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

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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).

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

TABLE 1
Characteristics of diet formulas

	Test diets*		AIN†
	H-AGE	L-AGE	(recommended)
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; Alteon, 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+/+ Lepr^{db} 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 are 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 CO_2 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 mice exposed to H-AGE and L-AGE diets

		H-AGE		L-AGE	
		Baseline	End	Baseline	End
Body weight (g)	*db/db*	51 ± 0.7	53 ± 0.5	50 ± 0.5	51 ± 0.6
	non-*db*	22.7 ± 0.4	23.8 ± 0.3	22 ± 0.4	23.1 ± 0.4
Blood glucose (mg/dl)	*db/db*	358 ± 34	424 ± 43	293 ± 26	373 ± 25
	non-*db*	114 ± 7	110 ± 6.5	117 ± 5	106 ± 5
Food intake (g/day)	*db/db*	5 ± 1	6.2 ± 0.5	5.2 ± 0.4	6 ± 0.5
	non-*db*	4 ± 0.3	4.3 ± 0.1	3.9 ± 0.4	4.1 ± 0.2
sCML (units/ml)	*db/db*	69 ± 4.1	104 ± 7.2*†	71 ± 2.1	65 ± 2.8†
	non-*db*	53.4 ± 2.5	64.3 ± 5.5*	52.2 ± 2.7	48.7 ± 2.3
sMG (nmol/ml)	*db/db*	1.7 ± 0.1	2.4 ± 0.1*†	1.7 ± 0.1	1.6 ± 0.1
	non-*db*	1.4 ± 0.09	1.8 ± 0.1*†	1.4 ± 0.06	1.4 ± 04
Urinary CML excretion (units/min)	*db/db*	334 ± 29	186 ± 19*†	278 ± 17	285 ± 20
	non-*db*	79.2 ± 20.5	187.5 ± 62.3	71 ± 8	94.6 ± 23
Urinary MG excretion (nmol/min)	*db/db*	2.4 ± 0.2	1.14 ± 0.3*†	2 ± 0.1	2.04 ± 0.1
	non-*db*	1.9 ± 0.6	3.2 ± 0.5*	1.5 ± 0.2	1.8 ± 0.3
Urinary albumin/creatinine ratio (μg/mg)	*db/db*	1.6 ± 0.1	3.9 ± 0.3†	1.6 ± 0.2	1.9 ± 0.2
	non-*db*	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 $P \leq 0.05$. 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. 1*A* 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. 1*C* 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. 2*A* and Fig. 3*A–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. 2*A*).

