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Delayed healing of oral mucosa in a diabetic rat model: Implication of TNF-α, IL-1β and FGF-2

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1. Introduction

Diabetes mellitus is a group of diseases characterized by persistent hyperglycemia, which culminates in various pathological changes, including neuropathy, retinopathy, nephropathy, cardiovascular diseases and impaired wound healing. There is a lack of resolution of the healing phases (inflammatory, proliferative and remodeling) resulting in delayed healing or in a chronic wound. [1,2].

In normal patitens, during the inflammatory phase of healing (24–48 h post-wounding), neutrophils are the first cells that migrate into the damaged tissue; these cells comprise approximately 50% of the cells at the wound site, where they play a protective role due to phagocytosis. At the fifth day post-wounding, the number of neutrophils has declined, concomitantly with the increase in epithelialization, and monocytes/macrophages and lymphocytes become the main cells at the ulcer site. Those cells play a critical role in the repair process by producing cytokines and growth factors [3]. DM appears to modulate the inflammatory phase of wound healing by hampering the initial recruitment of inflammatory cells and lowering the leukocyte-mediated bactericidal activity and the levels of the inflammatory cytokines interleukin (IL)-1β and tumor necrosis factor-alpha (TNF-α) [4]. DM also hinders the proliferative phase (re-epithelialization, fibroplasia and angiogenesis) of wound healing, during which the wound is closed, through causing the decreased/delayed expression of growth factors, such as tumor growth factor -beta (TGF-β), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), which negatively affects the recruitment and proliferation of fibroblasts and vascular cells, collagen deposition and angiogenesis [5]. FGF is a potent mitogen produced by fibroblasts and endothelial cells that promotes angiogenesis and granulation-tissue formation. The FGF-2 isoform is present in the basal, parabasal and superficial epithelial layers and in connective cells (eosinophils, mast cells, macrophages and fibroblasts) but its distribution may change under certain pathological conditions [6]. The remodeling phase of normal wound healing is marked by the maturation of and changes in the extracellular matrix, such as the deposition of proteoglycans and collagen therein. During this phase, fibroblasts differentiate into myofibroblasts, which can contract like smooth muscle cells, leading to the reorganization of the provisory extracellular matrix to form the definitive extracellular matrix and to wound closure (ulcer contraction). Thus, a delicate balance between the fibroblasts and myofibroblasts at a wound site is critical for effective tissue repair [7]. In the remodeling phase of wounds in diabetic patients there is increased apoptosis of keratinocytes, fibroblasts and endothelial cells, leading to maturation and collagen deposition and a reduction in the levels of several growth factors [8].

Most studies of wound healing have focused on cutaneous healing [9] rather than healing of the oral mucosa, particularly those concerning pathological conditions such as diabetes. Moreover, there is no established protocol for the treatment of traumatic ulcerated oral lesions in diabetic patients, who are treated only to ameliorate their symptoms [10]. Thus, the aim of this study was to investigate the differences in the healing of ulcerated oral mucosal lesions in diabetic rats compared with those of normal rats.

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2. Methods

2.1. Chemicals

Alloxan monohydrate was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Animals

This study was approved by the Ethics Committee for Animal Research of the State University of Ceará (CEUA/UECE; protocol no. 12066654-5/17) and was performed in accordance with the Ethical Principles for Animal Experimentation adopted by the Brazilian College of Animal Experimentation (COBEA).

Adult male Wistar rats (2–3 months old; 200–300 g; 6–8 animals per group) were maintained in plastic cages containing pine-sawdust covered floors, at 24 °C with a dark-light cycle of 12 h and with free access to water and commercial food (Bio-base, Águas Frias, Santa Catarina, Brazil).

2.3. Induction of diabetes and oral ulcers

Diabetes was induced by intravenous injection of alloxan (45 mg/kg) diluted in 0.15 M NaCl. Control animals received sterile saline. Two milliliters of blood was collected from the retro-orbital plexus at 48 h after diabetes induction for determination of the glucose levels. Animals were considered diabetic when their blood glucose level was ≥200 mg/dL.

Oral mucosal ulceration of the diabetic and normoglycemic animals was performed at 48 h post-treatment. Oral mucosal antisepsis was achieved using cotton pellets soaked in 0.12% chlorhexidine gluconate. The left buccal mucosa was ulcerated by abrasion with a no. 15 scalpel blade using a marker (8-mm diameter) after the animals were anesthetized via an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (8 mg/kg) [11].

2.4. Clinical analysis

The animals were weighed at day zero (initial weight) and at 1, 5, 10, 15, 20 days (final weight) after ulceration. Their body mass was evaluated according to the following formula: final weight − initial weight. The animals were sacrificed at 1, 5, 10, 15 and 20 days after ulceration. The ulcers were measured using a 0.5-mm precision digital paquimeter (D = largest diameter and d = smaller diameter) and the sizes of the ulcerated areas were expressed using the following formula: $A = \pi \cdot D/2 \cdot d/2$. The area of a contracted wound was calculated using the equation $100 \times (Wi - Wo) / Wo$ (Wo = initial area; Wi = final area) and was expressed as the percentage of contraction [12].

2.5. Histopathological analysis

Cheek mucosal specimens were collected at 1, 5, 10, 15 and 20 days post-ulceration, fixed in 10% formol for 24 h, dehydrated using a graduated alcoholic series, diaphanized using xylol, and impregnated with paraffin. The specimens were placed in molds at room temperature and were cut into 4-μm thick sections, which were stained with hematoxylin and eosin (HE). The sections were analyzed using an optical microscope (Motic BA310 microscope equipped with a Moticam 2000 camera 2.0 M Pixel and the Motic 2.0 program), and the histological characteristics of the ulcers and the corresponding cicatrization phases were described.

The histopathological parameters of the ulcers were scored using a scale of 0 to 4, as follows: (0) no ulcer/remodeled connective tissue (mature, homogeneous and parallel collagen fibers); (1) no ulcer/fibrosis (remarkable and diffuse deposition of collagen fibers) , with mild chronic inflammation; (2) ulcer/fibrosis, with moderate chronic inflammation; (3) ulcer/chronic inflammation (granulation tissue); (4) ulcer/acute process (dilated vessels and mixed inflammatory infiltrate containing neutrophils) [11].

2.6. Histomorphometric analysis (neutrophil infiltration, fibroplasia, angiogenesis and collagenesis)

To assess PMN (polymorphonuclear) infiltration and fibroplasia, three fields of the ulcerated areas in 3 or 4 slides from different animals (HE staining) per group were photographed (using a Motic BA310 microscope equipped with a Moticam 2000 camera 2.0 M Pixel and the Motic 2.0 program; 400× magnification). Within each field, 8 microscopic fields (250 × 250 μm) were chosen for counts of the number of neutrophils (cells with a segmented and multilobular nucleus) and fibroblasts/myofibroblasts (cells with a fusiform morphology) [13]. For quantitative analysis of the extent of angiogenesis (blood vessels excluding endothelial sprouts), images of 4 fields (3–4 slides per group; 100× magnification) including the region spanning the epithelium to the connective tissue were captured. The plugin tool "Cell Counter" in ImageJ (RSB) software was used for this analysis [14].

Collagen deposition was evaluated by examining sections (3-μm thickness) of oral ulcers that had been stained using picrosirius dye. Five fields of these sections were photographed (using a Motic BA310 microscope equipped with a Moticam 2000 camera 2.0 M Pixel and the Motic 2.0 program; 200× magnification). The photomicrographs were quantitatively analyzed using ImageJ software (National Institutes of Health, USA) after calibration using the Color Threshold command (Image > Adjust > Color Threshold) of the RGB function for red (minimum 71, maximum 255), green (minimum 0, maximum 69) and blue (minimum 0, maximum 92) [14].

