Pre-clinical evaluation of thermosensitive decellularized adipose tissue/platelet-rich plasma interpenetrating polymer network hydrogel for wound healing

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ABSTRACT

Wound healing remains a challenge worldwide, and an ideal wound dressing that promotes healing is urgently needed. In this study, we developed a thermosensitive injectable hydrogel known as the thermosensitive decellularized adipose tissue/platelet-rich plasma interpenetrating polymer network (t-DPI) hydrogel based on decellularized adipose tissue (DAT) and temperature-controlled platelet-rich plasma (t-PRP). Abundant platelets, growth factors (GFs), and bioactive substances from the decellularized extracellular matrix (dECM) in the t-DPI hydrogel had positive effects on wound healing. The morphology, thermosensitivity, and GFs release properties of the t-DPI hydrogel were studied. In vitro, the t-DPI hydrogel showed ideal cytocompatibility and the abilities to promote the proliferation, migration and tube formation of human umbilical vein endothelial cells (HUVECs). Moreover, M2 macrophage polarization was enhanced after treated with t-DPI hydrogel. In vivo, the t-DPI hydrogel notably accelerated the full-thickness wound healing. The positive role of the t-DPI hydrogel on proangiogenesis, macrophage polarization and collagen deposition were validated in the nude mouse full-thickness skin defect model. In addition, the clinical application potential was confirmed using a pre-clinical porcine full-thickness wound model. Overall, this study demonstrated that the t-DPI hydrogel achieves fast and ideal wound healing in full-thickness wound defects and provides a potential clinical treatment strategy.

1. Introduction

Around 14.5% of Medicare beneficiaries (8.2 million patients) suffered from at least one type of wound or wound-related infection in 2014, and $28.1 to $96.8 billion were spent for all kinds of wounds [1]. When skin injuries occur for various reasons, wound healing involves four consecutive overlapping steps: hemostasis, inflammation, proliferation, and dermal remodelling [2]. Various types of cells, including endothelial cells, macrophages, keratinocytes, fibroblasts, are involved in the healing process. The migration, infiltration, proliferation, and differentiation of these cells are closely related to the inflammatory response, tissue regeneration, and finally wound healing. Multiple growth factors (GFs), cytokines, and chemokines participate in regulating the biological behavior of these cells and the complex healing process [3–5].

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Angiogenesis plays an essential role in wound healing [6]. Proangiogenesis methods have been reported as therapeutic strategies for promoting wound healing [7]. Moreover, M2 macrophage polarization at a certain period is necessary during tissue regeneration, including wound repair [8,9]. Dysregulation these biological processes can delay healing, which severely affects patients’ quality of life. Currently, a wound dressing that promote wound healing at a low cost, and is clinically feasible, is urgently needed.

Platelet-rich plasma (PRP), which contains platelets, multiple GFs, and a fibrin network, has been shown to improve wound healing [10]. Various methods for preparing PRP exist [11], and a novel convenient preparation method without exogenous thrombin and anticoagulant, named temperature-controlled PRP (t-PRP) was presented [12]. In this method, the activation of PRP and the formation of the fibrin network were controlled by temperature changes. Evidences shows that PRP could accelerate wound healing owing to its capacity for pro-angiogenesis and regulation of macrophage polarization [13–15]. In addition, a new mechanism for PRP in promoting skin regeneration, in which platelets could improve the pro-angiogenic ability of mesenchymal stem cells (MSCs) via respiratory-competent mitochondrial transfer, was presented [16]. However, the therapeutic effectiveness of PRP is restricted by the poor stability of its fibrin network and the burst release of GFs [17,18].

The interpenetrating polymer network (IPN) of collagen–fibrin hydrogel sheds some light on reinforcing the structure of PRP. The principle of fibrin network formation in PRP is similar to the fibrin hydrogel gelation process, in which thrombin converts fibrinogen to fibrin, which assembles as fibrin gels. Fibrin hydrogels are transitory materials in vivo that degrade rapidly compared to collagen hydrogels [19]. Moreover, strong interactions between collagen and fibrin exist in collagen–fibrin hydrogel after mixing the pre-polymerized form of the collagen and fibrin hydrogels [19]. The addition of extra collagen during

Scheme 1. Fabrication and application of the t-DPI hydrogel. (A) Preparation of t-DPI hydrogel. (B) Characterization of t-DPI hydrogel. (C) Formation process of t-DPI hydrogel. (D) Application of t-DPI hydrogel in wound healing.
the formation of the fibrin network in PRP might enhance its poor stability and trap GFs in the interpenetrating polymer network, which could lead to better sustained release.

Compared with single collagen, decellularized extracellular matrix (dECM) comprises collagens (I, III, IV, VI and VII), glycosaminoglycans, laminin, elastin, and fibronectin and exhibits better biological activity in tissue engineering [20,21]. The decellularized extracellular matrix (dECM) from adipose tissue, decellularized adipose tissue (DAT), can be harvested in vast amounts from liposuction surgical waste [22]. The formation of DAT hydrogels, as well as other dECM hydrogels involves collagen self-assembly processes regulated in part by glycosaminoglycans, proteoglycans, and (extracellular matrix) ECM proteins [23,24]. DAT contributes to various forms of tissue regenerations, including wound healing, bone regeneration and adipose tissue regeneration, owing to its abundant biologically active substances [22,25–27].

