Taohong Siwu (TSW) decoction enhances angiogenesis by regulation of VHL/HIF-1α/VEGF pathway

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Research

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Abstract

Background: The incidence of bone fracture and bone-related diseases is increasing year by year. Angiogenesis plays vital role in fracture healing and bone repair. In this study, we assessed the effect of Taohong Siwu (TSW) decoction on angiogenesis by treatment of isolated rat aortic endothelial cell with TSW-containing serum.

Method: First, TSW-containing serum was prepared from male Sprague-Dawley by intragastrically administration of TSW decoction. Then, isolated aortic endothelial cells were treated with different dose of TSW-containing serum. The effect of TSW-containing serum on the viability/proliferation of aortic endothelial cells were examined by MTT assay. The effects of TSW-containing serum on endothelial cell invasion, migration/spreading were detected by trans-well assay and scratch/wound-healing assay respectively. In addition, the effect of HIF-1α inhibitor on TSW-induced cell invasion and migration was also assessed. Moreover, the effect of TSW-containing serum on the expression of HIF-1α signaling pathway related protein, including HIF-1α, VHL and VEGF was detected by qRT-PCR and western-blot.

Results: Our results showed that TSW decoction significantly increased the endothelial viability, invasion and wound-healing in a dose-dependent manner. Importantly, these enhanced effects induced by TSW were attenuated by HIF-1α inhibitor. Furthermore, our data demonstrated that TSW-containing serum increased the expression of HIF-1α and VEGF in a dose-dependent manner, while decreased the expression of VHL slightly. These effects induced by TSW were reversed by HIF-1α inhibitor.

Conclusion: In summary these results for the first time suggested that TSW decoction enhances the angiogenesis by regulating of VHL/HIF-1α/VEGF pathway.

Background

The incidence of bone fractures and bone-related diseases has been high over the years, and delayed healing and non-union of fractures are still frequently encountered in fracture treatment [1]. Fracture healing and bone repair are very complicated pathological process. The local microenvironment of the fracture, such as oxygen saturation, pH, and cytokines and growth factors are all critical factors involved in and affect the fracture healing [2]. These factors directly affect the formation of blood vessels, the proliferation and differentiation of bone cells, and the deposition of minerals [2]. Fractures must be accompanied by rupture and embolization of blood vessels, and cause local and systemic hemorheological changes, increased blood viscosity, slowed local microcirculation blood flow, and decreased tissue oxygen saturation [1]. This pathological process has dual significance for fracture healing. Although It might be unfavorable to the metabolism, proliferation, and differentiation of tissue cells, however, a certain degree of hypoxia can start the body's compensatory response to hypoxia and the transcription of many genes in the cell and the expression changes of different genes, such as genes encoding vascular endothelial growth factor (VEGF), erythropoietin (EPO), tyrosine hydroxylase, and many enzymes involved in glycolysis, etc. [3, 4]. These genes are called hypoxic response genes hypoxia
responsive genes (HRG). Studies have shown that this response is related to hypoxia-inducible factor-1α (HIF-1α) and HIF-1α protein expression is the main determinant of its activity [5, 6].

One of the direct consequences of a fracture is local hypoxia caused by rupture of the blood vessel [4, 7]. The repair process of the fracture end is largely dependent on the formation of new blood vessels at the fracture site, and angiogenic factors have been shown to be necessary for the initiation and maintenance of fracture healing [8]. Under hypoxia, HIF-1α is a core regulator of angiogenesis [9, 10]. It directly participates in the entire process of angiogenesis by affecting the expression of related growth factors [5, 10]. The stability of HIF-1α activity may be related to the sustained maintenance of angiogenesis [9, 10]. Many basic studies have shown that HIF-1α cell-associated signaling pathway is closely related to microangiogenesis in osteogenesis [11, 12]. Traditional Chinese medicine for promoting blood circulation and removing blood stasis can improve local and systemic blood circulation in the early stage of fracture healing, improve local blood oxygen status, and create a good internal environment for fracture healing [13–16]. The traditional Chinese medicines such as Taohong Siwu (TSW) Decoction for promoting blood circulation and removing blood stasis are used to treat orthopedic diseases [17, 18].

