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A study of the effects of hydroxyapatite bioceramic extract on Ang/Tie2 system of umbilical vein endothelial cells

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Abstract.
OBJECTIVE: We aimed to investigate the effects of hydroxyapatite bioceramic extract on Ang/Tie2 system and cell proliferation of umbilical vein endothelial cells.
METHODS: Human umbilical vein endothelial cells (HUVECs) were used in this research. There are two induvial groups, control group and hydroxyapatite bioceramics extract treatment group. Cell Counting Kit-8 (CCK-8) was used to evaluate cell proliferation. Western blot and real time quantitative PCR (Q-PCR) were used to evaluate the protein and mRNA expression levels of Ang1, Ang2 and Tie2 in Ang/Tie2 system, respectively. All the results were statistically analyzed by Spss19.0. All data were presented as mean ± standard error of mean (SEM). Student's t-test was performed to determine the differences among grouped data.
RESULTS: Hydroxyapatite bioceramics extract showed no effect on the cell morphology and cell proliferation of HUVECs. Interestingly, we found that both Ang2 and Tie2 protein and mRNA level were markedly increased by hydroxyapatite bioceramics extract.
CONCLUSIONS: Hydroxyapatite bioceramic extract showed no cytotoxicity to HUVECs, and might regulate vascular remodeling by mediating Ang/Tie2 system.

Keywords: Ang, Tie2, umbilical vein endothelial cells, hydroxyapatite bioceramics extract

1. Introduction

Bone defect repair is a major and important issue in the clinical work of oral cavity. The alveolar bone of adult orthodontic patients is thin, which causes the maxillary sinus easily perforating through the maxillary sinus bottom wall. Bone regeneration is need to be taken in the bone defect site of the implant area and the alveolar bone absorption place which are caused by multiple reasons [1]. Additionally, bone regeneration allows clinicians to place implants or miniscrews with greater diameter and length, thus allowing better clinical performance and durability over time [2,3]. With the development of materials science, clinicians gradually use bone graft materials instead of autologous bone for small-scale bone grafting, and have achieved good bone regeneration effect [4].

Hydroxyapatite bioceramics is one kind of widely used bone graft materials. The main component of Hydroxyapatite bioceramics is hydroxyapatite (HA) that is similar to inorganic materials in biological

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bone tissue [5]. It has been reported that hydroxyapatite bioceramics, as a bone graft material and with certain biocompatibility, could increase the bone thickness and density of alveolar bone [6].

The vascular system can provide oxygen and nutrition for the regenerated tissue and maintain the growth of the tissue. Therefore, vascular remodeling is an essential procedure during the formation of bone tissue. Tissue necrosis and poor tissue formation can be caused by the insufficient blood supply or slow angiogenesis [7]. Vascular tissue reconstruction is closely related to the proliferation of vascular endothelial cells. It has been found that angiopoietin/tyrosine kinase receptors 2 (Ang/Tie2) system plays a critical role in maintaining vascular maturity and stability, as well as repairing microcirculation after vascular injury [8]. Until now, the effect of hydroxyapatite bioceramics on the proliferation and Ang/Tie2 system of human umbilical vein endothelial cells (HUVECs) has not been reported yet. Here, in this study, firstly, we found that hydroxyapatite bioceramic extract showed no cytotoxicity to HUVECs, and might regulate vascular remodeling by mediating Ang/Tie2 system.