The combination of PRP and DAT may form an IPN hydrogel with an ideal role in tissue regeneration, which could be applied to promote wound healing. To prove this hypothesis, we developed a thermosensitive injectable hydrogel, named thermosensitive DAT/PRP interpenetrating polymer network (t-DPI) hydrogel, based on DAT hydrogel and t-PRP (Scheme 1A, C). Both DAT and t-PRP are derived from the human body and have ideal biological activity and low rejection risk. The IPN in the t-DPI hydrogel was more stable compared to the fibrin network in PRP. When the temperature increased from 4 °C to 37 °C, PRP was activated, and the t-DPI hydrogel started gelling, which favors irregular wound treatment. Moreover, abundant platelets, GFs, and bioactive substances from the dECM in the t-DPI hydrogel were significant promoting wound healing. In this study, the morphology, thermosensitive properties and GFs release of the t-DPI hydrogel were investigated. The cytocompatibility and the effect of the t-DPI hydrogel on M2 macrophage polarization and pro-angiogenesis of t-DPI hydrogel were demonstrated in vitro. Furthermore, these biological functions were validated in a nude mouse full-thickness skin defect model. Finally, a porcine full-thickness skin defect model was established to simulate the clinical applications of the t-DPI hydrogel (Scheme 1D).

2. Materials and methods

2.1. Preparation of t-PRP

Temperature-controlled PRP was prepared using a variant of method described in a previous study [12]. Venous blood was obtained from healthy adult volunteers with precooled vacuum blood collection tubes, which contained only blood separating gel without an anticoagulant. The whole blood was centrifuged for 10 min (200 g, 4 °C), and the plasma was transferred to new precooled blank tubes and centrifuged again for 10 min (1550 g, 4 °C). The supernatant plasma was partly discarded, and unactivated t-PRP was obtained by resuspending the precipitated platelets with the remaining plasma at the bottom, and were stored temporarily in hypothermic conditions for further use. Unactivated t-PRP (1 mL) was obtained from 10 mL whole blood. Activated t-PRP was obtained after incubation at 37 °C for 15 min. Venous blood from a Bama miniature pig was obtained for preparing t-PRP following the aforementioned procedure.

2.2. Preparation of DAT and DAT hydrogel

Human lipoaspirate was decellularized, lypophilized, and milled to obtain DAT powder according to a previous described method [28]. The DAT powder was digested in HCl and pepsin solutions. The mixed solution was stirred at room temperature until DAT was completely dissolved. Then, the pH was adjusted to 7.4 with NaOH and PBS to obtain the DAT pre-gel solution. The hydrogel was formed after the pre-gel solution was incubated at the 37 °C.

2.3. Preparation of t-DPI hydrogel

Unactivated t-PRP and DAT pre-gel were mixed with screw syringes and Luer-Lok connectors under hypothermic conditions to obtain t-DPI pre-gel. And t-DPI hydrogels with 30%, 50%, and 70% t-PRP (v/v) were fabricated (30 t-DPI, 50 t-DPI, and 70 t-DPI, respectively) for further studies. The final concentration of DAT in the hydrogel was controlled at 8 mg/mL by adjusting the concentration of the DAT pre-gel before mixing.

2.4. Biocompatibility of the hydrogel

2.4.1. Hemolysis assay

Hemolysis assay was conducted according to previous studies [29]. Fresh blood from healthy volunteers was drawn to obtain red blood cells (RBCs), which were then resuspended in saline at a volume concentration of 5% (v/v). Then, the RBCs suspensions were incubated with t-PRP gel, DAT hydrogel and t-DPI hydrogels (with 30%, 50%, and 70% t-PRP (v/v)) at 37 °C for 1 h. Saline and 0.1% Triton X-100 served as the negative and positive controls, respectively. All samples were centrifuged at 2000 rpm for 10 min to obtain the supernatants. Finally, 100 μL of supernatant from each sample was transferred to a 96-well plate and the absorbance was measured at 540 nm by a microplate reader (Tecan, Infinite 200 PRO, Switzerland). The hemolysis ratio was calculated by: ([A]test-[A]negative)/([A]positive-[A]negative) × 100%. The RBCs were observed under a microscope (Nikon, ECLIPSE, Ts2-RFL, Japan).

2.4.2. Live/dead assay

For the live/dead assay, human umbilical vein endothelial cells (HUVECs) were seeded in 24-well plates (5 × 10^4 cells per well). The cells were incubated with different interventions which were added in the upper chamber of the Transwell (NEST, 725,301, China). After 72 h of treatment, a Calcein-AM/PI Double Stain Kit (YEASEN, 40747ES76, China) was used to detect the viability of HUVECs. Images were captured by a fluorescence microscope (Nikon, ECLIPSE, Ts2-RFL, Japan).

2.5. Cell proliferation assay

2.5.1. Cell Counting Kit 8 assay

HUVECs were seeded in 96-well plates (3 × 10^3 cells per well) and cultured with the extract medium of hydrogels. After being treated for 1 day and 3 days, the culture medium was replaced with 100 μL of fresh medium with 10% (v/v) Cell Counting Kit 8(CCK-8) solution (BOSTER, AR1160, China), and incubated for 2 h at 37 °C. The absorbance was measured by a microplate reader (Tecan, Infinite 200 PRO, Switzerland) at 450 nm.

