Original Research

The effect of Klotho protein complexed with nanomaterials on bone mesenchymal stem cell performance in the treatment of diabetic ischaemic ulcer

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Abstract
A lack of angiogenesis is the key problem in the healing of diabetic foot ulcers. Stem cells have already been proven to have a high potential for angiogenesis. The most important aspects of stem cell therapy are improving the microenvironment, cell homing and continuous factor stimulation. We investigated the effect of Klotho protein to heal wounds by promoting the proliferation and migration of bone mesenchymal stem cells and endothelial cells in vitro. Based on the above study, we produced a compound material by using poly(lactic-co-glycolic acid) (PLGA), chitosan microspheres and gelatin through electro spinning technology. The structure of the compound material, just like a sandwich, is that two pieces of PLGA nanofiber films clamped gelatin film which contained chitosan microspheres. In the in vitro release experiment, we could detect the release of Klotho after seven days in the compound material, but the release time was approximately 40 hours for the chitosan microspheres. After seeded bone mesenchymal stem cells (BMSCs) on the surface of the compound material, we observed morphologies of the chitosan microsphere, the PLGA nanofiber and BMSCs by scanning electron microscopy. The nanofiber mesh biological tissue materials could supply an appropriate microenvironment and cell factors for the survival of BMSCs. Compared with the control group, the biological tissue material seeded with BMSCs significantly promoted angiogenesis in the lower limb of diabetic C57BL/6J mice and accelerated diabetic foot wound healing. The compound biomaterial which could continuously stimulate BMSCs through releasing Klotho protein could accelerate wound healing in the diabetic foot and other ischemic ulcers.

Keywords
BMSCs, diabetic foot ulcer, klotho protein, nanomaterial

1 | Introduction

Diabetes mellitus (DM) is one of the most common diseases; nearly 422 million people worldwide suffer from diabetes [1]. DM damages several systems, including the vascular, neural and cardiac systems, and these damages impose a heavy burden on patients via numerous aspects [2]. Nearly 25% of these patients suffer from diabetic foot ulcers (DFU) [3]. DFUs are considered to be primarily due to changes in the vascular system. Large vessel lesions are primarily due to atherosclerosis, while basement incassation is more common for capillary lesions [4]. These variations will induce anoxia and ischaemia as well as skin ulcers. Conventional foot ulcer therapy mainly aims to improve the oxygen supply and microcirculation [5, 6], but the curative effects of these approaches are far from satisfactory. Effective therapies are imperative to reduce the pain associated with DFUs. Stem cell transplantation constitutes a new therapeutic strategy for tissue reconstruction.

Due to their pluripotency and immunomodulatory properties, bone marrow mesenchymal stem cells (BMSCs) have been widely used in tissue engineering strategies [7, 8]. BMSC transplantation can be utilised in the repair of ischaemic and...
MATERIALS AND METHODS

2.1 | The controlled release of Klotho protein

2.1.1 | Chitosan microsphere

We purchased chitosan from Golden-Shell Pharmaceutical Co, LTD. The molecular weight was approximately $2 \times 10^5$, and the degree of de-acetylation ranged 85% from 90%. Two hundred and 50 mg of the chitosan and 5.0 mg of Klotho protein were then dissolved in 10 ml of 2% ethylic acid and magnetically stirred for 1–2 h. Emulsifying agent (100 ml) was then slowly added, followed by stirring for another 2 h. Twenty five ml of 5% Triphenyl phosphate solution was then dropped into the solution, which was subsequently stirred for 24 h. The solution was centrifuged at 4°C 15,000 r/min for 25 min, the supernatant was discarded, and the pellet was decanted. Isopropyl alcohol was added to wash the pellet, which was then lysed using ultrasound and centrifuged again at 4°C, followed by freeze-drying at $-80^\circ C$.

2.1.2 | Electrospinning of PLGA

We purchased PLGA form Daigang Pharmaceutical Co, LTD. The molecular weight was approximately $6-8 \times 10^4$. The PLGA was dissolved in a 9:1 mixture of chloroform and dimethyl formamide and magnetically stirred at room temperature for 2 h to obtain a stable solution. The solution was transferred into an injector and then electrospun under at 25°C, 65% humidity, and 12 kV. The injector was placed at a distance of 12 cm from the plate, and the solution was ejected at a rate of 0.05 ml/h. After 12–14 h, we harvested a PLGA film.

