

Full Length Research Paper

Study of the effect of PAPA NONOate on the rate of diabetic wound healing

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To investigate the effect of exogenous nitric oxide donor (PAPA NONOate) a drug which spontaneously release nitric oxide on the rate of wound healing and collagen synthesis on impaired wound healing in experimental diabetes. 12 male Sprague – Dawley rats were transferred to separate metabolic cages. Nine days before wounding, the rats were injected intraperitoneally (IP) 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 given full thickness dermal wound. The test group (n = 6) was treated with 100 µmole PAPA NONOate in phosphate buffer while control wounds (n = 6) received sterile phosphate buffer on the same day and every three days. Daily urine samples were collected at every 24 h intervals. To inhibit bacterial growth, 5 ml of 3 M HCl was added to each urine collection (pH = 1) and urine samples were kept frozen until analyzed (-70 °C). Urinary nitrate (NO⁻³) 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. PAPA NONOate treatment increased the rate wound healing in test group as compared to the control group. The nitric oxide donor PAPA NONOate may represent a potential treatment for impaired wound healing in diabetes by increasing the rate of collagen synthesis at the wound site.

Key words: Wound healing, PAPA NONOate, diabetic wound.

INTRODUCTION

Wound healing is an orchestrated sequence of biochemical changes which results in tissue repair Timothy and Margret, (2008). In diabetes all the stages of the complex wound healing process including inflammation, proliferation, angiogenesis and matrix formation, are impaired (Dashti et al., 2004). During the early phases of wound repair, chemotaxis, phagocytosis, bacterial killing and antioxidant levels are decreased in diabetes (Witte et al., 2002; Bitar et al., 1996; Beer et al., 1997). Also, growth factor depletion, increased glucocorticoids levels (Bitar et al., 1997), decreased cell proliferation (Hehenberger et al., 1998; Goldstein et al., 1979) and up regulation of apoptosis (Darby et al., 2000) characterize the later phases of healing in diabetics, resulting in poorer granulation tissue formation. The rate of collagen

synthesis is decreased in diabetic wound healing (Witte et al., 2002). Based on previous findings, the role of nitric oxide as a mediator of wound healing has been determined (Witte et al., 2002, 2000).

In diabetes, the level of nitric oxide is decreased in the wound environment. In diabetes, an endogenous deficiency in nitric oxide synthase (NOS) enzyme leads to decreased wound nitric oxide (NO) production and a wide range of related pathologies, such as impaired cutaneous vasodilation, decreased neurogenic vascular response, diabetic neuropathy, endothelial cell function that inhibit the processes necessary for granulation tissue formation. Accumulation of reparative collagen is also reduced. As a mediator of tissue repair, NO plays an important role in promotion of angiogenesis and cellular migration, increase wound collagen deposition and collagen cross linking, regulate vasodilation, inhibit platelet aggregation, inhibit endothelial - leukocyte cell adhesions, modulate endothelial proliferation and

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Figure 1. Metabolic cages.

apoptosis, increase the viability of random cutaneous flaps, and enhance cellular immunomodulation and bacterial cytotoxicity (Dashti et al., 2004). NO is critically involved in the entire continuum of events associated with collagen synthesis in fibroblasts by an unknown mechanism and accelerates wound closure when applied topically at the wound site (Witte and Thornton, 2000). Reduced nitric oxide production in diabetic wounds has been shown to be associated with impaired healing and reduced collagen deposition (Thimothy et al., 2008).

Failure of wound healing is a major cause of morbidity and mortality in diabetes (Terezelain and Hanson., 2009). Collagen molecule is one of the most fundamental constituents of connective tissue with a triple helical structure (Bitar et al., 1997; Hehenberger et al., 1998; Goldstein et al., 1979; Darby et al., 2000; Burgeson and Marcel, 1992). Based on previous findings that diabetes is characterized by reduced nitric oxide levels in the wound environment, this study investigated whether exogenous nitric oxide supplementation with nitric oxide donor PAPA NONOate could reverse impaired healing in diabetes. The 24 h urine samples were collected throughout the healing period (21 days). Wound closure profiles were examined by video image every three days and urinary nitrate (NO_3^-) output was measured by Griess reagent.

