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Original Article

Effect of β-glucan from *Aureobasidium* on dermal wound healing in diabetic C57BL/KsJ-db/db mouse model

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The objective of the present study was to examine the effects of β-glucan originating from *Aureobasidium* on full-thickness skin wound healing in diabetic C57BL/KsJ-db/db mouse models. In the diabetic C57BL/KsJ-db/db model, test articles were topically applied twice a day for 20 days starting from 1 day after wounding. The results were compared to that of Madecassol™ ointment (madecassol; 1% *Centella asiatica* extracts) topically applied at a concentration of 100 mg/kg. Treatment with β-glucan resulted in significant ($p<0.01$ or $p<0.05$) and dose-dependent decreases in wound size compared with that of vehicle control showing increased wound size (WS, %). In addition, 50% contraction time (CT_{50}) was dramatically and dose-dependently reduced, and inflammatory cells in granulation tissues of the wound area were significantly ($p<0.01$ or $p<0.05$) and dose-dependently reduced compared with that of vehicle control showing increased numbers of micro-vessels and fibroblasts as well as re-epithelialization. In the madecassol group, similar changes in inflammatory cells and fibroblasts with re-epithelialization were also observed, but madecassol did not influence angiogenesis. No meaningful changes in body weight were detected in all tested groups compared with the vehicle control. Therefore, these data suggest that β-glucan has a beneficial effect on diabetic delayed skin wound healing and may be useful to manage incurable skin wounds in diabetic animals.

Key words: glucan, dermal, wound healing, diabetic, mouse

Introduction

Wound healing is a fundamental response to tissue injury. Healing involves epithelialization, granulation, and tissue remodeling caused by inflammatory responses [1]. Several innate substances are known to promote wound healing [2]. Among them, β-glucan is a fiber-type complex sugar (polysaccharide) found on cell walls of baker's yeast, oat and barley fibers, and many medicinal mushrooms. β-glucan promotes immune system activity [3, 4] and lowers blood cholesterol levels [5, 6]. Similar to other polysaccharides [7-9], β-glucan has been reported to accelerate wound healing [10-12]. Previously, we showed that β-glucan can stimulate proliferation and migration of human dermal fibroblast cells in a *in vitro* fibroblast populated collagen lattice (FPCL) system mediated by TGF-β [13] as well as promote full-thickness wound healing in an infection murine model [14]. However, the *in vivo* effect of β-glucan on wound healing under diabetic conditions has not yet been evaluated. For this reason, the stimulatory effect of β-glucan on dermal wound healing should be assessed in a diabetic animal model, which displays delayed wound healing.

Therefore, the objective of the present study was to examine the effects of β-glucan originating from *Aureobasidium* on full-thickness skin wound healing in diabetic C57BL/KsJ-db/db mouse models.

Materials and Methods

Animals and husbandry

One hundred male genetically diabetic C57BL/KsJ-db/db mice (7-wk old upon receipt, Jackson Laboratory, USA) were used after 7 days of acclimatization. Five animals were allocated per polycarbonate cage in a temperature (20~25°C) and humidity (45~50%) controlled room. Light:dark cycle was 12 hr:12 hr and feed (Samyang, Korea) and water were supplied free to access. All mice were wounded, and about half of the animals were selected 1 day after wounding according to body weight, blood glucose level, and wound size. All laboratory animals were treated according to the national regulations

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of the usage and welfare of laboratory animals, and the procedure was approved by the Kyungpook National University Experimental Animal Ethical Committee.

Measurement of blood glucose levels

For detecting blood glucose levels, blood samples were collected from wounds at the orbital plexus and deposited into NaF glucose vacuum tubes (Becton Dickinson, USA), after which plasma was separated. Blood glucose levels were detected using an automated blood analyzer (Toshiba 200 FR, Japan) (Table 2).

Full-thickness skin wound preparation

Full-thickness dermatotomies (round; 10 mm) were prepared on the shaved dorsal wall of the diabetic mice. One day after wounding, about half of the animals showing similar wound areas were selected.

Preparations of test materials

β-glucan was purified from *Aureobasidium pullulans* SM-2001, which is a UV-induced mutant of *A. pullulans* [15]. Madecassol™ (Dongkook Pharm. Co., Korea) was used in this study. β-glucan (2.5% solution) was diluted in distilled water and topically applied at dosages of 10, 50, and 100 mg/kg and at a volume of 10 ml/kg starting from 1 day after wounding, twice a day for 20 days. In addition, Madecassol™ was also topically applied at 100 mg/kg, twice a day for 20 days. The administered dose and schedule of these drugs are shown in Table 1. β-glucan were sterilized at 121°C for 10 min before use.

