

Original Article

NeutroPhase® in chronic non-healing wounds

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Abstract: Chronic non-healing wounds, such as venous stasis ulcers, diabetic ulcers, and pressure ulcers are serious unmet medical needs that affect a patient's morbidity and mortality. Common pathogens observed in chronic non-healing wounds are *Staphylococcus* including MRSA, *Pseudomonas*, *Enterobacter*, *Stenotrophomonas*, and *Serratia* spp. Topical and systemically administered antibiotics do not adequately decrease the level of bacteria or the associated biofilm in chronic granulating wounds and the use of sub-lethal concentrations of antibiotics can lead to resistant phenotypes. Furthermore, topical antiseptics may not be fully effective and can actually impede wound healing. We show 5 representative examples from our more than 30 clinical case studies using NeutroPhase® as an irrigation solution with chronic non-healing wounds with and without the technique of negative pressure wound therapy (NPWT). NeutroPhase® is pure 0.01% hypochlorous acid (i.e. >97% relative molar distribution of active chlorine species as HOCl) in a 0.9% saline solution at pH 4-5 and is stored in glass containers. NovaBay has three FDA cleared 510(k)s. Patients showed a profound improvement and marked accelerated rates of wound healing using NeutroPhase® with and without NPWT. NeutroPhase® was non-toxic to living tissues.

Keywords: NeutroPhase®, hypochlorous acid, chronic non-healing wounds, 510(k), negative pressure wound therapy (NPWT)

Introduction

Chronic non-healing wounds, such as venous stasis ulcers, diabetic ulcers, and pressure ulcers cause tremendous patient suffering. Treatment of such wounds presents a serious unmet medical need. The chronic non-healing wound is a complex interplay of extrinsic and intrinsic factors that has a direct and profound effect on a patient's morbidity and mortality [1-4]. Normal wound healing is a highly interactive process comprising four defined phases of coagulation, inflammation, tissue formation, and then tissue re-modeling. Factors inhibiting or delaying wound healing are: 1) Extrinsic factors such as excessive bacterial bioburden (bacteria plus biofilm) or fungal infection, the presence of dead tissue or foreign bodies; 2) Exacerbating issues such as a deficiency of growth factors, pathogen virulence factors, tissue maceration, ischemia, venous stasis, or circulatory issues; and 3) Host factors such as malnutrition, renal disease, compliance and

advanced age. Chronic non-healing wounds can persist for months or years versus acute wounds that can heal in days to weeks.

Critical infections in these wounds can be caused by multiple bacterial pathogens *Staphylococcus* including MRSA, *Pseudomonas*, *Peptoniphilus*, *Enterobacter*, *Stenotrophomonas*, *Finnegoldia*, and *Serratia* spp. [5, 6]. These pathogens form a robust biofilm that become a major obstacle for wound closure and the healing process. Bacterial biofilm in the wound bed is difficult to penetrate by traditional antibiotics and bacterial virulence factors can overwhelm the host's resistance that can result in additional tissue damage. If the physician cannot control the infection in these chronic wounds, the patient may become further compromised by additional tissue damage, bacteremia, sepsis or deeper wound infections that may require the surgeon to consider additional surgery or possible amputation of a portion of a limb to spare adjacent viable tissues or to save the patient.

Topical and systemically administered antibiotics do not effectively decrease the level of bacteria or the associated biofilm in a chronic granulating wound. Topical antimicrobials and temporary biologic dressings have been the methods of choice but still do not fully provide effective alternatives or significant relief. Topical antiseptics have a long history of use, such as sodium hypochlorite (Dakin's solution), hydrogen peroxide, acetic acid and povidone-iodine that remain in widespread use today. Used at typical concentrations, these agents have been reported to impede wound healing and are discouraged by experts for use on chronic ulcers. Therefore, the standard of care for chronic ulcers is debridement and moisture retentive dressings.

Pure hypochlorous acid in solution (the protonated form; HOCl) has been described as being 80-100 times more potent as a germicide than sodium hypochlorite (the hypochlorite anion) [7]. This is because pure hypochlorous acid as a neutral/uncharged species can penetrate microbial cells and spore walls while the charged hypochlorite anion cannot penetrate cell walls. Understanding the chemistry of chlorine species in aqueous solution is important because the amount of pure hypochlorous acid (the protonated form) in a 0.9% saline solution is dependent upon pH. Between pH 4 - 6, >97% of the active chlorine species exists as HOCl. At neutral pH, a mixture of hypochlorous acid and hypochlorite are present in solution, while at basic pH (pH > 8) the hypochlorite anion is the predominant species (unpublished results). NeutroPhase® is pure 0.01% hypochlorous acid (i.e. >97% relative molar distribution of active chlorine species as HOCl) in a 0.9% saline solution at pH 4-5 and is stored in glass containers.

