# L-Arginine Supplementation Enhances Diabetic Wound Healing: Involvement of the Nitric Oxide Synthase and Arginase Pathways

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**Diabetes impairs wound healing and there are few therapeutic options to reverse it. Previous work has demonstrated the importance of nitric oxide for successful wound healing. In diabetes, NO synthesis is reduced in the wound milieu. The amino acid L-arginine is the only substrate for NO synthesis. We hypothesized that L-arginine supplementation would enhance wound healing by restoring NO synthesis. Thirty-six male Sprague-Dawley rats (body weight, 225 to 250 g) were separated in 4 groups: 20 rats were rendered diabetic 7 days prior to wounding by intraperitoneal streptozotocin (STZ) injection (70 mg/kg). Sixteen rats served as controls. Half of the animals of each group received 1 g/kg supplemental L-arginine administered by gavage twice daily. Control rats were gavaged with water. Treatment was started 3 days before wounding. All rats underwent a dorsal skin incision and subcutaneous implantation of polyvinyl alcohol (PVA) sponges. The rats were killed 10 days post wounding and wound breaking strength, hydroxyproline content of the sponges, nitrite/nitrate ($NO_x$) concentration, arginase activity, and amino acid composition of the wound fluid and plasma were analyzed. Wound fluid $NO_x$ concentrations and wound breaking strength were significantly reduced in the diabetic group compared to the controls. L-Arginine treatment restored diabetic $NO_x$ levels toward normal values and significantly enhanced wound breaking strength. Wound fluid arginase activity and ornithine concentrations were significantly lower in the diabetic animals but unaffected by treatment. The data demonstrate that the impaired NO synthesis in the diabetic wound milieu can at least partially be reversed by arginine supplementation. In view of previous results on the importance of NO for wound healing, the data suggest that arginine supplementation restores impaired healing in this acute wound model by normalizing the NO pathway but without affecting arginase activity.**



THE AMINO ACID L-arginine is known to have several modulatory functions on the endocrine and immune system. Arginine stimulates T-cell proliferation and insulin release and improves nitrogen balance after trauma.[1] Supplemental arginine improves wound healing by a yet unknown mechanism in healthy and elderly humans,[2] as well as in rodents.[3] Arginine becomes a semi-essential amino acid after trauma and wounding, with often undetectably low concentrations in the wound fluid.[4-7] Serum arginine levels decrease with age when diabetes is predominant.[8] However, this is not due to decreased arginine synthesis in kidney with age, since renal arginine production is maintained even in end-stage renal disease.[9]

Arginine is the only and unique substrate for nitric oxide synthesis, which is involved in many regulatory mechanisms relevant to wound healing such as angiogenesis, cell proliferation, collagen synthesis, epithelialization, etc by its inducible isoform (iNOS).[10] iNOS knock-out mice supplemented with L-arginine do not demonstrate improved incisional healing, suggesting that the effect of arginine involves, at least partially, the arginine/iNOS pathway.[11] In wounds, arginine can also be metabolized to urea and ornithine by arginase. This pathway has been postulated to be important for wound healing by providing proline as a substrate for collagen synthesis[12] and polyamines as stimulators of cell proliferation.[13] Polyamine concentrations are high in wound fluid (T.K. Hunt, personal communication).

Diabetic healing is impaired, but the mechanisms are not well understood. High blood glucose lowers cell proliferation[14] and affects collagen synthesis. Cross-linking of collagen fibers can be reduced, leading to increased susceptibility for collagenase digestion.[15] Growth factors, mediators of collagen synthesis, are reduced in the wound milieu[16,17] probably secondary to the diminished and delayed inflammatory reaction. Phagocytosis is reduced leading to increased rates of infectious complications[18,19] and apoptosis is increased,[20] potentially reducing granulation tissue formation.

In the present study, we investigated whether supplemental arginine can restore diabetes-impaired healing in an acute incisional wound model and whether it affects the NO synthase/arginase pathways.

