行政院國家科學委員會專題研究計畫　成果報告

人工關節溶解性鈦微粒對肺部及心血管系統的作用(2/2)

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行政院國家科學委員會專題研究計畫成果報告

人工關節溶解性鈦微粒對肺部及心血管系統的作用

計畫編號：NSC 91-2314-B-002-248 (1/2)
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主持人：楊榮森 教授
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一、 中文摘要

目前人工關節的置換已是骨科臨床上治療關節炎的常見手術，由於鈦具有高惰性及耐腐蝕性，因此鈦合金(Ti_6Al_4V)是其中一種廣被採用於手術醫材及人工關節的材質，但是使鈦植入物後，會釋出溶解態腐蝕產物及磨損微粒。本研究小組也曾證實，發生人工關節鬆脫病患的血液內及尿液的金屬離子含量(包括鈦)遠比正常人及未發生鬆脫者為高，顯見金屬元素可經循環系統分佈全身組織。這些金屬可在體內各處散佈，這些又可能再引起該部位的局部巨噬細胞進行各種反應，包括釋出一氧化氮，細胞動力素、分解酵素等，因此可能會對全身各處引起各種可能的生理或病理反應，例如肺臟、心血管組織等。近年來有關此方面的研究很少。第一年研究：為利用已建立的體外細胞培養模式，以探討人工關節鈦金屬釋放之溶解態鈦所引起的細胞生化學酵素變化及其互動關係。結果發現植入人工關節同材質的金屬(Ti_6Al_4V)塊動物2和4周後，其血液及肺部鈦元素含量明顯增高，且分離出之肺泡巨噬細胞對於細菌內毒素引起之一氧化氮生成及對於誘導型一氧化氮生成酶表現上有減弱現象。外加溶解態二氧化鈦至大鼠肺泡巨噬細胞亦可看到LPS刺激NO釋放及iNOS蛋白和mRNA量增加的情形皆會受到抑制。這些結果似乎暗示著肺部巨噬細胞在鈦作用下對於細菌內毒素引發之免疫反應會改變。另外，我們也發現當以溶解態二氧化鈦處理人類臍靜脈內皮細胞時eNOS蛋白表現會減少，且合併細菌內毒素處理時eNOS減少情形更明顯，但cyclooxygenase-2蛋白表現卻增加，此也似乎暗示著心血管系統功能可能會受到影響。第二年研究：主要研究重點在於利用動物模式進行研究，以探討鈦合金人工關節釋放之溶解態鈦，對肺臟及心血管系統的影響，以進一步探討人工關節金屬微粒所引起的全身性反應，以供推論人工關節置換手術後可能引起全身之反應。結果發現Wistar鼠埋鈦合金植入物4週後，血液及肺部之Ti元素明顯上升；而合併處理細菌內毒素6小時後發現老鼠肺部水種情形明顯增強（與單獨細胞內毒素組比較）且肺部病理現象亦較對照組來得顯著，這些現象可被一氧化氮抑制劑（iNOS inhibitor）aminoguanidine所拮抗。另外在肺部支氣管肺泡抽出液及血液

中皆可偵測到一氧化氮（NO）的存在（以Nitrite表示），這些作用也可被aminoguanidine 所拮抗。而在西方墨點法實驗中，鈦合金合併內毒素處理組的肺部組織可偵測到較對照組明顯的誘導型一氧化氮生成酶（iNOS）蛋白表現量。這些結果顯示鈦合金釋放出來的鈦原素會加強細菌內毒素感染時肺部的發炎反應。由於人口老化及關節炎病患的日漸增多，可預知二十一世紀會有更多骨科病患接受人工關節(例如選用鈦合金材質)的置換手術，因此深入探討人工關節置換手術長期之後，可能引起的全身組織變化，可供吾人明白這些可預見的重要資料及可能重要機轉，因此極具重要臨床意義。

