Arginine plus proline supplementation elicits metabolic adaptation that favors wound healing in diabetic rats

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Raynaud-Simon A, Belabed L, Le Naour G, Marc J, Capron F, Cynober L, Darquy S. Arginine plus proline supplementation elicits metabolic adaptation that favors wound healing in diabetic rats. *Am J Physiol Regul Integr Comp Physiol* 303: R1053–R1061, 2012. doi:10.1152/ajpregu.00003.2012.—Diabetic patients with wounds are at risk of protein malnutrition, have low arginine plasma levels, and suffer from delayed wound healing. We sought to determine the efficacy of arginine plus proline supplementation on protein and amino acid metabolism and on wound repair in a model of diabetic rats. Eighteen 11-wk-old Zucker diabetic fatty *fa/fa* male rats underwent a 7-cm abdominal skin incision with implantation of sponges and daily excision of full-thickness round sections of dorsal skin for 5 days. They were randomized to be fed with either a standard formula (S group, Clinutren Iso), a high-protein and arginine (ARG) plus proline (PRO)-enriched formula (ARG+PRO group, Clinutren Repair), or an isonitrogenous isoenergetic control formula (IC group). Nitrogen balance was calculated daily. The rats were euthanized on *day 5*, and plasma glucose, insulin, amino acids, skin epithelialization, and angiogenesis were measured. In macrophages, we assessed inducible nitric oxide synthase (iNOS) and arginase expression, production of nitric oxide (NO) and amino acid metabolism. Both the ARG+PRO and IC groups showed improved nitrogen balance. ARG plus PRO supplementation increased proline and branched-chain amino acid plasma concentrations and improved angiogenesis. Arginase and iNOS expressions in macrophages were reduced, together with NO and citrulline production. In diabetic rats, ARG plus PRO supplementation improves wound angiogenesis and favors whole body protein metabolism. Low macrophage iNOS expression at *day 5* may reflect a low inflammatory state in the wounds, favoring wound closure.

amino acids; epithelialization; angiogenesis; macrophage

DIABETIC FOOT ULCERS ARE ESTIMATED to occur in 15% of all patients with diabetes (37) and precede 84% of all diabetes-related lower-leg amputations (36). In individuals with diabetes, many physiological factors contribute to wound healing failure. These factors include decreased or impaired growth factor production, angiogenic response, macrophage function, collagen accumulation, and reduced quantity of granulation tissue and keratinocyte and fibroblast migration and proliferation (5). Reduced arginine availability may also play an important role, given that in diabetic rats, in which arginine plasma levels are reduced (3, 41), arginine supplementation improves wound healing (38, 42). In diabetic rats with dorsal skin incision and implantation of polyvinyl alcohol sponges, supplementation with arginine has been shown to en- hance wound breaking strength, hydroxyproline deposition, and procollagen I and III mRNA content in the sponges (38, 42). In diabetic animals with burn wounds, arginine supplementation has led to an increase in the healing rate and in angiogenesis (16).

Arginine may improve wound repair through various mechanisms. It plays a key role as a substrate for protein synthesis, in collagen deposition, cell proliferation, and T-lymphocyte function, and it promotes positive nitrogen balance (39, 43). Arginine-supplemented patients compared with nonsupplemented controls demonstrated significantly greater hydroxyproline content—an indicator of collagen deposition—and protein accumulation at the wound site (21). Arginine is also the only single direct endogenous substrate for the synthesis of nitric oxide (NO), which is involved in many regulatory mechanisms relevant to wound healing, such as angiogenesis, cell proliferation, collagen synthesis, and epithelialization (12). Diabetic rats have been found to have less NO in wound fluid than controls. Molsidomine, a drug that spontaneously releases NO, has been shown to increase NO urinary excretion, wound-breaking strength, and hydroxyproline deposition in wounds of diabetics, although plasma and wound NO fluid concentrations were not modified (42).

Macrophages play a critical role in wound healing (10, 25, 31). In diabetic mice, macrophages appear dysfunctional, displaying altered morphological appearance, sparsity, blunted phagocytal activity, and reduced cytokine production (26, 27, 47). Metabolism of arginine through NO synthase (NOS) produces citrulline together with NO. Citrulline is reused for arginine synthesis (6, 30). This arginine-citrulline cycle is activated in peritoneal macrophages from diabetic rats, favoring arginine recycling and, therefore, NO production (44); NO production by macrophages is increased in both diabetic mice and rats (47). The production of NO by activated macrophages is regulated by the level of inducible NOS (iNOS) transcription and by substrate availability. The iNOS is induced by a range of cytokines, growth factors, and inflammatory stimuli (22). Besides its metabolism through iNOS, arginine may be metabolized by arginase, producing ornithine, a precursor for aliphatic polyamine and proline synthesis (1). Polyamines promote cell proliferation and matrix synthesis (29). When arginase expression is upregulated, the amount of arginine available to iNOS is reduced, and vice versa. In diabetic rats, arginase activity has been shown to be either reduced in wound fluid (42), or abnormally elevated in wound tissue (19) but has not been specifically explored in macrophages.

It is not known whether supplying proline, an important substrate for collagen, helps to improve skin wound healing. How-

ever, proline, together with lysine and ascorbic acid, was found to reduce tibial shaft fracture healing time in diabetic rats (18).

There is no currently available efficient treatment of impaired wound healing in diabetes. Considering the high medical cost of impaired wound healing (e.g., diabetic foot), research into new therapeutic strategies is urgent. We hypothesized that supplying arginine and proline together might be useful for promoting wound healing in diabetes. We set out to determine the efficacy of arginine plus proline supplementation on protein metabolism and wound repair as assessed by a histological study (epithelialization and angiogenesis) and macrophage function in a model of diabetic rats.

MATERIALS AND METHODS

Animals

Animal care complied with the French regulations for the protection of animals used for experimental and other scientific purposes (D 2001-486) and with all applicable European Community regulations (Official Journal of the European Community, L538 12:18:1986). Luc Cynober is authorized to perform experiments with rodents (authorization no. 75.461).