Body weight changes

Changes in body weight were calculated at 0, 2, 4, 8, 12, 16, and 20 days after test article application. In addition, body weight gains were calculated as follows (Equation 1).

Equation 1. Body weight gains (g)=Body weight gains throughout the whole experimental period (day 20~day 0)

Determination of wound sizes

Wound areas were measured on the initial applied day (just immediate before test article application) as well as 2, 4, 8, 12, 16, and 20 days after test article application after wounding as mm^2 using an automated image analyzer (analySIS Image Processing; SIS, Germany) attached to a stereoscope. The 50% contraction time (CT_{50}) was calculated using probit methods, and WS % changes (% changes in wound area between wounding and sacrifice) were calculated as follows (Equation 2).

Equation 2. Changes in wound size (%)=% changes in wound size during experiment (wound sizes at day 20~wound sizes at day 0)

Histopathological procedures

After measuring last wound sizes, wounded area of skin containing dermis was sampled. Sampled skin was fixed in 10% neutral-buffered formalin. After paraffin embedding, 3~4 μm sections were prepared. Representative sections were stained with Hematoxylin and Eosin (H&E) for light microscopic examination, after which the histological profiles of individual skin were observed. For histomorphometric evaluation, the numbers of inflammatory cells such as polymorphonuclear cells (PMNs), macrophages, lymphocytes, and fibroblasts in granulation tissues (N/mm^2) were counted at 21 days after wounding using automated image analysis (analySIS Image Processing; SIS, Germany). The number of micro-vessels in granulation tissues (N/mm^2) was counted at 21 days after wounding using automated image analysis. The degree of re-epithelialization was estimated as a percent (%) of the re-epithelialized filled length in each wound tissue using automated image analysis.

Statistical analyses

All data were calculated as mean ± S.D. (n=10). Statistical analyses were conducted using Mann-Whitney U-Wilcoxon Rank Sum W test (MW test), and CT_{50} was calculated based on measured wound sizes using Probit

Table 1. Experimental design used in this study

	Group	Dose
	Control	Vehicle only
Test groups	β-glucan	10 mg/kg
	β-glucan	50 mg/kg
	β-glucan	100 mg/kg
	Madecassol™	100 mg/kg

*β-glucan and vehicle were topically applied at a volume of 10 mL/kg for 20 days; all animals were sacrificed after application for 20 days; Madecassol™ was topically applied at 100 mg/kg.

Table 2. Blood glucose levels of diabetic animals used in this study upon wounding

	Group	Blood glucose levels (mg/dL)
	Control	548.40 ± 65.46
Test groups	Madecassol™ 100 mg/kg	554.80 ± 50.66
	β-glucan 10 mg/kg	552.10 ± 51.32
	β-glucan 50 mg/kg	540.20 ± 60.72
	β-glucan 100 mg/kg	555.80 ± 60.64

n=10; (Mean ± S.D.).

methods with SPSS for Windows (Release 6.1.3., SPSS Inc., USA). Inhibition rates compared to that of vehicle control were calculated to determine the effects of test materials on differences between control and test groups (Equation 3).

Equation 3. Percentage of changes compared control (%)=((data of tested groups − data of control)/data of control) × 100

Results

Effects of β-glucan on changes in body weight

There were no significant changes in body weight among all tested groups compared to the control, except for slight decreases in all test article applied groups (Table 3).

Body weight during experimental periods showed that it underwent changes of − 6.15, − 7.08, − 9.31, and − 6.52% in the madecassol and 10, 50, and 100 mg/kg of β-glucan groups compared with control group, respectively.

