รายงานวิจัยฉบับสมบูรณ์

โครงการ

พยาธิกำเนิดและผลของยาต่อการติดเชื้อพยาธิ *Schistosoma mekongi*

Pathogenesis and drug treatment effect in *Schistosoma mekongi* infection

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Wannee Jiraungkoorskul

ภาษาไทย พยาธิกำเนิดและผลของยาต่อการติดเชื้อพยาธิ *Schistosoma mekongi*

ภาษาอังกฤษ Pathogenesis and drug treatment effect in *Schistosoma mekongi* infection

ABSTRACT

Schistosoma mekongi infection is one of the Asian shistosomiasis forms. Not many studies were done on this infection until 1992 when a focus of shistosomiasis mekongi was rediscovered in the Northeast of Cambodia.

The first objective of this study was to investigate pathogenesis of experimental schistosomiasis mekongi in mice given in high (30 cercarias) and low (10 cercarias) intensity infections at various time interval. The results showed that the longer exposure time and higher cercarias infection resulted in significant decrease ($p < 0.05$) in body weight, increase in hepatosomatic and spleen index, decrease hematological parameters, increase serum aspartate and alanine aminotransferase and alkaline phosphatase. Total protein level was unaffected while albumin level increased. The main histopathological alterations in the liver were the deposition of the eggs and egg granulomas, proliferation of fibrosis tissues in the portal areas and infiltration of the inflammatory cells. Spleen showed large numbers of megakaryocytes, thickening of capsule, fibrosis around splenic arteries, and congestion with marked dilatation of the splenic sinus. These results revealed that *S. mekongi* infection might suggest invasion of the mice immunity system.

The second objective of this study was to investigate the efficacy of praziquantel and artesunate on tegumental changes in adult *S. mekongi* in mice were compared using scanning electron microscopy (SEM). Forty-five mice infected with *S. mekongi* for 49

days were treated intragastrically with either 300 mg/kg praziquantel or 300 mg/kg artesunate. Mice were sacrificed 1, 3 or 7 days post-treatment. Worms were collected by perfusion and examined by SEM. One to 7days after administration of artesunate, the tegument of *S. mekongi* showed severe swelling, vacuolization, fusion of the tegumental ridges and loss or shortening of the spines on the trabeculae, collapse and peeling. Praziquantel induced similar lesions on the tegument as those observed after administration of artesunate, but were less severe. Seven days post-treatment, there was evidence of recovery only in the case of praziquantel. The study thus suggested that artesunate was more effective than praziquantel in causing tegumental damage in adult *S. mekongi*.

The last objective of this study was to investigate the efficacy of praziquantel and artesunate on adult *S. mekongi in vitro* were investigated by monitoring worm motility and compared tegumental alteration using SEM. Fifty mice were infected with *S. mekongi* cercaria for 49 days. Adult worms were collected by perfusion method and prepared for *in vitro* study. After treatment with 40μg/ml artesunate, contraction and decrease motor activity were observed as early as 3h incubation. Some of the worms died 24 h after exposure, and almost died within 48h. The tegument of *S. mekongi* showed severe swelling, vacuolization, disruption, fusion of the tegumental ridges, collapse, and peeling. After treatment with 80μg/ml praziquantel for 3 to 6h, no apparent effect was seen. After 12 to 48h incubation, lesions on the tegument were similar to those treated with artesunate, but less severe. The direct observation of the worm motility and scanning electron microscopic study highly suggested that artesunate was more effective than praziquantel treatment for the schistosomiasis infection.

ภาษาไทย พยาธิกำเนิดและผลของยาต่อการติดเชื้อพยาธิ *Schistosoma mekongi*

ภาษาอังกฤษ Pathogenesis and drug treatment effect in *Schistosoma mekongi* infection

บทคัดย่อ

การติดเชื้อพยาธิใบไม้เลือดชนิด *Schistosoma mekongi* ซึ่งเป็นโรคสำคัญที่พบในแถบทวีปเอเชีย วัตถุประสงค์ข้อที่หนึ่งของการวิจัยนี้เพื่อศึกษาพยาธิกำเนิดต่อการติดเชื้อพยาธิชนิดนี้ในเรื่องของปริมาณเชื้อ และระยะเวลาของการติดเชื้อ โดยศึกษาการเปลี่ยนแปลงทางโลหิตวิทยา การเปลี่ยนแปลงทางเคมีของการทำงานของตับ การเปลี่ยนแปลงทางพยาธิสภาพ โดยใช้หนูเป็นสัตว์ทดลอง พบว่าเมื่อมีการติดเชื้อในปริมาณที่มากและในระยะเวลานานจะพบการเปลี่ยนแปลงอย่างมีนัยสำคัญทางสถิติ ประกอบด้วย การลดลงของน้ำหนักตัว ตับและม้ามมีขนาดใหญ่ขึ้น ค่าชี้วัดทางโลหิตวิทยามีค่าลดลง ในขณะที่ตัวชี้วัดทางเคมีของการทำงานของตับมีค่าสูงขึ้นบางตัว ได้แก่ aspartate และ alanine aminotransferase, alkaline phosphatase และ albumin ในขณะที่ total protein มีการเปลี่ยนแปลงอย่างไม่มีนัยสำคัญทางสถิติ การเปลี่ยนแปลงทางพยาธิสภาพที่เห็นเด่นชัด ได้แก่ การเกิด egg granuloma ในตับซึ่งทำให้เกิด fibosis และ การแทรกตัวของเซลล์ในขบวนการอักเสบตามมา นอกจากนั้นในม้ามพบว่าผิวชั้นนอกมีขนาดใหญ่ขึ้น หลอดเลือดมีการขยายตัว แสดงว่าการติดเชื้อพยาธิชนิดนี้น่าจะมีผลกระทบต่อระบบภูมิคุ้มกันของหนู

วัตถุประสงค์ข้อที่สองของการวิจัยนี้เพื่อเปรียบเทียบประสิทธิภาพระหว่างยา praziquantel และ artesunate ต่อการเปลี่ยนแปลงของผิวชั้นนอกของพยาธิชนิดนี้ในหนูทดลอง โดยใช้กล้องจุลทรรศน์แบบส่องกราด การทดลองเริ่มจากแบ่งหนูจำนวน 45 ตัวที่มีการติดเชื้อพยาธิชนิดนี้เป็นเวลา 49 วันออกเป็น3กลุ่ม กลุ่มที่1เป็นกลุ่มควบคุม กลุ่มที่2 ได้รับยา praziquantel ขนาด 300

มก/กก และกลุ่มที่3 ได้รับยา artesunate ขนาด 300 มก/กก หลังจากได้รับยาเป็นเวลา1, 3 และ7 วัน หนูแต่ละกลุ่มจะถูกทำให้สลบและเก็บพยาธิเพื่อนำมาตรวจด้วยกล้องจุลทรรศน์แบบส่องกราดต่อไป พบว่าผิวชั้นนอกของพยาธิที่เก็บมาจากหนูที่ได้รับยา artesunate มีอาการบวม มีช่องว่าง หนามมีขนาดสั้นลง มีการหลุดลอกและฉีกขาด ส่วนผิวชั้นนอกของพยาธิที่เก็บมาจากหนูที่ได้รับยา praziquantel จะมีอาการคล้ายคลึงกันแต่มีความรุนแรงน้อยกว่าและพบว่าพยาธิมีการกลับคืนสู่สภาพปกติภายในวันที่7 หลังจากที่ได้รับยา แสดงว่า artesunate มีประสิทธิภาพมากกว่า praziquantel ต่อการเปลี่ยนแปลงของผิวชั้นนอกของพยาธิชนิดนี้ในหนูทดลอง

วัตถุประสงค์ข้อสุดท้ายของการวิจัยนี้เพื่อเปรียบเทียบประสิทธิภาพระหว่างยา praziquantel และ artesunate ต่อพยาธิชนิดนี้ในหลอดทดลอง โดยดูการเคลื่อนที่ของพยาธิ และการเปลี่ยนแปลงของผิวชั้นนอกของพยาธิโดยใช้กล้องจุลทรรศน์แบบส่องกราด การทดลองเริ่มจาก แบ่งพยาธิจำนวน 90ตัวที่เก็บจากหนูที่มีการติดเชื้อพยาธิชนิดนี้เป็นเวลา 49 วันออกเป็น3 กลุ่ม กลุ่มที่1เป็นกลุ่มควบคุม กลุ่มที่2 ได้รับยา praziquantel ขนาด 80 มคก/มล และกลุ่มที่3 ได้รับยา artesunate ขนาด 40 มคก/มล หลังจากได้รับยาเป็นเวลา3, 6, 12, 24 และ48 ชั่วโมง นำพยาธิมาตรวจดูการเคลื่อนที่ และตรวจด้วยกล้องจุลทรรศน์แบบส่องกราดต่อไป พบว่าพยาธิที่ได้รับยา artesunate จะมีการหดตัวและการเคลื่อนไหวลดลงภายใน 3ชั่วโมง พยาธิบางตัวตายภายใน24 ชั่วโมงและตายเกือบหมดภายใน 48 ชั่วโมง ผิวชั้นนอกของพยาธิมีอาการบวม มีช่องว่าง หนามมีขนาดสั้นลง มีการหลุดลอกและฉีกขาด ส่วนพยาธิที่ได้รับยา praziquantel จะมีอาการคล้ายคลึงกันแต่มีความรุนแรงน้อยกว่า แสดงว่า artesunate มีประสิทธิภาพมากกว่า praziquantel ต่อการเคลื่อนที่ของพยาธิและการเปลี่ยนแปลงของผิวชั้นนอกของพยาธิชนิดนี้ในหลอดทดลอง

