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Research Article

Effects of Vascular Endothelial Growth Factor (VEGF) Alone and in Combination on Rapidly Dividing Dental Pulp Stem Cells (DPSC)

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Abstract

Background: Dental pulp stem cells (DPSC) are known to possess many of the properties of pluripotent stem cells. Many researchers have focused their efforts to refine the range of growth factors that influence and modulate DPSC growth and differentiation. More specifically, some evidence has emerged that demonstrated Vascular Endothelial Growth Factor (VEGF) may be sufficient to enhance odontogenic and angiogenic potential of some DPSC isolates. Based upon this information, the primary objective of this study was to evaluate whether VEGF administration or concomitant VEGF and Bone Morphogenic Protein (BMP) administration induced any measurable effects on DPSCs.

Methods: This study used six (n = 6) previously isolated and characterized DPSCs, cells, which were sorted into rapid doubling time (rDT ~1 - 2 days, n = 3) and intermediate doubling time (iDT ~4 - 6 days, n = 3). Each DPSC isolate was plated into 96-well assay plates for three days using media with and without VEGF, BMP-2 alone and in combination. Cellular growth and viability were measured for comparison.

Results: Administration of VEGF reduced growth in two of the rDT DPSC isolates (dpsc-3882, dpsc-5653), with no effect on the iDT DPSC isolates (dpsc-8124, dpsc-9894, dpsc-17322). A corresponding increase in cellular viability was noted among all the rDT DPSC isolates along with corresponding changes to cellular morphology, with no effect on the iDT DPSCs. BMP-2 exhibited no effects on either rDT or iDT DPSC isolates. The combination of VEGF and BMP-2 in combination had similar effects to the administration of VEGF in isolation.

Discussion: These data provide significant preliminary results that clearly demonstrate significant and pronounced effects of VEGF on at least one subset of rapidly dividing DPSC isolates. These effects include changes to cellular viability and growth, which are supported by clear changes to cellular adhesion and morphology. However, these data strongly suggest more reseach is needed to determine the underlying pathways triggered by VEGF administration in the cells and the pathophysiologic mechanisms that determine the responsiveness of these DPSC isolates but not others.

Keywords: *Dental Pulp Stem Cells (DSPC); Vascular Endothelial Growth Factor (VEGF)*

Abbreviations

DPSC: Dental Pulp Stem Cell; VEGF: Vascular Endothelial Growth Factor; BMP: Bone Morphogenic Protein; OPRS: Office for the Protection of Research Subjects; IRB: Institutional Review Board; UNLV: University of Nevada, Las Vegas; SDM: School of Dental Medicine; RMPI: Roswell Park Memorial Institute; FBS: Fetal Bovine Serum; rDT: Rapid Doubling Time; iDT: Intermediate Doubling Time; sDT: Slow Doubling Time

Introduction

Dental pulp stem cells (DPSC) are known to possess many of the properties of pluripotent stem cells [1,2]. Recent studies have demonstrated that DPSCs may have therapeutic potential as multipotent stem cells with the capacity for reprogramming and bioengineering applications [3,4]. However, the ability to control and transform DPSC into specific lineages with precision and accuracy remains an elusive and motivating goal [5-7].

Many researchers have focused their efforts to refine the range of growth factors that influence and modulate DPSC growth and differentiation [8]. For example, some studies have demonstrated specific effects of fibroblast growth factor (FGF) as a transforming growth factor on DPSCs to facilitate odontoblast differentiation and dentin formation [9,10]. Others have focused attention on vascular endothelial growth factor (VEGF), which may play a critical role in DPSC regeneration, differentiation and pluripotency [11,12].

More specifically, some evidence has emerged that demonstrated VEGF may be sufficient to enhance odontogenic and angiogenic potential of some DPSC isolates [13,14]. In fact, research from this group recently demonstrated VEGF may have the potential to induce osteogenic phenotypes in some subsets of DPSC - although these effects were not uniform or consistent and were mainly restricted to the most rapidly dividing DPSCs [15]. Alternatively, one recent study demonstrated that VEGF administration or temporal "priming" of DPSC with VEGF may enhance their odontogenic and osteogenic differentiation potential in combination with other growth factors, such as bone morphogenic protein (BMP) [16].

