

STUDY PROTOCOL AND STATISTICAL ANALYSIS PLAN

Document date: 20.01.2020

Title study: Cellular Proliferation, Dermal Repair, and Microbiological Effectiveness of Ultrasound-Assisted Wound Debridement (UAW) Versus Standard Wound Treatment in Complicated Diabetic Foot Ulcers (DFU): An Open-Label Randomized Controlled Trial.

An open-label randomized and controlled parallel clinical trial was performed involving 51 outpatients with complicated DFU that were admitted to specialized diabetic foot unit between November 2017 to December 2019. This study protocol received full approval from the local Ethics Committee of the Hospital Clínico San Carlos, Madrid, Spain (C.P.-C.I. 16/484-P). All patients provided written informed consent before inclusion. The present study was registered retrospectively in ClinicalTrials.gov (Registration no.: NCT04633642).

2. Methods

2.1. Trial Design

An open-label randomized and controlled parallel clinical trial was performed involving 51 outpatients with complicated DFU that were admitted to specialized diabetic foot unit between November 2017 to December 2019.

2.2. Participant

We enrolled patients in which the following inclusion criteria were implemented:

- Male and female patients over 18 years old

Type 1 or type 2 diabetes with levels of HbA1c ≤ 85.8 mmol/mol (10%) within 30 days of the beginning of the study, based on a previous international, multicenter, randomized controlled trial [11]

- Wound stages IB, IIB, ID, and IID according to the University of Texas Diabetic Wound Classification [12]
- Wound duration of 1–24 months
- Wound size among 1–30 cm² after debridement

- Diabetic foot ulcers showing mild or moderate infection, according to the criteria of the European Wound Management Association (EWMA) [13] and the Infectious Disease Society of America Guidelines [14]
- Ankle-brachial index (ABI) > 0.9 or $\text{ABI} \leq 0.9$ and ankle systolic blood pressure (ASBP) > 70 mmHg, or toe systolic blood pressure (TSBP) > 50 mmHg. In patients with medial arterial calcification ($\text{ABI} > 1.4$) we considered Peripheral Arterial Disease (PAD) a toe-brachial index (TBI) < 0.7 [15,16]

We considered exclusion criteria:

- Chronic kidney disease (glomerular filtration rate $< 60\text{mL/min per } 1.73 \text{ m}^2$ during at least three months) or dialysis [17]
- Non-treated osteomyelitis
- Necrotizing soft tissue infections
- Critical limb ischemia patients with $\text{ABI} < 0.5$ and $\text{ASBP} < 70\text{mmHg}$ or $< 50\text{mmHg}$ [15,16]

Life expectancy < 6 months due to malignant DFU

- Pregnancy and lactation
- Patients diagnosed with human immunodeficiency virus (HIV) or hepatitis
- Patients showing local or systemic conditions that could impair tissue regeneration.

2.3. DFU Assessment

A senior clinician in the management of diabetic foot (F.J.Á.-A.) always carried the baseline assessment of patients' DFU. Sensorimotor neuropathy of DFUs was diagnosed using a biothesiometer (both from Novalab Iberica, Madrid, Spain) and Semmes-Weinstein 5.07/10g monofilament. Patients who did not feel one of the two tests were diagnosed with neuropathy [18,19].

Brachial and ankle systolic pressure were evaluated using a manual 8MHz Doppler (Doppler II, Huntleigh Healthcare Ltd., Cardi4, UK). Toe systolic pressure was taken via digital plethysmography (Systoe, Atys Medical, Madrid, Spain). Wound tissue oxygen levels were measured using transcutaneous oxygen readings (Radiometer Medical, Brønshøj, Denmark).

2.4. Intervention

2.4.1. DFU Debridement and Wound Management Patients were randomly assigned to receive either surgical debridement or UAW debridement every week during a six-week treatment period. All debridement procedures were performed by the same surgeon (J.L.L.-M.), who is specialist in the field of diabetic foot surgery with more than 21 years of experience.

Surgical debridement involved removal of all necrotic and devitalized tissue that was incompatible with healing, as well as surrounding callus.

UAW debridement was performed using an UAW SONOCA 185 device (Söring GmbH, Quickborn, Germany) by a senior clinician (J.L.L.-M.) with more than three years of experience applying this type of debridement. The UAW device is equipped with three UAW instruments with different sonotrode shapes and generates an ultrasound low frequency of 25 kHz. The choice of sonotrode depends on ulcer depth (ranges from superficial to deep). The UAW device piezoelectrically transforms the electrical energy delivered from the UAW device into mechanical oscillations in the sonotrode tip. In the majority of diabetic foot ulcers in the UAW group, a two-minute treatment with 40% intensity was made by holding the sonotrode in contact mode, holding it perpendicular to the wound bed and moving it across in an up-and-down pattern. For diabetic foot ulcers measuring $> 15 \text{ cm}^2$, the debridement treatment was increased to three minutes. In addition to UAW debridement, a scalpel was used for careful tissue removal, but only in cases where periwound tissue exhibited calluses or maceration.