Eighteen 11-wk-old Zucker diabetic fatty (ZDF) *fa/fa* male rats (358 ± 4 g; Charles River, L'Arbresle, France) were brought to our central animal facility and caged individually for a 2-wk acclimatization period in metabolic cages that allowed measurement of food intake and urine collection. The cages were maintained at constant temperature (21 ± 1°C) and humidity on a 12:12-h light-dark cycle. ZDF rats show hyperphagia and obesity-related diabetes, dyslipidemia, and hypertension (40). During this first 2-wk period, the rats were given free access to laboratory chow (17% protein, 3% fat, 59% carbohydrate, 21% water, vitamins and minerals) supplying 320 kcal/100 g (Purina, Genobios, Laval, France), and water.

Wounding

On *day 1*, after an overnight fast, the rats were anesthetized with isoflurane (3% in oxygen; Minerve, Esternay, France) and kept anesthetized by continuous isoflurane inhalation (1.5% in oxygen) throughout surgery. The abdominal area was shaved, and a 7-cm abdominal skin incision was performed. Ten preweighed (50 mg)

saline-moistened, polyvinyl alcohol sponges were inserted into subcutaneous pockets on each side of the incision, which was closed with 3.0 silk surgical sutures (Ethicon, Johnson and Johnson International, St-Stevens-Woluwe, Belgium). Two full-thickness round sections of dorsal skin, each 3 mm in diameter, were cut with a biopsy punch and excised using scissors. Each wound was treated with a spray wound dressing that formed a transparent film (Urgo, Chenôve, France). The rats received a single subcutaneous injection of analgesic ~20 min before the end of surgery (Temgesic, 0.05 mg/kg; Schering-Plough, Levallois-Perret, France).

Two full-thickness round sections of dorsal skin were then cut every day from *day 2* to *day 5*, with 1.5-cm intervals of healthy skin between wounds to allow full analysis of the wound repair process. The rats were anesthetized with isoflurane and received a subcutaneous injection of analgesic each day.

Thus, all of the rats underwent both the abdominal skin incision and a total of 10 dorsal excision wounds over the 5-day study period. The experimental design is shown in Fig. 1.

Postoperative Care and Nutrition Program

On *day 1*, the rats were randomized to be fed with either a standard formula (Clinutren Iso, Nestlé Clinical Nutrition, Noisiel, France), an arginine plus proline-enriched formula (Clinutren Repair Nestlé Clinical Nutrition), or an isonitrogenous isoenergetic control [Clinutren Iso with added nonessential amino acids histidine, asparagine, serine, alanine, and glycine in equimolar amounts]. Diet compositions are described in Table 1. The rats were fed via a 120-ml plastic bottle with a steel teat. The formulas were diluted in water (80:20, vol/vol) to prevent clogging in the teat. Every day for 4 days, 100 ml of formula was made available, and the amounts left were recorded to calculate actual intake. The animals also had free access to water.

On *day 5*, nutrition was stopped and the rats were decapitated immediately after anesthesia with isoflurane and wounding.

Sample Processing

From *day 1* to *day 5* (Fig. 1), the rats were weighed, and urine was collected daily in a container on a preservative (Amukin, Gifrer Barbezat, France) for nitrogen balance. Blood, skin, and wound fluid (sponges) were collected at the fasting state immediately after death.

Blood. Blood was sampled in heparin tubes and immediately centrifuged (10 min, 2500 *g*, 4°C). Part of the plasma was deprotein-
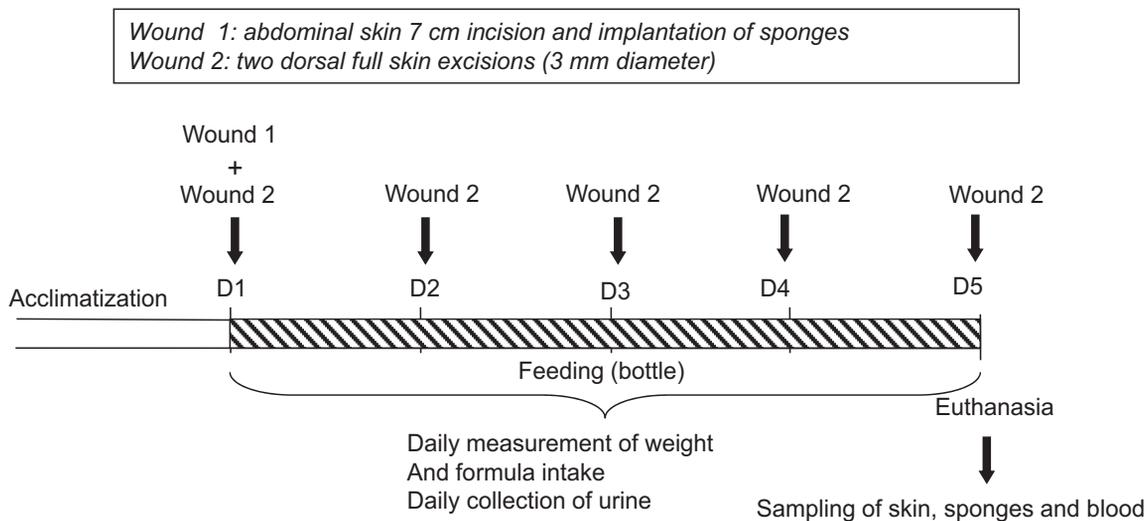
Fig. 1. Experimental design. On D1, rats underwent both a 7-cm skin abdominal incision to implant sponges (*wound 1*) and two dorsal full-skin excisions, 3 mm in diameter (*wound 2*). These two full-skin dorsal excisions (*wound 2*) were repeated each day to D5 for assessment of the progression of epithelialization and angiogenesis. Each day from D1 to D5, weight and formula intake were measured and urine was collected. At death, skin strips containing *wound 2*, sponges from *wound 1*, and blood were sampled.

