Nanotechnology promotes the full-thickness diabetic wound healing effect of recombinant human epidermal growth factor in diabetic rats

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ABSTRACT

We utilized a modified double-emulsion method with poly(lactic-co-glycolic acid) as the carrier to prepare recombinant human epidermal growth factor (rhEGF) nanoparticles. The morphology of the nanoparticles was detected by a transmission electron microscope. The particle size distribution was measured by a laser analyzer with a zeta potential meter. Enzyme-linked immunosorbent assays were performed to determine the rhEGF encapsulation efficiency and release model, and the proliferation of the mouse fibroblasts was analyzed by the MTT method. Diabetic rats with full-thickness wounds were divided into four groups according to different treatments: rhEGF nanoparticles, rhEGF stock solution, empty nanoparticles, and phosphate-buffered saline. Photographs were taken after the treatments to calculate the wound healing rates, and the granulation tissue of the wounds was sampled for pathologic slides. Proliferating cell nuclear antigen was assayed by immunohistochemistry. Our results showed that the rhEGF nanoparticles were around 193.5 nm (diameter), and the particle size distribution was uniform and dispersible. The encapsulation efficiency was 85.6% and rhEGF release lasted 24 hours. Compared with other groups, the rhEGF nanoparticles promoted the highest level of fibroblast proliferation, and this group showed the fastest healing rate. The number of proliferating cell nuclear antigen positive cells in the rhEGF nanoparticles group was higher than the other groups. We concluded that controlled release of rhEGF encapsulated in the nanoparticles enhanced rhEGF effects to stimulate cell proliferation and shorten the wound healing time.

Nonhealing diabetic foot (DF) ulcers have become a problem to health care systems all over the world. Fifteen percent of diabetic patients suffer from DF in their lifetime.[1] It is accepted that long-term hyperglycemia causes damage of blood vessels, nerves, and the immune system resulting in recurrent, persistent, and refractory ulcers in DF patients. Deficiency of epidermal growth factor (EGF) is thought to be one of the pathophysiologic fundamentals in DF ulcers.[2] The local administration of exogenous recombinant human EGF (rhEGF) in DF ulcers has proven to be effective but its short biological half-life has limited the efficiency of this treatment. Moreover, rapid dilution by tissue fluid, leakage from the wound surface, and degradation by enzymes make it difficult for rhEGF to achieve effective concentrations to treat DF ulcers.[3] In order to overcome these shortcomings and optimize the rhEGF treatment, we used a modified double-emulsion method to prepare rhEGF nanoparticles. The nanoparticles were sprayed onto the full-thickness diabetic wounds of rats, and the wound healing effects were evaluated compared with the rhEGF stock solution, the empty nanoparticle, and phosphate-buffered saline (PBS) (solvent for nanoparticles). We also began preliminary investigations into the underlying mechanisms responsible for the wound healing effects of the nanoparticles.

METHODS

Preparation, morphology, and particle size distribution of rhEGF nanoparticles

A modified double-emulsion method was used to prepare the rhEGF nanoparticles.[4] The rhEGF stock solution was purchased from Hua-sheng-yuan Genetic Engineering Company (Shenzhen, China). Fifty microliters of the rhEGF stock solution (0.1 μg/μL) was mixed completely with 200 μL of 3% PEG2000 in PBS to make the inner aqueous phase. Under ultraphonic dispersion (80 W, 2 minutes), 250 μL of the inner aqueous phase was added to 500 μL of 5% poly(lactic-co-glycolic acid) (PLGA) in the organic phase (acetone : dichloromethane = 1 : 1) forming W/O preemulsion. Two milliliters of the outer aqueous phase containing 0.3% F-68 in PBS was added to the preemulsion in a dropwise fashion forming W/O/W complexes under ultraphonic dispersion (80 W, 5 minutes). The W/O/W complexes were evaporated for 4 hours under stirring, and solid particles were obtained after a centrifugation (12,000 × *g*). The particles were washed three times with double distilled water, frozen at −20 °C overnight, and applied in a lyophilizer for 24 hours. The preparation

of empty nanoparticles followed the same procedure, except rhEGF was not added in the inner aqueous phase. A transmission electron microscope (TEM) was used to detect the morphology of the rhEGF nanoparticles. Laser particle size appearance/zeta electric potential appearance was used to analyze the particle size distribution of the nanoparticles.