2.7. Immunohistochemistry

Samples of tissues containing the oral ulcers were cut into 3-μm thick sections, which were placed on silanized slides and processed. Briefly, the samples were deparaffinized, rehydrated and subjected to antigen-recovery using a citrate buffer (pH 6.0). To inactivate the endogenous peroxidase, the specimens were incubated (10 min; r.t.) with 6% H_2O_2 in methanol (1:1), washed with Tris buffer at pH 7.6, incubated for 1 h (r.t.) with primary antibodies (Ab) directed against α-smooth muscle actin (α-SMA) (monoclonal; Dako "DAKOM0851"; 1:200); FGF-2 (polyclonal; Santa Cruz Biotechnology "SC-79"; 1:100); TNF-α (monoclonal; Abcam "AB1793"; 1:50) and IL-1β (monoclonal; Abcam "AB9787"; 1:100), washed and then incubated (30 min; r. t.) with biotinylated immunoglobulin (Ig; DAKO E0468) and streptavidin (DAKO P0397). Subsequently, diaminobenzidine chromogen (DAKO K3469) was applied to the specimens for 10 min. Mayer's hematoxylin was used as a counterstain, after which the specimens were dehydrated (using ethanol and xylene) and cover-slipped using a permanent mounting medium. Parallel sections were treated with control IgG instead of a primary Ab.

In the case of α-SMA immunostaining, blood vessels in the connective tissue (or granulation tissue) adjacent to the ulcer were considered the internal control. Myofibroblasts were identified as cells with a fusiform morphology (non-vascular and non-inflammatory cells) showing brown cytoplasmic staining. In the case of the FGF-2, TNF-α and IL-1β immunostaining of several type of cells (epithelial, mesenchymal and inflammatory cells), epithelial and connective tissue cells in samples of non-ulcerated rat oral mucosa served as the positive controls (data not shown).

Semiquantitative immunohistochemical evaluation was performed by three different observers. Five randomly selected fields (400× magnification) in regions with a higher concentration of immunostained cells located in connective tissue (or granulation tissue) next to the ulcerated area or in the epithelial tissue of ulcerated area were examined. The percentages of cells with cytoplasmic or nuclear immunostaining

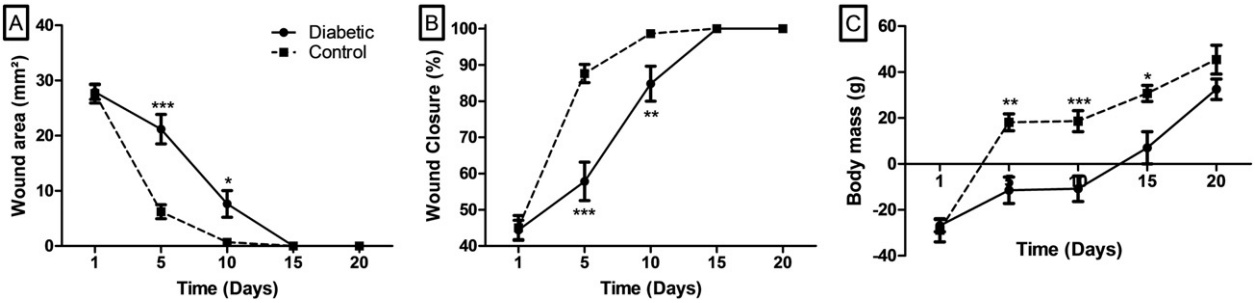

Fig. 1. Values of the Wound Area, Wound Closure and Variation in body mass on the time- course of oral ulcers in the Control and Diabetic groups. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (versus control in same day), Two-way-ANOVA/Bonferroni (Mean ± SE).

were scored on a scale of 0 to 3, as follows: (0) no positive cells; (1 — mild) 1–33% positive cells; (2 — moderate) 34–66% positive cells; (3 — intense) 67–100% positive cells. The final score was considered the same score given by two blinded observers (kappa = 0.821) [15].

2.8. Vascular permeability

Measurements of vascular permeability, not previously conducted in this model, were obtained using the Evans-blue extravasation assay. For this assay, Evans blue (25 mg/kg; i.v.) was injected 1 h before euthanasia was performed. Ulcerated and non-ulcerated oral mucosal samples (control and diabetic) were removed on the 1st, 5th and 10th days post-wounding. The wound edge and control samples were harvested, and mucosal rings of 3-mm in diameter were weighed. The Evans-blue dye was extracted by incubating the mucosal samples in formamide at 37 °C for 48 h, and the amount of extracted dye was photometrically measured at $A_{60}0$ nm (μg Evans blue/g tissue) [16].

2.9. Statistical analysis

The results were expressed as the mean values ± SE (standard error). For multiple comparisons of parametric data, an analysis of variance (ANOVA, one-way or two-way, with the Bonferroni correction) was performed. The results of the histopathological and immunohistochemical evaluations were expressed as the median (maximum and minimum) value and were analyzed using the Mann-Whitney test. The Pearson correlation method was applied to the linear data (ulcer area and weight loss) and the Spearman correlation method was applied to the nonlinear data (histological scores). Differences were considered significant at $p < 0.05$. The analyses were performed using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Clinical parameters

There was a significant delay in the wound healing of the diabetic rats compared with that of the control rats, as demonstrated by measuring the areas of the wounds on the 5th and 10th days post-wounding. A significant reduction in wound closure was observed in the diabetic animals at day 5 compared with that observed at day 10 post-ulceration. The body mass of the diabetic animals was significantly decreased at days 5, 10 and 15 (Fig. 1).

3.2. Histopathological parameters

On the first day post-wounding, the histopathological profiles of the lesions of the diabetic and control rats were similar, with ulcers, vascular ectasia, signs of acute inflammation, neutrophil infiltrates

Table 1
Histopathological and immunohistochemistry assessment on the time–course of oral ulcers in the control and diabetic groups.

		Experimental day				
	Group	1st	5th	10th	15th	20th
Histologic scores	Control	4 (4–4)	3 (1–4)	1 (0–2)	0 (0–1)	0 (0–0)
	Diabetic	4 (4–4)	4 (3–4)*	1 (1–4)*	1 (0–1)*	0 (0–0)
Anti-AML scores	Control	–	3 (2–3)	1.5 (1–2)	2 (1–2)	1 (1–2)
	Diabetic	–	1.5 (1–2)*	3 (2–3)*†	2 (1–3)‡	2 (1–3)
Anti-FGF2 scores (epithelium)	Control	–	3 (3–3)	3 (3–3)	3 (2–3)	3 (2–3)
	Diabetic	–	1 (1–2)*	1 (1–2)*	3 (2–3)‡	2.5 (2–3)
Anti-FGF2 scores (connective tissue)	Control	–	2 (1–2)	2 (1–2)	2 (1–2)	2 (1–2)
	Diabetic	–	1 (0–1)*	1 (0–1)*	1 (1–1)	1.5 (1–2)
Anti-TNFα scores (epithelium)	Control	2 (1–3)	1 (1–2)	1 (1–2)	1 (0–2)	1 (0–1)§
	Diabetic	2 (1–3)	1.5 (1–3)	2 (1–3)	2 (2–3)*	1.5 (1–3)*
Anti-TNFα scores (connective tissue)	Control	3 (3–3)	2 (0–3)	1 (0–3)	2 (1–2)§	1.5 (1–2)§
	Diabetic	3 (3–3)	3 (3–3)*	1 (0–3)†	2 (2–3)	2 (1–2)
Anti-IL-1β scores (epithelium)	Control	3 (2–3)	1.5 (1–3)	0 (0–1)§	1 (0–2)§	0 (0–1)§
	Diabetic	3 (2–3)	3 (1–3)	3 (2–3)*	3 (2–3)*	2.5 (1–3)*
Anti- IL-1β scores (connective tissue)	Control	3 (2–3)	1 (1–2)	1 (0–1)§	1 (1–1)§	0 (0–1)§
	Diabetic	3 (2–3)	3 (2–3)*	2 (2–3)*	1 (1–3)	1 (0–1)§

FGF-2 = anti-fibroblast growth factor-2; α-SML = smooth muscle actin; TNF-α = tumor necrosis factor-alfa; IL-1β = inteleukin-1beta. Mann-Whitney (Median (Minimum–Maximum)).

