

Adverse Effects of Dietary Glycotoxins on Wound Healing in Genetically Diabetic Mice

Melpomeni Peppia,¹ Harold Brem,² Paul Ehrlich,³ Jian-Gang Zhang,² Weijing Cai,¹ Zhu Li,¹ Anca Croitoru,⁴ Swan Thung,⁴ and Helen Vlassara¹

Advanced glycoxidation end products (AGEs) are implicated in delayed diabetic wound healing. To test the role of diet-derived AGE on the rate of wound healing, we placed female *db/db* (+/+) (*n* = 55, 12 weeks old) and age-matched control *db/db* (+/-) mice (*n* = 45) on two diets that differed only in AGE content (high [H-AGE] versus low [L-AGE] ratio, 5:1) for 3 months. Full-thickness skin wounds (1 cm) were examined histologically and for wound closure. Serum 24-h urine and skin samples were monitored for N^ε-carboxymethyl-lysine and methylglyoxal derivatives by enzyme-linked immunosorbent assays. L-AGE-fed mice displayed more rapid wound closure at days 7 and 14 (*P* < 0.005) and were closed completely by day 21 compared with H-AGE nonhealed wounds. Serum AGE levels increased by 53% in H-AGE mice and decreased by 7.8% in L-AGE mice (*P* < 0.04) from baseline. L-AGE mice wounds exhibited lower skin AGE deposits, increased epithelialization, angiogenesis, inflammation, granulation tissue deposition, and enhanced collagen organization up to day 21, compared with H-AGE mice. Reepithelialization was the dominant mode of wound closure in H-AGE mice compared with wound contraction that prevailed in L-AGE mice. Thus, increased diet-derived AGE intake may be a significant retardant of wound closure in diabetic mice; dietary AGE restriction may improve impaired diabetic wound healing. *Diabetes* 52:2805–2813, 2003

Wound healing is impaired in diabetes and constitutes a major cause for increased morbidity and mortality in patients with diabetes (1). The majority of nonhealing wounds often lead to amputation. The percentage of amputees increases with age as do the direct costs of their care, rehabilitation, and lost productivity (2).

The exact cellular and molecular mechanisms underlying the pathogenesis of this complication are not fully

elucidated (3,4). However, a number of hyperglycemia-dependent factors have been identified, including the progressive accumulation of advanced glycation end products (AGEs) (5).

Two well characterized compounds, N^ε-carboxymethyl-lysine (CML) and methylglyoxal (MG), derivatives of glucose-protein or glucose-lipid interactions, serve as markers for AGE in a wide range of disorders related to diabetes, renal failure, and aging (6–8). According to recent observations, AGEs can be introduced in the body by exogenous sources such as diet and possibly in amounts that exceed those caused by hyperglycemia alone. A direct correlation is shown between the amount of AGEs consumed and that found in the circulation (9,10). In vitro data show that food-derived AGEs, which include CML and MG derivatives, can mimic the actions of endogenously formed AGEs and can induce intracellular oxidative stress and inflammatory cell activation, in a manner reversible by antioxidants or anti-AGE agents (11). Animal studies have revealed a significant contribution to the total AGE pool and related pro-oxidant or proinflammatory processes by dietary AGE intake, including tissue damage; this seems to be preventable by restricting dietary AGE intake (12–16). These findings were further supported by clinical studies, showing a reduction of circulating AGE levels and a suppression of inflammatory markers by dietary AGE restriction in patients with diabetes and normal renal function (17) and in patients without diabetic and with renal failure (18). In addition, a cross-sectional study in patients with renal failure demonstrated a significant correlation between dietary AGE intake and circulating AGE levels (19).

Skin, like other tissues, accumulates glycoxidation products in diabetes (20–22), which account for alterations in physicochemical and other characteristics of wound repair (5,23). The latter include altered tissue oxygen delivery (24); growth factor activity (5,25–28); vascular, skin fibroblast, and inflammatory cell dysfunction (5,29–32); increased metalloproteinase production (5,33); and defective collagen remodeling (5,23). In skin, inhibitors of AGE formation, such as aminoguanidine, prevent AGE accumulation and subsequent collagen cross-linking, improve angiogenesis, and restore various growth factor activity (27,34–37). In addition, receptor for AGE (RAGE) blockade results in accelerated wound healing in *db/db* mice (5).

Taken together, these considerations prompted us to determine whether dietary AGE intake, by influencing the total AGE pool, constitutes a determinant in the delayed healing of diabetic wounds. The diabetic *db/db* (+/+) mouse, a model commonly used for these studies, was chosen (38).

From the ¹Division of Experimental Diabetes and Aging, Department of Geriatrics, Mount Sinai School of Medicine, New York, New York; the ²Angiogenesis and Wound Healing Laboratory, Department of Surgery, Mount Sinai School of Medicine, New York, New York; the ³Division of Plastic Surgery, Hershey Medical Center, Hershey, Pennsylvania; and the ⁴Department of Pathology, Mount Sinai School of Medicine, New York, New York.

Address correspondence and reprint requests to Helen Vlassara, Mount Sinai School of Medicine, Box 1640, New York, NY 10029. E-mail: helen.vlassara@mssm.edu.

Received for publication 31 March 2003 and accepted in revised form 22 July 2003.

AGE, advanced glycoxidation end product; ELISA, enzyme-linked immunosorbent assay; CML, N^ε-carboxymethyl-lysine; MG, methylglyoxal; RAGE, receptor for AGE.

© 2003 by the American Diabetes Association.

TABLE 1
Characteristics of diet formulas

	Test diets*		AIN† (recommended)
	H-AGE	L-AGE	
Nutrients (%)			
Protein	25.6		20
Fat	5.12		5
Carbohydrate	50.3		65
Total calories (kcal/g)	3.4		4
AGE content			
CML (units/mg)‡	535	107	
MG (nmol/mg)§	18	3.6	

*Refer to Picolab Rodent Diet 5010. †Nutrient Requirements of Laboratory Animals, The National Academy of Sciences, Washington, DC, National Academy Press, 1995 (41). ‡Determined by 4G9 mab (42). §Determined by MG-3D11 mab (11).

RESEARCH DESIGN AND METHODS

Mouse dietary formulas. PicoLab Rodent Diet 5010 (Labdiet; Purina Mills, St. Louis, MO) is an autoclavable diet that is exposed to heat during pelleting and is fortified with supplements to offset heat-depleted micronutrients (Table 1). In this study, we used two forms of this standard 5010 Rodent Diet; one form was prepared without exposure to heat, and the second form consisted of the same chow exposed to an autoclaving cycle (121.5°C for 30 min), as per standard procedure. The autoclaving cycle included exposure to vacuum, an autoclaving step, and a drying step. Both dietary formulas were nutritionally equivalent (39), differing only in AGE content, based on assessment of CML-BSA and MG derivatives by ELISA, using monoclonal anti-CML-KLH (4G9; Alton, Northvale, NJ) (40) and anti-MG-ovalbumin (MG3D11), as previously described (11). The autoclaved standard formula contained 535 units of CML and 18 nmol of MG derivatives per mg, herein termed high AGE diet (H-AGE); the formula prepared in the absence of exposure to heat contained fivefold lower levels of CML (107 units/mg) and 4.5-fold lower MG derivatives (3.6 nmol/mg), termed low-AGE diet (L-AGE; Table 1). Both dietary formulas were pelleted by the manufacturer and kept at 4°C (12–16).

Mice. Twelve-week-old diabetic female BKS.Cg-m^{+/+} Lep^{ob} mice (*db/db*+/+, *n* = 55, 40–50 g; Jackson Laboratories, Bar Harbor, ME) and age-matched, female, nondiabetic mice from the same colony (heterozygous for the diabetes allele; *db/db*+/-, *n* = 45, 20–25 g) were used for our study. The *db/db* (+/+) mice carry a single autosomal recessive mutation on chromosome 4 and exhibit characteristics similar to those of human type 2 diabetes and thus are considered a reasonable choice for the study of chronic, nonhealing diabetic wounds (38). All mice were housed in a temperature-controlled animal facility (23°C) with a 12-h light/dark cycle and provided with food and water ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee. Baseline body weights and blood and urine samples were obtained, and mice were randomly assigned to either H-AGE diet (*db/db*+/+ [*n* = 21] and *db/db*+/- [*n* = 16]) or L-AGE diet (*db/dn*+/+ [*n* = 34] and *db/db*+/- [*n* = 29]) for up to 28 weeks of age. Food and water intake were recorded daily for 1 week and biweekly thereafter. Fasting blood glucose levels obtained from tail vein were measured by Glucometer (Elite XL 3901E; Bayer, Tarrytown, NY) weekly. The diabetic mice were markedly hyperglycemic (fasting blood glucose ~300–400 mg/dl), compared with the nondiabetic animals (90–120 mg/dl). Blood and 24-h urine samples were collected at baseline and at the end of the study.

