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Original Article

Effect and Mechanism of *Ganoderma lucidum* Polysaccharides on Human Fibroblasts and Skin Wound Healing in Mice*

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ABSTRACT **Objective:** To investigate the effects of *Ganoderma lucidum* polysaccharides (GL-PS) on human fibroblasts and skin wound healing in Kunming male mice and to explore the putative molecular mechanism. **Methods:** Primary human skin fibroblasts were cultured. The viability of fibroblasts treated with 0, 10, 20, 40, 80, and 160 μg/mL of GL-PS, respectively were detected by 3-4,5-dimethyl-2-thiazolyl-2,5-diphenyl-2-H-tetrazolium bromide (MTT). The migration ability of fibroblasts treated with 0, 10, 20, and 40 μg/mL of GL-PS were measured by transwell assay. The secretion of the C-terminal peptide of procollagen type Ⅰ (CICP) and transforming growth factor-β1 (TGF-β1) in the cell supernatant was tested by enzyme-linked immunosorbent assay. The expression of β-catenin was detected by Western blot. Furthermore, the Kunming mouse model with full-layer skin resection trauma was established, and was treated with 10, 20, and 40 mg/mL of GL-PS, respectively as external use. The size of the wound was measured daily, complete healing time in each group was recorded and the percentage of wound contraction was calculated. **Results:** Compared with the control group, 10, 20, and 40 μg/mL of GL-PS significantly increased the viability of fibroblasts, promoted the migration ability of fibroblasts, and up-regulated the expressions of CICP and TGF-β1 in fibroblasts ($P<0.05$ or $P<0.01$). The expression of β-catenin in fibroblasts treated with 20 and 40 μg/mL of GL-PS was significantly higher than that of the control group ($P<0.01$). Furthermore, after external use of 10, 20, and 40 mg/mL of GL-PS, the rates of wound healing in mice were significantly higher and the wound healing time was significantly less than the control group ($P<0.05$ or $P<0.01$). **Conclusion:** A certain concentration of GL-PS may promote wound healing via activation of the Wnt/β-catenin signaling pathway and up-regulation of TGF-β1, which might serve as a promising source of skin wound healing.

KEYWORDS *Ganoderma lucidum* polysaccharides, human fibroblast, wound healing, transforming growth factor-β1, Wnt/β-catenin signaling pathway

The process of skin wound healing can be divided into several steps, including hemostasis, inflammation, proliferation, angiogenesis, extracellular matrix (ECM) formation, re-metaplasia, and tissue remodeling.[1-3] The proliferation and migration of fibroblasts and the synthesis of collagen and other ECM play critical roles in tissue remodeling.[4] In the process of wound healing, fibroblast proliferation, migration, and secretion are regulated by several cytokines and signaling pathways. Cheon, et al[5-8] demonstrated that up-regulation of β-catenin could promote the proliferation and invasive ability of fibroblasts; the expression level of β-catenin in fibroblasts increased during the proliferative phase of wound healing stage. It was also found that transforming growth factor-β1 (TGF-β1) promoted the proliferation ability of fibroblasts in wound healing. Thus, both Wnt/β-catenin signaling pathway and TGF-β1 play crucial roles in wound healing.

Ganoderma lucidum (GL), a Chinese herbal medicine, has long been used in China to prevent and treat various diseases, such as hepatitis,[9] chronic

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bronchitis,[10] bronchial asthma,[11] diabetes,[12] cancer and others.[13] *Ganoderma lucidum* polysaccharides (GL-PS) is one of the main bioactive components in GL. It has been shown to exert a variety of pharmacological effects, such as immune regulation,[14] reducing blood glucose and lipids,[15] protecting nerve cells,[16] anti-oxidant,[17] anti-tumor,[18] anti-microbial,[19] and improving the damage to intestinal mucosa.[20] The study by Sun, et al[20] suggested that GL-PS promoted the proliferation, migration, and differentiation ability of intestinal epithelial cells during wound healing process. Tie, et al[21] showed that the oral administration of GL-PS could improve skin wound healing and wound angiogenesis by suppression of the cutaneous manganese superoxide dismutase nitration and mitochondrial oxidative stress.

