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# High-Voltage Pulsed Current Stimulation Enhances Wound Healing in Diabetic Rats by Restoring the Expression of Collagen, $\alpha$-Smooth Muscle Actin, and TGF-$\beta$1

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Impaired wound healing is a common complication of diabetes mellitus and a major morbidity that leads to pain and severely diminished quality of life. Diabetic wounds are commonly associated with defective immune cell responses or abnormality of extracellular matrix. Various types of electrical stimulation interventions have been used to promote tissue healing. However, it is unclear whether high-voltage pulsed current stimulation (HVPCS) enhances diabetic wound healing. In this study, the effects of HVPCS on wound healing were investigated in diabetic rats. Three groups of rats (10 per group) were used: non-diabetic control, diabetic control, and diabetic rats that were administered HVPCS for 40 minutes daily for 1 week. Rats from control groups were administered sham interventions. Dorsal incision wounds were generated in all animals, and wound-healing rate was determined during one-week intervention. After interventions, we measured the relative expression levels of collagen type I (collagen-I), $\alpha$-smooth muscle actin ($\alpha$-SMA), and transforming growth factor-$\beta$1 (TGF-$\beta$1) mRNAs in the wounded skin. Wound closure was delayed in diabetic control rats compared to the non-diabetic control rats, and the diabetic control rats showed the reduced expression levels of collagen-I, $\alpha$-SMA and TGF-$\beta$1 mRNAs. Importantly, compared to diabetic control rats, rats with HVPCS showed accelerated wound closure and healing ($p < 0.01$) and restored expression levels of collagen-I ($p = 0.02$), $\alpha$-SMA ($p = 0.04$), and TGF-$\beta$1 ($p = 0.01$) mRNAs. In conclusion, HVPCS may be beneficial for enhancing the healing of diabetic wounds by restoring the expression levels of TGF-$\beta$1, collagen-I, and $\alpha$-SMA.

**Keywords:** diabetes; high-voltage pulsed current stimulation; rat; streptozotocin; wound healing
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## Introduction

Impaired wound healing is a common complication of diabetes mellitus and a major morbidity that leads to pain and a severely diminished quality of life (Becker et al. 2012). Diabetic wounds are characterized by impaired angiogenesis, occlusion of blood vessels within the wound bed, and decreased collagen production (Brem and Tomic-Canic 2007). In the various causes of poor wound healing, defective growth factors are thought to contribute to impaired tissue repair. However, certain growth factors are known to reverse the detrimental effects of diabetes and glucocorticoid on wound healing (Tepper et al. 2002; Brunner and Blakytny 2004).

Previous studies have attempted to identify the electro-biologic mechanism responsible for wound healing acceleration by electrical stimulation (ES) (Magnoni et al. 2013; Kawasaki et al. 2014). Normal skin surface maintains a negative charge with respect to deeper epidermal layers. The electrical fields (EFs) generated by ES can promote multiple signaling pathways essential for wound healing and provide a directional signal for cell migration during wound healing (Zhao et al. 2010; Vieira et al. 2011). These studies provided insights into the cellular and physiological mechanisms whereby ES enhances wound healing. ES has also been reported to attract keratinocytes to wounds (Nishimura et al. 1996), and to facilitate the proliferation and migration of keratinocytes during the wound healing process (Hinsenkamp et al. 1997). However, recent studies of the application of ES to wound healing focused on normal skin wounds, and as a result, little information is available on the benefits of ES applied to diabetic wounds. Furthermore, the few studies that addressed the efficacy of ES in healing diabetic wounds failed to demonstrate a

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significant benefit of this intervention, and the analytical methods employed have been limited (Lundeberg et al. 1992; Cullum et al. 2001).

In the present study, we investigated the effects of high-voltage pulsed current stimulation (HVPCS) on wound healing in diabetic rats, and determined the expression of collagen type I (collagen-I), $\alpha$-smooth muscle actin ($\alpha$-SMA), and transforming growth factor-$\beta$1 (TGF-$\beta$1) mRNAs and wound healing during the intervention period.