關鍵字：鈦植入物；一氧化氮；肺部組織；內毒素；人類臍靜脈內皮細胞

Abstract

Total joint prosthesis replacement has become one of the most common procedures to treat arthritic patients in the clinical orthopedic practice. Titanium alloy (Ti_6Al_4V) is one of the materials widely used in common orthopaedic practice. The use of Ti implant results in the dissemination of soluble metallic corrosion products and particulate metallic wear debris in the long run. Previous reports focused on the release of metal debris that has been reported to be an important underlying mechanism for prosthesis-related late complications, such as loosening of prosthesis, local osteolysis, release of inflammatory factors. The first year uses *in vitro* cell culture model (easier to control the experimental conditions). We have found that an increase in the levels of elemental Ti in the blood and lung tissues of rats with an alloyed-Ti implant also has been found. The cellular reaction to elevated elemental Ti in the circulation remains unclear. We furth- er performed experiments to examine the changes of inducible nitric oxide synthase（iNOS）expression in alveolar macrophages from alloyed-Ti-implanted rats. The ele- vation of nitrite and iNOS expression induced by lipopolysaccharide（LPS）was suppr- essed. The *in vitro* effect of a soluble form of Ti was further investigated. Ti（0.01- 0.06 mM）inhibited the LPS-induced nitrite production and iNOS expression in alve- olar macrophages from normal rats without any cytotoxic effects. LPS induced protein tyrosine phosphorylation, tyrosine-phosphorylation of *lyn*（a CD14-receptor-associa- ted-tyrosine kinase）, and degradation of I_KB-α protein（inhibitor of NF-$_K$B）in alve- olar macrophages. These events were inhibited by co-incubation with Ti. These results indicate that elemental Ti may impair iNOS expression in alveolar macrophages thro- ugh the alteration of protein tyrosine

phosphorylation and NF-$_K$B activation. The inhibitory action of Ti on cellular responses of alveolar macrophages may be anti-inflammatory and thus may depress local defense mechanisms related to microbial killing.

The second year uses animal model to investigate the effect of Ti on the lung tissue. We have found that the Ti alloy implant enhanced lung injury rel ated to endotoxin from Gram-negative bacteria (lipopolysaccharide, LPS), which was characterized by lung edema and other histo- logical changes such as recruitment of neutrophils, interstitial edema, and alveolar hemorrhage in the lung. In the presence of endotoxin, an increase of nitrite production was shown in the plasma and bronchialveolar lavage fluid of rats implanted with a Ti alloy. Moreover, the Ti alloy implant further enhanced the induction of inducible nitric oxide (NO) synthase (iNOS) protein expression induced by LPS in the lung. These endotoxin-related responses in the presence or absence of the Ti alloy implant could be inhibited by aminoguanidine (an iNOS inhibitor). These results provide the first experimental evidence that circulating Ti released from Ti alloy implants has an ability to affect pulmonary iNOS protein expression, and enhance the pathogenesis of acute lung injury during endotoxemia.

Keywords: titanium；alveolar macrophages；inducible nitric oxide synthase；protein tyrosine phosphorylation；titanium alloy, lung tissue, human endothelial cells, nitric oxide synthase, endotoxin.

二、 緣由與目的

隨著骨科醫學的進展，使人工關節的研發廣泛應用於嚴重關節炎病患的治療，多年來改善眾多病患的關節功能及生活品質，造福無數關節炎病患，臨床績效十分彰顯。可是部分病患在置換人工關節後，發生一些始料未及的併發症，引起人工關節周圍組織的局部反應，包括骨吸收及人工關節鬆脫，進而影響其臨床成果，嚴重者可能須接受二度人工關節置換手術。先前的許多研究皆注重這些局部組織的反應，這些研究探討引起人工關節局部反應的可能病因，結果證實前述變化可能與人工關節的磨損微粒間具有密切關係，其中例如金屬微粒、高分子量聚乙烯微粒，及骨水泥微粒等，皆可能扮演重要的角色，因而稱為微粒疾病。由於人口老化及關節炎病患的日漸增多，可預知二十一世紀會有更多骨科病患接受人工關節(例如選用鈦合金材質)的置換手術，因此深入探討人工關節置換手術長期之後，可能引起的全身組織變化，可供吾人明白這些可預見的重要資料及可能重要機轉，因此極具重要臨床意義。鈦(Ti)曾被建議對於無骨水泥式人工髖關節

