CED
Clinical and Experimental Dermatology

Effect of topical insulin on cutaneous wound healing in rats with or without acute diabetes

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doi:10.1111/j.1365-2230.2009.03419.x

Summary

Background. Diabetes is a condition known even in its early stages to impair the normal course of wound healing, thus leading to chronic wounds. The role of insulin in the regulation of energy metabolism, protein synthesis, cell differentiation and growth suggests that this hormone could also play an essential role in regulation of wound healing.

Aim. To determine the effects of topical insulin administration on wound healing in rats with or without acute diabetes.

Methods. This study was conducted using four groups of male Sprague–Dawley rats: (i) nondiabetic rats receiving topical insulin (n = 7), (ii) nondiabetic rats receiving topical sterile water (n = 7), (iii) diabetic rats receiving topical insulin (n = 7) and (iv) diabetic rats receiving topical sterile water (n = 7). Wound healing was assessed by wound contraction rate, complete epithelialization time and histological results.

Results. Topical insulin enhanced wound healing by shortening the time needed for complete epithelialization in both the nondiabetic and acute diabetic groups. The histological observations supported the planimetric results in both groups.

Conclusions. This study revealed that topical insulin application to cutaneous wounds accelerates wound healing in rats with or without acute diabetes.

Introduction

Diabetes is a condition known even in its early stages to impair the normal course of wound healing, thus leading to chronic wounds. The wound-healing impairment in diabetes can be attributed to several factors including inadequate blood supply, decreased proliferative potential of fibroblasts, and decreased inflammatory changes.[1]

The role of insulin on regulation of energy metabolism, protein synthesis, and cell differentiation and growth suggests that this hormone could also play an essential role in regulation of wound healing. There is evidence for a positive effect of systemic insulin replacement on diabetic wound healing,[2,3] but a few reports on topical insulin administration in diabetic wound healing gave conflicting results.[4–6]

This study aimed to determine the effects of topical insulin administration on wound healing in both rats with or without acute diabetes. To our knowledge, this is the first study examining the effects of topical insulin application on cutaneous wound healing in acute diabetes.

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Conflict of interest: none declared.

Accepted for publication 30 September 2008

Methods

Animals

Male Sprague–Dawley rats (Istanbul University Institute of Experimental Medicine) weighing 216–315 g were used. All the rats were kept in plastic cages and fed with

standard laboratory pellets and tap water *ad libitum* until the day of the experiment. After wound creation, the rats were housed individually in separate cages.

Experimental groups

Animals were randomly assigned to diabetic and nondiabetic groups. After diabetes induction and wound creation, animals within each group were assigned to the treatment and the control groups by a second randomization. The study was carried out on these four groups. Group 1: nondiabetic animals that received topical insulin (regular human insulin; Humulin R®; Eli Lilly Turkey, Istanbul, Turkey) (non-DM-I; $n = 7$); group 2: nondiabetic control animals that received topical sterile water (water for injection) (non-DM-C; $n = 7$); group 3: diabetic animals that received topical insulin (DM-I; $n = 7$); and group 4: diabetic control animals that received topical sterile water (DM-C; $n = 7$).

Diabetes induction

Rats randomized to the diabetic group were injected with a single dose of intraperitoneal (IP) streptozotocin 50 mg/kg body weight in saline (0.9% NaCl w/v) to induce diabetes. The solutions were prepared freshly and injected without any delay. All the rats were checked for symptoms of polydipsia, polyuria and weight loss. A blood glucose measurement was performed 48 h after streptozotocin injection. Blood was drawn from the tail vein and the glucose level was determined using a glucometer (Accu-Chek; Roche Diagnostics, Mannheim, Germany). Rats with blood glucose levels > 250 mg/dL were considered to be diabetic. Rats in the nondiabetic group were injected with a single dose of IP saline.