## Methods

*Animals and experimental design*

Thirty male Sprague-Dawley rats weighing between 240-260 g (KOATECH, Pyongtaek, Korea) were used. Rats were housed in cages (two per cage) under controlled conditions (22°C, relative humidity 40%, and a 12-h dark/12-h light schedule), and were allowed free access to food and tap water. All animal studies were approved by the ethics committee of Sahmyook University, in compliance with the current guidelines regarding the care and use of animals in research and education. Rats ($n$ = 30) were divided into three groups of 10 as follows: non-diabetic control rats with sham stimulation (NC group); diabetic control rats with sham stimulation (DC group); and diabetic rats that were administered HVPCS daily (ES group). Diabetes was induced in the rats allocated to the DC and ES groups by injecting pancreatic $\beta$-cell toxin streptozotocin (STZ) (S0130, Sigma-Aldrich, St. Louis, MO, USA) intraperitoneally at 65 mg/kg of body weight in cold 0.1 M citrate buffer (pH 4.5). Glucose concentration in blood of diabetic rats was measured 2 days after injection. Rats that failed to develop hyperglycemia (blood glucose > 250 mg/deciliter) were excluded. Ten rats (NC group) were injected with buffer intraperitoneally.

*Diabetic wound model*

After 7 days of acclimatization, rats were anesthetized by halothane inhalation, maintained at a concentration of 2-3%. After shaving the prospective site of the wound and scrubbing with organic iodine solution, all 30 rats received two 15 mm full-thickness incision wounds on the back. Incisions were cleaned with sterile gauze and were not sutured or covered.

*Intervention*

Rats were wrapped with elastic bandages and placed in clear plastic restrainers. The linear wounds were covered with a carbon-silicone rubberized electrode superimposed on saline-soaked 2 × 2 cm gauze pads moistened with 0.9% sodium chloride. The first electrode was placed on the wound and the second electrode was placed distally to the area above the wound. Rats in the ES group received HVPCS at a pulse rate of 100 pulses per second (pps) for 40 min from a Pulsed High Volt Stimulator (ECG® 100-SL, Electro-Med Industries, Miami, FL, USA). The waveform consisted of monophasic, twin-peak pulses of 140 $\mu$s. Stimulation was performed such that palpable contractions were barely evident; voltages ranged between 35 and 50 V (Table 1). The polarity of the electrode on the wound was negative for the first 3 days of intervention (the inflammation phase) and positive for the following 4 days (the proliferation phase). Rats in the control groups (NC and DC) received sham stimulation with electrodes not generating electricity (Lee et al. 2004).

Table 1. High-voltage pulsed current stimulation (HVPCS) protocol.

| | |
|---|---|
| Wave form | Monophasic and Twin-peak pulse |
| Pulse rate | 100 pulse per second |
| Pulse duration | 140 $\mu$s |
| Voltages range | 35-50 V |
| Treatment time | 40 min |
| Intervention period | 7 days |
| Active electrode | Negative charge (day 1 to 3)<br>Positive charge (day 3 to 7) |

*Measurements*

To assess wound-healing rate (WHR), wound lengths were observed and photographed with a digital camera daily following 1, 3 and 7 days of intervention. Wound length immediately after incision was considered as the baseline length (100%) and all subsequent measurements were expressed as percentage of baseline length.

WHR = (Original wound length − Final wound length)/Original wound length × 100%

Reverse transcription-polymerase chain reaction (RT-PCR) was used to measure the expression levels of collagen-I, $\alpha$-SMA and TGF-$\beta$1 mRNAs in the wounded skin. Total RNA was extracted with TRIzol (Ambion Co., Austin, TX, USA) from wounded skin of rats at the 7th day of intervention period. Superscript II RNAse Reverse transcriptase (Invitrogen, Carlsbad, CA, USA) was used to reverse transcribe 1 $\mu$g of RNA, and collagen-I, $\alpha$-SMA, TGF-$\beta$1 and $\beta$-actin cDNAs were amplified on an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany). Primer pairs used for the amplification of each product are as follows: 5′-TGGAGACAGGTCAGACCTG-3′ and 5′-TATTCGATGACTGTCTTGCC-3′ (collagen-1); 5′-TGGCTGATGGAGTACTTC-3′ and 5′-GATAGAGAA GCCAGGATG-3′ ($\alpha$-SMA); 5′-CAAGCAGAGTACACACAGCA-3′ and 5′-GATGCTGGGCCCTCTCTCCAGC-3′ (TGF-$\beta$1); 5′-GTG GGCCGCCCTAGGCACCA-3′ and 5′-CGGTTGGCCTTA GGGTTCAG-3′ ($\beta$-actin). The sizes of the PCR products of collagen-1, $\alpha$-SMA, TGF-$\beta$1 and $\beta$-actin cDNAs were 409, 494, 481 and 245 base pairs, respectively. $\beta$-Actin expression was used to normalize the expression of each mRNA. The PCR products were detected and analyzed with an EDAS-290 system (Eastman Kodak, Rochester, NY, USA). Results represent mRNA expression ratio at the 7th day of intervention (the proliferation phase).