In this report we show several clinical case studies using NeutroPhase® with and without NPWT with our easy-to-use protocol for the treatment of chronic non-healing wounds with a variety of wound types.

Materials and methods

Minimum bactericidal concentration

The bactericidal activity of hypochlorous acid was determined using a modification of CLSI M7-A7 and M26-A methods as previously

described [5]. Briefly, microorganism stocks were diluted 100-fold in sterile unbuffered saline pH 4 containing serial 2-fold dilutions of hypochlorous acid to give a final inoculum of 10^5 - 10^6 CFU/mL. After 1 h of incubation at room temperature 0.1 mL aliquots from each tube were transferred into 0.9 mL of Dey and Engley (D/E) neutralizing broth (Hardy Diagnostic, Santa Maria, CA) followed by plating for quantification at 37°C for 24-48 h.

Microbial strains

Microorganisms used in these studies were purchased from Eurofins Global Infectious Diseases Services (Chantilly, VA) and ATCC (Manassas, VA), grown and propagated according to the recommendations for each organism by ATCC.

Minimum biofilm eradication concentration

Minimum biofilm eradication concentration (MBEC) values provide estimates on the concentration of an antimicrobial product required to kill bacterial biofilm. The method was adapted from Harrison *et al.* [8]. *S. aureus* culture was grown and diluted in TSB to approximately 1×10^7 CFU/mL before inoculation of the Calgary Biofilm Device (CBD) plate. The CBD plate was incubated for 24 hr at 35°C on a rocking table. The CBD lid containing biofilm was first rinsed to remove planktonic cells prior to treatment with hypochlorous acid for 60 min at room temperature. The CBD lid was neutralized with D/E broth before incubation in fresh Tryptic Soy Broth (TSB). Bacterial quantification was performed by measuring absorbance at 650 nm (A_{650}). By definition, A_{650} reading of less than 0.1 indicates biofilm eradication. Bacterial enumeration was also performed as above and plate counts were correlated to A_{650} readings for MBEC values.

Activity against a robust biofilm

The CDC biofilm reactor was used to produce a robust and highly reproducible biofilm growth on glass coupons [9]. After inoculation with 1 mL of approximately 1×10^8 CFU/mL *P. aeruginosa* ATCC 27853, the reactor was placed on a stir plate at 125 rpm for 24 hr at room temperature. Media flow was then initiated at 12 mL/min for another 24 hr. The reactor was taken apart, and coupons were removed aseptically.

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Coupons were treated with 0.01% hypochlorous acid for 15 min prior to rinsing with PBS.

For microscopy, the treated biofilms were stained with BacLight LIVE/DEAD (SYTO 9 and propidium iodide) and images were collected using confocal laser scanning microscopy. SYTO 9 stains both intact and damaged membranes, whereas propidium iodide (PI) only stains cells with damaged membranes (Molecular Probes/Invitrogen, Eugene, OR) [10]. Therefore, viable cells will fluoresce green and dead cells will fluoresce red.

Clinical microbiology methods

The clinical microbiology testing of the patient samples were done by using tissue culture swabs obtained from the target lesion for each study patient. Identification of the pathogens present in the wounds was done at Seton Medical Center, California.

Clinical consent

Written informed consent was obtained prior to treatment for all patients. A combination of NeutroPhase® as the irrigation, cleansing and debridement solution and an appropriate non-adhering wound mesh such as Sorbact® (Pioneer Technology, Nashville, TN 37209, USA) as the wound mesh dressing was used to treat patients with chronic non-healing wounds.

Step-wise protocol

The step-wise protocol is representative for NPWT treatment in a variety of wound cases studies. Before NPWT the wound is cleaned and debrided. A variety of wound dressings can be used with NPWT in an effort to reduce tissue maceration such as a hydrophobic mesh, black or white foam. Transparent thin-film adhesive dressings provide the wound care specialist the flexibility to properly dress the wounds, or edge drape the wounds, and to secure the inlet port, wound dressings, and exit ports for NPWT. The inlet port is placed at the wound bed while the exit port is placed distal to the inlet port and on top of the wound dressings so as to provide the proper flow of NeutroPhase® across and through the chronic non-healing wound prior to exiting at the exit port with NPWT. A standard NPWT step-wise protocol is illustrative:

The patient's wound is moistened with NeutroPhase® via a standard cotton gauze compress immediately prior to physical cleaning, debridement and drying.

