Relaxin improves multiple markers of wound healing and ameliorates the disturbed healing pattern of genetically diabetic mice

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Abstract

Diabetic mice are characterized by a disrupted expression pattern of VEGF (vascular endothelial growth factor), and impaired vasculogenesis during healing. Experimental evidence suggests that RLX (relaxin) can improve several parameters associated with wound healing. Therefore we investigated the effects of porcine-derived RLX in diabetes-related wound-healing defects in genetically diabetic mice. An incisional wound model was produced on the back of female diabetic C57BL/KsJ-m$+/+Lept^{db}$ (db$^+$/db$^+$) mice and their normal littermates (db$^{+/+}$m). Animals were treated daily with porcine RLX (25 μg/mouse per day, subcutaneously) or its vehicle. Mice were killed on 3, 6 and 12 days after skin injury for measurements of VEGF mRNA and protein synthesis, SDF-1α (stromal cell-derived factor-1α) mRNA and eNOS (endothelial NO synthase) expression. Furthermore, we evaluated wound-breaking strength, histological changes, angiogenesis and vasculogenesis at day 12. Diabetic animals showed a reduced expression of VEGF, eNOS and SDF-1α compared with non-diabetic animals. At day 6, RLX administration resulted in an increase in VEGF mRNA expression and protein wound content, in eNOS expression and in SDF-1α mRNA. Furthermore, the histological evaluation indicated that RLX improved the impaired wound healing, enhanced the staining of MMP-11 (matrix metalloproteinase-11) and increased wound-breaking strength at day 12 in diabetic mice. Immunohistochemistry showed that RLX in diabetic animals augmented new vessel formation by stimulating both angiogenesis and vasculogenesis. RLX significantly reduced the time to complete skin normalization and this effect was abrogated by a concomitant treatment with antibodies against VEGF and CXCR4 (CXC chemokine receptor 4), the SDF-1α receptor. These data strongly suggest that RLX may have a potential application in diabetes-related wound disorders.

Key words: angiogenesis, diabetes, matrix metalloproteinase (MMP), relaxin, vascular endothelial growth factor (VEGF), wound healing

INTRODUCTION

Patients suffering from diabetes show a disturbed wound-healing process that may enhance the overall morbidity and mortality of this population [1,2]. A complex programmed sequence of cellular and molecular processes including inflammation, cell migration, angiogenesis, provisional matrix synthesis, collagen deposition and re-epithelization characterizes the normal skin repair [1,3]. Angiogenesis plays a central role in wound healing and is associated with the expression of several cytokines, and angiogenic factors, such as VEGF (vascular endothelial growth factor) [4,5].

Healing impairment in people with diabetes is characterized by delayed cellular infiltration and granulation tissue formation,

decreased collagen organization and, more interestingly, reduced angiogenesis [6,7]. As far as angiogenesis is concerned a defect in VEGF regulation, characterized by an altered expression pattern of VEGF mRNA during skin repair, has been shown in diabetic mice [8]. In addition, it has been reported that AAV (adeno-associated viral vector)-mediated human VEGF$_{165}$ gene transfer, stimulates angiogenesis and wound healing in genetically diabetic mice [9].

Besides angiogenesis, vasculogenesis may also play a crucial role in the healing process. Vasculogenesis, the *in situ* differentiation of the primitive endothelial progenitors known as angioblasts into endothelial cells that aggregate into a primary capillary plexus, has been shown to be responsible for the development of the vascular system during embryogenesis [10]. However, vasculogenesis is also present in adults and occurs through the action of circulating or resident BM (bone marrow)-derived cells called EPCs (endothelial progenitor cells), and may also be primed by VEGF [11]. Further cell lineages not BM derived may be found at different sites and have been demonstrated to differentiate into endothelial cells under hypoxic conditions or during physiological replenishment of skin and gut [12]. Moreover, vasculogenesis is more prevalent and effective when angiogenesis is failing: this is the case of the healing of diabetic ulcers in which there is an impairment of haemostasis, inflammation, matrix deposition and most of all angiogenesis [13]. EPCs circulating and wound-level numbers are also decreased in diabetes, implying an abnormality in EPC mobilization and homing mechanisms [14]. The deficiency in EPC mobilization is presumably because of the impairment in the eNOS (endothelial NO synthase)–NO cascade in the BM, and the failure of EPCs to reach the wound tissues is partly a result of a down-regulated production of SDF-1α (stromal cell-derived factor-1α) in the wounds [14]. In fact SDF-1α, by binding to its receptor CXCR4 (CXC chemokine receptor 4) on EPCs, allows the recruitment and homing of these cells in hypoxic tissues [14].

RLX (relaxin) is a peptide hormone of the insulin super-family that has a long history as a reproductive hormone since its discovery in 1926 [15]. Like insulin, RLX is a 6 kDa protein processed from a preproform to the mature hormone containing A and B peptides connected by two inter-chain disulfide bridges, and one inter-chain disulfide within the A chain. Several RLX-like peptides exist. Two RLX genes are present in humans, encoding protein known as H1 and H2 RLX, but only H2 RLX is known to circulate. RLX has been shown to induce VEGF expression and angiogenesis selectively at wound sites in an experimental *in vitro* model [16]. Furthermore, RLX may also increase the expression of eNOS, thus modulating NO production. Besides angiogenesis, RLX may also modulate collagen synthesis and extracellular matrix homoeostasis: in fact it increases the expression of MMPs (matrix metalloproteinases) and degrades collagen, thus antagonizing the exaggerated fibrosis of the wounds (anti-scarring effect) [17].

All of these experimental observations make RLX a logical candidate for treatment to speed up wound closure. Indeed, intraperitoneal administration of a crude preparation containing porcine RLX improved wound healing and increased tensile strength in a rodent model [18] and recombinant H2 RLX enhanced wound healing and prevented scar formation in a pig excision wound model [19]. However, the effects of RLX in diabetes-impaired wound healing have not been fully investigated. We therefore investigated the effects of a porcine derived RLX in an incisional wound-healing model in genetically diabetic mice [6,7].

MATERIALS AND METHODS

Animals

All animal procedures were in accordance with the Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985) and authorized by our National Institution. Genetically diabetic female (30–35 g) C57BL/KsJ-m$+/+Lept^{db}$ mice (db$^+$/db$^+$) and their normal littermates (22–25 g) (db$^{+/+}$ m) were obtained from Jackson Laboratory. Animals were 10 weeks old at the start of the experiments. During the experiments the animals were maintained one per cage, under controlled environmental conditions (12 h light–dark cycle, 23 °C), and provided with standard food and water *ad libitum*. After general anaesthesia with sodium pentobarbital (80 mg/kg of body weight, intraperitonealy), hair on the back was shaved and two parallel 4-cm incisions were produced with the use of a scalpel (Figure 1A), on the back of all mice as described previously [20].

