Azelnidipine, a New Calcium Channel Blocker, Promotes Skin Wound Healing in Diabetic Rats


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**Objective.** Impaired wound healing in diabetes is associated with decreased nitric oxide (NO) bioavailability in wound tissue. We hypothesized azelnidipine (AZL), a new calcium channel blocker with antioxidant properties, would enhance wound healing in streptozotocin-induced diabetic rats by restoring NO synthesis.

**Methods.** Twelve male rats were taken as non-diabetic group. Twenty four rats were taken and caused to be diabetic by a single streptozotocin injection. Diabetic rats were divided randomly to two groups: control and treatment. Half of non-diabetic and also diabetic rats (in each group of control and treatment) randomly served as excisional-wound model and the other half as nitrite-measurement model. Six weeks after causing diabetes, the excisional wound model underwent dorsal full-thickness excisional wounds (1 × 1 cm). After wound healing completion, full-thickness skin samples (1 × 1 cm) were taken from the wound sites for evaluation of stereological parameters. The nitrite-measurement model (6 wk after causing diabetes) underwent insertion of subcutaneous polyvinyl alcohol sponges in dorsum. The rats were killed 2 wk post-wounding, and wound fluid was analyzed. In the study, after wounding, the treatment groups were gavaged with AZL (3 mg/kg/d) and control and non-diabetic groups with AZL vehicle till euthanasia.

**Results.** AZL accelerated wound healing rate and also improved wound fluid NO level toward normal value in diabetic rats. Volume density of collagen fibers, numerical density of fibroblasts, and length density of vessels were increased in AZL-treated rats compared with control group.

**Conclusion.** AZL administration promotes diabetic wound healing by stimulating NO production and enhancing histologic processes central to normal wound healing.

Key Words: diabetes; wound healing; nitric oxide; azelnidipine.

**INTRODUCTION**

It is estimated that there are more than 170 million people with diabetes in the world [1]. One of the common complications of diabetes is delayed wound healing and chronic ulcer formation often leading to amputation [2, 3]. Wound healing impairment in diabetic patients represents a particularly challenging clinical problem for which there is currently no efficacious treatment regimen [4].

The etiology of delayed diabetic wound healing is multifactorial, and among them vasculopathy and neuropathy are two major contributors [4, 5]. Compelling evidence indicates that reduced wound nitric oxide (NO) synthesis in diabetes may contribute to impairment of wound healing [6]. NO has several key roles in normal wound repair [7, 8]. NO promotes processes central to wound healing, including angiogenesis [9–11] and migration and proliferation of fibroblasts, [12] epithelial cells, [13] endothelial cells [9, 10], and...
keratinocytes [11]. Sustained hyperglycemia leads to increased vascular superoxide (O2−) production, which inactivates NO and causes vascular dysfunction in diabetes [14–16].

Azelnidipine (AZL) is a new long-acting dihydropyridine calcium channel blocker (CCB) with selectivity for L-type voltage-operated calcium channels, and recently has been approved in Japan for the treatment of patients with hypertension [17]. It has previously been shown that CCBs have antioxidant activity and can improve vascular endothelial function by increasing endothelial NO synthase (eNOS)-catalyzed NO production [18]. Among CCBs, AZL possesses more potent antioxidant properties [19, 20]. AZL can reduce expression of nicotinamide-adenine-dinucleotide phosphate (NADPH) oxidase, the key enzyme for superoxide (O2−) production [21]. Finally, it has previously been shown that AZL enhances basal NO production in human umbilical vein endothelial cell (HUVEC) [18] and increases eNOS expression in the brain, heart, and aorta [22].

The aim of the present study was to evaluate the effect of AZL on wound tissue NO production, and macroscopic and microscopic aspects of cutaneous wound healing in an experimental diabetes model.

MATERIALS AND METHODS

Animals

Thirty six male Sprague-Dawley rats weighing 180–250 grams were chosen for this study. The animals were kept in controlled temperature condition (25°C) with 12 h light-dark cycles and free access to water and food (except the times of fasting necessary for biochemical assays). The current study was conducted according to the World Medical Association Declaration principles for animal studies, revised in October 2008 [23] and was approved by the Ethics Committee of Shiraz University of Medical Sciences.

