Doxycycline and Metronidazole Exhibit a Synergistic Antibacterial Activity against *Porphyromonas gingivalis*

นิพนธ์ต้นฉบับ

Original Article

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บทคัดย่อ

วัตถุประสงค์: ยาปฏิชีวนะมีข้อจำกัดในการนำมาใช้กับร่างกาย ซึ่งข้อเสีย ได้แก่ การเกิดอาการไม่พึงประสงค์จากการใช้ยา การแพ้ยา และทำให้เชื้อแบคทีเรียบางชนิดเกิดการดื้อยา ด้วยเหตุนี้จึงมีการศึกษาความเป็นไปได้ในการใช้ยาปฏิชีวนะหลายชนิดร่วมกันเพื่อให้เกิดการเสริมฤทธิ์ของยาซึ่งจะทำให้สามารถเพิ่มฤทธิ์ต้านจุลชีพ ลดความเป็นพิษ และลดความเสี่ยงในการเกิดเชื้อดื้อยาลง วัตถุประสงค์ของการศึกษานี้คือการทดสอบว่ายาคู่ผสมระหว่างด็อกซีไซคลินไฮเคลต, ซิโปรฟล็อกซาซิน ไฮโดรคลอไรด์, เมโทรนิดาโซล สามารถออกฤทธิ์เสริมกันเพื่อยับยั้งเชื้อชนิดต่างๆได้หรือไม่ **วิธีการศึกษา:** ศึกษาฤทธิ์ต้านจุลชีพของยาด็อกซีไซคลินไฮเคลต, ซิโปรฟล็อกซาซิน ไฮโดรคลอไรด์, เมโทรนิดาโซล และคู่ผสมของยาเหล่านี้ต่อเชื้อแบคทีเรียชนิดต้องการออกซิเจน ได้แก่ *Staphylococcus aureus* กับ *Escherichia coli* และต่อเชื้อแบคทีเรียชนิดที่ไม่ต้องการออกซิเจน ได้แก่ *Streptococcus mutans* และ *Porphyromonas gingivalis* **ผลการศึกษา:** ผลของการศึกษาพบว่ายาคู่ผสมระหว่างด็อกซีไซคลินไฮเคลตกับเมโทรนิดาโซลแสดงผลยับยั้งเชื้อแบบเสริมฤทธิ์ต่อเชื้อแบคทีเรีย *Porphyromonas gingivalis* โดยมีค่า fractional inhibitory concentration (FIC_I) เท่ากับ 0.4 **สรุป:** ยาคู่ผสมระหว่างด็อกซีไซคลินไฮเคลตกับเมโทรนิดาโซลสามารถแสดงผลยับยั้งเชื้อแบบเสริมฤทธิ์ต่อเชื้อแบคทีเรีย *Porphyromonas gingivalis* การเสริมฤทธิ์ของยาคู่ผสมนี้อาจมีประโยชน์สำหรับการรักษาโรคปริทันต์อักเสบ

คำสำคัญ: ด็อกซีไซคลิน, เมโทรนิดาโซล, พอร์ไฟโรโมแนส จิงจิวาลิส, การเสริมฤทธิ์ของยาปฏิชีวนะ

Abstract

Objective: Administration of antibiotics is not without limitations. Certain drawbacks include adverse drug reactions, drug allergies and the emergence of antibiotic-resistant bacteria. To this end, antibiotic combinations with synergistic antimicrobial effects are sought after to establish combinations which exhibit an increased antimicrobial activity, decreased toxicity and lowered chance of antibiotic resistance development. This study aimed to test a synergistic antibacterial activity against selected pathogens of the combination of selected drugs, i.e. doxycycline hyclate, ciprofloxacin hydrochloride and metronidazole. **Methods:** Antibacterial activity of doxycycline hyclate, ciprofloxacin hydrochloride, metronidazole and their combinations against aerobic microbes (*Staphylococcus aureus* and *Escherichia coli*) and anaerobic microbes (*Streptococcus mutans* and *Porphyromonas gingivalis*) were evaluated using checkerboard method. **Results:** Our result indicated that doxycycline hyclate-metronidazole combination exhibited synergistic antimicrobial activity (fractional inhibitory concentration; FIC_I = 0.4) against the anaerobic pathogen *Porphyromonas gingivalis*. **Conclusion:** Doxycycline hyclate-metronidazole combination exhibited synergistic antimicrobial activity *Porphyromonas gingivalis*. This synergistic activity may be exploited in the management of periodontitis.

