Extracorporeal Shock Wave Treatment Improves Incisional Wound Healing in Diabetic Rats

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Impaired wound healing in surgical patients with diabetes increases the incidence of infection, prolongs hospitalization, and even increases the rate of mortality. Low-energy extracorporeal shock wave treatment (ESWT) was reported to accelerate chronic wound healing by promoting revascularization and tissue regeneration; however, it is not known if ESWT could also improve healing of acute surgical incisional wounds in diabetes. In this study, using a rat model of diabetes, we investigated the effect of low-energy ESWT on collagen content in wound tissues and its efficacy in incisional wound healing. A single dorsal incisional wound was inflicted in streptozotocin-induced diabetic rats, and they received ESWT at different time post-wounding. Rats were sacrificed on days 7 and 14 post-wounding. Wound breaking strength, hydroxyproline content, histological characteristics and the expression of transforming growth factor beta 1 (TGF-β1) were analyzed. As a result, the wound breaking strength was significantly enhanced and the hydroxyproline content in wound tissues was increased at each time point examined. The number of fibroblasts was significantly increased, and the new collagen fibers were more abundant at the wound site after ESWT. Furthermore, the expression of TGF-β1 was up-regulated after ESWT on day 7 post-wounding. These results suggest that low-energy ESWT can increase collagen content, enhance wound breaking strength and improve the healing of incisional wound in diabetic rats. The increased collagen content may be attributed, at least in part, to the up-regulation of TGF-β1 expression in wound tissues.

Keywords: collagen; diabetes; extracorporeal shock wave; transforming growth factor beta 1; wound healing


Diabetic-induced wound healing impairment is a significant challenge to surgeons. In patients with diabetes, wounds tend to heal much more slowly than those in normal individuals, and the healing is typically of poorer quality. Even well-controlled diabetics are at an increased risk of post-surgical wound complications (Hoogwerf 2001). At present, diabetes is commonly seen in surgical patients. Impaired wound healing not only increases patient-experienced pain and extends hospital stay, but also places a significant burden on the health care system.

Wound healing is a dynamic and well-orchestrated process that involves overlapping steps of hemostasis and inflammation, cellular proliferation, angiogenesis, collagen synthesis and tissue remodeling (Singer and Clark 1999). Healing of a surgical incision is mainly dependent on the tissue collagen deposition (Chang et al. 2010). A major element of healing in suture-closed wounds is the integration of newly deposited collagen with the residual dermal collagen fibers at the edges of the wound; therefore collagen contributes a lot to the restoration of wound strength.

Collagen is mainly secreted by fibroblasts and this process is modulated by various cytokines, including transforming growth factor beta 1 (TGF-β1), which plays a central role (Beanes et al. 2003). In patients with diabetes, decreased fibroblasts proliferation, reduced collagen synthesis and decreased TGF-β1 expression were observed and thought to be the primary reasons of wound healing impairment (Spanheimer et al. 1988; Hehenberger et al. 1998).

Extracorporeal shock wave (ESW) is a longitudinal acoustic wave generated by electrohydraulic, electromagnetic, or piezoelectric methods that propagate in tissue with a sudden rise from ambient pressure to its maximum pressure at the wave front, followed by lower tensile amplitude. Initially, high-energy ESW was used to fragmentize urinary stones. Recently, low-energy ESW was shown to be beneficial in the treatment of various chronic wounds, including bedsores, burn wounds, diabetic and vascular ulcers (Meirer et al. 2005; Schaden et al. 2007; Saggini et al. 2008; Larking et al. 2010; Wolff et al. 2011). Clinical studies demonstrated that low-energy ESW treatment (ESWT)
accelerated the healing of chronic diabetic foot ulcers and displayed no treatment-related toxicity, infection, or deterioration of wounds (Moretti et al. 2009). In a prospective study, Wang et al. (2009) compared the effect of ESWT with that of traditional hyperbaric oxygen therapy on chronic diabetic foot ulcers, and concluded that ESWT achieved better clinical results than hyperbaric oxygen therapy. Although the mechanism underlying low-energy ESWT-mediated improvement of wound healing is not completely known, it is widely thought that its positive effect is mainly related to its role in promoting the revascularization and tissue regeneration in wounds (Kuo et al. 2009a).

