The efficacy of non-thermal gas plasma in the treatment of diabetic foot ulcers stalled by subclinical, biofilm-related wound infection

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New concepts and strategies for managing wound infection, biofilm and impaired wound healing are urgently needed. This study examines the effects of non-thermal gas plasma (NTGP) in the management of diabetic foot ulcers stalled by subclinical, biofilm-related wound infection. Patients either received local standard of care or standard care plus 5 minutes NTGP twice weekly). Qualitative analysis of assessed parameters (wound closure, bioburden, inflammation) showed that overall outcomes were good to very good. The data obtained strengthens the evidence of the beneficial effect of NTGP on wound healing in patients with diabetic foot ulcers.

Impaired wound healing and chronic, non-healing wounds are a burden for the affected patients and the healthcare system. There were an estimated 3.8 million patients with a wound managed by the NHS in 2017/18; an increase in the annual prevalence of wounds of 71% between 2012/13 and 2017/18 (Guest et al, 2020). Most of these are associated with ischaemia, diabetes, venous stasis disease or pressure.

Diabetic foot ulcers (DFU) are a frequent complication leading to hospitalisation of (Ulbrecht et al, 2004; Boulton et al, 2005). As the body's healing process is impaired, it leaves ulcers susceptible to infection, which may become severe and persistent. These infections can be due to a wide variety of aerobic and anaerobic microorganisms, the most prominent being Gram-positive cocci in new wounds and *Pseudomonas aeruginosa* and *Staphylococcus aureus* in older wounds (Lipsky et al, 2012; Jockenhöfer et al, 2014; Bus et al, 2020).

Topical antimicrobials have been suggested to decrease bioburden and control infection (Nasir et al, 2016; Dumville et al, 2017; Akbiyik et al, 2020). However, these therapies are often not selective to micro-organisms and some may also damage healthy tissue when used over a prolonged period of time, impeding granulation and re-epithelisation. Other antimicrobial agents may exhibit reduced penetration in wound tissue and biofilm layers, and thus be incapable of reaching deeper infections (Dumville et al, 2017; Akbiyik et al, 2020).

New concepts and strategies capable of managing wound infection and improving healing are urgently needed. One promising strategy is the application of cold plasma or non-thermal gas plasma (NTGP) with its multimodal mechanisms of action (Yousfi et al, 2011; Emmert et al, 2020).

Physical plasma may be regarded as the fourth physical state of matter, with the highest energy density after solids, liquids and gases. The plasma state is created by adding energy to a neutral gas. Physical plasmas can be divided into thermal (hot) or non-thermal (cold) plasmas.

The antimicrobial properties of NTGP were the first biomedical effects that were investigated in various groups as part of the plasma medical use of NTGP (Emmert et al, 2020). The use of microwave-induced argon successfully sterilised biofilms and removed planktonic bacteria at atmospheric pressure (Mee et al, 2009). Besides reducing the bacterial load on the wound, NTGP has been found to re-initiate the stalled healing process (Boeckmann et al, 2020; Stratamann et al, 2020). It was reported that cold plasma promoted proliferation and growth factor secretion of endothelial cells and fibroblasts *in vitro* (Kalghatgi et al, 2010; Shao et al, 2016). Positive results with

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cold argon plasma were obtained when applied to chronic wounds (Isbary et al, 2012; García-Alcantara et al, 2013; Kramer et al, 2013; Ulrich et al, 2015).

In addition to the demonstrated antimicrobial effectiveness, NTGP has a remarkable capability to access narrow and confined spaces (Assadian et al, 2019). However, the efficacy of the generated plasma depends on chemical composition and physical characteristics, which vary with a number of variables, such as pressure, gas mixture, design of the device, physical stimuli, and surrounding environmental factors.

Several types of NTGP sources are available for medical purposes, operating with either noble gases (Ar, He) or ambient air (Heinlin et al, 2013). When argon plasma has contact with the ambient air, reactive species are produced including reactive oxygen and nitrogen species, OH radicals, ions, electrons, and UV light photons, and these are delivered to the object by the gas flow. One advantage of the system is that the distance between the device and the target area can be varied to a certain degree (Heinlin et al, 2010).

