J Infect Chemother 22 (2016) 426–429

Contents lists available at ScienceDirect

Journal of Infection and Chemotherapy

journal homepage: http://www.elsevier.com/locate/jic

Note

In vitro antimicrobial activity of benzoyl peroxide against *Propionibacterium acnes* assessed by a novel susceptibility testing method

Kazuaki Okamoto [a, c, *], Fumiaki Ikeda [a], Shoji Kanayama [a, c], Akiko Nakajima [a], Tatsumi Matsumoto [a], Ritsuko Ishii [a], Masatoshi Umehara [b], Naomasa Gotoh [c], Naoki Hayashi [c], Takako Iyoda [d], Kaoru Matsuzaki [d], Satoru Matsumoto [d], Makoto Kawashima [e]

[a] Drug Development Research Laboratories, Kyoto R&D Center, Maruho Co., Ltd., Japan
[b] CMC Research Laboratories, Kyoto R&D Center, Maruho Co., Ltd., Japan
[c] Department of Microbiology and Infection Control Science, Kyoto Pharmaceutical University, Japan
[d] Clinical Trial Testing Department, LSI Medience Co., Japan
[e] Department of Dermatology, Tokyo Women's Medical University, Japan

ARTICLE INFO

Article history:
Received 10 June 2015
Received in revised form
17 November 2015
Accepted 9 December 2015
Available online 21 January 2016

Keywords:
Propionibacterium acnes
Acne vulgaris
Benzoyl peroxide
MIC
MBC
Bactericidal

ABSTRACT

Benzoyl peroxide (BPO), a therapeutic agent for acne vulgaris, was assessed for *in vitro* antimicrobial activity against *Propionibacterium acnes* using a novel broth microdilution testing that improved BPO solubility.

We searched for a suitable culture medium to measure the minimum inhibitory concentration (MIC) of BPO against *P. acnes* and finally found the Gifu anaerobic medium (GAM) broth supplemented with 0.1(v/v)% glycerol and 2(v/v)% Tween 80, in which BPO dissolved up to 1250 μg/mL and *P. acnes* grew well. The MICs and minimum bactericidal concentrations (MBCs) of BPO against 44 clinical isolates of *P. acnes* collected from Japanese patients with acne vulgaris were determined by our testing method using the supplemented GAM broth. The MICs of BPO were 128 or 256 μg/mL against all isolates of *P. acnes* regardless of susceptibility to nadifloxacin or clindamycin. The MBCs of BPO were also 128 or 256 μg/mL against the same isolates. Moreover, BPO at the MIC showed a rapid bactericidal activity against *P. acnes* ATCC11827 in time-kill assay.

In conclusion, we could develop a novel assay for the MIC and MBC determinations of BPO against *P. acnes*, which is reliable and reproducible as a broth microdilution testing and the present results suggest that BPO has a potent bactericidal activity against *P. acnes*.

Propionibacterium acnes is one of the primary factors involved in the pathogenesis of acne vulgaris [1]. In the Japanese guidelines for the treatment of acne vulgaris, topical or oral antimicrobial agents are strongly recommended for the treatment of inflammatory lesions including papules and pustules [2]. However, it has been reported that the long-term use of the antimicrobial agents is associated with an increase in the population of antimicrobial resistant *P. acnes* [3].

Benzoyl peroxide (BPO) has been commonly used as a topical drug for the treatment of acne vulgaris in the United States and Europe since the 1960s. In the American [4] and European [5] guidelines for the treatment of acne vulgaris, BPO is considered as a standard therapeutic drug. In 2014, BPO-containing topical gel (BEPIO® Gel 2.5%, Maruho Co., Ltd., Osaka, Japan) was first approved as an ethical drug for acne vulgaris treatment in Japan [6].