In preliminary experiments, RLX (IBSA Rizhao-Lanshan) dose was titrated against the effects on VEGF expression. Normoglycaemic animals ($n = 24$), following skin incision, were implanted subcutaneously with osmotic pumps delivering different RLX concentrations (6.25, 12.5, 25 and 50 μg/mouse per day). The pumps were implanted on the neck right above the mid-scapular line (Figure 1A) and the treatments lasted 6 days. This experiment identified 25 μg/mouse per day of RLX as the optimal dose to be used in the further experiments, indeed the highest dose (50 μg/mouse per day) did not show additional improvements.

The animals ($n = 72$) were divided into groups of six animals each. Normoglycaemic and diabetic mice received either RLX (25 μg/mouse per day, subcutaneously) or its vehicle (6 μl of 0.9 % NaCl/mouse per day) for up to 12 days. A total of ten animals from each strain were killed after 3, 6 and 12 days after surgery, and the wounds removed by using a scalpel to cut the shape of an ellipse around the lesion. Of the two wounds, one was used for NO products (only at day 6) or tensile strength (only at day 12), and the other one sectioned for the other analysis as shown in Figure 1(A). All animals killed at day 12 were also tested for glycaemic blood levels, by using a colorimetric glucose oxidase assay (One-Touch; Lifescan).

To better understand if the angiogenetic or the vasculogenetic pathway were affected by RLX, two additional groups ($n = 7$ for each group) of diabetic animals underwent wounding and received RLX (25 μg/mouse per day, subcutaneously) together with the anti-VEGF murine antibody (10 mg/kg of body weight, intraperitonealy daily; Abcam) or with the anti CXCR4 antibody (0.4 mg/kg of body weight, intraperitonealy daily; Enzo Lifescience). Additional normoglycaemic ($n = 18$) and diabetic ($n = 18$) mice were subjected to wounding, implanted with pumps and used to investigate the time needed to complete wound closure.
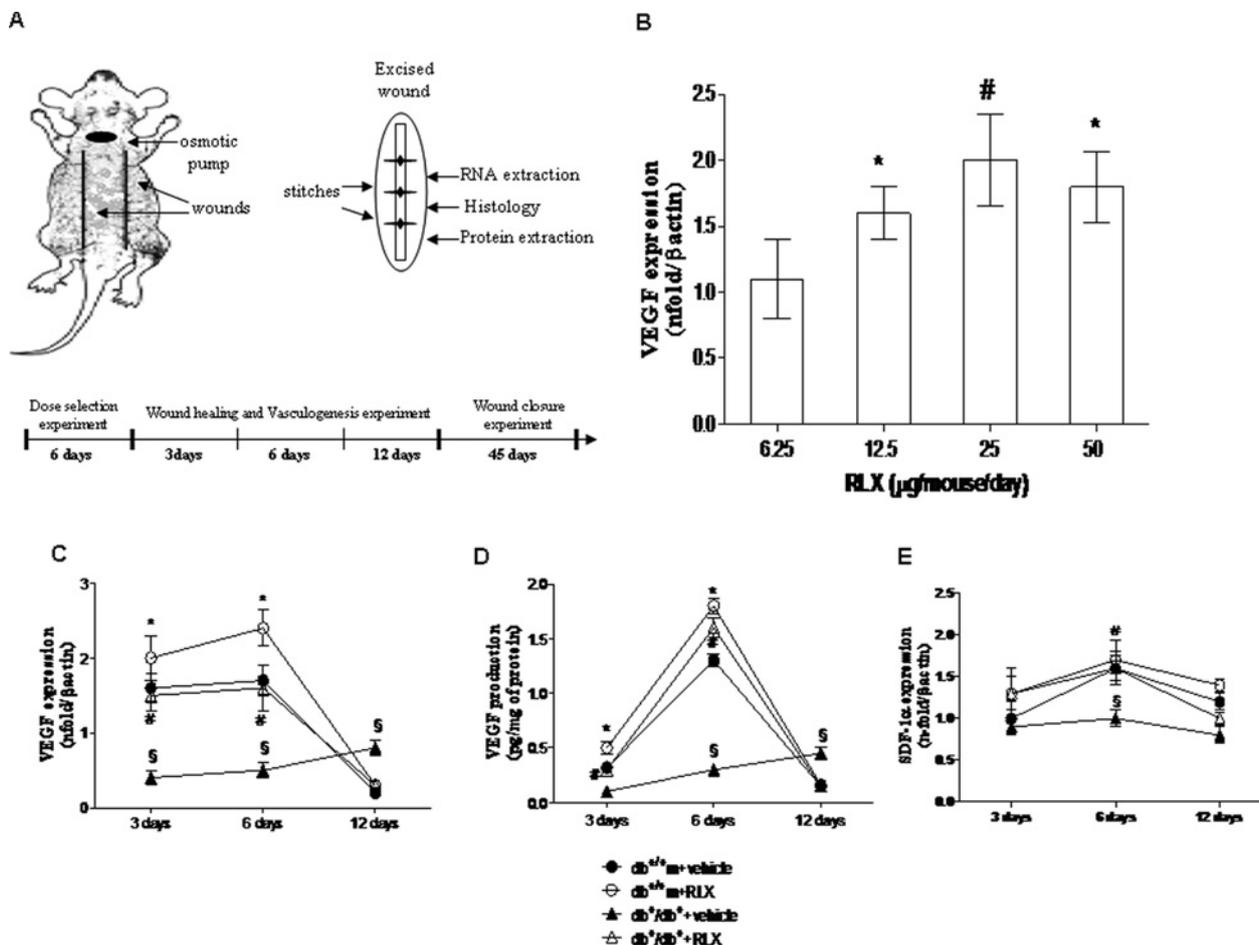
Figure 1   Experimental approach and angiogenetic and vasculogenetic molecule expression
(**A**) The cartoon represents the back of a mouse with the site of the two wounds and of the implanted pump indicated; on the right side is represented an excised wound (ellipse shaped) that is subdivided in pieces to perform all the analyses. The breaking strength analysis is performed on an entire wound, following excision. At the bottom the timeline of the experiment is represented. (**B**) VEGF mRNA expression in skin wound samples collected from normoglycaemic (db$^{+/+}$m) mice treated with different RLX doses (6.25, 12.5, 25 and 50 μg/mouse per day) at day 6. Each bar represents the mean $\pm$ S.D. of six animals. *$P < 0.05$ compared with RLX 6.25; #$P < 0.001$ compared with RLX 6.25. VEGF (**C**) and SDF1-α (**E**) mRNA expression in skin wound samples collected from either normoglycaemic (db$^{+/+}$m) and diabetic mice (db^{+}/db^{+}) given either RLX (25 μg/mouse per day, subcutaneously) or vehicle (6 μl of 0.9% NaCl/mouse per day) at 3, 6 and 12 days. Each point represents the mean $\pm$ S.D. of six animals. §$P < 0.001$ compared with db$^{+/+}$m + vehicle; *$P < 0.05$ compared with db$^{+/+}$m + vehicle; #$P < 0.001$ compared with db^{+}/db^{+} + vehicle. (**D**) VEGF protein levels studied by ELISA in skin wound samples collected from either normoglycaemic (db$^{+/+}$m) or diabetic mice (db^{+}/db^{+}) given either RLX (25 μg/mouse per day, subcutaneously) or vehicle (6 μl of 0.9% NaCl/mouse per day) at 3, 6 and 12 days. Each point represents the mean $\pm$ S.D. of six animals. §$P < 0.001$ compared with db$^{+/+}$m + vehicle; *$P < 0.05$ compared with db$^{+/+}$m + vehicle; #$P < 0.001$ compared with db^{+}/db^{+} + vehicle.