TSW Decoction has been widely used and recognized by the Department of Orthopedics in China. Our research team conducted a series of studies on TSW Decoction and its active ingredients, and extracted the main active ingredients of TSW Decoction, including four major parts: total glycosides, total volatile oils, and total alkaloids, and total polysaccharides. Previous studies showed that TSW containing serum enhanced the expression of VEGF in human endothelial cell through PI3K/Akt-eNOS axis [19]. In addition, another group also demonstrated that TSW decoration promoted angiogenesis in early pregnancy rat by increasing the expression of Ang-1/2 and Tie-2 [20]. Through experimental research, our group confirmed that TSW Decoction and its active ingredients can promote microvascular growth and VEGF expression during bone formation, thereby promoting fracture healing (Data not shown, published by Chinese Journal). However, the mechanism of TSW Decoction for the treatment of fractures, ischemia and hypoxia to the regeneration of blood vessels and the promotion of fracture healing after fracture are still unclear. Therefore, in this study, our hypothesis is TSW may promote the expression of VEGF via regulation of the expression of HIF-1α to further enhance the endothelial cell angiogenesis and promotes the bone fracture healing.

Material And Methods

Preparation of Taohong Siwu Decoction (TSW):

Taohong Siwu Decoction was prepared from six different medicinal plants, including: 20 g of peach red, 10 g of saflower, 20 g of angelica, 20 g of raw land, 20 g of red oak, 10 g of Chuanxiong. The medicinal materials are provided by the Chinese Pharmacy of the First Affiliated Hospital of Hunan University of Traditional Chinese Medicine. Preparation method of traditional Chinese medicine: Weigh the traditional Chinese medicine decoction pieces according to the prescription dose, add the decoction pieces to the medicine tank, add 10 times of double distilled water of raw medicine, soak for 30 min, fry the fire with
boiling fire until it is boiled, and simmer for 30 min, filter out the liquid medicine. Add 5 times of double-distilled water to boil, and decoct for 30 minutes. Mix 2 times of the drug solution, concentrate to 2 g of raw drug per 1 ml, and store in a refrigerator at 4 °C.

Animals and breeding environment

Ten male Sprague-Dawley rats weighing 200–260 g were purchased from Hunan Slack Jingda Experimental Animal Co., Ltd., and the experimental animal production license number is SCXK (Xiang) 2013-0004. The rats were raised at the Experimental Animal Center of Hunan University of Traditional Chinese Medicine. They were housed in a standard animal room with temperature of 21 °C -25 °C, humidity at 50% ± 5, 12 hours of day and night light and dark alternate time. The cages are cleaned once a week and the litter are replaced as needed. Animals are free to water and food. Rat food was provided by the Experimental Animal Center of Hunan University of Traditional Chinese Medicine. Rats were fed ad libitum for 1 week, and 1 week later, TSW was administered to the stomach for 1 week. All the animal experiments and protocols were approved by the Local ethics committee.

Preparation of serum containing TSW

Ten male Sprague-Dawley rats weighing (200–250) g were administered TSW according to the following formula: Rat dose (g/kg) = 6.25 × [adult dose (g) / adult body mass (60 kg)] × 3. Rats were administered at a dose of about 25 g/kg/d intragastrically twice each day. The rats in the blank group were given the same volume of normal saline for 10 days. After the last 2 hours of gavage, they were anesthetized with 10% chloral hydrate.

The blood was collected from the abdominal aorta. Briefly, the animals were anesthetized, placed on the surgical frame on the back, and the abdominal cavity was opened to expose the abdominal aorta clearly. Gently pry open the fat around the blood vessels with a small forceps, and then wipe the excess fat covering the blood vessels with a cotton ball until the blood vessels are clearly seen. The abdominal aorta is above the spine, next to the inferior vena cava, the inferior vena cava is thick, the color is dark, and the abdominal aorta is light in color. After finding it, clamp a 1.5 cm abdominal aorta with 2 arterial clips, and gently lift the artery wall with a small forceps at the distal end. Then use the blood collection tube for abdominal aortic puncture to see the blood return, and the other end is inserted into the vacuum tube. When you open the proximal blood vessel clamp, blood will flow out. 5 ml of blood was collected and left at 4 °C overnight, centrifuged. Then the serum was collected, sterilized by filtration using a 0.22 µM filter, and was inactivated at 56 ° C, and stored at -80 ° C until use.