2.5.2. Edu proliferation assay

HUVECs were in 24-well plates (5 × 10^4 cells/well) and treated with different interventions, which were added to the upper chamber of the Transwell (NEST, 725,301, China). After 72 h of intervention, Beyo-Click™ Edu Cell Proliferation Kit (Beyotime, C0078S, China) was applied to determine the proliferation of HUVECs. The experiments were performed following the manufacturer's instructions. Fluorescence was detected using a fluorescence microscope (Nikon, ECLIPSE, Ts2-RFL, Japan).

2.6. Migration and tube formation assay

2.6.1. Scratch wound assay

The scratch assay was performed for assessing the migration of HUVECs. The cells were seeded in 6-well plates (1 × 10^5 cells/well) and cultured until the confluence of cells reached 90%. Artificial wounds were created using 200-μl pipette tips. After the plates were washed...
gently with PBS, the culture medium was replaced with fresh serum-free medium. Different treatments were added to the upper chamber of the Transwell (NEST, 725,301, China) and cocultured with cells. Bright-field images were collected by a microscope (Nikon, ECLIPSE, Ts2R-FL, Japan) at 0 and 24 h. Images were assessed by the Image J software (NIH, Image J 1.8, USA).

2.6.2. Tube formation assay

Angiogenesis in vitro was evaluated via tube formation assays. The wells in 24-well plates were precoated with Matrigel (BD, 354,230, USA). HUVECs were seeded in the wells (5 x 10^4 cells/well) in serum free medium. The interventions were performed by adding them to the upper chamber of the Transwell (NEST, 725,301, China). Bright-field images were captured with a microscope (Nikon, ECLIPSE, Ts2R-FL, Japan) at 12 h.

2.7. Macrophage regulation in vitro

For the macrophage polarization assay, RAW 264.7 cells were seeded in 24-well plates (5.0 x 10^4 cells/well). The cells were treated by adding different interventions to the upper chamber of the Transwell (NEST, 725,301, China), and cocultured for 48 h.

2.7.1. Immunofluorescence staining

The samples were fixed in 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with 5% BSA for 30 min. Next, the primary antibodies anti-F4/80 antibody (1:100, Servicebio, GB113373, China), and anti-CD206 antibody (1:100, Servicebio, GB113497, China) were added to the cells at 4 °C for 12 h and subsequently washed off. The samples were incubated with Cy3-conjugated goat anti-rabbit IgG (1:200, Servicebio, GB21303, China), and FITC-conjugated goat anti-rabbit IgG (1:200, Servicebio, GB22303, China) at room temperature for 1 h, followed by DAPI staining for 10 min. Fluorescence images were collected using a fluorescence microscope (Nikon, ECLIPSE, Ts2R-FL, Japan).

2.7.2. Quantitative real-time PCR

Total RNA from cells was extracted using TRIzol reagent (Invitrogen, 15,596,018, USA). It was then transcribed into first-strand cDNA and subjected to qRT-PCR. GAPDH and the M2 markers Arg1, Fizz1, and Ym1 were used. The primer sequences of the target genes are provided in Table S1.

2.8. Characterization of hydrogel

2.8.1. Scanning electron microscopy analysis

For scanning electron microscopy (SEM) analysis, the hydrogels (fresh samples without lyophilizing or gold-coating) were placed on a stub and sputter-coated with gold before their application. In the hemolytic test, the positive control group was treated with 1% Triton. The hydrogels were dried at 15 kV. Phosphomonoesterase (PME) activity was measured in the absence or presence of EDTA in each sample. All animal experiments were approved by the Ethics Committee of the Fourth Military Medical University. Thirty-two BALB/c nude mice were randomly assigned to four groups (control, t-PRP, DAT hydrogel, and t-DPI hydrogel). The mice were anaesthetised by inhalation of isoflurane. An 8 mm biopsy punch was used to create two circular full-thickness wounds on the back skin of each mouse after preoperative sterilisation. After treated with different interventions in the four groups, the wounds were covered with transparent dressing (3 M, Tegaderm, USA), and the interventions were applied once a week. On day 0, 3, 7 and 14, the wound closure was captured using a digital camera and analysed using Image J software (NIH, Image J 1.8, USA).

2.10. Full-thickness wound healing study using the porcine model

All animal experiments were approved by the Ethics Committee of the Fourth Military Medical University. The Bama miniature pig was anaesthetised with Zoletil and maintained with propofol. Sixteen full-thickness wounds (3 x 3 cm^2) were created by surgical scalpel and divided into four groups (control, t-PRP, DAT hydrogel, t-DPI hydrogel). Autologous venous blood was obtained from Bama miniature pig for the preparing the t-PRP and t-DPI hydrogel. After treated with different interventions in the four groups, the wounds were covered with transparent dressing (3 M, Tegaderm, USA), and the interventions were applied weekly. On day 0, 7, 14, 21, 28 and 35, the wound closure was captured with a digital camera and analysed by Image J software (NIH, Image J 1.8, USA).

