Available online at www.sciencedirect.com
ScienceDirect
Experimental Gerontology 42 (2007) 523–531
Experimental Gerontology
www.elsevier.com/locate/expgero

# The synergism of age and db/db genotype impairs wound healing ☆

Harold Brem [a,*], Marjana Tomic-Canic [b], Hyacinth Entero [c], Andrew M. Hanflik [d], Vincent M. Wang [e], John T. Fallon [f], H. Paul Ehrlich [g]

[a] Department of Surgery, Columbia University College of Physicians and Surgeons, New York, NY, USA
[b] Tissue Engineering, Regeneration and Repair Program, Hospital for Special Surgery at Weill Cornell Medical College, New York, NY, USA
[c] Ross University School of Medicine, Roseau, Dominica
[d] Keck School of Medicine, University of Southern California, Los Angeles, CA, USA
[e] Department of Orthopedic Surgery, Rush University Medical Center, Chicago, IL, USA
[f] Department of Pathology, Mount Sinai School of Medicine, New York, NY, USA
[g] Department of Surgery, Milton S. Hershey Medical Center, Hershey, PA, USA

Received 5 May 2006; received in revised form 20 November 2006; accepted 21 November 2006
Available online 1 February 2007

**Abstract**

Both diabetes and advanced age have been implicated in delaying wound repair. However, the contribution of age alone has not been shown clinically to significantly impair the ability to heal. To determine the contribution of age and db/db genotype multiple wound healing parameters were determined in young db/db mice, aged db/db mice, age-matched non-db/db control and wild-type C57BL/6 mice. Biomechanical properties (breaking load and tensile stiffness), epithelialization, and collagen deposition were determined for the four groups of mice 14 days after wounding with suture-closed incisional wounds. While neither hyperglycemia nor age alone caused impairment in biomechanical properties, the combination of age and db/db genotype resulted in a 36% reduction in stiffness and a 42% reduction in breaking load, when compared to young control mice, suggesting poor quality of healing. Statistically significant differences in the volume of granulation tissue deposited within the wound site were also observed, with the aged db/db mice displaying more than any other group, suggesting greater dermal loss from the dermal edges of incisional wounds in aged db/db mice, suggesting that the combination of age and diabetes act synergistically to impair healing in mice with type 2 diabetes. Interestingly, the impairment occurs independently of the prevailing glycemia, supporting the hypothesis that diabetes in synergy with advanced age has downstream effects, leading to further impairment, necessitating initiation of early and aggressive intervention in elderly patients with diabetic foot ulcers.


*Keywords:* Effects of aging; Diabetes; Wound healing; Pathology; db/db mouse model

## 1. Introduction

The prevalence of diabetes is rapidly rising and is expected to grow from 2.8% to 4.4% by the year 2030, with the worldwide population expected to reach 366 million persons (Wild et al., 2004). Over the years 1997–2003 the yearly incidence of newly diagnosed diabetes climbed 52% (Data and Trends, 1997–2003). The elderly represent an enormous portion of this population, and by the year 2030, there will be over 130 million people over the age of 65 afflicted by diabetes (Wild et al., 2004). Foot ulcers are estimated to occur in 11.8% of all patients with diabetes (History of foot ulcer. . ., 2003) and the elderly account for over 54% of the annual 82,000 diabetes related lower limb amputations in the United States (Data and Trends, 1980–2002).

Since the beginning of the 20th century, it has been widely believed that a delay in wound healing occurs in advanced age (Du Nouy, 1916). Rabbits, rats and mice

☆ This work was supported in part by the National Institutes of Health: NIDDK 059424, AR45974, NR08029 and the American Diabetes Association.
* Corresponding author. Tel.: +1 212 932 4325; fax: +1 212 658 9806.
*E-mail address:* hb2133@columbia.edu (H. Brem).

0531-5565/$ - see front matter © 2007 Published by Elsevier Inc.
doi:10.1016/j.exger.2006.11.018

all show age-related wound healing impairments, most prominently towards the very end of their lifespan. This impairment is recognized as being a delay, rather than an inability to heal (Davidson, 1998; Gosain and DiPietro, 2004). It is believed that the encumbrance is due to an alteration or reduction in the activity of the proliferative aspects of wound healing (i.e. keratinocyte proliferation and migration as well as deposition of granulation tissue) (Gilchrest et al., 1982; Swift et al., 1999) and that the immune response is altered (Yoshikawa, 1997; Van den Biggelaar et al., 2004; Colonna-Romano et al., 2004). Angiogenesis is required for optimal repair and experimental studies show that angiogenesis is impaired in advanced age, with capillary density significantly reduced in aged wound specimens from multiple species (Swift et al., 1999; Rivard et al., 1999; Yamaura and Matsuzawa, 1980). In old age, the composition of growth factors increases the time of healing in rats, as demonstrated by a decrease in granulation tissue deposition, collagen organization and functional integrity (assessed by wound breaking load) at 10 and 14 days post-wounding (Ballas and Davidson, 2001). Despite these experimental findings, we have previously shown that aging does not empirically inhibit a person's ability to close a wound, and we hypothesized that the elderly have a higher incidence of diabetes but not necessarily delayed healing (Brem et al., 2003).

The effects of diabetes in delayed healing has been established experimentally (Davidson, 1998; Keswani et al., 2004). Full thickness wounds in diabetic animals show a marked decrease in angiogenesis, epithelialization, collagen deposition and granulation tissue formation (Keswani et al., 2004; Lee et al., 2004; Galeano et al., 2004). In diabetes associated wounds, there are multiple physiological processes that result in decreased granulation tissue deposition, such as endothelial dysfunction resulting, in several metabolic anomalies, including oxidative stress (Johansen et al., 2005) and decreased expression of growth factors (Lee et al., 2004; Frank et al., 1995). In the healing of suture-closed wounds, the effects of diabetes are similar to those of advanced age, contributing to a decrease in wound breaking load (Galeano et al., 2004).

