17β-Estradiol Modifies Diabetic Wound Healing by Decreasing Matrix Metalloproteinase Activity

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Abstract: Postmenopausal women are more susceptible to poor wound healing. This phenomenon can be reversed by estrogen replacement therapy in non-diabetic individuals. Postmenopausal women with type 2 diabetes are more susceptible to wound healing complications, potentially secondary to an estrogen deficiency. Few studies have examined the mechanism of action and effects of estrogens on diabetic wound healing in females. It appears that multiple factors influence delayed wound healing among individuals with diabetes including an imbalance in cytokines, growth factors, extracellular matrix (ECM) turnover, and oxidant stress (OS). Estrogens have been shown to regulate the expression of genes important for extracellular matrix turnover, including collagen and matrix metalloproteinases (MMP).

Methods. For this reason, the effects of 17β-estradiol (E2) on MMP-2, MMP-13, and MMP-14 and estrogen receptor alpha and beta (ER-α and -β) expression in the wound tissue of estrogen-deficient female mice with established type 2 diabetes mellitus (C57BL/6J-m Leprdb/2+) were studied.

Results. Topical E2 upregulates ER in wound tissue thereby improving and accelerating diabetic wound healing in estrogen deficient mice. Conclusion. The mechanism appears to decrease MMP-2, MMP-13, and MMP-14 mediated tissue matrix destruction and increasing collagen content.

It has been shown clinically that postmenopausal women are more susceptible to wound complications. The decline of postmenopausal estrogens leads to dysregulation of extracellular matrix turnover (ECM) and increased inflammation in wounds. This process is prevented or reversed by topical and systemic 17β-estradiol (E2) replacement therapy. Skin is a direct estrogen target tissue and expresses both ERα and ERβ. In humans, both topical and systemic estrogens have been shown to improve wound healing through a number of mechanisms: inflammatory response modulation, cytokine stimulation, matrix deposition, re-epithelialization rate enhancement, angiogenesis stimulation, and proteolysis regulation. In addition,
Ashcroft et al\textsuperscript{1} have demonstrated that estrogen is the major reproductive hormone involved in dermal fibroblast proliferation. During the inflammatory phase of wound healing, estrogen has been shown to enhance the oxidative metabolism of activated neutrophils during phagocytosis and decrease local levels of inflammatory cell-derived proteases.\textsuperscript{1} Pirila et al\textsuperscript{6} further demonstrated that decreased wound collagen deposition was associated with matrix metalloproteinase (MMP)-mediated collagenolysis in ovariectomized rats. Estrogen replacement could reverse these effects thereby suggesting an important role for estrogen in the balance between matrix degradation and synthesis.

Multiple studies have been published on estrogen’s role in improving wound healing.\textsuperscript{1,2,4-6,7,9} However, there is a paucity of data on its ability to improve/accelerate healing in women with diabetes. This group has been found to be at a greater risk for diabetic complications when compared to men with diabetes.\textsuperscript{5,10}

Diabetic wounds have a prolonged inflammatory phase.\textsuperscript{11} This is most likely due to bacterial contamination and associated repetitive painless tissue injury. This leads to an increased response from proteases, especially MMPs and elastase. MMPs can either degrade the ECM or regulate numerous biological activities through specific cell signaling mechanisms once activated and left uninhibited.\textsuperscript{11} MMPs, expressed by fibroblasts, inflammatory cells, and keratinocytes play an essential role in wound debridement, angiogenesis, epithelialization, and scar remodeling. Under ideal conditions, there is controlled and timed expression of specific MMPs into the wound area.\textsuperscript{12-15} Crucial balance between proteases and their inhibitors is required for successful wound healing; thus, an imbalance may be the etiology of poor wound healing.

The authors hypothesize that \( E_2 \) treatment of diabetic wounds would accelerate healing by normalizing the balance between synthesis of collagen and degradation by MMPs. Therefore, female db/db mouse (a model of Type 2 diabetes) were treated with either topical \( E_2 \) or placebo. Db/db mice on a C57Bl/6J background (C57Bl/6J Lepr\textsuperscript{ob}/Lepr\textsuperscript{ob}) serve as a model of type 2 diabetes mellitus (DM) in which stable DM develops between 6 to 8 weeks of age. Female B6 db/db mice have shown to exhibit markedly impaired healing of open wounds and are infertile because of a defective hypothalamic-pituitary-ovarian axis.\textsuperscript{14} This animal model permits studying the effects of ovarian failure (estrogen deficiency/ menopause) on the healing of wounds and the potential effect of estrogen treatment in the absence of confounding factors such as aging and hypergonadotropic blood levels. The present study was able to demonstrate that topical \( E_2 \) increased ER\( \beta \) expression and was effective in accelerating wound healing in these mice by altering ECM turnover through regulation of MMP-2, -13, and -14.

