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Loading of VEGF to the heparin cross-linked demineralized bone matrix improves vascularization of the scaffold

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Abstract Deficient vascularization is one of the prominent shortcomings of porous tissue-engineering scaffolds, which results in insufficient oxygen and nutrients transportation. Here, heparin cross-linked demineralized bone matrices (HC-DBM) pre-loaded with vascular endothelial growth factor (VEGF) were designed to promote cells and new microvessels invasion into the matrices. After being chemical crosslinked with heparin by *N*-hydroxysuccinimide and *N*-(3-di-methylaminopropyl)-*N'*-ethylcarbodiimide, the scaffold could bind more VEGF than the non-crosslinked one and achieve localized and sustained delivery. The biological activity of VEGF binding on heparinized collagen was demonstrated by promoting endothelial cells proliferation. Evaluation of the angiogenic potential of heparinized DBM loaded with VEGF was further investigated by subcutaneous implantation. Improved angiogenesis of heparinized DBM loaded with VEGF was observed from haematoxylin-eosin staining and immunohistochemistry examination. The results demonstrated that heparin cross-linked DBM binding VEGF could be a useful strategy to stimulate cells and blood vessels invasion into the scaffolds.

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1 Introduction

Demineralized bone matrix (DBM), derived from native bone tissues, is a biocompatible biomaterial and has been used in clinical applications in bone defect treatment [1–6]. With a three-dimensional structure, DBM can provide cells anchorage sites, mechanical stability and structural guidance. Furthermore, this porous scaffold can also result in new blood vessels invasion after being implanted [7]. In order to improve biological functions of this scaffold, several growth factors, such as bone morphogenetic protein-2 (BMP-2) and basic fibroblast growth factor (bFGF), were immobilized on this porous scaffold and resulted in more bone formation and significantly improved wound healing [8–10]. However, deficient vascularization limits its utilization on reconstruction of large skeletal defects [11]. Because of the lack of viable capillary networks, neither the nutrients and oxygen can be transferred to the cells, nor can the metabolic waste be removed. Failure of delivery and maintenance of oxygen and nutrients at the wound site results in apoptosis of the newly invaded cells. Hence, vascularization of implanted scaffolds may be essential for the improvement of healing in clinical application of DBM.

Angiogenesis is a complex process which is resulted from subtle interactions among cells, extracellular matrix components, and regulatory molecules. It results in ingrowth of new blood vessels into biomaterials from pre-existing vasculatures. A series steps are involved into this process such as the localized proteolytic degradation of the subendothelial basement membrane, migration of endothelial cells, the

formation of sprouts, remodeling of extracellular matrix and the formation of anastomoses [12]. All of these steps together precede the establishment of blood flow. Neovascularization by angiogenesis, stimulated by stimuli, is considered a key step in success of bone graft healing [13]. The new blood vessels, invading the scaffolds after implantation and incorporating between the newly formed woven-bone trabeculae, function on both providing nutrient for newly formed bone and forming the hematopoietic bone narrow [14].

A variety of growth factors (GFs) are demonstrated to play roles in the formation, prolonging and maturation of new blood vessels [15–19]. A widely used pro-angiogenic growth factor in tissue engineering is vascular endothelial growth factor (VEGF), which functions on endothelial cells proliferation, migration, tube formation, and neovessels stabilization by acting on VEGF receptors (VEGFR) [20]. VEGF is a key mediator in the process of angiogenesis [21]. It is reported that VEGF has been used for promoting angiogenesis in vivo by associating with biomaterials [22–24]. However, VEGF burst release from porous bi-layered poly (lactide-co-glycolide) (PLG) scaffold just resulted in small and immature blood vessels after 14 days implantation in vivo [25]. For decreasing the burst diffusion and increasing the local concentration of VEGF in injury site, sustained and localized delivery system of VEGF using biomaterial scaffold was developed in the presence of heparin.