2.11. Histology analysis and immunostaining

Wound tissue samples from the BALB/c nude mice were collected on day 7 and 14 after injury, and fixed in 4% paraformaldehyde. All the tissues were embedded in paraffin blocks and sliced into paraffin sections. These sections were processed and stained with hematoxylin and eosin (H&E), and Masson staining. For immunofluorescence staining, anti-F4/80 (1:100, Servicebio, GB113373, China), anti-CD206 (1:100, Servicebio, GB113497, China), anti-α-SMA (1:400, BOSTER, BM0002, China) antibodies were used. The following secondary antibodies applied were used: Cy3-conjugated secondary antibody (1:200, Servicebio, GB21303, China), FITC-conjugated secondary antibody (1:200, Servicebio, GB22303, China), Cy3-conjugated secondary antibodies (1:200, BOSTER, BA11513-3, China) and FITC-conjugated secondary antibody (1:200, BOSTER, BA1101, China). Wound tissue samples from the Bama miniature pig were collected on day 35, and fixed in 4% paraformaldehyde. All the tissues were embedded in paraffin blocks and sliced into paraffin sections. Then, the sections were then processed and stained with hematoxylin and eosin (H&E), and Masson staining. Images were captured with a microscope (Nikon, C2, Japan) and a slide scanner microscope (Olympus, VS200, Japan), and analysed by the Image J software (NIH, Image J 1.8, USA).

2.12. Statistical analyses

Statistical analysis was performed using the Prism software (GraphPad Software, GraphPad Prism version 6.02, USA). Data from the experiments were expressed as means ± standard deviation (M ± SD). One-way ANOVA was used to determine the statistically significant differences, and the statistically significance was determined when p < 0.05.

3. Results

3.1. In vitro hemocompatibility and cytocompatibility evaluations

Assessment of the biocompatibility of materials is indispensable before their application. In the hemolytic test, the positive control group (Triton X-100) showed an apparent red color, whereas the DAT hydrogel group and t-DPI (30 t-DPI, 50 t-DPI and 70 t-DPI) hydrogel groups...
showed similar color to the negative control group (PBS) (Fig. 1A). The integrality of red blood cells (RBCs) in experimental groups was consistent with that in the negative control (treated with PBS) (Fig. 1B). In addition, the statistical graph in Fig. 1C demonstrated that the hemolysis ratio in experimental groups was significantly lower than 5%, exhibiting excellent hemocompatibility. The cytocompatibility of the t-DPI hydrogel was validated by the live/dead assay of HUVECs. Live/dead staining indicated that most cells were viable (green), and only a few dead cells (red) exist in control group, DAT hydrogel group and t-DPI (30 t-DPI, 50 t-DPI and 70 t-DPI) hydrogel groups (Fig. 1D). As a nature source hydrogel derived from the adipose tissue and blood of the human body, the t-DPI hydrogel showed ideal biocompatibility.

3.2. Cell proliferation assay

Cell counting kit 8 and EdU proliferation assays were conducted to assess the effect of the t-DPI hydrogel on the proliferation of HUVECs. The proliferation of HUVECs for up to 3 days was determined using the
CCK 8 assay (Fig. 1E). The optical density (OD) values in all groups were not statistically different on day 1. However, on day 3, the OD values increased with the raise of t-PRP proportion in t-DPI hydrogel, and no statistically difference was observed in the 50 t-DPI hydrogel and 70 t-DPI hydrogel groups. Moreover, the results showed a similar tendency in the EdU proliferation assay (Fig. 1F and G), with the increasing proportion of t-PRP (v/v) in t-DPI hydrogel, the proportion of EdU-positive cells was gradually increased compared to the DAT hydrogel and control groups. The proportion of EdU positive cells was not statistically different between the 50 t-DPI hydrogel (52.98 ± 6.67%) and 70 t-DPI (55.02 ± 7.18%) hydrogel groups (P = 0.9872). The t-DPI hydrogels presented strong capability in promoting the proliferation of HUVECs. The biocompatibility of the t-DPI hydrogel was further confirmed in these two assays. Moreover, the above results provide some clues on selecting the appropriate proportion of t-PRP (v/v) in the t-DPI hydrogel.

3.3. Angiogenesis assay in vitro

Timely and adequate angiogenesis is crucial during wound healing process [30,31]. The capability of the t-DPI hydrogel to promote angiogenesis in vitro was determined by the migration and tube formation assays. The scratch assay was performed to determine the migration of HUVECs. The results indicated that HUVECs migrated significantly faster in the 30 t-DPI, 50 t-DPI and 70 t-DPI hydrogel groups than in the DAT and control groups, and no significant difference existed between the 50 t-DPI and 70 t-DPI hydrogel groups (Fig. 2A and B). In the tube formation assay, more vessel-like structures were observed in t-DPI hydrogels groups (Fig. 2C). The total tube lengths in the 50 t-DPI (7.098 ± 0.654 mm) and 70 t-DPI (7.645 ± 0.898 mm) hydrogel groups were significantly longer than those in other groups, while no significant difference existed between the 50 t-DPI and 70 t-DPI hydrogel groups (P = 0.8196) (Fig. 2D). Both the migration and tube formation assays indicated the well ability of the t-DPI hydrogel to promote angiogenesis in vitro.
To minimise the expected harm to patients in clinically applications, we expect to achieve a maximum effect with a minimum amount of autologous blood from patients. Therefore, combined with the results of the aforementioned cell proliferation and angiogenesis assays in vitro mentioned earlier, the 50 t-DPI hydrogel (50% t-PRP (v/v), DAT final concentration: 8 mg/mL) was selected for subsequent experiments.