ABBREVIATION

% = percent

ALT = alanine aminotransferase

AST = aspartase aminotransferase

ALP = alkaline phosphatase

ALB = albumin

BW = body weight

°C = degree Celsius

cm = centrimeter

DALYs = disability adjusted life years = the sum of years of potential life lost due to premature mortality and the years of productive life lost due to disability

EDTA = ethylene diamine tetra acetic acid

g = gram

g/L = gram per liter

h = hour

Hb = hemoglobin

HBSS = hanks' balanced salt solution

Hct = hematocrit

IU/mL = International unit per milliliter

kV = kilo voltage

M = mole

µL = microliter

µm = micrometer

ABBREVIATION (cont.)

mg/kg = milligram per kilogram

mg/mL = milligram per milliliter

min = minute

mL = milliliter

mL/kg = milliliter per kilogram

mm = millimeter

mm/sec = millimeter per second

mol/L = mole per liter

n = number

nm = nanometer

PI = post infection

rpm = round per minute

S.D. = standard deviation

TP = total protein

U/L = unit per liter

WBC = white blood cell

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EXECUTIVE SUMMARY

1. ความสำคัญและที่มาของปัญหา

โรคที่เกิดจากพยาธิใบไม้เลือดเรียกว่า Schistosomiasis คาดว่ามีผู้เป็นโรคพยาธินี้ทั่วโลกประมาณ 200 ล้านคน และอีกประมาณ 600 ล้านคนที่มีโอกาสเสี่ยงที่จะเป็นโรคนี้ ในประเทศไทยพยาธิใบไม้เลือดที่เป็นปัญหาคือ *Schistosoma mekongi*

Schistosoma mekongi

วงจรชีวิต

วงจรชีวิตคล้ายคลึงกันกับพยาธิใบไม้เลือดชนิดอื่นๆตัวเต็มวัยเพศผู้และเพศเมียอาศัยอยู่เป็นคู่ที่เส้นเลือดดำของลำไส้เล็ก (superior mesenteric vein) และ portal vein ผสมพันธุ์แล้วออกไข่เป็นจำนวนมากในแต่ละวัน เมื่อไข่ออกมาจากมดลูกระยะแรก ตัวอ่อนไมราซิเดียมยังเจริญไม่เต็มที่จะใช้เวลาประมาณ 6-7 วัน ในการไชทะลุผ่านผนังเส้นเลือดและผนังลำไส้ออกมาอยู่ในช่องว่างของลำไส้เล็กและปนออกมากับอุจจาระ เมื่อไข่ตกสู่น้ำ ไมราซิเดียมจะออกจากไข่และว่ายเป็นอิสระอยู่ในน้ำ และถ้าหากไชเข้าโฮสต์กึ่งกลางที่เหมาะสม ได้แก่ *Neotricula aperta* จะเจริญพัฒนาไปเป็นสปอโรซิสต์รุ่นที่หนึ่ง ซึ่งต่อมาจะผลิตสปอโรซิสต์รุ่นที่สองตามลำดับ สปอโรซิสต์รุ่นที่สองนี้จะเจริญพัฒนาไปเป็นเซอร์คาเรีย ออกจากหอยว่ายน้ำเป็นอิสระ การเจริญเติบโตในหอยใช้เวลาประมาณ 5-7 สัปดาห์ เซอร์คาเรียเมื่อออกจากหอยและว่ายเป็นอิสระอยู่ในน้ำเพื่อหาโฮสต์เฉพาะ เมื่อพบจะไชเข้าสู่ผิวหนังของโฮสต์โดยสลัดหางทิ้ง จากนั้นจะไชไปสู่เส้นเลือดฝอยโดยอาศัยการช่วยย่อยของ proteolytic enzyme ซึ่งหลั่งออกมาจาก penetration gland ตัวอ่อนในระยะนี้เรียกว่า schistosomule ต่อมาเข้าสู่ระบบเลือดดำหรือระบบน้ำเหลืองเพื่อไปยังหัวใจและปอด จากปอดจะย้อนกลับมายังหัวใจและไปที่ตับสู่ระบบไหลเวียนโลหิตภายในตับ (intrahepatic portal circulation) พยาธิตัวอ่อนจะเจริญเติบโตเป็นตัวเต็มวัย จับคู่และผสมพันธุ์ จากนั้นจะเคลื่อนย้ายไปอยู่ที่ superior mesenteric vein และเพศเมียจะเริ่มวางไข่ วงจรชีวิตทั้งหมดนี้ใช้เวลาประมาณ 5-7 สัปดาห์

ระบาดวิทยา

สัตว์เลี้ยงลูกด้วยนมเช่น สุนัข หมู วัว ควาย แพะ แกะ สามารถติดเชื้อพยาธินี้ได้ตามธรรมชาติ ในถิ่นระบาดหลายแห่งสัตว์เหล่านี้ถือว่าเป็นโฮสต์สะสมเชื้อที่มีความสำคัญต่อวงจรชีวิตและการแพร่ระบาดของพยาธินี้ ในปัจจุบันพบว่าสุนัขและหมูเป็นโฮสต์เฉพาะของ *S. mekongi* การระบาดของพยาธิใบไม้เลือดอาศัยปัจจัยที่สำคัญได้แก่ แหล่งน้ำซึ่งเป็นที่ให้ตัวอ่อนในไข่พยาธิเติบโตและมีหอยเป็นโฮสต์กึ่งกลาง มีอุณหภูมิที่พอเหมาะให้ไมราซิเดียมออกมาจากไข่และสามารถไชเข้าสู่หอยที่เหมาะสมและมีการเจริญพัฒนาในหอยจนเป็นเซอร์คาเรียซึ่งเป็นระยะติดเชื้อในคน โดยต้องมีการติด

เชื้อเข้าสู่คนหรือสัตว์เพื่อให้ครบวงจรชีวิต การติดเชื้อพยาธิใบไม้เลือดพบได้ในประชากรทุกกลุ่มอายุที่มีส่วนเกี่ยวข้องกับน้ำ เช่น การกสิกรรมและเกษตรกรรม การประมง รวมทั้งมีการใช้น้ำจากแหล่งน้ำที่มีการแพร่เชื้อเพื่อการบริโภคและอุปโภค

พยาธิสภาพ พยาธิวิทยา และอาการ

พยาธิสภาพจะเกิดมากที่สุดในตับและลำไส้ โดยทั่วไปความรุนแรงของโรคขึ้นอยู่กับจำนวนของพยาธิตัวเต็มวัยโดยมีไข่เป็นสาเหตุหลักของการเปลี่ยนแปลงทางพยาธิสภาพทั้งหมดถ้าเป็นการติดเชื้อจำนวนมากอาจทำให้ผู้ป่วยเสียชีวิตได้ พยาธิวิทยาและอาการของการติดเชื้อ *S. mekongi* คล้ายกันกับ *S. japonicum* แต่ความรุนแรงของโรคน้อยกว่า อาจมีอาการอุจจาระร่วงเล็กน้อย ที่พบบ่อยคือตับโตและม้ามโต บางรายอาจเกิดความดันเลือดในระบบ portal สูง แต่จะไม่พบอาการแทรกซ้อนทางสมองและ หัวใจกับปอด

การรักษา

การรักษาทำได้ลำบากเนื่องจากไข่ของพยาธิเท่านั้นที่จะทำให้เกิดพยาธิสภาพและเป็นอันตรายต่อโฮสต์ สำหรับการทำลายตัวเต็มวัยใช้ยา praziquantel ในขนาด 30 มก./กก. กินวันละ 2 เวลา

2. วัตถุประสงค์

2.1 เพื่อศึกษาพยาธิกำเนิดต่อการติดเชื้อพยาธิ *Schistosoma mekongi* โดยศึกษาการเปลี่ยนแปลงดังต่อไปนี้

2.1.1 ศึกษาการเปลี่ยนแปลงทางโลหิตวิทยา

2.1.2 ศึกษาการเปลี่ยนแปลงทางเคมีของการทำงานของตับ

2.1.3 ศึกษาการเปลี่ยนแปลงทางพยาธิสภาพของผิวนอกของพยาธิด้วยกล้องจุลทรรศน์ธรรมดาและกล้องจุลทรรศน์อิเลคตรอนแบบแสงส่องผ่านและแบบแสงส่องกราด

2.2 เพื่อศึกษาเปรียบเทียบผลของยา Praziquantel และ Artesunate ที่มีต่อผิวนอกของพยาธิ *Schistosoma mekongi* ในสัตว์ทดลอง (*in vivo*)

2.3 เพื่อศึกษาเปรียบเทียบผลของยา Praziquantel และ Artesunate ที่มีต่อผิวนอกของพยาธิ *Schistosoma mekongi* ในหลอดทดลอง (*in vitro*)

