

# The Effect of Nitric Oxide Donor in Diabetic Wound Healing

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## Abstract

Diabetes is characterized by a nitric oxide deficiency at the wound site. Diabetes is a factor that influences all stages of wound healing. In animals with acute experimental diabetes induced by streptozotocin (STZ), the early inflammatory responses after wounding is impaired, fibroblast and endothelial cell proliferation is reduced as well as accumulation of reparative collagen and gain in wound breaking strength. This study investigated whether exogenous nitric oxide supplementation with nitric oxide donor DETA NONOate could reverse impaired healing in diabetes. The results suggest nitric oxide donor DETA NONOate can reverse impaired healing associated with diabetes ( $P < 0.001$ ) and urinary nitrate (NO<sub>3</sub>) output may reflect the extent of repair in this wound model ( $P < 0.001$ ).

**Keywords:** DETA NONOate, Diabetes, Wound healing

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## Introduction

Nitric oxide (NO) is a unique, gaseous free radical that is an important physiologic mediator for autonomic functions such as vasodilation, neurotransmission, and intestinal peristalsis. Recent wound healing studies of NO - mediated shows enhanced tissue repair (1, 2). In diabetes, an endogenous deficiency in NOS enzyme leads to decreased wound NO production and a spectrum of related pathologies, (3, 4) such as impaired cutaneous vasodilation, decreased neurogenic vascular response, diabetic neuropathy, endothelial cell function that inhibit the processes necessary for granulation tissue formation and accumulation of reparative collagen is reduced. As a mediator of tissue repair, NO has shown to promote angiogenesis (5) and cellular migration (6), increase wound collagen deposition and collagen cross linking (7), regulate vasodilation (8), inhibit platelet aggregation (9), inhibit endothelial - leukocyte cell adhesions (10), modulate endothelial proliferation and apoptosis (11), increase the viability of random cutaneous flaps (12), and enhance cellular immunomodulation and bacterial cytotoxicity (9). Based on previous findings that diabetes is characterized by

reduced nitric oxide levels in the wound environment (13), this study investigated whether exogenous nitric oxide supplementation with nitric oxide donor DETA NONOate could reverse impaired healing in diabetes. The 24 hour urine samples were collected through out the healing period (21 days). Wound closure profiles were examined by video image every 3 days and urinary nitrate (NO<sub>3</sub>) output was measured by Griess reagent.

## Materials and Methods

DETA NONOate (Alexis Co., Switzerland) Low nitrate diet 2% L-arginine, (Pasture Institute, Tehran Iran). Potassium Nitrate (99.9%) (Merck chemical Company, Germany). Vanadium (III) Chloride (99%) (Johnson Mattley GmbH). Glucose oxidase kit (Zist Chimy Chemical Co. Tehran, Iran) Griess Reagent (Alexis Biochemicals, Switzerland). Blood glucose levels were measured with glucose oxidase kit. All procedures used for animal experimentation was approved by the animal care committee of Tehran University of Medical Sciences. Male Sprague-Dawley Rats (Tehran University of Medical Sciences animal house, Tehran, Iran) were acclimatized for one

week given water and libitum, and fed a low nitrate containing diet (2% L-arginine). Animals were transferred to separate metabolic cages. Nine days before wounding, 12 rats were injected intraperitoneally (IP) with streptozotocin (STZ in citrate buffer 0.1 mol/L, pH 4.5, 55 mg/kg body weight) to induce diabetes. Evidence of diabetes was confirmed by blood glucose levels greater than 250 mg/dL and excessive urination. Daily urine samples were collected at every 24 hour intervals. To inhibit bacterial growth, 5ml of 3 M HCl was added to each urine collection (pH=1) and urine samples were kept frozen until analysed (-70°C). Before wounding, the rats were anesthetized with Nembutal (40 mg/Kg i.p.). The dorsal surface of each rat was properly shaved and given full thickness dermal wounds approximately 1 cm × 1 cm. The test group (n=6) was treated with 100 μmole DETA NONOate in phosphate buffer while control wounds (n=6) received sterile phosphate buffer on the same day and every 3 days. Urinary nitrate (NO<sub>3</sub><sup>-</sup>) was quantitated daily prior to wounding, and during wound healing (21 days) following external wound closure. The rate of wound healing was determined by video image analysis. Forty eight hr following wounding and every 3 days, wounds were video taped using Nikon Colpix 5000. The urinary nitrate levels was determined using Griess reagent. The principle of this assay is reduction of nitrate by vanadium (III) combined with detection by acidic

