行政院國家科學委員會專題研究計畫　成果報告

骨質疏鬆症的動物模式中 Pg-LPS 與蝕骨細胞生成間的關係

計畫類別：個別型計畫
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執行期間：93 年 08 月 01 日至 94 年 07 月 31 日
執行單位：臺北醫學大學牙醫學系

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*Porphyromonas gingivalis*脂多醣對停經後骨質疏鬆症之鼠科動物模式血清中OPG/RANKL、Interleukin-6 調節之影響

Effect of *Porphyromonas gingivalis* lipopolysaccharide on the regulation of OPG/RANKL and Interleukin-6 in the serum of simulating murine model of postmenopausal osteoporosis

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Abstract

Periodontitis is a chronic inflammatory disease which results in the breakdown of tooth supporting structures, especially alveolar bone destruction. Deep periodontal pocket, attachment level loss, and alveolar bone loss are found under clinical examination. *Porphyromonas gingivalis* (*P. gingivalis*), a black-pigmented gram-negative anaerobic bacterial rods, is a vital pathogen for adult periodontitis. *P. gingivalis* has been proved to contribute to bone resorption in many vivo studies. However, unlike the clear relationship between osteoporosis and tooth loss, controversy still exists concerning the association between osteopenia/opteoporosis and the periodontal pathogen. The purpose of the present study is to clarify the etiologic relationship of *P. gingivalis* lipopolysaccharide (*P. gingivalis* LPS) and the expression of OPG, RANKL, IL-6 in the serum of postmenopausal osteoporosis mice. Injection of *Escherichia coli* LPS (*E. coli* LPS) was need as the control.

Ninty 10-week old ICR female mice were divided into ovariectomized group (experimental group) and sham-operation group (control group). After operation 4 weeks, we collect the serum as baseline. 100 μg *P. gingivalis* LPS and *E. coli* LPS were separately injected into the peritoneum of experimental group (30 mice) and control group (30 mice). Subsequently, we collected the serum 1,3,6,24 and 48 hours after the injection of both LPS. Then the concentrations of IL-6, OPG and RANKL of serum will be estimated by using sandwich ELISA. The femur bone was dissected for TRAP stain analysis. Besides, 4 weeks after operation, 15 experimental mice and 15 control mice was sacrificed for TRAP stain to compare lipopolysaccharide effect. Mann-Whitney U test was applied to study the difference of OPG, RANKL, OPG/RANKL ratio, IL-6 between *P. gingivalis* LPS and *E. coli* LPS. Wilcoxon signed rank test was used to study the difference of OPG, RANKL, OPG/RANKL ratio, IL-6 between each time interval and baseline in group. The strength of correlations between IL-6 and OPG, RANKL, OPG/RANKL ratio at each time interval in group were determined by Spearman rank correlation coefficients. The difference of TRAP positive cell count in the specimen among was compared by using Kruskal-Wallis test. Results with $p< 0.05$ were defined statistically different.

In the injection of *P. gingivalis* LPS of experimental and control group, OPG/RANKL ratio decreased at 3 hours ($p< 0.05$), then trended to rising tendency. IL-6 rose at 1、3 hours ($p< 0.05$). Besides, the changes of OPG, RANKL, OPG/RANKL ratio, IL-6 were greater in injection of *E. coli* LPS than *P. gingivalis* LPS. There is stronger TRAP positive cells distribution in experimental group than control group ($p< 0.05$). No matter single booster of *P. gingivalis* LPS or *E. coli* LPS did not effect the expression of TRAP positive cells.

It should be noticed that only single shot of bacterial LPS was applied in this animal model. *P. gingivalis* LPS directly effected the expression of IL-6, but not OPG/RANKL system. It indicated that OPG/RANKL ratio of experimental group injected with *P. gingivalis* LPS might also be regulated by a more complex system. Removal of ovary did not effect the expression of IL-6; our data also indicated there were no correlation of IL-6, expression of OPG, RANKL, and OPG/RANKL ratio. In addition, the stimulatory effect of *E. coli* LPS was stronger than *P. gingivalis* LPS.

Keywords: postmenopausal osteoporosis, osteoprotegerin, RANKL, Interleukin-6, tartrate-resistant acid phosphatase.

