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Wound Fluid Extracellular Microvesicles: A Potential Innovative Biomarker for Wound Healing

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Background: Extracellular vesicles, or microvesicles, are a large family of membrane-bound fluid-filled sacs that cells release into the extracellular environment. Extracellular microvesicles (EMVs) are essential for cell-to-cell communications that promote wound healing. We hypothesize a correlation between the concentration of EMVs in wound fluid and the percentage of wound healing in treated chronic, nonhealing, wounds. A prospective, multicenter, randomized, single-blind clinical trial was conducted to evaluate EMV concentration in relation to wound healing percentages.

Methods: Wound fluid samples were obtained from 16 patients with stage IV trunk pressure ulcers. Patients were divided equally into two groups: (1) control group on negative pressure wound therapy (NPWT) alone and (2) study group with NPWT plus porcine extracellular matrix dressing. NPWT was replaced two times a week, and porcine extracellular matrix applied once weekly for all subjects. An NPWT canister device, called a wound vacuum-assisted closure, containing wound fluid was collected from each patient every 4 weeks. EMVs were isolated and the concentration measured by nanoparticle tracking analysis.

Results: The study group's total healing percentage was around 89% after 12 weeks compared with the control group's percentage of about 52% ($P \leq 0.05$). Using R programming software, simple linear regression was carried out to investigate the hypothesis. Data demonstrated significant positive correlation ($R^2 = 0.70$; $P = 0.05$) between EMV concentrations and the healing percentage.

Conclusions: There is a positive correlation between EMV concentration and wound healing percentages. Results propose that the EMVs in wound fluid could serve as a biomarker for healing. *(Plast Reconstr Surg Glob Open 2024; 12:e5781; doi: 10.1097/GOX.0000000000005781; Published online 3 May 2024.)*

INTRODUCTION

Wound healing is an active process in which tissue repairs itself. Moreover, all tissues in the human body have the ability of healing, either by regeneration or repair mechanisms. Regeneration is a simple process of substituting damaged tissue with native tissue and is dependent on regenerative capacity of the tissue.[1] The repair is the primary mechanism by which injured tissues are replaced by connective tissue, which results in scar formation.[2] Wound healing is frequently compared to an orchestra playing or to a play, with cells, cytokines, and growth factors interacting to cause the skin to heal. However, recent data suggest that even when this delicate balance between cells and mediators might be altered, the lack of a certain cell type or the absence of a mediator can be compensated by others that are participating in wound healing so that the repair can still occur.[3] The wound healing process advances through a series of time-dependent, collaborative, and overlapping phases: homeostasis and coagulation phase, inflammatory phase, proliferation, and remolding. Throughout this process, homegrown cells, inflammatory mediators, and extracellular matrix actively contribute to the four stages of healing.[4] Also, overlaid on the healing process is a series of precisely controlled processes corresponding

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to different cell types in the wound during each specific phase of healing.[5]

Chronic Wounds and Pressure Ulcers

Chronic wounds are considered to be wounds that do not heal within 3 months. Furthermore, given that they impact more than 1% of the western population, chronic wounds pose a significant health care concern. Furthermore, by placing a substantial financial burden on the healthcare system, chronic wounds cost the US healthcare system more than $25 billion annually.[6] Pressure ulcers, diabetic foot ulcers, venous ulcers, and ischemic ulcers are common types of chronic wounds.[7] "A pressure injury is localized damage to the skin and underlying soft tissue typically over a bony prominence or associated to a medical or other device," according to The National Pressure Ulcer Advisory Panel. Soft tissue may respond differently to pressure and shear depending on factors such as tissue perfusion, environment, patients' nutritional state, comorbidities, and soft tissue condition.[8]

The word "deep tissue injury" was adopted because a high level of pressure at the bone-soft tissue crossing point was the most possible culprit of these pressure ulcers.[9] Pressure ulcers can develop in a variety of body regions, including the trunk, limbs, and buttocks, but the sacrum and heel are the most susceptible because they contain a thin layer of soft tissue and are more susceptible to develop pressure ulcers.[10] As a chronic wound, these ulcers are believed to be "stuck" in the inflammatory stage of wound healing. The current theory holds that the wound bed has a localized increase in the level of proteases, such as matrix metalloproteinases. Furthermore, the hypoxic microenvironment degrades crucial growth factors and precludes the wound from proceeding to the proliferative phase of healing, where granulation tissue and a new provisional matrix for remolding and healing are formed.[7]