Detection of rhEGF nanoparticle encapsulation efficiency, rhEGF release model, and biologic activities

The encapsulation efficiency of the rhEGF nanoparticles was evaluated by enzyme-linked immunosorbent assays (ELISA). The anti-rhEGF ELISA kits were purchased from Jing-Mei Biology Product Company (Shanghai, China). The supernatant and washing buffers were collected throughout the entire rhEGF loading procedure, and the amounts of rhEGF were calculated according to the standards supplied in the kit. The amounts were summed and named as the loss of rhEGF. The encapsulation efficiency was calculated as the amount of rhEGF before loading loss of rhEGF/amount of rhEGF before loading. Release activities were also detected by the anti-rhEGF ELISA kits. Five milligrams of rhEGF nanoparticles was suspended in 5 mL of PBS and incubated at 37 °C with shaking ($72 \times g$). At the designed intervals, the nanoparticles were centrifuged and supernatants were collected and analyzed for rhEGF concentrations. Cellular experiments were carried out in the L929 mouse fibroblast cell line, and the MTT assay was used to investigate the effects of the rhEGF nanoparticles on cell proliferation.[5]

Establishment of a full-thickness diabetic wound model in rats

One hundred and thirty male Sprague-Dawley rats (8-week old, 220–260 g) were purchased from the Chinese Academy of Medical Sciences Laboratory Animal Center. All the protocols for animal experiments were approved by the Tianjin Medical University Animal Care and Use Committee. They were individually housed in cages on a 12-hour light/dark cycle (lights on 06:00–18:00 hours) under constant temperature (20–22 °C) with ad libitum access to food and water. The rats were injected with streptozotocin (45 mg/kg in citrate buffer, Sigma-Aldrich, St. Louis, MO) via their tail veins to induce diabetes. After 7 days, 116 out of 130 rats were defined as diabetic with blood glucose levels over 16.7 mmol/L. The remaining rats were injected twice with streptozotocin to reach the same blood glucose level as the diabetic rats. After diabetes modeling, they were anesthetized with 10% chloral hydrate (30 mg/kg) via an abdominal cavity injection and a round, full-thickness dermal wound (1.8 cm in diameter) was made in the left part of the backbone of each rat using a hole puncher. The wounds were subsequently covered by dressings soaked with saline.

Animal groups and treatments

The diabetic rats with the full-thickness wounds were divided into four groups according to different treatments: the rhEGF nanoparticle group ($n=32$), the rhEGF stock solution group ($n=32$), the empty nanoparticle group ($n=32$), and the PBS control group ($n=32$). Each group was subdivided and marked with 1, 2, 3, or 4 representing 3, 7, 14, and 21 days after the treatments, respectively. All treatments were administered locally (spray) to the wounds once a day. The rhEGF nanoparticle group was administered rhEGF nanoparticles containing 1 μg rhEGF (500 U), and the rhEGF stock solution group was administered 1 μg rhEGF stock solution. The empty nanoparticle group was administered empty particles (quality similar to rhEGF nanoparticles group, only without rhEGF) and the PBS control group was administered PBS only (PBS served as the solvent in the other three groups).

Collection of samples and determination of wound healing

Wound healing in the diabetic rats was calculated by photographs taken at different time points. The nonhealing area of the wound was calculated by a computer image analytical system. The healing rate was then calculated with the following equation:

$$\text{Healing rate} = [(\text{primitive area} - \text{nonhealing area})/\text{primitive area}] \times 100\%$$

In each group, pathology observations were made on rats that were sacrificed 3 days ($n=8$), 7 days ($n=8$), 14 days ($n=8$), and 21 days ($n=8$) after treatment. Tissue samples were taken from the wounds to prepare pathological slides and observed under a light microscope.

Immunohistochemical staining for proliferating cell nuclear antigen (PCNA)

Paraffin was removed from the slides and they were washed twice with PBS for 2 minutes each and incubated in 3% H_2O_2 at room temperature for 20 minutes. PCNA was recovered with citrate buffer (by microwave). Primary monoclonal antibodies against PCNA (Ai-Bo-Sen Biologic Company, Beijing, China) were diluted 1 : 100, and incubated with the slides at 4 °C overnight. The slides were washed twice with PBS for 5 minutes each and then incubated with secondary antibodies for 15 minutes at 37 °C. Washed slides were developed by 3,3′ diaminobenzidine solution for 5 minutes. After developing, the slides went through washing (tap water for 2 minutes and distilled water for 1 minutes), counterstaining, anhydration, and mounting. Each group included eight slides. Eight random areas and 100 cells from each slide were counted under high magnification to calculate PCNA-positive fibroblast cells.