It has been reported that BPO has an antimicrobial activity against *P. acnes*, and additionally possesses keratolytic/comedolytic and anti-inflammatory effects [7]. The antimicrobial activity of BPO

* Corresponding author. 93, Awata-cho, Chudoji, Shimogyo-ku, Kyoto, 600-8815, Japan. Tel.: +81 (0) 75 325 3255; fax: +81 (0) 75 325 3222.
E-mail address: okamoto_dix@mii.maruho.co.jp (K. Okamoto).

http://dx.doi.org/10.1016/j.jiac.2015.12.010
1341-321X/

is thought to mediate via free radicals generated by degradation of BPO, and to cause damage to the bacterial cell wall and cytoplasmic membrane [8]. Moreover, BPO showed the potent antibacterial activity against bacteria resistant to antimicrobial agents that have been commonly used for acne treatment [9]. In addition, no reports about bacterial resistance to BPO were published to date [10,11] although many kinds of BPO-containing topical products have been widely used in acne patients in other countries.

The minimum inhibitory concentrations (MICs) of BPO against *P. acnes* significantly varied in the range of ≤0.78 from 800 μg/mL among previous reports using different assays [9,12–14]. The major reason for this is probably that BPO is insoluble in water and instable in a solution containing a reducing compound because of its powerful oxidizing activity [7,15]. Therefore, a reliable and reproducible method is needed for the MIC measurement of BPO.

In the present study, we first searched for a suitable culture medium to measure the MIC of BPO against *P. acnes.* Although Brucella broth containing 5% lysed horse blood is recommended to use in broth microdilution testing for obligate anaerobic bacteria including *P. acnes* [16], BPO did not dissolve in this medium at all. Therefore, we next tested Brucella broth (Nippon Becton Dickinson Company, Ltd., Tokyo, Japan) containing 5% lysed horse blood (Nippon Bio-Test Laboratories Inc., Tokyo, Japan) supplemented with 0.1(v/v)% glycerol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 2(v/v)% Tween 80 (Kanto Chemical Co., Inc., Tokyo, Japan). As shown in Table 1, BPO was dissolved in this supplemented medium up to 1250 μg/mL. However, BPO was immediately precipitated when this solution was left at 35 °C under anaerobic condition using AnaeroPack® system (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan). Then, we tested Mueller Hinton (MH) broth (Nippon Becton Dickinson Company, Ltd.) containing 5% lysed horse blood, Brain Heart Infusion (BHI) broth (Nippon Becton Dickinson Company, Ltd.) and Gifu anaerobic medium (GAM) broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). Using each basal media supplemented with 0.1% glycerol and 2% Tween 80, the solubility of BPO and the growth of *P. acnes* ATCC11827 were evaluated (Table 1). All the supplemented basal media solubilized BPO up to 1250 μg/mL. However, precipitation of BPO was immediately observed in the supplemented MH broth containing 5% lysed horse blood at 35 °C under anaerobic condition. The growth of *P. acnes* in the supplemented BHI broth was less than those in the other supplemented media. Therefore the supplemented GAM broth was considered as a suitable medium in which BPO did not precipitate and *P. acnes* grew well. Using this supplemented GAM broth, we determined the MICs and minimum bactericidal concentrations (MBCs) of BPO against clinical isolates of *P. acnes*.

Forty four isolates were selected from *P. acnes* collected from Japanese patients with acne vulgaris during a period from 2012 to 2013. The isolates were chosen to include clindamycin (CLDM)-nonsusceptible (NS) strains that were either susceptible (S) or nonsusceptible (NS) to nadifloxacin (NDFX). This resulted in four phenotypic patterns with respect to NDFX and CLDM: S/S (23 strains), S/NS (13 strains), NS/S (4 strains), and NS/NS (4 strains). Due to the limited total number of isolates, the numbers of isolates in all phenotypes were not equal. All clinical isolates in this study were used according to The Ethical Guidelines for Epidemiological Research in Japan, and patient's information was unlinkable anonymized.

