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Basic Research

Is Implant Coating With Tyrosol- and Antibiotic-loaded Hydrogel Effective in Reducing *Cutibacterium* (*Propionibacterium*) *acnes* Biofilm Formation? A Preliminary In Vitro Study

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Abstract

Background Studies have suggested that *Cutibacterium acnes* (formerly known as *Propionibacterium*) is the most frequently isolated pathogen after shoulder arthroplasty. To address the burden of periprosthetic joint infections associated with this pathogen, new prevention methods are needed. Tyrosol has a promising record of effectiveness in the field of biofilm-associated infections; however, to our knowledge, it has not been tested against *C. acnes* thus far.

Questions/purposes In this in vitro study, we asked: (1) Is tyrosol effective in inhibiting and eradicating *C. acnes*

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planktonic growth? (2) Is there synergy between tyrosol and rifampicin? (3) Is supplementation of hydrogel with tyrosol at the minimum inhibitory and subinhibitory concentrations efficacious in reducing free-floating *C. acnes* growth? (4) Is implant hydrogel coating (either alone or combined with tyrosol, rifampicin, or vancomycin) beneficial in reducing *C. acnes* biofilm formation? (5) Is the administration of soluble tyrosol an effective measure against *C. acnes* biofilm formation?

Methods We assessed *C. acnes* planktonic growth and eradication by inspecting visually the results of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays. We also evaluated macroscopically the presence of synergy among tyrosol and rifampicin by means of the MIC checkerboard testing. Thereafter, we addressed colorimetrically the efficacy of tyrosol-loaded Defensive Antibacterial Coating (DAC®) hydrogel against the *C. acnes* free-floating form by means of the XTT cell proliferation reduction assay. Then, we explored photometrically the effect of hydrogel and soluble tyrosol at reducing *C. acnes* biofilm formation on titanium alloy disks that simulated orthopaedic implants by using the minimum biofilm inhibition concentration assay. In particular, 16 disks were sequentially allocated to each of the following testing conditions: (1) hydrogel alone; (2) tyrosol-loaded hydrogel; (3) rifampicin-supplemented hydrogel; (4) vancomycin-loaded hydrogel; and (5) soluble tyrosol. Subsequently, implants were sonicated and cell viability was evaluated in terms of the XTT assay.

Results Tyrosol was effective in inhibiting *C. acnes* planktonic (free-floating) growth demonstrating MIC values of 63 mM (9 mg/mL) and MBC values of 250 mM (35 mg/mL). Concerning synergy assessment, the checkerboard testing revealed additivity among tyrosol and rifampicin with a fractional inhibitory concentration index of 0.56. In addition, a hydrogel coating with tyrosol at the MIC showed no difference in the inhibition of free-floating *C. Acnes* form over control (median absorbance [MA] for tyrosol-supplemented hydrogel versus control groups were 0.21 [interquartile range {IQR}, 0.19–0.24] versus 0.26 [IQR, 0.23–0.31], p = 0.066). Furthermore, loaded hydrogel with tyrosol at 597 mg/mL (1 M) was no more effective than control in reducing *C. acnes* biofilm formation (MAs for tyrosol versus control were 0.12 [IQR, 0.11–0.13] versus 0.14 [IQR, 0.12–0.16], respectively; p = 0.076). This was also the case when we considered hydrogel in conjunction with vancomycin and rifampicin (MAs for vancomycin at 2% and 5% and rifampicin at 1% versus biofilm control were 0.139 [IQR, 0.133–0.143] and 0.141 [IQR, 0.133–0.143] and 0.135 [IQR, 0.128–0.146] versus 0.142 [IQR, 0.136–0.144], correspondingly). In contrast, soluble tyrosol at 597 mg/mL (1 M) inhibited biofilm formation compared to control (MAs for tyrosol and control groups were 0.11 [IQR, 0.09–0.13] versus 0.13 [IQR, 0.12–0.14], p = 0.007).

Conclusions Although the implant coating with hydrogel (either pure or supplemented with antimicrobial agents) did not diminish *C. acnes* biofilm development in vitro, soluble tyrosol at 597 mg/mL (1 M) exceeded the meaningful biofilm inhibition threshold of 80%.

Clinical Relevance The results of the current preclinical investigation did not support the use of a fast, bioresorbable hydrogel as a coating method against *C. acnes* biofilms. Instead, direct local administration of soluble tyrosol at high concentrations should be further tested in future animal studies.