However, currently, the effects of GL-PS on human fibroblasts and whether the external use would be beneficial to skin wound healing have not been well documented. Also, whether GL-PS could activate the Wnt/β-catenin signaling pathway to promote wound healing has not been reported. In this study, we aimed to investigate the effects of GL-PS on human fibroblasts and skin wound healing in Kunming male mice and to explore the putative molecular mechanism.

METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (USA). Fetal bovine serum (FBS) was purchased from Hyclone (USA). Dimethyl sulfoxide (DMSO) and 3-4,5-dimethyl-2-thiazolyl-2,5-diphenyl-2-H- tetrazolium bromide (MTT) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), TGF-β enzyme-linked immuno sorbent assay (ELISA) kit from Boster Biological Engineering Co., Ltd. (Wuhan, China), and C-terminal propeptide of procollagen type Ⅰ (CICP) ELISA kit from Xinyu Biological Technology Co., Ltd. (Shanghai, China). Rabbit anti-human β-catenin polyclonal antibody and rabbit anti-human β-actin polyclonal antibody was procured from Cell Signaling Technology (USA), while horseradish peroxidase (HRP) labeled goat anti-mouse IgG was purchased from Proteintech (USA).

Preparation of GL-PS

Purified GL-PS was purchased from Yangling Ciyuan Biotech Co., Ltd. (Shaanxi, China; batch No. CY170505). The polysaccharides were extracted and purified from GL according to the procedure as previously reported.[22] In brief, GL was pulverized into powder after slicing. The powder was extracted with distilled water at a ratio of 1:20 (w/v) in 80 ℃ thermostat water bath for 2 h. The supernatant was collected and concentrated in vacuo after the residue was extracted 3 times repeatedly. The protein was removed by the Sevage method.[23] About 20 mL of Sevage reagent was added to each 100 mL of concentrated solution. The solution was shaken vigorously for 30 min and centrifuged at 4,000 r/min for 15 min. The supernatant was taken, and absolute ethanol was added until the solution concentration reached 95%. The precipitate was collected after the solution was allowed to stand overnight. Then, each was washed twice with 95% ethanol, diethyl ether and acetone to obtain a crude polysaccharide. The crude specimens dissolved in distilled water were processed with dialysis, concentration, ethanol precipitation, and then freeze-dried to obtain purified GL-PS. The obtained GL-PS was stored in sealed containers at cool and dry place. It appeared as a deep brown powder. The content of the polysaccharides was 90.3%, which was analyzed by ultraviolet method.

Isolation and Culture of Human Dermal Fibroblasts

Normal human dermis and epidermis were obtained from a healthy 20-year-old male donated foreskin following circumcision, with informed consent. The foreskin was soaked in iodine for 15 min and subsequently rinsed with phosphate buffer saline (PBS). The subcutaneous tissue was removed and the remaining tissue was cut into small pieces (<5 mm). Trypsin was used to isolate fibroblasts, and all cells were subsequently collected, washed, and cultured in DMEM containing 10% FBS. When cells were 80%–90% confluent, they were passaged at a ratio of 1:3. Exponential growth phase cells from passages 4–8 cells were used in the subsequent experiments.

Cell Grouping

GL-PS (1 mg/mL) was prepared in PBS and diluted to the corresponding concentration with complete medium or medium without serum. The cells were divided into a control group and GL-PS groups, in which GL-PS was added to the culture medium at different concentrations.

Fibroblast Viability Measured by MTT Assay

The cells in the logarithmic growth phase were

inoculated at the density of 1×10^4 cells/well in a 96-well plate. Subsequently DMEM-high glucose was used for culture during the cell starvation for 12 h in the presence of various concentrations of GL-PS (0, 10, 20, 40, 80 and 160 μg/mL) for 24 h. Then, 20 μL of MTT reagent was added (stock 5 mg/mL) per well and incubated for an additional 4 h at 37 ℃. The reaction was ceased by 150 μL of DMSO, and absorbance measured at 490 nm.

Migration Ability of Fibroblasts Measured by Transwell Assay

The starved cells for 24 h with different concentrations of GL-PS (0, 10, 20 and 40 μg/mL) were suspended at a density of 5×10^5/mL in the upper chamber. A 100-μL cell suspension was inoculated into the 24-well plate, and 600 μL containing 10% fetal calf serum was added to the lower chamber avoiding air bubbles, followed by incubation at 37 ℃, 5% CO_2 for 24 and 48 h, respectively. Subsequently, the transwell chamber was removed, abandoning the culture medium, washed twice with PBS, then fixed with 10% methanol for 30 min at room temperature, followed by 0.1% crystal violet staining for 20 min. A cotton swab was used to gently wipe the upper non-migrated cells, followed by PBS washes. Five random visual fields were observed, imaged and cells enumerated at 200× magnification.

