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Hypoxia-Mediated Upregulation of Semaphorin 3A during Pulp Revascularization

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Abstract

Semaphorin 3A could be involved in angiogenesis and also enhanced bone formation was investigated in many researches. In our current study, we firstly investigated that canal obliteration might be present in some regenerative endodontic procedures cases clinically. After the establishment of the model of apical periodontitis in the maxillary first molars of rats, pulp revascularization was performed in mesial root canal. Histological sections showed that most of the tissues growing into the root canal were not real pulp tissue, but cementoid, osteoid and periodontal-like membrane. Moreover, we detected that the expression of Semaphorin 3A increased in the mesial root canal. When we used $CoCl_2$ to induce hypoxic environment, the expression of genes and proteins, Hypoxia inducible factor-1α, Vascular endothelial growth factor, and Semaphorin 3A in dental pulp stem cells were both upregulated. In conclusion, hypoxia mediated the high expression of Semaphorin 3A in DPSC might be involved tissue regeneration during pulp vascularization.

Keywords

Dental Pulp Stem Cells, Hypoxia, Pulp Revascularization, Regenerative Endodontic Procedures, Semaphorin 3A

1. Introduction

Pulp revascularization is a new method for the treatment of immature permanent teeth with necrotic pulps, which aims to replace inflamed/necrotic pulp tissue with regenerated pulp-like tissue to achieve the maximum conservation of tooth vitality and the continued development of immature tooth [1]. A well-functioning vascular system is vital, as it ensures gas exchange, nutrient supply, and waste removal for all organs, including the tooth [2]. So it is a very important step that vascularization occurs in the sterile root canal. Three key elements

which are essential for tissue regeneration are stem cells, bioactive molecules, and scaffolds and hypoxia can be considered a common driving force. Stem cells included dental pulp stem cells (DPSCs) under hypoxic stress release proangiogenic factors, with vascular endothelial growth factor (VEGF) which involved in angiogenesis being one of the most potent [3]. Moreover, hypoxia activation influences genetic cascades effecting osteoprogenitor cell recruitment and activity [4]. Evidence showed that under hypoxia condition mesenchymal stem cells (MSCs) expressed VEGF could induce itself differentiation towards osteoblasts rather than adipocytes via an intracrine signalling loop [5]. Hypoxia thus is intricately involved in coupling angiogenesis and osteogenesis during tissue regeneration. In previous studies, the tissue formed in root canals was fibrous connective and cementum like tissues instead of a maturedentin-pulp complex. Study suggested that the cementoid/osteoid tissue deposited in the canal and the apex could be caused by cementoblasts/osteoblasts differentiated from the stem cells in the periodontal ligament [6]. However, within pulp revascularization, how the bioactive molecules secreted by MSCs balance angiogenesis and osteogenesis under hypoxic conditions in dental pulp cavity is unknown.

Semaphorin 3A (Sema3A) is a secreted protein characterized by its role in migration and guidance of axons in the developing nervous system [7]. Sema3A can only bind to neuropilin 1 receptor (NRP1), and NRP1 serves as co-receptors for VEGFA which is a proangiogenic factor widely known. Palodetto ascertained that sema3A partially reversed VEGF effects through binding to NRP1 [8]. Hayashi *et al.* identified a unique dual regulator Sema3A, which suppresses osteoclastic bone resorption while enhancing osteoblastic bone formation [9]. Recently, Yoshida found Sema3A induced cell migration, chemotaxis, proliferation, and odontoblastic differentiation of DPSCs clones [10]. However, the role of the Sema3A in pulp vascularization is still not fully understood.