* $p < 0.05$ (versus control in same day).
† $p < 0.05$ (versus 5th day in same group).
‡ $p < 0.05$ (versus 10th day in same group).
§ $p < 0.05$ (versus 1st day in same group).

and necrotic foci present. Significant differences in the median histopathological scores of the lesions of the two groups were observed at days 5, 10 and 15 after wounding, showing that the lesions of the control animals healing more rapidly (Table 1, Fig. 2). At day 20, histology showed that the lesions of the two groups were healed.

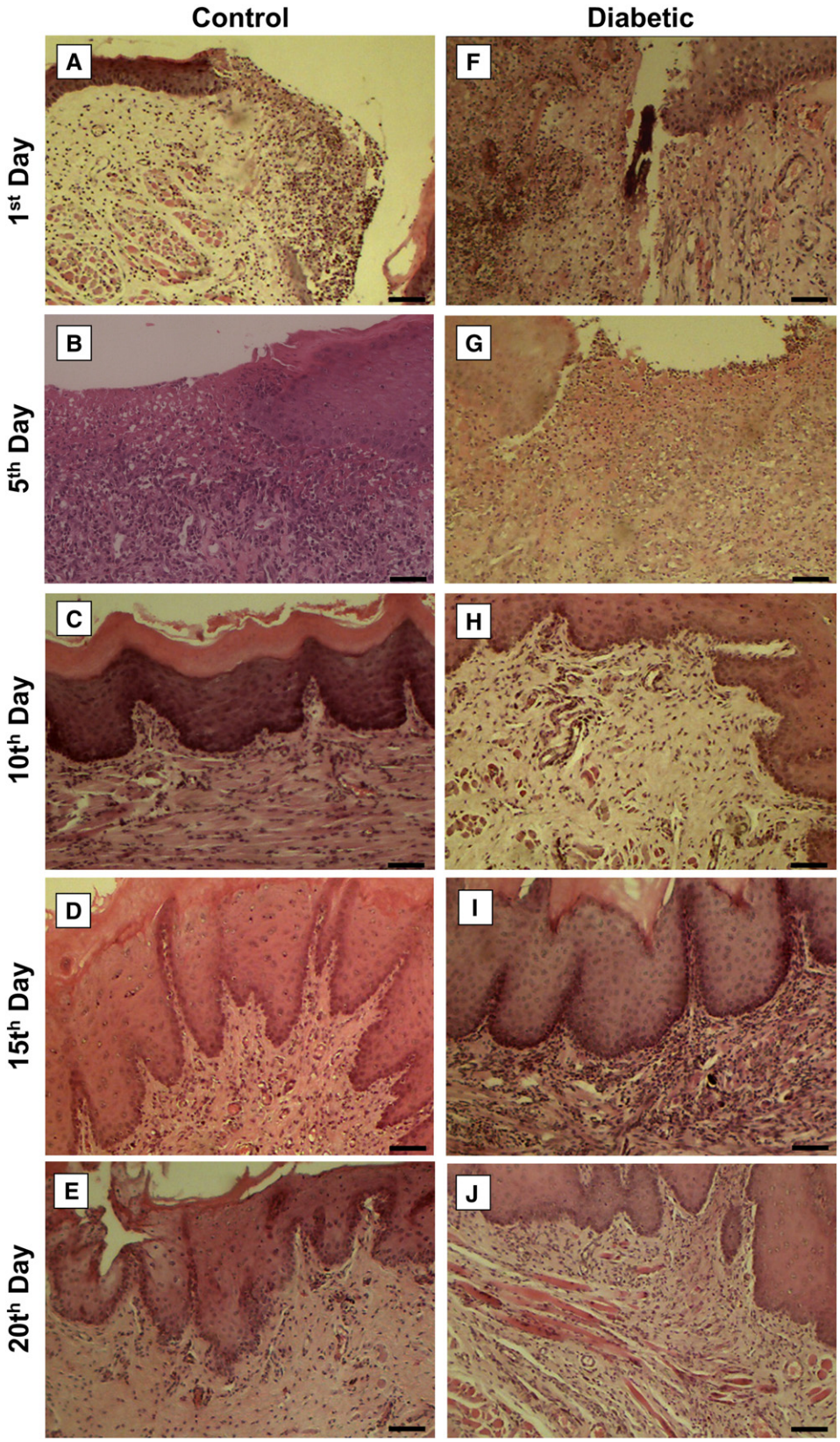

Fig. 2. Photomicrography of ulcer area in rat cheek mucosa. Control (A–E) and diabetic (F–J) at the 1st, 5th, 10th, 15th and 20th days after ulceration (HE, 200×) (bars = 100 μm).

Table 2
Histomorphometric assessment of neutrophil infiltration, fibroplasia and angiogenesis on the time-course of oral ulcers in the control and diabetic groups.

		Experimental day				
	Group	1st	5th	10th	15th	20th
Number of neutrophil	Control	254.3 ± 35.6	63.3 ± 3.5	22.0 ± 3.6	9.7 ± 0.3	6.3 ± 0.3
	Diabetic	314.7 ± 57.1	249.7 ± 27.7***	50.0 ± 4.7	14.0 ± 1.7	4.3 ± 0.3
Number of blood vessels	Control	23.2 ± 3.2	50.2 ± 2.0††	61.2 ± 5.9†††	45.7 ± 5.4†	30.0 ± 4.0
	Diabetic	25.2 ± 2.7	26.0 ± 1.5***	22.7 ± 1.5***	49.7 ± 4.9†	32.0 ± 2.3
Number of fibroblast/miofybroblast	Control	37.7 ± 2.6	72.7 ± 2.7††	91.3 ± 8.2†††	96.3 ± 0.7†††	74.0 ± 4.7†††
	Diabetic	40.0 ± 1.1	41.0 ± 2.1***	44.7 ± 4.5***	73.7 ± 2.3**†††	56.7 ± 5.0*
Percentage of collagen deposition area (%)	Control	–	42.2 ± 2.7	46.9 ± 1.8	49.1 ± 0.9	49.3 ± 2.8
	Diabetic	–	24.6 ± 0.8*	23.2 ± 1.8*	28.3 ± 1.4***	34.2 ± 2.5*

$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (versus control in same day), two-way-ANOVA/Bonferroni.
$^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ (versus 1st day in same group), one-way-ANOVA/Bonferroni (Mean ± SE).

