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News & Views

May Dietary Supplementation Augment Respiratory Burst in Wound-Site Inflammatory Cells?

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Abstract

Persistent infection contributes to wound chronicity. At the wound site, NADPH oxidase (NOX) activity in immune cells fights infection to enable the healing process. Fermented papaya preparation (FPP) is a carbohydrate-rich nutritional supplement that has demonstrated ability to bolster respiratory burst in experimental rodent systems. In FPP, glucose coexists with fructose and maltose in addition to multiple other sugar alcohols such as inositol. We have previously reported that FPP supplementation augments wound healing in diabetic mice *via* improvement of respiratory burst activity of wound innate immune cells. In this clinical study (clinicaltrials.gov: NCT02332993), chronic wound patients were orally supplemented with FPP daily. Inducible production of reactive oxygen species was significantly higher in wound-site immune cells from patients supplemented with FPP and on standard of care (SoC) for wound management compared with those patients receiving SoC alone. Wound closure in FPP-supplemented patients showed improvement. Importantly, the consumption of this mixture of carbohydrates, including significant amounts of glucose, did not increase HbA1c. These observations warrant a full-length clinical trial testing the hypothesis that FPP improves wound closure by augmenting NOX activity in immune cells at the wound site. *Antioxid. Redox Signal.* 00, 000–000.

Keywords: ROS, fermented papaya preparation, nutritional supplement, wound management, immune cells

Introduction

Chronic wound infections account for substantial morbidity and result in significant increases in the cost of healthcare (2). Persistent infection and associated excessive inflammation are major causes of wound chronicity (2). The cells of the innate immune system, monocytes/macrophages and neutrophils, are the first line of host defense against invading pathogens (8). NADPH oxidase (NOX)-dependent ''oxidative burst'' by innate immune cells represents a key mechanism of pathogen killing at the wound site (2, 8). Patients with deficiency in NOX suffer from chronic granulomatous disease (CGD), characterized by inability of phagocytes to adequately generate reactive oxygen species (ROS). CGD patients suffer from impaired healing (8).

Dietary and nutritional supplements are known for their ability to bolster respiratory burst function in phagocytes. Fermented papaya preparation (FPP) is a product of yeast fermentation of *Carica papaya* Linn (3). Previous reports from our laboratory have demonstrated that FPP supplementation

Innovation

Based on sound preclinical body of evidence, this is the first testing of fermented papaya preparation (FPP) in chronic wound patients. That FPP, taken orally, is able to bolster inducible NADPH oxidase activity of cells suspended in the wound fluid constitutes first patient-based evidence.

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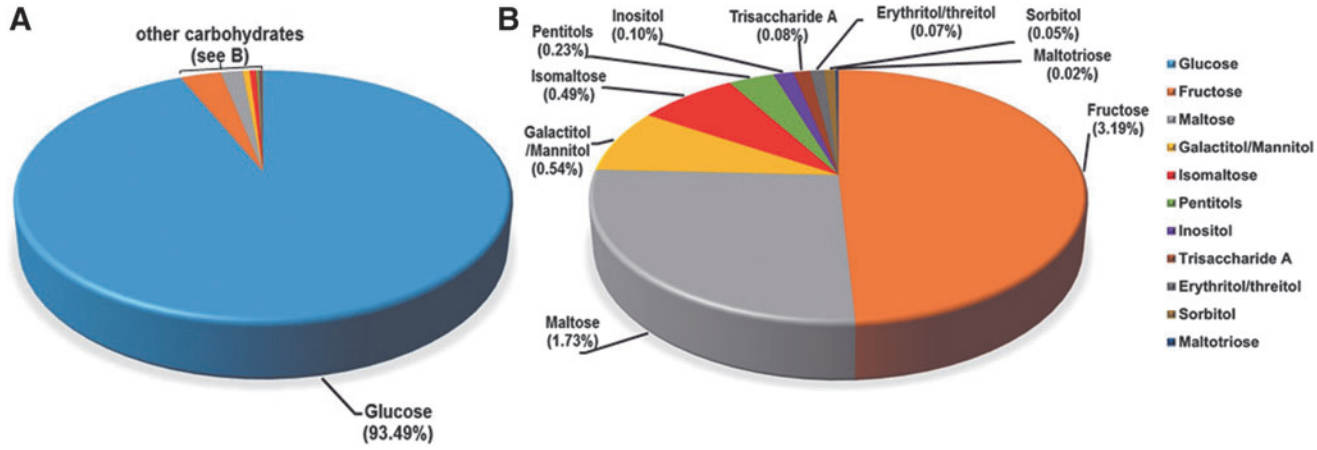