To minimize surrounding tissue maceration, transparent thin-film adhesive dressing is used to either edge dress the wound or by covering the entire wound followed by carefully excising the area over the wound to expose the to-be-treated area.

An inlet-port is placed at the base of the wound bed which can easily be prepared from a standard silicone IV extension tube (or equivalent) fitted at one end with a standard valve (i.e. one-way valve) such that the cut end is positioned at the wound bed base.

A hydrophobic mesh, black or white foam is cut and fitted into the wound and secured into place with transparent thin-film adhesive dressing.

A moderate to small-sized hole is made in the transparent thin-film adhesive dressing immediately above the hydrophobic mesh, black or white foam.

An exit-port tube is secured with transparent thin-film adhesive dressing or by the use of a self-adhesive exit-port leading to the vacuum pump for NPWT.

Stomahesive paste can be used to secure any edges from leakage at creased or angled areas (as needed).

The entire system is then placed under a mild vacuum (50 mm to 125 mm Hg PSI) to check for any air leaks.

NeutroPhase® (e.g. 5 mL or a predetermined amount) is instilled via syringe through the inlet-port into the wound bed with the vacuum on (50 mm to 125 mm Hg PSI). The procedure can also be done with the vacuum off. Care should be taken in either procedure so as to not overextend the clear thin-film dressing causing fluid leakage. If the vacuum is turned off it should then be turned back on to remove the fluids and exudates in 5 minutes.

The wound area is then wrapped in cotton gauze (e.g. Kerlix gauze) and a secondary dressing (e.g. ACE bandage) as ordered by the physi-

Table 1. Activity of hypochlorous acid against *S. aureus* clinical isolates

Pathogen	Study Isolate ID	MBC (µg/mL)
<i>Staphylococcus aureus</i> (MRSA)	1674616	0.25
<i>Staphylococcus aureus</i> (MRSA)	1674625	0.5
<i>Staphylococcus aureus</i> (MRSA)	1674631	0.5
<i>Staphylococcus aureus</i> (MRSA)	1674635	0.5
<i>Staphylococcus aureus</i> (VISA)	1674604	0.25
<i>Staphylococcus aureus</i> (VISA)	1674612	0.5
<i>Staphylococcus aureus</i> (Mupiricin R)	1674607	0.5
<i>Staphylococcus aureus</i> (Mupiricin R)	1674611	0.5

Bac Light™ Live/Dead stain where live cells are green and dead cell are red.

Biofilm treated with saline (control) was viable (**Figure 1A**) (mainly green fluorescence), whereas biofilm treated with 0.01% hypochlorous acid was mainly dead (red fluorescence) (**Figure 1B**). Treatment with

cian. The wound care specialists or informed patients can instill NeutroPhase® with vacuum removal as instructed by the attending surgeon or wound care nurse.

Adverse events

Adverse events (AE) were determined by the investigator and assessed to be unrelated to treatment, possibly related or probably related to treatment.

Results

Antimicrobial activity of hypochlorous acid

MBCs of hypochlorous acid against clinical isolates of *S. aureus* including MRSA, mupirocin-resistant and vancomycin-intermediate sensitive *S. aureus* (VISA) were tested. Hypochlorous acid was bactericidal against these pathogens with MBC range of 0.25 to 0.5 µg/mL (**Table 1**). These results confirm previously reported data [5].

Activity of hypochlorous acid against bacterial biofilms in the Calgary Biofilm Device (CBD)

Calgary Biofilm Device (CBD) was used to test susceptibility of *S. aureus* biofilm to hypochlorous acid. MBEC values against both *S. aureus* ATCC 6538 and *S. aureus* MRSA 1674631 expressed as the mode of MBECs obtained from 5 plates for each organism were 4 µg/mL.

Activity of hypochlorous acid against robust bacterial biofilms in the CDC reactor

P. aeruginosa biofilm was formed on glass coupons in the CDC reactor. The 48 hr. biofilm was treated with saline or 0.01% hypochlorous acid for 15 min. Bacterial killing in biofilm was shown by confocal imaging of coupons stained with

0.01% hypochlorous acid appeared to have disrupted biofilm structure.