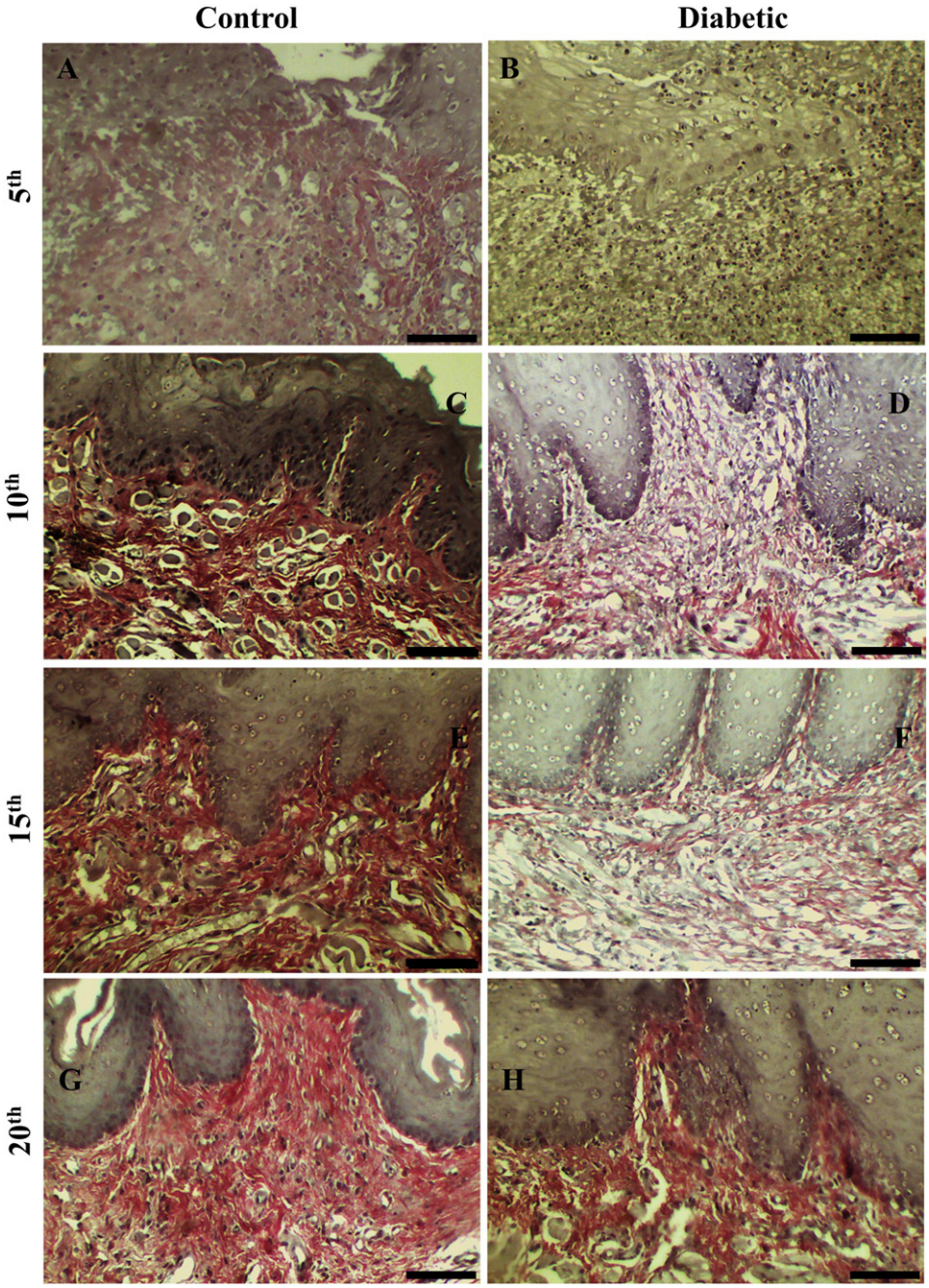

Fig. 3. Photomicrography of ulcer area in rat cheek mucosa. Collagen (red color) in control and diabetic animals on the 5th, 10th, 15th and 20th days (Picrosirius, 200×) (bars = 100 μm).

3.3. Histomorphometry of the PMN infiltrates and the extents of angiogenesis, fibroplasia and collagenesis in the wounds

The lesions of the diabetic animals contained significantly more PMNs on the 5th day post-wounding than those of the control animals. Regarding angiogenesis in the healing wounds, the number of blood vessels in the lesions of the control group at days 5, 10 and 15 post-wounding was significantly greater than that observed at day 1 in the same group. A similar profile was observed in the healing wounds of the diabetic animals, except that the number of blood vessels within them was significantly greater only at day 15 than at day 1. Comparing the healing lesions of the control and diabetic animals showed that those of the control animals had a greater number of blood vessels at days 5 and 10 post-wounding. Similarly, increased fibroplasia (number of fibroblasts/myofibroblasts) was observed in the healing lesions of the control group at days 5, 10, and 15 post-wounding, as well as at day 20 compared with that at day 1 in the same group. In the diabetic animals, the level fibroplasia in the healing lesions was also increased at day 15 post-wounding compared with that at the day 1 in the same group. A greater number of fibroblasts / myofibroblasts was found in the healing wounds of the control animals than in those of the diabetic animals at days 5, 10, 15 and 20 post-wounding (Table 2).

