Bio-inspired Polydopamine Coated Hemoglobin as Potential Oxygen Carriers for Wound Healing

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Abstract

Adequate oxygen is identified as one of major factors in the processes of wound healing. Various strategies of oxygen therapy have been developed to improve wound healing, such as oxygen carriers. Herein polydopamine coated hemoglobin (Hb-PDA) nanoparticles as one of Hemoglobin-based Oxygen Carriers (HBOCs) for oxygen supply is proposed and synthesized, Hb-PDA nanoparticles accelerate the wound repair process in an excisional full-thickness mouse model. And inflammatory phased of wounds can be shortened by antioxidant properties of polydopamine (PDA) modification. Moreover, Hb-PDA nanoparticles efficiently promote the formation of blood vessel by upregulating expression of vascular endothelial growth factor (VEGF) and angiogenesis related protein. Interestingly, Hb-PDA nanoparticles improve the oxygen supply of wounds in the wound area, as well as down-regulated expression of Hypoxia-inducible factor-1α (HIF-1α). The results indicate the Hb-PDA nanoparticles might be a promising approach for efficient wound healing with the capacity to improve oxygen supply.

Introduction

As one of the largest organs, there are many critical functions with skin including protection, sensor, secretory and excretion.[1] A series of phases for wound healing are initiated, once the barrier function of skin is broken due to trauma or burns. The complex process involves four stages: bleeding, inflammation, proliferation and remodeling.[2,3] It is noted that oxygen is of great significance for basic metabolism and life activities of cells. Adequate oxygen is identified as having a great influence in the processes of wound healing.[4] On the one hand, it is demonstrated that wound healing is a process of energy dissipation. Oxygen is essential for adenosine triphosphate (ATP) synthesis, which provide biological energy for proper cellular function.[5] On the other hand, reactive oxygen species (ROS) play a vital role to prevent the wound from infection because of the oxidative killing of bacteria.[6] Oxygen is critical to the production of ROS by neutrophils and macrophages. However, it is the blood hypo-perfusion and the long diffusion distance from vessel to tissue that influence the wounds oxygen delivery greatly. Meanwhile, increased oxygen consumption result in lower level of oxygen content at wounds site.[7] It is commonly considered local hypoxia to be an important factor limiting wound healing. The reduction of PO_2 from 40-45 mmHg to 28-30 mmHg slowed wound healing in rabbit ear ischemia models by 80%.[8] It is reported that the bacterial killing capacity of Neutrophils can be lost in vitro at a PO_2 level below 40 mmHg because of the decreasing production of ROS.[9, 10]

In recent years, various strategies of oxygen therapy have been developed to improve wound healing.[11] Hyperbaric oxygen therapy (HBOT) increases the dissolved oxygen content in plasma to promote the growth of granulation tissue and wound healing.[12] But it has some shortcomings such as expensive, time consuming, side effects like fatigue, dizziness or lung failure.[13] In addition, compared with the HBOT, topical oxygen therapy (TOT) is characterized by oxygen administration independent on the wound's microcirculation. It can be used either by pure oxygen stored in the dressing (e.g., Oxygeneses, Oxyband) or by biochemical reactions in the dressing (e.g., Oxyzyme).[14] Although gaseous oxygen can spread to

the surface of wound, the wound exudate can affect oxygen diffusion. And oxygen supply requires more efficient to improve therapeutic effect in the wound bed.[15] Therefore, it is fulfilling that develop a strategy of oxygen therapy with a highly effective capacity to delivering oxygen to deeper parts of the wounds.

Hemoglobin is mainly responsible for delivering oxygen to tissues and organs, which provides a new idea for improving the local oxygen supply in wound tissues. The total amount of oxygen in this person's systemic arterial blood is 206.3 mL/liter blood, since 3 mL are in solution and 203.3 mL are bound to hemoglobin. Ping et al. demonstrated that systemic administration of modified bovine hemoglobin (IKOR 2084) can protect skin cells from hypoxia-induced apoptosis and promote the proliferation of antigenic cells and collagen synthesis for efficient wound healing.[16] Liposome-coated hemoglobin (LEH) has been shown to reduce inflammatory cell infiltration, increase granulation, and accelerate wound healing in mice treated with LEH after traumatic damage in skin.[17, 18] Hemoglobin spray (Granulox®) clinical trial studies proved that 50 patients were treated with hemoglobin spray for chronic wounds, 90% of which healed completely, compared with 38% of the 50 control patients.[19] High oxygen levels are prone to more reactive oxygen species (ROS), which can not only damage the structure of extracellular proteins, but trigger the signal transduction pathways resulting in an increasing expression of inflammatory cytokines. Therefore, there is a prerequisite for wound healing, which is a delicate balance between the amount of oxidants and antioxidants.

However, the free hemoglobin (Hb) are peculiarly prone to turn into methemoglobin (MetHb) by oxidation, which could impair the oxygen-carrying capacity. In our previous work, polydopamine coated hemoglobin (Hb-PDA) nanoparticles was found to have significant antioxidant activity during the process of oxygen-carrying as novel oxygen carriers.[21] Polydopamine (PDA), a original coating material, can be simply, effectively and strongly attached to substrate surfaces.[20] PDA could be acted as an antioxidant agent which could scavenge free radicals caused by the distinct hydroquinone moiety. On the one hand, PDA coating could keep Hb from turning into MetHb. On the other hand, PDA coating on Hb surface with ROS scavenging ability is significantly reduce inflammation for wound healing. Therefore, Hb-PDA could contribute to the oxygen-delivering of the wound and is essential to guard against oxygen oversupply to avoid inflammatory response.

This study focuses on therapeutic efficacy of the Hb-PDA with the fulfilling properties in vivo wound. Normal wound healing analysis was characterized by the change of wound closure. Anti-inflammatory capacity and angiogenesis evaluation of the wounds were evaluated by histological analysis and immunohistochemistry. Oxygenation effects were detected by Visisens imaging system. Gene expression analysis of hypoxia inducible factor 1-alpha (HIF-1α) and vascular endothelial growth factor (VEGF) were investigated by quantitative polymerase chain reaction (qPCR) assay.

