科技部補助專題研究計畫成果報告
期末報告

牙周病嚴重程度與牙齦液胱抑素活性關係之流行病學追蹤研究（第3年）

計 畫 類 別 ： 個別型計畫
計 畫 編 號 ： NSC 102-2314-B-040-013-MY3
執 行 期 間 ： 104年08月01日至105年07月31日
執 行 單 位 ： 中山醫學大學口腔科學研究所

計 畫 主 持 人： 胡素婉
共 同 主 持 人： 林育誼
計畫參與人員： 學士級-專任助理人員：江芷吟
博士班研究生-兼任助理人員：鄒欣樺

中 華 民 國 105 年 10 月 23 日

中 文 摘 要 ： 牙周病致病菌會釋放出半胱胺酸蛋白酶，我們最近的研究發現牙周病患者牙齦液的半胱胺酸蛋白酶活性在治療後會降低。牙周病菌所引起的組織破壞，大部份是宿主本身降解酶作用的結果。宿主的組織蛋白酶也是屬於半胱胺酸蛋白酶，在骨頭破壞上扮演重要角色，它的天然抑制物-胱抑素，存在於唾液和牙齦液中。研究指出牙周病患牙齦液中具有較高的組織蛋白酶，發炎牙齦組織胱抑素表現較低。然而有關研究仍然相當缺乏。在牙齦液中的激肽釋放酶系統也可能受到胱抑酶所調控，因為激肽的產生也是藉由半胱胺酸蛋白酶活化，我們先前研究顯示，激肽可以增加血管通透性，幫助牙周病菌進入循環系統。本研究的目的為探討牙周狀態和宿主半胱胺酸蛋白酶抑制能力的關連性、以及唾液和牙齦液中胱抑素的濃度，同時也將測量牙周病人的組織蛋白酶含量、激肽濃度以及牙周病菌的半胱胺酸蛋白酶水解能力。本追蹤式調查之研究對象為在牙周病科門診求診且符合研究納入條件的患者，參加者接受問卷訪談，並在4個時間點(治療前及治療後3、6、9個月)接受口腔檢查並採集唾液和牙齦液，牙齦液將採自兩顆有最嚴重牙周病的牙齒(牙周病區)和兩顆正常的牙齒(健康區)。本計畫的結果如下：(1)有142位牙周病患者參加本研究，共完成467人次的調查。(2)總共收集467個唾液樣品與1868個牙齦液樣品。(3)在治療前收集的牙齦液樣品，牙周病區的半胱胺酸蛋白酶的平均濃度顯著高於健康區(平均值(標準差)：0.47 (0.11) vs. 0.40 (0.06))；牙周病區牙齦液的半胱胺酸蛋白酶的平均濃度，在治療前顯著高於治療後3個月。(4)牙周病區的組織蛋白酶生物活性平均顯著高於健康區((4.70 (0.43) pU/μl vs. 3.57 (0.52) pU/μl)。(5)大部分患者牙齦液或唾液中組織蛋白酶濃度並不顯著表現於西方點墨法中。少數顯著表現的患者，病情發展與治療預後，對於治療的反應似乎不如其他患者。(6)唾液胱抑素濃度在嚴重牙周病患者與輕微牙周病患者中並無顯著差異。然而分析11位嚴重牙周病患在開刀前後的唾液胱抑素濃度，發現其平均值由86 units/ml降為71 units/ml。(7)病人牙齦液或唾液中均無法測出激肽。(8)牙齦液中與發炎相關的細胞激素，IL-1α與IL-8，牙周病區顯著高於健康區。此差異在開刀治療3個月後即無法察覺。其他相關細胞激素(TNF, IFN, IL-12,與IL-17)在牙周病區和健康區牙齦液中並沒有顯著差異。本研究之結果發現牙周病活躍的區域，細菌半胱胺酸蛋白酶與宿主組織蛋白酶生物活性均顯著增加。治療後獲得改善。IL-1α與IL-8能夠反應患病部位發炎狀況。患者胱抑素濃度與其牙周病嚴重程度並無相關。然而治療前後若持續表現組織蛋白酶蛋白質，其治療預後較差。

中 文 關 鍵 詞 ： 牙周病、半胱胺酸蛋白酶、組織蛋白酶、胱抑素、激肽

英 文 摘 要 ： Previous studies suggested the proteinase activities in gingival crevicular fluid (GCF) and maybe in saliva from patients with periodontitis can be used as an indicator of disease severity. However, the studies concerning cathepsin and cystatin and their association with periodontal disease are still limited. This study investigated the association between periodontal status and bacterial cysteine proteinase activities and host cysteine protease inhibition

activities in saliva and GCF. One hundred and forty-two patients were recruited from the department of periodontics. The participants had an oral examination and their saliva and GCF samples were collected from four tooth sites (two diseased sites and two healthy sites) in four visits. The samples were analyzed for host cystatin, cathepsin and kinin levels, and bacterial protease activities using ELISA and substrate chromatography. Bacterial proteinase activity was significantly different between the diseased and healthy sites before treatment. The mean cathepsin protease activity in GCF of the diseased sites was significantly higher than those of the healthy sites. Patients who expressed marked cathepsin protein responded poorly to surgical treatment. Cystatin protein concentrations were not significantly different between patients with severe or mild periodontitis. Kinin was not detected in saliva and GCF. IL-1 and IL-8 levels were significantly elevated in diseased sites from patients with severe periodontitis. TNF, IFN, IL-12, and IL-17 showed no differences. In conclusion, our study shows that bacterial gingipain and host cathepsin activities are positively associated with the severity of periodontitis and the levels of IL-1 and IL-8 can reflect the inflammatory status of diseased sites. The protein levels of cathepsin may relate to the disease prognosis. The data obtained from this study greatly improve our understanding about physiopathogenesis mechanisms of periodontal disease development.