Real-time PCR for VEGF and SDF-1α

Briefly, the total RNA was extracted from wound samples, and quantified as described previously [21]. RNA was reverse-transcribed to cDNA and used to quantify the amount of VEGF and SDF-1α mRNA by real-time PCR; β-actin was used as endogenous control. The results are expressed as the n-fold difference relative to normal controls (relative expression levels).

Determination of eNOS by Western blot analysis

eNOS expression was evaluated at day 6, by Western Blot, as described previously [21]. Primary antibodies for eNOS and phospho-eNOS (Ser^{1177}) were purchased from Chemicon and

Cell Signaling respectively. Secondary peroxidase-conjugated antibodies were obtained from Pierce. The protein signals were quantified by scanning densitometry, using a bio-image analysis system (Bio-Profil Celbio), and are expressed as integrated intensity in comparison with β-actin (Cell Signaling) measured on stripped blots [21].

Determination of NO$_2^-$/NO$_3^-$

At day 6, pieces of wound samples were removed and frozen in liquid nitrogen until use. NO products were determined in wound lysates using the Griess reaction, as described previously [22].

All samples and standards were assayed in triplicate. Data are expressed as means $\pm$ S.D. of NO_2^-/NO_3^-.

Determination of VEGF in wounds

The amount of VEGF in wounds was determined at 3, 6 and 12 days, by a commercially available VEGF-specific ELISA assay kit (R&D Systems) as described previously [22]. The amount of VEGF was expressed as pg/mg of protein.

Histological examination and time to complete wound closure

Wound samples were fixed in 10% neutral buffered formalin, and processed as described previously [20]. All slides were examined by a pathologist blinded to the previous treatment, by means of an eye-piece grid under the microscope from ×20 to ×100 magnification. The following parameters were evaluated and scored: re-epithelialization, dermal matrix deposition and regeneration, granulation tissue formation and remodelling. The histological specimens were evaluated according to the score reported in the literature concerning wound healing in experimental models [20,22–24].

Time to complete wound closure, evidenced by a closed linear healing ridge, was monitored as described previously [20].

Immunohistochemistry for VEGFR (VEGF receptor)-1, VEGFR-2, VE (vascular endothelial)-cadherin, CD31, CD34 and MMP-11

Paraffin-embedded tissues were sectioned (5 μm), rehydrated, and antigen retrieval was performed using 0.05 M sodium citrate buffer. Slides were incubated overnight with primary antibody to detect CD31, CD34, MMP-11, VE-cadherin, VEGFR-1 (all from Abcam) and VEGFR-2 (Cell Signaling) as described previously [20]. DAB (3-3′ diaminobenzidine, Sigma) was used to reveal the reaction and counterstaining was performed with haematoxylin where needed.

To assess new blood vessel formation, MVD (microvessel density) was estimated after CD31 staining. Briefly, three hot spots or areas with the highest visible blood vessel density (marked by the vessel marker) per section were selected, in the dermis just proximal to the wound site, and the number of small calibre blood vessels having a visible lumen were counted per high-power field (×40 magnification) by three pathologists blinded to the samples. To assess positive CD34, VEGFR-1, VE-cadherin and VEGFR-2 staining six areas per section were randomly selected in the dermis and the positive endothelial cells were counted per high-power field (×40 magnification) by three pathologists blinded to the samples. To evaluate positive MMP-11 staining five areas per section were randomly selected in the subepithelial tissue and positive spots were counted per high-power field (×40 magnification) by three pathologists blinded to the samples. For negative controls, the primary antibodies were replaced by citrate buffer (pH 6.0).

Breaking strength

At day 12, the maximum load (breaking strength) tolerated by wounds was measured blindly on coded samples using a calibrated tensometer (Sans) as described previously [20]. The ends of the skin strip were pulled at a constant speed (20 cm/min), and breaking strength was expressed as the mean maximum level of tensile strength in Newtons before separation of wounds.

Statistical analysis

All data are expressed as means $\pm$ S.D.. Comparisons between different treatments were analysed by one-way ANOVA followed by Tukey's multiple comparison test. In all cases, a probability error of less than 0.05 was selected as the criterion for statistical significance. Graphs were drawn using GraphPad Prism (version 5.0 for Windows).

RESULTS

Identification of the RLX dose

Normoglycaemic animals were treated for 6 days after wounding with RLX at different concentrations (6.25, 12.5, 25 and 50 μg/mouse per day) in order to identify the optimal dose to be used in further experiments. Figure 1(B) shows that the most effective RLX dose in increasing VEGF expression was 25 μg/mouse per day. A greater dose did not further increase VEGF expression.

VEGF expression and protein content in wounds

In the wounds of non-diabetic animals, a strong induction of VEGF mRNA (Figure 1C) was found at days 3 and 6 when compared with basal values (uninjured skin $= 0.05 \pm 0.001$ n-fold/β-actin; $P < 0.001$). VEGF protein levels in uninjured skin from non-diabetic and diabetic mice were very low (results not shown). In the wounds of non-diabetic animals, VEGF content increased at days 3 and 6 compared with basal values (Figure 1D). Thereafter, VEGF expression and protein levels declined at day 12. Administration of RLX enhanced VEGF mRNA and protein content at days 3 and 6 in normoglycaemic animals (Figures 1C and 1D).