Results And Discussion

Normal Wound Healing Analysis. An excisional full-thickness mouse model was established to investigate *in vivo* wound therapeutic efficacy of Hb-PDA nanoparticles. A 6 mm round wound was made

on the back of Kunming mice. The animals were randomly divided into saline-treated group (NS), Hb-treated group (Hb) and Hb-PDA-treated group (Hb-PDA). During the healing process, the changes of wounds as illustrated in the digital images were observed and recorded (Figure 1A). The Hb and Hb-PDA presented a relatively smaller wound size than that of the NS from Day 3 to Day 10 (Figure 1B). As illustrated in Figure 1C, a significantly higher wound closure rate of Hb-PDA was measured compared with Hb from Day 3 to Day 10. The wound closure rate of the Hb-PDA reached over 90% on Day 10, while that in Hb was 84.11±1.57%. These results indicate the Hb and Hb-PDA accelerated the wound repair process and the Hb-PDA exhibited a better recovery compared with Hb.

To investigate microscopically therapeutic efficacy of different treatments, the wound pathology was further evaluated. Hematoxylin-eosin (H&E) staining assay was performed from Day 3 to Day 10 after the treatments, as seen from Figure 2. The NS showed a large number of inflammatory cells from Day 3 to Day 10 and granulation tissue was observed until Day 7. By contrast, granulation tissue was found in other groups from Day 3. Nevertheless, Coherent epidermis was observed in Hb-PDA on Day 7, while that was found in Hb on Day 10, indicating Hb-PDA could accelerate healing process with fewer inflammations. Additionally, masson's trichrome staining was carried out to assess the collagen deposition during tissue remodeling.[24] As shown in Figure 3, the Hb-PDA displayed more collagen deposition than the Hb. All the results indicated that the Hb-PDA showed superior efficiency in wound healing in terms of fewer inflammations and more collagen deposition.

Anti-inflammatory effects. Under normal circumstances, inflammatory cells will produce ROS in the wound tissue, which at low concentration are thought as cells messages to stimulate key processes associated with wound healing. While breakdown products further promote oxidative stress occurs when the oxidative capacity exceeds the antioxidant capacity, prolonging the inflammation phase. To validate the ability to suppress the inflammatory, immunohistochemistry staining of interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α), which is known as cytokines with inflammatory property were carried out.[25] As shown in Figure 4A, the Hb-PDA presented a relatively lower expression of IL-6 than that of the NS and Hb from Day 3 to Day 10. In addition, in consistency with the trend of IL-6, the expression of TNF-α in Hb-PDA presented a relatively lower level than that in NS and Hb group, reconfirming anti-inflammatory effects due to the PDA modification on Hb surface with a shortened inflammatory phase at the early stage of wound healing.

The existence of oxidative stress should be taken into account in the inflammatory response stage of wound healing, and timely antioxidation treatment will contribute to wound healing. Parameters, such as superoxide dismutase (SOD) and malondialdehyde (MDA) plays a key role in damaged tissue timely caused by oxidative stress.[26] In comparison, the pression of SOD in Hb-PDA still remain higher level concentration from Day 3 to Day 10 (Figure 4C). The expression level of MDA was found to significantly decrease in Hb and Hb-PDA from Day 3 to Day 10, especially in the Hb-PDA (Figure 4D). It was reasonably understandable that the PDA modification on surface presented anti-inflammatory effects during wound healing. The distinct free radical character allows PDA to act as radical trap in the

biological system. Its interesting abilities to scavenge reactive free radicals have conferred to strong antioxidant effects in the organism.[27]

Angiogenesis evaluation of the wounds. Angiogenesis is an essential contributor in the process of healing, which is the foundation of nutrient and oxygen supply. Platelet endothelial cell adhesion molecule-31 protein (CD-31) is involved in many pathways, including collagen synthesis and angiogenesis, re-epithelization. Platelet endothelial cell adhesion molecule-34 protein (CD-34) is generally used to indicate the expression in endothelial cell tissue and to assess angiogenesis.[28] Immunohistochemical staining analysis shows that the Hb and Hb-PDA accelerates wound closure by upregulating expression of CD-31 and CD-34, indicating the formation of blood vessel increases significantly in the oxygen treatment groups, especially in the Hb-PDA (Figure 5A). The expression of CD-31 and CD-34 was clearly observed in all groups on Day 7. It is worth noted that the expression of CD-31 and CD-34 experienced a time dependent manner. The NS showed higher expression of CD-31 and CD-34 on Day 7 compared to Day 10, demonstrating the wound was still under proliferation phase of wound repair. By contrast, the down-regulation of CD-31 and CD-34 was observed in the Hb and Hb-PDA on Day 10 (Figure 5B, C). This indicated the Hb and Hb-PDA group entered the remodeling phase as evidenced contraction of granulation tissues and thus to prevent extraordinary scar formation.

VEGF promotes multilevel processes of the angiogenic activity and vascular permeability. The immunohistochemistry staining indicated that Hb-PDA secretes much more VEGF to promote angiogenesis, demonstrating Hb-PDA enhances angiogenesis to promote the vascular stabilization stage and accelerate wound healing (Figure 5A). Elisa and Real-time quantitative PCR (qRT-PCR) was carried out to measure the expression of VEGF.[29] The Level of VEGF in the Hb and Hb-PDA group rapidly reached above 100 pg/mL on Day 3 and remained higher level until Day 7. As shown in Figure 5B, the Hb-PDA group presented a significant higher VEGF production than that of the Hb group on Day 7. In contrast, the expression of VEGF in the NS showed a slowly increasing trend and reached above 100 pg/mL on Day 10. In consistency with the trend of results from Elisa, the expression of VEGF in Hb-PDA presented a relatively higher level than that in NS and Hb group from Day 3 to Day 7 (Figure 5C). Based on the above results, due to the lack of oxygen delivery without vascular, it's insufficient to provide energy critical for angiogenesis in the wound site. It should be noted that the addition of Hb with the oxygen loading and unloading capacity contributes to effectively enhance oxygen concentration to show the better therapeutic effects.