FIG. 1. Sugar and sugar alcohols in FPP. Sugar and sugar alcohol contents of FPP were quantified using liquid chromatography and mass spectrometry. Sugar and sugar alcohols in FPP **(A)**, with glucose **(B)**, without glucose. FPP, fermented papaya preparation.

improved respiratory burst activity of wound-site innate immune cells and improved wound closure in diabetic mice with phagocyte dysfunction and impaired NOX activity (2). FPP treatment corrected diabetes-impaired respiratory burst activity in the peripheral blood mononuclear cells derived from type 2 diabetes mellitus patients, both *in vivo* (3 g/dose, 3 doses/day) (3) and *ex vivo* (4). In this work, we sought to test in chronic wound patients whether oral supplementation of FPP is (i) safe for patients with chronic wounds and (ii) effective in influencing ''respiratory burst'' activity of wound-site immune cells derived from human chronic wounds.

Results and Discussion

Sugar and sugar alcohol components

Using liquid chromatography/tandem mass spectrometry (LC-MS/MS), we investigated the composition of the carbohydrates in FPP. Glucose (~94%) was the primary carbohydrate present in FPP followed by fructose (3.2%) and maltose (1.7%; Fig. 1). Interestingly, multiple sugar alcohols were also detected in FPP (Fig. 1). Among the sugar alcohols detected in FPP, inositol has been reported to prime neutrophils for respiratory burst activation (5). On preincubation with inositol hexakisphosphate (InsP6), the production of inducible (N-formyl-methionyl-leucyl-phenylalanine [FMLP], phorbol 12-myristate 13-acetate (PMA), or phagocytic particles) ROS was enhanced in neutrophils (5). This study supports the notion that the presence of inositol in FPP is likely to contribute to the enhanced respiratory burst activity as observed following FPP supplementation.

Improved inducible "respiratory burst" activity of wound-site immune cells

To determine whether oral supplementation of FPP induced respiratory burst in wound-site immune cells, patients with chronic wounds undergoing negative pressure wound therapy (NPWT; Table 1) were subjected to oral supplementation with 9 g (3 g/dose, 3 doses/day) of FPP daily. The patients received standard of care (SoC) for their wound managements. Control group only received SoC and no supplementation. The wound-site immune cells were harvested from the NPWT dressing using Ficoll density centrifugation (Fig. 2). The study design and sample collection scheme have been presented in Figure 2A. Inducible superoxide anion (O_2^-) as a product of NOX activity was measured using dichlorodihydrofluorescein diacetate (H_2DCF-DA)-based assay (Fig. 2B) after 2 weeks of FPP supplementation (3). Inducible ROS production was significantly high in wound-site immune cells derived from patients supplemented with FPP compared with patients receiving SoC alone for their wound care (Fig. 2C). These data support the notion that increased NOX-dependent ROS production at the wound site improved wound closure (Fig. 3). A transient inflammatory response aimed at cleansing the wounds from pathogens is a prerequisite for successful wound closure (8). Improved wound closure outcome in response to FPP supplementation was also reported in a model of wound healing in diabetic mice (2). At the wound site, NOX-derived ROS are known to be implicated in multiple mechanisms of healing, including angiogenesis (8). Indeed, in diabetic mice supplemented with FPP, wound angiogenesis was improved (2). These observations lead to the contention that FPP may influence multiple aspects of wound healing ranging from wound infection management to perfusion. Such foundation makes room for a full-length randomized clinical trial addressing these testable hypotheses.

Oral supplementation of FPP by chronic wound patients

Metabolic syndrome (dyslipidemia and hyperglycemia) is often associated with patients with chronic wounds. To test the effects of carbohydrate-rich FPP, HbA1c, glucose, and

Table 1. Demographic Characteristics of Study Participants

Sample size	22
Completed study	19
Age (years)	50.7 ± 2.7
Sex (Male:Female)	13:6
Ethnicity—Caucasian	12
Ethnicity—African American	7
BMI (kg m^{-2})	35.9 ± 2.8

Age and BMI are expressed as mean ± standard error of the mean.
BMI, body mass index.