## MATERIALS AND METHODS

Male Sprague-Dawley rats weighing between 225 and 250 g were used. Animals were allowed to acclimatize for 1 week and were kept at constant temperature (25°C) and humidity on a 12-hour light-dark cycle. Animals were separated into 4 groups: 20 rats were rendered diabetic by intraperitoneal streptozotozin (STZ) injection (70 mg/kg body weight in 0.1 mol/L citrate buffer, pH 4.0); 16 rats served as controls and received citrate buffer injection only. Blood glucose measurements were performed from tail vein blood using glucose oxidase strips (Chemistrip, Boehringer Mannheim, Mannheim, Germany) the first and second day after STZ injection and on the day of harvest. Rats with blood glucose levels less than 250 mg/dL were excluded from the study.

Rats were fed a complete pelleted laboratory diet containing 1.3% arginine (Teklad LM-485 Diet, Harlan Teklad, Madison, WI) and had free access to water. Half the animals of each group received supplemental L-arginine (1 g/kg) by gavage twice daily (given as L-arginine

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*Submitted October 6, 2001; accepted April 5, 2002.*

*Supported by Grants No. GM54566 from the National Institutes of Health (A.B.) and the Deutsche Forschungsgemeinschaft (Wi 1499/1-1) (M.W.B.).*

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*0026-0495/02/5110-0008$35.00/0*

*doi:10.1053/meta.2002.35185*

hydrochloride in water). Control animals were gavaged with water alone.

Arginine supplementation was started 4 days after STZ injection and 3 days prior to wounding. Wounding consisted of a 7-cm dorsal skin incision cut down to the paniculus carnosus under pentobarbital anesthesia (45 mg/kg. Ten preweighed, saline-moistened, polyvinyl alcohol (PVA) sponges (M-Pact, Eudora, KS) were inserted into subcutaneous pockets on each side. The skin was closed with staples. The animals were killed 10 days later by pentobarbital overdose.

The dorsal pelt containing the healing scar was excised and cut into equal strips using a multibladed guillotine. Four strips from each animal were assessed immediately for fresh wound breaking strength using a tensiometer (WC Dillon, Inc, Van Nuys, CA). The 2 most cephaled sponges from each animal were used for hydroxyproline analysis, an index of collagen synthesis, as described previously.[21]

The remaining sponges from each animal were pooled and squeezed to obtain wound fluid. This was rendered acellular by centrifugation at 400 × *g* for 10 minutes and subsequently at 1,600 × *g* for 20 minutes at 4°C. Wound fluid was stored at -20°C until analysis.

Wound-derived cells were harvested from the subcutaneously implanted sponges as described.[22] Briefly, sponges were minced with scissors, squeezed, and passed through a stainless steel mesh. The red cells were lysed in hypotonic buffer, the pellet was washed and the cells were resuspended in Dulbecco's modified Eagles medium (DMEM) containing 1% albumin, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, and 1 μCi [2,3 $^{3}$H]-L-arginine per milliliter (NEN Dupont, Boston, MA) and cultured for 24 hours. The capacity of wound cells to synthesize NO was assessed by measuring the conversion of radiolabeled arginine to citrulline. Newly formed radiolabeled L-citrulline was separated from $^{3}$H-arginine using cationic exchange chromatography (Dowex AG-50, Biorad Laboratories, Richmond, CA). Quantification was performed using a beta-counter (liquid scintillation Analyzer 1600-TR, Packard, Downers Grove, IL).

The cells were lysed in sodium chloride-sodium citrate buffer containing 0.02% sodium dodecyl sulfate, and DNA concentrations were measured using the Hoechst 33258 dye technique on a TKO 100 minifluorometer (Hoefer, San Francisco, CA). Newly formed citrulline was expressed as nanomoles of cold citrulline formed per microgram of over 24 hours.