Introduction

Cutibacterium acnes (historically known as *Propionibacterium*) is a low-virulence, slow-growing, Gram-positive rod, which is linked to spine [4, 5, 41] and shoulder [19, 35, 36] infections after orthopaedic surgery. A recent meta-analysis has suggested that this is the most frequently isolated pathogen after shoulder arthroplasty, representing 38.9% of all such infections [45]. Given the estimated economic burden of USD 36,500 per patient for the management of a periprosthetic joint infection (PJI) [32], the need for new prevention methods is great. Antibacterial coatings seem a promising avenue to explore because they may prevent bacterial adhesion to orthopaedic biomaterials [63], an early stage in the process of infection. The pathogenesis of PJI of *C. acnes* is tightly related to its ability to grow in biofilms [3, 11], which are defined as "structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface" [18]. The bacteria enclosed by exopolysaccharides in the biofilm state are less susceptible to immune system defenses and antimicrobial therapy, thus rendering these low-grade infections difficult to diagnose and eradicate [3].

To achieve the objectives of inhibiting *C. acnes* biofilm formation on orthopaedic implants and confronting growing multidrug resistance, use of nonantibiotic agents in terms of low-cost, seemingly safe, and effective compounds has been recommended [28]. Tyrosol, a quorum-sensing molecule (that is, a molecule that regulates cell-to-cell communication) [7, 14] present in olive oil and produced by *Candida albicans* [6], appears to fulfill the previously mentioned criteria because this agent is an inexpensive natural phenolic compound [7, 10, 37] with substantial antimicrobial/antibiofilm activity against multiple aerobic microorganisms [1, 2] and in vitro studies suggest, preliminarily, that the compound may be safe to use [7, 10, 37]. To our knowledge, this particular molecule has not been previously tested against *C. acnes*. To achieve satisfactory tyrosol release in a controlled fashion, use of antibacterial coatings such as collagen sponges [29], cancellous bone [13], elution systems [44], and hydrogel can be considered [26]. One example of a

novel and quickly bioresorbed hydrogel is the Defensive Antibacterial Coating (DAC®; Novagenit Srl. Mezzolombardo, Italy), which consists of covalently linked hyaluronan and poly-D,L-lactide and possibly resists declothing in press-fit fixation [20]. This particular coating reduced aerobic bacterial colonization and biofilm formation in an *in vitro* model of infection, apparently by acting as a physical barrier to biofilm development [20].

In the current preclinical (in vitro) investigation, we sought answers to the following questions: (1) Is tyrosol effective in inhibiting and eradicating *C. acnes* planktonic growth? (2) Is there synergy between tyrosol and rifampicin? (3) Is supplementation of hydrogel with tyrosol at the minimum inhibitory and subinhibitory concentrations efficacious in reducing free-floating *C. acnes* growth? (4) Is implant hydrogel coating (alone or loaded with tyrosol, rifampicin, or vancomycin) beneficial in reducing *C. acnes* biofilm formation? (5) Is the administration of soluble tyrosol an effective measure against *C. acnes* biofilm development in vitro?

Materials and Methods

An in vitro model was implemented because our objective to bridge the literature gap in this field rendered our research preliminary in nature. The initial experiment pertaining to confocal laser scanning microscopy (CLSM) study was conducted in the Laboratory of Clinical Chemistry and Microbiology, IRCCS Orthopedic Institute Galeazzi (Milan, Italy) and the remainder of the investigations were undertaken at the 1st Department of Pharmacology of the Medical School of Aristotle University of Thessaloniki (Thessaloniki, Greece). We used standard *C. acnes* ATCC 11827, which is the most extensively investigated strain in pertinent literature [27, 51, 57].

Local Antimicrobial Agents

Among the available nonantibiotic compounds, we chose tyrosol (2-[4-hydroxyphenyl] ethanol) (purchased from Sigma St Louis, MO, USA) because its antimicrobial/antibiofilm activity against aerobic pathogens is well documented [1, 2].

In terms of antibiotic agents, we opted for vancomycin (Fresenius, Kabi, Bad Homburg, Germany), which is one of the most widely used compounds when it comes to local intraoperative administration [21, 31], as well as rifampicin (Sanofi-Aventis, Tours, France), which is highly effective in eradicating *Staphylococcus* and *C. acnes* biofilms according to earlier preclinical and in vivo studies [54, 57]. We dissolved each antibiotic in distilled water to achieve the concentration of 10% and preserved a stock solution at -20° C for up to 1 month. Further dilutions were performed to achieve the predetermined working concentrations of 1% and 2% to 5% for rifampicin and vancomycin, respectively.