Heparin belongs to a class of highly sulfated glycosaminoglycans, which are known primarily for its antithrombogenic function. With high affinity for some GFs, it is capable of storing and stabilization of GFs and maintaining their bioactivity [26, 27]. The action mechanism between heparin and GFs is related partly to shape recognition, but primarily to electrostatic attractions between N- and O-sulfated residues of heparin and the lysine and arginine residues of the GFs [28, 29]. The association/dissociation reaction between heparin and GFs is reversible, so the immobilized GFs can be released from heparin. A number of GFs are known as heparin-binding GFs such as BMP-2, transforming growth factor-beta 1 (TGF-β1), VEGF and bFGF [8, 26, 30]. Heparin can stabilize GFs active conformation, protect GFs from both chemical and physiological degradation, and limit GFs availability within the regions of active cells invasion [26, 29, 31]. Furthermore, after crosslinked with N-hydroxysuccinimide (NHS) and n-(3-di-methylaminopropyl)-N′-ethylcarbodiimide (EDC) on tissue engineered matrices, heparin can greatly improve the absorption ability of the scaffolds to exogenous GFs with heparin-binding sites, and control the GFs in the way of sustained and localized release which leads to more significant angiogenic response [32, 33]. Heparin modified collagen matrices loaded with VEGF showed character of enhancing angiogenic effect [34]. After immobilizing VEGF on heparinized hyaluronan hydrogels, the release of growth factor in vitro could be sustained over 42 days and the vascularization in vivo was sustained over 28 days in mouse model [30].

In this study, we hypothesize that VEGF, loaded on heparinized DBM, can promote angiogenesis of the scaffolds in vivo. To test this hypothesis, we examined the binding ability and the sustained releasing ability of VEGF on heparin cross-linked DBM (HC-DBM). The biological activity of immobilized VEGF, promotion cells proliferation in vitro and improvement vascularization of scaffolds in vivo, were also evaluated.

2 Materials and methods

2.1 DBM matrices and heparin immobilization

DBM, obtained from bovine sponge bone, was provided by Zhenghai Biotechnology Inc. (Shandong, China). The DBM was prepared as described before [9].

Heparin was crosslinked to DBM using EDC and NHS as described with slight modification [8]. Before crosslinking, the reaction of heparin (2 mg/ml) (H-4784, sigma, USA), NHS (1.2 mg/ml) (14405, Sigma, USA) and EDC (2 mg/ml) (39391, Sigma, USA) in 2-(N-morpholino) ethanesulfonic acid (MES) buffer was proceeded for 15 min at 37°C. Then after two washes with filtration sterilized MES buffer, DBM were immersed in that solution containing heparin, NHS and EDC in MES buffer for 4 h at 37°C. When the reaction completed, heparin crosslinked DBM (HC-DBM) and the control (DBM) were extensively washed respectively with 0.1 M $NaHPO_4$ (four times in 2 h), 4 M NaCl (eight times in 8 h) and distilled water (eight times in 8 h).

2.2 Amount of immobilized heparin

The amount of immobilized heparin, physically adsorbed or chemical crosslinked on the DBMs was determined using toluidine blue method [35]. The DBMs used here was $4 \times 4 \times 1$ mm in volume and 2 mg in weight. The heparin was physically adsorbed or chemical crosslinked on DBMs. These two kinds of DBMs were immersed in 100 µl toluidine blue solution (0.2% NaCl, 0.01 M HCl) in 96-well plate for 15 min at room temperature (RT) respectivelly, followed by adding 100 µl hexane anhydrous (95%, ACDRICH) for 6 min at RT. Then 50 µl reacted solutions were transferred to another 96-well plate respectively and determined by absorption at 620 nm with a plate reader (TECAN, SUNRISE, Australia). The amount of heparin immobilized on DBMs was calculated from standard curve.

2.3 Immobilization of VEGF on HC-DBM and DBM

VEGF was prepared by us as described [36]. The entire scaffolds of HC-DBM group and DBM group were immersed in 100 μl VEGF solution with different concentration. The reaction was preceded for 1 h at RT in a 96-well plate. Then the materials were washed by fresh phosphate buffer saline (PBS) (five washes of each at least 5 min) to remove free VEGF. For the main component of DBM was type I collagen, we also examined the VEGF immobilized on extracted rat tail collagen crosslinked with or without heparin.

2.4 Quantification of immobilized VEGF on HC-DBM and DBM

The amount of VEGF bound to HC-DBM and DBM was quantified by a direct VEGF enzyme-linked immunosorbent assay (ELISA) technique. Briefly, the scaffolds, with or without VEGF, were blocked with 200 μl bovine serum albumin (BSA, 2.5 mg/ml) in PBS for 1 h in 96-well plate at RT, after which they were washed by PBST (PBS plus 0.05% Tween-20) for two times (5 min per time). The scaffolds were then incubated with 100 μl mouse anti-VEGF antibody (0.8 μg/ml, Santa Cruz) for 1 h at 37°C. After three washes by PBST (5 min every time), 100 μl anti-mouse-alkaline phosphatase antibody (1:10,000 dilutions by PBS, Sigma, USA) was added for 1 h at 37°C, following three washes by PBST as above. Bound protein were detected with 200 μl 2 mg/ml *p*-nitrophenyl phosphate-hexahydrate (*p*-NPP, Ameresco) in alkaline phosphatase buffer (100 Mm Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, pH 9.6) for 20 min. 80 μl colored substrate solution was removed from each well and transfered to a new plate, and measured at 405 nm in an ELISA reader. The quantification of immobilized VEGF on HC-collagen and collagen was assayed with resemble method.