2.1.3 | Gelatin film

Gelatin was dissolved in 25°C water for 30 min and then heated to 37°C to yield a gelatin solution. Chitosan microspheres were added to the gelatin solution and stirred for 1 h to obtain a stable solution. The solution was then coated on the PLGA thin film. Another PLGA film was then electrospun on the gelatin film to obtain a complex film that was structured like a sandwich.

2.1.4 | Scanning electron microscope (SEM)

The chitosan microspheres, PLGA film, gelatin film and cells on the material were observed using electron microscopy at the Third Military Medical University.

2.1.5 | Release curve

A piece of material (diameter = 1 cm) was submerged in a well filled with Phosphate Buffered Saline (PBS); the material remained moist to mimic skin conditions. The solubility of klotho at different times was estimated using ELISA; the OD was measured at A450, and a release curve was generated.

2.2 | Cell culture and cell group

We purchased BMSCs from Saiye and endothelial cells (EC) from American type culture collection (USA). The cells were cultured according to the supplier's instruction. We selected cells between passages 4 and 6 for the experiments. We established four groups: the control group, high glucose, high glucose with Klotho protein, and Klotho protein group.
2.3 | The effect of Klotho protein on bone mesenchymal stem cells and ECs

2.3.1 | MTT assay

Cells were harvested with 0.25% trypsin-EDTA when they reached 80% confluency and transferred cells to a 96-well plate at 8000 cells per well. After culture for 36 h in the medium, the cells were used in the experiment. 3-(4,5)-dimethylthiazoliodiazoo (-z-y1)-3,5-di- phenyntetrazoliumromide (MTT) was dissolved in PBS at 5 mg/ml, and 20 μL of the MTT solution was added to each well. The cells were then incubated for 4 h at 37°C. The supernatant was then removed, and 150 μL of Dimethyl sulfoxide was then added. The plate was placed on an orbital shaker for 15 min before measuring the absorbance at a wavelength of 492 nm using a plate reader.

2.3.2 | EDU test

Seed cells in the logarithmic growth phase were cultured in 96-well plates at 4 × 10^4 ~ 8 × 10^4 cells per well until they reached the normal growth phase. The cells were grouped and treated with the indicated drugs for the group. After 36 h, 100 μL of medium containing 1:1000 diluted 5-Ethynyl-2'- deoxyuridine (EDU) was added to each well, and the cells were then incubated for 2 h at 37°C. The cells were then fixed, stained with Apollo and 2'-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (for DNA staining), and observed under a fluorescence microscope.

2.3.3 | Transwell chamber experiment

The cells were harvested with 0.25% trypsin-EDTA when they reached 80% confluency. The upper of a 24-well transwell migration insert (pore size is 5 mm) was then seeded with 8000 cells. Conditioned medium was added to the lower chamber, and normal medium was added to the upper chamber. After 36 h, the cells on the upper side of the membrane were wiped away, and those on the lower side were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. The cell morphology was observed using light microscopy, and the number of cells that had migrated to the lower chamber was counted.

2.3.4 | Cell scratch experiment

The cells were seeded in six-well plates at 1 × 10^6 cells per well and cultured with normal medium for 24 h. A central scratch was created by scraping cells away with a pipette tip. The cells were cultured in conditioned medium and incubated at 37°C for 24 h. The cells were then fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. The scratches were observed with an OLYMPUS BX50 microscope, and the scratch area was calculated using the IPP software (Media Cybernetics).

2.4 | Animal experiment

We obtained ethical committee approval for the animal experiment from the Third Military Medical University. Five-to 8-week old male C57BL/6j mice from the Laboratory Animal Center were selected for experiment. We divided the mice into 4 groups. Each group contained 20 mice. Streptozotocin (50 mg/kg, Sigma) was intraperitoneally injected for 4 days. The blood glucose content was estimated after 3 days, and mice with a blood glucose content exceeding 20 mmol/L were selected for the experiment. After ligating the arteria cruralis, a full-thickness excisional wound was created (5 × 5 mm), as described previously in detail [25].

A Nikon D90 digital camera was used to record the images of the wounds 0, 3, 7 and 10 days after surgery, and the areas were calculated using the IPP software. The wound areas were harvested for immunohistochemistry.

2.5 | Statistics

Each experiment was repeated at least three times. SPSS for Windows (Version 18.0, SPSS, Inc.) was used to perform the t test and variance analyses. All experimental data are expressed as the means ± SE; all p values are two-tailed; and p < 0.05 was considered statistically significant.