Biomaterials

To simulate orthopaedic implants, sandblasted titanium alloy (Ti6Al4V) disks (diameter 4 mm, height 2 mm) were purchased by commercial industry (see Figure, Supplemental Digital Content 1, http://links.lww.com/CORR/A138). We considered this particular biomaterial on the grounds that titanium and its alloys have been widely used in shoulder and spine-related orthopaedic surgery since 1950 [23]. Before use, implants were mechanically rinsed and autoclave-sterilized at 121° C.

Evaluation of Minimum Inhibitory and Bactericidal Concentrations

For the purpose of this study, minimum inhibitory concentration (MIC) was visually determined using broth microdilution assay in accordance with the Clinical and Laboratory Standards Institute guidelines and defined as the lowest concentration of antibacterial agent in the presence of which *C. acnes* was not capable of growing [16]. Of note, to ensure reliability in our evaluation, all MIC testing conditions were technically (that is, within the same experiment) replicated three times. Briefly, a suspension of *C. acnes* recovered from Schaedler agar plates (bioMérieux, Marcy l'Etoile, France) was prepared in thioglycolate to an optical density of 0.5 McFarland (approximately 1.5 x 10^8 colony-forming units [CFU]/mL) and successively inoculated to a final concentration of 10^5 CFU/mL in a 96-well microplate containing serial twofold dilutions of the testing molecules. MIC values, corresponding to the lowest concentration exhibiting no visible bacterial growth, were read after 48 hours. Optimal growth conditions were achieved by using anaerobiosis-generating sachets and the respective jar (Oxoid™ AnaeroGen™ 2.5 L; ThermoScientific, Waltham, MA, USA) [57]. We addressed the effects of the following antimicrobial compounds against planktonic *C. acnes*: (1) rifampicin; (2) vancomycin; (3) tyrosol; (4) pure hydrogel; (5) tyrosol-loaded hydrogel; (6) daptomycin (Novartis Pharma AG, Bern, Switzerland); (7) clindamycin (Fresenius); (8) ciprofloxacin (Cooper, Athens, Greece); and (9) penicillin (Cooper, Athens, Greece).

We also determined tyrosol minimum bactericidal concentration (MBC) as the lowest concentration that killed > 99.9% of the cells of the initial inoculum. After completing the visual assessment of the MIC assay, an aliquot of 10 μL was withdrawn from each well and subjected to plating. After anaerobic incubation, any absence of turbidity into Schaedler agar plates was assessed.

Synergy Exploration

To investigate the presence of synergy among tyrosol and rifampin, we proceeded with an MIC checkerboard testing and successively evaluated the results in a visual manner. In particular, we calculated the fractional inhibitory concentration index (FICI) after implementing the following formula [56]: $FIC_{tyrosol} + FIC_{rifampin} = FICI$ ($FIC_{tyrosol}$ = MIC of tyrosol in combination/MIC of tyrosol alone; and $FIC_{rifampicin}$ = MIC of rifampin in combination/MIC of rifampin alone. To interpret the retrieved FICI value, we considered the following classification: FICI $\leq$ 0.5 indicated synergy; FICI of > 0.5 to < 1 denoted additivity; 1 < FICI < 4 revealed no interaction; and FICI > 4 demonstrated antagonism.

Coating of Biomaterials

The experiments presented subsequently were performed using the DAC hydrogel, which is a hydrolytically biodegradable medical device intended for the intraoperative coating of orthopaedic implants. Currently, notwithstanding the fact that this device is Conformité Européenne-marked, it is not approved by the US Food and Drug Administration for sale.

Each titanium disk was weighed in a sterile fashion using analytical balance. DAC was then reconstituted with respect to sterilization as per the manufacturer's instructions with or without supplementation with antimicrobials depending on our preplanned protocol. After complete product hydration, the titanium disks were dipped in hydrogel and subsequently placed at the bottom of each well. Afterward, each coated implant was weighed again and the obtained value was subtracted from the initial measurement to specify the exact coating weight. At the end of this procedure, an increase of 7 mg (SD = 2) was documented.

Impact of Tyrosol-loaded Hydrogel on the C. acnes Free-floating Form

To address the effect of hydrogel (used either alone or in conjunction with tyrosol) on *C. acnes* planktonic growth, the following coating conditions were considered: (1) hydrogel alone; (2) tyrosol-loaded hydrogel at the MIC (that is, 63 mM [9 mg/mL]); and (3) tyrosol-loaded hydrogel at subinhibitory concentrations (that is, 31 mM [4 mg/mL] and 16 mM [2 mg/mL]).