Wound preparation and macroscopic examination. At 21 weeks and for the ensuing study period, mice were housed individually. At 24 weeks, they were shaved with an electric shaver (Oster 76, 0.05-mm detachable blade). After anesthesia induction (50 mg/kg ketamine and 5 mg/kg xylazine intraperitoneally) and skin sterilization with povidone (Betadine; Purdue Frederick, Norwalk, CT) rinsed with an ethanol swab, a full-thickness wound was made with a punch of 10 mm diameter (George Tiemann and Co, Long Island City, NY) over the mid-dorsum of the mouse, 15 mm below the skull base measured by a caliper (Bel-Art Products, Pequannock, NJ). The marked area was excised with scissors to include the epidermis, dermis, and panniculus carnosus. The wound was covered by a transparent, bio-occlusive dressing (Tegaderm 3 mol/L; Health Care), thereby creating a moist wound chamber environment. For improving adherence of the wound dressing, tincture of benzoin (Paddock Laboratories, Minneapolis, MN) was applied to the perimeter of the wound and allowed to dry. On day 7 after wounding, the Tegaderm was removed and the wound was left open thereafter. The wounds were

observed daily after wound induction for signs of infection and healing or until 100% closure with full epithelialization and no drainage was found.

Analysis of wound closure. Wound closure was documented with a digital camera (Coolpix 950; Nikon) at a fixed distance from the wound on days 0, 7, 14, and 21. A photo ruler (Shamrock, Bellwood, IL) was placed in the field of the photograph, labeled with the mouse's identification number and date. Digital photos were used to access wound area measurements. The wound area was calculated using the Med-Data System Woundimager software (version 1.0.3) by tracing the wound margin with a fine-resolution computer mouse and calculating pixel area (41). Measurements were performed in duplicate, and mean values of consecutive tracings were computed and expressed as percentage of closure from the original wound according to the following formula: % healing rate (rate of contraction) = (wound area day 0 – wound area day *n*)/(wound area day 0).

Three animals from each group were excluded because of bacterial infection. Animals were excluded from the study when signs of bacterial infection were observed. Animals were killed by CO₂ on days 7, 14, and 21 (H-AGE: *db/db*+/+, *n* = 7, 8 and 6, respectively, and *db/db*+/-: *n* = 5, 6, and 5, respectively; L-AGE: *db/db*+/+, *n* = 12, 11, and 11, respectively, and *db/db*+/-: *n* = 8, 9, and 9, respectively). The entire wound, including a 5-mm margin of the surrounding normal skin, was excised to the level of fascia for immunohistochemical analyses (see below), and the remaining skin from the back of the mice was removed for tissue AGE determination.

Histopathological analyses. Wound biopsies were fixed in 10% buffered formalin and embedded, and 4-μm sections were cut from the mid-portion of the wound. The sections were cut perpendicular to the anterior-posterior axis and perpendicular to the surface of the wound. Tissue sections were stained with hematoxylin and eosin (H&E) and Sirius red (42,43). The evaluation of collagen organization was made by the intensity of birefringence from polarized light examination of Sirius red stained sections. The more mature the collagen fibers, the greater the birefringence intensity. The parameters evaluated from H&E staining were degree of reepithelialization, density of granulation tissue, inflammatory cell infiltration, vascularity, and collagen deposition. Histological scores ranged as follows: 1–3, minimal epithelialization, minimal inflammation, sparse granulation tissue, no vascularity, no collagen deposition; 4–6, minimal epithelialization, predominantly inflammatory cells, minimal granulation tissue with few fibroblasts, capillaries, little collagen; 7–9, completely reepithelialized, more granulation tissue with fibroblasts and inflammatory cells, moderate vascularization; 10–12, thick epithelial layer, thick granulation tissue with moderate fibroblast density, extensive collagen deposition, dense vascularization; 13–15, thick epithelium, uniformly thick granulation tissue with low density of fibroblasts in scar, dense collagen deposition, and mature blood vessels.

Deparaffinized sections were immunostained for AGEs using a previously characterized rabbit anti-AGE-KLH antibody, as previously described (44–46). Goat anti-rabbit was used as secondary antibody, and for detection, we used the ABC peroxidase system (DAKO, K0355). Each slide was given a histological score related to the intensity of staining of epithelial cells (score 1–4), collagen (score 1–4), fibroblasts and/or inflammatory cells (score 1–4), and endothelial cells of blood vessels (score 1–4). Sections that were stained with Sirius red had their collagen birefringence staining intensity semiquantified by scoring 1–3: 1, minimal (poor); 2, moderate; and 3, dense bright orange-red birefringence. Scoring was performed by two trained staff pathologists in a blinded manner. Quantification of immunohistology was performed as follows: sections were selected by a blinded observer and imaged using a Zeiss microscope and an attached Sony video camera. For quantitative evaluation of days 7 and 14, sites for study were chosen at the edge of the wound, at the margin between normal and wounded skin. For evaluation of day 21, sites for study were selected at the center of the closed wounds. To quantify collagen content, we selected sites just below the epithelium to encompass the granulation tissue/healing wound observed on each slide.

Metabolic studies. At baseline and at the end of the study, serum and 24-h urine were tested for CML and MG by ELISA, as described above (10,38). Renal function was monitored by the urinary albumin:creatinine ratio (12). Urinary albumin was measured with an anti-mouse albumin-based ELISA Kit (Bethyl Laboratories, Montgomery, TX). Urinary creatinine was measured in the same samples by a standard colorimetric method (12).

Skin tissue AGE assays: total protein AGE levels. Segments of wounds and skin surrounding the wound were taken, rinsed in PBS, dried, and homogenized in PBS. Samples were digested with proteinase-K digestion (20:1 wt/vol; Roche Diagnostics, Indianapolis, MN); after overnight incubation at 37°C, samples were centrifuged and the supernatants were stored for AGE determination. Protein concentration in the samples was measured by Bio-Rad Protein Assay (Hercules, CA). Total protein-linked AGE immunoreactivity was determined by competitive ELISA using monoclonal anti-CML (4G9) or

TABLE 2

Characteristics of *db/db* and non-*db* mice exposed to H-AGE and L-AGE diets

		H-AGE		L-AGE	
		Baseline	End	Baseline	End
Body weight (g)	<i>db/db</i>	51 ± 0.7	53 ± 0.5	50 ± 0.5	51 ± 0.6
	non- <i>db</i>	22.7 ± 0.4	23.8 ± 0.3	22 ± 0.4	23.1 ± 0.4
Blood glucose (mg/dl)	<i>db/db</i>	358 ± 34	424 ± 43	293 ± 26	373 ± 25
	non- <i>db</i>	114 ± 7	110 ± 6.5	117 ± 5	106 ± 5
Food intake (g/day)	<i>db/db</i>	5 ± 1	6.2 ± 0.5	5.2 ± 0.4	6 ± 0.5
	non- <i>db</i>	4 ± 0.3	4.3 ± 0.1	3.9 ± 0.4	4.1 ± 0.2
sCML (units/ml)	<i>db/db</i>	69 ± 4.1	104 ± 7.2*†	71 ± 2.1	65 ± 2.8†
	non- <i>db</i>	53.4 ± 2.5	64.3 ± 5.5*	52.2 ± 2.7	48.7 ± 2.3
sMG (nmol/ml)	<i>db/db</i>	1.7 ± 0.1	2.4 ± 0.1*†	1.7 ± 0.1	1.6 ± 0.1
	non- <i>db</i>	1.4 ± 0.09	1.8 ± 0.1*†	1.4 ± 0.06	1.4 ± 0.04
Urinary CML excretion (units/min)	<i>db/db</i>	334 ± 29	186 ± 19*†	278 ± 17	285 ± 20
	non- <i>db</i>	79.2 ± 20.5	187.5 ± 62.3	71 ± 8	94.6 ± 23
Urinary MG excretion (nmol/min)	<i>db/db</i>	2.4 ± 0.2	1.14 ± 0.3*†	2 ± 0.1	2.04 ± 0.1
	non- <i>db</i>	1.9 ± 0.6	3.2 ± 0.5*	1.5 ± 0.2	1.8 ± 0.3
Urinary albumin/creatinine ratio (μg/mg)	<i>db/db</i>	1.6 ± 0.1	3.9 ± 0.3†	1.6 ± 0.2	1.9 ± 0.2
	non- <i>db</i>	0.85 ± 0.2	0.92 ± 0.2	0.91 ± 0.2	0.9 ± 0.1