More recently, studies showed that ESWT could enhance proliferation and collagen synthesis in cultured tenocytes and dermal fibroblasts (Chao et al. 2008; Berta et al. 2009). Considering that synthesis of collagen is essential to regain wound strength, we hypothesized that low-energy ESWT would increase collagen content in wound tissue and thus accelerate the healing of incision in surgical patients with diabetes. In this study, a streptozotocin-induced diabetic rat model was used to investigate the effect of low-energy ESWT on the healing of incisional wounds.

Materials and Methods

Animals

This study was approved by the Animal Research Committee of Shanghai Jiao Tong University School of Medicine. Forty-eight adult male Sprague-Dawley rats (250-280 g) were randomly divided into four groups (n = 12): (1) Non-diabetic group; (2) Diabetic group, diabetic rats receiving no ESWT; (3) ESWT-1 group, diabetic rats receiving one session of ESWT on day 1 post-wounding; and (4) ESWT-2 group, diabetic rats receiving three sessions of ESWT on days 1, 3 and 5 post-wounding. After arrival, the rats were housed two per cage on a 12:12 h light-dark schedule and habituated to the laboratory environment for 1 week prior to use. Rats were given regular standard rat chow and water ad libitum throughout the experiment.

Induction of Diabetes

Diabetes was induced by injection of streptozotocin (STZ) as described by Ozkaya et al. (Ozkaya et al. 2007). Rats were fasted for 12 hours before a single intraperitoneal injection of STZ (60 mg/kg; Sigma Co., USA) dissolved in citrate buffer (0.1 mol/L; pH 4.5). 12 rats in non-diabetic group were injected with an equivalent volume of citrate buffer. Diabetes was defined as a blood glucose level of greater than 300 mg/dL at 3 days after STZ injection. Blood glucose levels were monitored using a One Touch ultra glucometer (Johnson & Johnson Co., USA) twice a week.

Wounding

Three weeks after STZ administration, rats were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg; Fujian Gutian Pharmaceutical Co., China). The dorsal hair was shaved and the surgical site was wiped with 75% alcohol. A 5-cm full thickness skin incision was made on the midline of dorsum. Then, wounds were closed with interrupted 4-0 nylon sutures (1 cm apart). Wounds were kept open throughout the entire experiment without dressing.

Extracorporeal Shock Wave Treatment

Before ESW was applied, a sterile ultrasound transmission gel (Pharmaceutical Innovations Inc., USA) was used as contact medium between the wound and ESW apparatus (Orthospec, Medispec Ltd., Israel). According to the results of our own pilot study, 100 impulses of focused ESW (flux density level: 0.11 mJ/mm²; 3 Hz) per centimeter wound length were applied on the wound in ESWT-1 and ESWT-2 groups. In non-diabetic and diabetic groups, wounds were also smeared with the transmission gel, but no shock wave impulse was administered.

Wound Harvesting and Tissue Processing

On days 7 and 14 post-wounding, six rats per group were sacrificed using an overdose of ketamine. The entire wound and its 2 cm margins were excised and cut transversely into 4 strips of equal width (1 cm). The cranial strip was fixed in 10% formaldehyde for histological examination; the caudal strip was kept in liquid nitrogen for further hydroxyproline content analysis and molecular study, while the central two strips were used for evaluating wound breaking strength immediately after harvest.

Breaking Strength Testing

Breaking strength was measured with an Instron Tensometer (Model No. 5569; Instron Co., USA). After being harvested and processed, wound strips were loaded between the holders of the machine and elongated from zero length at the constant speed of 2 cm/min till they were ruptured. The breaking strength (Newton) was defined as the minimum force required to rupture the wound completely.

