Alleviation of Renal and Pulmonary Injury by Immunomodulation in leptospirosis: Hamster Model

Kearkiat Praditpornsilpa MD*, Noppadon Sangjun DVM**,
Wipawee Kittikowit MD***, Duangporn Phulsuksombati DVM***,
Yingyos Avihingsanon MD*, Talerngsak Kansanabuch MD*,
Kriang Tungsanga MD*, Somchai Eiam-Ong MD*

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** Division of Nephrology,Department of Medicine,Faculty of Medicine,Chulalongkorn University Hospital*
*** Thai Component-Armed Forces Research Institute of Medical Sciences*
**** Department of Pathology, Faculty of Medicine, Chulalongkorn University Hospital*

Objective: *Severe leptospirosis manifestations include acute renal failure, caused by acute interstitial nephritis and pulmonary hemorrohage. Spirochete invasion and toxicity of outer membrane cause robust inflammatory host responses. These responses lead to the generation of cytokines, chemokines, and inflammatory cell infiltrations which result in severe organ dysfunctions. The immunomodulation by the modulation of host immune response may alleviate the renal and pulmonary injury. The authors determined whether the current immunosuppressive agents could alleviate the inflammation and minimize the organ injury in hamster model.*
Material and Method: *The animal experiments were conducted with the approval of The Ethical Research Committee of Chulalongkorn University Hospital. The leptospira interrogan serovar pyrogenese was isolated from a wild rat. The spirochete was grown in Fletcher's semisolid media and after subcultures were transferred to the Fletcher's liquid media. An amount of 0.5 ml of the spirochete culture media containing 1 x 10^8 leptospires/ml was intraperitoneally injected to golden Syrian hamsters (Mesocrietus auratus), age 4-6 weeks, weighing 60-80 grams. The hamsters were randomed into 5 groups (n = 4 in each group) namely, 1) Normal group (Control group), 2) Leptospira group, 3) CsA group (leptospira with cyclosporine feeding, 100 mg/kg/day), 4) Rapa group (leptospira with rapamicin feeding, 0.6 mg/kg/day), and 5) Irra group (leptospira with irradiation). Cyclosporine and rapamicin were started at day 0 after the spirochete injection. Gamma ray dose 200 cGy was irradiated to the hamster 3 days before the spirochete inoculation. The animals were autopsied or euthanized if expired or at day 5 post inoculation. The blood samples for BUN, and creatinine were drawn before the inoculation and at autopsy or euthanasia.*
Results: *The inoculation of L Interrogan 0.5 ml (1 x 10^8 leptospires/ml) without immunomodulation cause mortality of all animals at day 4 or day 5 post inoculation. The blood chemistry showed acute severe azotemia. The autopsy findings revealed severe interstitial nephritis and severe pulmonary hemorrhage. The hamsters in the Rapa group had only minimal pulmonary hemorrhage and minimal focal interstitial inflammation of kidney. There were cytoadherance of inflammatory cells to the endothelial cells in lungs and kidneys without the intrusion into the interstitium. The blood chemistry in Rapa group showed mild elevation of BUN and Cr. The immunomodulation by cyclosporine and irradiation did not alleviate the disease. On the contrary, cyclosporine and irradiation caused more severe histopathology.*
Conclusion: *The immunomodulation by rapamicin in leptospirosis in hamsters could alleviate the kidney and pulmonary injuries. The up-regulation of IL-2 in peripheral blood lymphocytes did not result in the kidney and pulmonary injuries.*

Keywords: *Hamster leptospirosis, Cyclosporine, Rapamicin, Irradiation*

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Correspondence to : Praditpornsilpa K, Division of Nephrology, Department of Medicine, Faculty of Medicine, Chulalongkorn University Hospital, Bangkok 10330, Thailand. Phone & Fax: 0-2252-6920, E-mail: kearkiat@hotmail.com

Leptospirosis is a zoonotic disease caused by Leptospira interrogans. The disease is seen worldwide, but is common in the tropics[1]. Various mammals namely, humans, rodents, pigs, goats, dogs, foxes, marsupials, and livestock can be infected with this spirochetes[2]. The microorganisms are transmitted from animals to human via infectious urine or a water-bourn environment that is contaminated with spirochetes. Although the etiology of severe leptospirosis is unclear, host response to the invasion of the spirochetes may play an important role in the organ dysfunction[3]. The different host response may explain the variety of infection outcome. Among mammal species infected with microorganisms, not all species manifested the disease. This phenomenon is obviously seen in rodents. Only specific species of rodents namely hamsters guinea pigs, and specific strains of murine (C3H/Hej) manifest leptospirosis disease. Other rodents, especially rats are natural reservoir. Reservoir rodents can carry the microorganisms for a long time in the proximal tubules and shed them in their urine without hazard[4]. The differences of the disease manifestation from reservoir to fatality in different animal species make the issue of host response important[5]. Leptospires enter the host through the abraded skin or mucosa and spread through the bloodstream to various organs. The immune response of the host to the spirochetes dictates the course of the infection[6,7]. The purpose of the present study was to address the role of modulation of host response by immunosuppressive agents in severe leptospirosis by using the hamster model.

Material and Method

Bacteria

Isolation of Leptospira interrogan serovar pyrogenese was obtained from blood cultures of a wild rat. The spirochete was grown in Fletcher's semisolid media. Later, the subcultures were transferred to the Fletcher's liquid media. The leptospire serovar was identified by using microscopic agglutination test. The leptospira antibodies were given by Pattama Eakpo PhD, Division of Immunology, Faculty of Medicine Siriraj Hospital.