3. ระเบียบวิธีการวิจัย

3.1 การศึกษาพยาธิกำเนิดของการติดเชื้อ *Schistosoma mekongi* ในสัตว์ทดลอง

โดยใช้หนู mice เพศผู้อายุ 6 สัปดาห์ จำนวน 180 ตัว แบ่งออกเป็น 3 กลุ่ม

กลุ่มที่ 1 เป็นกลุ่มควบคุม ไม่ได้รับเซอร์คาเรีย มีจำนวนหนู 60 ตัว

กลุ่มที่ 2 เป็นกลุ่มทดลองได้รับเซอร์คาเรียจำนวน 15 ตัว มีจำนวนหนู 60 ตัว

กลุ่มที่ 3 เป็นกลุ่มทดลองได้รับเซอร์คาเรียจำนวน 30 ตัว มีจำนวนหนู 60 ตัว

วิธีทำ

สัตว์ทดลองแต่ละตัวจะถูกอดอาหารก่อนการให้เซอร์คาเรียเป็นเวลา 16 ชั่วโมงและ2 ชั่วโมง หลังการให้เซอร์คาเรีย เฝ้าสังเกตอาการ พฤติกรรมและชั่งน้ำหนักทุกวัน ภายในเวลา 3, 7, 21, 35, และ 49 วันหลังจากให้เซอร์คาเรีย หนูในแต่ละกลุ่ม จำนวน 12 ตัว จะถูกนำมาสลบ เปิดช่องท้อง ล้างด้วย 0.85% NaCl 10 มล. น้ำล้างช่องท้องจะนำมาตรวจหาพยาธิ ต่อไป ปอด ตับและ ม้าม จะถูกนำมาศึกษาการเปลี่ยนแปลงทางพยาธิสภาพ เจาะเลือดจากหัวใจ เพื่อศึกษาการเปลี่ยนแปลงทางโลหิตวิทยาและการเปลี่ยนแปลงทางเคมีของการทำงานของตับ

1. การศึกษาการเปลี่ยนแปลงทางโลหิตวิทยา (Hematology) โดยศึกษาการเปลี่ยนแปลงของค่าต่างๆ ทางโลหิตวิทยาดังนี้คือ ค่าปริมาตรเม็ดเลือดแดงอัดแน่น, ฮีโมโกลบิน, จำนวนเม็ดเลือดขาว, จำนวนของเม็ดเลือดขาวแต่ละชนิด
2. การศึกษาการเปลี่ยนแปลงการทำงานของตับ (Liver function test) โดยศึกษาการเปลี่ยนแปลงของระดับเอ็นซัมย์ดังต่อไปนี้ Alanine aminotransferase, Aspartate aminotransferase, Alkaline Phosphatase, Total protein, และ Albumin
3. การศึกษาการเปลี่ยนแปลงทางพยาธิสภาพของผิวนอกของพยาธิในระดับกล้องจุลทรรศน์ธรรมดา (Light microscope)
4. การศึกษาการเปลี่ยนแปลงทางพยาธิสภาพของผิวนอกของพยาธิในระดับจุลทรรศน์อิเลคตรอนแบบแสงส่องผ่าน (Transmission electron microscope)

3.2 การศึกษาผลของยาต่อการติดเชื้อพยาธิ *Schistosoma mekongi* โดยแบ่งออกเป็น

3.2.1 การศึกษาเปรียบเทียบผลของยา Praziquantel และ Artesunate ต่อการเปลี่ยนแปลงลักษณะผิวนอกของพยาธิ *Schistosoma mekongi* ในสัตว์ทดลอง (*in vivo*)

ยาที่ใช้ได้แก่ Praziquantel (Atlantic Laboratories Corp. Ltd.) ที่ระดับความเข้มข้น 300 mg/kg และ Artersunate (Atlantic Laboratories Corp, Ltd.) ที่ระดับความเข้มข้น 300 mg/kg ซึ่งยาทั้งหมดจะถูกละลายใน 7% Tween80+3% ethanol

สัตว์ทดลองที่ใช้ได้แก่ mice เพศผู้อายุ 6 สัปดาห์ จำนวน 45 ตัว แบ่งออกเป็น 3 กลุ่มกลุ่มละ 15 ตัว

กลุ่มที่ 1 เป็นกลุ่มควบคุม ได้รับ 7% Tween80+3% ethanol

กลุ่มที่ 2 เป็นกลุ่มทดลองได้รับ Praziquantel ที่ระดับความเข้มข้น 300 mg/kg

กลุ่มที่ 3 เป็นกลุ่มทดลองได้รับ Artersunateที่ระดับความเข้มข้น 300 mg/kg

สัตว์ทดลองแต่ละตัวจะถูกอดอาหารก่อนการให้เซอร์คาเรียเป็นเวลา 16 ชั่วโมงและ2 ชั่วโมง หลังการให้เซอร์คาเรีย เฝ้าสังเกตอาการ พฤติกรรมและชั่งน้ำหนักทุกวัน ภายในเวลา 49 วันหลังจากให้เซอร์คาเรีย หนูในแต่ละกลุ่ม จะได้รับยาตามที่กำหนด ภายหลังจากการให้ยา 1, 3, 7 วัน หนูในแต่

ละกลุ่มจำนวน 5 ตัว จะถูกนำมาสลบ เปิดช่องท้อง ตับจะถูกนำมาแช่ใน 0.85% NaCl ที่อุณหภูมิห้อง เพื่อศึกษา motility ของ พยาธิตามเกณฑ์มาตรฐานที่กำหนดไว้ ต่อไป

3.2.2 การศึกษาเปรียบเทียบผลของยา Praziquantel และ Artesunate ต่อการเปลี่ยนแปลงลักษณะผิวนอกของพยาธิ *Schistosoma mekongi* ในหลอดทดลอง (*in vitro*)

ยาที่ใช้ได้แก่ Praziquantel (Atlantic Laboratories Corp, Ltd.) ที่ระดับความเข้มข้น 80 μg/ml และ Artersunate (Atlantic Laboratories Corp, Ltd.) ที่ระดับความเข้มข้น 40 μg/ml ซึ่งยาทั้งหมดจะผสมอยู่ใน M199 culture medium ซึ่งมี ยาปฏิชีวนะได้แก่ penicillin 50 IU/ml, streptomysin 50 mg/mL พยาธิในระยะตัวอ่อนและตัวเต็มวัยจะถูกเลี้ยงอยู่ในอาหารเลี้ยงพยาธิที่ผสมยาตามที่กำหนดแล้วนำเข้าตู้อบที่อุณหภูมิ 37 องศาและ 5% ก๊าซคาร์บอนไดออกไซด์ พยาธิแต่ละกลุ่มนำมาศึกษา motility ของพยาธิตามกฎเกณฑ์มาตรฐานที่กำหนดไว้ นอกจากนั้นที่เวลา 3, 6, 12, 24, และ 48 ชั่วโมงหลังจากเข้าตู้อบ พยาธิจำนวน 6ตัว ในแต่ละกลุ่มจะถูกนำมาศึกษา การเปลี่ยนแปลงทางพยาธิสภาพในระดับกล้องจุลทรรศน์อิเลคตรอนแบบแสงส่องกราด

4. ผลงาน/หัวข้อเรื่องที่คาดว่าจะตีพิมพ์ในวารสารวิชาการระดับนานาชาติในแต่ละปี

ชื่อเรื่อง : Effects of praziquantel and artesunate on the tegument of adult *Schistosoma mekongi* harboured in mice

ชื่อวารสาร : Parasitology International 2005: Article in press; Impact Factor - 2001 = 1.045

5. งบประมาณโครงการ (ตามระยะเวลาโครงการที่ได้เสนอรับทุน)

	ปีที่ 1	ปีที่ 2	รวม
1.หมวดค่าตอบแทน			
-ค่าตอบแทนหัวหน้าโครงการ	120,000	120,000	240,000
2.หมวดค่าวัสดุ			
-ค่าเครื่องแก้ว สารเคมีและวัสดุวิทยาศาสตร์ และสำนักงาน	60,000	60,000	120,000
3.หมวดค่าใช้สอย			
-ค่ายานพาหะ/เชื้อเพลิงเดินทางและค่าที่พักในการเก็บตัวอย่าง	15,000	15,000	30,000
-ค่าชั่วโมงและค่าใช้จ่ายในการใช้เครื่องมือพิเศษ ได้แก่ กล้องจุลทรรศน์อิเลกตรอน ฯลฯ	25,000	25,000	50,000
-ค่าเอกสารและค่าส่งไปรษณีย์สิ่งพิมพ์ เพื่อการวิจัย	10,000	5,000	15,000
-ค่าใช้สอยเบ็ดเตล็ด	10,000	15,000	25,000
รวมงบประมาณโครงการ	**240,000**	**240,000**	**480,000**

CHAPTER I

INTRODUCTION

Figure 1. Dr. Theodore Maximillian Bilharz (1825-1862), German physician (http://www.path.cam.ac.uk/~schisto/History/History.html)

Schistosomiasis: It is the second most devastating parasitic disease in tropical countries, after malaria and is of great public health and socio-economic importance in the developing world. In some parts of the world, it is also known as bilharzia in honor of Theodore Bilharz. He first identified the etiological agent for *Schistosoma heamatobium* in Egypt in 1851 (Figure 1).

