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Geniposide promotes wound healing of skin ulcers in diabetic rats through PI3K/Akt pathway

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ABSTRACT

Continuously hyperglycation-induced lesion and poor blood flow contributed to the wound incurable and susceptible to infection. About fifteen percent of people with diabetes would develop ulcers during their lifetime, especially on the feet, which could lead to severe tissue destruction and eventual amputation. Various strategies were limited to accelerate wound healing in diabetic patients for high cost and unsatisfied effects. Geniposide is well-known for its anti-inflammation and anti-apoptosis in several pathological tissues. This study is to explore the protective effect of geniposide on wound healing rate, inflammatory response, nutritional function and cellular apoptosis in diabetic rats. Diabetic rats was induced by streptozotocin and defined as plasma glucose >300 mg/dl. Western blot and immunostaining technologies were performed to mark and quantify the target proteins. The oral administration of geniposide (200 mg/kg and 500 mg/kg) could significantly promote wound healing by the increment of lesion retraction in diabetic rats compared to model group. In the apoptotic study of skin wound in diabetic rats, the TUNEL-positive cells were greatly decreased in geniposide subgroups ($P < 0.05$). The levels of TNF-α, IL-1β and IL-6 were significantly inhibited by geniposide with the IC_{50} value of 470 mg/kg, 464 mg/kg and 370 mg/kg body weight respectively, which might be related to the enhancement of the phosphorylation of PI3K and Akt proteins. Geniposide enhanced the repairment of skin wound in diabetic rats by inhibiting inflammatory response and apoptosis.

1. Introduction

Long-term high glucose exposure in the diabetes can cause continuous damage in tissues and organs, finally contributing to diabetic peripheral neuropathy, diabetic nephropathy and diabetic skin ulcer (DSU), as the major complications of diabetes [1]. Diabetic foot is a representative manifestation of DSU, and can even lead to amputation [2]. Continuously hyperglycation-induced lesion contributed to the wound incurable and susceptible to infection [3]. In view of the limited strategies for wound healing, there are many new methods to promote wound healing in diabetic patients, such as local application of recombinant human collagen hydrogel, closed negative pressure drainage, traditional Chinese medicine external washing combined with hyperbaric oxygen, etc., but all of them are limited by high cost and unsatisfactory effect [[4,5]].

Geniposide is one of the major iridoid glycosides from *Gardenia jasminoides* J. Ellis [[6,7]]. Extensive literatures reported that the pharmacological activities of geniposide could be introduced as anti-oxidation, anti-inflammation, anti-thrombosis and anti-ischemic stroke via mitogen-activated protein kinase (MAPK)/nuclear factor-kappa B (NF-κB) [8] and phosphatidylinositol-3-kinase (PI3K)-

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protein kinase B (Akt) signaling pathways [9]. Beside, many previous studies implied that aqueous extract from *Gardenia jasminoides* and geniposide possessed remarkable antithrombotic activities, increased autophagy and inhibited apoptosis by regulating the function of mTOR [[10,11]]. Our former study demonstrated that geniposide-induced IL-10 expression contributed to its anti-inflammatory action by inhibiting TNF-α, IL-1β and IL-6 [12]. The pharmacokinetics of geniposide belonged to the one-compartment model with short half-life ($t_{1/2} \approx 1$ h) eliminated fast in in plasma [13]. However, the effect of geniposide and its underlying mechanism on DSU was still unclear. We suspect that geniposide may also improve the repairment of skin wound in diabetic rats by inhibiting inflammatory response and cellular apoptosis via PI3K/Akt signaling pathway. Besides, we are also unclear whether geniposide possess nutritional function by modulating the endothelial growth factors in wound tissue. Thus in this study, we try to explore the protective effect of geniposide on wound healing rate, inflammatory response, nutritional function and cellular apoptosis in diabetic rats.

2. Materials and methods

2.1. Animal and chemical agents

Healthy male Wistar rats (8 weeks old, body weight 250 ± 20 g, Experimental Animal Center of Jiangxi University of Traditional Chinese Medicine, laboratory animal certificate number: SCXK (Gan) 2019–0001, certificate number: 025–4622); Geniposide (HPLC >95 %, Chengdu Dicotyledon Chinese Medicine Resources Co., Ltd., batch number 20191210); streptozotocin (Sigma); blood glucose testing paper; IL-6, IL-1β, TNF-α detection Kit (R&D Systems); epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) ELISA kits (Bioss Biotechnology Co., LTD, Beijing, China); total nitric oxide test kit (Beyotime Biotechnology, Shanghai, China); p-PI3K, PI3K, p-Akt, Akt primary antibody (U.S., CST). All animal experiment operations were approved and implemented by the Ethics Committee of Jiangxi University of Science and Technology.