Prior to the MIC measurement, the isolates were precultured on anaero columbia agar containing 5% of rabbit blood (Nippon Becton Dickinson Company, Ltd.) for 48 h at 35 °C under anaerobic condition. BPO (Sigma–Aldrich, St. Louis, MO, USA), NDFX (LKT Laboratories, Inc., St. Paul, MN, USA) and CLDM (Sigma–Aldrich) were commercially available. The MICs of BPO, NDFX and CLDM against *P. acnes* isolates were determined by broth microdilution testing according to the Clinical and Laboratory Standards Institute (CLSI) [16]. The supplemented GAM broth with 0.1% glycerol and 2% Tween 80 was used as a medium for the MIC measurement. BPO was dissolved in and then diluted by dimethyl sulfoxide (DMSO, Kanto Chemical Co., Inc.) in a 2-fold serial manner. The final concentration of BPO in the culture medium ranged from 2 to 1024 μg/mL and that of DMSO in the culture medium was 3% or less which was the maximum concentration did not influence the growth of *P. acnes*. NDFX was dissolved in 0.1 N sodium hydrate and diluted by sterile purified water in a 2-fold serial manner. CLDM was dissolved in and diluted by sterile purified water in a 2-fold serial manner. The final concentration of NDFX and CLDM in the culture medium ranged from 0.06 to 128 μg/mL. The clinical isolates with MIC ≥8 μg/mL were defined as CLDM-NS according to CLSI [17]. Since there are no CLSI criteria for NDFX, we defined the clinical isolates with MIC ≥8 μg/mL as NDFX-NS. Apart from the microbiological experiment, the stability of BPO in the supplemented GAM broth was measured after incubation for 2 h at 35 °C under anaerobic condition. As a result, the concentration of BPO in the culture medium was lowered approximately 50% of the initial concentration (data not shown). Therefore, bacterial suspension was inoculated into the supplemented GAM broth within 1 h after dissolving BPO in DMSO.

The MBCs were determined according to Clinical Microbiology Procedures Handbook 3rd edition [18]. Briefly, 10 μL aliquots of the bacterial suspension were removed from all wells showing no visible growth after determination of the MICs, and were plated onto anaero columbia agar containing 5% of rabbit blood. After incubation for 72 h at 35 °C under anaerobic condition, the number of colonies on the subculture plates was counted. The MBCs were defined as the lowest concentration showing ≥99.9% killing of the initial MIC inoculum.

To confirm the accuracy of the MIC and MBC of BPO measured by our testing method, time-kill assay was performed using the supplemented GAM broth. The bacterial suspension of *P. acnes* ATCC11827 and BPO at 1 and 2 times the MIC were added to each well of 96 well microplate, and incubated at 35 °C under anaerobic condition. The bacterial inoculums were adjusted to approximately 3×10^6 CFU/mL in each well. After incubation for 1 or 2 h, the

Table 1
Different basal media supplemented with 0.1% glycerol and 2% Tween 80 evaluated for susceptibility testing of BPO.

Basal medium[a]	Solubility of BPO	Precipitation of BPO[b]	Growth of *P. acnes*[c]
Brucella broth with 5% lysed horse blood	up to1250 μg/mL	+[d]	Larger
Mueller Hinton broth with 5% lysed horse blood	up to1250 μg/mL	+[d]	Middle
Brain Heart Infusion broth	up to1250 μg/mL	−	Small
Gifu anaerobic medium (GAM) broth	up to1250 μg/mL	−	Larger

[a] Each of four basal media was supplemented with 0.1% glycerol and 2% Tween 80.
[b] Each of the media containing BPO at 1250 μg/mL was incubated up to 48 h at 35 °C under anaerobic condition.
[c] Each medium was evaluated by testing growth of *P. acnes* ATCC11827.
[d] Precipitation was immediately observed when the medium containing BPO at 1250 μg/mL was incubated under anaerobic condition.