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Animal models of ischemic limb ulcers: a systematic review and meta-analysis

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ABSTRACT

The aims of this systematic review were to assess the clinical relevance and quality of previously published animal models of ischemic ulceration and examine the available evidence for interventions improving ulcer healing in these models. Publicly available databases were searched for original studies investigating the effect of limb ischemia on wound healing in animal models. The quality of studies was assessed using two tools based on the Animal research: Reporting of In Vivo Experiments (ARRIVE) guidelines and the clinical relevance of the models. A total of 640 wounds (ischemic=314; non-ischemic=326) were assessed in 252 animals (92 mice, 140 rats, 20 rabbits) from 7 studies. Meta-analyses showed that wound healing was consistently delayed by ischemia at all time-points examined (day-7 standard median difference (SMD) 5.36, 95% CI 3.67 to 7.05; day-14 SMD 4.50, 95% CI 2.90 to 6.10 and day-21 SMD 2.53, 95% CI 1.25 to 3.80). No significant difference in wound healing was observed between 32 diabetic and 32 non-diabetic animals with ischemic wounds. Many studies lacked methods to reduce bias, such as outcome assessors blinded to group allocation and sample size calculations and clinically relevant model characteristics, such as use of older animals and a peripheral location of the wound. Five different interventions were reported to improve wound healing in these models. The impaired wound healing associated with limb ischemia can be modeled in a variety of different animals. Improvements in study design could increase clinical relevance, reduce bias and aid the discovery of translatable therapies.

INTRODUCTION

Chronic wounds, or ulcers, are a worldwide problem estimated to affect about 1%–2% of the population and an important contributor to disability and early mortality.[1 2] Treatment of ulcers is expensive. In the USA, for example, it has been estimated that the cost of managing diabetes-associated foot ulcers is between US$28.1 and 96.8 billion per year.[3] The 5-year mortality of people with diabetes-associated lower limb ulcers is comparable to some cancers.[4]

Ischemia plays an important role in the etiology of about a quarter of ulcers.[5 6] Ischemic ulcers are frequently very difficult to heal and are an important cause of major lower limb amputation.[7] Ischemic ulcers are usually managed by open or endovascular revascularization surgery. Revascularization, however, has a number of deficiencies, including a risk of perioperative complications, failure to successfully heal the ulcer and lack of suitability for some patients.[8 9] There is therefore a need for alternative and adjunctive therapies.[10 11]

Animal models of ischemic ulcers are needed to test potential new therapies. For preclinical studies to be valuable, it is important that the animal models mimic the underlying pathophysiological mechanisms for delayed wound healing seen in patients. Currently, there is no consensus on which animal model best represents human lower limb ischemic ulcers. The focus of this study was to systematically review the published animal models of ischemic ulcers. The first aim was to assess whether there was evidence that the published animal models simulate the ability of ischemia to delay wound healing. The second aim was to assess the relevance of available animal models to patients and examine the quality of past animal research. The third aim was to assess what evidence there was for the efficacy of different interventions to heal ischemic wounds within the animal models examined. The intention was to inform the most appropriate model of ischemic ulceration for future research and advance the approach to research designed to develop new treatments for ischemic ulcers.

METHODS

Search strategy

This systematic review was performed according to the 2015 Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols (PRISMA-P) statement.[12] The protocol was registered in the PROSPERO database (Registration Number: CRD42020126521). The literature search was conducted by one author (ST) to identify animal studies that evaluated the effect of ischemia on wound healing in animal models. The databases PubMed, Scopus and Web of Science were searched to include all

relevant publications until the 23 February. The search strategy used both medical subject headings (MeSH) and keywords as given in the online supplementary data.

Inclusion and exclusion criteria

Included articles were identified by one author (ST). Studies included were reported as original articles comparing wound area of ischemic and non-ischemic ulcers in animal models that were published in the English language. Only ischemia induced through ligation of the femoral, iliac or saphenous arteries and wounds created through excision methods in the extremities were included to represent the clinical setting. Exclusions included clinical studies, reviews or case reports.