Based upon this information, the primary objective of this study was to evaluate whether VEGF administration or concomitant VEGF and BMP administration induced any measurable effects on DPSCs.

Methods

Protocol approval

This study was approved through the Office for the Protection of Research Subjects (OPRS) and the Institutional Review Board (IRB) at the University of Nevada, Las Vegas (UNLV) under protocol OPRS#763012-1 "Retrospective analytsis of dental pulp stem cells (DPSC) from the UNLV School of Dental Medicine (SDM) pediatric and clinical population. The DPSC isolates were originally collected and obtained under OPRS#0907-3148 "Isolation of Non-Embryonic Stem Cells from Dental Pulp". Each DPSC isolate was given a unique identifying number to prevent bias and disallow any patient identifying information from being disclosed.

DPSC culture

All DPSC isolates were originally cultured for a minimum of ten (10) passages to ascertain the rate of growth or doubling time (DT). The average time between passaging for each DPSC isolate was characterized as either rapid doubling time (rDT) between 0 - 2 days and intermediate doubling time (iDT) between 4 - 6 days. All cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 Medium with the addition of 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptocmycin from Fisher Scientific. Cells were maintained in 25 cm^2 tissue culture flasks at 5% CO_2 in humidified tissue culture chambers.

Experimental growth factors

The previous study from this group determined that only rapdily dividing or rDT DPSC isolates responded to VEGF administration, therefore - to determine any effects on DPSC isolates, rDT DPSC isolates were plated into 96-well assay plates for three days using media with and without additional growth factors. An additional set of experiments with intermediate or iDT DPSC isolates was used for comparison. Vascular Endothelial Growth Factor (VEGF) obtained from Fisher Scientific (PCH9394) was used at an experimental concentration of 10 ng/mL and Bone Morphogenic Protein (BMP-2) from Fisher Scientific (RP-8638) at a similar concentration of 10 ng/mL. Cells were either plated without any growth factor as a negative control (CTL), with VEGF, BMP-2 or both VEGF and BMP-2 concomitantly.

Proliferation and viability

Cells were grown for three days in each experimental condition (n = 24 wells/plate) and each experiment was replicated in triplicate. Experimental assays were fixed with 10% buffered formalin for 24 hours. Following fixation, cells were stained with Gentian Violet and

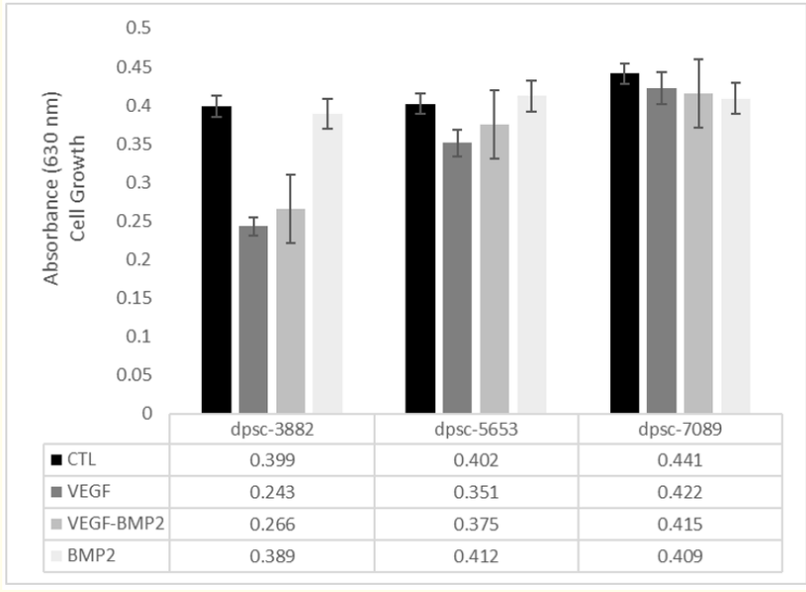
absorbance (growth) measured using a BioTek 808x 96-well plate reader. Higher absorbance readings correspond with higher growth measurements and larger cell numbers, as previously described [17,18]. Viability was measured using the Trypan Blue exclusion assay and a BioRad TC20 automated cell counter, as previously described [19,20]. Cells were imaged at 20X using an AxioVert inverted microscope from Ziess.