Extracellular Microvesicles

Extracellular vesicles (EVs), also known as microvesicles (MVs) and exosomes, are a varied family of membrane-bound vesicles that cells release into the extracellular space and are loaded with different proteins, nucleic acids, and lipids.[11] Wolf first identified EVs in the extracellular space in 1967.[12] EVs were first mentioned by Pan and Johnstone in 1983 as a component of the disposal system for cell debris.[11] The International Society for Extracellular Vesicles recently proposed using the word "extracellular microvesicles" (EMVs) to refer to all varieties of vesicles found in the extracellular fluid. EVs are particles with sizes ranging from 20 nm to 2 mm that are enclosed in phospholipid bilayer membranes and released into the extracellular space via special synthesis and release mechanisms. The release and content of EVs are determined by the cell from which they originated and the trigger for release, such as exposure to lipopolysaccharide, hypoxia, oxidative damage, or shear force stress.[12] MVs are formed by the outward budding of cytoplasmic protrusions and its emission occurs in all cells at rest or upon stimulation by physical or chemical stress such as hypoxia, oxidative stress, shear stress, or soluble agonists.[13]

Takeaways

Question: Can the level of microvesicles found in wound fluids predict wound healing?

Findings: The level of microvesicles in wound fluids correlated with the healing rate.

Meaning: The level of circulating microvesicles in wound fluids may be used as a biomarker that may predict healing of the existing wound.

Depending on their physical characteristics and mode of synthesis, three distinct categories of EVs have been identified: MVs, exosomes, and apoptotic bodies. MVs range in size from 100 to 1000 nm, with a density of 1.04–1.07 cell surface (g/mL), are formed by outward cell membrane blebbing, and are composed of RNA, miRNA, other noncoding RNA, cytoplasmic protein, and cell organelles. Furthermore, its membrane is impermeable (PI negative), and cellular markers are annexin V positivity and origin cell-specific markers. In addition, there is no one unique marker to recognize each type of EV, including MVs; however, proteins that are not specific to each EV group have been exploited as markers. Major histocompatibility complex molecules, heat shock proteins, Tsg101, tetraspanins such as CD9, CD63, CD81, and CD82; 14-3-3 proteins, and the endosomal sorting complex required for transport binding protein Alix are among those surface markers.[14] MVs can be characterized via using high sensitivity flow cytometry and imaging flow cytometers as tiny as 100 nm in diameter. Nevertheless, a flow cytometer is not able to characterize smaller sizes such as exosomes. Dynamic light scattering and nanoparticle tracking analysis (NTA) are two cutting-edge technologies that have demonstrated the ability to identify and characterize EVs with diameters smaller than 100 nm.[12] MVs activate cells through a variety of methods, including direct communication with receptors and molecules expressed on their surfaces that attach to ligands expressed on recipient cells. Following interactions with ligands, MVs could be able to deliver their contents to the recipient cells.[13] EVs, which transport signaling molecules such as lipids, proteins, mRNAs, and miRNAs, are crucial for cell-to-cell communication. Poste et al[15] demonstrated for the first time in 1980 that a poorly metastatic B16 cell line may acquire plasma membrane content from a highly metastatic B16 cell line, increasing the metastatic capacity of the latter. Zitvogel et al[16] showed in 1998 that EVs (exosomes) produced from dendritic cells boost T-cell antitumor response in vivo. Bruno et al[13] demonstrated that MVs derived from human liver stem cells stimulate in vitro proliferation and apoptosis resistance in human and rat hepatocytes. They also demonstrated that in vivo MVs hasten the functional and morphological recovery of the liver in a model of 70% hepatectomy in rats.[13] Rats given a single dose of endothelial progenitor cell-derived MVs (EPC-MVs) immediately following renal ischemia and reperfusion injury could produce morphological and functional protection from acute kidney injury by increasing proliferation and lowering apoptosis of renal tubular cells.[13] Furthermore, EPC-MVs demonstrated the

ability to protect against chronic kidney disease by inhibiting glomerulosclerosis, capillary rarefaction, and tubulointerstitial fibrosis. Depletion of miRNA content in MVs reduced EPCs' renal protective effect.[13] Furthermore, in a mouse model of hind limb ischemia, EPC-MVs enhanced reperfusion and minimized injury. Recently, He et al[17] demonstrated that mesenchymal stem cell–derived MVs protect against renal damage in the mouse remnant kidney model (5/6 partial nephrectomy). Purified MVs have also been shown to prevent gentamicin-induced acute renal damage, according to Reis et al.[18] Finally, EVs are no longer seen as "cell dust" but rather as effective messengers in intercellular signaling that have an impact on both nearby and distant cells.[12]

MATERIAL AND METHODS

Experimental Design

This project was part of a prospective, multicentered, randomized, single-blinded clinical trial that was permitted by the ethics committee of the Copernicus.[19] Written informed consent was obtained from all patients. We discussed and answered patients' concerns before obtaining the consent. Wound fluid samples were obtained from 16 patients with stage IV trunk pressure ulcers. The patients were divided in two groups (eight in each group): a control group on negative pressure wound therapy (NPWT) alone and a study group with NPWT plus porcine extracellular matrix dressing (Oasis Ultra; Table 1).