Mature Biofilm Production

Before undertaking any biofilm experiments, we ensured that *C. acnes* was capable of producing mature biofilm. Accordingly, not only did we proceed with colony-forming unit (CFU) counting of the sonicated fluid obtained from biofilm control samples, but also evaluated biofilm formation on sandblasted titanium disks by means of confocal laser microscopy after 72 hours of anaerobic incubation (Fig. 1). More specifically, for the CFU counting, an aliquot of 10 μL was withdrawn and subjected to plating (Schaedler agar plates by bioMérieux, Marcy I'Etoile, France), spreading, and anaerobic incubation for 72 hours. For CLSM analysis, biofilms grown on titanium disks were gently washed with sterile saline and stained for 15 minutes at room temperature in the dark with a Filmtracer™ LIVE/DEAD™ Biofilm Viability Kit (ThermoScientific, Waltham, MA, USA) according to the manufacturer's instructions. The staining solution was prepared by adding 3 μL of SYTO9 (able to permeate intact membranes of live cells) and 3 μL of propidium iodide (able to stain only dead cells with damaged membranes) to 1 mL of filter-sterilized water. After incubation, samples were washed with sterile saline and examined with an upright TCS SP8 (Leica Microsystems CMS GmbH, Mannheim, Germany) using a 20x dry objective (HC PL FLUOTAR 20x/0.50 DRY). A 488-nm laser line was used to excite SYTO9, whereas a 552-nm laser line was used to excite propidium iodide. Bacterial count revealed significant bacterial growth (> 10^5 CFU/mL) suggesting strong biofilm production, also confirmed by the images acquired by CLSM, which displayed organized structures of clustered cells all over the titanium surface (Fig. 1).

Evaluation of Minimum Biofilm Inhibitory Concentration

For the purpose of the current study, the minimum biofilm inhibition concentration was defined as the concentration

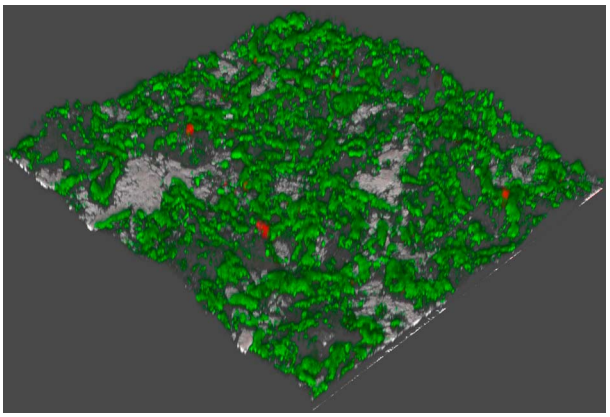

Fig. 1 Three-dimensional representation of a 72-hour *C. acnes* biofilm. Live cells are depicted in green, whereas dead ones are red. The titanium disk surface is illustrated in gray.

of a substance in a sequential twofold dilution series that inhibited biofilm growth by 80% as a minimum when compared with controls. This particular assay was adjusted in a fashion similar to that of a broth microdilution pattern.

At the beginning of each reported biofilm study, *C. acnes* was cultured on Schaedler agar plates and incubated for 72 hours at 37° C. Then, *C. acnes* colonies were suspended until a turbidity of 0.5 McFarland standard, corresponding to a bacterial concentration of approximately 10^8 CFU/mL, was obtained. For all the experiments, we used thioglycolate broth (ThermoScientific), which is suitable for anaerobic cultures.

To assess the biofilm inhibitory activity of the study substances, we sequentially allocated 16 disks to each of the following testing conditions: (1) hydrogel alone; (2) tyrosol-loaded hydrogel (at 1 M [597 mg/mL], 250 mM [149 mg/mL], 125 mM [75 mg/mL], and 63 mM [9 mg/m:]); (3) rifampicin-supplemented hydrogel (at a concentration of 1%); (4) vancomycin-loaded hydrogel (at 2% and 5%); and (5) soluble tyrosol (at 1 M [597 mg/mL], 500 mM [299 mg/mL], 250 mM [149 mg/mL], 125 mM [74.6 mg/mL], 63 mM [9 mg/mL], 31 mM [4 mg/mL], and 15 mM [2 mg/mL]). For all the reported investigations, we also implemented positive and negative control groups to ensure reliability in the estimation of absorbance reduction.