Statistical analysis

As continuous (parametric) data measurements were made using absorbance readings at 630 nm, differences between experimental and control treatments were evaluated using two-tailed t-tests. An alpha level of $\alpha = 0.05$ was used to determine statistical significance.

Results

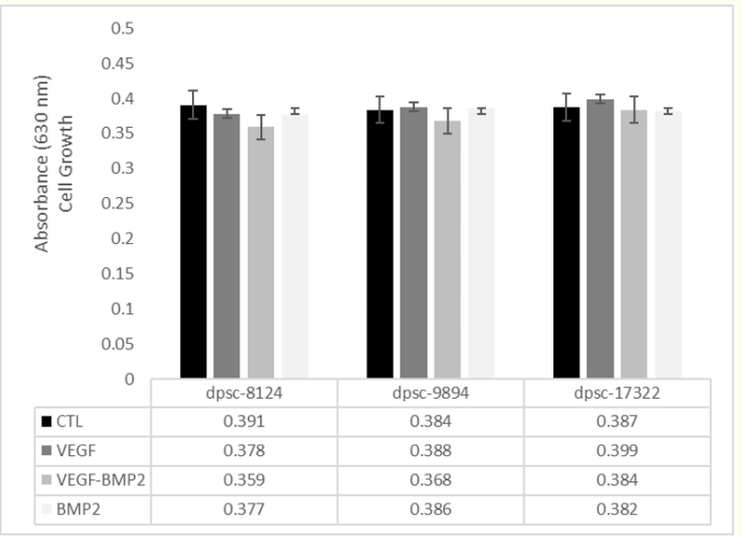
The results of the initial experimental assay using rapidly dividing or rDT DPSC isolates demonstrated that two DPSC isolates responded to VEGF administration (Figure 1). More specifically, VEGF administration induced significant, measurable decreases in cellular growth among dpsc-3882 (-39.1%, p = 0.018) and dpsc-5653 (-12.6%, p = 0.039) over three days compared with the negative control - with no measurable differences observed among dpsc-7089 (-4.3%, p = 0.852). In contrast, administration of BMP-2 did not significantly alter cellular growth among any of the rDT DPSC isolates, including dpsc-3882 (-2.5%, p = 0.948), dpsc-5653 (+2.4%, p = 0.823), and dpsc-7089 (-7.2%, p = 0.742). However, concomitant administration of VEGF in combination with BMP-2 appeared to have a more modest effect on rDT DPSC isolates, reducing growth in dpsc-3882 (-33.3%, p = 0.0241), dpsc-5653 (-6.7%, p = 0.592), and dpsc-7089 (-5.8%, p = 0.691).



	dpsc-3882	dpsc-5653	dpsc-7089
CTL	0.399	0.402	0.441
VEGF	0.243	0.351	0.422
VEGF-BMP2	0.266	0.375	0.415
BMP2	0.389	0.412	0.409

Figure 1: *Growth factor effects on rapidly dividing (rDT) DPSC isolates. Administration of VEGF reduced growth in dpsc-3882 and dpsc-5653 significantly (-39.1%, p = 0.018; -12.6%, p = 0.039) with no effect on dpsc-7089 (-4.3%, p = 0.852). Administration of BMP-2 alone exhibited no significant effects on growth in rDT DPSC isolates, although VEGF-BMP-2 in combination had a modest effect on dpsc-3882 (-33.3%, p = 0.0241).*