Infection is widespread with a relatively low mortality rate, but a high morbidity rate, causing severe debilitating illness in millions of people. It is estimated that at least 200 million people are currently infected with schistosomiasis and another 600 million are at risk of infection from the five species affecting man, *S. haematobium*, *S. intercalatum*, *S. japonicum*, *S. mansoni* and *S. mekongi*. Estimates suggest that 85% of all schistosomiasis cases are now in sub-Saharan Africa (Chitsulo *et al.*, 2000). Schistosomiasis is still endemic, but at very low levels, in North Africa and several countries of the Middle East. In Asia, schistosomiasis is also endemic along parts of the Yangtze River Basin in China, in the middle reaches of the Mekong River in Lao People's Democratic Republic, Cambodia, and Thailand, and on several islands of the Philippines. It is also endemic in several eastern states of Brazil, and in several foci in Venezuela and Suriname.

Mortality due to schistosomiasis estimated at 11,000 deaths per year and the burden of disease at 1.7 million DALYs lost per year (WHO, 1997). These calculations did not consider late sequelae and indirect morbidity/mortality due to schistosomiasis infection such as liver disease, portal hypertension, hematemesis, hydronephrosis, non-functioning kidney, cervical and squamous cell bladder carcinoma. The global distributions of schistosomiasis are shown in Figures 2 and 3.

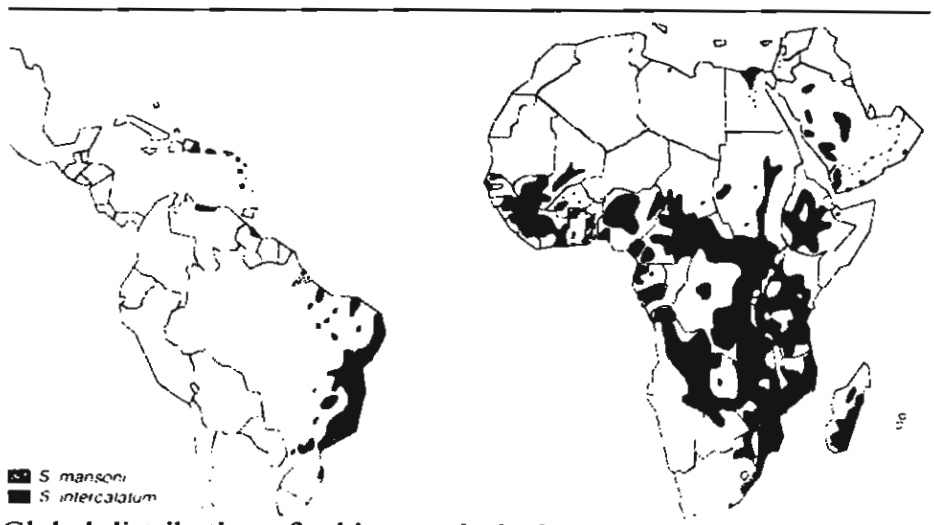


Figure 2. Global distribution of schistosomiasis due to *S. mansoni* and *S. intercalatum*, in 1993 (WHO, 1997)

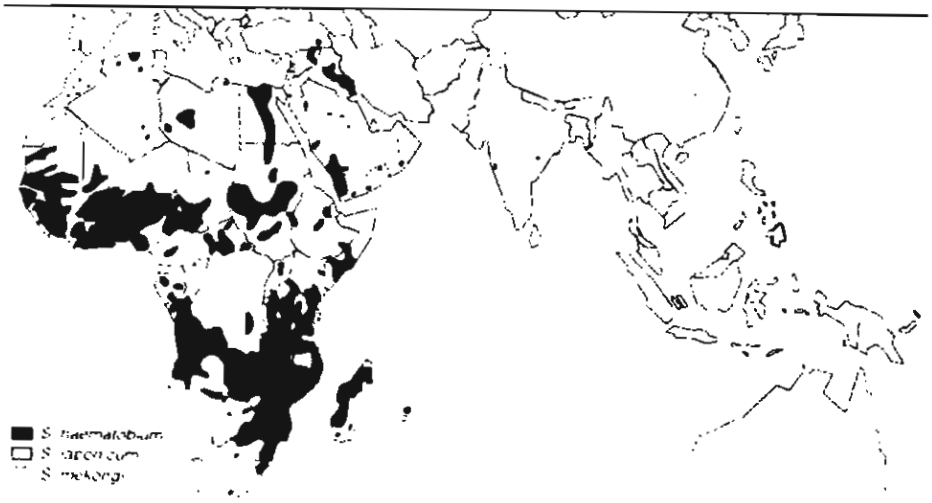


Figure 3. Global distribution of schistosomiasis due to *S. haematobium*, *S. japonicum* and *S. mekongi*, in 1993 (WHO, 1997)

Mortality/Morbidity:

Acute schistosomiasis: The acute stage, often known as Katayama fever, is normally found in young children or young adults with no previous exposure to the disease, and is particularly prevalent in individuals with *S. japonicum* infections. The acute reaction is in response to the sudden high level of antigen exposure and is usually associated with the onset of egg deposition. Clinical symptoms consists of skin rashes, asthma-like episodes, daily fever, malaise, diarrhea, swollen lymph nodes and aching joints and a number of other non-specific symptoms.

Chronic schistosomiasis: The patient experiences diarrhea and fevers. In children the infection can depress their growth rate. The infection also leads to enlargement of the liver and spleen. Fibrosis of the liver can result in portal hypertension, hepatosplenomegaly, ascites formation, esophageal varices leading to fatal hematemesis.

Gastrointestinal schistosomiasis: The most common complication is periportal fibrosis, also termed Symmers clay pipestem fibrosis. This leads to portal hypertension and gastrointestinal hemorrhage. Liver failure is uncommon unless concomitant chronic hepatitis or cirrhosis is present.

Urinary tract schistosomiasis: This can lead to renal failure due to obstructive uropathy, pyelonephritis, or bladder carcinoma, occurring usually 10-20 years after the initial infection.

Cerebral schistosomiasis: Most cases of cerebral schistosomiasis are observed with *S. japonicum*, constituting 2-4% of all *S. japonicum* infections. However, cerebral schistosomiasis also can occur with other species. Spinal schistosomiasis usually presents as transverse myelitis and primarily is due to *S. mansoni* infection.

Taxonomy:

Phylum Platyhelminthes

Sub Phylum Euplatyhelminthes

Super Class Rhabditophora

Class Trematoda

Sub Class Digenea

Super order Anepitheliocystida

Order Strigeatida

Suber Family Schistosomatoidea

Family Schistosomatidae

Subfamily Schistosomatinae

Genus Schistosoma

Species Schistosoma mekongi

Life cycle and transmission:

The worms also are called blood flukes because they live in the vascular system of humans and other vertebrates. *S. haematobium* lives in the venous plexus near the urinary bladder and ureters, *S. mansoni* lives in the inferior mesenteric vein, and *S. japonicum* lives in the superior mesenteric vein of both the large and small intestines.

The life cycle involves a sexual stage in the human and an asexual stage in the fresh water snail host. The adult worms are small, 12-26mm long and 0.3-0.6mm wide, and vary with the different species. Adult worms mate and lay eggs. The eggs are non-operculate, possess a spine, and contain a miracidium. The microscopic appearance of the egg allows diagnostic differentiation of the 5 species. An adult *S. haematobium* produces 20-200 round, terminally spined eggs per day; *S. mansoni* produces 100-300

ovoid, laterally spined eggs per day; and *S. japonicum* produces 500-3500 round, small, laterally spined eggs per day. The eggs of *S. intercalatum* have prominent terminal spines, and *S. mekongi* have small lateral spines.

On reaching water, the eggs excreted by an infected person hatch to release a tiny parasite (miracidium) that swims actively through the water by means of fine hairs (cilia) covering its body. The miracidium survives for about 8 - 12 hours, during which time it must find and penetrate the soft body of a suitable freshwater snail in order to develop further. Once inside the snail, the miracidium reproduces many times asexually until thousands of new forms (cercariae) break out of the snail into the water. Depending on the species of snail and parasite, and on environmental conditions, this phase of development may take 3 weeks in hot areas, and 4 - 7 weeks or longer elsewhere. The fork-tailed cercariae can live for up to 48 hours outside the snail. Within that time they must penetrate the skin of a human being in order to continue their life cycle. As the cercaria penetrates the skin, it loses its tail. Within 48 hours it penetrates the skin completely to reach the blood vessels. The illustration of Schistosome's life cycle is shown in Figure 4 (CDC, 2003).

This process sometimes causes itching, but most people do not notice it. Within seven weeks the young parasite matures into an adult male or female worm. Eggs are produced only by mated females. Male and female adult worms remain joined together for life, a period of less than five years on average but 20 years has been recorded. The more slender female is held permanently in a groove in the front of the male's body. Once eggs are produced, the cycle starts again. In intestinal schistosomiasis the worms attach themselves to the blood vessels that line the intestines; in urinary schistosomiasis, they live in the blood vessels of the bladder. Only about half of the eggs leave the body

in the feces or urine; the rest remain embedded in the body where they cause damage to organs.

