行政院國家科學委員會專題研究計畫 期末報告

廣東住血線蟲感染鼷鼠造成血腦障壁功能喪失之機轉

計 畫 類 別 ：個別型
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計 畫 主 持 人 ：賴世展

公 開 資 訊 ：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中 華 民 國 102 年 10 月 17 日

中 文 摘 要 ： 由上皮細胞形成的血腦脊髓液障壁（blood-cerebrospinal fluid barrier）可調節離子、大分子、免疫細胞及病原體等物質的傳輸。本試驗利用鼷鼠感染廣東住血線蟲（Angiostrongylus cantonensis）當作動物模型，探討基質金屬蛋白酶（Matrix metalloproteinase, MMP）-9 在感染時，造成緊密連結蛋白降解之角色為何?結果顯示，在廣東住血線蟲感染鼷鼠誘發嗜伊紅性腦膜腦炎（eosinophilic meningoencephalitis），會增加核轉錄因子（nuclear factor kappa-light-chain-enhancer of B cells, NF-κB）及 IκB 的磷酸化。在給予 MG132 處理組發現，經 MG132 處理後，會減少 NF-κB 的磷酸化及 MMP-9 的活性，顯示 MMP-9 的活化是藉由核轉錄因子這個路徑。然而廣東住血線蟲感染鼷鼠會造成緊密連結的破壞，會使緊密連結蛋白 claudin-5 在腦部表現量是減少的，但在腦脊髓液中增加的 claudin-5，是因腦部損傷而進入腦脊髓液中。claudin-5 的降解與血腦脊髓液障壁通透性的改變是相關的。在給予廣效型基質金屬蛋白酶抑制劑（GM6001）組，會減少因感染造成 claudin-5 的破壞。這些結果顯示，在鼷鼠感染廣東住血線蟲誘發嗜伊紅性腦膜腦炎中，claudin-5 的破壞，是 MMP-9 所造成的。Claudin-5 在感染廣東住血線蟲造成血腦脊髓液障壁的破壞及緊密連結瓦解提供一個病理診斷之方針。

中文關鍵詞： 廣東住血線蟲、claudin-5、嗜伊紅性腦膜腦炎、基質金屬蛋白酶-9、核轉錄因子 κB

英 文 摘 要 ： The epithelial barrier regulates the movement of ions, macromolecules, immune cells and pathogens. The objective of this study was to investigate the role of the matrix metalloproteinase (MMP)-9 in the degradation of tight junction protein during infection with rat nematode lungworm Angiostrongylus cantonensis. The results showed that phosphorylation of IκB and NF-κB was increased in mice with eosinophilic meningoencephalitis. Treatment with MG132 reduced the phosphorylation of NF-κB and the activity of MMP-9, indicating upregulation of MMP-9 through the NF-κB signaling pathway. Claudin-5 was reduced in the brain but elevated in the cerebrospinal fluid (CSF), implying that A. cantonensis infection caused tight junction breakdown and led to claudin-5 release into the CSF. Degradation of claudin-5 coincided with alteration of

the blood-CSF barrier permeability and treatment with the MMP inhibitor GM6001 attenuated the degradation of claudin-5. These results suggested that degradation of claudin-5 was caused by MMP-9 in angiostrongyliasis meningoencephalitis. Claudin-5 could be used for the pathophysiologic evaluation of the blood-CSF barrier breakdown and tight junction disruption after infection with A. cantonensis.

英文關鍵詞： Angiostrongylus cantonensis, claudin-5, eosinophilic meningoencephalitis, matrix metalloproteinase-9, NF-κB

行政院國家科學委員會補助專題研究計畫 ∨成果報告　□期中進度報告

（計畫名稱）

Mechanisms of blood-brain barrier dysfunction in mice infected with *Angiostrongylus cantonensis*

廣東住血線蟲感染鼷鼠造成血腦障壁功能喪失之機轉

計畫類別：∨ 個別型計畫　□ 整合型計畫

計畫編號：NSC 101-2320-B-040-021-

執行期間：2012 年 08 月 01 日至 2013 年 07 月 31 日

計畫主持人：賴世展

共同主持人：

計畫參與人員：

成果報告類型(依經費核定清單規定繳交)：□精簡報告　∨完整報告

本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份

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□涉及專利或其他智慧財產權，□一年∨二年後可公開查詢

執行單位：中山醫學大學

中　華　民　國　2013　年　10　月　12　日

目錄

中文摘要

由上皮細胞形成的血腦脊髓液障壁（blood-cerebrospinal fluid barrier）可調節離子、大分子、免疫細胞及病原體等物質的傳輸。本試驗利用鼷鼠感染廣東住血線蟲（*Angiostrongylus cantonensis*）當作動物模型，探討基質金屬蛋白酶（Matrix metalloproteinase, MMP）-9 在感染時，造成緊密連結蛋白降解之角色為何?結果顯示，在廣東住血線蟲感染鼷鼠誘發嗜伊紅性腦膜腦炎（eosinophilic meningoencephalitis），會增加核轉錄因子（nuclear factor kappa-light-chain-enhancer of B cells, NF-κB）及 IκB 的磷酸化。在給予 MG132 處理組發現，經 MG132 處理後，會減少 NF-κB 的磷酸化及 MMP-9 的活性，顯示 MMP-9 的活化是藉由核轉錄因子這個路徑。然而廣東住血線蟲感染鼷鼠會造成緊密連結的破壞，會使緊密連結蛋白 claudin-5 在腦部表現量是減少的，但在腦脊髓液中增加的 claudin-5，是因腦部損傷而進入腦脊髓液中。claudin-5 的降解與血腦脊髓液障壁通透性的改變是相關的。在給予廣效型基質金屬蛋白酶抑制劑（GM6001）組，會減少因感染造成 claudin-5 的破壞。這些結果顯示，在鼷鼠感染廣東住血線蟲誘發嗜伊紅性腦膜腦炎中，claudin-5 的破壞，是 MMP-9 所造成的。Claudin-5 在感染廣東住血線蟲造成血腦脊髓液障壁的破壞及緊密連結瓦解提供一個病理診斷之方針。

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The epithelial barrier regulates the movement of ions, macromolecules, immune cells and pathogens. The objective of this study was to investigate the role of the matrix metalloproteinase (MMP)-9 in the degradation of tight junction protein during infection with rat nematode lungworm *Angiostrongylus cantonensis*. The results showed that phosphorylation of IκB and NF-κB was increased in mice with eosinophilic meningoencephalitis. Treatment with MG132 reduced the phosphorylation of NF-κB and the activity of MMP-9, indicating upregulation of MMP-9 through the NF-κB signaling pathway. Claudin-5 was reduced in the brain but elevated in the cerebrospinal fluid (CSF), implying that *A. cantonensis* infection caused tight junction breakdown and led to claudin-5 release into the CSF. Degradation of claudin-5 coincided with alteration of the blood-CSF barrier permeability and treatment with the MMP inhibitor GM6001 attenuated the degradation of claudin-5. These results suggested that degradation of claudin-5 was caused by MMP-9 in angiostrongyliasis meningoencephalitis. Claudin-5 could be used for the pathophysiologic evaluation of the blood-CSF barrier breakdown and tight junction disruption after infection with *A. cantonensis*.