The presented pilot study revisits the potential effects of NTGP generated by the SteriPlas® device (Adtec Europe Ltd, Twickenham, UK) in the management of DFUs, including wound closure progression, control of bioburden and reduction of inflammation. Therefore, this small placebo-controlled, investigator-blinded study was conducted to assess the effect of application of NTGP in addition to standard care treatment compared with placebo on healing of diabetic wounds.

Patient well-being and subjective perceptions of wound pain were also evaluated during treatment. Fifteen patients were randomised to receive either local standard of care wound treatment (SOC) or SOC plus 5 minutes of plasma treatment (NTGP) twice weekly. For placebo control, patients in the SOC group received a (blinded) sham 5 minute treatment with the SteriPlas® device (Adtec Europe Ltd, Twickenham, UK) without the plasma being turned on. DFUs were classified according to the University of Texas system (Parisi et al, 2008).

Materials and methods

Study design and patient recruitment

This study was performed as a prospective, randomised clinical trial including two wound centres (Salford Royal NHS Foundation Trust, Salford, UK; Leeds Teaching Hospitals NHS Trust, Leeds, UK). Eligible subjects who had diabetic foot ulcers (DFUs) were randomised using sequentially numbered, opaque, sealed envelopes for receiving either SOC or SOC plus 5 minutes plasma treatment.

The trial was conducted under the Declaration of Helsinki principles and received HRA approval (IRAS project ID: 198288, REC reference: 16/EM0476).

Patients were included if they met all of the following criteria:

- Aged 18+
- Had diabetes with HbA_{1c} <10% (<86mm/mol) recorded within the previous 3 months
- Foot ulcers with University of Texas grade/size A1, A2 or B1 sited below the ankle, including plantar, dorsal and heel ulcers
- Had a wound/ulcer currently, or in the last 7 days, with symptoms consistent with mild diabetic foot infection (Lipsky et al, 2012), including pus or inflammation, inflammation extending <2 cm from the wound and infection limited to skin/soft tissue
- Had an ulcer where there is <40% decrease in wound surface area in the previous 4 weeks
- Ulcers not present for >2 years
- Adequate blood supply determined by palpable foot pulses or, if not palpable, a TBPI >0.5 (or ABPI >0.8)
- Loss of protective sensation to a 10 g monofilament.

Exclusion criteria were

- Age <18 year
- HbA_{1c} >12% (>108 mmol/mol).
- Malignancies or other immunosuppressive diseases
- Receiving radiotherapy or medications that actively delay healing (e.g. steroids, NSAIDs or antimetabolites
- Pregnancy or breast feeding
- Women of child-bearing age who were not using reliable contraception
- TBPI <0.5
- Clinical evidence of gangrene at any location
- Participation in other clinical studies in the last 4 weeks.

With respect to the latter, all patients had a washout period prior to the study start to avoid effects of previous interventions.

The trial recruited 15 patients (2 women, 13 men) with recalcitrant, chronic wounds *[Table 1]*. In total, eight patients were randomised to receive NTGP treatment and seven patients received SOC. The average patient age was 62.6 years (± 10.8 years).

Treatment procedure

All patients received local SOC wound treatment according to the International Working Group on the Diabetic Foot guideline (Lipsky et al, 2016). The plasma group (NTGP) received 5 minutes plasma treatment in addition to SOC twice a week. The study timeline is given in *Figure 1*.

NTGP was generated using the SteriPlas® device

Table 1: Demographic data, wound characteristics, wound size and treatment randomisation for the study.

ID	Facility	Age	Sex	Wound location	Wound aetiology	Treatment	Wound size day 1 (cm^2)	Wound size day 29 (cm^2)
1	Salford	54	M	Left heel	Diabetic	SOC	12	11.9
2	Salford	82	F	Right foot, first toe	Diabetic	NTGP	1.7	1.3
3	Salford	59	M	Right foot, plantar	Diabetic	SOC	305.1	205.7
4	Salford	66	F	Left foot, left heel	Diabetic	NTGP	6.3	2.3
5	Salford	69	M	Left foot, first toe	Diabetic	SOC	2.7	0.8
6	Salford	69	M	Left foot, first toe	Diabetic	NTGP	2.4	2
7	Salford	55	M	Left foot, second toe	Diabetic	NTGP	39.1	25.9
8	Leeds	70	M	Sole, left, second metatarsal head	Diabetic	NTGP	3.1	0.5
9	Leeds	78	M	Sole, right, tarsal	Surgical	NTGP	7.7	2.4
10	Leeds	63	M	Sole, right, plantar surface	Diabetic	NTGP	4.2	2.9
11	Leeds	54	M	Foot, right, third digit	Surgical	SOC	2.8	2.1
12	Leeds	51	M	Foot, right, medial malleolus	Diabetic	SOC	16.9	17.4
13	Leeds	69	M	Foot, right, posterior ankle	Pressure ulcer	SOC	2.4	3.1
14	Leeds	39	M	Foot, right, first digit	Diabetic	SOC	1.1	0.8
15	Leeds	57	M	Foot, right	Diabetic	NTGP	9.8	4.2