Effect of β-glucan on wound size

Wound size (WS) significantly decreased in a dose-dependent manner in a β-glucan-treated groups compared to the control over a treatment period of 16 or 20 days ($p<0.01$ or $p<0.05$). Wound size was significantly reduced at β-glucan concentrations of 50 and 100 mg/kg starting from day 8 when Madecassol™ had no effect. Wound size also significantly decreased in the madecassol group starting from day 12 ($p<0.01$ or $p<0.05$). The reducing effect of β-glucan on wound size was stronger than that of Madecassol™. In addition, changes (%) in wound size significantly increased among all tested groups compared to the control ($p<0.01$ or $p<0.05$). There were also sig-

Table 3. Changes in body weight

Body weight (g)	Control	Madecassol™ 100 mg/kg	β-glucan-applied groups 10 mg/kg	50 mg/kg	100 mg/kg
Day 0[1)]	38.67 ± 1.53	38.63 ± 2.17	38.79 ± 2.09	38.70 ± 1.55	38.66 ± 1.57
Day 2	39.70 ± 2.58	39.51 ± 2.46	39.63 ± 1.73	39.84 ± 1.59	39.54 ± 1.67
Day 4	40.56 ± 2.37	40.66 ± 1.99	40.43 ± 1.80	41.09 ± 2.37	40.55 ± 1.88
Day 8	41.16 ± 2.21	41.61 ± 2.28	41.61 ± 1.70	41.64 ± 1.98	41.62 ± 2.46
Day 12	42.76 ± 1.96	42.48 ± 2.17	42.26 ± 1.68	42.28 ± 1.36	42.39 ± 2.23
Day 16	43.47 ± 1.96	42.53 ± 2.21	43.25 ± 1.73	43.12 ± 1.32	42.81 ± 2.21
Day 20[2)]	44.04 ± 2.44	43.67 ± 2.02	43.78 ± 2.10	43.57 ± 1.27	43.68 ± 1.89
Gains (g)[3)]	5.37 ± 2.86	5.04 ± 2.10	4.99 ± 2.31	4.87 ± 2.01	5.02 ± 1.69

n=10; (Mean ± S.D.); [1)] At initial application of test article 1 day after wounding; [2)] At sacrifice; [3)] Body weight gains (g)=Body weight gains throughout the whole experimental period (day 0~day 20).

Table 4. Changes in wound size (WS)

Wound sizes (mm^2)	Control	Madecassol™ 100 mg/kg	β-glucan-applied groups 10 mg/kg	50 mg/kg	100 mg/kg
Day 0[1)]	86.07 ± 5.34	86.16 ± 4.80	86.34 ± 4.81	86.58 ± 3.84	86.31 ± 4.40
Day 2	84.10 ± 5.93	83.15 ± 4.89	83.05 ± 4.69	83.77 ± 4.42	84.07 ± 5.45
Day 4	81.16 ± 6.17	78.58 ± 3.75	80.47 ± 5.22	78.08 ± 7.30	77.62 ± 5.20
Day 8	76.88 ± 5.78	69.89 ± 7.49	73.85 ± 6.97	66.01 ± 7.14*	63.17 ± 5.39*
Day 12	69.16 ± 5.41	60.13 ± 9.53**	63.38 ± 8.58	58.81 ± 8.97*	51.51 ± 6.86*
Day 16	57.75 ± 4.77	48.12 ± 9.51**	51.60 ± 6.72**	47.21 ± 10.01**	43.62 ± 7.51*
Day 20[2)]	44.71 ± 7.08	35.25 ± 8.05*	37.16 ± 7.40**	33.41 ± 8.14*	31.70 ± 6.30*
WS % changes (%)[3)]	− 47.97 ± 8.03	− 59.03 ± 9.25*	− 57.13 ± 6.97**	− 61.44 ± 8.78*	− 63.24 ± 7.02*
CT_{50} (days)[4)]	28.35 ± 10.65	19.82 ± 5.68**	20.55 ± 3.27**	17.58 ± 3.75*	15.57 ± 2.55*

n=10; (Mean ± S.D.); [1)] Before initial application of test article 1 day after wounding; [2)] Before sacrifice; [3)] % changes in wound size from day 0 to day 20; [4)] 50% contraction time of wound; *$p<0.01$ and **$p<0.05$ compared to that of control by MW test.

nificant decreases in CT_{50} (p<0.01) (Table 4).

Effect of β-glucan on histopathology

There were dose-dependent increases of angiogenesis, re-epithelialization, and reconstruction of the dermis in all β-glucan groups compared to the control. Except for angiogenesis, skin wounds showed similar reconstruction between the madecassol-treated group and β-glucan-treated groups (Fig. 1). There were dose-dependent and significant decreases in the numbers of inflammatory cells such as PMNs, macrophages, and lymphocytes in the 50 and 100 mg/kg of β-glucan groups compared to the control (p<0.01). Further, there were significant increases in the numbers of micro-vessels and fibroblasts as well as the rate of re-epithelialization (p<0.01). In the 10 mg/kg of β-glucan group, significant (p<0.01 or p<0.05) reduction of inflammatory cells and elevation of fibroblasts were observed compared to the control. In the madecassol-applied group, similar changes in inflammatory cells and fibroblasts were observed with a significant (p<0.01) increase in re-epithelialization, although no meaningful changes were observed in angiogenesis (Table 5).