Wounds from diabetic mice showed markedly reduced expression and protein levels of VEGF when compared with non-diabetic ones at days 3 and 6 following the surgery. VEGF mRNA and protein levels were still significantly detectable at day 12 in diabetic mice (Figures 1C and 1D). Daily treatment with RLX enhanced VEGF expression and protein levels at days 3 and 6, thus restoring the disturbed pattern of VEGF secretion (Figures 1C and 1D).

SDF1-α expression in wounds

In the wounds of non-diabetic animals, SDF1-α mRNA increased significantly at days 3 and 6 when compared with basal values (uninjured skin $= 0.02 \pm 0.005$ n-fold/βactin; $P < 0.001$). SDF1-α expression remained sustained at day 12 (Figure 1E). Administration of RLX slightly, but not significantly, enhanced SDF1-α mRNA at days 3 and 6 in normoglycaemic animals.

Wounds from diabetic mice showed a markedly reduced expression of SDF1-α when compared with non-diabetic ones at day 6 following surgery (Figure 1E). Daily treatment with RLX augmented SDF1-α levels; thus restoring the impaired pattern of SDF1-α mRNA expression (Figure 1E).
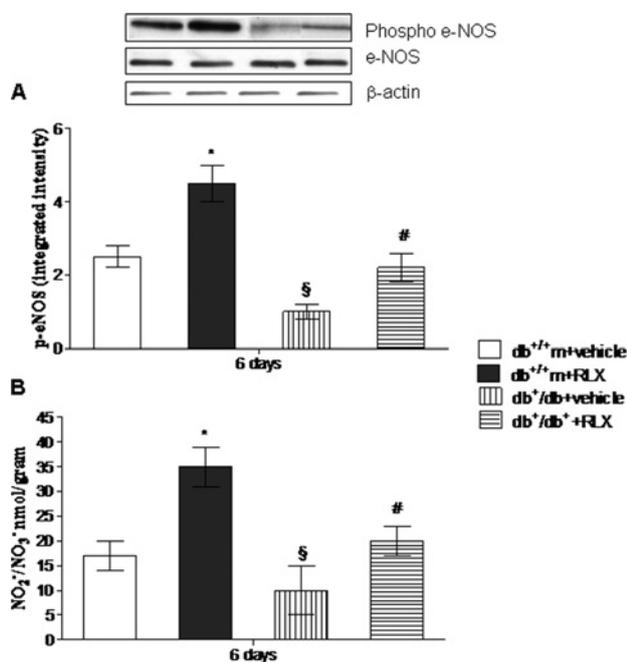
Figure 2 NO production

eNOS expression (constitutive and phosphorylated form) (**A**) and NO products (**B**) in skin wound samples collected from either normoglycaemic (db$^{+/+}$m) or diabetic mice (db$^+$/db$^+$) given either RLX (25 μg/mouse per day, subcutaneously) or vehicle (6 μl of 0.9% NaCl/mouse per day) at day 6. Each bar represents the mean $\pm$ S.D. of six animals. *$P < 0.01$ compared with db$^{+/+}$m + vehicle; §$P < 0.05$ compared with db$^{+/+}$m + vehicle; #$P < 0.05$ compared with db$^+$/db$^+$ + vehicle.

Figure 3 Histological pictures and score

(**A**) Histological scores in wound samples collected from either normoglycaemic (db$^{+/+}$m) or diabetic mice (db$^+$/db$^+$) given either RLX (25 μg/mouse per day, subcutaneously) or vehicle (6 μl of 0.9% NaCl/mouse per day) at day 12. Each bar represents the mean $\pm$ S.D. of six animals. §$P < 0.001$ compared with db$^{+/+}$m + vehicle; #$P < 0.001$ compared with db$^+$/db$^+$ + vehicle. (**B–E**) Histological photomicrographs, haematoxylin–eosin, of the several experimental groups at day 12. (**B**) Vehicle-treated normoglycaemic (db$^{+/+}$m) wounds. Re-epithelialization is almost complete. Granulation tissue is well-formed without inflammatory infiltrates. (**C**) RLX-treated normoglycaemic (db$^{+/+}$m) wounds. Complete re-epithelialization and restoration of normal architecture in dermis. (**D**) Vehicle-treated diabetic (db$^+$/db$^+$) wounds. Incomplete re-epithelialization and poorly formed granulation tissue. (**E**) RLX-treated diabetic (db$^+$/db$^+$) wounds. Re-epithelialization is almost complete, well-organized granulation tissue and no evidence of inflammation, oedema or erythrorrhagies.

Expression of eNOS in wounds

Uninjured skin specimens showed a very low content of phospho-eNOS (0.7 ± 0.04 and 0.4 ± 0.06 integrated intensity in normoglycaemic and diabetic mice, respectively). Wounding enhanced expression of phospho-eNOS in both strains of animals at day 6 (Figure 2A), but this expression was significantly lower in diabetic animals. In wounds treated with RLX, a greater increase in phospo-eNOS was detected in both non-diabetic and diabetic animals compared with vehicle-treated animals (Figure 2A).

NO products in wounds

Wounding produced a remarkable increase in NO$_2^-$/NO$_3^-$ content compared with control uninjured skin at day 6 (1.3 ± 0.4 and 0.8 ± 0.04 nmol/g of tissue in normoglycaemic and diabetic mice respectively). However, NO$_2^-$/NO$_3^-$ levels were significantly reduced in the wounds of diabetic animals. A significant higher enhancement in wound NO products was observed in RLX-administered diabetic and non-diabetic mice compared with vehicle-treated animals (Figure 2B).

Histology

Figure 3 shows the histological scores (A) and representative histological pictures (B–E) of the several experimental groups at day 12. In normoglycaemic animals, dermis remodelling and wound closure processes were complete (Figures 3A and 3B). However,
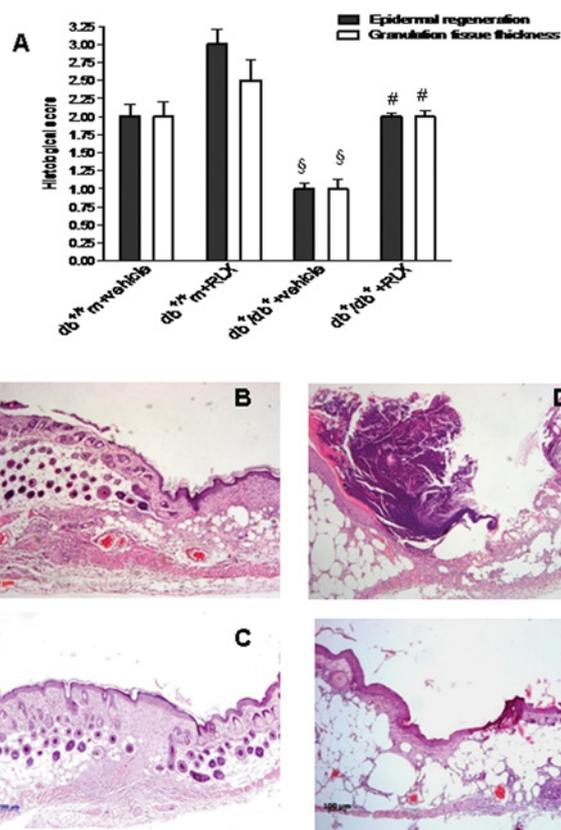
the administration of RLX qualitatively improved wound healing (Figures 3A and 3C).