Animals

Golden Syrian hamsters (Mesocrietus auratus), weighing 60-80 grams, 4-6 weeks of age, were housed in individual cages and fed standard chow and water ad libitum. The hamsters were randomized into 5 groups (n = 4 in each group) namely, 1) Normal hamster (Control group), 2) leptospira group, 3) CsA group (leptospira with cyclosporine feeding), 4) Rapa group (leptospira with rapamicin feeding), and 5) Irra group (leptospira with irradiation). Hamsters were injected intraperitoneally with 0.5 10^8 of leptospires in a final volume of 0.5 ml. Negative control animals were injected with Fletcher's liquid media alone. In experimental groups, cyclosporine, rapamycin or irradiation was given to the animals. Animals were monitored daily for signs of illness including weight loss and loss of mobility, and were euthanized when they appeared moribund. Also, the animals that survived after day 5 post inoculation were euthanized for pathological examinations. Before the euthanasia, blood samples were collected for renal function studies.

Immunosuppression

Cyclosporine dosage of 100 mg/kg/day or rapamycin dosage of 0.6 mg/kg/day was started at day 0 immediately after the spirochete injection. Gamma ray dose 200 cGy was irradiated to the hamster 3 days before the spirochete inoculation. Ad lip neomycin concentration 0.7 gram/L in water drinking for bowel sterilization was given to the Irra group hamster 3 days before the irradiation.

Light microscopic studies

Normal and infected hamster kidneys and lungs were fixed in neutral-buffered 4% formaldehyde, processed routinely, embedded in paraffin, cut into 4-μm serial sections and stained with hematoxylin and eosin (H&E).

Statistical analysis

Descriptive results were compared and reported by pictures. ANOVA and post hoc comparisons were used to compare the mean $\pm$ SE between groups. A p-value of less than 0.05 was considered statistically significant.

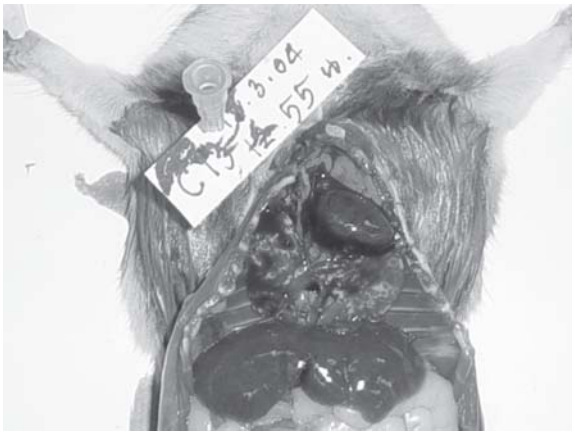
Results

Virulence of Leptospira interrogan serovar pyrogenese

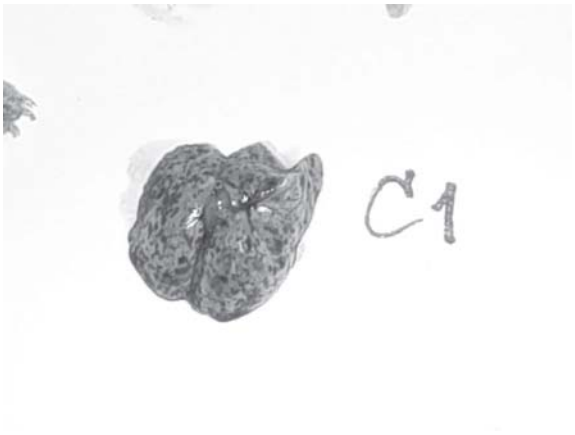
Leptospira interrogan serovar pyrogenese isolated from a wild rat proved highly virulent when injected intraperitoneally into hamsters at doses of 0.5 10^8 of leptospires in a final volume of 0.5 ml. The spirochete inoculation caused mortality of all animals at day 4 or day 5 post inoculations. The gross autopsy revealed distinct massive area of hemorrhage on the surfaces of the lungs (Fig. 1) and peritoneal surfaces. The hemorrhage did not grossly involve the kidneys, liver or spleen. There was marked congestion of the

liver. The blood chemistry showed acute severe azotemia. Both BUN and serum creatinine were elevated by the time of autopsy. The histopathology of the infected hamster demonstrated numerous changes compared with the normal control. The microscopic pulmonary sections showed filled blood lake in the alveolar space (Fig. 2). The alveolar and interalveolar capillaries were distended and engorged with red blood cells and inflammatory cells predominantly by polymorph nuclear cells (Fig. 2).

There was thickening of interalveolar septum. The thickened alveolar septum consisted of an increased number of neutrophils, plasma cells, pulmonary macrophages and hemosiderin in the form of brown granules. There were stagnation and cytoadherence of the inflammatory cell to the endothelial walls of the arterioles.