Between debridement sessions, sterile saline was used to clean all wounds prior to evaluation and all patients received standard of care for their diabetic foot ulcers, which consisted of moist wound dressings for wound management and proper off-loading (a removable walker cast based on the functioning and ambulatory status of the patient) as per the International Working Group of the Diabetic Foot guidelines [1,20]. When necessary, patients with moderate infections took empirical antibiotics during the treatment period, based on IDSA guideline recommendations [14], until the results from deep tissue culture were available [21]. After we received tissue culture results, we adjusted the antibiotic therapy to target the bacteria that were isolated during tissue culture.

2.4.2. Analysis of Tissue Samples

Soft tissue punch biopsies (3 mm) were taken after wound debridement sessions at week zero (day 0) and week six (day 42). After tissue collection, samples were immediately transported to the laboratory for cellular proliferation and microbiological analyses.

2.5. Outcome Measures

2.5.1. Main Outcome Measure: Cellular Proliferation Analysis of Wound Tissue Samples. The same senior clinician interpreted all samples to evaluate the cellular proliferation. The microvascular structure of CD31, an endothelial marker, was subjected to immunohistochemical analysis and quantification to understand the effects of debridement on neo-angiogenesis, [22]. Sections of tissue sample were immunohistochemically stained with the CD31 marker. Light microscopy was used to count the number of microvessels/endothelial cells in a standardized grid, with the results expressed as microvessel density (Leica DMD 800 morphometric system). Microvessel density was scored in proportion to the following scale: 0 (absent), 1 (low, at least one microvessel), 2 (moderate) and 3 (more than two microvessels).

To differentiate collagen content from other components, such as muscle fibrin and erythrocytes, in tissue samples we used Massons's trichome staining. Collagen content was scored according to the following scale: 0 (absent), 1 (mild), 2 (moderate) and 3 (severe) [23].

Actin staining was used to evaluate the presence of myofibroblasts involved in wound healing. These cells increase in number during wound healing. The number of stained cells was semi-quantitatively analyzed using a 0–3 scaling score (0 = no myofibroblasts, 1 = myofibroblasts in low quantities, 2 = myofibroblasts in moderate quantities, 3 = myofibroblasts in high quantities).

2.5.2. Secondary Outcome Measure: Microbiological Analysis of Wound Tissue Samples. Specimens of wound tissue were homogenized in 0.5 mL volumes of sterile phosphate buffered saline (PBS, Sigma Aldrich, St Louis, MO). After mechanical homogenization, the specimens were seeded in Columbia agar (BD, Sparks, MD), MacConkey agar (BD), Sabouraud dextrose agar (BD) and Columbia

agar supplemented with nalidixic acid and colistin (BD) using a spiral plater Workstation (Don Whitley Scientific, Shipley, UK). Quantitative and qualitative microbiological analyses were performed after incubation of plates at 37 °C for 24 h. Isolated microorganisms were identified by standard methods and susceptibility testing was performed in accordance with Clinical and Laboratory Standards by the disk diffusion method [24]. The results were expressed as CFU per gram of tissue (CFU/g) and the limit of detection was 10 colony-forming units (CFU).

2.5.3. Third Outcome Measure: Evaluation of Wound Conditions

Diabetic foot ulcers were evaluated at patient admission and weekly before and after each debridement treatment. A validated wound scoring system was used to assess the wound bed tissue according to quality, presence and consistency of granulation tissue [25]. Furthermore, diabetic foot ulcers were evaluated for amounts of wound exudate and periwound skin conditions such as skin maceration by the same senior clinician (F.J.Á.-A.) according to the triangle wound assessment [26].

Wound healing was supervised weekly during the treatment period (6 weeks) and the wound size was assessed using Visitrak (Smith & Nephew, Hull, UK), determining the area of the lesion with an approximation of $\pm 5 \text{ mm}^2$.

2.6. Follow-Up

Patients were followed up for 6 months after inclusion. During the follow-up period, we recorded ulcer healing. Ulcer healing was defined as complete epithelialization without any sustained drainage up to 24 weeks after the end of the study follow-up.

2.7. Sample Size

Granmov.12 program (Municipal Institute of Medical Research, Barcelona, Spain) (<https://www.imim.cat/ofertadeserveis/software-public/granmo/>) was used to calculate the sample size. Thus, we analyzed 51 patients (24 in surgical group and 27 in UAW group) with a statistical power of 0.80 and an alpha of 0.05, with a

power of the clinical difference of 37% to detect a statistically significant between groups.

2.8. Randomization

A computer-generated random number table was used to carry out the randomization of the patients into the two groups by an investigator who was unaware of the identity of the participants.

2.9. Blinding

None of the participants, care providers and outcome adjudicators were blinded to the interventions after assignment.

2.10. Statistical analysis

Data were analyzed, based upon an intention-to-treat analysis, using the software package SPSS for IOs version 21.0 (SPSS, Inc. Chicago, IL, USA). Kolmogorov–Smirnov test was used to verify the assumption of normality of all continuous variables. Chi-square test was performed to calculate differences between groups and, if applicable, Fisher’s exact test for categorical variables. Student’s t-test and Mann–Whitney U test were performed for normally and abnormally distributed quantitative variables, respectively. Graphics to evaluate the differences among decrease in bacterial load and cellular proliferation between groups were done using GraphPad® for Mac OS.

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