是選擇性材質。但是有些研究也發現合金材質人工關節磨損導致可溶性金屬(包括鈦)釋出且會隨著循環到達全身；本研究小組也曾證實，發生人工關節鬆脫病患的血液內及尿液的金屬離子含量(包括鈦)遠比正常人及未發生鬆脫者為高，顯見金屬元素可經循環系統分佈全身組織。這些可溶性金屬可能進而在體內各處散佈，這些又可能再引起該部位的局部巨噬細胞進行各種反應，包括釋出一氧化氮，細胞動力素、分解酵素等，因此可能會對全身各處引起各種可能的生理或病理反應，例如肺臟、心血管組織等。近年來有關此方面的研究很少，因此，進一步探討人工關節磨損微粒的全身生理反應，可有助於我們明白這些長期置入體內的人工關節，所可能會造成的影響。所以本研究計畫藉體外細胞和組織培養模式(肺泡巨噬細胞，血管內皮細胞及血管)及動物植入人工關節金屬塊(Ti_6Al_4V)之體內實驗模式，希望可以了解有關人工關節植入後的全身組織病理變化。目前有關此方面的研究報告很少，因此，更顯得本研究的臨床意義十分重大。

本二年期研究計畫之執行，在第一年係利用體外細胞及組織培養模式（條件較便利控制）探討人工關節相關金屬離子對動物組織所造成的影響，第二年再利用動物體內模式深入評估其所造成的全身性反應。本研究的重要研究工作分述如下：第一年研究：為利用已建立的體外細胞和組織培養模式，已探討人工關節鈦金屬釋放之溶解態鈦所引起的細胞和組織生化學酵素變化及其互動關係。主要探討肺部巨噬細胞、血管內皮細胞、血管組織等在未受刺激及接受磨損微粒或溶解態二氧化鈦刺激時（配合與細菌內毒素的相互作用），其釋出一氧化氮及其合成酵素及可能牽涉到的訊息傳遞相關路徑變化。對主動脈環而言，並且測試血管平滑肌收縮變化，以評估心血管的變化。第二年研究：為利用植入人工關節金屬塊(Ti_6Al_4V)之動物模式，以探討人工關節鈦金屬釋放之可溶性鈦所引起動物肺臟及心血管系統的影響。利用動物體內模式施行長期間置入（配合與細菌內毒素的相互作用），以觀察血壓變化、離體血管收縮情形及肺水腫程度，並測量分離之肺和心血管組織內所含有的鈦元素量，一氧化氮，及可能牽涉到的訊息傳遞相關路徑變化。

經由此研究我們可藉以推論臨床上關節炎病患在接受人工關節置換手術後的可能反應，對於人工關節置換病患的臨床意義而言，本研究可提供非常珍貴的資料供參考，意義重大。

三、 結果與討論

第一年研究：試驗結果發現植入人工關節同材質的金屬(Ti_6Al_4V)塊動物(Wistar rats) 2 和 4 周後，其血液及肺部鈦元素含量明顯增高 (Fig. 1)，且分離出之肺泡巨噬細胞(alveolar macrophages)對於細菌內毒素(lipopolysaccharide, LPS)引起

之一氧化氮(nitric oxide, NO)生成及對於誘導型一氧化氮生成酶(iNOS)表現上有減弱現象(Figs 2 and 3)。外加溶解態二氧化鈦(TiO_2) (0.01-0.06 mM)至大鼠肺泡巨噬細胞亦可看到 LPS 刺激 NO 釋放及 iNOS 蛋白和 mRNA 量增加的情形皆會受到抑制。這些結果似乎暗示著肺部巨噬細胞在鈦作用下對於細菌內毒素引發之免疫反應會改變。另外，我們也發現當以溶解態二氧化鈦(Ti, 0.06mM)處理人類臍靜脈內皮細胞(HUVECs)時，eNOS(endothelial nitric oxide synthase)蛋白表現會減少，且合併細菌內毒素(LPS, 10μg/mg)處理時 eNOS 減少情形更明顯，但 COX-Ⅱ蛋白表現卻增加 (Fig.4)，此也似乎暗示著心血管系統功能可能會受到影響。