Comprehensive GLP preclinical package

The following studies have been completed showing the topical tolerability of 0.01% hypochlorous acid: 28 day wound toxicity (rat, mini-pig); dermal sensitization (guinea pig); primary skin irritation study (rabbit); and primary eye irritation study (rabbit).

Photographic example of the treatment of a diabetic foot following TMA

Case #1 was a diabetic foot following post transectatarsal amputation (TMA; **Figure 2**) where the following sequential discussion highlights the case and the clinical outcome: (a) The patient was a 75 year old male who is a diabetic with chronic renal insufficiency, atrial fibrillation, cardiovascular disease, renal transplant and has had a femoral-tibial bypass. (b) TMA wound tested positive for MRSA 1 week post amputation. (c) The patient's wound was cleaned, debrided and the wound prepared for Negative Pressure Wound Therapy (NPWT) by a wound care specialist. An instillation port placed at the base of the wound proximal to the exposed bone and at the base of the skin flap. Black foam was used distal to the instillation port at the distal edge of the wound and an exit port was placed over the top of the black foam and secured with transparent thin film adhesive dressings. (d) NeutroPhase® was instilled (15cc's) through the instillation port while the vacuum for NPWT was left on @ 125 mm Hg throughout the treatment period as well. (e) Treatment with NeutroPhase® and NPWT was performed twice (2x/day) a day for 1 week. (f) After the wound bed showed significant response with no signs/symptoms of active

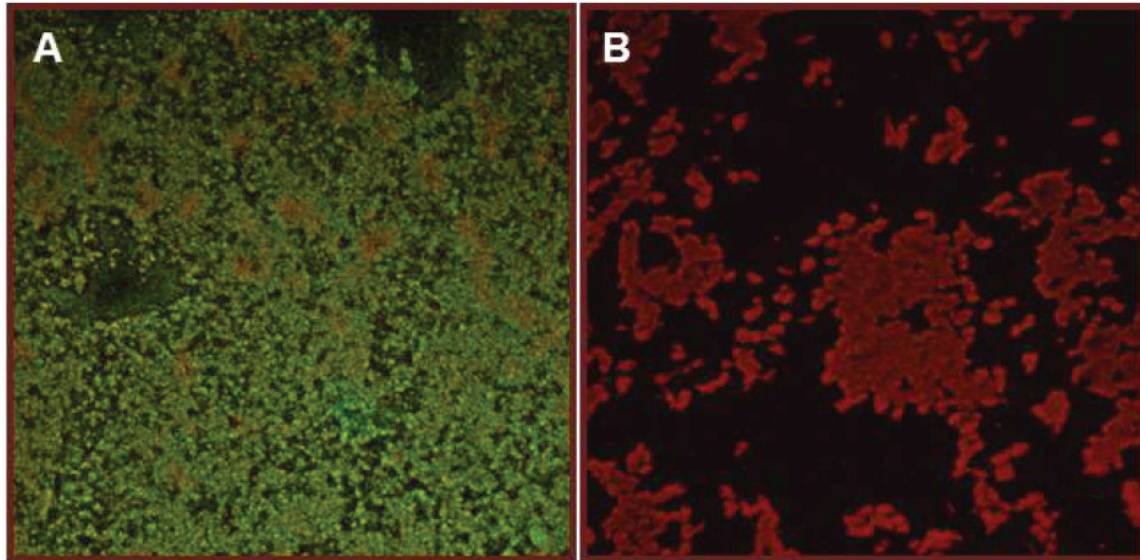


Figure 1. Fluorescent microscopy of *P. aeruginosa* ATCC 27853 biofilm grown in CDC reactor (live = green) and treated with 0.9 % saline pH 4 (A) or 0.01% hypochlorous acid in saline pH 4 (B) for 15 min (red = dead).



Figure 2. A: Case #1 shows a one (1) week post transmetatarsal amputation (TMA) with revascularization and an MRSA infection. A viable skin flap and transmetatarsal bone exposure is shown. B: The same TMA wound reclosed one (1) week after. C: Shows the wound five (5) weeks post initiation of treatment.

infection, the TMA site was surgically closed. (g) Outcome: TMA site healed.