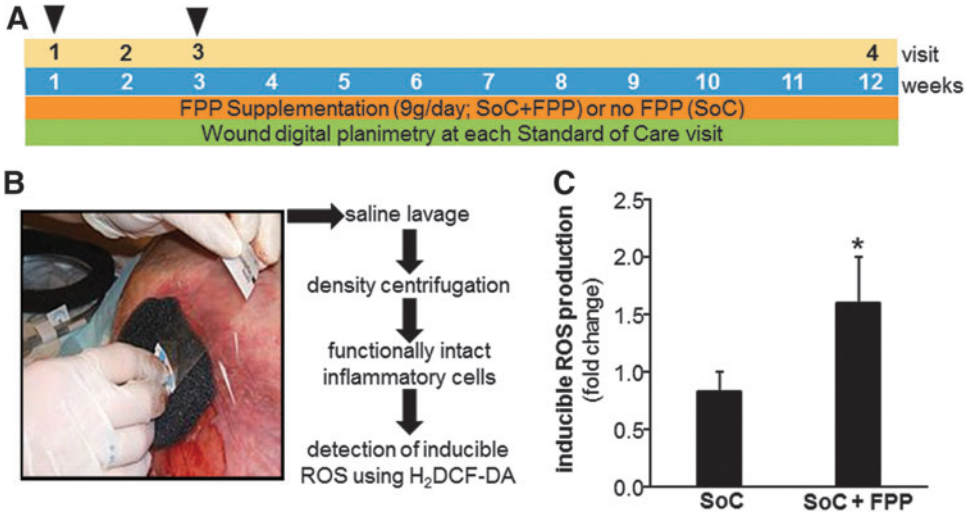

FIG. 2. Improved inducible "respiratory burst" activity in human chronic wound inflammatory cells on FPP supplementation. (A) Study design of FPP supplementation in chronic wound patients. Subjects were presented with the necessary information, and proper consent was obtained if subject agreed to enroll. Subjects were randomly assigned to two test groups, one without supplementation (SoC) and the other with a controlled dose of FPP (SoC+FPP; 3 g/dose, 3 dose/day). Wound dressings and wound images were collected at the initial visit to provide a baseline, and again at visit 3 (indicated with *black arrows*). The final visit was 11 weeks after the baseline visit. Blood was collected at visit 3. **(B)** Workflow for detection of inducible ROS from wound-site inflammatory cells lavaged from sponges of human chronic wound patients undergoing NPWT. **(C)** Wound cells were isolated from sponge of patients undergoing NPWT at baseline (0 weeks) and after 2 weeks of FPP supplementation. Inducible ROS production was measured after PMA (1 μg/mL) stimulation for 30 min. Data are expressed as fold change compared with the baseline (0 weeks). Data are presented as mean ± SEM ($n = 6$ for SoC; $n = 4$ for SoC+FPP). *$p < 0.05$ compared to SoC. H_2DCF-DA, dichlorodihydrofluorescein diacetate; NPWT, negative pressure wound therapy; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; SEM, standard error of the mean; SoC, standard of care.

lipid parameters in peripheral blood were studied as available from medical records or measured for the purposes of this study. FPP supplementation (9 g/day: 3 g/dose, 3 doses/day) did not influence the HbA1c, glucose, and total lipid profile after 2 weeks of supplementation (Fig. 4).

Nutritional deficiencies often underlie impaired wound healing. A systematic review and meta-analysis of nutritional supplementation in chronic lower extremity wounds identified 23 randomized controlled trials involving nutritional supplementation, which was found to be overall favorable (9). While the overall case for dietary supplements in wound care has strength, results on their efficacy to improve wound outcomes are equivocal making room for well-designed interventions (7).

