

LETTER TO THE EDITOR.

Working title: Evaluating STZ-induced impaired wound healing in rats

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To the editor,

A vast array of *in vivo* experimental models are utilised within the wound healing field. There remains little agreement as to the optimal *in vivo* experimental approach to mimic human chronic wounds (Wilhelm et al., 2017). Moreover, animal models of impaired healing have performed particularly poorly at translating drug based therapies to the clinic over many years, leading many to question their effectiveness (Gordillo et al., 2013). We have previously shown that the wound type (i.e. incision or excision) and subsequent analysis method will determine the sensitivity, and hence the likelihood of a statistically significant difference being correctly identified using a specific healing model (Ansell et al., 2014). When considered alongside our demonstration that rodent hair cycle can significantly alter the speed of repair (Ansell et al., 2011), careful experimental design of *in vivo* wound models becomes critical for maximising the probability of achieving statistical significance. Indeed the paucity of new drug based therapies emerging for the treatment of chronic wounds may be, at least in part, due to sub-optimal preclinical models.

Diabetes mellitus (DM) is a major cause of human chronic wounds (Eming et al., 2014). Several rodent models of DM are available (Ansell et al., 2012, Boyko et al., 2017, Davidson, 1998), however, the Streptozotocin (STZ)-induced DM model is almost exclusively used to model type 1 DM (Goodson and Hung, 1977). Like other wound models there is an inherent lack of consistency between published STZ-DM studies with variation in animal gender, wound size and wound type, but also in the length of time between DM-induction and subsequent injury (Table 1). We predict this latter variable to be crucial given that numerous effects of hyperglycaemia can take many weeks to manifest, such as advanced glycation end-product accumulation (Chen et al., 2009), or structural features of neuropathy (Biessels et al., 2014). To our knowledge, a rigorous assessment of the degree of healing impairment in the STZ model linked to time post induction has not been published.

To begin to fill this knowledge gap, we first examined wound healing following STZ-induced DM in male Wistar rats. We compared healing in rats at 3- (n=6 rats) or 6-weeks (n=4 rats) post-DM induction to non-diabetic (n=6 rats) sham control rats (6mm punch biopsy wound harvested at 5 days post-wounding; see supplemental methods for full details), to assess the influence of time post-induction on healing outcome.

We collected wound photographs at day 5 (Figure 1A), which when assessed revealed a significant delay in healing (larger wound surface area) versus control only in 6-week post-DM induction animals (Figure 1B). To confirm this observation we profiled standardised histological wound parameters from tissue sections from the centre of each wound (Figure 1C). We found no statistically significant difference in histological wound width (Figure 1D) or the area of wound granulation tissue (Figure 1E). Taken together, these data suggest that

planimetry is a more reliable measure of overall healing delay in the STZ-DM model. Histological analysis, however, has merits with the parameter of re-epithelialisation demonstrating a statistically significant delay reduction (i.e. delayed wound closure) following 6 weeks of DM (Figure 1F). Again, there was only a trend towards delayed re-epithelialisation in rats 3 weeks post-DM induction. Thus, re-epithelialisation appears to provide a sensitive histological readout for the onset of impaired healing with DM.

The inability to statistically demonstrate any aspect of delayed healing at 3 weeks post-STZ-DM induction suggests that this (or any earlier) time point is insufficient, despite being used frequently in the literature (Table 1). We do however note a trend towards reduced re-epithelialisation at 3 weeks, which might become statistically detectable with increased sample sizes (Table S1). Many studies do not confirm whether significantly delayed repair exists with their chosen impaired healing model and sample size (Table 1). Furthermore, almost one third of studies do not indicate the DM induction timeframe used.

That only some wound parameters are demonstrably altered at 6 weeks post-STZ-DM induction suggests that the rate at which individual repair processes become impaired following loss of blood glucose control differs. To further explore this point we assessed changes in inflammation, collagen deposition and angiogenesis, with time-post STZ-DM induction. We find a strong increase in the number of wound macrophages in the 6 week STZ-DM group (Figure 1G), with no detectable difference in collagen deposition or angiogenesis (Figure 1H, 1I). While these data suggest that impairment of angiogenesis and collagen deposition take longer than 6 weeks post-STZ-DM, we cannot exclude our single analysis timepoint (day 5) providing a poor readout for these later phases of repair.

Pain withdrawal time in DM rats only declines 4-6 weeks post STZ administration (Kambiz et al., 2015), so few wounding studies will examine neuropathic healing. It would be interesting to assess chronic diabetes effects on healing, though this would necessitate use of insulin pellets, which will impact on the rate of healing (Goodson and Hung, 1977), and would preclude direct comparison to our earlier timepoints.

Our assessment of the literature revealed a strong preference for using male animals (Table 1), though the rationale for this remains unclear. To assess gender-specific effects on the STZ-DM model we conducted a second experiment comparing 6 weeks post-STZ-DM (n=9) versus non-diabetic (n=8) groups of female rats. Our macroscopic assessment reveals a significant delay after 6 weeks of diabetes, although the magnitude of difference was smaller than in males (Figure S1A). We could find no delay to repair by any histological measure in female rats (Figure S1B-D). Re-epithelialisation data were surprising given the pronounced delay observed in males (Compare Figure S1D to Figure 1F). Our data indicate that DM impaired healing is less pronounced in female rats. The underlying cause of this gender dichotomy remains unclear, though it may be related to the effects of sex steroid hormones (Ashcroft et al., 1997, Gilliver et al., 2009).