Accumulating evidence has encouraged investigations on the potential roles of Sema3A in bone metabolism and angiogenesis. In present study, we conducted a preliminary study on the role of Sema3A in pulp revascularization. We first identified root canal calcification in clinical cases through Cone Beam Computed Tomography (CBCT) image analysis of pulp vascularization. Then, we established the model of apical periodontitis in the maxillary first molars of rats and carried out pulp revascularization in mesial root canal. We detected the expression of VEGF and Sema3A in 4 weeks after pulp vascularization through IHC. Finally, we simulated a hypoxic environment in the body to detect the expression of genes and proteins, Hypoxia inducible factor-1α (HIF-1α), VEGF, and Sema3A in DPSCs.

2. Materials and Methods

2.1. CBCT Image Analysis Clinically

CBCT Images of patients undergoing the pulp revascularization before and 24 months after treatment were searched from the NNT software in department of

Radiology, Xuzhou Stomatology Hospital.

2.2. Induction of Apical Periodontitis in a Rat Model and Pulp Revascularization

All experiments were performed according to the Guidelines for the Treatment of Animals at Xuzhou Medical University. Six male Wistar rats weighing approximately 110 g were used in this study. Dental surgery under intraperitonealanaesthesia of 10% chloral hydrate (30 mg/kg) was performed on all rats. The first molars in right maxillas was drilled using a #1/8 dental round bur (Dentsply, USA) until the pulp was exposed, and the left molar as control. After three weeks, the canal was minimally instrumented and irrigation with 20 mL 2.5% sodium hypochlorite and then dried with sterile paper points and a triple antibiotic paste containing metronidazole, ciprofloxacin, and minocycline in the ratio 1:1:1 by weight was placed into the mesial root canal using a syringe. The access cavity was then closed with temporarily restored materials Caviton. After three weeks, a sterile #8 K-file (Dentsply, USA) was introduced into the canal beyond the apical foramen to stimulate the bleeding in the root tips. At last the access opening was covered with Theracal-LC (BISCO, USA), and sealed with resin (Matsushita, Japan).

2.3. Haematoxylin and Eosin (H&E) Staining

After four weeks pulp revascularization, maxillas were fixed with 4% paraformaldehyde immediately after they were dissected from the rats. Forty-eight hours later, the samples were decalcified with 10% ethylene diaminetetraacetic acid (EDTA) disodium salt for at least 3 months and then trimmed into 8 mm × 6 mm × 4 mm blocks for dehydration and paraffin embedding. Four-micrometer thickness serial sections were cut in the mesiodistal direction and stained with H&E. The slices were performed under Olympus microscpe (Osaka, Japan) by two independent investigators.

2.4. Immunohistochemical Staining

To analyse the protein expression level of VEGF and Sema3A after four weeks pulp revascularization, immunohistochemistry was performed each sample. Specifically, sections were incubated at 4˚C for 24 hours with primary antibodies: mouse anti-VEGF (1:1000, Abcam) and rabbit anti-Sema3A (1:1000, Affnity) antibody. Goat anti-rabbit IgG was used as secondary antibody. Negative controls were obtained by replacing primary antibodies with phosphate-buffered saline. The slices were performed under Olympus microscpe (Osaka, Japan) by two independent investigators.

2.5. Dental Pulp Stem Cells Cultured and Induction of Hypoxia Condition

After obtaining informed consent, DPSCs were isolated from freshly extracted human third molars with immature roots (aged 18 - 25 years) as described pre-

viously. The DPSCs were cultured in α-modified essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1% Penicillin-Streptomycin (Invitrogen, Carlsbad, CA) in a humidified incubator at 37˚C with 5% CO_2. The medium was changed every three days and DPSCs of passages3-6 were used in the subsequent studies.

For hypoxic conditions, DPSCs were seeded at a density of 3.5 × 105 cells/mL in six-well plates and cultured for 3 days to achieve 80% - 90% confluence. Then, the cells were starved with serum-free medium for 12 h followed by incubation with different concentrations of $CoCl_2$ (Sigma, 100 mmol/L, 300 mmol/L, 500 mmol/L). Then we chose 500 mmol/L $CoCl_2$ in subsequent studies. After hypoxia treatment for 0, 6 and 12 hours, mRNA and proteins were harvested from the cells for subsequent analyses. All experiments were performed in triplicate.

