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Streszczenie w języku angielskim

Title: Translation of the studies results on animal wound models into their application to the clinical practice of wound healing in patients with diabetes

Introduction

In an ageing population, the problem of treating hard-to-heal wounds is becoming increasingly clinically important. The generators of delayed tissue healing are all diseases with an underlying reduction in tissue oxygenation, both at the microvascular level and in the bed of large blood vessels. The leading disease entity complicating wound healing is diabetes. Basic research is directed towards the search for both molecules and methods to optimize the healing process in wounds associated particularly with long-term uncompensated diabetes. The essence of such therapies is to reduce the risk of tissue infection by decreasing the size of ulcerations and therefore influencing the regeneration process. A wound without infectious features also means a lower risk of complications, such as sepsis, including possible death. The persistence of infectious features is also a risk of potential irreversible loss not only of the foot, but also of the entire lower limb, as a result of necrosis. However, before new forms of therapy can be applied to humans, clinical trials based on properly developed animal models are required.

The aim of the research forming part of this dissertation was to create an experimental animal model of chronic wounds. The initially created model was then modified, obtaining conditions that reflected as closely as possible the pathophysiological changes present in the tissues of patients affected by hyperglycaemia. Once such a model had been produced, the impact of two different therapeutic models on wound healing efficacy was assessed.

Papers 1 and 2 of this dissertation are original and were designed after an in-depth analysis of the available literature on the design of animal model studies. The first paper is a description of a pilot study with the authors' proposed model of chronic wound healing in Wistar rats, in which diabetes was produced with streptozotocin. The second paper was based on the conclusions from the experiments described in the first paper. In the second paper, the conclusions of study 1 were structured by modifying the designed animal model of chronic

wounds and two separate therapeutic regimens were used to compare their effects on the wound healing process itself, thus exploiting the non-hypoglycaemic effects of molecules routinely used in diabetes therapy per se. The third paper summarizes the collected experience. The observations in the paper provide a signpost for researchers who will evaluate the efficacy of new molecules based on an animal model of wound healing with associated diabetes.

Materials and Methods

The studies described in papers 1 and 2 were carried out after obtaining approval from the 2nd Local Ethical Committee for Animal Experiments in Warsaw. The studies were conducted in collaboration with the Department of Experimental and Clinical Pharmacology, WUM. Both studies used Wistar rats, male, with out-bred characteristics, weighing approximately 300 g (+/-30g).

Study 1 was a pilot study. Its aim was to obtain moderately severe diabetes with concomitant established neuropathy to mimic analogous conditions in subjects with long-term, uncompensated diabetes. The study protocol was to ultimately prepare such a model, which could in future serve as a model for various studies evaluating the efficacy of different molecules with potential use in the treatment of difficult-to-heal wounds in diabetes. This includes molecules administered systemically as well as topically to the wound bed. In order to induce diabetes, animals received streptozotocin intramuscularly (dose 38mg/kg body weight). The presence and development of neuropathy was assessed 7, 14, 21 and 28 days after streptozotocin administration, respectively, with a mean reduction in antinociception of 43.6 - 44% from baseline. For the actual part of the pilot study, 14 individuals which maintained glycaemias in the range of 250-350 mg/dl were selected. These individuals were divided into two groups. Group I received subcutaneous injections of NPH insulin at a dose of 5 IU/kg, while group II (control group) received injections of saline (0.9% NaCl). After 21 days of glycaemic stabilization in group I (glycaemias in the range 80-150 mg/dl), a superficial wound was made in both groups. A thin layer of epidermis and dermis measuring 1.5 cm x 2.5 cm was removed from the dorsum using a scalpel, then injected with a 5mg/dl solution of lipopolysaccharide from *Pseudomonas aeruginosa* (to mimic local inflammatory conditions) and finally the wound was protected with a secondary dressing. Dressings were changed every three days in order to take wound measurements and wound biopsies for histopathological evaluation.