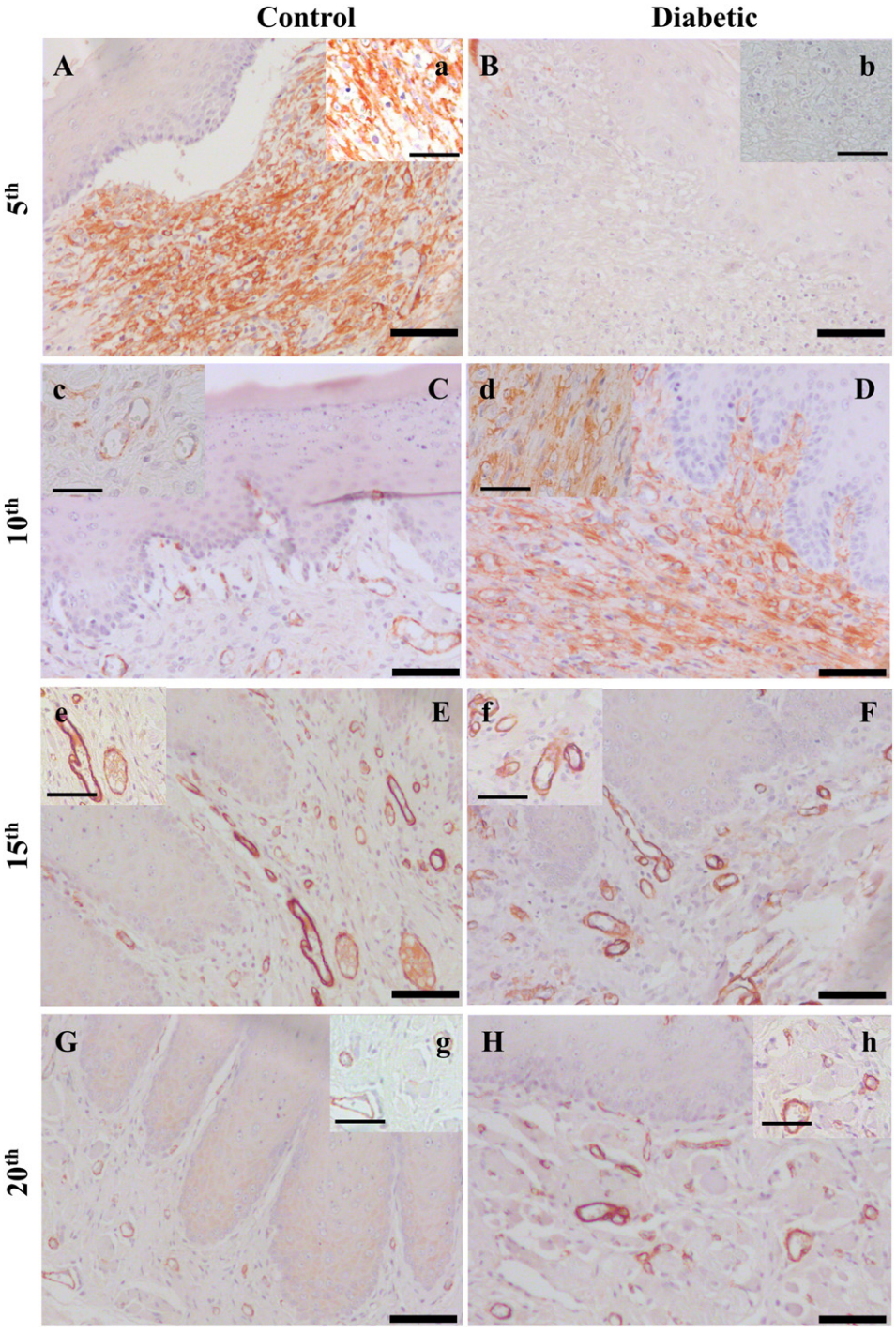

Fig. 4. Photomicrograph of traumatic ulcers in cheek mucosa of diabetic rats. Expression of positive α-SMA cells (5, 10, 15, 20 days) in control (A, C, E, G) and diabetic animals (B, D, F, H) (200×) (thin bars = 50 μm; thick bars = 100 μm).

As expected, the percentage of the healing wounds of the control animals that was occupied by deposited collagen was greater than that in the wounds of the diabetic animals at days 5, 10, 15 and 20. Moreover, qualitative assessment revealed denser, more compact and more organized collagen fibers in the healing wounds of the control animals than in those of the diabetic animals (Table 2, Fig. 3).

3.4. Immunohistochemistry

Cytoplasmic α-SMA immunostaining was observed in vascular cells and in the myofibroblasts in the connective tissue (Fig. 4). The α-SMA-positive cells in the wounds of the normoglycemic group had a higher level of expression on the 5th day post-ulceration. In contrast, the α-SMA-immunostaining of cells in the wounds of the diabetic group was more intense on the 10th day. On the 15th and 20th days, the level of α-SMA staining was mild to moderate in both groups (Table 1, Fig. 4).

FGF-2 immunostaining was observed in the cytoplasm of epithelial cells and in the nuclei and cytoplasm of connective tissue cells (fibroblasts and inflammatory cells) (Fig. 5). Intense FGF-2 immunostaining was observed in the keratinocytes in the supra-basal layers of the ulcerated areas in the normoglycemic animals on all days that evaluation was performed, which differed from that observed in the wounds of the

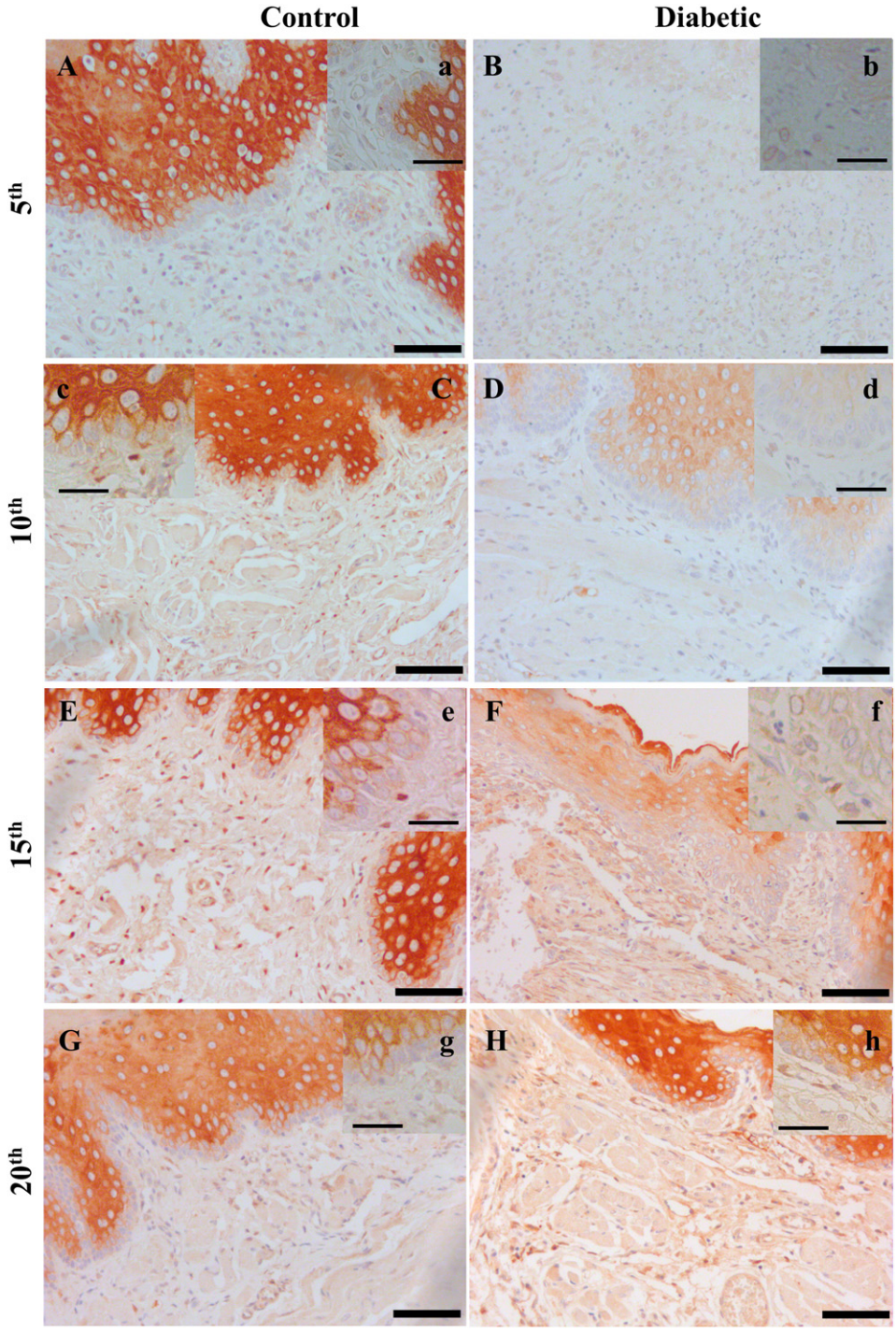

Fig. 5. Photomicrograph of traumatic ulcers in cheek mucosa of diabetic rats. Expression of FGF-2 (epithelium and connective tissue) (5, 10, 15 and 20 days) in control (A, C, E, G) and diabetic animals (B, D, F, H) (200×) (thin bars = 50 μm; thick bars = 100 μm).

diabetic animals on the 5th and 10th days post-wounding. A moderate level of FGF-2 expression was observed in cells within the connective tissue of the healing wounds of the normoglycemic group on all days that evaluation was performed, which differed from that observed in the wounds of the diabetic group on days 5 and 10 post-ulceration (Table 1, Fig. 5).

