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# Porcine derived relaxin stimulates new vessel formation and improves the disturbed wound healing of the genetically diabetic mice

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Summary

Diabetic mice are characterized by an altered expression pattern of VEGF, and impaired vasculogenesis during healing. We investigated the effects of porcine derived relaxin 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 RLX (25$\mu$g mouse/day subcutaneously) or its vehicle. Mice were killed on days 3, 6 and 12 after skin injury for measurements of vascular-endothelial-growth-factor (VEGF) mRNA and protein synthesis. Furthermore, we evaluated wound-breaking strength, histological changes, and angiogenesis at day 12.
At day 6, RLX administration resulted in an increase in VEGF mRNA expression and protein wound content. Furthermore the histological evaluation indicated that RLX improved the impaired wound healing, and increased wound breaking strength at day 12 in diabetic mice. Immunohistochemistry showed that RLX in diabetic animals augmented new vessel formation. These data strongly suggest that RLX may have a potential application in diabetes-related wound disorders.

Key words

**A**ngiogenesis, diabetes, relaxin, wound healing

Healing impairment in diabetics is characterized by delayed cellular infiltration and granulation tissue formation, decreased collagen organization and, more interestingly, reduced angiogenesis. As far as angiogenesis is concerned an altered expression pattern of VEGF mRNA during skin repair, has been shown in diabetic mice (Altavilla et al., 2001). Relaxin has been shown to induce VEGF expression and angiogenesis selectively at wound sites in an experimental in vitro model (Unemori et al., 2000). However the effects of relaxin in the diabetes impaired wound healing have not been fully investigated. We therefore investigated the effects of a porcine derived relaxin in an incisional wound healing model in the genetically diabetic mice (Altavilla et al., 2001).

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All animal procedures were in accordance with the Principles of Laboratory Animal Care (NIH publication no.85-23, revised 1985) and authorised by our National Institution. Genetically diabetic female (30-35g) C57BL/KsJ-m+/+*Lept*$^{db}$ mice (db$^{+}$/db$^{+}$) and their normal littermates (22-25g) (db$^{+/+}$m) were obtained from Jackson Laboratory (Bar Harbor, USA). After general anesthesia with sodium pentobarbital (80 mg/kg/i.p.), 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 previously described (Altavilla et al., 2011). Normoglycaemic and diabetic mice, received either RLX (25$\mu$g/mouse/day s.c.) or its vehicle (6$\mu$l/mouse/day of 0.9% NaCl) for up to 12 days. 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. Skin was used for molecular, functional and histological analysis.

Wounds obtained at each time point were analysed for VEGF mRNA content by Real-Time PCR using b-actin as endogenous control. The results were expressed as the n-fold difference relative to normal controls (relative expression levels). 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, USA) as previously described (Galeano et al., 2006). The amount of VEGF was expressed as picograms per milligram of protein.

For the histological analysis wound samples were fixed in 10% neutral buffered formalin, and processed as previously described (Altavilla et al., 2011). All slides were examined by a pathologist blinded to the previous treatment, and the following parameters were evaluated and scored: re-epithelialization, dermal matrix deposition and regeneration, granulation tissue formation and remodelling, according to the literature data concerning wound healing in experimental models (Altavilla et al., 2011; Galeano et al., 2008; Galeano et al., 2011). For the immunohistochemical study paraffin-embedded tissues were sectioned (5 mm), re-hydrated, and antigen retrieval was performed using 0.05M sodium citrate buffer. Slides were incubated overnight with primary antibody to detect CD-31, VEGFR-1 (all from Abcam, UK), and VEGFR-2 (Cell Signaling, USA) as previously described (Altavilla et al., 2011). DAB (3-3' Diaminobenzidine, Sigma, USA) was used to reveal the reaction and counterstain was performed with haematoxylin where needed. To assess new blood vessel formation, microvessel density (MVD) was estimated after CD-31 staining. To assess positive VEGFR-1 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.

At day 12, wound breaking strength was evaluated by the maximum load tolerated by wounds using a calibrated tensometer (Sans, Italy) as previously described (Altavilla et al., 2011). The ends of the skin strip were pulled at a constant speed (20 cm min$^{-1}$), and breaking strength was expressed as the mean maximum level of tensile strength in newton (N) before separation of wounds.

All data were expressed as means and standard deviations (mean ± SD). 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).

