAGGAAAAGGAG-3' (sense) and 5'-CTGGTGGTATCTTGGGCACT-3' (antisense), expected product size, 213 bp; for DSPP, 5'-GACCCCTTCATTGACCTCAACT-3' (sense) and 5'-TGCCATTTGCTGTGATGTTT-3' (antisense), expected product size, 181 pb); and for the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-GACCCCTTCATTGACCTCAACT-3' (sense) and 5'-CACCACCTTCTTGATGTCATC-3' (antisense), expected product size, 683 bp. The following reverse transcription and amplification conditions were used: 30 minutes at 55°C (reverse transcription), followed by 2 minutes at 94°C,

35 cycles of 94°C for 30s, 55°C for 30s, and 72°C for 1 min, with a final extension period of 10 minutes at 72°C. PCR products were analyzed by 2% agarose gel electrophoresis.

Statistical Analysis

Statistical analysis was performed using the Statistica™ software, version 11.0 (StatSoft Inc. Tulsa, USA). Cell viability and proliferation data were analyzed by two-way ANOVA, followed by Tukey test. Migration data were analyzed by one-way ANOVA, followed by Tukey test. Differences were considered statistically significant when $p<0.05$.

Results

TGFβ-1 treatment increases SHED proliferation, with no loss of cell viability.

MTT assay results showed that treatment with TGFβ-1 for 1, 3, 5 and 7 days did not affect SHED viability, when compared with the untreated negative control (shown in Fig. 1). Growth in 20% FBS (positive control) led to statistically higher MTT absorbance values after 7 days, compared with all other groups ($p=0.000$ vs all TGFβ-1-treated samples, and $p=0.022$ vs the negative control; shown in Fig. 1).

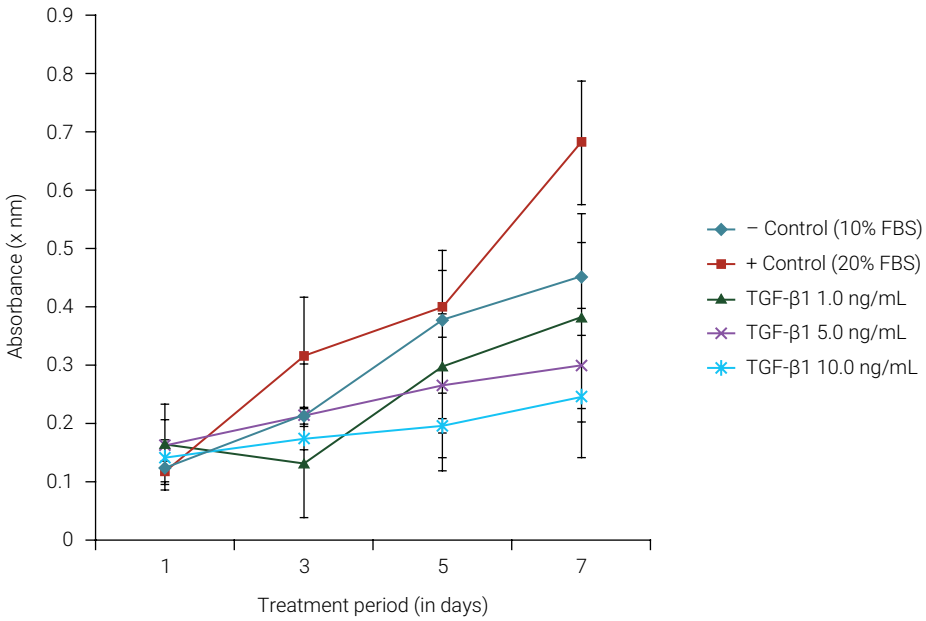

Figure 1. Effect of TGF-β1 on the cell viability of SHED (MTT assay). Cells were treated with 1.0, 5.0 or 10.0 ng/mL TGF-β1 for up to 1, 3, 5, and 7 days. Negative and positive controls represented untreated cells kept in 10 or 20% FBS, respectively. Data represent mean ± SD/SEM of x independent experiments, performed in triplicates. * $p<0.05$ vs the untreated control (- control) in the same treatment period (ANOVA followed by Tukey).

Cell proliferation analysis using the SRB assay showed that TGF-β1 treatment increased the proliferation of SHED from day 3 of treatment, compared with the negative control (shown in Fig. 2; p≤0.001). The proliferation of cells treated with TGF-β1 was similar to that observed in the positive control of cells grown in 20% FBS, and different concentrations of TGF-β1 had similar effects on cell proliferation (shown in Fig. 2).