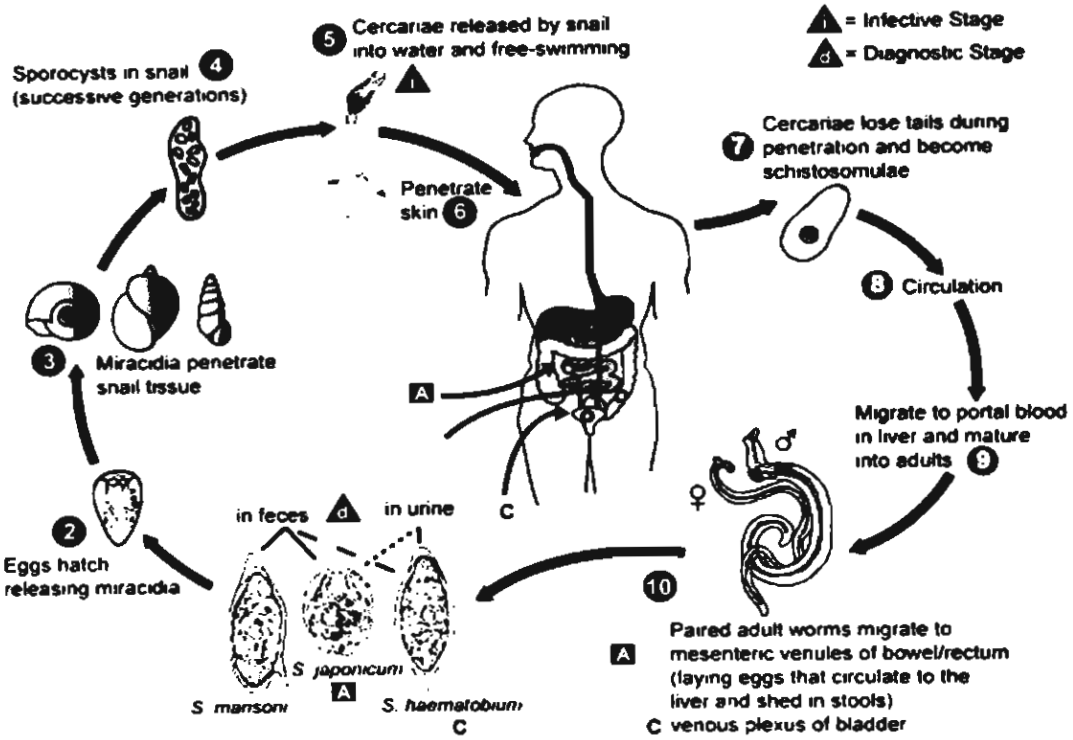


Figure 4. The illustration of Schistosome's life cycle (CDC, 2003)

Eggs:

The eggs of *S. mansoni* and *S. haematobium* are of the same size; aproximately 140 x 60μm. Their similarity however, ends there; *S. mansoni* eggs are laid singly by the female, they have a lateral spine in addition to their surface being covered in very small spines of about 0.3μm long. *S. haematobium* eggs have terminal spines and are laid gregariously. *S. japonicum* eggs have a very reduced terminal spine in a small depression of the shell and are rounder and smaller in shape (~80 x 63μm) but do share with *S. mansoni* the very small spines covering the surface. Of the more minor species *S. mekongi* eggs are similar to those of *S. japonicum*, whereas *S. interculatum* share the

terminal spine characteristic of *S. haematobium*. The schistosome egg, as opposed to related digenean parasites, is non-operculate and have terminal or lateral spines. The shells are sclero-proteic with an inside lining of vitelline membrane adhering to the shell via vacuoles that press on the developing larva. The eggs of *S. mansoni* enter the environment via the feces of their definitive host; man. To do this they must pass through the mesentery and intestinal walls to arrive in the lumen of the gut. This is most likely achieved through a number of interacting factors: the eggs, with the help of their spine, anchor themselves into the tissue and the delayed hypersensitivity reaction that occurs has an inflammatory effect on the host tissue. This results in ulceration at the affected site and the egg ulcerates into the intestinal lumen allowing it to enter the environment via the feces. The action on the egg spine by host blood pressure and peristalsis of the gut also aids this passage. The miracidium itself has been shown to release proteolytic enzymes and their digestion of host tissue will also play a part. There is about a six day period from the eggs being laid to the time they are fully embryonated and leave the host. On entering the water in the feces the eggs hatch according to three principle factors: temperature (25-30°C), light, and osmotic pressure. The increase in osmotic pressure inside the egg as water enters the two or more vacuoles that lie under the vitelline membrane, cause them to swell, and the activation of the leucine amino peptidase enzyme, splits the egg laterally so releasing a highly motile (2mm/sec) miracidium larva. Their eggs are shown in Figure 5.

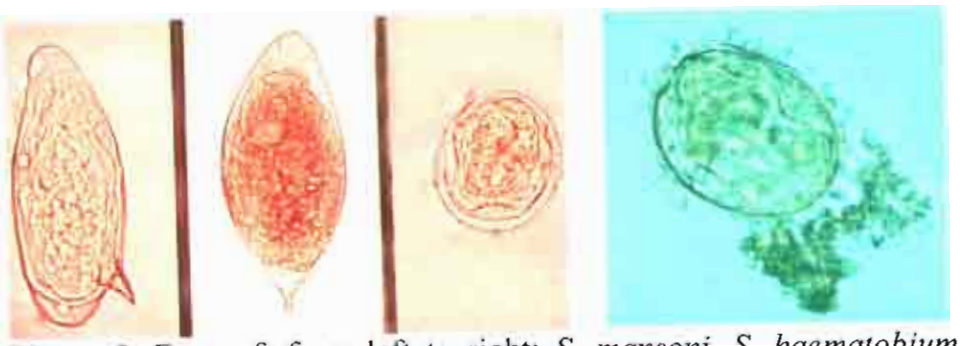


Figure 5. Eggs of, from left to right; *S. mansoni*, *S. haematobium*, *S. japonicum* and *S. mekongi* (http://www.med-chem.com/Para/Prob%20of%20Month/Prob%20of%20Month%2026%20Feb%2003.htm)

Miracidia:

A miracidium is about 150-180μm in length by 70-80μm wide with its outer four epidermal layers covered cilia enabling its movement through water. It has no gut and is unable to feed until it penetrates an intermediate snail host, its energy requirements up to this point are met by glycogen stores. Its excretory system comprises of posterior and anterior flame cells. The apex of the parasite consists of gland cells, the trebratorium - a number of membranous folds, and ciliated sensory organelles, all of which aid in the penetration of the snail. It also has a nervous system involving a number of sensory organs allowing it to respond to its environment and so show its host finding behavior. The miracidium is sex determined; it is either male or female and all resulting cercariae and subsequent adults will be either all male or all female. It is infective for 4-6 hours, after which its glycogen stores are depleted, and in that time must find an intermediate snail host, which for *S. mansoni* is the *Biomphalaria spp*, for *S. haematobium* is the *Bulinus spp*, and for *S. japonicum* is *Oncomelania spp*. To increase the chance of the miracidia finding the host it becomes negatively geotrophic - swimming against gravity, and positively phototrophic - swimming towards the light,

placing it in the environment of the snail. Chemical attractants produced by the snail including mucus, long chain fatty acids and amino acids attract the miracidia until it makes contact with the snail. After contact the miracidia shows exploratory behaviour and searches for the prefered site of penetration. Penetration through the foot is the most common with 70% of miracidia appearing to do so. Penetration is achieved via mechanical motion of the apical papillae and histolytic secretions from the lateral glands. After penetration the cilia are lost as are the muscle layers and it becomes a primary sporocyst, the location of which depends on the species. The miracidium of *S. mansoni* is shown in Figure 6.

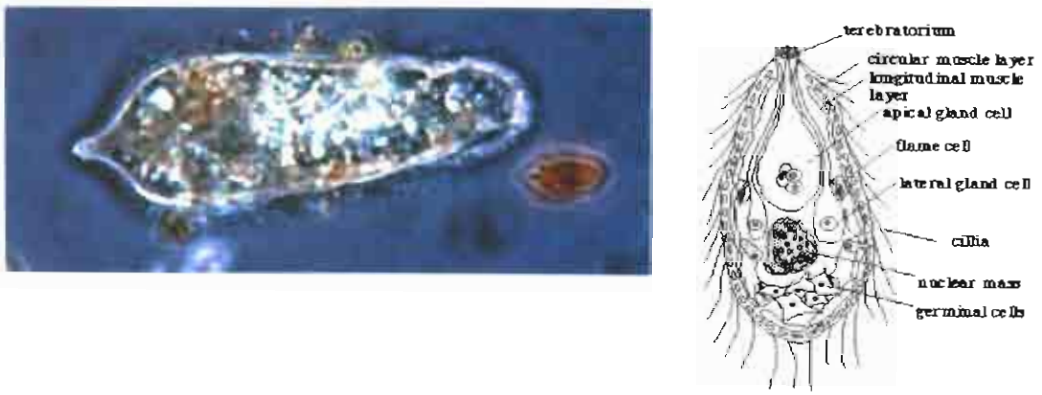


Figure 6. Miracidium of *S. mansoni*

(http://www.path.cam.ac.uk/~schisto/SchistoLife/Miracidium.html)

Sporocyst:

Within the molluscan host, the primary sporocyst develops as a hollow, fluid filled germinal sac with a birth pore at its conical anterior from which subsequent generations emerge. The daughter sporocyst starts inside the mother as a single cell, totipotent embryo that develops into a germ ball. This germ ball becomes the secondary sporocyst, 35-600 of which can be produced after 3 weeks of this asexual budding process. The secondary sporocysts migrate through the host tissues finally coming to

rest near the digestive diverticulatum and producing the mammal infective stage; the cercaria. Nutrients at the sporocyst stage are obtained by passage of soluble material across the tegument. The mean output of cercariae from an infected snail has been estimated at about 1500 every day lasting for up to about 18 days. The time from penetration of the snail by the miracidia to the release of cercariae is about 4 weeks. They are released depending on the time of day their host is most abundant. The sporocyst is not seen to cause any pathology in its host but does induce reproductive and metabolic changes, measures taken in order to preserve the energy of the developing sporocyst. The growth and development of the sexual organs are reduced, inciting a reduction in reproductive potential. The parasitised snail's metabolic rate is increased causing it to eat more but an infected snail has been found to be smaller and slower than an uninfected snail. To avoid being attacked by the host's immune system the parasite has evolved a number of ways; host mimicry, acquisition of host components allowing the sporocyst to been seen as 'self' by the snail immune system, and direct interference with the immune response through chemical secretions. The sporocyst is shown in Figure 7.

