pH regulates the lumen diameter of tissue-engineered capillaries

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Abstract. Angiogenesis is vital in tissue engineering and the size of the capillary lumen diameter directly affects vascular function. Therefore, the involvement of the pH in the regulation of the capillary lumen diameter was investigated in the present study. The cytosolic pH of different pH medium groups was measured using flow cytometry. Bromodeoxyuridine staining and wound-healing assays were performed to detect cell proliferation and migration, respectively. The expression of angiogenesis-related genes was detected using reverse transcription-quantitative PCR. In addition, cell tube formation under different pH conditions was assessed using a tube formation assay and a 3D Matrigel® model. The results indicated that a change in the pH value of the culture medium affected the cytosolic pH of the endothelial cells, which then led to a change in vascular diameter. When the medium's pH ranged from 7.4 to 7.6, the diameter of the lumen formed in the Matrigel was suitable for capillary formation in tissue engineering. The present results revealed an important role for the pH in the process of capillary formation and provided insight for pH regulation during endothelial cell tube formation and angiogenesis in tissue engineering.

Introduction

The lumen diameter directly affects the physiological function of capillaries and understanding its regulatory mechanism is important for the construction of functional tissue-engineered capillaries (1,2). If the diameter of the capillary lumen is too small, the passage of blood cells will be affected, causing damage to blood and vascular endothelial cells (3). When the lumen diameter is too large, it may result in an ineffective exchange of nutrients and metabolites between tissues and blood, leading to tissue hypoxia and accumulation of metabolites (4,5). However, the mechanisms underlying regulation of the capillary lumen diameter remain to be fully elucidated.

During wound healing, the pH of the surface layer of the granulation tissue is low (pH 5.8-6.0); the pH of the granulation tissue in the proliferative stage is 6.4-7.0 and the pH of the deep layer is close to that of the plasma (pH 7.3-7.6) (6,7). In tumor specimens, the capillaries near the center of the tumor tissue (pH 5.8-6.6) are relatively small in diameter or even without any definite lumen formation, while those near the edge of the tumor tissue (plasma pH 7.3-7.4) have larger lumen diameters (7,8). This suggests that the pH of the peripheral environment is one of the key factors that regulate the lumen diameter of newly formed capillaries.

However, pH, as a parameter for assessing the overall outcome, is not well studied, as the effect of the pH on the diameter of capillaries during neovascularization is rarely reported. Therefore, the present study aimed to explore the role of the pH in determining the vascular lumen diameter of new vessels.

Materials and methods

Cell culture. Human dermal microvascular endothelial cells (HDMECs; cat. no. 2000; ScienCell Research Laboratories, Inc.) were cultured according to the manufacturer's protocol in an endothelial cell medium (cat. no. 1001; ScienCell Research Laboratories, Inc.) supplemented with 5% fetal bovine serum (ScienCell Research Laboratories, Inc.), 100 U/ml penicillin-G, 100 U/ml streptomycin and 1% endothelial cell growth supplement (ScienCell Research Laboratories, Inc.). The cells were cultivated in 35-cm^2 culture flasks at 37°C under 5% CO_2 in air. The culture medium was replaced every 24 h. The cells in passage 4 to 5 were used for experimentation. All experiments reported in this study were performed in triplicate.

Preparation of different pH media. Different concentrations of NaOH and hydroxyethyl piperazine ethanesulfonic acid were added into the endothelial cell medium and the pH of the medium was adjusted to 6.4, 6.6, 6.8, 7.0, 7.2, 7.4, 7.6 and 7.8 using a pH meter (model PB-10; Sartorius AG). A 0.22-μm membrane was used to filter the medium and remove bacteria, after which it was stored at 4°C until use.

Cytosolic pH determined using flow cytometry. Cytosolic pH was assessed through flow cytometry using the pH-sensitive

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fluorescent probe 2,7-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM; cat. no. ab143463; Abcam). The cells incubated in media with different pH for 12 h and were harvested by centrifugation and incubated in a culture medium containing 4 μM BCECF-AM in the dark at 37˚C for 30 min. The cells were washed with PBS and quantitative analysis of intracellular fluorescence was performed using a flow cytometer (BD FACSCalibur™; BD Biosciences). Data analysis was performed using the CELLQuest software tool (version 5.1; BD Biosciences).