Keywords: doxycycline, metronidazole, *Porphyromonas gingivalis, antibiotics synergy*

Introduction

Infectious diseases are serious health concerns caused by an invasion of microorganisms into the human body. A considerable proportion of infectious diseases are caused by bacteria. The severity of bacterial infections ranges from mild, topical infections to life-threatening, systemic infections involving multiple vital organs. Multiple classes of antibiotics have been developed and effectively used in the combat against these pathogens. However, the increased usage of antimicrobial agents has evoked the emergence of drug-resistant microbes including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), multidrug-resistant (MDR)-*Pseudomonas aeruginosa*, extended spectrum beta-lactamase (ESBLs)-producing Enterobacteriaceae and MDR-*Acinetobacter baumannii*[1-3], which are considered to be emerging global crises associated with refractory response, increased mortality and higher cost of therapy. This has led to the development of antimicrobial combinations with a goal to seek combinations which exhibit synergistic effects to increase antimicrobial activity while also decreasing toxicity and the chance of bacteria to develop resistance.

To assess the synergistic effects of antibacterial agents, three model drugs with diverse mechanisms of action were selected and evaluated against four microbes with different

molecular and metabolic backgrounds. Doxycycline is a broad-spectrum antibiotic in the class of tetracycline. It possesses a bacteriostatic activity through the inhibition of protein synthesis by preventing the binding of aminoacyl-tRNA to 30S ribosome[4] and, at a subantimicrobial dose, inhibits matrix metalloproteinases (MMPs).[5,6] The antibacterial activity of doxycycline is documented against a wide range of bacterial pathogens including *Staphylococcus aureus*[7], *Escherichia coli*[8], and *Porphyromonas gingivalis*[9], while the sensitivity of *Streptococcus mutans* to tetracycline has been demonstrated.[10,11] Ciprofloxacin is a member of fluoroquinolone antibiotics which inhibit DNA gyrase and topoisomerase IV resulting in the blockade of DNA synthesis.[12,13] The antibacterial activity of ciprofloxacin against *Staphylococcus aureus*[14], *Escherichia coli*[15,16] and *Porphyromonas gingivalis*[17] has been reported. Metronidazole is a nitroimidazole antimicrobial agent that inhibits DNA replication of anaerobic bacteria.[18-20] The transformation of metronidazole to its active, reduced form is favored in an anaerobic condition.[19,20] The reduced metronidazole triggers DNA strand breakage.[19,20] Metronidazole is routinely prescribed for the management of anaerobic bacterial infection. Antibacterial activity of metronidazole against the anaerobes *Streptococcus mutans*[21] and *Porphyromonas gingivalis*[9,17] has been demonstrated.

Four possible outcomes are expected from antimicrobial combinations.[22] The interaction is classified as synergistic when the effect of the combined drugs is greater than the sum of each agent used individually. Additivity is when the drug combination possesses the antimicrobial activity equal to the sum of the activity of each drug used separately. Indifference is the relationship where the activity of the combination is equal to the activity of one of the two agents used individually. Lastly, antagonism is a phenomenon when the combination is less effective than each agent used individually. A strategy which may be employed to study the activity of antimicrobial combinations is the checkerboard test.[22-24]

To assess the synergistic effect of antibacterial agents, the antibacterial activity of doxycycline hyclate, ciprofloxacin hydrochloride, metronidazole and their combinations against aerobic microbes (*Staphylococcus aureus* ATCC 6853P and *Escherichia coli* ATCC 25922) and anaerobic microbes (*Streptococcus mutans* ATCC 27175 and *Porphyromonas gingivalis* ATCC 33277) were evaluated using the checkerboard method while the standard fungal pathogen *Candida albicans* ATCC 17110 was used as negative control.