第二年研究：Wistar 鼠埋鈦合金植入物 4 週後，血液及肺部之 Ti 元素明顯上升(Fig.1)；而合併處理細菌內毒素 6 小時後發現老鼠肺部水種情形明顯增強（與單獨細胞內毒素組比較）且肺部病理現象亦較對照組來得顯著(Fig.5)，這些現象可被一氧化氮抑制劑（iNOS inhibitor）aminoguanidine 所拮抗，顯示一氧化氮參與其中的反應。另外，在肺部支氣管肺泡抽出液及血液中皆可偵測到一氧化氮（NO）的存在（以亞硝酸物 Nitrite 表示）(Fig.6)，這些作用也可被 aminoguanidine 所拮抗。而在西方墨點法實驗中，鈦合金合併內毒素處理組的肺部組織可偵測到較對照組明顯的誘導型一氧化氮生成每（iNOS）蛋白表現量(Fig.7)。這些結果顯示鈦合金釋放出來的鈦元素會加強細菌內毒素感染時肺部的發炎反應，而一氧化氮在其中扮演重要的媒介角色。

由於人口老化及關節炎病患的日漸增多，可預知二十一世紀會有更多骨科病患接受人工關節(例如選用鈦合金材質)的置換手術，因此深入探討人工關節置換手術長期之後，可能引起的全身組織變化，可供吾人明白這些可預見的重要資料及可能重要機轉。經由此研究我們可藉以推論臨床上關節炎病患在接受人工關節置換手術後的可能反應，對於人工關節置換病患的臨床意義而言，本研究可提供非常珍貴的資料供參考，意義重大。

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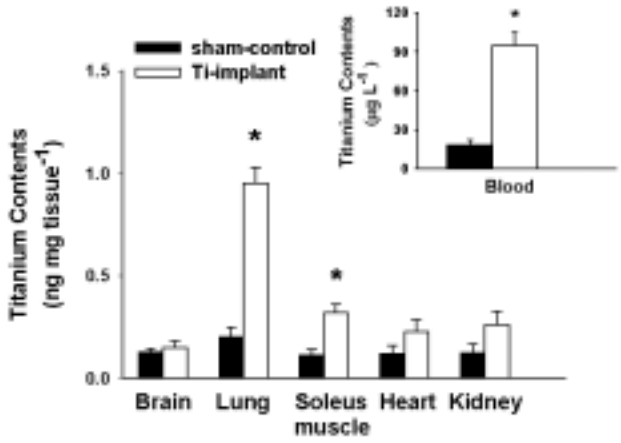

Fig. 1. Ti content in the blood and various tissues of titanium alloy implanted rats. Rats were implanted with titanium alloy discs 4 weeks prior to measurement of Ti content. Data are presented as means±SEM for four to six animals. * P < 0.05 as compared with

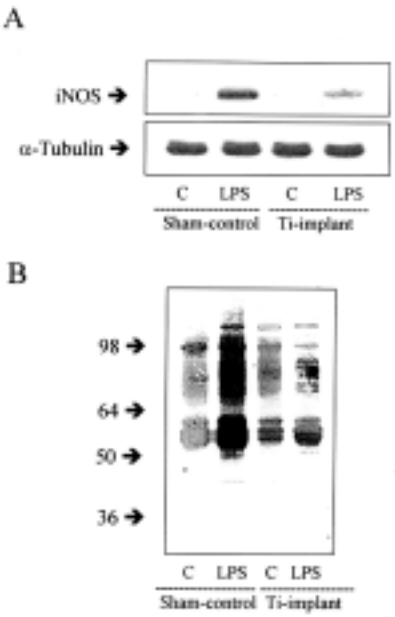

Fig.2. Alteration of LPS-induced iNOS protein expression and protein tyrosine phosphorylation in alveolar macrophages from titanium alloy implanted rats. Rats were implanted with titanium alloy discs 4 weeks prior to bronchoalveolar lavage to obtain alveolar macrophages. Alveolar macrophages were exposed in culture to 10 μg/ml LPS for 6 h prior to assay of iNOS and protein tyrosine phosphorylation. (A). Total cell lysates were prepared and subjected to Western blot analysis for macrophage iNOS as described in Methods. (B). Tyrosine phosphorylation of multiple proteins were detected using an anti-phosphotyrosine antibody.