Cell proliferation assay. The cells ($5x10^5$ cell/ml) were grown on polylysine-coated glass coverslips and stained with an anti-bromodeoxyuridine (BrdU) antibody (1:500 dilution; cat. no. B35128; Thermo Fisher Scientific, Inc.) at 4˚C overnight. The coverslips were washed with PBS and the cells were fixed with 4% paraformaldehyde for 15 min at 23˚C. The coverslips were placed in 2 mol/l HCl for 30 min and washed with PBS. The cells were incubated with goat anti-mouse Cy3-conjugated secondary antibodies (1:1,000; cat. no. ab97035; Abcam) at 37˚C for 2 h and then blocked with PBS containing 5% goat serum (cat. no. ab7481; Abcam) at room temperature for 1 h. Finally, the cells were stained with 4',6-diamidino-2-phenylindole for 10 min at room temperature and examined using a fluorescence microscope (Olympus IX 71 fluorescence microscope; Olympus Corporation).

Wound-healing assay. A wound-healing assay was used to evaluate the migratory behavior of HDMECs in media at varying pH. In brief, the cells were labeled with CellTracker™ (cat. no. C2925; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, and then confluent cells were scratched with a 200-μl micropipette tip to create a consistent wound gap in the middle. The cells were washed with PBS to remove any cell debris and media of varying pH without serum were added to allow wound healing. Images of the wound gap were acquired using a fluorescence microscope (Olympus IX 71; Olympus Corporation) at 0, 24 and 48 h at three random locations in each well to examine the distance of wound closure. Image-Pro Plus software (version 6.0; Media Cybernetics, Inc.) was used to calculate the migration rate.

Reverse transcription-quantitative PCR (RT-qPCR). RT-qPCR was performed to detect the expression of endothelial angiogenesis-related genes [VEGFA, angiopoietin 1 (ANG1) and CD31] at different pH. Total RNA was extracted from the different groups of cells using a Takara MiniBEST Universal RNA Extraction kit (cat. no. 9767; Takara Bio, Inc.), according to the manufacturer's protocol. RT reactions were performed with PrimeScript™ RT Master Mix (cat. no. RR036A; Takara Bio, Inc.) according to the manufacturer's protocol. qPCR was performed using TB Green™ Premix Ex Taq II (cat. no. RR820A; Takara Bio, Inc.) according to the manufacturer's protocol. The PCR amplifications were performed in Real Time Instrument (CFX Connect Real Time PCR system, Bio-Rad Laboratories, Inc.). Thermocycling conditions were: Initial denaturation at 95˚C for 2 min; 40 cycles of denaturation at 95˚C for 15 sec, annealing and elongation at 60˚C for 1 min; then a

final extension at 72˚C for 3 min. The mRNA expression levels of the target genes were quantified using the $2^{-\Delta\Delta Cq}$ (9) method and normalized to those of GAPDH. The primers used for qPCR are listed in Table SI.

Tube formation on Matrigel®. Endothelial cells rapidly attach, align and form capillary-like tubules on a reconstituted basement membrane matrix (10,11). Cold liquid Matrigel (cat. no. 354234; BD Biosciences) was added to 96-well plates at a volume of 50 μl/well and incubated at 37˚C in an incubator containing humidified air with 5% CO_2 for 1 h. After the Matrigel had solidified, fluorescently-labeled (CellTracker™; cat. no. C2925; Invitrogen; Thermo Fisher Scientific, Inc.) HDMECs ($2x10^4$ cells/100 μl) were seeded into each well and incubated in the conditioned medium at 37˚C. Images were acquired after 24 h with a fluorescence microscope (Olympus IX 71; Olympus Corporation) to evaluate the degree of tube formation, which was quantified by measuring the branch length in three randomly selected fields using ImageJ software (version 1.51; National Institutes of Health).

Tube formation in a 3D Matrigel model. The cell suspensions with different pH values were mixed with Matrigel and cultured in 48-well plates under 5% CO_2 at 37˚C. The cell tubes were stained using toluidine blue at room temperature for 10 min after 48 h cultured, as described previously (12). To observe tube formation, the cells were photographed under a microscope (Olympus IX 71; Olympus Corporation) after 3 days. The degree of tube formation was quantified by measuring the lumen diameter using ImageJ software (version 1.51; National Institutes of Health).

Statistical analysis. Values are expressed as the mean ± standard error of the mean of at least three independent experiments and all experiments were performed at least three independent repeats. Differences among groups were analyzed using one-way analysis of variance followed by Bonferroni correction for multiple comparisons. Statistical analysis was performed using SPSS 23.0 software (IBM Corporation). Figures were plotted using Origin 8.5 software (OriginLab Corporation).