2. Materials and methods

2.1. Reagents and instruments

Hydroxyapatite bioceramics (synthetized by Beijing Yihuajian science and Trade Co., Ltd., China), endothelial cell culture medium ECM (1001, ScienCell, USA), Potassium phosphate buffer (PBS) (SH30256 01B, Hyclone, USA), clean bench (sw-cj-ifd, Suzhou Antai, China), low-speed centrifuge (sc3614, Zhongjia, China), inverted optical microscope (CKX41, Olympus, Japan), cell incubator (Heracell 150i, Thermo Scientific, USA) and CCK-8 solution (Kga317, Jiangsu Kaiji, China), plate reader (Multiscan MK3, Thermo Fisher Scientific, USA), Ang1 and Tie2 antibody (251671, Beijing Zen bio, China), Ang2 antibody (24613-1-ap, Proteintech, USA), RIPA buffer (P0013B, Shanghai Beyotime, China), secondary antibody (111-035-003, Jackson ImmunoResearch, USA), luminescent solution (wbkls0500, Millipore, USA), BCA Protein Assay Kit (PA115, Beijing Tiangen, China), PVDF membrane (ipvh00010, Millipore, USA), TRIzol reagent (1556018, Invitrogen, USA), SYBR GREEN qPCR Super Mix (C11733046, Invitrogen, USA), fluorescent PCR (1725274, ABI, USA).

2.2. Methods

2.2.1. Groups

There are two induvial groups, control group and hydroxyapatite bioceramics extract treatment group. Control group is HUVECs alone, and hydroxyapatite bioceramics extract treatment group is defined as experimental group.

2.2.2. Hydroxyapatite bioceramics extract preparation

The concentration of hydroxyapatite bioceramics extract solution was prepared according to the protocol in part 12 of iso10993.12-2008 biological evaluation of medical devices. Briefly, 0.2 g hydroxyapatite bioceramics was dissolved into 1 ml complete culture medium and extracted in an inert container at 37°C for 5 days.

2.2.3. Cell STR identification

Umbilical vein endothelial cell line was purchased from Shanghai FUHENG Biotechnology Co., Ltd. Briefly, total DNA was extracted by axygen genomic extraction kit and amplified according to 21-str amplification protocol. The STR locus and sex gene amelogenin were detected by using genetic analyzer

Table 1
RT-PCR primer sequences for target genes and reference gene

Gene name	Primer sequence
Ang1	F:5'-TGCTGATAATGACAACTGTA-3'
	R:5'-AAGGTCGAATCATCATAGTT-3'
Ang2	F:5'-GAGAATATTGGCTGGGAAAT-3'
	R:5'-CCTGTAAGTCCTTTAAGGTG-3'
Tie2	F:5'-TTATGAGAGGCCATCATTTG-3'
	R:5'-CTCATAAAGCGTGGTATTCA-3'
GAPDH	F:5'-GGGAAACTGTGGCGTGAT-3'
	R:5'-GAGTGGGTGTCGCTGTTGA-3'

(ABI 3730xl, Thermo Scientific, USA). The results showed that there were no multiple alleles which indicates the good quality of cell typing.

2.2.4. Cell culture

HUVECs were cultured in endothelial cell culture medium ECM. Under normal conditions, cells were cultured at 37°C with 5% CO_2, and 95% humidity. Cells were passaged every 2 days. Briefly, wash the cells with PBS for 2 times, and then add 0.05% trypsin. After that, incubate the cells in 37°C for 2 minutes, and then add the medium with serum to terminate the digestion process. Centrifuge the cells at 300 g for 5 minutes, and then passage the cells to new culture dish according to the ratio of 1:2.

2.2.5. Cell viability assay

HUVECs cells were plated at 8,000/well in 96-well plates, and cell viability assay was assessed on the indicated time points (Day1, Day3, Day5 and Day7) according to the manufacturer's instructions (Kga317, Jiangsu Kaiji, China). Cell viability was expressed as the percentage of viable cells relative to untreated cells using the absorbance at 450 nm and absorbance was measured using auto microplate reader. All experiments were performed in three individual experiments.

