นิพนธ์ต้นฉบับ • Original Article

ประสิทธิภาพของออเรียนตินต่อการยับยั้งโปรตีน HMGB1 เมื่อใช้ในการร่วมรักษาโรคติดเชื้อเมลิออยด์แบบติดเชื้อในกระแสเลือดในหนูทดลอง

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Efficiency of Orientin Inhibits HMGB1: Potential Adjunct Therapeutic Agent for Septicemic Melioidosis in a Mouse Model

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หลักการและวัตถุประสงค์: โปรตีน HMGB1 เป็นตัวกระตุ้นการอักเสบที่มีบทบาทสำคัญในผู้ป่วยที่ติดเชื้อเมลิออยด์ในกระแสเลือด ระดับของ HMGB1 ถูกพบว่าสัมพันธ์กับความรุนแรงของโรค การศึกษานี้มีวัตถุประสงค์เพื่อสืบสวนประสิทธิภาพของสารออเรียนติน (orientin) ที่สามารถยับยั้ง HMGB1 ในการร่วมรักษาหนูที่ติดเชื้อเมลิออยด์ในกระแสเลือด

วิธีการศึกษา: หนู BALB/c จำนวน 4 กลุ่ม (กลุ่มละ 6 ตัว) ถูกทำให้ติดเชื้อ *Burkholderia pseudomallei* เพื่อชักนำให้เกิดภาวะติดเชื้อในกระแสเลือด และให้การรักษาด้วยสารต่างๆ ดังนี้ กลุ่มที่ 1 Phosphate Buffered Saline (PBS) กลุ่มที่ 2 ออเรียนติน (Ori) ความเข้มข้น 36 ไมโครกรัมต่อตัว กลุ่มที่ 3 เซฟตาซิดีมที่มี dose ต่ำ (CTZ) ความเข้มข้น 300 มิลลิกรัมต่อกิโลกรัม และกลุ่มที่ 4 Ori ร่วมกับ CTZ (Ori/CTZ) ที่ความเข้มเข้นเดียวกันกับกลุ่ม 2 และ 3 จากนั้น สังเกตความมีชีวิตรอด และเก็บตัวอย่างเลือดเพื่อตรวจดูจำนวนแบคทีเรียในเลือดและปริมาณของ HMGB1 ในพลาสมา

ผลการศึกษา: กลุ่มที่ให้การรักษาด้วย CTZ และ Ori/CTZ มีอัตราการมีชีวิตรอดสูงสุด ร้อยละ 33.3 รองลงมาคือกลุ่ม Ori ร้อยละ 11.1 ค่ากลางเวลามีชีวิตรอดสูงสุด คือ กลุ่มที่ให้การรักษาด้วย CTZ และ Ori/CTZ แบคทีเรียในเลือดถูกตรวจพบเพียงในกลุ่มที่ให้การรักษาด้วย Ori และกลุ่มควบคุม PBS ส่วนระดับ HMGB1 พบสูงสุดในกลุ่ม Ori

สรุป: การรักษาด้วย Ori ร่วมกับ CTZ ไม่สามารถเพิ่มประสิทธิภาพในการรักษาหนูที่ติดเชื้อเมลิออยด์ในกระแส

Background and Objective: HMGB1 is a potent proinflammatory cytokine in septicemic melioidosis patients. The HMGB1 level was found to be correlated with severity of the disease. The aim of this study was to investigate the effective of the potential of HMGB1-inhibitor, orientin, as an adjunct therapeutic agent in septicemic melioidosis mice.

Methods: **Four** groups of BALB/c mice were infected with *Burkholderia pseudomallei* to induce septicemic melioidosis. The infected mice were then treated with various substances including group I) Phosphate Bufferred Saline (PBS), group II) Ceftazidime (CTZ), 300 mg/kg, group III) Orientin (Ori) 36 μg/mouse and group IV) Orientin combines with Ceftazidime (Ori/CTZ) at the same concentrations with group I) and II) and observed their survival. Blood samples of infected mice were collected to determine the number of bacteria in blood and HMGB1 level in plasma.

Results: CTZ and Ori/CTZ-treated groups gave the highest survival rate at 33.3%, followed by Ori-treated group with 11.1%. The highest median survival time was found in CTZ- and Ori/CTZ-treated groups. The bacteria count in blood was found only in Ori-treated group and PBS-control group. HMGB1 showed the highest level in Ori-treated group.