Induction of Type 1 Diabetes

Twenty four rats were given a single intraperitoneal (IP) injection of streptozotocin (STZ) (60 mg/kg, Sigma, St. Louis, MO) dissolved in sterile citrate buffer (0.05 mol/L sodium citrate, pH 4.5). Twelve rats were injected intraperitoneally with equal volumes of sterile citrate buffer as nondiabetic (non-DM) group. Five days later, 1 mL blood sample was taken from each rat’s tail vein, after an overnight fasting. The samples were centrifuged, and plasma glucose levels were measured by a glucose analyzer (Easy Gluco Infopia Co., Ltd., Anyang, Gyeonggi-do, Korea). The rats with plasma glucose level higher than 280 mg/dL were considered to be diabetic and used for the next steps of the experiment. Half of diabetic and half of non-DM rats were chosen randomly for analyzing wound healing rate. Blood pressure (BP) of the diabetic rats was measured 1 d before wound creation and after wound healing completion by indirect tail-cuff method (NIBP controller, ML 125; ADInstruments Pty Ltd., Castle Hill, NSW, Australia). The other half of the rats was used for plasma and wound fluid nitrite measurement. The rats’ fasting plasma glucose levels were measured once again 1 d before euthanasia.

Excisional Wound Model

Six weeks after STZ or its vehicle injection, 12 diabetic and six non-diabetic rats were anesthetized with ketamine (100 mg/kg IP; Sigma Chemical Co., St. Louis, MO, USA) and xylazine (10 mg/kg IP; Sigma), and the dorsum was clipped free of hair. Under sterile condition, a full-thickness skin sample (1 × 1 cm) was created with fine scissors. The wounds were covered with a Bioclusive transparent dressing (Johnson and Johnson Medical, Ascot, UK), which is a hypoallergenic adhesive film dressing that is impermeable to fluids but permeable to vapors and gases and provides a bacterial/viral barrier to maintain sterility. Thereafter, 12 diabetic rats were divided into two control and treatment groups, randomly. The treatment group was gavaged with AZL (3 mg/kg/d, Sankyo Co., Tokyo, Japan) till euthanasia. AZL was suspended in polysorbate 80 (1:5 in water). Control and six non-DM rats were gavaged with equal volumes of AZL vehicle.

To determine the wound closure rate, the wounds were visited every other day till complete healing. At each visit, the wounds were debrided and a photograph was taken of each rat’s wound with a single lens, 12.1-megapixel digital Camera (Canon, Tokyo, Japan) and the wounds were covered with new dressings. To calibrate the magnification of photographs, the camera was held at the distance of 20cm from the wound and a fine ruler was laid at the level of the wound. Dressings were changed at these times. The wound area (mm²) at each visit was estimated, using a software composed of a point grid (designed at Histomorphometry and Stereology Research Centre, Shiraz University of Medical Sciences) using the following formula:

\[
\text{Area} = \sum P \times a/p, \quad \text{where} \quad 2P \text{ was the total points laid on the favorable area of the wound and } a/p \text{ was the area associated with each point (mm²).}
\]

Thereafter, wound closure rate was calculated as:

\[
\text{Wound closure rate (\%) = \left[ \frac{\text{area at visit 1} - \text{area at each visit}}{\text{area at visit 1}} \right] \times 100}
\]

Tissue Preparation and Processing

Fourteen days after wounding (the day which has been shown, in our pilot studies, most of the wounds are closed by then) the rats were euthanized by ether overdose. After clipping the dorsum of the rats free of hair, full thickness skin samples (1 × 1 cm) were taken from the wound sites. The wound tissues were fixed in buffered formaldehyde (pH = 7.2), and then cut into small pieces. In a systematic random sampling manner (first with a random start and others with equal distances), nine pieces were chosen for histologic analysis, each one about 1 mm². The pieces were embedded in a cylindrical paraffin block to obtain isotropic systematic random sections. These sections are necessary in some stereological analyses including length density estimation. The cylindrical blocks were sectioned using orientator methods for generating isotropic uniform random sections. Briefly, each cylindrical block was placed on a circle that was divided into 10 equal pieces using radial lines. The blocks were sectioned along the lines bearing a randomly selected number. The sectioned surface of the bar was placed on the 0-0 direction of the circle with 10 unequal cosines-weighted divisions and the second cut was done. The new surface was sectioned (5 and 16 µm thickness) and stained with Hedenhain’s azan.