Keywords：*Angiostrongylus cantonensis*, claudin-5, eosinophilic meningoencephalitis, matrix metalloproteinase-9, NF-κB

Introduction

The rat nematode lungworm *Angiostrongylus cantonensis* undergoes obligatory intracerebral migration in its hosts and induces the parasitic disease angiostrongyliasis (Mackerras and Sandars, 1954). Infection with this parasite induces severe central nervous system (CNS) disease, especially eosinophilic meningitis (Hsu et al., 1990) or meningoencephalitis (Gardiner et al., 1990) in non-permissive hosts (human or mice). Matrix metalloproteinase (MMP)-9 activity is closely associated with angiostrongyliasis meningitis caused by infection with *A. cantonensis* (Lai et al., 2004; Lee et al., 2004). This enzyme is associated with disruption of the blood-CNS barrier in mice with angiostrongyliasis meningitis and triggers increased cellular infiltration of the subarachnoid space (Chen et al., 2006).

The CNS can exclude circulating cells and harmful compounds from blood via the blood-brain barrier (BBB) and the blood-CSF barrier, which are formed by tight junction proteins around cerebral epithelial cells of the choroid plexus (Vorbrodt and Dobrogowska, 2003). Claudins are integral membrane tight junction proteins localized at tight junctions and sufficient for the formation of tight junctions at cell-cell contacts (Furuse et al., 1998). Claudin 1, 2 and 5 were reported to be present in epithelial cells of the choroid plexus (Lippoldt et al., 2000). Also, claudin-5-deficient mice displayed size-selective loosening of the BBB and died shortly after birth (Nitta et al., 2003). In viral infection, brain microvascular endothelial cells exposed to the human immunodeficiency virus-1 Tat protein decreased expression of claudin-5 and induced a redistribution of claudin-5 from cell-cell borders (András et al., 2003). In bacterial infection, lipopolysaccharide can decrease expression of claudin-5 protein, resulting in increased permeability of rat brain microvascular endothelial cells (Deng et al., 2011). In parasite infection, BBB impairment with enhanced expression of claudin-5 was reported in experimental cerebral toxocariasis (Liao et al., 2008).

In an attempt to characterize the mechanisms underlying *A. cantonensis*-induced blood-CSF barrier dysfunction, we investigated the association of NF-κB, MMP-9 and claudin-5 in the choroid plexus of the mouse brain after infection with *A. cantonensis*. The objective of the present study was to investigate alteration of claudin-5 via the NF-κB-MMP-9 pathway in mice with eosinophilic meningoencephalitis.

Materials and methods

Experimental animals

Five-week-old male BALB/c mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan) and maintained in a temperature-controlled environment with a 12 h light/12 h dark cycle and provided with Purina Laboratory Chow and water *ad libitum*. Mice were kept in a specific pathogen-free room at the Animal Center, Chung-Shan Medical University (Taichung, Taiwan) for more than one week before the experimental infection. All procedures were done in accord with the protocols approved by the Institutional Animal Care and Use Committee of Chung-Shan Medical University and the institutional guidelines for animal experiments.

Antibodies

Anti-mouse monoclonal antibodies IκB-α, p-IκB-α, NF-κB and p-NF-κB generated in rabbits were purchased from Cell Signaling Technology (Beverly, MA). Rabbit anti-mouse MMP-9 polyclonal antibody was purchased from Abcam (Cambridge, UK). Goat anti-mouse MMP-9 polyclonal antibody was purchased from R&D systems (Minneapolis, MN, USA) and goat anti-mouse claudin-5 polyclonal antibody was purchased from Santa Cruz Biotechnology (CA, USA). Mouse anti-mouse β-actin monoclonal antibody was purchased from Sigma (St. Louis, MO, USA). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG, HRP-conjugated anti-goat IgG, HRP-conjugated anti-mouse IgG, Rhodamine Red X (RRX)-conjugated anti-rabbit IgG and DyLight 488-conjugated anti-goat IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Larval preparation

Infective larvae (L_3) of *A. cantonensis* were obtained originally from wild giant African snails (*Achatina fulica*) that were propagated for several months and infected with *A. cantonensis* L_1 by rats (the definitive host) at the Wufeng Experimental Farm (Taichung, Taiwan). The L_3 larvae were recovered essentially as described (Parsons and Grieve, 1990) but with some modification. Briefly, snail shells were crushed and the tissues were homogenized in a pepsin-HCl solution (pH 1-2, 500 IU pepsin/g tissue) and digested with agitation at 37℃ for 2 h. The larvae in the sediment were collected by serial washes in double-distilled water and counted under a microscope. The identity of the L_3 larvae of *A. cantonensis* was confirmed as described (Hou et al., 2004).

Animal infection

A total of 120 male BALB/c mice were randomly allocated to five experimental groups (D_5, D_{10}, D_{15}, D_{20} and D_{25}) and a control group of 20 mice

each. Mice did not have access to food or water for 12 h before infection. Mice in the experimental groups D_5, D_{10}, D_{15}, D_{20} and D_{25} were each infected with 50 *A. cantonensis* larvae by oral inoculation, and were sacrificed on days 5, 10, 15, 20 or 25 post-inoculation (PI), respectively. The control mice received only water and were sacrificed on day 25 PI. Brains were rapidly removed and frozen in liquid nitrogen.

