An in vivo Investigation on the Wound-Healing Effect of Two Medicinal Herbs Using an Animal Model with Foot Ulcer

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Abstract

Background/Aim: Over 190 million people in the world suffer from diabetes mellitus. Diabetics are 25 times more likely to have a leg amputated because of unhealing foot ulcers. Herbal medicine has been used in China to salvage the ulcerated limb. With the aim to study the efficacy of two commonly used herbs for ulcer healing, namely Radix Astragali and Radix Rehmanniae, a good animal model needs to be developed for a proper in vivo investigation. ***Methods:*** Firstly, a diabetic animal model was established by streptozotocin injection. Then standard wounds were created on the feet of the diabetic rats. Digital photographs were taken and analyzed by a novel image analysis software. ***Results:*** The average ulcer area in the Radix Rehmanniae treatment group was 11.45 mm^2, which was significantly smaller than the 15.12 mm^2 in the water treatment group (p = 0.04). Radix Astragali, on the other hand, was found to have no significant effect on ulcer shrinkage. ***Conclusion:*** Further investigation is needed for the identification of the active principles of Radix Rehmanniae.

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Introduction

Diabetes mellitus is an endocrine disorder which was first described in the ancient book of Chinese medicine 'Huangdi Neijing' in China around 500 BC and was called 'thirsty disease'. Diabetes mellitus is now defined as a metabolic disorder in which the body's capacity to utilize glucose, fat and protein is disturbed due to insulin deficiency, insulin resistance or both [1]. According to the information of the International Diabetes Federation, there are currently at least 194 million diabetics in the world and the World Health Organization estimates that there will be 300 million people with diabetes mellitus by 2050 [2].

People with diabetes are 25 times more likely to have a leg amputated than those without the condition, according to the International Diabetes Federation [3]. The combination of peripheral neuropathy, peripheral arterial disease and infections would result in unhealing ulcers, gangrene and amputation [4]. Amputation leads to significant morbidity and mortality [5, 6].

Literature search revealed that no animal model of foot ulcer had been established for the study of ulcer healing in diabetes mellitus. For the purpose of animal model establishment, various parameters have to be considered. Firstly, the diabetic condition is certainly a prereq-

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uisite for the animal model; therefore, a reliable and well-validated diabetic model is required. It is also sensible to induce the wound on the foot rather than on other parts of the body in order to mimic the diabetic foot ulcer condition in humans. Regarding the animal species used, rats were chosen in our study rather than mice or guinea pigs since the size of the rat foot is appropriate for wound induction and subsequent measurements. Secondly, wound area measurements would be a critical factor in the establishment of the animal model as it involves many factors to be considered and adjusted.

Wound area assessment is a very important parameter for predicting and estimating the time of healing [7, 8]. The wound area has long been studied by measuring the length and width, having the shape of a rectangle or an ellipse [9]. Currently, the usual way is the acetate method that makes use of grid paper for area estimation by counting the number of squares inside the traced wound parameter [10, 11]. As a result, the wound margin determination is difficult and subject to rater judgment that leads to individual variation. Moreover, a vast number of manual wound measurements would be a great burden to the health care practitioners. Some modern methods use digital planimetry, in which the area is calculated by computer software once the wound margin is traced [12]. However, the photograph taking and tracing methods have never been standardized and optimized considering many factors, such as light intensity, white balance, scale calibration and photograph pixel density. As a result, it is necessary to explore and develop an accurate and efficient methodology for wound area determination. During the development of our animal model, the importance of wound area determination was not neglected. Hence we designed a specific image analysis computer program (IACP) to demonstrate a systematic and automatic determination of wound area. By connecting every aspect of investigation, we, therefore, ventured to develop a diabetic rat model with standard wound induction and area determination.

Many herbs have long been used to treat diabetes mellitus and its complications. In many of these successful attempts Radix Rehmanniae (RR) and Radix Astragali (RA) have frequently been used in combination with other herbs to form complex formulae such as 'blood-house blood stasis-dispelling decoction with additive ingredients' and 'pills of six drugs with Rehmannia' [13]. Drinks consisting of these herbs as major components also rescued 87% of the legs condemned to amputation due to nonhealing diabetic ulcers [14].