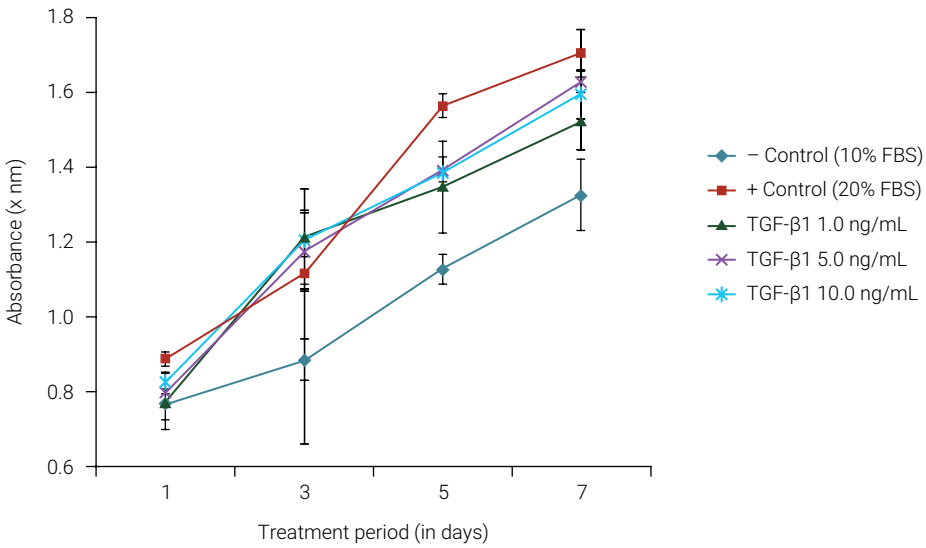

Figure 2. Effect of TGF-β1 on the cell proliferation of SHED (SRB assay). Cells were treated with 1.0, 5.0 or 10.0 ng/mL TGF-β1 for up to for 1, 3, 5, and 7 days. Negative and positive controls represented untreated cells kept in 10 and 20% FBS, respectively. Data represent mean ± SD/SEM of x independent experiments, performed in triplicates. * p<0.05 vs the untreated control (- control) of the same treatment period (ANOVA followed by Tukey).

TGF-β1 stimulates SHED migration, in a serum-independent manner

For cell migration analysis, SHED was placed in 24-well companion plates and allowed to migrate towards medium with TGF-β1 for period of 24h. Then, migrated cells were harvested, stained with a fluorescent dye and the fluorescence intensity was measured by spectrophotometer. Cell migration was higher towards media containing TGF-β1 (1-10 ng/mL) than towards negative and positive control media, with 10 or 20% FBS, respectively (p=0.000). Migration rates were higher towards medium with 20% FBS, compared with 10% FBS (p=0.002); however, supplementation with 10% FBS did not alter significantly the migration rates towards media containing TGF-β1 (shown in Fig. 3).

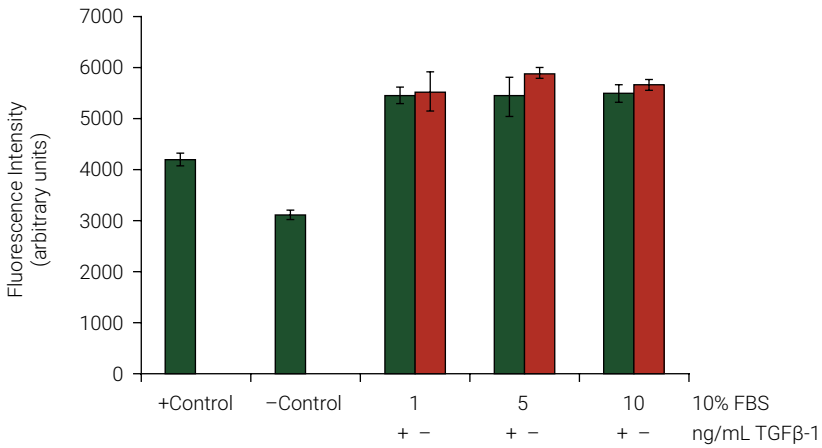

Figure 3. Effect of TGF-β1 on the migration of SHED. Cells were treated with 1.0, 5.0 or 10.0 ng/mL TGF-β1. Negative and positive controls represented untreated cells kept in 10 and 20% FBS, respectively. Data represent mean ± SD/SEM of x independent experiments, performed in triplicates. * p<0.05 vs the untreated control (- control) in the same treatment period (ANOVA followed by Tukey).