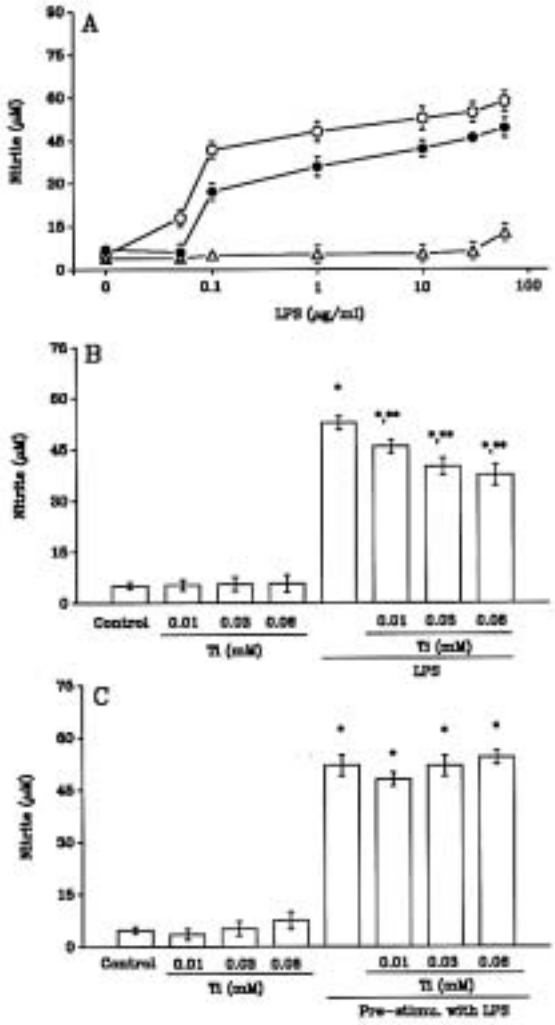

Fig 3. Inhibition of LPS-induced NO production in alveolar macrophages from normal rats with titanium. (A). Effects of Ti (0.06 mM) and anti-CD14 antibody (10 μg/ml) on production of nitrite from macrophages exposed to various LPS doses for 24 h. (B). Effects of increasing concentrations of Ti on production of nitrite from macrophages in the absence or presence of LPS (10 μg/ml) for 24 h. (C). Macrophages were pre-stimulated (pre-stim) with LPS (10 μg/ml) for 24 h. After which LPS was removed, and macrophages were subsequently incubated in medium containing Ti for a further 24 h prior to measurement of nitrite products in conditioned medium. Data are presented as means±SEM of 3-5 separate experiments performed in triplicate. * P<0.05 as compared with controls. ** P<0.05 as compared with LPS alone.

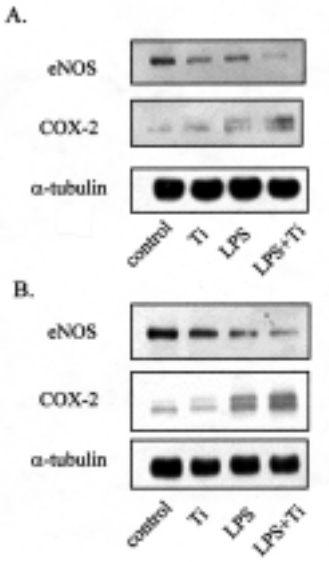

Fig.4. Western blot analysis of eNOS and COX-2 proteins in HUVECs. HUVECs were co-incubated with Ti (0.06 mM) and LPS (10贡g/ml) for 6 hr (A) and 24 hr (B). These proteins were separated by SDS-PAGE and analyzed by immunoblotting.