Introduction

骨質疏鬆症和牙周疾病間的相關性目前還不是十分清楚。某些研究支持低的骨密度或是骨質疏鬆是降低齒槽骨高度、牙周附連高度的指標，還有許多研究認為牙齒的喪失 (tooth loss) 和系統性的骨質疏鬆症 (systemic osteoporosis) 有關[i,ii,iii,iv,v,vi,vii,viii]。此外也有研究提出骨骼密度和牙周疾病間並無顯著相關性[ix,x]。會產生以上之爭議可能導因於上述研究多屬橫向之臨床研究，缺乏縱向動物實驗與臨床追蹤。

因此本實驗的設計是將牙周致病菌 *Porphyromonas gingivalis* 的脂多醣 (lipopolysaccharide)(*P. gingivalis* LPS) 注入切除卵巢老鼠的腹膜腔 (停經後骨質疏鬆症動物模型)，觀察與骨表現有關的 osteoprotegerin (OPG)、receptor activator of nuclear factor κB ligand (RANKL)、Interleukin-6 (IL-6) 與 tartrate-resistant acid phosphatase (TRAP) 等生物標誌之表現。假設 *P. gingivalis* LPS 引起停經後骨質疏鬆症老鼠的噬骨細

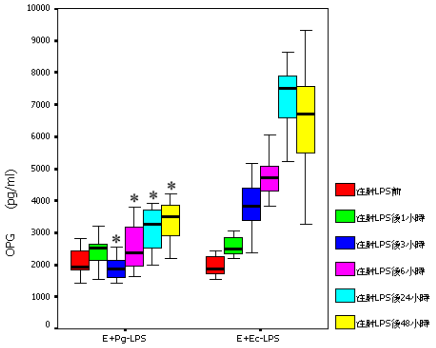
胞生成 (osteoclastogenesis) 反應是透過與骨吸收表現有關的 OPG/RANKL 系統表現而加速噬骨細胞生成，則患有停經後骨質疏鬆症老鼠注入 *P. gingivalis* LPS 後，血清中 OPG 的濃度會下降、RANKL 上升、代表噬骨細胞生成的 OPG/RANKL ratio 會下降。如果是透過 IL-6 來調控噬骨細胞生成，則血清中 IL-6 的濃度會上升。並且取下老鼠的股骨組織切片作 TRAP 染色，觀察 TRAP 染色呈陽性的細胞數量。假設在切除卵巢並且注入 *P. gingivalis* LPS 的老鼠會有較多的 TRAP 染色呈陽性的細胞。

Materials and methods

選擇 90 隻 10 週大的 ICR 雌性小白鼠 (由國立台灣大學醫學院實驗動物中心提供)，將 90 隻老鼠分成六組，每組 15 隻，第一組切除卵巢 (實驗組一) 後 4 週，將 100 μg 的 *P. gingivalis* LPS 注入老鼠的腹膜；第二組假性切除卵巢 (對照組一) 後 4 週，將 100 μg 的 *P. gingivalis* LPS 注入老鼠的腹膜； 第三組切除卵巢 (實驗組二) 後 4 週，將 100 μg 的 *Escherichia coli* 的 LPS (*E. coli* LPS) 注入老鼠的腹膜；第四組假性切除卵巢 (對照組二) 後 4 週，將 100 μg 的 *E. coli* LPS 注入老鼠的腹膜。老鼠是以 Pentobarbital (6.0 mg/100g body weight；腹腔注射)麻醉，採背側進入腹腔的方法進行卵巢切除。切除卵巢 4 週後在眼窩抽血，離心後取血清以分析 IL-6、OPG 及 RANKL，做為骨吸收反應的 biomarker，作為注入脂多醣的基準點。分別在注入後 1 小時、3 小時、6 小時、24 小時、48 小時抽血，之後犧牲動物，取下右側股骨做 TRAP 染色觀察。剩下的第五組則是切除卵巢 (實驗組三)、第六組假性切除卵巢 (對照組三)，手術後 4 週犧牲動物，取下右側股骨做 TRAP 染色觀察，作為注入 *P. gingivalis* LPS、*E. coli* LPS 的組織切片對照。