Data are expressed as mean ± SE. **P* related to statistically significant differences in both diet groups at the end of the study; †*P* related to statistically significant differences in each diet group between baseline and end of the study.

anti-MG ovalbumin antibodies (3D11), as previously described (11,41). Tissue AGE values were expressed as CML units/mg protein or MG nmol/mg protein. **Skin collagen AGEs.** Portions of homogenized skin were depilated with acetone and chloroform (1:1 vol/vol) and digested with 1:100 (wt/vol) type VII collagenase (Sigma Chemical Co., St. Louis, MO) for 48 h at 37°C. The digest was centrifuged, and the supernatants were stored for CML and MG determinations by competitive ELISA. An aliquot of the supernatant was hydrolyzed separately in 6 N HCl, and hydroxyproline content was measured using a colorimetric assay (44).

Statistical analysis. All data are given as mean ± SE. Differences of variables between the H-AGE and L-AGE groups were analyzed by the Mann-Whitney *U* unpaired test. Differences of variables at different time points on either group were analyzed by a two-sided Wilcoxon's matched pair signed-rank test. Statistically significant differences were defined as *P* ≤ 0.05. Data analysis was performed using the SPSS statistical program (SPSS 10.0 for Windows).

RESULTS

Body weight, hyperglycemia and serum AGE levels.

Body weights and fasting blood glucose were similar within the diabetic and nondiabetic mice, groups in both H-AGE- and L-AGE-fed groups (Table 2). No significant differences in food intake were observed between the diabetic or nondiabetic groups on either diet type (Table 2). While maintaining a similar intake of food nutrients and energy equivalents (Table 1), H-AGE-fed mice ingested ~5 times more AGEs than the L-AGE-fed mice. This was reflected in fasting serum AGE, which by the end of the study were significantly higher in the H-AGE-fed compared with the L-AGE-fed mice (*P* = 0.000; Table 2). With the H-AGE diet, serum CML and MG increased significantly (52.8% [*P* = 0.003] and 48.5% [*P* = 0.008], respectively), whereas with the L-AGE diet, serum AGE remained close to baseline despite the presence of diabetes (CML, 7.8%; MG, 1.4%, respectively; *P* = 0.037, NS; Table 2). Similar findings were observed in the control mice. At the end of the study, CML and MG levels were higher in the H-AGE-fed mice than in the L-AGE-fed mice (CML, *P* = 0.048; MG, *P* = 0.010; Table 2).

Effect of dietary glycotoxins on renal function and urinary AGE levels. High dietary AGE intake by diabetic mice over the study period was associated with a decrease

in urinary CML and MG excretion (42.2% [*P* = 0.011] and 53.5% [NS], respectively), compared with the unchanged excretion in L-AGE-fed mice (CML, *P* = 0.007; MG, NS; Table 2). In nondiabetic mice, the H-AGE diet was associated with higher levels of urinary MG and CML excretion, compared with L-AGE-fed mice (*P* = 0.011 and NS, respectively; Table 2). In *db/db* mice, renal function based on albumin/creatinine ratio remained unchanged in L-AGE-fed mice, whereas it deteriorated in H-AGE-fed mice (*P* = 0.000; Table 2).

Dietary glycotoxins and skin-tissue AGE deposition.

In diabetic mice, the H-AGE diet was associated with increased protein-linked tissue deposition of MG- and CML-like AGE, compared with L-AGE-fed mice (*P* = 0.001 and *P* = 0.001, respectively), whereas no AGE increases were observed, despite prolonged diabetes (Fig. 1A and B). Similarly, collagen-associated AGE accumulation was greater in the H-AGE-fed mice compared with L-AGE-fed mice (*P* = 0.011 and *P* = 0.001, respectively; Fig. 1C and D). Also, nondiabetic mice on the H-AGE diet showed greater, albeit nonsignificant, protein- or collagen-linked CML and MG skin deposits (Fig. 1).

Effect of dietary glycotoxins on wound closure in diabetic mice. Beginning by day 7 after wounding, diabetic L-AGE-fed mice displayed faster closure (32.3 ± 2.3%) compared with H-AGE-fed mice (19.2 ± 2.8%; *P* = 0.003). This more rapid pace of TC continued to day 14 for L-AGE mice (86.4 ± 2% vs. 72 ± 2.7%; *P* = 0.001). On day 21 the wounds of L-AGE mice were completely healed (mean healing time, 19.2 ± 1.8 days) compared with incompletely healed wounds of H-AGE mice (81.3 ± 4.1%; mean healing time, 24.5 ± 0.9 days; *P* = 0.013; Figs. 2A and Fig. 3A–H). In the control mice, no differences were observed with regard to timing of healing or mean time wound closure between the diet groups (H-AGE, 13.7 ± 0.3 days; L-AGE, 13.9 ± 0.4 days; Fig. 2A).

Histology. On day 7, wounds retrieved from L-AGE mice displayed higher cellularity at the wound-dermis interface, consistent with a greater inflammatory response at the