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

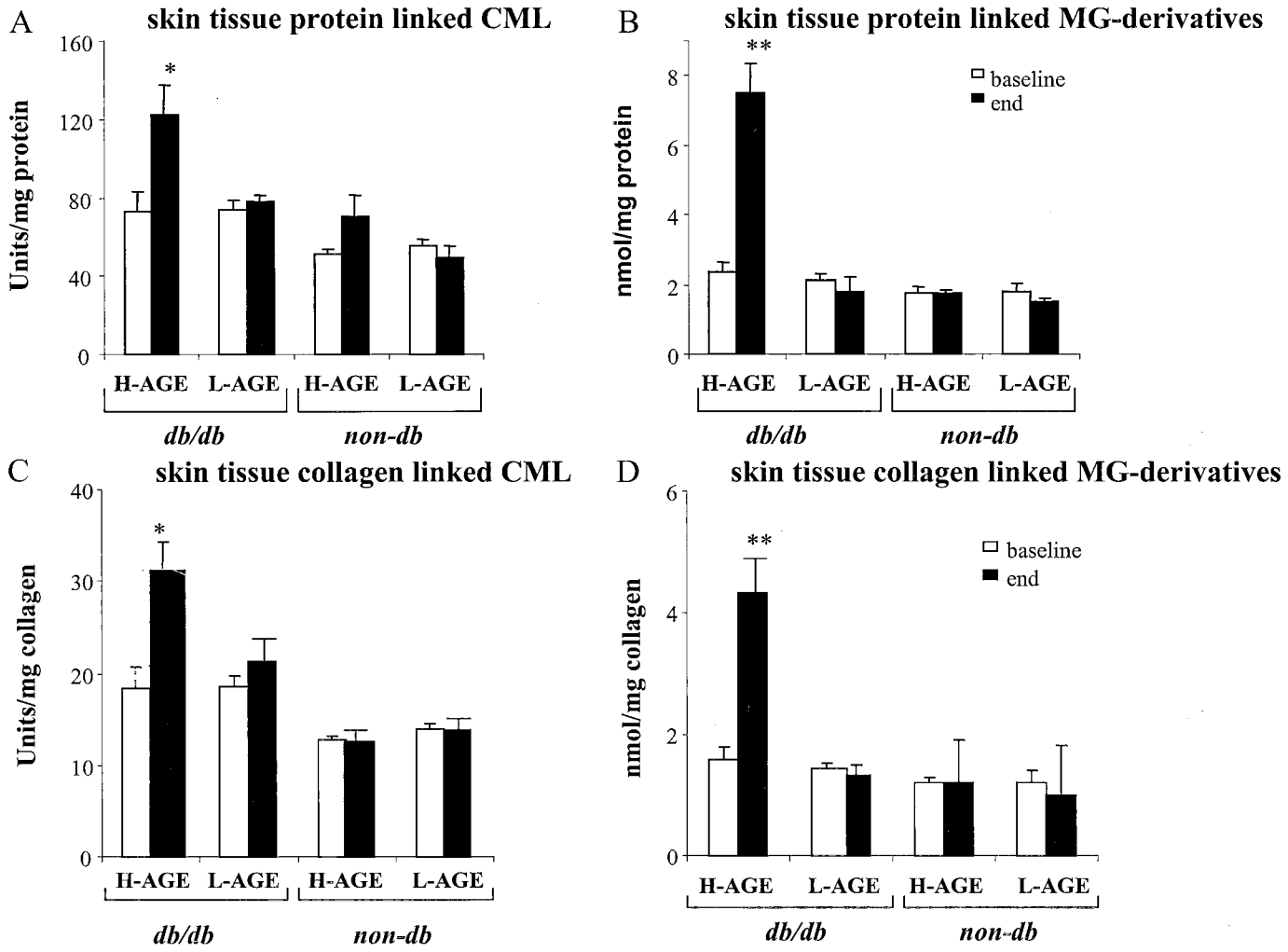

FIG. 1. Skin tissue AGE levels in diabetic and nondiabetic *db/db* mice correlate with dietary AGE intake. At baseline (12 weeks of age) and 12 weeks after exposure to either H-AGE or L-AGE diet, skin tissue sections were homogenized and digested with proteinase-K (protein-linked AGE; *A* and *B*) or 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 ± 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. 4*B* and *D* and 5*A* and *C*). On day 14, L-AGE–fed mouse wounds displayed robust reepithelialization, granulation tissue deposition, inflammatory cell infiltration, and angiogenesis (Fig. 4*A*–*C*). In contrast, in the wounds from H-AGE–fed mice, all of these parameters were delayed and attenuated (Figs. 4*B* and *D* and 5*A* 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. 4*A*–*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. 4*B* and 5*B* and *D*), fewer blood vessels, and a lower degree of reepithelialization (Fig. 4*A*). 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. 2*B* and 6*E* and *F*).