Materials and Methods

Microbial strains and antimicrobial agents

Doxycycline hyclate was purchased from Huashu pharmaceutical corporation, Shijiazhuang, China (Batch No. 20071121). Ciprofloxacin hydrochloride and metronidazole were kindly supplied by T. MAN Pharma Ltd, Bangkok, Thailand. Standard microbes included *Staphylococcus aureus* ATCC 6538P, *Escherichia coli* ATCC 25922, *Candida albicans* ATCC 17110, *Streptococcus mutans* ATCC 27175 and *Porphyromonas gingivalis* ATCC 33277.

Bacterial culture

Each microbe was cultured in appropriate media. Tryptic soy broth (lot no. 8091999, Difco™, USA) was used for *S. aureus* and *E.coli*. Sabouraud dextrose broth (lot no. 6345690, Difco™, USA) was used for *C. albicans*. Brain heart infusion broth (lot no. 0270845, Bacto™, USA) was used for *S. mutans* and *P. gingivalis*. Microbes were incubated in an aerobic incubator (for aerobic microbes *S. aureus*, *E.coli and C. albicans*) or anaerobic incubator (Forma Anaerobic System, Thermo Scientific, Ohio, USA, for anaerobic microbes *S. mutans* and *P. gingivalis*) at 37 °C, for 24 - 48 h. The relationship between the number of microbes and optical density at 530 or 540 nm was determined using a UV-vis spectrophotometer (Perkin-Elmer, Germany) (n = 3). The suspension was further diluted to obtain a final inoculum density of $2x10^5$ cfu/mL.

Minimal inhibitory concentration (MIC) determination

MICs of each antimicrobial agent against standard microbes were determined by broth microdilution method. MIC was defined as the lowest concentration of antimicrobial agents which inhibited the growth of the tested microbe (n = 6).

Assessment of antimicrobial combination by checkerboard test

Checkerboard test was performed in microtiter plates (96 well plates, U-bottom with lid, Corning Incorporated, USA). Antimicrobial activity of the drug combinations was determined by microdilution method. Antimicrobial agents were prepared at 1.25, 6.4, 4.0 and 12.8 mg/mL, respectively. Fifty µL of

diluted microbial agents were distributed to each well. Then, 200 µL of $1x10^5$ cfu/mL microbial suspension were added to each well and incubated at 37 °C for 24 h. The antimicrobial efficacy of each drug in the combination treatment was expressed in terms of fractional inhibitory concentration (FIC), which is defined as the MIC of the drug used in combination divided by the MIC of the drug used alone. Finally the FIC of each drug in the combination was used to calculate the fractional inhibitory concentration index (FIC_I) using the following formula:

$$FIC_I = FIC_A + FIC_B$$

where FIC_A and FIC_B are FIC of drug A and B, respectively. $FIC_I < 0.5$ signified a synergistic antimicrobial effect of the drug combination, $0.5 \leq FIC_I < 1.0$ was interpreted as additive, $1 \leq FIC_I < 4.0$ was interpreted as indifference and $FIC_I > 4$ was interpreted as antagonistic.[22-24]

Results

The efficacy of doxycycline hyclate, ciprofloxacin hydrochloride, metronidazole and their combinations against aerobic microbes (*S. aureus*, *E. coli* and *C. albicans*) and anaerobic microbes (*S. mutans* and *P. gingivalis*) were evaluated by MICs and FIC index. To establish reference efficacies of each individual antimicrobial agent, the MICs of doxycycline hyclate, ciprofloxacin hydrochloride and metronidazole against *S. aureus*, *E. coli*, *C. albicans*, *S. mutans* and *P. gingivalis* were determined by broth microdilution method (Table 1). The standard curves for each microbe were presented in Figure 1. The good relationship was evident by R^2 in the range of 0.9950 - 0.9979.