Figure 7. The sporocyst of *Schistosome spp*

(http://www.cvm.okstate.edu/~users/jcfox/ htdocs/Disk1/Images/Img0087d.jpg)

Intermediate snail host:

The snail host for *S. mansoni* is of the *Biomphalaria spp,* but the specific species involved differs in various countries. In the western hemisphere, the main host is *B. glabrata.* In the west countries of Africa, the host is *B. pfeifferi.* It is *B. sudanica* in Cameroon and the Congo. Others include *B. alexandrina* in Egypt and *B. rueppellii* in the Sudan and Tanzania. The intermediate snail species for *S. haematobium* are of the *Bulinus spp. B. truncatus* is the main host of infection in northern Africa, the East and the Mediterranean. Other important species are *B. angolensis, B. guernei, B. natalensis, B. tropicus* and *B. rohlfsi.* The *S. japonicum* snail species are of the genus *Oncomelania,* and unlike other intermediate snail hosts the males and females are separate and amphibious. The species that transmit infection are *O. nosophora* in Japan, *O. quadrasi* in the Philippines, *O. formosana* in Taiwan and *O. hupensis* in China. Inside the soft tissue of the snail it takes the miracidium 5-7 weeks to develop into the bifurcate cercariae via the two stages of sporocysts. The snail host for *S. mekongi* is *Neotricula aperta* (*Tricula aperta*), consisting of three strains (alpha, beta and gamma). Both the alpha and gamma strains are commonly found in the main stream of the Mekong from Khong Island to Kratie province (Yasuraoka *et al.*, 1994). However, the beta strain is found only in the Mun River, a tributary of the Mekong River in Northeast Thailand. Although all the three strains show varied degree of capability of transmitting *S. mekongi,* the gamma strain alone is known to be naturally infected and of epidemiological significance (Kitikoon *et al.*, 1973). A seasonal cycle is observed, consisting of a period of transmission in the dry season, from February and April in Cambodia, and from March to June in Lao People's Democratic Republic and Thailand. The intermediate snail hosts are shown in Figure 8.

Figure 8. Intermediate snail host of, from left to right; *S. mansoni, S. haematobium, S. japonicum* and *S. mekongi*

(http://www.path.cam.ac.uk/~schisto/SchistoLife/Host.Snails.html)

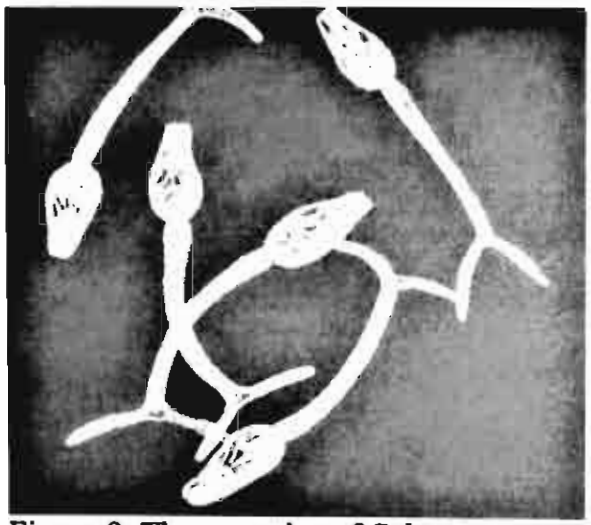
Cercariae:

Cercariae are very active and survive on glycogen stores that decline exponentially with time after release. Glycogen reserves can vary greatly and may depend on the health of the snail, affected by both nutritional state and level of infection. Once released, the cercariae go in search of their definitive host through bursts of swimming towards the water surface. They, like the miracidia show behaviour that gets them closer to their host: they are negatively geotrophic, positively phototrophic, shadows cause activity and when close the host's chemicals cause the cercariae to swim continuously and frequently change direction, this is known as klinokinetic behaviour. Once a suitable host has been located the cercaria must penetrate its skin, this occurs in three phases:

1. Attachment
2. Exploratory behavior involving creeping over the skin. These two behaviors are stimulated by chemical and thermal triggers.
3. Epidermal penetration, in response to chemical stimuli from the skin, involving physical and secretory mechanisms, can take only a few minutes to complete the initial phase. Subsequent structural and physiological

changes transform this free-living infective stage into a parasitic schistosomulum larva.

The pattern of cercarial shedding from the snail host has been found to be dependent on the behavior of the primary host. A study in Guadeloupe island where there was both human and sylvatic primary parasitic hosts, found three different shedding patterns in three different regions. Early morning shedding was seen in the urban area where humans were the focus of infection. The remote country area, where animals were the hosts, shedding occurred in late afternoon. An intermediate pattern of shedding was found in a rural community where the rodents and humans were parasitized. The cercariae are shown in Figure 9.



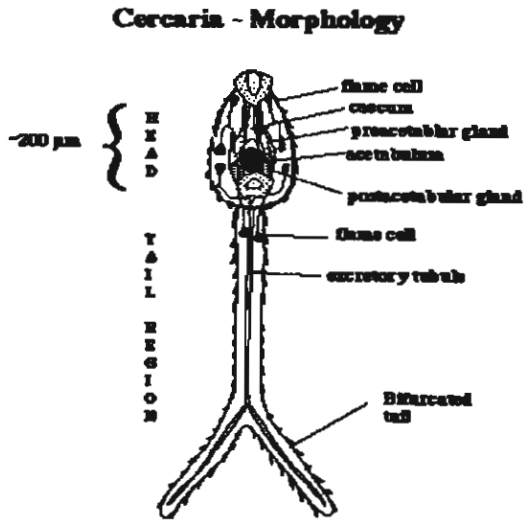


Figure 9. The cercariae of *Schistosome spp.*

(http://www.path.cam.ac.uk/~schisto/SchistoLife/Cercaria.html)

Schistosomula:

For the cercariae to become schistosomula a number of structural and biochemical changes must occur.

The structural changes: The tegument, bound by a trilaminate plasma membrane that has a 1-2μm thick glycocalyx, is mostly shed along with the tail at penetration. The various penetration glands empty. Tegumental cell bodies lying beneath the muscle layer release membranous vesicles that move into tegumental cytoplasm and replace the trilaminate membrane with a multilaminate tegumental surface. Esophageal glands discharge their contents into the esophageal lumen allowing feeding to start. These structural changes require the physiological triggers of elevated temperatures and iso-osmotic conditions. The loss of the glycocalyx, thought to control the surface permeability in water, coincides with osmotic sensitivity.

The biochemical changes: A switch from an aerobic glycogen based metabolism to a principally anaerobic one causing increased lactate production, over the first 24 hours. Also during this time, and up to 48 hours, there is an extraordinary turnover of surface molecules, which mask antigenic epitopes causing the schistosomulum to be 'unseen' by the host and unaffected by antibody mediated immune cell cytotoxicity. As well as making its own 'masking molecules' it can coat itself with host molecules, absorbing erythrocyte surface glycolipids onto its own surface, hiding it from the host's defense system.

Migration through the body occurs in three phases:

1. Penetration of the skin and the skin migratory phase

This involves initial attachment to the skin by the cercaria and its subsequent creeping over it in search of a suitable penetration site, often a hair follicle, resulting in penetration into the epidermis using proteolytic secretions from the pre- and post-acetabular glands, and transformation into the schistosomulum. It takes less than 30 min

to enter the initial epidermal skin layer where they reach the dermis and rest for about 40 hours. Once through the dermis it takes 10 hours locate a venule (or more rarely a lymphatic vessel) and a further 8 hours to penetrate its wall, when it is then carried through the right side of the heart to the first capillary bed in the lungs.

2. Lung stage schistosomulum

In the lungs, over the next 3-8 days, the surface area of the schistosomulum greatly increases, but not its mass, as it becomes long and slender. At this point the parasite is feeding on plasma but not the blood cells. To further develop the schisosomulum migrates to the liver directly through the diaphragm, or via the circulatory system, through the left side of the heart to the hepatic portal vein

3. Liver stage schistosomulum and immature adult

Now in the liver the parasite grows properly, development of the gut is completed along with maturation of the gonads. The sexually mature adults pair up and migrate, in the case of *S. mansoni,* back along the hepatic portal vein, against the blood flow, to the mesenteries around the intestine, where egg laying begins. The time taken from infection of cercaria to the commencement of egg laying is about 25-35 days.