Arginase activity in wound fluid was measured using a previously described method.[23] Briefly, 25 μL wound fluid was mixed with equal volumes activation buffer (10 mmol/L MnCl in 50 mmol/L Tris, pH 7.5) and incubated for 10 minutes at 55°C. Equal volumes of 0.5 mol/L L-arginine, pH 9.7, were added and the mixture incubated at 37°C for 1 hour. The reaction was stopped using a phosphoric/sulfuric acid mixture. Urea formation was detected by colorimetric reaction after isonitrosopropiophenone (IPSF) addition and boiling for 45 minutes. Urea alone was run as standard. Arginase activity was expressed as nanomoles of urea formed per minute per milligram of protein in wound fluid. Wound fluid protein concentrations were measured by colorimetric reaction using Coomassie blue technique (Biorad, Hercules, CA).

**Table 1. Body Weight and Blood Glucose Levels on the Day of Sacrifice (mean ± SEM)**

| | Body Weight (g) | Blood Glucose (mg/dL) |
|---|---|---|
| Control | 333.4 ± 4.4 | 69.3 ± 14 |
| Control + Arg | 332.5 ± 4.1 | 71.5 ± 17 |
| DM | 265.8 ± 7.6* | 568.0 ± 15.7* |
| DM + Arg | 276.2 ± 11.0* | 595.1 ± 18.2* |

Abbreviation: DM, diabetes mellitus.
*$P < .05$ *v* control.

**Table 2. Wound Breaking Strength and Hydroxyproline Content of Subcutaneously Implanted PVA Sponges in the Experimental Groups (mean ± SEM)**

| | Wound Breaking Strength (g) | OHP (μg/100 mg sponge) |
|---|---|---|
| Control | 320 ± 20 | 1,701.1 ± 245 |
| Control + Arg | 383 ± 35 | 1822.2 ± 137 |
| DM | 109 ± 12* | 860.7 ± 124* |
| DM + Arg | 187 ± 21*† | 1,025.3 ± 62* |

Abbreviation: OHP, hydroxypoline.
*$P < .05$ *v* control.
†$P < .05$ *v* DM.

The stable end products nitrite and nitrate were measured in wound fluid as described previously.[24] Plasma protein concentrations were measured by standard technique in the clinical laboratory of Sinai Hospital of Baltimore.

Amino acid analysis in wound fluid and plasma was performed by chromatography. Wound fluid samples were passed through polysulfone filters (Ultrafree, 10 kd; Millipore Corp, Marlborough, MA), mixed with internal standards and derivatized with polyisothiocyanate (Waters Pico Tag Vacuum Station, Millipore). Derivatized amino acids were separated by reverse-phase chromatography (high-performance liquid chromatography [HPLC] System; Waters Chromatography Division, Millipore) and quantified by computerized analysis.

Data are reported by means ± SEM. Significance was analyzed by 1-way analysis of variance (ANOVA) performing Fisher's post-hoc test using the Statview v4 computer program (Abacus Concepts, Berkeley, CA). Significance was achieved at $P < .05$.

## RESULTS

Treatment was well tolerated as demonstrated by equal weight in the arginine-fed rats compared to controls. Diabetic animals lost weight over the experimental period (Table 1). Blood glucose levels on the day of sacrifice were significantly elevated in the diabetic groups (Table 1). Arginine supplementation did not influence body weight change or blood glucose levels.

Although treatment with arginine increased wound breaking strength, this reached statistical significance only in the diabetic group (Table 2). Hydroxyproline content of PVA sponges, an index of new collagen deposition in the wound, was higher in the arginine-supplemented groups but these differences did not reach statistical significance.

The amino acid and $NO_x$ concentrations in wound fluid and plasma are shown in Fig 1. Arginine levels in wound fluid were low and unaffected by treatment. However, plasma arginine levels were significantly increased by arginine feeding in both groups. $NO_x$ wound fluid levels were significantly lower in diabetic rats compared to controls and reversed by treatment. Ornithine, the product of arginase activity, was significantly lower in wound fluid from diabetic animals compared to controls. Arginine treatment did not affect ornithine wound fluid levels, suggesting that arginine is mainly metabolized via the NO pathway. Plasma levels for $NO_x$ were not different between groups, showing the specificity of the effect at the wound site.