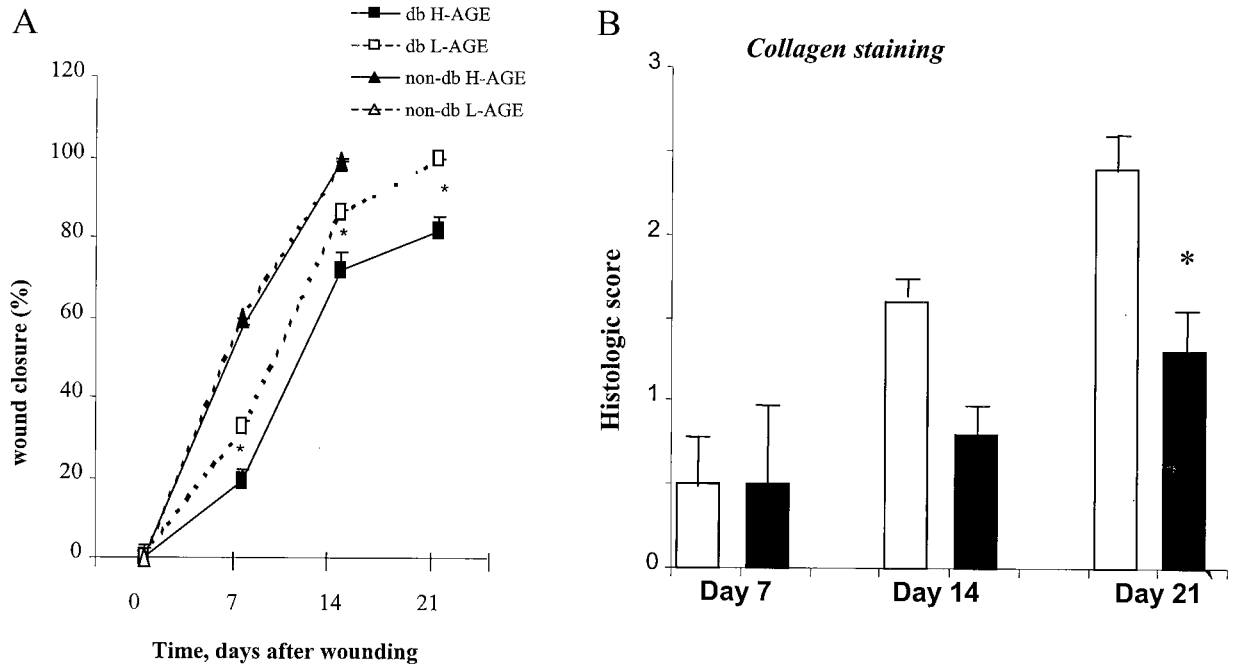

FIG. 2. Time to closure and histologic changes of diabetic wounds correlate with dietary AGE intake. *A*: After 12 weeks' exposure to either L-AGE or H-AGE diets, full excisional wounds (1 cm) were created at the back of the mice and the time to closure was evaluated at days 7, 14, and 21 after wounding (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 ± 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,