Results

圖 1. 實驗組老鼠注入 *P. gingivalis* LPS、*E. coli* LPS 的 OPG 值比較

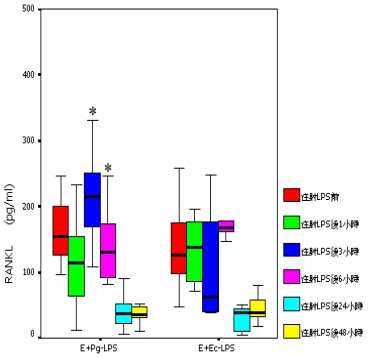


說明：

1. **E+Pg-LPS 為實驗組注射 *P. gingivalis* LPS 100 μg**
   E+Ec-LPS 為實驗組注射 *E. coli* LPS 100 μg

2. **實驗組注射 *P. gingivalis* LPS 後 3、6、24、48 小時比注射 *E. coli* LPS 低。(*為實驗組注射 *P. gingivalis* LPS、*E. coli* LPS 在各時間點相比有差異，Mann-Whitney U test，$p < 0.05$。)**

圖 2. 實驗組老鼠注入 *P. gingivalis* LPS、*E. coli* LPS 的 RANKL 值比較

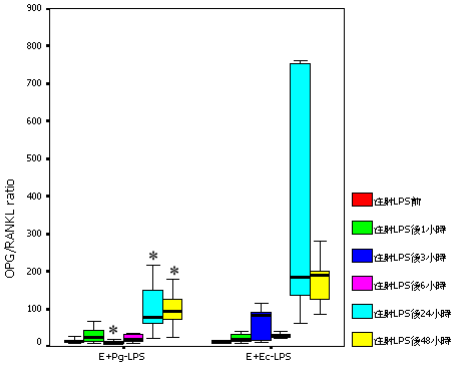


說明：

1. **E+Pg-LPS 為實驗組注射 *P. gingivalis* LPS 100 μg**
   E+Ec-LPS 為實驗組注射 *E. coli* LPS 100 μg

2. **實驗組注射 *P. gingivalis* LPS 後 3 小時比注射 *P. gingivalis* LPS 高，6 小時時比注射 *E. coli* LPS 低。(*為實驗組注射 *P. gingivalis* LPS、*E. coli* LPS 各時間點相比有差異，Mann-Whitney U test，$p < 0.05$。)**

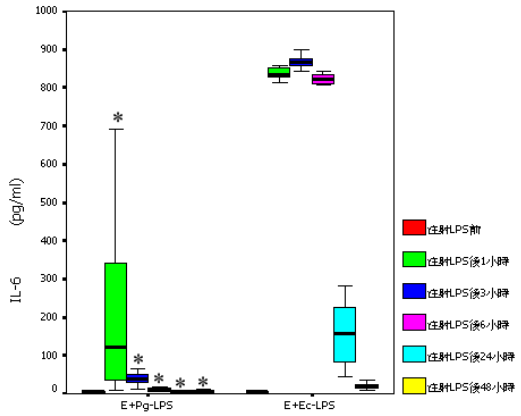
圖 3. 實驗組老鼠注入 *P. gingivalis* LPS、*E. coli* LPS 的 OPG/RANKL ratio 值比較



說明：

1. E+Pg-LPS 為實驗組注射 *P. gingivalis* LPS 100 μg
   E+Ec-LPS 為實驗組注射 *E. coli* LPS 100 μg
2. 實驗組注射 *P. gingivalis* LPS 後 3、24、48 小時比注射 *E. coli* LPS 低。 (*為實驗組注射 *P. gingivalis* LPS、*E. coli* LPS 各時間點相比有差異，Mann-Whitney U test，$p < 0.05$。)

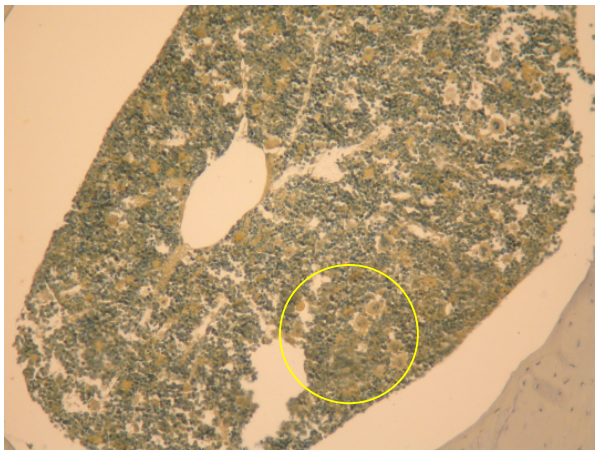
圖 4. 實驗組老鼠注入 *P. gingivalis* LPS、*E. coli* LPS 的 IL-6 值比較