Statistical analysis

The statistical analysis was made with SPSS 16.0 (SPSS Inc., Chicago, IL). The variables were tested for normal distribution and homogeneity. The data in the normal distribution were presented as mean ± standard deviation. The multigroup variances were analyzed by the general linear model with repeated measures. The variance between two groups was compared with the LSD post hoc test. A p-value < 0.05 was considered to be significant.

RESULTS

Characteristics of the rhEGF nanoparticles

The diameter and dispersion indexes determined by laser particle size appearance/zeta electric potential appearance were 193.5 nm (mean) and 0.176, respectively (Figure 1A). The morphology of the rhEGF nanoparticles was detected by the TEM and showed spherical, uniform, and well-dispersed particles (Figure 1B). Furthermore, the encapsulated efficiency was 85.6%. The rhEGF-releasing curve was examined by the first-class-releasing kinetic model and the Higuchi-releasing kinetic model. The Higuchi-releasing kinetic model displayed a higher correlation coefficient, and the rhEGF release was calculated with the following kinetic model: $Q=46.80738+10.09451t^{1/2}$ ($R=0.94341$). This model indicated that the rhEGF release time lasted 24 hours (Figure 1C).

The effect of rhEGF nanoparticles on cell proliferation in vitro

The proliferation of L929 mouse fibroblast cells was measured by the MTT method and presented as absorbance (OD). Different concentrations of rhEGF nanoparticles (1–100 μg/L) accelerated the cell proliferation to various degrees compared with the control group, which was not treated with rhEGF nanoparticles ($p < 0.05$). The OD values continually increased when the concentration of the particles was lower than 10 μg/L, whereas the OD values were decreased when the concentration of particles was higher than 10 μg/L. This suggested that the rhEGF nanoparticle promoted the proliferation of L929 cell in a saturable manner (Table 1). Because of this finding, we used 10 μg/L of rhEGF nanoparticles for the following experiments. The abilities of different treatments to stimulate cell proliferation were compared among the different treatment groups (Figure 2), and the rhEGF nanoparticles promoted the largest amount of fibroblast proliferation ($p < 0.05$). The empty particles did not show any effects on the cells similar to the PBS control group ($p=0.231$).

Wound healing in vivo

There was no evidence of wound healing in any of the groups on the third day of treatment (Table 2). On the seventh day of treatment, however, the wounds of the rats in the rhEGF nanoparticle group showed an accelerated healing rate compared with the empty nanoparticle-treated group and the PBS control group ($p < 0.01$). The healing rate was also higher than the rhEGF stock solution group at 14 and 21 days after the treatments ($p < 0.05$ and < 0.01, respectively).

Pathological description

Both the rhEGF nanoparticle group and the rhEGF stock solution group showed capillary and inflammatory cells on the third day of treatment. There were a few fibroblasts but most of the cells were monocytes. In addition, there were mostly inflammatory cells in the empty nanoparticle group and the PBS control group (data not shown).