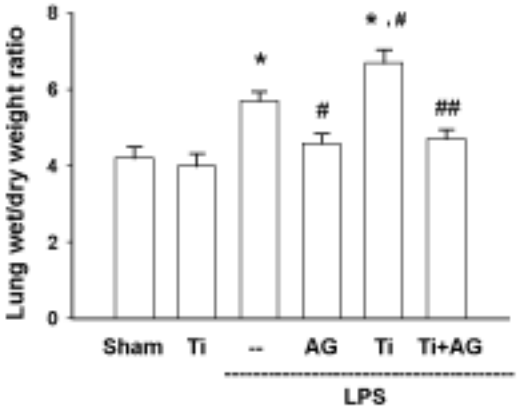

Fig. 5. LPS-induced lung edema in titanium alloy implanted rats. Lung edema was measured as wet/dry weight ratio in rats without or with Ti implants, which were injected peritoneally with bacterial endotoxin (LPS) for 6 h. Some rats were pretreated with aminoguanidine (AG). Data are presented as means+SEM of three experiments (three rats per group in each experiment). *: $P < 0.05$ as compared with sham control. $^{\#}$ $P<0.05$ as compared with LPS alone. $^{\#\#}$ $P<0.05$ as compared with Ti+LPS alone.

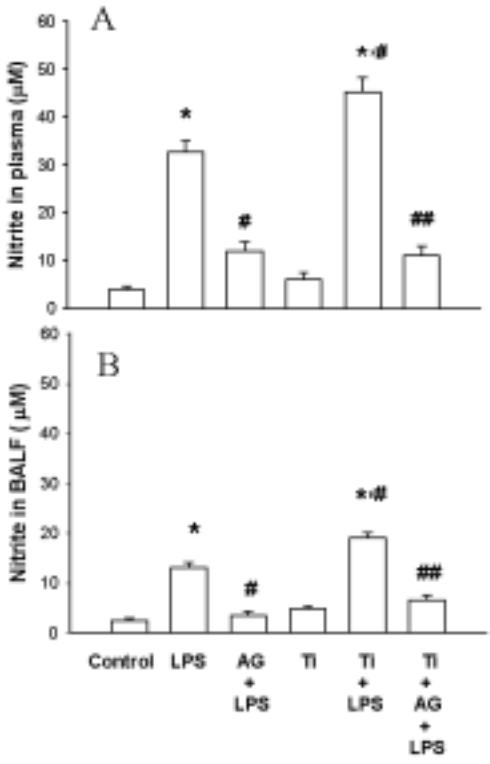

Fig. 6. Ti implants enhance NO production related to LPS in plasma and bronchoalveolar lavage fluid (BALF). Rats without or with Ti implants were injected peritoneally with bacterial endotoxin (LPS) for 6 h to measure plasma (A) and BALF (B) nitrite levels. Some rats were pretreated with aminoguanidine (AG). Data are presented as means+SEM of three experiments (three rats per group in each experiment). *: $P < 0.05$ as compared with sham control. $^{\#}$ $P<0.05$ as compared with LPS alone. $^{\#\#}$ $P<0.05$ as compared with Ti+LPS.

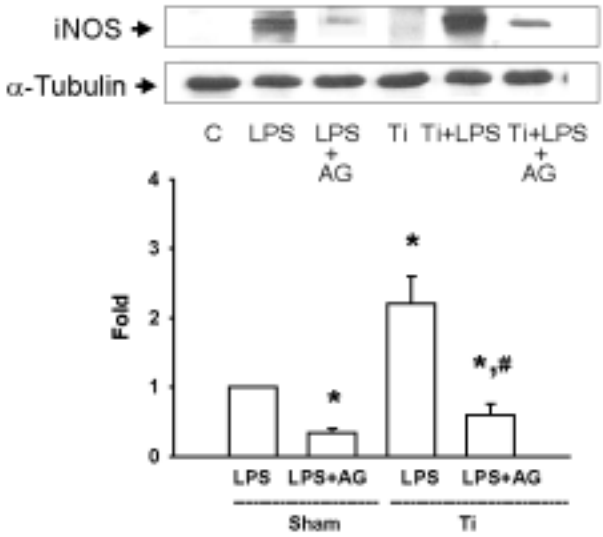

Fig. 7. Alteration of iNOS protein expression in the lung of Ti alloy implanted rats. Lung samples in rats without or with Ti implants, which were treated intraperitoneally with LPS for 6 h, were prepared and subjected to Western blot analysis as described in Methods. α-Tubulin served as control for sample loading and integrity. Quantification of the expression of iNOS protein expression was performed by densitometric analysis. Data are presented as means±SEM of three experiments. *: $P < 0.05$ as compared with LPS alone. #: $P < 0.05$ as compared with Ti+LPS.