Oxygenation effects of Hb-PDA. It should be mentioned that adequate oxygen is prerequisite for successful wound healing, which can promote cell proliferation and tissue remodel. Therefore, attempts to promote wound healing for tissue repair can tend to focus on the study of oxygen-delivering.[30] Due to the vascular rupture in the wound, the blood supply to the skin tissue was affected, leading to the lack of oxygen in the wound area. In addition, the increased oxygen consumption in the process of wound repair, such as normal operation of inflammatory cells, proliferation of fibroblasts and synthesis of collagen, leads to hypoxia at the wound site. Thus, providing adequate oxygen supply to the wound tissue is the key factor in wound treatment. Three different groups have been compared through measuring the

changes of fluorescent intensity by VISISENS fluorescence imaging device. Oxygen saturation was further calculated with time. Compared to the pristine wound on Day 0, a clear rise of oxygen saturation in each group was observed from Day 1 to Day 10, which was attributed to the inflammatory mediators and immune response cells inducing the dilatation of blood vessels during the inflammatory phase of wound healing (Figure 7A). It was obvious that oxygen saturation shows enlacement from Day 1 to Day 8, while it began to decline on Day 10. Compared with other groups, a higher level of oxygen saturation of Hb-PDA group was also measured from Day 1 to Day 10 (Figure 7B).

Hypoxia-inducible factor 1 (HIF-1) is an oxygen-regulated transcriptional activator that plays essential roles in mammalian development, physiology and disease pathogenesis. Due to the oxygen delivery, the immunohistochemistry staining demonstrated that Hb-PDA secretes lower HIF-1α (Figure 7C). The expression of HIF-1α in the Hb and Hb-PDA group showed a decreasing trend on Day 7 and Day 10 (Figure 7D). The expression of HIF-1α in Hb-PDA presented a relatively lower level than that in NS and Hb group on Day 10. Tsuyoshi applied liposomal-coated hemoglobin (m-LEH) with high oxygen affinity to the skin ulcer wound in mice, which could improve the aerobic metabolism of skin ulcer in mice and accelerate wound healing. Using hemoglobin to carry oxygen and release oxygen can improve the utilization rate of oxygen and promote wound healing, and become a potential application of oxygen therapy. In addition, the expression of HIF-1α in the Hb-PDA group was significantly lower than that in Hb group on Day 10. The reason may be that the ability to carry and release oxygen of Hb in the Hb-PDA nanoparticles was maintained for a longer period of time by the antioxidant activity of PDA than the free Hb. The PDA surface modification technology could maintain its oxygen-carrying and oxygen-releasing function, which is essential for cell proliferation, antimicrobial activity, angiogenesis and collagen generation.

Conclusion

In summary, our study prepared Hb-PDA, which preserves the primary function of hemoglobin and not only improves the oxygen utilization rate of wound site but also relieve oxidative stress on wound surface and reduce inflammation. By establishing full-thickness skin injury model in Kunming mice, and it suggested that Hb-PDA can effectively reduce the skin cutting wound area and improve the wound healing rate. Hb-PDA can improve the oxygen supply of wounds in Kunming mice, down-regulate the expression of HIF-1α, and then promote the expression of VEGF, thereby promoting angiogenesis. In addition, it can accelerate the elimination of inflammation and promote the repair of wound tissue by down-regulating the proinflammatory factor such as IL-6 and TNF-α. It can also resist oxidative damage, reduce oxidative stress response via SOD and MDA. Therefore, our prepared Hb-PDA showed good practical performance in the treatment of full-thickness skin wounds, indicating that they have broad application prospects in wound healing and related biomedical fields.

Experimental Section

Materials. Dopamine hydrochloride was purchased from Sigma-Aldrich (USA). Normal saline was provided by Shijiazhuang SiYao Co. Ltd (China). IL-6, TNF-α and VEGF assay kits were bought from by RnDsystems Co. Ltd (USA). SOD and MDA assay kits were bought from by FineTest Co. Ltd (China). Tris-HCl buffer solution (1 M, pH 8.5) was bought from Beijing Leagene Biotech Co. Ltd (China). Phosphate buffer saline (1xPBS, pH 7.2-7.4) solution was purchased from Thermo Fisher Scientific (USA). All chemicals were used without further purification.

Preparation of Hemoglobin. Hemoglobin was collected from bovine red blood cells. Packed fresh bovine whole blood (acquired from JinXiuDaDi Agricultural Park, China) was centrifuged at 4000 g for 10 min at 4 ^{o}C. The Hb solution was obtained by hypotonic hemolysis after the ultrafiltration and concentration. The Hb solution was prepared via anion-exchange chromatography assay and were stored at -80 ^{o}C. The MetHb concentration of the Hb solution was less than 5% measured by a blood gas analyzer (Radiometer ABL90COOX, Denmark).

Preparation of Hb-PDA. The Hb was prime-coated with PDA by the oxidative polymerization of dopamine. Hb was incubated with dopamine hydrochloride solution for 210 min at 4^{o}C in Tris-HCl buffer (10 mM, pH 8.5) with the slight stirring. The total volume of reaction system was 2 mL and then the mixture was dialyzed in PBS solution to remove additional dopamine hydrochloride. Dopamine concentration was fixed at 4.88 mg/mL unless specified otherwise.[21]

In vivo Animal Experiment. All the experimental protocols were approved by the Institutional Animal Care and Use Committee of the Academy of Military Medical Sciences. Healthy female KunMing mice (35±5 g) were obtained from Beijing SIPEIFU biotechnology corporation. The mice were randomly divided into three groups: saline-treated group (NS), Hb-treated group (Hb), and Hb-PDA-treated group (Hb-PDA). After anesthetization with 1% pentobarbital sodium solution (50 mg/kg), full thickness skin wounds (6 mm) were created on the back. A silicone ring was placed around each skin defect and eight stitches were stitched with monofilaments to prevent skin shrinkage. 10 uL NaCl, Hb and Hb-PDA solutions were added on the surface of the wound, respectively. After 10 days treatment, the wounds was photographed with a digital camera and the wound surface areas of the mice were calculated with ImageJ software.