英文關鍵詞：periodontal disease; cysteine proteinases; cathepsin; cystatin; kinin

中文摘要

牙周病致病菌會釋放出半胱胺酸蛋白酶，我們最近的研究發現牙周病患者牙齦液的半胱胺酸蛋白酶活性在治療後會降低。牙周病菌所引起的組織破壞，大部份是宿主本身降解酶作用的結果。宿主的組織蛋白酶也是屬於半胱胺酸蛋白酶，在骨頭破壞上扮演重要角色，它的天然抑制物-胱抑素，存在於唾液和牙齦液中。研究指出牙周病患牙齦液中具有較高的組織蛋白酶，發炎牙齦組織胱抑素表現較低。然而有關研究仍然相當缺乏。在牙齦液中的激肽釋放酶系統也可能受到胱抑酶所調控，因為激肽的產生也是藉由半胱胺酸蛋白酶活化，我們先前研究顯示，激肽可以增加血管通透性，幫助牙周病菌進入循環系統。本研究的目的為探討牙周狀態和宿主半胱胺酸蛋白酶抑制能力的關連性、以及唾液和牙齦液中胱抑素的濃度，同時也將測量牙周病人的組織蛋白酶含量、激肽濃度以及牙周病菌的半胱胺酸蛋白酶水解能力。本追蹤式調查之研究對象為在牙周病科門診求診且符合研究納入條件的患者，參加者接受問卷訪談，並在4 個時間點(治療前及治療後 3、6、9 個月)接受口腔檢查並採集唾液和牙齦液，牙齦液將採自兩顆有最嚴重牙周病的牙齒(牙周病區)和兩顆正常的牙齒(健康區)。本計畫的結果如下：(1)有 142 位牙周病患者參加本研究，共完成 467 人次的調查。(2)總共收集 467 個唾液樣品與 1868 個牙齦液樣品。(3)在治療前收集的牙齦液樣品，牙周病區的半胱胺酸蛋白酶的平均濃度顯著高於健康區(平均值(標準差)：0.47 (0.11) vs. 0.40 (0.06))；牙周病區牙齦液的半胱胺酸蛋白酶的平均濃度，在治療前顯著高於治療後 3 個月。(4)牙周病區的組織蛋白酶生物活性平均顯著高於健康區((4.70 (0.43) pU/μl vs. 3.57 (0.52) pU/μl)。(5)大部分患者牙齦液或唾液中組織蛋白酶濃度並不顯著表現於西方點墨法中。少數顯著表現的患者，病情發展與治療預後，對於治療的反應似乎不如其他患者。(6)唾液胱抑素濃度在嚴重牙周病患者與輕微牙周病患者中並無顯著差異。然而分析 11 位嚴重牙周病患在開刀前後的唾液胱抑素濃度，發現其平均值由 86 units/ml 降為 71 units/ml。(7)病人牙齦液或唾液中均無法測出激肽。(8)牙齦液中與發炎相關的細胞激素，IL-1α與 IL-8，牙周病區顯著高於健康區。此差異在開刀治療 3 個月後即無法察覺。其他相關細胞激素(TNFα, IFNγ, IL-12,與 IL-17)在牙周病區和健康區牙齦液中並沒有顯著差異。本研究之結果發現牙周病活躍的區域，細菌半胱胺酸蛋白酶與宿主組織蛋白酶生物活性均顯著增加。治療後獲得改善。IL-1α與 IL-8 能夠反應患病部位發炎狀況。患者胱抑素濃度與其牙周病嚴重程度並無相關。然而治療前後若持續表現組織蛋白酶蛋白質，其治療預後較差。

關鍵詞：牙周病、半胱胺酸蛋白酶、組織蛋白酶、胱抑素、激肽

Abstract

Previous studies suggested the proteinase activities in gingival crevicular fluid (GCF) and maybe in saliva from patients with periodontitis can be used as an indicator of disease severity. However, the studies concerning cathepsin and cystatin and their association with periodontal disease are still limited. This study investigated the association between periodontal status and bacterial cysteine proteinase activities and host cysteine protease inhibition activities in saliva and GCF. One hundred and forty-two patients were recruited from the department of periodontics. The participants had an oral examination and their saliva and GCF samples were collected from four tooth sites (two diseased sites and two healthy sites) in four visits. The samples were analyzed for host cystatin, cathepsin and kinin levels, and bacterial protease activities using ELISA and substrate chromatography. Bacterial proteinase activity was significantly different between the diseased and healthy sites before treatment. The mean cathepsin protease activity in GCF of the diseased sites was significantly higher than those of the healthy sites. Patients who expressed marked cathepsin protein responded poorly to surgical treatment. Cystatin protein concentrations were not significantly different between patients with severe or mild periodontitis. Kinin was not detected in saliva and GCF. IL-1α and IL-8 levels were significantly elevated in diseased sites from patients with severe periodontitis. TNFα, IFNγ, IL-12, and IL-17 showed no differences. In conclusion, our study shows that bacterial gingipain and host cathepsin activities are positively associated with the severity of periodontitis and the levels of IL-1α and IL-8 can reflect the inflammatory status of diseased sites. The protein levels of cathepsin may relate to the disease prognosis. The data obtained from this study greatly improve our understanding about physiopathogenesis mechanisms of periodontal disease development.

Keywords: periodontal disease; cysteine proteinases; cathepsin; cystatin; kinin

BACKGROUND AND SIGNIFICANCE

Periodontal disease is one of the two major oral diseases that affect populations worldwide at high prevalence rates (Petersen 2003; Petersen and Ogawa 2012). Gingival bleeding and calculus (the Community Periodontal Index, CPI, score = 1 or 2) is highly prevalent among adult populations and advanced periodontal disease (CPI score = 4) affects 10% to 15% of adults worldwide (Petersen and Ogawa 2012). A nationwide survey of adults and seniors in Taiwan during 2003-2005 reported that only 35.8% of the subjects had healthy periodontal conditions (CPI score = 0), while 64.2% of the subjects had gingival bleeding, calculus, 3-5 mm pocket, or >6 mm pocket (CPI score >= 1) (Taiwan Ministry of Health and Welfare 2015a).

Ischemic heart disease, stroke, chronic obstructive pulmonary diseases (COPD), and diabetes are among the 10 leadings causes of death in the world in 2012 (World Health Organization 2015). In Taiwan, heart disease, cerebrovascular disease, diabetes mellitus, and chronic lower respiratory diseases, respectively, are the second, third, fifth, and seventh leading causes of death in 2014 (Taiwan Ministry of Health and Welfare 2015b).

Periodontal disease, cardiovascular diseases (CVD), diabetes, and chronic lower respiratory diseases are highly prevalent worldwide. The World Health Organization reports pointed out that oral diseases share common risk factors with CVD, diabetes, cancer and COPD and has recommended the oral diseases preventive strategies basing on the common risk factors approach (Petersen 2003; Petersen and Ogawa 2012). Furthermore, periodontal diseases have been reported in many epidemiological studies to be risk factors for or be associated with systemic conditions, including CVD, diabetes, respiratory diseases (such as COPD), preterm delivery of low birth weight, osteoporosis, and rheumatoid arthritis (Cullinan and Seymour 2013; Inaba and Amano 2010; Otomo-Corgel et al 2012).