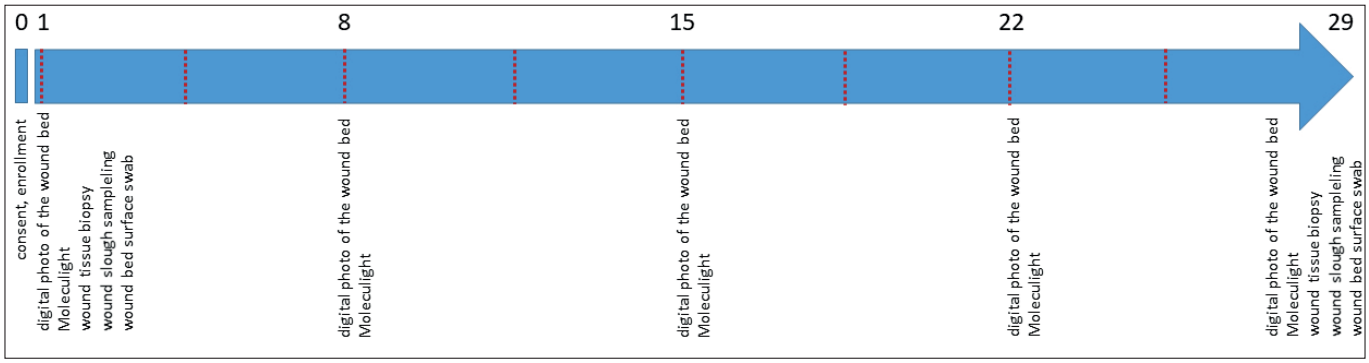

Figure 1. Schematic presentation of the study timeline.

manufactured by Adtec Europe Ltd, Twickenham, UK *[Figure 2]*. The treatment head includes ionisation chambers that use electrodes to activate argon gas and accelerate the resulting plasma through an electric grid.

Data collection and outcome measures

The primary outcome criterion was the reduction of the wound size over the trial period. Secondary outcome measures included the decrease of bioburden, inflammatory processes and subjective pain relief. Digital photographic images were taken either with the Eykona system (Eykona Medical, Oxford, UK) or the WoundWorks eKare camera (WoundWorks eKare Europe, Nieuw-Vennep, the Netherlands). These images were to record and measure the wound dimensions as well as provide the basis for the evaluation of macroscopic wound progression by the blinded investigator. The MolecuLight i:X Imaging Device (MolecuLight, Toronto, Canada) was used to record autofluorescence images of bacteria on the wounds.

Wound surface swab samples were taken to determine the microbial colonisation of the wounds and were processed at the associated microbiology labs of the included wound centres. For more extensive bacteriological procedures, debrided wound tissue was subjected to microbial DNA extraction using the QIAamp DNA Kits (Qiagen, Hilden, Germany). Afterwards, the samples were directly analysed for the presence of specific microbial species in real-time PCR (qPCR) using the microbial DNA assays (Qiagen, Hilden, Germany) for *S. aureus* (BISD00314AR), *P. aeruginosa* (BPID00288AR), *Escherichia coli* (BIPD00146AR, *Acinetobacter baumannii* (BPID00004AR) and *Proteus mirabilis* (BPID00719AR).

Figure 2. Treatment with the SteriPlas® device. Courtesy of Adtec Europe Ltd.