Diabetic wounds of mice administered with vehicle at day 12 showed poor-to-mild re-epithelialization with partially organized granulation tissue (Figures 3A and 3D). In contrast, moderate to complete re-epithelialization and well-formed granulation tissue were observed in the diabetic wound of mice treated with RLX (Figures 3A and 3E).

Wound closure

The time to complete wound closure was indicated by a closed linear ridge, with no open areas between stitches. Complete skin normalization in untreated normoglycaemic animals occurred at day 23 ± 3 after wounding and RLX did not modify this parameter
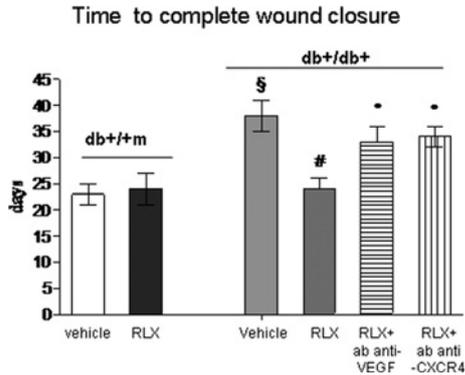
Time to complete wound closure

Figure 4 Complete healing time

Time to complete the wound closure in normoglycaemic (db$^{+/+}$m) and diabetic (db^{+}/db^{+}) mice given either RLX (25 μg/mouse per day, subcutaneously) or vehicle (6 μl of 0.9% NaCl /mouse per day). Two additional groups of diabetic animals were administered with RLX (25 μg/mouse per day, subcutaneously) and treated with an anti-VEGF murine antibody (10 mg/kg of body weight, intraperitoneally daily) or with anti CXCR4 antibody (0.4 mg/kg of body weight, intraperitoneally daily). Each point represents the mean $\pm$ S.D. of six animals. §$P < 0.001$ compared with db$^{+/+}$m + vehicle or RLX; *$P < 0.05$ compared with db$^{+/+}$m + vehicle; #$P < 0.001$ compared with db^{+}/db^{+} + vehicle; °$P < 0.05$ compared with db^{+}/db^{+} + RLX.

(Figure 4). Wound closure in diabetic animals was observed at day 38 ± 3 after the surgical procedure and RLX markedly reduced the time to complete wound closure (Figure 4). A concomitant treatment with antibodies raised against either VEGF or CXCR4 abated this effects, thus confirming that both VEGF and SDF1-α mediate the beneficial RLX effects on wound healing.

Evaluation of new blood vessel formation

CD31 immunostaining was investigated at day 12 to confirm neo-vessel formation (Figure 5). In fact, this protein represents a highly specific marker for endothelial cells. Positive staining was present in the wounds from vehicle administered non-diabetic animals (Figure 5C). This staining appeared markedly reduced in the wounds from diabetic animals injected with vehicle (Figure 5A). Administration of RLX augmented CD31 immunostaining in both strains of mice (Figures 5B and 5C). The effect was remarkable in diabetic wounds where the positive staining was mostly localized in small vessels and capillaries (Figure 5B). Using the images obtained from CD31 staining, the tissue samples were assessed for MVD (Figure 5C). Treatment with RLX markedly increased MVD in both non-diabetic and diabetic animals (Figure 5C).

Assessment of CD34 and VEGFR-1 immunostaining

To confirm neo-angiogenesis, CD34 and VEGFR-1 immunostaining was also studied at day 12 (Figure 6). Positive staining was evident in the wounds from vehicle injected non diabetic animals (Figures 6C and 6F). This staining was significantly reduced in the wounds from diabetic animals injected with vehicle (Figures 6A and 6D). Administration of RLX enhanced CD34 (Figures 6B and 6C) and VEGFR-1 (Figures 6E and 6F) immunostaining in both strains of mice. In RLX-treated diabetics a marked staining in the subepithelial layer was observed, VEGFR-
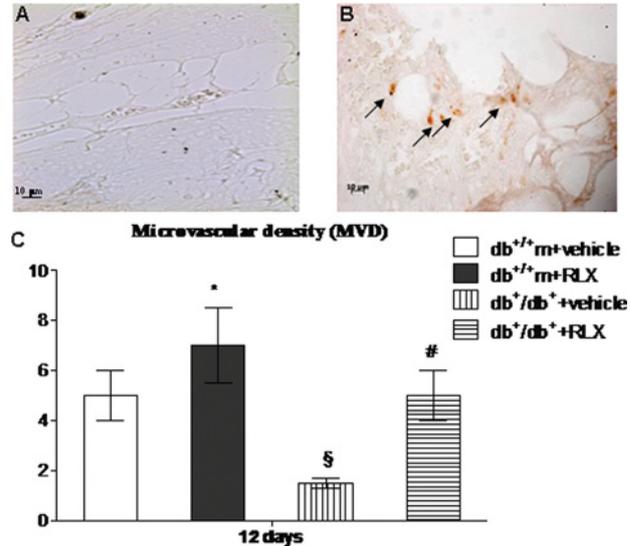

Figure 5 CD31 staining and MVD score

(**A**, **B**) Representative immunohistochemical CD31 staining of skin wounds samples from from diabetic (db^{+}/db^{+}) mice treated with vehicle (6 μl of 0.9% NaCl/mouse per day) or RLX (25 μg/mouse per day, subcutaneously) at day 12. Arrows indicate staining positivity. (**C**) Comparison of semi-quantitative assessment of microvascular density (MVD) between each group. Each bar represents the mean $\pm$ S.D. of six animals. *$P < 0.05$ compared with db$^{+/+}$m + vehicle; §$P < 0.01$ compared with db$^{+/+}$m + vehicle; #$P < 0.01$ compared with db^{+}/db^{+} + vehicle.