2.2.6. Western blot

Total proteins were extracted from HUVECs by using cell lysis buffer and passed the tissue through a 21-gauge needle more than 30 times. The cell lysate was centrifuged at 4°C and 14000 g for 15 minutes to eliminate large aggregates. Protein concentrations were evaluated by BCA protein assay kit according to the manufacturer's instructions (PA115, Beijing Tiangen, China). The protein was denatured with 4X SDS loading buffer at 96°C for 10 minutes. After that, the samples were loaded for electrophoresis on SDS-PAGE gel. The gel containing proteins was then transferred to a PVDF membrane. The membrane was blocked with 5% milk in 1 × TBST for 1 hours, and incubated with the primary antibody at 4°C overnight. Wash the membrane with 1 × PBST 5 times at room temperature, and incubate the membrane with a secondary antibody (1:5000 dilution) for 1 hour. The blots were detected by chemiluminescence (ChemiDoc XRS+, Bio-Rad, USA). The band intensity was quantified by Image lab. All experiments were performed in four individual experiments [9].

2.2.7. Real-time PCR

Total mRNA of HUVECs was extracted by using TRIzol reagent according to the manufacturer's protocols. The concentration of mRNA wad detected by BioPhotometers plus (Eppendorf, Germany). The quantitative real-time PCR using SYBR GREEN qPCR Super Mix was performed in ABI one step Plus (1725274, ABI, USA). All experiments were performed in nine individual experiments. All primers are listed in Table 1.

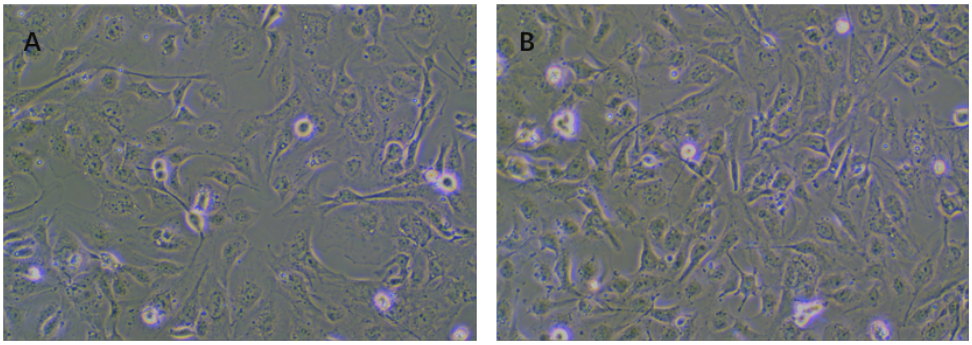

Fig. 1. The morphology shape of HUVECs (A) the morphology of HUVECs in control group (B) the morphology of HUVECs in hydroxyapatite bioceramic extract treatment group.

2.3. Statistical treatment

Spss19.0 statistical software (IBM, USA) was used to analyze the data. All data were presented as mean $\pm$ standard error of mean (SEM). Student's t-test was performed to determine the differences among grouped data. * indicates statistically significant with $P < 0.05$, ** indicates statistically significant with $P < 0.01$, *** indicates statistically significant with $P < 0.001$.

3. Results

3.1. Hydroxyapatite bioceramics extract showed no effect on the cell morphology of HUVECs

We first test the effect of hydroxyapatite bioceramics extract on the cell morphology of HUVECs. The images were captured by inverted microscope. As shown in Fig. 1A and B, the cell morphology of both control group and hydroxyapatite bioceramics extract treatment group (48 h) showed the "paving stone" shape, which was reflected by spindle shaped and small angle shaped (as shown by the white arrow).

3.2. The effect of hydroxyapatite bioceramics extract on cell proliferation of human HUVECs

To investigate whether hydroxyapatite bioceramics extract could affect the viability of HUVECs, cell proliferation was assessed by CCK-8 assay after hydroxyapatite bioceramics extract treatment at the indicated time points (Day1, Day3, Day5 and Day7). The results in Fig. 2 showed that there was no significant difference in cell proliferation rate between control and hydroxyapatite bioceramics extract treatment group ($P > 0.05$), which indicates that hydroxyapatite bioceramics extract is not cytotoxic to cells.