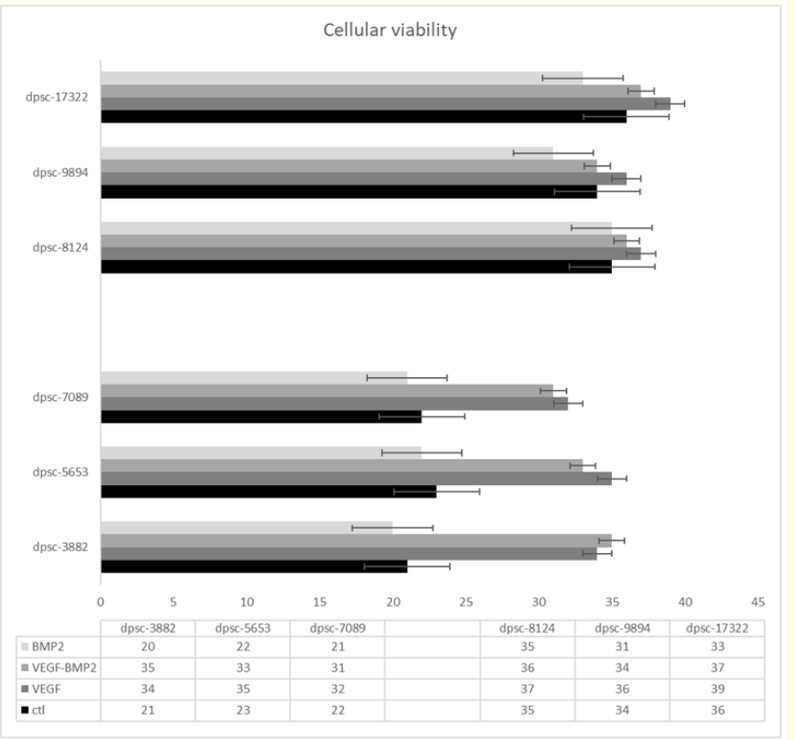
The results of the subsequent experimental assay using intermediate dividing or iDT DPSC isolates demonstrated no significant or measurable effects on these DPSC isolates (Figure 2). More specifically, the administration of VEGF did not exhibit any significant effect on cellular growth in any of the iDT DPSC isolates, including dpsc-8124 (-3.3%, p = 0.137), dpsc-9894 (+1.3%, p = 0.539), and dpsc-17322 (+3.1%, p = 0.165). The concomitant administration of VEGF and BMP-2 also had no significant effect on cellular growth in dpsc-8124 (-8.1%, p = 0.0579), dpsc-9894 (-4.2%, p = 0.441), and dpsc-17322 (-0.8%, p = 0.887). These results were similar to the observations with the administration of BMP-2 on dpsc-8124 (-3.6%, p = 0.189), dpsc-9894 (+0.5%, p = 0.653), and dpsc-17322 (-1.3%, p = 0.794).



	dpsc-8124	dpsc-9894	dpsc-17322
CTL	0.391	0.384	0.387
VEGF	0.378	0.388	0.399
VEGF-BMP2	0.359	0.368	0.384
BMP2	0.377	0.386	0.382

Figure 2: *Growth factor effects on intermediate dividing (iDT) DPSC isolates. Administration of VEGF exhibited no significant effect on iDT DPSC growth: dpsc-8124 (-3.3%, p = 0.137), dpsc-9894 (+1.3%, p = 0.539), and dpsc-17322 (+3.1%, p = 0.165) - similar to the effects of BMP-2: dpsc-8124 (-3.6%, p = 0.189), dpsc-9894 (+0.5%, p = 0.653), dpsc-17322 (-1.3%, p = 0.794). Combined VEGF and BMP-2 also had no significant effects: dpsc-8124 (-8.1%, p = 0.0579), dpsc-9894 (-4.2%, p = 0.441), dpsc-17322 (-0.8%, p = 0.887).*

To evaluate whether any of the observations in cellular growth were associated with any changes to survival, cellular viability was evaluated under each control and experimental condition (Figure 3). These data demonstrated an overall higher viability for the iDT DPSC isolates at baseline: dpsc-8124 (34%), dpsc-9894 (33%), dpsc-17322 (36%) compared with the rDT DPSC isolates: dpsc-3882 (21%), dpsc-5653 (23%), dpsc-7089 (22%), which was statistically significant, p = 0.0067.