Synthesis of Type Ⅰ Collagen and TGF-β1 in Fibroblasts Measured by ELISA

The starved cells were inoculated at a density of 1×10^5 cells/well in 6-well plate according to the above experimental results, respectively, with GL-PS (0, 10, 20 and 40 μg/mL) in serum-free medium for 24 h, in triplicate. The supernatants were harvested by centrifugation for 10 min at 4,000 r/min, followed by CICP ELISA and TGF-β1 ELISA tests. The absorbance was determined at 490 nm.

Expression of β-Catenin in Fibroblasts Assessed by Western Blot

The cells were cultured in culture dishes until reaching 60%–70% confluence. After 12-h serum starvation, cells were treated with different concentrations of GL-PS (0, 10, 20 and 40 μg/mL) for 24 h. The protein was extracted from the cells, subjected to gel electrophoresis, and transferred on a membrane that was blocked and probed with anti-β-catenin antibody overnight at 4 ℃. Subsequently, the membranes were incubated for 40–60 min with HRP-conjugated goat anti-rabbit IgG antibody. The expression of target protein was measured by color-development and exposure method. Anti-β-actin used as an internal reference.

Animals

Thirty-two Kunming male mice (8 weeks old; 20–24 g; specific pathogen free grade) used in the wound healing experiment were provided by the Animal Experiment Center of the Third Xiangya Hospital of Central South University [certification No. SYXK (xiang) 2014-0013]. Mice were kept in animal laboratories where the indoor temperature and humidity were kept at 23 ℃ and 56%, respectively, and were randomly given standard laboratory food and water. The present study was approved by the Institutional Review Board of the Third Xiangya Hospital of Central South University, and was conducted according to the Laboratory Animal Administration Rules of China.

Skin Healing Assay in Mice

The back hair of the Kunming mouse was removed by using 4% sodium sulfide before the intraperitoneal injection of anesthesia with 4% chloral hydrate (1 mL/100 g). The site was rinsed with distilled water, dried, and disinfected with iodine. The self-moving paper (diameter about 1 cm) was pasted on the right rear back of the mice. The circle was marked with a marker pen. The center of the circle on the mice skin was picked up with sterile forceps and the skin of the back annihilated along the marking to obtain the same size depth round wound. Then, the ring was stitched using Kiesel gel onto the edge of the wound to reduce the error produced from skin shrinkage, and the suture was away from the edge of the wound by about 0.2 cm. Lastly, the surgical towels were pasted onto the wound.

According to completely randomized design, all the wounds were divided into a control group and 3 experimental groups (n=8 per group). The control group was given 0.05 mL of physiological saline outside the wound. The experimental groups were given a physiological saline solution containing 10, 20 and 40 mg/mL of GL-PS, respectively outside the wound in a volume of 0.05 mL. The medicine was replaced daily. Wound healing time referred to the number of days in which a wound healed more

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than 95%. Wound healing rate (%)=(initial wound area–unhealed area)/initial wound area × 100%. The wounds were observed and photographed at 3, 6 and 10 days after wounding. Computer image analysis system was used to calculated the unhealed area, and each wound healing rate was calculated.

Statistical Analysis

Data were represented as mean ± standard deviation ($\bar{x} \pm s$), and analyzed using SPSS 19.0 software. All the experiments were repeated thrice and the results were tested by the homogeneity of variance. The comparisons between groups were tested by one-way analysis of variance. $P<0.05$ indicated the difference with statistical significance.

RESULTS

GL-PS Increased Viability of Fibroblasts

The viability of cells was significantly increased by 10, 20 and 40 μg/mL of GL-PS after 24-h treatment compared with the control group ($P<0.01$). The viability of the cells was increased by 80 μg/mL of GL-PS and was decreased by 160 μg/mL of GL-PS after 24-h treatment, but there was no statistical significance compared with the control group ($P>0.05$, Figure 1).