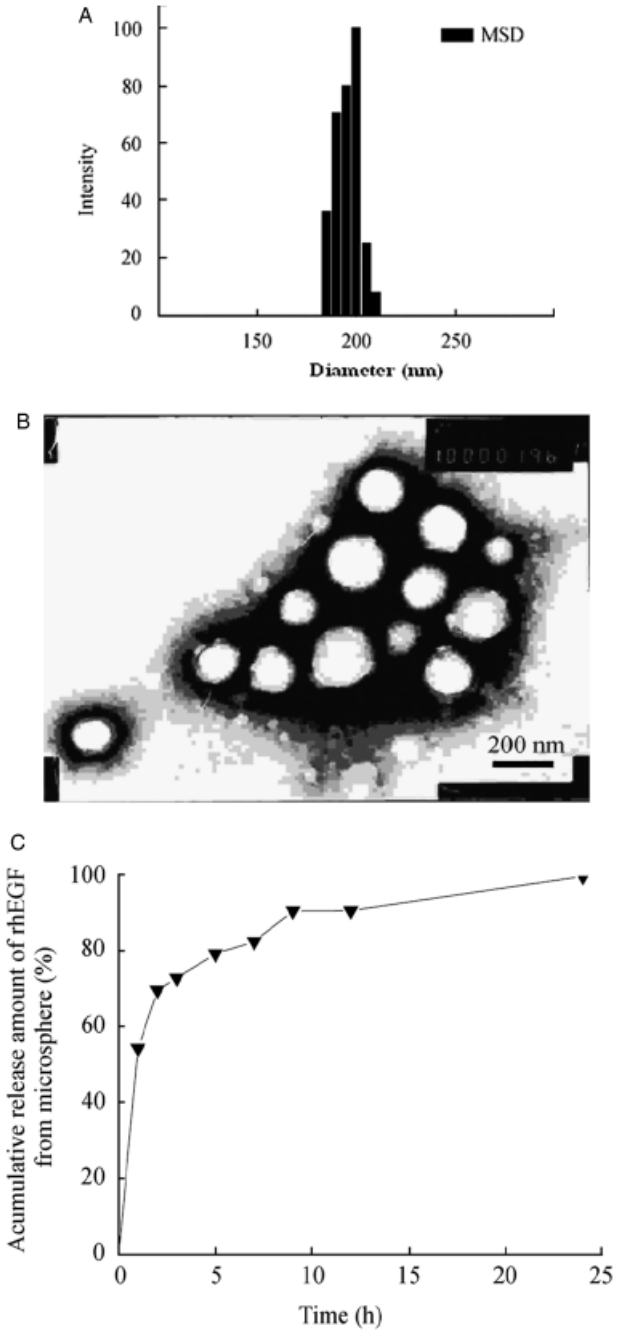

Figure 1. (A) Size distribution of recombinant human epidermal growth factor (rhEGF) nanoparticles. The size distribution was measured by the laser particle analyzer. Average particle size was 193.5 nm with the polydispersity index of 0.176; (B) transmission electron microscopy of rhEGF nanoparticles. Microspheres showed global, regular contour with homogenous size and distribution, and no adhesion; (C) release behavior of rhEGF nanoparticles in phosphate-buffered saline (pH 7.4). After an initial burst in the first hour, the accumulative release time could extend up to 24 hours. PSD, particles size distribution.

Table 1. Effects of different concentrations of the recombinant human epidermal growth factor nanoparticles on cell proliferation

Concentrations (μg/L)	Mean of OD	The increase rate (%)
Control	0.255 ± 0.022	
1	0.301 ± 0.023	18.02
5	0.302 ± 0.042	18.29
10	0.336 ± 0.040	31.41*
50	0.310 ± 0.014	21.42
100	0.297 ± 0.012	16.33

Values represent means ± SD.
*$p < 0.05$ vs. control group.

On the seventh day of treatment, both the rhEGF nanoparticle group and the rhEGF stock solution group showed new epidermis on the edge of the wound. The granulation tissue was primarily composed of fibroblasts and capillary cells (data not shown).

On the 14th day of treatment (Figure 3A–D), the rhEGF nanoparticle group showed a complete epidermis on the edge of the wound. In addition, the rhEGF stock solution group had less epidermis on the edge of the wound and incomplete cornification compared with the rhEGF nanoparticle group. Both the empty nanoparticle group and the PBS control group had an incomplete epidermis on the edge of the wound and relatively immature granulation tissue with plenty of capillary cells but few fibroblasts.

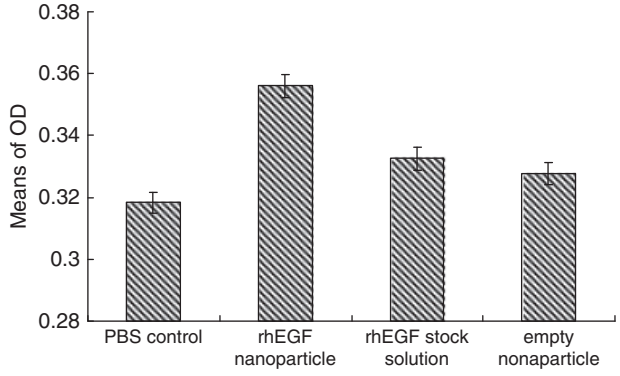

Figure 2. The effects of different treatments on cell proliferation. L929 cells were treated by the dissolvent phosphate-buffered saline; the recombinant human epidermal growth factor (rhEGF) nanoparticles (containing 10 μg/L rhEGF); the rhEGF stock solution (10 μg/L); and same amount of nanoparticles without rhEGF loading. The absorbance (OD) measured by MTT method represented the level of cell proliferation. The data were presented as means with SEM bars. The rhEGF nanoparticles group showed the highest level of cell proliferation ($p < 0.05$ vs. the PBS control group, the rhEGF stock solution group, and the empty nanoparticle group).