At approximately 1, 2, and 3 months, wound specimen canisters were collected and taken to the laboratory at Wright State University department of Pharmacology and Toxicology for analysis of the drained fluids from all wounds. Specimen canisters were secured in biohazard bags and transported to Wright State University by a member of the study team within 24 hours of collection.

For all wound fluid samples, canisters were obtained in a sterile fashion. Part of the fluid was used for the study and the other part was stored in liquid nitrogen at −80°C for potential later use, possibly unrelated to this study. If a subject refused to allow their samples to be used outside of this study, that subject's extra fluid sample was destroyed.

To maintain blinding for the assessment laboratory personnel, specimen canisters and stored specimens were identified by a coded label. Pressure reduction beds and patient repositioning were employed throughout the study as a standard of care for those patients.

Following the conclusion of the study, results of the clinical trial were published.[20]

Table 1. Demographics of the Patients Who Joined the Clinical Trial

Variable	NPWT (Control)	NPWT +Oasis Ultra (Study)
Total number	8	8
Male	3	4
Female	5	4
Average age, y	62.5	63.5
Wound location	7 sacrum, 1 left ischium	7 sacrum, 1 left buttock
Average wound size, cm^3	64.13	176.40
Smoking history	None	None

Sample Preparation

An NPWT canister device, called a wound vacuum-assisted closure, was collected from the patients every 4 weeks of the study and brought to the laboratory where a hole was made in the canister by using a drill to drain the fluids, which were aliquoted to 1.5-mL eppendorf tubes. Protease inhibitor was added to the fluid before the fluid stored in an −80°C freezer.

MV Isolation

MVs were isolated using the differential ultracentrifugation method. Patient samples were mixed in 5 mL of 20-nm filtered (Whatman, Pittsburgh, Pa.) phosphate-buffered saline (PBS). Samples were transferred to a centrifuge tube; 5 mL of filtered PBS was added to dilute the samples. The wound fluid samples were centrifuged at 4°C (500*g* for 10 min followed by 2000*g* for 20 min) to remove intact cells and cell debris. New tubes were used after every centrifugation stage. The supernatant centrifuged at 10,000*g* for 2 hours to isolate MVs. MV pallets were suspended in 1 mL of filtered PBS. We took 5 µL from the sample and diluted it in 695-µL filtered PBS for analysis by using an NTA machine. MV concentration was measured by the NTA.

Nanoparticle Tracking Analysis

Purified MV samples were analyzed by NTA using the Malvern Panalytical NanoSight NS300 with a 405-nm laser instrument (Malvern Instruments, UK). Five microliters

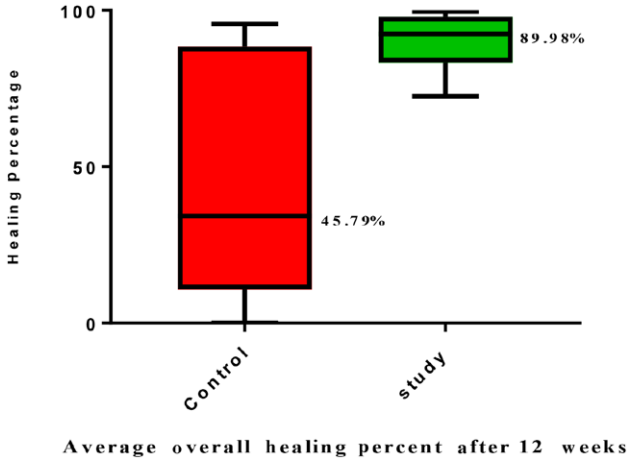

Fig. 1. Average percentage healing in the control and study groups. The box and whisker plot shows the difference in healing between the two groups. The control showed a wide interquartile range and an average healing rate of 45.79% after 12 weeks. In comparison, Oasis Ultra had an average healing rate of 89.98% after 12 weeks. Reprinted with permission from Mari W, Younes S, Naqvi J, et al. Use of a natural porcine extracellular matrix with negative pressure wound therapy hastens the healing rate in stage 4 pressure ulcers. *Wounds*. 2019;31:117-122.[20]

from the samples were diluted in 695-µL filtered PBS, and three 30-second videos were recorded using a camera level of 12–15 with a frame rate of 30 frames per second. The data were analyzed using NTA software 3.0 (Malvern Instruments) which was optimized to first identify and then track each particle on a frame-by-frame basis.[21] The detection threshold was optimized for each sample and screen gain at 10 to track as many particles as possible with minimal background.[22]

Statistical Analyses

Analyses were performed using R programming software. Simple linear regression was employed to study the correlation between the healing percentage and EMV concentration. Furthermore, a *P* value of less than or equal to 0.05 was considered statistically significant.