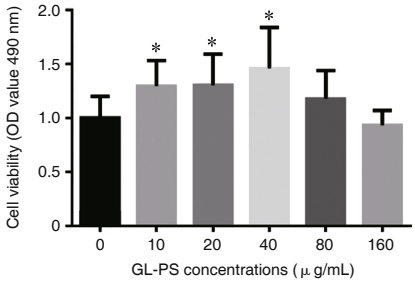

Figure 1. Comparison of Fibroblasts Viability among Different Groups (n=3, $\bar{x} \pm s$)

Notes: *$P<0.01$ *vs*. the control group. The results are represented as a percentage of absorbance relative to the control cells (100%). GL-PS: *Ganoderma lucidum* polysaccharides

GL-PS Enhanced Migration Ability of Fibroblasts

After 24-h and 48-h culture, compared with the control group, the migration rates of fibroblasts were significantly increased in the 10, 20, and 40 μg/mL of GL-PS groups compared with the control group ($P<0.05$ or $P<0.01$, Figure 2).

GL-PS Promoted Synthesis of Type Ⅰ Collagen and TGF-β1 in Fibroblasts

The expressions of CICP and TGF-β1 in fibroblasts treated with 10, 20 and 40 μg/mL of GL-PS were significantly higher than those of the control group ($P<0.05$ or $P<0.01$, Figure 3).

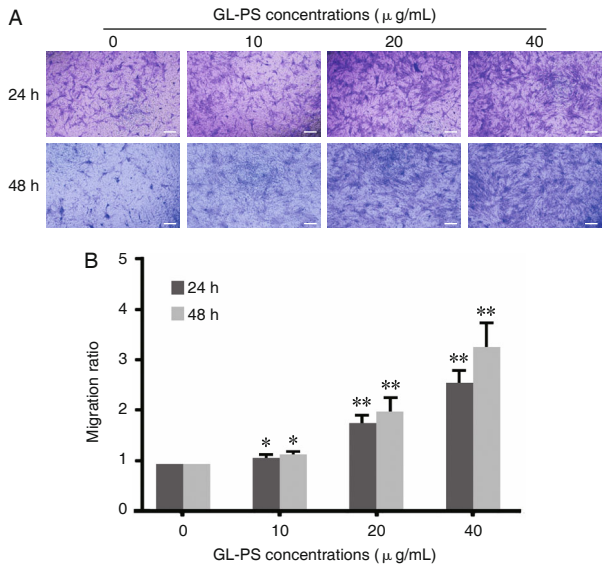

Figure 2. Comparison of Migration Ability of Fibroblasts among Different Groups (n=3, $\bar{x} \pm s$)

Notes: A: Cells stained by 0.1% crystal violet (×400); scale bar= 50 μm; B: results are represented as a percentage of fibroblast cell number relative to control cells (100%). *$P<0.05$, **$P<0.01$ *vs*. control group. GL-PS: *Ganoderma lucidum* polysaccharides

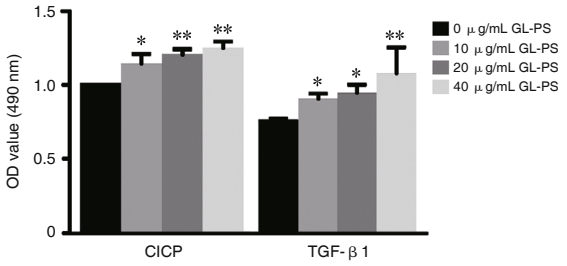

Figure 3. Comparisons of Expressions of CICP and TGF-β1 in Fibroblasts among Different Groups (n=3, $\bar{x} \pm s$)

Notes: *$P<0.05$, **$P<0.01$ *vs*. control group. GL-PS: *Ganoderma lucidum* polysaccharides; CICP: C-terminal peptide of procollagen type Ⅰ; TGF-β1: transforming growth factor-β1

GL-PS Promoted Expression of β-Catenin in Fibroblasts

The expression of β-catenin in fibroblasts treated with 10 μg/mL GL-PS had no significant difference compared with the control group ($P>0.05$). However, 20 and 40 μg/mL of GL-PS significantly increased the expression of β-catenin in fibroblasts ($P<0.01$, Figure 4).

