Final Report

Potential effects of K21 in chronic wound healing process in a pre-clinical model.

Proposal # BRAG 12-2023

18 March 2024

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The problem: Uncontrolled diabetes may also affect circulation, causing blood to move more slowly, which makes it more difficult for the body to deliver nutrients to wounds. As a result, the injuries heal slowly or may not heal at all. There is no existing effective treatment in the field for chronic wound healing in diabetics.

Hypothesis: To test whether K21 can have beneficial effects in chronic wound healing process in a pre-clinical model.

Materials and methods

- **Animal model:** We used db/db mice which are well known to model phase 1 to 3 of diabetes type II and obesity. Mice that are homozygous for the diabetes spontaneous mutation ($Lepr^{db}$) demonstrate morbid obesity, chronic hyperglycemia, pancreatic beta cell atrophy and come to be hypoinsulinemic. They show an uncontrolled rise in blood sugar, severe depletion of insulin-producing beta-cells of the pancreatic islets, peripheral neuropathy, myocardial disease and death by ten months. Exogenous insulin results in loss of control of blood glucose levels, while gluconeogenic enzyme activity increases. They are the best model to study diabetes and its complications such as non-healing chronic wound in a pre-clinical setting.

- **Procedure and experimental model:** Mice were anesthetized using a gaseous mixture consisting of 30% oxygen, 70% N2O and 2.5% isoflurane using a vaporizer. For maintenance of anesthesia, isoflurane concentration was reduced to 1.5%. Mice breathed spontaneously via breathing mask throughout the surgical procedure. Surgical instruments were autoclaved prior to initiating the procedure. During the procedure, for multiple animals, the instruments were sterilized using a bead sterilizer. We kept at least two sets of instruments were available to alternate use between animals before touching them to tissue. Gloves, lab coat or disposable gown were always on during the whole procedure. Face mask, head cap and safety goggles were used during surgery. The hair on the dorsal part of each mouse were removed

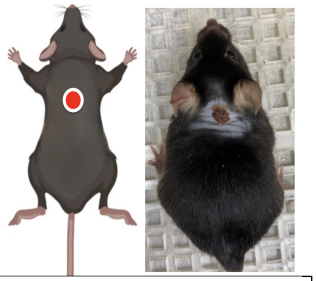

Fig 1. Schematic diagram of wound model

before surgery. The dorsal part of ea ouse was disinfected with iodophors, followed by a

rinse with 75 % (v/v) ethanol. This disinfection procedure was repeated 3 times alternating wipes of the iodophor and 75% (v/v) ethanol (alcohol). For optimal pain control, Buprenorphine SR were administered prior to the start of procedure. A one wound model was created using a sterile biopsy punch (Fig 1). The wound was created using a sterile disposable biopsy punch, 6 mm diameters with plunger available commercially (BrainTree Scientific Inc, USA). As post-operative management and care protocol, analgesic agent (Buprenorphine SR,) was administered once every 24 to72 hours for post-operative pain relief. All animals were individually caged (Single Housed Animal) for the rest of experimental time.
The wounds were then treated with topical application of K21 (0.46%) or vehicle control every other day until complete wound closure was achieved in 10 days. Animals were monitored twice daily for manifestations of pain and weight loss. In case of any observed adverse effect, after initial consultation and detailed examinations, mice were sacrificed. All animals were maintained and monitored for the period of maximum 4 weeks before they get sacrificed for further histologic and flow cytometric analysis.

- **Visual observation and direct measurement:** To assess and monitor the healing process, all subjects were photographed using a digital camera at 4 periodic timepoints accordingly. Photographic images were compared and differences were recorded.

- **Histologic evaluation.** Histology assessment was carried out by performing hematoxylin and eosin (H&E) and additional Masson Trichrome staining (MTR). Briefly, the dorsal skin tissues from wound areas were harvested and were paraffin-embedded. Next, tissue sections were cut in 5 µm thickness and stained using H&E and MTR. All stained sections were mounted in Faramount and were covered. All preparations were analyzed and imaged using bright-field microscopy, measured using ImageJ software (version 1.53e).