2.6. CCK8 Assay

DPSCs were plated at a density of 1×10^4 cells per well and a volume of 100 μL in 96-well plates. The cells were starved for 24 h with serum-free medium at 37˚C in a humidified 5% CO_2 atmosphere before the test. Then the serum-free medium was replaced with 500 mmol/L $CoCl_2$. At each time point (0, 6, 12, and 24 h after seeding cells), 10 μL of CCK8 assay solution was added to each well and incubated for 4 h at 37˚C. Cell number and viability were calculated by measuring absorbance at a wavelength of 550 nm on a multi-plate reader (BIO-TEK, USA). The experiments were performed in triplicate.

2.7. Western Blot Analysis

To assess the effects of hypoxia on the synthesis of HIF-1*α* with the increase of the $COCl_2$ concentration, and the expression of HIF-1*α*, VEGF, Sema3A after hypoxia treatment for 0, 6, 12 hours, western blot analysis was performed. Total protein was isolated from DPSCs by lysis with NP 40 buffer (Beyotime, China). The protein concentration was determined using the bicinchoninic acid protein assay reagent (Thermo Scientific, USA). The total proteins (60 μg) were resolved on 8% or 10% SDS-PAGE gel and transferred to nitrocellulose membranes. The membranes were blocked in Tris Buffered Saline with Tween containing 3% bovine serum albumin for 1h at room temperature and further incubated with special antibodies, rabbit anti-HIF-1*α* (1:1000, Affnity), mouse anti-VEGF(1:1000, Abcam) and rabbit anti-Sema3A (1:1000, Affnity) antibody at 4˚C overnight. After the membranes were washed with Tris Buffered Saline with Tween for three times, they were incubated with secondary antibodies (1:15,000) for 1h at room temperature. At last, the blots were scanned, and target bands were analyzed using Image J software.

2.8. Real-Time Polymerase Chain Reaction

After hypoxia treatment for 0, 6, 12 hours, the total RNA of DPSCs was extracted using RNA Mini kit (Qiagen, Valencia, CA), with the first-strand cDNA

reversely transcribed using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). Real-time PCR was performed using a Light Cycler 480 SYBR Green I Master kit (Qiagen, Dusseldorf, Germany). Primers used for the desired sequence are shown in Table 1. The relative mRNA expression levels of Hif-*α*, Vegf, Sema3a were quantified compared to GAPDH using the 2-ΔΔCT method.

2.9. Immunofluorescence Staining

To determine the specific localizations and the amount of VEGF and Sema3A at normoxic and hypoxia conditions during DPSCs cultured, immunofluorescence was performed. After 6 hours cultured with 500 mmol/L $COCl_2$, the DPSCs washed using PBS, fixed by 4% formaldehyde solution, and treated with 0.1% triton for 3 minutes. After three extensive washes with PBS, the samples were blocked with 5% BSA for 1h at room temperature, incubated with primary antibody, mouse anti-VEGF (1:1000, Abcam) and rabbit anti-Sema3A (1:1000, Affnity) antibody at 4˚C overnight, and then incubated with a secondary antibody conjugated with DyLight 488 or Dylight 594 (Bioworld, St. Louis) at 37˚C for 1h. Subsequently, the samples were stained with DAPI (Beyotime, China) to detect the cell nuclei. The coverships were mounted on glass slides, and the images were viewed with an Olympus BX43F fluorescence microscope (Tokyo, Japan).

2.10. Statistical Analysis

Data were analyzed using the Student's t-test to compare between two groups as appropriate. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Root Canal Calcification Occurred 24 Months after Pulp Revascularization Clinically

A sagittal plane of CBCT image of #21 with apical periodontitis in a 10-year-old girl (Figure 1(B)). 11 was a normal tooth (Figure 1(A)). #11 and #21 were both

Table 1. Primers for real-time polymerase chain reaction.