Treatment of animals

Forty mice were randomly allocated to four treatment groups (10 mice/group). Two groups of GM6001-treated mice were infected with 50 larvae and inhibited with specific MMPs inhibitor, GM6001 (2 mg/kg/day, Chemicon International, USA) on days 5 and 15 PI for seven consecutive days intraperitoneally, respectively. Mice were sacrificed on day 22 PI and the CSF was collected for claudin-5 analysis. Two groups of MG132-treated mice were infected with 50 larvae and treated with 1.5 or 3.0mg/kg/day MG132 (Cayman Chemical, Ann Arbor, MI) for twenty consecutive days. Mice were sacrificed on day 22 PI and the brain was collected for NF-κB p-p65 and MMP-9 analysis.

CSF collection

Mice were anesthesized by intraperitoneal urethane (1.25g/kg) injection. Mouse placed in a stationary instrument with 135 degree from the head and body. Skin of neck shaved and swabbed with 70% ethanol (three times). Subcutaneous tissue and muscles were separated. Capillary tube through dura mater into citerna magna and CSF well inpoured capillary tube. Inject CSF into a 0.5 ml eppendorf tube and centrifuged at 3000×*g* at 4 ℃ for 5 min. Collection of supernatant in a 0.5 ml eppendorf tube and kept at -80℃ freezer.

Western blot analysis

The mouse brains were homogenized in RIPA lysis buffer (150 mM sodium chloride, 1% (v/v) NP-40, 0.5% (v/v) deoxycholic acid, 0.1% (w/v) SDS and 50 mM Tris, pH 7.5) containing Protease Inhibitor Cocktail (Sigma, St. Louis, MO, USA). The homogenates were centrifuged at 12,000*g* for 10 min at 4℃. Protein concentration was determined with protein assay kits (Bio-Rad, CA, USA) using bovine serum albumin (BSA) as the standard. Samples were mixed with an equal volume of loading buffer (62.5 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% SDS, 5% (v/v) 2-mercaptoethanol and 0.05% (w/v) bromophenol blue) and heated for 5 min at 95℃. The mixture was subjected to SDS-PAGE and transferred electrophoretically to nitrocellulose membranes at a constant current of 190 mA for 90 min. Membranes were blocked with 5% (w/v) non-fat milk in PBS containing 0.1% (v/v) Tween 20 (PBST) for 1 h at room temperature.

Membranes were reacted with primary antibodies at 37℃ for 1 h. Membranes were washed three times with PBST, HRP-conjugated secondary antibody (1:10,000 dilution) was added and incubated at 37℃ for 1 h to detect primary bound antibody. Reactive proteins were detected by enhanced chemiluminescence (Amersham, Little Chalfont, Bucks, UK) and the density of specific immunoreactive bands was quantified by densitometric scanning.

Co-immunoprecipitation

rProtein G agarose (Invitrogen, Carlsbad, CA) was washed in PBS and centrifuged for 2 min at 10,000*g*. The supernatant was discarded and the residue was subjected to two more washing cycles. Brain homogenates (1 mg) and claudin-5 antibody were added to rProtein G agarose and incubated at 4℃ overnight. The agarose-immuno complexes were washed twice in dissociation buffer (0.5 M Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% (v/v) Triton X-100). The agarose-immuno complexes were mixed with an equal volume of loading buffer (62.5 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 0.05% (w/v) bromophenol blue), heated at 95℃ for 5 min and subjected to SDS-PAGE. The target protein (MMP-9) detection was determined by western blotting.

Immunohistochemistry

Sections of wax-embedded brain were dewaxed and rehydrated by standard procedures and blocked with 3% BSA before incubation with a 1:100 dilution of goat anti-mouse claudin-5 polyclonal antibody in 3% BSA at 37℃ for 1 h. Sections were washed in PBS three times for 5 min each, avoiding light, to incubate with the DyLight 488-conjugated secondary antibody diluted 1:500 in 1% BSA at 37℃ for 1 h, and washed again in PBS. For co-localization, sections were incubated with rabbit anti-mouse MMP-9 polyclonal antibody in 3% BSA at 37℃ for 1 h, washed in PBS three times for 5 min each, avoiding light, to incubate with the RRX-conjugated secondary antibody diluted 1:500 in 1% BSA at 37 ℃ for 1 h, and washed again in PBS. Sections were mounted with Vectashield Hard Set Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA) before visualization by laser scanning confocal microscopy (Zeiss LSM 510 META; Carl Zeiss Inc., Heidelberg, Germany).

Gelatin zymography

Brain homogenates were subjected to SDS-PAGE (7.5% (w/v) polyacrylamide gel containing 0.1% (w/v) gelatin (Sigma, St. Louis, MO, USA) in running buffer (25 mM Tris, 250 mM glycine, 1% SDS) at room temperature. The gel was washed twice in double-distilled water containing 2.5% Triton X-100 for 30 min each time,

then incubated in reaction buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10 mM $CaCl_2$, 0.02% (w/v) Brij® -35, 0.01% (w/v) NaN_3) at 37℃ for 18 h. The gel was stained with 0.25% (w/v) Coomassie brilliant blue R-250 (Sigma, St. Louis, MO, USA) for 1 h and then destained in 15% (v/v) methanol, 7.5% (v/v) acetic acid. Gelatinase activity was detected as unstained bands on a blue background. Quantitative analysis was done with a computer-assisted imaging densitometer system (UN-SCAN-IT™ gel version 5.1, Silk Scientific, UT).

Eosinophil counts in the CSF

The CSF was collected and centrifuged at 400*g* for 10 min, the sediment was recovered and mixed gently with 100 μL of Unopette buffer (Vacutainer System; Becton Dickinson, Franklin Lakes, NJ) and 2 μL of acetic acid, and placed into the cell counting chamber of a hemocytometer (Paul Marienfeld, Lauda-Koenigshofen, Germany) to count the eosinophils.

Assay of blood-CSF barrier permeability

Blood-CSF barrier permeability was evaluated by assessing the concentration of Evans blue in CSF. At 2 h before sacrifice, mice were injected with 2% Evans blue (100 mg/kg body weight; Sigma, St. Louis, MO, USA) in saline into a tail vein. The average concentration of Evans blue in CSF was calculated from measurement of the absorbance at 620 nm in a spectrophotometer (Hitachi U3000; Tokyo, Japan).

Statistical analysis

Results for the different groups were compared with the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison of means. All results are presented as mean±standard deviation (S.D.). Statistically significant difference was set at $P < 0.05$.