Histological Analysis and Immunohistochemistry. On days 3,7,10, the basal tissues of the wounds on backs were taken and fixed with 4% paraformaldehyde for 24 hours. Next, the skin tissue is encapsulated in paraffin and stained using hematoxylin and eosin (H&E) and Masson trichromatic (Masson). Immunohistochemical staining (CD31, CD34, VEGF, HIF-1α) was implemented to authenticated the effect of different treatment on angiogenesis and inflammatory response.

Measurement of Oxygen Content. O_2 sensor foils with a size of 6 mm were slided against the tissue surface to squeeze out the air, and waiting at least 2 min to develop a steady state between the sensor and wounds surface before measurement. Luminescence 2D imaging was demonstrated with the VisiSens imaging systems (PreSens, Regensburg Germany). After defining the image, the mean values

were calculated in each region of interest (ROI). Data recording and evaluation is analysed by the VisiSens Analytical Software (PreSens, Regensburg, Germany).[22]

Gene Expression Analysis. The total ribonucleic acid (RNA) was isolated from the samples (200 mg) crushing in Trizol reagent with the homogenizer according to the manufacturer's protocol. The concentration of RNA in each sample was determined by measuring absorbance at 260 nm using a spectrophotometer. The first strand complementary deoxyribonucleic acid (cDNA) was synthesized using an *in vitro* transcription kit. The reaction mixture was made up to consisting of SYBR Green/Fluorescein PCR Premix Mix, forward primer, reverse primer, cDNA and nuclease-free water.

Primer sequences of the genes were as follows:

VEGF F: 5′-CTCGCAGTCCGAGCCGGAGA-3′

R: 5′-GCAGCCTGGGACCACTTGGC-3′

HIF-1α F: 5′-GATTCAAGTGGTCTTCCTGCTTCAGC-3′
R: 5′-GGGACTCATCCCAGGCGGG-3′

The gene of interest was normalized against the reference geneglyceraldehyde-3-phosphate dehydrogenase (GAPDH):
F: 5′-ACGGCACAGTCAAGGCCGAG-3′

R: 5′-ACCCTTCAAGTGGGCCCCGG-3′

The protocol included initial denaturation at 50^{o}C for 2 min, 95^{o}C for 10 min, followed by 40 cycles with denaturation at 95^{o}Cfor 15 s, annealing at 55^{o}C for 30 s, followed by a melt curve 30 s at 72 ^{o}C. PCR was performed in triplicate. The expression level of each target gene was calculated as $2^{-\triangle Ct}$.

Expression of Cytokines. Two mice from each group were euthanized on day 3,7 and day 10 after wounding. The wounds with edges were harvested by sterile cut. Wound tissue was stored in liquid nitrogen and treated according to histochemical methods.[23] The wound tissues were then homogenized. The samples were centrifuged at 14000×g and stored at -80 ^{o}C before assaying. The Cytokines (VEGF, IL-6, TNF-α, SOD, MDA) were assayed with ELISA kits respectively.

Statistical Analysis. All the data are evaluated as mean ± standard deviation (SD) based on at least three tests and contrasted with Kruskal-Wallis one-way analysis of variance (ANOVA).

Declarations

Acknowledgements

Not applicable.

Authors' contributions

Ning Ma and Bingting Li were major contributors in this study. All authors read and approved the manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interest.

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Figures

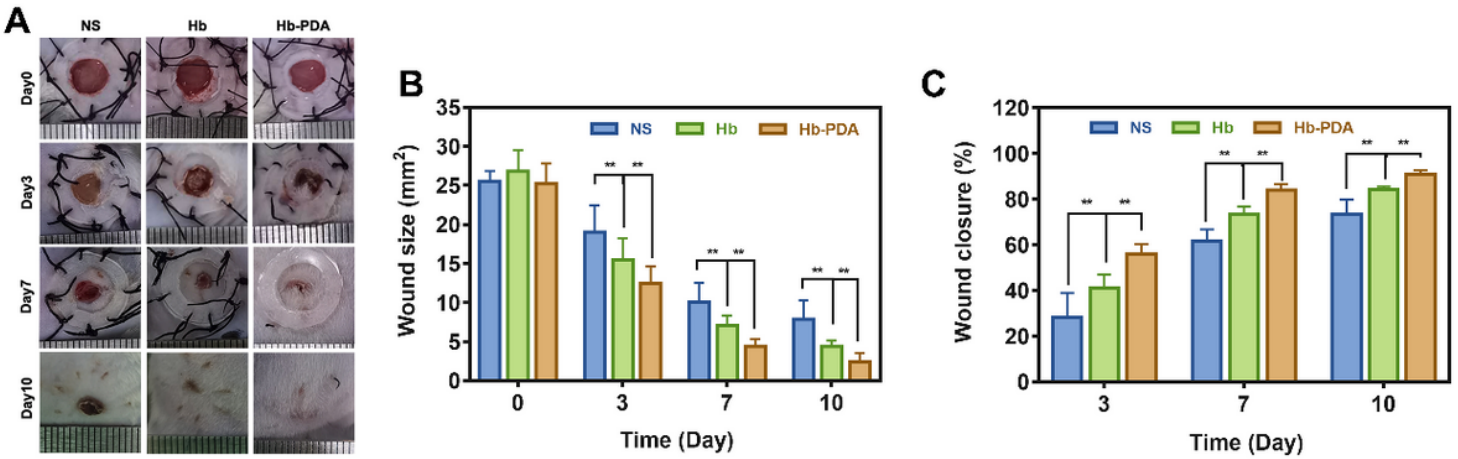

Figure 1

In vivo effects of Hb-PDA in a full-thickness mouse model. (A) Representative photographs of the wounds treated with NS, Hb, Hb-PDA from Day 0 to Day 10. (B) Change in wound size after various treatments

from Day 0 to Day 10. (C) Wound closure rate from Day 3 to Day 10. Data are reported as means±SD (**p<0.01).

