Identification and characterization of lipopeptides as a potential wound healing agent produced by *Bacillus subtilis*

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Abstract

Wound healing is an intricate and orchestrated process composed of inflammation, tissue formation, and remodeling phases, and is delayed in some cases, such as diabetes. Although some drugs have been applied to clinical use, the availability of drugs to promote the wound healing is required. Lipopeptides produced by *Bacillus subtilis* are famous for their antimicrobial activities, however, no report of lipopeptides has been found on affecting wound healing. The aim of our study is to determine the possibility of lipopeptides on wound healing. Results indicated that lipopeptides produced by *B. subtilis* markedly accelerated wound healing on the rabbit skin, and regulated inflammation reaction at the wound site by increasing the expression of TGF-β2 and IL-6. In conclusion, the function of lipopeptides might indicate the possibility of its clinical use for wound therapy.

1 Introduction

Wound healing is a physiological process that induced by tissue injured, to repair the damaged skin tissue and restore skin structure and function. The process of wound healing is complex and intricate, which can be divided into three phases: inflammation, tissue formation, and remodeling according to cytokines and growth factors secretion, cell proliferation, extracellular matrix [1]. In some cases, diabetes, smoking, aging, starvation and surgery can delay the wound repair leading to acute and chronic wound [2]. Although many drugs including fibroblast growth factor (FGF) and epidermal growth factor (EGF) have been developed to promote wound healing, the availability of drugs to promote the wound healing process is sorely lacking [3]. Thus searching and exploiting natural compounds and polymers is quite meaningful to reconstruct and regenerate tissues [4].

Bacillus strains represent renowned producers of lipopeptides as secondary metabolite with an importance for their antimicrobial activity. The lipopeptides have both hydrophilic group (peptides moiety) and hydrophobic group (saturated or unsaturated fatty acid moiety). It has been widely found that most of the *bacillus* can produce at least one type of lipopeptide via fermentation [5]. Several lipopeptide applications have been well-documented and related to antimicrobial efficacy. Recent study has reported a hydrogel loaded with lipopeptide used for antimicrobial treatment of burn wound infection [6]. Another study revealed that the lipopeptide could affect inflammatory response and the release of inflammatory cytokines by macrophages [7]. Considering the close relationship between wound healing and inflammation, we speculated that lipopeptides may have the potential to promote wound healing. As far as we know, there have been no reports on lipopeptides affecting wound healing. Therefore, the aim of our study was to reveal the role of lipopeptides in wound healing.

In the present study, we demonstrated that lipopeptides accelerated the wound healing of rabbit skin in dose-dependent effect. Moreover, lipopeptides significantly increased the expression of inflammatory cytokines in rabbit model and showed a good wound healing quality. Taken together, our results suggest that lipopeptides might be a cheap, effective and potential alternative drug to promote wound healing.

2 Materials and methods

2.1 Bacterial cultures and lipopeptides extraction

Bacillus subtilis CCTCC M207209, previously isolated from soil, was used to produce lipopeptides in this study, and now stored in the China Center for Type Culture Collection (Wuhan, China). Lipopetides with wound healing potential were collected from the fermentation broth of *B. subtilis* according to the previous method with slightly modification [8]. In brief, the strain *B. subtilis* was inoculated in nutrient broth medium and cultivated at 32 °C for 48 h under agitation at 160 rpm. After fermentation, bacterial culture medium was centrifuged at 10000 × *g* for 10 min to remove the bacteria. The supernatant was adjusted to pH 2.0 with 6 M HCl and then set at 4 °C for overnight precipitation. Next day, the acid precipitates was collected by centrifugation at 10000 × *g* for 30 min and extracted by methanol at 4 °C. Methanol extract was concentrated using a vacuum evaporator and the obtained lipopeptides were dissolved in pH 7.0 phosphate buffer solution, freeze-dried to powder and stored at 4 °C for further application [9].