Hydroxyproline Content Analysis

Hydroxyproline content was measured using a published method (Reddy and Enwemeka 1996). Specimens were first kept in an oven at 60°C and their dry weight was noted; samples were then hydrolysed in alkali at 120°C for 20 min. The hydrolysate was neutralized to pH 7.0 and subjected to chloramine-T oxidation for 25 min at room temperature. The reaction was terminated by the addition of 0.4 M perchloric acid. Color was developed with the help of the Ehrlich reagent at 60°C and measured at 550 nm using a spectrophotometer (Model No. 8500; Keda Instrument Factory, China). The amount of hydroxyproline was determined by comparison to a standard curve.

Histological Evaluation

After fixation 24 h at room temperature, specimens were embedded in paraffin and sectioned in a plane perpendicular to the incision. Sections were stained with hematoxylin and eosin (H&E) or Masson’s trichrome. Evaluation of all sections was performed by an experienced pathologist without knowledge of the previous treatment. For each specimen, three separated sections were randomly selected for histological evaluation. The fibroblast proliferation, neovascularization, granulation tissue, epithelialization and collagen deposition were evaluated semi-quantitatively using accepted wound scoring protocols published previously (Muchberger et al. 2005; Qiu et al. 2007). Wound healing scores were assigned a value between 0 and 4 as follows: 0 = none; 1 = rare or minimal; 2 = moderate; 3 = abundant; and 4 = severe or marked.

Analysis of TGF-β1Expression by Immunohistochemical Staining

TGF-β1 staining was performed on paraffin-embedded wound
sections using a streptavidin–biotin–peroxidase complex technique. Sections were deparaffinized in xylene, and rehydrated through a graded series of ethanol. Then 3% hydrogen peroxidase was used for to block endogenous peroxidase activity 30 min. Thereafter, the sections were incubated with anti-TGF-β1 primary antibody (Santa Cruz Biotechnology Inc., USA) at a dilution of 1:100 at 4°C overnight. After washing in PBS, the sections were then incubated with biotinylated goat anti-rabbit IgG (Zhongshan Jinqiao Co., China) for 2 h, washed in PBS, and incubated with an avidin horseradish peroxidase complex diluted in accordance with the manufacturer’s instructions. The chromogenic reaction was visualized using 3-3′-diaminobenzidine-4HCL. Positive staining was indicated by a cytoplasmic brown color. To quantify assess the expression of TGF-β1, the digital photomicrographs were obtained, and the number of TGF-β1-positive fibroblasts in the wound sites per quantified unit area (0.04 mm²) in either section were measured using the Image-pro plus software (Version 6.0; Media Cybernetics, USA).

**Analysis of TGF-β1 Protein Expression by Western-blots**

Frozen tissues were homogenized in a lysis buffer (50 mM pH 8.0 Tris, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 100 mM phenylmethylsulfonyl fluoride, 1 mg/mL leupeptin, 1 mg/mL apro tinin, and 1 M dithiolthretol). Homogenates were centrifuged at 13,000 rpm for 10 min at 4°C. Protein from the supernatant was quantified with a Bio-Rad Bradford kit (Bio-Rad Laboratories Inc., USA), then equal amount of proteins were loaded onto 10% SDS-PAGE, and transferred to nitrocellulose membranes and nonspecific binding sites blocked with 5% skim milk powder for 2 h at room temperature. The membranes were incubated with polyclonal antibody to TGF-β1 (Santa Cruz Biotechnology Inc., USA) in blocking buffer for 1 h, and incubated with a horseradish peroxidase-conjugated secondary TGF-β1 anti-mouse IgG. The bands were visualized by using the enhanced chemiluminescent reagent kit (Amersham Biosciences Inc., USA). Signal intensities were quantified by image analyzer (Las 3000, Fuji, Japan).

**Statistical Analysis**

Experimental data were expressed as the mean ± standard deviation (s.d.). All statistical analyses were performed using SPSS 17.0 (SPSS Inc., USA). Comparisons between different groups were analyzed by one-way analysis of variance (ANOVA) and followed by Student Newman Keuls test. A P < 0.05 was considered statistically significant.