1 in fact exists also in soluble form and the diffuse staining suggests an enhanced production. Figures 6(C) and 6(F) represent a quantitative representation of these results.

Assessment of VEGFR-2 and VE-cadherin immunostaining

At day 12, immunostaining was performed for VEGFR-2 and VE-cadherin, markers that identify endothelial precursors cells. These cells are not only circulating and BM derived, but they are also present in subcutaneous adipose tissue. A slight staining for VEGFR-2 (Figures 7A and 7C) and VE-cadherin (Figures 7D and 7F) was observed in both strains of mice administered with vehicle. Injection of RLX significantly increased VEGFR-2 (Figures 7B and 7C) and VE-cadherin (Figures 7E and 7F) staining in both non-diabetic and diabetic animals. Figures 7(C) and 7(F) show a quantitative representation of these results.

Assessment of MMP-11 immunostaining

To ascertain whether RLX modulates collagen synthesis and extracellular matrix homoeostasis, MMP-11 immunostaining was investigated. A slight staining for MMP-11 was observed in both strains of mice administered with vehicle (Figures 8A and 8C). Injection of RLX significantly augmented MMP-11 staining in both non-diabetic and diabetic animals (Figures 8B and 8C). In this latter group, a strong positive stain was shown in the subepithelial tissue and in endothelial cells from newly formed capillaries, thus suggesting that RLX may antagonize the exaggerated fibrosis of the wounds. Figure 8(C) shows a quantitative representation of these data.
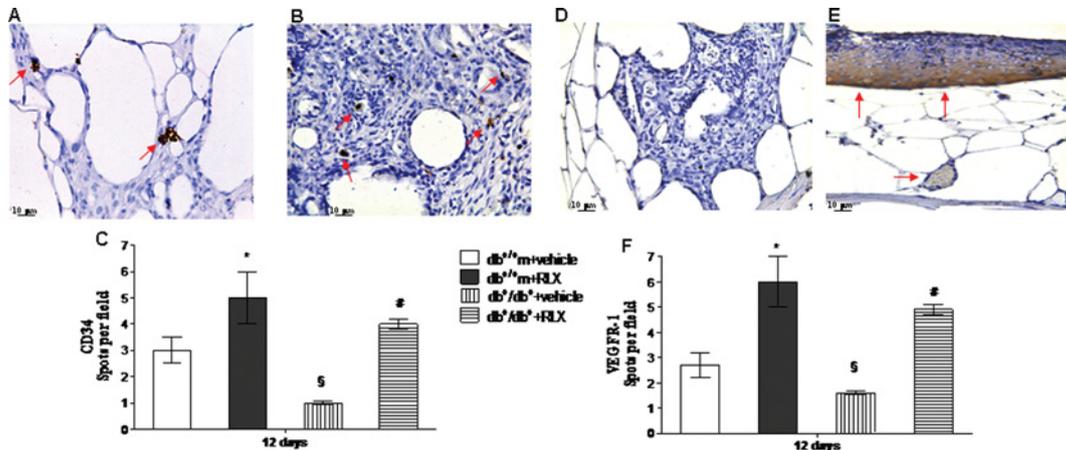
Figure 6   CD34 and VEGFR-1 staining
(**A**, **B**) Representative immunohistochemical CD34 staining of skin wound samples from diabetic (db$^+$/db$^+$) mice treated with vehicle (6 μl of 0.9% NaCl/mouse per day) or RLX (25 μg/mouse per day, subcutaneously) at day 12. Arrows indicate staining positivity. (**D**, **E**) Representative immunohistochemical VEGFR-1 staining of skin wound samples from diabetic (db$^+$/db$^+$) mice treated with vehicle (6 μl of 0.9% NaCl/mouse per day) or RLX (25 μg/mouse per day, subcutaneously) at day 12. Arrows indicate staining positivity. (**C**, **F**) Quantification of CD34- and VEGFR-1-positive cells per field in normoglycaemic (db$^{+/+}$m) and diabetic (db$^+$/db$^+$) mice treated with vehicle (6 μl of 0.9% NaCl/mouse per day) or with RLX (25 μg/mouse per day, subcutaneously) at day 12. Each bar represents the mean $\pm$ S.D. of six animals. *$P < 0.05$ compared with db$^{+/+}$m + vehicle; §$P < 0.01$ compared with db$^{+/+}$m + vehicle; #$P < 0.01$ compared with db$^+$/db$^+$ + vehicle.
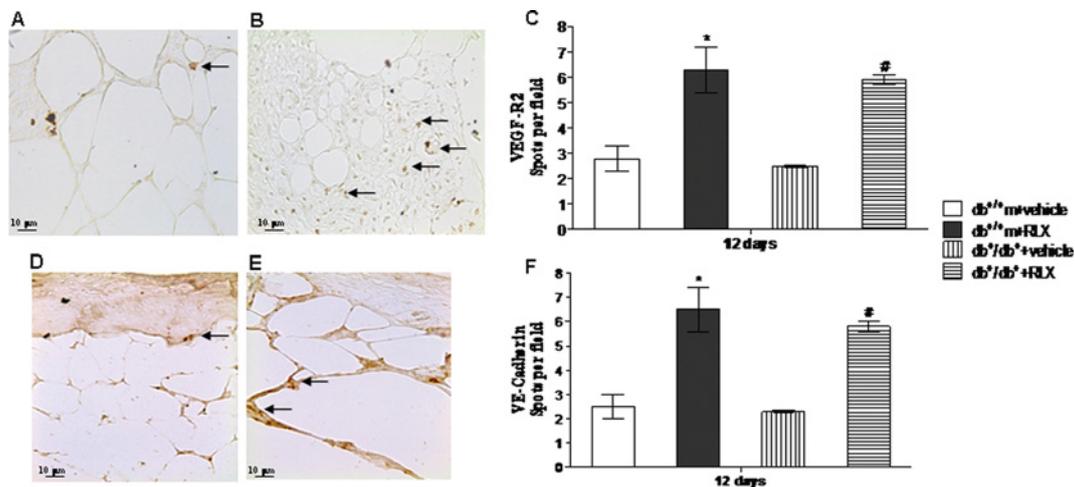

Figure 7   VEGFR-2 and VE-cadherin staining
(**A**, **B**) Representative immunohistochemical VEGFR-2 staining of skin wounds samples from diabetic (db$^+$/db$^+$) mice treated with vehicle (6 μl of 0.9% NaCl/mouse per day) or RLX (25 μg/mouse per day, subcutaneously) at day 12. Arrows indicate staining positivity. (**C**, **D**) Representative immunohistochemical VE-cadherin staining of skin wounds samples from diabetic (db$^+$/db$^+$) mice treated with vehicle (6 μl of 0.9% NaCl/mouse per day) or RLX (25 μg/mouse per day, subcutaneously) at day 12. Arrows indicate staining positivity. (**C**, **F**) Quantification of VEGFR-2- and VE-Cadherin-positive cells per field in normoglycaemic (db$^{+/+}$m) and diabetic (db$^+$/db$^+$) mice treated with vehicle (6 μl of 0.9% NaCl/mouse per day) or with RLX (25 μg/mouse per day, subcutaneously) at day 12. Each bar represents the mean $\pm$ S.D. of six animals. *$P < 0.05$ compared with db$^{+/+}$m + vehicle; §$P < 0.01$ compared with db$^{+/+}$m + vehicle; #$P < 0.01$ compared with db$^+$/db$^+$ + vehicle.