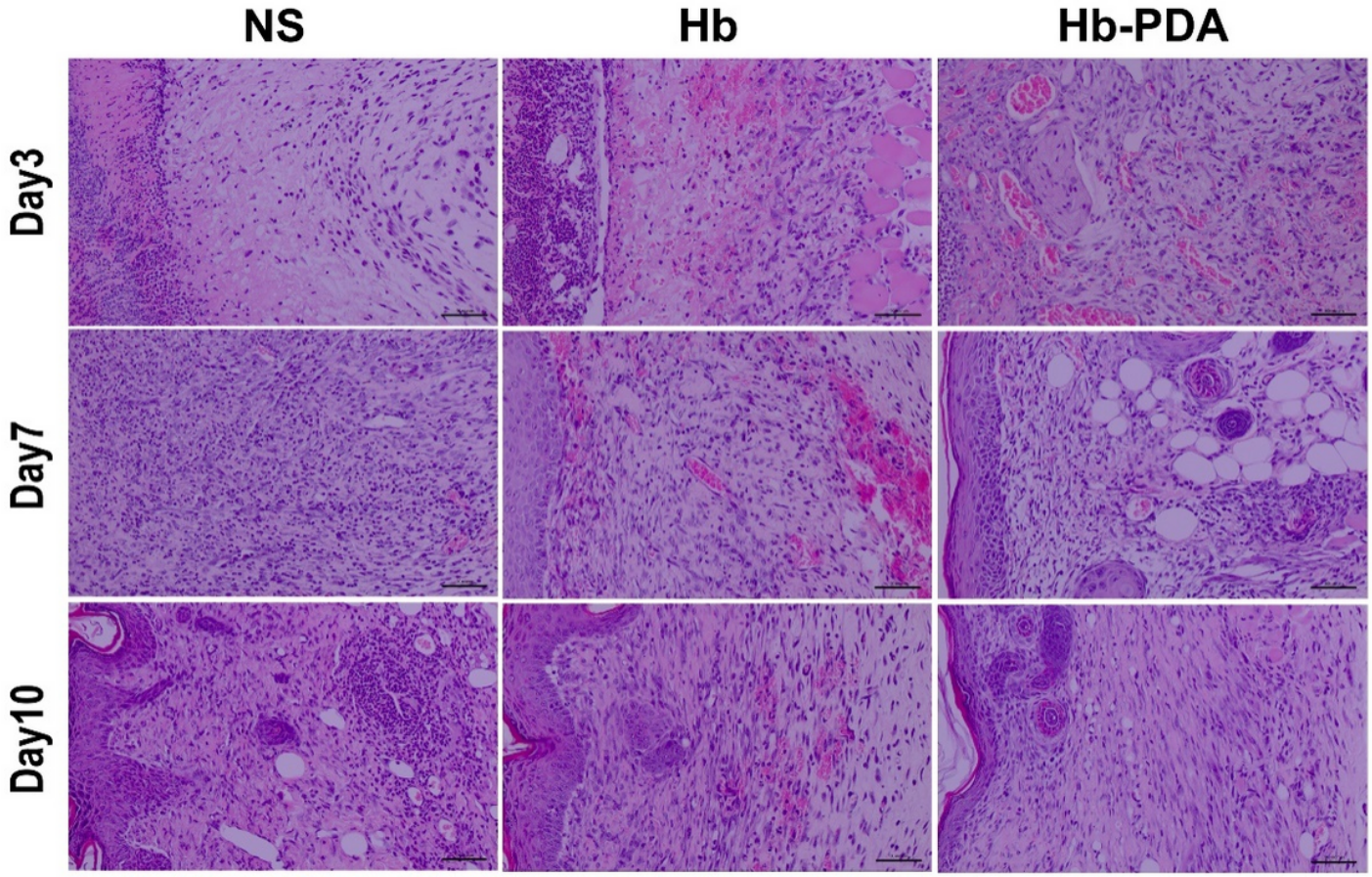

Figure 2

Histological evaluation of the wounds treated with Hb-PDA. H&E stained images of wound on Day 3, Day 7 and Day 10.