TNF-α immunostaining was observed in the nuclei and cytoplasm of epithelial and connective tissue cells (fibroblasts and inflammatory cells). The wounds of the diabetic group showed increased TNF-α expression in the epithelium compared with that of the control group on days 15 and 20 post-ulceration. A difference in the level of TNF-α expression in the connective tissue of the wounds in the diabetic animals was apparent as early as the 5th day post-wounding (Table 1, Fig. 7).

IL-1β immunostaining was seen in the nuclei and cytoplasm of epithelial and connective tissue cells (fibroblasts and inflammatory cells). The levels of IL-1β expression in the epithelium of the wounds in the two groups were not significantly different at days 1 and 5 post-ulceration. However, at days 10, 15 and 20, the wounds of the diabetic group showed an increased level of IL-1β expression compared with those of the normoglycemic group. The levels of IL-1β expression in the connective tissue of the wounds of the two groups were significantly different only at days 5 and 10 post-ulceration (Table 1, Fig. 6).

3.5. Vascular permeability

At the first day after ulceration, there was no difference in the levels of protein extravasation (mg of Evans blue/g tissue) observed in the cheek mucosal lesions of the control and diabetic animals. However, ulcerated cheek mucosa of both of these groups were significantly different from the cheek mucosa of the non-ulcerated groups. At the 5th day post-wounding, the ulcerated cheek mucosa of the diabetic animals showed more protein extravasation (approximately 2 times more) than those of the normoglycemic animals, and at the 10th day post-wounding, the ulcerated cheek mucosa of the diabetic animals showed even more protein extravasation (approximately 3 times more) than those of the normoglycemic animals. At the 5th and 10th days post-wounding, the level of protein extravasation in the ulcerated mucosa of the normoglycemic animals was restored to that of the non-ulcerated animals on the same days (Fig. 8).

3.6. Correlation analysis

Examining the linear values for the ulcer areas and the body mass variations showed that they were inversely correlated, such that the larger the ulcer area, the greater the weight loss (control group: $p < 0.001, r = -0869$; diabetic group: $p < 0.001, r = -0.712$). However, the non-linear data were directly correlated, such that the higher the histological score, the greater the ulcer area (control group: $p < 0.001$, $r = 0.893$; diabetic group: $p < 0.001$, $r = 0.848$) and the weight loss (control group: $p < 0.001$, $r = -0.830$; diabetic group: $p < 0.001$, $r = -0.703$).

4. Discussion

This study revealed that healing of cheek mucosal lesions in the diabetic animals was delayed, as shown by macroscopic and microscopic parameters. This delayed process was associated with a delay in the inflammatory phase of healing in parallel with increased vascular permeability and decreased angiogenesis, fibroplasia and collagenesis in the wounds compared with those of the control animals. Similar results have been reported for wounds in the skin [17] and mucosa [18] of diabetic animals.

In our study, deficient healing of the lesions of the diabetic animals was most obvious on the 5th and 10th days of the healing processes. Previous studies using the same model showed healing at 10 days after ulceration in normoglycemic animals [11], whereas in our study, healing in the diabetic animals occurred later, at 15 days post-ulceration.

Additionally, several studies have demonstrated weight loss in diabetic animals that began the first day after diabetes induction and ranged from 11 to 24% of the body mass [19]. Our data showed significant reduction in the weight of the diabetic animals at the 5th, 10th and 15th days. It is known that diabetic animals present clinical features that are the same as those of diabetic humans, such as hyperglycemia, polyuria, glycosuria, polydipsia and weight loss [19]. The weight loss observed in the model animals may be due to the anorexigenic effect of alloxan, which occurs via modulation of the activity in the area of the brain that controls feeding in response to the increased level of insulin released by the Langerhans beta cells [20]. The ulceration process itself is another cause of body mass loss. The pain caused by ulcerative oral lesions may limit the nutrient and water intake, leading to anorexia and dehydration [21]. In this study, both the diabetic or normoglycemic subjects showed body mass loss one day after ulceration, suggesting that the oral mucosal ulcers contributed to dysphagia. This hypothesis is reinforced by the correlation seen between the ulcer size, histological score and weight variation, which demonstrated that the greater the area of ulceration and the higher the histological score, the greater the weight loss.

Furthermore, we demonstrated that the diabetic state increased the level of vascular permeability in the cheek mucosa of the ulcerated animals and that this alteration appeared to be related to the diabetic state per se and not to the ulceration because a similar level was observed in the cheek mucosa of the normoglycemic ulcerated animals and the non-ulcerated control animals on the fifth day post-wounding, a feature not observed in the diabetic animals. Moreover, in the diabetic state, the basal membrane of the capillaries in the microcirculation thickens and the capillary size decreases with the constant increase in hydrostatic pressure, which promotes inflammation of the microvascular endothelium, which in turn maintains the increased level of vascular permeability and tissue edema [22]. Some of the mediators responsible for maintaining this increased vascular permeability have been identified, including VEGF-A [23], prostaglandin E2 [24] and cytokines (TNF-α and IL-1β). TNF-α and IL-1β have a synergistic effect on the capillary endothelium, leading to increased vascular permeability during inflammation both in vivo and in vitro [25]. Several studies have attributed the changes in vascular permeability, particularly that occurring in the diabetic state, to the overexpression of TNF-α [13,26]. This cytokine is known to induce reorganization of the endothelial cytoskeleton, which increases the level of vascular permeability, causing leakage of fluid and plasma proteins and thereby contributing to edema [27]. Our study demonstrated that there were high levels of expression of IL-1β (at the 5th day post-wounding in the connective tissue and at the 10th day in the epithelium and connective tissue) and TNF-α (at the 5th day in the connective tissue) in the ulcers of the diabetic animals, coincident with the increase in vascular permeability.

Several factors may be responsible for the impaired healing in diabetics, including deficits in the migration and production of extracellular matrix components [28], keratinocytes, fibroblasts and inflammatory cells (macrophages and neutrophils) [29], and the production of granulation tissue [30], angiogenic factors [31] and growth factors.

This study demonstrated increased PMN infiltration of the oral ulcers of the diabetic animals compared with those of control animals, particularly at the 5th day post-wounding. The delayed wound healing in diabetics has been associated with an increased inflammatory response and increased cytokine expression and with the recruitment and improper functioning of inflammatory cells, particularly neutrophils and macrophages [13,32]. Several studies demonstrated changes in the metabolic and functional profiles of these cells, as well as their decreased levels of chemotaxis [33] and phagocytosis [34], which prolongs the inflammatory process and increases the risk of infection [4]. An immunohistochemical (CD15) evaluation of the cellular infiltrate of