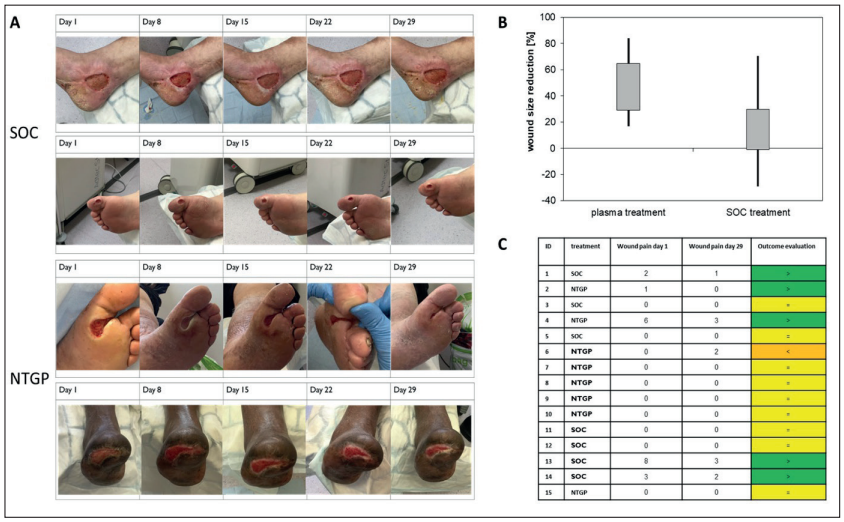

Figure 3. A. Representative images of treatment results for two patients of the SOCand one in the NTGP group. B Reduction of wound size (%). C. Effect on wound pain.

Punch biopsies taken of the wound bed on day 1 and day 29 were obtained for histology and immunohistochemically analysis. The tissue samples were fixed in 4% formalin solution, embedded in paraffin, and cut into 4 µm sections. Sections were placed onto glass slides and stained for routine haematoxylin and eosin staining. The sections were stained for CD45 using an automated system for immunohistochemistry (Ventana NexES). In addition, sections were subjected to PAS and Gram staining for the identification of microbial components and bacteria. Images were acquired using the VHX 950F digital microscope (Keyence Deutschland, Neu-Isenburg, Germany).

Statistical evaluation

Due to the small group sizes and high variance, the non-parametric Mann-Whitney U test and a χ^2 test for association between categorical variables were used for statistical evaluation of the results (SPSS software, IBM, Armonk, US).

Results

Clinical outcomes

Wound photographs were taken on day 1, day 8, day 15, day 22 and day 29. Eleven out of 15 patients showed a subjective clinical improvement of the wound with less slough being present and an improved presentation of the wound bed *[Figure 3A]*. This was confirmed by an overall decrease in wound sizes. Of these 11 patients, seven received NTGP treatment and four received SOC. In four cases, distinct signs of inflammation and infection were still present at the end of the trial and no decrease in wound size was observed. Of these four patients, one received NTGP and three received SOC. Although these results indicate a trend toward beneficial effects of NTGP over SOC, the difference was not statistically significant (χ^2 0.1847).

At the beginning of the trial, the median wound size in the NTGP group was 5.3 cm^2 and in the SOC group, the median wound size was 2.8 cm^2 *[Table 1]*. Over the course of treatment, wound size was reduced by 45.5 % in the NTGP group and 25.0 % in the SOC group *[Figure 3B]*. Differences in wound size reduction between plasma treatment and SOC did not reach statistical significance (p=0.0618).

Several patients experienced a decrease in wound pain during both treatments *[Figure 3C]*. However, changes were minimal as the visual analogue scale reported at the beginning of the trial was generally very low, which is consistent with the high amount of DFUs in the trial. Only one patient reported an increase in wound pain during the treatment with NTGP.

Inflammation and matrix formation in the wound tissue

Tissue biopsies were taken for histopathological evaluation on day 1 and at the end of the study (day 29). Mostly, granulation tissue with lymphocyte infiltrates was observed in the samples together with fibrin, elastin and collagen deposition *[Figure 4]*. In addition, microbial components were visible in the HE stained sections, most likely presenting bacterial biofilms.

