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Evaluation of *Pseudomonas aeruginosa* Antibiofilm Activity of Chlorogenic Acid-Protamine Sulfate Combination Using *Ex Vivo* Porcine Skin Model

Nisreen Ahmad Dahshan[1], Suha Mujahed Abudoleh[2]*, Ahmad Talhouni[1], Zahira Alkhani[1]

[1] Department of Applied Pharmaceutical Sciences, Faculty of Pharmacy, Isra University, Amman, Jordan

[2] Department of Basic Pharmaceutical Sciences, Faculty of Pharmacy, Isra University, Amman, Jordan

Abstract: *Pseudomonas aeruginosa* plays an important role in chronic wound infection due to the development of biofilms. Biofilm infections are characterized by decreased antibiotic susceptibility and resistance to host immune responses. The purpose of this research was to investigate the effect of combining chlorogenic acid and protamine sulfate on *Pseudomonas aeruginosa* biofilm, looking for a novel, safe strategy to fight biofilm formation by *Pseudomonas aeruginosa* without using antibiotics to reduce the dependency on antibiotics, with improving efficacy. This combination's biofilm inhibitory activity was evaluated against *Pseudomonas aeruginosa* biofilm cultured on *an ex vivo* porcine skin explant model that mimics wound conditions. The activity was detected using a sessile viability count. No significant difference in the bacterial load was detected when the explants were treated either with chlorogenic acid (12 mg/mL) or protamine sulfate (0.5 mg/mL). On the other hand, a combination of chlorogenic acid (12 mg/mL) and protamine sulfate (0.5 mg/mL) showed a significant decrease in the bacterial load with 3 Log cycle reduction compared to the control untreated group. This combination was not tested before, and it is a promising alternative therapy inhibiting biofilm formation from being clinically translated in wound management.

Keywords: porcine skin explant, antibiofilm, chlorogenic acid, protamine sulfate.

使用離體豬皮膚模型評價綠原酸-酸魚精蛋白組合的銅綠假單胞菌抗生物膜活性

摘要：由於生物膜的形成，銅綠假單胞菌在慢性傷口感染中起重要作用。生物膜感染的特徵是抗生素敏感性降低和對宿主免疫反應的抵抗力。本研究的目的是研究綠原酸和硫酸魚精蛋白組合對銅綠假單胞菌生物膜的影響，尋找一種新的、安全的策略來對抗銅綠假單胞菌的生物膜形成，而不使用抗生素，以減少對抗生素的依賴，提高療效。該組合的生物膜抑制活性針對在模擬傷口條件的離體豬皮膚外植體模型上培養的銅綠假單胞菌生物膜進行了評估。使用固著活力計數檢測活性。當外植體用綠原酸 (12 毫克/毫升) 或硫酸魚精蛋白 (0.5毫克/毫升)處理時,沒有檢測到細菌負荷的顯著差異。另一方面，與對照組未處理組相比，綠原酸 (12毫克/毫升) 和硫酸魚精蛋白 (0.5毫克/毫升) 的組合顯示細菌負荷顯著降低，3個對數循環減少。這種組合以前沒有經過測試，它是一種有前途的替代療法，可以抑制生物膜形成在傷口管理中的臨床轉化。

关键词：豬皮外植體、抗生物膜、綠原酸、硫酸魚精蛋白。

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About the authors: Nisreen Ahmad Dahshan, Department of Applied Pharmaceutical Sciences, Faculty of Pharmacy, Isra University, Amman, Jordan; Suha Mujahed Abudoleh, Department of Basic Pharmaceutical Sciences, Faculty of Pharmacy, Isra University, Amman, Jordan; Ahmad Talhouni, Zahira Alkhani, Department of Applied Pharmaceutical Sciences, Faculty of Pharmacy, Isra University, Amman, Jordan

Corresponding author Suha Mujahed Abudoleh, suha.abudoleh@iu.edu.jo

1. Introduction

Pseudomonas aeruginosa is very difficult to control and eradicate compared with other pathogens as it exerts high resistance to a wide variety of antibiotics [1-4]. *Pseudomonas aeruginosa* plays a central role in chronic wound infection [5-6]. *Pseudomonas aeruginosa* infection delays wound healing and comprises a challenge for treatment due to the development of biofilms [5, 7-9]. Biofilm infections are characterized by a decreased susceptibility to antibiotics and resistance to host immune response [6-10]. Antibiotics can decrease the number of sessile cells, but biofilm eradication is still difficult [11]. So, inhibiting *Pseudomonas aeruginosa* biofilm formation is an efficient strategy in wounds that could become chronic when infected.

Chlorogenic acid is one of the most important phenolic acids with valuable biological activities [12-14]. It has antibacterial activity against various microorganisms [15-17] and antimicrobial activity against *Pseudomonas aeruginosa* [18-19]. Wang et al. reported quorum-sensing quenching activity against *Pseudomonas aeruginosa* and biofilm formation inhibition [20].