Isolation, Identification and Culture of Rat Aortic Endothelial Cells

For the aortic endothelial cells preparation, use dissection scissors to open the abdomen from the midline, and expose the abdominal aorta. Open the chest cavity and expose the heart and lungs. Cut the abdominal aorta at the middle with dissection scissors to release the blood. Fill a 1 mL syringe (with 25 G needle) with 1 mL of PBS containing 1,000 U/mL of heparin. Inject PBS containing 1,000 U/mL of
heparin to the left ventricle and perfuse the aorta. Push the heart and the lung with forceps at the great arteries to the right side of the mice to expose the thoracic aorta. Quickly remove the thoracic aorta using micro-dissection forceps and put it in ice-cold 1x PBS (sterile), then transport the container into a laminar airflow hood. Insert a 1 mL syringe fitted with 25 G needle into one end of the aorta, and gently flush the aorta with ice-cold PBS to remove the blood. Use micro-dissection forceps to remove as much of the attached adipose tissue and small lateral vessels as possible. Immediately transfer the aorta to endothelial growth medium. Cut the aorta into 1 mm rings using a sterile scalpel blade. Harvest about 8–10 rings per aorta. Open each aortic ring using a pair of micro-dissection scissors. The rat aorta was selected, separated, placed in a petri dish, and the morphology of the cells was observed with a fluorescent inverted microscope, and the cells were stained with an immune factor-related antigen.

MTT assay

The MTT method was used to detect the effect of serum containing serum of Taohong Siwu Decoction on the cell viability of vascular endothelial cells. Briefly, endothelial cells were plated into 96-well plate with 3000 cells/well and cultured for 6 h. Then cells were added with 10% KB blank, 2.5% TSW serum, 5% TSW serum, and serum 10% TSW serum and cultured for overnight. Then cells were added with 100 µl of MTT solution (Cat#: M2128, Sigma, USA) and incubated for another 4 hours. After incubation, the solution was aspirated and the cells were added with DMSO, 100 µl/well, and the absorbance was measured by a microplate reader at a wavelength of 490.

Transwell assay

The Transwell chamber migration experiment was used to detect the effect of the serum containing TSW and HIF-1α inhibitor on the migration of vascular endothelial cells. The cells were seeded into 24-well plates with 5 × 10^3 cells/well. The drug-containing serum or TSW serum together with HIF-1α inhibitor (CAY10585, cat#: ab144422, Abcam, USA) were used to treat the cells as described above. After overnight treatment, the cells at the bottom chamber were fixed with paraformaldehyde, and then stained with the 0.1% crystal violet. Then migrated cells were observed and quantified by inverted microscope. At least 5 different fields with more than 200 cells were counted and the images were acquired.

Tube formation assay

96-well plate were coated with gel 50 µl/well Matrigel. Then the isolated endothelial cells were plated into 96-well plates with 1.5 × 10^4 cell/well. Cells were treated with TSW-containing serum in the presence and absence of HIF-1α inhibitor as described before. 24 hours after incubation, the tube formation was checked, and images were acquired by C5 microplate reader. Images from at least 5 different fields were acquired the IPP software was used to calculate the length of the lumen.

Wound-healing assay
The isolated endothelial cells were plated into 24-well plates and then treated with TSW-containing serum in the presence and absence of HIF-1α inhibitor as described before. Different groups of cells were scratched with a 20-µL pipette to create the wound. Cells were washed with PBS buffer to remove the debris. Images were acquired at 0, 24 and 48 hours after treatment to determine the wound healing at 3 random locations. The wound area in each group was quantified.

Quantitative real-time PCR (qRT-PCR)

Total RNAs were extracted from treated cells and then reverse transcribed into cDNA with reverse transcriptase (Cat#: K1622, Thermo USA). Then the relative expression levels of HIF-1α, pVHL and VEGF were detected by qRT-CPR by using T100™ Thermal Cycler. GAPDH was used as endogenous control and the relative expression of target genes was calculated by using the $2^{-\Delta\Delta Ct}$ method. The primes used for qRT-PCR were listed in the Table 1.

| Name   | Forward Primer | Reverse Primer |
|--------|----------------|---------------|
| HIF-1α | ACGTTCCCTTCGATCAGTTGTCAC | GCCAGTGAGTGGTGACCATTAG |
| pVHL  | TTTGTGCCATCTCTCATAATGTTG | GCCATCGCTTTTCAGAGTATA |
| VEGF  | ATCGAGTACATCTTTCAGCCAT | GTGAGGTTTGATCCGCATAATC |
| GAPDH | AGTCCACTGGCGTCTTCAC | GAGGCATTGCTGATGATCTTG |

Western-blot analysis

Total proteins were prepared by digested the cells treated with different treatment with lysis buffer (P0013, Beyotime Bio, China). Then, total of 30 µg proteins from each group were separated by sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels and then transferred onto polyvinylidene difluoride membranes (Millipore, USA). After the membranes were blocked with 5% fat-free milk in PBS buffer, the membranes were incubated with anti-HIF-1α (Abcam, USA), VEGF (Abcam, USA), VHL (Abcam, USA) and β-actin (Proteintech, USA) antibodies for overnight at 4 °C. Then, membranes were washed four times with PBST buffer with 5 minutes for each time. The secondary antibodies were incubated with membranes for 1 hour at room temperature. After the membranes washed with PBST buffer, the protein expression was detected using enhanced chemiluminescence (Advans Group). The quantification of protein expression was evaluated by ImageJ (National Institutes of Health).