Data extraction

The full texts of included studies were independently assessed by two investigators (ST and JP) to extract data on animal species, age, sex, weight and number, method of ischemia and wound induction, wound area at days 7, 14 and 21. Extracted data were discussed in a meeting with another researcher (JG) and finally agreed through consensus. In studies where numerical data of wound area was not reported, the corresponding authors of the studies concerned were contacted to obtain original data. In studies where the data was unobtainable, a screenshot of the graphical data was exported into ImageJ V.1.48 (National Institutes of Health, USA) and relevant values were extracted. From the screenshot, the length covered between the base of the graph to the plotted area of day 0 of the respective group was calculated as the baseline wound area and the relative percentage change in the length covered in the graph at the plotted points of day 7, 14 and 21 of respective groups were calculated and the results were presented in terms of percentage. In studies where SE of the mean (SEM) was provided, SD was manually calculated by multiplying the square root of number of animals used in the study to the SEM value. If no p value were reported, a two tailed unpaired t test was performed using Graphpad Prism 7 (San Diego, California, USA) where there was evidence of normal distribution of data reported in the original study.

Quality assessment

Each included study was assessed for risk of bias and reporting quality and clinical relevance by one researcher (ST). Risk of bias and reporting quality was assessed using the Animal research: Reporting of In Vivo Experiments (ARRIVE) guideline criteria.[13] Each study was assessed as to whether the following items were reported: ethics statement, study design, animal characteristics, detailed experimental procedure, animal randomization to different groups, experimental measurements of wound area, baseline wound area data for comparison, data estimates reported as mean or median with variance, statistical analysis used and adverse events. The clinical relevance of the models were assessed in relation to the following parameters: the location of the ischemic wound in the peripheral part of the limb, the presence of comorbidities (diabetes and older age) and whether complete wound healing data were reported. In both risk of bias and reporting quality and clinical relevance assessments, scoring was answered in binary form (yes=1/no=0) and reported in terms of percentage. Studies with scores less than 50%, 50%–80% and >80% were considered as low, moderate and high quality, respectively.

Outcomes and data analysis

The primary outcome was the healing rate in ischemic compared with non-ischemic wounds. Wound healing was estimated using extracted wound area data at 7, 14 and 21 days. Wound area was compared with baseline in terms of percentage and the relative wound area at different time-points were compared between ischemic and non-ischemic animals during healing. The secondary outcome of the number of days taken for full closure of wounds were also compared between the ischemic and non-ischemic animals. The third outcome was to compare healing rate and wound closure between animals receiving an interventional agent, or with an induced comorbidity, with controls. A minimum of three studies at any given time-point were required to be eligible for meta-analysis. Meta-analyses were performed using Review Manager 5.3.5 (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014). Due to anticipated statistical heterogeneity, random-effects models were used. Data were expressed as standardized mean difference (SMD) with 95% CI. Leave one out sensitivity analyses, by removing studies individually, were performed to assess the consistency of findings. The I^2 index was used to assess the degree of heterogeneity between studies, with I^2>50% accepted to denote statistical heterogeneity. Funnel plots of the effect size versus the SEM of the log-transformed effect were constructed to assess potential publication bias. P<0.05 was considered as statistically significant.

RESULTS

Study selection

Of 5844 screened studies, a total of 7 were included (online supplementary figure 1).[14–20]

Study characteristics

The included studies investigated mouse (n=3),[15 19 20] rat (n=3)[14 17 18] and rabbit (n=1)[16] ischemic ulcer models. All included studies induced hind limb ischemia by ligating either the femoral (n=5)[15 17–20] or external iliac (n=2)[14 16] arteries (online supplementary table 1).

Five studies investigated ischemic ulcers in animals where diabetes was also induced by intraperitoneal injection of streptozotocin (STZ).[15 17–20] Wounds were created immediately following ischemia induction in all studies except one where the wound induction was performed 3 weeks after the ischemia surgery.[16] No other comorbidities were induced within any of the included studies. Wounds were created in either the dorsal (n=5) or lateral (n=2) surface of the thigh (n=4)[15 16 19 20] or the paw (n=3).[14 17 18] All data were reported in terms of mean±SD.