Results

3.1. Kinetic studies for p-IκB-α and IκB-α in mouse brain

p-IκB-α and IκB-α in the brain of mice infected with *A. cantonensis* were analyzed by western blotting. p-IκB-α and IκB-α in infected mouse brains were elevated significantly on days 5, 10, 15, 20 and 25 PI compared to the levels in the uninfected mice brains (Fig. 1).

Kinetic studies for p-NF-κB and NF-κB in mouse brain

Levels of p-NF-κB and NF-κB proteins in mouse brain at six time points were analyzed by western blotting . p-NF-κB and NF-κB proteins were increased significantly on days 10, 15, 20 and 25 in infected mice brains compared to the levels in uninfected mice brains (Fig. 2).

Kinetic studies for claudin-5 in mouse brain and CSF

Detection of claudin-5 protein levels

in the brains and CSF by western blotting. Claudin-5 in the brains were significantly decreased ($P < 0.05$) on days 10, 15 and 20 of infected mice than uninfected mice. However, claudin-5 in CSF was significantly increased ($P < 0.05$) on days 10, 15 and 20 PI of infected mice than uninfected mice (Fig. 3).

Interaction of MMP-9 and claudin-5 in brain homogenate

Co-immunoprecipitation is commonly used to demonstrate protein-protein interaction. We examined the interaction between claudin-5 and MMP-9 in *A. cantonensis*-infected mouse brain homogenates and found MMP-9 interacted with claudin-5 (Fig. 4).

Immunofluorescent staining of claudin-5 and MMP-9 in epithelial cells of mouse choroid plexus on day 20 PI

Localization of claudin-5 and MMP-9 was analyzed by immunofluorescent staining of *A. cantonensis*-infected mouse brain sections. Claudin-5 was stained around the epithelium of the choroid plexus and MMP-9 was localized in epithelial cells. Merged images showed the expression of MMP-9 and claudin-5 at the same sites around the epithelium in the choroid plexus after infection with *A. cantonensis* (Fig. 5).

Influence of NF-κB after treatment with MG132

The effect of MG132 was investigated in a murine angiostrongyliasis model. Western blotting measured the levels of protein p-p65, which was increased significantly in the brain of infected mice compared to the level in uninfected controls. The levels of protein p-p65 were reduced significantly after treatment with MG132. Additionally, gelatin zymography demonstrated the protein activity of MMP-9, which was elevated significantly in the brain of infected mice compared to the level in uninfected controls. The protein activity of MMP-9 was decreased significantly after treatment with MG132 (Fig. 6).

Influence of claudin-5 after treatment with GM6001

The effects of GM6001 were investigated in a murine angiostrongyliasis model. Western blotting measured the level of claudin-5 protein, which was increased significantly in CSF of infected mice compared to the level in uninfected controls. The levels of claudin-5 protein were reduced significantly after treatment with GM6001 (Fig. 7).

Correlation between claudin-5 and Evans blue

Evans blue is normally excluded from CSF by an intact blood-CSF barrier but it can enter CSF when the integrity of the blood-CSF barrier is

disrupted. This method is often used to assess simple alterations in the blood-CSF barrier. The blood-CSF barrier permeability assays for the mouse CSF after infection with *A. cantonensis* showed a significant correlation with the degradation of claudin-5 (Fig. 8).

Correlation of claudin-5 with CSF eosinophils

CSF eosinophilia was found only in infected mice. The level of claudin-5 was correlated significantly with the eosinophil count in CSF (Fig. 9).

Discussion

Infection of mice with *A. cantonensis* causes brain injury and increased expression of NF-κB in the brains of ICR (Lee et al., 2000) and BALB/c strains of mice (Lan et al., 2004). Exposure of human bone marrow endothelial cells to *Trypanosoma brucei gambiense* induced NF-κB translocation to the nucleus and CNS barrier dysfunction (Girard et al., 2005). In addition, over-expression of IκB-α completely blocked NF-κB binding to the MMP-9 promoter, demonstrating that NF-κB activity mediated expression of MMP-9 in primary rabbit and human dermal fibroblasts (Bond et al., 1998). In the present study, significant elevation of p-IκB-α and p-NF-κB was demonstrated in brain samples from mice with eosinophilic meningoencephalitis caused by infection with *A. cantonensis*. Mice treated with MG132 had decreased NF-κB phosphorylation and MMP-9 production in eosinophilic meningoencephalitis. These results indicate NF-κB is a mediator of the upregulation of MMP-9 during infection with *A. cantonensis*.

MMPs contributed to the inflammatory breakdown of the blood-CSF barrier in porcine choroids plexus epithelial cells (Zeni et al., 2007). MMP-12 might have an important role in the degradation of elastin and participate in the breakdown of blood-CSF barrier in mice with eosinophilic meningoencephalitis caused by *A. cantonensis* (Wei et al., 2011). These studies demonstrated that MMPs might be associated with the disruption of blood-CSF barrier during inflammation. In this study, we showed that the degradation of claudin-5 is correlated significantly with blood-CSF barrier permeability. MMP-9 interacted with claudin-5 by co-immunoprecipitation in mice with angiostrongyliasis meningoencephalitis. In addition, the degradation of claudin-5 was reduced significantly when mice were treated with GM6001 and *A. cantonensis*-induced disruption of blood-CSF barrier was prevented by treatment with this MMP inhibitor. These results demonstrate that *A. cantonensis* can activate MMP-9 and leukocyte-derived MMP-9 can contribute to tight junction damage and impairment of the blood-CSF barrier.

Tight junctions control the paracellular permeability of epithelial cells and dysregulated permeability is associated with inflammatory diseases. In a cell model for blood-CSF barrier consisting of porcine choroid plexus epithelial cells infected with *Streptococcus suis*, which causes bacterial meningitis, the bacteria induced loss of blood-CSF barrier function and might facilitate trafficking of leukocytes across the barrier (Tenenbaum et al., 2005; Wewer et al., 2011). There are two possible routes to explain a traversal mechanism during bacterial meningitis: (1) leukocyte infiltration-directed transcellular migration; and (2) leukocytes crossing the barrier by paracellular migration (Wewer et al., 2011). In this study, we demonstrated that claudin-5 was decreased in brain and increased in CSF of mice with angiostrongyliasis meningoencephalitis. Reduction of claudin-5 implies increased blood-CSF barrier permeability and leukocyte migration across the blood-CSF barrier in choroid plexus after infection with *A. cantonensis*. We suggest that leukocytes migrate across the blood-CSF barrier via the paracellular route and subsequently reach inflammatory space in mice infected with *A. cantonensis*. Therefore, the traversal mechanism of leukocytes across blood-CSF barrier could depend on the type of pathogen.