Wound model and treatment

In order to study wound healing on an acute diabetes model, the wounds were created on day 3 after streptozotocin or saline administration. Rats were anaesthetised with IP ketamine 10 mg/kg body weight, then the dorsal hair of the rats was shaved with an electrical clipper. After cleansing with clorhexidine solution (5 mg/mL), two round, full-thickness excision wounds, 0.6 mm in diameter, were created with a punch biopsy instrument on the dorsal thoracic area of each rat. Wounding day was coded as day 0. The wounds were left open throughout the study.[7] After the wound excision, 20 μL of the test and control solutions were applied to each wound. Each animal received insulin and sterile water in aliquots of 20 μL twice a day until the end of experiment (day 15). Test and control solutions were applied under light ether anaesthesia, allowing some time for the absorption of the drug from the wound site.

Wound closure measurements

Wound-healing rate was calculated by wound contraction rate and complete epithelialization time. Wound contraction was monitored by measuring wound area planimetrically. To determine the wound contraction rate, wounds were traced on transparent paper with millimetre scaling. Traces on the transparent paper were transferred to photocopy papers of 80 g/m^2 weight. The wound traces were cut out and weighed by a precision balance for more accurate measurements, and the weights were converted to the corresponding surface area values (mm^2). Wound closure was reported as percentage closure and calculated using the formula:[8] $\%\,\text{closure} = [(\text{area on day 0-open area on day } n)/\text{area on day 0}] \times 100$, n represents each of the measurement days.

Presence of complete epithelialization was monitored by visual observation throughout the experiment. Complete epithelialization was said to be reached when the scar fell off the skin, leaving no raw wound behind. The planimetric measurements were performed on both wounds of each animal, thus a total of 14 wounds were studied for each group.

Termination of the experiment

At the end of the experiment (day 15), the rats were anaesthetised with ketamine, and wound samples were harvested. Blood samples were drawn by cardiac puncture and all animals were killed by exsanguination. Levels of blood glucose and serum creatinine, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured in these blood samples.

Histological evaluation

For histological evaluation, skin tissue samples were fixed in 10% formaldehyde and processed routinely for embedding in paraffin wax. After dehydration in an ascending series of ethyl alcohol, tissue samples were cleared in toluene. Paraffin wax-embedded sections 5–6 μm thick were stained with haematoxylin and eosin to identify histological degeneration, and with Masson trichrome stain for collagen distribution.

Statistical analysis

Continuous variables are expressed as mean ± SE of the mean, or median (range). The difference between the two groups were analysed using the nonparametric Mann–Whitney test. Binomial data are expressed as *n* (%) and analysed using the Pearson χ^2 test. Significance was set at $P < 0.05$. The analyses were performed using the statistical software package SPSS (version 11.5; SPSS Inc., Chicago, IL, USA).

Results

Induction of diabetes

All the rats that received streptozotocin injection displayed symptoms of diabetes such as polydipsia, polyuria and weight loss. Induction of diabetes was confirmed by a blood glucose measurement of > 250 mg/dL performed 48 h after streptozotocin injection in all animals in the diabetic groups. All the nondiabetic animals had blood glucose values < 250 mg/dL. The median preoperative glucose level was 457.5 mg/dL (range 343–583) for the diabetic and 175.5 mg/dL (148–201) for the nondiabetic rats. It was found that blood glucose stayed high throughout the study period for the diabetic animals. Glucose level on day 15 was 539.1 mg/dL (465–589) for the diabetic and 187.8 mg/dL (155–224) for the nondiabetic rats. Weight of the diabetic control rats was initially a mean of 248 g (216–259) and decreased to 233 g (196–243 g) by day 15 ($P < 0.05$). Serum creatinine, AST and ALT levels measured in samples drawn on day 15 were significantly higher for the acute-diabetic controls compared with the nondiabetic controls (Table 1).

Effect of acute diabetes on wound healing

The effect of acute diabetes on wound healing was tested by comparing the nondiabetic and diabetic control groups. There was no difference between the diabetic and nondiabetic groups in planimetric measurements at any time point (Table 2). The histological evaluation of tissue samples taken on day 15 showed presence of granulation tissue in diabetic control rats, but not in nondiabetic control rats (Fig. 1a,e). The presence of granulation tissue in samples of diabetic control rats is indicative that healing was impaired, although there was no overt delay in wound healing.

Table 1 Renal and hepatic function.