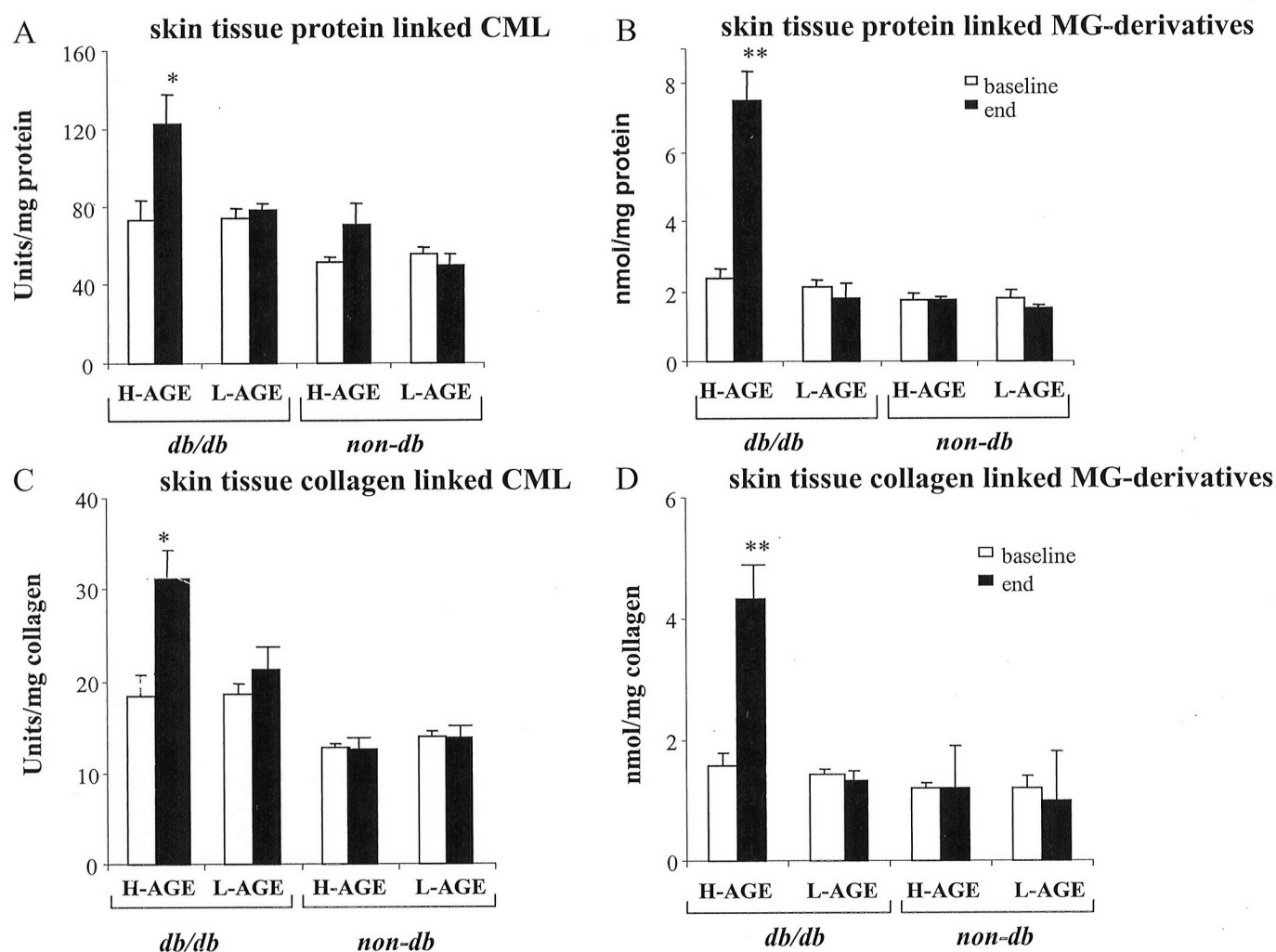


FIG. 1. Skin tissue AGE levels in diabetic and nondiabetic *db/db* mice correlate with dietary AGE intake. At baseline (12 weeks of age) and 12 weeks after exposure to either H-AGE or L-AGE diet, skin tissue sections were homogenized and digested with proteinase-K (protein-linked AGE; A and B) or collagenase type VII (collagen-linked AGE; C and D) and tested for CML and MG by ELISAs (10,15). Total protein and hydroxyproline levels were tested based on standard assays. Data are expressed as mean \pm SE CML units/mg protein or collagen CML units/mg collagen and as MG nmol/mg protein or MG nmol/mg collagen. * $P < 0.001$, ** $P < 0.011$, between diet groups at the end of the study.

wound site of the L-AGE diabetic mice at this early phase compared with that of H-AGE-fed mice (3 ± 0.4 vs. 1.3 ± 0.3 , respectively; $P = 0.033$; Figs. 4B and D and 5A and C). On day 14, L-AGE-fed mouse wounds displayed robust reepithelialization, granulation tissue deposition, inflammatory cell infiltration, and angiogenesis (Fig. 4A–C). In contrast, in the wounds from H-AGE-fed mice, all of these parameters were delayed and attenuated (Figs. 4B and D and 5A and C). By day 21, wounds from L-AGE-diabetic mice began to show a change, from a high density of inflammatory cells to a high density of fibroblasts with numerous new blood vessels and extensive reepithelialization (Fig. 4A–C). In contrast, in the H-AGE-fed mice, wounds displayed sustained inflammation (3.7 ± 0.3 vs. 2.1 ± 0.2 , respectively; $P = 0.017$; Figs. 4B and 5B and D), fewer blood vessels, and a lower degree of reepithelialization (Fig. 4A). By this time, L-AGE-fed mouse wounds were completely covered by a differentiated epidermis (Figs. 3 and 6). In contrast, wounds from the H-AGE-fed mice showed incomplete reepithelialization with the edges covered with undifferentiated epidermis. Nondiabetic control mice on either diet showed no differences in either

inflammatory cell response or the histological score evaluated on days 7, 14, and 21.

Evaluation of AGE deposits in H-AGE wound skin segments showed extracellular and intracellular tissue AGE staining, most intense on day 21, that co-localized with endothelial cells, fibroblasts, and inflammatory cells, compared with the L-AGE skin sections, which, by day 21, were virtually negative for AGE, with the exception of the epidermis (Fig. 7). No differences in AGE accumulation were observed in either diet group of nondiabetic mice.

At day 21, birefringence intensity and patterns of deposited collagen fibers were consistent with thick uniform collagen fibers in L-AGE-fed mouse wounds. In the H-AGE-fed mouse wounds, the birefringence intensity was less, consistent with finer, less organized collagen fibers throughout (mean histological score: L-AGE, 2.4 ± 0.2 ; H-AGE, 1.3 ± 0.3 ; $P = 0.032$). In L-AGE-fed mice, wound closure was the result of reepithelialization, and these wounds appeared completely reepithelialized at ~ 19 days after wounding. Wounds from H-AGE mice were not closed for up to ~ 24 days (Figs. 2B and 6E and F).

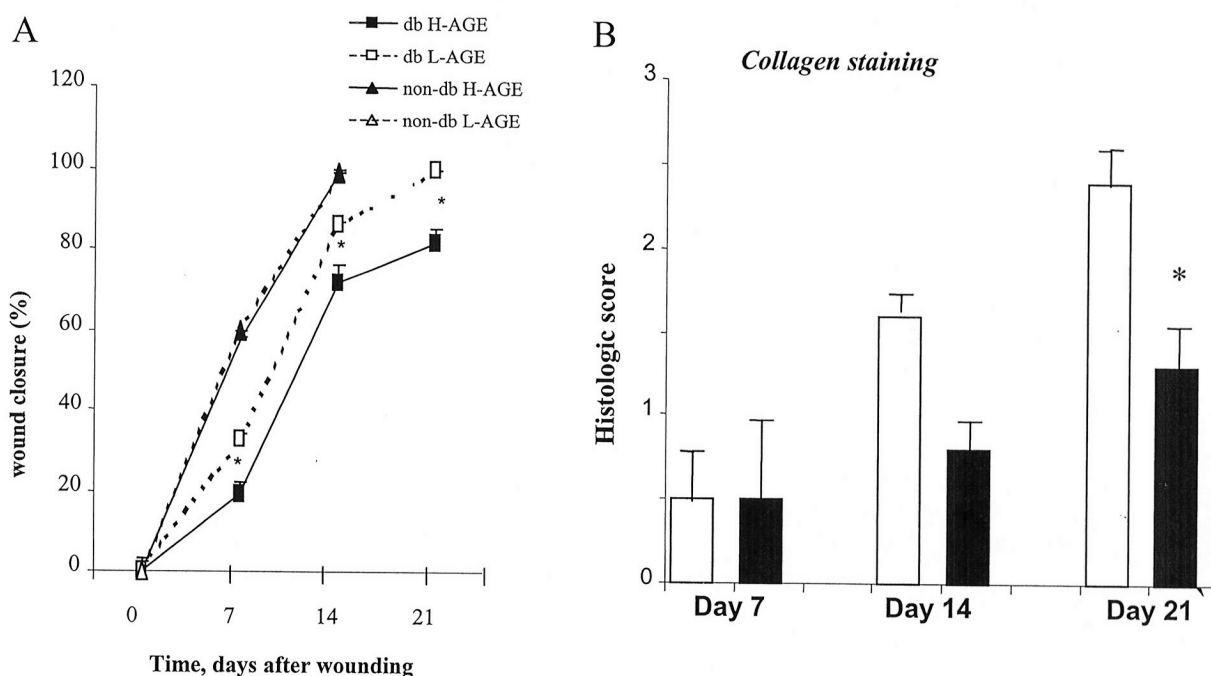


FIG. 2. Time to closure and histologic changes of diabetic wounds correlate with dietary AGE intake. *A*: After 12 weeks' exposure to either L-AGE or H-AGE diets, full excisional wounds (1 cm) were created at the back of the mice and the time to closure was evaluated at days 7, 14, and 21 after wounding (data shown as percentage of change from baseline). *B*: Sections were stained with Sirius red, and collagen deposition was scored according to the intensity of the staining (score 1–3). Data are expressed as mean \pm SE. * $P < 0.05$ between diet groups at the end of the study.