Next, the effects of antimicrobial combinations were evaluated by the checkerboard method. The FIC_I of each combination was shown in Table 2. As expected, all antibacterial combinations demonstrated an indifferent effect against the fungus *C. albicans* which was used as a negative control. Doxycycline hyclate-ciprofloxacin HCl combination exhibited an indifferent relationship against all aerobic microbes (*S. aureus*, and *E. coli*) and against *S. mutans* while exhibiting an additive effect against *P. gingivalis* (Table 2). Doxycycline hyclate-metronidazole and ciprofloxacin HCl-metronidazole combinations exhibited additive effects against *S. aureus* and *S. mutans* but demonstrated indifferent relationship against *E. coli*. Ciprofloxacin HCl-metronidazole combination showed an indifferent effect against *P. gingivalis*. Interestingly, doxycycline hyclate-metronidazole combination demonstrated a synergistic effect against the anaerobic pathogen *P. gingivalis* (Table 2). Further research into this synergism is warranted to assess its usefulness in the combat against *P. gingivalis* infections such as in periodontitis.

Table 1 Minimal inhibitory concentrations (MICs) of antimicrobial agents against *S. aureus* ATCC 6538P, *E. coli* ATCC 25922, *C. albicans* ATCC 17110, *S. mutans* ATCC 27175 and *P. gingivalis* ATCC 33277 determined by broth microdilution method (n = 3).

MIC (µg/mL)	Drugs		
	Doxycycline hyclate	Ciprofloxacin	Metronidazole
S. aureus	0.08±0.00	0.25±0.02	1.60±0.05
E. coli	0.20±0.01	0.27±0.03	5.69±0.05
C. albicans	2.50±0.04	1.06±0.02	11.38±0.06
S. mutans	0.21±0.01	0.97±0.00	3.20±0.21
P. gingivalis	1.88±0.04	0.31±0.01	0.80±0.02

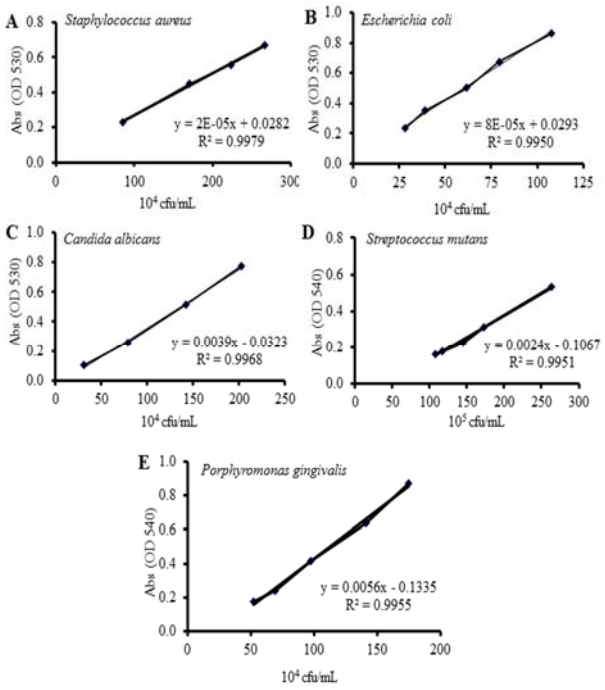

Figure 1 (A) Calibration curve of *Staphylococcus aureus* ATCC 6853P, (B) *Escherichia coli* ATCC 25922, (C) *Candida albicans* ATCC 17110, (D) *Streptococcus mutans* ATCC 27175 and (E) *Porphyromonas gingivalis* ATCC 33277

Table 2 Fractional inhibitory concentration (FIC) index values of the drug combinations.

Combined drugs	FIC in the combination		FIC_I	Interpretation
	FIC_A	FIC_B		
S. aureus				
D + C	0.67	0.40	1.07	ID
D + M	0.40	0.40	0.80	AD
C + M	0.50	0.33	0.83	AD
E. coli				
D + C	0.40	0.75	1.15	ID
D + M	0.50	0.83	1.33	ID
C + M	1.00	0.25	1.25	ID
C. albicans				
D + C	1.00	0.25	1.25	ID
D + M	1.00	0.20	1.20	ID
C + M	0.83	0.20	1.03	ID
S. mutans				
D + C	0.50	0.63	1.13	ID
D + M	0.30	0.20	0.50	AD
C + M	0.10	0.75	0.85	AD
P. gingivalis				
D + C	0.10	0.40	0.50	AD
D + M	0.10	0.30	0.40	S
C + M	0.30	0.75	1.05	ID

Note: D: Doxycycline hyclate; C: Ciprofloxacin; M: Metronidazole; S: Synergy; AD: Additive; ID: Indifference.