Grown biofilms on titanium disks were carefully rinsed three times using NaCl 0.9% to remove any unbound bacteria. To achieve watertightness, the working microtiter plates were sealed with Parafilm before being introduced into a sonicator set at 35 kHz (Transsonic 570 Elma, Singen, Germany). Subsequently, for the retained bacteria to be reliably dislodged from the biomaterials, the disks were sonicated for 5 minutes at 37° C [12, 33] and thereafter subjected to shaking by means of a microtiter shaker. Afterward, a volume of 100 μL of the supernatant was transferred from each well to a new microtiter plate and the XTT ([2,3-bis{2-methoxy-4-nitro-5-sulfophenyl}-2H-tetrazolium-5-carboxanilide]) (ThermoScientific) reduction assay was executed. To be more specific, we quantified bacterial biofilms using this well-established colorimetric assay, which allowed us to reliably discriminate between living and dead microorganisms [34, 47, 48] as well as accounting for multiple replicates. At the end of this assay, absorbance was read spectrophotometrically (Epoch™ BioTek, Winooski, VT, USA). In particular, the difference in absorbance values between treatment and control groups was proportional to the decline in metabolically active *C. acnes* cells [52].

From a methodological perspective, for biofilm studies addressing the results of hydrogel, we accounted for any potential turbidity generated by hydrogel, which may have obscured our findings by considering not only measurements obtained at 450 nm, but also 650 nm.

Endpoints of Interest

We assessed *C. acnes* planktonic growth in the presence of various antimicrobial agents by means of visual inspection of the results of the MIC assay. We also assessed the *C. acnes* eradication potential of tyrosol in terms of the MBC assay. Furthermore, we investigated the presence of synergy among the primary study substance (tyrosol) and rifampicin using an MIC checkerboard testing, aiming to deal with the issue of antimicrobial monotherapy resistance development [50]. Additionally, we assessed the efficacy of hydrogel coating (implemented on titanium disks either alone or in conjunction with tyrosol) on *C. acnes* planktonic (free-floating/free-swimming) cells by spectophotometry. Finally, we colorimetrically addressed the reduction of biofilm development after considering soluble tyrosol as well as implant coating with hydrogel (either alone or supplemented with various antimicrobial agents) utilizing a minimum biofilm inhibition concentration (MBIC) assay.

Sample Size Calculation

To ensure validity and reliability in our results, a sample size calculation was performed as per published guidelines [40]. Given the fact that the desired prevention rate for *C. acnes* growth ranges between 80% and 100% [46], a sum total of 213 disks was predetermined after setting statistical power at 0.8 and α and β errors at 5% and 20%, respectively. We also prespecified interim analyses [58] at 30%, 50%, and 70% of our initial sample size estimation. This sequential testing enabled us to terminate recruitment at an interim stage (that is approximately 30%), because sufficient data were available to draw robust conclusions. At that stage, we did not expect our findings pertaining to hydrogel coating to achieve the meaningful biofilm inhibition rates of 80% even if we had included more disks.

Statistics and Interpretation of the Results

Statistical analyses were performed using SPSS 25.0 software (SPSS, Chicago, IL, USA). To begin with, the depended variables represented the absorbance measurements, whereas the independent ones the intervention groups. For all the analyses, a normality test was executed and depending on its results, either parametric or nonparametric tests were applied. To be more exact, in cases following normal distribution, comparisons of mean values for two and multiple groups were performed in terms of the independent-sample t-test and analysis of variance, respectively. On the other hand, if nonnormal

distribution was revealed, medians were compared using Mann-Whitney and Kruskal-Wallis tests for two and multiple groups, respectively. Finally, Prism 6 (GraphPad Software, Inc, La Jolla, CA, USA) was utilized for graph creation.

To interpret our findings, we considered not only the p values we obtained after executing the aforementioned statistical tests, but also the biofilm growth reduction rates. To achieve this objective, we determined a p value of < 0.05 in advance to indicate significance and specified a threshold of 80% in the amount of biofilm reduction to reflect a meaningful inhibition.

Results

Assessment of Minimum Inhibitory and Bactericidal Concentrations

Tyrosol was effective in preventing and eradicating *C. acnes* planktonic growth, demonstrating MIC and MBC values of 9 mg/mL (63 mM [9 mg/mL]) and 250 mM (35 mg/mL), respectively. Among the tested antibiotics, rifampicin and vancomycin inhibited *C. acnes* development at 0.008 μg/mL and 0.06 μg/mL, in that order (Table 1). On the other hand, clindamycin and ciprofloxacin appeared to be less efficacious against free-floating *C. acnes* form (MICs were 1 μg/mL and 0.3 μg/mL, respectively).

Synergy Assessment

After combining tyrosol with rifampin, an FICI of 0.56 was calculated, which is suggestive of additivity. More specifically, the MICs of tyrosol considered in conjunction with rifampicin and rifampicin administered in combination with tyrosol were lower than those of the standalone interventions (31 mM [4 mg/mL] and 0.0005 μg/mL, respectively).