DISCUSSION

The present report demonstrates that modification of orally ingested glycotoxins can alter significantly the time and the quality of healing in wounds of diabetic animals using the *db/db* (+/+) mouse as a model. Increased dietary

AGE intake delayed time to closure and interfered with angiogenesis and granulation tissue maturation, which were in contrast to those associated with a low-AGE diet. The effects of this intervention are directly related to parallel changes in circulating and tissue-retained AGE,

H - AGE Diet

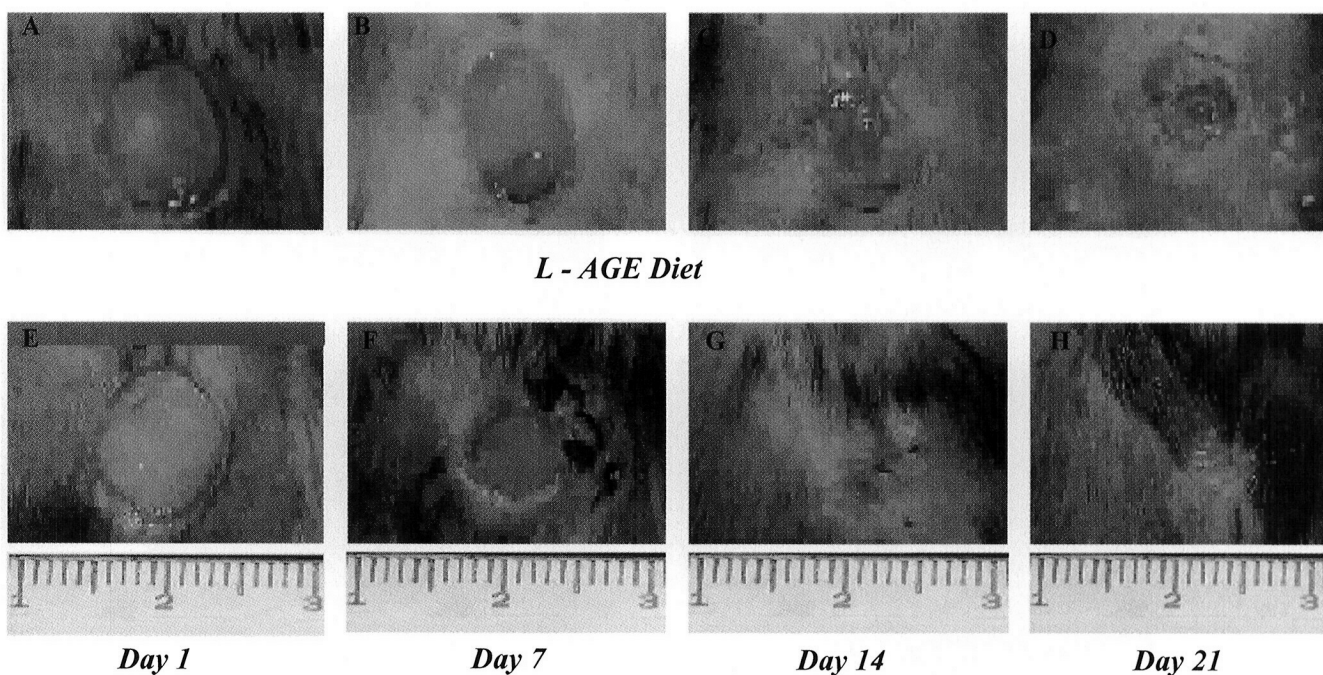


FIG. 3. Wound closure in *db/db* mice is modulated by exogenous (dietary) AGE. After exposure to L-AGE or H-AGE diets, wound closure was documented with a digital camera at a fixed distance from the wound at different time points. *A* and *E*: Day 1. *B* and *F*: Day 7. *C* and *G*: Day 14. *D* and *H*: Day 21. *A*–*D*: H-AGE diet. *E*–*H*: L-AGE diet.

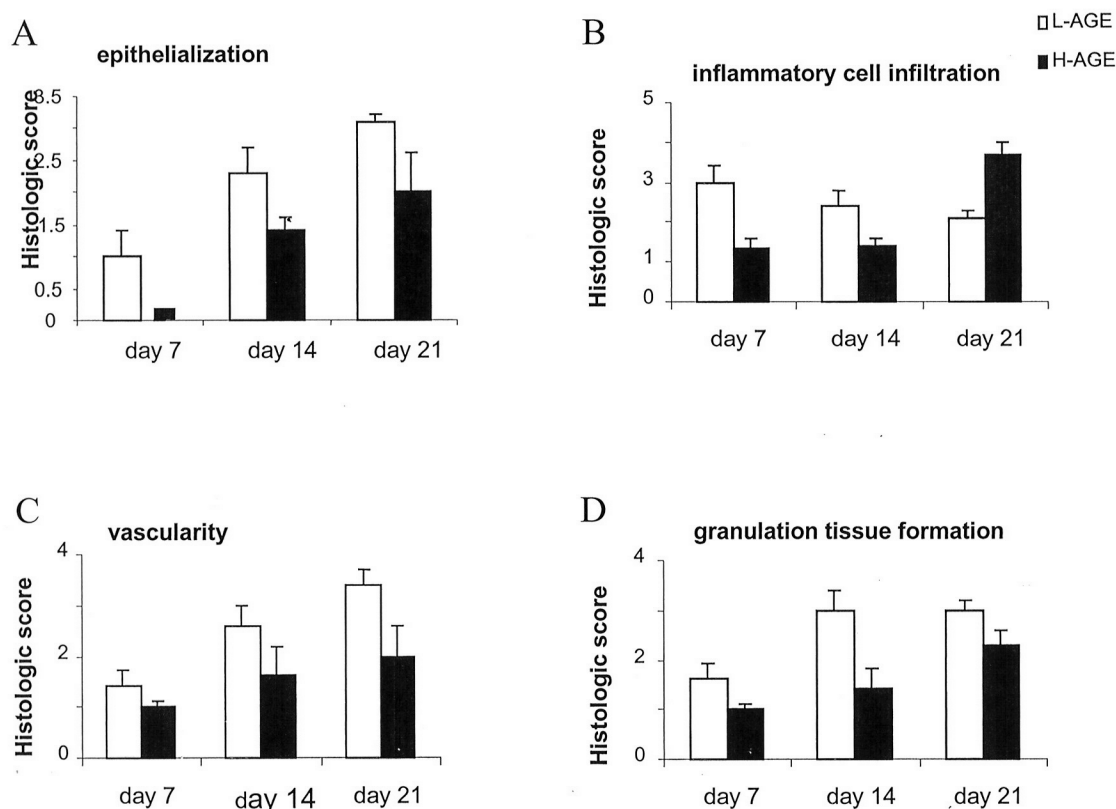


FIG. 4. Histochemical evaluation of wounds from diabetic (*db/db*) mice; association with dietary AGE intake. After 12 weeks on the diets, sections were stained with H&E and epithelialization (A), inflammatory cell infiltration (B), vascularity (C), and granulation tissue formation (D) were evaluated. Data were calculated based on histological score, as described and expressed as mean \pm SE ($n = 5-8$ per H-AGE group and $n = 8-12$ per L-AGE group, diabetic versus nondiabetic, respectively). * $P < 0.02$.

confirming that systemic effects of toxic derivatives extend to skin tissue.

The findings could be attributed only to the diet, as no

other variables were altered. The observed changes were not due to nutrient composition, as the diets used were of a single origin, with equivalent macronutrient, micronutri-

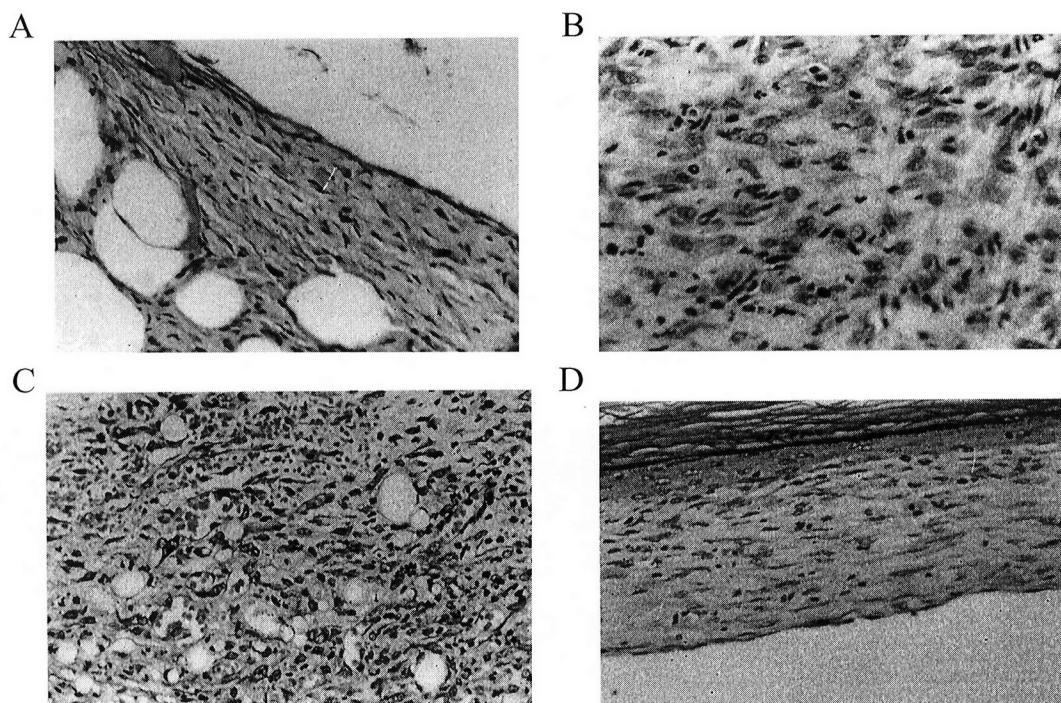


FIG. 5. Skin wound from diabetic mice exposed to H-AGE diet exhibit delayed but sustained nonsubsiding inflammatory response. Wound skin sections from diabetic *db/db* mice exposed to either H-AGE diet (A and B) or L-AGE diet (C and D), stained with H&E. Note distinct pattern of inflammatory cell infiltrate at 7 and 21 days postwound between the two diets. Magnification $\times 200$.