RESULTS

In the previous randomized control trial, the difference in healing percentage between the study group and the control group was analyzed by one-way analysis of variance statistical test. The differences between the mean percentages ± SD of control (45.8% ± 38.7%) versus small intestinal submucosa extracellular matrix–treated (90.0% ± 9.5%) wounds were found to be significant at a *P* value of less than 0.01 at 12 weeks. Using these results, a box and whisker plot was created to show the average of overall healing percentage after 12 weeks in both groups (Fig. 1).

The primary objective of this project was to determine if wound fluid MV concentration would correlate with the percentage healing or wound healing rate. To study this, we examine the number of EMVs in the wound exudate at different time points. To analyze this further, the EMV concentration (EMV concentration × 10^8 particles/mL) was plotted against percentage healing, and the data for each time point were analyzed by dots and color coding (Fig. 2). From these plots, we determined that there was a higher EMV concentration that correlated with higher percentage healing in the wounds treated with the combination of Oasis Ultra dressing and NPWT (Fig. 3), as

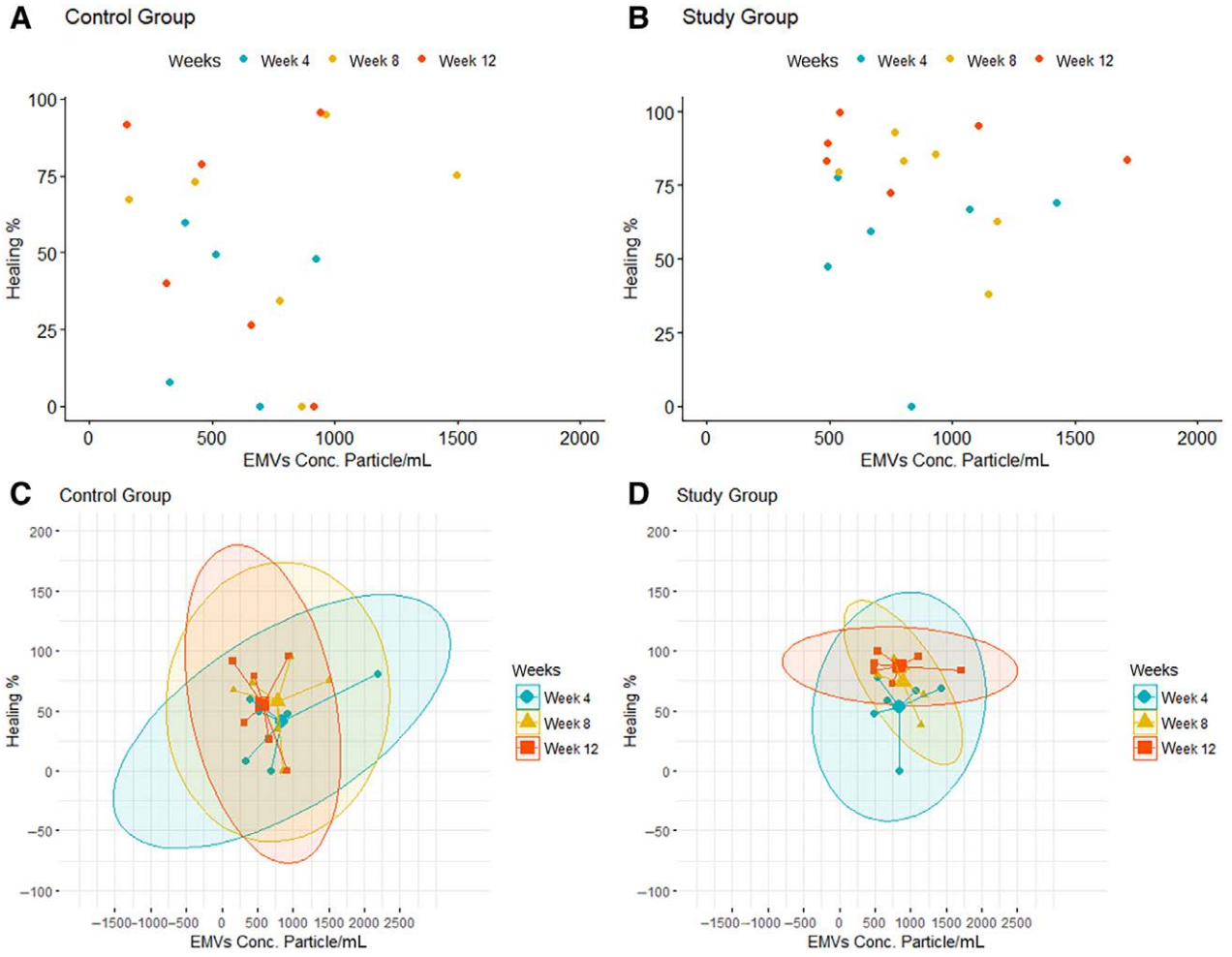

Fig. 2. Corrolation of wound fluid MV concentration with the percentage healing or wound healing rate. A-B, Scatter plot of EMV concentration vs wound healing percentage in the control and study groups at 4, 8, and 12 weeks. Each dot represents data of one patient. C and D, Star plot (ellipse) with the mean (central point) of EMV concentration vs wound healing percentage in the control and study groups at 4, 8, and 12 weeks. Each dot represents data of one patient.