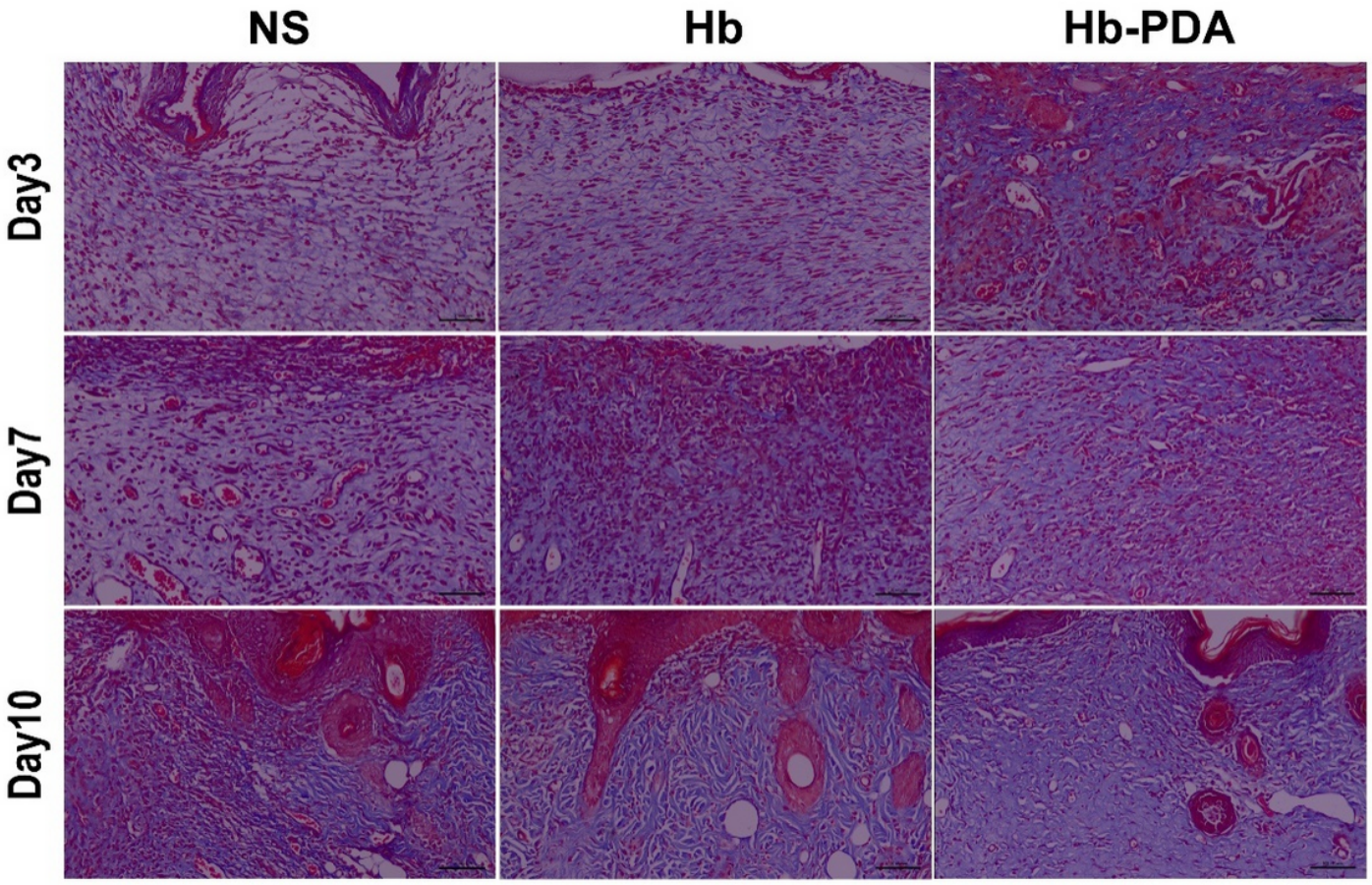

Figure 3

Histological evaluation of the wounds treated with Hb-PDA. Masson's trichrome stained images of the wound on Day 3, Day 7 and Day 10.

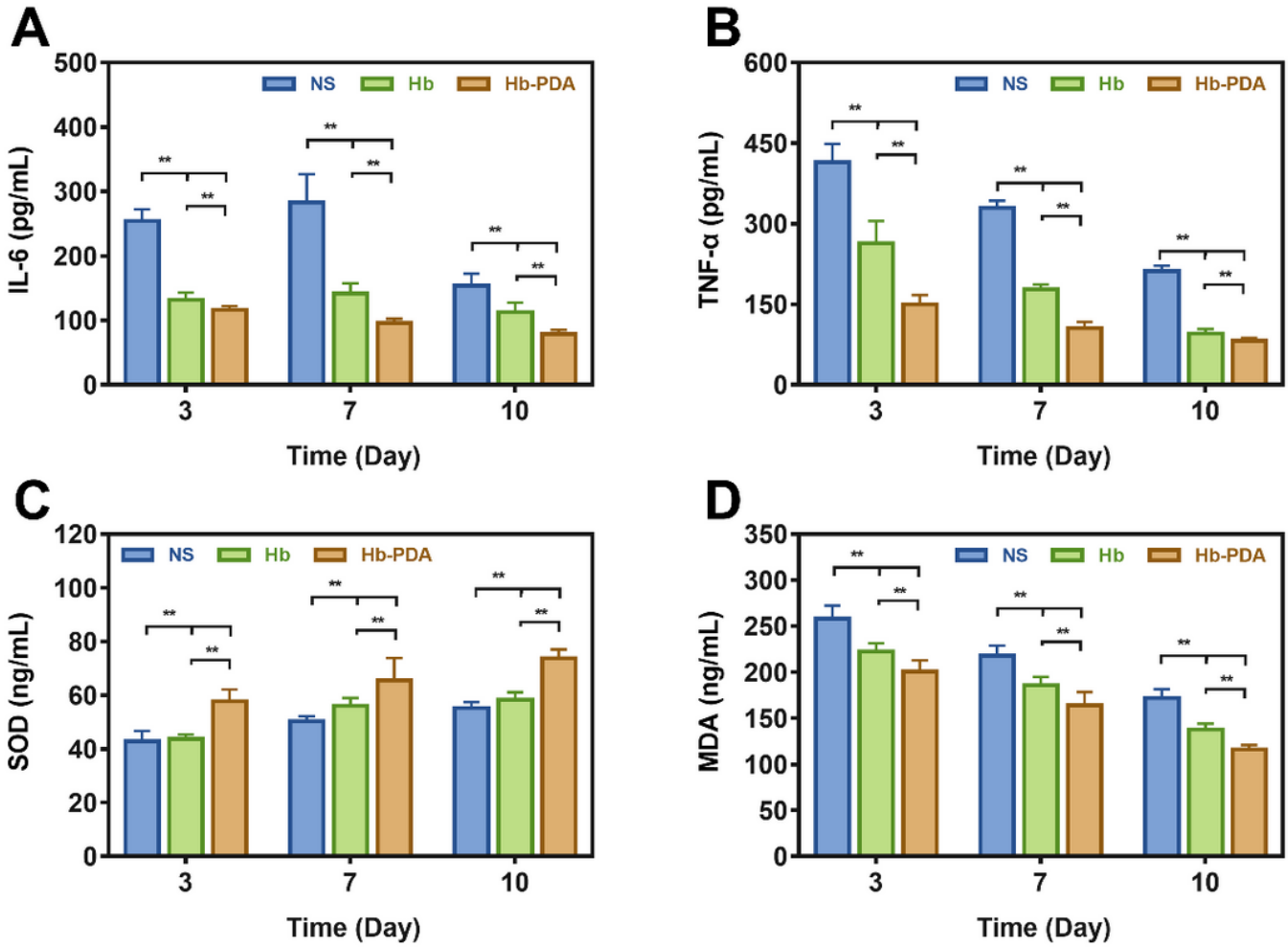

Figure 4

Anti-inflammatory effects of the Hb-PDA. Quantitative analysis of (A) IL-6, (B) TNF-α, (C) MDA, (D) SOD in the harvested tissues by Elisa on Day 3, Day 7, Day 10. Data are reported as means±SD (**p<0.01).