Conclusion: Ori as an adjunctive to suboptimal dose of CTZ could not improve the potential of treatment in mice

เลือด มากไปกว่านั้น การให้Ori อย่างเดียวทำให้จำนวนแบคทีเรียในเลือดและระดับ HMGB1 ในพลาสมาเพิ่มขึ้น
คำสำคัญ: ออเรียนติน, โรคเมลิออยโดสิส, โปรตีน HMGB1, แบคทีเรีย *Burkholderia pseudomallei*

with Septicemic melioidosis. Moreover, administration of orientin alone trended to increase number of bacteria in blood and HMGB1 level in a mouse model of melioidosis.
Keywords: Orientin, melioidosis, HMGB1, *Burkholderia pseudomallei*

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Introduction

Burkholderia pseudomallei is a motile Gram-negative bacterium that commonly found in soil and water in endemic areas of Southeast Asia and Northern Australia. It resists to several antibiotics and no approved vaccine available in the market[1]. The bacterium is a causative agent of melioidosis, the third most common cause of death among infectious disease in northeastern Thailand[2]. People can get infection via percutaneous inoculation, inhalation or ingestion. Risk factors of melioidosis were found in patients that have an underlying disease including diabetes, alcohol addict, chronic pulmonary or renal disease, thalassemia and people with immunosuppressive therapy[2]. Melioidosis can manifest as various clinical forms, septicemic is the most important form with 40% mortality rate despite the use of potent antibacterial agents[1,3].

High mobility group box 1 (HMGB1) is a 30 kDa nuclear protein produced by almost all mammalian cell types[3]. It can be actively released during the innate immune response by inflammatory cells[4]. It was also passively released from dying cells and migrate out of cells, where it functions as efficient proinflammatory mediator[3,5]. Established molecular mechanisms of HMGB1 binding and signaling through Toll like receptor (TLR)-4 revealed signaling pathways that mediate cytokine release and tissue damage via Nuclear Factor-kappa B (NF-kB) pathway[5,6]. At present, HMGB1 was identified as a cytokine mediator of lethal systemic inflammation, arthritis and local inflammation[7]. In 2014, Charoensup and colleagues reported that HMGB1 level in human plasma was significantly correlated with the clinical severity score (SOFA score) in septicemic melioidosis patients and suggested that its level could be used as a marker of late severe sepsis[8].

Orientin, HMGB1-inhibitor, is one of flavonoid C-glycosyl compound with anxiolytic properties. Orientin inhibits HMGB1-induced inflammatory response in HUVEC and murine polymicrobial sepsis has been reported and the research suggested that orient in maybe a candidate therapeutic agent for the treatment of vascular inflammatory diseases via inhibition of the HMGB1 signaling pathway[9]. Although orientin could increase the survival rate of mice with polymicrobial sepsis, but the potential of orientin on mice with *B. pseudomallei* infection has not been studied. Therefore, in this study, orientin was investigated as an adjunct treatment with suboptimal ceftazidime in order to improve the survival mice.

Materials and Methods

1. Bacterial strain

B. pseudomallei strain A2[10] was used to establish septicemic melioidosis in BALB/c micewith septic shock[8].

2. Animals

Forty eight, 6-8 weeks-old male, BALB/c mice were ordered from the National Laboratory Animal Center, Mahidol University. The animal ethics were submitted and approved by the Animal Ethics Committee of Khon Kaen University, Thailand (ACUC-KKU-47/2559).

3. Orientin

Commercially available orientin was obtained from Sigma-Aldrich Company (Sigma-Aldrich Pte Ltd, Singapore).

4. Infection of BALB/c mice with *B. pseudomallei* to establish septic shock

*B. pseudomallei*was subculture on to Nutrient Agar plate. After incubation at 37°C for 24 hours, a single colony was transferred into 20 ml of Tryptic Soy Broth (TSB), then incubated at 37°C, 200 rounds per minute (rpm) in shaking incubator for 16 hours and transferred culture 200 µl into 20 ml of new TSB and incubated at 37°C, 200 rpm for 3 hours, measure and adjust optical density (OD) (OD of 0.1 at 550 nm). Appropriate dilutions were prepared in Phosphatebuffered saline (PBS) to get a concentration of 1.0×10^3 CFU/ml. The BALB/c mice were infected with $5LD_{50}$ (LD_{50} = 20 CFU)[10] intraperitoneally in 100 µl of PBS. Numbers of colonies were checked later by Drop plate method after 24 hours incubation at 37°C.

5. The potential of HMGB1 inhibitors for adjunct treatment of septicemic melioidosis in a mouse model

Sixto eight-week-old BALB/c mice were used. Four groups of mice (six mice per group) were infected with 5 LD_{50} of *B. pseudomallei* on day 0. The treatments were performed on groups II - IV. Group II was treated solely with 300 mg/kg of ceftazidime (1/4 of optimal dose). Group III and IV were treated with 36 µg of orientin at 12 and 50 hours after infection without or with 300 mg/kg of ceftazidime. Ceftazidime was administered intraperitoneally 12-hourly for 10 days[11]. Group I was treated with PBS as negative control. Mice were observed daily for 30 days and the probability of surviving mice were plotted against times.