Apart from the clinical efficacy observed, the herbs RA and RR were previously found to be biologically active in related aspects of diabetes and its complications. In fact, RA has been used in inflammation control and prevention of insulin resistance [15, 16], and RR has been shown to elicit antihyperglycemic and anti-inflammatory effects [17–21]. However, the direct effects of RA and RR on diabetic foot ulcer have not been studied. Hence our objective of this study was the establishment of a systematic and reliable in vivo ulcer assessment model. In order to demonstrate the practicability of this animal model, we investigated the efficacy of two traditional Chinese medicines, RA and RR, for their potential ulcer-healing effects.

Materials and Methods

Animal Model

Various animal models of diabetes have been established, either chemically or genetically induced. The streptozotocin (STZ)-induced diabetic rat model was chosen in our study, since STZ has been widely used for induction of diabetes mellitus in animal experiments [22–24]. The model in our study used neonatal STZ-diabetic rats [25–28]. This condition could mimic severe diabetes in patients with a high incidence of developing or having foot ulcers. Neonatal non-insulin-dependent diabetes mellitus was induced by intraperitoneal administration of STZ (70 mg/kg) to Wistar neonatal rats within 5 days of age [28]. These rats, at 8 weeks of age, develop hyperglycemia, hypoinsulinemia and glucose intolerance, and this condition persists for 12 weeks. The procedures of diabetes induction were done under the animal license No. 208 (DHNTE 007/5 Pt. 24).

The adult diabetic rats were then used for the development of the ulcer model. The plasma glucose level was determined using a kit from BioSystems (Barcelona, Spain). Only those rats with severe diabetes (plasma glucose >300 mg/dl) were selected for wound induction. One day after glucose determination, wound induction was carried out. The day of wound induction was defined as day 0. The rats were anesthetized with an intraperitoneal injection of 75 mg/kg ketamine and 10 mg/kg xylazine. A rectangle was marked on the dorsal surface of the foot using a signet, and then a layer of skin in full thickness (standard area 2×5 mm) was removed. The procedures of ulcer induction (fig. 1) were performed under the animal license No. 14 (DHNTE 007/5 Pt. 26). One day after ulcer induction (day 1; initial wound size measurement), the wound became slightly larger due to the centrifugal pulling of the skin at the periphery [29, 30].

Apart from the diabetic foot ulcer model, normal rats were also used to test the herbal effects on normal wound healing using the same wound induction method.

Ulcer Area Measurement and Validation

Planimetric measurements were performed on digital photographs taken from each rat's foot, and the pictures were analyzed using the IACP. True-color images (24 bits) of the rat's foot were captured using a digital camera (D100; Nikon, Tokyo, Japan) positioned 20 cm above a graphics table, onto which vertical and hori-
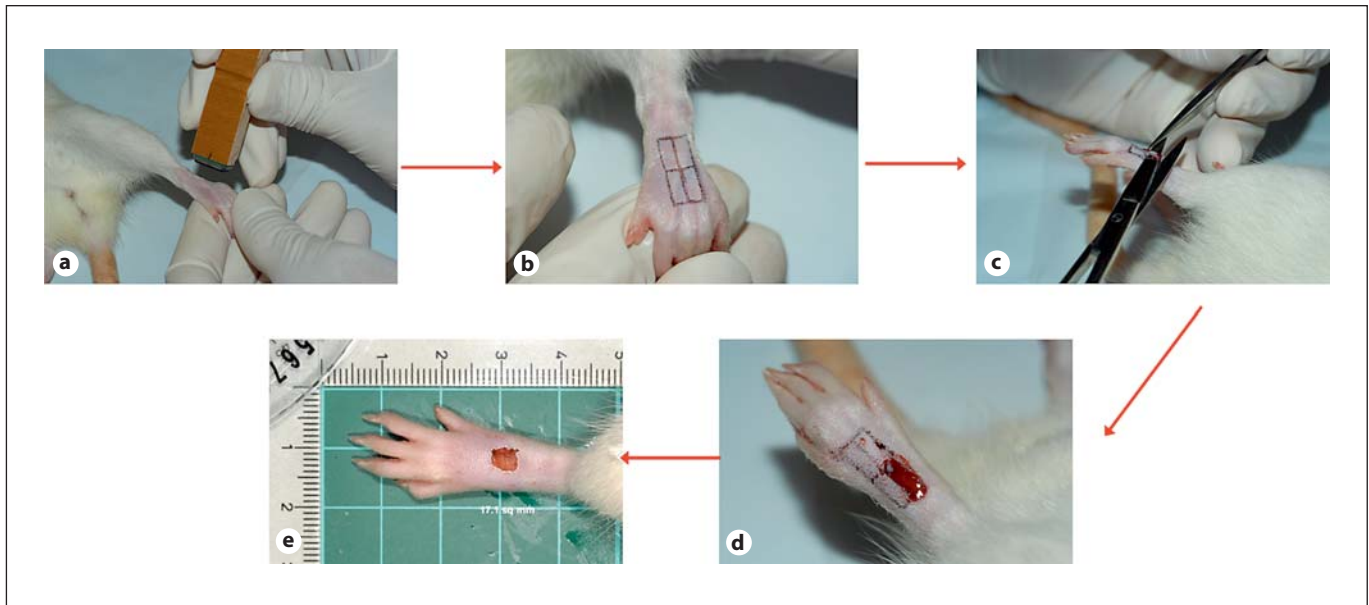
Fig. 1. Procedures of wound induction. **a** Signet marking. **b** Clear boundary for wound induction. **c** Removal of full-thickness skin. **d** Fresh wound area of 2 × 5 mm. **e** Slighty larger wound area after 1 day.