	Non-DM-C	DM-C
Serum Cr	0.34 (0.30–0.36)*	0.42 (0.35–0.48)*
ALT	48.0 (42–55)*	132.0 (121–155)*
AST	101.0 (73–139)*	199.0 (187–206)*

Data are expressed as median (range). ALT, alanine aminotransferase; AST, aspartate aminotransferase; Cr, creatinine; DM-C, diabetic control; non-DM-C, nondiabetic control. *$P < 0.001$.

Wound healing in nondiabetic groups

Nondiabetic rats receiving topical insulin had higher rates of wound closure rates than the nondiabetic control group; the difference was significant on days 3, 5, 7 and 9 ($P < 0.001$, $P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively) (Table 2). The median time of complete epithelialization was 11.5 days (range 9–13) for the nondiabetic controls, and 8 days (7–10) for the nondiabetic rats receiving topical insulin ($P < 0.001$). Topical insulin administration enhanced wound healing by shortening the time needed for complete epithelialization in the nondiabetic group (Table 3). Histological examination showed that there was an increase in the number of collagen fibres in the insulin-treated nondiabetic rats compared with nondiabetic control animals (Fig. 1a,c).

Wound healing in diabetic groups

Diabetic rats receiving topical insulin had higher rates of wound closure than the diabetic control group, and the difference was significant on days 7 and 9 ($P < 0.01$) (Table 2). The median time of complete epithelialization was 11.5 days (range 8–13) for the diabetic controls and 8 (7–12) days for the diabetic rats receiving topical insulin ($P = 0.001$). Topical insulin administration enhanced wound healing by shortening the time needed for complete epithelialization in the diabetic group (Table 3). Histological examination found the presence of a thick epidermis in the insulin-treated diabetic rats compared with the diabetic control animals (Fig. 1e,g).

Discussion

Diabetes is one of the factors affecting the normal course of wound healing. Diabetic wound healing is characterized by a delay in cellular infiltration and formation of granulation tissue, and diabetic wounds have a prolonged epithelialization time.[9] Experimental diabetes was shown to impair wound healing by decreasing collagen concentration and formation of granulation tissue,[10–12] and also by increasing activities of protease

Table 2 Wound closure rates of the groups.

	Percentage wound closure rate, median (range)					
Group	Day 3	Day 5	Day 7	Day 9	Day 11	Day 13
Non-DM-C	21.5* (8.8–37.7)	47.0* (25.4–55.2)	76.4† (55.7–88.1)	92.8‡ (87.2–100)	99.3 (91.4–100)	100 (100–100)
Non-DM-I	32.1* (23.4–47.7)	66.5* (55.8–74.0)	88.5† (51.8–100)	100‡ (91.4–100)	100 (100–100)	100 (100–100)
DM-C	21.1 (5.0–35.4)	45.3 (20.7–67.3)	75.2† (55.7–87.9)	92.2† (80.8–100)	99.4 (95.0–100)	100 (100–100)
DM-I	32.3 (7.1–49.1)	54.7 (9.3–73.7)	87.8† (76.9–100)	100† (96.0–100)	100 (100–100)	100 (100–100)

DM-C, diabetic control; DM-I, diabetic rats treated with topical insulin; non-DM-C, nondiabetic control; non-DM-I, nondiabetic rats treated with topical insulin. *$P < 0.001$; †$P < 0.01$; ‡$P < 0.05$.

Table 3 Complete epithelialization time of the groups.

	Complete epithelialization, *n* (%)		
Group	Day 7	Day 9	Day 11
Non-DM-C	0 (0)	2 (14.3)*	7 (50)*
Non-DM-I	0 (0)	9 (64.3)*	14 (100)*
DM-C	0 (0)	4 (28.6)†	7 (50)†
DM-I	2 (14.3)	10 (71.4)†	13 (92.9)†

DM-C, diabetic control; DM-I, diabetic rats treated with topical insulin; non-DM-C, nondiabetic control; non-DM-I, nondiabetic rats treated with topical insulin. *$P < 0.01$; †$P < 0.05$.

and collagenase.[4] Collagen provides tensile strength, organization and integrity to the connective tissues, and plays a role in haemostasis by interacting with thrombocytes.