Table 1 Risk of bias and reporting quality assessment using the ARRIVE guidelines

Criteria		Study 14	15	16	17	18	19	20
Method								
Ethical statement	Reported the ethics committee approval	1	1	1	1	1	1	1
Study design	Reported control vs Ischemia group	1	1	1	1	1	1	1
Animal characteristics	Animal age/weight	1	0	1	1	0	1	1
	Sex	1	0	1	1	0	1	1
	Strain	1	1	1	1	0	1	1
Experimental procedures	Detailed the ischemia protocol	1	1	1	1	1	1	1
	Detailed the wound development protocol	1	1	1	1	1	1	1
Animal randomization to different groups	Indicated methods employed to minimize selection bias between groups	0	0	1	0	0	0	0
Statistics	Reported the statistical methods used for each analysis	1	1	1	1	1	1	1
Experimental measurements	Reported the process employed for measurement of wound area	1	1	1	1	1	1	1
	Reported that the wound measurements were performed in duplicates or blinded to confirm reliability	0	1	1	0	0	0	0
Results								
Baseline data	Reported baseline data for all animal groups	1	1	1	1	1	1	1
Numbers analyzed	Reported the absolute number of animals used in each group	1	0	1	1	1	1	1
Data estimation	Reported the results of analysis with a measure of precision (SD or SEM)	1	1	1	1	1	1	1
Adverse events	Reported if there was no/any adverse events or infection arising from the wounds	0	0	0	0	0	0	0
Score of 15=100%		12	10	14	12	9	12	12
Total score (%)		80	66.6	93.3	80	60	80	80

1=Yes; 0=No.
ARRIVE, Animal research: Reporting of In Vivo Experiments.

Risk of bias and reporting quality of the included studies

An ethics statement, study design with ischemic and non-ischemic comparison groups, animal characteristics including age, sex and strain, experimental procedures including ischemia and wound creation protocols, wound area data and complete wound closure time for both comparison groups at baseline and available time-points (day 7, 14 and/or 21), and statistical analyses were reported in most included studies (table 1). Random allocation of animals to the different groups was reported in only one study[16] and assessment of outcomes by an observer blinded to group allocation was reported in two studies.[15 16] Five of the included studies were considered of high quality[14 16 17 19 20] and two were considered of moderate quality.[15 18] The mean quality assessment score was 77.1%±10.8%.

Clinical relevance of the included studies

As determined by the inclusion criteria, all studies reported ischemic wounds in the hind limb region (table 2). Only three studies created wounds in the paw region that was considered to represent the clinically relevant peripheral site.[14 17 18] Patients with ischemic wounds typically have comorbidities, such as diabetes and older age, which contribute to delay wound healing. Five of the seven studies investigated ischemic wound in which diabetes was induced.[15 17–20] Glucose levels were reported in only three of these studies.[15 19 20] The relative age of the animals studied was not comparable to patients ranging from 90 days in rats to 8 weeks in mice (online supplementary table 1). Blood flow in the ischemic limb was measured using laser Doppler imaging in four studies,[14 15 17 18] but not reported in the remaining three studies[16 19 20] (table 2). Three studies

Table 2 Clinical relevance assessment of the included studies

Criteria	Study						
	14	15	16	17	18	19	20
Species	0	0	0	0	0	0	0
Was ischemia created in a limb as generally seen in patients?	1	1	1	1	1	1	1
Was the ischemia created via ligation of an iliac, femoral or saphenous or popliteal artery?	1	1	1	1	1	1	1
Was the blood flow assessed?	1	1	0	1	1	0	0
Was the wound located in the paw to mimic a foot ulcer seen in patients?	1	0	0	1	1	0	0
Did the animals have diabetes?	0	1	0	1	1	1	1
Did the animals have peripheral neuropathy?	0	0	0	0	0	0	0
Were the animals aged an equivalent of over 50 years of human age?	0	0*	0*	0	0*	0	0
Did the study report complete wound healing data?	1	0	0	1	1	0	0
Score of 9=100%	5	4	2	6	6	3	3
Total score (%)	55.5	44.4	22.2	66.6	66.6	33.3	33.3

1=Yes; 0=No.
*Not reported.

reported data on full wound closure, which was considered a clinically relevant outcome.[14 17 18] The clinical relevance of the models was considered moderate in three studies[14 17 18] and low in remaining four studies.[15 16 19 20] The mean clinical relevance score was only 46.1%±17.5%.