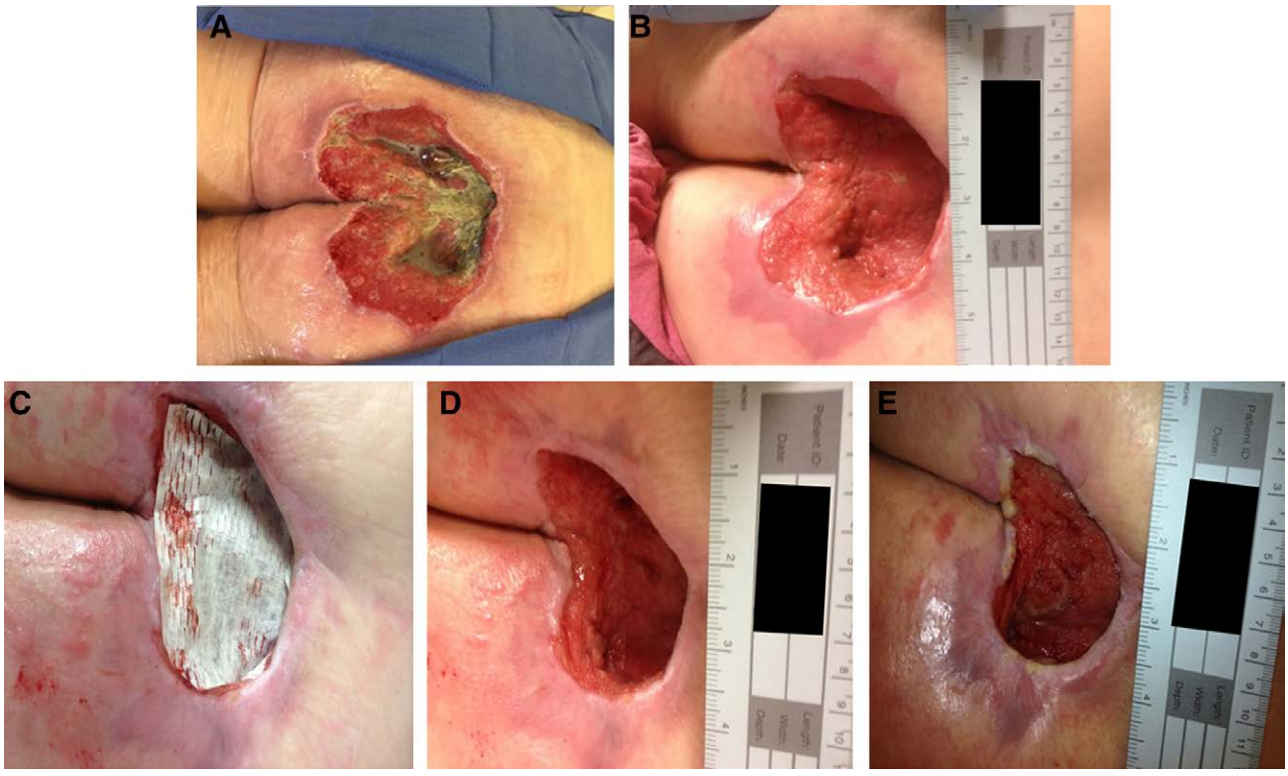

Fig. 3. Stage IV pressure wounds randomized as a study candidate received Oasis Ultra plus NPWT clinical outcomes of a stage 4 sacral and gluteal pressure ulcer treated with SIS-ECM and negative pressure wound therapy at the (A) initial visit at week 0 with the wound measuring 12.0 cm × 15.5 cm × 3.5 cm and (B) 4 weeks of treatment; (C) continued treatment at 8 weeks with SIS-ECM in place before NPWT application; (D) 10 weeks of treatment; and (E) 12 weeks of treatment. SIS-ECM, small intestinal submucosa extracellular matrix. Reprinted with permission from Mari W, Younes S, Naqvi J, et al. Use of a natural porcine extracellular matrix with negative pressure wound therapy hastens the healing rate in stage 4 pressure ulcers. *Wounds*. 2019;31:117-122.[20]

compared with the wounds that were treated with NPWT alone (Fig. 4). These findings suggest that Oasis Ultra may provide an environment conducive to increased EMV concentration.

To test if EMV concentration in wound fluid could impact wound healing percentage regardless of the wound treatment, a simple linear regression was conducted to see if EMV concentration could be a predictor for the healing percentage (Table 2). We observed that regardless of which treatment the patients received, there was positive correlation between EMV concentration in wound fluid and healing percentage. In another way, data showed an increase in healing percentage for every single unit change in the EMV concentration (Fig. 5). These findings also suggest that EMV concentration in wound fluid could predict the healing rate and potentially could serve as a biological biomarker for wound healing.

DISCUSSION

Wound healing is a complex, dynamic process that necessitates intricate cell-to-cell communication in the appropriate extracellular environment.[23] The wound healing process is started by a series of time-dependent, tightly coordinated, interactive, and overlapping steps. Extracellular matrix actively contributes to each stage of wound healing.[2,4] For a wound to heal successfully, highly coordinated signals via cytokines, growth factors, and chemokines are needed. These cytokines attach to their respective receptors and function via an endocrine, autocrine, or paracrine mechanism by initiating a particular biochemical cascade, which results in a change in the metabolism, growth, and differentiation of the target cells.[24] Accumulating evidence suggests that EVs can deliver growth factors, cytokines, and trophic factors (eg, vascular endothelial growth factor A, epithelial growth factor, platelets degradation growth factor A, tumor necrosis factor-α, interleukin-1β, and monocyte chemoattractant protein-1) to recipient injured cells, or induce recipient cells to release these factors.[25] EVs have natural biocompatibility, stability in circulation, low toxicity, and low immunogenicity, and serve as efficient carriers of molecular cargos, and are therefore ideal therapeutic candidates for regenerative medicine.[26] A recent study found that EVs played a large role in macrophage reprograming during the inflammatory phase, allowing them to have to have proresolving/healing properties called M2 macrophages.[27]

The main purpose of this study was to investigate the correlation between wound fluid MVs and wound healing percentage, and also to see whether the Oasis Ultra

