Effect of Topical Insulin Application on Wound Neutrophil Function

Xuelian Chen, MD; Xiong Zhang, MD, PhD; Yan Liu, MD, PhD

Abstract: This study observed the quantity and functions of wound neutrophils after topical insulin treatment and investigated the effect of insulin on the wound inflammatory response, as well as the mechanism of insulin-induced wound healing. Methods. Full-thickness excisional wounds were made on the dorsal symmetrical site of C57BL/6J mice. The wounds were treated with either 0.03U insulin/20 µL saline or 20 µL saline. The healing times and healing rates of the wounds were recorded. The wounds and adjacent tissues were collected during the first 3 consecutive days after the injury. Quantification of myeloid differentiation antigen Gr-1, myeloperoxidase (MPO), malondialdehyde (MDA), and macrophage inflammatory protein-2 (MIP-2) were measured by Western blotting, biochemical analysis, and real-time polymerase chain reaction (PCR), respectively. Results. The healing time of insulin-treated wounds was significantly decreased compared with the saline-treated wounds. The wound closure rates at days 5 and 7 after injury were significantly higher with insulin treatment than with saline treatment. Additionally, wound neutrophil infiltration was suppressed by insulin treatment within the first 2 days after injury. Real-time PCR revealed insulin treatment significantly inhibited MIP-2 expression within 2 days after injury (P <0.05), whereas MIP-2 expression in both groups decreased to a similar level at 3 days after injury. There was no statistical difference in MPO or MDA content between the insulin treatment and control group. Conclusion. Topical insulin application decreased neutrophil infiltration by inhibiting MIP-2 expression and advanced neutrophil resolution. It has been reported that insulin promotes neutrophil functions; therefore, the present results, along with previous findings, suggest there is a delicate regulation of insulin on wound inflammatory response during the healing process.
cal insulin application also promoted healing of thermal traumas in rats and incision wounds in rabbits.\textsuperscript{7,8} In the proliferation phase of wound healing, the authors’ previous research showed that low-dose topical insulin stimulated migration of keratinocytes and vascular endothelial cells through the insulin receptor-mediated PI3K-Akt-Rac1 signal pathway. These molecular events could trigger re-epithelialization and angiogenesis, and hence, promote wound healing.\textsuperscript{9,10}

In the inflammatory phase, the initiation of wound healing, which consists of a series of biological events including coagulation and release of vasoactive substances, chemokines, and cytokines, is important for regulating the consequent phases of proliferation and remodeling.\textsuperscript{11} Systemic insulin treatment was reported to alleviate the systemic inflammatory response via inhibiting the expression of monocyte chemoattractant protein-1 (MCP-1), cytokine-induced neutrophil chemoattractant 1 (CINC-1), and CINC-2.\textsuperscript{12} However, it remains unknown whether topical insulin application could help regulate the traumatic inflammatory response, and if so, whether this insulin-regulated inflammatory response was one of the underlying mechanisms of insulin-accelerated wound healing.

Neutrophils are the main type of cells that are involved in the inflammatory response. They clean exogenous pathogens through phagocytosis and release enzymes and reactive oxygen species (ROS) to kill bacteria and other intruders. Because macrophages also have the function of phagocytosis, neutrophils are not essential to wound healing, since it has been shown that anti-neutrophil antibodies do not interfere with healing.\textsuperscript{13} On the contrary, depletion of neutrophils facilitates wound healing and improves the quality of recovery.\textsuperscript{14} Additionally, prolonged neutrophil infiltration may contribute to impaired wound healing.\textsuperscript{15} These findings indicate that although neutrophils work as traumatic scavengers and help wound healing in some ways, neutrophils also have a negative impact on wound healing, particularly when excess and/or hyper-functional neutrophils are present in the wound area. In other words, suppressing neutrophil infiltration, especially in asepsis, might promote wound healing.

\textit{In vitro} experiments have shown that insulin regulates neutrophil functions through binding cell membrane receptors.\textsuperscript{16} Systemic insulin treatment enhances the expression of neutrophil adhesion molecules and various cell surface receptors to reinforce the cellular functions of migration, phagocytosis, and bactericidal actions.\textsuperscript{17} However, there have been no publications showing whether low-dose topical insulin application directly influences the quantity and function of wound neutrophils.