The results obtained showed that non-diabetic animals, have a strong induction of VEGF mRNA and protein at day 3 and 6, that decline at day 12. In this animals

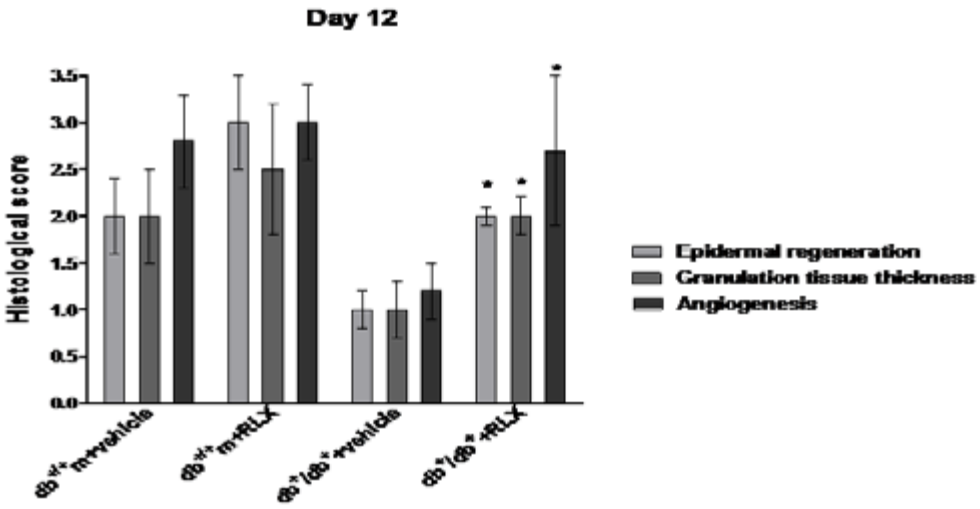


**Figure 1.** Histological scores in wound samples collected from either normoglycaemic ($db^{+/+}m$) and diabetic mice ($db^{+}/db^{+}$) given either RLX (25µg mouse/day/s.c.) or vehicle (6µl mouse/day of 0.9 % NaCl) at day 12. Each bar represents the mean ± S.D. of six animals. $^{*}p<0.001$ vs $db^{+}/db^{+}$ + vehicle.

administration of RLX enhanced VEGF mRNA and protein content. At day 3 and 6 wounds from diabetic mice showed a markedly reduced expression and protein levels of VEGF when compared with non-diabetic ones, and were still significantly detectable at day 12. Daily treatment with RLX enhanced VEGF expression and protein levels at days 3 and 6, thus restoring the disturbed pattern of VEGF secretion.

The histological scores depicted in the Figure show that normoglycemic animals completed the remodeling and wound closure process at day 12, however the administration of RLX qualitatively improved wound healing. By contrast, diabetic wounds of mice administered with vehicle at day 12 showed poor-to mild reepithelialization, with partially organized granulation tissue. Wounds of diabetic mice treated with RLX, showed moderate to complete reepithelialization and well-formed granulation tissue. CD-31 immunostaining was investigated at day 12 to confirm neo-vessel formation. In fact, this protein represents an highly specific marker for endothelial cells. Positive staining was present in the wounds from vehicle administered non diabetic animals. This staining appeared markedly reduced in the wounds from diabetic animals injected with vehicle. Administration of RLX augmented CD-31 immunostaining in both strains of mice. The effect was remarkable in diabetic wounds where the positive staining was mostly localized in small vessels and capillaries. To confirm neo-angiogenesis, VEGF-R1 immunostaining was also studied at day 12. Positive staining was evident in the wounds from vehicle injected non diabetic animals. This staining was significantly reduced in the wounds from diabetic animals injected with vehicle and increased by relaxin. In RLX-treated diabetics a marked staining in the sub-epithelial layer was observed, VEGF-R1 in fact exist also in soluble form and the diffuse staining suggest an enhanced production. At day 12, immunostaining was performed also for VEGFR-2 that identify endothelial precursors cells. These cells are not

only circulating and bone-marrow derived, but they are also present in subcutaneous adipose tissue. A slight staining for VEGFR-2 was observed in both strains of mice administered with vehicle. Injection of RLX significantly increased VEGFR-2 staining in both non-diabetic and diabetic animals. Using the images obtained from the immunoistochemichal staining, the tissue samples were assessed for micro-vessel density and angiogenesis quantification (Figure).

At day 12 the 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. The breaking strength of incisional diabetic wounds of mice treated with RLX was higher than that of diabetic mice treated with vehicle.

The present findings indicate that porcine relaxin improved the altered healing process, augmented new vessel formation and increased wound breaking strength in obese-diabetic animals. Relaxin also ameliorated the disturbed pattern of VEGF expression. Interestingly, relaxin stimulated also the expression of CD31, VEGFR-1 and VEGFR-2 in new vessels close to the wound site in genetically obese-diabetic mice. In diabetes, the need of neovascularization arises from the 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 addressed to the aim of stimulating the expression of the impaired angiopoietic factor. Our results clearly suggest that relaxin efficiently induces active angiogenesis, in turn improving the disturbed healing process.

In conclusion, results from this study demonstrate that relaxin administration can ameliorate the altered diabetic wound healing, by accelerating new vessel formation. Since a recombinant form of human relaxin has been studied in Phase II/III clinical trials and demonstrated promising results, particularly in the treatment of acute heart failure (Teerlink et al., 2009), that is more likely to occur in diabetic patients, we believe that our study might shed light on the possible use of relaxin in diabetic patients with peripheral artery diseases and local circulatory insufficiency like foot ulcers.

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