Results

pH of the medium is tightly associated with the cytosolic pH. First, the effect of media with different pH on the cytosolic pH was determined (Fig. 1). An association between the relative fluorescence and the pH of the medium was observed; the cytosolic pH increased steadily with the increase in the pH of the medium. The specific data for each group are presented in Fig. S1.

pH affects the proliferation and migration of HDMECs. To investigate whether pH affects the biological properties of vascular endothelial cells, the effects of media of varying pH on cell proliferation and migration were examined. The cell proliferation rate increased as the pH increased to 6.4-7.6, particularly at pH 7.2-7.6; however, the cell proliferation rate decreased at pH 7.8 (Fig. 2A and B). The result of cell
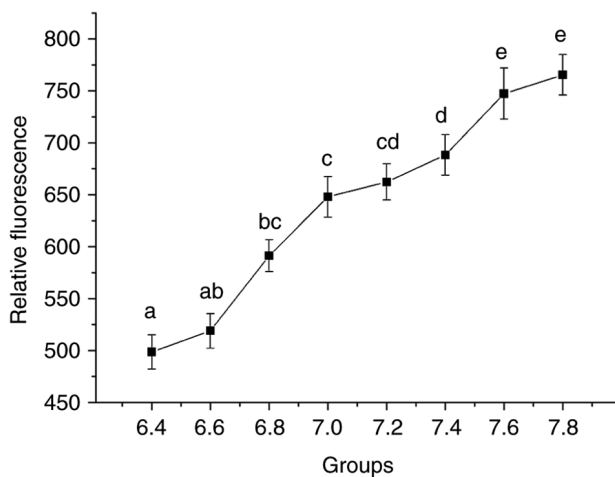
Figure 1. Relative fluorescence of each group in endothelial cells and different cytosolic pH levels analyzed using flow cytometry. The X-axis represents the different pH groups and the Y-axis the relative fluorescence. One-way analysis of variance and Bonferroni correction among groups was performed. [a]P<0.05 vs. 6.8, 7.0, 7.2, 7.4, 7.6 and 7.8; [b]P<0.05 vs. 7.0, 7.2, 7.4, 7.6 and 7.8; [c]P<0.05 vs. 7.4, 7.6 and 7.8; [d]P<0.05 vs. 7.6 and 7.8; [e]P<0.05 vs. 6.4, 6.6, 6.8, 7.0, 7.2 and 7.4.

migration is analogous to proliferation. The cell images after 24 and 48 h revealed that HDMEC migration was significantly influenced by the pH (Fig. 3A and B). At pH values between 6.4 and 6.8, the migratory rate of HDMECs into the wound gap after 24 h of culture was relatively low (~22%) and only moderate wound coverage (~50%) was achieved after 48 h. By contrast, HDMEC migration into the wound gap was markedly enhanced by increasing the pH from 7.0 to 7.6; the 'wound gap' was completely covered at pH 7.6 after 48 h. However, compared with pH 7.6, the migratory rate of HDMECs decreased significantly at pH 7.8 (P<0.05).

pH influences the expression of angiogenesis-related genes. RT-qPCR analysis of the expression of angiogenesis-related genes further confirmed that the pH influenced their expression (Fig. 4). The expression of ANG1 gradually increased with the increase in pH and reached a maximum at pH 7.6 (Fig. 4B). Similarly, the expression of VEGFA was significantly higher at pH 7.0-7.8 compared with at pH 6.4-6.8; however, it decreased from pH 7.6 to 7.8 (Fig. 4A). By contrast, the expression of CD31 was only slightly altered with the increase in the medium pH and the differences between the groups were not significant (Fig. 4C).

Medium pH affects tube formation. An HDMEC tube formation assay was performed to evaluate the effect of the media at different pH on tube formation. The cells were seeded on Matrigel and the formation of tube-like cell arrangements was assessed. The tube formation capacity of the cells was evaluated by determining the branch points and branch length of the tubes (13,14). It was revealed that tube formation was significantly reduced at pH 6.4-7.0 compared with higher pH values (Fig. 5A). The histogram indicated that in comparison with those at pH 6.4-7.0, the number of branch points and the capillary length at pH 7.2, 7.4, 7.6 and 7.8 were significantly increased, the highest being at pH 7.4 and 7.6

(P<0.05; Fig. 5B and C). Therefore, the results indicated that 7.4 and 7.6 are the optimal pH values for HDMEC tube formation.

pH regulates lumen diameter in Matrigel. HDMECs formed tube-like structures in a 3D Matrigel model in media with different pH (Fig. 6). HDMECs formed tubules (Fig. 6A) and the diameter of the vascular tubules increased with an increase in the pH of the medium; however, at pH 7.8, the diameter of the vascular tubules was decreased. The histogram in Fig. 6B indicated that the lumen diameter increased steadily from pH 6.4-7.6, and significantly decreased at pH 7.8 (P<0.05). These results indicated that the pH of the medium has an important role in lumen formation.