Epidemiological studies on the association of periodontal disease and systemic diseases

The associations between periodontal disease and systemic diseases (including CVD, diabetes, respiratory disease, osteoporosis, preterm birth, and chronic kidney disease) have been extensively evaluated in many reviews or meta-analysis of epidemiological studies. Several reviews or meta-analyses suggested that periodontal disease can be a risk factor for CVD (Belstrom et al. 2012; Inaba and Amano 2010; Seymour et al. 2007) or several systemic diseases (Inaba and Amano 2010; Kuo et al. 2008; Seymour et al. 2007). However, many review studies had inconclusive results about the associations between periodontal disease and CVD (Blaizot et al. 2009; Cullinan and Seymour 2013; Fisher et al. 2010; Kuo et al. 2008; Lockhart et al. 2012; Otomo-Corgel et al. 2012) or other systemic diseases (Blaizot et al. 2009; Cullinan and Seymour 2013; Fisher et al. 2010; Otomo-Corgel et al. 2012). Three reviews or meta-analysis found beneficial effects of periodontal treatment on markers of CVD (Orlandi et al. 2014; Teeuw et al. 2014; Ying Ouyang et al. 2011), but the effects of periodotnal therapy on prevention of CVD outcomes or other systemic diseases still remain unclear. Furthermore, most studies pointed out that more data are needed to confirm the causal relationship between periodontal disease and systemic diseases.

Potential biological mechanisms for the association between periodontal disease and systemic diseases

Oral cavity harboring diverse bacterial populations may act as a site of origin for stimulation of systemic inflammatory status or for spreading of pathogenic microorganisms to other body sites (Nagpal et al. 2015). Results from previous epidemiological studies

suggest that periodontal disease/infection is associated with increased risk of several systemic diseases, but whether the relationship is causal is inconclusive. Furthermore, the biological mechanisms are not well understood. Several hypotheses (Seymour et al. 2007) have been proposed, including common susceptibility for both periodontal disease and systemic diseases, direct infection/invasion of the vascular endothelium by periodontal bacterial pathogens, systemic inflammation (i.e. periodontal inflammation increases circulating cytokines, which damage the endothelium and lead to atherosclerosis), and cross-reactivity between bacterial antigens and self-antigens.

Beck et al. (1996) indicated several possible pathways of an infectious inflammatory origin of systemic diseases. Among the mechanisms periodontal pathogens might use to exert systemic effects, these microorganisms may leave their local oral habitat and disseminate to other parts of the infected hosts (Beck et al. 2001). Porphyromonas gingivalis (P. gingivalis) is a principal etiologic agent of chronic adult periodontal disease (Zambon 1996; Zambon and Haraszthy 1995). Its purported virulence traits include production of proteinases, fimbriae (Njoroge et al. 1997; Sojar et al. 2002), hemagglutinin (Shi et al. 2000), hemolysins (Sakurai et al. 2000; Chen et al. 2000) and the ability to disseminate and/or invade host tissues (Lamont et al. 1995; Meyer et al. 1997). Periodontal diseases manifested by P. gingivalis infection was characterized by the elevation of bacterial cysteine protease (gingipain) activity and ensuing host lysosomal cysteine cathepsin production (Elkaim et al. 2008). Together, gingipain of P. gingivalis and host cathepsin can destroy periodontal connective tissue and alveolar bone structure and further stimulate plasma contact system by cleaving kininogen into kinin via kallikrein enzyme activity (Cox et al. 2006). There is compelling evidence (Imamura et al. 1994, 1995) indicating that kinin is rapidly generated after proteolytic tissue injury by P. gingivalis infection and kinin per se seems to modulate many of the events observed during the inflammatory processes including vasodilatation, increase of vascular permeability, plasma extravasation, and cell migration (Hall 1997; Blais et al. 2000; Travis et al. 1994).

Previous studies of kinin, cathepsin, and cystatin in the pathogenesis of periodontal diseases

Our previous animal study (Hu et al. 2006) suggested that increased transvascular dissemination of *P. gingivalis* may be explained by the potent action of kinin in causing the intercellular junctions of endothelium at post capillary venules to open allowing the bacteria to invade the circulatory system. This study provides evidence for a strain specific ability of *P. gingivalis* to disseminate to remote sites through activation of the vasoactive kinin system. Such ability might be expected to be a virulence property of the clinical isolates associated with systemic manifestations including cardiovascular pathologies and abnormal pregnancy outcomes. The possession of such traits should be considered in studies examining the association of periodontopathogens with systemic diseases (Hu et al. 2006).

Previous reports have documented that the expression of gingipains by *P. gingivalis* can vary in the hosts (Genco 1995; Brochu et al. 2001). Since the majority of *P. gingivalis* strains examined appeared to produce gingipains (Persson et al. 2005), which can digest *N*-α-benzoyl-dl-arginine-p-nitroanilide (BANA) in vitro, it has been postulated that the involvement of these proteinases in bacterial virulence may be due to differential regulation and enhanced expression in virulent strains.

The work by Rapala-Kozik described the ability of *P. gingivalis* to concentrate the host plasma-derived kinin-forming system on its cell surface to trigger kinin production (Rapala-Kozik et al. 2011). Their analysis of kinin release from human plasma on contact with *P.*

gingivalis clearly demonstrated that the generation of kinin by the proteolytic action of the gingipains. Kinin is powerful proinflammatory mediator, and it has been implicated in the pathogenesis of periodontitis due to its potential to strongly upregulate bone resorption (Brechter and Lerner 2007; Brechter et al. 2008). However, kinin production in the inflamed gingival tissue is poorly characterized.

The host kinin-forming cascade plays an important role in the inflammatory processes (Sprague and Khalil 2009). Kinins are produced in large amounts at local inflammatory foci to induce vasodilation, vascular permeability enhancement, cell migration, and pain. Activated plasma Hagemam factor converts prekallikrein into the active enzyme which subsequently releases kinin from high-molecular-weight kininogen (Fujisawa et al. 1995). This kinin-forming cascade is activated and controlled by a series of host cysteine proteases and can by hijacked by bacterial pathogens (eg, in the case of periodontal diseases, *P. gingivalis*), which take advantage of the kinin-induced increase in vascular permeability for dissemination (Hu et al. 2006). The gingipains of *P. gingivalis* generate kinin via prekallikrein activation and release kinin directly from its precursor.