Impact of Tyrosol-supplemented Hydrogel on the Free-floating C. acnes Form

Tyrosol loading at the inhibitory concentration of 63 mM (9 mg/mL) did not result in inhibition of *C. acnes* planktonic growth (median absorbances for tyrosol versus planktonic control groups were 0.21 [interquartile range {IQR}, 0.19–0.24] versus 0.26 [IQR, 0.23–0.31], $p = 0.07$) (Table 2).

Impact of Hydrogel on C. acnes Biofilms

With the numbers available, we did not find any differences between tyrosol-supplemented hydrogel at 1 M (597 mg/mL) and biofilm controls (median absorbances were 0.12 [IQR, 0.11–0.13] versus 0.14 [IQR, 0.12-0.16],

Table 1. The inhibitory effect as well as class and action mechanism of various antimicrobial agents against planktonic *Cutibacterium acnes* is presented

Antimicrobial agent	Class/action mechanism	Molecular weight (g/mol)	MIC (μg/mL)	MIC (mM)
Tyrosol	Phenolic quorum-sensing molecule produced by *Candida albicans*	138	8,636	63
Rifampicin	Rifamycin: DNA-dependent RNA polymerase inhibition	823	0.0078	9.5E-06
Vancomycin	Glycopeptide: incorporation of the antibiotic into the cell wall during synthesis, resulting in destruction of the structure of the cell wall	1449	0.0625	4.3E-05
Ciprofloxacin	Fluoroquinolone: bacterial gyrase inhibition, thus interfering with bacterial DNA supercoiling	331	0.25	0.00075
Penicillin	β-lactam antibiotic: bactericidal action achieved through binding to the penicillin-binding proteins, which are involved in cell wall development	243	0.0156	6.4E-05
Clindamycin	Lincosamide: either bacteriostatic or bactericidal action depending on pathogen concentration, bacterial species and drug concentration	425	1	0.002
Daptomycin	Ribosomal 50S RNA inhibition	1620	0.125	7.7E-05

DNA= deoxyribonucleic acid; MIC = minimum inhibitory concentration; mM= millimolar; RNA= ribonucleic acid

Table 2. The antimicrobial activity of hydrogel (considered either alone or supplemented with tyrosol at the minimum inhibitory and subinhibitory concentrations)

Treatment group	Mean absorbance difference* (SD)	p values for parametric tests†	Median absorbance (IQR)		p values for nonparametric tests†
			Intervention group	Control group	
Hydrogel alone	0.027 (0.096)	0.188	0.24 (0.21-0.25)	0.26 (0.23- 0.31)	0.221
Tyrosol-loaded hydrogel at 62.5 mM (8.6 mg/mL)	-	-	0.21 (0.19-0.24)		0.066
Tyrosol-loaded hydrogel at 31.25 mM (4.3 mg/mL)	0.022 (0.097)	0.292	0.23 (0.2-0.27)		0.298
Tyrosol-loaded hydrogel at 15.625 mM (2.2 mg/mL)	0.024 (0.104)	0.283	0.25 (0.19-0.27)		0.408

No statistically significant differences are demonstrated, indicating absence of planktonic growth inhibition.
*between intervention and untreated control groups.
†$p < 0.05$ indicates significance; IQR= interquartile range.

p = 0.076) (see Table, Supplemental Digital Content 2 http://links.lww.com/CORR/A137). In terms of antibiotic-loaded hydrogel, neither did vancomycin at 2% and 5% nor rifampicin at 1% inhibit *C. acnes* biofilm formation (median absorbances were 0.139 [IQR, 0.133–0.143], 0.141 [IQR, 0.133–0.143], and 0.135 [IQR, 0.128–0.146] versus 0.142 [IQR, 0.136–0.144); p values were 0.342, 0.459, and 0.519, in that order) (see Table, Supplemental Digital Content 2 http://links.lww.com/CORR/A137).

Soluble Tyrosol Anti-Biofilm Activity

Soluble tyrosol at 1 M (597 mg/mL) was more efficacious than controls in inhibiting *C. acnes* biofilm formation (median absorbances were 0.11 [IQR, 0.09–0.13] versus 0.13 [IQR, 0.12–0.14], p = 0.007) (Table 3; Fig. 2). To elaborate further, only when the aforementioned concentration was considered was the meaningful biofilm inhibition threshold of 80% exceeded. Notably, differences in between-group comparisons were revealed after executing the Kruskal-Wallis test (median absorbances for soluble tyrosol at 1 M was 0.22 [IQR, 0.2–0.23] versus at 500 mM, which was 0.24 [IQR, 0.22–0.25] versus at 250 mM, which was 0.24 [IQR, 0.23–0.25] versus at 125 mM, which was 0.246 [IQR, 0.23–0.25] versus at 63 mM [8.6 mg/mL], which was 0.248 [IQR, 0.23–0.25] versus at 31 mM [4.3 mg/mL], which was 0.24 [IQR, 0.22–0.25] versus at 15 mM, which was 0.25 [IQR, 0.21–0.26]; p = 0.018).