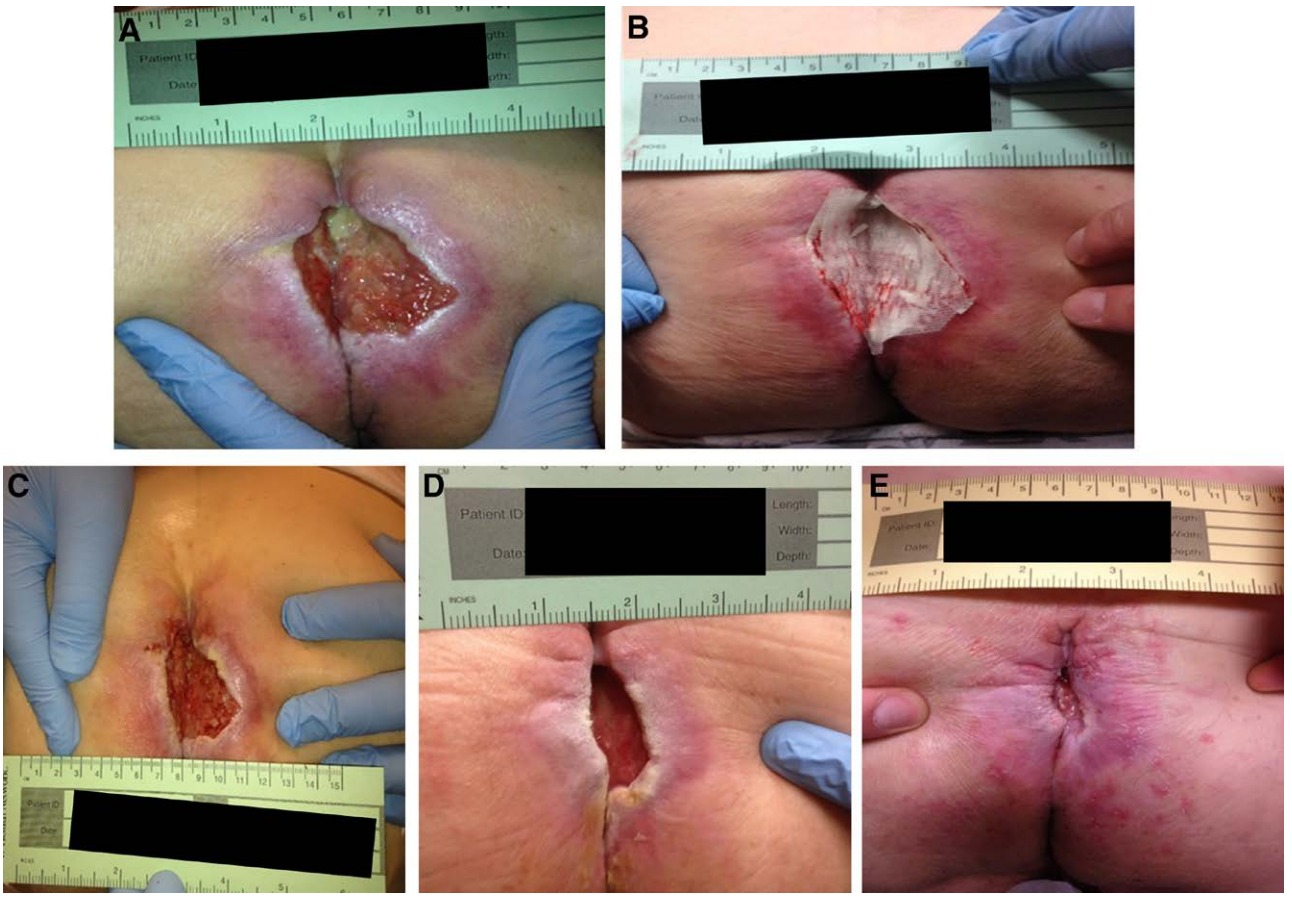

Fig. 4. Stage IV sacrococcygeal pressure wounds randomized as a study candidate received Oasis Ultra plus NPWT clinical outcomes of ulcer treated with SIS-ECM and negative pressure wound therapy during the 12-week study period. Clinical outcomes at the initial visit (A-B) at week 0 with the wound measuring 5.0 cm × 4.0 cm × 1.2 cm; C, 1 week of treatment; D, 4 weeks of treatment; and E, 12 weeks of treatment of a patient with a stage 4 sacral pressure ulcer treated with small intestinal submucosa extracellular matrix and negative pressure wound therapy. Reprinted with permission from Mari W, Younes S, Naqvi J, et al. Use of a natural porcine extracellular matrix with negative pressure wound therapy hastens the healing rate in stage 4 pressure ulcers. *Wounds*. 2019;31:117–122.[20]

Table 2. The Effect of EMV Concentrations on Healing Percentage (H%)

Variable	Simple Linear Regression