Volume Density of the Collagen Fibers

A video-microscopy system made up of a microscope (E-200; Nikon, Taguig, Japan), a video camera, a computer, and a flat monitor was used for microscopic analyses of the sections. The volume density (\(V_v/collagen/dermis\)) of the collagen fibers was estimated using 5 µm sections. A grid of points [24, 25] was laid on the live image of dermis at
final magnification of 450 (Fig. 1A) and $V_{v\text{(collagen/dermis)}}$ of the collagen fibers was calculated as:

$$V_{v\text{(collagen/dermis)}} = \frac{P_{\text{collagen}}}{P_{\text{dermis}}}$$

Where the $P_{\text{collagen}}$ was the number of points hitting the profiles of the collagen and $P_{\text{dermis}}$ was the number of points hitting the dermis.

**Length Density of the Vessels**

Five $\mu$m sections were used for estimation of length density ($L_V$) of the vessels. An unbiased counting frame [24, 25] was laid randomly on the monitor image of wound dermis at final magnification of 450. Any vessels lied in the counting frame or touched the inclusion borders without touching the exclusion borders was selected (Fig. 1B). The $L_V$ of the vessels was calculated as:

$$L_V = 2 \times \frac{\Sigma Q}{(a/f) \times \sum f}$$

Where $\Sigma Q$ was the total number of the sectioned vessels, $(a/f)$ was the area of the counting frame ($130 \times 130 \mu m^2$), and $\sum f$ was the total number of frames counted in each animal.

**Numerical Density of the Fibroblasts**

Sixteen $\mu$m thickness sections and a high numerical aperture (60×, NA = 1.4) oil immersion lens at magnification of 2000 were used to estimate the numerical density ($N_V$) of the fibroblasts. An unbiased counting frame was superimposed on the monitor image of the sections to estimate the numerical density ($N_V$) of the fibroblasts. The nuclei were unclear at the first 5 $\mu$m optical section (height of disector) (left), but appeared within the next traveling 5 $\mu$m optical section (right). As above, any nucleus lied in the counting frame or touched the inclusion borders (yellow dotted lines) and did not touch the exclusion borders (bold yellow lines) were counted. In this figure the fibroblast nucleus with black mark was ignored and the nucleus with yellow mark was counted. Hedenhain’s azan x2000.

**Nitrite Measurement Model**

Six weeks after STZ or its vehicle injection, 12 diabetic and six non-DM rats were anesthetized with ketamine (100 mg/kg IP) and xylazine (10 mg/kg IP). Each rat underwent a 7 cm longitudinal, midline, dorsal skin incision under sterile condition. One sterile polyvinyl
alcohol sponge (10 mm × 4 mm; M-PACT, Eudora, US) was used per rat. Sponges were inserted in subcutaneous pocket, and the wounds were closed with surgical staples. The studied groups and the dose of administered compounds were the same as stated above in wound healing study. Two weeks later, when the excisional wounds were completely healed, the rats were euthanized by ether overdose, and harvested sponges were pulled out from the subcutaneous pockets. The implanted sponges from each rat was squeezed and wound fluid and plasma was filtered (10 kDa Ultrafree-PF filter; Millipore Corp., Marlborough, MA). Plasma and wound fluid nitrite, the stable and non-volatile breakdown product of NO, was measured using Griess reagents (Sigma) [26].

**Statistical Analysis**

Values are expressed as mean ± standard error (SE) in the text and figures. Statistical analyses were performed using SPSS software (Ver. 16). One-way analysis of variance (ANOVA) followed by Tukey post test, repeated measurement and Mann-Whitney U-test were used to analyze the data. \( P < 0.05 \) was considered significant.

In all steps of the study, investigators, including research assistants, pharmacologists, immunologists, stereologists, and statisticians were completely blinded to the studied groups.

**RESULTS**

All STZ-injected rats showed gross hyperglycemia and maintained it during the study. AZL did not influence plasma glucose levels compared with the control group (430 ± 56 mg/dL 5 d after STZ injection *versus* 367 ± 13 mg/dL before euthanasia in treatment group, \( n = 12 \), and 425 ± 59 mg/dL *versus* 363 ± 10 mg/dL in control group, \( n = 12 \)). AZL did not induce significant changes in the BP of the treatment group compared with the control group during the study (121.94 ± 4.20 mm Hg 1 d before wound creation *versus* 125.6 ± 5.00 mm Hg after completion of wound healing in the treatment group, \( n = 6 \), and 137.75 ± 3.41 mm Hg *versus* 137.07 ± 2.68 mm Hg in the control group, \( n = 6 \)).