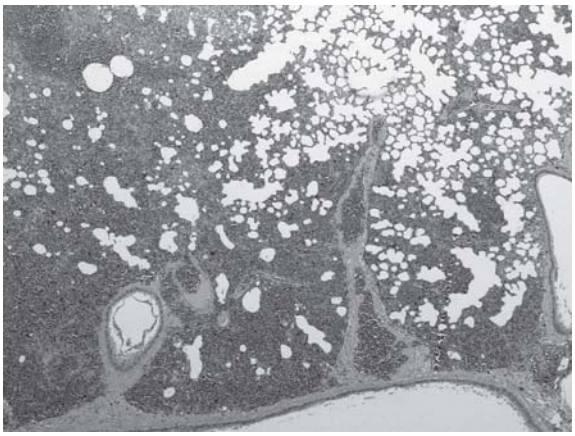


A



B

Fig. 1 The gross anatomy of a hamster infected with Leptospira interrogan serovar pyrogenese isolated from a wild rat (A) revealed massive pulmonary bleeding (B)



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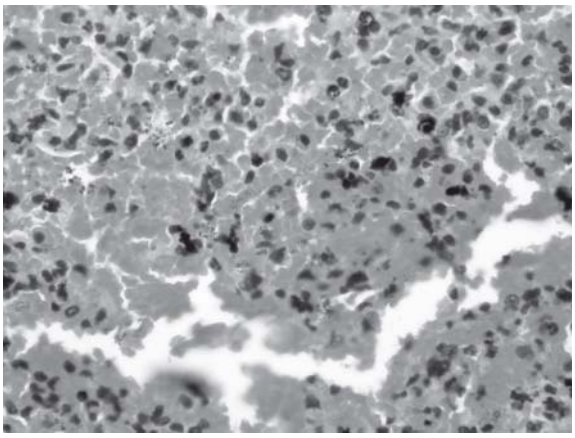

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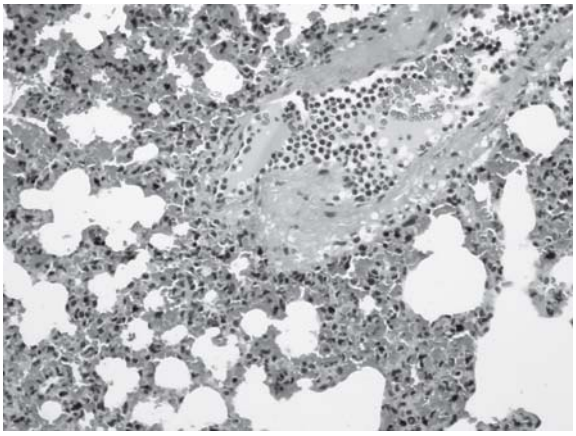


C

Fig. 2 The pulmonary histopathology of hamster infected with Leptospira interrogan serovar pyrogenese at doses of 0.5 x 10^8 of leptospires; blood lake in the alveolar space (A, B), thickening of interalveolar septum with stagnation and cytoadherence of the inflammatory cell to the endothelial walls of the arterioles (C)