Gene	Primers
Hif-*α*	For: GAAACGACCACTGCTAAGGCA Rev: GGCAGACAGCTTAAGGCTCCT
Vegf	For: CCACGTCAGAGAGAGCAACATCA Rev: TCATTCTCTCTATGTGCTGGCTTT
Sema3a	For: CAGCCATGTACACCCAGTG Rev: ACGGTTCCAACATCTGTTCC
Gapdh	For: ATCATCCCTGCATCCACT Rev: GTCATCATACTTGGCAGGTTTC

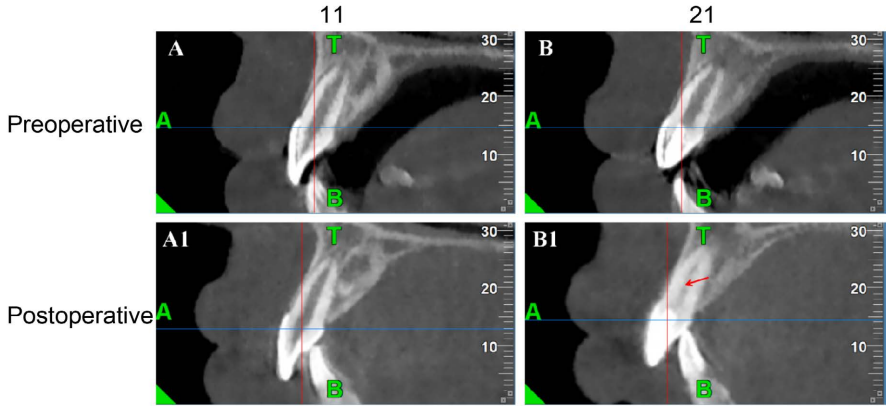

Figure 1. Root canal calcification occurred 24 months after pulp revascularization clinically. A 11 was a normal tooth. B #21 with apical periodontitis in a 10-year-old girl. C 24 months follow-up CBCT image of #11. D 24 months follow-up CBCT image of #21. red arrow indicated root canal calcification.

exhibited blunderbuss root apex. After pulp revascularization 24 months, we found #11 and #21 Apical closure (**Figure 1(C)**). In addition, the root dentin was thickened, and the image of root canal was not clear in #21 (**Figure 1(D)** red arrow).

3.2. The Expression of VEGF and Sema3A Increased after Pulp Revascularization in Root Canal

Histological analysis by H&E staining (**Figure 2(A)** and **Figure 2(B)**) revealed that after pulp revascularization, there was no normal pulp tissue, and most of the tissues present in the root canal were osteoid (five-pointed star indicated), cementoid (dotted line indicated), and periodontal-like tissues (triangle indicated). Immunohistochemical staining (**Figure 2(C)** and **Figure 2(D)**) analysis revealed that in control groups, VEGF and Sema3A were slightly expressed, mainly secreted by odontoblast, fibroblast and vascular endothelial cells. But after pulp revascularization (**Figure 2(E)** and **Figure 2(F)**), the number of VEGF and Sema3A-positive cells was dramatically increased in root cannal compared with control groups ($P < 0.05$) (**Figure 2(G)**). Especially surrounding the osteoid and cementoid, Sema3A was highly expressed.

3.3. A Hypoxic Environment Was Established in DPSCs Cultured

Different concentrations of $COCl_2$ could promote the expression of HIF-1α by DPSCs (**Figure 3(A)**), and high concentrations of $COCl_2$ promoted cells proliferation within 12 hours, but inhibited the proliferation during 12 to 24 hours (**Figure 3(B)**).