Insulin is one of the potential hormonal mediators of altered collagen production. It is known to stimulate collagen synthesis in skin fibroblasts in a strong and selective manner.[13,14] Pellegrinelli *et al.*[9] reported the presence of insulin receptors in keratinocytes of the epidermis and in hair follicles, and identified signalling pathways through which insulin can promote growth in the skin. It was shown that human keratinocytes are dependent on insulin for their growth.[15] Several *in vitro* experiments suggest that growth factors such as insulin can act as chemoattractants and mitogens for the cells involved in wound healing,[8,16,17] and that growth factors can stimulate angiogenesis, extracellular matrix formation and degradation, and cytokine release.[8] Therefore, we aimed to determine the effects of topical insulin administration on wound healing in both rats with or without acute diabetes.

In this study, we confirmed the presence of wound-healing impairment in the early stages of diabetes by histological examination. Nondiabetic control rats had a regular epidermal and dermal structure with organized collagen fibres and hair follicles, whereas the diabetic control rats had a thin epidermal layer in localized areas, narrow hair follicles, irregular collagen fibres and increased angiogenesis in the dermis and hypodermis. In addition, granulation tissue was found in samples from diabetic control rats but not from nondiabetic control rats. These findings suggest that in diabetic rats healing was impaired, although there was no overt delay in wound healing. It has been suggested that alterations in the wound-healing process are present even at the onset of diabetes and that early diabetes can be associated with deficiencies in the defence cells involved in normal wound healing[18] and also with a marked decrease in the production of collagen.[19]

In our study, it was found that topical insulin administration enhanced wound healing by shortening the time needed for complete epithelialization in the nondiabetic group. The histological findings of an increased number of collagen fibres in the topical insulin-treated nondiabetic group supported the planimetric results. This result is in accordance with other reports in which topical insulin was found to enhance wound healing by affecting myofibroblasts[20] and increasing collagen deposition.[21] The most recent study on the effects of topical insulin in wound healing was conducted on human skin cell cultures. In their study, the skin wounds of the rats treated topically with insulin healed faster, the surface cells in the epidermis covered the wound more quickly, and the cells in the dermis rebuilt blood vessels more rapidly. It was also shown that topical insulin stimulated the proliferation and migration of keratinocytes and the migration of microvascular endothelial cells.[22]

We found that topical insulin administration enhanced wound healing by shortening the time needed for complete epithelialization in the diabetic group as well. The histological results, showing a thick epidermal layer in the topical insulin-treated diabetic group, supported the planimetric results. Our results also concur with those of a similar study in which topical