Table 1. *Composition of diets (/100 ml)*

	Standard Diet (Clinutren Iso)	ARG+PRO Diet (Clinutren Repair)	Isonitrogenous Control Diet (Clinutren Iso + NEAA)
Energy, kcal	100	125	125
Protein, g	3.8	9.3	9.3
Arginine, g	0.13	0.47	0.13
Proline, g	0.31	1.15	0.31
Asparagine, g	0.34	0.61	1.28
Histidine, g	0.09	0.24	0.82
Serine, g	0.21	0.46	1.71
Glycine, g	0.08	0.16	1.15
Alanine, g	0.16	0.26	1.43
Leucine, g	0.37	0.83	0.37
Isoleucine, g	0.21	0.43	0.21
Valine, g	0.23	0.54	0.23
Tyrosine, g	0.15	0.45	0.15
Phenylalanine, g	0.15	0.41	0.15
Cysteine, g	0.05	0.05	0.05
Methionine, g	0.08	0.24	0.08
Threonine, g	0.20	0.35	0.20
Lysine, g	0.31	0.66	0.31
Glutamic acid + Glutamine, g	0.72	1.87	0.72

ized with 30 mg/ml sulfosalicylic acid, and samples were stored at −80°C for amino acid analysis (23).

Skin. The dorsal skin was excised and cut into 1-cm square strips. Each strip contained a healing scar. The strips were fixed on a polystyrene board and placed in 10% formaldehyde. The healing scars were cut in half and embedded in paraffin. Sections 2 μm thick were stained with hematein-eosin-safran, Weigert hemalum-phloxine-safran, and picro sirius red. Microscopic study of the strips was used to score epithelialization and angiogenesis; we used one scar corresponding to one rat on one day. Epithelialization was assessed semiquantitatively on a scale from 0 to 4 on each side of the wound: 0 (no epithelialization), 1 (~25% epithelialization), 2 (50% epithelialization), 3 (75% epithelialization), and 4 (total epithelialization), giving a 0 to 8 score for the whole wound (Fig. 2). Angiogenesis was scored according to the number of new vessels within the scar tissue. About 20 fields (×20) were screened for new blood vessels in each scar. We used a semiquantitative method on a scale from 0 to 3: 0 (no angiogenesis), 1 (few new blood vessels), 2 (moderate number of new blood vessels), or 3 (many new blood vessels, Fig. 3). For both epithelialization and angiogenesis, results were validated by comparative analysis of two independent observers blinded to the nutritional supplementation.

Wound fluid. The subcutaneously implanted sponges were pooled, cut, and squeezed to obtain wound fluid. Wound fluid was centrifuged, and cells were resuspended in 20 ml of DMEM culture medium (Sigma-Aldrich, La Verpillière, France) and supplemented with FBS (10%), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Sigma-Aldrich). Macrophages were selected according to their capacity to adhere to wells: macrophage-rich cultures were obtained after a 2-h incubation period (37°C, 5% CO_2), and removal of nonadherent cells was done by washing twice with DMEM. Macrophages were incubated in DMEM medium overnight, and this supernatant was removed for NO determination. For protein extraction, the macrophages were incubated at a density of 10^6 cells/ml in 6-well polystyrene culture plates (4 wells per experimental condition). Macrophages in microplates were washed with PBS (Sigma) and dried before storage at −80°C for protein extraction.

Analytical Methods

Urinary variables. Urinary nitrogen was quantified using a pyrochemiluminescence-based method (Antek 7000, Antek, Houston, TX) (17). Nitrogen balance corresponded to the difference between daily total nitrogen intake and daily total nitrogen urinary output. Cumulative nitrogen balance on D2 was expressed as nitrogen balance on D1+D2; cumulative nitrogen balance on D3 was expressed as nitrogen balance on D1+D2+D3, and so on.