MATERIALS AND METHODS

PAPA NONOate [1- propamine, 3- (2-hydroxy-2-nitroso-1-propyl hydrazine)] was purchased from Alexis Co. (Switzerland). Low nitrate diet (2% L-arginine) was obtained from Pasture Institute, Tehran, Iran. Blood glucose levels were measured with glucose oxidize kit (Zist Chimy Chemical Co. Tehran, Iran). Male Sprague – Dawley rats (Tehran University of Medical Sciences animal house, Tehran, Iran) were acclimatized for one week, they were given water *ad libitum*, and fed a low nitrate containing diet (2% L-arginine). Animals were transferred to separate metabolic cages (Figure 1). Nine days before wounding, the test groups were injected intra peritoneally (IP) 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 blood glucose levels 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 properly shaved and given full thickness dermal wound approximately 1×1 cm. The test group ($n = 6$) was treated with 100 μmole PAPA NONOate in phosphate buffer while control wounds ($n = 6$) received sterile phosphate buffer on the same day and every three days. Daily urine samples were collected at every 24 h intervals. To inhibit bacterial growth, 5 ml of 3 M HCl was added to each urine collection (pH = 1) and urine samples were kept frozen until analyzed (-70°C). Urinary NO_3^- 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. 48 h following wounding and every three days,

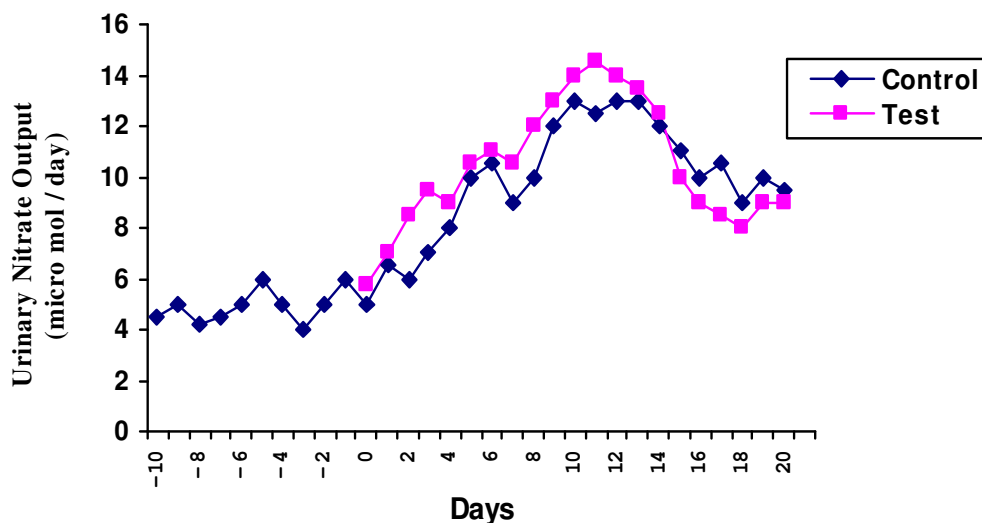


Figure 2. Urinary nitrate output profiles ($\mu\text{mol}/\text{day}$) for diabetic wounds treated with PAPA NONOate.

Table 1. Urinary nitrate output (mmol/day) for diabetic normal wounds treated with PAPA NONOate

Urinary nitrate output	Control (mmol/day)	Test (mmol/day)	p-Value	Significance
Pre-wound	4.9 ± 0.21	4.9 ± 0.21	-	-
Early-wound	8.80 ± 0.70	9.59 ± 0.67	0.001	S
Post-wound	8.80 ± 0.90	9.60 ± 0.93	0.001	S

wounds were videotaped using Nikon Colpix 5000. The urinary nitrate levels were 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

