The effect of DETA NONOate, a nitric oxide donor, on the rate of collagen synthesis in rat as an animal model of diabetes

Dashti, N.*; Einollahi, N.; Zarebavani, M. and Kiani, F.

Faculty of Allied Health Sciences, Tehran University of Medical Sciences, Tehran, Iran.

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Correspondence
Dashti, N.,
Faculty of Allied Health Sciences, Tehran University of Medical Sciences,
P. O. Box: 14155-6111, Tehran, Iran.
Tel:+98(21)64432525
Email: dashti@tums.ac.ir

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Abstract

Exogenous nitric oxide donors such as DETA NONOate, spontaneously release nitric oxide. This study aimed to investigate the effect of DETA NONOate as a nitric oxide releasing drug on the rate of collagen synthesis during the impaired wound healing in a rat model of diabetes. Twelve male Sprague–Dawley rats were transferred into separate metabolic cages. Nine days before wounding, the rats were injected intraperitoneally with streptozotocin (STZ; 55 mg/kg body weight in citrate buffer 0.1 mol/L, pH 4.5) to induce diabetes. The dorsal surface of each rat was properly shaved and a full thickness dermal wound was made. The test group (n=6) was treated with 100 µM DETA NONOate in phosphate buffer while the control wounds (n=6) received sterile saline (PBS) only on the same day as wounding and every three days for one week. After the skin incision, polyvinyl alcohol (PVA) sponges were implanted subcutaneously on the dorsal of each animal under sterile conditions for the collection of wound fluid. Electrophoresis (current: 20 mA) was performed on the wound fluid. The gel was stained with Coomassie blue G-250, destained, and photographed. DETA NONOate treatment increased the rate of collagen synthesis in the diabetic test group compared to the control group. The nitric oxide donor, DETA NONOate, may represent a potential treatment for impaired wound healing in diabetes by increasing the collagen synthesis at the wound site.

Introduction

The process of wound repair following surgery is an extremely complex phenomenon, which involves a number of well-orchestrated programs (Prathiba and Suryan Arayanan, 1999). Wound healing starts immediately after an injury and proceeds with a series of complicated but well-organized interactions among various types of tissue and cells (Qing Lin and Kondo, 2003). The full-thickness wound is immediately filled by clots in the presence of platelet aggregates. Thereafter, the inflammatory phase occurs; leukocytes, such as neutrophils and monocytes, infiltrate the site in order to remove the breakdown products from injured cells and clots and release various growth factors and cytokines (Singer and Clark, 1999; Martin, 1997). The proliferative phase then starts in which epidermal cells migrate and proliferate to fill the wound gap, displace the remnants of the original clots, and secrete basement membrane components such as collagen (Dashti *et al*., 2004). The collagen molecule is one of the most fundamental constituents of connective tissue with a triple helical structure (Mathews, 1975; Piez, 1976; Ramachandran, 1976; Miller, 1976 and Burgenson; Marcel, 1992). Nitric oxide (NO) plays an important role in the inflammatory phase of healing.

Failure of wound healing is a major source of morbidity and mortality in diabetes (Tereze Laing and Hanson, 2009). In patients with diabetes, the levels of NO are decreased in the environment surrounding the wound. Additionally, NO plays an important role in collagen synthesis by fibroblasts through an unknown mechanism; it accelerates wound closure when applied topically at the wound site. Therefore, the reduced production of NO in wounds of patients with diabetes has been shown to be associated with impaired healing and reduced collagen deposition (Witte *et al*., 2002).

In this study, we investigated the effect of an exogenous NO donor, DETA NONOate, which is a drug that spontaneously releases NO, on the rate of collagen synthesis during wound healing in an experimental animal model of diabetes.

Materials and Methods

DETA NONOate (Z-1-[2-(2-Aminoethyl)-N-(2-aminoethyl) amino] diazen-1-ium-1, 2-diolate was purchased from Alexis Co. (Switzerland). The low nitrate

diet (2% L-arginine) was obtained from the Pasteur Institute, Tehran, Iran. Blood glucose levels were measured with a glucose oxidase kit (Zist Chimmy Chemical Co., Tehran, Iran). Polyvinyl alcohol (PVA) sponges were purchased from M-PACT Eudora (Kansas, USA).

Male Sprague–Dawley rats (Animal House, Tehran University of Medical Sciences, Tehran, Iran) were acclimatized for one week; they were given water *ad libitum* and were fed a diet that contained low levels of nitrate (2% L-arginine). Animals were then transferred to separate metabolic cages. Nine days before wounding, 12 rats were injected intraperitoneally (i.p.) with streptozotocin (STZ; 55 mg/kg body weight in citrate buffer 0.1 mol/L, pH 4.5) to induce diabetes. Evidence of diabetes was confirmed by the occurrence of blood glucose levels that were greater than 250 mg/dL and excessive urination.