On the 21st day of treatment, the rhEGF nanoparticle group and the rhEGF stock solution group both had a complete epidermis on the edge of the wound with little granulation tissue. The epidermis in the empty nanoparticle group and the PBS control group was close to being complete, and there were more capillary cells and fibroblasts in the granulation tissue (supporting information Figure S1).

Immunohistochemical staining for PCNA

PCNA was expressed in the cell nucleus and immunostained in brown (Figure 4A–D). PCNA-positive fibroblast cells represented the amount of proliferation that occurred after different treatments. In the rhEGF nanoparticle group, the largest number of PCNA-positive fibroblast cells was observed on Day 14 and slightly decreased by Day 21 (Table 3). Compared with other groups, the expression of PCNA in the rhEGF nanoparticle group was significantly higher after 14 days ($p < 0.05$ vs. other groups).

DISCUSSION

Multiple factors cause recurrent, persistent, and refractory DF ulcers.[6] Among them, impaired growth factors and their receptors have been considered to be important.[7] Many studies have indicated that the local application of rhEGF in DF ulcers promotes fibroblast proliferation, collagen fiber build-up, and granulation tissue formation.[8] rhEGF has shown positive efficacy in the treatment of ulcers and has been approved for use.[9,10] However, there are also some problems associated with the use of rhEGF. rhEGF has a short biological half-life (only a few hours) and it can be leaked from the DF wound surface and degraded by enzymes in DF ulcer tissues.[3,11] Thus, we wanted to introduce an appropriate protective technique to retain the biological activity of rhEGF and maintain continuous contact of rhEGF with DF wound surfaces.

PLGA, which was used as the rhEGF carrier to form nanospheres, has been proven to be a safe, nontoxic, drug-delivery method that does not inactivate growth factors.[12–16] It is also characterized as a biocompatible and biodegradable polymer that is used in the clinic for medical sutures and microencapsulation of compounds for injection.[17,18]

In this study, we used the modified double-emulsion method to prepare rhEGF nanoparticles.[4] They were uniform, well-dispersed smooth nanoparticles, which allowed them to have a large contact area with the wound surface. The high encapsulation efficiency (85.6%) is essential for potential industrial processing of these nanoparticles. The release of the rhEGF nanoparticles was in accordance with the Higuchi-releasing kinetic model and exhibited a slow-release behavior after an initial burst during the first hour. The total release time could extend up to 24 hours and indicates that the nanoparticles can induce a controlled release of rhEGF, which is the fundamental factor to assure that rhEGF contacts granulation tissue. The local administration of rhEGF could be simplified by the use of nanoparticles because they protect its biological activity.

Nanoparticles containing rhEGF stimulated mouse fibroblast proliferation illustrating that rhEGF maintains

Table 2. Comparison of wound healing rate at different time points of the treatments

Groups	Third day (%)	Seventh day (%)	14th day (%)	21st day (%)
PBS control	38.02 ± 7.36	60.16 ± 9.27	88.10 ± 3.04	89.85 ± 3.69
rhEGF nanoparticle	41.86 ± 5.94	$68.06 \pm 6.06^{\#,\dagger}$	$93.04 \pm 1.15^{\#,\dagger,\blacktriangle}$	$96.81 \pm 1.86^{\#,\dagger,\ddagger}$
rhEGF stock solution	35.68 ± 9.02	$66.98 \pm 3.38^{\#,\dagger}$	$90.63 \pm 1.28^{*,\dagger}$	$92.84 \pm 2.25^{*}$
Empty nanoparticle	35.54 ± 7.49	53.65 ± 5.72	87.70 ± 2.79	91.34 ± 3.93

The data are means $\pm$ SD.
$^{*}p < 0.05$ vs. PBS control group.
$^{\#}p < 0.01$ vs. PBS control group.
$^{\dagger}P < 0.01$ vs. empty nanoparticle group.
$^{\blacktriangle}p < 0.05$ vs. rhEGF stock solution group.
$^{\ddagger}p < 0.01$ vs. rhEGF stock solution group.
rhEGF, recombinant human epidermal growth factor; PBS, phosphate-buffered saline.