Plasma variables. Glycemia was measured using a hexokinase endpoint method at 340–380 nm (Olympus AU600, Rungis, France).
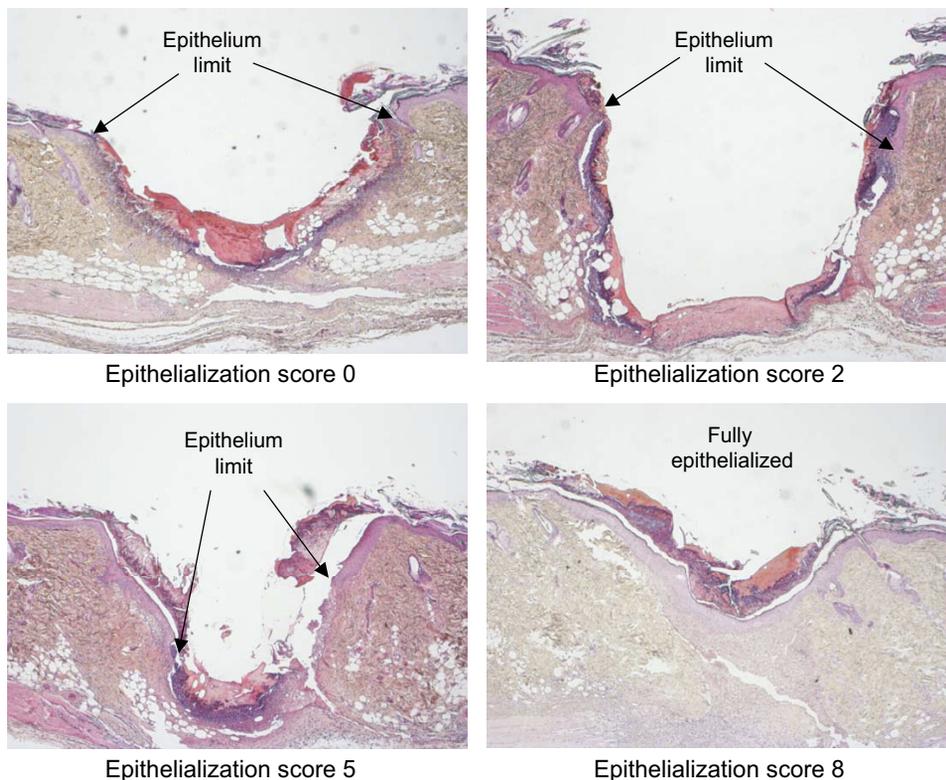
Fig. 2. Histological study: epithelialization. Microscopic study of the skin strips was used to score epithelialization semiquantitatively on a scale from 0 to 4 on both sides of the wound: 0 (no epithelialization), 1 (~25% epithelialization), 2 (50%), 3 (75%), and 4 (total epithelialization), giving a score from 0 to 8 for the whole wound.
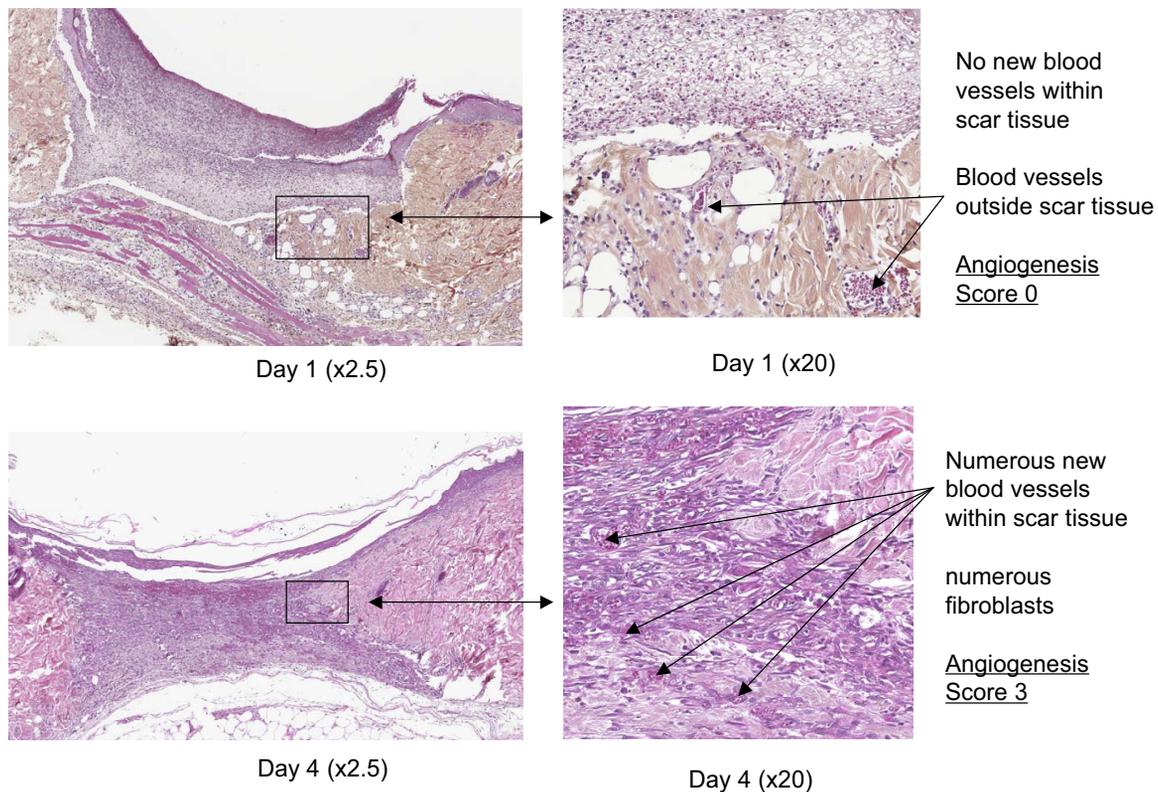
Fig. 3. Histological study: microscopic study of the skin strips was used to score angiogenesis semiquantitatively from 0 (no new blood vessels in the scar tissue) to 3 (many new blood vessels in the scar tissue).

Insulinemia was determined by radioimmunoassay using an INSIK-5 kit (Diasorin, Saluggia, Italy) and rat insulin (Linco, Saint-Charles, MO) as a standard.

Amino acid concentrations were determined by ion-exchange chromatography with ninhydrin detection (35a) using a fully automated apparatus (Jeol JLC-500V, Tokyo, Japan). Our laboratory is registered under the European Quality Control Program (ERNDIM, Brussels, Belgium); measurement reliability is, therefore, guaranteed for all the amino acids studied. Amino acid concentrations were measured both in the plasma (results expressed in micromoles per liter of plasma) and the macrophage medium cultures (results expressed in nanomoles per hour per 5×10^5 cells). For amino acid concentrations in the macrophage medium cultures, culture supernatants corresponding to each rat included in the study were deproteinized with a 30% (wt/vol) sulfosalicylic acid solution before quantification by ion exchange chromatography. Citrulline and ornithine productions correspond to their concentrations in the medium at the end of the experiment, since the culture medium did not contain these amino acids. For other amino acids, consumption was calculated by subtracting the final concentration from the initial concentration in the medium.

NO assay. Nitrites and nitrates (final products of NO, noted NO_x) were measured in cell supernatants by a modified Griess reaction (RD Systems, Lille, France). NO_x was measured once for every rat, and the mean value in each group was expressed in nanomoles per hour per 5×10^5 cells.

Protein extraction and Western blot analysis. The macrophages were homogenized in 150 µl of Laemmli extraction buffer [62.5 mmol/l Tris·HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue (Bio-Rad, Marnes-la-Coquette, France) with 5% β-mercaptoethanol (Sigma)]. The homogenates were incubated for 5 min at 100°C and centrifuged at 12,000 rpm for 10 min at 4°C. The total protein extracts underwent a 10% PAGE containing 0.1% SDS using 30 mA/gel for 40 min and were transferred to nitrocellulose membranes (Hybond-C Extra, Amersham Biosciences, Orsay, France) at 120 mA for 2 h. Proteins were fixed on the membrane with Ponceau red solution.