Protamine sulfate is a cationic peptide used as a heparin antidote [21]. Protamine sulfate has antimicrobial activity against *Pseudomonas aeruginosa* [22]. It was previously reported to be effective in combinations with antibiotics and nonantibiotics against *Pseudomonas aeruginosa* [23-26].

This research aimed to investigate the effect of combining chlorogenic acid and protamine sulfate on *Pseudomonas aeruginosa* biofilm grown in *an ex vivo* porcine skin explant model that mimics wound conditions, looking for a novel, safe strategy to fight biofilm formation by *Pseudomonas aeruginosa* without using antibiotics to reduce the dependency on antibiotics, with improving efficacy.

2. Materials

Chlorogenic acid was purchased from Biosynth-Carbosynth Ltd, and protamine sulfate was purchased from Sigma-Aldrich, USA. Porcine skin explants were collected freshly from local slaughtering markets in Jordan and kept in a freezer at -20ºC for preservation. *Pseudomonas aeruginosa* ATCC 27853 was purchased from the American Type Culture Collection and preserved at -20ºC in 30 % glycerol.

3. Methods

3.1. Porcine Explant Preparation

The *ex vivo* porcine skin explant biofilm model was prepared as previously described by Phillips and co-authors with some modifications [27-28]. Explants were prepared by cutting frozen porcine skin with a round cutter to obtain 12 mm diameter explants approximately 3-4 mm thick. A high-speed drill (Louxor, China) with a round cutter was utilized to form a wound bed in the center of each explant 3 mm in diameter and 1.5 mm in depth. Explants were washed three times with normal saline and sterilized by chlorine gas for 45 minutes. Chlorine gas was generated by mixing 40 mL acetic acid with 20 mL commercial grade bleach (Chlorox®) in a covered plastic reaction chamber.

3.2. Biofilm Formation over Porcine Explant

Each explant was washed three times with normal saline and aseptically placed in 24-well plates having soft nutrient agar (0.5% agar). The wound bed was inoculated with 10 µl (ca. 6×10^6 CFU/ml) of bacterial suspension-cultured overnight in a nutrient broth that allowed for 48h growth at 37°C.

3.3. Groups and Treatments

The porcine explants were divided and grouped as the following: Chlorogenic acid-treated group (12 mg/mL), protamine sulfate treated group (0.5 mg/mL), chlorogenic acid-protamine sulfate combination-treated group (12 mg/mL chlorogenic acid and 0.5 mg/mL protamine sulfate), and untreated control group. Each group was composed of three explants. The treatments (100 µL) were applied twice at zero time and 24 h of bacterial inoculation. Explants were incubated at 37°C for 48 hr.

3.4. Explant Processing and Numeration of Biofilm

The explant was washed three times with sterile PBS and then aseptically placed into a 15 mL test tube containing 5 ml of cold, sterile PBS with 5 µl/L of Tween 80. In order to liberate the bacteria from the biofilm, explants were sonicated for 30 seconds and vortexed for another 30 seconds. The bacterial suspension was serially diluted then plated on nutrient agar plates and incubated overnight at 37ºC to determine the bacterial load (CFU/ml). Bacterial Load (CFU/mL) was log-transformed to determine log cycle reduction (Fig. 1).

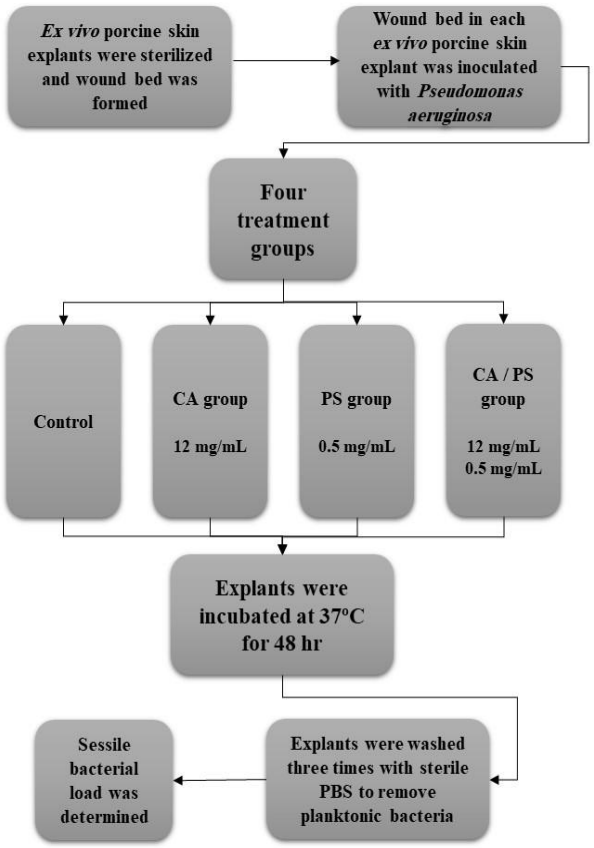

Fig. 1 Schematic of the *ex vivo* porcine skin biofilm model preparation (CA: Chlorogenic acid, PA: Protamine sulfate)

3.5. Statistical Analysis

Bacterial Load (CFU/mL) was log-transformed and calculated as the average of three trials ± SD. Analysis of variance was conducted, and differences between groups were tested for significance by one-way ANOVA using Microsoft Excel. Differences at $P < 0.05$ were considered statistically significant.