- **Immunohistochemistry evaluation.** Fresh dorsal skin tissues from wound areas were fixed with 10% neutral buffered formalin, processed, embedded and subsequently cut into 4 µm sections for immunohistochemical assessment. After blocking endogenous peroxidase activity with hydrogen peroxide diluted 1:10 in distilled water for 10 min, sections were treated with Proteinase K for 10 min and washed twice in PBS. Slides were then incubated with antibodies against VEGF (vascular endothelial growth factor). The significance of VEGF is the fact that VEGF stimulates angiogenesis and also influences wound closure and epidermal repair, granulation tissue formation, and the quality of repair, both in terms of the strength of the healed wound and the amount of scar tissue that is deposited. Biotinylated immunoglobulins (Biogenex) were added to all slides for 20 min. After two washes with PBS, all slides were incubated with peroxidase-conjugated streptavidin (Biogenex) for 20 min followed by two washes in PBS. All slides then treated with chromogen (Dako)
until clearly detectable color appearance. Excess chromogen was decanted and all slides were washed by distilled water. All preparations were counterstained with

hematoxylin for 3 minutes and mounted in aqueous mountant (LERNER laboratories) prior to the analysis using bright field Zeiss (AXIO Imager M2) light microscope.

- **Analytical flow cytometry analysis.** For flow cytometry analysis, fresh tissues from wound areas were placed in a tissue culture dish with 1 mL PBS + 2% FCS, 2 mg/mL of collagenase type II, and 1 mg/mL of DNase type I for 30 minutes at 37°C. Samples

  were then sieved through a cell strainer (BD Biosciences), followed by centrifugation (252g, 5 minutes, 4°C) to prepare single-cell suspensions. Cells were then fixed and permeabilized using fix/perm concentrate before incubation with antibody for VEGF and CD26. CD26 is an ecto-enzyme, functionally involved in skin wound healing by regulating cell proliferation, migration, and collagen synthesis in fibroblasts. Samples were then washed and subjected to flow cytometry using NovoCyte Quanteun and analyzed by FlowJo analytical software. Isotype-matched controls were analyzed to set the appropriate gates for each sample.

Results

- **K21 expedited the wound's closure time in murine diabetic model.** Visual observation and measuring the wound size demonstrated that K21 could expedited the wound's closing time in db/db mice compared to the vehicle (control) treated group (Fig 2). As shown in figure 2, the assessment was done at day 4 and 12 post operation, with day 12 to have the most significant outcomes.

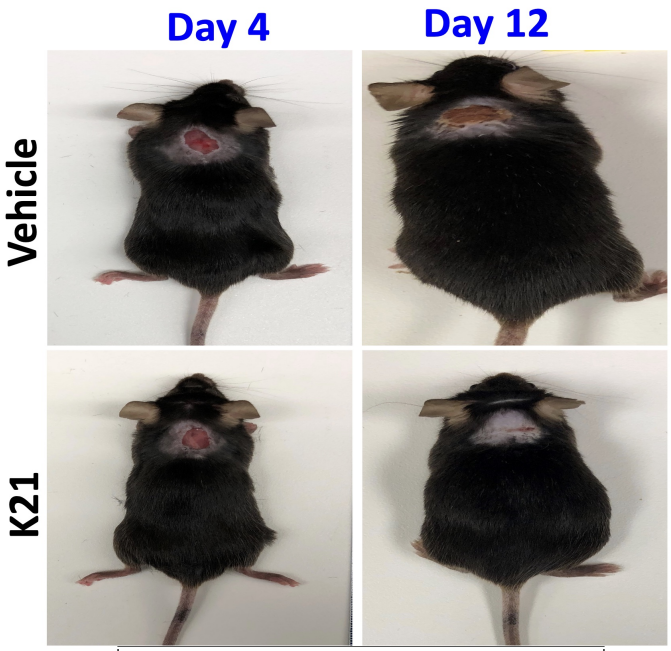

Fig 2. K21 accelerated the wound closure

K21 improved tissue remodeling and structural recovery. H&E and Masson trichrome (MTR) staining showed the tissue remodeling and structural integrity were

improved, consistent with clinical outcomes ‹pedited wound closure in K21 treated wounds compared to the vehicle treated (Fig 3).