Griess reaction. The Griess reaction entails formation of a chromophore from the diazotization of sulfanilamide by acidic nitrite followed by coupling with bicyclic amines such as N-1-naphthyl ethylenediamine. SPSS computer software was used for data management and analysis.

## Results

Promising results have been obtained from studies using non-soluble, polymeric DETA NONOate as NO donating agent during cutaneous healing in rats. The urinary nitrate (NO<sub>3</sub><sup>-</sup>) profiles for diabetic rats with DETA NONOate and controls is shown in Figure 1. Control diabetic rats had significantly less urinary nitrate (NO<sub>3</sub><sup>-</sup>) output than the test group (*P*<0.001). A significant peak in nitrate (NO<sub>3</sub><sup>-</sup>) output occurred between days 12-13 when the external wound was approximately 65% closed. Diabetic rats whether treated with DETA NONOate or not, exhibited a significant increase in urinary nitrate (NO<sub>3</sub><sup>-</sup>) output within 24 to 48 hr post wounding period. During a 3 day period, all the rats were removed from their cages and video imaged. The wound closure profiles for all the rats are shown in Figure 2. There is a significant difference (*P*<0.001) in wound closure profiles between the test and the control group. Photographs of full thickness thermal wounds for the control and the test group on days 0, 12, 21 are shown in Figures 3 and 4 respectively.

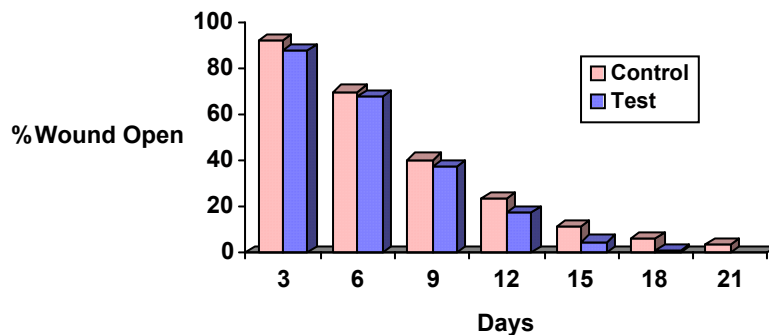


Fig. 1: Urinary Nitrate (NO<sub>3</sub><sup>-</sup>) output profiles for wounded diabetic treated and control rats