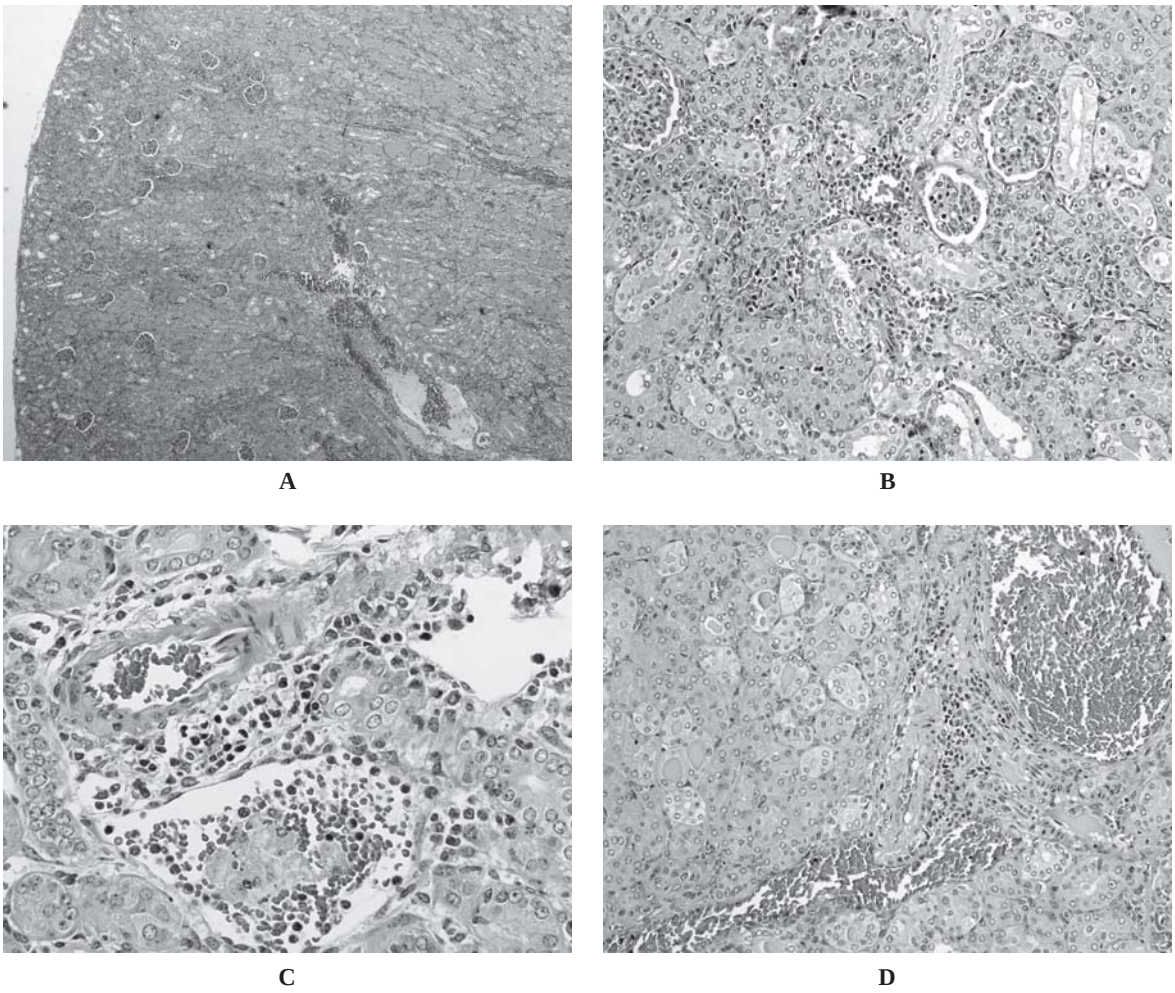


Fig. 3 The renal histopathology of hamster infected with Leptospira interrogan serovar pyrogenese at doses of 0.5 x 10^8 of leptospires; diffuse congestion with focal interstitial hemorrhage (A), accumulation of red blood cell and inflammatory cells in renal interstitium (B), margination of inflammatory cells (C, D)

The renal histopathology showed diffuse congestion with focal interstitial hemorrhage (Fig. 3). There was accumulation of red blood cell and inflammatory cells in renal interstitium.

The margination of inflammatory cells was shown in perivascular area). There was mild congestion of glomeruli (Fig. 4). Mononuclear cells and red blood cells were frequently seen in tubular lumen (Fig. 5). Some tubular cells were degenerating and sloughed off into tubular lumen.

Cyclosporine and irradiation did not alleviate the organ injury from leptospirosis

Cyclosporine daily dose 100 mg/kg/day orally or pre-inoculation irradiation 200 cGy did not alleviate

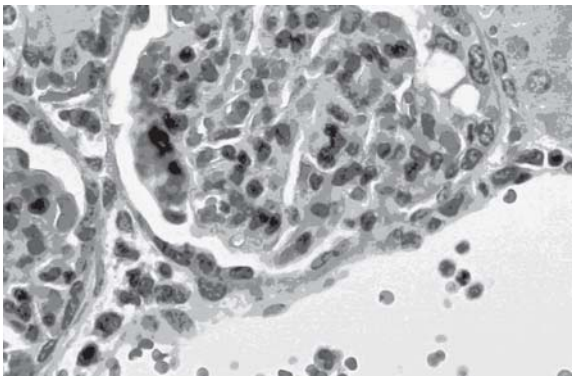


Fig. 4 The glomerular histopathology of hamster infected with Leptospira interrogan serovar pyrogenese at doses of 0.5 x 10^8 of leptospires

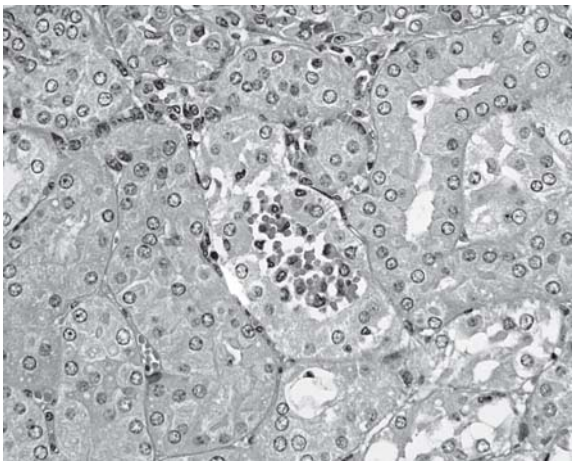


Fig. 5 The tubular histopathology of hamster infected with Leptospira interrogan serovar pyrogenese at doses of 0.5 x 10^8 of leptospires

the hazard effect of the spirochetes. In the contrary, the concomitant oral cyclosporine and pre-inoculation irradiation caused more severe pulmonary hemorrhage as seen in the autopsy (Fig. 6). The histopathology studies showed more severe blood filled alveoli and interseptum pulmonary inflammatory cell infiltration (Fig. 7). There was a robust accumulation of inflammatory cells along the vascular endothelial cells.

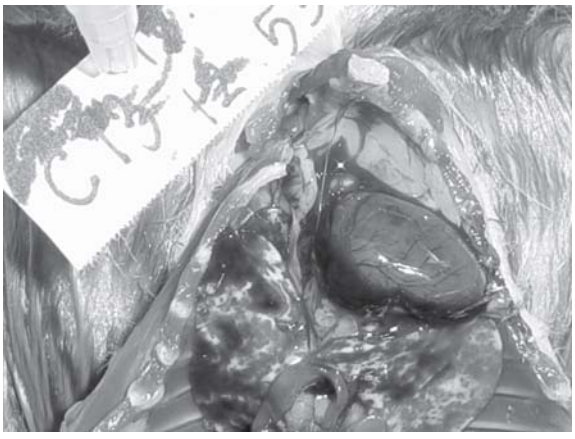
There were no significant changes of renal histopathology compared between the hamster which received the spirochetes inoculation alone or spirochetes inoculation with cyclosporine administration or pre-inoculation irradiation.

Rapamicin alleviated the pulmonary and kidney injury in leptospirosis

Concomitant administration of rapamicin 0.6 mg/kg/day with leptospires inoculation alleviated the pulmonary bleeding. The autopsy gross findings in the Rapa group showed minimal pulmonary bleeding (Fig. 8). There were small spots of minute bleeding on the lungs surface. The histopathology of pulmonary showed minimal focal perivascular bleeding (Fig. 9). There was cytoadherance of inflammatory cells along the surface of endothelial cell. However, the adherence did not result in the penetration of cells into the lung parenchyma. There were hemosiderin depositions in the area of bleeding (Fig. 9C). The renal histopathology (Fig. 10) showed near normal renal architecture. There was minimal perivascular infiltration of inflammatory cells and some areas of cytoadherence were seen.