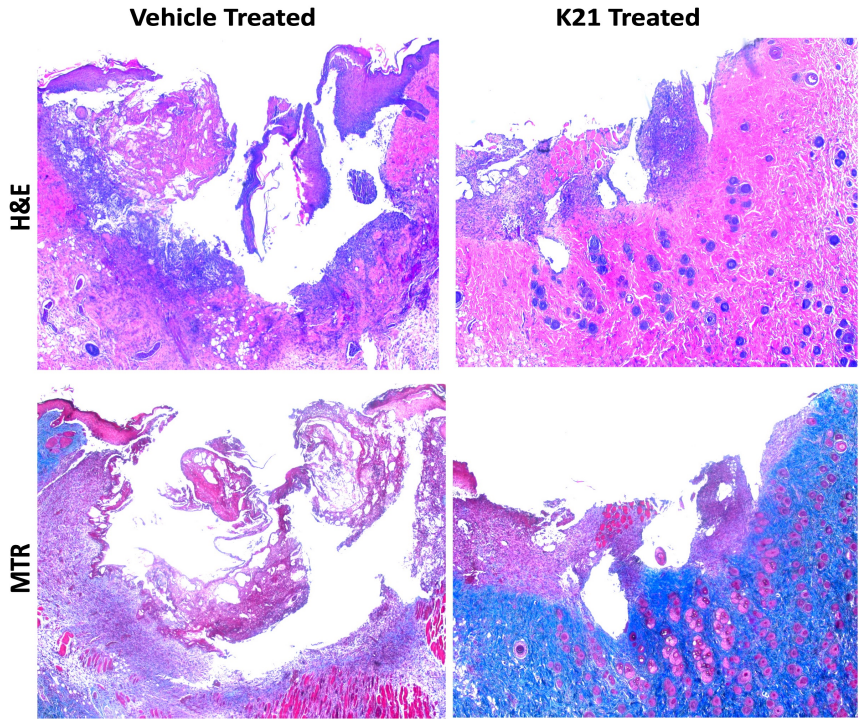

Fig 3. H&E and TCR staining of wound tissues showed that K21 expedited tissue remodeling and structural recovery compared to the vehicle treated wounds.

K21 increased VEGF expression during wound healing process in diabetic mice. As shown in figure 4, K21 increased VEGF expression in tissues from wound in db/db mice compared to vehicle treated (control) tissues. The red/brownish chromogen staining indicates the VEGF expression in the dorsal skin tissues with wounds in db/db mice with high adipose tissues as shown in figure 4.