### Keypoints
- The authors’ previous research showed that low-dose topical insulin stimulated migration of keratinocytes and vascular endothelial cells through the insulin receptor-mediated PI3K-Akt-Rac1 signal pathway. These molecular events could trigger re-epithelialization and angiogenesis, and hence, promote wound healing.\textsuperscript{9,10}
- Systemic insulin treatment enhances the expression of neutrophil adhesion molecules and various cell surface receptors to reinforce the cellular functions of migration, phagocytosis, and bactericidal actions.\textsuperscript{17} However, there have been no publications showing whether low-dose topical insulin application directly influences the quantity and function of wound neutrophils.

### Materials and Methods

**Equipment/materials.** The following materials and equipment were used: neutral insulin injection; Tegaderm\textsuperscript{TM} film (3M, St. Paul, MN); anti-Ly-6G (Gr-1, Gr1); HRP-conjugated secondary antibody; RIPA lysis buffer, BCA protein assay kit, SDS sample loading buffer and ECL reagents; MDA assay kit and MPO assay kit; Trizol\textsuperscript{15®} Invitrogen, Grand Island, NY); PrimeScript RT reagent kit and SYBR Premix Ex Taq (Takara, Shiga, Japan); SDS-PAGE running tank and Western blot membrane transferring equipment (BIO-RAD); 7500 real-time PCR system (Applied Biosystems, Carlsbad, CA); Spectra Max\textsuperscript{®}190 (Molecular Devices, Sunnyvale, CA); and cornea trephine.

**Experimental animals and tissue samples.** A total of 42, 6- to 8-week-old mice (SPF C57BL/6J; 21 female and 21 male) were purchased from the Department of Laboratory Animal Science, Fudan University (Shanghai, China). Mice gender was randomly assigned to control or insulin treatment group. Mice were anesthetized with 0.5% pentobarbital sodium (50 mg/kg). Depilatorium (barium sulfide: pulvistalci: washing powder = 2:1:1, volume ratio) was used on the dorsal skin to remove hair after shaving. Six full-thickness wounds on the top, middle, and bottom areas of the...
dorsal skin, 3 at each side symmetrically, were created using a 5-mm diameter cornea punch. Wounds were treated with 0.03-U insulin in 20-µL saline or 20-µL saline immediately after injury; absorption was allowed for 5 minutes and then the wounds were covered with film dressing. Insulin was applied once every day. Tissue from the wounded areas and 5-mm adjacent normal skin tissue (n = 12) were collected at 1, 2, and 3 days after injury, and then were frozen in liquid nitrogen. These samples were later examined by Western blotting, real-time polymerase chain reaction (PCR), myeloperoxidase (MPO), and malondialdehyde (MDA) biochemical quantification assays.

**Healing times and healing rates.** The area without epithelium was identified as the wounded surface, and the period of time between the injury and total epithelium recovery was identified as the wound healing time (n = 6). The wounded surfaces were drawn on transparent tracing paper and scanned for analysis. The pictures were analyzed with ImageJ software to calculate the area of the wounded surface:

\[
\text{Wound closure rate} = \left( \frac{\text{area of the wounded surface at the day of injury} - \text{area of the wounded surface at the day of examination}}{\text{area of the wounded surface at the day of injury}} \right) \times 100\% 
\]

**Western blot assay for Gr-1 expression.** Tissue samples from the wounded area were lysed with RIPA lysis buffer. Total protein was detected by bicinchoninic acid assay (BCA). A total of 50 µg of total protein from each sample was mixed with sample buffer and loaded into each well of a sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) gel. Samples were then run by SDS-PAGE on 12% gradient gels, and transferred to polyvinylidene fluoride (PVDF) membrane. Transferred PVDF membranes were blocked by 5% BSA in tris-buffered saline and Tween 20 (TBST) at room temperature for 1 hour, followed by overnight incubation with a primary anti-Gr-1 antibody at 4°C. On the second day, membranes were washed 3 times and then incubated in secondary antibody for 2 hours at room temperature. Visualization was processed by ECL reagents and compatible scanners. Blots then were re-probed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to show equal loading. Band intensities were analyzed using ImageJ software.

**Biochemical analysis of MPO and MDA expression.** The experimental protocol followed the manufacturer’s instructions. The unit of MPO quantification was units/g (wet sheet). The unit of MDA quantification was nmol/mg (protein amount).

**Real-time quantitative PCR assay for MIP-2 mRNA expression.** Total mRNA was extracted using Trizol (Life Technologies, Grand Island, NY). The ratio of OD 260 nm to OD 280 nm was detected. If the values were higher than 1.8, RNA samples were reverse transcribed.