GL-PS Increased Skin Wound Healing Rate and Shortened Wound Healing Time in Mice

On Day 3, 6 and 10 after wounding, the rates of wound healing in 10, 20 and 40 mg/mL of GL-PS

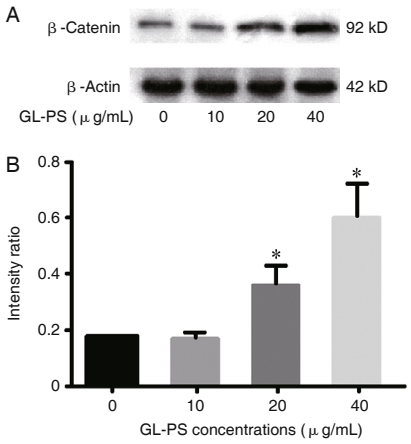

Figure 4. Comparison of Protein Expression of β-Catenin in Fibroblasts among Different Groups (n=3, $\bar{x} \pm s$)

Notes: intensity ratio: β-catenin to β-actin. *P<0.01 *vs*. control group. GL-PS: *Ganoderma lucidum* polysaccharides

groups were significantly higher than the control group (P<0.05 or P<0.01, Figure 5A). The average wound healing time in 10, 20 and 40 mg/mL of GL-PS groups was significantly less than the control group (P<0.05 or P<0.01, Figure 5B). Wound healing in skin injury model mice is shown in Figure 5C, and all wounds were not infected with good wound care. On Day 3, the wound contraction of different concentrations of GL-PS became faster than the control group. And from Day 6 to Day 10, the wound contraction of different concentrations of GL-PS was almost healed.

DISCUSSION

Wound healing is composed of 3 stages, including inflammation, proliferation, and remodeling. The inflammatory reaction occurred within a few hours after injury. During the proliferation period, the wounds were covered by granulation tissue, followed by a collagen-based ECM that replaced the granulation tissue. In the remodeling phase, the collagen protein of the ECM was primarily composed of type Ⅰ collagen.[24] After skin injury, the fibroblasts promote wound healing not only by proliferation and migration but also through the synthesis and secretion of the collagen-based type Ⅰ ECM.[25] Therefore, finding a drug that can potentiate the ability of proliferation, migration, and synthesis of type Ⅰ collagen in fibroblasts would significantly promote wound healing. In this study, we utilized different concentrations of GL-PS to treat human fibroblasts and found that 10, 20, and 40 μg/mL of GL-PS promoted the viability and migration ability of fibroblasts, as well as, the secretion of type Ⅰ collagen. These findings suggested that GL-PS promoted wound

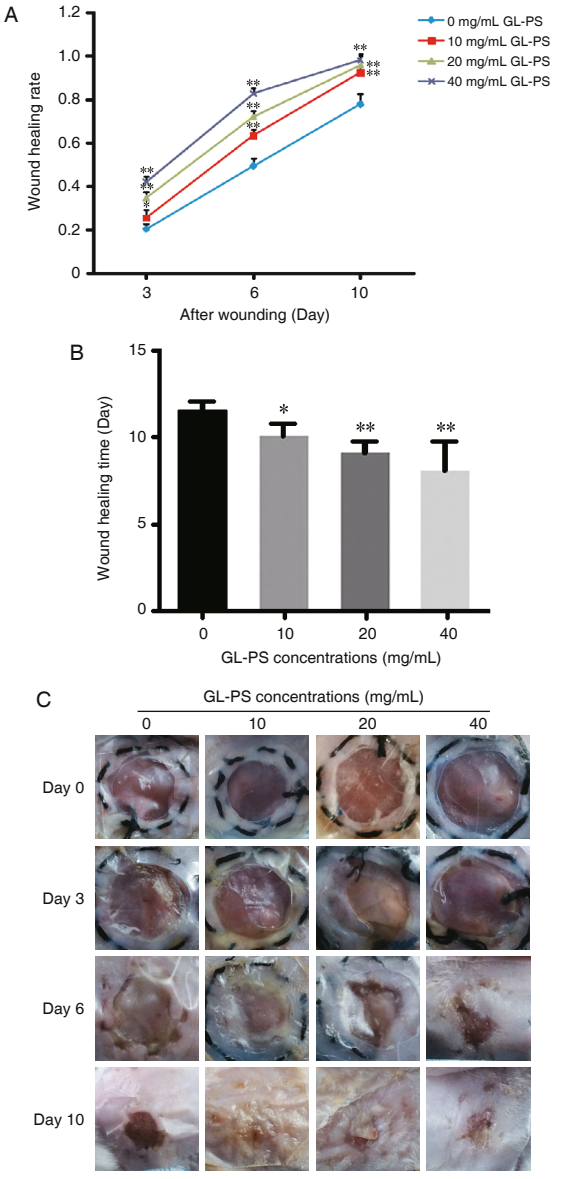

Figure 5. Wound Contraction at Different Time Points (n=8)