Photographic example of the treatment of a chronic venous insufficiency

Case #2 was a chronic venous insufficiency (**Figure 3**) where the following sequential discussion highlights the case and the clinical outcome: (a) 65 year old female with a history of chronic venous insufficiency who presented to our wound center with bilateral ankle ulcers ongoing for 2 years. (b) Wounds noted as having recurrent MRSA colonization. (c) Treatment involved sharp debridement, wet-to-moist dressing with NeutroPhase® and gauze, and compression with Ace wraps. (d) After 2 ½ weeks of treatment, a very healable granulat-

ing ulcer was achieved, and wound culture was negative for MRSA. (e) Treatment progressed with application of biological dressings and compression therapy. (f) Outcome: Patient healing without NPWT.

Photographic example of the treatment of a pressure ulcer

Case #3 was a pressure ulcer (**Figure 4**) where the following sequential discussion highlights the case and the clinical outcome: (a) 75 year old male with dementia presented with a deep Stage IV Pressure Ulcer on the left heel with undermining and bone exposure. (b) Ulcer surgically debrided up to viable tissue, but wound culture was positive for MRSA. (c) Patient underwent systemic antibiotics, and the wound



Figure 3. A: Case #2 is a patient with a chronic venous girdle ulcer on the left leg. B: After 2 ½ weeks of treatment resolving a chronic venous girdle ulcer to a very healable granulating ulcer.



Figure 4. A: This case #3 shows a deep decubitus heel ulcer. Note the Achilles tendon showing in the upper right and the calcaneus excision (center). B: After 3 weeks of NeutroPhase® & NPWT. Notice the Achilles tendon is covered, granulation tissue (center) with reepithelialization around the wound edges. C: Healed after 85 days of therapy.

was prepared and treated with NPWT using black foam fitted to the wound. Neutrophase was instilled while the vacuum remained on @ 125 mmHg PSI. NeutroPhase® was used to irrigate the wound followed by NPWT vacuum 2x/day. (d) Outcome: Healable granulation tissue with epithelial tissue starting to form at the edges in 3 weeks and the wound healed in 85 days.

Photographic example of the treatment of an infected surgical flap

Case #4 was an infected surgical flap (**Figure 5**) where the following sequential discussion high-

lights the case and the clinical outcome: (a) 89 year old male with a history of basal cell carcinoma who had undergone removal on the left parietal area. (b) The tissue flap failed from MRSA infection. (c) The patient was admitted to the hospital and a wound consult referral made. (d) Patient started treatment with debridement, antibiotics, and NeutroPhase® infusion with NPWT. (e) Once the MRSA infection was controlled, patient was discharged to home care and followed as an outpatient at the wound center. Outpatient treatment involved conservative sharp debridement, wound cleansing with NeutroPhase®, NPWT and topical dressing consisting of collagen. (f) Outcome: Patient

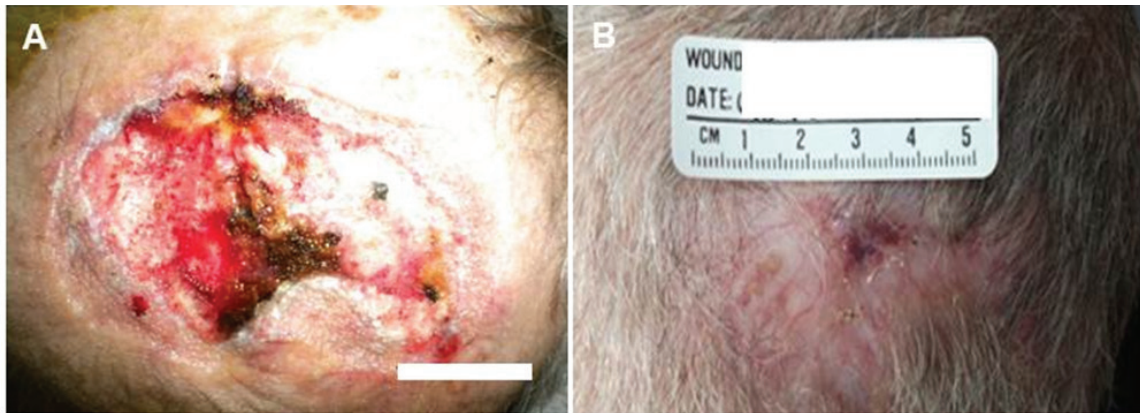


Figure 5. A: Case #4, an infected left parietal scalp surgical flap following a radical excision for squamous cell cancer leaving exposed cranial bone. A tissue flap had been put in place to cover the exposed bone. Unfortunately the flap became infected with MRSA and the flap was lost. B: Treatment with NeutroPhase®, a hydrophobic mesh and NPWT allowed the skin to grow back over the exposed bone in almost a year.