Periodontitis is an inflammatory disease initiated by anaerobic bacteria. In vitro and clinical studies have provided evidence that the destructive process in periodontitis is a consequence of degradative enzymes, such as lysosomal cysteine proteinases, cathepsins (Elkaïm et al. 2008). The concentration of cathepsin has been shown to elevate significantly in gingival crevicular fluid from persons with periodontitis as compared with that in healthy individuals (Ito et al. 2008). Cathepsin participate in multiple host systems that is active in healthy and disease situations, such as tissue remodeling, turnover of the extracellular matrix, immune system function, and apoptosis, or in antigen and pro-protein processing (Wiesner and Vilcinskas 2010). However, we know relatively little about the role of the cathepsin in periodontitis.

Many of the characteristics of periodontal diseases such as inflammation and attachment loss are associated with proteolytic events. Oral tissue destruction can result from the release of an array of proteolytic enzymes by colonizing bacteria. The biochemical events that ensue can lead to the release of host lysosomal cathepsins, which lead to further tissue degradation and are currently thought to be major contributors to periodontal tissue destruction. Both host cathepsins and bacterial gingipains belong to a group of proteolytic enzymes that cleave peptide bonds by use of a reactive cysteine residue at the catalytic site. Host proteinases that activate the forming of kinins are also members of cysteine proteinases.

Cystatins are potent physiological inhibitors of cysteine proteinases and involved in the control of protein degradation (Baron et al. 1999). Levels of cystatin in patients with periodontal disease have been shown altered as compared with healthy individuals (Ganeshnarayan et al. 2012). Inhibition of cystatin expression was also observed in inflamed gingiva and fluids (Fábián et al. 2012). Cystatins have been shown to inhibit the activities of gingipains from *P. gingivalis*, cathepsins of osteoclasts and epithelial cells, and the conversion of active kinin in plasma.

Specific aims

The main purpose of this study was to investigate the association between periodontal status and host cysteine protease inhibition activities, especially the levels of cystatin, in saliva and gingival crevicular fluid (GCF). We also looked into host cathepsin levels, host kinin contents and bacterial cysteine proteinase activities in patients with periodontal disease.

The specific aims were (1) to assess the association between periodontal disease and host cysteine protease inhibition activities in saliva and GCF, (2) to assess the effects of periodontal treatment on the cysteine protease inhibition of hosts, (3) to evaluate host cathepsin levels in saliva and GCF from patients with periodontal disease, (4) to evaluate the kinin expression in saliva and GCF from patients with periodontal disease, (5) to assess the association between periodontal disease status and bacterial proteinase activities in saliva and GCF, and (6) to assess the changes in bacterial proteinase activities in saliva and GCF after periodontal treatment. The data obtained from this study will greatly improve our understanding about physiopathogenesis mechanisms of periodontal disease development.

METHODS

Study design and study subjects

This study had a cross-sectional part and a longitudinal follow-up component. All study subjects were included in the baseline cross-sectional survey at recruitment and had three follow-up visits. Study subjects were recruited from patients visiting the dental clinics of a teaching hospital. The inclusion criteria were: females or males, aged 20 years and over, seeking care at the periodontal department, having at least 10 natural teeth, and being diagnosed with periodontitis. The study protocol has been approved by the Institutional Review Board of Chung Shan Medical University Hospital. Eligible subjects signed an informed consent before participation.

Oral examination and classification of periodontal disease status

For each study subject the data was collected in four occasions: prior to any periodontal treatment and three-, six-, and nine-month after periodontal treatment. The periodontal condition, including bleeding on probing, probing depths and attachment levels at six sites of each tooth, was recorded by the same dentist specialized in periodontics. Classification of each subject's periodontal disease status was performed according to the report from the International Workshop for Classification of Periodontal Diseases and Conditions (Armitage 1999). For this study, we will include subjects with >=1 teeth with >=3 mm clinical attachment loss (CAL). Furthermore, the severity of CAL, extent of CAL in whole mouth and probing depth were used to further categorize these subjects into subgroups (Armitage 1999; Anonymous 2005).

Collection of saliva and GCF samples

Saliva (2 min production) was collected with a sterile syringe, avoiding contact with the epithelia. No stimulation or spitting was practiced. Saliva was placed into ice-chilled graded tubes and brought immediately to the laboratory. For each participant, the GCF was sampled using paper points from gingival sulci of the four targeted teeth: two teeth mostly affected by periodontal disease (diseased sites) and two "normal" teeth with probing depth < 3 mm (healthy sites).

Laboratory analyses of saliva and GCF samples

Bacterial protease activity assay

GCF samples collected from aseptic paper points were immersed immediately after collection into 1 ml of Tris buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM dithiothreitol, pH 7.4). For saliva samples, 0.2 ml of saliva was diluted in Tris buffer to 1 ml in final volume. The proteinase activities of above samples then were determined by using *N*-α-benzoyl-dl-arginine-p-nitroanilide (BANA) (Sigma Chemical Co., St Louis, MO, USA). Samples were preincubated in 0.5 ml of 0.1 M Tris-HCl, 0.2 M Gly-Gly, 5 mM $CaCl_2$, 10 mM cysteine, pH7.6, for 5 min at 37^{o}C, and then 0.5 ml of 2 mM BANA were added. The

formation of p-nitroaniline was read spectrophotometrically at 410 nm.

Quantification of cystatin, cathepsin and kinin

The cystatin content was estimated by enzyme-linked immunosorbent assay (ELISA) (Fábián et al. 2012). The assay of cathepsin activity was performed by the development of methylcoumarin based on the method described by Chen et al (1998). Kinin will be conjugated to cytochrome C with the linker molecular, N-succinimidyl 3-(2-pyridyldithio) propionate before subjected to ELISA assay (Cassim et al. 2009).

Cysteine protease inhibitory activity

The cysteine protease inhibitory activity of total cystatin present will be measured using the extent of inhibition of papain (a cysteine protease). The activity of papain will be followed by continuously monitoring the rate of production of the fluorescent product 4-amino-methyl-coumarin, released by the cleavage of the substrate N-α-benzoyl-L-agrinine-7-amido-4 methyl coumarin (BA-AMC) in a fluorimeter at room temperature. In each case papain was pre-incubated with samples for 4 min at room temperature and the assay started by the addition of substrate (Baron et al. 1999).

Cytokines measurement

TNF-α, IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, and IL-12 levels in samples were determined by using a colorimetric commercial kit (Qiagen), according to the manufacturer's instructions. All cytokine concentrations were expressed as pg/mg tissue.