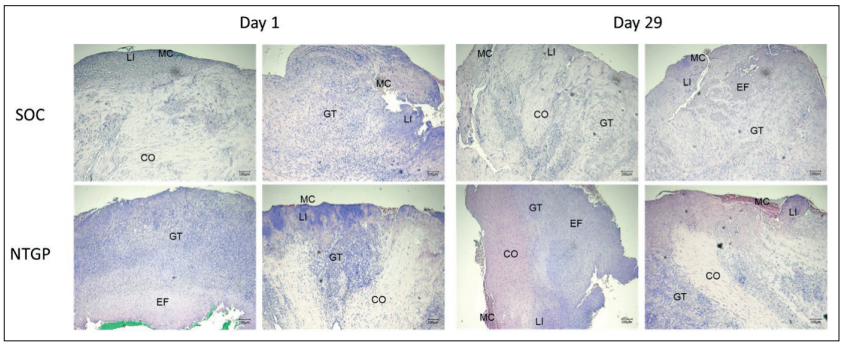

Figure 4. Representative results for biopsy sections of patients receiving SOC or NTGP after HE staining. Granulation tissue (GT) with lymphocyte infiltrates (LI) was observed in the samples together with fibrin, elastin, and collagen (CO) deposition. Also, microbial components (MC) were visible in the HE stained sections.

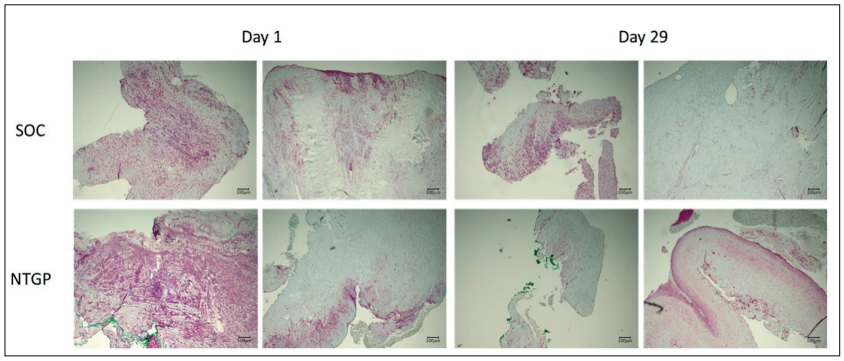

Figure 5. Immunohistochemical analysis for infiltration of immune cells was performed using anti-CD45 antibodies. Presence of lymphocyte infiltrates indicated ongoing inflammation in most of the samples, however, a more distinct reduction was observed in sections from patients receiving NTGP treatment.

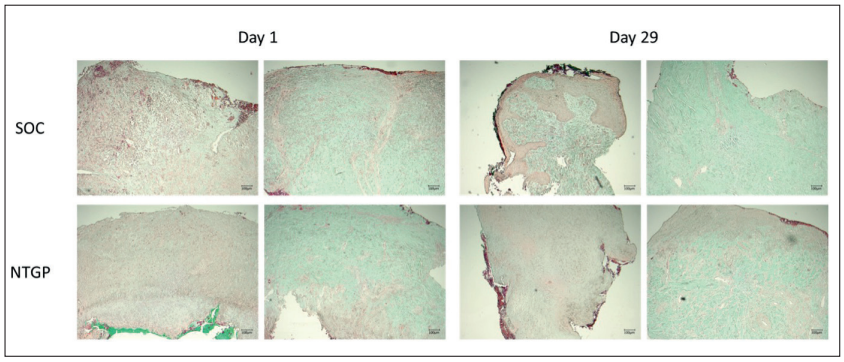

Figure 6. NTGP treatment stimulated collagen synthesis in the dermal layer, encouraging its reformation from the granulation tissue compared to SOC.

Immunohistochemically analysis for infiltration of immune cells was performed using anti-CD45 antibodies *[Figure 5]*. The presence of lymphocyte infiltrates indicated ongoing inflammation in most of the samples. For five patients, a decrease of inflammatory cells between biopsies at day 1 and day 29 was noted, of which two patients received SOC and three patients were NTGP treated. Plasma treatment further stimulated the production of collagen in the dermal layer, encouraging its reformation from the granulation tissue, in three patients at day 29 compared to day 1 while only two patients receiving SOC demonstrated an increase in collagen *[Figure 6]* . The other patients showed no change in the collagen amounts in the samples from day 1 compared to day 29. However, these patients already exhibited high collagen proportions in the samples from day 1.