*Statistical analysis*

The distributions of continuous variables were analyzed using the Shapiro-Wilk test. ANOVA was used to compare parameters between the NC, DC and ES groups. All values are expressed as means ± SEMs, and the analysis was performed using SPSS v 16.0 (IBM, Armonk, NJ, USA). Differences were considered statistically significant for 2-tailed $p < 0.05$.

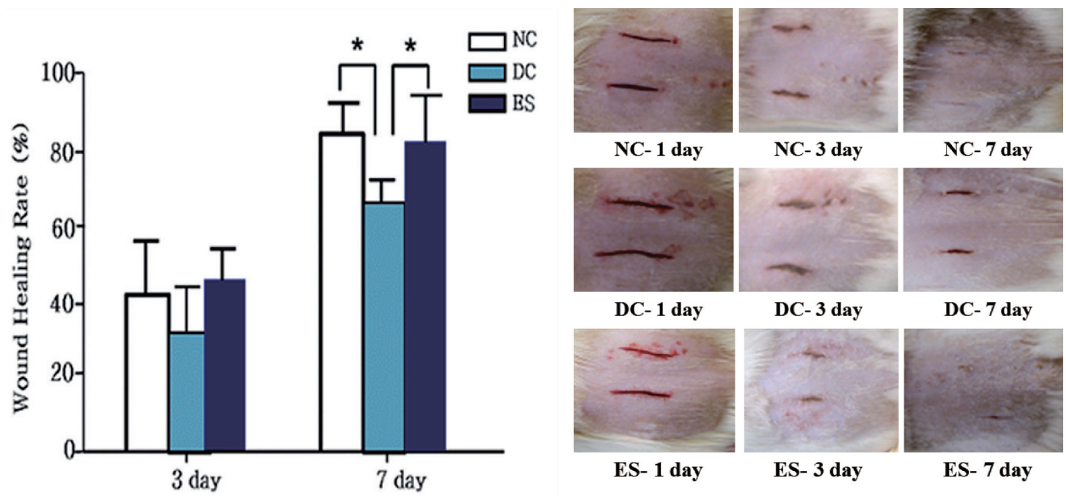
## Results

Body weight and blood glucose level were monitored after wounding (Table 2). Body weight and blood glucose level were similar in all groups before administration of HVPCS. However, body weight of diabetic rats significantly decreased ($F = 82.48$, $p < 0.001$), and blood glucose levels ($F = 100.20$, $p < 0.001$) significantly increased during

Table 2. Blood glucose levels in normal and diabetic rats during the intervention period.

| ($n$ = 30) | | NC ($n$ = 10) | DC ($n$ = 10) | ES ($n$ = 10) | $p$ |
|---|---|---|---|---|---|
| Body Wight (g) | Base line | 249.31 ± 8.42 | 249.50 ± 1.58 | 251.00 ± 13.08 | .64 |
| | 1 day | 256.56 ± 6.57†‡ | 229.00 ± 8.75 | 234.00 ± 12.43 | .00 |
| | 3 days | 266.67 ± 6.12†‡ | 211.50 ± 14.15 | 214.00 ± 11.74 | .00 |
| | 7 days | 289.44 ± 9.82†‡ | 187.20 ± 22.15 | 195.70 ± 21.77 | .00 |
| Blood Glucose Level (mg/dl) | Base line | 117.88 ± 11.72 | 116.10 ± 10.14 | 115.60 ± 13.68 | .91 |
| | 1 day | 116.78 ± 9.34†‡ | 364.10 ± 32.70 | 318.10 ± 70.98 | .00 |
| | 3 days | 115.50 ± 11.05†‡ | 410.82 ± 45.62 | 415.36 ± 53.68 | .00 |
| | 7 days | 119.78 ± 12.08†‡ | 418.90 ± 66.04 | 428.00 ± 61.37 | .00 |

Values are means ± S.D. (95% CI, mg/dl). †$p$ < 0.05 between the NC and DC groups and ‡$p$ < 0.05 between the NC and ES groups. There was no significant difference between the DC and ES group. NC, non-diabetic control rats with sham stimulation; DC, diabetic control rats with sham stimulation; ES, diabetic rats administered HVPCS.