Wound-breaking strength and blood glucose levels

Figure 9 shows wound-breaking strength (Figure 9A) and blood glucose levels (Figure 9B) for each group at day 12. Wound-breaking strength of diabetic mice was significantly lower than that of normoglycaemic animals. Administration of RLX did not significantly change this parameter in non-diabetic animals (Figure 9A). The breaking strength of incisional diabetic wounds

of mice treated with RLX was higher than that of diabetic mice treated with vehicle (Figure 9A).

At the end of the experiment, blood glucose levels (Figure 9B) in diabetic animals treated with vehicle were 660 ± 32 mg/dl. Blood glucose levels in non-diabetic animals averaged 120 ± 12 mg/dl. RLX administration did not modify glycaemia in non-diabetic animals (106 ± 10 mg/dl),
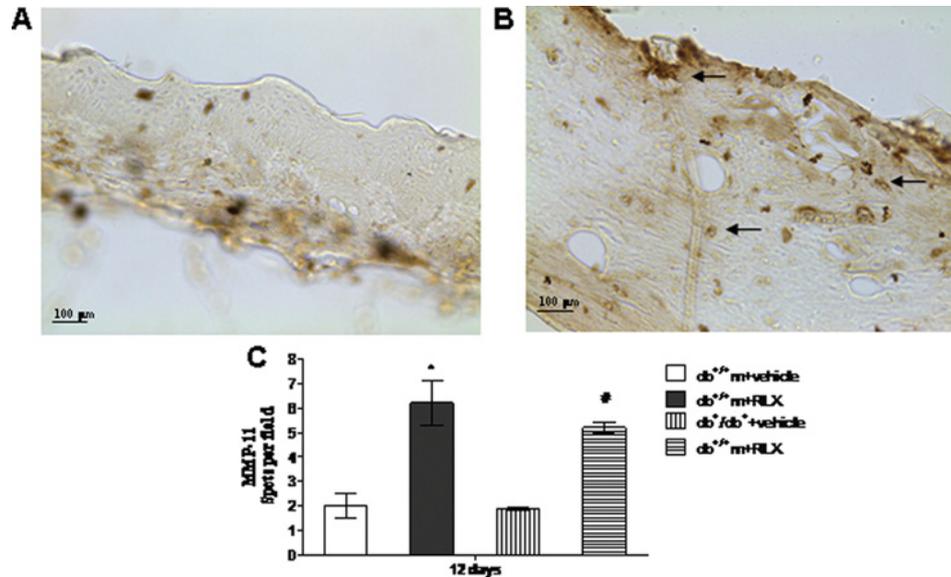
Figure 8   MMP-11 staining and scoring

(**A**, **B**) Representative immunohistochemical MMP-11 staining of skin wound samples from diabetic (db$^+$/db$^+$) mice treated with vehicle (6 μl of 0.9% NaCl/mouse per day) or RLX (25 μg/mouse per day, subcutaneously) at day 12. Arrows indicate staining positivity. (**C**) Quantification of MMP-11 staining per field in normoglycaemic (db$^{+/+}$m) and diabetic (db$^+$/db$^+$) mice treated with vehicle (6 μl of 0.9% NaCl/mouse per day) or with RLX (25 μg/mouse per day, subcutaneously) at day 12. Each bar represents the mean $\pm$ S.D. of six animals. $*P < 0.05$ compared with db$^{+/+}$m + vehicle; $^\#P < 0.01$ compared with db$^+$/db$^+$ + vehicle.
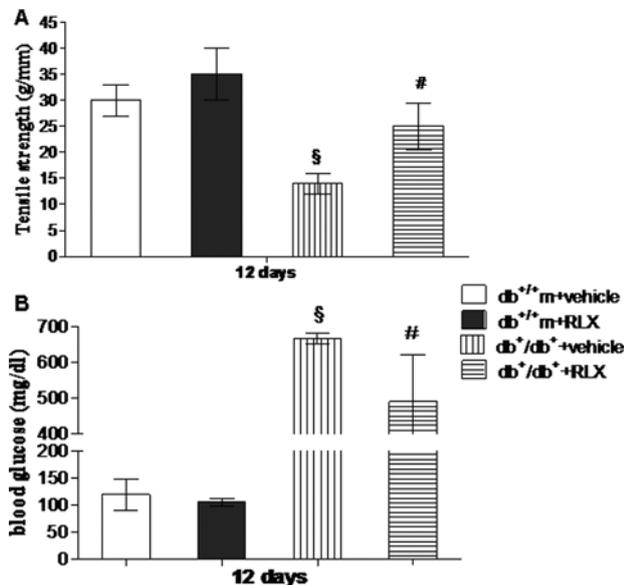

Figure 9 Tensile strength and glucose levels

Tensile strength of wounds (**A**) and blood glucose levels (**B**) obtained at day 12 from normoglycaemic (db$^{+/+}$m) and diabetic (db$^+$/db$^+$) mice administered with either RLX (25 μg/mouse per day, subcutaneously) or vehicle (6 μl of 0.9% NaCl/mouse per day). Each bar represents the mean $\pm$ S.D. of six animals. $^\S P < 0.01$ compared with db$^{+/+}$m + vehicle; $^\#P < 0.05$ compared with db$^+$/db$^+$ + vehicle.

but it significantly reduced blood glucose in diabetic animals (490 $\pm$ 60 mg/dl).

DISCUSSION

There is a complex cascade of events in skin repair, but angiogenesis is considered a key process during wound healing. A hostile micro-environment in diabetes, mainly because of enhanced production of reactive oxygen species [8], causes a defect in VEGF-driven angiogenesis that impairs the sequential and co-ordinated phases of the healing process. The final outcome of skin healing has been suggested to be improved only when the micro-environment is appropriately modified to be favourable for new vessel formation [9,20–24].