3. Experimental method

3.1. Preparation of diabetic skin ulcer model

All rats were adaptively fed with normal rodent food for one week, and allowed *ad libitum* to water. High-fat feed was switched as continuous feeding for ten weeks. The rats were fasted for one night before a single intraperitoneal injection of streptozotocin (55 mg/kg, 0.1 M buffered saline, pH = 4.5). Forty-eight hours later, fasting Blood glucose level was tested in tail vein blood. The rats with fasting plasma glucose (>300 mg/dl) would be recruited as diabetic rats [14].

For rodent modeling of diabetic skin ulcer, diabetic rats were anesthetized by intraperitoneal injection of 1 % sodium pentobarbital injection (40 mg/kg). After removing residual hair with depilatory cream on the right-sided back, a 2 cm diameter wound reaching the lower fascia layer was created by a hole puncher. In order to prevent the gauze being ripped by rats after the operation, the gauze was wrapped with a thin thread and the rat was raised in separate cage. The skin around the wound was swollen with dull red on Days 3 after the operation and the skin ulcer was extensively induced on Day 5–7 after wounding onset.

3.2. Experimental intervention

The recruited rats were separated into normal group, model group, geniposide subgroups (Gen-L: 200 mg/kg; Gen-M: 400 mg/kg; Gen-H: 500 mg/kg), considering that geniposide at a single dose of close to 600 mg/kg could induce hepatotoxicity in rats after oral administration [15]. All rats were divided into each group randomizedly according to body weight (n = 6). The diabetic rats were administered with saline solution or one dose of geniposide by gavage. The drug was administered once a day, and the wound healing and inflammation of each group were recorded every day. The fasting blood glucose of the rats in each group was measured on the Day −14, −7, 0, 1, 21. All experimental procedures were performed by two operators blindly, and finally the data were collected and analyzed by another one.

3.3. Wound healing test

The electronic picture quantification assay was used to trace the ulcer edges of diabetic rats in each group, and the wound area was quantitated and calculated with computer-assisted image analysis (ImageJ Software, Wayne Rasband, NIH, USA). The wound healing rate could be calculated in the formula as follows: Percentage value (%) = (wound area before treatment-wound area after treatment)/wound area before treatment * 100 %.

3.4. Detection of inflammatory factors and endothelial growth factors in wound tissue

The ulcer wound tissue on the Day 7 after trauma onset was extracted and homogenized in PBS-buffered protease inhibitor cocktail (Sigma-Aldrich) by sonication. The homogenate was centrifuged at 12000 rpm for 15 min at 4 °C. The supernatants were collected for commercial enzyme-linked immunosorbent assay (ELISA). ELISA kits (R&D Systems, Inc. Minneapolis, MN, USA) were performed to determine pro-inflammatory or inflammatory factors (interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α)), according to the manufacturer's instructions. The expression of EGF and VEGF were tested by ELISA kits (Bioss Biotechnology Co.,

LTD, Beijing, China). The NO level was measured by Total nitric oxide test kit from Beyotime Biotechnology.

3.5. TUNEL staining for the evaluation of apoptotic cells

The skin tissue was isolated and dehydrated in gradient ethanol solutions (75–95 %) at 4 °C within 3–5 days. The skin tissue was fixed in paraffin wax at 55–65 °C and cut into 10-μm sections by paraffin microtome. The skin sections were prepared and attached on glass slide by free-floating sections method. Coronal sections were suffered from xylene deparaffinize and rehydrate by gradient ethanol solutions (95-75 %) before fluorescent staining in TUNEL reactive resolution for 30 min at 37 °C. After washing with 0.01 M PBS buffer (pH = 7.4) for three times, DAPI was added to counterstain cell nucleus in room temperature for 5 min. The slices were observed under fluorescent microscope with × 100 times magnification. The number of apoptotic cells was quantified in each slice and the mean value of three samples was used as the representative data.

3.6. Western blotting assay

It was applied to detect the expression of PI3K and Akt protein in wound tissues according to the reported method [16]. Proteins were extracted and detected by BCA kit. The corresponding p-PI3K, PI3K (1:1000), p-Akt, Akt (1:1000) primary antibodies were obtained from Cell Signaling Technology to incubate membrane overnight at 4 °C. After washing with Tris-HCl Buffered saline containing 0.1 % Tween-20 (TBST), the secondary antibody was added and incubated for 2 h at room temperature. After washing with TBST, chemiluminescence reagents were added and the gel imaging was quantified by using Image J software to analyze the grey value of the corresponding protein bands.

3.7. Statistical methods

The GraphPad Prism software 7.0 was used to perform the statistical analysis. In wound healing test and detection of inflammatory factors, the distribution of continuous variables was evaluated by the Shapiro-Wilk normality test. The data in these experiments were found to be distributed normally and presented as the mean ± standard deviation. In TUNEL staining and Western blot tests, normality test was not appropriate for the small sample size, thus data were shown in the median and interquartile range [min to max]. Statistical significance was assessed by using nonparametric *t*-test. For the data passing through normality test, both ANOVA followed by Bonferroni (Two-way) or Tukey's multiple comparison (One-way) post hoc test and unpaired and two-tailed Student's *t*-test (if equal variation $P > 0.05$ by Bartlett test) were performed to check the significance between model group and treatment subgroups, and P value less than 0.05 was considered to be statistically significant.