Statistical analysis

All the data were presented as mean ± SEM from at least three independent experiments. All the results were analyzed by using GraphPad Prism 8 software (GraphPad Prism Software Inc., San Diego, USA).
The difference between two groups was analyzed by using \( t \) test. P < 0.05 was considered statistically significant.

**Results**

**TSW-containing serum enhanced the vascular endothelial cell viability**

Aortic Endothelial Cells were isolated from Rat and then treated with different concentration of TSW-containing serums for 24 hours and then the cell viability was detected by MTT assay. The results indicated that lower concentration of TSW-containing serum (2.5%) had no statistical effect on the viability of endothelial cell viability, while higher concentration (5% and 10%) of TSW-containing serum significantly increased the viability of endothelial cell viability, suggesting its potential involvement in angiogenesis.

**TSW-containing serum regulates angiogenesis by promoting migration via HIF-1\( \alpha \) signaling.**

We next sought to investigate how TSW-containing serum modulates endothelial cell angiogenesis. Transwell assay was performed to detect the effect of TSW-containing serum on the migration capability of endothelial cells. The results showed that TSW-containing serum enhanced the migration of endothelial cell in a dose-dependent manner (Fig. 2). After fracture, ischemia and hypoxia occur locally. Hypoxia-induced bone tissue initiates HIF-1\( \alpha \) signaling pathway, and HIF-1\( \alpha \) expression level is increased, thereby activating vascular endothelial growth factor. HIF-1\( \alpha \) is a positive correlation regulator of VEGF, and HIF-1\( \alpha \) can promote the expression of VEGF. Therefore, we next determine the HIP inhibitor effect on TSW-promoted angiogenesis. Transwell results demonstrated that higher dose of TSW-induced angiogenesis was markedly blocked by HIF inhibitor, indicating that HIF-1\( \alpha \) involved in the TSW-induced angiogenesis in endothelial cells (Fig. 2).

**TSW-containing serum regulates angiogenesis by increasing endothelial cell tube formation via HIF-1\( \alpha \) signaling.**

Tube formation assay was carried out to examine the effect of TSW-containing serum on endothelial cell angiogenesis. The results showed that the TSW-containing serum enhanced the tube formation in a dose-dependent manner (Fig. 3A). HIF-1\( \alpha \) inhibitor treatment attenuated higher concentration of TSW-containing serum-induced tube formation (Fig. 3A). Quantitative data showed that 2.5% of TSW-containing serum increased the tube number in each field to 5 from 2.6, 5% of the TSW-containing serum increased the tube number to 10 from 2.6, and the 10% TSW-containing serum increased the tube number to 12.5 from 2.6, while HIF inhibitor blocked 10% TSW containing serum-induced the tube number from 15 to 4. In addition, we also measured the tube length and the results showed that TSW-containing serum enhanced the tube formation by 10% in 2.5% TSW group, 30% in 5% TSW group and 55% in 10% TSW group, while HIF inhibitor attenuated high concentration of TSW-induced increase in tube formation (Fig. 3C). These results suggested that TSW-containing serum regulates angiogenesis by increasing endothelial cell tube formation via HIF-1\( \alpha \) signaling.
TSW-containing serum regulates angiogenesis by increasing endothelial cell wound healing via HIF-1α signaling.

Wound healing assay was carried out to examine the effect of TSW-containing serum on endothelial cell spreading. The results showed that the TSW-containing serum enhanced the wound healing in a dose-dependent manner (Fig. 3A) after the endothelial cells treated for 24 hours and 48 hours. HIF inhibitor treatment attenuated higher concentration of TSW-containing serum-induced tube formation (Fig. 3A). Quantitative data showed that 2.5% of TSW-containing serum increased the wound healing by 25.9% for 24 h and 51.5% for 48 h, 5% of the TSW-containing serum increased the wound healing by 26.6% for 24 h and 52.76% for 48 h, and the 10% TSW-containing serum increased the wound healing by 57.7% for 24 h and 73.5% for 48 h, while HIF-1α inhibitor blocked 10% TSW containing serum-induced wound healing by 95.5% for 24 h and 86.7% for 48 h. These results suggested that TSW-containing serum regulates angiogenesis by increasing endothelial cell wound healing via HIF-1α signaling.