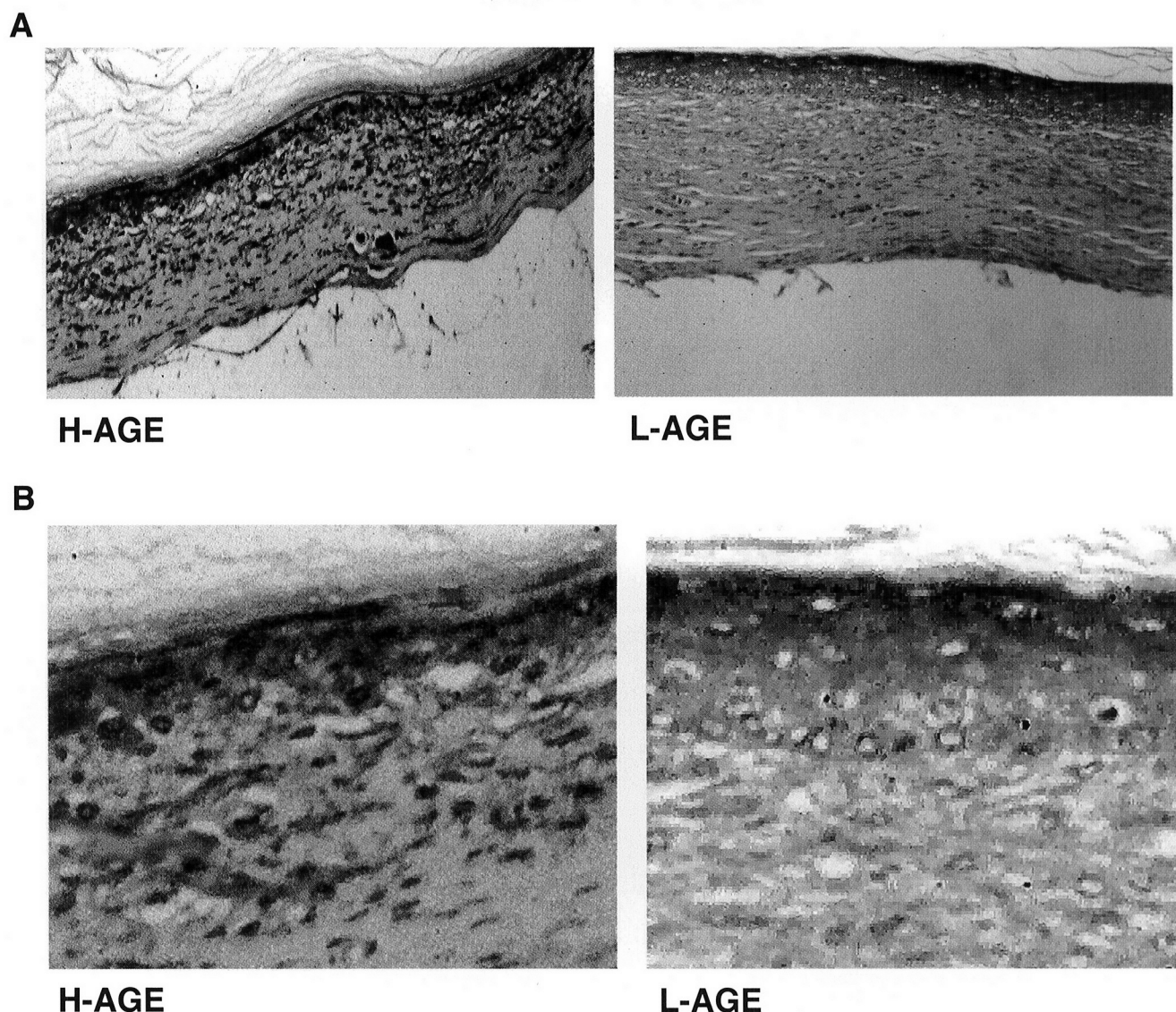


FIG. 6. Collagen staining in wounds from diabetic (*db/db*) mice exposed to L-AGE or H-AGE diet. Skin tissue samples from full-thickness excisional wounds from diabetic (*db/db*) mice were stained with Sirius red and scored at different time points. A and B: Day 7. C and D: Day 14. E and F: Day 21. A, C, and E: H-AGE diet. B, D, and F: L-AGE diet.

ent, vitamin, and energy profiles, according to American Institute of Nutrition recommendations (39). A prominent characteristic of these diets is their distinctly different glycotxin content: the formation of a substantial proportion of AGEs present in the H-AGE diet is avoided in the L-AGE diet as a result of reduced exposure to heat during preparation. Indeed, methods of food processing (heating, sterilization, pasteurization) increase the production of diverse, unstable compounds, which include reactive α - β -dicarbonyl derivatives such as CML and MG derivatives (47–49).

The relationship of dietary glycotoxins to those in serum and in urine has been shown earlier (9,10,12–16) and was confirmed in this study: H-AGE-fed diabetic mice exhibited increased levels of circulating CML, a terminal, stable oxidation product, as did MG derivatives, albeit to a lesser degree possibly as a result of their unstable nature. Thus, dietary glycotoxins influence circulating AGE levels, independent of hyperglycemia. However, hyperglycemia was

important, as evidenced by the higher AGE levels in diabetic compared with nondiabetic mice.

Greater AGE deposition was also shown in skin tissue from H-AGE-compared with L-AGE-fed mice. Skin constitutes a tissue where glycoxidation products have been known to accumulate (20–22). Herein, skin AGE and AGE-related toxicity were found modulated by exogenous glycotoxins to a greater extent than by hyperglycemia itself, a finding corroborated on other organs in previous reports (12–16). Similar, albeit less prominent, were the findings on nondiabetic mice.

An inverse correlation between circulating AGE and time to healing was observed. Wound healing is a multifactorial process involving inflammation, tissue remodeling, and restoration of healthy tissue. AGE are shown to regulate a variety of inflammatory cell responses and growth-promoting events, which to a certain extent occur normally during wound healing (5,29–32). Excessive AGEs, however, could promote overt oxidant stress (5,26),