Few, if any studies to date have examined the combined effects on wound healing of aging and diabetes in db/db mice. This may be, at least in part, due to difficulties in the husbandry of these animals. Both diabetes and advanced age have been independently shown to lead to a depression of essential components of wound healing such as growth factor secretion, angiogenesis, epithelialization, collagen deposition and biomechanical properties such as wound breaking load. The present investigation tested the hypothesis that diabetes and advanced age work in concert to impede the normal healing of an incisional wound. This was tested in a db/db aged mouse model and compared to young db/db as well as young and aged C57BL/6 mice. Using biomechanical and histological measurements, it was concluded that indeed age and diabetes act in synergy, causing an impairment in wound healing.

## 2. Materials and methods

### 2.1. Animal protocols

The Institutional Animal Care and Use Committee approved all animal procedures. Female BKS·Cg-m+/+ Lepr$^{db}$ mice (separate 2- and 16-month-old groups) were obtained from The Jackson Laboratory (Bar Harbor, ME); the 16-month-old db/db animals were aged in our facility. After 6 months of aging, the aged animals were separated into three distinct groups based on glycemic control (12 h fasting glucose levels (mg/dL) and HbA1C (%) $p < 0.001$ between all groups). These differences in glucose levels remained consistent up until the point when the mice were sacrificed. The groups were examined separately, terming them aged normoglycemic (HbA1c values of $3.2\% \pm 0.4$ and fasting glucose values of 76.7 mg/dL $\pm$ 16) ($n = 11$), aged borderline ($4.7\% \pm 0.7$ and 180.7 mg/dL $\pm$ 41) ($n = 11$) and aged hyperglycemic ($7.8\% \pm 1.0$ and 459.2 mg/dL $\pm$ 88) ($n = 7$). Age matched, wild-type C57BL/6 female mice at 8 weeks old ($n = 11$) and 15–16 months old ($n = 11$) were purchased from Charles River (Wilmington, MA: Area K96 Kingston, NY). In younger animals, both db/db and control mice were acclimated for a period of 2 weeks prior to wounding. Animals were individually caged.

Each animal was randomly assigned an identification number for glucose and body weight monitoring purposes. Mice were maintained in a temperature controlled animal facility with a 12-h light/dark cycle. They were fed and drank ad libitum with Lab Diet 5001 Rodent Diet (PMI Nutrition, Inc., Brentwood, MO), with the exception of the 12 h fasting prior to glucose measurement, when only water was given. Fasting and non-fasting glucose were measured using a Glucometer Elite (Bayer Corp., Elkhart IN). Hemoglobin A1c (HbA1c) was measured to determine long-term glycemic control using a DCA 2000 (Bayer Diagnostics, Pittsburgh, PA). Monthly, then weekly body weight in grams and glucose measurements were assayed near the time of wounding (13 months in aged animals).

### 2.2. Wounding experiments

Wounds were created in the young and old mice (2 and 15–16 months, respectively). Animals were shaved at least one day prior to wounding, and then anesthetized by an intraperitoneal injection with a mixture of ketamine HCl (54 mg/kg) (Ketaset; Fort Dodge, IA), saline and xylazine (5.4 mg/kg) (Rompun; Bayer Corporation, Shawnee, KS). The shaved skin surface was sterilized using a gauze sponge soaked in Betadine solution (Purdue Frederick, Norwalk, CT). Full thickness linear incision wounds 30 mm in length were made on the dorsum of mice with a scalpel in the proximal-distal direction. Each incision was closed with six #5 non-absorbable nylon sutures (Ethicon, Somerville, NJ) at 5-mm intervals, as depicted in (Fig. 1A). Sutures were removed at 7 days post-wounding. Animals were sac-

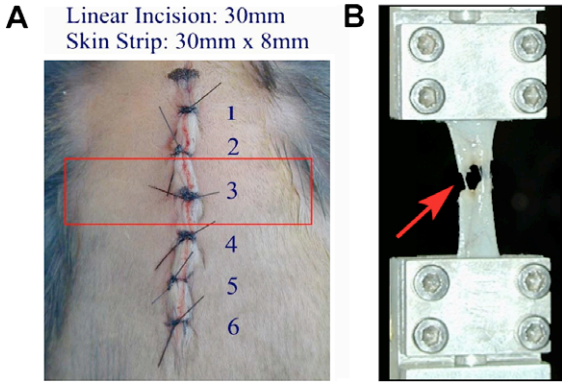



Fig. 1. Experimental design. (A) Incisional wound model is shown. Red rectangle demarcates the segment analyzed in experiments described below. (B) Tensile strength testing is shown with the segment marked in (A).

rificed at 14 days post wounding by carbon dioxide asphyxiation. From the freshly excised wounds, a $30 \times 8$ mm skin strip perpendicular to the incision at the 3rd suture site was obtained for biomechanical testing along with an adjacent sample of identical dimensions for histology.

### 2.3. Biomechanical testing

For biomechanical testing, specimens were kept moist using phosphate-buffered saline (PBS). Each skin sample was secured in custom grips and loaded to failure in tension at a constant rate of 0.1 mm/s using a servo-hydraulic materials testing system (Instron 8841, Canton, MA). For each biomechanical test, the tensile stiffness (N/mm) was computed as the slope of the linear portion of the force-deflection curve, while the breaking load (N) was determined as the maximum force sustained by the sample prior to rupture (Fig. 1B).

### 2.4. Histology evaluations

For histology of freshly excised wounds, each skin strip was fixed overnight in 4% paraformaldehyde. The tissue was processed for histological analysis (paraffin embedding) and stained with hematoxylin and eosin (H&E) and Sirius red (Constantine and Mowry, 1968). H&E stained sections were used to determine degree of epithelialization, granulation tissue volume and the presence of inflammatory cells. Sirius red stained sections were viewed with polarized light to evaluate the maturation of the collagen fibers and the difference in the birefringence patterns of the collagen fiber bundles deposited within granulation tissue (Junqueira et al., 1979; Ehrlich et al., 2005; Salmon-Her et al., 2000; Ehrlich et al., 1994).