Rapamycin alleviated the azotemia in the hamster leptospirosis

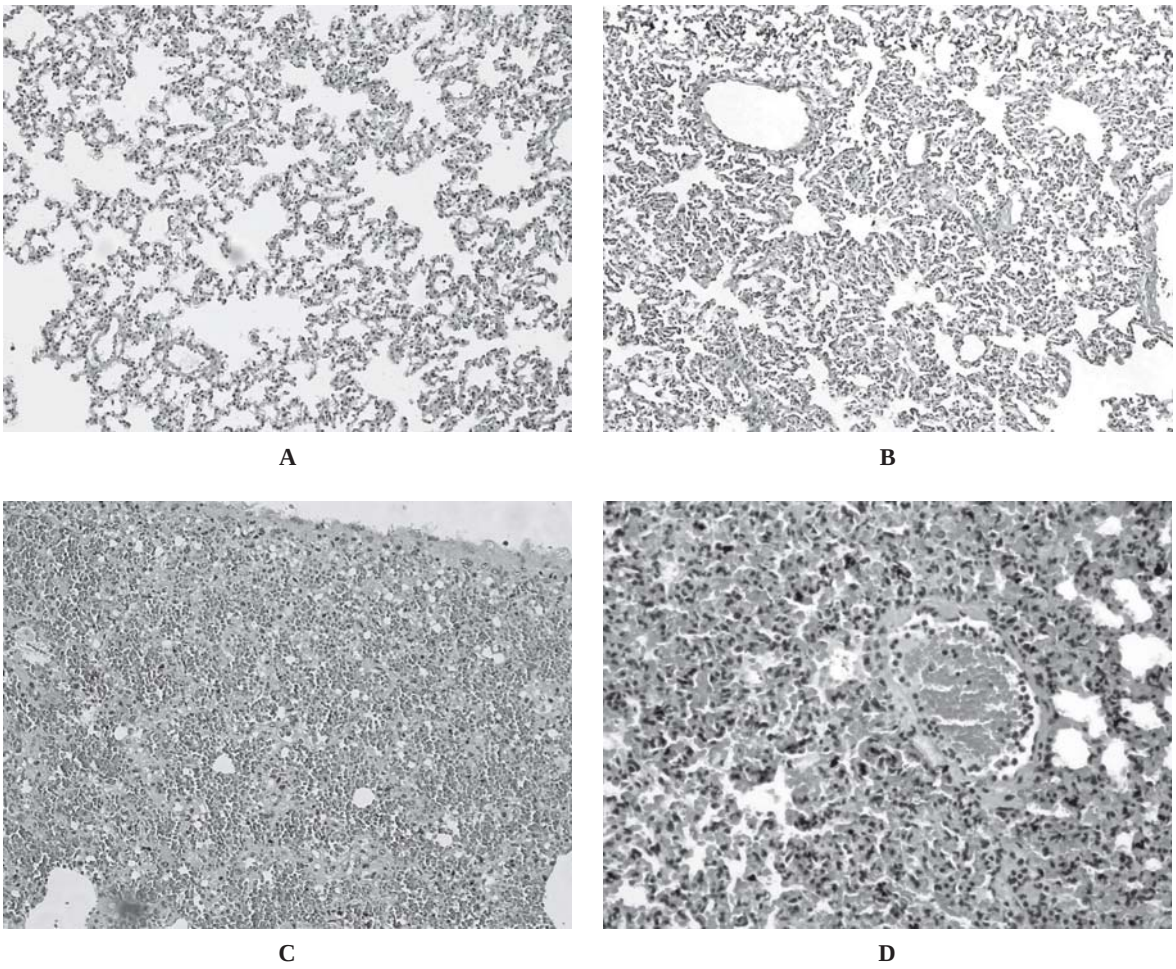
The leptospires inoculation caused severe azotemia in the animals. There were no significant differences of degree of azotemia compared between the animals which received either cyclosporine or pre-inoculation irradiation and the animals which received leptospires alone (Fig. 11). There were significant differences of serum creatinine and blood urea nitrogen compared between the animals which received rapamycin with the spirochetes injection and the animals which received leptospires alone.



A

B

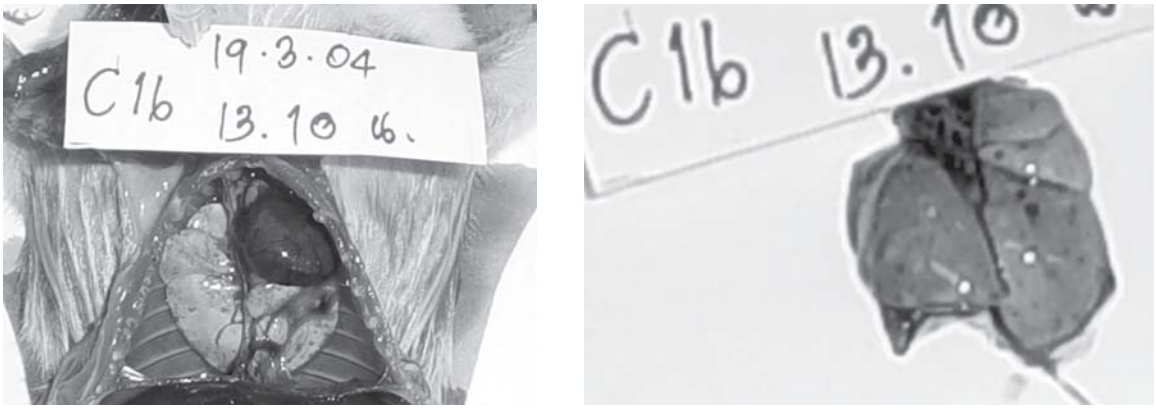
Fig. 6 The gross anatomy of hamster infected with Leptospira interrogan serovar pyrogenese and treated with cyclosporine (A) or pre-inoculation irradiation (B)