(a) Compression of WHR at 3 days and 7 days (b) Wound length changes

Fig. 1. The effect HVPCS on wound-healing rates (WHR).
WHR was significantly higher in rats from the ES and NC group than in those from the DC group (*$p$ < 0.05). There was no significant difference between the ES group and the NC group at 3 day and 7 day. NC, non-diabetic control rats with sham stimulation; DC, diabetic control rats with sham stimulation; ES, diabetic rats administered HVPCS.

Table 3. Effect of HVPCS on wound-healing rates in diabetic rats.

| ($n$ = 30) | NC ($n$ = 10) | DC ($n$ = 10) | ES ($n$ = 10) | $p$ |
|---|---|---|---|---|
| 1 day | 0 | 0 | 0 | |
| 3 days | 40.44 ± 16.30 | 29.40 ± 10.99 | 42.60 ± 12.02 | 0.08 |
| 7 days | 85.15 ± 8.35† | 65.11 ± 7.47 | 82.94 ± 10.33‡ | 0.00 |

Values are means ± S.D. (95% CI), Rate (%). †$p$ < 0.05 between the NC and DC groups, and ‡$p$ < 0.05 between the ES and DC groups. There was no significant difference between the ES and NC group. NC, non-diabetic control rats with sham stimulation; DC, diabetic control rats with sham stimulation; ES, diabetic rats administered HVPCS.

the intervention period.

Mean group wound lengths were used to determine the changes at each time point versus baseline (Fig. 1). Rats from the NC and ES groups exhibited good wound healing, whereas healing was poor in rats the from DC group (Table 3). During the last day of intervention, mean WHR values were significantly different between the 3 groups ($F$ = 15.17, $p$ < 0.001; Table 3). Multiple comparison analysis revealed that WHR in rats from the ES and NC groups was significantly higher than in rats from the DC group (both $p$ < 0.05; Table 3).