Microbiological assessment

As seen in the HE stained sections, microbial components were present in most of the samples. To further elucidate the manifestation of bacteria and probably bacterial biofilms as well as the treatment effects, Gram-staining was included for the identification of bacteria in the samples *[Figure 7]*. Wounds treated with NTGP were not only generally well-healing, but also showed a distinctly decreased staining for bacteria presence at day 29 compared to day 1. Six patients in total exhibited a reduction in bacteria, of which two received SOC and four were NTGP treated. In the other cases, no change was detected from day 1 to day 29 with the exception of two patients receiving SOC, where an increase of Gram staining in sections at day 29 compared to day 1 was found. No patients treated with NTGP showed a worsening of the conditions in this respect. These results were affirmed by the measurement of bacterial autofluorescence using the MolecuLight device *[Figure 8]*.

Standard bacterial swabs were taken from the wound beds of all patients on day 1 and day 29 for identification of bacterial species present in the wound. From these results, an overview of the distribution of bacteria at the beginning and the end of the study for wounds receiving SOC and those treated with NTGP was generated *[Figure 9A]*. It was observed that NTGP treatment decreased the number of bacteria present, resulting in a proportional increase of *Staphylococcus* species in the wounds while decreasing the proportion of *Corynebacteria, Enterococci*, coliform, *Diphteroid* species and *Proteus* species. Over the 29 days of SOC, a comparable increase of *S. aureus* in the wounds was noted, but not of other staphylococci, and *Corynebacteria* and coliforms increased as well. Interestingly, all wounds randomised to SOC featured distinctly less *P aeruginosa* compared to those that later received NTGP treatment on day 1.

To further validate these results, DNA isolation from the slough samples for microbiological assessment was performed. The samples were then subjected to qPCR analysis for the five relevant wound species *S. aureus*, *P. aeruginosa*, *E. coli*, *P. mirabilis* and *A. baumannii*. *E. coli* was only identified in one of the samples, although cultural assessment reported several cases of coliforms. In contrast, *A. baumannii* was identified in four patients on day

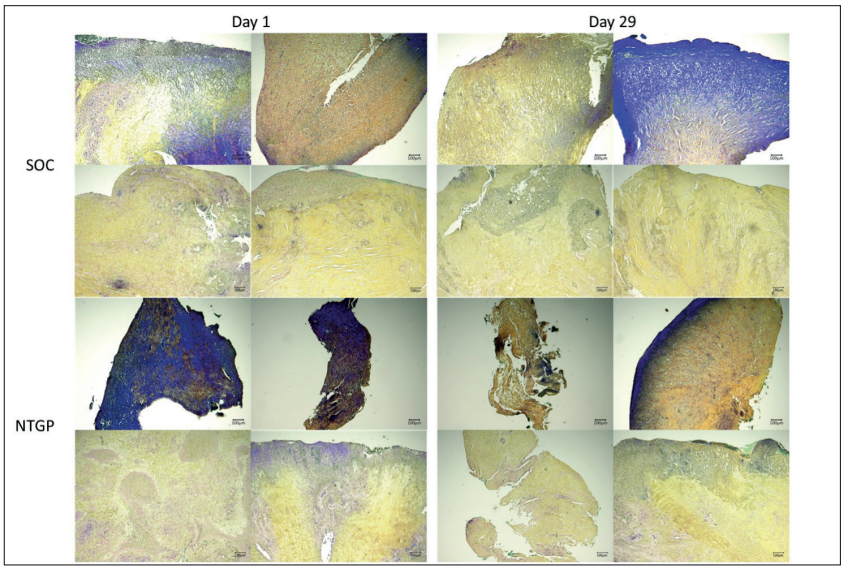

Figure 7. Gram-staining was included for identification of bacteria in the samples. Wounds treated with NTGP showed a distinctly decreased staining for bacteria presence at day 29 compared to day 1.

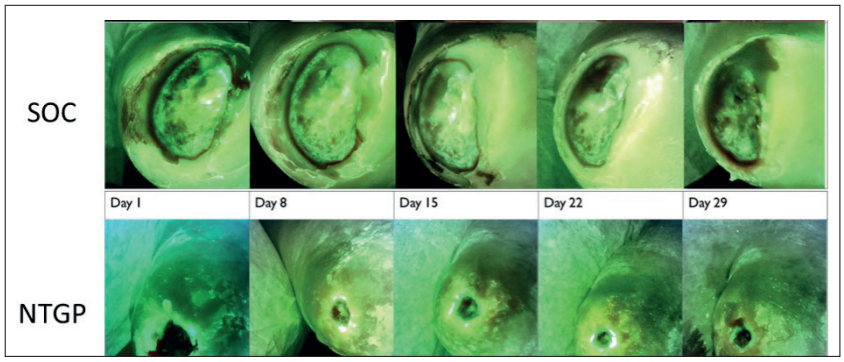

Figure 8. The MolecuLight i:X Imaging Device (MolecuLight, Toronto, Canada) was used to record autofluorescence images of bacteria on the wounds.