A **B**

C **D**

Fig. 7 The pulmonary histopathology of hamster infected with Leptospira interrogan serovar pyrogenese and treated with cyclosporine or irradiation: cyclosporine alone (A), irradiation alone (B), cyclosporine with leptospires inoculation (C), and pre-inoculation irradiation with leptospires inoculation (D)



A **B**

Fig. 8 The gross anatomy of hamster infected with Leptospira interrogan serovar pyrogenese treated with rapamycin

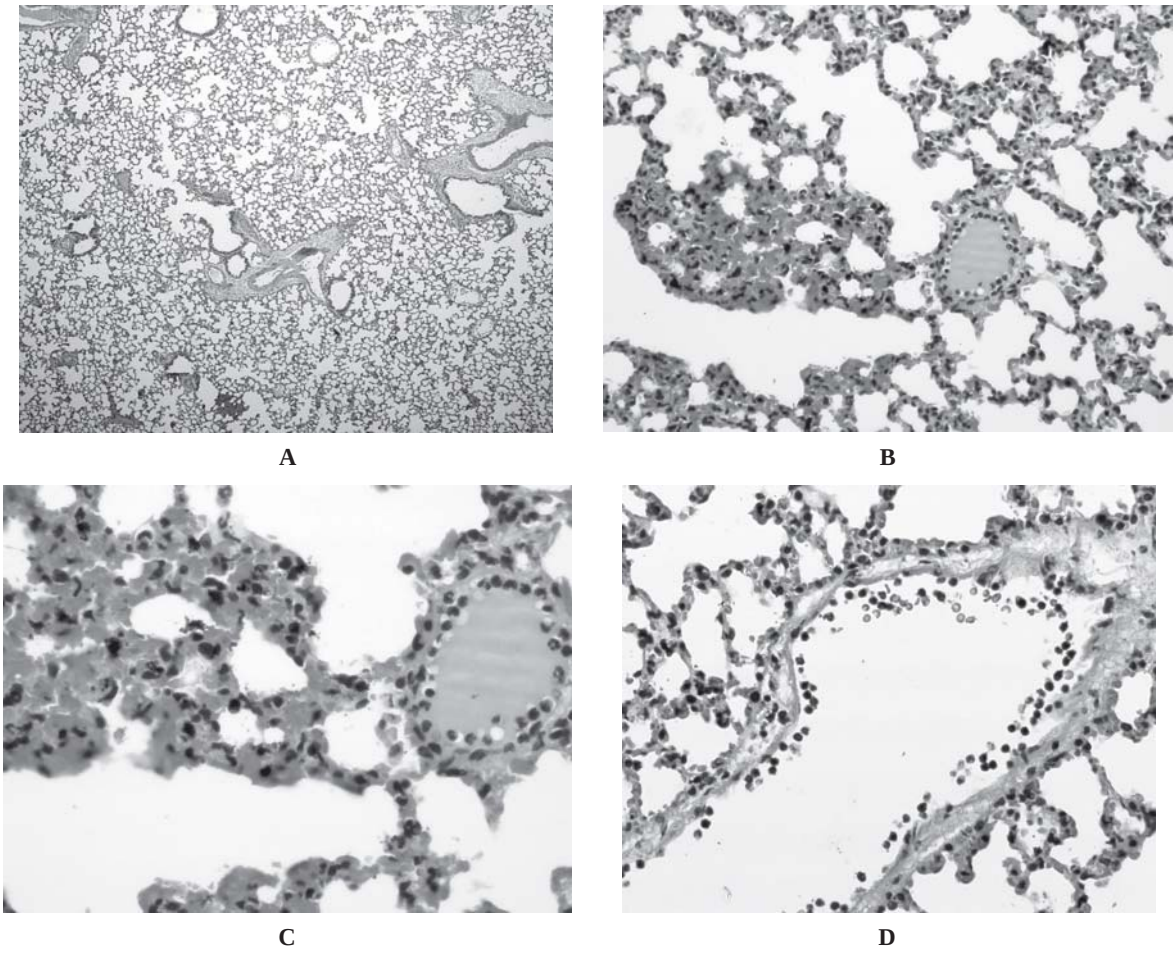


Fig. 9 The pulmonary histopathology of a hamster infected with Leptospira interrogan serovar pyrogenese and treated with rapamycin (A: 4X, B: 10X, and C, D 100X)