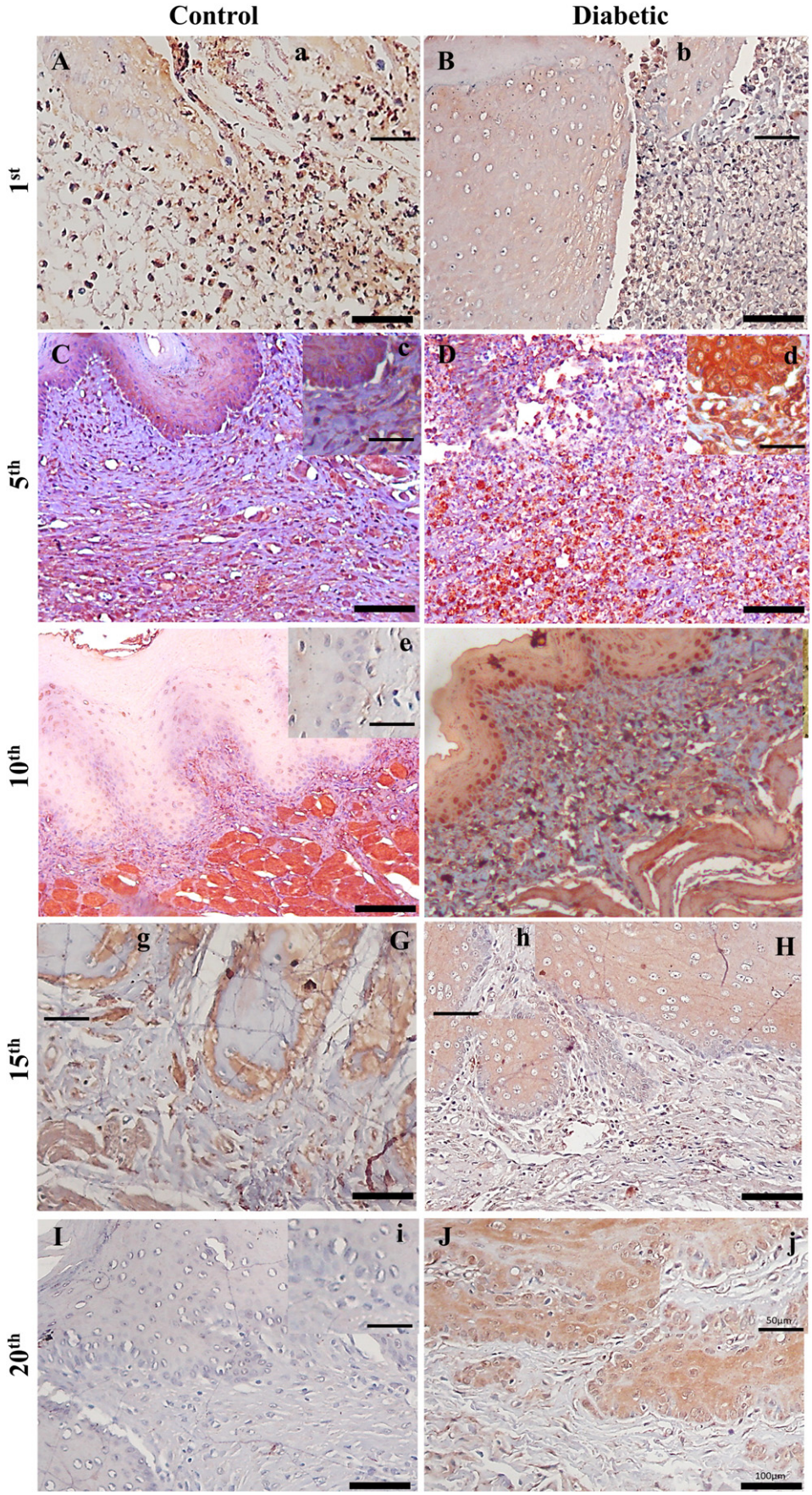

Fig. 6. Photomicrograph of traumatic ulcers in cheek mucosa of diabetic rats. Expression of IL-1β (epithelium and connective tissue) (1, 5, 10, 15 and 20 days) in control (A, C, E, G, I) and diabetic (B, D, F, H, J) animals (200×) (thin bars = 50 μm; thick bars = 100 μm).

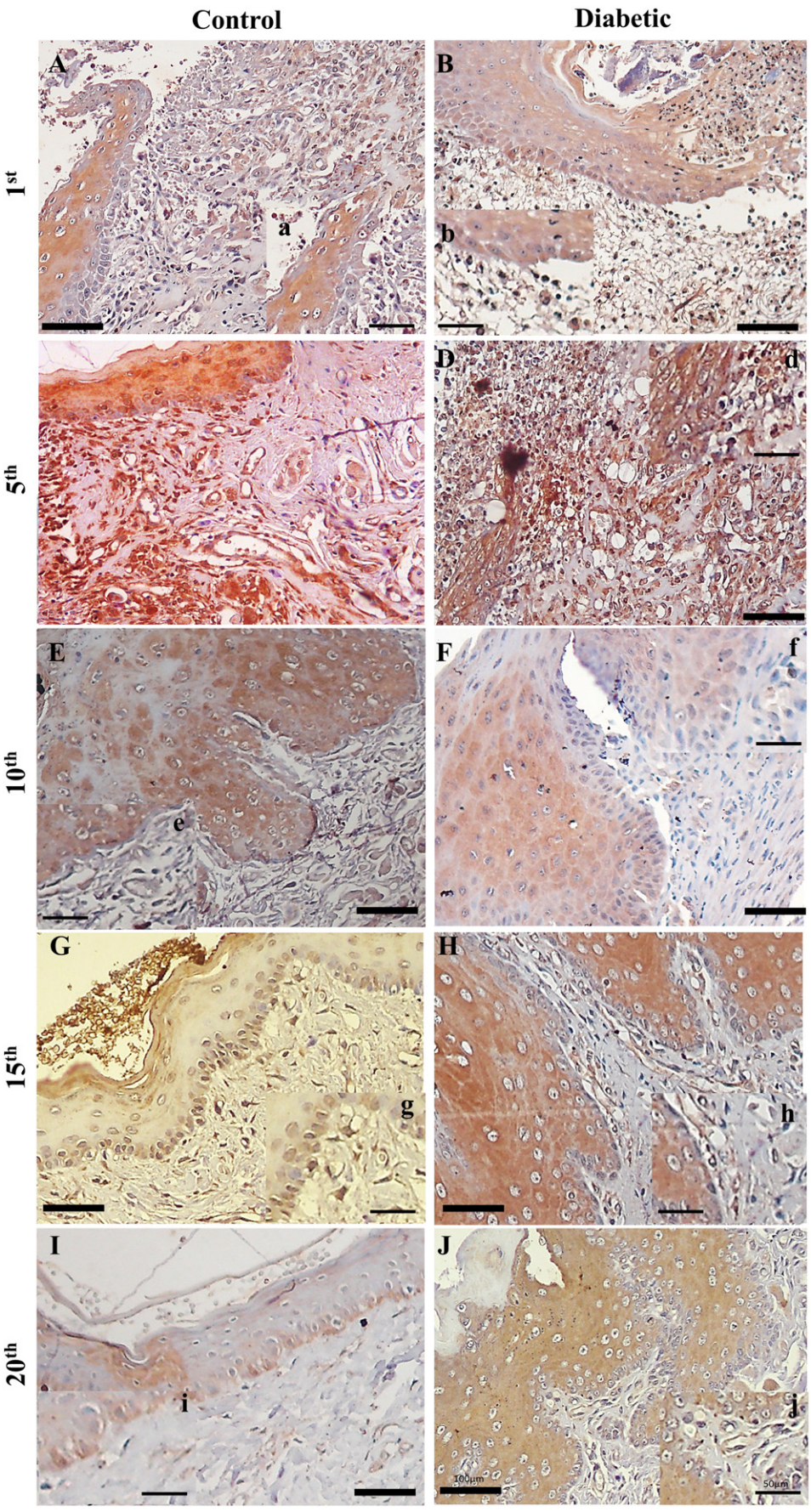

Fig. 7. Photomicrograph of traumatic ulcers in cheek mucosa of diabetic rats. Expression of TNF-α (epithelium and connective tissue) (1, 5, 10, 15 and 20 days) in control (A, C, E, G, I) and diabetic (B, D, F, H, J) animals (200×) (thin bars = 50 μm; thick bars = 100 μm).

