Protocatechuic Acid as a Topical Antimicrobial for Surgical Skin Antisepsis

Preclinical Investigations

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Background: There is a need for novel skin antiseptic agents to combat the health-care burdens associated with surgical site infection (SSI) and bacterial resistance. The purpose of this proof-of-principle pilot study was to investigate the potential of the phenolic compound protocatechuic acid (PCA) as a topical antimicrobial for surgical skin antisepsis.

Methods: The Kirby-Bauer method of disc diffusion was used to investigate the in vitro antimicrobial activity and comparative effectiveness of PCA and 7 related compounds against SSI pathogens. To explore the in vivo efficacy of topical PCA for providing deep, penetrating skin antisepsis, living Cutibacterium acnes was intradermally injected into the skin of female BALB/c mice. Mice were assigned to treatment with daily applications of topical PCA at 3 doses (78, 39, and 19.5 mM) or no treatment (n = 2 mice per group). After 96 hours, infected skin samples were harvested to compare mean C. acnes counts by treatment.

Results: Compared with other polyphenols, PCA demonstrated the broadest spectrum of antimicrobial activity against tested SSI pathogens, including drug-resistant organisms. At 96 hours following infection, the mean C. acnes burden in untreated mice was 6.65 log colony-forming units (CFUs) per gram of skin. Compared with the untreated group, daily topical application of 78 mM of PCA was associated with a significantly lower C. acnes CFU burden in mice skin (mean, 5.51 log CFUs per gram of skin; p = 0.0295). Both lower dosages of topical PCA failed to show an effect.

Conclusions: PCA demonstrated laboratory efficacy against pathogens implicated in SSI, including drug-resistant organisms. In vivo, topical PCA demonstrated dose-dependent skin penetration and antimicrobial activity against mouse skin C. acnes loads. Human clinical studies exploring the antimicrobial efficacy of topical PCA for preoperative shoulder skin antisepsis are warranted.

Clinical Relevance: Topical PCA may have the potential to improve current shoulder SSI treatment and prevention protocols.

Surgical site infection (SSI) causes substantial patient morbidity1. Since two-thirds of SSIs are confined to the incision area, much focus for SSI prevention has been on optimizing the strategy for preoperative skin antisepsis.2 Rises in bacterial resistance3-5 and insufficient skin-penetration properties6-8 have been shown to limit the efficacy of existing skin antisepsis protocols in the prevention of SSI. As a result, there is a continuing need for alternative skin antiseptic agents, with the hope of identifying reagents with a broad spectrum of utility and adequate skin penetration and without a history of overuse or resistance.

Natural substrates with antimicrobial properties have been identified as possible sources of novel antimicrobials9. There is mounting evidence that the phenolic compound protocatechuic acid (PCA) functions as a broad-spectrum antimicrobial agent, including activity against drug-resistant organisms10-12, and demonstrates potent skin-penetration properties and deep dermal bioavailability when applied topically13,14. In exerting their antibacterial activity, phenolic compounds have been shown to enhance the generation of reactive oxygen species in bacteria15 and disrupt bacterial cell-membrane integrity, causing the inhibition...
and leakage of intracellular contents. However, most recent investigations involving PCA have focused on plant extracts, of which the exact ingredients and dosages are unknown, limiting the feasibility for studies of potential clinical applications of the antimicrobial effects observed with this compound.

In vitro studies of natural compounds containing PCA have demonstrated its dose-dependent antimicrobial activity against several pathogens implicated in SSI, including Cutibacterium acnes (formerly Propionibacterium Acnes), a gram-positive anaerobe responsible for infections following shoulder surgery. In vivo, plant extracts containing PCA were shown to inhibit C. acnes-induced inflammation in mouse skin through the inhibition of lipase activity and the suppression of proinflammatory cytokines. No study, to our knowledge, has examined the efficacy of topical PCA in reducing C. acnes bacterial loads in living skin, a particularly noteworthy consideration when evaluating its potential to prevent SSI with this pathogen.

The purpose of this proof-of-principle pilot study was to investigate the potential of PCA as a topical antimicrobial for surgical skin antisepsis. Our study aims were to (1) investigate the spectrum of antimicrobial activity of PCA, focusing on its activity against skin and wound pathogens, and (2) explore the in vivo application of PCA as a topical agent for deep, penetrating skin antisepsis using a mouse model of C. acnes dermal skin infection.