The claudin family, integral components of tight junctions, are responsible for determining the permeability of paracellular transport within epithelial cell layers. The present study showed that MMP-9 might contribute to the degradation of claudin-5 in the blood-CSF barrier. MMP-9 production was correlated with claudin-5 degradation and blood-CSF barrier permeability in angiostrongyliasis meningoencephalitis. Therefore, we propose a possible mechanism (Fig. 8) to explain the contribution of MMP-9 to blood-CSF barrier permeability. In mice infected with *A. cantonensis*, the parasite-induced eosinophilia and inflammation might lead to the induction of the phosphorylation levels of IκB-α and NF-κB. The activation of IκB-α and NF-κB could upregulate MMP-9 production. Blocking IκB-α and NF-κB signaling by MG132 could reduce MMP-9 production. Further, blocking MMP-9 activity by GM6001 could reduce claudin-5 degradation and blood-CSF barrier permeability during angiostrongyliasis meningoencephalitis. MMP-9 could cause claudin-5 degradation and promote leukocyte infiltration into CSF via the paracellular route during infection with *A. cantonensis* in mouse choroid plexus.

In summary, increased blood-CSF barrier permeability is associated with disruption of tight junction proteins elicited by activation of MMP-9. Claudin-5 degradation and blood-CSF barrier dysfunction in brain with

angiostrongyliasis is mediated by MMP-9 *via* the IκB-α/NF-κB/MMP-9 signaling pathway. These mechanistic insights could be used for the pathophysiologic evaluation of blood-CSF barrier breakdown and provide the basis for therapeutic strategies for *A. cantonensis*-induced tight junction disruption.

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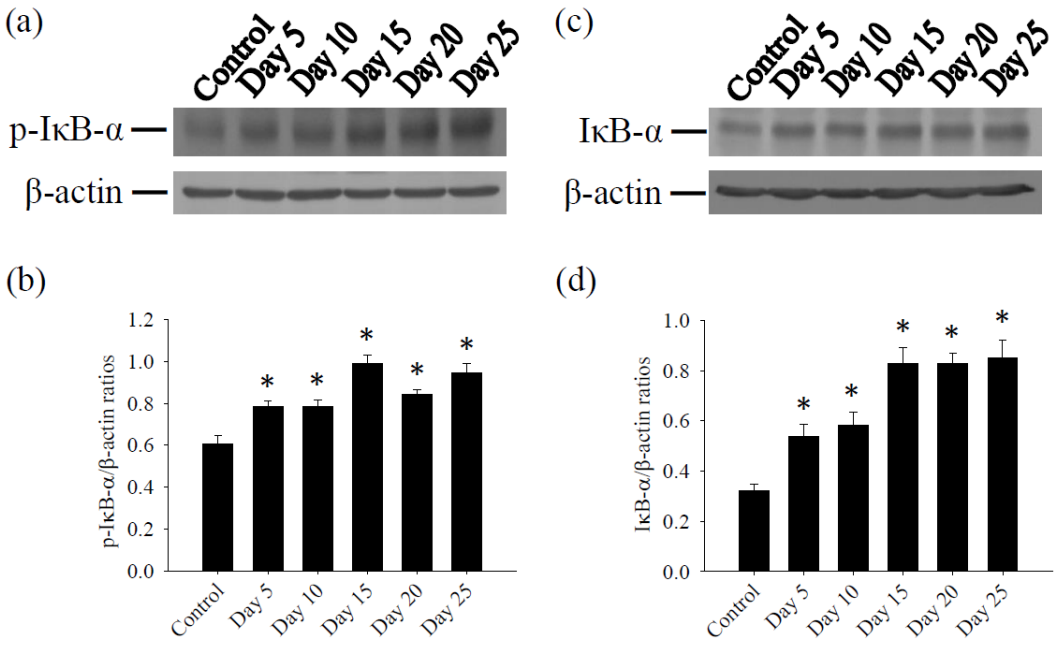

Figure 1. Time-course studies for p-IκB-α and IκB-α in mouse brain. Protein bands of (a) p-IκB-α and (c) IκB-α from the brain of mice infected with *A. cantonensis*. β-actin was used as a loading control. (b and d) Quantification and normalization to β-actin showed significant increases (*$P < 0.05$) in p-IκB-α and IκB-α.