SHED treated with higher concentrations of TGF-β1 express both DSPP and DMP-1 odontoblast markers.

The odontoblast markers DSPP and DMP-1 were not detected in untreated samples, but were readily detected in a positive control of odontoblasts obtained from freshly extracted human third molars (shown in Fig. 4). Stimulation with TGF-β1 at concentrations of 1.0 and 5.0 ng/mL led to a progressive increase in the expression of DMP-1, from 1 to 14 days of treatment (shown in Fig. 4), while stimulation with 10 ng/mL of TGF-β1 led to marked DMP-1 expression as early as 1 day after treatment initiation. DSPP expression was readily detected only after 14 days of stimulation with 10.0 ng/mL of TGF-β1, while no DSPP expression was detected after stimulation with lower concentrations of TGF-β1 (shown in Fig. 4).

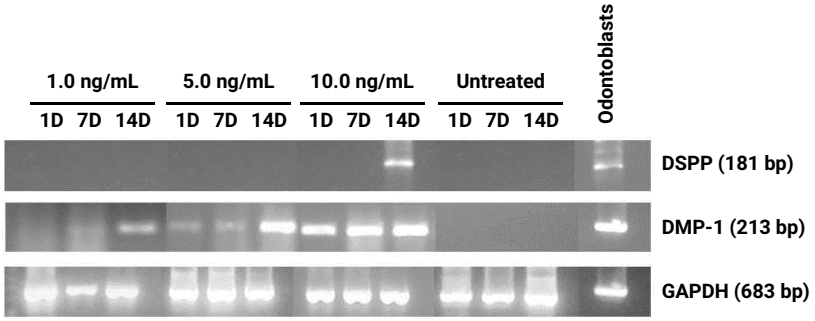

Figure 4. Effect of TGF-β1 on the differentiation of stem cells from exfoliated deciduous teeth (STEM). Cells were treated with 1, 5, and 10 ng/mL TGF-β1 and then differentiation was analyzed by RT-PCR for the expression of the odontoblast markers DSPP (181 bp product) and DMP-1 (213 bp product), using the constitutively expressed gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference. (683 bp product). Untreated cells and odontoblasts (from freshly extracted third molars) were used as negative and positive controls, respectively.

Discussion

SHED represent interesting candidates for stem cell sources in human regenerative therapies[11-13,16,17]. Given the clear odontogenic properties of TGF-β1, indicative of its potential for dentin-pulp complex regeneration[16,28-36], this study examined the effects TGF-β1 on SHED proliferation, viability, migration, and differentiation with different TGF-β1 concentration, because the effective dose of this factor required for pulp stem cell differentiation *in vivo* remains unclear.

Cell viability was higher in the positive control than in samples treated with TGF-β1 (1.0, 5.0 and 10.0 ng/mL), and in the untreated negative control, at the end of the 7-day treatment period. The treatment with different TGF-β1 concentration did not show statistically significant differences in cell viability compared with the untreated negative control. Similarly, He et al.[27] (2008) reported no statistically significant differences in cell viability relative to the negative control after treatment of DPSCs with 5.0 ng/mL of TGF-β1; however, these authors did not compare different TGF-β1 concentrations, and the stimulation period was relatively short (4 days). In contrast, Nie et al.[33] (2006) observed an increase in the viability of dental pulp cells using 5.0 ng/mL of TGF-β1, after 3 and 6 days of stimulation, when compared with the negative control (untreated). Farea et al.[16] (2014) reported that stimulation of SHED with 10.0 ng/mL TGF-β1 for 3 days led to a peak in cell viability, compared with the use of 2.5, 5.0 or 20.0 ng/mL of this factor. In contrast, we did not observe statistically significant differences in cell viability between treatments with 1.0, 5.0 and 10.0 ng/mL TGF-β1.