Discussion

Preventing *C. acnes* device-associated infections poses a great challenge for orthopaedic surgeons not only as a result of the increased incidence of isolation of this Gram-positive bacillus in shoulder and spine surgery, but also owing to the dreadful complications resulting from such infections. We attempted to bridge this gap by exploring the inhibitory impact of a promising derivative of tyrosine [15] (that is, tyrosol) against *C. acnes* planktonic and biofilm forms after considering compelling published data supporting the antimicrobial effects of quorum-sensing molecules produced by *C. albicans* [61]. To achieve this objective, we implemented an in vitro model, which allowed for a simple, species-specific, and comprehensive analysis of the study substances [49, 62]. The decision on the most suitable study design also depended on the paucity of literature addressing the efficacy of tyrosol against anaerobic bacteria. The major finding of the current investigation indicated that soluble tyrosol at high concentrations was effective in inhibiting *C. acnes* biofilm formation on titanium implants. In light of this discovery, we claim that this particular molecule can be considered a candidate in the field of prevention of implant-related infections, provided that its antibiofilm activity and safety will be first validated in future studies.

We recognize that there are several limitations in the current article. First, we advise that caution should be exercised when extrapolating preclinical study results back to the biology of a living organism. As such, feasibility of local tyrosol administration should be further tested in animal studies, particularly in terms of rat models, which are evidenced to be suitable for such investigations [38]. Second, we emphasize that safety for use in humans cannot be established from in vitro studies performed thus far [7, 10, 37]. Third, we recognize that the evaluation of more intervention arms in our study would have been of value because the results of various antimicrobial combinations could have been tested. Combining interventions may not only result in improved infection eradication, but also decrease the possibility of antibiotic resistance development.

Table 3. The antibiofilm activity of soluble tyrosol

Concentration of soluble tyrosol	Mean absorbance difference* (SD)	p value for parametric test†	Median absorbance (IQR)		p value for nonparametric test†
			Intervention group	Untreated control group	
1 M (597.1 mg/mL)	0.04 (0.44)	**0.0002**	0.219 (0.2-0.23)	0.252 (0.24–0.28)	**0.0003**
500 mM (298.5 mg/mL)	0.019 (0.44)	**0.03**	0.241 (0.22-0.25)		**0.03**
250 mM (149.3 mg/mL)	0.019 (0.4)	**0.028**	0.239 (0.23-0.25)		**0.04**
125 mM (74.6 mg/mL)	-	-	0.246 (0.23-0.25)		0.127
62.5 mM (8.6 mg/mL)	0.014 (0.04)	0.116	0.248 (0.23-0.25)		0.169
31.25 mM (4.3 mg/mL)	0.019 (0.44)	**0.039**	0.24 (0.22-0.25)		0.09
15.125 mM (2.2 mg/mL)	0.019 (0.05)	0.058	0.247 (0.21-0.26)		0.187

*The findings on statistical comparisons between intervention groups and untreated controls are shown.
†p < 0.05 denotes statistical significance and displayed in bold; IQR = interquartile range.

From a mechanical perspective, we did not expand our experiments to include commonly used component biomaterials other than titanium alloy (other materials include cobalt-chromium, polyethylene, polymethylmethacrylate, or ultrahigh-molecular-weight polyethylene). The rationale behind the use of titanium alloy was that this particular biomaterial presents a higher affinity to *C. acnes* adhesion when compared with stainless steel and cobalt chromium [55]. Biomechanically, titanium presents a lower modulus of elasticity (ranging from 110 to 55 GPa) in constrast to chromium-cobalt alloys (240 GPa) and 316 L stainless steel (210 GPa) [23]. In the clinical setting, orthopaedic surgeons should also bear in mind that a major determinant of bacterial adherence to implants is the response of each individual patient to the infective microorganism rather than the actual biomaterials [25]. Concerning the surface of the disks, we opted for using roughened titanium implants as a result of the fact that there is a strong correlation between roughness of biomaterials and degree of bacterial attachment [30, 42, 59]. On top of that, it is evidenced that roughened titanium surfaces are correlated with improved osteointegration compared with smoother ones [23].