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2.2 Wound healing model in vivo

To identify the effects of lipopeptides on skin wound healing, a wound healing model was established on the rabbit skin [10]. Before asceptic surgery, female rabbits (weighing ≈ 5 kg) were anesthetized with pentobarbital sodium, had their back hair depilated, and were disinfected using 75% ethanol. A 5 cm incised wound reaching to the myometrium was made on the rabbits' backs by a scalpel. Then the rabbits were caged individually with the wounds covered. Lipopeptides were dissolved in vehicle (DMSO mixed with glycerin, 1:1, v/v), and the incisions immediately treated with either vehicle, lipopeptide (0.1 mg/mL), or no treatment after the wounds were made. Wound areas were recorded by camera at 24 h, 48 h, and 72 h. All protocols used in the study were approval by the Animal Care and Experimental Committee of Northwestern Polytechnical University School of Life Science.

2.3 Determination of traction stress

After wound healing, traction stress test can be applied to evaluate the quality of healing. Traction stress was measured as previously described. In brief, rabbits were inducted to deep general anesthesia, then the skin on both sides of the wound were grasped with forceps. Traction stress of wound was calculated and used directly for comparison as previously described [11].

2.4 Real-time quantitative reverse transcriptase-PCR

Total RNA was isolated from the rabbit skin samples using TRIzol plus RNA purification kit (Invitrogen, Life Technologies, Carlsbad, CA), and cDNA was synthesized using HiScript® II Q RT SuperMix (Vazyme, Nanjing, China) according to the manufacturer's instructions [12]. Quantitative real-time PCR was performed using SYBR qPCR Master Mix (Vazyme, Nanjing, China) with a CFX96-PCR instrument (Bio-rad, MN, USA). PCR amplifications were performed as previously described [13]. The PCR primers used for amplifying TGF-β2 and IL-6 are as follows: TGF-β2, sense 5'-ATGTGCAGGATAATTGCTGCC-3', antisense 5'-TGTGCTGAGTGTCTGAACTC-3'; IL-6, sense 5'-TGGTGGTGGCTACCGCTTTC-3', antisense 5'-GCTGGCTTGAGGGTGGCTTC-3'; β-actin, sense 5'-GCCAACACAGTGCTGTCTG-3', antisense 5'-CACATCTGCTGGAAGGTGG-3'.

2.5 Statistical analysis

All statistical analyses were performed using GraphPad Prism software (version 6.0; GraphPad Software, La Jolla, CA). Each experiment was repeated at least three times. All values are shown as the mean ± SD. Student's t-test was performed for comparisons of data with paired samples, and one-way ANOVA was applied for multiple group comparisons.

3 Results

3.1 Lipopeptides affect the wound closure in rabbit

The role of lipopeptides in skin wound healing was preliminarily investigated by applying lipopeptides in a rabbit model with a 5 cm incised wound on the back. As shown in **Figure 1**, lipopeptides could accelerate the wound closure in a concentration-dependent manner. In comparison, 0.1mg/mL treatment had the best wound healing effect on rabbit skin.

3.2 Lipopeptides promote the wound healing in rabbit skin

After determining the optimal concentration, we further determined the promoting effect of lipopeptide on wound healing. As shown in **Figure 2**, *B. subtilis* lipopeptides exhibited a remarkable promotion of wound healing as compared with the control. After 24 h treatment, callus was obvious in the wound treated with lipopeptides as compared with the other two treatments. At 72 h, treatment with lipopeptide showed significant reduction in wound size as compared with the untreated and solvent treated groups.

3.3 Traction stress of wounds

As for evaluating the effect of agents on wound healing, traction stress is an important indicator but easily overlooked. Traction stress test measures tissue tightness and laxity, which directly provided valuable insight into the process of cell adhesion and contraction, indicating the quality of wound healing. As shown in **Figure 3**, the healed wound treated with lipopeptides revealed a great traction stress that was negatively correlated with the concentration of lipopeptides.