Effect of ischemia on wound healing

A total of 640 wounds (Ischemic=314; Non-ischemic=326) were assessed from all included studies at days 7 (n=252), 14 (n=244) and 21 (n=144) in 252 animals (92 mice, 140 rats, 20 rabbits) from 7 included studies. Diabetes was present as a comorbidity in 322 wounds (Ischemia=161; Non-ischemia=161) during assessments performed at days 7 (n=144), 14 (n=136) and 21 (n=42). All included studies reported wound area at days 7 and 14,[14–20] whereas four studies reported wound area at day 21.[14 16–18] All time-points were eligible for meta-analysis. Wound healing was consistently delayed by ischemia at all time-points (day 7 SMD=5.36 (95% CI 3.67 to 7.05), day 14 SMD=4.50 (95% CI 2.90 to 6.10) and day 21 SMD=2.53 (95% CI 1.25 to 3.80), figure 1).

Comparisons of wound healing between diabetic and non-diabetic groups in the presence of ischemia were eligible for meta-analysis at time-points of day 7 and 14. This analysis suggested that diabetes did not significantly delay wound healing (online supplementary figures 2 and 3). The number of studies reporting data at day 21 was less than three and therefore data from this time-point were not eligible for meta-analysis.

Effect of ischemia on complete wound closure

Three studies reported the number of days taken for complete closure of the wounds.[14 17 18] Alizadeh *et al*[14] reported that the wound was completely closed by day 17 in all animals within the non-ischemic group as compared with day 27 in the ischemic group. Tobalem *et al*[17] reported that, under normoglycemic condition, full wound closure took a mean of 19.3±2.11 days in ischemic as compared with 15.1±1.05 days in non-ischemic wounds ($p<0.05$). On the other hand, under hyperglycemic condition, ischemic ulcers took a mean of 36±8.73 days to heal as compared with 17.2±1.32 days for non-ischemic wounds ($p<0.05$) respectively. Andre-Levinge *et al*[18] reported that non-ischemic wounds closed in a mean of 27.3±3.1 days as compared with 33.4±7.6 days for ischemic wounds ($p=0.002$) under normoglycemic condition. The meta-analysis showed that ischemia significantly delayed the time to full wound closure (SMD=1.97 (95% CI 1.59 to 2.34)) (online supplementary figure 4).

Effect of interventions on ischemic wounds

Five of the included studies investigated the effect of different interventions on wound healing and reported significant improvement in healing under both diabetic and non-diabetic conditions (table 3). These interventions included hyperbaric oxygen therapy,[18] angiogenic self-assembling nanofiber hydrogel scaffold seeded with *Akkermansia muciniphila*,[19] bone marrow aspirate and peripheral blood derived platelet-rich plasma,[16] embryonic artery CD133+ cells loaded with the Sirt1 agonist SRT1720[20] and knockout of Redox Enzyme p66Shc.[15] The interventions were reported to improve wound healing via multiple different mechanisms including increased collagen content, granulation tissue thickness, myofibroblast differentiation and capillary density in addition to increased expression levels of vascular endothelial growth factor (VEGF-A), interleukin-8 and basic fibroblast growth factor. Furthermore, these