2.5 Sustained release of VEGF bound on HC-DBM and DBM

HC-DBM and DBM binding VEGF were separately put into 1 ml PBS in 24-well plate at 37°C shaking with 54 rpm.The buffer was changed into fresh one every 12 h. The amount of VEGF retained on HC-DBM and DBM were measured by ELISA.

2.6 Growth factor activity on scaffolds in vitro

The activities of VEGF on collagen-coated plates were tested using human umbilical vein endothelial cells (HUVECs). Acid soluble collagen prepared from rat-rail tendon was neutralized and added to 48-well plates (150 mg/ml). The plate was airdried in superclean bench overnight at RT. After three washes with PBS, collagen was crosslinked with heparin by NHS/EDC as mentioned previously and 5 μg VEGF solved in PBS was loaded to heparin crosslinking collagen (HC-collagen). After 3 washes with PBS, 300 μl cells (1×10^4 cells in 1 ml of DMEM-10% fetal bovine serum) were added and maintained at 37°C in a humidified atmosphere of 5% CO_2 for 4 days. The cells number was determined by MTT assay.

2.7 In vivo experiment

A rat subcutaneous model, which had been proven to be a relevant model to evaluate cellularization and vascularization of DBM absorbing with GFs [8, 9], was utilized to evaluate the functionalization of scaffolds in the presence or absence of VEGF. Sprague-Dawley (SD) rats weighting 180–200 g were employed for implantation. Chinese Ministry of Public Health (CMPH) guideline for the care and use of laboratory animals was observed. SD rats were anesthetized by pentobarbital sodium (30 mg/kg) and the dorsal hair was shaved. Four incisions (two incisions each side) with 1 cm length were made on the back of rats. Four groups of materials were employed: (1) DBM loading with PBS (DBM); (2) HC-DBM loading with PBS (HC-DBM); (3) DBM loading with 12.9 μg VEGF (DBM/VEGF); (4) HC-DBM loading with 12.9 μg VEGF (HC-DBM/VEGF). In order to avoid individual error, each rat had been implanted with four different materials. After operation, the rats were housed in separate cages and given standard food and water until they were sacrificed.

At 3 weeks post-surgery, the animals were sacrificed and the samples were excised, followed by fixation in 10% (v/v) neutral-buffered formalin overnight. Then the samples were embedded in paraffin wax, sectioned in the same direction to 5 μm thicknesses with Microm HM 325 Microtome (Microm, German Medical Equipment, Waldorf, Germany) and stained with haematoxylin-eosin (H.E.).

2.8 Immunohistochemical examination

Anti-actin (Clone ZCA34) antibody was utilized to perform immunohistochemical staining, paralleling with the H.E. staining. Smooth muscle actin (α-SMA) present in blood vessel walls can be utilized to determine blood vessels present in tissue specimen. Briefly, the samples were deparaffinized, rehydrated and blocked by H_2O_2 (3% solution in methanol, 15 min) to remove the intrinsic peroxidase activity. Sections were treated with pronase (Sigma; 0.1%, 30 min, at room temperature) and incubated with mouse anti-actin antibody (1:100 dilution by PBS for 2 h at 37°C). A Histostain^{TM}-Plus Kit (Zymed Laboratories, San Francisco, CA) was used for staining, according

to the manufacturer's instructions. The histomorphometrical evaluation was performed using a Nikon calibrated lens micrometer (Nihon Kogaku Co., Tokyo, Japan) and nine locations per specimen (four specimens one group) were selected at random for counting the number of blood vessels. The microvessel density was calculated from microvessels quantity per location.

2.9 Statistical analysis

All results were reported as means ± standard deviation (SD). The significance of difference was determined by two-tail *t*-test. Values were considered to be statistically significant at a value of $P < 0.05$.

3 Results and discussion

3.1 Porous structure of DBM

The porous structure of DBM was visualized with a HITACHI Model S-2500 SEM (Fig. 1). The pore sizes ranged from 350 to 750 μm, which were the expected sizes for cells and new blood vessels invasion. The porous structure should be beneficial for the penetration of immobilized growth factor which functioned on cells proliferation and new blood vessels prolongation.