The repair site between the dermal edges of the incisional wound at 2 weeks post-wounding contained granulation tissue. Digital images of the wound site and surrounding skin were captured with a Nikon D50 camera mounted on a BH2 Olympus microscope with bright and polarizing light optics. The width of the granulation tissue between the dermal edges of the suture-closed wounds was measured by analyzing the digital images from the H&E stained sections in Adobe Photoshop. Those measurements were made in relative distance units (rdu) and were analyzed and compared between treatment groups. The statistical differences of the distances between the dermal edges of the wound dermis were evaluated using the Mini Tab, Inc. (State College, PA) statistical package.

### 2.5. Statistical analyses

Statistical analysis of hemoglobin A1c levels, tensile stiffness and breaking load among test groups, was performed using a non-parametric one-way analysis of variance (Kruskal–Wallis test), followed by Dunn's post-hoc tests. All reported $p$ values have been adjusted to account for all possible pairwise comparisons among the various treatment groups. For HbA1c, only the aged db/db groups were statistically compared. Statistical analyses for these parameters were performed using GraphPad Prism software (version 3.0, San Diego, CA). Significance was assumed for $p < 0.05$.

## 3. Results

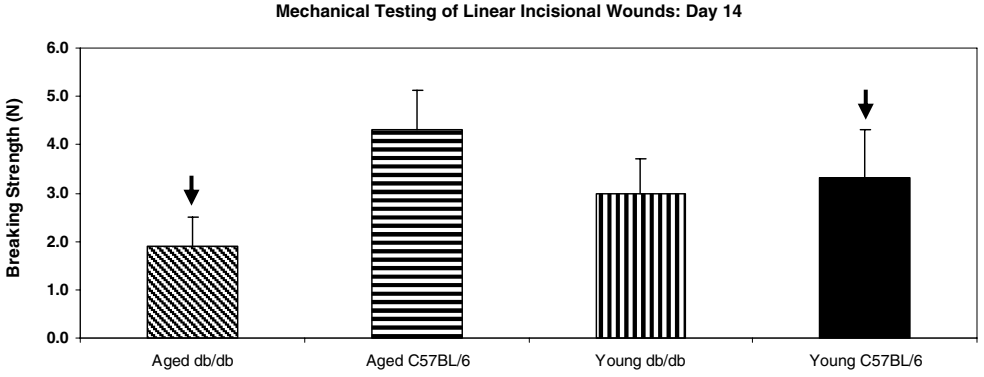
To determine how age and db/db genotype individually and in combination affect wound healing, biomechanical testing of incisional wounds were performed 14 days post wounding among the following groups of mice: aged normoglycemic, aged borderline (hyperglycemic with normal HbA1c), aged hyperglycemic, young db/db as well as young and aged C57BL/6 mice. Biomechanical testing showed that there were no statistically significant ($p > 0.05$) differences within the aged db/db group between the animals with normal, borderline or elevated blood glucose, either in breaking load or stiffness (data not shown). Hence the data from these 3 subgroups of animals were pooled for the purpose of comparing them to the other 3 groups. This pooled group of 29 mice, had a mean breaking load of $1.9 \pm 0.7$ N and a stiffness of $0.7 \pm 0.3$ N/mm. There was a significant difference between the aged db/db mice and all other groups. This included the young db/db ($p < 0.05$), aged C57BL/6 ($p < 0.001$) and the young C57BL/6 ($p < 0.001$) mice (Table 1). Aged db/db mice displayed a 42% reduction in breaking load, when compared to young C57BL/6 mice without diabetes (Fig. 2). Differences in stiffness were also seen when comparing the aged db/db with the young db/db ($p < 0.01$), aged C57BL/6 ($p < 0.001$) and the young C57BL/6 ($p < 0.001$) mice. A 36% reduction in stiffness was seen between the young C57BL/6 mice and the aged db/db mice. When each of the aged groups (aged normoglycemic, aged borderline, aged hyperglycemic) was compared separately against the other three groups, there was no significant difference between each of the groups and the young db/db group for breaking load, nor for tensile stiffness of the young

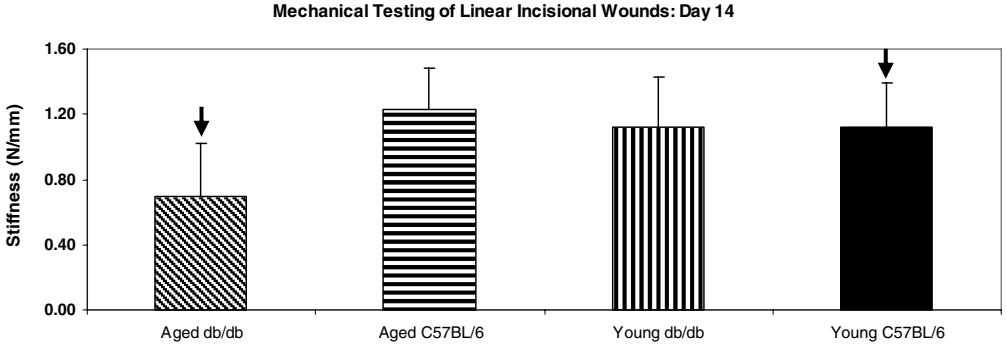
Table 1
Biomechanical testing data

| Group | Stiffness (N/mm) | Breaking strength (N) |
|---|---|---|
| Aged db/db $n = 29$ | $0.70 \pm 0.3$ | $1.90 \pm 0.7$ |
| Aged C57BL/6 "control" $n = 11$ | $1.23 \pm 0.3$ | $4.32 \pm 1.0$ |
| Young db/db $n = 11$ | $1.12 \pm 0.2$ | $3.00 \pm 0.9$ |
| Young C57BL/6 "control" $n = 11$ | $1.12 \pm 0.3$ | $3.33 \pm 0.8$ |

C57BL/6 group. No significant differences were demonstrated in either breaking load or stiffness among the young db/db group, the young C57BL/6 and aged C57BL/6 groups. Taken together, these data suggest that hyperglycemia does not correlate with impaired healing in db/db mice, but rather the synergism of db/db genotype and age.