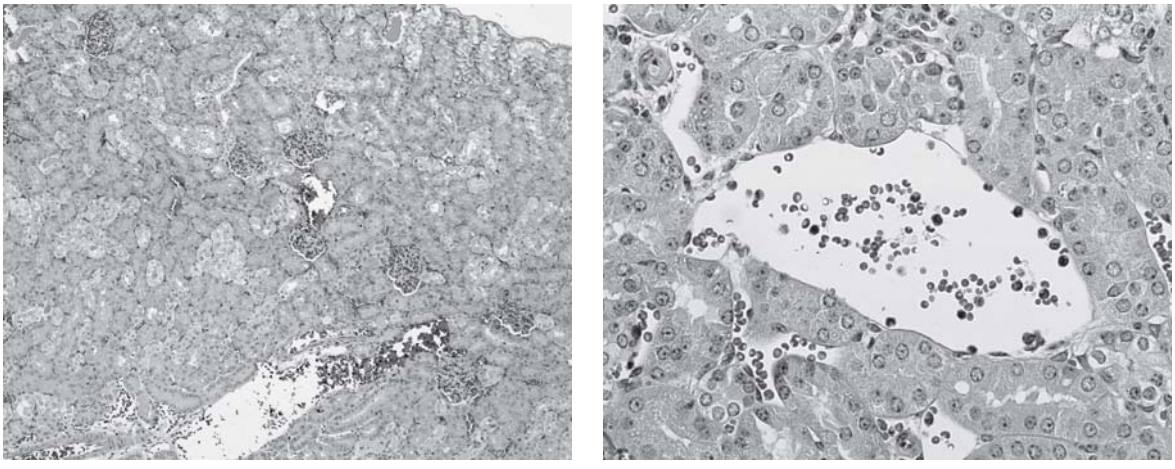


Fig. 10 The renal histopathology of hamster infected with Leptospira interrogan serovar pyrogenese and treated with rapamycin (A: 4X, B: 10X)

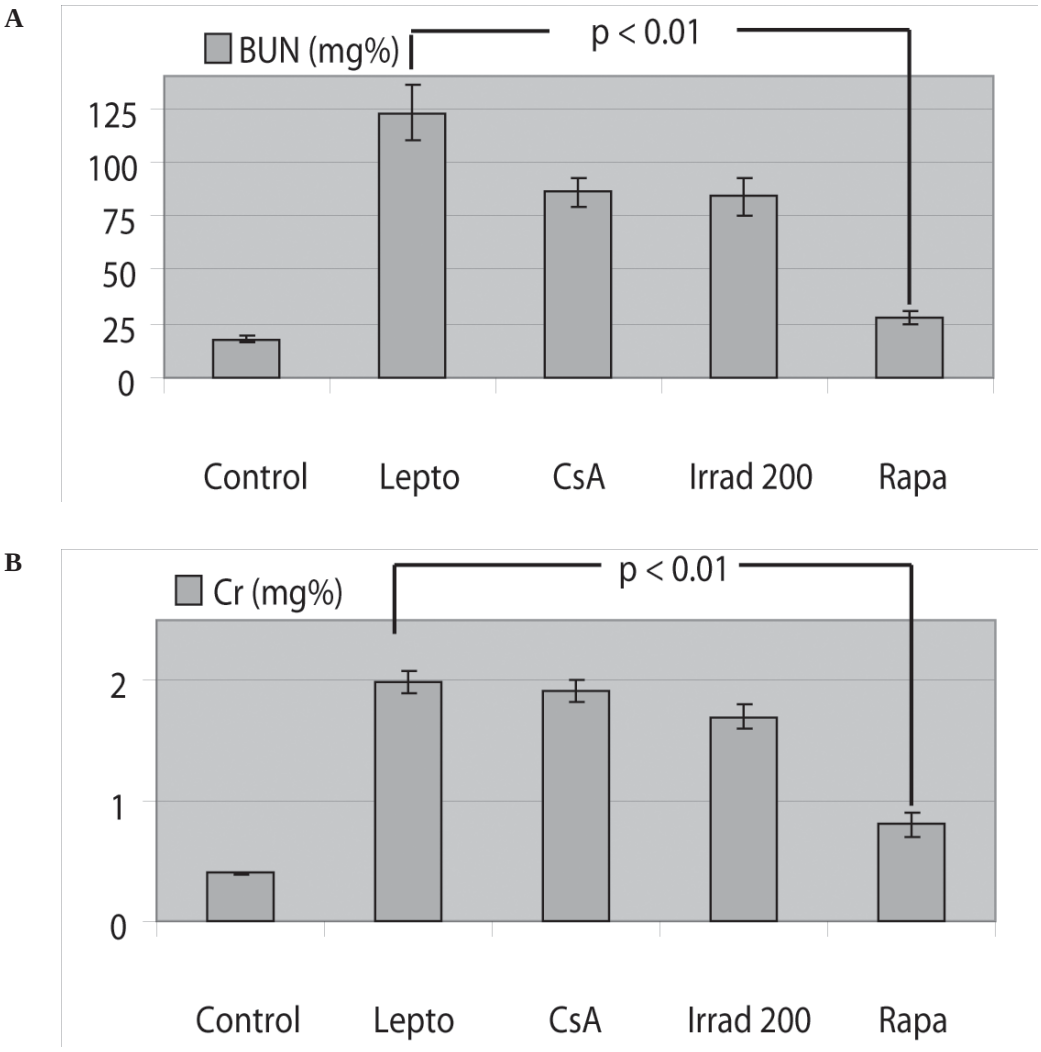


Fig 11. The serum creatinine and blood urea nitrogen of animal received leptospires inoculation alone and animals received leptospires inoculation and different immunomodulations (A: BUN, B: Cr) The figures were presented as mean ± standard error of mean

Discussion

The host response to the leptospires invasion throughout the humans after the host gets the infection from the environment has been a major focus to elucidate the pathogenesis of the disease[8,9]. The potential hazardous host responses include the activation of the cellular immune component and humoral immune component. Study of the mechanism of the severe pulmonary bleeding in guinea pig leptospirosis showed the deposition of immune complex and complements at the alveolar wall[5]. The mechanical effect of the organism invasion has been inadequate to explain the mechanism of the disease as the same virulent organism which causes the organ dysfunction in one species, yet produces no effect in another though the spirochetes has the same migration path after the introduction to the body mostly via abraded skin[10]. The natural host excretes the leptospires via urine as the accidental host. The organ dysfunctions in the accidental host include pulmonary hemorrhage, acute interstitial nephritis, and jaundice. Previous studies[11] have demonstrated several potential bacterial components as the initiator of hazard host response. Those components mainly are the outer membrane of the organisms.