3.2 Crosslinking of heparin to DBM using NHS and EDC as linkages

Heparin was conjugated with DBM surface amine groups via EDC/NHS chemistry. Standard curve was utilized to calculate the amount of heparin cross-linked on DBM. As shown in Fig. 2, about 0.62 ± 0.13 μg heparin was covalently conjugated on DBM by NHS and EDC. The amount of heparin cross-linked on DBM was about seven times more than that absorbed physically. The data showed that NHS and EDC could effectively crosslink heparin on DBM.

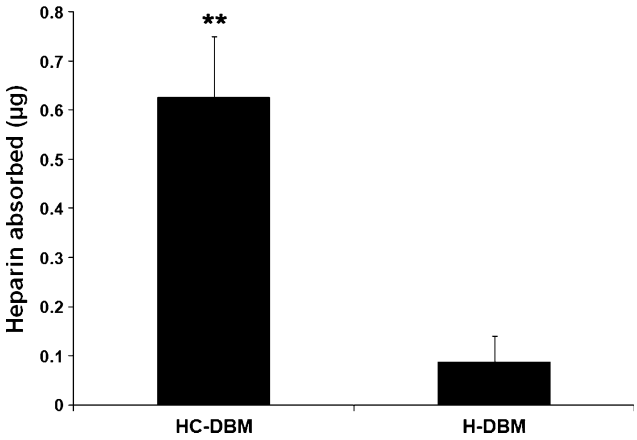

Fig. 2 Heparin immobilization on DBM in the presence or absence of crosslinkages. The columns show the mean values, and the error bars represent the corresponding standard deviation (n = 4). ** Statistically significant difference, $P < 0.01$

3.3 Heparinized materials could immobilize more VEGF

As we have showed above, more heparin was crosslinked on DBM by NHS and EDC, we thought that high amount of heparin cross-linked on DBM would improve the binding capability of VEGF to the scaffold. VEGF was loaded on the nonmodified and heparinized scaffolds by simple dipping method and the amounts of VEGF immobilized on scaffolds were assessed by a direct ELISA. As shown in Fig. 3, the amounts of immobilized VEGF on all these scaffolds were concentration-dependent. There were statistically significant difference ($P < 0.01$) between HC-DBM and DBM with 9.5, 4.75, 2.38 and 1.19 μg of VEGF (Fig. 3a). Specially, at the 9.5 μg VEGF loaded, the amount of VEGF bound on HC-DBM was 2.36 ± 0.04 μg compared to 1.80 ± 0.09 μg on DBM. It was shown that HC-DBM could bind more VEGF than DBM.

Because the main component of DBM was type I collagen, we also studied the VEGF on heparinized collagen (HC-collagen). As shown in our study, HC-collagen also

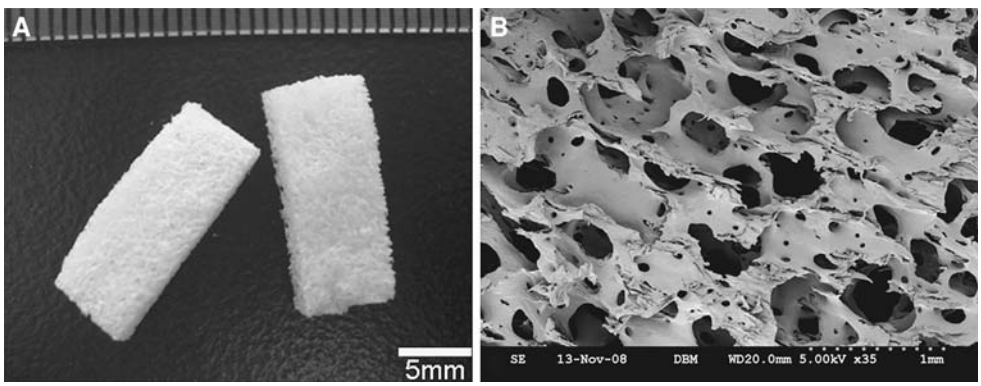

Fig. 1 The structure of DBM. (**a**) Macroscopic view of DBM porous structure. (**b**) The SEM of DBM. The pore size of the scaffold was measured as visualized by scanning electron microscopy. Scale bar = 5 mm (**a**) and 1 mm (**b**), respectively