SDS-PAGE and Western blotting to detect cystatin, cathepsin and kinin

Samples were mixed with 4× sample buffer (8% SDS, 10% 2-mercaptoethanol, 30% glycerol, 0.02% bromophenol blue in 0.25M Tris–HCl, pH 6.8) and incubated at 100°C for 15 min. The solution was then loaded into each well and run at 120 V for 3 h in 12% SDS–PAGE gels. The human polyclonal antibody against cystatin and cathepsin purchased from BD Biosciences were used in this study. HRP-conjugated goat anti-human IgG was used as the secondary antibody (Mäntylä et al. 2012)

Questionnaire survey for important factors

Information regarding subjects' medical history, oral health, oral hygiene habit, demographics factors, and history of areca nut chewing, cigarette smoking and alcohol drinking were collected by interview using a structured questionnaire. One trained interviewer conducted the interviews of all of the study subjects.

Statistical analysis

The paired t test or Wilcoxon signed-rank test were used to compare proteinase activities, cystatin, cathepsin, and kinin, respectively, between groups (healthy sites vs. diseased sites). The repeated measurement analysis was performed to compare proteinase activities, cystatin, cathepsin, and kinin, respectively, measured in different occasions and from teeth with different severity of periodontal disease, taking into account intra-individual correlation and potential confounding factors. The SAS version 9.4 software (SAS Institute Inc., NC, USA) was applied for the data analyses. An alpha level of 0.05 was used for all statistical tests.

RESULTS AND DISCUSSION

One hundred and forty-two patients, 93 females and 49 males, participated in this study.

The mean (standard deviation, SD) age was 48.99 (9.74) (range: 31.32-73.78) years, and 92.20% of the subjects had high school or higher education. Twelve (8.45%) participants were taking medicine for hypertension, and four (2.82%) were on medication for diabetes. Ninety-five percent of the subjects reported good perception of general health, while only 38% perceived good oral health. As to the smoking habit, 11 (7.75%) subjects were current smokers and 17 (11.97%) were former smokers.

After 9 months of follow-up for each participant, 92 completed data collection for four visits, and 50 had data for two or three visits. In total, 467 saliva samples and 1868 GCF samples were collected from these participants.

Pocket depth of the diseased sites significantly decreased at three months after treatment ($p < 0.05$, paired t test). The frequency for bleeding on probing at the diseased sites also significantly decreased after treatment ($p < 0.05$, McNemar's test). There was no significant differences in either pocket depth or bleeding on probing for the healthy sites before and after treatment.

Bacterial proteinase detection (BANA assay)

For GCF samples collected in the first visit (before treatment), the mean (SD) proteinase activity (optical density, without unit) were 0.47 (0.11) and 0.40 (0.06), respectively, for the diseased sites and the healthy sites, and the difference was statistically significant ($p < 0.05$, paired t test). The bacterial proteinase activity in the diseased sites was significantly decreased at three month and six month after treatment ($p < 0.05$, repeated measurement regression analysis). Bacterial proteinase activity of saliva collected in different visits was not significantly different.

Saliva cystatin activity

Saliva from the periodontal patients were collected. There were no significant differences in saliva cystatin activities in patients between severely and mild diseased groups. The mean cystatin activity in 11 pre-surgery subjects' saliva was 86 units/ml. However, in the post-surgery saliva of these patients, cystatin activity was detected as 71 units/ml. There was a decreasing trend of cystatin activity, when comparing the post-surgery saliva with the pre-surgery saliva.

Inflammation associated cytokines

GCF taken from the diseased sites showed increases in the concentrations of IL-1α and IL-8 compared to those of healthy sites. All of them have been strongly implicated associated with clinical status of periodontal inflammation. The levels of these two cytokines were lowered after surgical intervention. However, we were unable to find any differences in the levels of TNFα, IFNγ, IL-12, and IL-17 between these two groups. As for the TH2 cytokines such as IL-4 and IL-10, there were marginally significant differences between these two groups.

Figure 1 displays the levels of TNFα (in ng/ml) in gingival crevicular fluids collected before surgery and after surgery. There was no significant difference between these two time points. It was obvious from the data shown above that the levels of TNFα before treatment were close-packed ranging between 10 to 30 ng/ml. On the other hand, after receiving surgery intervention, most of the levels of TNFα decreased except for some patients, who exhibited even higher titers of TNFα.

Cathepsin B activity in GCF

Significant differences were found between the healthy and diseased GCF for cathepsin values. The mean cathepsin values for the diseased GCF were significantly higher than those of the healthy sites (4.70 ± 0.43 versus 3.57 ± 0.52 pU/μl, $p < 0.001$, paired t test)

Cathepsin B protein quantification by Western blot

GCF cathepsin B contents in non-surgical patients

Most non-surgical patients showed very low levels of cathepsin B protein (less than 0.2 μg/ml) in their GCF. There were no significant differences between diseased sites and their corresponding control sites. GCF cathepsin B levels remained low after non-surgical periodontal treatment (scaling and root planning) indicating this protein was irrelative to this kind of treatment in this group of patients. However, a few exceptions caught our attention, in that diseased sites apparently demonstrated elevated concentrations of cathepsin B when compared to their corresponding control sites. Some non-surgical patients also showed raised levels of cathepsin B in their saliva, although the protein levels in their GCF still stayed low.

GCF cathepsin B contents in surgical patients

We found that the levels of GCF protein cathepsin B were lower than 0.2 μg/ml before and after surgical procedure, and there were no statistically significant differences between diseased sites and control sites.

Figure 2 presents the host cysteine proteinase levels of cathepsin B in saliva collected from patients with severe periodontitis, both before and after surgery. The data showed that there was no association between cathepsin B and the severity of periodontitis. Some patients demonstrated exceptional high levels of cathepsin B after receiving surgery intervention, which coincided with their poor responsiveness to treatment.

As a whole, patients showed very low levels of cathepsin B in their GCF (whether diseased or control sites) and in their saliva either in surgical or non-surgical group of patients. Some patients did show elevated levels of cathepsin B in their saliva and remained high through the period of treatment and monitoring. These patients responded poorly to surgical intervention and pocket depth were not improved, suggesting that cathepsin B released by host may be served as marker for periodontitis treatment future prognosis.