TSW-containing serum regulates angiogenesis by increasing the expression of VHL/HIF-1α/VEGF axis.

We identified the involvement of the HIF in regulation of TSW-induced angiogenesis. Previous studies showed that the on Hippel-Lindau (VHL) protein can target HIF-1α. While, HIF-1α can induce the expression of VEGF. Therefore, we next sought to investigate the effect of TSW on the expression of VHL/HIF-1α/VEGF axis. To better understand the role of HIF-1α signaling in endothelial cell angiogenesis, we examined the mRNA expression of VHL, HIF-1α and VEGF in each group treated with different dose of TSW-containing medium and the HIF-1α inhibitor in the presence of 10% of TSW. qRT-PCR results showed that the expression of HIF-1α and VEGF was increased while the expression of VHL was slightly decreased in endothelial cells upon treatment with TSW-containing serum in a dose-dependent manner, while HIF-1α inhibitor alleviated the 10% TSW-containing serum-induced the expression of HIF-1α and VEGF and increased the expression of VHL (Fig. 5A and B). Then, we detected the protein level of VHL, HIF-1α, and VEGF by Western-blot. The results showed that the protein levels of HIF-1α, and VEGF were increased whereas the expression of VHL was slightly decreased in endothelial cells after treatment with TSW-containing serum, while HIF-1α inhibitor attenuated the TSW-induced protein expression change (Fig. 5C and D). Altogether, these results demonstrated that TSW-containing serum regulates angiogenesis by increasing the expression of VHL/HIF-1α/VEGF axis.

Discussion

Modern researches proved that the traditional Chinese medicine for promoting blood circulation and removing blood stasis accelerates bone fracture healing by improving blood rheology, accelerating angiogenesis in injured areas and improving blood circulation at the injured site [13–16]. Bone healing usually requires four steps: blood vessel nonunion (breakage), proliferation of vascular endothelial cells (VEC), VEC moving buds, and three-dimensional structure reconstruction of the lumen [21, 22]. The growth of VEC is closely related to vascular endothelial cell growth factor (VEGF) [21, 22]. In the early stage of the bone fracture, a local bleeding and hematoma appear after the fracture [23]. Along with the
absorption and mechanization of the hematoma, an exudate will be produced, and this exudate can promote the secretion of vascular proliferation regulators, thereby accelerating the sprouting of micro blood vessels, and then differentiate into blood vessels [22, 23]. Angioplasty is the formation of tubules of vascular endothelial cells. After the tubules are formed, they are interwoven into a network and penetrate into each other, and penetrate into the membrane for the growth of fractured ends and bone structure remodeling [24]. Therefore, detection of vascular endothelial cell tubule formation as a marker of angiogenesis is also a prerequisite and basis for healing [24].

After the fracture, the periosteal and vascular damage happened in the fracture site, and the local blood supply is reduced or even interrupted, resulting in a hypoxic state of the fracture site. The bone tissue in a hypoxic environment can activate HIF (hypoxia-inducible factor) -1α, by up-regulating HIF-1α levels, thereby activating the HIF/vascular endothelial growth factor (VEGF) signaling pathway, participating in vascular reconstruction of fracture ends, and accelerating the process of fracture healing [25, 26]. In this study, we detected the effects of Chinese traditional medicine TSW decoction on isolated mouse aortic endothelial cell angiogenesis by using well known assays such as transwell assay, wound healing assay and tube formation assay. Our results first showed that TSW-containing serum increased the endothelial cell viability, indicating the efficacy of TSW decoction on endothelial cells. Following studies demonstrated that TSW-containing serum enhanced the angiogenesis of endothelial cells by increasing the migration, spreading and tube formation of endothelial cells.