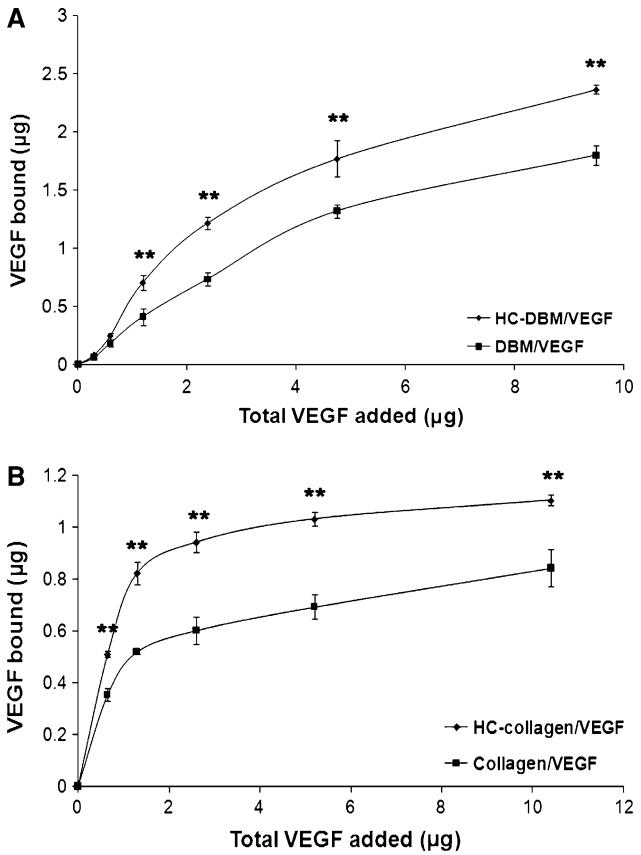

Fig. 3 The amount of VEGF bound on scaffold. (**a**) Immobilization of VEGF on HC-DBM and DBM. (**b**) VEGF amount on HC-collagen and collagen. ** Statistically significant difference, $P < 0.01$

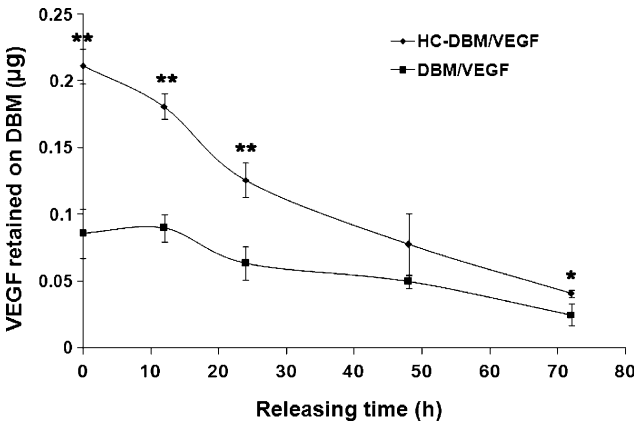

Fig. 4 VEGF release from HC-DBM and DBM over 3 days time period in vitro (n = 3). * Statistically significant difference, $P < 0.05$. ** Statistically significant difference, $P < 0.01$

displayed higher capability of immobilizing VEGF than collagen.

When the VEGF was added on the non-heparin treated DBM, the immobilized just depended on some kind of physical absorbing, it could be easier washed away by PBS in vitro, and by the body fluid in vivo. There was just limited absorbing capability for DBM to maintain VEGF.

However, like several other growth factors, there exists some special binding ability between VEGF and heparin. Heparin is a kind of extracellular matrix. Its binding with the growth factors is usually as a kind of regulatory mechanism for those growth factors to exert their special biological function in the body. When DBM was crosslinked with heparin, its binding ability with VEGF was greatly improved. Specific interaction between VEGF and heparin played a key role in increasing the amount of VEGF immobilizing on heparinized scaffolds [32]. In our study, we proved that heprinized DBM immobilized more VEGF.

3.4 VEGF on HC-DBM released in a sustained manner

The different scaffolds loaded with VEGF were placed into sterilized PBS of pH 7.2 at 37°C. Figure 4 shows the release profiles of VEGF from HC-DBM and DBM over 3 days. The release profile of VEGF on HC-DBM was in sustained manner. 14.2% VEGF immobilized on HC-DBM was released after 12 h incubation with PBS, while after 3 days incubation 80.6% VEGF was released. After 3 days release, the amount of VEGF retained on HC-DBM and DBM were 40.9 ± 2.8 ng and 24.8 ± 8.3 ng, respectively. The quantity of VEGF released from HC-DBM and DBM mainly based on the initial total mass of VEGF immobilization. Hence, the thermodynamic equilibrium between free VEGF in release medium and VEGF immobilized on scaffolds mainly governed the dissociation of VEGF from VEGF-heparin complex in scaffolds [37].