Ex vivo citrulline synthesis by wound-derived cells, an index of NO synthase activity, was decreased in diabetic wound cells compared to controls. The specific activity was significantly lowered by arginine treatment in both groups (Fig 2A). Wound

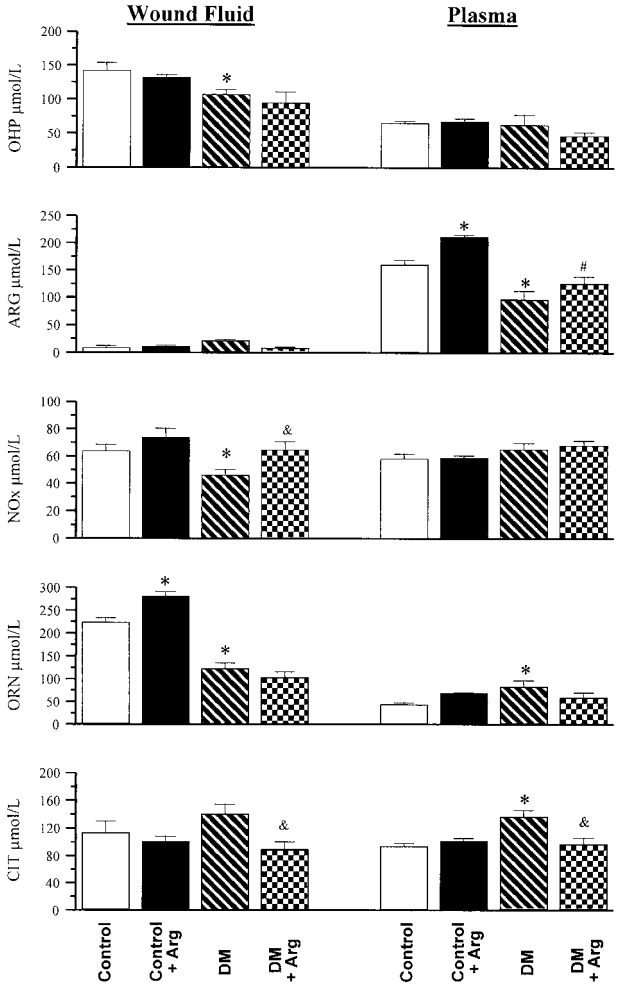


**Fig 1. Wound fluid (left) and plasma (right) amino acids (citrulline, ornithine, and hydroxyproline) and $NO_x$ concentrations assessed by HPLC (mean ± SEM). *$P < .05$ *v* control, #$P < .05$ *v* control + Arg, &$P < .05$ *v* diabetes mellitus (DM).**

fluid arginase activity was also significantly lower in diabetic rats, but unaffected by arginine treatment (Fig 2B).

Wound fluid and plasma protein concentrations were significantly lower in diabetic rats, probably reflective of the decreased inflammatory reaction at the wound site (Table 3).

## DISCUSSION

The results of these experiments demonstrate that arginine supplementation to acutely diabetic rats improves incisional healing by increasing breaking strength. Although hydroxyproline levels in subcutaneously implanted sponges were increased by arginine supplementation, this did not reach statistical significance. In accordance with previous results,[25] the levels of nitrite and nitrate ($NO_x$) were lower in diabetic animals compared to controls. This suggests that a NO-deficient state exists in diabetes at the wound site. This deficiency could occur because of a decreased inflammatory reaction with lower cellular infiltrates with lesser iNOS activity in the diabetic wounds.[26] The finding of lower wound fluid protein concentrations further underscores this reduced inflammatory reaction.