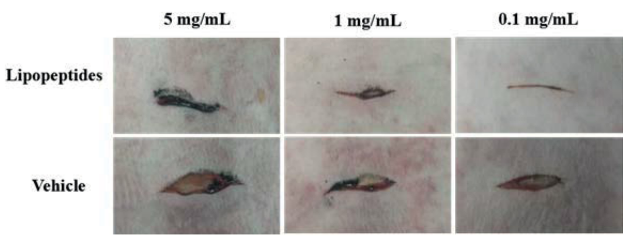

Figure 1 Effect of different concentrations of lipopeptides on wound healing in rabbit. Images indicate *B. subtilis* lipopeptides could accelerate the wound closure in a dose-dependent manner. Vehicle (DMSO: glycerin = 1:1, v/v); concentration of lipopeptides was 5, 1 and 0.1 mg/mL, respectively.

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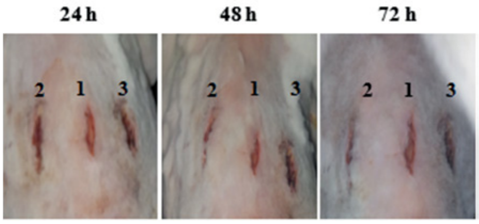

Figure 2 Effect of lipopeptides on wound healing in rabbit with time. Images indicate *B. subtilis* lipopeptides could promote wound healing. 1, no treatment; 2, vehicle (DMSO: glycerin = 1:1, v/v); 3, lipopeptide (0.1 mg/mL). Photos were taken post injury, at 24 h, 48 h, and 72 h

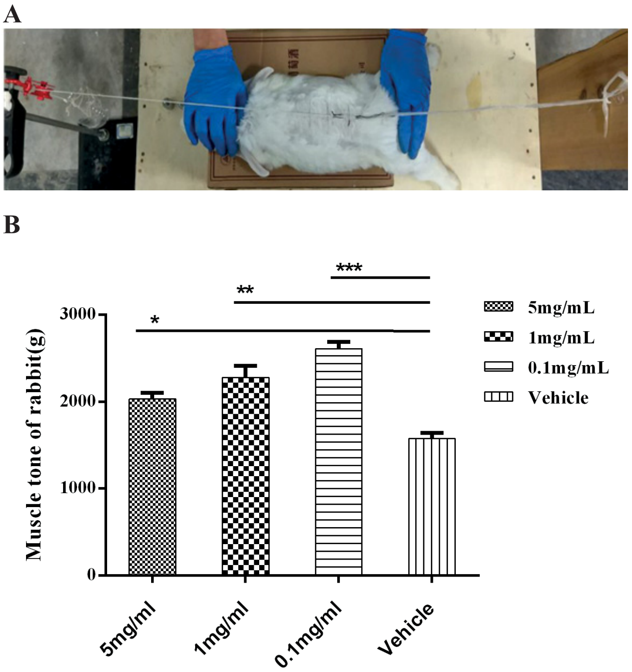

Figure 3 Effects of lipopeptides on the average integrated traction stress of rabbit skin wound. Images indicate *B. subtilis* lipopeptides had a great muscle tension. *P < 0.05, **P < 0.01, ***P<0.001

3.4 Lipopeptides affect inflammatory cytokines secretion

In skin wound healing, macrophages at the wound site can produce cytokines, such as TNF-α, IL-6 and TGF-β, acting as a chemical attractant that recruit additional macrophages and stimulate the migration of keratinocytes to close the wounds [14]. To identify lipopeptides' effects on inflammatory response, real time-qPCR was used to quantify the inflammatory cytokine secretion in the wound sites. As illustrated in **Figure 4A** and **4B**, after 72 h treatment with lipopeptides, relative expression levels of both TGF-β2 and IL-6 were up-regulated, meanwhile, showed a significantly up-regulation with a dose-dependent manner. Results indicated that lipopeptides could increase the expression of TGF-β2 and IL-6 in the injured skin.