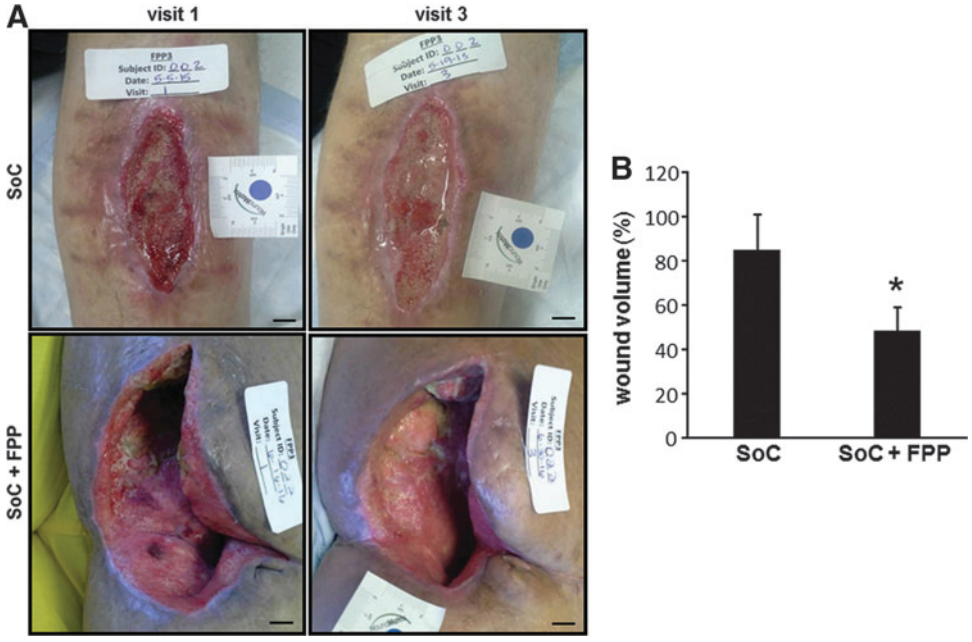

FIG. 3. Improved wound closure of human chronic wounds on FPP supplementation. Wounds were imaged on each visit, and the wound volume was calculated by using approved clinical practices. **(A)** Digital images of representative wounds at baseline and after 2 weeks of FPP supplementation. **(B)** Wound volume after 2 weeks of FPP supplementation (visit 3) calculated as percentage of initial wound volume (visit 1). Data are expressed as mean ± SEM ($n = 8$ for SoC; $n = 7$ for SoC+FPP). *$p < 0.05$ compared to SoC.

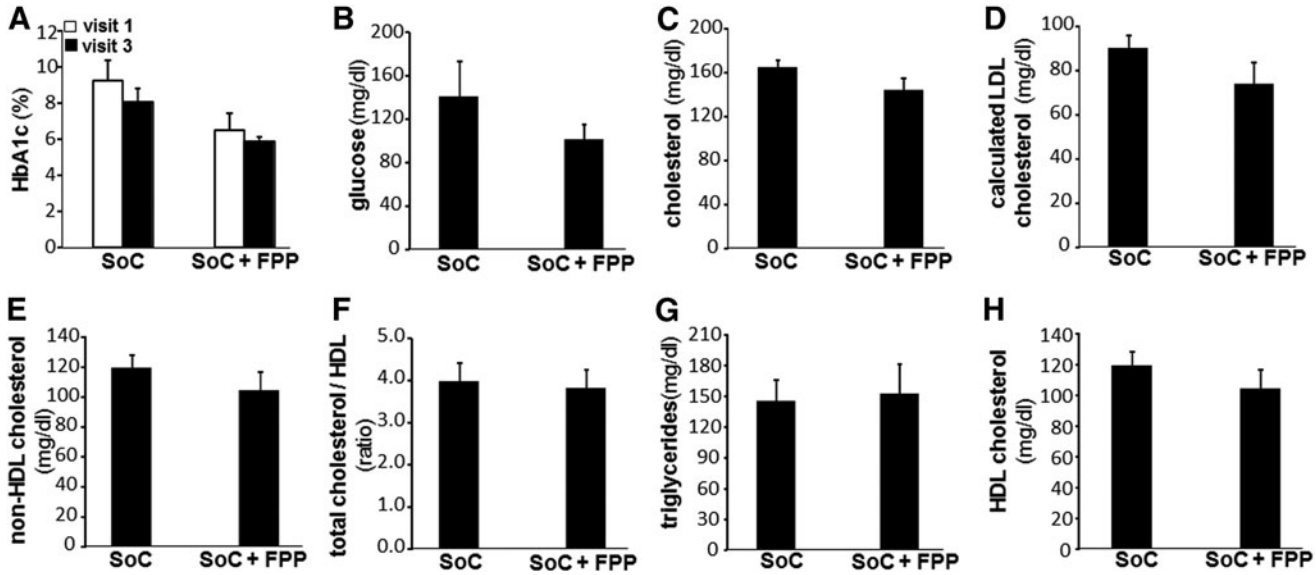

FIG. 4. Oral consumption of FPP by chronic wound patient is safe. Peripheral blood was drawn from patients suffering from chronic wounds 2 weeks after supplementation. **(A)** HbA1c was measured to determine safety of FPP supplementation. Those patients for whom HbA1c measurements were performed as part of SoC, baseline data were collected from electronic medical records. Data are expressed as mean ± SEM ($n = 6$ for SoC; $n = 3$ for SoC+FPP). **(B–H)** Glucose and lipid parameters were measured to determine safety of FPP supplementation. Data are expressed as mean ± SEM ($n = 9$ for SoC; $n = 8$ for SoC+FPP). HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Notes

Ethics, consent, and permissions

The Western Institutional Review Board approved the study protocols (clinicaltrials.gov: NCT02332993) and materials. All subjects provided written informed consent before participation in the study.