CONCLUSION

To sum up, our study shows that bacterial proteinase activity, bacterial gingipain and host cathepsin activities are positively associated with the severity of periodontitis and the levels of IL-1α and IL-8 can reflect the inflammatory status of diseased sites. The protein levels of cathepsin may relate to the disease prognosis of these recruited patients. The data obtained from this study greatly improve our understanding about physiopathogenesis mechanisms of periodontal disease development.

REFERENCES

Anonymous. 2005. Epidemiology of periodontal diseases. J Periodontol 76:1406-1419.

Armitage GC. 1999. Development of a classification system for periodontal diseases and conditions. Ann Periodont/the American Academy of Periodontology 4:1-6.

Baron AC, Gansky SA, Ryder MI, Featherstone JDB. 1999. Cysteine protease inhibitory activity and levels of salivary cystatins in whole saliva of periodontally diseased patients. J

Periodont Res 34:437–444.

Beck J, Garcia R, Heiss G, Vokonas PS, Offenbacher S. 1996. Periodontal disease and cardiovascular disease. J Periodontol 67:1123-1137.

Beck JD, Elter JR, Heiss G, Couper D, Mauriello SM, Offenbacher S. 2001. Relationship of periodontal disease to carotid artery intima-media wall thickness: The atherosclerosis risk in communities (ARIC) study. Arteriosclerosis, Thrombosis, and Vascular Biology 21:1816-1822.

Belstrom D, Damgaard C, Nielsen CH, Holmstrup P. 2012. Does a causal relation between cardiovascular disease and periodontitis exist? Microbes Infect 14:411-418.

Blais, Jr., Marceau F, Rouleau JL, Adam A. 2000. The kallikrein-kininogen-kinin system: Lessons from the quantification of endogenous kinins. Peptides 21:1903-1940.

Blaizot A, Vergnes J-N, Nuwwareh S, Amar J, Sixou M. 2009. Periodontal diseases and cardiovascular events: Meta-analysis of observational studies. Int Dent J 59:197-209.

Brechter AB, Lerner UH. 2007. Bradykinin potentiates cytokine-induced prostaglandin biosynthesis in osteoblasts by enhanced expression of cyclooxygenase 2, resulting in increased RANKL expression. Arthritis Rheum 56:910–923.

Brechter AB, Persson E, Lundgren I, Lerner UH. 2008. Kinin B1 and B2 receptor expression in osteoblasts and fibroblasts is enhanced by interleukin-1 and tumour necrosis factor-alpha. Effects dependent on activation of NF-kappaB and MAP kinases. Bone 43:72–83.

Brochu V, Grenier D, Nakayama K, Mayrand D. 2001. Acquisition of iron from human transferrin by porphyromonas gingivalis: A role for arg- and lys-gingipain activities. Oral microbiology and immunology 16:79-87.

Cassim B, Shaw OM, Mazur M, Misso NL, Naran A, Langlands DR, et al. 2009. Kallikreins, kininogens and kinin receptors on circulating and synovial fluid neutrophils: role in kinin generation in rheumatoid arthritis. Rheumatology (Oxford) 48:490–496.

Chen HY, Cox SW, Eley BM. 1998. Cathepsin B, Alpha2-Macroglobulin and Cystatin Levels in Gingival Crevicular Fluid From Chronic Periodontitis Patients. J Clinical Periodontology 25(1):34–41.

Chen T, Dong H, Yong R, Duncan MJ. 2000. Pleiotropic pigmentation mutants of porphyromonas gingivalis. Microbial Pathogenesis 28:235-247.

Cox SW, Rodriguez-Gonzalez EM, Booth V, Eley BM. 2006. Secretory leukocyte protease inhibitor and its potential interactions with elastase and cathepsin B in gingival crevicular fluid and saliva from patients with chronic periodontitis. J. Periodont. Res. 41:477–485.

Cullinan MP, Seymour GJ. 2013. Periodontal disease and systemic illness: Will the evidence ever be enough? Periodontol 2000 62:271-286.

Elkaïm R, Werner S, Kocgozlu L, Tenenbaum H. 2008. P. gingivalis Regulates the Expression of Cathepsin B and Cystatin C. J Dent Res 87:932–936.

Fábián TK, Hermann P, Beck A, Fejérdy P, Fábián G. 2012. Salivary defense proteins: their network and role in innate and acquired oral immunity. Int J Mol Sci 13:4295–4320.

Fisher MA, Borgnakke WS, Taylor GW. 2010. Periodontal disease as a risk marker in coronary heart disease and chronic kidney disease. Curr Opin Nephrol Hypertens 19:519-526.

Fujisawa H, Ito H, Kashiwagi S, Nomura S, Toyosawa M. 1995. Kallikrein-kinin system in chronic subdural haematomas: its roles in vascular permeability and regulation of fibrinolysis and coagulation. J Neurol Neurosurg Psychiatr 59:388–394.

Ganeshnarayan K, Velliyagounder K, Furgang D, Fine DH. 2012. Human salivary cystatin SA exhibits antimicrobial effect against Aggregatibacter actinomycetemcomitans. J Periodont Res 47:661–673.

Genco CA. 1995. Regulation of hemin and iron transport in porphyromonas gingivalis. Adv Dent Res 9:41-47.

Hall JM. 1997. Bradykinin receptors. General Pharmacology 28:1-6.

Hu S-W, Huang C-H, Huang H-C, Lai Y-Y, Lin Y-Y. 2006. Transvascular dissemination of Porphyromonas gingivalis from a sequestered site is dependent upon activation of the kallikrein/kinin pathway. J Periodont Res 41:200–207.

Imamura T, Pike RN, Potempa J, Travis J. 1994. Pathogenesis of periodontitis: A major arginine-specific cysteine proteinase from porphyromonas gingivalis induces vascular permeability enhancement through activation of the kallikrein/kinin pathway. Journal of Clinical Investigation 94:361-367.

Imamura T, Potempa J, Pike RN, Travis J. 1995. Dependence of vascular permeability enhancement on cysteine proteinases in vesicles of porphyromonas gingivalis. Infection and Immunity 63:1999-2003.

Inaba H, Amano A. 2010. Roles of oral bacteria in cardiovascular diseases--from molecular mechanisms to clinical cases: Implication of periodontal diseases in development of systemic diseases. J Pharmacol Sci 113:103-109.

Ito T, Komiya-Ito A, Arataki T, Furuya Y, Yajima Y, Yamada S, et al. 2008. Relationship Between Antimicrobial Protein Levels in Whole Saliva and Periodontitis. J Periodontol 79:316–322.