**Figure 1.** A) Excision wounds were created in C57BL/6J mice, and the healing process was monitored at different time points. Representative images of wounds treated with 20-µL saline and 0.03-U insulin (in 20-µL saline) everyday. B) Wound area was quantified every 2 days and expressed as wound healing rate. Statistics are shown as comparisons between the treatment and control (*P <0.05; n = 6). Insulin significantly facilitated wound healing rate.

**KEYPOINTS**

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transcribed. The system volume of reverse transcription was 20 µL containing 1 µg RNA. The reaction conditions of the reverse transcription were 37°C for 15 minutes and 80°C for 5 seconds. The system volume of the real-time PCR was 20 µL containing 10 µL of SYBR Premix Ex Taq™ II (2x), 8 µL of PCR forward primer (10 µm), 8 µL of PCR reverse primer (10 µm), 0.4 µL of ROX Reference Dye or Dye II (50x), 2 µL of cDNA solution, and 6.8 µL of dH2O. The primers for MIP-2 and GAPDH were designed by BioTNT (Wanchai, Hong Kong). The reaction conditions of real-time PCR were 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds, and 60°C for 30 seconds.

Statistical Analysis

The ΔΔC(T) method, as described previously, was use for data analysis. Data were reported as mean ± standard deviation. Comparisons between groups were made by paired t-tests. Comparisons between different time points were made by one-way analysis of variance. The differences were considered significant when P <0.05. Data were analyzed using SPSS 13.0 (Chicago, IL).

Results

Full-thickness skin wounds made using a 5-mm diameter cornea punch on the dorsal skin of mice were treated using 0.03-U insulin or saline. The dose of insulin, chosen based on the authors’ previous work, accelerated healing without an obvious effect on blood glucose.9,10 Healing progress was inspected daily, and healing times were recorded. No scab was observed during the wound healing processes with a film dressing cover, whereas neoeplithelium had formed under the film (Figure 1A). The healing time of wounds treated with insulin was 6.67 ± 0.52 days, which is significantly shorter than that of the saline treated wounds (8.17 ± 0.75 days; P<0.05). The closure rate of wounds treated with insulin was also significantly higher than that of the control wounds at 5 and 7 days after injury (P<0.05; Figure 1B).

Expression of Gr-1, a specific marker of neutrophils, was detected by Western blotting. At day 1 after injury, neutrophil infiltration was observed in the wound area, whereas the extent of neutrophils infiltration was similar with or without insulin treatment. At day 2 after injury, significantly fewer neutrophils were found in the insulin-treated wounds compared with control wounds (P<0.05). At day 3 after injury, in both groups of wounds

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<th>Table 1. Primers used in real-time quantitative PCR.</th>
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Figure 2. Insulin decreases neutrophil infiltration into wound area and induces the resolution of the inflammatory response. Levels of the Gr-1 were quantified by determining the ratio of the integrated density of Gr-1 to GAPDH, the loading control, using ImageJ software. Data are shown as mean ± SD. *P<0.05.

KEYPOINTS

- The healing time of wounds treated with insulin was 6.67 ± 0.52 days, which is significantly shorter than that of the saline-treated wounds (8.17 ± 0.75 days; P<0.05).
neutrophil infiltration was attenuated to a similar level, compared to 2 days after injury ($P < 0.05$) (Figure 2). This phenomenon suggested that topical insulin application suppressed neutrophil infiltration.

MIP-2 expression was analyzed at the mRNA level to check the correlation between MIP-2 level and the amount or time phase of neutrophil infiltration. At 1 and 2 days after injury, MIP-2 expression was significantly lower with insulin treatment than the saline control ($P < 0.05$). At 3 days after injury, MIP-2 expression decreased to similar levels in both groups. In the saline control group, the expression of MIP-2 decreased over time, whereas in the insulin treatment group, the expression of MIP-2 was comparatively stable over time (Figure 3).

Wounded and adjacent tissues were collected at different days after injury. Tissue samples were homogenized in saline, and MPO and MDA expression levels in the supernatant were detected. There were no significant differences in MPO (Figure 4B) or MDA (Figure 4A) expression between the insulin treatment and control groups. Considering there were fewer neutrophils in insulin-treated wounds, similar MPO and MDA levels suggest neutrophil function was augmented after insulin treatment.