Discussions and Conclusion

Due to the limited number of antibiotics available and the emergence of antibiotic resistance at alarming rate, novel antibiotics as well as innovative strategies to use them against infections are areas of active research. Given the challenges in the discovery of novel antibiotics, synergy between antibacterial agents has been proposed as an alternative strategy for the management of infection. Extensive researches in this area have extended the synergism beyond those routinely prescribed antibiotics to include the synergy between existing antibiotics and plant-derived compounds and other non-traditional antibacterial agents.[25] However, an intrinsic limitation of antibiotic combination studies is that it requires tremendous effort to study all possible combinations of antibiotics against pathogens of interest, not to mention the genetic variability of bacteria even within species which adds yet another layer of complication and possibly accountable for the discrepancies in experimental results between research groups. Nevertheless, our results provide a proof-of-concept that antibiotic combination may be beneficial in the combat against bacterial pathogens, at least in the case of doxycycline hyclate and metronidazole combination against *P. gingivalis*.

Despite the fact that the mechanisms of antibacterial synergism are likely case-specific, the interaction of antibiotics in a combination can be classified into three main categories: (1) drugs which target different pathways within bacteria, (2) drugs that inhibit different targets in the same pathways and (3) drugs which target the same molecules in different ways.[25,26] Unanticipated antibacterial effects of antibiotics normally ineffective against tested pathogens have been reported when used in combination with other compounds. The mechanisms of such events are largely explained by the ability of the added compound in the sensitization of the pathogen to the antibiotics by, but not limited to, two main mechanisms: one involves an inhibition of enzymes which target the antibiotics for destruction such as the restoration of the effectiveness of beta-lactam antibiotics against MRSA in the presence of epigallocatechin gallate which inhibits bacterial beta-lactamase enzyme, and the other involves an increase in the intracellular concentration of antibiotics by the modulation of bacterial barriers or pumps such as the sensitization of MRSA to tetracycline by reserpine and the restored effectiveness of ciprofloxacin against gram positive and gram negative bacteria by epigallocatechin gallate.[25] Moreover, it may be important to mention that antibacterial synergy can also result from compounds that have no antimicrobial activity against the organism by themselves, but can sensitize the organism to antibiotics, as is the case for beta-lactamase inhibitors and beta-lactam antibiotics.[27]

Despite the attractive benefits of antibiotic combinations, case-by-case considerations must be made in order to speculate clinical performances. Many factors affect the interpretation of *in vitro* antibiotic interaction, including but not limited to (1) test methods, i.e. checkerboard microdilution, time-kill method and Etest, (2) outcomes, i.e. inhibition or killing, and (3) experimental setting, e.g. concentration of bacteria, treatment time, etc, as exemplified by the interaction between polymixins and carbapenem.[28] Moreover, pharmacokinetic factors, site of infection and time frame governed by treatment regimen can be anticipated to complicate the extrapolation of *in vitro* data to predict clinical outcomes.

Periodontitis is the inflammation of the periodontium caused by the accumulation of bacteria in the periodontal pocket.[29] Common pathogens causing periodontitis include *Porphyromonas gingivalis, Actinobacillus actinomycetem-comitans, Fusobacterium nucleatum, Bacteroides forsythus*