3.4. Macrophage polarization assessment in vitro

M2 macrophages are associated various tissue regeneration process, including wound healing [32,33]. Immunofluorescence staining and quantitative real-time PCR were performed in vitro to confirm the effects of the t-DPI hydrogel on M2 macrophage polarization. As Fig. 2E and F shown, the treatment of DAT hydrogel and t-PRP could promoted M2 macrophage polarization, as the intensity of CD206 increased compared with that in the control group (P = 0.0365, P = 0.0066, respectively). Moreover, the t-DPI hydrogel group demonstrated the highest staining intensity of CD206 compared to the DAT hydrogel and t-PRP groups (P = 0.0121, P = 0.0025, respectively). The results of qPCR on M2 macrophage markers (Arg1, Fizz1, Ym1) further demonstrated these trends (Fig. 2G). These results indicated the capability of t-DPI hydrogel on promoting M2 macrophage polarization, and activating the process of regeneration.

3.5. Characterization of the t-DPI hydrogel

For the t-DPI hydrogel preparation, unactivated t-PRP and DAT pre-gel were mixed under hypothermic conditions to obtain t-DPI pre-gel, which could be gelled and activated at 37 °C (Scheme 1A). The 50 t-DPI hydrogel (50% t-PRP (v/v), DAT final concentration: 8 mg/mL) was chosen based on the in vitro results described earlier. The t-PRP, DAT hydrogel and t-DPI hydrogel can be injected with 23G needles (Fig. S1). For the t-DPI hydrogel preparation, unactivated t-PRP and DAT pre-gel were mixed under hypothermic conditions to obtain t-DPI pre-gel, which could be gelled and activated at 37 °C (Scheme 1A). The 50 t-DPI hydrogel (50% t-PRP (v/v), DAT final concentration: 8 mg/mL) was chosen based on the in vitro results described earlier. The t-PRP, DAT hydrogel and t-DPI hydrogel can be injected with 23G needles (Fig. S1). As Fig. 3A shown, t-PRP, DAT hydrogel and t-DPI hydrogel were in the liquid states at 4 °C. After being injected into heart-shape molds, and maintained at 37 °C for 15 min, t-PRP did not retain its shape upon removal from the mold with liquid dropout, whereas the DAT and t-DPI hydrogels remained heart-shape. In addition, the t-DPI hydrogel was more stereoscopic, with sharp edges compared to the DAT hydrogel. As an injectable hydrogel with thermosensitivity and good shapeability, the t-DPI hydrogel is suitable for irregular wound therapy.

Fresh samples without lyophilization or gold-coating were subjected to SEM analysis to obtain more realistic structure images. The fibrin polymers network in t-PRP was shown in higher magnified image (Fig. 3B, upper right). The DAT hydrogel presented an irregular structure and interconnected pores with different sizes (Fig. 3B, middle). Although collagen I is the main component of the DAT hydrogel, multiple peptides and small molecular weight peptide fragments exist, and the micro-structures of the DAT and collagen I hydrogels remain different [28]. A 3D morphology with smaller and more uniform pores was observed in t-DPI hydrogel (Fig. 3B, below). Fibrin meshes were found on the pore connections and walls in the dense network of the t-DPI hydrogel, indicating the formation of the IPN.

Various GFs in PRP, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), are major contributors to its capability of promoting wound healing. VEGF is known as a key dynamic molecule of angiogenesis [34]. PDGF participates in inflammation, granulation tissue formation, reepithelialization, matrix formation and remodelling [3]. Various studies showed that EGF promotes epithelization and accelerates the healing process [35]. The rapid release of GFs from PRP limits its clinical therapeutic effects, thus, the t-DPI hydrogel was devised achieve the sustained release effect. ELISA assay was conducted to further determine the release curves of VEGF, PDGF-BB, and EGF. As Fig. 3 C, D and E shown, in t-PRP group, more than half of PDGF-BB and EGF were released in the first 6 h. The release curve of VEGF is relatively gentle by comparison, but the accumulative release still reached 50% within 12 h. The t-DPI hydrogel exhibited lower burst release and better sustained-release properties of VEGF, PDGF-BB, and EGF compared to t-PRP. The IPN formation in t-DPI hydrogel with GFs trapped in may contribute significantly to this effect.

3.6. Wound healing assessment in the nude mouse full-thickness wound model

The above results illustrated the favorable biocompatibility, the abilities to promote cell proliferation, angiogenesis and M2 macrophage polarization, and the characterization of the t-DPI hydrogel in vitro, which indicated that the t-DPI hydrogel is suitable for cutaneous wound treatment.

For the determination of the wound healing ability in vivo, a nude mice model was used to evaluate the efficacy of t-DPI hydrogel. As shown in Table 1, the wound closure rate was significantly higher in the t-DPI hydrogel group compared to the control group (P = 0.0025, respectively). The results of qPCR on M2 macrophage markers (Arg1, Fizz1, Ym1) further demonstrated these trends (Fig. 2G). These results indicated the capability of t-DPI hydrogel on promoting M2 macrophage polarization, and activating the process of regeneration.