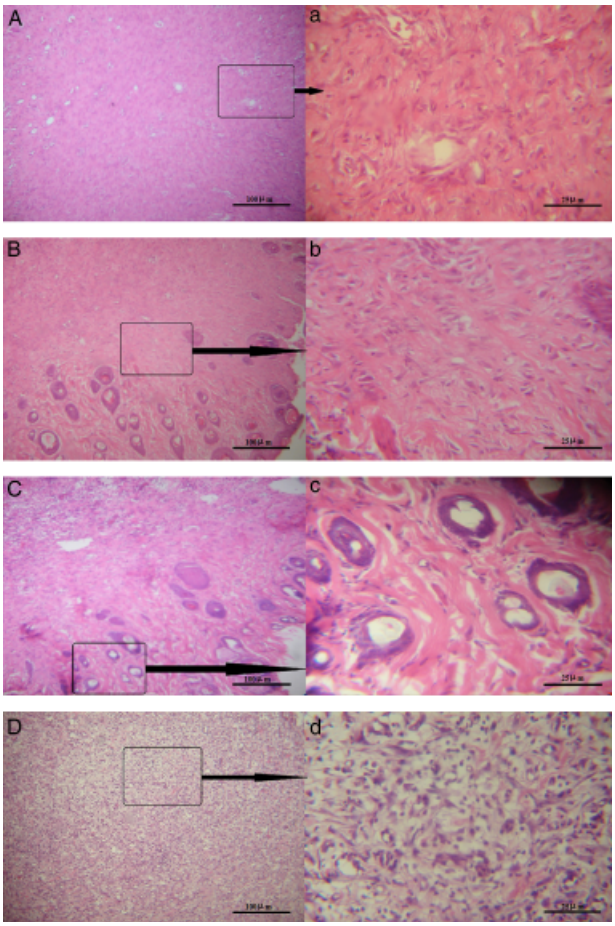

Figure 3. (A–D) The HE-stained pathologic slides from four groups 14 days after the treatments: the phosphate-buffered saline control (A: ×100, a: ×400); the recombinant human epidermal growth factor (rhEGF) nanoparticles (B: ×100, b: ×400); the rhEGF stock solution (C: ×100, c: ×400); and the empty nanoparticles (D: ×100, d: ×400).

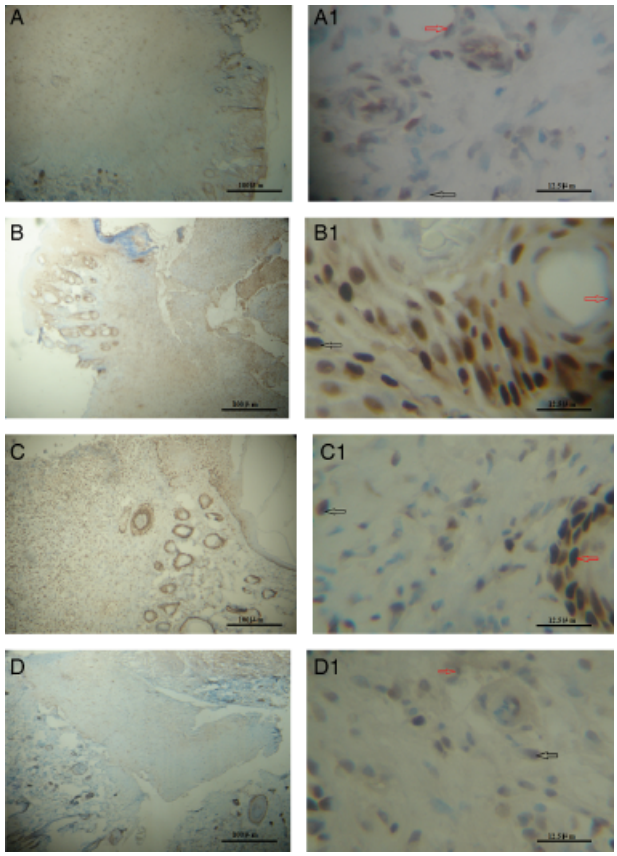

Figure 4. (A–D) The proliferating cell nuclear antigen expression from four groups 14 days after the treatments: the phosphate-buffered saline control (A: ×100, A1: ×800); the recombinant human epidermal growth factor (rhEGF) nanoparticles (B: ×100, B1: ×800); the rhEGF stock solution (C: ×100, C1: ×800); the empty particles (D: ×100, D1: ×800). The black arrow shows a fibroblast, the red arrow shows an endothelial cell.