**Wound Healing Rate**

The mean initial area of the ulcers was 113.23 ± 1.11 mm² (range 94.12–148.03 mm²). There was no significant difference in primary wound surface area among the three groups. The rate of wound closure in diabetic rats was significantly slower compared with that of non-DM group (\( P < 0.001 \)). Wound closure rate was significantly increased in AZL-treated rats compared with control group (\( P < 0.001 \)), without any significant difference with non-DM animals (Table 1).

**Histologic Results**

\( V_{V_{\text{collagen/dermis}}} \) of collagen fibers, \( N_{V} \) of fibroblasts, and \( L_{V} \) of vessels were significantly decreased in wound tissue of control rats compared with non-DM animals (\( P < 0.001 \)). \( V_{V} \) of collagen fibers and \( N_{V} \) of fibroblasts were significantly increased in wound tissue of AZL-treated rats compared with the control group (\( P < 0.001 \)), without any significant difference with non-DM animals. AZL increased \( L_{V} \) of the vessels in the AZL-treated group compared with both control (\( P < 0.001 \)) and non-DM groups (\( P < 0.001 \)) (Table 1).

**Nitrite Level**

Plasma nitrite concentration of the control group was significantly reduced compared with non-DM animals (\( P < 0.001 \)), without any significant difference with non-DM rats. AZL increased \( L_{V} \) of the vessels in the AZL-treated group compared with both control (\( P < 0.001 \)) and non-DM groups (\( P < 0.001 \)) (Table 1).
Wound fluid NO level was also decreased in the control group compared with non-DM; however, this reduction was not statistically significant ($P = 0.17$). Wound fluid nitrite level was significantly increased in AZL-treated rats compared with the control group ($P = 0.039$), without any significant difference with non-DM animals. AZL had no significant effect on the plasma nitrite level (Fig. 4).

**DISCUSSION**

The present study demonstrates that AZL, a new dihydropyridine CCB, increases wound fluid NO level, enhances collagen accumulation and fibroblast proliferation, and promotes angiogenesis, resulting in accelerated wound healing in type 1 diabetic rat. Since diabetic wound problems are common throughout the world and in addition to causing pain and morbidity, the economic consequences are major, AZL can reduce this burden by accelerating wound healing in diabetes. In the applied dose, AZL has no effect on BP and plasma glucose level, so the observed effects on wound healing cannot be attributed to these parameters.

Previous studies have highlighted the importance of NO as an essential mediator in normal wound healing. NO promotes angiogenesis [9–11], and proliferation of fibroblasts [12], epithelial cells [13], endothelial cells [9, 10], and keratinocytes [11]. Endothelial NO expression, eNOS activity, and cutaneous NO bioavailability is significantly decreased during the wound healing process in diabetes [27]. There are growing data which suggest that restoring NO bioavailability may ameliorate delayed diabetic wound healing [27]. In previous studies, it was shown that different therapeutic regimens and interventions such as L-arginine supplementation, propranolol or pravastatin administration, and gene therapy of eNOS could enhance wound healing rate by increasing NO bioavailability in wound tissue [27–30]. We have also shown in this study that wound healing enhancement by AZL administration was paralleled by restoring NO-deficient state in diabetic rats. AZL increased wound fluid nitrite level to the values similar to those in non-DM animals. However, AZL did not change the decreased plasma NO level in diabetic rats. This may suggest that other mechanisms are involved in determining systemic NO bioavailability, which could not be modulated effectively by AZL. Similar results have also been observed in a study in which L-arginine supplementation in diabetic rats could enhance NO production in wound tissue without influencing plasma NO level [29]. Previously, it has been shown that AZL enhances NO production by endothelial cells [18].

One of the shortcomings of the present study was the failure to measure eNOS expression. However, in previous studies it has been shown that AZL can increase the expression of this enzyme in brain, heart, and aorta of spontaneously hypertensive rats and HUVECs [18, 22]. In addition to increase NO production, AZL can reduce NO degradation by suppression of $O_2^-$ production. Increased $O_2^-$ generation especially by NADPH oxidase is thought to be responsible for impaired NO bioavailability in diabetic vasculature [16, 31]. The capability of AZL to stimulate NO production in wound fluid may be due to its antioxidant effects. AZL is proven to have several antioxidant properties without any influence on blood pressure when used at antioxidant dose [21, 32, 33]. AZL administration (0.1 and 3 mg/kg/d) can reduce the expression of NADPH oxidation.