Discussion

The regulation of capillary lumen diameter is important in the formation of tissue-engineered capillaries (15-17). The lumen diameter directly influences the physiological function of capillaries and its regulation is of great significance for the construction of tissue-engineered organs (18). Capillaries in normal tissues have diameters of 5-10 μm (19,20). If the capillary lumen diameter is too small, it will affect the passage of blood cells, resulting in blood cell and vascular endothelial cell damage (21). On the other hand, if the capillary lumen diameter is too large, the velocity of blood flow decreases, affecting the rate of delivery of oxygen, nutrients, growth factors and circulating cells necessary for the body (22,23).

In the present study, it was observed that the pH of the peripheral environment is a key factor in regulating the lumen diameter of newly formed capillaries. The extracellular pH affects the pH of the cytoplasm (24,25). Furthermore, the relationship between the pH of the medium and that of the cell cytoplasm was studied. In HDMEC cultures, an increase in the pH of the culture medium led to an increase in the pH of the cytoplasm. However, these corresponding changes were not always consistent. This may be attributed to the buffering capacity of HDMECs. Therefore, a change in the pH of the culture medium was able to affect the cytoplasmic pH of HDMECs, leading to a change in vascular diameter.

In *in vivo* vascular systems, capillaries that are between 5 and 10 μm in diameter are the only regions where nutrients and metabolites are able to be exchanged with tissues (19). The lumen diameter of capillaries from HDMECs formed in Matrigel was ~10 μm when the pH of the medium was 6.4-6.8. This size is suitable for forming capillaries in tissue engineering. This possibly suggests that smaller capillaries may be constructed using a medium with lower pH. A higher cytosolic pH is associated with increased cell growth and proliferation in mammalian cells (26), which is