Figure 6. A: Shows case #5 a non-healing pilonidal Cyst. B: Shows 70 days post treatment with NeutroPhase® and NPWT at home with home health care.

healed and was discharged from wound program.

Photographic example of the treatment of a pilonidal cyst

Case #5 was a pilonidal cyst (**Figure 6**) where the following sequential discussion highlights the case and the clinical outcome: (a) 20 year old male with a non-healing ulcer from a pilonidal cyst surgical excision infection. The patient presented to the wound center referred by a surgeon after poor response to NPWT. (b) Treatment included wound culture and sensitivity and appropriate oral antibiotic regimen. Regular appointments for sharp debridement, and NPWT resumed with NeutroPhase® instillation 2x/day via inlet port. (c) Wound progressed with improved granulation tissue filling in depth after 3 weeks. Choice of dressing changed to calcium alginate with NeutroPhase® cleansing

during dressing changes. (d) Outcome: Patient healed.

Discussion

The treatment of chronic non-healing wounds is a serious unmet medical need. In these cases it is the presence of a bacterial infection plus the associated biofilm that appears to have significantly retarded wound repair making the wound non-healing and chronic.

Hypochlorous acid is a naturally occurring well-known broad-spectrum, fast-acting antimicrobial agent produced as part of the innate immune system's response to infection during oxidative burst by neutrophils and monocytes [11]. Hypochlorous acid has beneficial effects in addition to its antimicrobial activity such as disruption of biofilms [12], activating signal transduction [13, 14] as a pro-inflammatory

agent [15], penetrating microbial cells, spore walls and amoeba cysts [16, 17], wound repair, tissue regeneration, and its use in chronic non-healing wounds [5, 6, 18]. While there is a therapeutic window observed, at higher concentrations hypochlorous acid can cause necrosis and apoptosis [19, 20]. There are several common chemical sources for hypochlorous acid/hypochlorite anion.

Pure hypochlorous acid has been described as being 80-100 times more potent as a germicide than the hypochlorite anion [10]. This is because pure hypochlorous acid as a neutral/uncharged species can penetrate microbial cells and spore walls while the charged hypochlorite anion cannot penetrate cell walls. Our MBC and biofilm disruption data supports our previous reports clearly showing that hypochlorous acid has broad-spectrum antibacterial activity against Gram-positive and Gram-negative pathogens including drug-resistant bacteria such as MRSA, VISA and mupirocin-resistant *S. aureus* with MBC ranging from 0.1 - 2.8 µg/mL and also demonstrated fungicidal activity against *C. albicans* and *A. niger* [5]. It is important to realize that hypochlorous acid in the protonated form (HOCl) which predominates at a pH 4-6 disrupts biofilms and kills the pathogens.

In these and other clinical case studies we used NeutroPhase® topically or with NPWT with marked accelerated wound repair in our patients (Crew JR, et al. NeutroPhase® with Sorbact® dramatically enhances the speed of wound healing. Poster presented at Symposium on Advanced Wound Care /Wound Healing Society 2011). This easy to use protocol resulted in a significant change in the wound character from chronic to acute allowing formation of brisk granulation tissue with better use of biological, surgical closure or other wound care modalities to finalize healing. NeutroPhase® can be a significant tool in our battle against infection, the number one restraint to wound healing.

We have more than 30 clinical case studies where we used NeutroPhase® with and without negative pressure wound therapy (NPWT). Patients in these clinical case studies showed profound improvement and accelerated rates of wound healing using NeutroPhase® and NPWT. NovaBay has three FDA cleared 510(k)s.

NeutroPhase® and NPWT are easy to use and show a marked significant acceleration in the healing of chronic non-healing wounds with a variety of wound types.

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Statement for conflict of interest

Dmitri Debabov, Lu Wang, Azar Najafi, Suriani Abdul Rani and Ron (Ramin) Najafi are employees of NovaBay Pharmaceuticals, Inc. Dr. John Crew is a paid consultant, speaker and stock holder with NovaBay Pharmaceuticals, Inc. Randell Varilla and Thomas Allandale Rocas have no conflict of interests. Mark Anderson was the Chief Scientific Officer and is a stockholder with NovaBay Pharmaceuticals; and is a free-lance consultant and founder of Biotech Pharma Solutions.

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