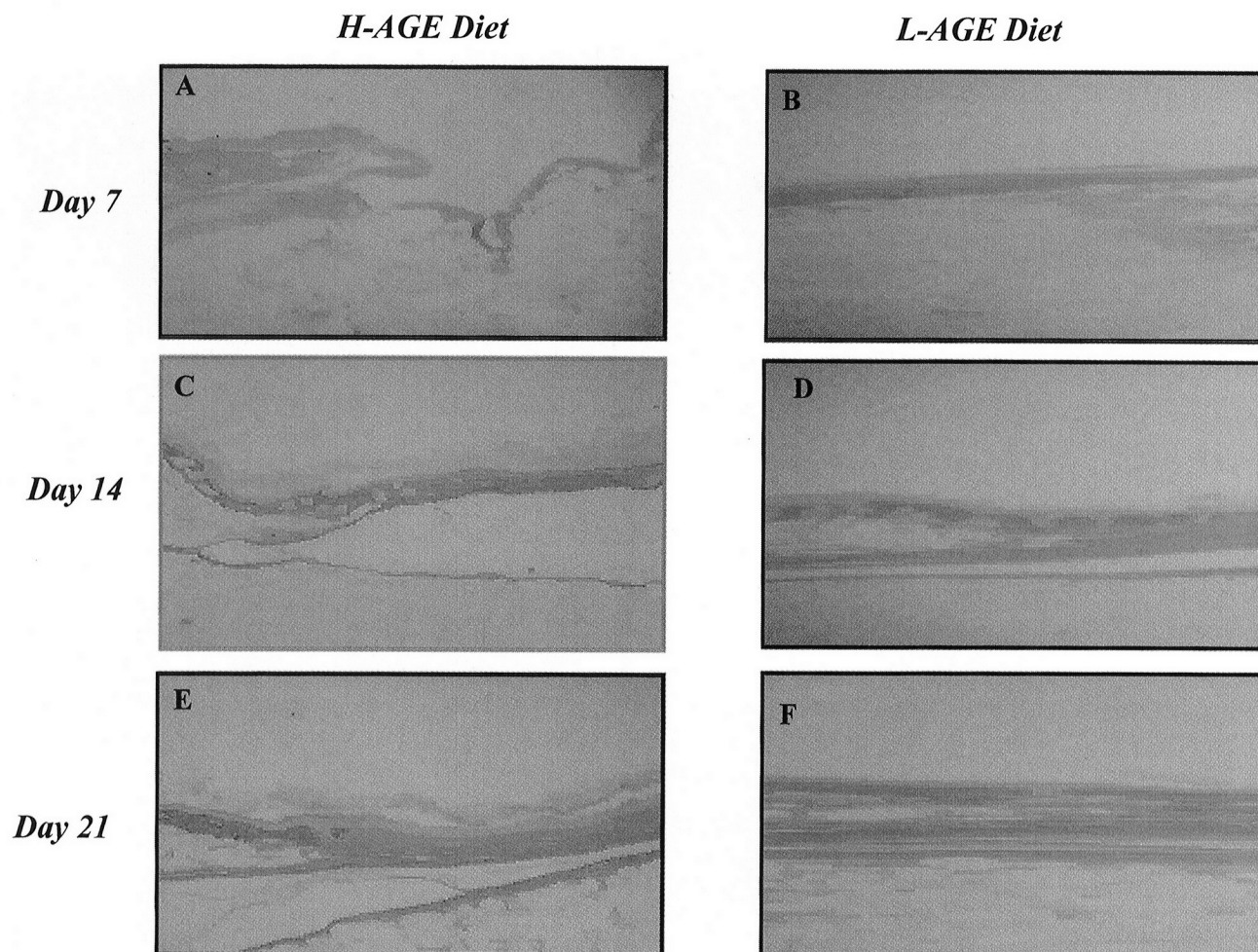


FIG. 7. AGE deposition in skin wounds from diabetic mice is influenced by diet-derived AGE. A: Wound skin tissue sections from diabetic *db/db* mice exposed to H-AGE or L-AGE diet were stained with anti-AGE antibody at day 21 postwounding. Magnification $\times 40$. B: Enlargement of sections shown in A displays AGE distribution in skin tissue components. Magnification $\times 200$. Note the marked differences in intra- and extracellular AGE deposits between sections from diabetic mice exposed to different AGE diets, despite similar levels of hyperglycemia.

which could in turn impair normal cellular functions and wound tissue remodeling (5,23–33). Thus, diabetic H-AGE-fed mice healed slowly with thicker scars and with a persistent, nonresolving inflammatory infiltrate. In contrast, in L-AGE-fed diabetic mice, skin wounds healed with a near-normal rate, resulting in healthier healed tissue. Similar findings were reported by agents that reduce AGE toxicity, such as aminoguanidine, an AGE inhibitor (27,34–37), or soluble RAGE (5).

The harmful effects associated with the H-AGE diet were not limited to wound healing; similar effects were demonstrated with respect to renal function, based on albumin/creatinine ratio, consistent with previous studies (12). On the basis of these data, accelerated renal dysfunction and delayed wound healing may be interrelated processes in diabetes, the connecting links being AGE-dependent inflammatory response. Moreover, diet-originating AGEs are important determinants of the total AGE pool and, consequently, of systemic AGE toxicity. Dietary AGE lowering may prove to be an effective approach for promoting normal wound healing, as already suggested for other diabetic complications (12,14–17).

ACKNOWLEDGMENTS

This work was supported, in part, by grants from the National Institute of Diabetes and Digestive and Kidney Diseases (59424 and 60214), the Eastern Paralyzed Veterans Association, and the American Diabetes Association.

We thank Ina Katz for invaluable editorial assistance and Min Lu for technical assistance.

REFERENCES

1. Singer A, Clark R: Cutaneous wound healing. *N Engl J Med* 341:738–746, 1999
2. Moss SE, Klein R, Klein BE: Long-term incidence of lower-extremity amputations in a diabetic population. *Arch Fam Med* 5:391–398, 1996
3. Nwomeh BC, Yager DR, Cohen IK: Physiology of the chronic wound. *Clin Plast Surg* 25:341–356, 1998
4. Brem H, Balledux J, Bloom T, Kerstein M, Hollier L: Healing of diabetic ulcers and pressure ulcers with human skin equivalent: a new paradigm in wound healing. *Arch Surg* 135:627–634, 2000
5. Goova MT, Li J, Kislinger T, Qu W, Lu Y, Bucciarelli LG, Nowygrod S, Wolf BM, Caliste X, Yan SF, Stern DM, Schmidt AM: Blockade of receptor for advanced glycation end-products restores effective wound healing in diabetic mice. *Am J Pathol* 159:513–525, 2001
6. Vlassara H, Palace MR: Diabetes and advanced glycation endproducts. *J Intern Med* 251:87–101, 2002