For dose-response curve analysis, values of response (Y) were fitted by non-linear least squares curves to the relation: $Y = a + b\chi$, where $\chi = [D]^n/(ED_{50}^n + [D]^n)$, to calculate the parameters [maximum effect (E_{max}) and half-effective dose (ED_{50})] from individual dose-response curves. To calculate these parameters, response values were transformed and fitted by non-linear least squares curves according to the literatures [11].

4. Results

4.1. Prevention of geniposide on wound healing

In all diabetic rats, the blood glucose level of rats decreased dose-dependently with significant difference on Day 8 and 21 after continuous administration of geniposide by gavage compared to model group (Table 1, $P < 0.05$).

Among the selected rats, seven rats were excluded due to modeling failure or died of high blood sugar or infection. Finally, six normal rats and eighteen diabetic rats were included (Fig. 1A). Oral administration of geniposide in diabetic rats could significantly retract wound size compared to model group since Day 7 after surgery, until the termination of experimental surgery (Day 15, Fig. 1B and C). The data in all groups were analyzed by two-way ANOVA followed by the Bonferroni post-hoc test. The two-way ANOVA was performed after Shapiro-Wilk normality test. In Bonferroni posttests, treatment subgroups were also significantly different from model group in each time-point (Model vs Gen(L): $P < 0.05$; Model vs Gen(H): $P < 0.001$). To further confirm the difference between model group and treatment subgroups, pairwise comparison by unpaired *t*-test was also performed after normality and equal variances test.

Inhibition of geniposide on skin cell apoptosis by terminal dexynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining.

According to the results showed in Fig. 1, the skin ulcer in diabetic rat was induced on Day 7 after surgery, which was identical with

Table 1
Fasting blood glucose level in non-diabetic and diabetic rats (mg/dl).

Groups	Day −14	Day −7	Day 1	Day 8	Day 21
Normal rats	103.88 ± 5.49	105.73 ± 6.12	99.11 ± 5.76	105.91 ± 6.14	109.82 ± 7.18
Model rats in control	104.14 ± 3.55	494.15 ± 5.09	487.42 ± 3.96	517.21 ± 3.22	523.56 ± 7.10
Gen L group	106.86 ± 6.04	485.63 ± 6.47	485.50 ± 6.35	421.35 ± 7.79*	377.65 ± 4.68**
Gen H group	106.21 ± 5.15	501.22 ± 3.79	498.26 ± 7.26	376.03 ± 5.49**	297.54 ± 6.00**