1. After NTGP treatment as well as SOC for 29 days, the bacteria were no longer found in the wound samples. *P. mirabilis* was also successfully treated. However, it was found that a high proportion of *S. aureus* and *P. aeruginosa* still remained on the wound *[Figure 9B]*.

Discussion

The purpose of this study was to evaluate the efficacy of NTGP in the treatment of patients with DFUs that are below the ankle and that are stalled by subclinical, biofilm-related wound infections as measured by changes in wound size. The secondary objective was to correlate the clinical presentation of these wounds with wound microbiology and histology. A qualitative analysis of all assessed parameters showed that overall outcomes for 12 of the patients were "good" to "very good" *[Figure 10]*. Seven of these patients received NTGP treatment versus five patients who had SOC. If this was evaluated for 'very good' outcome only, the ratio was five patients receiving NTGP versus none receiving SOC. The five patients with 'good' outcomes receiving SOC were expanded by two patients with 'good' outcomes receiving NTGP. Moreover, only one patient receiving NTGP showed no or only slight overall improvements, while this was the case for two patients receiving SOC.

These results are in line with previous results on the application of plasma treatments to accelerate wound healing in DFUs. So far, significant improvements in wound healing in terms of wound size, reduction of exudate produced, and wound grading in the plasma group compared to the control group have been reported (Mirpour et al, 2020; Stratmann et al, 2020; Samsavar et al, 2021). This was accompanied by a slight reduction in bacterial load. Others also found that both plasma and placebo treatments reduced microbial load. As all wounds received standard wound care procedures, including systemic antibiotic treatment if indicated, regular wound debridement, local disinfection, off-loading, and moist wound care, they concluded that these procedures may account for the microbial reduction seen in both groups and may dilute the plasma effect on microbial load (Stratmann et al, 2020). Here, no direct quantitative assessment of microbial counts was performed. The qPCR-based evaluation showed that a high proportion of *S. aureus* and *P. aeruginosa* still remained on the wounds independent of the treatment. However, this may be because the examined debrided tissue, which is by definition "bad" tissue and must be removed for the wound to improve, contains bacteria and biofilm. However, a qualitative but distinct decrease in the presence of bacteria and microbial components at day 29 compared to day 1 could be shown by Gram staining.

Based on these results and those of others (Mirpour et al, 2020; Stratmann et al, 2020; Samsavar et al, 2021), it can be concluded that NTGP is an effective and safe treatment modality for the therapy of DFUs.

Previous findings also corroborate the supportive effect of plasma in wound healing (Isbary et al, 2012; García-Alcantara et al, 2013; Kramer et al, 2013 Ulrich et al, 2015). One source is the well-known broad-spectrum bactericidal effect of NTGP (Assadian et al, 2019). Another reason is that NTGP may directly stimulate the regeneration of damaged tissue at the wound surface (Kalghatgi et al, 2010; Shao et al, 2016).

Significant improvement in burn wound healing and skin graft integration after treatment with NTGP is thought to be due to the increased production of extracellular matrix (Frescaline et al, 2020). Collagen type I is the most abundant