Blood vessels form through two distinct processes: vasculogenesis and angiogenesis [7]. In vasculogenesis, EPCs differentiate *de novo* from mesodermal precursors, whereas in angiogenesis new vessels are generated from pre-existing ones. Angiogenesis and neovascularization can be unwanted processes in certain diseases, including cancer. However, formation of the new blood vessels can also help alleviate some states, as in the formation of collateral circulation in ischaemic myocardium and limbs as well as during the healing of wounds. There is also in diabetes a well-documented disturbance of the mechanisms underlying vasculogenesis including altered SDF-1α expression, blunted eNOS–NO cascade and reduced EPC mobilization and homing [14].

The present findings suggest that a combined stimulation of both angiogenesis and vasculogenesis might represent a rational approach for the management of diabetes-induced impairment in wound healing, and justify the search of compounds able to achieve this. In this regard the polypeptide hormone RLX may be of interest. RLX has a relevant effect on peripheral and coronary vasculature [25], promotes growth of blood vessels [26] and

induces angiogenesis at wound sites [20]. It is also able to regulate the immune system [27,28].

In our experiment, RLX improved the altered healing process, augmented new vessel formation and increased wound breaking strength in obese diabetic animals. RLX also ameliorated the disturbed pattern of VEGF expression. Interestingly, RLX also stimulated the expression of CD31, CD34 and VEGFR-1 in new vessels close to the wound site in genetically obese-diabetic mice. In diabetes, the need for neovascularization arises from inadequate VEGF production and release in wounds; thus, the classic angiogenetic process is extremely delayed and poor and, as a consequence, therapeutically valuable approaches have been assessed with the aim of stimulating the expression of the impaired angiopoietic factor. Our results clearly suggest that RLX efficiently induces active angiogenesis, in turn improving the disturbed healing process. However, vasculogenesis is also impaired in diabetes and may contribute to the reduced pattern of new vessel formation [14].

EPCs present the surface marker VEGFR-2 (or *flk-1*) specific for the committed as well as for the young endothelial cells and, driven by growth factors, migrate from the lateral plate mesoderm towards the dorsal aorta to the site of neovasculogenesis [11]. In addition VE-cadherin, an endothelial-specific cell–cell adhesion protein of the adherence junction complex, may represent an additional marker of vasculogenesis [29]. We found significantly reduced VEGFR-2 and VE-cadherin expression in the wounds of obese diabetic animals treated with vehicle, whereas RLX administration markedly augmented VEGFR-2 and VE-cadherin staining in the vessels close to the surgical injury. Interestingly, we found SDF-1α expression and eNOS–NO cascade to be expressed at a low level at the wound site in db^+/db^+ mice; RLX treatment succeeded in reversing these defects. These observations confirm that, in diabetic wound healing, there is also a defect in vasculogenesis that might also contribute to impaired skin repair. Furthermore, the present results led us to hypothesize that RLX may have the ability to generate new vessel formation by a dual mechanism of action including both angiogenesis and vasculogenesis. These effects might represent the main mechanisms by which RLX ameliorates the skin repair process. To confirm this hypothesis, we evaluated the time to complete wound closure that represents the 'main outcome' for any therapeutic intervention aimed at improving the depressed wound healing in diabetes. RLX markedly reduced the time needed to complete skin normalization. Interestingly, the concomitant administration of neutralizing anti-VEGF [30] and SDF-1α receptor CXCR4 antibodies [31] (to interrupt the signalling events involved in the recruitment and homing of EPCs into the ischaemic tissue) abated RLX effects on wound closure, thus confirming our hypothesis. Taken together, the immunohistochemical observation and the neutralization experiment demonstrate that RLX acts through both these pathways to promote angiogenesis and vasculogenesis.

However, a well co-ordinated healing process does not have to generate an excessive fibrosis that may contribute to 'disorders' in the skin repair process such as hypertrophic wounds and finally scar formation [32]. RLX has been suggested to be therapeutically useful for regulating excessive collagen deposition and promoting tissue remodelling in diseases characterized by fibrotic

deposit [33,34]. The mechanism underlying the antifibrotic action of RLX is still far from being fully understood; however, the polypeptide hormone has been shown to reduce the activity of some pro-fibrotic factors (i.e. transforming growth factor-β1 and interleukin-1β) [35,36] and to cause MMP production in some tissues [37]. In agreement with this previous observation, we found that RLX treatment markedly augmented the staining of MMP-11 in the wounds. This may represent a further positive feature of RLX treatment that may well balance collagen turnover, thus avoiding the uncontrolled fibrosis seen in hypertrophic healing. The beneficial effect of RLX on the disturbed diabetic wound healing was also accompanied by a reduction in blood glucose levels. This effect might be of benefit in the overall management of diabetic patients, even if it is unlikely that this significant, but 'not yet clinically relevant' reduction in blood glucose might contribute to the RLX-induced improvement in skin repair.

In conclusion, results from the present study demonstrate that RLX administration can ameliorate the altered diabetic wound healing not only by accelerating new vessel formation through a 'double' mechanism of action, but also by regulating MMP expression, thus avoiding an unwanted and exasperated fibrosis. Since a recombinant form of human RLX has been studied in Phase II/III clinical trials and demonstrated promising results, particularly in the treatment of acute heart failure [38], which is more likely to occur in diabetic patients [39,40], we believe that our study might shed light on the possible use of RLX in diabetic patients with peripheral diseases and local circulatory insufficiency such as foot ulcers.

CLINICAL PERSPECTIVES

- Patients suffering from diabetes show a disturbed wound-healing process that may enhance the overall morbidity and mortality of this population. Experimental evidence suggests that RLX (relaxin) can improve several parameters associated with wound healing. Therefore we investigated the effects of porcine-derived RLX in diabetes-related wound-healing defects in genetically diabetic mice.
- The results of the present study show that RLX is able to improve both angiogenesis and vasculogenesis, via the stimulation of specific molecular targets, including VEGF and SDF1-α.
- These findings suggest that in altered wound healing, as during diabetes, RLX administration could represent an alternative therapeutic strategy. The application in a clinical setting may not be that far away, as RLX had no side effects and the positive effects were visible after a few days of treatment.

AUTHOR CONTRIBUTION

Alessandra Bitto, Natasha Irrera and Letteria Minutoli conceived and designed the study. Margherita Calò, Patrizia Lo Cascio, Paolo Caccia, Antonio Micali, Gabriele Pizzino, Giovanni Pallio, Antonio Micali, Mario Vaccaro and Antonino Saitta analysed and interpreted

data. Francesco Squadrito and Domenica Altavilla led the design and drafted the paper.

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