Kuo L-C, Polson AM, Kang T. 2008. Associations between periodontal diseases and systemic diseases: A review of the inter-relationships and interactions with diabetes, respiratory diseases, cardiovascular diseases and osteoporosis. Public Health 122:417-433.

Lamont RJ, Chan A, Belton CM, Izutsu KT, Vasel D, Weinberg A. 1995. Porphyromonas gingivalis invasion of gingival epithelial cells. Infection and Immunity 63:3878-3885.

Lockhart PB, Bolger AF, Papapanou PN, Osinbowale O, Trevisan M, Levison ME, et al. 2012. Periodontal disease and atherosclerotic vascular disease: Does the evidence support an independent association?: A scientific statement from the american heart association. Circulation 125:2520-2544.

Meyer DH, Mintz KP, Fives-Taylor PM. 1997. Models of invasion of enteric and periodontal pathogens into epithelial cells: A comparative analysis. Critical Reviews in Oral Biology and Medicine 8:389-409.

Nagpal R, Yamashiro Y, Izumi Y, Nagpal R, Yamashiro Y, Izumi Y. 2015. The Two-Way Association of Periodontal Infection with Systemic Disorders: An Overview. Mediat. Inflamm. 2015:1–9.

Njoroge T, Genco RJ, Sojar HT, Hamada N, Genco CA. 1997. A role for fimbriae in porphyromonas gingivalis invasion of oral epithelial cells. Infection and Immunity 65:1980-1984.

Orlandi M, Suvan J, Petrie A, Donos N, Masi S, Hingorani A, et al. 2014. Association between periodontal disease and its treatment, flow-mediated dilatation and carotid intima-media thickness: A systematic review and meta-analysis. Atherosclerosis 236:39-46.

Otomo-Corgel J, Pucher JJ, Rethman MP, Reynolds MA. 2012. State of the science: Chronic periodontitis and systemic health. J Evid Based Dent Pract 12:20-28.

Persson GR. 2005. Immune responses and vaccination against periodontal infections. J Clin Periodontol 32 Suppl 6:39-53.

Petersen PE, Ogawa H. 2012. The global burden of periodontal disease: Towards integration with chronic disease prevention and control. Periodontol 2000 60:15-39.

Petersen PE. 2003. The World Oral Health Report 2003: Continuous improvement of oral health in the 21st century - the approach of the WHO Global Oral Health Programme. Geneva:WHO.

Rapala-Kozik M, Bras G, Chruscicka B, Karkowska-Kuleta J, Sroka A, Herwald H, et al. 2011. Adsorption of components of the plasma kinin-forming system on the surface of Porphyromonas gingivalis involves gingipains as the major docking platforms. Infect Immun 79:797–805.

Sakurai Y, Yamaguchi M, Abiko Y. 2000. Inhibitory effect of low-level laser irradiation on lps-stimulated prostaglandin e2 production and cyclooxygenase-2 in human gingival fibroblasts. Eur J Oral Sci 108:29-34.

Seymour GJ, Ford PJ, Cullinan MP, Leishman S, Yamazaki K. 2007. Relationship between periodontal infections and systemic disease. Clin Microbiol Infect 13 Suppl 4:3-10.

Shi Y, Kong W, Nakayama K. 2000. Human lactoferrin binds and removes the hemoglobin receptor protein of the periodontopathogen porphyromonas gingivalis. Journal of Biological Chemistry 275:30002-30008.

Sojar HT, Sharma A, Genco RJ. 2002. Porphyromonas gingivalis fimbriae bind to cytokeratin of epithelial cells. Infection and Immunity 70:96-101.

Sprague AH, Khalil RA. 2009. Inflammatory cytokines in vascular dysfunction and vascular disease. Biochem Pharmacol 78:539–552.

Taiwan Ministry of Health and Welfare. 2015a. 臺灣地區成年與老年人口腔健康調查 2003-2005 (計畫主持人：蔡吉政), available at: http://www.mohw.gov.tw/cht/DOMHAOH/DM1_P.aspx?f_list_no=189&fod_list_no=0&doc_no=48300, accessed 2015/12.

Taiwan Ministry of Health and Welfare. 2015b. 民國 103 年死因統計結果分析, available at: http://www.mohw.gov.tw/cht/DOS/Statistic.aspx?f_list_no=312&fod_list_no=5488, accessed 2015/12.

Teeuw WJ, Slot DE, Susanto H, Gerdes VE, Abbas F, D'Aiuto F, et al. 2014. Treatment of periodontitis improves the atherosclerotic profile: A systematic review and meta-analysis. J Clin Periodontol 41:70-79.

Travis J, Pike R, Imamura T, Potempa J. 1994. The role of proteolytic enzymes in the development of pulmonary emphysema and periodontal disease. American Journal of Respiratory and Critical Care Medicine 150.

Wiesner J, Vilcinskas A. 2010. Antimicrobial peptides: the ancient arm of the human immune

system. Virulence 1:440–464.

World Health Organization. 2015. The top 10 causes of death, available at: http://www.who.int/mediacentre/factsheets/fs310/en/, accessed 2015/12.

Ying Ouyang X, Mei Xiao W, Chu Y, Ying Zhou S. 2011. Influence of periodontal intervention therapy on risk of cardiovascular disease. Periodontol 2000 56:227-257.

Zambon JJ, Haraszthy VI. 1995. The laboratory diagnosis of periodontal infections. Periodontol 2000 7:69-82.

Zambon JJ. 1996. Periodontal diseases: Microbial factors. Annals of periodontology / the American Academy of Periodontology 1:879-925.

Figure 1. Cytokine levels of TNFα (ng/ml) in gingival crevicular fluids collected before surgery (A) and after surgery (B).