![Fig. 3. Characterization of t-DPI hydrogel. (A) Photographs of t-PRP, DAT hydrogel and t-DPI hydrogel at 4 °C and 37 °C. (B) SEM images of t-PRP, DAT hydrogel and t-DPI hydrogel. (C) Release curve of VEGF in vitro. (D) Release curve of PDGF-BB in vitro. (E) Release curve of EGF in vitro.](image-url)
mouse full-thickness wound model (8 mm diameter, round) was estab-
lished. We prepared the DAT hydrogel, t-PRP and t-DPI hydrogel for the
interventions of the full-thickness wounds. The healing process in the
control, t-PRP group, DAT hydrogel, and t-DPI hydrogel groups was
shown Fig. 4A and B. Consistent with expectations, the t-DPI hydrogel
group exhibited the fastest wound closure rate compared with other
groups throughout the wound healing process. As shown in Fig. 4C, by
the third day, the wound closure rate in the t-DPI hydrogel and t-PRP
groups was significantly higher than in the control group. Moreover, the
wound healed faster in the t-DPI hydrogel group than in the t-PRP group
(\( p = 0.0324 \)). On day 7, the DAT hydrogel and t-PRP groups exhibited
faster wound closure rates than the control group. The wound closure
rate in the t-DPI hydrogel reached 73.63 ± 4.80%, which was
significantly higher than the t-PRP (65.10 ± 6.41%) and DAT hydrogel
(62.43 ± 5.82%) groups. On day 14, the wound area in the DAT
hydrogel, t-PRP and t-DPI hydrogel groups was markedly reduced.
Wounds in the t-DPI hydrogel group were almost completely healed
(97.55 ± 2.95%). These results indicated the excellent capability of the t-
DPI hydrogel in promoting wound healing.

H&E staining was conducted to further evaluate the morphological
changes during the healing process (Fig. 4D). H&E staining of healthy
skin from nude mouse was showed in Fig. S3A. On day 7, the t-DPI
hydrogel group exhibited thick neo-epidermis, whereas the epidermis in
t-PRP and DAT hydrogel groups was incomplete, and the neo-epidermis
was found in the margin of the granulation tissue in the control group. On
day 14, compact and dense epidermis, and well-structured dermis existed

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**Fig. 4.** Wound healing capability assessment in the nude mouse full-thickness wound model. (A) Representative photos of wounds during healing (The inner diameter of the white ring: 9 mm). (B) Schematic diagram. (C) Quantitative data of wound closure rate. (D) H&E staining of wound samples from four groups on day 7 and 14 (Scale bar in low magnification: 1 mm, scale bar in high magnification: 200 μm). \( *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; n = 8 \).
in t-PRP and t-DPI hydrogel groups. Moreover, the neogenic hair follicles in the t-DPI hydrogel group indicated the most optimal skin maturation. The synthesis and deposition of collagen are crucial for tissue reconstruction during wound healing [36]. Masson staining was performed to reveal the differences in collagen deposition and remodelling during the healing process among the four groups (Fig. 5A). Collagen deposition and remodelling in the t-DPI hydrogel group performed the most ideal phenotype among the four groups. The collagen fibers were compact and in an ordered arrangement, indicating more similar phenotype to normal skin (Fig. S3B). In the control group, the collagen fibers were loose and immature compared to those in other three groups. As shown in the statistical plot (Fig. 5B), collagen deposition in the DAT hydrogel and t-PRP groups was significantly higher than in the control group. Moreover, collagen deposition in the t-DPI hydrogel group reached 71.23 ± 7.30%, which showed significant differences to other groups. The results of H&E and Masson staining demonstrated the therapeutic effects of the t-DPI hydrogel in promoting reepithelialization, collagen deposition and remodelling.

As appropriate angiogenesis is indispensable during wound healing, various interventions to promote angiogenesis are commonly utilized [37,38]. Immunofluorescence staining of CD31 was performed to evaluate the angiogenesis in vivo (Fig. 5C and E). The number of CD31 positive vessels decreased from day 7–14, and the diameter increased from day 7–14. The DAT hydrogel and t-PRP groups indicated superior effect in promoting angiogenesis than the control group. The blood vessel density in the t-DPI hydrogel group increased significantly compared to that in other groups on both day 7 and 14. In addition, immunofluorescence staining for α-SMA was performed to evaluate the mature blood vessels (Fig. 5C, F). The number of SMA positive vessels increased from day 7–14. Similarly, the t-DPI hydrogel group exhibited most abundant mature blood vessels. These results indicated that the t-DPI hydrogel promoted wound healing by enhancing vascular regeneration and maturation.

M2 macrophage polarization has been widely reported as beneficial in promoting tissue regeneration, including wound healing [39,40]. The F4/80, a pannaphagocyte marker, and CD206, a surface marker of M2 macrophages, were chosen to display the M2 macrophage polarization in vivo (Fig. 5D, G). The DAT hydrogel and t-PRP groups exhibited higher percentage of M2 macrophages than the control group (P = 0.0194, P = 0.0053, respectively). The percentage of M2 macrophages in the t-DPI hydrogel reached 47.49 ± 2.62%, which was significantly higher than those of the t-PRP and DAT hydrogel groups (P = 0.0391, P = 0.0101, respectively). The above results indicated that the t-DPI hydrogel promoted the M2 macrophage polarization in vivo owing to the t-PRP and DAT in the hydrogel.