3.3. The protein levels of Ang2 and Tie2 were up-regulated by hydroxyapatite bioceramic extract

Evidence suggests that the Ang/Tie2 signaling pathway is essential for maintaining vascular homeostasis, and its dysregulation is associated with several diseases [10]. Until now, the relevance between hydroxyapatite bioceramic extract and Ang/Tie2 is unkown, therefore, it is of great interest to investigate whether hydroxyapatite bioceramic extract could affect Ang/Tie2 signaling pathway. Data in Fig. 3 showed that there was no significant difference in the protein level of Ang1 ($P > 0.05$) compared with the

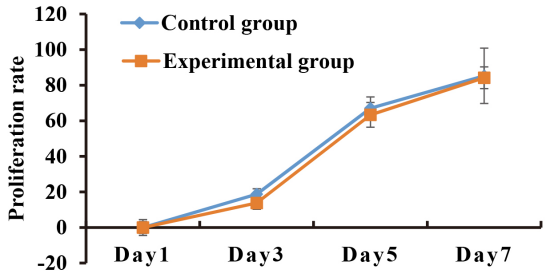

Fig. 2. Cell proliferation curve. HUVECs were treated with hydroxyapatite bioceramic extract for the indicated time points (Day1, Day3, Day5 and Day7), and then assessed by CCK-8 assay.

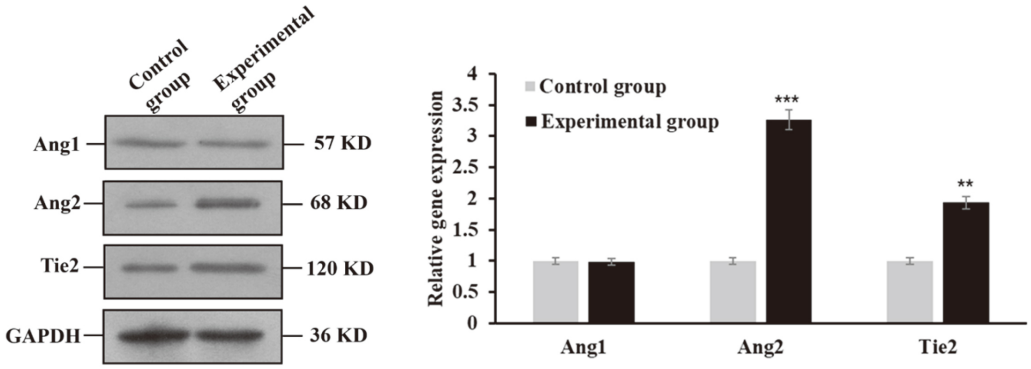

Fig. 3. Ang2 and Tie2 were up-regulated by hydroxyapatite bioceramic extract (**$P < 0.01$, ***$P < 0.001$).

control group. However, surprisingly, the protein levels of Ang2 and Tie2 were significantly up-regulated in hydroxyapatite bioceramic extract treatment group (Fig. 3), which hints that hydroxyapatite bioceramic extract might affect angiogenesis by regulating Ang/Tie2 system.

3.4. The transcriptional level of ANG2 and TIE2 were up-regulated by hydroxyapatite bioceramic extract

Consistently, the results of real-time quantitative PCR in Fig. 4 showed that there was no significant difference in the transcriptional level of *ANG1* ($P > 0.05$) compared with the control group. However, surprisingly, the transcriptional level of *ANG2* ($P < 0.01$) and *TIE2* was up-regulated ($P < 0.001$) in hydroxyapatite bioceramic extract treatment group (Fig. 4).