Analysis of histology showed that all but 2 wounds were completely reepithelialized in all sections studied (total of 82 slides) 2 weeks after surgery. There were no remarkable differences in the inflammatory response between the studied groups of mice. Prominent numbers of inflammatory cells were found in only 3 sections of all slides examined. Those 3 slides were found in 3 separate groups, which indicated there was no relationship with excess inflammation and a specific aged or db/db group. The increased density of inflammatory cells was attributed to a local infection. Overall, wounds from aged wild-type mice when compared to the other experimental groups, appeared to have the lowest density of cells within granulation tissue, whereas the young db/db incisional suture-closed wounds had a greater cell density than all other experimental groups (Fig. 3).




**Statistical Significance:**

| GROUP: | Breaking strength P= | Stiffness P= |
|---|---|---|
| **Aged db mice groups** | | |
| Normoglycemic vs. Borderline | >.05 | >.05 |
| Normoglycemic vs. Hyperglycemic | >.05 | >.05 |
| Borderline vs. Hyperglycemic | >.05 | >.05 |
| | | |
| Aged db vs Aged C57BL/6: | <.001 * | <.001 * |
| Aged db vs Young db: | <.05* | <.01* |
| Aged db vs Young C57BL/6: | <.001* | <.01* |
| Aged C57BL/6 vs Young db: | >.05 | >.05 |
| Aged C57BL/6 vs Young C57BL/6: | >.05 | >.05 |
| Young db vs Young non-db: | >.05 | >.05 |

* Significant

Fig. 2. Aged db/db group shows decreased biomechanical properties. Bar graphs show a significant difference between the aged db/db mice and all other groups, for both breaking strength and stiffness (aged db/db and young C57BL/6 are indicated by arrows). Additionally, neither age nor db/db genotype alone showed a significant difference when compared to the young C57BL/6 control mice. Statistical analyses are shown below the figure.

To document differences in the expanses between the dermal edges of these suture-closed wounds, the distance between the wound edges as identified by sub-epidermal appendages was measured in all H&E sections. As done for the biomechanical data, the data from the 3 aged groups with different glycemia were combined together and referred to as the aged db/db mouse group. The volume of granulation tissue deposited within the incisional wound site in the order of greatest to least was aged db/db, young db/db, aged C57BL/6, young C57BL/6 (Table 2). The distance between the dermal edges of healing wounds of aged db/db mice group was 8.1 times greater than the young wild-type mice and 3.7 times greater than young db/db mice group. The mean for the aged db/db mouse group was 10.43 relative distance units (rdu) whereas from wild-type young mice it was 1.29 (rdu). When

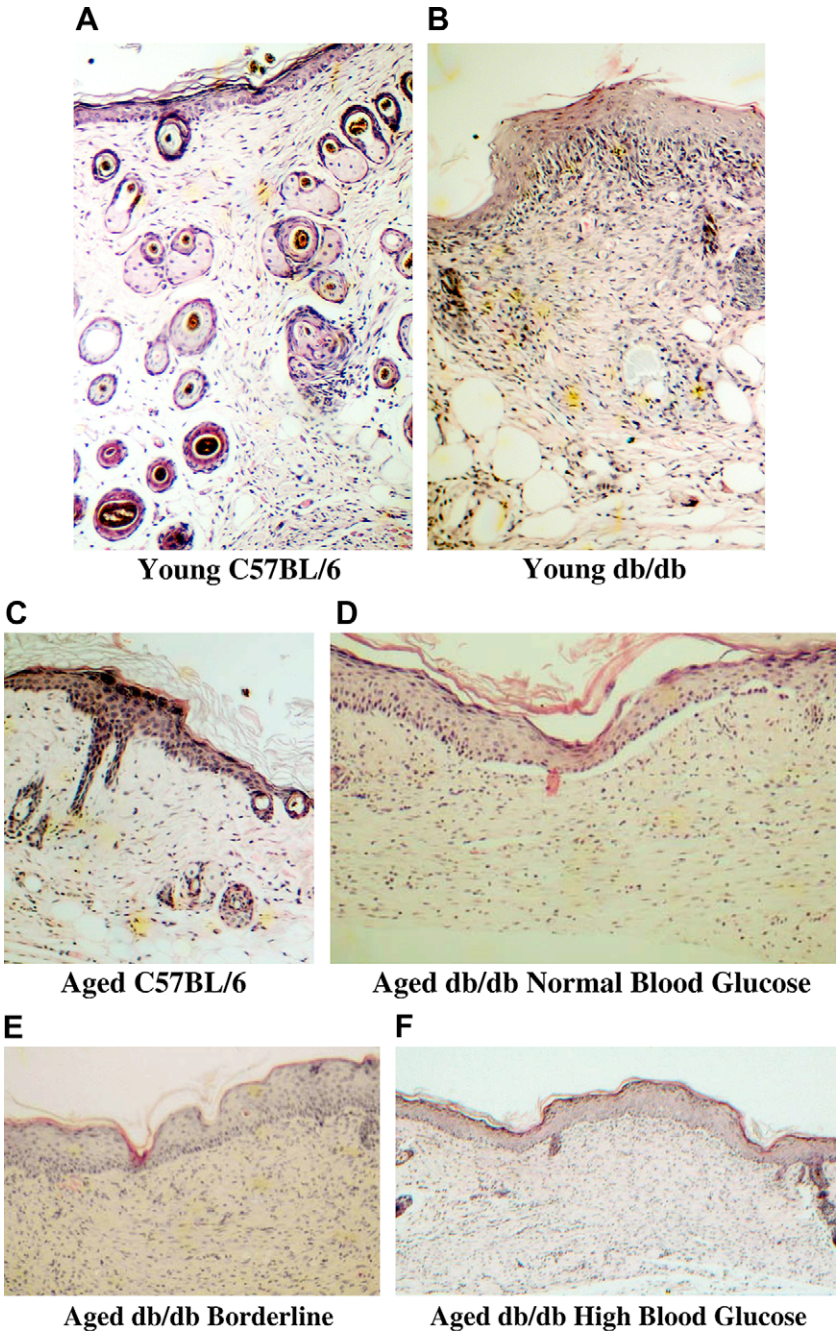



Fig. 3. Histology of the 21-day-old incisional wounds. Wounds with surrounding skin were prepared for histological microscopic evaluation by H&E staining. A typical section of a healing wound is shown from: (A) a young C57BL/6 mouse; (B) a young db/db mouse; (C) an aged C57BL/6 mouse; (D) an aged db/db "normal blood glucose" mouse; (E) an aged db/db mouse with a "borderline" (hyperglycemic with a normal HbA1c) blood glucose level and (F) an aged db/db mouse with a high blood glucose and an elevated HbA1c. These samples were also used to measure the distance between granulation tissue edges in the 6 panels (Table 2). Magnification 10×.