EMV concentration	0.070*
	(0.008)
No. samples	32
R^2	0.704
F statistics	80.853*

In this table we report results of simple linear regression of healing percentage (H%) as a response variable and EMV concentrations as a key predictor variable. The dataset consists of 12 patients who finished the study period at the time of this analysis. The estimated regression coefficient (0.070), *P* value, * Indicates significant at the 1%. Robust standard errors are shown in parentheses below the estimated regression coefficients.

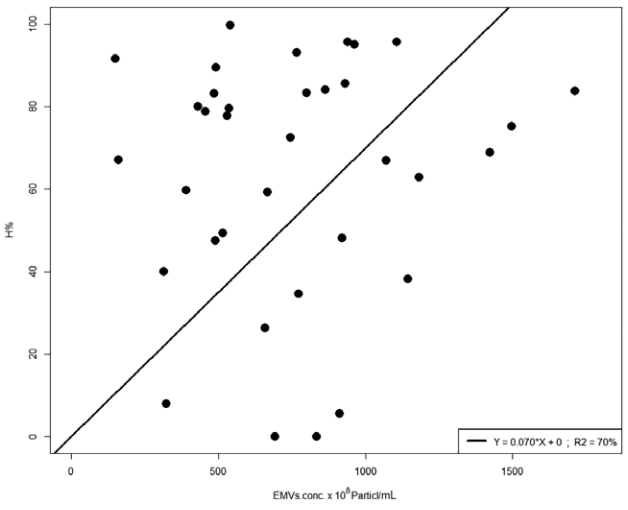

Fig. 5. Analysis of percentage healing vs EMV concentration**.** Linear regression using R Statistical software was conducted to analyze the data of all patients regardless of the treatment, and data showed that there is a positive correlation between MV concentration and wound healing percentage; the R^2 was 70%, and the *P* value was ≤0.01.