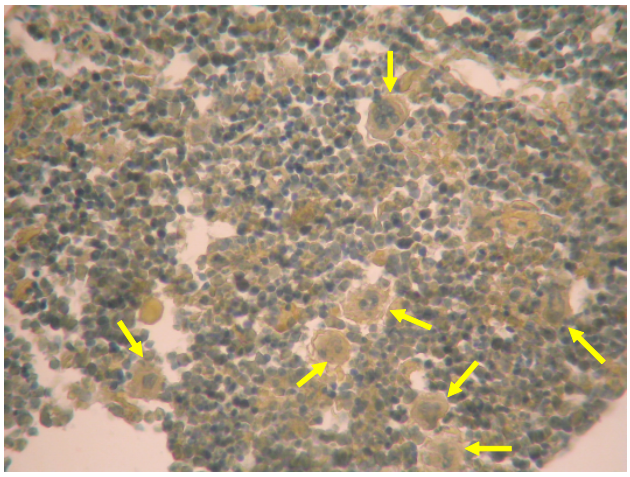
說明：

1. E+Pg-LPS 為實驗組注射 *P. gingivalis* LPS 100 μg
   E+Ec-LPS 為實驗組注射 *E. coli* LPS 100 μg
2. 實驗組注射 *P. gingivalis* LPS 後 1、3、6、24、48 小時比注射 *E. coli* LPS 低。(*為實驗組注射 *P. gingivalis* LPS、*E. coli* LPS 各時間點相比有差異，Mann- Whitney U test，$p < 0.05$。)

圖 5. 實驗組老鼠注入 *P. gingivalis* LPS 的 TRAP 染色



200 倍



400倍

說明: TRAP染色呈陽性的細胞 (黃色箭頭處)。

表 1. 實驗組老鼠注入 *P. gingivalis* LPS 在各個時間點時 IL-6 與 OPG、RANKL、OPG/RANKL ratio 的相關性

	OPG	RANKL	OPG/RANKLratio
IL-6 Baseline	r = 0.300	r = -0.200	r = 0.350
IL-6 After injection 1 hour	r = 0.121	r = 0.168	r = -0.218
IL-6 After injection 3 hours	r = 0.125	r = -0.200	r = -0.082
IL-6 After injection 6 hours	r = 0.125	r = -0.293	r = 0.236
IL-6 After injection 24 hours	r = -0.007	r = 0.226	r = -0.138
IL-6 After injection 24 hours	r = 0.114	r = 0.146	r = 0.025

各個時間點的 IL-6 與 OPG、RANKL、OPG/RANKL ratio 沒有相關性。(*p* 值皆大於 0.05)

表 2. 對照組老鼠注入 *P. gingivalis* LPS 在各個時間點時 IL-6 與 OPG、RANKL、OPG/RANKL ratio 的相關性

	OPG	RANKL	OPG/RANKL ratio
IL-6 Baseline	r = 0.742*	r = -0.538	r = 0.509
IL-6 After injection 1 hour	r = 0.016	r = -0.049	r = 0.147
IL-6 After injection 3 hours	r = -0.049	r = -0.203	r = 0.005
IL-6 After injection 6 hours	r = 0.148	r = 0.231	r = -0.154
IL-6 After injection 24 hours	r = -0.060	r = 0.070	r = -0.098
IL-6 After injection 24 hours	r = 0.038	r = -0.084	r = 0.105

***為有相關性 (Spearman rank correlation coefficients，*為 *p*< 0.05)。**

Conclusion

由本動物模式發現：無論是實驗組或對照組，由***Pg-LPS***所誘導之IL-6的表現，和OPG、RANKL、OPG/RANKL ratio皆沒有相關性。因此可推論牙周致病菌對停經後骨質疏鬆症之骨代償機制可能沒有直接全身性的影響。本實驗可推翻牙周炎之感染會加劇停經後骨質疏鬆之假說。

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