The heparinized DBM not only immobilized more VEGF, it also could release it in a sustained manner. The consistent release of VEGF from HC-DBM at the rate of no less than 30 ng/day could provide sustained stimulation on the surrounding tissue, which was beneficial for angiogenesis in vivo [38].

3.5 The VEGF in the scaffolds/VEGF system promoted the proliferation of HUVECs in vitro

In our study we used collagen and DBM to investigate their immobilizing ability with VEGF. Due to the main component of DBM is collagen, no matter witch kind of scaffolds, the essential interactions are among VEGF, heparin and collagen. Did the biological activity of VEGF affected by this kind of treatment? The ability of VEGF immobilized on collagen to promote proliferation of HUVECs was tested by MTT method. As shows in Fig. 5, there was significant difference between VEGF groups (collagen/VEGF and HC-collagen/VEGF groups) and corresponding control groups (collagen and HC-collagen groups), while there wasn't significant difference between collagen/VEGF and HC-collage/VEGF groups.

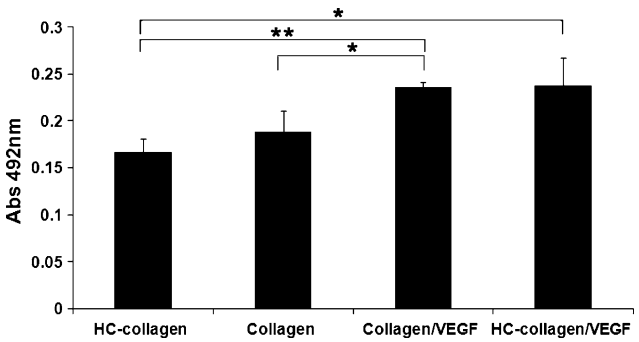

Fig. 5 Biological activity of VEGF bound on scaffolds. The scaffolds were loaded with or without 5 μg VEGF. The VEGF releasing from the scaffolds promoted the proliferation of HUVECs. Columns show mean values and error bars represent the corresponding standard deviations (n = 3). * Statistically significant difference, $P < 0.05$. ** Statistically significant difference, $P < 0.01$

The difference between VEGF groups and control groups indicated that the absorption of VEGF on collagen with or without heparin didn't affect its biological activity to promote the proliferation of HUMECs in vitro. It could retain its biological activity after the absorption treatment and the interaction between heparin and the heparin binding domain of VEGF did not suppress the biological activity of VEGF.

Figure 5 also showed that there wasn't significant difference between collage/VEGF and HC-collagen/VEGF groups. That might be related with saturation of VEGF receptor by overdose VEGF or the contact inhibition of the cells make the further proliferation unavailable.

Here we proved that VEGF immobilized on collagen could still have the biological activity. With this in mind, we executed the study in vivo.

3.6 Cellularization and vascularization of VEGF loaded scaffolds were improved in vivo

In order to investigate the cellularization and vascularization of the scaffolds, Haemalaun & Eosin (H.E.) staining and immunohistochemistry examination were performed after 3 weeks implantation.

Cells infiltration and new blood vessels invasion of each implant are shown by H.E. staining (Fig. 6). As shown in representative photographic images, cells seemed to be randomly distributed within whole matrices and capillaries with intact borders were also present. Several kinds of cells took part in the infiltration of scaffolds beneath the dermis, mainly macrophages and fibroblasts [23]. The localized release VEGF could promote a local guidance of cells invasion. The red cells confined within defined capillary walls showed the functional new blood vessels. Few new blood vessels were found in DBM group. On the other hand, more functional blood vessels were present in heparin modified scaffolds loaded with VEGF, similar to the findings of Steffens et al. [34]. The result showed that HC-DBM/VEGF group produced significant angiogenic response, while DBM group just resulted in moderate angiogenic response. All of the growth factors, VEGF, fibroblast growth factor (FGF) and platelet derived growth factor (PDGF), participate the new blood vessels maturation [39]. A single growth factor seems not sufficient to develop a normal vasculature [40, 41]. There were two possible reasons for heparin modified DBM with VEGF resulting in more new blood vessels formation. One was that sustained VEGF might be capable of upregulating the expression of other GFs which functioned together with VEGF to develop new blood vessels formation. The other