study group had more MVs than the NPWT control group. Furthermore, we looked for specific factors that could either affect wound healing or act as an indicator of the wound healing process, either positively or negatively.

Numerous studies have identified MVs as an indicator of risk and having the power to promote or positively impact wound healing. In a study by Zheng et al,[28] the use of low-intensity ultrasound stimulation as a new method

for promoting significant EV secretion in diabetic wounds promoted markedly increased wound healing in vitro and in vivo. In diabetic mice, Wei et al[29] found that EVs promote wound healing by reducing yes-associated protein 1 phosphorylation and activating the phosphoinositide 3-kinase, protein kinase B oncogene, mammalian target of rapamycin kinase pathway, therefore increasing vascular markers and fibroblast proliferation. MVs derived from mesenchymal stem cells and platelet-rich plasma improved burn wound healing via regulating scar formation and antioxidant mechanism.[30] In a study by Gan et al,[31] the use of MVs was able to suppress multiple anti-apoptotic/cardioprotective molecules in cardiomyocytes, preventing ischemia and reperfusion injuries in diabetic mice. Furthermore, MVs were found to reverse kidney ischemia reperfusion in rats by maintaining renal vascular and epithelial networks, preventing renal oxidant stress and apoptosis, and restrained activation of proinflammatory and profibrogenic pathways.[32]

In vivo studies in chronic diabetic wounds found the use of adipose-derived mesenchymal stem cell exosomes in the form of a hydrogel significantly enhanced the healing efficiency of diabetic full-thickness cutaneous wounds, characterized by enhanced wound closure rates, fast angiogenesis, reepithelization, and collagen deposition within the wound site.[33] MVs studied in the hepatic reperfusion injury demonstrated that mesenchymal stem cell EVs decreased serum transaminase levels, reduced hepatic necrosis, increased the amount of Ki67-positive hepatocytes, and repressed the transcription of inflammation-associated genes.[34]

In a study by Ramirez-Carracedo et al,[35] it was found that ivabradine-stimulated MV release in vivo had significant cardiac protection by increasing left ventricle ejection fraction and a significant reduction of the necrotic area. Furthermore, in stroke patients, it was found that circulating MVs may allow better monitoring of the response to antiplatelet therapy in patients after stroke. In addition, the link between platelet-derived MVs and neutrophil granulocytes might become therapeutic targets in the future.[36] MVs on liquid biopsy have also been found to potentially predict future major ischemic events in genetically characterized familial hypercholesterolemia patients.[37]

We demonstrated that when wounds were treated with Oasis Ultra as compared with NPWT alone, the overall concentration of MVs increased with time. The data clearly showed a positive correlation between wound fluid EMV concentration and wound healing percentage. Data also demonstrated that, regardless of therapy, an increased healing rate was associated with an increase in MV concentration, which appeared to be enhanced with Oasis Ultra. These findings suggest using wound fluid EMVs as a potential novel biomarker for wound healing. Further research is needed to fully understand the role of EMVs in wound healing.

CONCLUSIONS

According to our data, the percentage of wounds that have healed and the EMV concentration in wound fluid are positively correlated. Our results suggest that EMVs in wound fluid could serve as a potential diagnostic biomarker for wound healing. This could potentially be used to pave the way for future studies to delineate the precise mechanism of EMVs in wound healing. Additionally, our findings suggest the possibility for using wound fluid EMV as a therapeutic tool to accelerate wound healing.

LIMITATIONS

The limitations of this study include the relatively small sample size that was able to finish the study. Additionally, the population of patients who experience chronic stage 4 pressure ulcers is usually very debilitated and has multiple other comorbidities impacting healing rates. Although the findings in this study demonstrate significant improvement in wound healing with the use of porcine extracellular matrix dressing as adjunctive therapy to NPWT, a larger study would allow for more definitive findings. Moreover, the exact mechanism of action of this combination therapy for chronic pressure ulcers should be further investigated.

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DISCLOSURE

The authors have no financial interest to declare in relation to the content of this article.

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