Interesting and promising results have been obtained from studies using non-soluble, polymeric PAPA NONOate as NO donating agent during cutaneous healing in rats. Topical application of NONOate was made on days 0, three, six, nine, 12, 15, 18 and 21. The mean pre wound urinary nitrate output for NONOate and control group was $4.9 \pm 0.21 \mu\text{mol}/\text{day}$. The mean early wound urinary nitrate output for control and test group was 8.80 ± 0.70 and $9.59 \pm 0.67 \mu\text{mol}/\text{day}$, respectively. The mean post wound urinary nitrate output for control and test group was 8.80 ± 0.90 and $9.60 \pm 0.93 \mu\text{mol}/\text{day}$, respectively. The urinary NO^3 profiles for diabetic rats with PAPA NONOate and controls is shown in Figure 2 and Table 1. Control diabetic rats had significantly less

urinary NO^3 output than the test group ($P < 0.001$). A significant peak in NO^3 output occurred between days 12 to 13 when the external wound was approximately 65% closed. Diabetic rats whether treated with PAPA NONOate or not, exhibited a significant increase in urinary NO^3 output within 24 to 48 h post wounding period. During a three 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 3 and Table 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 and 21 are shown in Figures 4 and 5, respectively.

Therefore, PAPA NONOate treatment can increase the rate of wound healing and collagen synthesis in diabetic test group as compared to the control group.

DISCUSSION

NO is important in the process wound healing and tissue repair. NO potentiates antiseptic effects, minimizing intraoperative wound contamination. It, besides, stimulates endothelial and basal cells of epidermis

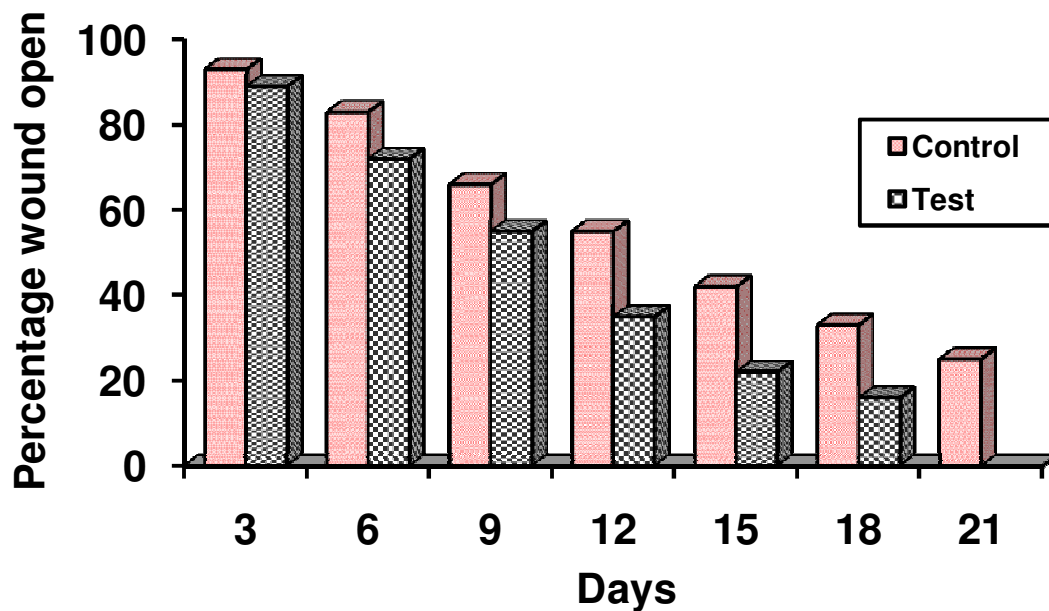


Figure 3. Wound closure profiles for diabetic wounds treated with PAPA NONOate and control rats.

Table 2. Percent wound open for diabetic normal wounds treated with PAPA NONOate.

Percent wound open (days)	Control (%)	Test (%)	p-Value	Significance
3	93 ± 0.33	89 ± 0.15	0.001	S
12	55 ± 0.11	35 ± 0.43	0.001	S
21	25 ± 0.27	0	0.001	S

proliferation (Larichev et al., 2011).