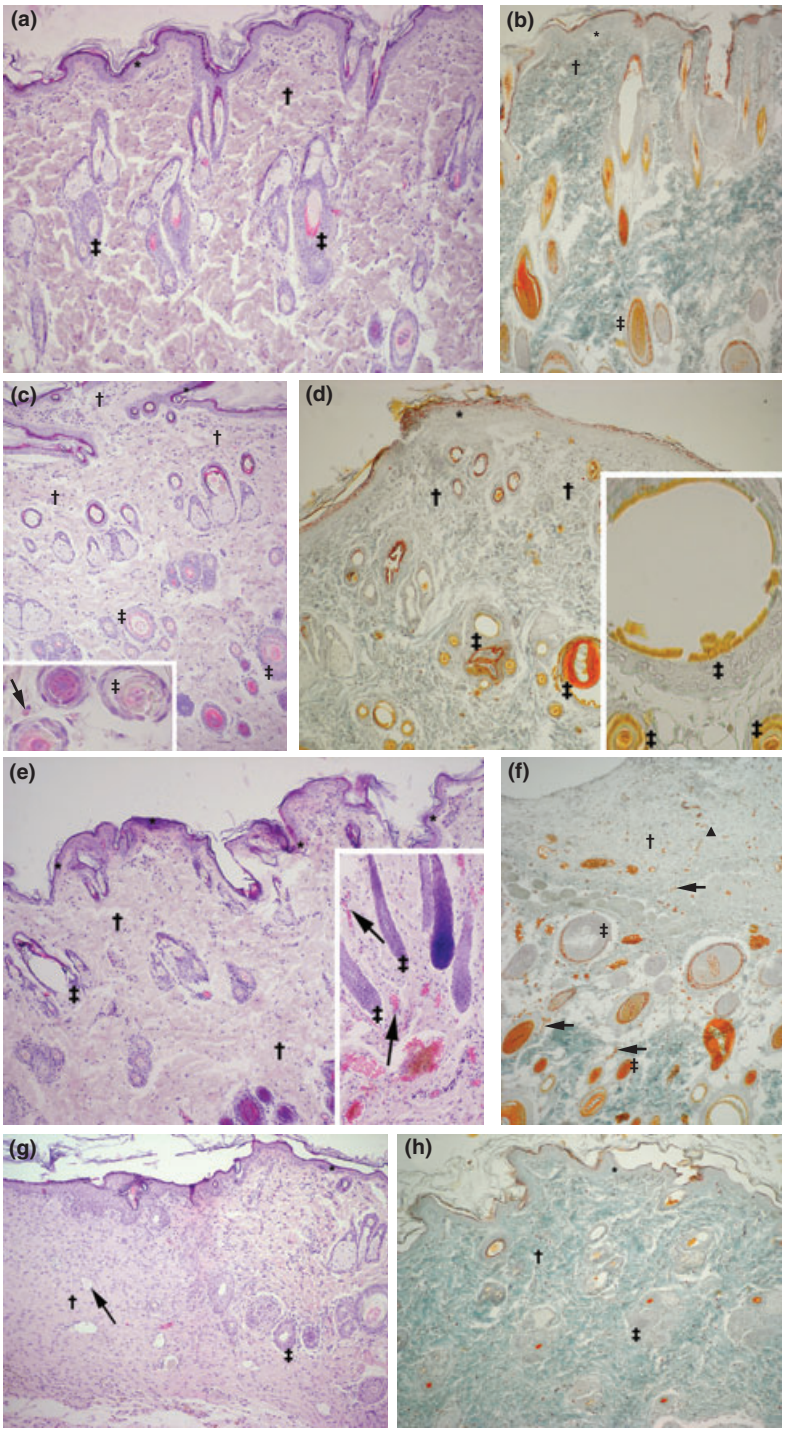

Figure 1 (a,b) Group of nondiabetic control animals: *regular epidermis; †regular collagen fibres; ‡regular hair follicles in dermis. (c,d) Group of nondiabetic animals that received topical insulin: *regular epidermis morphology; †localized increase in collagen fibres; ‡presence of hair follicles of different diameters in dermis. (e,f) Group of diabetic control animals: *localized thinning of epidermis; †irregularly increased collagen fibres in dermis and hypodermis; ‡thinning of hair follicles; increased angiogenesis in dermis (arrow), formation of granulation tissue (arrowhead). (g,h) Group of diabetic animals that received topical insulin: *presence of thick epidermis in localized areas; †increased collagen fibre; ‡absence of hair follicles; angiogenesis in dermis (arrow). (a,c,e,g) Haematoxylin and eosin; (b,d,f,h) Masson's trichrome. Original magnifications: main pictures × 100; inserts × 400.

insulin provided better rates of wound healing than treatment with base or control preparations,[5] and one in which diabetic rats that received topical insulin alone or in combination with epidermal growth factor (EGF) had lower collagenase activity than both control and diabetic EGF groups.[4] Our results were not in accordance with those from the study of Grotendorst *et al.*,[6] which suggested that insulin had no effect on the rate of new tissue formation. However, in that study, a combination of platelet-derived growth factor (PDGF) and insulin

caused an even more rapid increase in collagen deposition than that found with PDGF alone.

In conclusion, this study supports the notion that even in the early stages of diabetes, the wound-healing process in impaired, and revealed that topical insulin application to nondiabetic as well as acute diabetic cutaneous wounds accelerates wound healing in rats. We suggest that this practice could be used as an alternative to or in combination with currently used wound-healing treatments.

Acknowledgements

This project was financially supported by Marmara University Scientific Research Projects Committee (file no. SAG-DKR-290107–0023).

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