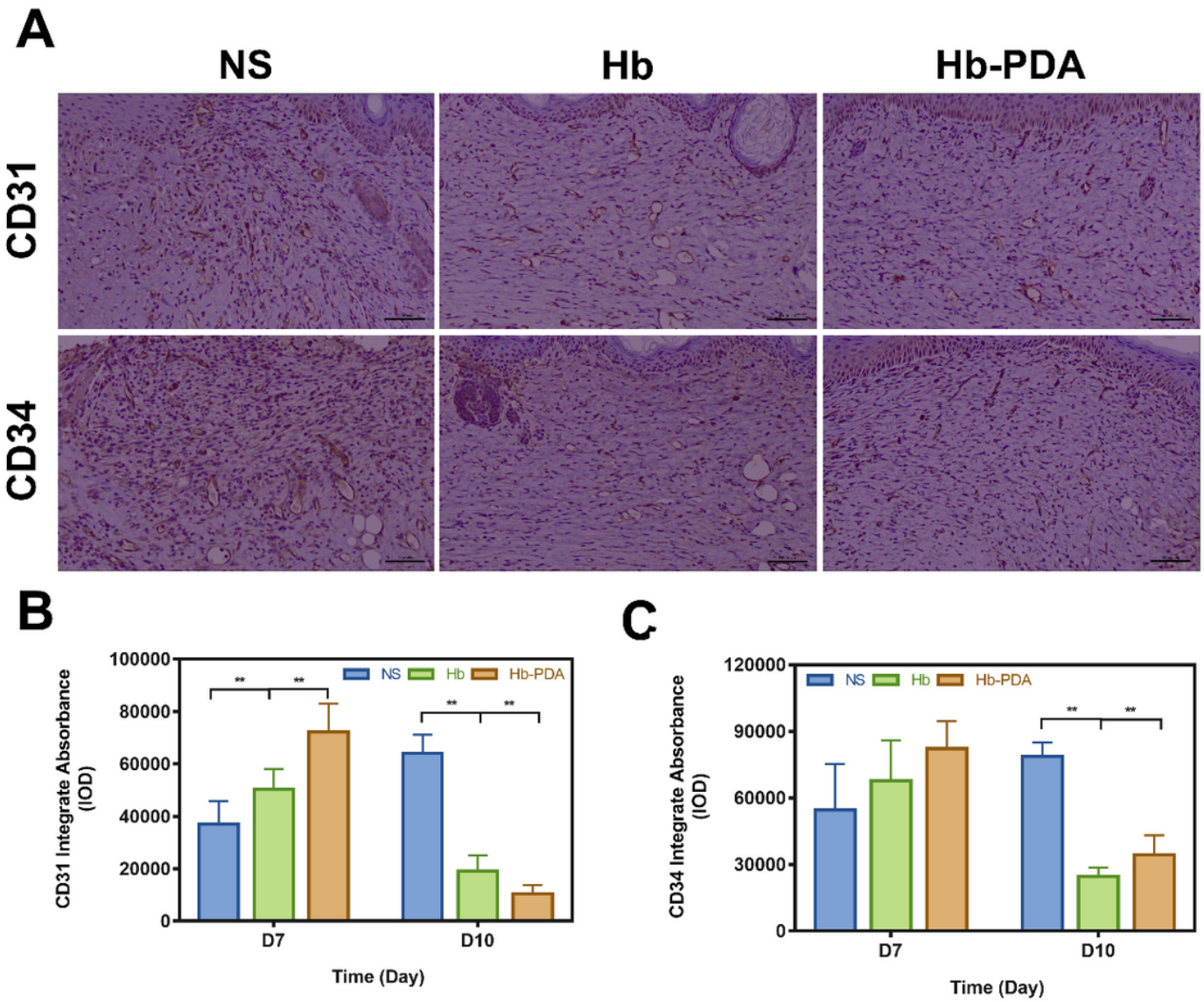

Figure 5

Angiogenesis evaluation of the wounds treated with Hb-PDA. (A) CD-31and CD-34 immunohistochemical staining images on Day 10. Quantitative analysis of the relative density of (B) CD-31 and (C) CD-34 from immunohistochemically stained wound section on Day 7 and Day 10. Data are reported as means±SD (**p<0.01).