### Table 1. Values for Wound Breaking Strength and Hydroxyproline Content on Days 7 and 14 Post-wounding.

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Breaking strength (N)</td>
<td>Hydroxyproline (μg/mg)</td>
</tr>
<tr>
<td>Non-diabetic</td>
<td>6.83 ± 1.10⁴</td>
<td>17.79 ± 2.12⁴</td>
</tr>
<tr>
<td>Diabetic</td>
<td>2.38 ± 0.67</td>
<td>8.60 ± 1.79</td>
</tr>
<tr>
<td>ESWT-1</td>
<td>3.29 ± 0.94⁹</td>
<td>12.10 ± 1.70⁹</td>
</tr>
<tr>
<td>ESWT-2</td>
<td>5.12 ± 0.80⁶</td>
<td>15.66 ± 1.89⁶</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.D. of six incisional wounds.

⁴ P < 0.05 when compared to diabetic group.

⁵ P < 0.05 when compared to ESWT-1 group.
neovascularization, granulation tissue, epithelialization and collagen deposition. In the wounds of diabetic group, granulation was thin; only a very few vessels, fibroblasts and collagen fibers were sparsely distributed in the incisional space. After ESWT, healing of diabetic wounds was markedly improved as indicated by histological scores in Table 2. Compared with that in diabetic group, epithelialization in ESW-treated wounds was more rapid and the epithelialization process was completely finished on day 14 post-wounding; granulation became thicker and a larger number of new capillary vessels can be observed in the granulation tissue. Between incision edges, the number of fibroblasts was significantly increased and the new collagen fibers presented more intensively in the wounds of ESWT groups in

<table>
<thead>
<tr>
<th>Day 7</th>
<th>Non-diabetic</th>
<th>Diabetic</th>
<th>ESWT-1</th>
<th>ESWT-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulation tissue</td>
<td>2.61 ± 0.78</td>
<td>0.72 ± 0.46</td>
<td>1.83 ± 0.38</td>
<td>2.56 ± 0.51</td>
</tr>
<tr>
<td>Neovascularization</td>
<td>1.67 ± 0.69</td>
<td>0.56 ± 0.51</td>
<td>1.50 ± 0.71</td>
<td>2.33 ± 0.49</td>
</tr>
<tr>
<td>Epithelialization</td>
<td>3.83 ± 0.52</td>
<td>2.22 ± 0.43</td>
<td>3.00 ± 0.59</td>
<td>3.11 ± 0.58</td>
</tr>
<tr>
<td>Fibroblast proliferation</td>
<td>2.44 ± 0.51</td>
<td>1.44 ± 0.51</td>
<td>2.17 ± 0.62</td>
<td>2.28 ± 0.67</td>
</tr>
<tr>
<td>Collagen deposition</td>
<td>2.83 ± 0.38</td>
<td>1.11 ± 0.32</td>
<td>1.83 ± 0.38</td>
<td>2.50 ± 0.51</td>
</tr>
<tr>
<td>Day 14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granulation tissue</td>
<td>3.17 ± 0.38</td>
<td>1.89 ± 0.47</td>
<td>2.94 ± 0.54</td>
<td>3.56 ± 0.51</td>
</tr>
<tr>
<td>Neovascularization</td>
<td>3.22 ± 0.55</td>
<td>1.61 ± 0.61</td>
<td>3.17 ± 0.38</td>
<td>3.56 ± 0.62</td>
</tr>
<tr>
<td>Epithelialization</td>
<td>4.00 ± 0.00</td>
<td>3.17 ± 0.51</td>
<td>4.00 ± 0.00</td>
<td>4.00 ± 0.00</td>
</tr>
<tr>
<td>Fibroblast proliferation</td>
<td>3.89 ± 0.32</td>
<td>1.94 ± 0.87</td>
<td>2.83 ± 0.71</td>
<td>3.22 ± 0.72</td>
</tr>
<tr>
<td>Collagen deposition</td>
<td>3.61 ± 0.50</td>
<td>1.94 ± 0.24</td>
<td>2.78 ± 0.55</td>
<td>3.06 ± 0.24</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± s.d. of six incisional wounds.  
*P < 0.05 when compared to diabetic group.  
**P < 0.05 when compared to ESWT-1 group.