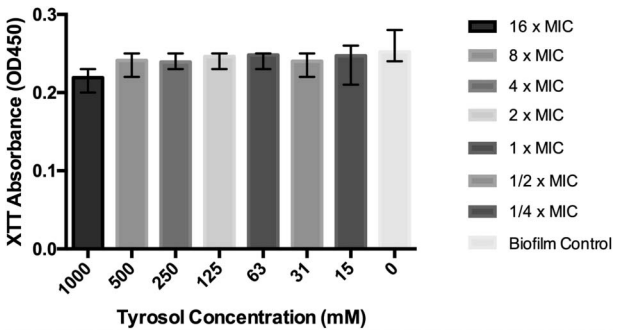

Fig. 2 The inhibitory effect of soluble tyrosol against *C. acnes* biofilms is demonstrated. On the y-axis, XTT median absorbances with their interquartile ranges are depicted. OD = optical density; MIC= minimum inhibitory concentration.

We demonstrated that tyrosol is capable of inhibiting and eradicating the *C. acnes* free-floating form. We believe that tyrosol is a promising candidate molecule in the battle against multidrug resistance and local tissue toxicity associated with conventional antibiotics [8, 22, 43]. Other factors worth considering—which may also favor tyrosol over locally administered agents—may be diminished osseointegration interference and perhaps improved antibiofilm activity, though further studies on these endpoints are needed. From a biologic perspective, it is postulated that this molecule presents low cytotoxicity levels [7, 10, 37] as well as antigenotoxic, antiinflammatory, and cancer-preventing properties [60]. Based on our promising findings, we consider that future studies should specifically focus on nonantibiotic approaches addressing multiple types of bacteria [19].

We found evidence of synergy between tyrosol and rifampicin. Taking advantage of such combinations is important to further increase the PJIs prevention potential while minimizing antibiotic resistance. Importantly, in the setting of a biofilm-associated infection, implementation of rifampicin in conjunction with other antimicrobial agents has been supported and is considered good clinical practice [50]. Based on our study, we recommend that future animal models investigate the impact of combined interventions against *C. acnes* biofilms.

Concerning the results of our passive and active antibacterial coating [63] with hydrogel, we highlight that this method was neither effective in reducing *C. acnes* biofilm formation on titanium alloy disks nor beneficial in inhibiting planktonic growth of this slow-growing rod. It should be mentioned that DAC undergoes complete hydrolytic degradation within 72 hours and, like other coating options [13, 29, 44], allows for local release of antimicrobial compounds in a controlled fashion. Intriguingly, contradictory results have been demonstrated in prior in vitro studies, which have underlined the inhibitory potential of DAC against multiple aerobic bacteria [20, 24]. Likewise, we did not observe any additional benefits over biofilm controls when we loaded hydrogel with vancomycin, although the latter antibiotic demonstrates considerable antistaphylococcal activity and is widely used in shoulder and spine surgery [31]. Notably, earlier randomized trials have indicated that antibiotic-loaded hydrogel implementation yielded reduced post-surgical site infections compared with control after internal fixation for closed fractures as well as lower limb arthroplasty [39, 53]. According to those studies, DAC neither resulted in unintended events nor compromised bone healing.

By contrast, a biofilm reduction of > 80% was achieved when tyrosol at 597 mg/mL (1 M) was considered, suggesting a strong inhibitory potential. In addition, others have found that this natural phenolic molecule presents considerable antibiofilm activity against *Staphylococcus aureus, Pseudomonas aeruginosa, C. albicans, Candida glabrata,* and *Streptococcus mutans* [2, 9, 17]. We also note that the exact mechanism of antibiofilm action of tyrosol still remains unclear [9]. In terms of reconstituting this particular compound, the desired concentrations can be achieved by dilution of the readily available tyrosol powder. Nevertheless, we advise that unwarranted extrapolations of our positive findings related to soluble tyrosol be avoided because it is still unknown whether intraoperative implantation of an orthopaedic device affects local agent delivery and, therefore, antibiofilm activity.

In conclusion, tyrosol presented not only a substantial antimicrobial activity against the *C. acnes* free-floating form, but also additivity with rifampicin. On the other hand, implant coating with hydrogel did not diminish *C. acnes* planktonic and biofilm development in vitro regardless of supplementation with antimicrobial compounds at various concentrations. Conversely, when soluble tyrosol at high concentrations was used, the minimum biofilm reduction threshold of 80% was exceeded, thereby achieving meaningful inhibition. We also underline that further validation in animal studies would be of essence and, if those deliver promising results, then human clinical trials will be called for.

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