RT-PCR was performed to measure the relative

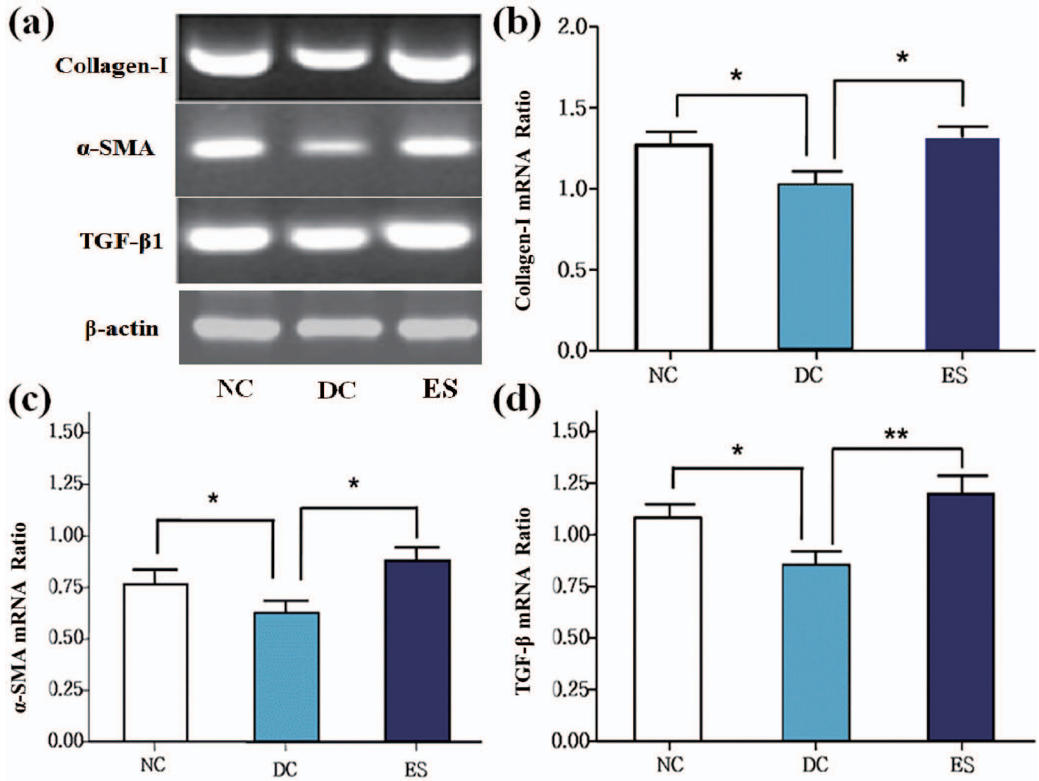



Fig. 2. Difference in the relative expression levels of mRNAs.
(a) Gel electrophoresis of PCR products for the expression of collagen-I, α-SMA, TGF-β1 and β-actin mRNA in rats from the NC, DC and ES groups after intervention. There was no significant difference in β-actin mRNA expression among three groups. Also shown are the relative expression levels of collagen-I mRNA (b), α-SMA mRNA (c), and TGF-β1 mRNA (d). The relative expression level of collagen-I, α-SMA, or TGF-β1 mRNA was significantly higher in the ES group and the NC group than that in the DC group (*$p < 0.05$ and **$p < 0.01$). There was no significant difference in the relative expression level of collagen-I, α-SMA, or TGF-β1 mRNA between the ES group and the NC group. NC, non-diabetic control rats with sham stimulation; DC, diabetic control rats with sham stimulation; ES, diabetic rats administered HVPCS.

Table 4. Effect of HVPCS on collagen-I, $\alpha$-SMA, and TGF-$\beta$1 mRNA ratios in diabetic rats.

| ($n = 30$) | NC ($n = 10$) | DC ($n = 10$) | ES ($n = 10$) | $p$ |
|---|---|---|---|---|
| Collagen-I | $1.28 \pm 0.23^{\dagger}$ | $1.00 \pm 0.29$ | $1.31 \pm 0.22^{\ddagger}$ | 0.02 |
| $\alpha$-SMA | $0.76 \pm 0.23^{\dagger}$ | $0.63 \pm 0.19$ | $0.88 \pm 0.20^{\ddagger}$ | 0.04 |
| TGF-$\beta$1 | $1.08 \pm 0.21^{\dagger}$ | $0.85 \pm 0.21$ | $1.20 \pm 0.29^{\ddagger}$ | 0.01 |

Values are means ± S.D. (95% CI); values are OD ratios versus $\beta$-actin. $^{\dagger}p < 0.05$ between the NC and DC groups, and $^{\ddagger}p < 0.05$ between the ES and DC groups. There was no significant difference between the ES and NC group. NC, non-diabetic control rats with sham stimulation; DC, diabetic control rats with sham stimulation; ES, diabetic rats administered HVPCS.

expression levels of collagen-I, $\alpha$-SMA, and TGF-$\beta$1 mRNAs in wound tissues collected at the 7th day of intervention (Fig. 2). The relative mRNA expression levels are shown in Table 4. Significant differences in mRNA expression were found between the 3 groups [collagen-I ($F = 4.96$, $p = 0.02$), $\alpha$-SMA ($F = 3.79$, $p = 0.04$) and TGF-$\beta$1 ($F = 5.64$, $p = 0.01$)]. The ES and NC groups showed significantly higher mRNA expression of collagen-I, $\alpha$-SMA and TGF-$\beta$1 than the DC group. In particular, rats from the ES group exhibited significantly higher expression levels of collagen-I ($p < 0.05$), $\alpha$-SMA ($p < 0.05$) and TGF-$\beta$1 ($p < 0.01$) than rats from the DC group. Moreover, there were no significant differences between the ES group and the NC group, suggesting that HVPCS might have restored the expression of these proteins.

## Discussion

Although the positive influence of ES on wound healing is well established, its role in diabetic wound healing is not well understood (Cullum et al. 2001; Zhao et al. 2010).