Table 2
Glycemic control and summary of the measurements of distances between dermal edges of the healing wounds

| Group | Means (RDU) | SE of the means | 12-h fasting glucose (mg/dL) | Non-fasting glucose testing (mg/dL) | HbA1c (%) | Sections examined |
|---|---|---|---|---|---|---|
| Aged db/db normal glucose | 10.35 | ±0.88 | 76.7 ± 15.8 | 73.0 ± 24.9 | 3.2 ± 0.4 | $N = 14$[a,b,c] |
| Aged db/db borderline | 11.36 | ±0.54 | 180.7 ± 40.6 | 131.8 ± 55.6 | 4.7 ± 0.7 | $N = 13$[a,b,c] |
| Aged db/db high glucose | 9.59 | ±0.72 | 459.2 ± 88.1 | 394.7 ± 186.4 | 7.8 ± 1.0 | $N = 11$[a,b,c] |
| Aged C57BL/6 | 2.80 | ±0.24 | 120.0 ± 5.0 | 114.7 ± 12.9 | 3.0 ± 0.1 | $N = 12$[a] |
| Young db/db | 4.62 | ±0.40 | 397.0 ± 53.7 | 509.1 ± 77.4 | 4.4 ± 0.3 | $N = 16$[a] |
| Young C57BL/6 | 1.29 | ±0.10 | 118.1 ± 4.6 | 141.3 ± 14.6 | 3.4 ± 0.1 | $N = 16$[b] |

[a] $p \leqslant 0.0001$ compared to young wild-type.
[b] $p \leqslant 0.0001$ compared to young db/db mice.
[c] $p \leqslant 0.0001$ compared to aged wild-type mice.

compared to the young wild-type mice, all other groups demonstrated an expansion in the size of the healing wound (Table 2). An increase by 2.2 times (2.80 rdu) was shown in aged wild-type mice and by 3.6 times (4.62 rdu) in young db/db mice. These differences were highly statistically significant. The distance between the dermal walls was markedly greater in the suture-closed wounds of aged db/db mice (Fig. 3D, E and F). Since all wounds were initially sutured closed, which brought the cut dermal edges in contact with each other, the expansion in separation between the wound edges must have occurred after the sutures were removed at day 7.

To further evaluate granulation tissue, the collagen organization was determined by Sirius red staining. The organization of collagen fiber bundles in the granulation tissue of the all experimental groups was similar as documented by the birefringence patterns from Sirius red stained sections viewed with polarized light (Fig. 4). The birefringence pattern from all groups showed fine collagen fibers within the granulation tissue, deposited between the dermal edges of the wound. In contrast, the birefringence pattern of collagen fibers in the dermis at the edges of the healing wounds showed typical thick collagen bundles, which were arranged in a basket weave pattern. Like granulation tissue, the dermis of all experimental groups had similar birefringence dermal collagen patterns. The increase in volume of granulation tissue in the aged db/db mouse wounds was also demonstrated in these Sirius red stained sections, when viewed with polarized light. The length of the fine collagen fiber birefringence pattern between the edges of dermis was greater in the aged db/db mice (Fig. 4). Since the volume of granulation tissue and its birefringence pattern were identical within the aged db/db mice with differing glycemic levels, control of blood glucose in the aged db/db mice did not alter the volume of granulation tissue deposited.

## 4. Discussion

The combination of db/db genotype and aging significantly impairs wound healing relative to young mice, (independent of the presence of hyperglycemia and db/db genotype in young mice). This surprising finding was confirmed by both biomechanical testing as well as histological analysis. Both stiffness and breaking load were significantly decreased in aged db/db mice, analogous to the elderly clinical population with type 2 diabetes. Likewise, the granulation tissue volume in the aged db/db mice was greater than all other experimental groups. Mechanical testing is sensitive to changes that occur during the progression of wound healing, and can be used as a tool to measure the quality of healing. Mechanical property data provide a clinically relevant, functional assessment of wound healing quality. Histological analyses highlight cellular and connective tissue adaptation at the ultrastructural level in the repair process. When compared to the young mice without diabetes, aged db/db mice showed a statistically significant difference in both breaking load ($p < 0.001$) and stiffness ($p < 0.01$). In addition, the histological parameters did not show a deficit in collagen deposition. There were no obvious differences in the connective tissue organization as demonstrated by the birefringence pattern of collagen fiber bundles using polarized light. These results demonstrate that aging and db/db genotype act synergistically, and our data expand upon previous literature reporting diabetes and advanced age as individual factors responsible for impairment of healing in mouse wounds (Ballas and Davidson, 2001; Keswani et al., 2004).

The most remarkable observation was the disparity in the volume of granulation tissue deposited amongst the different groups. A typical control suture-closed wound has little granulation tissue accumulating between the dermal edges of the wound, (see Fig. 3A). The distance between the dermal edges in the other groups was greater, demonstrating that a larger volume of granulation tissue had accumulated there. Surprisingly, the amount of granulation tissue seen in the aged db/db mice was great enough that it gave the impression of being a full thickness wound.