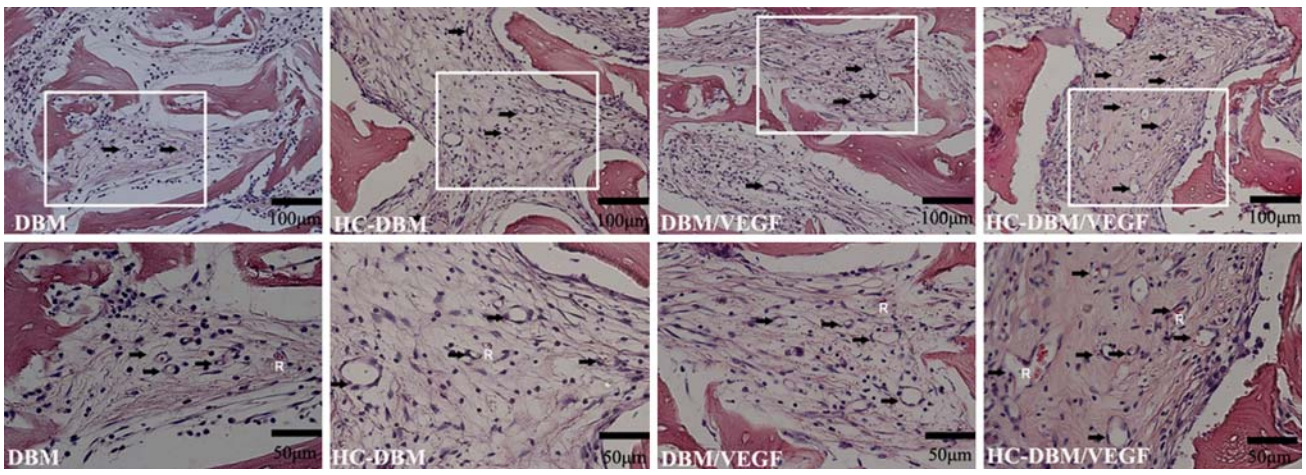

Fig. 6 Histological evaluation of scaffold explants after 3 weeks. Haematoxylin-eosin staining of DBM group, HC-DBM group, DBM/VEGF group and HC-DBM/VEGF group show cells and new blood vessels (*arrow*) invasion into the scaffolds. Capillaries with intact borders were present and red cells (marked by R) confined within defined capillary walls show the functionalization of new blood vessels

possible reason was that, after releasing VEGF, heparinized DBM could bind other free GFs in vivo which functioned on the formation of new blood vessels.

The vascularization of the biological constructs was showed by representative photographic images of immunohistochemical evaluation (Fig. 7a) and was quantified by assessing the new blood vessels density (Fig. 7b). Here we found there existed significant difference not only between the experiment groups (DBM/VEGF and HC-DBM/VEGF) and control groups (DBM and HC-DBM), but also the DBM/VEGF group and HC-DBM/VEGF group. Furthermore the HC-DBM/VEGF group showed the greatest blood vessels density (31.4 ± 3.9). There was significant difference between HC-DBM/VEGF group and DBM/VEGF (22.0 ± 2.6) group in microvessels density ($P < 0.01$). These results indicated that VEGF on DBM could promote the vascularization and the heparin cross-linked DBM could absorb more VEGF to enhance this promoting effect than the natural absorbing.

VEGF burst releasing from scaffold could be degraded by proteolysis within 10–20 min in vivo [18, 42, 43]. While the sustained delivery system of VEGF from HC-DBM could protect VEGF from degradation by protease in vivo, maintain its biological activity and result in significant angiogenic response. Furthermore, the sustained release of VEGF from HC-DBM could provide a sustained promotion of new blood vessels formation and result in more vessels formation in the porous matrices than that of burst release. The localized delivery of VEGF also confined its function within the construct and prevented VEGF dispersing to unexpected tissues, avoiding the adverse reactions. These results, along with data of HUVECs proliferation and release on the collagen, directly or indirectly demonstrated that as a kind of scaffold, the biological