zontal rulers had been fixed. Each digital image was taken with a horizontal and vertical resolution of 1,504 and 1,000 pixels, respectively. All images were captured at a shutter speed of 1/180 s using a ring flash in order to provide a uniform and constant light source. A white-colored 1-cm² reference card was positioned in the same location to allow for checking of the uniformity of the light between digital images. Prior to picture taking, the leg of the rat was gently pinched manually, so that the ulcer site was positioned at the intersection of the vertical and horizontal grid lines on the graphics table. All digital images were captured by same investigator.

The digital images were deconstructed into their primary components using a specifically written Pascal language image analysis computer software (Borland Delphi version 6.0; Borland, Austin, Tex., USA). The IACP employed conventional image analysis techniques such as color thresholding, edge detection and color filtering. The program also divided each image into the relevant regions of interest, namely the rulers, rat foot and wound (fig. 2). The two regions containing the horizontal and vertical rulers were firstly segmented and then further analyzed to identify the horizontal and vertical gradation marks on the rulers to allow determination of the horizontal and vertical resolutions of the images in pixels per millimeter. The foot was segmented from the background of graphic table by applying a color filter using the prior knowledge that the table used was green in color. The foot was then further segmented into three regions, 'raw wound', 'new epithelization' and 'undamaged intact skin', using the color gradient differences between adjacent pixels. This was done based on the assumption that pixels in the wound and undamaged intact skin regions would have similar color densities as other pixels within that specific region. The new epithelization region was then further processed to determine the precise boundary of the wound. This was done using color differentiation based on wheth-

er the pixels were red (wound) or skin color. The thresholds for determining a pixel color were derived from the analysis of an initial series of images taken for the development of a working algorithm being used in the IACP. The area of the ulcer (mm²) was determined by counting the number of pixels contained within the region bound by the wound edge (fig. 2).

In order to check the reliability of the IACP in assessing the wound surface area, a comparison of manual measurements versus computer measurements was done to find out whether the measurements obtained were statistically similar. A total of 123 wounds on days 1 and 8 were used for comparisons. A rater entered all the data of the photographs of the rats' feet into a computer using the 'Spot' software (Diagnostic Instruments Inc., Sterling Heights, Mich., USA) and then manually traced the border of each wound. Then the 'Spot' program provided a calculated wound surface area according to the border (fig. 3). In the meantime, the data of all these 123 wounds were also entered into a computer running the IACP for automatic recognition and determination of the areas. Using statistics by intraclass correlation coefficient, the associations between the two wound measurement systems was analyzed.

Herbal Experiments

RR and RA were purchased from a quality herb shop in Hong Kong. They were authenticated by organoleptic methods and thin-layer chromatography using criteria recommended by the Chinese Pharmacopoeia Commission [31]. Voucher specimens were deposited in the museum of the Institute of Chinese Medicine, Chinese University of Hong Kong (Nos. 2003-2452 and 2003-2457, respectively).