The blots were incubated with the following primary antibodies in 5% milk-1 × TBST (Tris·HCl buffer saline Tween): purified polyclonal rabbit anti-iNOS at 1:2,000 dilution (BD Transduction Laboratories, Lexington, UK), and anti-arginase I at 1:500 dilution (Interchim, Montluçon, France). The blots were washed in 1× TBST and incubated for 60 min with peroxidase-conjugated anti-rabbit IgG (dilution 1:10,000) in 5% milk-1× TBST. They were washed in 1× TBST, and antibody binding was visualized using an ECL Western blotting kit (Amersham Biosciences) and Kodak X-MAT films (Eastman Kodak, Rochester, NY). Exposure time was selected after testing a broad range of exposure times to select the conditions in which the band densities were below saturation (data not shown). Films were scanned, and the intensity of the bands was estimated using the Gene Genius Bio Imaging System (Syngene, St.-Quentin-en-Yvelines, France). GAPDH was used as a standard.

Statistical analysis. Data are expressed as means ± SE. Between-group comparisons were performed using an ANOVA test. When a variable was measured several times during the study (body weight, daily energy and protein intake, and nitrogen balance), the effect of time was analyzed using an ANOVA on repeated measures. All tests were performed at the 5% type I error level, using StatView software (Abacus Concepts, Berkeley, CA). The number of rats used for the study ($n = 6$ per group) was set for practical reasons; no statistical power analysis was performed before the study.

RESULTS

One arginine and proline-supplemented rat died during the study from severe hyperglycemia and dehydration. Accordingly, the data from 17 rats were analyzed: 6 fed with the

Table 2. *Daily energy and nitrogen intake*

	S	ARG+PRO	IC
Energy intake, kcal·kg^{-1}·day^{-1}	213 ± 13	228 ± 27	219 ± 16
Nitrogen intake, g·kg^{-1}·day^{-1}	1.30 ± 0.08	2.65 ± 0.32*	2.61 ± 0.19*

S, standard formula; ARG+PRO, arginine plus proline high-energy and high-protein formula; IC, isonitrogenous isoenergetic control formula. *$P < 0.0001$ vs. S.

standard diet (S), 5 fed with the arginine and proline-supplemented diet (ARG+PRO), and 6 with the isonitrogenous control diet (IC).

Rats consumed a mean of 73 ± 1, 60 ± 1, and 62 ± 1 ml/day of formula in the S, ARG+PRO, and IC groups, respectively, over the study period. The rats' mean daily energy and protein intakes are given in Table 2. Owing to the composition of the diets, protein intake was significantly higher in the ARG+PRO and IC diet groups than in the S group. All of the rats lost weight during the study (−39 ± 6 g from *day 1* to *day 5*), but there was no significant difference between groups for weight at any time (data not shown). There was also no difference between groups for glycemia (19.8 ± 5.6, 19.3 ± 2.3, and 23.3 ± 4.9 mmol/l for S, ARG+PRO, and IC, respectively) or insulinemia (109 ± 23, 117 ± 37, and 95 ± 19 μU/ml for S, ARG+PRO, and IC, respectively) on *day 5*.

Protein accretion was assessed by cumulative nitrogen balance: both ARG+PRO and IC groups, fed with a diet richer in nitrogen than that of the S group, had better cumulative nitrogen balance than the S group (Fig. 4).

Plasma amino acid profile was modified in the ARG+PRO and IC groups compared with the S group (Table 3). ARG+PRO supplementation increased proline but not arginine plasma concentrations. Branched-chain amino acid (leucine, isoleucine, valine) plasma concentrations were also significantly higher in the ARG+PRO group than in either the S or IC groups. As a result of nonessential amino acid supplementation in the IC group, histidine, asparagine, serine, and glycine plasma concentrations (but not alanine) were higher in the IC group than in the S or ARG+PRO groups.

Table 3. *Plasma amino acid concentrations (nmol/ml)*

	S	ARG+PRO	IC
Arginine	147 ± 19	154 ± 36	101 ± 11
Proline	168 ± 19	465 ± 84*§	168 ± 17
Citrulline	63 ± 5	65 ± 2	52 ± 3#*
Ornithine	52 ± 4	77 ± 17	41 ± 5
Asparagine	51 ± 5	63 ± 9	132 ± 36#*
Histidine	54 ± 2	54 ± 6	73 ± 10#*
Serine	187 ± 9	185 ± 13	378 ± 80#*
Glycine	168 ± 10	104 ± 4	369 ± 63#*
Alanine	516 ± 41	677 ± 69	612 ± 89
Leucine	247 ± 26	455 ± 19*§	248 ± 20
Isoleucine	134 ± 13	230 ± 26*§	139 ± 9
Valine	303 ± 28	585 ± 51*§	297 ± 23
Tyrosine	84 ± 7	148 ± 43*§	58 ± 6
Phenylalanine	75 ± 4	84 ± 7	69 ± 5
Cysteine	40 ± 2	31 ± 3*	35 ± 1
Methionine	41 ± 2	62 ± 9*§	41 ± 2
Threonine	191 ± 16	223 ± 22	153 ± 7#
Lysine	432 ± 38	484 ± 71	334 ± 15#
Asparate	15 ± 1	23 ± 5*§	15 ± 2
Glutamic acid	127 ± 10	148 ± 26	118 ± 21
Glutamine	440 ± 35	405 ± 20	424 ± 15

*$P < 0.05$ vs. S. §$P < 0.05$ vs. IC. #$P < 0.05$ vs. ARG+PRO.