Refractory wound is a severe complication that leads to limb amputation in diabetes, and is also a leading cause of hospitalizations in diabetic patients with limited treatment regimens. However, the underlying mechanisms remain to be fully elucidated. Various factors contribute to delayed diabetic wound healing, such as growth factor, nitric oxide (NO), reactive oxygen species (ROS), matrix metalloproteinases (MMPs), microRNA, endothelial progenitor cells (EPCs), etc (Sheng Li et al., 2010).

NO and its interrelationship with essential growth factors is involved in the entire events associated with wound repair. NO is often impaired in rats with diabetes. Diabetic rats have a reduced ability to generate NO from L-arginine which is reflected by direct measurements of plasma nitrate and nitrite levels. NOS from which NO is derived is a pH dependent enzyme which is active at slightly alkaline (basic) conditions but is suppressed by acidic conditions. In diabetes, glycolysis and ketoacidosis force pH towards acidic conditions and this may account, in part, for the reduced production of NO (Jian-dong et al., 2005).

Adequate oxygen is necessary for the activity of NOS and therefore NO. Circulation is notoriously impaired in diabetic patients, which limits available NOS and NO. Lastly, people with diabetes often experience elevated glucose levels. Some of this glucose becomes incorporated into hemoglobin and is measured as glycosylated hemoglobin (Hob) or HgbA1C. Glycosylated hemoglobin binds to NO as nitrosothiols very tightly so that any NO that is formed cannot be easily released from red blood cell (RBC) to help maintain blood flow through smooth muscle cell relaxation. When available NO is tightly bound to glycosylated hemoglobin its release is limited in smooth muscle cells where NO is required for essential cellular functions. Similarly, the NO donor molsidomine (N-ethoxycarbonyl-3-morpholinylsidnonimine) or NO releasing poly (vinyl alcohol) hydrogel dressings are also shown to partially restore such healing impairment in STZ-induced diabetic rats. Collectively, impairment of skin NO function represents an important factor for delayed wound healing in diabetes and strategies aimed at restoring cutaneous NO bioavailability with NO donors or NOS gene therapy may serve as effective means for diabetic wound healing



Figure 4. Photographs showing normal diabetic control wounds on day 0, 12 and 21 respectively.



Figure 5. Photographs showing normal diabetic wounds treated with PAP NONOate on day 0, 12 and 21, respectively.

(Jian-dong et al., 2005).

The capacity of a nitric oxide-releasing nanoparticle (NO-np) to treat wounds infected with *Acinetobacter baumannii* (Ab) was examined. It was found that the NO-nps were therapeutic in an experimental Ab murine wound model. Treatment with NO-nps significantly accelerated healing of infected wounds (Mihu et al., 2010).

Collagen is one of the principle structural proteins which play an important role in wound healing (Bildien and Oktay, 1999). Collagens are a large family of structural proteins in the extra cellular matrix of eukaryotes (Bulfield., 1990). During wound healing, the collagen molecules, after being secreted by the cells, assemble to form fibers for the functional integrity of tissues (Freeman et al., 1988). Diabetes is characterized by a nitric oxide – deficient state accompanied by decreased collagen deposition at the wound site.

The nitric oxide donor PAPA NONOate may represent a potential treatment for impaired wound healing in diabetes by increasing the rate of collagen synthesis at the wound site. It has been shown that diabetic wounds are more susceptible to nitric oxide donor treatment since the wound is deficient in nitric oxide (Witte et al., 2002).

Furthermore, the previous results show that topical application of S-nitrosoglutathione (GSNO) is effective in the treatment of ischemic wounds, leading to a significant improvement in the wound healing (Georgii et al., 2010).

Also, our previous study on DETA NONOate showed, this compound can partially improve impaired healing in diabetes by increasing the rate of collagen synthesis (Dashti et al., 2010).

In the previous studies, it was shown that the effect of nitric oxide donor administration may be dependent on a threshold rather than the dose. So therefore, further studies should be performed to demonstrate a correlation between levels of nitric oxide donors at the wound site and its outcome. In summary, administration of PAPA NONOate can partly restore the impaired healing in diabetes by increasing the rate of collagen synthesis. This may have therapeutic potential and needs further evaluation.

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