It was expected that the reduction in wound stiffness and breaking load would be related to a deficit in granulation tissue deposition in the aged db/db mouse suture-closed wounds. Surprisingly a greater volume of granulation tissue in the aged db/db mouse wounds was found when com-

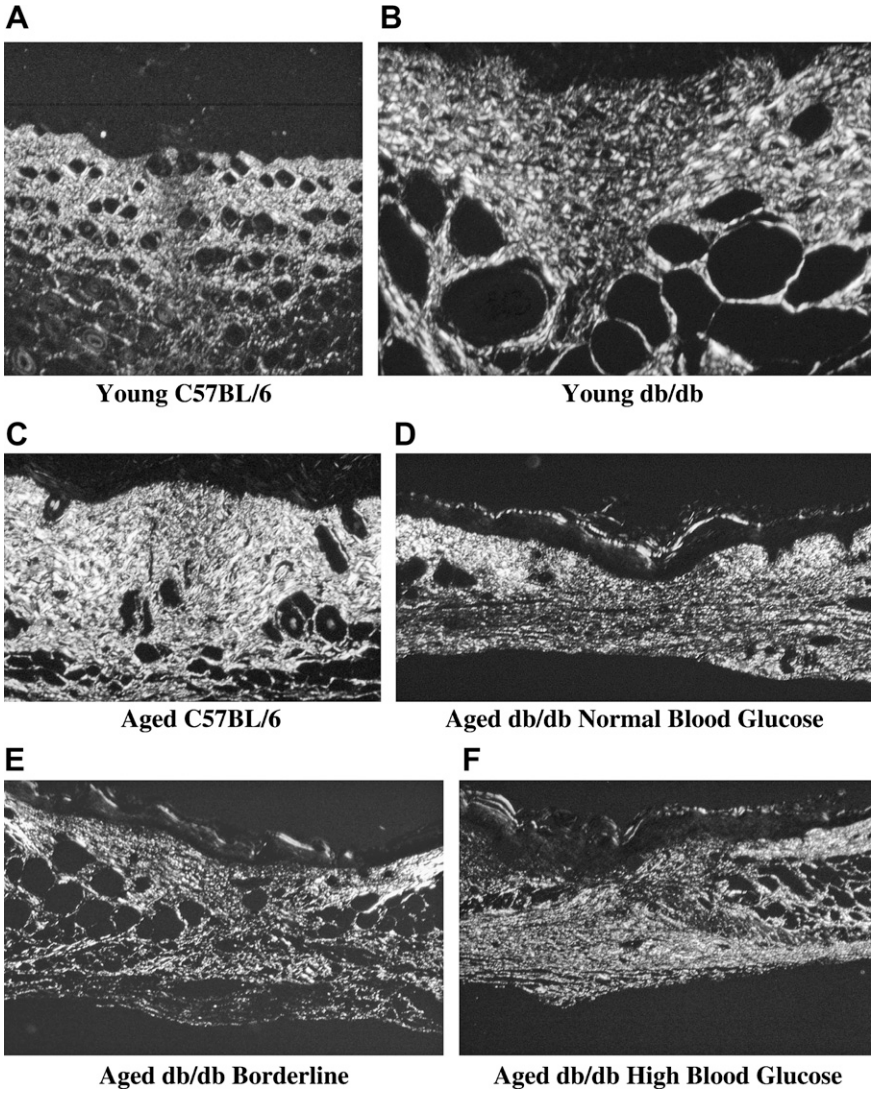



Fig. 4. Sirius red staining with polarized light microscopy. The wound areas with surrounding skin were Sirius red stained and viewed with polarizing optics. The panels show the weak birefringence pattern of collagen fiber bundles within granulation tissue, which are situated between the stronger birefringence responses of normal dermis at the edges of the incisional wounds. A typical birefringence pattern of a healing wound from: (A) a young C57BL/6 mouse; (B) a young db/db mouse; (C) an aged C57BL/6 mouse; (D) an aged db/db normal blood glucose mouse; (E) an aged db/db mouse with a borderline blood glucose level; (F) an aged db/db mouse with a high blood glucose. Compare the widths of the weak birefringence response of granulation tissue and the strong birefringence response at the edges of each section. Magnification 4×.

pared to those of the young db/db and aged wild-type mice. Though a greater volume of granulation tissue was deposited within the aged db/db mouse wounds, the tissue exhibited inferior biomechanical properties. This suggests that healing in aged db/db mice occurs via deposition of an increased quantity of a lesser quality. We speculate that these data may reflect a biological compensatory mechanism whereby a deficit in the ability of the matrix to generate a functional tissue is "offset" by an increase in the amount of tissue deposition. Our data further suggest that the volume of tissue loss increases in the wounds of aged db/db mice. The removal of sutures at 7 days may allow the skin on either side of the incision to pull the wound edges out from the wound site. More granulation tissue may be deposited in response to those forces. Future studies are required to assess the relative contributions of biochemical, biomechanical, ultrastructural and organizational factors in the formation of wound granulation tissue. Taken together, these data suggest an alteration not in the quantity, but the quality of granulation tissue deposition in aged db/db mice.

While the combination of aging and db/db genotype impaired wound healing in mice, neither hyperglycemia nor age alone led to an impediment in wound tensile properties at 14 days. At 16 months of age, db/db mice are elderly, while wild-type C57BL/6 controls are not (JAX Mice Data Sheet, 2006; Danon et al., 1989). In many mouse models, elderly mice are generally regarded as those over 2 years (Agah et al., 2004), and C57BL/6 mice as old as 18 months are considered "middle aged" (Meydani

et al., 1998), as these mice can be expected to live an average life of 27–28 months and are regularly studied later in life (Danon et al., 1989). Here the aged C57BL/6 controls represent the amount of aging that would take place in a mouse in the absence of diabetes. It demonstrates the hastening of the aging process in the presence of diabetes. The wild-type C57BL/6 is not the BKS strain matched or an *m*+/+ *Lepr*$^{db}$ heterozygote, because at the time of the experiment aged-matched BKS or *m*+/+ *Lepr*$^{db}$ heterozygote controls were not available. There are restrictions in developing BKS breeding colonies and there is a lack of availability of aged heterozygotes. While it is recommended that this experiment be repeated utilizing the heterozygote control used in many previous experiments, the control used in the current study provides as much insight as these mice are both of C57 background and share 84% of all alleles (Naggert et al., 1995). While the use of the C57BL/6 may not represent the ideal control, it is assumed adequate for illustrating the effects of aging and diabetes.