consistent with the results of the present study. Cell proliferation tests indicated that the proliferation ratio increased with an increase in the medium pH from 6.4 to 7.6, particularly at pH 7.2-7.6, but it gradually decreased at pH 7.8. The expression of ANG1 and VEGFA exhibited a similar trend. These results reflect the difficulty in constructing capillaries with small diameters; however, they further confirmed that a medium pH of 7.2-7.6 is optimal for *in vitro* neovascularization with HDMECs. The expression of CD31 remained unaffected. Therefore, the pH possibly does
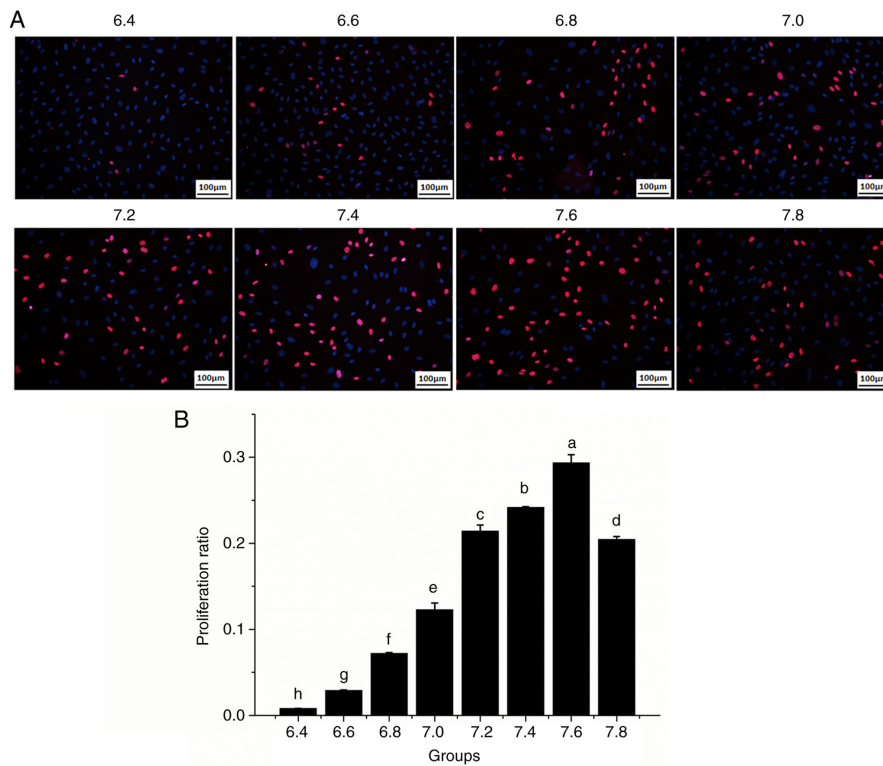
Figure 2. Proliferation of human dermal microvascular endothelial cells under different pH conditions. (A) Cells were subjected to bromodeoxyuridine immuno-fluorescence staining. Proliferating and normal cell nuclei were stained in red and blue, respectively (scale bars, 100 μm). (B) Quantification of the proliferation rate in each group. The X-axis represents the different pH groups. One-way analysis of variance and Bonferroni correction among groups was performed. [a]P<0.05 vs. 7.4; [b]P<0.05 vs. 7.2; [c]P<0.05 vs. 7.8; [d]P<0.05 vs. 7.0; [e]P<0.05 vs. 6.8; [f]P<0.05 vs. 6.6; [g]P<0.05 vs. 6.4; [h]P<0.05 vs. 6.6, 6.8, 7.0, 7.2, 7.4, 7.6 and 7.8.
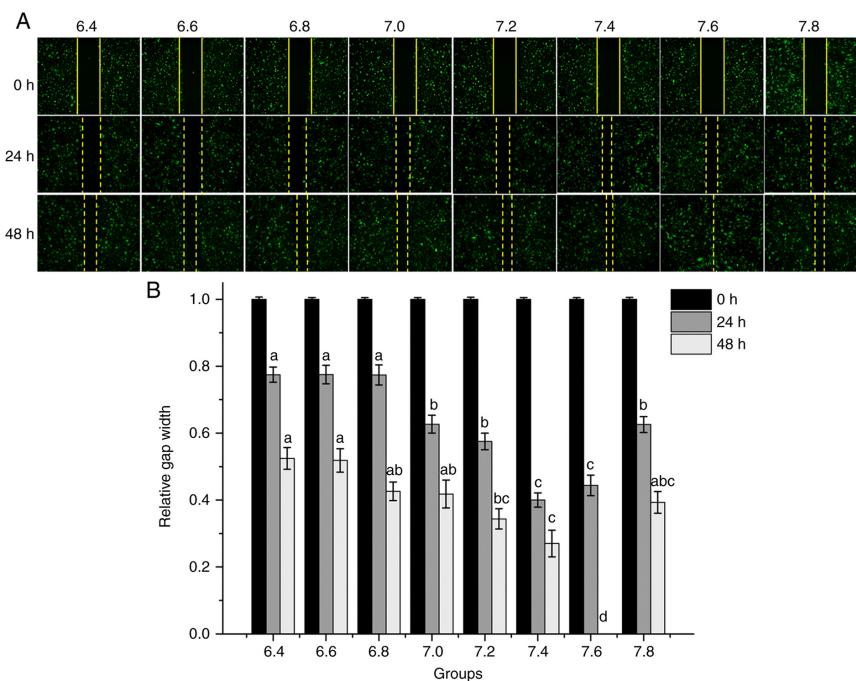

Figure 3. Migration of human dermal microvascular endothelial cells at different pH levels determined using a wound-healing assay. (A) Representative migration images (magnification, x40). Cells were allowed to migrate after the wound gaps were created and visualized after 24 and 48 h. (B) Quantification of the wound gap distance between the front lines of migrating cells. The X-axis represents the different pH groups. One-way analysis of variance and Bonferroni correction among groups was performed. 24 h: [a]P<0.05 vs. 7.0, 7.2, 7.4,7.6 and 7.8; [b]P<0.05 vs. 7.4 and 7.6; 48 h: [a]P<0.05 vs. 7.2, 7.4 and 7.6; [b]P<0.05 vs. 7.4 and 7.6; [c]P<0.05 vs. 7.6; [d]P<0.05 vs. 6.4, 6.6, 6.8, 7.0, 7.2, 7.4 and 7.8.