3.4. DPSCs Secreted More VEGF and Sema3A under Hypoxia Conditions

The expressions of VEGF and Sema3A up-regulated with the extension of hypoxia time both at gene (**Figure 4(A)**) and protein (**Figure 4(B)** and **Figure 4(C)**) level. In the normal state, VEGF and Sema3A were mainly distributed in

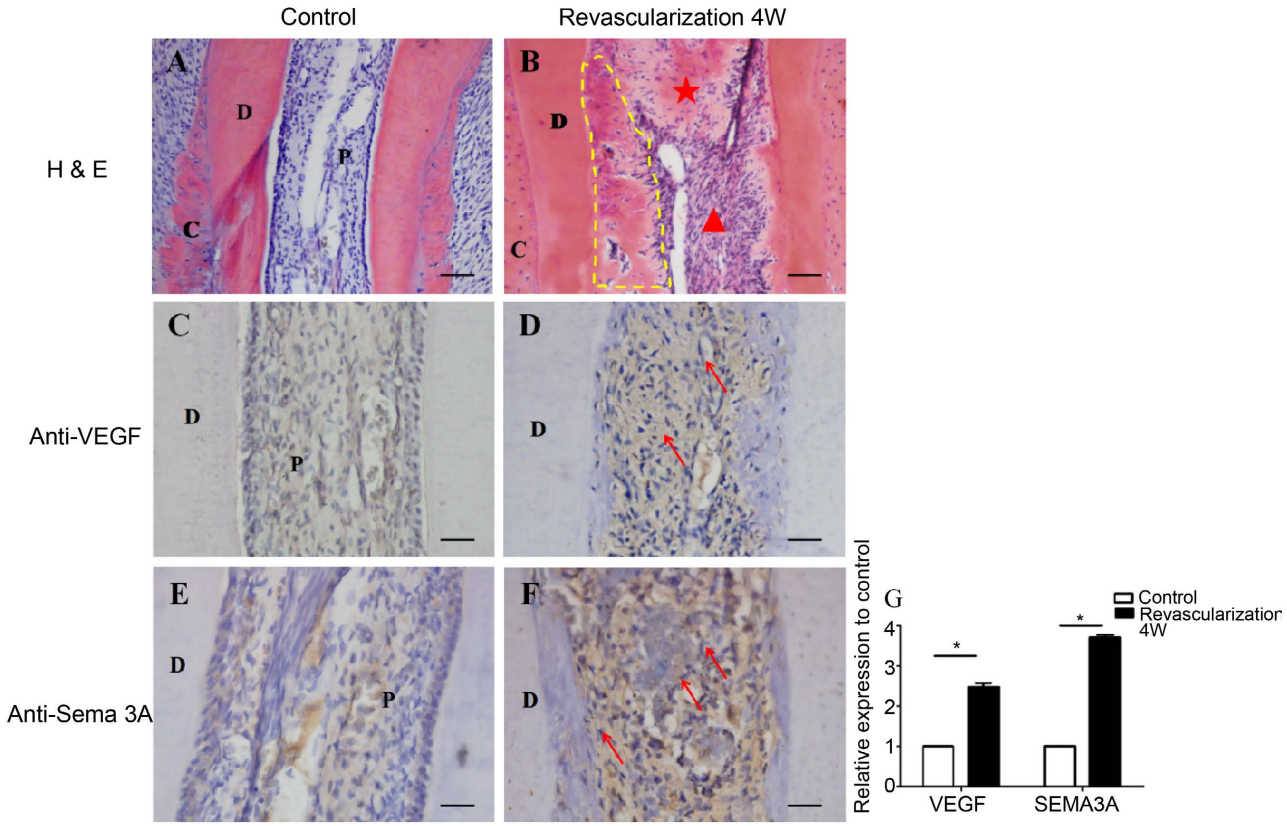

Figure 2. The expression of VEGF and Sema 3A increased after pulp revascularization in root canal. (A) normal root canal tissue. (B) four weeks after pulp revascularization root canal tissue. five-pointed star indicated osteoid; dotted line indicated cementoid; triangle indicated periodontal-like tissues. (C), (D) VEGF-positive cells in normal and pulp revascularization root canal tissue. (E), (F) Sema3A-positive cells in normal and pulp revascularization root canal tissue. (G) Quantitative analysis through Image J software. Bars = 25 mm.

the cytoplasm with low content in DPSCs, But after 6 hours of $COCl_2$ treatment, the expression of VEGF and Sema3A in the cytoplasm increased (**Figure 4(D)**).