Discussion

It has been reported that insulin regulates systemic inflammatory responses, whereas the regulation of traumatic inflammation by topical insulin has not been studied. The present study showed that low-dose topical insulin application decreased wound neutrophil infiltration and advanced wound neutrophil attenuation, which is closely related to insulin-regulated, decreased MIP-2 expression. Additionally, insulin enhanced the cellular functions of neutrophils in the wound area. These results demonstrate that insulin regulates traumatic inflammatory responses in a self-constriction manner through the regulation of both cell quantity and cell function.

Neutrophils assemble at the wounded area at the initiation wound healing. A day after injury, wound neutrophil infiltration was observed in both insulin- and saline-treated wounds. Neutrophil attenuation was observed at 3 days after injury in the saline group, whereas topical insulin advanced this phenomenon to 2 days after injury. Advanced neutrophil attenuation alleviated the negative effects of neutrophils, mainly the neutrophil-mediated tissue damage caused by a blast of enzymes and neutrophil-produced reactive oxygen (RO), which might be related to accelerated, insulin-induced healing.

MIP-2, a member of the CXC chemokines family,
strongly induced neutrophil chemotaxis.\textsuperscript{20} At 2 days after injury with topical insulin application, traumatic MIP-2 expression significantly decreased. Similarly with the change of MIP-2, wound neutrophils had notably decreased. This observation suggests topical insulin regulated the inflammatory response in the wounded area by restraining wound neutrophil infiltration through inhibition of chemokine MIP-2 expression. Vascular permeability also regulates inflammatory cell recruitment. Despite regulating MIP-2 expression, insulin stabilizes vessel endothelial barrier function.\textsuperscript{21} More stable microvessels that undergo insulin treatment might hamper neutrophil transmigration to the wound area, and thus limit the quantity of neutrophils in the wound area. Meanwhile, insulin itself acts as a chemoattractant, recruits more macrophage infiltration, and facilitates macrophage phagocytosis (unpublished data), which could also be involved in advanced neutrophil attenuation.

MPO is a peroxidase enzyme most abundantly present in the azurophilic granules of polymorphonuclear neutrophils (PMN).\textsuperscript{22} MPO is a marker of PMN activation because MPO enzyme activity significantly correlates with PMN function.\textsuperscript{23} Activated PMNs release a significant quantity of ROS to induce lipid peroxidation of multiple unsaturated fatty acids in cell membranes via the respiratory burst. MDA is one of the products of lipid peroxidation; hence, the level of MDA marks the level of lipid peroxidation by ROS and further indicates how severely cells are damaged.\textsuperscript{24} The present study did not show significant differences in MPO and MDA levels between the insulin treatment group and saline control group. However, at 2 days after injury, the number of neutrophils in the wounds treated with insulin was significantly lower than in the wounds that received only saline treatment. Combining the results mentioned above, one might draw the conclusion that neutrophils in wounds with topical insulin receive enhanced cellular functions. Similar MPO and MDA levels with a lesser number of inflammatory cells indicate that insulin can boost neutrophil function and might help clean exogenous pathogens. Increased neutrophil function and restrained wound neutrophil infiltration, as well as advanced resolution of neutrophils, suggest a delicate regulation of insulin on wound inflammatory response during the healing process.

Although insulin affects the inflammatory response, the overall effects on wound healing are still mediated by repairing cells (keratinocyte and endothelial cells, and the like) and are revealed only in later phases of healing, as evidenced by the significant difference in the wound closure rate at the later and not the early stage of healing. Moreover, the differences in healing times and wound closure rates between the 2 groups, although statistically significant, appear to be minor. Statistical significance between groups strongly suggests the positive effects of insulin on healing. However, larger wounds that take longer to heal might magnify the difference between groups.

Injured patients with diabetes suffer from more infectious wounds and prolonged healing times. It has been reported that diabetic wounds have elevated neutrophils and prolonged neutrophil infiltration.\textsuperscript{15} Conversely, neutrophils in wounds of patients with diabetes are dysfunctional in terms of phagocytosis, migration, and bactericidal actions.\textsuperscript{25} The present findings of insulin-induced stimulation of neutrophil functions may help advance the clinical treatment of chronic, nonhealing diabetic wounds.

Conclusion
Topical insulin application decreased neutrophil infiltration by inhibiting MIP-2 expression and advanced neutrophil resolution. Insulin promotes neutrophil function, and the present results, along with previous findings, suggest insulin delicately regulates the inflammatory response during wound healing.

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References