All TGF-β1 concentrations tested increased SHED proliferation relative to that observed in the negative control, and this effect was apparent from the 3 days of stimulation. These results agree with those of other studies showing that TGF-β1 treatment increases SHED proliferation[16,32,33,37]. An increase in the proliferation rate is mandatory if both SHED and TGF-β1 are to be used in clinical applications involving tissue engineering[38]. In the present study, statistically significant differences were not observed between the proliferation rates obtained with the distinct TGF-β1 concentrations tested. The findings of the present study agree with those reported by Farea et al.[16] (2014) who showed that TGF-β1 significantly increased SHED proliferation, survival ability, and osteogenic differentiation; however, that study did not compare the experimental groups with a positive control. In this study, the proliferation increase was significantly less pronounced than in the positive using the concentration of 1.0 ng/mL TGF-β1, while higher concentrations of this stimulus led to similar proliferation increases compared to the positive control, by the end of the study period. Although previous studies employed different methodologies to assess cell proliferation, all verified that TGF-β1 stimulation increased the proliferation rate of stem cells and dental pulp cells when compared with the absence of the growth factor[16,32,33,37].

The migration activity of progenitor cells towards lesion sites after a stimulus is of fundamental importance for tissue regeneration[39,40]. This study shows that TGF-β1 was capable of boosting SHED migration when compared with the positive and negative controls, regardless of the concentration used. These findings agree with

the studies of Melin et al.[32] (2000) and Howard et al.[40] (2010) who also observed that TGF-β1 had chemotactic activity in stem cells, and suggested that extracellular matrix proteins, particularly laminin, and chemotactants, particularly sphingosine-1-phosphate and TGF-β1, are important promoters of DPSC migration. Howard et al.[40] (2010) observed that the presence of FBS in the culture medium inhibited DPSCs migration towards the TGF-β1, which differs from our results showing that the stimulation of SHED migration by TGF-β1 was independent of the presence of FBS, demonstrating that the growth factor accounted for the cell migration stimulation.

The treatment with TGF-β1 induced the expression of the odontoblast markers DSPP and DMP-1 in SHED cells, showing that TGF-β1 is capable of triggering SHED differentiation. Different expression patterns were obtained depending on the TGF-β1 concentration used, and the length of the treatment period: DMP-1 expression occurred at all TGF-β1 concentrations tested, while DSPP expression occurred at the concentration of 10.0 ng/mL, after 14 days of treatment. These data show that TGF-β1 was capable of stimulating SHED differentiation, corroborating previous studies on the use of this growth factor in dental or pulp stem cells[16,27,30,33,34,41,42].

Although our data corroborate broadly that of others showing that TGF-β1 stimulates cell differentiation, we noticed differences to published data regarding the concentrations required for differentiation marker expression. This variation may be explained by the use of cell types different from SHED. At the concentration of 5.0 ng/mL of TGF-β1, Nie et al.[33] (2006) observed DMP-1 expression only at day 14, in dental pulp cells, while in our study DMP-1 expression increased progressively from 1-14 days at this concentration. Also, a number of studies[27,33] observed inhibition or lack of DSPP expression at 10 μg/mL of TGF-β1 in dental pulp cells, DPSCs and rat odontoblastic cells (MDPC-23), rather than the induction of DSPP expression observed here, at this TGF-β1 concentration only. However, Nie et al.[33] (2006) observed DSPP expression in pulp cells after treatment with 5.0 ng/mL TGF-β1 after 2 weeks, and He et al.[27] (2008) observed increasing expression of DSPP over time (7 and 14 days) at 5.0 ng/mL of TGF-β1 (with or without FGF2), in DPSCs. Therefore, TGF-β1 promoted the key regeneration-associated events required for successful regenerative endodontics and tissue engineering.

In conclusion, these results suggested that different concentrations of TGF-β1 on SHED promoted positive effect on proliferation and migration, with no loss of cell viability. The concentrations of 10.0 ng/mL TGF-β1 for 14 days induced the expression of the odontoblast markers DMP-1 and DSPP.

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Conflicts of interest

None.

Author Contribution

Ana Paula Fernandes, Leandro Borges Araújo, and **Fábio Antonio Colombo** performed the design, performance, and laboratory analysis. **Eloá Cristina Passucci Ambrosio, Ana Beatriz Vieira Silveira, Mariel Tavares Oliveira Prado Bergamo, Paula Karine Jorge, Maria Aparecida Andrade Moreira Machado, Thais Marchini Oliveira**, and **Vivien Thiemy Sakai** contributed substantially to the statistical analysis, interpretation of data for the work, proofreading and paper writing. All authors actively revised and approved the final version of the manuscript.

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