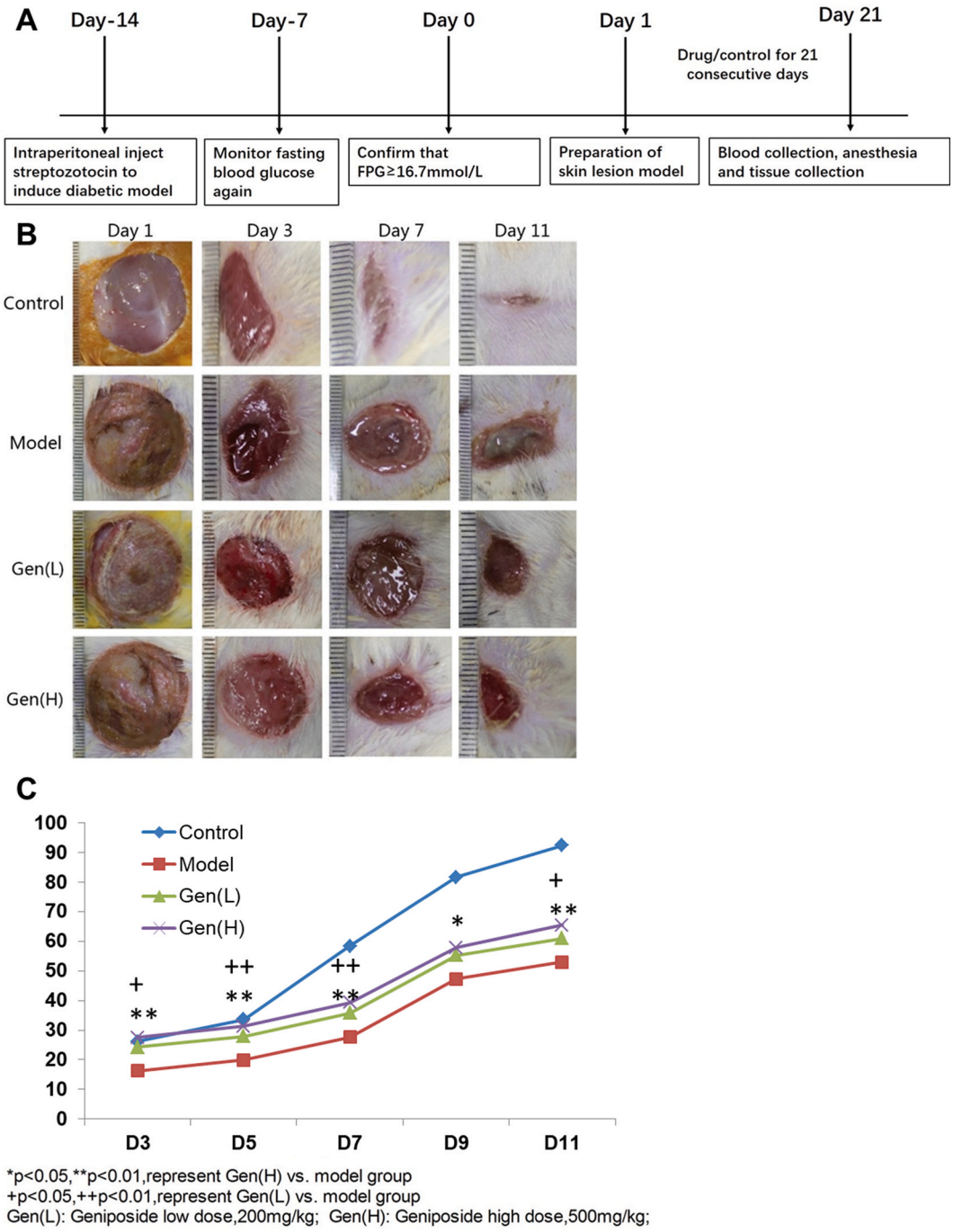

Fig. 1. Geniposide imporved wound healing at different time points in diabetic rats. A: The time course of skin ulcers modeling in diabetic rats and involvement of geniposide. B: The situation of wound healing in each group at Day 1, 3, 7 and 11 after surgery. C: The area of wound healing was quantified by ImageJ Software and results showed that administration of geniposide improved wound healing of skin ulcers in diabetic rats ($n = 6$ in each group). $^{*/+}P < 0.05$, $^{**/++}P < 0.01$ denoted statistical significance compared with model group, analyzed by two-way ANOVA followed by the Bonferroni post-hoc test, and finally unpaired *t*-test after normality and equal variances test.

the expectation in rodent model of diabetic foot ulcer [17]. As a chronic skin disorder, the biomedical change in skin ulcer was commonly stable with slow recovering rate, thus we got used to evaluate the efficacy at one time-point according to our experience. In this rodent model of diabetics-induced skin ulcer, the subsequent tests on cell apoptosis and biochemical indicators were assessed on Day 7 and Day 11 after wounding onset.

The fluorescent signal in control rats was weak in both epidermis and dermis of rat skin with a few TUNEL-positive apoptotic cells.

The apoptotic cells in the dermis are mainly concentrated around the hair follicles. For the limitation of small sample size, Shapiro-Wilk normality test could not be performed, thus finally nonparametric *t*-test was done. The TUNEL-positive cells were greatly decreased in diabetic rats treated with geniposide on both Day 7 and Day 11 after surgery (Model vs Gen(L) at Day 7: $t = 8.36$, $P < 0.0001$; Model vs Gen(H) at Day 7: $t = 12.84$, $P < 0.001$; Model vs Gen(L) at Day 11: $t = 9.31$, $P < 0.0001$; Model vs Gen(H) at Day 11: $t = 13.28$, $P < 0.001$, Fig. 2A–I). The TUNEL-positive cells in treatment subgroups were mainly in the epidermis, related to those in the dermis.

4.2. Activation of geniposide on PI3K-Akt pathway in wound tissues

For the limitation of small sample size, Shapiro-Wilk normality test could not be performed, thus finally nonparametric *t*-test was done. Based on grey bend analysis of Western blot, the results showed that the phosphorylation levels of both PI3K and Akt were significantly decreased in diabetic rats on Day 7 after surgery (pPI3K/PI3K: $t = 4.52$, $P = 0.011$ for Model vs Gen(L); $t = 6.96$, $P = 0.0022$ for Model vs Gen(H); pAkt/Akt: $t = 8.01$, $P = 0.0013$ for Model vs Gen(L); $t = 13.21$, $P = 0.0002$ for Model vs Gen(H)), which was reversed in the geniposide-treated diabetic rats (Fig. 3A–C). It was suggested that geniposide promoted wound healing at least partially through PI3K/Akt signaling pathway.

Nutritional function of geniposide and its inhibition on pro-inflammatory or inflammatory factors in ulcerative tissues.

The protection of skin wounds is the joint action of repairment and anti-apoptosis. Endogenous factors such as VEGF, NO and EGF play a key role, among which EGF can promote the repair of skin wounds by activating the exchange of Na^+/H^+ in epithelial cells. The effect of 400 mg/kg geniposide on the expression of EGF, VEGF and NO level was quantified by the significant increment of 38.2 %, 27.2 % and 42.7 %, respectively (Fig. 4A–C).