not affect cell adhesion. Both the capillary length and branch points of the tubes were associated with an increased cytosolic

pH. This is particularly important for the construction of capillaries in tissue-engineered organs. Furthermore, in a
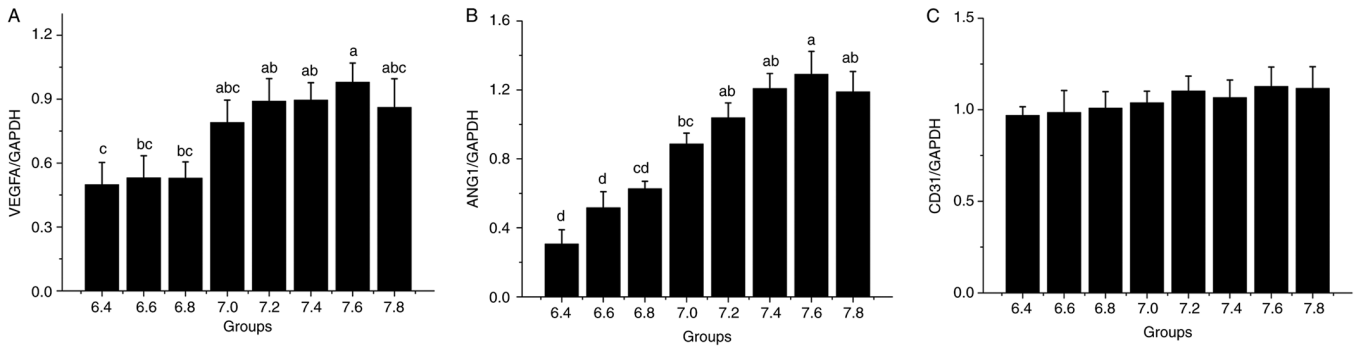
Figure 4. Reverse transcription-quantitative PCR analysis of genes related to angiogenesis. The X-axis represents the different pH groups. Relative levels of (A) VEGFA ([a]P<0.05 vs. 6.4, 6.6 and 6.8; [b]P<0.05 vs. 6.4; [c]P<0.05 vs. 7.2, 7.4 and 7.6), (B) ANG1 ([a]P<0.05 vs. 6.4, 6.6, 6.8 and 7.0; [b]P<0.05 vs. 6.4, 6.6 and 6.8; [c]P<0.05 vs. 6.4 and 6.6; [d]P<0.05 vs. 7.0, 7.2, 7.4, 7.6 and 7.8) and (C) CD31. ANG1, angiopoietin 1.
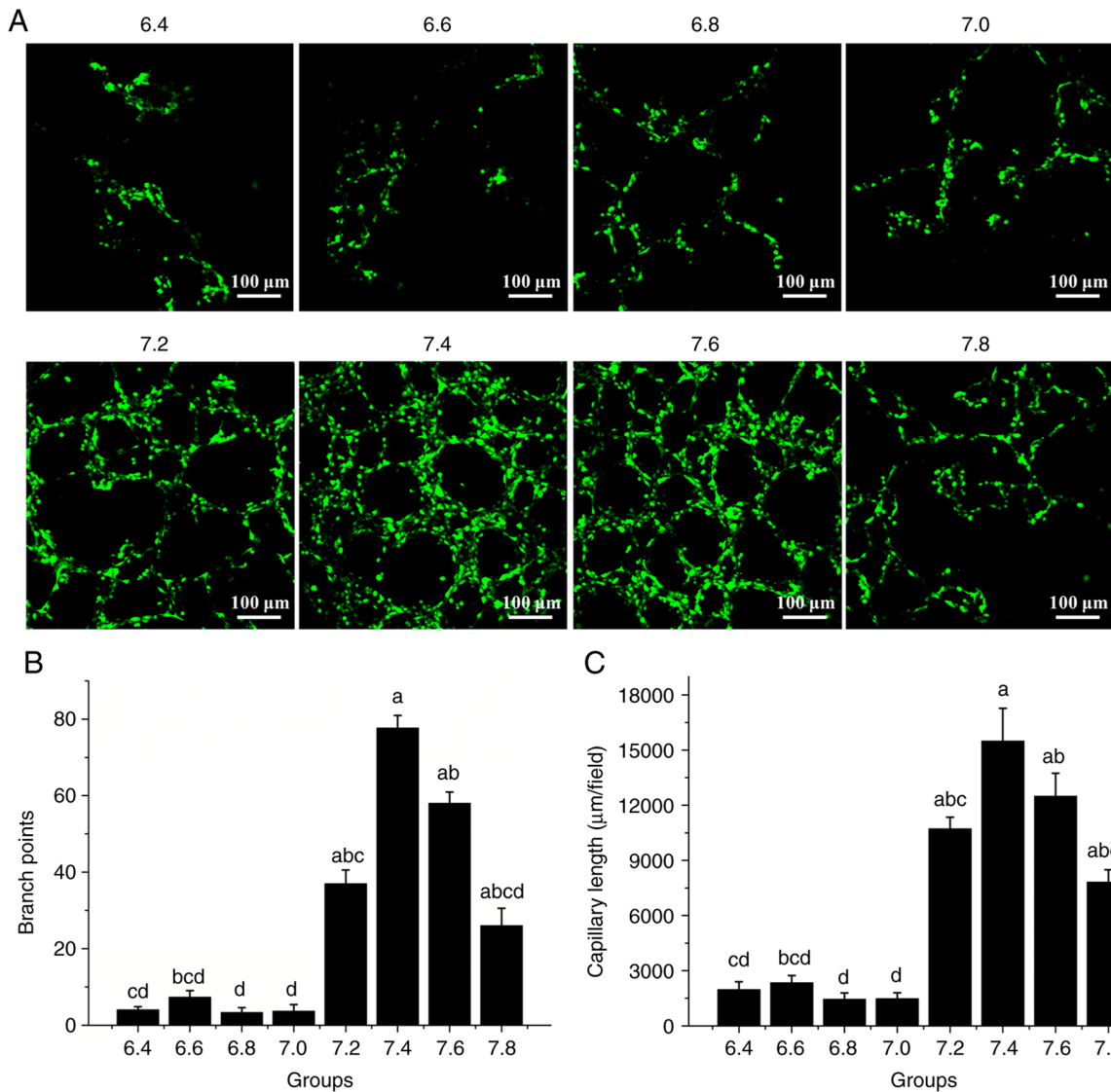