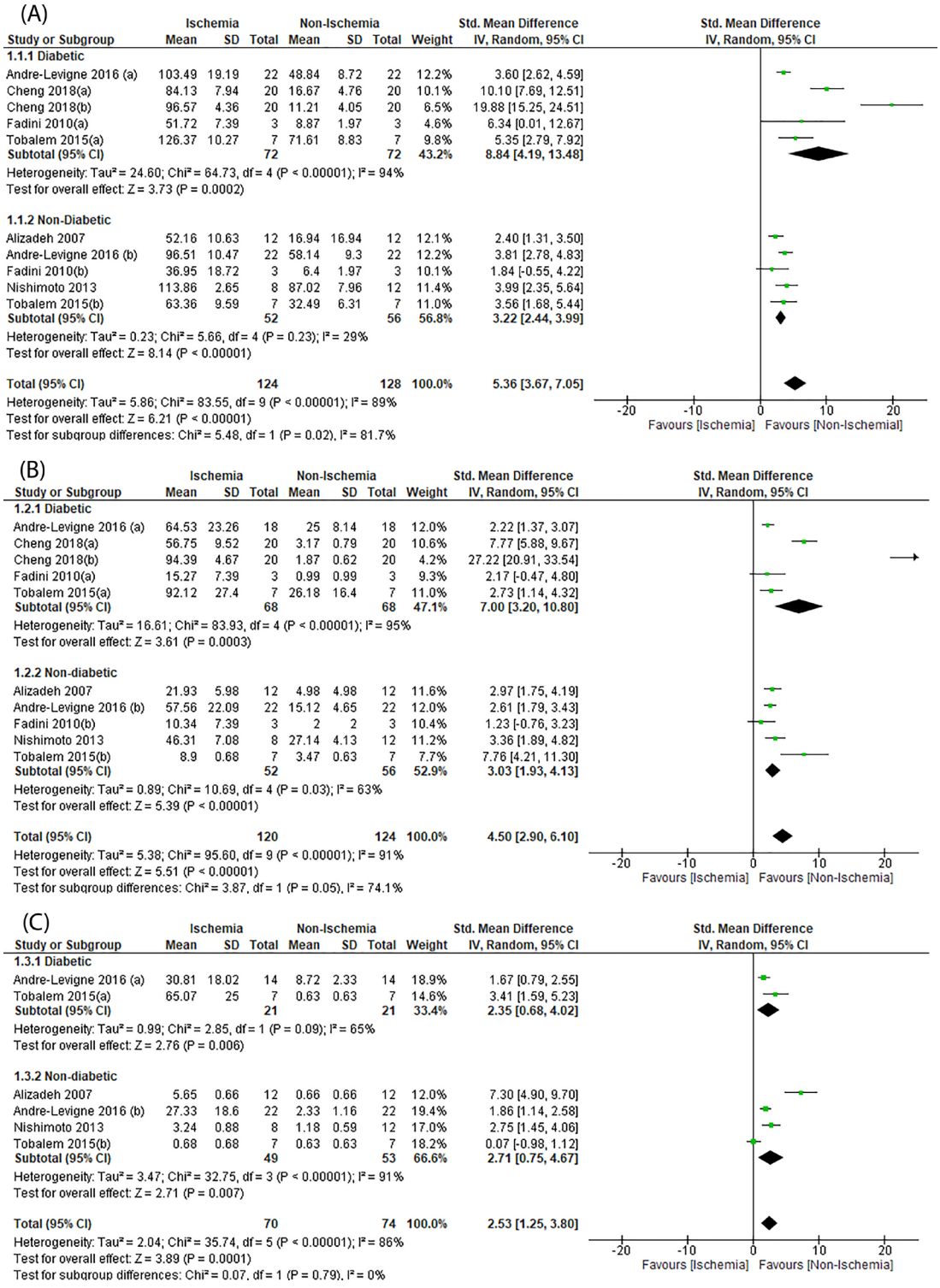

Figure 1 Wound healing at day 7 (A), 14 (B) and 21 (C) following ischemic wound induction. Studies were grouped in relation to whether diabetes was induced are not. A random effects model was used for analysis. Heterogeneity was assessed using the I^2 statistic. Pooled estimates of standard mean difference and 95% CIs were calculated using Review Manager 5.3.5. IV, inverse variance; I^2, heterogeneity.

interventions were reported to reduce apoptosis and leukocyte infiltration in addition to reducing expression levels of tumor necrosis factor (TNF) and β-catenin expression (table 3). Due to variations in interventions studied, meta-analyses were not possible. The risk of bias and reporting quality was considered high for three of these studies[16 19 20] and moderate[15 18] for two studies. The mean risk of bias and reporting quality score was 76.0%±13.0%. The clinical relevance of four of the studies was considered low[15 16 19 20] and moderate for one study.[18] The mean clinical relevance score was 40.0%±16.8%.

Table 3 Factors involved in wound healing as reported in the included studies

Reference	Species	Reported factors that delayed wound healing	Treatment	Reported factors that improved wound healing
14	Rat	↓ Myofibroblast quantification ↓ Collagen type1 mRNA ↓ Wound contraction ↓ Neovascularization	None	NA
15	Mice	↓ Collagen content ↓ Granulation tissue thickness ↑ Apoptosis ↑ c-myc and β-catenin expression ↓ Capillary density	P66Shc Knockout	↑ Collagen content ↑ Granulation tissue thickness ↓ Apoptosis ↓ c-myc and β-catenin expression ↑ Capillary density
16	Rabbit	↓ Myofibroblast differentiation	Bone marrow aspirate & Platelet rich plasma	↑ Myofibroblast differentiation
17	Rats	↓ Myofibroblast differentiation ↓ Wound contraction	None	NA
18	Rats	↓ Wound contraction and re-epithelialization ↓ Collagen deposition	Hyperbaric oxygen therapy	↑ Blood flow and wound closure ↑ Wound contraction and re-epithelialization ↑ Collagen deposition
19	Mice	↓ Capillary density ↑ Leukocyte infiltration	*Akkermansia muciniphila*	↑ Capillary density ↓ Leukocyte infiltration
20	Mice	↑TNF-α ↓VEGF-A ↓Interleukin-8 ↓ b-FGF ↓ Cell migration ↓ Cell invasion	Sirt1 agonist SRT1720	↓ TNF-α ↑ VEGF-A ↑ Interleukin-8 ↑ b-FGF ↑ Cell migration ↑ Cell invasion