Fig. 1. Representative wound sections of Hematoxylin and eosin staining on day 7 post-wounding. Compared with that in diabetic group (A. C), wounds in ESWT-2 group (B. D) were markedly improved after treating with three sessions of low energy ESW. The thickness of the granulation tissue at wound site was significantly increased with prominent fibroblast proliferation (arrow), neovascularization (triangle) and epithelialization. EP, epithelium; GT, granulation tissue.
Improvement of Diabetic Wound Healing by ESWT

Comparison with that in diabetic group both on days 7 and 14 (Fig. 1).

ESWT Up-regulates TGF-β1 Expression in the Diabetic Wound

The immunohistochemical staining showed that the TGF-β1-positive cells were mainly spindle-shaped fibroblasts which aggregated at wound edges (Fig. 2). Compared with that in diabetic group, the number of TGF-β1-positive fibroblasts in ESWT groups was significantly increased on day 7 post-wounding (P < 0.05). Similarly, the results of western-blot showed that the TGF-β1 protein expression in wounds was increased in ESWT groups compared with that in diabetic group (Fig. 3). Thus, ESWT up-regulates the expression of TGF-β1 in diabetic wound.

Discussion

The results of this experiment demonstrated that low-energy ESWT could increase collagen content, enhance wound breaking strength and improve the healing of incisional wound in diabetic rats. The mechanism of the effect of ESWT on diabetic wounds probably lies in its role of up-regulating the expression of TGF-β1 in wound tissue.

In this study, a single dorsal incisional wound was inflicted in STZ-induced diabetic rats and used to simulate the clinical surgical incision healing process in diabetic patients. Beginning at the third day post-STZ injection, rats in the diabetic group exhibited a sustained hyperglycemic state, and this state was being maintained throughout the whole experiment. In order to stimulate the metabolic and pathologic features of long-standing diabetes in humans, wound was created 3 weeks after the successful induction of diabetes. As a result, the wound breaking strength, hydroxyproline content and histological scores in diabetic rats were significantly lower than those in non-diabetic rats. These results confirmed the previously published findings and were similar with the characteristics of healing deficiencies in human diabetes (Spanheimer et al. 1988; Falanga 2005).

Wound breaking strength is an important index for evaluating the healing of incisional wounds (Franz et al. 2000). In the present study, the wound breaking strength of diabetic rats was significantly enhanced after receiving ESWT. This indicated that low-energy ESWT could enhance incisional wound healing quality and prevent wound dehiscence; this could reduce the risk of infection and the length of a hospital stay for diabetic patients. Collagen synthesis and deposition are important for wound healing and development of wound strength (Falanga 2005). Various studies have shown that collagen production could be promoted by some mechanical stimuli, such as ultrasound (Olerud et al. 1990), pulsating electromagnetic field (Murray and Farndale 1985) and mechanical pressure or stretch force (Chan et al. 2010). In this study, we observed that the enhanced wound breaking strength correlated well with the increased hydroxyproline content in both ESWT groups. Meanwhile, the increased collagen deposition was also verified by Masson’s trichrome staining. These results suggested that low-energy ESWT exerted a beneficial effect on the healing of diabetic incisional wound.
wound by increasing the content of collagen and enhancing wound breaking strength.