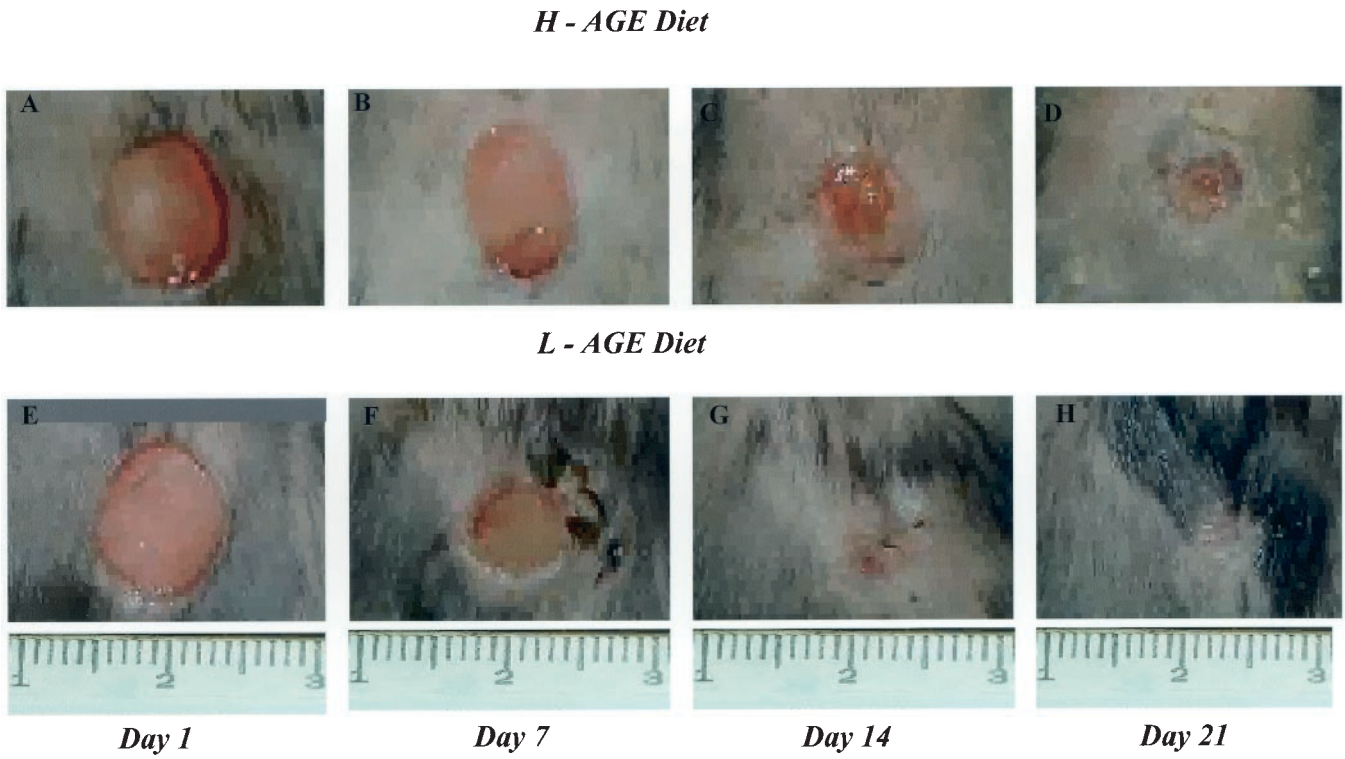

FIG. 3. Wound closure in *db/db* mice is modulated by exogenous (dietary) AGE. After exposure to L-AGE or H-AGE diets, wound closure was documented with a digital camera at a fixed distance from the wound at different time points. *A* and *E*: Day 1. *B* and *F*: Day 7. *C* and *G*: Day 14. *D* and *H*: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