Before wounding, the rats were anesthetized with Nembutal (40 mg/kg, i.p.). The dorsal surface of each rat was fully shaved and a full thickness dermal wound was created in each rat that was approximately 1 cm × 1 cm. The test group (n=6) was treated with 100 µM DETA NONOate in phosphate buffer solution (PBS), while rats in the control group (n=6) were treated with sterile PBS on the same day and every three days.

After the skin incision was made, polyvinyl alcohol (PVA) sponges were implanted subcutaneously under sterile conditions on the dorsum of each animal next to the incision site, avoiding contamination or infection at the wound site itself. The skin incision was then closed using surgical clips.

All sponges were harvested six days after implantation; the fluid contained within the sponges was removed by with squeezing the sponge with forceps. The wound fluid was then centrifuged at 400 g for 10 min at 4°C. The cell-free supernatants were aliquoted and stored at -80°C until it was assayed.

The relative molecular weight profile for wound fluid collagen was determined according to the method of Laemmli (Laemmli, 1970) with the use of 150 g/L separation gel and 30 g/L stacking gel. The sample was dissolved in 24 mmol/L Tris-HCl buffer (pH 6.8) that contained 10 g/L SDS (sodium dodecyl sulfate) , 100 mL/L glycerol, 20 mL/L 2 mercaptoethanol, and 0.4 g/L bromophenol blue. Each sample was then boiled for 5 min prior to electrophoresis. 20 µL of wound fluid (1 mg/ml) and 20 µL (1 mg/ml) of plasma were loaded onto the gel. Electrophoresis was performed at a current of 20 mA. The gels were stained with Coomassie blue G-250, destained, and photographed.

Results

Wound fluid and plasma contained bands of similar molecular weights. Wound fluid electrophoresis of samples from the test group showed five protein bands from the point of origin to the migration side by SDS-PAGE, which corresponds to the different subunits of collagen. Significant difference between wound treated with and without DETA NONOate were obtained for the wound collagen content.

Wounds treated with DETA NONOate had larger collagen bands when compared to the control group (Figure 1), which suggests that DETA NONOate treatment increased the rate of collagen synthesis in the diabetic test group compared to the control group.

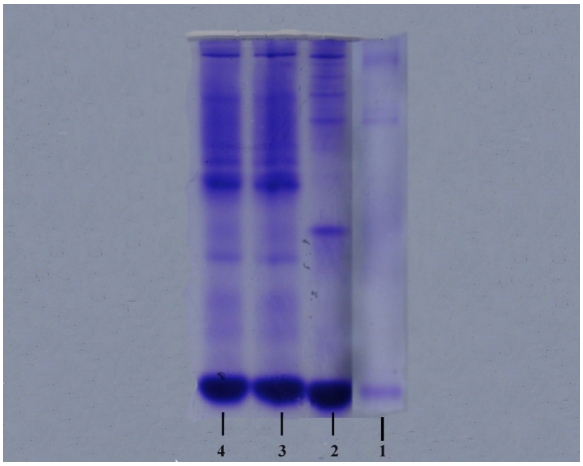

Figure 1: SDS-PAGE elestrophoresis of wound fluid.
Lane 1: Test wound fluid, Lane 2: Control wound fluid, Lane 3, 4: Plasma

Discussion

Collagen is one of the principle structural proteins that play an important role in wound healing (Bilden and Oktay, 1999). Collagens comprise a large family of structural proteins in the extracellular matrix (ECM) of eukaryotes (Bulfield, 1990). During wound healing, the collagen molecules are secreted from cells in the ECM and assemble to form fibers that enhance the functional integrity of tissues (Freeman, 1988). It has been shown that diabetic wounds are more susceptible to treatment with NO donors since the wound is deficient in nitric oxide (Singer and Clark, 1999). The previous studies confirm that diabetes is characterized by a NO-deficient state, which is accompanied by decreased collagen deposition at the wound site (Witte *et al*., 2002).

The NO donor, DETA NONOate, may therefore represent a potential treatment for impaired wound healing that is a feature of diabetes by increasing the rate of collagen synthesis at the wound site. In previous studies, it has been shown that the effect of NO donor administration may be dependent on a threshold rather that the dose. Therefore, further studies should be performed to demonstrate a correlation between levels of NO donors at the wound site and its outcome.

In summary, the administration of DETA

NONOate can partially improve impaired healing in diabetes by increasing the rate of collagen synthesis. This may have therapeutic potential and requires further evaluation.

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