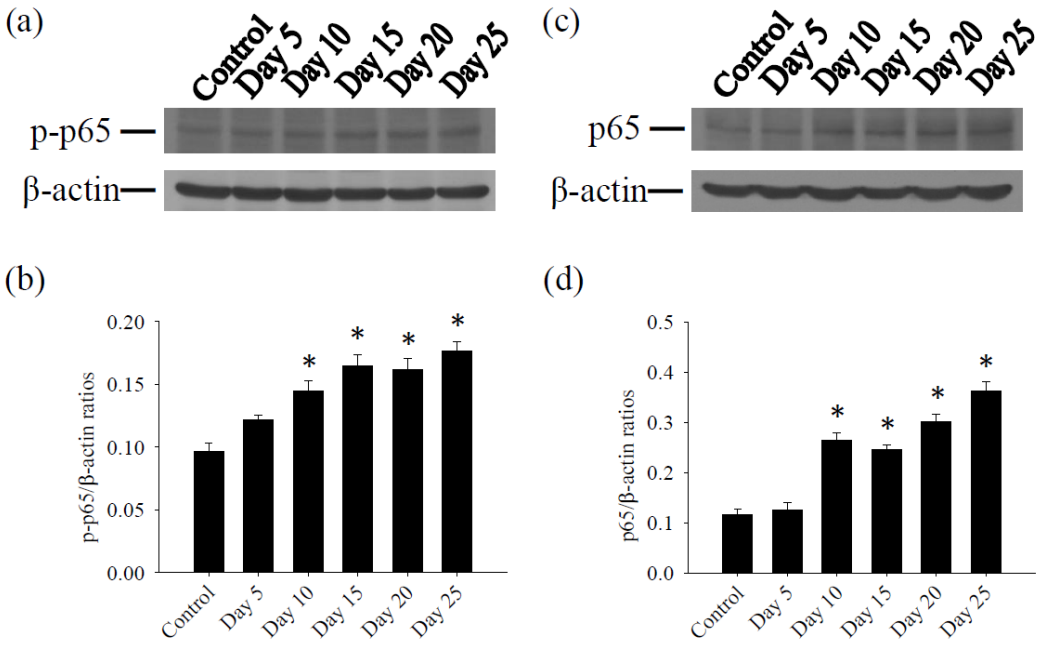

Figure 2. Time-course studies for p-NF-κB p65 and NF-κB p65 in mouse brain. Protein bands of (a) p-NF-κB p65 and (c) NF-κB p65 from the brain of mice infected with *A. cantonensis*. β-actin was used as a loading control. (b) and (d) Quantification and normalization to β-actin showed significant increases (*$P < 0.05$) of p-NF-κB p65 and NF-κB p65.

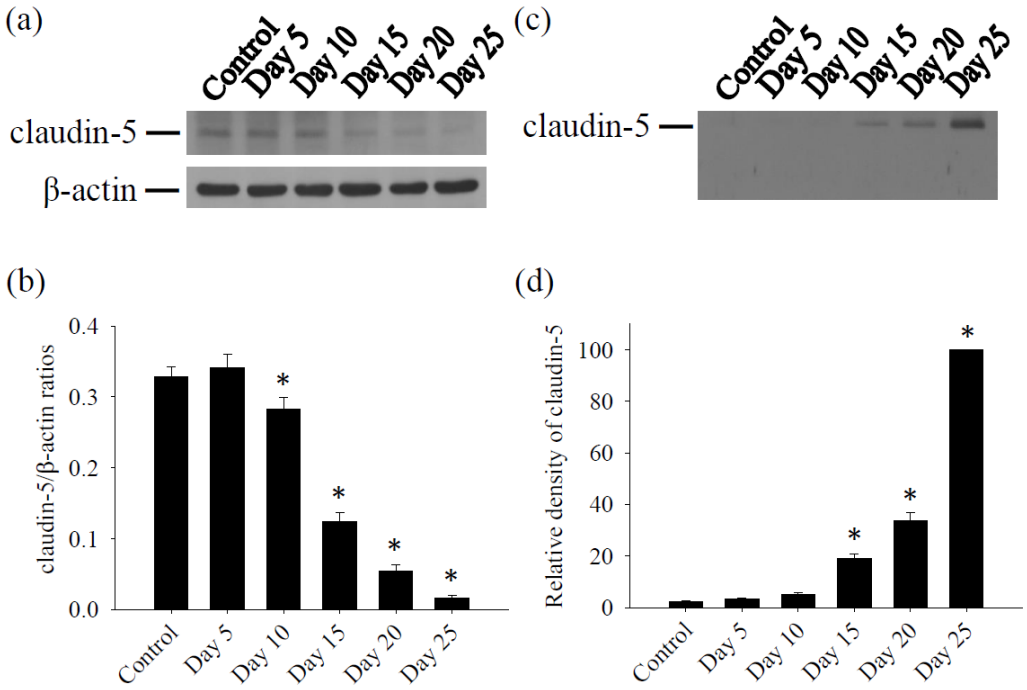

Figure 3. Time-course studies for claudin-5 in mouse brain and CSF. Protein bands of (a) brain claudin-5 and (c) CSF claudin-5 from mice infected with *A. cantonensis*. β-actin was used as a loading control. (b) and (d) Quantification and normalization to β-actin showed significant decrease (*$P < 0.05$) in claudin-5.

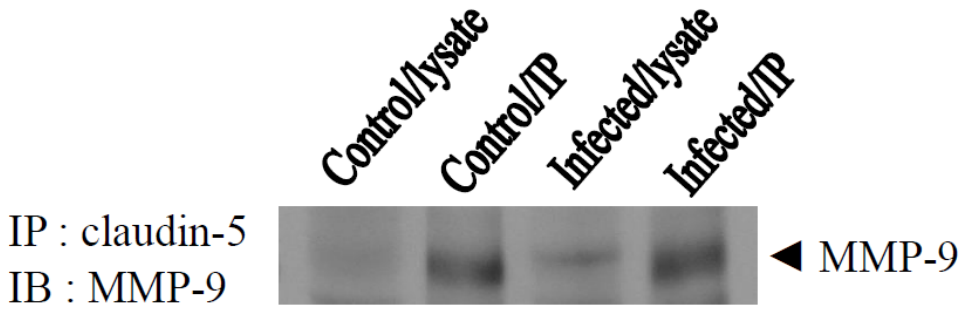

Figure 4. Interaction of claudin-5 and MMP-9 in the brain homogenates of mice. The brain homogenates were immunoprecipitated (IP) with anti-mouse claudin-5 antibody, followed by immunoblotting (IB) with anti-mouse MMP-9 antibody. Control, uninfected brain homogenates; Infected, infected with *A. cantonensis* on day 20 PI; MMP-9, matrix metalloproteinase-9; H, heavy chain.

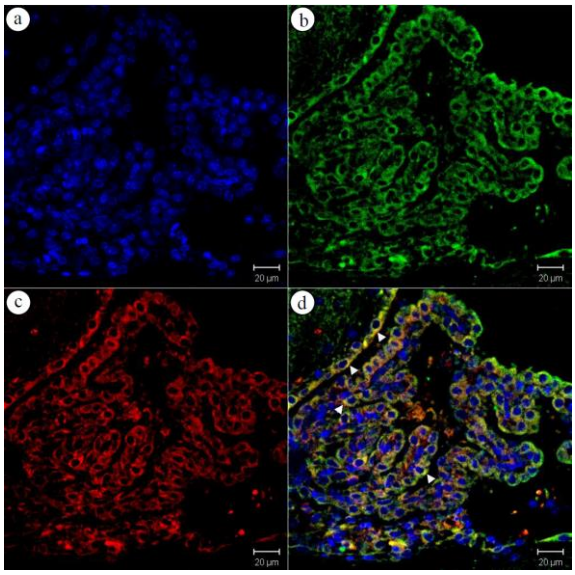

Figure 5. Localization of claudin-5 and matrix metalloproteinase (MMP)-9 in choroid plexus of mouse brain. (a) DAPI-stained for nuclei; (b) DyLight 488-stained for claudin-5 (green); (c) Rhodamine red-stained for MMP-9 (red); (d) the merged image (yellow) showed claudin-5 and MMP-9 were co-localized in the epithelial cells of choroid plexus (white arrowheads).