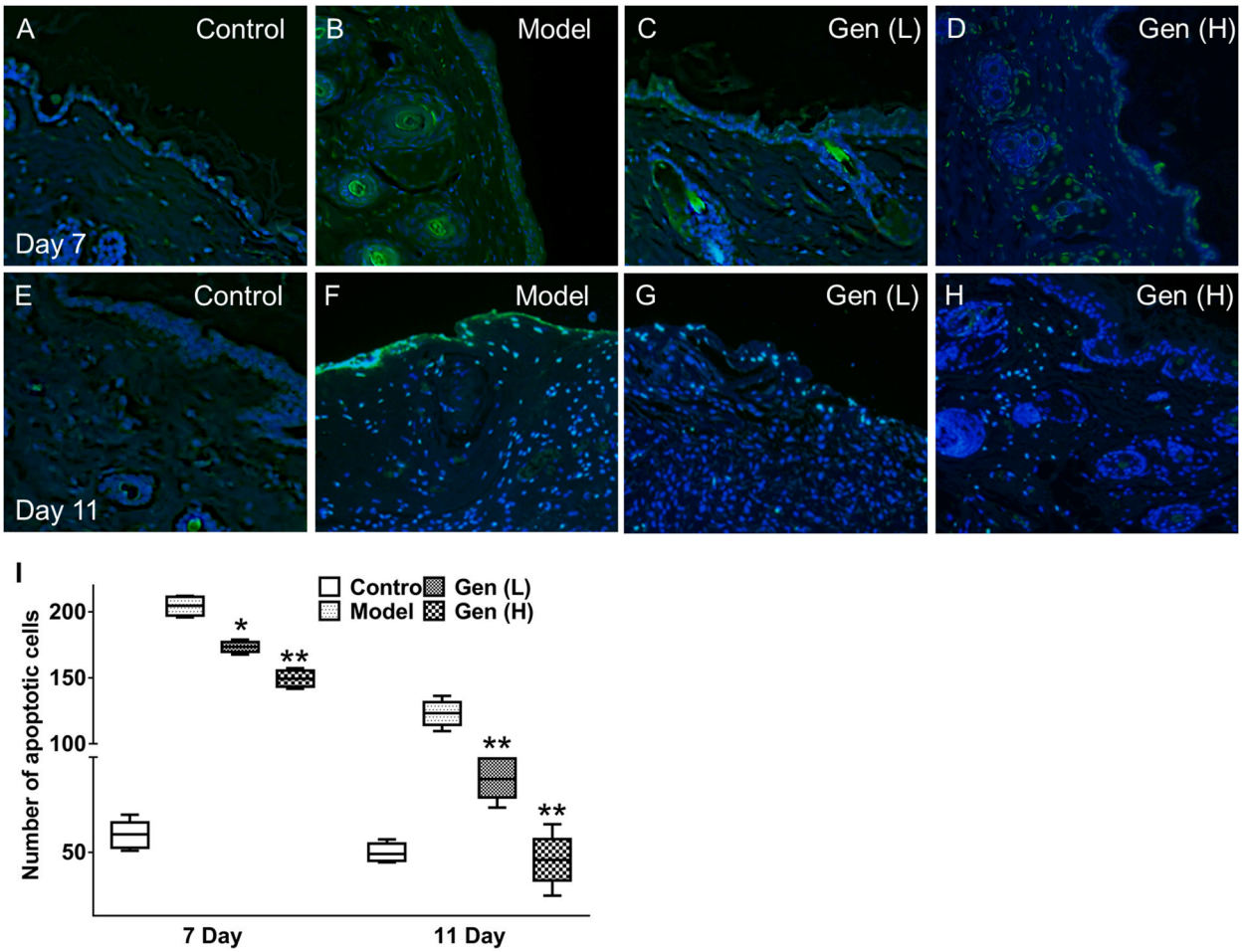

Fig. 2. Geniposide inhibited cellular apoptosis in skin tissues of diabetic mice on Day 7 and Day 11 after surgery. A–D: The representative images of TUNEL-positive apoptotic cells with green color in each group. E: The quantitative analysis of apoptotic cells number in each group. Data are shown as the median and interquartile range [min to max] (n = 5 in each group). *P < 0.05, **P < 0.01 denoted statistical significance compared with model group, analyzed by one-way ANOVA followed by the post-hoc Tukey's multiple comparison test, and finally nonparametric *t*-test for failure of normality test.