3 | RESULTS

3.1 | The material properties experiment

The microspheres release chitosan in a controlled manner over approximately 40 h, but this release time is extended to approximately 7 days for the self-made material. The material showed a better delay release profile than the chitosan microsphere. Additionally, BMSCs could adhere to the surface of the biomaterial and retained a normal morphology (Figure 1).

3.2 | Klotho protein promotes MSC proliferation and migration

In order explore the effect of Klotho protein on the growth of BMSCs and EC in a high-glucose medium, we used the MTT assay to examine proliferation (Figure 2). The OD value of Klotho protein-treated BMSCs was 123% that of the control value, which suggests that Klotho protein promotes the proliferation of BMSCs. The OD value of HG + Klotho-treated BMSCs was 131% of the HG group, which showed that Klotho protein can protect BMSCs from a high glucose environment. The OD value of Klotho protein-treated ECs was 157% of the control value. The OD value of HG + Klotho-treated ECs was 137% of the HG group, which showed that Klotho protein can protect ECs from high a glucose environment.
The release curve (a) showed that the release time of only microsphere is about 40 h, but in the complex biomaterial is about 7 days (b). \( p < 0.05 \) (n = 4). The values are the mean ± SE. The form of chitosan microsphere (c), and the microsphere (arrow) in gelatin film (d). The form of poly lactic-co-glycolic acid (PLGA) nanofiber (e), and morphology of bone mesenchymal stem cells (BMSCs) seeded on the surface of material (f). The sketch map of the compound material (g).
The EDU test indicated the proliferation ratio (Figure 3). The proliferation ratio of Klotho protein-treated BMSCs was 117% that of the control group. The ratio of HG + Klotho-treated BMSCs was 126% of the HG group. The ratio of Klotho protein-treated ECs was 142% that of the control group. The ratio of the HG + Klotho-treated ECs was 122% that of the HG group.
that of the HG group, which showed that Klotho protein can protect ECs from a high-glucose environment.

We used a scarification test to measure the migration rate (Figure 4). Klotho protein significantly promoted the migration of BMSCs, and the migration rate was 133% that of the control group. The migration rate of the HG + Klotho group was 151% that of the HG group, which shows that klotho could promote the migration of BMSCs in a high-glucose environment. The Klotho protein promoted the migration of ECs, and the migration rate was 123% that of the control group. The migration rate of the HG + Klotho group was 181% the rate of the HG group, which shows that klotho could promote the migration of BMSCs in a high-glucose environment.

We used a transwell test to measure the number of cells that migrated to the lower chamber (Figure 4). The Klotho protein significantly promoted the migration of BMSCs; the number of cells measured was 143% of those measured for the control group. The number of cells in the HG + Klotho group was 125% of the value measured for the HG group, which shows that klotho could promote the migration of BMSCs in a high-glucose environment. The Klotho protein promoted the migration of ECs, and the cell number was 189% of that of the control group. The number of cells in the HG + Klotho group was 114% of the number in the HG group, which shows that klotho could promote the migration of ECs in a high-glucose experiment.

3.3 | The contribution of new complex of bone mesenchymal stem cells and biomaterials to wound healing

To determine whether Klotho protein and BMSCs promote healing in the diabetic foot, we devised four experimental groups: material with mesenchymal stem cells (MSCs) and

![Figure 4](https://example.com/figure4.png)

_Figure 4_ (a) Cell scratch test showed that under high-glucose, the migration ability of bone mesenchymal stem cells (BMSCs) was damaged. Klotho protein promote BMSCs migration but cannot totally prevent damage from high-glucose. (b) Transwell test showed the same result as the a. (c) Cell scratch test showed that under high-glucose, the migration ability of ECs was damaged. Klotho protein promote ECs migration, but cannot totally prevent damage from high-glucose. (d) Transwell test showed the same result as (c) *p < 0.05 (n = 5). The values are the mean ± SE.
Klotho, material with Klotho, and material without Klotho and the control group. The wound area was measured 0, 3, 7 and 10 days after treatment. The wound treated with Klotho protein and BMSCs healed significantly faster than the control wound and the wound treated with only material and not Klotho protein. The wound treated with Klotho protein healed faster than the control and material without Klotho protein wound, but slower than the group treated with BMSCs and Klotho protein. The healing speed did not significantly differ between the control and material without klotho group. These findings indicate that Klotho and BMSCs play important roles in treating intractable diabetes ischaemic ulcers, and Klotho protein can also promote wound healing by protecting EC. Moreover, the function of EC in tissue repair has already been proven (Figure 5).