4. Discussion

Through CBCT imaging, we first found that the root canal of the affected teeth was significantly thinner and the root canal image was not clear 24 months after pulp revascularization, compared with the normal adjacent teeth. Chen *et al.* [11] suggested canal obliteration as one of the healing outcomes, and various types of canal obliteration have been reported in some pulp revascularization cases [12] [13]. We then tested it in animal models, after establishment of the model of apical periodontitis in the maxillary first molars of rats, pulp revascularization was performed in mesial root canal. Histological sections showed that most of the tissues growing into the root canal were not real pulp tissue, but cementoid, osteoid and periodontal-like membrane. Martin *et al.* [6] considered canal obliteration was an ectopic bone formation and cementogenesis inside the lumen of the root canals because of revascularization with induced bleeding from root tips area. Bleeding from periapex would carry periodontal ligament

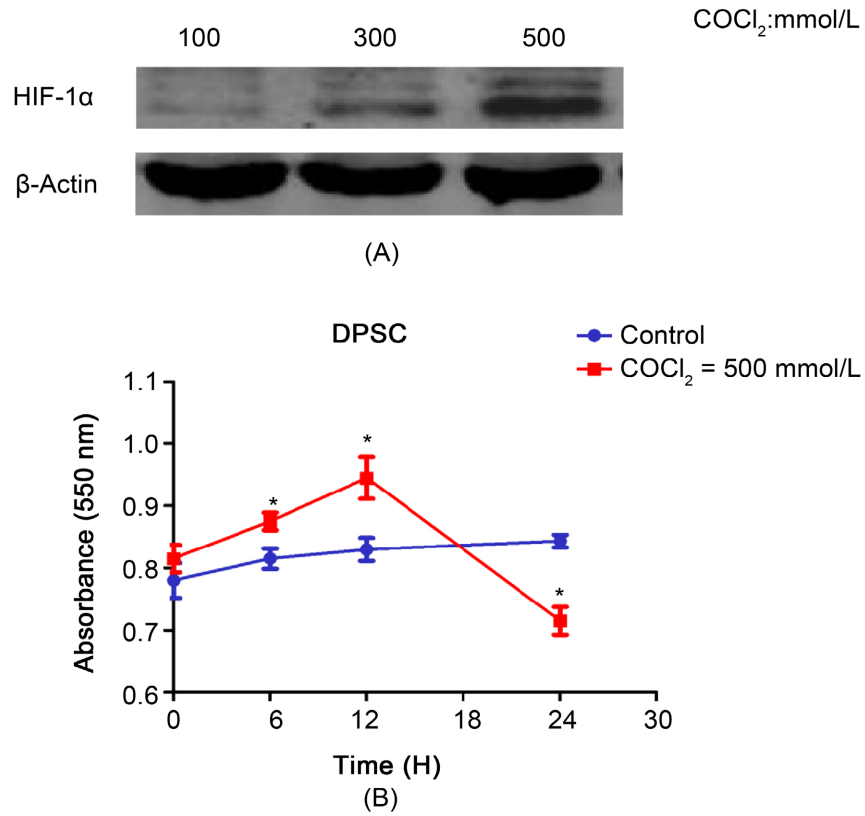

Figure 3. A hypoxic environment was established in DPSCs cultured. (A) the expression of HIF-*α* of DPSCs at three different concentrations of $COCl_2$ (100 mmol/L, 300 mmol/L, 500 mmol/L) treatment. (B) DPSCs proliferation after 0, 6, 12, 24 hours at high concentrations of $COCl_2$ treatment.

stem cells and bone marrow stem cells which with cementogenic and osteogenic differentiation capacities from alveolar bone [14]. Through immunohistochemical staining staining, we found a very interesting phenomenon: after pulp revascularization, VEGF was highly expressed in the root canal of the affected tooth, but not many blood vessels were produced. The expression of Sema3A also increased, mainly around the osteoid and cementoid in the root canal. We believed that the microenvironment affected the secretion of VEGF and Sema3A by stem cells, and further affected the tissue for repair and regeneration inpulprevascultation.