For extract preparation, 500 g of each herb was boiled twice under reflux in 2.5 liters of distilled water for 2 h. The supernatant
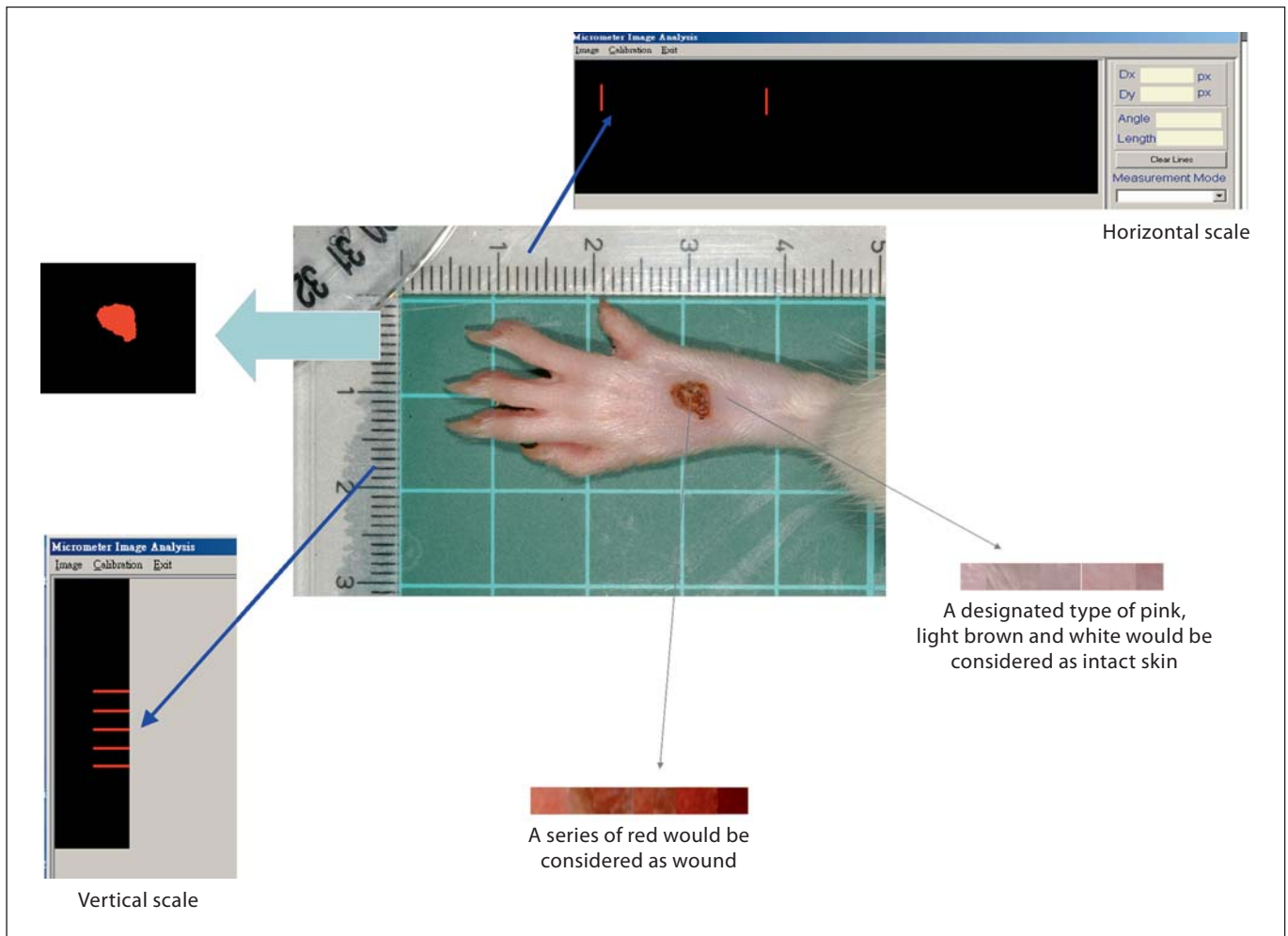
Fig. 2. Diagram showing the procedures of wound area measurement using IACP. Horizontal and vertical charting rulers on the stand copier platform were recognized and interpreted by the IACP as horizontal scale and vertical scale. In the aspect of wound color differentiation, some template photographs with a series of red color were employed to enable the program to recognize a wound and calculate the area automatically.

Fig. 3. Area measurement by a manual tracing method. A white plate was used for adjusting white balance to ensure the consistency of background color.

was filtered through cheese cloth and then subjected to freeze-drying (Thermo Savant Modulyo freeze dryer; E-C Apparatus, Holbrook, N.Y., USA).