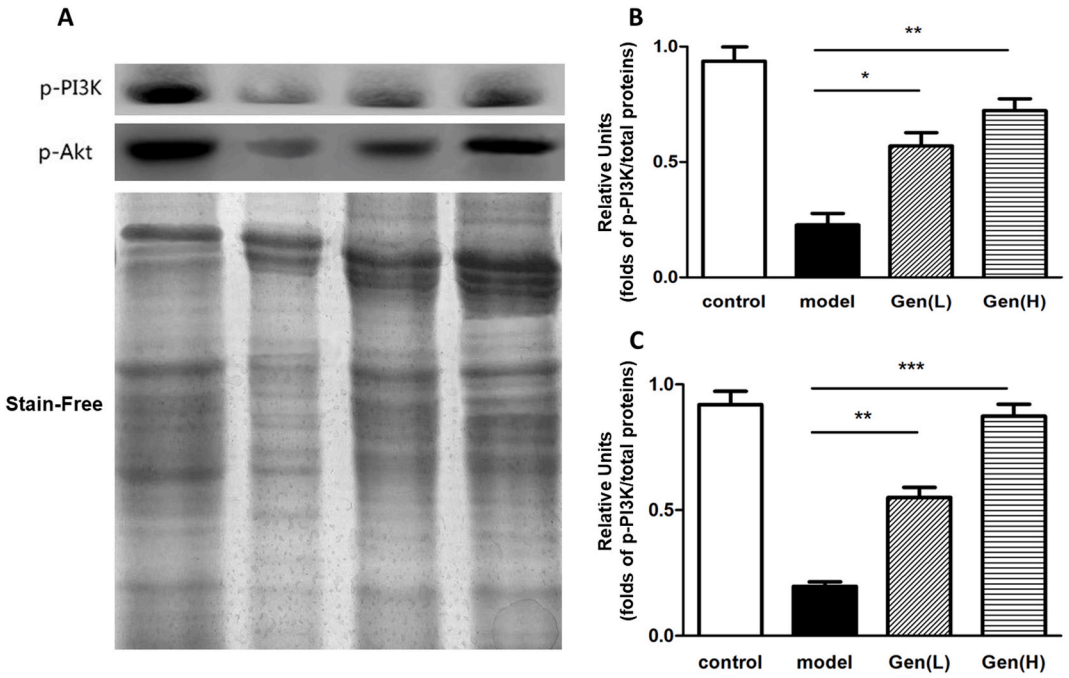

Fig. 3. Geniposide enhanced the protein phosphorylation of PI3K and Akt in wound tissues on Day 7 after surgery. A: The bands of PI3K/Akt proteins and their phosphorylation in each group. B–C: The ratio of PI3K/Akt proteins and their phosphorylation was quantified and analyzed by GraphPad Prism software. Data are shown as the median and interquartile range [min to max] (n = 3 in each group). *P < 0.05, **P < 0.01, ***P < 0.001 denoted statistical significance compared with model group, analyzed by one-way ANOVA followed by the post-hoc Tukey's multiple comparison test, and finally nonparametric *t*-test for failure of normality test.

The pro-inflammatory or inflammatory factors in ulcerative tissue were detected by ELISA assay. For the pathogenic infection, the levels of TNF-α, IL-1β and IL-6 in ulcerative wound were significantly increased on Day 7 after wounding onset. After oral administration of 500 mg/kg geniposide, the levels of pro-inflammatory factors (TNF-α, IL-1β) and IL-6 were significantly reduced by 27.2 ± 2.0 %, 29.2 ± 6.3 %, 31.2 ± 7.2 % ($P < 0.05$, Fig. 5A–C). The levels of TNF-α, IL-1β and IL-6 were significantly inhibited by geniposide with the IC_{50} value of 470 mg/kg, 464 mg/kg and 370 mg/kg body weight, respectively.

5. Discussion

The healing process of skin wound can be divided into the following three stages: 1) the inflammation stage due to the high release of pro-inflammatory mediators and the immune system damage; 2) the proliferation stage mainly including the proliferation of fibroblasts, the deposition of collagen fibers and the formation of collagen formation; 3) the stage of angiogenesis and remodeling of ulcer tissue [18].

Literatures indicated that hyperglycemic wounds could induce a decrease in the expression of antiinflammatory cytokines (IL-10 and TGF-β), along with the increment of proinflammatory and inflammatory cytokines (such as TNF-α, IL-1β and IL-6) for their susceptibility to infections [19]. Furthermore, pro-inflammatory cytokines could also inhibit cell proliferation and differentiation. In the activated stratum corneum cells, IL-6 could stimulate the formation of granulation and epithelial angiogenesis [20]. Besides, TNF-α possessed a dual role in inhibiting both granulation and collagen fiber [21].