Histological studies showed that the progression of epithelialization was similar in the S and ARG+PRO groups but was significantly reduced in the IC group on *day 5* compared with the two other groups (Fig. 5). Angiogenesis on *day 5* was significantly more marked in the ARG+PRO group than in the S and IC groups (Fig. 5).

Macrophages collected from the sponges in the abdominal wound were studied. First, amino acid concentrations in the macrophage culture medium were measured. There was no difference between groups for ARG and PRO consumption, but CIT production was significantly lower in the ARG+PRO group than in the S and IC groups (Fig. 6). Second, scanning of the Western blot bands showed reduced iNOS and arginase I expressions in macrophages from the ARG+PRO treated
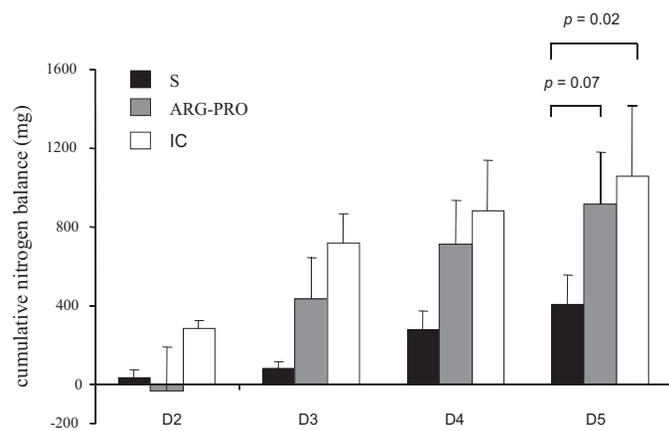
Fig. 4. Cumulative nitrogen balance. Cumulative balance at D2 was expressed as nitrogen balance at D1+D2; cumulative nitrogen balance on D3 was expressed as nitrogen balance on D1+D2+D3 and so on. S, standard diet; ARG+PRO, Clinutren Repair; IC, isonitrogenous control.
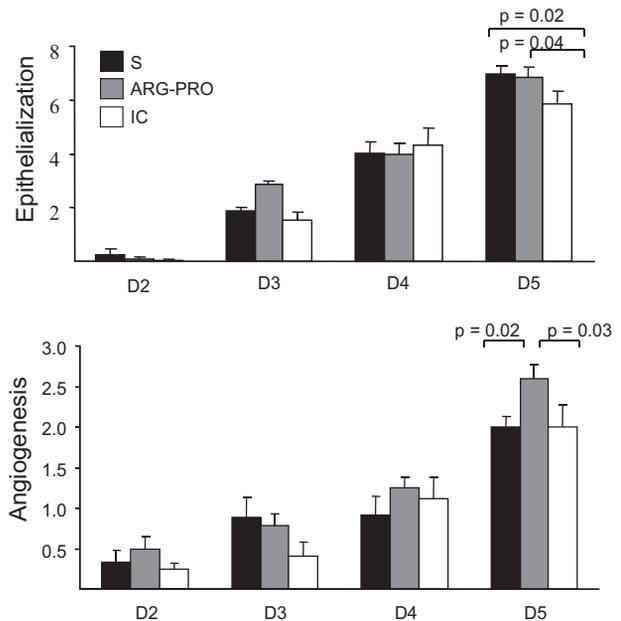
Fig. 5. Histologic study: epithelialization and angiogenesis.
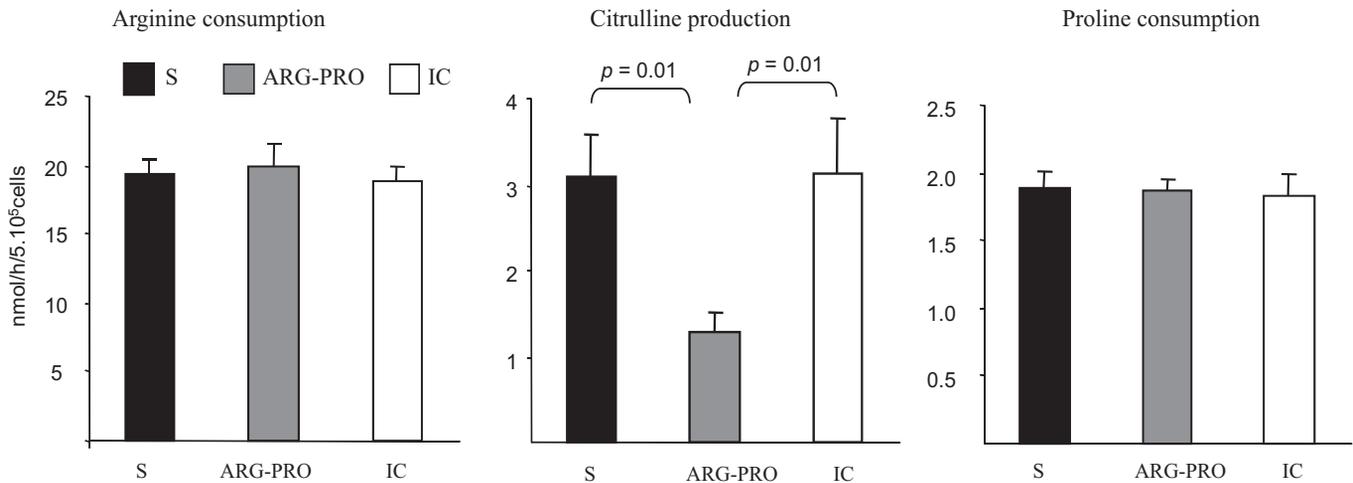
Fig. 6. Macrophage amino acid consumption and production. Citrulline production corresponds to its concentration in the medium and the end of the experiment, since the medium culture did not contain citrulline. For arginine and proline, consumption was calculated by subtracting the final concentration from the initial concentration in the medium.

group at *day 5*; the difference was statistically significant between ARG+PRO and S for iNOS and between ARG+PRO and IC for arginase 1 (Fig. 7). Third, the macrophages in the medium culture of rats fed the ARG+PRO diet produced significantly less NO than that in rats fed the S or IC diets (Fig. 8).