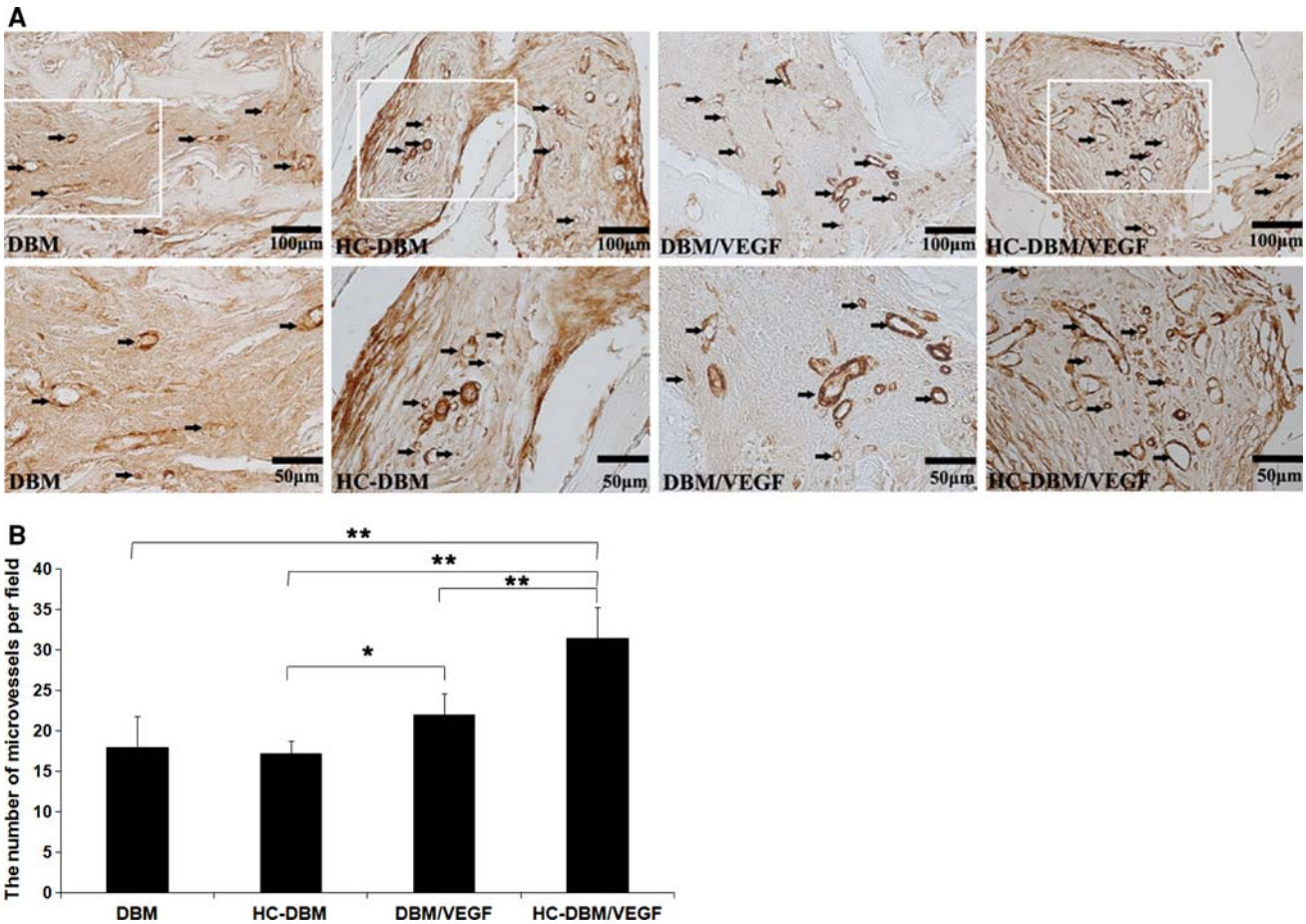

Fig. 7 Immunohistochemical evaluation of the implants after 3 weeks. (**a**) Representative images of DBM group, HC-DBM group, DBM/VEGF group and HC-DBM/VEGF group show new blood vessels (*arrow*) in scaffolds. HC-DBM/VEGF group displayed highest density of new blood vessels compared to other three groups. (**b**) The blood vessels densities were calculated from immunohistochemical staining. HC-DBM/VEGF group showed significantly more blood vessels density than other three groups. Columns show mean values and error bars represent the corresponding standard deviations (n = 4). * Statistically significant difference, $P < 0.05$. ** Statistically significant difference, $P < 0.01$

function of DBM could be significantly improved by heparin modification while loading with VEGF.

4 Conclusions

Our results indicated that more VEGF could be immobilized on the heparin-modified DBM than bound naturally. The sustained and localized delivery system of VEGF from heparinized DBM was developed. After loading with VEGF, HC-DBM had greater biological activity of promoting cells infiltration and capillary invasion in scaffolds in vivo than unmodified DBM.

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