Adults:

When the worms have matured, the pair migrates out of the liver to the blood vessels their particular species inhabit, and begin egg laying. *S. mansoni* are most often found in the small inferior mesenteric blood vessels, which surround the large intestine and caecal region, while *S. japonicum* and *S. mekongi* reside in the superior mesenteries associated with the ileo-caecal area, and *S. haematobium* inhabit the pelvic blood vessels of the vesicle plexus around the bladder.

The adult worms remain in permanent copula with the female lying inside the male's ventral groove, called the gynacophoric canal, laying eggs and receiving nutrients trans-membranously from the male. This is very important for if the female does not mate it fails to mature properly and remains stunted. They utilize hemoglobin as the main source of amino acids. It is ingested into their blind, bifurcated gut where a hemoglobinase enzyme digests the globin portion and detoxifies the heme into a pigment before regurgitating it back into the blood stream. Vitelline glands lie along the lateral margins of the body and provide material for the production of the egg shell.

The females are generally longer and thinner than the males: *S. mansoni* is ~1.1cm by 0.2mm, *S. haematobium* 10-15mm by 1mm and *S. japonicum* 2cm by 0.4mm. The females have a ventral sucker near to the genital pore and an oral sucker by the esophageal gland, which is much less developed than in the males. The caecal-junction position in the female corresponds to the male of the same species but the number of eggs produce do not relate to the number of testes.

The adult male worms' outer layer is called a tegument and varies among the species: *S. mansoni* tegument is covered by finger-like projections called papillae, *S. haematobium* have shorter, sparcer projections called tubercles, and *S. japonicum* and *S. mekongi* have neither, so sport smooth teguments. Males also have a ventral and oral sucker with which they maintain position and feed in the blood vessel. *S. mansoni* and *S. haematobium* are similar in size measuring ~1cm by 1mm, whereas *S. japonicum* is longer and thinner at ~1.2-2.8cm by 0.5mm. Reproductive and digestive tracts differ also: *S. mansoni* has 8-9 testes around a short vas deferens and a caecal-junction in the anterior half of the body, *S. haematobium* has 4-5 testes and a posterior caecal-junction,

and *S. japonicum* has 6-8 testes and the caecae join in the middle of the body. The adult male and female are shown in Figure 10 and 11.

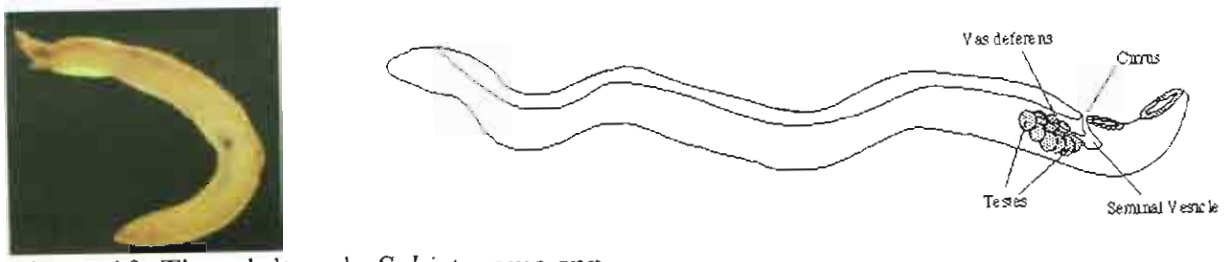


Figure 10. The adult male *Schistosome spp.*

(http://www.path.cam.ac.uk/~schisto/SchistoLife/Adult.worm.html)

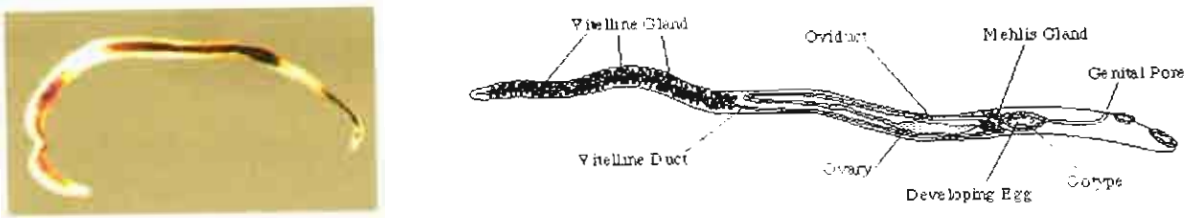


Figure 11. The adult female *Schistosome spp.*

(http://www.path.cam.ac.uk/~schisto/SchistoLife/Adult.worm.html)

Pathophysiology:

The pathophysiology of infection correlates with the life cycle of the parasite as follows:

Cercariae: Skin penetration of cercariae produces an allergic dermatitis at the site of entry. With prior sensitization, a pruritic papular rash occurs. This also is observed with nonhuman avian schistosomes.

Schistosomula: These are tailless cercariae that are transported through blood or lymphatics to the right side of the heart and lungs. Heavy infection can cause symptoms such as cough and fever. Eosinophilia may be observed.

Adult worm: They do not multiply inside the human body. In the venous blood, adult male and female worms mate, and the female lays eggs 4-6 weeks after cercarial

penetration. Adult worms rarely are pathogenic. The female adult worm lives for approximately 3-8 years and lays eggs throughout her life span.

Eggs: They cause Katayama fever and schistosomiasis.

Katayama fever: The exact pathophysiology is not known. It occurs 4-6 weeks after infection, at the time of the initial egg release. It is reported most commonly with *S. japonicum* but also has been reported with *S. mansoni*. Katayama fever is believed to be due to the high worm and egg antigen stimuli that result from immune complex formation and lead to a serum sickness-like illness. This syndrome is not due to granuloma formation.

Schistosomiasis: It is due to immunological reactions to *Schistosoma* eggs trapped in tissues. Antigens released from the egg stimulate a granulomatous reaction comprised of T cells, macrophages, and eosinophils that results in clinical disease. Symptoms and signs depend on the number and location of eggs trapped in the tissues. Initially, the inflammatory reaction is readily reversible. In the latter stages of the disease, the pathology is associated with collagen deposition and fibrosis resulting in organ damage that may be only partially reversible.

Schistosomiasis mekongi:

The first case of *S. mekongi* infection was reported as "*S. japonicum*" infection in a Laotian immigrant who lived along the middle Mekong River in Lao People's Democratic Republic in 1957 (Dupont *et al.*, 1957). Since then, some human cases have been reported from Thailand in 1959 (Chiyaporn *et al.*, 1959) and from Kratie province, Cambodia in 1968 (Auteband *et al.*, 1968). Heavily endemic areas were found in Khong Island, Lao People's Democratic Republic, and Kratie province, Cambodia. Schistosomiasis mekongi was, thus, described as a public health implication in the

middle Mekong River Basin, with population of more than 150,000. The map of Mekong River Basin is shown in Figure 12. During the period of civil war and revolution, clinical and epidemiological surveys were performed among refugees from Lao People's Democratic Republic or Cambodia. In 1983, 62 out of 24,619 Cambodian refugees were diagnosed as Schistosomiasis mekongi by stool examination. Some of the egg positive ones were the refugees from Battambang province, Cambodia or from Northern Lao People's Democratic Republic, where transmission of schistosomiasis had not been reported, suggesting the presence of new endemic foci in Cambodia and Lao People's Democratic Republic. There are habitats of the intermediate snail host in Northeastern Thailand, and schistosomiasis might become endemic in Thailand by infected immigrants (Ohmae *et al.*, 2004).

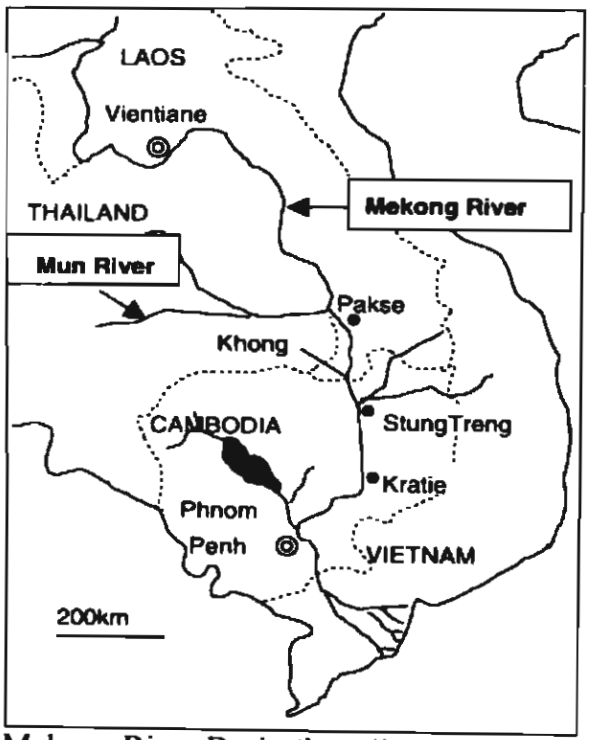


Figure 12. The map of Mekong River Basin (http://www.mekongexpress.com/)

Treatment:

Treatment of schistosomiasis is targeted to several aspects of the life cycle. The 1980's showed development of effective anti-helminthic chemotherapies for treating infected people and animals. Although treatment is highly effective and relatively uncomplicated, it does not lead to a completely immune state, so individuals living in highly endemic areas run a high risk of re-infection. The chemotherapy of choices are praziquantel and artesunate.