b-FGF, basic fibroblast growth factor; NA, not applicable; TNF, tumor necrosis factor; VEGF-A, vascular endothelial growth factor.

Heterogeneity, publication bias and consistency of meta-analyses

All meta-analyses had very high degrees of heterogeneity between studies (figure 1). Funnel plots suggested a high risk of publication bias (online supplementary figures 5–8). In the leave one out sensitivity analyses, removal of individual studies did not affect the significance of findings (online supplementary table 2).

DISCUSSION

This systematic review and meta-analysis suggests that hind limb ischemia delays wound healing in mice, rats and rabbits and can thus potentially model human ischemic ulcers. Induction of diabetes by STZ injection was included in some studies but did not significantly delay ischemic wound healing based on the meta-analysis. This finding likely reflects the small number of studies currently available. Findings were consistent in sensitivity analyses but there was noted to be a large amount of statistical heterogeneity and a high risk of publication bias.

The animal models reported did lack some features of human disease, such as the presence of older age and the generation of wounds in the periphery of the limb. The latter was only included in three studies, which created ulcers in the paws of rats.[14 17 18] All the included studies used younger animals but patients presenting with ischemic ulcers are usually aged over 60 years.[21] Given that angiogenesis and wound healing are impaired in older animals and humans, future studies would benefit from using older animals.[22 23] Other recommendations for the ideal ischemic ulcer model include induction of stable limb ischemia, which typically requires gradual artery occlusion,[24] and consideration of inclusion of typical patient comorbidities, such as diabetes (online supplementary table 3). This is supported by a recent systematic review that suggested the need for refinement of currently used mouse models of diabetes-associated ulcers in order to make them more clinically relevant.[25]

Five different interventions were tested in the included studies and reported to successfully improve healing rate through mechanisms such as promoting angiogenesis, collagen deposition, re-epithelialization and increased expression levels of proliferative markers such as VEGF and increased anti-inflammatory cytokines such as interleukin-8 in addition to reduction of leukocyte infiltration and reduced expression levels of inflammatory cytokines such as TNF[15 16 18–20] (table 3). The risk of bias and reporting quality of three of these studies was considered high but none of the models used were considered to be highly clinically relevant. Only one of the studies reported randomization of treatment groups and blinding of the outcome assessor.[16] Many of the studies failed to assess blood flow[16 19 20] did not create wounds at a peripheral site and failed to report complete wound closure.[15 16 19 20] Due to these weaknesses and the lack of repeat testing of common interventions, it remains unclear how likely the findings of these studies are to be translatable to patients.

A number of limitations of the research included in this systematic review should be acknowledged. Rodent skin anatomy differs from that of humans. Rodents have thin epidermis, loose skin adherence, dense hair which is thought to accelerate healing.[26] Furthermore, the age of animals used in the included studies were equivalent to teenage humans which is not representative of the population that present with ischemic ulcers.[27] The included studies also lack a number of important methods to reduce bias, such as blinding of outcome assessors. Improving the methods of preclinical ischemic ulcer research may lead to better translation of findings to patients.

In conclusion, previously reported models of ischemic ulceration have impaired wound healing representative of patients. Improvements in study design and models are possible to increase the likelihood of achieving findings which can be translated to patients.

Contributors ST was involved in the study conceptualization, keyword search, full text screening, data extraction, data analysis, and manuscript preparation and editing. JP contributed to the full text screening, data extraction, manuscript editing and critical assessment of the manuscript. JG was involved in the study conceptualization, data validation, data analysis, supervision, manuscript preparation, editing, critical assessment of the manuscript and funding acquisition.

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