Little is known about the composition of amino acids in wound fluid of diabetic rats. The results of our control groups correspond well with previous results.[4] Interestingly, $NO_x$ and ornithine levels were significantly lower in diabetes, suggesting a deficiency in both the iNOS and arginase pathways. This is underlined by the significantly lower arginase activity in wound fluid (Fig 2B). Since the arginase activity was normalized for wound fluid protein levels, we accounted for the lower inflammatory reaction. Arginine supplementation restored only the $NO_x$ levels in wound fluid toward normal, suggesting that the exogenously provided arginine is preferentially metabolized via iNOS in the wound. This is accordance with previous findings where arginine treatment in iNOS knockout mice failed to improve wound healing.[11] $NO_x$ levels are rather a reflection of the accumulation of $NO_x$ in the wound, whereas new citrulline formation in wound cells (Fig 2B) is a reflection of the actual NO formation.[27] These data together suggest that decreased NO levels in the wound milieu are due to both a lesser inflammatory reaction and lower iNOS activity per cell. Little is known about the overall metabolism of arginine in wounds. In healthy humans, plasma arginine turnover to urea was 10 times higher than plasma arginine turnover to NO formation.[28] The fact that plasma arginine increases significantly after arginine treatment but does not increased in the wound fluid could indicate a rapid turnover of administered arginine in the wound milieu. However, we cannot rule out that arginine treatment affects the composition of the cellular infiltrate[29] or the arginase pathway and the amino acid composition of wound fluid at earlier time points during healing since there was a single sample analysis at day 10 post wounding. Assuming that arginase is specifically impaired in diabetes, restoration of wound fluid ornithine by arginine feeding would not be expected and did not occur in our experiments. We did not account for intracellular arginase activity since arginase is known to be released in the extracellular wound environment by wound macrophages.[12,30] Arginase is relatively high in mucosa of the gut.[31] Hypothesizing that the arginase activity is in general deficient in diabetes, enteral administration of arginine in diabetes could account partially for the observed metabolic fate of arginine. This is underlined by the decreased proline levels in wound fluid and plasma in arginine-fed diabetic rats (data not shown). Proline is formed in tissue and the gut by arginase catabolism. Impaired arginase therefore leads to lower proline formation.

The results of this study demonstrate for the first time a simultaneous decrease at the wound site of 2 key enzymes of the arginine metabolism during diabetes. These results contrast with a previous report where iNOS and arginase were reduced in normal diabetic skin but increased in diabetic ulcers, possibly secondary to a decrease in transforming growth factor-beta (TGF-$\beta$) concentration at the wound site.[32] The different results may be related to the acute and chronic models studied, as well as to species differences.

Interestingly, NO activity in the wound cell population was not only lowered in the diabetes group but was also further decreased by arginine supplementation. Since these cells were