Totally 164 rats were used in this study. The rats were randomized into either the control group (water treatment, 5 ml/kg) or the herbal group (3.7 g/kg, 5 ml/kg). The herbal group was using either RA or RR. Hence only one of the herbal extracts was tested in each set of experiments. The effective herb was selected for testing again in the wound healing of normal rats. Repeated studies were done to confirm the results.
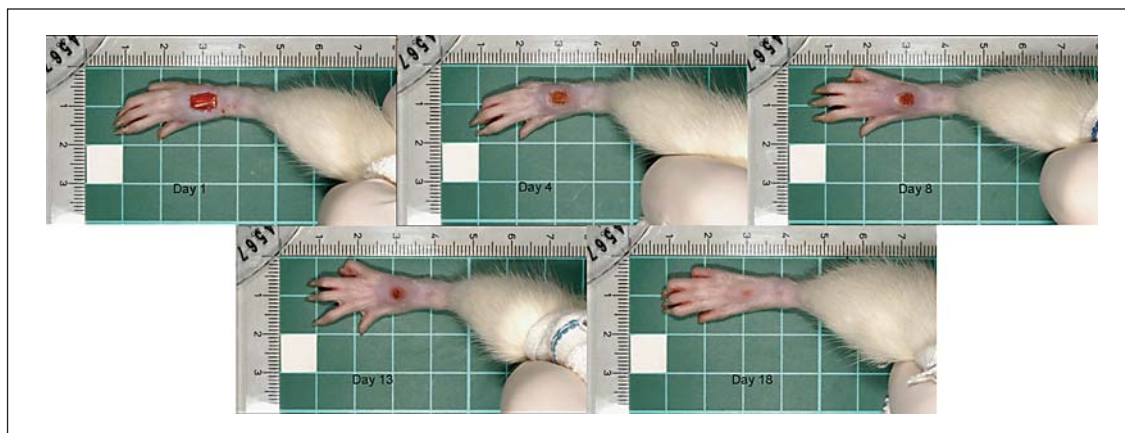
Fig. 4. Healing progress of the wound (days 1, 4, 8, 13 and 18) in the diabetic foot ulcer animal model.

In the preparatory stage of the model establishment and trial study, the wounds were photographed on days 1, 4, 8, 13 and 18 (fig. 4). We found that day 8 was the best cutoff time point for wound assessment in this animal model, as the greatest difference in wound area between treatment groups and control groups seemed to occur at around day 8 (fig. 5). We, therefore, adopted day 8 as the time point for wound comparison. More importantly, day 8 was usually adopted as the suitable time point for assessment of wound healing in other research studies [32–34]. The ulcer area determined on day 1 was considered to be the initial ulcer size and that on day 8 was defined as final ulcer size for comparison of treatment effects. The wound peripheral parts in the water treatment groups were usually too red and indicated an active inflammation below the epidermis, hence these areas were considered to be a part of a wound. During the treatment period, herbal extracts or water were fed to the rats using a syringe with a fine tubule from day 1 to day 7.

Statistical Analyses
Regarding the comparisons of manually traced wound measurements and IACP analyses, intraclass correlation coefficients were adopted for estimating the consistency between the two methods. For the effects of herbal treatment, the data are presented as mean ± SD for continuous variables and as frequencies (percentages) for categorical variables. Baseline characteristics between the herbal treatment groups and water treatment groups were assessed using t test or χ^2 test. A linear mixed-effects regression model was used to assess whether a herbal treatment was associated with ulcer area reduction after adjusting for probable confounding factors, namely plasma glucose concentration and body weight. Such a model took into account the intracorrelation of the ulcer areas of each rat and variations among the rats. The significance of the parameter estimates in the model was assessed by the Wald test. The linear mixed-effects regression model was fitted using MLwiN version 2.02 (Centre for Multilevel Modelling, University of Bristol, Bristol, UK). All the other statistical analyses were carried out using SPSS version 11.0 (SPSS, Chicago, Ill., USA). All statistical tests were two-sided, and p < 0.05 was taken as statistically significant difference.
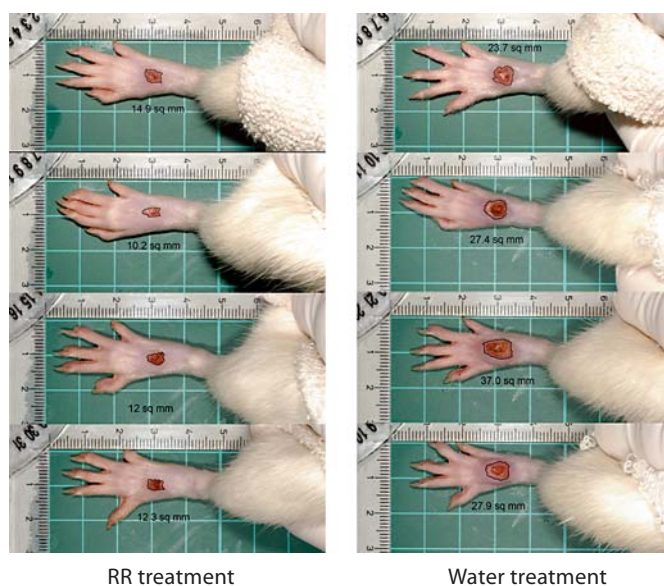