3.4 Effect of the new Klotho protein and bone mesenchymal stem cells complex on blood vessel restoration and angiogenesis

Angiogenesis is one of the key processes in ischaemic ulcer healing. In vivo, Klotho protein and BMSCs can significantly promote wound healing. To determine the relationship between angiogenesis, Klotho protein and BMSCs, we analysed the skin around the wounds in day 7. The animal was sacrificed with an overdose of anaesthetic. The capillary profiles were visualised using immuno-histochemical staining with rabbit CD31 antibody (LifeTechnologies, USA) followed by donkey anti-rabbit IgG antibody (LifeTechnologies, USA). The density of green fluorescence indicates the number of blood vessels. This fluorescence was highest in the MSCs + Klotho protein group. The staining was less pronounced in the Klotho protein group and lowest in the klotho group and control. The hematoxylin-eosin staining showed the same result (Figure 6).

4 DISCUSSION

Abundant, reliable and intact vessels are essential to promote wound healing. Unfortunately, the high-glucose environment in DM damages existing vessels, and this process is irreversible. Thus, angiogenesis has been considered a key process to heal DFU. Bone mesenchymal stem cells are pluripotent and can secrete several factors that promote angiogenesis, such as vascular endothelial growth factor (VEGF) and IGF-1 [26]. Moreover, BMSCs can also secrete several neurotrophic factors, such as Brain-derived neurotrophic factor and nerve growth factor [27]. These factors can promote angiogenesis by activating ECs. It was demonstrated that BMSCs can significantly promote wound healing by increasing the formation of epithelial cytoplasm and enhancing angiogenesis through secreting VEGF and accelerating migration and proliferation of HUVECs [28]. These findings can be exploited for new approaches to heal DFU. However, the survival of BMSCs is limited in high-glucose environments, which prevents them from promoting angiogenesis.

Klotho protein has been widely studied in renal diseases and life span research. In conjunction with FGF-23, it could promote recovery after the transplantation of several organs [29].

![Figure 5](Image)

**Figure 5** The therapeutic effects of mesenchymal stem cells (MSC) + Klotho protein on diabetic foot ulcer mice. (a) Typical photographs of wound healing for the above groups. (b) The wound healing rates of the control group, the MSC + Klotho protein group and the material contained Klotho protein group. *p < 0.05 (n = 20) compared with the MSC + Klotho group; #p < 0.05 (n = 20) compared with the material contained Klotho group. The values are the mean ± SE. (c) The wound healing rates of the control group and the material without Klotho protein group. *p > 0.05 (n = 20) compared with the material without Klotho group. The values are the mean ± SE.
However, its ability to protect stem cells has not been thoroughly examined. In vitro, our experiment shows that Klotho protein could promote the proliferation and migration of BMSCs and ECs. Moreover, it also protected BMSCs and ECs from high-glucose damage via its anti-apoptosis function [30].

Based on the above study, a solid material is required as a carrier to ensure that Klotho protein participates continuously in wound healing and not simply as a cell stimulant prior to implantation. Cells are more commonly stimulated by only the implanted material as opposed to a combination of a material and biological factors. The former approach is simpler but presents marked disadvantages because cells need to be continuously stimulated steadily to improve healing. We first attempted to electrospin Klotho protein into the nanofiber, but the toxic solvent used in this process and the high temperature caused by the high voltage reduced the activity of Klotho protein. Thus, we aimed to find a method that could maintain the activity of Klotho protein. PLGA, chitosan and gelatin are safe and durable. We produced the compound biomaterial due to its lack of toxicity, accessibility and most importantly, its controlled release profile. During the preparation, Klotho protein was protected from the damage mentioned above and retained its activity. Moreover, we needed to slow the release of the protein in order to coordinate the time of wound healing and the release time. Compared with the material that controls the release time using a single method, the compound material delayed the release time and stimulated BMSCs for a longer time. The SEM images show that the BMSCs could adhere to the surface of the biomaterial and retain a normal morphology.

In vivo, the material must remain moist during the healing process; as gelatin is hygroscopic, BMSCs require moisture to survive, and Klotho protein is not easily released from a dry environment. Interestingly, fewer mice died in the groups treated with compound material with or without BMSCs, which we attribute to the anti-infectious effect of Klotho protein; however, this hypothesis is not supported by the literature. Our future work will focus on developing a simpler-to-use material that improves the success rate. More importantly, future materials should be able to release protein, DNA or RNA in a quantitative and timed manner. Additionally, we aim to develop a material whose biomechanics can directly control the differentiation of cells [31, 32].

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