The classic process of wound healing consists of three phases: the inflammatory phase, proliferative phase and remodeling phase. Fibroblasts are the main cells involved in the last two phases. The migration and proliferation of fibroblasts is essential for collagen synthesis and extracellular matrix secretion (Singer and Clark 1999). However, the function of fibroblasts is impaired in diabetic patients (Hehenberger et al. 1998). In our study, histological examination revealed that only few fibroblasts scattered around the wound site in diabetic rats. However, the number of fibroblasts that migrated to the wound site increased notably after ESWT. This phenomenon was consistent with previous studies (Berta et al. 2009; Kuo et al. 2009b). Berta et al. (2009) reported that application of ESW enhanced human dermal fibroblasts proliferation in vitro. Results from Kuo’s study indicated that an appropriate dosage of ESWT could modulate fibroblasts recruitment, and thus, promote tissue remodeling in an ischemic skin flap rat model (Kuo et al. 2009b). Our data suggest that low-energy ESWT could promote proliferation of fibroblasts in diabetic wound and thereby increase collagen synthesis and wound breaking strength.
To further elucidate the mechanism of low-energy ESWT in increasing collagen content and wound breaking strength, the expression of TGF-β1 in the wound tissue was examined on day 7 post-wounding. TGF-β1 plays a pivotal role in mediating collagen synthesis and degradation. Studies also demonstrated that TGF-β1 promoted fibroblasts migration and proliferation in the process of wound healing (Falanga 2005). Exogenous administration of TGF-β1 could increase the amount of collagen in the wounds of STZ-induced diabetic rats, and partially reverse the decreased tensile strength in incisional wounds (Bitar and Labbad 1996). Increasing the endogenous release of growth factors is one potential mechanism by which ESWT facilitates tissue repair. Up-regulation of TGF-β1 expression following ESWT has been observed in various cell types, such as dermal fibroblasts (Berta et al. 2009), tenocytes (Chen et al. 2004a), mesenchymal stem cells, chondral cells and osteoblastic cells (Chen et al. 2004b). In the proliferative phase, TGF-β1 is mainly released from fibroblasts. In our study, there were significant differences in the expression of TGF-β1 between the diabetic group and ESWT-treated groups. Because TGF-β1 immune reactive cells were mainly fibroblasts at the wound edges, we believe that ESWT increases collagen synthesis by stimulating endogenous production of TGF-β1 in wound fibroblasts.

The ability of low-energy ESWT to increase neovascularization and to accelerate epithelialization has been well demonstrated in previous studies (Ito et al. 2009; Ottomann et al. 2010). In the present study, we also observed the improvement of wound histology in diabetic rats after ESWT, including the formation of new capillary vessels, thickening of the granulation tissue and acceleration of the epithelialization.

Although other investigators studied the effect of ESWT on diabetic wound healing (Kuo et al. 2009a; Zins et al. 2010), our study differed in many ways from these reports. In previous studies, an excisional wound model was used to explore the potential mechanism mainly focusing on ESWT-induced neovascularization and elevated angiogenic gene expression. In contrast, we examined the mechanism of ESWT-mediated wound healing by investigating its impact on collagen synthesis. An incisional wound model was used to simulate the impaired wound healing in diabetic surgical patients and to determine changes in the collagen content in wound. In addition, distinct from previous experimental studies, focused ESW was adopted and its effectiveness was proved in our experiment. Until recently, there is still no control study to compare the difference between focused and unfocused ESW in the treatment of wound healing.

There are two limitations of our study that should be mentioned. First, we did not determine whether low-energy ESWT could increase collagen deposition or up-regulate TGF-β1 expression in non-diabetic rats, because the main objective of our study was to investigate its effect on diabetic wounds. Nevertheless we are still curious about whether ESWT could accelerate incision healing in normal people. Second, we only examined different sessions of ESWT on the healing of incisional wounds. It is still controversial whether multiple doses of ESWT are beneficial for wound healing (Kuo et al. 2009a; Zins et al. 2010). Although the data from our study indicated that three sessions of ESWT were more efficient than a single-session treatment at enhancing wound breaking strength and collagen content, further experimental and clinical studies are still needed to determine the optimal energy level, number of ESW, treatment interval and type of ESW (unfocused versus focused) for treating surgical wounds.

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**Conflict of Interest**

The authors have no conflict of interest.

**References**