We hypothesized that ES might improve wound healing in diabetic patients by promoting the expressions of growth factors and collagen synthesis. In this study, we evaluated the effects of HVPCS on diabetic wound healing in STZ-induced diabetic rats. HVPCS was applied at 100 pps, 140 $\mu$s using twin peak pulses for 40 minutes to full-thickness incision wounds in the dorsal surface of rats, by applying negative polarity for the first 3 days and positive polarity for the following 4 days of a week-long intervention period. Lee et al. (2004) investigated the physiological effects of HVPCS on expression of growth factors, such as insulin-like growth factor-I and TGF-$\beta$, in wounds in normal rat. This study demonstrated that different physiological effects were obtained by opposite polarities, more specifically, negative polarity was found to enhance vascularization and granulation by attracting fibroblasts, and positive polarity was found to enhance DNA synthesis in fibroblasts and epithelial cell migration. Brown et al. (1995) determined the effects of HVPCS on wound tensile strength properties and wound closure in guinea pigs. This study suggested that HVPCS promotes faster wound healing by increasing collagen deposition and epithelialization, and concluded that HVPCS can be used as an effective treatment to facilitate wound healing. In the present study, HVPCS produced significant improvements in wound healing rate and in the mRNA expression of collagen-I, $\alpha$-SMA, and TGF-$\beta$1.

Diabetes is characterized by impaired wound healing. A large number of molecular changes are associated with delayed wound healing in people with diabetic ulcers (Blakytny and Jude 2006). Our results show that the delayed wound healing observed in diabetic rat was associated with a significant increase of blood glucose levels. When we compared diabetic control rats with normal controls, significant differences in wound healing were observed. These results support previous studies demonstrating that diabetes inhibited wound closure and contraction by diabetes disease including diminished TGF-$\beta$ levels in wounds (Haase et al. 2003; Peplow and Baxter 2012; Peplow and Chatterjee 2013).

The significant reduction in wound size observed after HVPCS treatment also correlated with our pathological findings. The WHR indicated wound contraction, which initially occurs without myofibroblast involvement. About 1 week after wounding, fibroblasts stimulated by TGF-$\beta$ differentiate into myofibroblasts, cells responsible for wound contraction (Todd et al. 2001; Ugarte and Brandan 2006). In addition, TGF-$\beta$ directly stimulates collagen synthesis and decreases extracellular matrix degradation by fibroblasts (Blakytny and Jude 2006). Collagen is critical for the strength and integrity of extracellular matrix and for epithelialization during the later stages of wound healing (Brancato and Albina 2011; Ebaid et al. 2011). In our study, WHR was significantly higher in rats from the ES group than in those from the DC group, and the accelerated wound healing observed in the ES group might be due to increased growth factor synthesis. Since TGF-$\beta$1 is a major stimulator of collagen synthesis, we examined TGF-$\beta$1 mRNA expression at the proliferation phase as an indicator of wound healing, and examined the mRNA expression of collagen-I and $\alpha$-SMA, which are stimulated by TGF-$\beta$1 and are involved in the maintenance of cutaneous structure and integrity. Although TGF-$\beta$1 plays a vital role in the inflammatory phase of wound healing, it stimulates many vital interactions throughout the wound healing process. In the proliferative phase, TGF-$\beta$1 produced by activated macrophages has positive influence on angiogenesis and epithelialization by stimulating keratinocytes (Douglas 2010).

Our results show that TGF-$\beta$1, collagen-I, and $\alpha$-SMA mRNA expression in rats from the ES group was significantly greater than in rats from the DC group, which may account for the enhanced tissue regeneration in rats from the ES group. Importantly, we observed that the improved WHR by HVPCS occurred in parallel to the restoration of TGF-$\beta$1 expression in wounded tissues. Therefore, we hypothesize that HVPCS upregulates $\alpha$-SMA expression by inducing TGF-$\beta$1, thereby promoting collagen-I expression secondary to transformation of fibroblasts into myofibroblasts. As a consequence, HVPCS facilitates wound closure by inducing collagen expression.

In conclusion, this study suggests that HVPCS promotes the healing of diabetic wounds by regulating the levels of TGF-$\beta$1, collagen-I, and $\alpha$-SMA. However, these findings must be interpreted in light of the limitations of the experimental design. First, although TGF-$\beta$1 stimulates angiogenesis and collagen deposition during the proliferative phase, we could not shed light on the role of TGF-$\beta$1 during the early inflammatory phase. Second, we did not assess the quantity of collagen synthesis obtained at different phases of wound healing. Therefore, further research on the effects of HVPCS on wound healing at different phases of this process is warranted.

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## Conflict of Interest

The authors declare no conflict of interest.

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