DISCUSSION

Diabetic patients suffer from delayed wound healing, and the presence of wounds carries a risk of malnutrition. Our rats underwent multiple wounds and daily anesthesia, inducing stress and a metabolic response that might impair nutritional status and wound healing. The protocol did not prevent wound healing (wounds were almost completely epithelialized on *day 5*), but mean energy intake was lower than that of nonstressed ZDF *fa/fa* rats (310 kcal·kg^{-1}·day^{-1}, laboratory observation) and the rats lost weight during the study. However, this only mimics the clinical situation of patients that suffer from chronic wounds, presenting with inflammation and malnutrition (4, 13).

Because of the alterations in amino acid metabolism in diabetic patients, nonspecific protein supplementation may not suffice to correct delayed wound healing. For example, standard protein and energy-rich oral supplements failed to improve wound healing in diabetic patients with foot ulcers (13). Similarly, in our study, the group of rats supplemented with a mixture of nonessential amino acids showed no improvement in wound epithelialization or angiogenesis compared with the standard diet group. Only the ARG-PRO group showed better angiogenesis than either standard or isonitrogeneous control groups. Thus, a specific amino acid approach (i.e., a disease-based diet) merits consideration.

In our model of experimental wounds in diabetic rats, a hyperprotein diet enriched with arginine plus proline significantly improved angiogenesis in wounds and favored whole body nitrogen accretion. First, the ARG+PRO group exhibited better nitrogen balance than the S group. However, this effect appeared to be nonspecific, since nitrogen balance was also better in the IC group. This incidentally enabled us to dismiss a possible limitation of our study, namely the fact that the

ARG+PRO group was richer not only in both arginine and proline but also in several other amino acids, including leucine, which is known to stimulate protein synthesis (20). Interestingly, plasma amino acid concentrations were not necessarily related to their intake, suggesting that intake of the amino acids concerned did not match requirements (7). In particular, plasma alanine was not higher in the IC group (in contrast to the other nonessential amino acids given in supplementation) despite a large intake. This may be related to its use in gluconeogenesis and may explain why glycemia tended to be higher in this group than in the other two groups. In addition, plasma arginine was not higher in the ARG+PRO group than in the others. Arginine is largely extracted by the splanchnic area, and the peripheral availability of arginine after an arginine load is limited compared with what is observed following loads of related amino acids (8). We note that Wu et al. (45) recently obtained very promising results for metabolic syndrome using a citrulline-enriched diet in non-wounded diabetic rats.

In our study, arginine plus proline supplementation significantly improved wound repair, as assessed by angiogenesis. Many studies have focused on the beneficial effect of arginine on wound healing, as assessed by wound-breaking strength or the hydroxyproline content of implanted sponges, but the effect of arginine on other markers of skin wound repair has been insufficiently researched. Only one study assessed the effect of arginine on epithelialization: in experimental wounds in healthy elderly subjects, arginine did not influence rate of epithelialization (21). Our model allowed an assessment of the daily progression of epithelialization, but as in the study by Kirk *et al.* (21), we observed no effect of arginine plus proline supplementation on epithelialization in our diabetic rats. However, angiogenesis did improve, and this is necessary for the process of tissue repair. The vasculature accounts for up to 60% of tissue repair (11). An abundant blood supply is mandatory to meet the enormous local metabolic demands; also, the endothelial cell plays a role in the organization and regulation of healing (2). Importantly, endothelial cell proliferation and angiogenesis are stimulated by NO, which can be produced only by the metabolization of arginine (24). However, data on the effect of arginine on angiogenesis are scarce. Angiogenesis
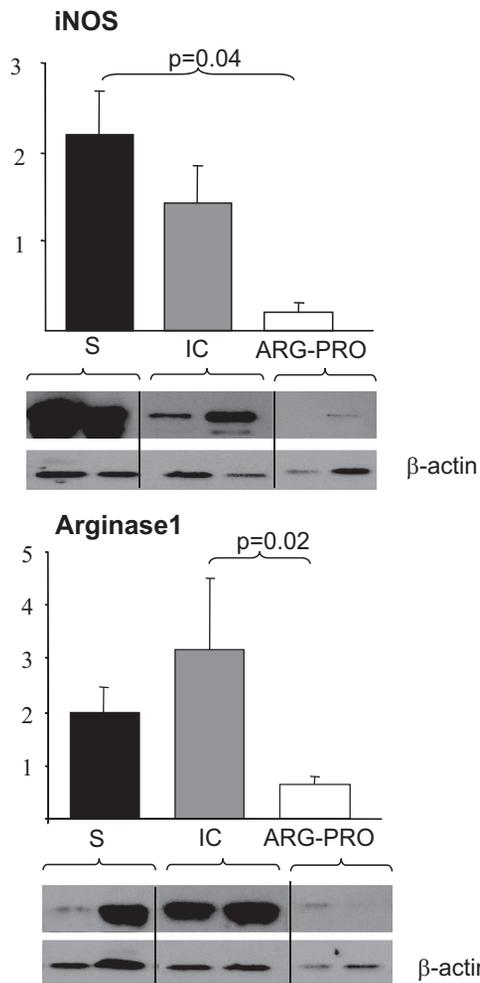
iNOS

Arginase1

Fig. 7. After quantification of the relative intensity of iNOS and arginase 1, membranes were stripped using a stripping buffer (0.5% acetic acid) and then were probed with β-actin. In the images of the blots, the solid lines inserted between lanes indicate that some lanes were removed for presentation. The blots were quantified by scanning, and results are expressed as a ratio of arginase1-β-actin and iNOS-β-actin. Values are expressed as means ± SE of iNOS and arginase 1 measured once for each rat in each group ($n = 6$ in group S: standard diet, $n = 5$ in group ARG ± PRO: Clinutren Repair, and $n = 6$ in group IC: isonitrogenous control).

has previously been assessed by measuring the number of capillaries in light microscopic histological sections of muscle in rabbits subjected to unilateral hindlimb ischemia (33) and by measuring microvascular density by CD34 immunohistochemistry staining in diabetic rats with burns (16). In both studies, arginine appeared beneficial to angiogenesis. We created a simple semiquantitative scale to assess the number of new capillaries and formation of granulation tissue buds, with comparative analysis by two independent observers to validate our results. Our results extend those of the two previously cited studies and show that arginine plus proline supplementation significantly improves angiogenesis in a model of excision skin wounds in diabetic rats.