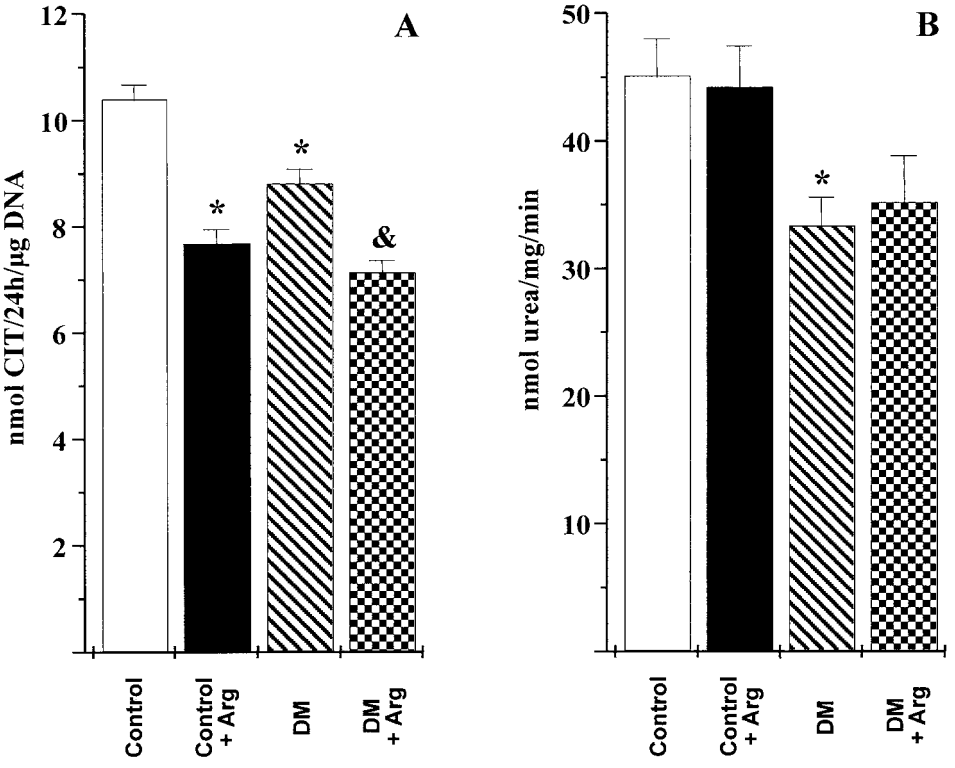


**Fig 2. (A) NO synthase activity in wound derived cells under ex vivo conditions measured as new radiolabeled citrulline formation (nmol CIT/24 h/μg DNA), mean ± SEM. (B) Arginase activity in wound fluid measured spectrophotometrically as new urea formation (nmol urea/min/mg protein in wound fluid), mean ± SEM. *$P < .05$ *v* control, &$P < .05$ *v* DM.**

isolated from the wound milieu and cultured under similar conditions (equal concentrations of arginine), this is somewhat troublesome. Many of the byproducts and endproducts of the 2 pathways are known to inhibit the counterparts.[33] Even though the cells were cultured ex vivo, they may still be under inhibitory factors from the wound milieu.[34] We did not analyze intracellular amino acid concentrations.

In accordance with previous results, arginine feeding significantly improved incisional healing in normal rats as measured by wound breaking strength (Table 1). However, we were not able to detect improved hydroxyproline content in PVA sponges. This could be accounted for by a $\beta$-error, because there were only 8 animals per control group. The administration of arginine via gavage ruled out intake differences between animals.

The paradox of NO in diabetes lies in the fact that NO is the mediator of autoimmune disease in diabetic onset and development. Increased NO excretion in diabetes-prone rats,[35] delayed onset of diabetes under administration of NO inhibitors,[36] or delayed onset of diabetes in iNOS knockout mice[37] on one hand and the fact that administration of arginine attenuates diabetes[38] as well as diabetic complications on the other hand underlies this paradox. It was recently suggested that elevated systemic NO in diabetes is not due systemic activation by the autoimmune disease but to decreased insulin levels leading to a downregulation of TGF-$\beta$ known to inhibit NO synthase.[39] If indeed there is an inverse relation between insulin and NO, then the previously demonstrated observations of insulin release under arginine stimulation[40] should lead to decreased NO levels. In our study as well as in a previous study from this laboratory, we found decreased $NO_x$ levels in the wound milieu which were partially restored by insulin administration or arginine supplementation.[25,41] However, the wound may represent a separate compartment that may not fully reflect the systemic disease of diabetes. The increased plasma citrulline levels in diabetic rats accompanied by decreased plasma arginine levels suggest systemic activation of the NO pathway with substrate depletion. This would support the concept that the wound site and the plasma reflect two distinct compartments of arginine metabolism and that studies of amino acid supplementation in wound healing need to account for this compartmentalization.

**Table 3. Protein Concentrations in Plasma and Wound Fluid (mean ± SEM)**

| | Plasma Protein (g/dL) | Wound Fluid Protein (g/dL) |
|---|---|---|
| Control | 5.10 ± 0.07 | 10.7 ± 0.3 |
| Control + Arg | 5.15 ± 0.14 | 13.7 ± 0.7* |
| DM | 4.23 ± 0.08* | 7.6 ± 0.5* |
| DM + Arg | 4.52 ± 0.13* | 8.1 ± 0.3* |

*$P < .05$ *v* control.

In summary, our data indicate that arginine supplementation improves wound healing assessed as breaking strength of incisional healing and collagen content of subcutaneously implanted sponges in acutely impaired diabetic healing. Restoring the NO-deficient state in diabetic animals in the wound milieu may be one factor. However this work presents for the first time that arginase activity is also deficient in diabetic healing, a factor that needs to be investigated further.

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