**TABLE 1**

<table>
<thead>
<tr>
<th>Groups</th>
<th>N$_V$ of fibroblasts</th>
<th>V$_V$ (collagen/dermis) of collagen fibers</th>
<th>L$_V$ of vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-DM</td>
<td>56.8 ± 8.3*</td>
<td>90 ± 1*</td>
<td>21.3 ± 1.1*</td>
</tr>
<tr>
<td>Control</td>
<td>20.8 ± 3.6</td>
<td>74 ± 5</td>
<td>10.6 ± 3.3</td>
</tr>
<tr>
<td>AZL-treated</td>
<td>53.6 ± 1.0**</td>
<td>88 ± 4**</td>
<td>41.5 ± 11.2***</td>
</tr>
</tbody>
</table>

Mean ± SE of the numerical density ($N_V$) of the fibroblasts ($\times 10^4$ per mm$^3$), volume density ($V_V$ (collagen/dermis)) of the collagen fibers (%), length density ($L_V$) of the vessels (mm/mm$^3$) in non-DM, control and AZL-treated groups.

* $P < 0.001$, non-DM versus control.
** $P < 0.001$, AZL-treated versus control.
*** $P < 0.001$, AZL-treated groups versus non-DM.
oxidase subunits and NADPH oxidase-mediated O$_2^-$ generation [21, 32, 33]. The other anti-oxidant effects of AZL are its ability to decrease lipid hyperoxides in erythrocyte membrane as an oxidative marker in the plasma [34] and to improve glucose intolerance by modulating intracellular signaling of insulin and angiotensin II, a major mediator of oxidative stress [32].

Dermal reconstruction depends on formation of granulation tissue, which includes cell proliferation, extracellular matrix deposition, wound contracture, and angiogenesis [35]. In the present study, angiogenesis, fibroblast number, and collagen accumulation were impaired significantly in diabetic rats. AZL normalized the fibroblast number and collagen accumulation to the values of non-DM group; angiogenesis observed in the wound tissue of the AZL-treated rats was even more than that in the non-DM animals. Promoting fibroblast proliferation and collagen accumulation, and increased angiogenesis by AZL administration may be the result of enhancing NO bioavailability in wound tissue. Previous studies have shown that administration of NO donors such as L-arginine, and other therapeutic agents such as pravastatin could improve wound breaking strength and collagen deposition probably by stimulating NO synthesis [29, 30]. Propranolol was also shown to improve wound healing in diabetic rats through greater NO production, fibroblast proliferation, collagen deposition, and increase in the blood vessel density [28].

The favorable effect of AZL on diabetic wound healing may not be only due to its antioxidant properties, but also due to its anti-inflammatory effects. Stronger expression of proinflammatory cytokines such as monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α) leads to delay in the tissue repair in diabetic wounds due to decreased collagen expression, and prolonged persistence of neutrophils and macrophages in the wound area [28, 36, 37]. TNF-α also up-regulates MCP-1 mRNA level in endothelial cells and stimulates intracellular reactive oxygen species (ROS) generation [38]. Previous studies have shown that AZL inhibits TNF-α induced MCP-1 expression and also inhibits ROS generation in HUVECs probably by suppressing NADPH oxidase [20, 38]. It has also been shown that AZL decreases plasma MCP-1 and TNF-α in hypertensive type 2 diabetic patients [39]. However, we did not evaluate the effects of AZL on wound tissue MCP-1 and TNF-α expression; so further investigations are necessary to clarify the effect of AZL on expression of pro-inflammatory cytokines in diabetic wound healing. Using NO inhibitor (L-NAME) to clarify the contribution and magnitude of mechanisms other than increase in NO bioavailability in the significant effect of AZL on diabetic wound healing is advisable.

In summary, our data indicate that AZL improves impaired wound healing and enhances histologic processes central to normal wound healing, such as angiogenesis, fibroblast proliferation, and collagen accumulation in type 1 diabetic rats. These effects can be attributed, at least partly, to increase in NO bioavailability.

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