Notes: A: Rate of wound healing at different time points. B: Wound healing time in different groups. *P<0.05, **P<0.01 *vs*. control group. C: The images of wound contraction on 0, 3, 6, and 10 days after wounding. GL-PS: *Ganoderma lucidum* polysaccharides

healing at the cellular level. The results also showed that with the external use of GL-PS, the skin wound closure time had been shortened significantly.

In the process of wound healing, several signal transduction pathways were involved in cell proliferation, migration, synthesis, and secretion function. Among these pathways, Hippo, Notch, phosphoinositide 3-kinase (PI3K)/threonine-protein kinase (AKT1), extracellular signal-regulated kinase 1/2 (ERK1/2) in mitogen-activated protein kinase (MAPK), TGF-β/Smad, and Wnt/β-catenin pathway have been

investigated frequently. The Wnt signaling pathway is an evolutionarily highly conserved fundamental signaling system that is involved in many physiological and pathological processes, such as cell proliferation, cell migration, angiogenesis, and ECM formation.[26] It plays a controlling role in hair follicle growth.[27-31] It also plays a vital role in all stages of tissue regeneration and wound healing.[24] The key protein in this signaling pathway is β-catenin.[32] Fathke, et al[33] found that the activation of Wnt/β-catenin pathway during the skin wound healing process could improve the proliferation and migration ability of epidermal cells and shorten the time of wound healing. Collins, et al[34] suggested that the activation of Wnt/β-catenin pathway could increase the proliferation ability of fibroblasts and promote the remodeling of ECM in skin tissues. Pandit, et al[35] showed that with the combination therapy of vacuum and lithium treatment, the migration ability of the fibroblasts was improved via the activation of the Wnt/β-catenin signaling pathway that further accelerated wound healing. In the current study, we found that the expression of β-catenin in fibroblasts was promoted after treatment with a specific concentration of GL-PS, which indicated that the Wnt/β-catenin signaling pathway had been activated by GL-PS application.

TGF-β1 is a growth factor that can stimulate the formation and proliferation of granulation tissue and plays a critical role in wound healing process.[14] In the study of cell activation of fibroblasts or fibrosis by Caraci, et al,[36] TGF-β1 was demonstrated to activate β-catenin via ERK pathway; thus, wound healing could be induced by transient TGF-β signaling, which in turn, increased the level of β-catenin in the cells. Consequently, the accumulation of β-catenin was due to the activation of the TGF-β signaling pathway.[37] Furthermore, the ligands of Wnt (Wnt3a and Wnt5a) can activate TGF-β1 in fibroblasts and intestinal epithelial cells.[38] In addition, a large number of studies have confirmed a close correlation between TGF-β and Wnt/β-catenin signaling pathway. In the present study, we found that 10, 20 and 40 μg/mL of GL-PS promoted the secretion of TGF-β1, thereby suggesting that it might promote wound healing by promoting the secretion of TGF-β1.

In summary, the results of the current study showed that GL-PS could promote the viability and migration of fibroblasts, elevate the ability of fibroblasts to synthesize collagen, and significantly shorten the duration of skin wound healing in the mice model. Moreover, GL-PS could increase the expressions of TGF-β1 and β-catenin, suggesting that GL-PS promoted wound healing via up-regulation of TGF-β1 expression or activation of the Wnt/β-catenin signaling pathway, which might serve as a promising source of skin wound healing.

Conflict of Interest

The authors declared that they had no conflict of interests.

Author Contributions

Zhou JD conceived the idea. Hu F, Yan Y, Wang CW, Liu Y, Wang JJ and Zhou F conducted the experiments. Hu F drafted of the manuscript. Zeng QH, Zhou X, Chen J, Wang AJ and Zhou JD revised and proofed the manuscript. All authors participated in the conception, design of the study, acquisition of data, analysis, and interpretation of data. All authors read and approved the final manuscript.

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