Human subjects and sample collection

Subjects participating in the study were patients with wounds undergoing NPWT. Subject demographics are presented in Table 1. Patients were randomized to be part of the control group with no supplementation or treatment group with supplementation. Once obtaining informed consent, patients in the treatment group received FPP (Immun'Age; Osato International, Gifu, Japan; 3 g/dose three times per day). A baseline wound dressing collection and wound imaging were done. Patients returned after 1 week (week 2) during which the wound dressing and image were collected. The patients revisited the next week (week 3) for wound dressing collection, blood collection, and imaging. Patients continued their SoC appointments as necessary. The final research visit was 9 weeks after visit 3 for a final wound image only. Drawn peripheral blood (10 cm^3) was sent to The Ohio State University Wexner Medical Center clinical laboratories for analysis of blood chemistry. Those patients for whom HbA1c measurements were performed as part of SoC, baseline data were collected from electronic medical records.

Wound cell isolation

Wound cells were isolated from the foam dressing of NPWT device on wounds. Wound fluid and cells were derived from the NPWT dressing by lavaging the wound dressing with saline solution. The lavaged fluid was centrifuged to obtain wound cells. Wound-site leukocytes were isolated from NPWT sponge-derived wound cells using Ficoll density centrifugation as previously described (3).

Determination of intracellular ROS

Detection of ROS in PMA (Sigma-Aldrich, St. Louis, MO; Catalog no. P8139)-activated wound cells was performed by using H_2DCF-DA (Molecular Probes, Invitrogen, Carlsbad, CA; Catalog no. D399), as described (2–4). In brief, isolated wound cells were resuspended in sterile phosphate-buffered saline and incubated with 10 μM H_2DCF-DA for 20 min at 37°C. Unstimulated ROS production was measured, followed by stimulation with PMA (1 μg/mL) for 30 min, and then ROS measured again. To detect cellular fluorescence, fluorochrome-loaded cells were excited by using a 488-nm laser in a flow cytometer. Dichlorofluorescein emission was recorded at 530 nm. Inducible ROS production was determined as ratio of each sample's post-PMA ROS production to pre-PMA ROS production. At the time of measurement of ROS, the experiment was run using the human promyelocytic leukemia (HL-60) cell line to serve as a reference control for the effect of PMA in inducing respiratory burst. Fold change was calculated as the ratio of inducible ROS production of visit 3 to visit 1.

Wound volume measurements

The wound volume (in cm^3) was calculated from the wound surface area, and the depth as recorded during standard clinical practice (6).

Sugar and sugar alcohol extraction and quantification by LC-MS/MS

Sugars and sugar alcohols were extracted following the method described by Cocuron *et al.* (1). Lyophilized samples

were resuspended in 350 μL of ultrapure water and transferred onto 3 kDa filtering devices. The extracts were centrifuged at 14,000 *g* for 60 min at 4°C. Then, 100 μL of sample was added to a vial containing 900 μL of acetonitrile/water (60:40). Ten microliters of the mixture was injected through the LC-MS/MS system and sugars and sugar alcohols were separated, detected, and quantified as previously described (1).

Statistics

Data are reported as mean ± standard error of the mean as indicated in respective figure legends. The difference between means was tested by using Student's *t*-test. A *p*-value <0.05 ($p < 0.05$) was considered statistically significant.

Acknowledgments

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Author Disclosure Statement

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Abbreviations Used

BMI = body mass index
CGD = chronic granulomatous disease
FMLP = N-formyl-methionyl-leucyl-phenylalanine
FPP = fermented papaya preparation
H_2DCF-DA = dichlorodihydrofluorescein diacetate
HDL = high-density lipoprotein
LC-MS/MS = liquid chromatography/tandem mass spectrometry
LDL = low-density lipoprotein
NOX = NADPH oxidase
NPWT = negative pressure wound therapy
PMA = phorbol 12-myristate 13-acetate
ROS = reactive oxygen species
SEM = standard error of the mean
SoC = standard of care