4. Results

The activity of chlorogenic acid, protamine sulfate, and their combination was evaluated. The control group explant did not receive any treatment in this study and recorded the highest bacterial load. No significant difference in the bacterial load was detected when the explant was treated either with chlorogenic acid or protamine sulfate. On the other hand, a combination of chlorogenic acid and protamine sulfate showed a significant decrease in the bacterial load with three Log cycle reductions compared to the control untreated group (Fig. 2).

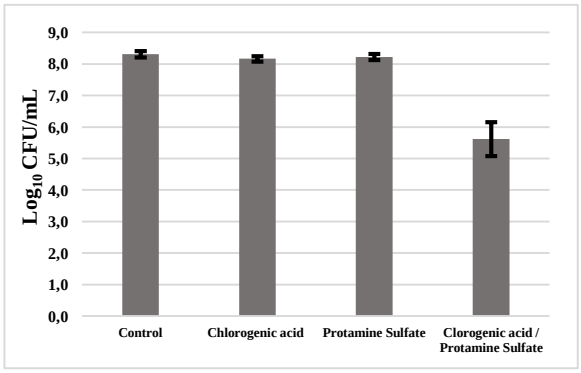

Fig. 2 Effect of chlorogenic acid (12 mg/mL) and protamine sulfate (0.5 mg/mL), and chlorogenic acid (12 mg/mL) and protamine sulfate (0.5 mg/mL) combination against *Pseudomonas aeruginosa* biofilm grown on *ex vivo* porcine skin explants, comparing with control untreated group

5. Discussion

This work aimed to evaluate the effect of chlorogenic acid and protamine sulfate combination against *Pseudomonas aeruginosa* biofilm grown on *ex vivo* porcine skin explant model mimicking wound conditions. Phenotypic characteristics of biofilm depend on surface-attached and growth conditions, which emphasizes the importance of the model [29]. This model was utilized to simulate conditions in wounds [27-30]. This combination was effective in inhibiting biofilm formation. Three log cycle reductions in sessile cell viability were observed (p-value < 0.05). This effect could be due to quorum sensing inhibitory activity of chlorogenic acid, as previously reported that attenuates virulence factors [20].

Moreover, this effect was enhanced by protamine sulfate activity. Protamine sulfate was previously proclaimed to enhance the activity of antibiotic and nonantibiotics against *Pseudomonas aeruginosa* [23-26]. Subinhibitory concentrations of protamine sulfate combined with ovotransferrin and EDTA were reported effective before reducing biofilm formation by *Pseudomonas aeruginosa* [24]. Protamine sulfate significantly enhanced the efficacy of ciprofloxacin against planktonic and sessile *Pseudomonas aeruginosa*, as previously reported [23]. Neither chlorogenic acid alone nor protamine sulfate has an inhibitory effect on *Pseudomonas aeruginosa* biofilm grown in *ex vivo* porcine skin. However, this combination effectively inhibited biofilm formation that could be clinically translated in wound management.

6. Conclusion

Antibiotic resistance is a major worldwide concern demanding new therapeutic options. *Pseudomonas aeruginosa* formation of biofilm represents a significant hurdle in chronic wound management. In this work, we have utilized a porcine skin *ex vivo*

wound model to simulate conditions in *Pseudomonas aeruginosa* biofilm grown on wounds as the growing conditions have a major influence on phenotypic characteristics of biofilms. A combination of chlorogenic acid and protamine sulfate effectively inhibited biofilm formation. Three log cycle reduction in sessile cell viability was attained. Chlorogenic acid was previously effective in attenuating *Pseudomonas aeruginosa* quorum sensing [20]. Protamine sulfate was previously reported to enhance the activity of antibiotic and nonantibiotics against *Pseudomonas aeruginosa* [23-26]. In this work, we have found that this combination effectively inhibited *Pseudomonas aeruginosa* biofilm formation. The wound model utilized in this study mimics conditions in wounds and provides a surface for biofilm adherence. Planktonic cells were removed before bacterial count determination not to exaggerate the biofilm inhibitory activity. Hence, the three-log cycle reduction observed is for sessile bacterial cells that are much resistant than planktonic cells. This combination is a promising alternative therapy inhibiting biofilm formation from being applied to wound management as it could be added to wound dressing to inhibit biofilm formation. The activity could be also be enhanced with multiple treatments and different dosage formulations. Other combinations with either chlorogenic acid or protamine sulfate could also be evaluated in the future as combination therapies are currently promising alternative therapies for biofilm formation.

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