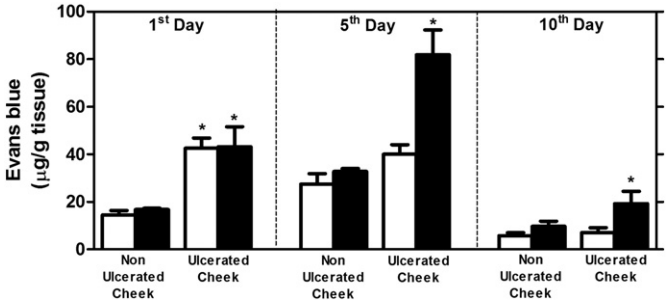

Fig. 8. Vascular permeability of traumatic ulcers in cheek mucosa of diabetic rats. Vascular permeability was assessed by quantification of Evans blue in rat ulcerated or non-ulcerated oral mucosa at the 1st, 5th, and 10th days. Mean ± S.E.M. (n = 6–7) *$p < 0.0001$ vs non-ulcerated; ** $p < 0.0001$ vs (One-way ANOVA/Bonfferroni).

diabetic ulcers in human skin showed results similar to ours, with the diabetic ulcers displaying sustained PMN infiltration at the 5th day [35]. A study of wounds in the gingival mucosa of diabetic animals found a sustained increase of neutrophil infiltration at the 5th day post-ulceration compared with that of the normoglycemic controls [[36]. Other studies, conducted using different methodologies, found an increased level of neutrophil infiltration in diabetic wounds, although along different time courses [13,18,37]. The sustained PMN profile in diabetic ulcers was attributed to the overexpression of IL-1β and TNF-α [37], cytokines that are associated with the expression of leukocyte adhesion molecules and the production of direct neutrophil-chemotactic substances [38], which may contribute to an increase in vascular permeability through direct endothelial damage or the release of vasoactive mediators. Those data are consistent with the results of our study, in which there was increased expression of TNF and IL-1β in the connective tissue at 5th day post-ulceration in the wounds of the diabetic animals, which correlated with the increased levels of neutrophil infiltration and vascular permeability.

It is well established that neovascularization within the provisional extracellular matrix of the granulation tissue is a key step in normal wound healing. This process occurs under both physiological and pathological conditions [39]. Studies of wound healing in DM mice have found that the angiogenesis and granulation-tissue formation processes are impaired] [40].

Our study demonstrated that the wounds of normoglycemic animals contained more vessels than did those of the diabetic animals, particularly at the 5th and 10th days post-ulceration, and that angiogenesis within the wounds was initiated earlier in the normoglycemic animals (day 5) than in the diabetic animals (day 15). FGF-2 immunostaining showed fewer positive cells in the epithelial tissue in the wounds of the diabetic animals than in those of the control animals (at 5 and 10 days). Similar findings were observed in the connective tissue of the healing wounds (fibroblasts and inflammatory cells). FGF is a potent mitogenic and angiogenic agent that is involved in morphogenesis, inflammation, tumorigenesis and tissue repair [41]. The increased level of vascularization (at 5 and 10 days post-wounding) seen in the healing ulcers of the normoglycemic animals in our study compared with those of the diabetic animals could be related to the relatively increased level of FGF-2 expression in the former animals [42]. Several experimental studies have shown that FGF-2 treatment improved the healing process by promoting the formation of granulation tissue and the deposition and maturation of collagen [43]. FGF- 2, which is ubiquitously expressed in human tissues and has a mitogenic effect on various inflammatory cells involved in the healing process, such as mast cells and macrophages. Fibroblasts, endothelial cells and epithelial cells are sources or targets of FGF- 2 [6]. Our study showed a decreased level of FGF-2 expression in the wounds of the diabetic animals that was associated with a significant delay in angiogenesis, fibroplasia and collagenesis therein [5]. In contrast, a single study showed that FGF-2 appeared to inhibit the differentiation of myofibroblasts and to impair wound contraction in the mouse palate without interfering with the cellular influx or vascularization processes in the wounds [42].

Significantly reduced levels of collagens type I and IV were observed in the skin ulcers of diabetic animals compared with those of normoglycemic animals, suggesting that these deficiencies are directly related to the delayed healing during the late stages of recovery and to the decreased percentage of the ulcer area occupied by collagen because collagen is the major component of the extracellular matrix [17]. Among the stains employed to visualize collagen (such as the Mallory and Masson & Van Gieson stains), Sirius Red was chosen for use in this study because it has been described as the most appropriate collagen stain (due to not staining reticular fibers or the basement membrane) and has the advantage of being visible under polarized light, allowing evaluation of the pattern and architecture of collagen fibers [44]. We found smaller amounts of and delayed fibroplasia and collagen deposition in the wounds of the diabetic animals compared with those of the normoglycemic animals. As expected, on the days that less fibroplasia was observed, less deposited collagen was observed.

Immunohistochemistry using the anti-α-SMA antibody showed reduced numbers of myofibroblasts and vascular cells in the ulcerated oral mucosa of the diabetic animals (day 5) compared with those of the normoglycemic animals, reinforcing the deficit in fibroplasia, angiogenesis and collagenesis in the lesions of the diabetic animals. It was only at the 10th day post-wounding that the diabetic animals show increased populations of myofibroblasts and vascular cells in their healing wounds, which can be associated with their delayed healing and wound contraction. Several other mechanisms might be associated with the reduced levels of fibroplasia and collagen deposition in the wounds of diabetics, such as defects in cellular differentiation [45] and proliferation [8] and an increased level of apoptosis in fibroblasts [13,36], which could be associated with the sustained increase in the level of TNF-α (in the epithelium at days 15 and 20 post-wounding) observed in the diabetic oral ulcers in the present study.

This study also demonstrated that a cheek mucosal ulcer is a reliable experimental model of the oral healing process [46], in contrast to traditional models using the palatal area [47], in which the exposure to bone and periosteum can interfere with the healing process, leading to necrosis, changes in the mucosal healing pattern and dysphagia. Some authors have used the tongue as an alternative model system to overcome these disadvantages [48], but due to its contractility, its specialized epithelium with a relatively high rate of turnover and its high level of vascularization, the latter of which may lead to bleeding, the healing process may be masked in this model. Thus, the results obtained in wound healing models of the oral mucosa have shown them to be accurate and effective and to lack variations that may cause difficulties in interpreting the results of oral healing studies.

The buccal mucosal ulcer model of diabetic rats utilized in this study provides easily reproducible results that can be used to better understand the pathophysiological mechanisms inferring with the wound repair process under hyperglycemic conditions and can contribute to the development of new therapeutic strategies for healing.

In conclusion, the present study demonstrated the delayed healing of the cheek mucosal wounds of diabetic animals relative to that of the controls. During the initial stage of repair, this delay manifested as the diminished contraction of the ulcerated area and the prolongation of the inflammatory phase of healing, which was associated with the overexpression of TNF-α and IL-1β. During the latter stages of healing, this delay manifested as decreased levels of angiogenesis, fibroplasia and collagenesis that was associated with a reduced level of FGF-2 expression and sustained TNF-α expression.

Authors' consent and conflict of interest

The authors also affirm that the publication of this article is approved by all authors and that, if accepted, it will not be published elsewhere in

the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

Additionally, the authors declare that there are no conflicts of interest.

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