Table 3. Comparison of PCNA positive cells rate in wound tissue at different time points of the treatments

Groups	0 day (%)	Third day (%)	Seventh day (%)	14th day (%)	21st day (%)
PBS control	27.4 ± 4.12	34.3 ± 4.90	42.0 ± 3.74	66.6 ± 5.42	55.6 ± 4.00
rhEGF nanoparticle	26.3 ± 4.54	$39.4 \pm 4.75^{\#,\dagger}$	$66.4 \pm 5.04^{\#,\dagger}$	$82.5 \pm 3.74^{\#,\dagger,\ddagger}$	$69.3 \pm 3.66^{\#,\dagger,\blacktriangle}$
rhEGF stock solution	25.9 ± 3.62	35.9 ± 2.80	$62.3 \pm 5.06^{\#,\dagger}$	$73.6 \pm 3.74^{\#,\dagger}$	$64.6 \pm 3.58^{\#}$
Empty nanoparticle	27.1 ± 3.99	31.6 ± 3.66	55.1 ± 5.62	64.5 ± 3.93	61.9 ± 5.44

The data are means $\pm$ SD.
$^{\#}p < 0.01$ vs. PBS control group.
$^{\dagger}P < 0.01$ vs. empty nanoparticle group.
$^{\ddagger}p < 0.01$ vs. rhEGF stock solution group.
$^{\blacktriangle}p < 0.05$ vs. rhEGF stock solution group.
rhEGF, recombinant human epidermal growth factor; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen.

its biological activity when inserted into a nanoparticle. In the animal experiments, the local administration of the rhEGF nanoparticles showed that the healing effects were not better than other groups on the third day of treatment. This may indicate that rhEGF has no wound healing effects at the acute inflammatory stage of the wound because the cells are mainly composed of inflammatory cells. However, on the seventh, 14th, and 21st days of the treatments, the healing rate in the rhEGF nanoparticle group was the fastest among all of the groups. Pathologic slides clearly showed that the rhEGF nanoparticle group and the rhEGF stock solution group had better granulation tissue formation and tissue repair than the other two groups. These results also support that the controlled release of the nanoparticles allows rhEGF to continually contact the wound surface and maintain an effective concentration to promote wound healing.

PCNA, a cofactor of DNA polymerases, is tightly connected with DNA synthesis.[19] PCNA was classified as a crucial endogenous cell proliferation marker based on the finding that PCNA expression varies with the cell cycle and reaches its highest level at S phase. Decreased amounts of fibroblasts induced by infection, hypoxia, and malnutrition in chronic ulcers can be related to the reduced expression of PCNA.[20,21] In the present study, there were no differences in the expression of PCNA between any of the groups in the acute inflammatory stage of the wound (on the third day of treatment). On the seventh day, PCNA expression in the rhEGF nanoparticle group was dramatically increased and was higher than the empty nanoparticle group and the PBS control group. On the 14th day, PCNA of the rhEGF nanoparticle group was notably higher than that of the rhEGF stock solution group. The results reveal that rhEGF application can be more effective for tissue repair at intermediate and advanced stages of a wound because the fibroblasts become predominant in the granulation tissue. Negative PCNA staining of fibroblasts was observed in the empty nanoparticle and PBS control groups more often than any of the other groups. Taken together, these results suggest that hyperglycemia induces a deficiency of endogenous EGF and a dysfunction of the cells required for wound repair resulting in delayed wound healing.

The rhEGF nanoparticles exhibit a controlled release of rhEGF for up to 24 hours without disturbing its biological activity. These nanoparticles possess better wound healing effects than that of rhEGF. The data suggest a new and more convenient method for clinical delivery.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. (A–D) The HE stained pathologic slides from four groups 21 days after the treatments: the PBS control (A: ×100, a: ×400); the rhEGF nanoparticles (B: × 100, b: ×400); the rhEGF stock solution (C: ×100, c: × 400); the empty nanoparticles (D: ×100, d: ×400).

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