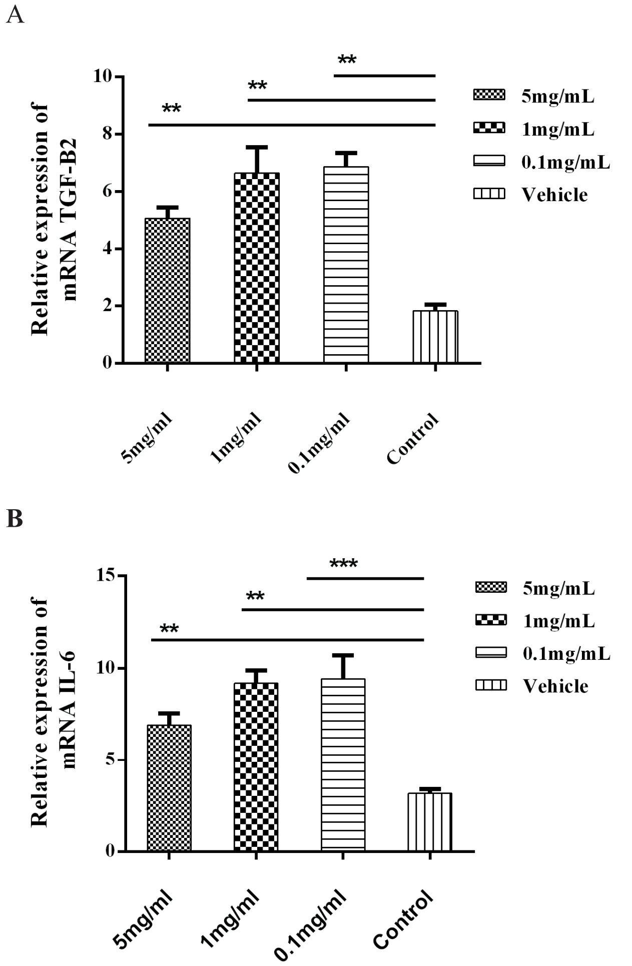

Figure 4 Effects of lipopeptides on inflammatory cytokine secretion in the wound site. Relative expression level of A) TGF-β2 gene and B) IL-6 gene in the injured skin of rabbit. Data are expressed as means ± standard error (SE). *P < 0.05, **P < 0.01, ***P<0.001.

4 Discussion

The wound healing is an intricate and orchestrated process. If broken by external factors, wound repair will be delayed causing severe complications or even death of patients. Currently, some drugs such as platelet-derived growth factor, basic fibroblast growth factor, keratinocyte growth factor have been used as clinical drugs, and natural products such as peptide from frog or salamander were found to be effective for cutaneous wound healing. However, high cost of extraction or synthesis of the wound healing drugs always brings serious economic burden to patients' treatment. Therefore, it makes sense to look for natural products that are both cheap and effective for wound therapy. *Bacillus* strains are typically isolated from different food sources, soil and ocean, most of

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which can produce one or more lipopeptides isoforms just via fermentation. Our study revealed that lipopeptides treatment markedly enhanced the wound healing on rabbit skin, suggesting that lipopeptides have a good development potential and application prospect in regenerative medicine and tissue repair. Our future investigation will focus on identifying the main components of lipopeptide that promoting wound healing. In the process of skin wound healing, the normal initiation of inflammatory response is crucial to the speed and quality of wound healing. A pro-inflammatory environment can contribute to pathogen phagocytosis and death during the early wound healing stage, and recruit additional macrophages and stimulate the migration of keratinocytes to close the wounds [14]. In skin wound healing, TGF-β plays a fundamental role in cell proliferation, formation of extracellular matrix, inflammation, angiogenesis, re-epithelialization, and connective tissue regeneration [15]. Hence, lipopeptides may regulate TGF-β production and release by MAKP signaling system, thus regulate inflammation reaction at the wound site.

5 Conclusions

The present study was conducted to evaluate the in vitro wound healing properties and efficiency of *B. subtilis* lipopeptides. As indicated above, *B. subtilis* lipopeptides exhibited a significant wound healing acceleration in a skin wound model of rabbit. Moreover, lipopeptides could increase the expression of TGF-β2 and IL-6 in the injured skin. In conclusion, *B. subtilis* lipopeptides have a strong capacity to promote wound healing in rabbit model likely by promoting the expression of pro-inflammatory factors in the wound site. *B. subtilis* lipopeptides could be considered as attractive complementary and alternative medicines for skin wound.

6 Acknowledgments

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