**Materials and Methods**

**Animal model and wounding.** All procedures were performed in accordance with recognized Institutional Animal Care and Use Committee (IACUC) guidelines. Each experimental animal was housed individually and given water and food ad libitum. Female db/db C57Bl/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Diagnosis of diabetes was confirmed weekly in each mouse by testing both blood and urine for glycemic control. Each 15-week-old mouse received 3 full-thickness wounds on the back. These wounds were made using an 8-mm punch biopsy after an adequate surface area of skin was shaved by clippers. Mice were subsequently randomized for administration of either \( E_2 \) or placebo. Topical \( E_2 \) (50 mg Estrogel 0.06%) or placebo gel (containing vehicle) was applied daily for 7 days to the wound. The wounds were left open to air and treated daily in accordance with their respective groups.

**Animal sacrifice.** Sacrifice was accomplished according to the protocol at 16 weeks of age. Wounds were harvested, and sections of skin were immediately frozen in OCT (Optimal Cutting Temperature) compound (Sakura Finetek USA, Torrance, CA) for embedding. Mice were submerged in paraformaldehyde overnight, and then wedge shaped excisions of the wound bed were removed and sent for histologic evaluation. Additional pieces of skin were flash frozen in liquid nitrogen. Uterus was removed and weighed as a measure of efficacy of estrogen depletion or replacement.

**Protein extraction, immunoprecipitation, and Western blotting.** Skin was homogenized in lysis buffer, vortexed for 1 minute, and centrifuged for 30 minutes. Supernatant was collected and the protein concentration was assayed using the BioRad DC protein kit (Hercules, CA). For immunoprecipitation experiments of ER\( \beta \), 80-g of protein extract was incubated with an antibody against ER\( \beta \) for 1 hour at 4°C, followed by the addition of protein G-Agarose overnight. Resulting protein-antibody conjugate was centrifuged at 4°C and washed four times in lysis buffer in the presence of protease inhibitors. Final pellet was re-suspended in Laemelli buffer under reducing conditions, boiled for 5 minutes, and centrifuged.
prior to analysis. Supernatant was analyzed as described above.

Western blot was used to examine protein expression as previously described.\textsuperscript{15,16} Protein samples were resolved by electrophoresis on 10% polyacrylamide gels. Blots were exposed to ER\textalpha{}, ER\beta{}, and MMP-13 antibodies. Immunoreactive bands were determined by exposing the nitrocellulose blots to Luminol chemiluminescence solution for 1 minute followed by exposure to X-Omat AR film (Eastman Kodak Co., Rochester, NY). Film was scanned and saved on computer disks for densitometric analysis using Image J software from the NIH (available by ftp at zippy.nimh.nih.gov/ or at http://rsb.info.nih.gov/nih-imageJ; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). All membranes were ink stained (India Ink) after Western blotting to ensure equal protein loading. Kalediscoscope\textsuperscript{™} marker (BioRad Laboratories, Hercules, CA) was run with each gel to detect protein weight.

Zymography. MMP-2 activity was assessed by zymography. Gelatin zymography was performed as previously described.\textsuperscript{19} Protein (5 μg) was applied to an SDS polyacrylamide gel co-polymerized with 10% gelatin. Gels were rinsed twice after electrophoresis in 2.5% Triton X-100 and then incubated for 18 hours at 37°C in incubation buffer (50 mM Tris-HCl, 5 mM CaCl\textsubscript{2}, 1 μM ZnCl\textsubscript{2}, 0.05% Brij\textsuperscript{™} 35 at 2°C–8°C). Following 1 minute of vortexing, the samples were centrifuged and supernatant collected. Briefly, 100 μL of peroxidase conjugate was added to 100 uL of unknown sample. One hundred microliters of this mix was added then plated. Plate was covered and incubated at room temperature for 2 hours. All wells were aspirated and washed 4 times with wash buffer and then 100 μL of TMB substrate was added to sample wells. Plate was recovered and allowed to incubate at room temperature for 30 minutes and read at 630 nm.