Materials and Methods
Antimicrobial Susceptibility Testing

The Kirby-Bauer method of disc diffusion was used for the testing of antimicrobial efficacy and specificity. Standard procedures recommended by the National Committee for Clinical Laboratory Standards were followed. A set of discs saturated with test or control compounds were placed onto inoculated agar plates. The plates were inoculated with gram-positive, gram-negative, aerobic, and anaerobic bacteria and the fungus Candida albicans (see Appendix Table 1 for a full list of organisms included in the analysis) to determine the spectrum of antimicrobial activity of the test compounds. As we sought to identify an antimicrobial agent with the potential to reduce SSI, the primary focus of our laboratory analysis was to determine the comparative effectiveness of test compounds against Staphylococcus aureus, Pseudomonas aeruginosa, methicillin-resistant S. aureus (MRSA), and C. acnes, which are clinically challenging organisms.

The primary disinfectant used for the in vitro investigation was a 100-mM PCA solution in a water vehicle. Vanillic acid, hippuric acid, delphinidin, pelargonidin, cyanidin chloride, 28% cyanidin-3-glucoside, and 2,4,6-trihydroxybenzoic acid were also tested (at the same concentration), as they are plant secondary metabolites related to PCA. The testing of related compounds was performed to determine which compound was the likely plant secondary metabolite responsible for the antibacterial effect demonstrated in previous plant-based therapies and studies. Amoxicillin served as a positive control.

Depending on the microorganism tested, plates were incubated for 18, 24, or 48 hours. The diameters of the zones of inhibition were measured using a sliding caliper and interpreted as follows: no inhibition (NI) of growth under the test sample, inhibition (I) of growth under the test sample, no zone (NZ) of inhibition surrounding the test sample, or a clear zone (CZ) of inhibition surrounding the test sample, with the width of the clear zone (in millimeters) recorded.

Testing Against C. acnes in Mouse Skin

All experimental procedures were carried out at an independent laboratory (TransPharm Preclinical Solutions), and were performed in compliance with the laws, regulations, and guidelines of the U.S. National Institutes of Health and with the approval of the TransPharm Institutional Animal Care and Use Committee. Eight female BALB/c (Harlan) mice weighing 17 to 19 g were obtained. Each mouse was housed with 1 partner in a static cage with corn-cob bedding and had free access to food and water. Table I outlines experimental procedures and time points.

Mice were randomly assigned to 1 of 4 treatment groups: (1) no treatment, (2) 78 mM of topical PCA, (3) 39 mM of topical PCA, and (4) 19.5 mM of topical PCA (n = 2 mice per group). On the day before treatment, mice were anesthetized with a 5% isoflurane induction chamber and a 2.0 × 2.0-cm region on the dorsal area was cleared of hair and delineated as the infection site. C. acnes was selected for testing as it is responsible for the majority of shoulder SSIs, and testing against this organism may allow for some assessment of the skin-penetration properties of test reagents.

Cultures of C. acnes (American Type Culture Collection 6919) were grown for 96 hours at 37°C, in an anaerobic atmosphere on trypticase soy (TS) agar plates supplemented

| Group | Intradermal C. acnes | Treatment | Treatment Schedule† | Skin Harvest† |
|-------|---------------------|-----------|---------------------|--------------|
| 1     | 2                   | 6.0 log CFUs | Untreated          | NA           | 96 hr |
| 2     | 2                   | 6.0 log CFUs | Topical PCA, 78 mM | 2, 24, 48, 72 hr | 96 hr |
| 3     | 2                   | 6.0 log CFUs | Topical PCA, 39 mM | 2, 24, 48, 72 hr | 96 hr |
| 4     | 2                   | 6.0 log CFUs | Topical PCA, 19.5 mM | 2, 24, 48, 72 hr | 96 hr |

*NA = not applicable, CFU = colony-forming unit, and PCA = protocatechuic acid. †Time defined relative to infectious challenge at 0 hour.
with 5% sheep blood. The culture was aseptically swabbed and transferred to tubes of TS broth and allowed to grow for 72 hours. Cultures were diluted to provide an infectious inoculum of 6.0 log colony-forming units (CFUs) per 50 mL in phosphate-buffered saline (PBS) solution. On day 0, mice were again anesthetized and 50 mL of the C. acnes suspension was administered via intradermal injection 25.