Numerous studies have confirmed that iridoid glycosides, the main constituents of *Gardenia jasminoides* J. Ellis, had effects of antioxidation, anti-inflammatory, anti-atherosclerosis, anti-ischemic brain injuries, anti-platelet aggregation, anti-hyperglycemia, anti-hyperlipidemia, anti-hypertension, and so on [22]. Geniposide was extensively studied as the main active component of iridoid glycosides in *Gardenia jasminoides* J. Ellis [6]. Our results showed that geniposide could promote the wound healing by prohibiting the apoptotic cells in the ulcerative tissue. Different from its anti-inflammatory action by MAPK/NF-κB pathway mentioned above, the anti-apoptotic mechanism of geniposide was confirmed specifically in this study by PI3K/Akt pathway. In previous study, it was demonstrated that geniposide could regulate macrophage polarization and enhance the expression of M2 phenotype proteins Arg-1 and IL-10 via the FOS/MAPK signaling pathway [23]. The anti-inflammatory and neuroprotective effect of geniposide was also proved in the identification of network pharmacology by modulating microglial polarization and down-regulating 5-LOX/CysLTs [24]. In the BV-2 microglial cells induced by oxygen-glucose deprivation/reoxygenation, geniposide suppressed the level of inflammatory cytokines via inhibiting the activation and expression of NLRP3 inflammasome and increasing autophagic activity to reduce cell death [25]. Besides, it was also reported that geniposide alleviated H_2O_2-induced oxidative damage in hepatocytes through regulating miR-27b-3p/Nrf2 Axis, which could be blocked by Nrf2 inhibitor ML385 [26].

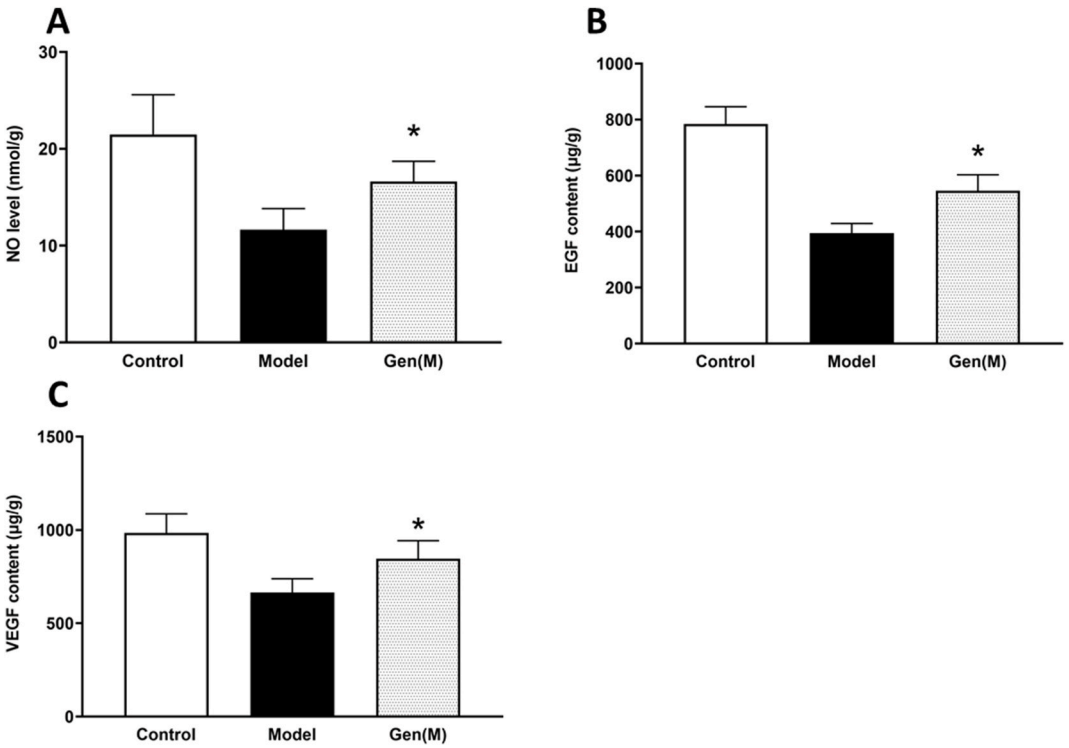

Fig. 4. Nutritional function of geniposide in ulcerative tissues. A–C: The expression of EGF, VEGF and NO level were measured on Day 7 in each group by ELISA kits. Data are shown as means ± SD. (n = 5–6 in each group). *P < 0.05 denoted statistical significance compared with model group, analyzed by one-way ANOVA followed by the post-hoc Tukey's multiple comparison test, and finally nonparametric *t*-test for failure of normality test.

6. Limitations

This study proved that geniposide enhanced the repairment of skin wound in diabetic rats by inhibiting inflammatory response and cellular apoptosis via PI3K/Akt pathway. Besides, the nutritional function of geniposide was also identified as the enhanced expression of EGF, VEGF and NO level. However, the effector cells of geniposide were still unknown: 1) For the anti-inflammatory activity of geniposide, the further studies were warranted to explore effect of geniposide on adjusting the polarization of microglia, and the expression of M2 phenotype proteins Arg-1 and IL-10, and 2) for its nutritional function, we found that geniposide adjusted the expression of EGF, VEGF and NO level for the first time. Thus the underlying mechanism of geniposide in driving the nutritional expression of fibroblast or endothelial cells need to be explained. It was hinted that geniposide might enhance angiogenesis in diabetic wound healing, at least partially, via regulating VEGF, EGF signaling [27]. Hence, the relationship of biological angiogenesis and nutritional function can be studied as well. 3) Besides, geniposide was reported to ameliorate liver fibrosis by enhanced the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) and decreased methane dicarboxylic aldehyde (MDA) levels in liver [28]. We speculate that geniposide may also adjust the expression of iNOS, GSH and so on to block skin ulcers-induced oxidative stress in diabetic rats as well. The further studies were warranted to verify these pharmacological effects and molecular mechanisms of geniposide.