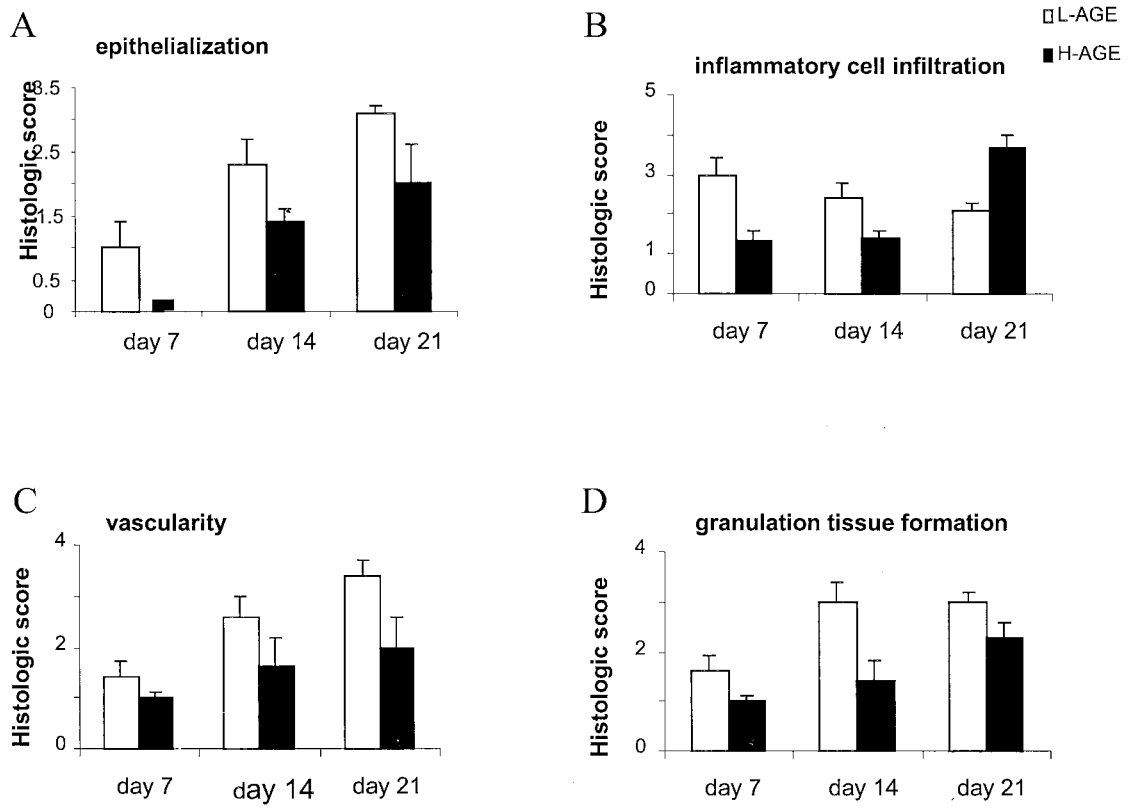

FIG. 4. Histochemical evaluation of wounds from diabetic (*db/db*) mice; association with dietary AGE intake. After 12 weeks on the diets, sections were stained with H&E and epithelialization (*A*), inflammatory cell infiltration (*B*), vascularity (*C*), and granulation tissue formation (*D*) were evaluated. Data were calculated based on histological score, as described and expressed as mean ± 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-

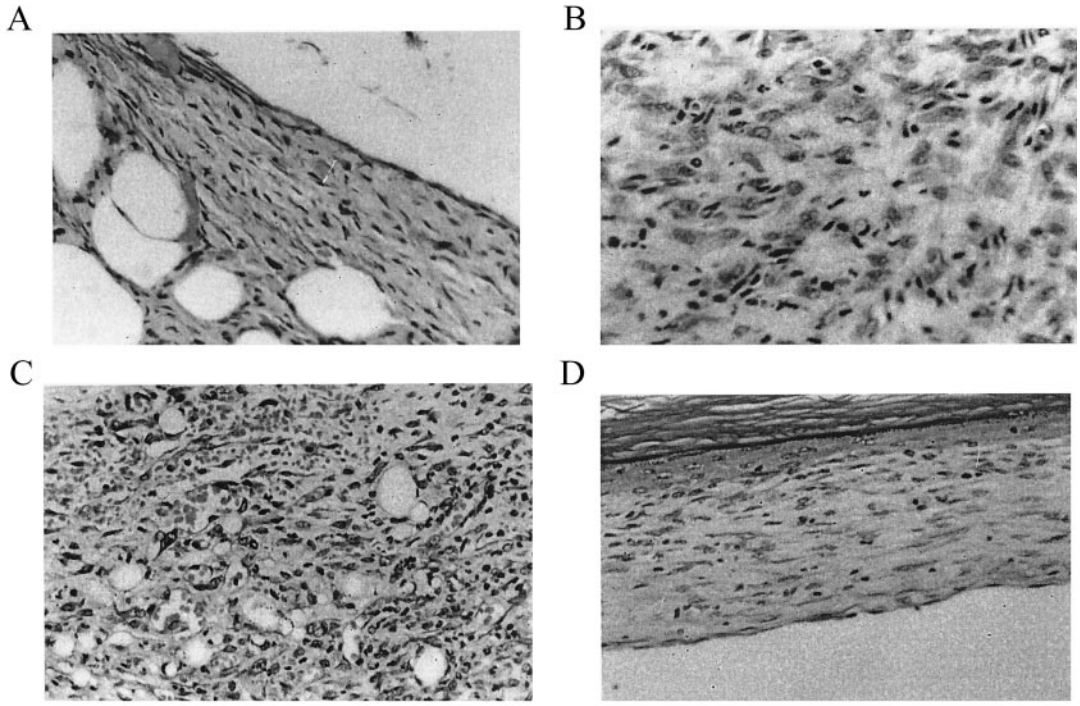

FIG. 5. Skin wound from diabetic mice exposed to H-AGE diet exhibit delayed but sustained nonsubsiding inflammatory response. Wound skin sections from diabetic *db/db* mice exposed to either H-AGE diet (*A* and *B*) or L-AGE diet (*C* and *D*), stained with H&E. Note distinct pattern of inflammatory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