In vitro study[12-13] has shown that the outer membrane can cause activation of inflammatory cells, proliferation of mesangial cell, and up expression of several cytokines such as tumor necrotic factor and interleukin.

The data in the present study have convinced the role of the host response to the disease as rapamycin as the immunomodulation agent can alleviate the organ injuries. On the contrary cyclosporine, a calcineurine inhibitor, inhibits the IL-2 synthesis without interference with other cytokines did not alleviate the severity of the disease. The failure of cyclosporine to alleviate the disease in the present study may reflect the pleiotrophy and redundancy of the cytokines as the inhibition of IL-2 may skew the host response to other cytokines such as IL-7 or IL-15. The irradiation in the present study also did not improve the pulmonary bleeding and interstitial nephritis. The effect of the irradiation is to ablate the immune cellular response. This ablation may not be adequate to suppress the inflammation. The irradiation dose in the present study did not totally ablate the circulating white blood cells (data not shown).

Rapamycin, a mammalian target of rapamycin (mTOR) inhibitor, inhibits mTOR which leads to inhibition of the growth factors and cytokines action, not the growth factors and cytokines production. In this regard, rapamycin has a more specific and more potent immunosuppressive activity for the cellular immune response. The uninterfered growth factor and cytokine levels allow the host to remain the baseline immune function, yet inhibit the robust inflammation as occurs after the leptospires invasion.

In conclusion: The immunomodulation by rapamycin in leptospirosis in hamster could alleviate the kidney and pulmonary injuries. This treatment should be further tested in other models to establish a new strategic treatment for severe leptospirosis.

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บทบาทของการกดภูมิคุ้มกันต่อการทำลายไตและปอดของหนูแฮมสเตอร์ที่ติดเชื้อเลปโตสไปโรซิส

เกื้อเกียรติ ประดิษฐ์พรศิลป์, นภดล แสงจันทร์, วิภาวี กิตติโกวิท, ดวงพร พูลสุขสมบัติ, ยิ่งยศ อวิหิงสานนท์, เถลิงศักดิ์ กาญจนบุษย์, เกรียง ตั้งสง่า, สมชาย เอี่ยมอ่อง

การติดเชื้อเลปโตสไปโรซิสเป็นสาเหตุของการเกิดภาวะไตวายเฉียบพลันและเกิดภาวะเลือดออกในปอดผู้ป่วย เชื้อเลปโตสไปโรซิสเมื่อเข้าสู่ร่างกายจะกระตุ้นปฏิกิริยาตอบสนองของร่างกาย ทำให้มีการหลั่งไซโตไค, คีโมไค และกระตุ้นเซลล์ภูมิคุ้มกันทำให้เกิดผลเสียต่ออวัยวะต่าง ๆ โดยเฉพาะอย่างยิ่งไตและปอด การยับยั้งปฏิกิริยาตอบสนองของร่างกายต่อการติดเชื้ออาจช่วยลดภาวะไตวายและภาวะเลือดออกในปอด การศึกษานี้เป็นการศึกษาในสัตว์ทดลองถึงบทบาทของการกดภูมิคุ้มกันต่อการทำลายไตและปอดของหนูแฮมสเตอร์ที่ติดเชื้อเลปโตสไปโรซิส การศึกษาทำโดยฉีดเชื้อเลปโตสไปโรซิสปริมาณ 1 x 10^8 ตัวต่อมิลลิลิตร จำนวน 0.5 มิลลิลิตร เข้าทางช่องท้องในสัตว์ทดลองกลุ่มต่าง ๆ ได้แก่ กลุ่มควบคุม (ฉีดเชื้อเพียงอย่างเดียว), กลุ่มไซโคสปอริน (ฉีดเชื้อร่วมกับให้กินยาไซโคสปอริน ขนาด 100 มิลลิกรัมต่อน้ำหนักตัวกิโลกรัมต่อวัน), กลุ่มราปามัยซิน (ฉีดเชื้อร่วมกับให้ยากินราปามัยซินขนาด 0.6 มิลลิกรัมต่อน้ำหนักตัวกิโลกรัมต่อวัน) และกลุ่มฉายแสง (ฉีดเชื้อร่วมกับการฉายแสงขนาด 200 เซนติเกรย์) ผลการทดลองพบว่า สัตว์ทดลองที่ได้รับการฉีดเชื้อเพียงอย่างเดียว และสัตว์ทดลองกลุ่มไซโคสปอรินและกลุ่มฉายแสงเสียชีวิตทั้งหมดวันที่ 4-5 หลังฉีดเชื้อ โดยพบว่าสัตว์ทดลองเกิดภาวะไตวายเฉียบพลันจากการอักเสบทางเนื้อไตส่วนอินเตอร์สติเธียมอย่างกว้างขวาง และมีภาวะเลือดออกในปอดอย่างรุนแรง ในทางตรงกันข้ามสัตว์ทดลองกลุ่มราปามัยซินมีภาวะเลือดออกในปอดเพียงเล็กน้อย และมีการอักเสบของเนื้อไตเป็นหย่อม ๆ เท่านั้น แสดงให้เห็นว่าการกดภูมิคุ้มกันโดยยาราปามัยซินช่วยบรรเทาผลของเชื้อเลปโตสไปโรซิสในการเกิดภยันตรายต่อไตและปอด