Statistical Analysis

Mean ± SD of the measures were calculated and probabilities (Student’s t-test) performed (Prism; GraphPad, San Diego, CA). Comparison of two groups within the same gel was analyzed with the Student’s t-test. Western blot, MMP-14 activity, and TIMP-2 activity values were calculated as a percentage of placebo values. A value of $P < 0.05$ was considered statistically significant. All experiments were performed in duplicate or triplicate with an $n \geq 3$.

Results

Animal Model. The application of topical E\textsubscript{2} accelerated wound healing (Figure 1). A difference in either the weight of the mice, glycemia, or glycosuria was not
found. Uterine weight increased after topical E2 treatment (0.065 ± 0.008) compared to placebo treatment (0.029 ± 0.009; P < 0.05). Wound histology revealed an increase in collagen deposition in the wound bed of the E2 treated mice as compared to the placebo treated animals (Figure 2). The wound bed was apparent by the disruption of the subcutaneous tissue in each slide.

**MMP-13 protein and activity levels.** MMP-13 regulates turnover of collagen types I, II, and III in acute and chronic wound healing. Topical E2 treatment decreased active MMP-13 protein levels 31% in the experimental models compared to the placebo (68.92 ± 6.11, n = 5 versus 100 ± 3.8, n = 3; P < 0.05 [Figure 3C]).

**MMP-2 and MMP-14 activity.** Pro- and active MMP-2 activity was reduced in wounds isolated from E2 treated mice compared to placebo treated mice (30.34 ± 4.76, n = 6 versus 100 ± 20.4, n = 4 [P < 0.05], and 55.08 ± 5.98, n = 6 versus 100 ± 11.22, n = 6, [P < 0.01], respectively, [Figure 3A, B]). MMP-14 activity was also decreased in the wounds of the E2 treated mice compared to the
placebo-treated mice (80.95 ± 11, n = 6 versus 100 ± 13.79, n = 5, respectively; P < 0.05 [Figure 4]).

**TIMP-2 activity.** TIMP-2 activity was not different between the placebo treated group (70.09 ± 15.03, n = 5) and the E2 treated group (102.6 ± 14.67, n = 4).

**Collagen assay.** Topical E2 treatment increased collagen content of the wounds compared to those wounds isolated from the mice treated with placebo (1.976 ± 0.1408, n = 5 versus 1.268 ± 0.1908, n = 4, respectively; P < 0.05 [Figure 5]).

**ER-α and ER-β protein levels.** E2-treatment increased ER-α protein expression in the wounds (181.5 ± 17.14, n = 6 versus 100 ± 26.17, n = 4; P < 0.05, [Figure 6]) compared to placebo. E2-treatment did not alter ER-β protein expression in the wounds (111.9 ± 2.688, n = 4 versus 100 ± 11.39, n = 3; P < 0.05 [Figure 6]) compared to placebo.

**Discussion**

E2 treatment accelerated wound healing in an experimental model of type 2 diabetes in female db/db mice. We were able to accomplish this by restoring the balance between MMP activity and collagen content. Our group had previously shown that E2 regulates ECM turnover in the eye, kidney, and aorta.16-19 Although there are other previous studies on estrogen’s role in improvement of wound healing, we are able to find little data describing its potential to improve/accelerate healing in the female postmenopausal diabetic patient population. This group has been clearly shown to be at a greater risk for diabetic complications when compared to diabetic men.19

Impairment of cutaneous wound healing in both diabetic and non-diabetic individuals is associated with an imbalance of ECM synthesis and degradation, which leads to alterations in the amounts or types of matrix accumulation.20 The present results showed that MMP-2, -13, and -14 decreased in E2-treated wounds. MMP-13, the main collagenase in mouse skin, degrades native triple helix collagen composed of collagen types I and III.21 Murine MMP-13 shares activity not only with human MMP-13 but also MMP-1 (collagenase-1) in acute wound healing.22 Lobmann et al23 found decreased MMP-1 in foot wounds of diabetic patients compared to traumatic wounds of non-diabetic patients. The authors went on to suggest that the increased proteolytic environment prevents wounds from healing in diabetic patients.