Collectively, these data indicate that numerous published studies have been performed using diabetic animals that display hyperglycaemia, but have not yet established a delayed healing phenotype, rendering the published observations invalid. While some may argue that in longer term post-wound studies rats will develop an impaired healing phenotype over the experimental window, early work showed that the initial wound response is critical for overall healing outcome (Seifter et al., 1981). Our study employed a small wound to correlate healing efficiency with a point in time, though this will not be the optimal approach for all

research questions. Our data highlight the importance of clear experimental design based on carefully validated wound models, in order to ensure that any treatment (e.g. drug) effect can be demonstrated, while reducing the overall requirements for animal use. Finally, our literature searches over the course of this study highlight an urgent need to improve the detail in reported experimental methodology, to include the age/weight, sex, strain and in the case of the STZ model the time post-STZ-DM induction, to allow the work to be properly compared to existing literature.

Conflict of interest

The authors declare no conflict of interest.

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Supplemental methods

Animal studies

Wistar rats weighing 250-300g were purchased from Harlan Labs. Animals were housed in trios and provided with food and water *ad libitum*. All studies were approved by The University of Manchester and conducted in accordance with UK Home Office regulations.

Diabetes was induced using a single 40mg/kg *i.p.* dose of Streptozotocin (Sigma; Paisley, UK), with a sham injection group acting as a non-diabetic control. In the first study 6 weeks and 3 weeks of diabetes was compared to controls (6 male animals per group), although 2 animals from the 6 week DM group were excluded; one as DM induction was unsuccessful, while the other animal presented with in anagen phase of hair growth cycle. Injections for the 3 week DM group were staggered such that all experimental animals were wounded on the

same day. In the second study using females 9 DM (with 6 weeks of induction) and 8 non-diabetic animals were compared.

Blood glucose tests were conducted 3 days following STZ administration. All animals were then weighed and monitored daily. Confirmation of a diabetic state during wound healing was through blood glucose readings (Table S2). Rats were anaesthetised via isoflurane inhalation and the dorsal skin shaved and swabbed with ethanol. Animals were wounded with two 6mm diameter (i.e. a surface area of 28.27mm²) full thickness excisions, approximately 6cm apart and separated by the dorsal midline. Wounds were left to heal via secondary intention.

Histology & Immunohistochemistry

Animals were sacrificed on day 5 following wounding by a rising concentration of CO₂. Wounds were photographed with a digital camera to measure the wound surface area (i.e. planimetry), and the wound tissue was excised. Wounds were bisected and formalin fixed for 24hrs before being processed for paraffin embedding as previously described (Ansell et al., 2014). 5µm tissue sections were dewaxed and rehydrated through an ethanol gradient before being stained using a Masson's trichrome stain kit (Atom Scientific) as per manufacturer's instructions. Immunohistochemistry was performed on 5µm sections using the Vector polymer method, with antigen retrieval using citrate buffer pH6 and blocking with 10% goat serum for 30 minutes. The primary antibodies used were Cd68 (Biorad AbD Serotec; MCA341R) at a concentration of 1mg/ml, Vwf (Dako; A0082) at 3.1mg/ml, with appropriate goat raised secondary antibody (Vector) and visualised with DAB reagent.

Image analysis

Stained slides were imaged using an Aperio Scanscope CS (Leica), and measurements of width, granulation tissue area and % re-epithelialisation were determined as indicated in Figure 1B. Measurements of collagen deposition and DAB intensity were determined using the entire granulation tissue as an area of reference.

Statistics

Measurements for the left and right hand wound were averaged, to give a mean value for each animal as a biological replicate, which served as our sample (n number). The experiment using males was analysed with a one way ANOVA, with posthoc Dunnetts tests between the control and other groups. The female experiment was analysed using a students t test. A P value of <0.05 was deemed significant.

Sample size power calculations were made using the actual mean and SD values of our non-diabetic control group for each healing measurement, and hypothetical means under different healing impairment scenarios. Alpha error was set at 5% and beta error was 20%.

Literature searches

Literature searches were made using PubMed (www.ncbi.nlm.nih.gov/pubmed). A total of 42 papers published in 2016 were detected under the search of “streptozotocin skin wound healing rat”. Of these, 28 studies had used an excisional skin wound model and were included for comparison. Of the 14 papers rejected, 5 papers had used wound models other than excision (burn, 2; skin flap, 1; incision, 1; laser, 1), 3 studies did not conduct wounding, 2 studies reported wounds to other tissues (cornea, 1; bone, 1). One study had coupled low dose

STZ with a high fat diet to mimic type 2 diabetes. The remaining 3 studies were excluded as had no English language version available,

Figure Legends

Figure 1. Profiling development of impaired wound healing following STZ induced diabetes. Wounds in male animals following 3 or 6 weeks of diabetes, were compared to a non-diabetic (ND) control. Representative macroscopic images of wounds at day 5 (A), were used to assess the wound surface area (B). Histology was taken through the centre of the wounds (C), to quantify the percentage of re-epithelialisation (D), wound width (E) and area of granulation tissue (F). Immunohistochemistry was used to assess for inflammation (Cd68; G), collagen deposition (Massons trichrome; H) and angiogenesis (Vwf; I). Bars expressed as mean +/- SEM, n=4 animals (6 week group) and 6 animals (ND and 3 week groups). * P<0.05, ** P<0.01, *** P<0.001.

Supplemental Figure 1. Females provide a less robust impaired wound healing model following STZ induced diabetes. Representative macroscopic images of wounds at day 5 (A), were assessed for wound closure (B). Histology of the percentage of re-epithelialisation (C), wound width (D), or the area of granulation tissue (E) was not significantly different. Bars expressed as mean +/- SEM, n=8 animals (ND group) and 9 animals (6 week group). ** P<0.01.