Young diabetic mice showed no healing impairments of wound stiffness and breaking load, which implies that the quality of granulation tissue is similar to young wild-type mice. In the db/db mouse model, insulin levels rise at 10–14 days of age, and glucose levels become elevated at 4–8 weeks (JAX Mice Data Sheet). The 8-week-old db/db mice displayed a HbA1c of 4.34%, far below the 8.4% demonstrated by others in the older (usually >10–14 weeks) more mature mice normally explored in wound healing experiments (Galeano et al., 2004). This lack of elevation in the glycosylated hemoglobin levels is emblematic of the fact that the long-term effects of diabetes are not fully realized in these mice. The experiments described here show that the downstream effects of db/db genotype and glycemic elevation are not truly seen at 8 weeks, as wound healing is not impaired as it is at later time points.

A very important finding is that healing impairment in aged db/db mice is independent of hyperglycemia suggesting that the hyperglycemia does not correlate with healing in db/db mice, although it is an important factor to control for. The aged db/db mice separated into three statistically distinct groups with different glycemia, with HbA1c's of $3.2 \pm 0.4$, $4.7 \pm 0.7$ and $7.8 \pm 1.0$, respectively ($p < .001$). Despite this, there was no significant difference in wound breaking load or stiffness. The volume of deposited granulation tissue in the healing wounds from these mice was the same. This also points towards the limitations of db/db as an animal model of human disease and further suggests that there may be yet unidentified factor(s) that may play a role such as AGE/RAGE (Peppa et al., 2003), increased adiposity (Ronti et al., 2006) and the deteriorated hormone profile of db/db mice(Aoki et al., 2003; Burkemper and Garris, 2006; Garris, 1999). One possible way that the combination of diabetes and age manifests itself is in the formation of Advanced Glycation End-products (AGE's) via the Maillard reaction. Increases in exogenous dietary AGE's is shown to impair wound healing in this mouse model (Peppa et al., 2003), and utilizing a soluble form of RAGE (SRAGE) significantly improves wound healing in the presence of diabetes (Goova et al., 2001). Importantly, the data reported in this manuscript reflect our clinical experience and strongly suggest that combination of advanced age and diabetes (not the glucose levels) compromise the healing. This further suggests that db/db mouse model has shortcomings for comparing diabetic healing in humans, but rather reflects the effects of db/db genotype.

Importantly, the data presented here demonstrates both that in this model, age and db/db genotype act synergistically to impair wound healing and that glucose control did not inhibit wound healing. A corollary to this finding is that aging alone does not impair quality of healing, suggesting that elderly patients are expected to heal. These findings confirm our clinical experience, where older patients (though no less likely to heal, i.e. close their wounds) appear to experience longer healing times and therefore complications from their wounds. Further study will determine which additional factors contribute to decreased healing in the wounds of elderly persons with diabetes.

Despite the fact that age and diabetes synergistically impair wound healing, it is important to delineate the difference between impairment and inability. In studies of over 20,000 persons, age as a variable is associated with a non-healing ratio of only 1.02, a ratio that is statistically but not necessarily clinically relevant (Margolis et al., 2003). Despite the fact that 54% of all diabetic amputations occur in the elderly, this population does not have an overall inability to heal (Data and Trends, 1980–2002). Taken together, this suggests that wounds in these patients can heal but the consequences of not treating them promptly are more grave, leading to amputations and death. The healing impairment in the elderly with diabetes necessitates early initiation of a comprehensive treatment plan, before more severe complications develop.

## References

Agah, A., Kyriakides, T.R., Letrondo, N., Bjorkblom, B., Bornstein, P., 2004. Thrombospondin 2 levels are increased in aged mice: consequences for cutaneous wound healing and angiogenesis. Matrix Biology 22, 539–547.

Aoki, K., Nakajima, A., Mukasa, K., Osawa, E., Mori, Y., Sekihara, H., 2003. Prevention of diabetes, hepatic injury, and colon cancer with dehydroepiandrosterone. The Journal of Steroid Biochemistry and Molecular Biology 85, 469–472.

Burkemper, K.M., Garris, D.R., 2006. Influences of obese (ob/ob) and diabetes (db/db) genotype mutations on lumber vertebral radiological and morphometric indices: skeletal deformation associated with dysregulated systemic glucometabolism. BMC Musculoskelet Disord 7, 10.

Ballas, C.B., Davidson, J.M., 2001. Delayed wound healing in aged rats is associated with increased collagen gel remodeling and contraction by skin fibroblasts, not with differences in apoptotic or myofibroblast cell populations. Wound Repair and Regeneration 9, 223–237.

Brem, H., Tomic-Canic, M., Tarnovskaya, A., Ehrlich, H.P., Baskin-Bey, E., Gill, K., Carasa, M., Weinberger, S., Entero, H., Vladeck, B., 2003. Healing of elderly patients with diabetic foot ulcers, venous stasis

ulcers, and pressure ulcers. Surgical Technology International 11, 161–167.

Colonna-Romano, G., Aquino, A., Bulati, M., Lio, D., Candore, G., Oddo, G., Scialabba, G., Vitello, S., Caruso, C., 2004. Impairment of gamma/delta T lymphocytes in elderly: implications for immunosenescence. Experimental Gerontology 39, 1439–1446.

Constantine, V.S., Mowry, R.W., 1968. Selective staining of human dermal collagen. II. The use of picrosirius red F3BA with polarization microscopy. Journal of Investigative Dermatology 50, 419–423.

Danon, D., Kowatch, M.A., Roth, G.S., 1989. Promotion of wound repair in old mice by local injection of macrophages. Proceedings of the National Academy of Science United States of America 86, 2018–2020.

Data and Trends. National Diabetes Surveillance System. Annual Number (in Thousands) of New Cases of Diagnosed Diabetes Among Adults Aged 18–79 Years, United States, 1997–2003. Centers for Disease Control (CDC). Available from http://www.cdc.gov/diabetes/statistics/incidence/fig1.htm Accessed 30 July, 2006.

Data and Trends. National Diabetes Surveillance System. Hospitalizations for Nontraumatic Lower Extremity Amputation, 1980–2002. Center for Disease Control (CDC). Available from: http://www.cdc.gov/diabetes/statistics/lea/byAgetable1_2.htm, Accessed 30 July, 2006.

Davidson, J.M., 1998. Animal models for wound repair. Archives of Dermatological Research 290 (Suppl), S1-11.