Topical E2 treatment decreased both pro- and active MMP-2 protein expression and activity to levels of that found in the skin of non-diabetic mice. These effects

![Figure 4.](Image) MMP-14 activity was decreased in E2-treated diabetic wounds compared to placebo after 7 days of topical application. MMP-14 was measured by commercial Biotrak activity assay. Data are graphed as the mean ± SEM. n = 4 mice/group *P < 0.05

![Figure 5.](Image) Total collagen content was increased in topical E2-treated wounds compared to placebo. Total collagen content was measured via dye-binding collagen assay, as described in methods. Data are graphed as the mean ± SEM. n = 4 mice/group *P < 0.05

![Figure 6.](Image) Topical E2 treatment increases ER-α protein expression in diabetic wounds. Data are graphed as mean ± SEM. Representative Western blot (inset). n = 4 mice/group *P < 0.05
could promote continual and non-delayed healing. In fact, MMP-1 (MMP-13 in rodents) and MMP-2 activity are increased in wounds from diabetic patients when compared to non-diabetic individuals. Increased MMP-2 activity could lead to continual breakdown of the wound bed. Such breakdown could possibly transition to chronic wounds.

The present study also found that E2 treatment regulates MMP-14. MMP-14 activity is crucial for both physiological and disease processes. Since proMMP-2, MMP-14, and TIMP-2 form a trimolecular complex resulting in MMP-2 activation; therefore, any change in the ratio of these components could lead to alterations in matrix turnover. Additionally, MMP-14 activates MMP-13, which directly regulates wound healing, angiogenesis, and inflammation. This occurs through the degradation of collagen types I, III (laminin and fibronectin). These components are found in skin layers. Decreasing MMP-14, therefore, not only regulates activation of MMP-2 and MMP-13, but also could prevent the eventual direct degradation of other ECM components.

The diabetic mice in both the placebo and E2 treated groups had extremely low levels of TIMP-2 activity. This correlates well with current clinical findings. Human diabetic wounds have been shown to have a two-fold reduction in TIMP-2 compared to nondiabetic wounds. However, recent data suggest this is due to changes in fibroblasts and not keratinocytes. Therefore, it may be difficult to clearly discern differences in whole tissue used in the present study rather than isolated cell lines. The present data, however, suggest that coupled with the E2-induced decrease in MMP-2 and MMP-14 activity, a change in TIMP would not result in a reduction in MMP-2 activation.

Evaluation of total collagen content (via ELISA) further confirmed the observed changes in MMPs correlated with an alteration in ECM turnover. We found a decrease in collagen in the placebo treated wounds, which was reversed, as expected, by E2 treatment. This was followed by accelerated healing, which correlates with previous studies evaluating the beneficial effects of E2 on normal wounds. Pirila et al. have shown that type I collagen content was increased in ovariecetomedized E2 treated rat wounds compared with ovariecetomy alone. Increased hyaluronic acid synthesis and mitotic activity have also been noted in murine epidermis.

Local estrogen levels play a role in cutaneous physiology. Both topical and systemic estrogens are known to increase the rate of wound healing in elderly men and women. In vivo, aging is associated with decreased keratinocyte migration, reduced cytokine expression, and increased responsiveness to inhibitory cytokines, which all delay epithelialization. E2 enhances wound healing via its effects on the following: inflammatory response, cytokines, matrix deposition, re-epithelialization, angiogenesis, wound contraction, and proteolysis. However, the exact mechanisms remain undefined.

Estrogen has been reported to have a mitogenic effect on keratinocytes, thereby promoting healing. Ashcroft et al. have also demonstrated that E2 is the major reproductive hormone involved in dermal fibroblast proliferation. During the inflammatory phase of wound healing, E2 enhances the oxidative metabolism of activated neutrophils during phagocytosis. Therefore, it decreases the risk of infection and transition to a nonhealing wound, especially in patients with diabetes.

Skin is a well documented end-organ target for estrogen. Skin is also important in cutaneous physiology, specifically the most potent form of estrogen, 17β-estradiol. Estrogen action is mediated through two receptor subtypes: estrogen receptor (ER) α and β, which are nuclear receptors in the steroid hormone receptor family. Predominant estrogen receptor in human skin is ERβ, while ERα is found in mouse skin. It is likely that the activation of murine ERα regulates signaling pathways and genes equivalent to those regulated by human ERβ. In the authors’ research, E2 treatment increased ERα protein levels in murine skin. The authors’ postulate that regulation of MMPs is an ER dependent event as shown in other organs. This finding has been documented in a previous human neonatal skin study. In-vitro experiments demonstrated upregulation of ERβ receptors in keratinocytes at physiologic levels of estradiol.

**Conclusion**

Topical E2 treatment of diabetic wounds improves and accelerates wound healing by decreasing MMP-2,-13, and -14 activity and increases collagen content. The most likely mechanism is through upregulation of ERα. Topical application of E2 may prove to be an important adjunct in the treatment of these difficult wounds. Further clinical studies will be required to elucidate this potential advantageous effect.

**References**