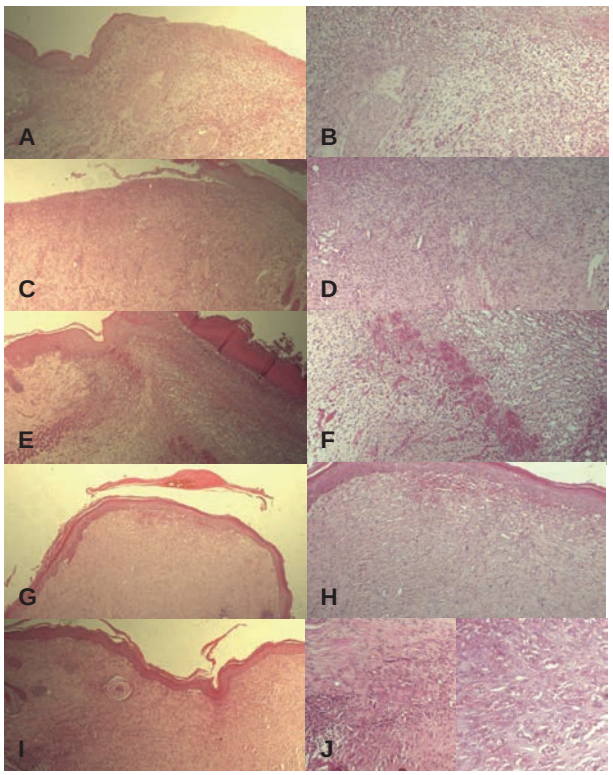

Fig. 1. Changes in histological profiles of granulation tissues at 20 days after application of vehicle (A, B), Madecassol™ (C, D), β-glucan 10 (E, F), 50 (G, H), and 100 (I, J) mg/kg in diabetic mice. Note that dermis reconstruction and re-epithelialization were dose-dependently detected in all β-glucan-applied groups compared to vehicle control showing angiogenesis, and 100 mg/kg of Madecassol™ showed similar promotion efficacy as 10 mg/kg of β-glucan except for angiogenesis, in which no meaningful changes were observed. All H&E stain; Scale bars=200 μm.

Discussion

In the present study, the direct effects of β-glucan on wound healing in diabetic mice were examined for the first time. As a result of treatment with β-glucan, significant (p<0.01 or p<0.05) and dose-dependent decreases in wound size were observed compared to the vehicle control showing increased WS%. In addition, CT_{50} was dramatically and dose-dependently reduced, and inflammatory cells in granulation tissues of wound areas were also significantly (p<0.01 or p<0.05) and dose-dependently decreased compared to the vehicle control showing increased numbers of micro-vessels and fibroblasts as well as re-epithelialization. In the madecassol-applied group, similar changes in inflammatory cells and fibroblasts with re-epithelialization were observed, although Madecassol™ did not affect angiogenesis. These data

Table 5. Changes in numbers of inflammatory cells, fibroblasts (fibrocytes), and micro-vessels in granulation tissues at 20 days after application with re-epithelialization

Histomorphometry	Control	Madecassol™	β-glucan-applied groups		
		100 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg
Cell counts (N/mm²)					
PMNs[1)]	98.60 ± 26.72	58.90 ± 14.40*	40.60 ± 12.52*	24.00 ± 8.82*	16.10 ± 6.82*
Macrophages	33.30 ± 9.21	23.80 ± 8.95**	20.90 ± 5.32*	10.30 ± 3.06*	8.00 ± 2.31*
Lymphocytes	40.30 ± 9.25	28.10 ± 10.91**	26.80 ± 12.53**	20.60 ± 4.62*	17.80 ± 8.13*
Fibroblasts	32.50 ± 10.86	76.00 ± 23.84*	63.10 ± 14.18*	102.80 ± 20.81*	125.20 ± 35.70*
Angiogenesis (N/mm²)					
Micro-vessels	67.30 ± 21.31	68.70 ± 26.30	90.50 ± 25.29	107.00 ± 25.63*	109.20 ± 17.30*
Re-epithelialization (%)	27.18 ± 10.64	46.67 ± 10.56*	30.22 ± 8.42	69.77 ± 12.39*	80.18 ± 11.49*

n=10; (Mean ± S.D.); [1)]Polymorphonuclear leukocytes; *p<0.01 and **p<0.05 compared to that of control by MW test.

suggest that β-glucan has properties that may be beneficial for wound healing, especially for intractable bedsores and other chronic ulcers frequently encountered in diabetic patients.