	dpsc-3882	dpsc-5653	dpsc-7089		dpsc-8124	dpsc-9894	dpsc-17322
BMP2	20	22	21		35	31	33
VEGF-BMP2	35	33	31		36	34	37
VEGF	34	35	32		37	36	39
ctl	21	23	22		35	34	36

Figure 3: *Growth factor effects on DPSC cellular viability. The addition of VEGF (alone or in combination) significantly increased viabilty among the rDT DPSC isolates (dpsc-3882, dpsc-5653, dpsc-8124) but had no effects on the iDT DPSC isolates (dpsc-8124, dpsc-9894, dpsc-17322). No changes in cellular viability were observed with the administration of BMP-2 in either the rDT or iDT DPSC isolates.*

The addition of VEGF significantly increased cellular viability among the rDT DPSC isolates, such as dpsc-3882 (35%, +61.9%), dpsc-5653 (35%, +52.1%), and dpsc-7089 (32%, +45.4%), p = 0.0081. However, no corresponding increase in viability was observed in any iDT DSPC isolate under, including dpsc-8124 (37%, +5.4%), dpsc-9894 (36%, +5.8%), and dpsc-17322 (39%, +8.3%), p = 0.102.

However, the addition of BMP-2 did not significantly alter cellular viabilty among the rDT DPSC isolates, inclduing dpsc-3882 (20%, -4.7%), dpsc-5653 (22%, -4.3%), and dpsc-7089 (21%, -4.5%), p = 0.287. Similarly, no effects were observed among the iDT DPSC isolates, such as dpsc-8124 (35%, no change), dpsc-9894 (31%, -8.8%), and dpsc-17322 (33%, -8.3%), p = 0.221.

The concomitant administration of VEGF and BMP-2 exhibited some effects on rDT but not iDT cellular viability. More specifically, VEGF-BMP-2 significantly increased viability among the rDT DPSC isolates, dpsc-3882 (35%, +66.7%), dpsc-5653 (33%, +43.4%), and dpsc-7089 (31%, +40.9%), p = 0.0036. In contrast, viability among the iDT DPSC isolates was not significantly changed under VEGF-BMP-2 administration, dpsc-8124 (36%, 2.8%), dpsc-9894 (34%, no change), dpsc-17322 (37%, +2.7%), p = 0.566.

Microscopy was performed to more closely evaluate the effects of VEGF on growth and viability of the rDT DPSC isolates (Figure 4). This analysis revealed that the increases in cellular viability and decrease in growth were associated with significant changes to cellular morphology in both dpsc-3882 (Figure 4A, Figure 4D) and dpsc-5653 (Figure 4B, Figure 4E). It was also noted that significant cell adhesion, cell spreading and increased cellular size was noted - although fewer overall numbers of cells were present. In addition, dpsc-7089 also contained a smaller proportion of cells with changes to adhesion, spreading and increased size (Figure 4C, Figure 4F). No changes to cell number, size or shape were noted among the iDT DPSC isolates (data not shown).