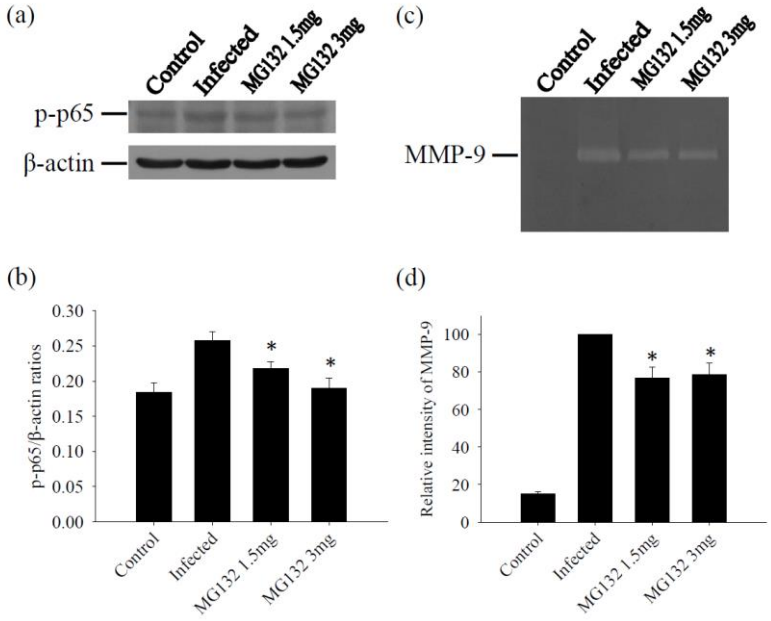

Figure 6. Influence of p-NF-κB p65 and matrix metalloproteinase (MMP)-9 in brain after treatment with MG132. The test groups were: uninfected mice (control); *A. cantonensis*-infected untreated mice (infected); mice treated with 1.5 mg of MG132; and mice treated with 3 mg of MG132. (a) The p-NF-κB p65 bands were analyzed by western blotting using specific antibody from the brains. (b) Ratios of p-NF-κB p65/β-actin showed significant decrease (*$P < 0.05$) in MG132 in treated mice versus infected mice. (c) The MMP-9 activities were analyzed by gelatin zymography from the brains. (d) The relative intensities of MMP-9 were decreased significantly (*$P < 0.05$) in MG132-treated mice versus infected mice.

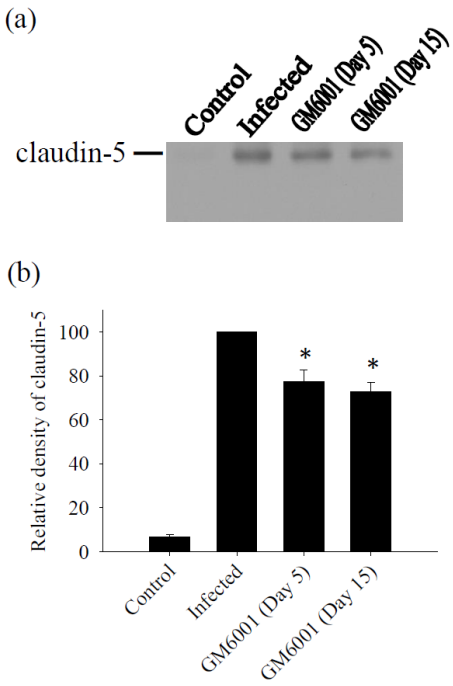

Figure 7. Influence of claudin-5 in CSF after treatment with GM6001. The test groups were: uninfected mice (control); *A. cantonensis*-infected untreated mice (infected); mice treated with GM6001 on day 5 PI; and mice treated with GM6001 on day 15 PI. (a) The claudin-5 bands were analyzed by western blotting using specific antibody from the CSF. (b) The relative densities of claudin-5 were decreased significantly (*$P < 0.05$) in GM6001-treated mice versus infected mice.

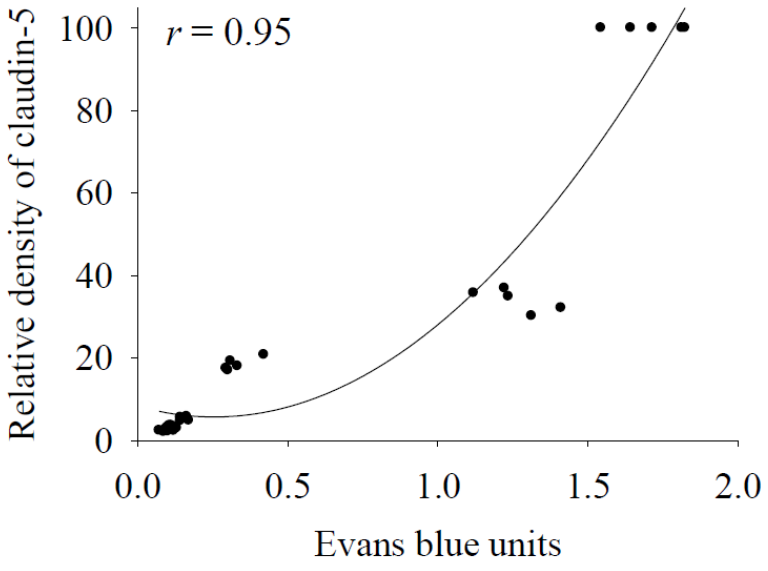

Figure 8. Correlation between claudin-5 and Evans blue. The claudin-5 levels were significantly correlated ($r = 0.95$; $P < 0.05$) with Evans blue.