4. Discussion

Bone tissue defect, which is caused by tissue defect, maxillofacial trauma, congenital malformation, tumor or alveolar bone defect, is a very common phenomenon in stomatology [1]. Bone regeneration allows clinicians to place implants or miniscrews with greater diameter and length, thus allowing better clinical performance and durability over time [2,3]. Recently, with the development of bone regeneration in surgery technology and bone substitute implantation, hydroxyapatite bioceramic has been widely used in clinical research. Hydroxyapatite bioceramic is mostly composed of hydroxyapatite which is about 90%, and the chemical composition it is similar to that of inorganic components in bone tissue. In oral

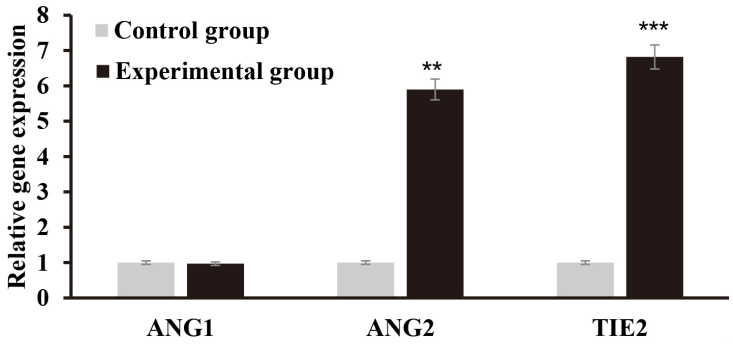

Fig. 4. The transcriptional level of *ANG2* and *TIE2* were up-regulated by hydroxyapatite bioceramic extract (**$P < 0.01$, ***$P < 0.001$).

clinic, it is commonly used in maxillary sinus elevation surgery and implantation bone defect repair. It has been reported that Fu as a bone implant material has a good effect on osteogenic [11]. Nowadays studies have demonstrated that hydroxyapatite-based biomimetic solutions are active for enamel and dentin remineralization but the reconstruction of hard tissues is still an open challenge [12,13].

Vascular remodeling is an essential procedure during the formation of bone tissue. Lack of blood supply and reconstruction can cause tissue undernutrition and necrosis during tissue repair process [14]. Moreover, too large size of the implanted tissue, which only relies on the neighborhood blood vessels to get nutrients, will suffer insufficient blood supply and undernutrition *in vivo*. Therefore, good blood cell compatibility, without cytotoxicity and promoting angiogenesis are the basal requirements of the bone graft materials. Therefore it is of great interest to investigate the effect of hydroxyapatite bioceramics on angiogenesis during bone regeneration.

Vascular remodeling is initiated by multiple cytokines, including angiopoietins like Ang1 and Ang2, VEGF, as well as TNF-α [15–18]. Angiopoietins are vascular regulators that act on Tie receptors, including Tie1 and Tie2. Ang1, a constitutive agonist for Tie2 phosphorylation (p-Tie2), contributes to vascular remodeling and maintenance of vascular stability [19–21]. *ANG1* or *TIE2* deletion could cause embryonic lethality [20]. The action of Ang1 on Tie2 can be competitively inhibited by Ang2 [21,22], hence, Ang2 can promote vascular remodeling by blocking Tie2 signaling [18,23,24]. Overexpression of Ang2 results in embryonic lethality, which is similar to the function of *ANG1* or *TIE2* deletion [22]. Paradoxically, Tie2 can be activated by Ang2 under certain conditions such as stress or inflammation [22,25,26]. Furthermore, under Ang1 deletion condition or on certain cell types, when Ang2 was used in high concentrations, it acts as a weak agonist of Tie2 [22,25,27]. It has been reported that under baseline conditions, Ang2 acts as a Tie2 agonist, however, under infection conditions, it acts as an antagonist of Tie2 [28]. What's more, Findings support the evidence that under normal conditions, both Ang1 and Ang2 act as Tie2 agonists, and elevate vascular stability and enlargement [29].

5. Conclusion

In this study, we firstly found that hydroxyapatite bioceramic extract, without cytotoxicity to HUVECs, can increase both Ang2 and Tie2 protein and mRNA levels, which hints that under hydroxyapatite bioceramic extract treatment condition, Ang2, here, may act as an agonist of Tie2, which might regulate vascular remodeling through Ang/Tie2 signaling. Therefore, the underling mechanism of how hydroxyapatite bioceramic extract regulates vascular remodeling needs further to be elucidated.

Conflict of interest

The authors state that there is no conflict of interest related to this article.

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