The C57BL/KsJ-db/db mouse is a mutant diabetic mouse model showing a delayed healing response in the dermis relative to normal mice [16]. These db/db mice also show obesity and hyperglycemia at 5-wk-old [17, 18]. In the present study, db/db mice showed hyperglycemia and obesity before wounding. No meaningful changes in body weight or gain were detected in the present study, except for a non-significant and slight decrease in body weight gain in all tested groups compared to the control. This result can be attributed to the stress of relatively long term (20 days) application of test articles, which means that topical application of β-glucan did not show any serious toxicity up to 100 mg/kg in diabetic mice.

In the present study, β-glucan potentially increased contraction of wounds in diabetic mice similar to normal mice. Increased wound contractions are considered as a basic characteristic of wound management agents. Reduction of CT_{50} and elevation of WS% detected in the β-glucan-applied groups can be considered as a direct evidence that β-glucan promoted wound contraction in diabetic mice.

Wound healing involves overlapping steps of inflammation, cell migration and proliferation, neovascularisation, and extracellular matrix production and remodeling, and collagen is a major component of the extracellular matrix [19]. Inflammation followed by tissue repair is a complex physiological process aimed at restoration of normal function after infection or wounding [20, 21]. Advances in growth factor discovery, biochemistry of the extracellular matrix, and immunology have enhanced our knowledge of inflammation and wound healing, including angiogenesis. While inflammation stimulates the production of angiogenic growth factors, angiogenesis is an independent event from inflammation [22, 23]. Angiogenesis occurs during later stages of tissue repair and is essential to restoration of tissue damaged by injury and inflammation [23-25]. Though many growth factors possess angiogenic properties and are commercially available in recombinant forms, their widespread clinical use is hindered by their prohibitive costs [26]. In addition, their half-lives in the bloodstream are likely to be short and unpredictable due to the presence of a variety of binding proteins. Therefore, identification and characterization of alternative compounds actively promoting wound healing are important to the development of new therapeutic strategies. Dose-dependent decreases in inflammatory cells such as PMNs, macrophages, and lymphocytes were detected in β-glucan-applied groups showing increased numbers of micro-vessels and fibroblasts as well as re-epithelialization in the present study. This result can be considered as direct evidence that β-glucan promoted wound reconstruction from granulation tissues in diabetic mice, similar to normal mice. Although the β-glucan used in this study did not influence collagen production of fibroblasts *in vitro*, it is possible that collagen production increased *in vivo* as a secondary effect of increased proliferation of fibroblast-mediated TGF-β1. In this study, dramatic increases in fibrosis and fibroblast numbers were observed.

The mechanism of action of β-glucan in the promotion of wound healing is complex. The anti-inflammatory activities of β-glucan in this study might be beneficial for dermal wound healing, similar to the effect of polysaccharide extract from *Phellinus gilvus* [27]. In addition, β-glucans are potent macrophage stimulators that enhance macrophage cytotoxicity and phagocytic capacity [28, 29]. Leibovich and Ross [30] reported that wound healing is delayed when wound macrophages are depleted. Therefore, it is possible that dermal wound healing may be promoted by the macrophage activity of β-glucan. Further, promotion of wound healing by β-glucan also stimulated fibroblasts and its specific cytokine TGF-β1 since proliferation of fibroblasts is promoted by β-glucan-mediated TGF-β1.

These data suggest that β-glucan has properties that may be beneficial for wound healing, as it promoted contraction of wounds and reconstruction of the dermis and epidermis. These data show that β-glucan has enough wound healing effects to be developed as a wound management agent or as an adjuvant for the treatment of skin wounds, especially intractable bedsores and other chronic ulcers frequently encountered in diabetic patients. However, additional studies regarding its mechanism of action will reveal its usefulness and limitations.

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