Figure 5. Tube formation of human dermal microvascular endothelial cells cultured on Matrigel. (A) Capillary tube formation on Matrigel (scale bars, 100 μm). Quantitative analysis of (B) branch points ([a]P<0.05 vs. 6.4, 6.6, 6.8 and 7.0; [b]P<0.05 vs. 6.4, 6.8 and 7.0; [c]P<0.05 vs. 6.8 and 7.0; [d]P<0.05 vs. 7.2, 7.4 and 7.6) and (C) total capillary length of the tubes formed on the Matrigel of different pH groups ([a]P<0.05 vs. 6.4, 6.6, 6.8 and 7.0; [b]P<0.05 vs. 6.4, 6.8 and 7.0; [c]P<0.05 vs. 6.8 and 7.0; [d]P<0.05 vs. 7.2, 7.4 and 7.6). The X-axis represents the different pH groups. One-way analysis of variance and Bonferroni correction among groups was performed.

previous study by Ye (27), wherein the acceptance rate of skin grafts in patients with acute and chronic wounds of different

origin was observed, 99% of the skin grafts were successfully taken in wounds at a pH of 7.4 and higher; however, no
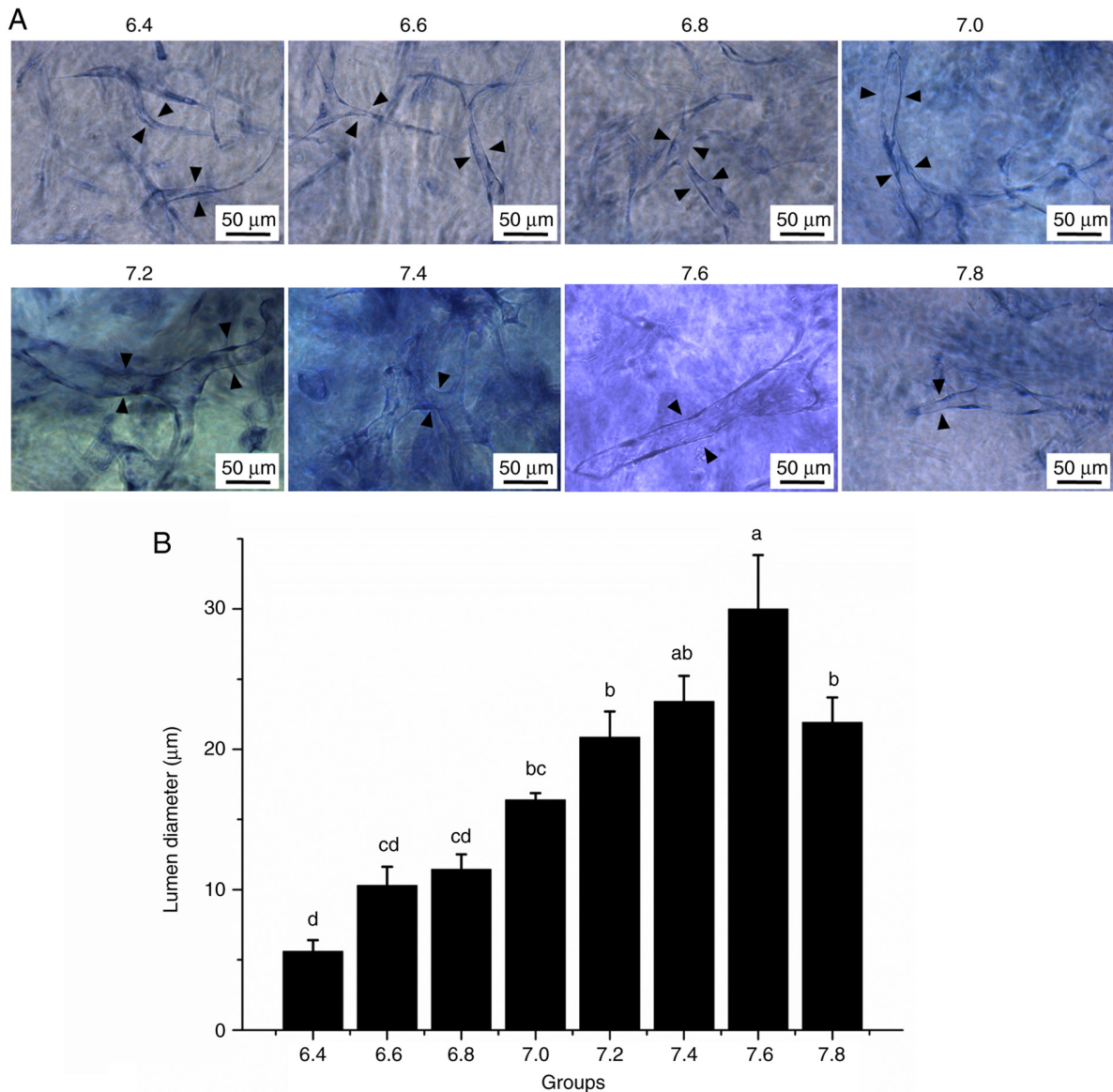
Figure 6. Three-dimensional HDMEC tubes in Matrigel. (A) Representative images of HDMEC tubes in Matrigel. The background of images may be different after toluidine blue staining due to staining in Matrigel, and the images with clear lumen were selected as the representative images. Black arrowheads indicate the cell tubes (scale bars, 50 μm). (B) Quantitative analysis of the lumen diameter of the endothelial cell tubes in each group. The X-axis represents the different pH groups. One-way analysis of variance and Bonferroni correction among groups was performed. [a]P<0.05 vs. 6.4, 6.6, 6.8, 7.0, 7.2 and 7.8; [b]P<0.05 vs. 6.4, 6.6 and 6.8; [c]P<0.05 vs. 6.4, 7.2, 7.4, 7.6, and 7.8; [d]P<0.05 vs. 7.0, 7.2, 7.4, 7.6 and 7.8. HDMEC, human dermal microvascular endothelial cell.

skin graft was successfully taken in wounds at a pH <7.0. In combination with the results of the present study, this may be due to the presence of appropriate growth conditions for capillaries at pH 7.4 and 7.6, which provide sufficient blood supply, leading to improved wound healing.

Considering the results of cell proliferation, migration and tube formation, it was determined that the most optimal pH of the medium was 7.2-7.6 for *in vitro* neovascularization with HDMECs. This may be attributed to the closeness to the physiological pH of blood and extracellular fluids, which is in the range of 7.35-7.45 (28-30). At this appropriate pH range, the various enzymes had maximum activity, enabling improved formation of new blood vessels.

In conclusion, the present study observed that the pH regulated the diameter of the capillary lumen in HDMECs; the optimal pH was 7.4-7.6. However, the mechanisms underlying the regulation of the lumen diameter of capillaries by the pH remain to be fully elucidated. This will be our next research direction.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XW, JinqL and XL designed the study. XW and JingL wrote the manuscript. XW, YB and CZ performed the experiments and JingL analyzed the data. All authors read and approved the final manuscript and confirm the authenticity of the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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