RR treatment   Water treatment

Fig. 5. Healing effects in the herbal treatment group (RR) and in the water treatment group on day 8.

Results

Animal Model Establishment
All STZ-diabetic rats had plasma glucose levels >300 mg/dl and weighed around 300 g. The baseline values of wound area, body weight and plasma glucose in the water treatment groups and herbal treatment groups matched well, as shown in tables 1 and 2.

By using a white square reference on each image capture, the IACP analysis showed that there was no color or intensity deviation among images from different trials.
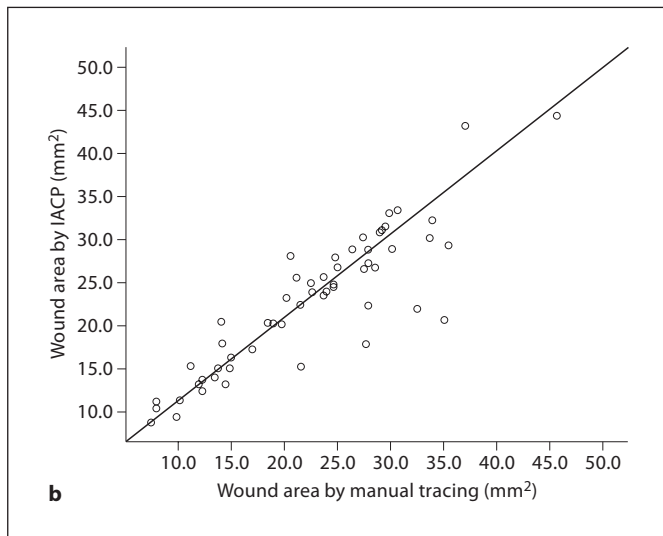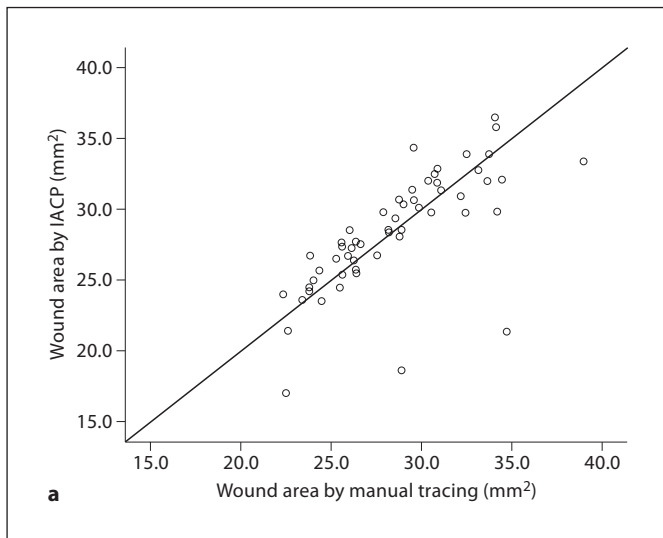
Fig. 6. Scatterplots of the wound area measured by manual tracing and IACP on day 1 (**a**) and day 8 (**b**). Most of the points are clustered along the diagonal line. As assessed using intraclass correlation coefficients, there was good agreement between the two methods of measurement.

The density of pixels on each image was found to be constant in different photographs, as the hardware setup was fixed.