The microenvironment in periapical tissue for repair and regeneration was dependent upon the spatial orientation of stem cells and signaling molecule on the suitable scaffold [15]. The first type of mesenchymal stem cells (MSCs) from dentoalveolar tissues was isolated from the human dental pulp, and it also had the function of angiogenic potential [16]. When the pulp was damaged, the vascular collapse resulted in local tissues being exposed to hypoxia and nutrient deprivation. In order to re-establish the vascular network to transport oxygen and nutrients, the pulp cells rapidly expressed HIF-1 [17]. Hif-1 mediated the production of a large number of genes that promote angiogenesis, such as vascular endothelial growth factor, platelet derived growth factor AB, placental growth

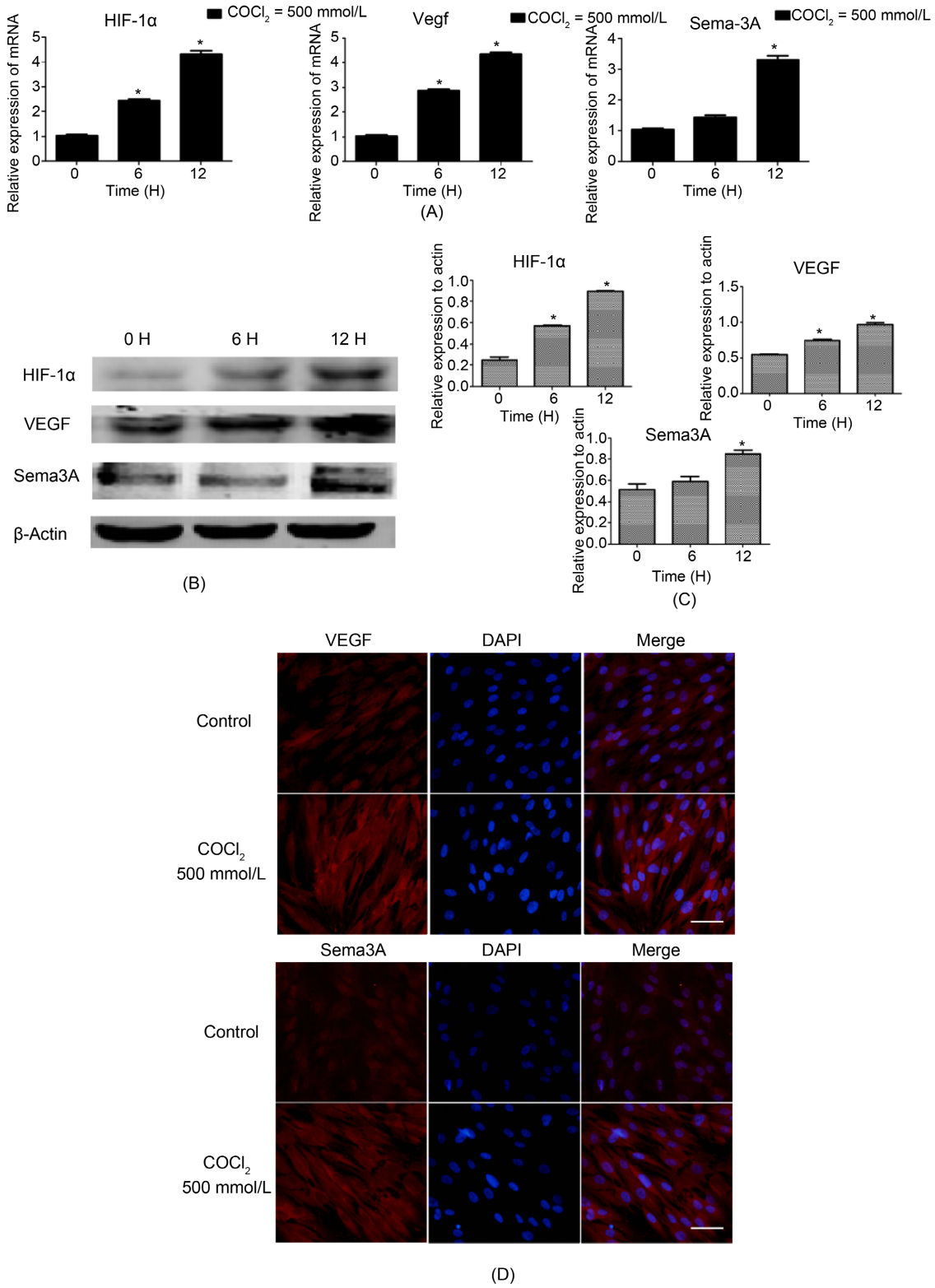

Figure 4. DPSCs secreted more VEGF and Sema3A under hypoxia conditions. (A) mRNA expression of HIF-1*α*, VEGF and Sema3A after 0, 6, 12 hours treatment with $COCl_2$. (B), (C) protein expression of HIF-1α, VEGF and Sema3A after 0, 6, 12 hours treatment with $COCl_2$. (D) Immunofluorescence staining of VEGF and Sema3A under control and hypoxia condition. Bars = 50 mm.

factor, and angiogenin. Hif-1 also regulates the expression of angiogenicchemokines such as stromal cell derived factor 1, sphingosinol-1-phosphoric acid and its receptors, which jointly promote neovascularization [18]. In our present study, for the sake of simulating the hypoxic environment in root canals, we added $COCl_2$ in the culture medium, which could inhibit the degradation of HIF-1α and allow it to accumulate in the cells. We found high concentrations of $COCl_2$ could up-regulate the expression of HIF-1α by DPSCs and promote cells proliferation within 12 hours, but inhibit the proliferation during 12 to 24 hours. It was consistent with other studies in the literature that a short time hypoxia could promote the proliferation of DPSCs for the purpose of building new vascular systems to transport oxygen and nutrients [19].