Following C. acnes inoculation, topical PCA in sterile water was administered in a dose volume of 0.1 mL at 2, 24, 48, and 72 hours post-challenge. Ninety-six hours following challenge, mice were humanely killed by carbon dioxide overexposure, and skin was aseptically removed from the infection site. Skin samples were placed in homogenization vials with 2.0 mL of PBS solution, weighed, and homogenized using a mini-bead beater. Homogenate was serially diluted and plated anaerobically on TS agar plates for the enumeration of CFUs, transformed to a decimal logarithm. To control for variations in harvested tissue weights, mean values were reported per gram of skin tissue.

**Statistical Analysis**

All statistical analyses were performed using Stata (version 13.0; StataCorp). Mean (and standard deviation) bacterial burdens per gram of skin tissue were calculated and were compared between untreated mice and mice treated with each dosage of PCA using a 2-tailed t test. A p value of <0.05 was considered significant for all tests.

**Results**

**Antimicrobial Susceptibility Testing**

The antimicrobial efficacies of the test compounds against S. aureus, C. acnes, MRSA, and P. aeruginosa are shown in Table II. Saturation with 100 mM of PCA resulted in complete zones of inhibition of at least 8 mm against each of the organisms selected for primary analysis, with the greatest activity shown against C. acnes (22 mm of growth inhibition). None of the other compounds tested demonstrated antimicrobial activity against all organisms. The control, amoxicillin, demonstrated growth inhibition against S. aureus, C. acnes, and MRSA, but failed to inhibit the growth of P. aeruginosa (see Appendix Table 1 for the results of the antimicrobial susceptibility testing against microbes not included in the primary analysis).

**Antimicrobial Activity Against C. acnes**

None of the mice displayed acute adverse events associated with the treatments, and no treatment group displayed adverse signs beyond those expected for mice that have received a superficial bacterial infection.

Ninety-six hours following infection, the mean bacterial burden for the untreated mice was 6.65 log CFUs per gram of skin. Compared with the untreated group, daily topical application of 78 mM of PCA was associated with a significantly lower C. acnes CFU burden in mice skin (mean, 5.51 log CFUs; \( p = 0.0295 \)). Both lower dosages of topical PCA failed to show an effect (Table III).

**Discussion**

The present study was undertaken to investigate the potential of PCA as a topical antimicrobial for surgical skin antisepsis. PCA demonstrated antimicrobial efficacy in vitro against pathogens prevalent in SSI, and topical PCA demonstrated a dose-dependent reduction of C. acnes loads in the skin and deeper

### Table II: Antimicrobial Efficacy of Plant Secondary Metabolites Against Skin and Wound Pathogens*

| Treatment (100 mM) | S. aureus | C. acnes | MRSA | P. aeruginosa |
|-------------------|-----------|----------|------|--------------|
| PCA               | I/CZ/8 mm | I/CZ/22 mm | I/CZ/9 mm | I/CZ/10 mm |
| Delphinidin       | I/CZ/10 mm | I/NZ     | I/CZ/7 mm | NI/NZ        |
| Pelargonidin      | I/CZ/3 mm  | I/CZ/1 mm | I/CZ/4 mm | NI/NZ        |
| Cyanidin Cl       | I/CZ/1 mm  | NI/NZ     | I/CZ/3 mm | NI/NZ        |
| 28% C-3-G         | I/NZ      | I/CZ/23 mm | I/CZ/4 mm | I/CZ/2 mm    |
| 2,4,6-THBA        | I/CZ/13 mm | NI/NZ     | I/CZ/14 mm | I/CZ/3 mm    |
| Vanillic acid     | I/CZ/1 mm  | NT        | I/CZ/1 mm | I/NZ         |
| Hippuric acid     | NI/NZ     | NT        | I/CZ/1 mm | NI/NZ        |

*PCA = protocatechuic acid, I = inhibition of growth under the sample, CZ = clear zone of inhibition surrounding the sample and zone width in millimeters, NZ = no zone of inhibition surrounding the sample, NI = no inhibition of growth under the sample, Cl = chloride, C-3-G = cyanidine-3-glucoside, 2,4,6-THBA = 2,4,6-trihydroxybenzoic acid, and NT = not tested.