Du Nouy, P.L., 1916. Cicatrization of wounds: III. The relation between the age of the patient the area of the wound, and the index of cicatrisation. Journal of Experimental Medicine 24, 461–470.

Ehrlich, H.P., Desmouliere, A., Diegelmann, R.F., Cohen, I.K., Compton, C.C., Garner, W.L., Kapanci, Y., Gabbiani, G., 1994. Morphological and immunochemical differences between keloid and hypertrophic scar. American Journal of Pathology 145, 105–113.

Ehrlich, H.P., Lambert, P.A., Saggers, G.C., Myers, R.L., Hauck, R.M., 2005. Dynamic changes appearing in collagen fibers during intrinsic tendon repair. Annals of Plastic Surgery 54, 201–206.

Frank, S., Hubner, G., Breier, G., Longaker, M.T., Greenhalgh, D.G., Werner, S., 1995. Regulation of vascular endothelial growth factor expression in cultured keratinocytes. Implications for normal and impaired wound healing. Journal of Biological Chemistry 270, 12607–12613.

Galeano, M., Altavilla, D., Cucinotta, D., Russo, G.T., Calo, M., Bitto, A., Marini, H., Marini, R., Adamo, E.B., Seminara, P., Minutoli, L., Torre, V., Squadrito, F., 2004. Recombinant human erythropoietin stimulates angiogenesis and wound healing in the genetically diabetic mouse. Diabetes 53, 2509–2517.

Garris, D.R., 1999. Estrogenic stimulation of hypothalamic-limbic system metabolism in ageing diabetic c57bl/ksj mice. Neuroendocrinology 69, 424–429.

Gilchrest, B.A., Murphy, G.F., Soter, N.A., 1982. Effect of chronologic aging and ultraviolet irradiation on langerhans cells in human epidermis. Journal of Investigative Dermatology 79, 85–88.

Goova, M.T., Li, J., Kislinger, T., Qu, W., Lu, Y., Bucciarelli, L.G., Nowygrod, S., Wolf, B.M., Caliste, X., Yan, S.F., Stern, D.M., Schmidt, A.M., 2001. Blockade of receptor for advanced glycation end-products restores effective wound healing in diabetic mice. American Journal of Pathology 159, 513–525.

Gosain, A., DiPietro, L.A., 2004. Aging and wound healing. World Journal of Surgery 28, 321–326.

History of foot ulcer among persons with diabetes – United States, 2000–2002, 2003. Morbidity and Mortality Weekly Report 52, 1098–1102.

JAX Mice Data Sheet; Strain:BKS.Cg-m +/+ Leprdb/J. The Jackson Laboratory. Available from http://jaxmice.jax.org/strain/000642.html Accessed 30 July, 2006.

Johansen, J.S., Harris, A.K., Rychly, D.J., Ergul, A., 2005. Oxidative stress and the use of antioxidants in diabetes: linking basic science to clinical practice. Cardiovascular Diabetology 4, 5.

Junqueira, L.C.U., Bignolas, G., Brentani, R.R., 1979. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. Histochemical Journal (Historical Archive) 11, 447–455.

Keswani, S.G., Katz, A.B., Lim, F.Y., Zoltick, P., Radu, A., Alaee, D., Herlyn, M., Crombleholme, T.M., 2004. Adenoviral mediated gene transfer of PDGF-B enhances wound healing in type I and type II diabetic wounds. Wound Repair and Regeneration 12, 497–504.

Lee, P.Y., Chesnoy, S., Huang, L., 2004. Electroporatic delivery of TGF-beta1 gene works synergistically with electric therapy to enhance diabetic wound healing in db/db mice. Journal of Investigative Dermatology 123, 791–798.

Margolis, D.J., Allen-Taylor, L., Hoffstad, O., Berlin, J.A., 2003. Diabetic neuropathic foot ulcers: predicting which ones will not heal. American Journal of Medicine 115, 627–631.

Meydani, M., Lipman, R.D., Han, S.N., Wu, D., Beharka, A., Martin, K.R., Bronson, R., Cao, G., Smith, D., Meydani, S.N., 1998. The effect of long-term dietary supplementation with antioxidants. Annals of the New York Academy of Science 854, 352–360.

Naggert, J.K., Mu, J.L., Frankel, W., Bailey, D.W., Paigen, B., 1995. Genomic analysis of the C57BL/Ks mouse strain. Mammalian Genome. 6, 131–133.

Peppa, M., Brem, H., Ehrlich, P., Zhang, J.G., Cai, W., Li, Z., Croitoru, A., Thung, S., Vlassara, H., 2003. Adverse effects of dietary glycotoxins on wound healing in genetically diabetic mice. Diabetes 52, 2805–2813.

Rivard, A., Fabre, J.E., Silver, M., Chen, D., Murohara, T., Kearney, M., Magner, M., Asahara, T., Isner, J.M., 1999. Age-dependent impairment of angiogenesis. Circulation 99, 111–120.

Ronti, T., Lupattelli, G., Mannarino, E., 2006. The endocrine function of adipose tissue: an update. Clinical Endocrinology 64, 355–365.

Salmon-Her, V., Ramont, L., Godeau, G., Birembaut, P., Guenounou, M., Bernard, P., Maquart, F.X., 2000. Implication of interleukin-4 in wound healing. Laboratory Investigation 80, 1337–1343.

Swift, M.E., Kleinman, H.K., DiPietro, L.A., 1999. Impaired wound repair and delayed angiogenesis in aged mice. Laboratory Investigation 79, 1479–1487.

Van den Biggelaar, A.H.J., Huizinga, T.W.J., de Craen, A.J.M., Gussekloo, J., Heijmans, B.T., Frolich, M., Westendorp, R.G.J., 2004. Impaired innate immunity predicts frailty in old age. The Leiden 85-plus study. Experimental Gerontology 39, 1407–1414.

Wild, S., Roglic, G., Green, A., Sicree, R., King, H., 2004. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 27, 1047–1053.

Yamaura, H., Matsuzawa, T., 1980. Decrease in capillary growth during aging. Experimental Gerontology 15, 145–150.

Yoshikawa, T.T., 1997. Perspective: aging and infectious diseases: past, present, and future. Journal of Infectious Diseases 176, 1053–1057.