and *Treponema denticola*.[30,31] Other gram negative anaerobic rods, certain gram positive bacteria and enteric rods/ pseudomonas may also play roles in the pathogenesis of periodontitis.[30,31] According to the clinical practice guideline issued by the American Dental Association, scaling and root planning (SRP) is the standard intervention for the management of chronic periodontitis.[9] Additionally, systemic subantimicrobial-dose of doxycycline (20 milligrams twice a day for 3 to 9 months) and systemic antimicrobials (amoxicillin and metronidazole, metronidazole, azithromycin, clarithromycin, moxifloxacin, and tetracyclines including doxycycline at an antimicrobial dose of 100 milligrams or greater per day) may be considered as adjunct therapies to SRP.[9] However, systemic antibiotics only provide little benefit, i.e. a small gain in tooth attachment[9], while also potentially giving rise to adverse reactions commonly associated with antibiotics such as rash, diarrhea, abdominal pain, nausea, vomiting and rare but life-threatening events i.e. the diverse types of allergic reactions. These limitations highlight the need for the development of new antimicrobial agents or new therapeutic strategies which are more effective and safer for the treatment of periodontitis.

A strategy which may be used to devise a more effective pharmacological intervention is to exploit the synergism between antimicrobial agents. Synergism is the phenomenon when two antimicrobial agents with complementary mechanisms of action are used against a pathogen the effect of the drug combination is greater than the sum of each individual agent used separately.[22,24,32] Synergism allows the usage of each agent at lower doses which provides a benefit in decreasing the adverse effects associated with the concentration of each molecule.[22,24,32] Our results demonstrated a synergistic relationship between doxycycline hyclate and metronidazole against *P. gingivalis*, a common pathogen causing periodontitis, suggesting that the combination may be useful for periodontitis treatment. Previously, the efficacy of metronidazole and doxycycline in the prevention of recurrent periodontitis was investigated by Atiken et al.[33] Briefly, patients suffering periodontitis were treated with bimonthly scaling and 3 weeks of systemic doxycycline (11 subjects) or scaling and placebo (12 subjects).[33] Patients were then monitored for signs of recurrent periodontitis, i.e. a present of periodontal abscess or loss of gingival attachment. Upon the detection of recurrent periodontitis, metronidazole was administered (250 mg every 8 hours) for 10 days. The investigators reported a 42% recurrent periodontitis in placebo group compared to 9% in doxycycline group, as well as a marked reduction in periodontitis pathogens *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Eikenella corrodens*, and *Fusobacterium nucleatum* before and one month after metronidazole administration in doxycycline group compared to placebo, suggesting that pre-exposure to doxycycline sensitized periodontitis pathogens to metronidazole.[33]

The mechanism of doxycycline and metro-nidazole synergy remains largely inconclusive. However, it will be interesting to investigate if the synergism is a specific effect of doxycycline and metronidazole or if the synergy is extended to other agents of the same class. A class effect would indicate that the synergism is attributable to the combinatorial inhibition of protein synthesis and DNA replication by tetracycline and nitroimidazole antibiotics, respectively; while a molecule-specific effect would suggest that the synergism results from certain molecular structure unique to doxycycline and/or metronidazole. Additionally, it may be worthwhile to examine the intracellular concentration of doxycycline and metronidazole when used individually and in combination, as changes in the intracellular concentration will suggest that the synergism may result from the ability of an agent to interfere with the drug export/import mechanism of the bacteria. Further research into this synergism is warranted to assess its usefulness in the management of periodontitis. Moreover, the efficacy of certain antimicrobial combinations is greater when sequentially administered as compared to a concomitant treatment.[34] The efficacy of doxycycline hyclate and metronidazole combination, both concomitantly and sequentially administered, against *P. gingivalis* and other periodontitis pathogens awaits further evaluation.

Conclusion

Using checkerboard method, antibacterial activity of doxycycline hyclate, ciprofloxacin hydrochloride, metronidazole and their combinations were evaluated against aerobic microbes (*Staphylococcus aureus* and *Escherichia coli*) and anaerobic microbes (*Streptococcus mutans* and *Porphyromonas gingivalis*). Our result indicated that the combination of doxycycline hyclate and metronidazole exhibited synergy (fractional inhibitory concentration; FIC_I = 0.4) against the anaerobic pathogen *Porphyromonas*

gingivalis, a common pathogen causing periodontitis, suggesting that the combination may be useful for the management of periodontal disease.

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