In the group of arginine plus proline-supplemented diabetic rats, the expressions of both iNOS and arginase 1 were clearly reduced in macrophages removed from the sponges on *day 5*. This unexpected finding was associated with a lower production of NO_x and citrulline, but with no change in arginine or

proline macrophage metabolism. To our knowledge, only one study has reported iNOS and arginase expression in macrophages in human wounds (9). In that study, iNOS and arginase 2 expressions were increased from *day 2* to *day 10*, with no decrease over time, compared with that of unwounded skin macrophages, whereas arginase 1 expression was unchanged. However, arginase expression by macrophages seems to be species-specific, as rat macrophages only express arginase 1, and the time course in rats may be faster than in humans. In wounded diabetic animals, arginase activity has been shown to be either reduced in wound fluid (42) or abnormally elevated in wound tissue (as well as arginase expression) (19). In nondiabetic rats, the highest iNOS expression in the wound occurs in the early phases of wound repair, between 6 and 24 h, persists for 1 to 5 days, and decreases over the next 10 days (1, 14, 35, 46). Thus, the low iNOS expression that we observed in macrophages from the wound in ARG+PRO-supplemented rats in our study at *day 5* may be due to a faster decrease in iNOS in the ARG+PRO group, reflecting lower cytokine stimulation and less inflammation. Given that a chronic inflammatory state in the wound is associated with poor progression of healing, low iNOS expression at *day 5* would suggest better regulation of the cytokine production and of the healing process. Both innate and alternative activation of the macrophages may stimulate arginase activity, but it seems that only the classic activation of the macrophages induces an increase in iNOS activity (28, 31). A limitation in our study is that we measured neither cytokine production, nor the markers that determine early vs. late, and innate and classic vs. alternative macrophage activation (28). Also, macrophages were selected according to their capacity to adhere to wells: we cannot exclude the possibility that adherence of the macrophages changes their activation state. Finally, arginine supplementation may also have induced an increase in endothelial nitric oxide synthase (eNOS) expression or activity in endothelial cells, not measurable in macrophages, resulting in a local production of NO that stimulates angiogenesis.

The interpretation of our results may be limited by the relatively small number of rats in each group. In particular, we found no effect on epithelialization. By contrast, in a model of Sprague-Dawley diabetic rats with burns, Ge et al. (15) observed that arginine increased the advancement of epithelial cells; in their study, there were 30 rats per group. Thus, our
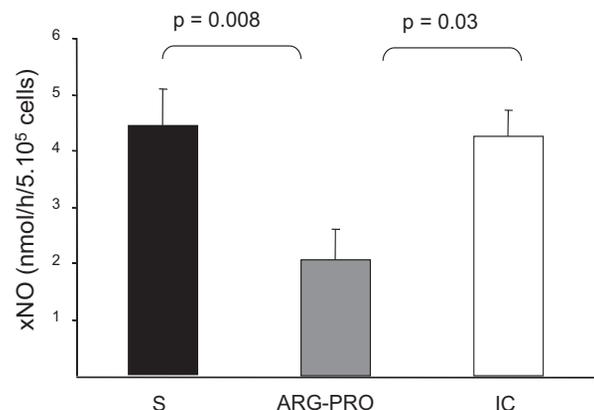
Fig. 8. Macrophage study: NO_x production in the medium culture. NO_x for nitrites and nitrates, final products of NO.

negative results on epithelialization may be due to a lack of statistical power. However, the different results could equally arise from the use of different models (streptozotocin-induced diabetes in Sprague Dawley rats vs. spontaneously diabetic Zucker diabetic fatty *fa/fa* rats).

Another limitation in our study is that for financial and technical reasons, we did not measure markers of collagen deposition in the wound, nor did we assess the quality of the scar. Thus, the present study cannot confirm the previously reported beneficial effect of arginine on collagen deposition and wound-breaking strength (38, 42). Also, given that proline is an important substrate for collagen, it would be interesting to determine whether the effect of proline plus arginine led to more collagen deposition than arginine alone; this should be the aim of a future study.

In conclusion, a hyperprotein formula enriched with arginine plus proline improved the process of wound repair in our model of diabetic rats. Clinical studies are now required to determine the efficacy of arginine plus proline-supplemented formulas in diabetic patients with wounds.

Perspectives and Significance

In the clinical setting, healing failure is common in diabetic patients and leads to chronic pain, infections, gangrene, and amputation. Malnutrition and low arginine availability may be involved in delayed wound healing in this population. We show that in wounded diabetic rats, a high-protein oral supplement enriched in arginine and proline improves nitrogen balance and amino acid profile (increase in branched-chain amino acids plasma concentrations) and favors angiogenesis better than an isonitrogenous control formula. Therefore, this oral nutritional supplement should now be tested in diabetic men and women that suffer from chronic wounds.

AUTHOR CONTRIBUTIONS

Author contributions: A.R.-S., L.B., G.L.N., F.C., L.C., and S.D. conception and design of research; A.R.-S., L.B., G.L.N., J.M., and S.D. performed experiments; A.R.-S., G.L.N., and S.D. analyzed data; A.R.-S. and S.D. interpreted results of experiments; A.R.-S. prepared figures; A.R.-S. drafted manuscript; F.C., L.C., and S.D. approved final version of manuscript; L.C. and S.D. edited and revised manuscript.

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