3.7. Wound healing assessment in the porcine full-thickness wound model

The structures and functions between human and porcine skins are similar, and porcine full-thickness wound model is considered as an ideal in vivo model for pre-clinical wound healing studies [41,42]. Sixteen wounds (3 × 3 cm², square) were excised on the back of the Bama miniature pig and divided into four groups (control, t-PRP, DAT hydrogel and t-DPI hydrogel group).

The healing process in the control, t-PRP, DAT hydrogel, and t-DPI hydrogel groups was shown in Fig. 6A and B. The t-DPI hydrogel group exhibited the best wound closure rate compared to other groups. The wounds were closed with complete epidermis in all groups on day 35. However, the scar area in the control group was larger than those in other groups. Statistical analysis suggested that the t-DPI hydrogel group exhibited a faster wound closure rate than control on day 7, and it showed better healing than any other groups from day 14–28. On day 28, the wound closure rate in the t-DPI hydrogel group reached 96.06 ± 3.05%, indicating the wounds in the t-DPI hydrogel group was almost completely closed. As the wounds were closed in all groups on day 35, statistical analysis was unnecessary.

As H&E staining in Fig. 6D shown, although the complete epidermis existed in all groups, the lengths of the existing granulation tissue (between two black dotted lines) are different. The t-DPI hydrogel group showed the best tissue regeneration, with the smallest portion of immature tissue indicating the highest histological wound healing percentage, while the length of existing granulation tissue in the control group is the longest. Statistical analysis (Fig. S2) further proved the above results. The results of Masson staining showed that there was no obvious difference in the collagen deposition of the existing granulation tissue (between two black dotted lines) at 35 days postoperatively (Fig. 6 E). However, in the entire regenerated skin area, the t-DPI hydrogel group showed a larger darkly stained blue area, indicating more mature healing.

Thus, the above results, which evaluated the effectiveness and feasibility of the t-DPI hydrogel for wound healing in the pre-clinical porcine full-thickness wound model, indicated its the potential for application in clinical patients.

4. Discussion

Wound healing remains serious clinical problems, that affects both the physical and mental health of patients, but also brings an economic burden [43,44]. A wound dressing that promotes the healing process at a low cost, and is clinically feasible is required. PRP, a low-cost and operationally simple blood product, enriched with GFs, has been utilized for a variety of human diseases treatment including wound therapy [10,45,46]. However, the clinical applications of PRP are limited owing to its unstable fibrin network and burst release of GFs. Strong interactions between collagen and fibrin would exist, if they are mixed in a pre-polymerized form. In IPN of collagen-fibrin hydrogels, fibrin and collagen networks are formed independently, with locally established physical or structural interactions [19,47,48]. Based on the above theory, we expect bring the extra pre-polymerized form collagen in unactivated PRP to reinforce the fibrin network with the formation of the collagen-fibrin IPN.

In this study we designed the t-DPI hydrogel, in which the collagen monomers polymerized during DAT hydrogel formation reinforced the fibrin network in t-PRP, and formed an IPN. The microstructure and the GFs release properties of t-DPI hydrogel were showed in previous section. In the t-DPI pre-gel (4 °C), platelets, thrombin and fibrinogen remained unactivated, while collagen from DAT existed in pre-polymerized form. When the temperature increased from 4 °C to 37 °C, fibrinogen converted to fibrin, collagen monomers polymerized owing to their self-assembly property, and ultimately the IPN of collagen-fibrin formed. In addition, platelets were activated with the change in temperature, and released GFs, which were partly trapped in the dense network of the t-DPI hydrogel for further sustained release (Scheme 1B). However, DAT contained collagens (I, III, IV, VI and VII), glycosaminoglycans, laminin, elastin, and fibronectin, more than only collagen. The collagen self-assembly process in ECM hydrogels were regulated in part by the presence of glycosaminoglycans, proteoglycans, and ECM proteins [20,49]. The IPN in t-DPI hydrogel was complex, and requires further investigations.