The reliability of the IACP was confirmed by comparing it with the manually traced measurements. A scatterplot of the ulcer areas measured by IACP and manual methods indicated reasonably good consistency between them; most of the points were clustered around the diagonal line, as assessed using an intraclass correlation co-

Table 1. Comparisons of wound area, plasma glucose level and body weight between the water treatment group and the Radix Astragali (RA) treatment group

	Water group 5 ml/kg (n = 33)	RA group 3.7 g/kg (n = 35)	p
Wound area, mm^2			
Day 1	35.05 ± 7.85	36.11 ± 6.94	0.56[a]
Day 8	17.08 ± 6.86	18.32 ± 7.60	0.94[b]
Plasma glucose, mg/dl			
Day 0	452.7 ± 82.8	471.6 ± 121.7	0.46[a]
Day 8	393.9 ± 109.9	390.5 ± 89.9	0.93[c]
Body weight, g			
Day 0	298.9 ± 43.2	289.9 ± 45.5	0.40[a]
Day 8	308.3 ± 48.0	297.7 ± 47.0	0.96[d]

[a] p value testing the baseline difference between the water treatment group and the RA treatment group.
[b] p value that represents additional change in wound area from day 1 to day 8 for the RA treatment group relative to the water treatment group, with probable confounding factors adjusted in the model.
[c] p value that represents additional change in plasma glucose from day 0 to day 8 for the RA treatment group relative to the water treatment group.
[d] p value that represents additional change in body weight from day 0 to day 8 for the RA treatment group relative to the water treatment group.

efficient. The coefficient on day 1 was 0.84 and that on day 8 was 0.94. This indicated that the two measurement methods were in close agreement (fig. 6).

Herbal Experiments

In the herbal extractions, the percentage yield of RA was 29.4% w/w and that of RR was 52% w/w. All the freeze-dried powder could be completely dissolved in distilled water prior to testing.

Radix Astragali. The average ulcer area in the water treatment group decreased from 35.05 mm^2 on day 1 to 17.08 mm^2 on day 8 and that in the RA treatment group from 36.11 mm^2 on day 1 to 18.32 mm^2 on day 8. A linear mixed-effects regression model was used to adjust the baseline difference in wound area, time effect and other probable confounding factors. The results revealed that there was no significant difference in the rate of change of wound area between the two groups (p = 0.94; table 1).

Radix Rehmanniae. The average ulcer area in the water treatment group decreased from 28.48 mm^2 on day 1 to 15.12 mm^2 on day 8 and that in the RR treatment

Table 2. Comparisons of wound area, plasma glucose level and body weight between the water treatment group and the Radix Rehmanniae (RR) treatment group in diabetic rats

	Water group 5 ml/kg (n = 27)	RR group 3.7 g/kg (n = 28)	p
Wound area, mm^2			
Day 1	28.48 ± 4.19	28.51 ± 3.43	0.98[a]
Day 8	15.12 ± 7.26	11.45 ± 4.46	0.04[b]
Plasma glucose, mg/dl			
Day 0	465.1 ± 116.5	424.4 ± 144.8	0.26[a]
Day 8	385.6 ± 83.6	367.5 ± 146.3	0.97[c]
Body weight, g			
Day 0	318.0 ± 61.6	335.0 ± 75.6	0.37[a]
Day 8	322.9 ± 49.4	321.7 ± 59.1	0.77[d]

[a] p value testing the baseline difference between the water treatment group and the RR treatment group.

[b] p value that represents additional change in wound area from day 1 to day 8 for the RR treatment group relative to the water treatment group, with probable confounding factors adjusted in the model.

[c] p value that represents additional change in plasma glucose from day 0 to day 8 for the RR treatment group relative to the water treatment group.

[d] p value that represents additional change in body weight from day 0 to day 8 for the RR treatment group relative to the water treatment group.

Table 3. Comparisons of wound area, plasma glucose level and body weight between the water treatment group and the Radix Rehmanniae (RR) treatment group in normal rats

	Water group 5 ml/kg (n = 20)	RR group 3.7 g/kg (n = 21)	p
Wound area, mm^2			
Day 1	18.7 ± 2.8	19.4 ± 1.7	0.291[a]
Day 8	8.6 ± 4.5	11.9 ± 4.7	0.115[b]
Plasma glucose, mg/dl			
Day 0	143.0 ± 11.9	129.2 ± 14.4	0.002[a]
Day 8	165.9 ± 17.2	160.5 ± 16.9	0.692[c]
Body weight, g			
Day 0	275.3 ± 29.4	244.3 ± 23.8	0.001[a]
Day 8	282.8 ± 33.7	254.8 ± 22.3	0.098[d]

[a] p value testing the baseline difference between the water treatment group and the RR treatment group.

[b] p value that represents additional change in wound area from day 1 to day 8 for the RR treatment group relative to the water treatment group, with plasma glucose and body weight adjusted in the model.

[c] p value that represents additional change in plasma glucose from day 0 to day 8 for the RR treatment group relative to the water treatment group.