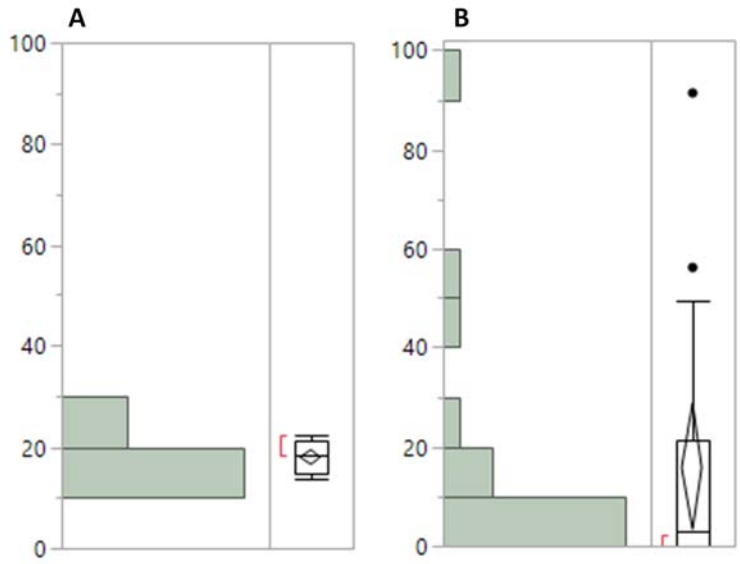
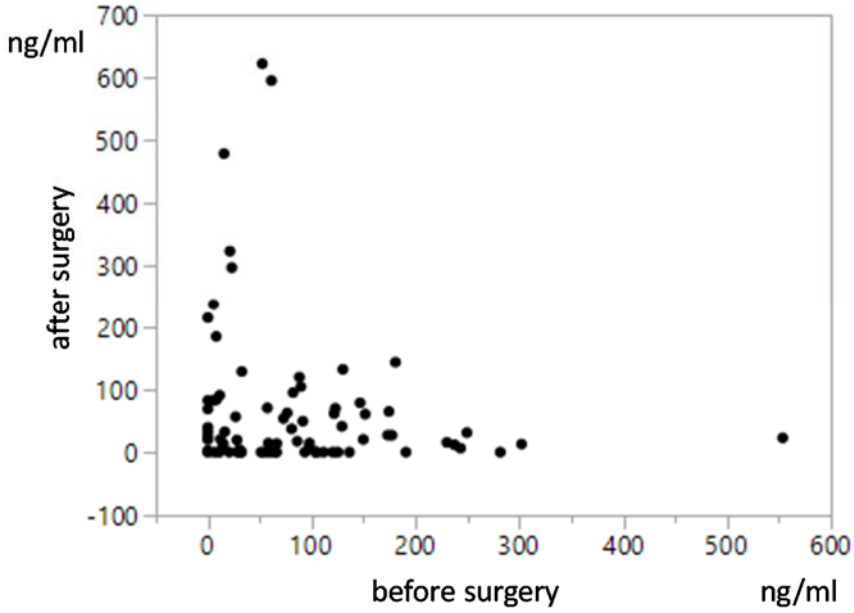


Figure 2. Host cysteine proteinase levels of cathepsin B in saliva collected from patients with severe periodontitis, before and after surgery.



科技部補助計畫衍生研發成果推廣資料表

日期:2016/10/23

科技部補助計畫	計畫名稱: 牙周病嚴重程度與牙齦液胱抑素活性關係之流行病學追蹤研究
	計畫主持人: 胡素婉
	計畫編號: 102-2314-B-040-013-MY3　　學門領域: 牙醫學

無研發成果推廣資料

102年度專題研究計畫成果彙整表

計畫主持人：胡素婉	**計畫編號：**102-2314-B-040-013-MY3
計畫名稱：牙周病嚴重程度與牙齦液胱抑素活性關係之流行病學追蹤研究	

成果項目					量化	單位	質化 （說明：各成果項目請附佐證資料或細項說明，如期刊名稱、年份、卷期、起訖頁數、證號...等）
國內	學術性論文	期刊論文			0	篇	
		研討會論文			0		
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		
	技術移轉	件數			0	件	
		收入			0	千元	
國外	學術性論文	期刊論文			1	篇	刊登於 Journal of periodontology. 2015;86(2):310-318.
		研討會論文			2		
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		

		其他	0		
	技術移轉	件數	0	件	
		收入	0	千元	
參與計畫人力	本國籍	大專生	0	人次	
		碩士生	1		學習並參與檢體的實驗室分析
		博士生	1		學習並參與檢體的實驗室分析
		博士後研究員	0		
		專任助理	1		負責問卷訪談、檢體的實驗室分析、與計畫行政工作。
	非本國籍	大專生	0		
		碩士生	0		
		博士生	0		
		博士後研究員	0		
		專任助理	0		
其他成果 （無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）					

科技部補助專題研究計畫成果自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現（簡要敘述成果是否具有政策應用參考價值及具影響公共利益之重大發現）或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   ■達成目標
   □未達成目標（請說明，以100字為限）
   　□實驗失敗
   　□因故實驗中斷
   　□其他原因
   說明：

2. 研究成果在學術期刊發表或申請專利等情形（請於其他欄註明專利及技轉之證號、合約、申請及洽談等詳細資訊）
   論文：■已發表　□未發表之文稿　□撰寫中　□無
   專利：□已獲得　□申請中　■無
   技轉：□已技轉　□洽談中　■無
   其他：（以200字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性，以500字為限）
   本研究探討牙周病患者牙齦液、唾液中細菌蛋白酶及宿主蛋白酶與疾病嚴重狀態的關係。兩種不同來源的蛋白酶皆會降解組織蛋白及破壞牙周組織，但何者才是牙周病的元凶，尚無定論，然而這答案攸關臨床治療方法的選擇。結果顯示細菌蛋白酶與牙周病的發生和嚴重程度有關，手術治療後，伴隨發炎程度好轉，也同步獲得改善。牙齦液中宿主組織蛋白酶生物活性與嚴重程度間無顯著關係。病人唾液及牙齦液的宿主組織蛋白酶表現並不明顯，但是表現明顯的病人，癒後往往較差。胱抑素在病情嚴重和病情輕微病人唾液中並無明顯差別，病情嚴重病人接受手術治療後，胱抑素呈現下降的趨勢。在發炎相關細胞素中，本研究發現第一間白素alpha和第八間白素有效反映牙周病病灶發炎程度，可以當作即時監控的發炎標記。總括來說，本研究結果顯示大部分牙周病引起的組織破壞與細菌來源的細菌蛋白酶較有相關，針對根除細菌及牙菌斑控制的處置，應該是足夠的。少部分病人口腔中宿主組織蛋白酶也被活化，同時伴隨癒後不良，針對這些病人，宿主組織蛋白酶的抑制與發炎免疫調控似乎也應該包括在治療方法中。藉由了解上述各因子與病情的關係，本研究更加完整了解牙周病的病理過程，能夠提供更有效的治療計畫。

4. 主要發現
   本研究具有政策應用參考價值：■否　□是，建議提供機關

（勾選「是」者，請列舉建議可提供施政參考之業務主管機關）
本研究具影響公共利益之重大發現：■否　□是
說明：（以150字為限）