7. Conclusions

Our outcomes indicated that geniposide could accelerate tissue repairment and reduce wound lesions of DSU in diabetic rats by promoting wound healing via PI3K/Akt pathway, which provided critically experimental evidence and theoretical support for the application of geniposide and other iridoid glycosides in the treatment of DSU.

Disclosure of potential conflicts of interest

All authors declared no competing interests in this work.

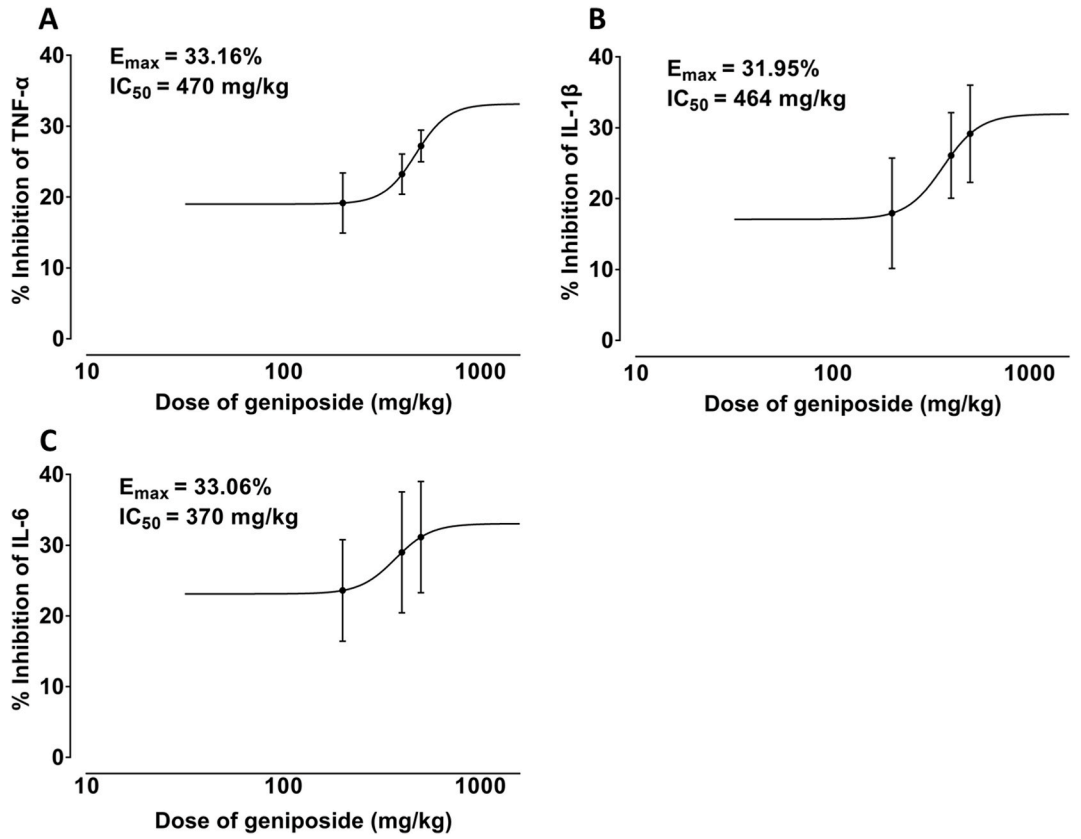

Fig. 5. Inhibition effect of geniposide (200 mg/kg, 400 mg/kg and 500 mg/kg) on the inflammatory factors in wound tissue on Day 7 after surgery. A–C: The levels of pro-inflammatory factors (TNF-α, IL-1β) and IL-6 were measured on Day 7 in each group by ELISA kits. Data are shown as means ± SD. (n = 5–6 in each group).

Research involving human participants and/or animals

The experimental animals were purchased from Experimental Animal Center of Jiangxi University of Traditional Chinese Medicine. All animal experiment operations were approved and implemented by the Ethics Committee of Jiangxi University of Science and Technology.

Informed consent

Not applicable.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Chun-juan Fang: Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing. **Xiao-juan Rong:** Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft. **Wen-wen Jiang:** Investigation, Resources, Software, Visualization. **Xiao-yan Chen:** Project administration, Resources, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. **Yan-ling Liu:** Investigation, Methodology, Resources.

Declaration of competing interest

All authors declared no competing interests in this work.

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