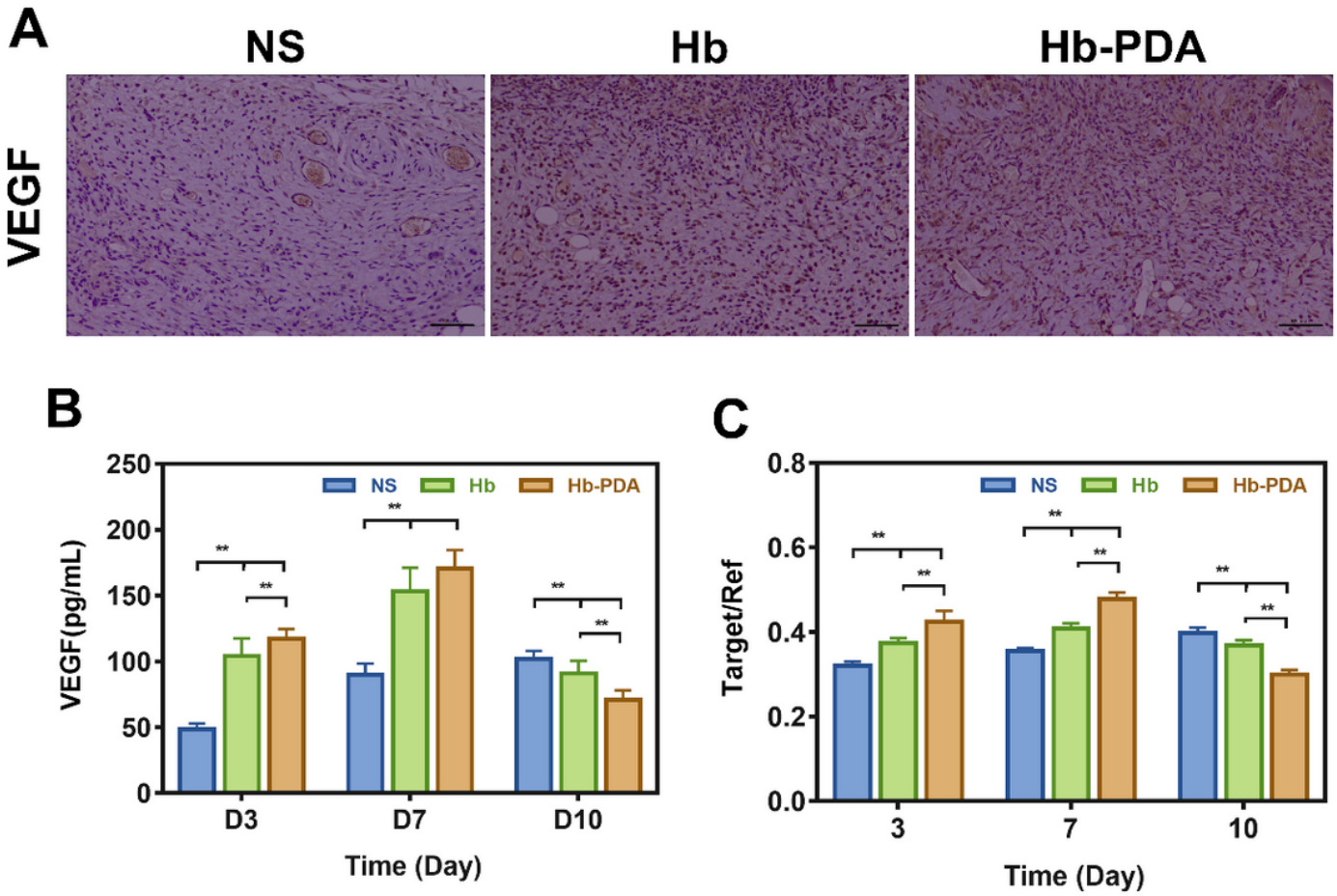

Figure 6

Expression changes of VEGF after different treatments. (A) VEGF immunohistochemical staining images on Day 10. (B) Elisa and (C) qRT-PCR validation results of VEGF after various treatments on Day 10. Data are reported as means±SD (**p<0.01).

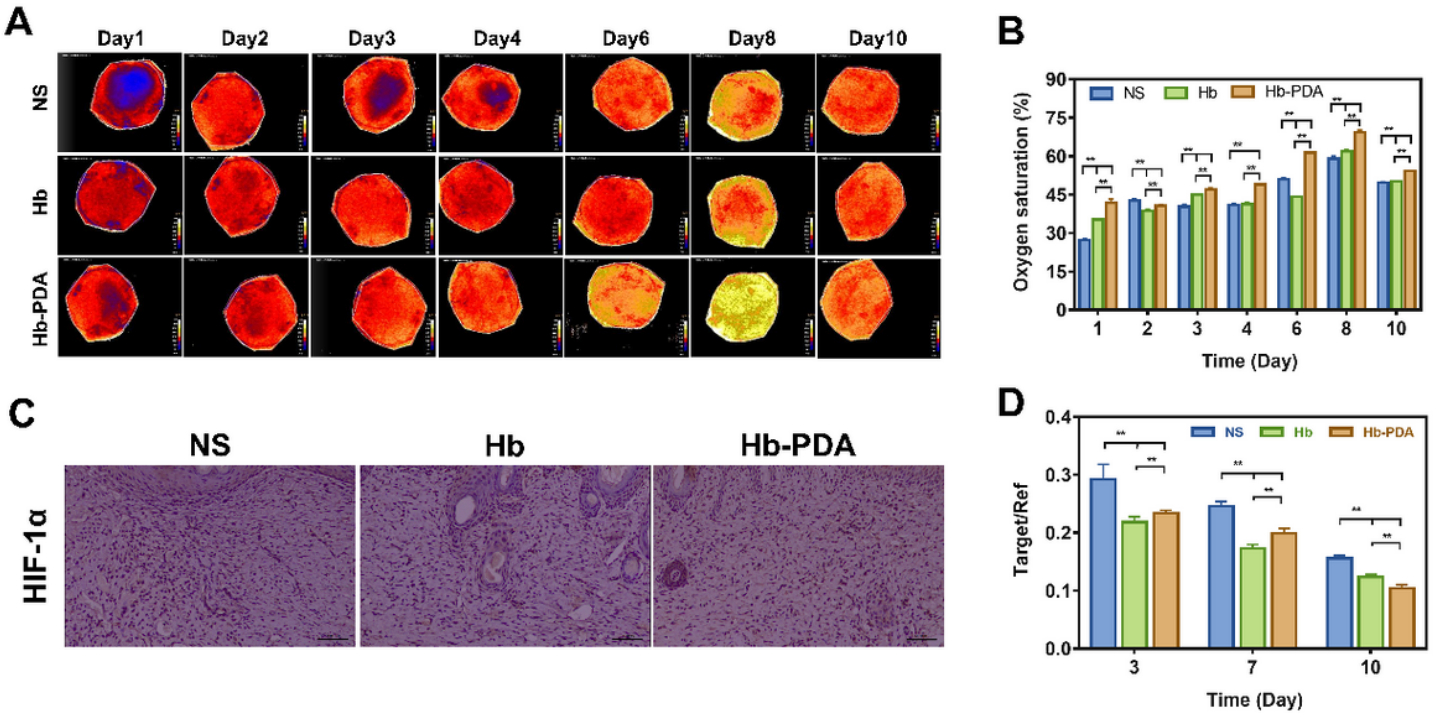

Figure 7

Oxygenation effects of the Hb-PDA. (A) Oxygen content changes of the wounds with different treatments from Day 1 to Day 10. (B) Oxygenation values of wounds from Day 1 to Day 10. (C) HIF-1α immunohistochemical staining images on Day 10. (D) qRT-PCR validation results of HIF-1α after various treatments on Day 10. Data are reported as means±SD (**p<0.01).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Graphicalabstract.png