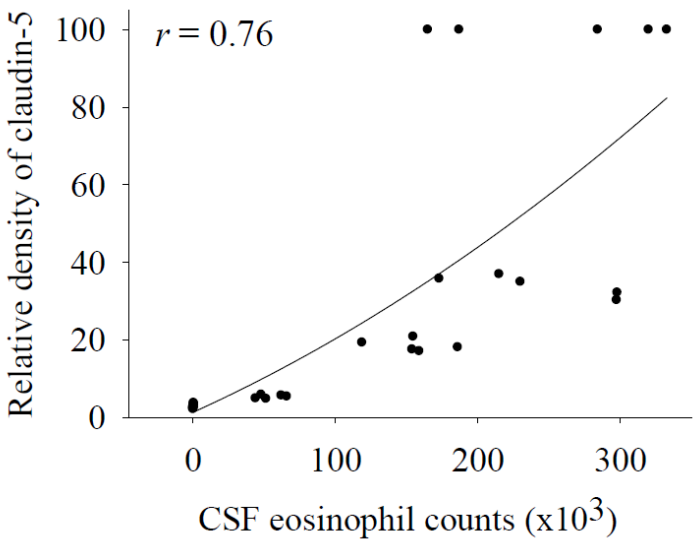

Figure 9. Correlation of claudin-5 with CSF eosinophils. The level of claudin-5 was correlated significantly (r = 0.76; P <0.05) with CSF eosinophil count, using Spearman's ranking correlation test.

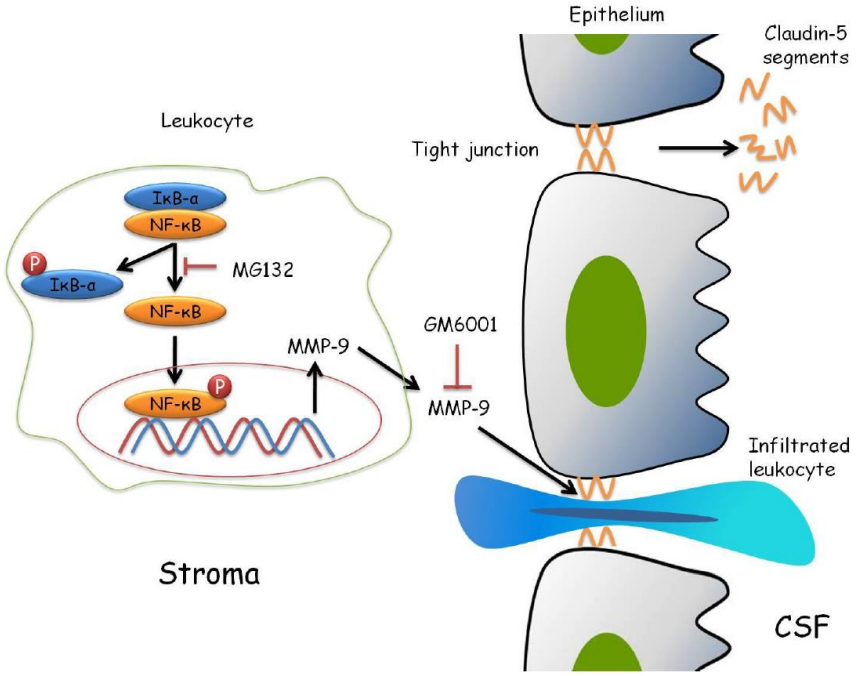

Figure 10. Possible mechanisms of matrix metalloproteinase (MMP)-9 leading to claudin-5 degradation via the NF-κB pathway. The activation of IκB-α and NF-κB could upregulate MMP-9 production in *A. cantonensis*-induced leukocytes. Blocking IκB-α and NF-κB signaling by MG132 could reduce MMP-9 production. Further, blocking MMP-9 activity by GM6001 could reduce claudin-5 degradation and blood-CSF barrier permeability during angiostrongyliasis meningoencephalitis. Therefore, we suggest that MMP-9 could cause claudin-5 degradation and promote leukocyte infiltration into CSF by the paracellular route during *A. cantonensis* infection in mouse choroid plexus.

計劃成果自評

一、 研究內容與原計畫相符程度達 90%

二、 達成預期目標情況：瞭解廣東住血線蟲感染鼷鼠後，IKK、IκBα及 NF-κB 的變化。使研究工作人員瞭解這種本土性寄生蟲(廣東住血線蟲)之致病機轉，此一試驗結果可成功的在蠕蟲建立生化研究的模式，對於寄生蟲之訊息傳遞與致病機轉的關係上將有相當貢獻與突破。對研究工作人員可獲得之訓練，有蛋白質操作、西方點墨法、EMSA 等技術之訓練。找出廣東住血線蟲感染時導致中樞神經系統發炎之致病機轉，由此模式建立預防及治療之管道。

三、 研究成果之學術或應用價值：找出廣東住血線蟲感染時導致腦炎之致病機轉，由此模式提供臨床上診斷此症之參考。

四、 預期完成之研究成果可投稿一篇 SCI 期刊論文。

五、 主要發現：NF-κB/MMP-9 訊息傳遞路徑與 BBB 通透性的關係。

國科會補助計畫衍生研發成果推廣資料表

日期:2013/10/17

國科會補助計畫	計畫名稱: 廣東住血線蟲感染鼷鼠造成血腦障壁功能喪失之機轉
	計畫主持人: 賴世展
	計畫編號: 101-2320-B-040-021- 學門領域: 寄生蟲學、醫事技術及實驗診斷

無研發成果推廣資料

101 年度專題研究計畫研究成果彙整表

計畫主持人：賴世展	計畫編號：101-2320-B-040-021-
計畫名稱：廣東住血線蟲感染鼷鼠造成血腦障壁功能喪失之機轉	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	1	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	1	0	100%	人次	
		博士生	1	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	1	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

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。)	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

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：

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 100 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)

一、 達成預期目標情況：瞭解廣東住血線蟲感染鼷鼠後，IKK、IκBα及 NF-κB 的變化。使研究工作人員瞭解這種本土性寄生蟲(廣東住血線蟲)之致病機轉，此一試驗結果可成功的在蠕蟲建立生化研究的模式，對於寄生蟲之訊息傳遞與致病機轉的關係上將有相當貢獻與突破。對研究工作人員可獲得之訓練，有蛋白質操作、西方點墨法、EMSA 等技術之訓練。找出廣東住血線蟲感染時導致中樞神經系統發炎之致病機轉，由此模式建立預防及治療之管道。

二、 研究成果之學術或應用價值：找出廣東住血線蟲感染時導致腦炎之致病機轉，由此模式提供臨床上診斷此症之參考。

三、 完成之研究成果已投稿 SCI 期刊論文。