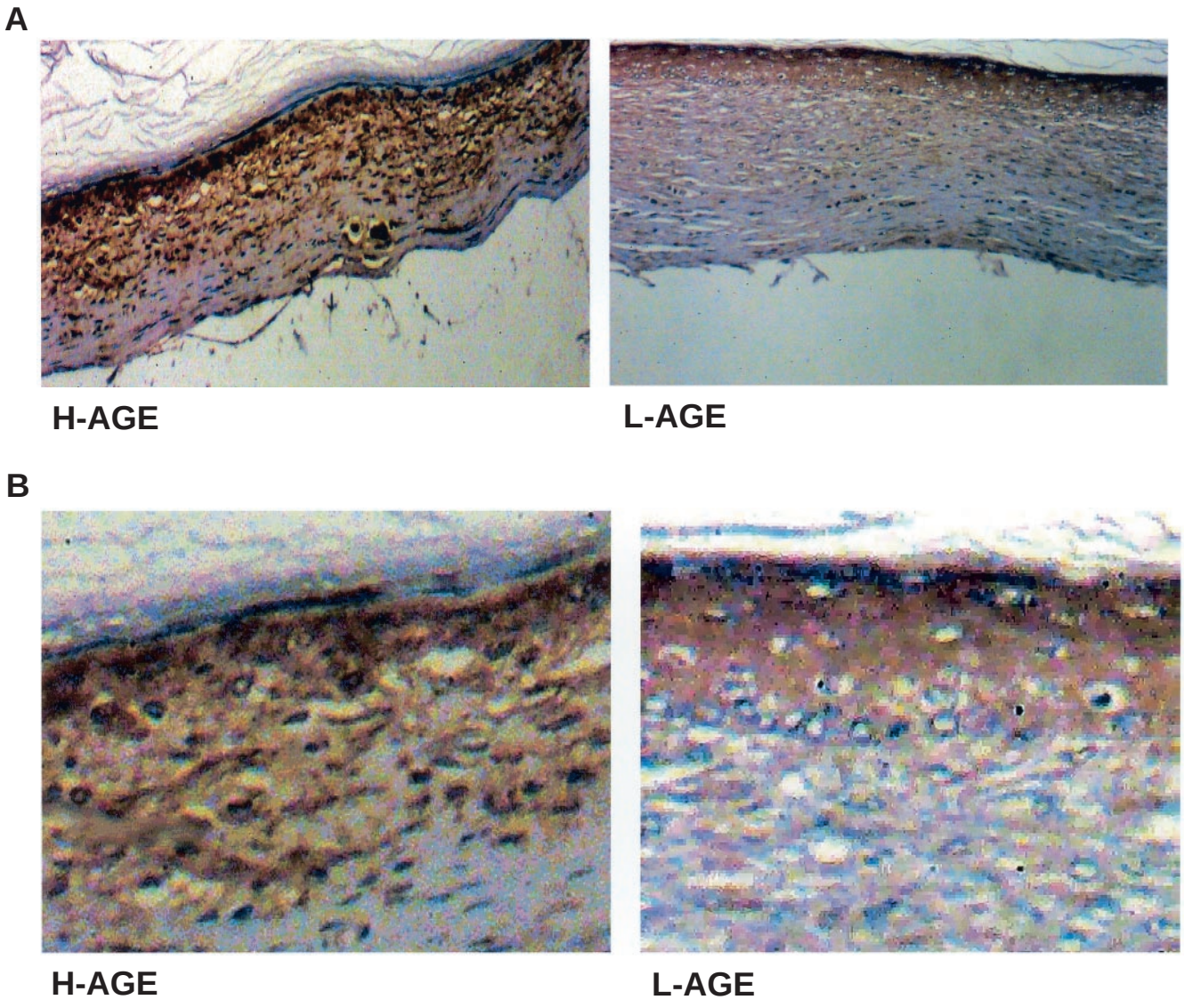

FIG. 6. Collagen staining in wounds from diabetic (*db/db*) mice exposed to L-AGE or H-AGE diet. Skin tissue samples from full-thickness excisional wounds from diabetic (*db/db*) mice were stained with Sirius red and scored at different time points. *A* and *B*: Day 7. *C* and *D*: Day 14. *E* and *F*: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