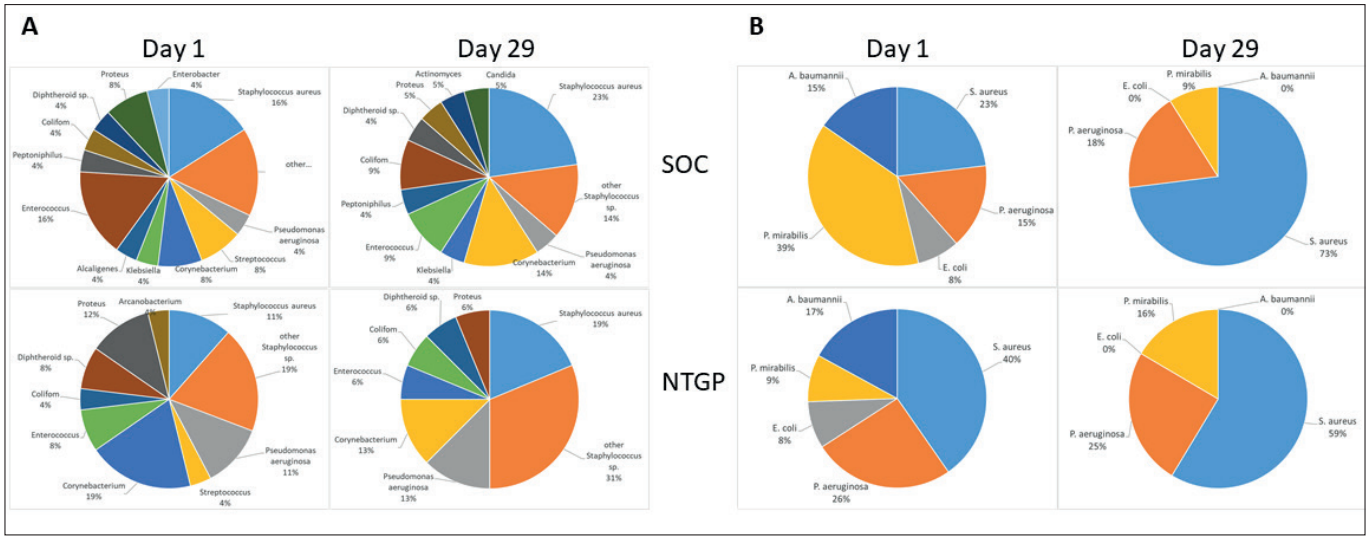

Figure 9. Comparison of the bacterial colonisation at day 1 and day 29 in the SOC group vs. NTGP by culture (A) and qPCR detection (B).

ID	treatment	wound size reduction	wound inflammation (clinic + histology), ECM production	reduction of microbial burden (histology, moleculight*, bacteria culture, qPCR)	overall outcome
1	SOC				
2	NTGP				
3	SOC				
4	NTGP				
5	SOC				
6	NTGP			no data	
7	NTGP				
8	NTGP				
9	NTGP				
10	NTGP				
11	SOC				
12	SOC				
13	SOC				
14	SOC				
15	NTGP				

Figure 10. Qualitative assessment of the study results concerning wound size reduction, wound pain, inflammation, and microbial burden ranging from 1 = red (worsening) to 10 = green (improvement) as well as a summary of the overall outcome ranging from 1 = very good (dark green blue), 2 = good (light green blue) to 3 = no/slight change (yellow green).

fibrous protein within this matrix (Eming et al, 2017) and its synthesis is crucial for cell migration, tissue maturation, and skin elasticity (Sun et al, 2014). Earlier reports have revealed that collagen I synthesis is enhanced by NTGP treatment in wounds, increasing wound strength (Arndt et al, 2013; Chatraie et al, 2018). Here, immunohistochemically results also indicated a slight improvement in the collagen content by NTGP vs. SOC.

In addition, others have shown that NTGP treatment stimulates cells to release TGF-β1, which is vital for diabetic wound healing (Fathollah et al, 2016). It further induces neovascularisation after 7 days of treatment (Fathollah et al, 2016), which is an essential prerequisite for diabetic wound healing (Costa and Soares, 2013) as one of the main clinical issues with diabetic ulcers is the poor blood circulation in the tissue leading to the lack of sufficient nutrients and oxygen in the wound area (Creager et al, 2003). In accordance, Matzkeit et al (2021) demonstrated improved cutaneous microcirculation in a standardised acute wound model after NTGP application with a significant increase in tissue oxygen saturation and capillary blood flow (Matzkeit et al, 2021).

Conclusion

To the best of our knowledge, the data obtained in this study strengthens the evidence obtained in other studies designed to demonstrate the effect of NTGP on wound healing in metabolic disorders, especially DFUs.

Conflict of interest

This study was supported by Adtec Europe Ltd, Twickenham, UK. JJ and MM are employees of Adtec Europe Ltd, Twickenham, UK.

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