6. Bacteria count in blood

Bacterial load was determined post 52 hours of infection, 200 µl of blood was collected from retro-orbital sinus of mice and kept in heparinized tubes. Blood was diluted by ten-fold serial technique form 10^{-1}-10^{-3} in PBS (pH 7.4). Each dilution was used to determine the number of bacteria using Drop plate method. A 20 µl of each dilution including undiluted blood samples were dropped on NA medium for 5 drops (duplicate). Plates were incubated at 37°C for 24 hours. Numbers of colonies were counted and calculated as CFU/mL of blood.

7. Measurement of plasma HMGB1

Mouse heparinized blood samples were collected from retro-orbital sinus of mice. Blood samples were centrifuged at 830xg to obtain plasma. The plasma samples were used as samples for determining of HMGB1 protein level using HMGB1 ELISA kit II (Shino-Test Corporation, Japan) according to manufacturer instruction and read the absorbance of each well at 450 nm using microplate reader (Tecan Group Ltd., Switzerland).

8. Statistical analysis

The statistical significance of differences was evaluated using the Kaplan-Meier survival analysis and One-Way ANOVA. Statistical significance was defined as $p < 0.05$.

Results

The survival graph of Orientin experiment is shown in Figure 1, CTZ and Ori/CTZ-treated groups gave the highest survival rate at 33.3%, followed by Ori-treated group with 11.1%, while all mice in PBS-control group were died. The highest median survival time was found in CTZ- and Ori/CTZ-treated groups followed by Ori- and PBS-treated groups as shown in Table 1. The bacteremia was found only in Ori-treated and PBS-control groups (Table 1). HMGB1 (mean±SE) showed the highest level in Ori-treated group (31.27±16.81 ng/ml), followed by CTZ (26.81±18.04 ng/ml), PBS (21.55±9.05 ng/ml) and Ori/CTZ-treated groups (14.60±3.95 ng/ml), respectively (Figure 2). For the results of blood culture, the bacteremia was found only in 2 groups including PBS- and Ori-treated groups (Table 1).

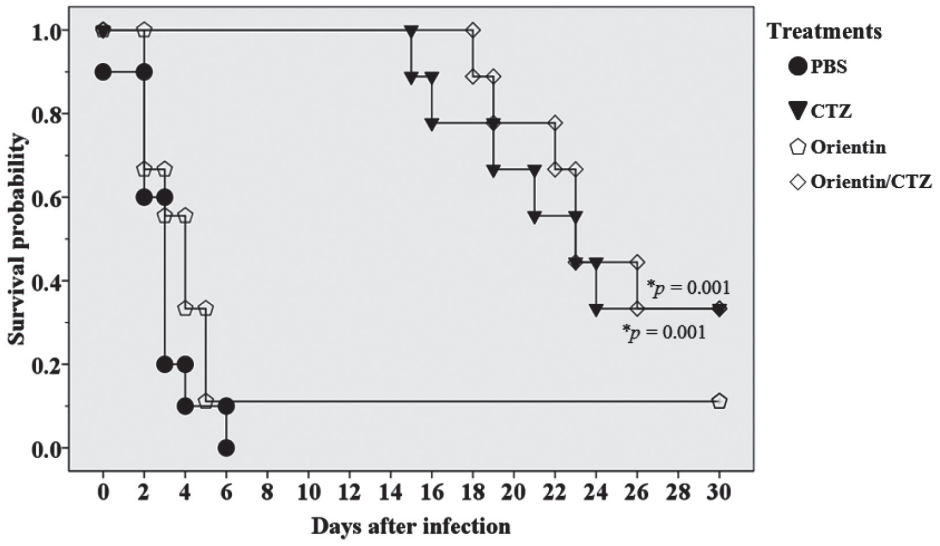

Figure 1 Survival patterns of infected mice with *B. pseudomallei* treated with various substances (PBS, CTZ, Ori or Ori/CTZ). Survival probabilities were plotted against times. The data represented the results of two independent experiments. The asterisks (*) indicate a significant difference ($p < 0.05$) versus PBS.

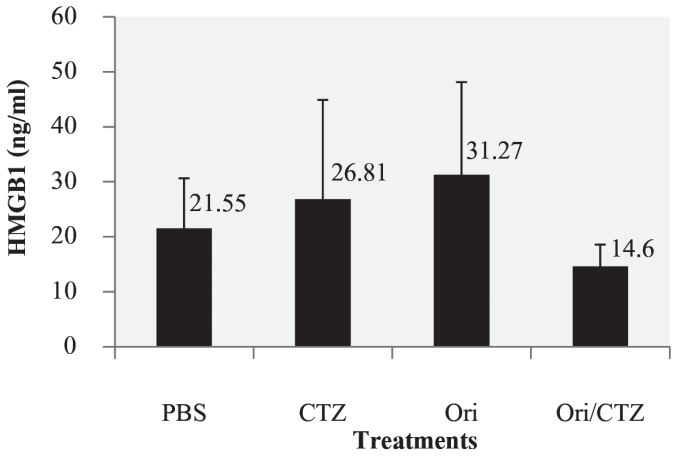

Figure 2 HMGB1 levels of infected mice with *B. pseudomallei* that treated with various substances including PBS, CTZ, Ori or Ori/CTZ. The plasma HMGB1 levels in all groups were detected at 52 hours after infection by ELISA. Results are expressed as mean ± standard error of two independent experiments.