7. Raj DSC, Choudhury D, Welbourne TC, Levi M: Advanced glycation end products: a nephrologist's perspective. *Am J Kidney Dis* 35:365–380, 2000
8. Dyer DG, Dunn JA, Thorpe SR, Lyons TJ, McCance DR, Baynes JW: Accumulation of Maillard reaction products in skin collagen in diabetes and aging. *Ann N Y Acad Sci* 663:421–422, 1992
9. Koschinsky T, He CJ, Mitsuhashi T, Bucala R, Liu C, Buening C, Heitmann K, Vlassara H: Orally absorbed reactive advanced glycation end products (glycotoxins): an environmental risk factor in diabetic nephropathy. *Proc Natl Acad Sci U S A* 94:6474–6479, 1997
10. He C, Sabol J, Mitsuhashi T, Vlassara H: Dietary glycotoxins: inhibition of reactive products by aminoguanidine facilitates renal clearance and reduces tissue sequestration. *Diabetes* 48:1308–1315, 1999
11. Cai W, Cao Q, Zhu L, Peppia M, He C, Vlassara H: Oxidative stress-inducing carbonyl compounds from common foods: novel mediators of cellular dysfunction. *Mol Med* 8:337–346, 2002
12. Zheng F, He C, Cai W, Hattori M, Steffes M, Vlassara H: Prevention of diabetic nephropathy in mice by a diet low in glycoxidation products. *Diabetes Metab Res Rev* 18:224–237, 2002
13. Peppia M, He CJ, Hattori M, McEvoy R, Feng Z, Vlassara H: Fetal or neonatal low-glycotoxin environment prevents autoimmune diabetes in NOD mice. *Diabetes* 52:1441–1448, 2003
14. Lin RY, Reis ED, Dore AT, Lu M, Ghodsi N, Fallon JT, Fisher EA, Vlassara H: Lowering of dietary advanced glycation endproducts (AGE) reduces neointimal formation after arterial injury in genetically hypercholesterolemic mice. *Atherosclerosis* 163:303–311, 2002
15. Lin RY, Choudhury RP, Cai W, Lu M, Fallon JT, Fisher EA, Vlassara H: Dietary glycotoxins promote diabetic atherosclerosis in apolipoprotein E-deficient mice. *Atherosclerosis* 168:213–220, 2003
16. Hofmann SM, Dong HJ, Cai WJ, Altomonte J, Thung SN, Zeng F, Fisher EA, Vlassara H: Improved insulin sensitivity is associated with restricted intake of dietary glycoxidation products in the db/db mouse. *Diabetes* 51:2082–2089, 2002
17. Vlassara H, Cai W, Crandall J, Goldberg T, Oberstein R, Dardaine V, Peppia M, Rayfield E: Inflammatory mediators are induced by dietary glycotoxins, a major risk factor for diabetic angiopathy. *Proc Natl Acad Sci U S A* 99:15596–15601, 2002
18. Uribarri J, Peppia M, Cai W, Goldberg T, Lu M, He C, Vlassara H: Restriction of dietary glycotoxins markedly reduces AGE toxins in renal failure patients. *J Am Soc Nephrol* 14:728–731, 2003
19. Uribarri J, Peppia M, Cai W, Goldberg T, Baliga S, Lu M, Vassalotti JA, Vlassara H: Dietary glycotoxins correlate with circulating advanced glycation end product levels in renal failure patients. *J Am Kidney Dis* 42:532–538, 2003
20. Monnier VM, Bautista O, Kenny D, Sell DR, Fogarty J, Dahms W, Cleary PA, Lachin J, Genuth S, the DCCT Skin Collagen Ancillary Study Group: Skin collagen glycation, glycoxidation, and crosslinking are lower in subjects with long-term intensive versus conventional therapy of type 1 diabetes: relevance of glycated collagen products versus HbA_{1c} as markers of diabetic complications. *Diabetes* 48:870–880, 1999
21. Schleicher ED, Wagner E, Nerlich AG: Increased accumulation of the glycoxidation product N (epsilon)-(carboxymethyl)lysine in human tissues in diabetes and aging. *J Clin Invest* 99:457–468, 1997
22. Beisswenger PJ, Makita Z, Curphey TJ, Moore LL, Jean S, Brinck-Johnsen T, Bucala R, Vlassara H: Formation of immunochemical advanced glycosylation end products precedes and correlates with early manifestations of renal and retinal disease in diabetes. *Diabetes* 44:824–829, 1995
23. Brennan M: Changes in solubility, non-enzymatic glycation, and fluorescence of collagen in tail tendons from diabetic rats. *J Biol Chem* 264:20947–20952, 1989
24. Watala C, Golanski J, Witas H, Gurbel R, Gwozdinski K, Trojanowski Z: The effects of in vivo and in vitro non-enzymatic glycosylation and glycoxidation on physico-chemical properties of haemoglobin in control and diabetic patients. *Int J Biochem Cell Biol* 28:1393–1403, 1996
25. Duraisamy Y, Slevin M, Smith N, Bailey J, Zweit J, Smith C, Ahmed N, Gaffney J: Effect of glycation on basic fibroblast growth factor induced angiogenesis and activation of associated signal transduction pathways in vascular endothelial cells: possible relevance to wound healing in diabetes. *Angiogenesis* 4:277–288, 2001
26. Ido Y, Chang KC, Lejeune WS, Björck RJ, Reiser KM, Williamson JR, Tilton RG: Vascular dysfunction induced by AGE is mediated by VEGF via mechanisms involving reactive oxygen species, guanylate cyclase, and protein kinase C. *Microcirculation* 8:251–263, 2001
27. Portero-Otin M, Pamplona R, Bellmunt MJ, Ruiz MC, Prat J, Salvayre R, Negre-Salvayre A: Advanced glycation end product precursors impair epidermal growth factor receptor signaling. *Diabetes* 51:1535–1542, 2002
28. Twigg SM, Joly AH, Chen MM, Tsubaki J, Kim HS, Hwa V, Oh Y, Rosenfeld RG: Connective tissue growth factor/IGF-binding protein-related protein-2 is a mediator in the induction of fibronectin by advanced glycosylation end-products in human dermal fibroblasts. *Endocrinology* 143:1260–1269, 2002
29. Imani F, Horii Y, Suthanthiran M, Skolnik EY, Makita Z, Sharma V, Sehajpal P, Vlassara H: Advanced glycosylation endproduct-specific receptors on human and rat T-lymphocytes mediate synthesis of interferon gamma: role in tissue remodeling. *J Exp Med* 178:2165–2172, 1993
30. Collison KS, Parhar RS, Saleh SS, Meyer BF, Kwaasi AA, Hammami MM, Schmidt AM, Stern DM, Al-Mohanna FA: RAGE-mediated neutrophil dysfunction is evoked by advanced glycation end products (AGEs). *J Leukoc Biol* 71:433–444, 2002
31. Bernheim J, Rashid G, Gavrieli R, Korzets Z, Wolach B: In vitro effect of advanced glycation end-products on human polymorphonuclear superoxide production. *Eur J Clin Invest* 31:1064, 2001
32. Abordo EA, Westwood ME, Thornalley PJ: Synthesis and secretion of macrophage colony stimulating factor by mature human monocytes and human monocytic THP-1 cells induced by human serum albumin derivatives modified with methylglyoxal and glucose-derived advanced glycation endproducts. *Immunol Lett* 53:7–13, 1996
33. Daoud S, Schinzel R, Neumann A, Loske C, Fraccarollo D, Diez C, Simm A: Advanced glycation endproducts: activators of cardiac remodeling in primary fibroblasts from adult rat hearts. *Mol Med* 7:543–551, 2001
34. Kochakian M, Manjula BN, Egan JJ: Chronic dosing with aminoguanidine and novel advanced glycosylation end product-formation inhibitors ameliorates cross-linking of tail tendon collagen in STZ-induced diabetic rats. *Diabetes* 45:1694–1700, 1996
35. Teixeira AS, Caliair MV, Rocha OA, Machado RD, Andrade SP: Aminoguanidine prevents impaired healing and deficient angiogenesis in diabetic rats. *Inflammation* 23:569–581, 1999
36. Teixeira AS, Andrade SP: Glucose-induced inhibition of angiogenesis in the rat sponge granuloma is prevented by aminoguanidine. *Life Sci* 64:655–662, 1999
37. Yavuz D, Tugtepe H, Kaya H, Celikel S, Deyneli O, Uyar S, Akalin S: Effects of aminoguanidine on wound healing in a diabetic rat model (Abstract). *Diabetes* 51 (Suppl. 2):A256, 2002
38. Davidson JM: Animal models in wound repair. *Arch Dermatol Res* 290 (Suppl.):S1–S1137, 1998
39. *Nutrient Requirements of Laboratory Animals*. Washington, DC, The National Academy of Sciences, National Academy Press, 1995
40. Mellinghoff AJ, Wuerth JP, Founds HW, Landgraf R, Hepp KD: Formation of plasma advanced glycosylation end products (AGEs) has no influence on plasma viscosity. *Diabet Med* 14:832–836, 1997
41. Draper B, Komurasaki T, Davidson M, Nannery L: Topical epiregulin enhances repair of murine excisional wounds. *Wound Repair Regen* 11:188, 2003
42. Yu W, Naim JO, Lanzafame RJ: Effects of photostimulation on wound healing in diabetic mice. *Lasers Surg Med* 20:56–63, 1997
43. Ehrlich HP, Desmouliere A, Diegelmann RF, Cohen IK, Compton CC, Garner WL, Kapanci Y, Gabbiani G: Morphological and immunochemical differences between keloid and hypertrophic scar. *Am J Pathol* 145:105–113, 1994
44. Vlassara H, Fuh H, Donnelly T, Cybulsky M: Advanced glycation endproducts promote adhesion molecule (VCAM-1, ICAM-1) expression and atheroma formation in normal rabbits. *Mol Med* 1:447–456, 1995
45. Stitt AW, Li YM, Gardiner TA, Archer DB, Vlassara H: Advanced glycation end products (AGEs) co-localize with AGE receptors in the retinal vasculature of diabetic and of AGE-infused rats. *Am J Pathol* 150:523–531, 1996
46. Nakamura Y, Horii Y, Nishino T, Shiiki H, Sakaguchi Y, Kagoshima T, Dohl K, Makita Z, Vlassara H, Bucala R: Immunohistochemical localization of advanced glycosylation endproducts (AGEs) in coronary atheroma and cardiac tissue in diabetes mellitus. *Am J Pathol* 143:1649–1656, 1993
47. O'Brien J, Morrissey PA: Nutritional and toxicological aspects of the Maillard browning reaction in foods. *Crit Rev Food Sci Nutr* 28:211–248, 1989
48. Pellegrino L, Cattaneo S: Occurrence of galactosyl isomaltol and galactosyl beta-pyranone in commercial drinking milk. *Nahrung* 45:195–200, 2001
49. Henle T: A food chemist's view of advanced glycation end-products. *Perit Dial Int* 21 (Suppl. 3):S125–S30, 2001