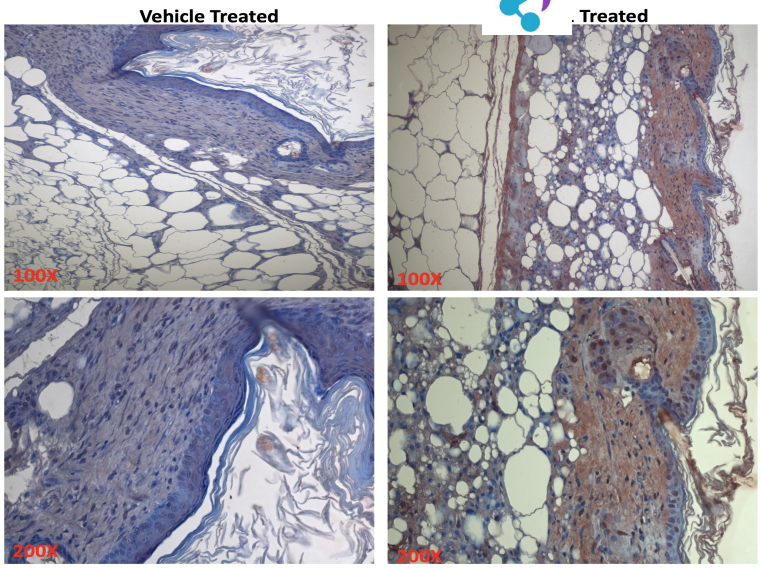

Fig 4. Immunohistochemical analysis of tissues from wound on dorsal skins in db/db mice shows that K21 increases VEGF expression compared to wound tissues treated with vehicle

K21 increased VEGF expression was associated with CD26 enhancement. Flow cytometry analysis showed that K21-induced increase in VEGF was associated with enhanced CD26 expression in wound tissues compared to vehicle (control) treated tissues (Fig 5).

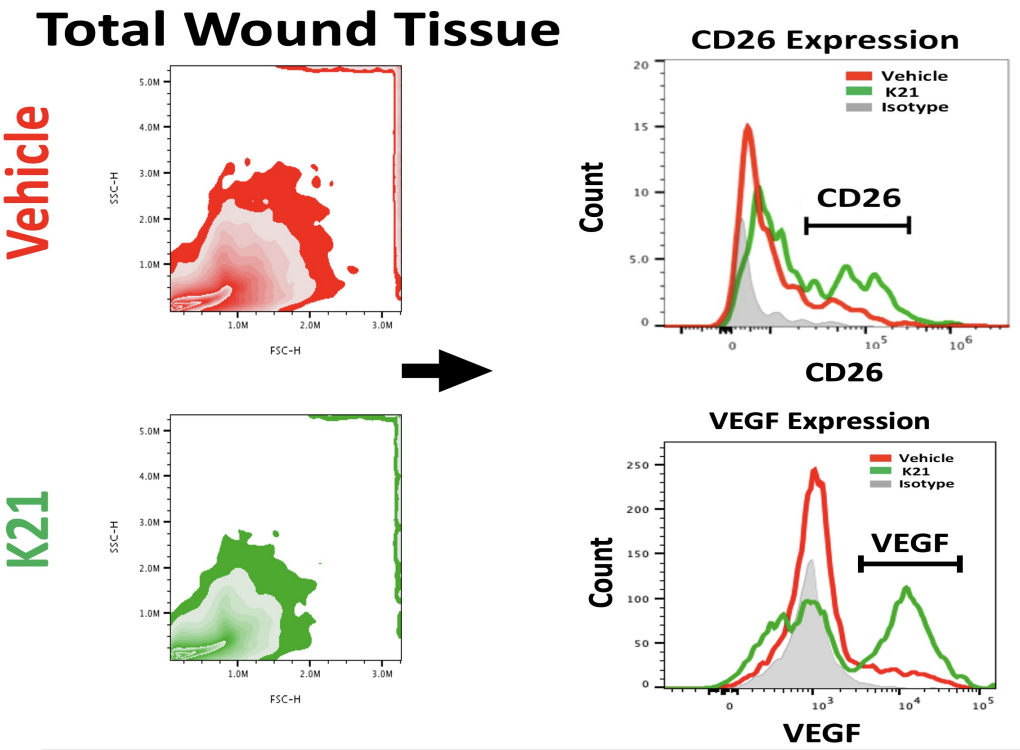

Fig 5. Flow cytometry from wound on dorsal skins in db/db mice showed that in addition to VEGF, K21 increased CD26 significantly compared to wound tissues treated with vehicle (control).

Summary of findings:

- K21 expedited wound closure in db/db mouse, a pre-clinical model for diabetes.
- K21 improves tissue remodeling and structural integrity in db/db mouse.
- K21 increased VEGF and CD26 expression in wound of a pre-clinical model for diabetes.

Conclusion:

K21 shows potentials in expediting and improving wound healing process which could be targeted as therapeutic modality in the treatment of chronic wound healing process, warrants further research.