The effects of the t-DPI hydrogel on wound healing were studied in vitro and in two full-thickness skin defect models. The ability of the t-DPI hydrogel in promoting cell proliferation were demonstrated in HUVECs using Cell Counting Kit 8 and EdU proliferation assays. The effects on macrophage polarization and pro-angiogenesis were validated in HUVECs, RAW 264.7 cells and in the nude mouse full-thickness skin defect model. The results exhibited previously indicated the ideal therapeutic capacity of the t-DPI hydrogel on wound healing via promoting cell proliferation, M2 macrophage polarization and angiogenesis. Moreover, t-DPI hydrogel group showed favorable effect on collagen deposition and remodelling. Finally, the optimal therapeutic effects of the t-DPI hydrogel on the pre-clinical porcine full-thickness wound model further validated its potential for clinical applications in wound treatment.
Fig. 5. Collagen deposition, angiogenesis and macrophage regulation assessment in the nude mouse full-thickness wound model. (A) Masson staining of wound samples from four groups on day 14 (Scale bar in low magnification: 1 mm, scale bar in high magnification: 100 μm). (B) Quantification of the collagen deposition on day 14. (C) Representative images of immunofluorescence (CD31, α-sm) on day 7 and 14 (Scale bar: 100 μm). (D) Representative images of immunofluorescence (F4/80, CD206) on day 7 (Scale bar: 100 μm). (E) Quantification of total blood vessels number in wound area on day 7 and 14. (F) Quantification of mature blood vessel number in wound area on day 7 and 14. (G) Statistical analysis of the percentage of M2 macrophages. (*p < 0.05, **p < 0.01, ***p < 0.001; n = 8).
GFs from the t-PRP in the t-DPI hydrogel greatly contributed to the aforementioned biological behavior. VEGF, well known as a pro-angiogenic factor, acts on Flt-1 (VEGF receptor-1) and KDR (VEGF receptor-2) at the endothelial surface of blood vessels, regulates the chemotaxis and proliferation of endothelial cells [3]. PDGF involves in the entire wound healing process, including promoting inflammatory cell recruitment, granulation tissue formation, collagen deposition, and blood vessel maturation [3,50]. PDGF-BB has been approved by the FDA for the clinical application in the last century [51]. EGF plays a crucial role in accelerating reepithelialization via increasing keratinocyte proliferation and cell migration in acute wounds [52]. It was reported that GFs, such as PDGF and Transforming growth factor (TGF) can promote the division and proliferation of the fibroblasts resulting in collagen deposition [13]. The serotonin and histamine released from platelets can increase the cellular permeability and further amplify the above effects [53]. Moreover, novel mechanisms of PRP in tissue regeneration have been reported recently, in which respiratory competent mitochondria from platelets were transferred to mesenchymal stem cells, and promoted their proangiogenic capabilities via increasing the cytosolic citrate level and stimulating of fatty acid synthesis [16]. Moreover, the effects of PRP on M2 macrophage polarization were validated both in this study and previous research [15]. It has been reported that activated platelets can...
drive monocytes into M2 macrophages through the interaction of platelet-derived CXCL12 with CXCR4 and CXCR7 [54].

Another ingredient of t-DPI hydrogel, DAT positively affected tissue regenerations [26,55,56]. As a biomaterial derived from the native ECM, DAT is in rich in a variety of biologically active ingredients, such as collagen, fibronectin, glycosaminoglycans and various GFs, which could recruit host stem cells, macrophages, fibroblasts, and endothelial cells to the wound area, and regulate reepithelialization, collagen deposition, and angiogenesis [56]. It has been reported that DAT showed the ability on M2 macrophage polarization [57]. Moreover, evidence indicated that dECM hydrogels from other decellularized tissues could promote M2 macrophage polarization [20]. Thus, we speculated and validated that the effect of t-DPI hydrogel on M2 macrophage polarization partly resource from DAT. Previous studies suggested the degradation products of dECM biological scaffolds could affect cell migration and proliferation [58], and showed antibacterial activity [59]. DAT in the t-DPI hydrogel may exert biological functions via its degradation products.

In addition, the t-PRP from human blood and t-PRP from Bama miniature pig were used in this study. Considering the feasibility of clinical application, the t-PRP from human blood and the DAT hydrogel from human adipose tissue were applied for in vitro studies. DAT hydrogel has been reported to be xenogenetically applied for wound healing [26]. However, biologically active substances and a few living cells derived from human exist in t-PRP. Therefore, the nude mouse full-thickness wound model was established to avoid immune reaction for in vivo mechanisms studies. Furthermore, in order to simulate the application of the t-DPI hydrogel in clinical patients (DAT and autologous t-PRP serve as raw materials), the autologous t-PRP from Bama miniature pig was applied in the porcine full-thickness wound model for the t-DPI hydrogel and t-PRP groups treatment.

In our envision, t-DPI hydrogel prepared with autologous blood and allogeneic DAT could be applied in patient with cutaneous wounds. First, DAT from liposapirate will be lyophilized, milled and sterilized. DAT pre-gel will be prepared in the sterile environment. Next, required volume of autologous blood could be calculated based on the area of the wounds. When treatment start, autologous blood will be obtained from the patients to produce unactivated t-PRP, which could be mixed with DAT pre-gel to produce t-DPI hydrogel. Clinical trials of t-DPI hydrogel in wound treatments are needed.

5. Conclusion

In this study, we developed the thermosensitive decellularized adipose tissue/platelet-rich plasma interpenetrating polymer network hydrogel (t-DPI) hydrogel based on DAT and t-PRP. The t-DPI hydrogel exhibited therapeutic effects on wound healing via promoting angiogenesis and M2 macrophage polarization. Furthermore, the clinical application potential of the t-DPI hydrogel was verified in the pre-clinical porcine full-thickness wound model. In general, as a novel nature source wound dressing, t-DPI hydrogel with injectable, thermostimulative and growth factors sustained release properties showed ideal therapeutic effects on wound healing, thereby providing novel low-cost potential options for patients with cutaneous wounds.

Ethics statement

All animal experiments were approved by the Ethics Committee of the Fourth Military Medical University (No. IACUC-20211250). Human tissue collection involved in this research were approved by the Ethics Committee of the Fourth Military Medical University (No. KY20213150-1).

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Credit author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mtbio.2022.100498.

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