Our results confirmed that under hypoxic conditions, DPSCs secreted more VEGF with the prolong of hypoxia time. This founding was same with Bakopoulou et al that they created a hypoxic (2% oxygen content), low serum and low sugar environment in vitro, and also found that cells derived from dental papilla, such as fibroblasts and dental pulp stem cells, could secrete a large amount of VEGE [20]. However, we didn't observe a lot of blood vessels in the previous histological sections instead of more osteoids and cementoids tissue. Many studies suggest that the microenvironment inside the root canal is more suitable for stem cell osteogenic differentiation than for the formation of real pulp tissue [21] [22]. In addition to the above hypothesis, we wondered whether stem cells secreted some signaling molecule that could antagonize the ability of VEGF to form blood vessels, so as to produce the phenomenon that new angiogenesis was still rare even though there was a large amount of VEGF in the microenvironment. It was also verified in this experiment. We found the expression of Sema3A also increased significantly in hypoxic conditions through gene and protein level. In turn, immunofluorescence determined that the secretion of Sema3A by DPSC increased in cytoplasm under hypoxic conditions.

5. Conclusion

In conclusion, as far as we know, the high expression of Sema3A was found after pulp revascularization in root cannal and in DPSC under hypoxic conditions had not been studied. The role of Sema3A in tissue repair and regeneration during pulp revascularization remains to be further studied.

Authorship Declaration

All authors have contributed significantly and agreed with the manuscript.

Conflicts of Interest

The authors deny any conflicts of interest related to this study.

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