### Table III: C. acnes Mouse Skin Loads by Treatment

| Treatment   | C. acnes Counts* | P Value† |
|-------------|------------------|---------|
| Untreated   | 6.65 ± 0.145     | —       |
| PCA, 78 mM  | 5.51 ± 0.249     | 0.0295  |
| PCA, 39 mM  | 7.5 ± 0.725      | 0.4513  |
| PCA, 19.5 mM| 5.86 ± 0.429     | 0.2235  |

*The values are given as the mean log CFUs (colony-forming units) per gram of skin tissue and the standard deviation. †Determined using a 2-tailed t test to compare C. acnes counts between untreated mice and mice treated with each dosage of PCA.
skThe in vitro antimicrobial efficacy of plant-derived phytochemicals against skin and wound pathogens has been demonstrated previously. Kuete et al. demonstrated the broad-spectrum activity of PCA in a crude extract of Ficus ovata against gram-positive bacteria, gram-negative bacteria, and fungi, including drug-resistant organisms. Liu et al. demonstrated the efficacy of PCA derived from Roselle calyx against the drug-resistant organisms MRSA, Klebsiella pneumoniae, and P. aeruginosac. Lim et al. demonstrated the antibacterial activity of PCA in Euphorbia supina against C. acnes. Our findings agree, as saturation with PCA resulted in clear zones of inhibition against C. acnes (22 mm), S. aureus (8 mm), MRSA (9 mm), and P. aeruginosan (10 mm). Through the comparison of the antimicrobial efficacy of PCA and that of related plant secondary metabolites, our results build on previous work by identifying PCA as the likely bioactive antibacterial compound contributing to the activity of these plant-based therapies against tested skin and wound pathogens.

It is estimated that up to 50.9% of SSIs in the United States are caused by microorganisms resistant to existing prophylactic antibiotics, and this rate is increasing. Compared with infection with antibiotic-susceptible strains, SSI caused by resistant pathogens has consistently been linked to poorer outcomes and increased costs following orthopaedic procedures. In the present study, PCA demonstrated activity against all tested drug-resistant organisms, including P. aeruginosac, MRSA, Legionella, K. pneumoniaec, and methicillin-resistant S. epidermidisc (MRSE). In contrast, P. aeruginosac, K. pneumoniaec, and MRSE were unaffected by saturation with the control (see xref ref-type="sec" rid="app1c"). amoxicillin, suggesting that some bacterial-resistance patterns developed in the presence of classic antimicrobials may not provide the same resistance to this compound. In conjunction with the extremely limited number of reported cases of microbial resistance to plant secondary metabolites, these findings provide further support for investigations currently underway exploring PCA, either as monotherapy or in combination with existing antimicrobial agents, to reduce infection with drug-resistant organisms. Taken together, our laboratory investigations suggest that research into the clinical application of PCA as a skin antiseptic agent may yield promise in reducing the health-care impact of SSI.

Despite promising results from in vitro findings, few studies have explored in vivo applications of the antimicrobial activity of PCA. Yim and Chao demonstrated its potential as a food preservative, as PCA inhibited growth of antibiotic-resistant Campylobacter and aerobic bacteria in a dose-dependent fashion in meat. The present study is the first, to our knowledge, to examine the efficacy of topical PCA at reducing C. acnes bacterial loads in living skin, an important therapeutic target for the prevention of SSI caused by this pathogen.

The C. acnes rodent injection model employed here was selected to simulate the chronic inflammatory process associated with C. acnes colonization in the dermis, the layer of the skin where commercial preoperative antiseptic agents have been shown to provide inadequate eradication of C. acnes. Our animal study tested the potential of PCA as a deep, penetrating topical skin antiseptic to combat shoulder SSI.

In conclusion, PCA demonstrated laboratory efficacy against a broad spectrum of organisms (full susceptibility data in Appendix Table 1) was necessary to identify the potential for PCA as a topical agent for skin disinfection. Ideally, comparisons to current skin-preparation solutions would have allowed for more robust conclusions regarding the potential of topical PCA application for skin disinfection. Another limitation relates to the small sample size in the in vivo model, predisposing our analysis to a type-II error and limiting the precision of our dosage analysis. Furthermore, only 1 microbe was examined in the in vivo model, perhaps limiting the generalizability to other pathogens of interest.
exploring PCA as a topical antimicrobial for human skin antisepsis are warranted.

Appendix

Supporting material provided by the authors is posted with the online version of this article as a data supplement at jbjs.org (http://links.lww.com/JBJSOA/A188).
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