Difficulties in, among other things, stabilizing the wound healing process in the first, pilot study (described in Chapter 4, which justifies combining the described papers into a

series) became the basis for modifying the protocol of study 2. Study 2, was conducted in a group of 200 male, inbred Wistar rats, in which diabetes was induced using the same method as in Study 1. In the initial phase, 120 rats were used to determine the appropriate dose of antidiabetic drugs to achieve glycaemia of 350-450 mg/dl. In the final phase of the study, 45 individuals with the most stable glycaemias were used. These individuals were divided into three groups: group I received human NPH insulin intraperitoneally (5 IU/kg b.w.) once daily, group II received metformin intragastrically (500 mg/kg b.w.) and group III (control) received saline intraperitoneally. After 14 days of antidiabetic treatment, a superficial wound was made in the rats. A thin layer of epidermis and dermis measuring 2x2 cm was cut on the dorsum of the rats and a 4 cm diameter silicone disc with a hole in the center was sutured to stabilize the skin and standardize the healing process. To monitor the wound healing process, both a photographic assessment of the wound size and a biopsy of the wound surface were taken every three days. The wound healing process was followed for 9 days. Biopsy samples were subjected to H+E staining and immunohistochemistry was performed to assess the expression of the proliferation marker Ki67 antigen.

Results

In study 1, a faster rate of wound healing was observed in group I (rats receiving insulin). The mean surface area at day 12 after wound formation for group I was 3808 px, while in group II (control) it was 13104 px, and the percentage ratio of final wound area to initial wound area was 8% in group I and 23% in group II, respectively. Analysis of the biopsy material showed a significantly higher accumulation of inflammatory cells and greater expression of local inflammation in group II (control). An enlargement of the wound area was initially observed in group I (after the first 3 days), which was not observed in group II. This result was most likely a consequence of the lack of wound stabilization due to the different mechanism of wound healing in rats (musculus *panniculus carnosus* contraction phenomenon) in such a way that the results could be ideally compared at any time point. The consequences of the effect of this mechanism on wound healing were avoided in paper 2 by using the silicone discs described above.

In paper 2, the mean glucose levels in the insulin or metformin treatment groups and the control group after 30 days of treatment were similar and ranged between 350 and 450 mg/dl to represent the metabolic unbalanced state of diabetes. These glycaemic ranges were maintained throughout the observation of wound healing, an important modification of the original model to better represent the state of chronic diabetic imbalance.

Mean wound area changes (± standard deviation) were -66.8% (± 9.7%) for group I (insulin), group II (metformin) -40.4% (± 15.8%) and group III (control) -48.0% (± 9.8%), respectively. Scheffé and Bonferroni post-hoc tests showed significant differences between the insulin group (group I) and the metformin group (group II), with $p < 0.002$ for both tests, and between the insulin group (group I) and the control group (group III), with $p < 0.03$ for both tests. On contrary, there were no significant differences between the metformin group (group II) and the control group (group III) ($p > 0.51$ for both tests). Therefore, the highest wound healing rate was for the insulin group (group I).

Biopsy material taken from group I (insulin-treated) rats on histopathological analysis showed significantly lower levels of inflammatory infiltration than those obtained from rats treated with metformin (group II) and the control group (group III). Immunohistochemical evaluation showed the highest density of proliferation centers (expressed with proliferation marker – Ki67 antigen) in the group of insulin-treated animals (proliferation index 27.5% vs 18%).

The results of studies 1 and 2 were used to prepare a review paper (paper 3) summarizing previous knowledge in the preparation of an animal model of chronic wounds with associated diabetes.

Conclusions

The authors were looking for a reproducible chronic wound model in diabetes, that could be used to study the effects of different pharmacological molecules on wound healing efficiency. This objective was achieved and the results of the second study conducted demonstrated the usefulness, versatility and reproducibility of the designed animal model of chronic wounds. In this study, insulin was shown to significantly stimulate cell proliferation more than metformin, as evidenced by increased expression of the Ki67 antigen. As the study was conducted under conditions of sustained hyperglycaemia, irrespective of the molecules used, the effect of wound area reduction was not dependent on changes in glycaemic values. This study demonstrates that the wound healing process in diabetes is influenced by the use of a specific antidiabetic drug. The material obtained may be the beginning of a consideration of the broad effects of antidiabetic drugs, including insulin, on wound healing mechanisms in diabetes. Establishing these mechanisms and attempting to molecularly explain the noticeable acceleration of wound healing under hyperglycaemic conditions in insulin-treated patients will be the aim of the next stage of our research.