Then, we explored the underlying mechanism involved in TSW-containing serum-induced endothelial cell angiogenesis. Interestingly, our results showed that HIF inhibitor dramatically attenuated TSW-containing serum-induced angiogenesis by inhibiting the migration, spreading and tube formation of endothelial cells, indicating the involvement of HIF signaling. Previous studies also shown the involvement of HIF-1α signaling pathway was involved in angiogenesis after bone fracture and the expression of HIF-1α and its downstream target, VEGF are both upregulated during the healing process [12, 27]. In addition, the expression level of HIF-1A was regulated by its upstream modulator VHL, an E3 ubiquitin ligase, which can induce the proteasome-dependent degradation of HIF-1α [27]. Therefore, in this study, we used qRT-PCR and western blot assays detected the expression of HIF-1α, VEGF and VHL after endothelial cells treated with TSW-containing serum. Our results showed that the expression of VEGF and HIF-1α was significantly increased, while the expression of VHL was decreased after endothelial cells treated with TSW-containing serum. However, these expression changes of HIF-1α, VEGF and VHL were reversed by HIF-1α inhibitor, indicating the involvement of HIF-1α pathway.

**Conclusion**

Altogether, our results for the first time demonstrated that the TSW-containing serum enhances angiogenesis of endothelial cells via regulation of VHL/HIF-1α/VEGF signaling pathway.

**List Of Abbreviations**
Declarations

Ethics approval and consent to participate

All the animal experiments and protocols were approved by the Local ethics committee.

Consent for publication

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

Availability of data and materials

All data generated or analyzed in this study are included in this manuscript and confirmed the accuracy of the data.

Competing interests

All the listed authors declared that they have no competing interests.

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Authors' contributions

Zhi Tang performed the experiment and analyzed the data; Wangyang Li performed the experiment and prepared the manuscript; Hongzan Xie, Shengping Jiang and Yunqing Pu performed the experiment; Hui Xiong guided the experiment, reviewed and edited the manuscript.
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MTT assay to detect the effect of serum containing TSW on the cell viability of isolated endothelial cells. The isolated endothelial cells were treated with control, 0%, 2.5%, 5%, and 10% TSW. 24 hours after treatment, the cell viability was measured by MTT assay. *P<0.05, 5% TSW treatment group compared with control group, and *p<0.05 10% TSW treatment group compared with control group.
Figure 4

Transwell assay to detect the effect of TSW-containing serum and HIF-1α inhibitor on vascular endothelial cell migration. A. The isolated endothelial cells were treated with control, 0%, 2.5%, 5%, and 10% TSW. 24 hours after treatment, the migrated cells were stained with crystal violet and then images were acquired under microscope. B. The migrated cells were counted and quantified. was measured by MTT assay. *P<0.05, **p<0.001, 2.5%, 5% and 10% TSW treatment groups compared with control group respectively. ##p<0.001, 10% TSW treatment group compared with HIF-1α inhibitor group.
Figure 6

Tube formation experiment to detect the intervention of TSW-containing serum and HIF inhibitor on vascular endothelial cell tubule formation. A. The isolated endothelial cells were treated with control, 0%, 2.5%, 5%, and 10% TSW. 24 hours after treatment, the tube formation was observed, and the images were acquired under microscope. B. The number of the tube in each group was quantified and analyzed. C. The length of the tube in each group was quantified. *P<0.05, **p<0.001, 2.5%, 5% and 10% TSW treatment groups compared with control group respectively. ##p<0.001, 10% TSW treatment group compared with HIF-1α inhibitor group.
Figure 8

Wound-healing experiments to examine the effect of TSW-containing serum and HIF-1α inhibitor on vascular-endothelial cell migration. A. The isolated endothelial cells were treated with control, 0%, 2.5%, 5%, and 10% TSW in the presence or absence of HIF-1α inhibitor and were scratched. 0 hour, 24 hours and 48 hours after treatment, the wound margins were imaged. B. The wound area in each group was calculated and analyzed. *P<0.05, **p<0.001, 2.5%, 5% and 10% TSW treatment groups compared with control group respectively. ##p<0.001, 10% TSW treatment group compared with HIF-1α inhibitor group.
Figure 10

qRT-PCR and western-blot analysis the effects of TSW-containing serum and HIF-1α inhibitor on the expression of VHL, HIF-1α and VEGF. A and B. The mRNA expression of VHL, HIF-1α and VEGF was determined by qRT-PCR after endothelial cells treated with different concentration of TSW-containing serum and HIF-1α inhibitor. C. The protein expression of VHL, HIF-1α and VEGF was examined by western-blot after endothelial cells treated with different concentration of TSW-containing serum and HIF-1α inhibitor. D. The protein expression of VHL, HIF-1α and VEGF was quantified. *P<0.05, **p<0.001, 2.5%, 5% and 10% TSW treatment groups compared with control group respectively. #p<0.05, ##p<0.001, 10% TSW treatment group compared with HIF-1α inhibitor group.