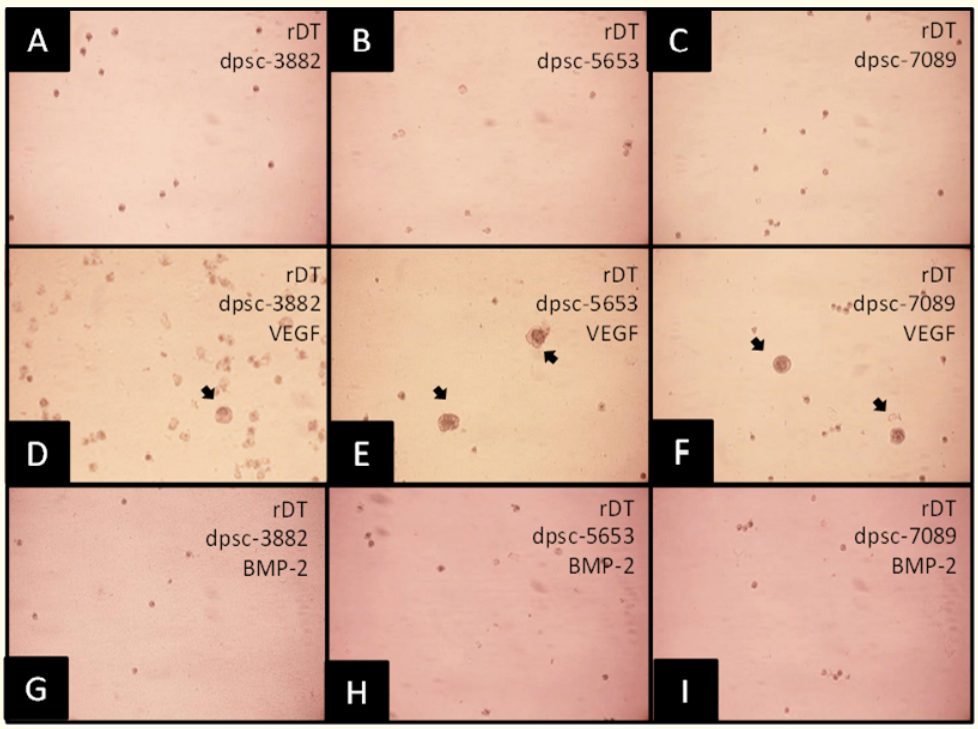


Figure 4: *Cellular morphology of rDT DPSC isolates in control and experimental conditions. The cellular morphology of rDT dpsc-3882 (A), dpsc-5653 (B), and dpsc-7089 (C) was marked altered by the addition of VEGF (D-F). Although an overall decrease in cell number was observed in dpsc-3882 and dpsc-5653 (D,E), an overall increase in cell size, cellular adherence and cell spreading was observed. No effects were observed with BMP-2 administration (G-I).*

Discussion

The main objective of this study was to evaluate whether VEGF administration or concomitant VEGF and BMP administration induced any measurable effects on DPSCs. These results demonstrated that administration of VEGF was sufficient to reduce growth in two of the rDT DPSC isolates, with no effect on any of the iDT DPSC isolates. Moreover, a corresponding increase in cellular viability was noted among all the rDT DPSC isolates along with corresponding changes to cellular morphology, with no effect on the iDT DPSCs. These results were in stark contrast to the effects of BMP-2, which exhibited no effects on either rDT or iDT DPSC isolates. In addition, the combination of VEGF and BMP-2 in combination had similar effects to the administration of VEGF in isolation, which may suggest VEGF may be primarily responsible for the observed changes to cellular phenotypes.

Although these data strongly suggest that VEGF may alter rDT cellular phenotypes, such as viability, proliferation and morphology, the limited scope of this study was not sufficient to allow for the elucidation of specific pathways responsible for these effects. The limited evidence that exists for MSC has recently suggested that VEGF may act on mesencymal stem cells through ERK- and FAK-dependent mechanisms that increase cellular migration - although there is no evidence to suggest these same pathways may be active among the rDT DPSC isolates [21,22].

The few studies that have explored VEGF in MSC or DPSC have been mainly restricted to studies of vasculogenic differentiation, an important topic for bioengineering and therapeutic applications [23,24]. However, the potential for VEGF to induce other cellular phenotypes and differentiation into lineages other than angiogenic and endothelial lineages remains an exciting and potentially revolutionary application for stem cell therapy [25,26].

Conclusions

These data provide significant preliminary results that clearly demonstrate significant and pronounced effects of VEGF on at least one subset of rapidly dividing DPSC isolates. These effects include changes to cellular viability and growth, which are supported by clear changes to cellular adhesion and morphology. Taken together, these data strongly suggest more reseach is needed to determine the underlying pathways triggered by VEGF administration in the cells and the pathophysiologic mechanisms that determine the responsiveness of these DPSC isolates but not others.

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