Table 1 Percent survival, median survival time and number of bacteria

Treatments	Survival rate (%)	Median survival time (days) ±SE	Average number of bacteria (CFU/mL) at 52 h after infection±SD
I: PBS	0.00	3.00 ± 0.31	$3.41 \times 10^4 \pm 4.53 \times 10^3$
II: CTZ	33.3	23.00 ± 2.98	0
III: Orientin	11.1	4.00 ± 0.71	$9.43 \times 10^4 \pm 3.92 \times 10^4$
IV: Orientin /CTZ	33.3	23.00 ± 0.75	0

SE is standard error.

SD is standard deviation.

Discussion

HMGB1 is a target of treatment in sepsis[12], the most common cause of death in melioidosis patients[2]. There are many evidences that used HMGB1 as a therapeutic target in several model of sepsis such as repeated administration of orientin significantly increased survival 60% in mice with polymicrobial sepsis. Anti-HMGB1 antibody administration after surgical induction of peritonitis significantly increased survival. Moreover, anti-HMGB1 antibody treatment effectively reduces inflammation and destruction during collagen induced arthritis and autoimmune hepatitis[13]. Taken together HMGB1 became the interesting target for sepsis treatment.

Although the Ceftazidime, the drug of choice for melioidosis, is an effective drug conferred the good results in melioidosis patients. Normally, melioidosis patients still died because of septic conditions resulting from the late admit to hospital. Septicemic melioidosis patients had high mortality rate[1]maybe due to Ceftazidime controls only the bacteremia, whereas excessive and uncontrolled levels of proinflammatory cytokines could not be controlled and lead to death[8]. There is the evidence confirmed this hypothesize that neutralizing antibody against HMGB1 treatment in acute melioidosis mice can not only reduce HMGB1 levels but could also improve the survival rate[8]. However, treatment using neutralizing antibody against HMGB1 adjunctive to Ceftazidime significantly improve survival approximately 30%[8]. Anti-HMGB1 antibodies as an adjunctive treatment can increase survival may be due to Ceftazidime controls the bacteremia and anti-HMGB1 antibodies controls excessive levels of HMGB1 and other proinflammatory cytokines[8].

In the current study, we therefore hypothesize using of Orientin, one of reported HMGB1-inhibitor, adjunctive to suboptimal dose of Ceftazidime could improve the survival of septic shock mice due to *B. pseudomallei* infection. In this study, the highly virulent, *B. pseudomallei* strain A2 (LD_{50} = 20 CFU) was selected[10] because that could induce acute sepsis in mice[8], which indicated by the signs such as fever, anorexia and weight loss within 1-2 days after infection. A potential of Orientin, as an adjunct therapy was tested as it was reported to be successfully used in other infections[8,9,14]. In this study, 2 experiments were done by the same protocols. Orientin plus suboptimal ceftazidime could increase the survival approximately 33%, which was not overcame the suboptimal Ceftazidime treatment alone. Unlike previous reported that the Orientin could increase higher survival approximately 60%[9]. One possible of such result might be due to the difference of pathogenic bacteria that used to establish sepsis, which our study used highly virulent strain of *B. pseudomallei,* whereas previous study using model of CLP-induced sepsis.

HMGB1 level trended to increase in Ori-treated group may be associated with increasing of bacteria in blood. This association may be due to in this group did not have Ceftazidime to control the number of bacteria like Ori/CTZ-treated group that showed lower level of HMGB1, because higher bacterial number can promote inflammation lead to release in high level of HMGB1[4]. These reason supported further study of improving septicemic melioidosis outcome using HMGB1-inhibitor additional to Ceftazidime or another drugs.

However, Orientin was notsucceed to improve septicemic melioidosis treatment in this study, but it is still interesting because this is the first study about potential of Orientin on melioidosis treatment. Thus, it could reduce HMGB1 level when compared between CTZ- and Ori/CTZ-treated groups. Further study may be improved the protocol of treatment such as repeat more times of administration or increase the concentration of Orientin.

Conclusion

Orientincould not be used as an adjunct therapy with Ceftazidime to improve the outcome of septicemic melioidosis in a mouse model.

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