[d] p value that represents additional change in body weight from day 0 to day 8 for the RR treatment group relative to the water treatment group.

group from 28.51 mm^2 on day 1 to 11.45 mm^2 on day 8. A linear mixed-effects regression model was used to adjust the baseline differences in area, time effect and other probable confounding factors. The results show that there was a significant difference in the rate of change of wound area between the two groups (p = 0.04). Hence, the RR treatment group on average had a faster reduction in ulcer area than the water treatment group (table 2).

In the wound-healing experiments of normal rats, RR was selected for further testing. Although there was a discrepancy between the two groups in baseline plasma glucose levels and body weight on day 0, our statistical program calculated and adjusted the differences which resulted in a reliable comparison of wound areas between the two groups. The results showed that there was no significant effect with p = 0.115 (table 3). Hence the effect of RR was found to be specific to diabetic ulcer healing in our animal model.

Discussion

In in vivo studies of ulcer healing, surgical ulcers were usually induced on the chest or back of a rat or a mouse [35–39]. A model of *Staphylococcus aureus* infection in the hindpaw of nonobese diabetic mice was used for the analysis of recalcitrant diabetic foot infections [40]. Ulcers induced on feet for the study of wound healing and its measurement method have never been reported. With the aim of mimicking more closely the clinical condition of diabetic foot ulcers, the ulcers were created on the feet of STZ-diabetic rats. The animal model was probably suitable for in vivo studies of ulcers in response to different medications. However, from the clinical viewpoint, there are still shortcomings in our animal model. First, an ulcer is defined as a depressed skin lesion in which at least the upper dermis is destroyed [41]. Actually the pathogenesis of diabetic foot ulceration involves neuropathy, vascular deficiency and infection [42]. We created our diabetic rat foot ulcer model by the removal of full-thickness skin. This artificially created ulcer is different from the ulcer in the human diabetic foot which usually

results from local pressure. Human diabetic foot ulcers usually extend beyond the surface boundaries and this subcutaneous extension does not occur in our animal model.

Wound measurement has long been used to assess the progress of wound healing [7, 8]. Various methods have been developed for wound area determination. They include the use of acetate tracing and point counting [11, 43]. Some new methods use specific software to determine a manually traced wound area from a photograph or an acetate sheet [44, 45]. A more advanced method has been developed using a laser scanner. With the surface's three-dimensional coordinates, curvature maps of the ulcerous area are calculated [46]. However, the common ways are acetate tracing and digital planimetry. The critical step of these methods is the wound-tracing procedure, it is, however, time-consuming and not consistent among different raters. Hence automatic wound recognition and determination would be a new direction for the development of wound measurement methods. In our animal model, a novel computer program, namely IACP, was used.

Manual tracing methods have long been used to compare and validate various analytical methods [47, 48]. In our study, a good consistency was found between the manual wound-tracing method and the IACP. The results showed that the IACP is reliable in wound area measurement. Hence in our animal model, the IACP can provide an effective tool for wound area analysis.

We used a visual assessment methodology IACP for refined ulcer measurements, including scale calibration, ulcer size detection, color differentiation and area calculation. A special setup for photographic capturing to achieve high-quality digital photographs and color recognition on the surface of healing wounds remained difficult. The different colors of red and brown were critical and needed to be well defined. After about 3 days of healing, the wound edges are covered by new epithelium which might blur the accurate color recognition. New epithelialization should be considered as a healed area, but red swollen tissue was occasionally found below this new layer of epithelium and thus gave an exaggerated wound size. In our study, such an area was considered an unhealed wound if it was too red, in fact showing active inflammation below the newly formed epidermis, which should be included as part of the wound area.

With regard to the healing properties of RA and RR, our results indicated that only RR was effective in ulcer-healing promotion. Both RA and RR were found to have no influence on the plasma glucose level during the ex-

perimental period. The wound-healing effects of RR, therefore, would not be the result of diabetes control. On the other hand, RR was found to have no significant wound-healing effects in normal rats. Hence it was interesting to note that RR could be applied in diabetic ulcer healing. RR has a long history of safe and effective usage and wide applications in clinical practice, hence the study of its effective compounds is worthwhile. Trials using these two herbs could help to explain their traditional usage and give a good demonstration of the application of our animal model, especially the wound measurement method. It is hence meaningful to further develop this animal model to enhance the efficiency and accuracy of wound measurement in pharmacological studies and clinical practice.

Acknowledgments

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