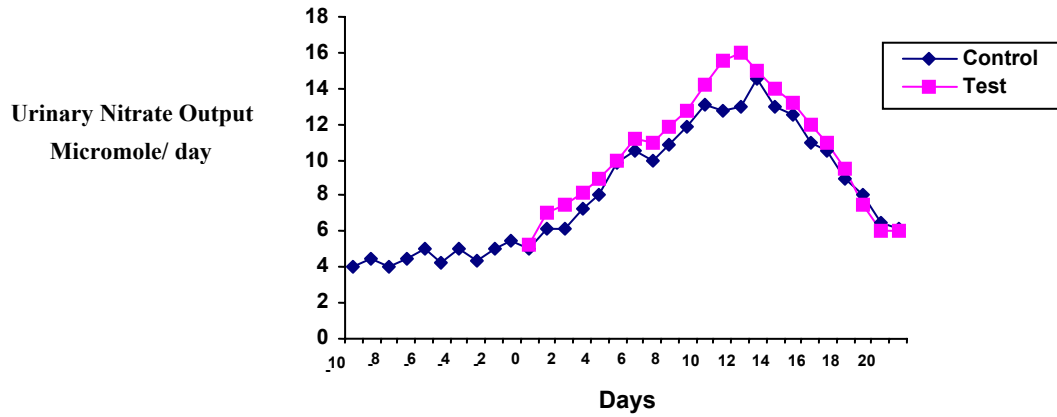


Fig. 2: Wound closure profiles for diabetic treated and control rats



Fig. 3: Photographs showing control wounds on days 0, 12 and 21 respectively

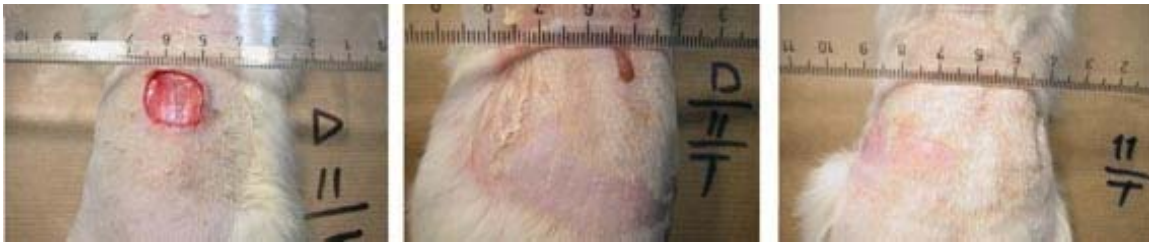


Fig. 4: Photographs showing treated wounds on days 0, 12 and 21 respectively

## Discussion

Diabetes is characterized by a nitric oxide-deficient state accompanied by decreased wound breaking strength, collagen deposition and a severely impaired inflammatory response. Diabetes mellitus is one of the most common metabolic disorders that well known to impair wound healing (14, 15, 16), and this represents a significant clinical problem. Based on previous findings diabetes is characterized by reduced Nitric Oxide levels in the wound environment (14, 17). In diabetic induced hyperglycemia there is an increased metabolism of glucose to

sorbitol via the polyol pathway. The increased activity of the enzyme aldose reductase requires and may deplete cellular NADPH, which is also a required cofactor for Nitric Oxide synthase (NOS) (16, 18, 24). It is possible that both of these mechanisms occur during diabetic wound healing and results in drastically reduced NO production. It is conceivable that diabetic wounds are more susceptible to nitric oxide donor treatment since the wound is deficient in nitric oxide. The Nitric Oxide donor DETA NONOate represents a potential treatment for acutely impaired healing in diabetes. NO donors

can partially reverse the impaired healing in diabetes and in parallel restore wound NO Levels towards more normal values (19, 20, 21). Urinary nitrate ( $\text{NO}_3^-$ ) output reflect the extent of wound repair, and is important throughout the wound healing (21, 22, 23). The results obtained indicate an increase in urinary  $\text{NO}_3^-$  output between days 12-13 though the external wound closure is almost complete. The results of this study shows that DETA NONOate applications could rise the urinary  $\text{NO}_3^-$  levels and the subsequent elevated levels compared to control rats indicates that significant amounts of NO were delivered to the wound site. This can be related to an increased biochemical activity and revascularization at the wound site and it is possible that urinary  $\text{NO}_3^-$  output had dropped to essentially pre-wounding levels. In summary, wound healing is a complex biological process and site specific delivery of nitric oxide via NO-donor DETA NONOate could be an effective therapeutic strategy to impaired wounds. Hence Nitric Oxide is vital to healing process. Novel therapies in wound care management rely heavily on our current knowledge of wound healing process. Recently investigators have implicated Nitric Oxide (NO) in the exertion of regulatory forces on various cellular activities of inflammatory and proliferative phases of wound healing. However a better understanding of the regulation and functions of Nitric Oxide supplementation as means of elevating Nitric Oxide at wound site is clearly required.

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