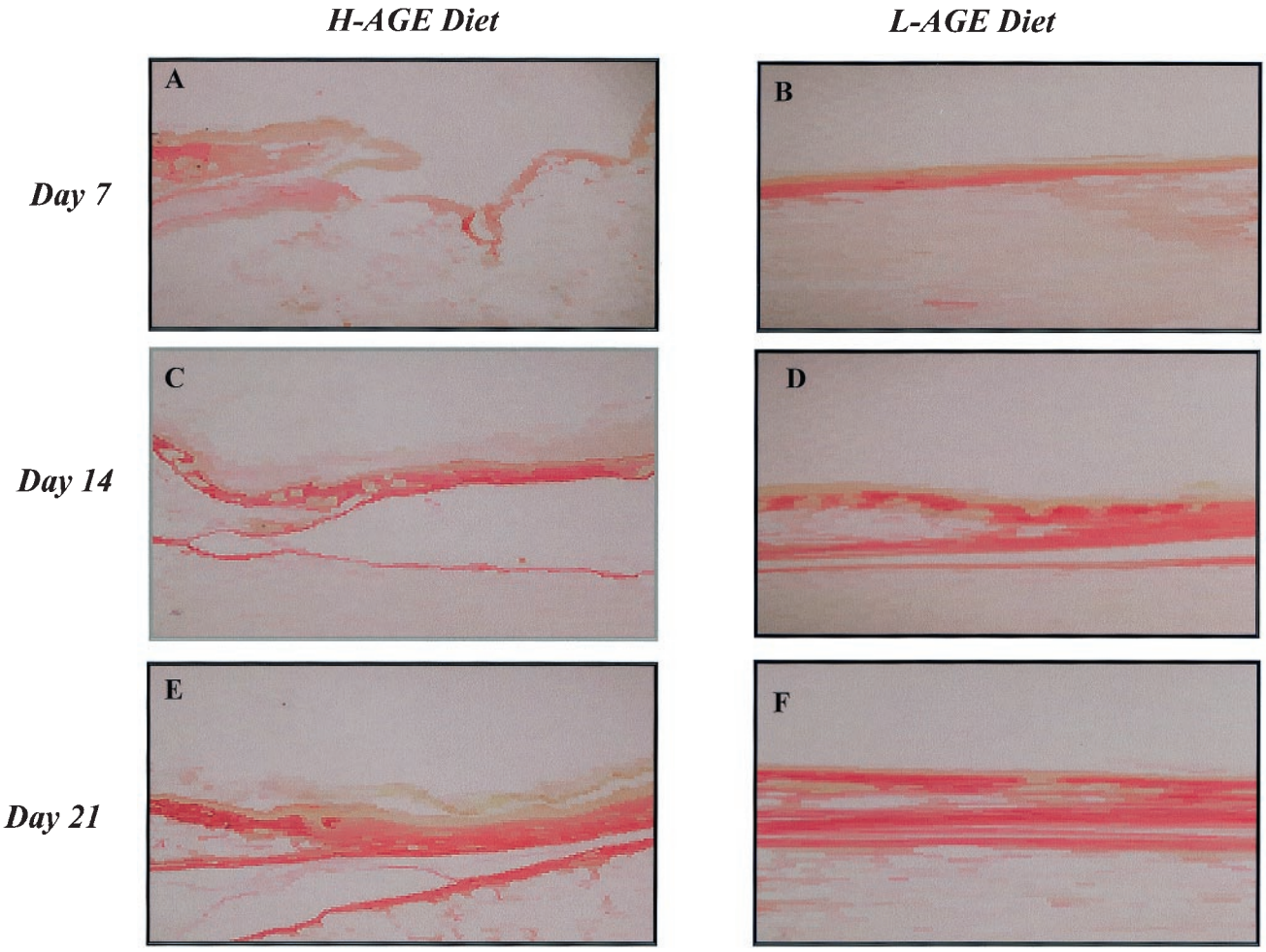

FIG. 7. AGE deposition in skin wounds from diabetic mice is influenced by diet-derived AGE. *A:* Wound skin tissue sections from diabetic *db/db* mice exposed to H-AGE or L-AGE diet were stained with anti-AGE antibody at day 21 postwounding. Magnification ×40. *B*: Enlargement of sections shown in *A* displays AGE distribution in skin tissue components. Magnification ×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).

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