Praziquantel:

Praziquantel is a drug that has been used for schistosomiasis in 1975. It has high effecticacy against all schistosome species; lack of serious short- and long-term side effects; administration as a single oral dose; and competitive cost. It is a member of the heterocyclic pyrazino-isoquinoline system and its chemical structure is 2-cyclohexylcarbonyl-1, 2, 3, 6, 7, 11 b-hexahydro-4H-pyrazino (2, 1-a) isoquinolin-4-one as shown in Figure 13. It is a colorless, odorless, bitter-testing crystalline power which melts at between 136 and 139°C. The active substance is hydroscopic and soluble in chloroform and dimethylsulfoxide, but insoluble in water and only soluble with difficulty in alcohol.

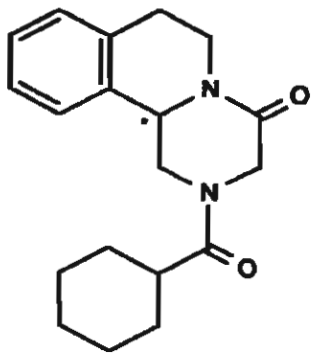


Figure 13. The chemical structure of praziquantel (http://www.infektionsnetz.at/test/bilder/strukturformeln/antiparasit/praziquantel.gif)

Praziquantel is rapidly absorbed from the intestinal tract, and its metabolites are excreted in the urine, mainly as glucuronide and sulphate conjugates. It is also rapidly taken up by all susceptible parasites *in vitro* but not metabolized, which proves that the compound itself is effective. The effects of praziquantel on schistosomes are generally grouped under four heading: (1) tegumental damage; (2) muscular contraction; (3) metabolic alteration; and (4) exposure of schistosome antigens at the parasite surface. All of the effects of praziquantel could be attributed either directly or indirectly to a single cause: an alteration of intracellular Ca^{2+} homeostasis at one or more sites in the worm. An alternative possibility is that praziquantel may directly interact with membrane phospholopids and alter the permeability properties of bilayer structures.

Artesunate:

The novel antimalarials - artemether and artesunate are derivatives of artemisinin from the plant *Artemisia annua*. Ching-hao refers to the tops of *A. annua* (the species name refers to its growth as an annual). The Chinese name (pinyin: qinghao) makes reference to the color green (qing); the dark green leaves at the top of the plant are the most active portion, which might account for this designation. The latter part of the herb's name, hao, indicates this type of plant, one characteristic being a tall stalk; the term is used to depict several other Artemisia species as well. Ching-hao is a common weed in southern China; it is also distributed in temperate regions across the globe, including in Europe and North America. There are numerous medicinal Artemisia species used worldwide, several of them having a reputation for dispelling worms, and hence the group is commonly called "wormwoods." Ching-hao is sometimes referred to by the English common name "sweet wormwood" the aroma is sweet. It is in the Asteraceae family, and has yellow flowers

A. annua is a vigorous weedy annual, a short day plant with a critical photoperiod of 13.5hr. The plant is usually single-stemmed reaching about 2m in height with alternate branches and alternate, deeply dissected, aromatic leaves ranging from 2.5 to 5.0cm in length. Tiny yellow nodding flowers (capitula) only 2 or 3mm across are displayed in lose panicles containing numerous, greenish or yellowish, bisexual central (disc) florets containing little nectar and pistillate marginal (ray) florets (Figure 14). The involucre is imbricated with several rows of bracts. The central flowers are perfect and can be either fertile or sterile. Ovaries are inferior and unilocular and each generates one achene, ca. 1mm in length and faintly nerved. The pistillate marginal florets in the capitulum produce numerous achenes without pappus. The pollen is tricolpate and smooth, typical of anemophilous species, and has vestigial or no spines. It has an internal, complex, columellae-tecta configuration in the exine, which is common to all taxa of the tribe Anthemideae and varies from two to three layers in *A. annua*. The plant is naturally cross-pollinated by insects and wind action, which is unusual in the Asteraceae.

Figure 14. The illustration of *A. annua* (http://www.itmonline.org/image/ching1.jpg)

Dihydroartemisinin is the product of the first step of chemical synthesis starting with artemisinin. Artesunate and artemether derive from futher synthesis steps and are rapidly converted *in vivo* back to dihydroartemisinin (Figure 15).

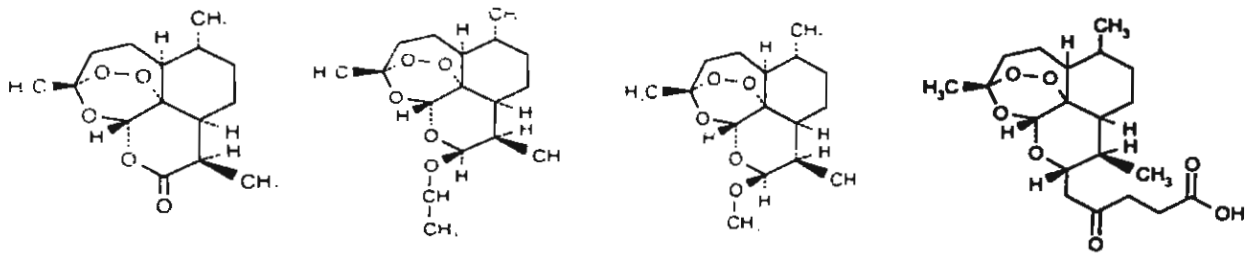


Figure 15. The chemical structure of, from left to right; artemisinin and derivaties: arteether, artemether, artesunate (http://www.artesunate.com/)

CHAPTER II

OBJECTIVES

The objectives of this study

1. To investigate the pathogenesis of *Schistosoma mekongi* infection in mice in the term of :
    1. Hematological studies
    2. Biochemical studies
    3. Light microscope studies
2. To investigate the effect of praziquantel and artesunate on tegument of *Schistosoma mekongi* in vivo
3. To investigate the effect of praziquantel and artesunate on tegument of *Schistosoma mekongi* in vitro

CHAPTER III

LITERATURE REVIEW

The need for alternatives to human biomedical studies has increased markedly during the last decades as a result of increasing ethical standards required in such studies. One alternative is to use experimental animal models, which have the advantage of allowing hypothesis testing under strictly controlled condition. According to the definition of Held (1983), an animal model is a living organism in which normative biology or behavior can be studied, or in which a spontaneous or induced pathological process can be the same phenomenon in humans or in other species of animals.

Like in humans, *S. japonicum* infection in experimental animals reported by Johansen and colleague (2000) established mainly in the large intestinal veins, with high fecal egg counts during the acute phase of infection, which varies greatly within and between days. Clinical signs as eosinophilia and diarrhea with mucus and blood, hepatomegaly, increased portal diameter, periportal fibrosis and ascites.

Clinical studies have demonstrated that the patients with hepatosplenic schistosomiasis are prone to develop complex hemostatic abnormalities that may be linked to the potential risk of bleeding from ruptured esophageal varices in these patients. The deficit in hemostatic parameters is more pronounced with the advancement of the disease and is maximal in the patients with experience of hematomesis. Evidences of enhanced generation of thrombin and plasmin indicate the presence of low-grade disseminated intravascular coagulation in advanced hepatosplenic schistosomiasis, which is considered as a principal cause of hemostatic abnormalities in this endemic disease (Tanabe, 2003).

In schistosomiasis, granuloma formation to parasite eggs signals the beginning of a chronic and potentially life-threatening disease. Hepatic granulomata were experimentally induced in mice, rats and hamsters by injecting viable exogenous eggs of *S. mansoni* via the mesenterico-portal system. Histopathological studies of livers of these animals showed that the lesions were similar to those in infections resulting from exposure to cercariae as occurs naturally in Mansonian schistosomiasis. Granulomatous response to schistosome eggs began in the livers of rats as early as 24h following intravenous injection of eggs. The mean sizes of hepatic granulomata were smallest in rats, intermediate in hamsters and largest in mice (100.7, 276.4 and 361.6μm, respectively). Comparable observations made of the lungs of animals that had received egg injections via their tail veins, showed striking differences with respect to timing of the occurrence of various histopathologic stages, mean size of granulomata, cellular composition and pathologic manifestations (Edungbola and Schiller, 1979).

Rheinberg and colleague studied the development of five schistosome species in mice by the recovery of schistosomula from chopped lung tissue and of adult worms by portal perfusion (Rheinberg *et al.*, 1998). Three development patterns appeared. (1) *S. japonicum* was unique in showing an early establishment of schistosomula in and a rapid departure from the lungs together with the highest worm recovery; (2) *S. haematobium* contrasted by establishing later and persisting in the lungs for at least 2 weeks; and (3) *S. intercalatum*, *S. mansoni*, and *S. rodhaini* had an intermediate pattern- they resided in the lungs for several days, then disappeared and produced intermediate numbers of adults. Lung petechiae, known to accompany the migration of *S. japonicum*, were never detected after infection with the other species. They speculate

that the three migration patterns of schistosomes are related to the size of the relative spectra of naturally infected definitive hosts.

Xiao and colleague studied the effect of artemether on *S. mansoni* infection in mice (Xiao *et al.*, 2000a; 2002a), *S. haematobium* infection in hamsters (Xiao *et al.*, 2000b; 2001a), *S. japonicum* infection in mice (Xiao *et al.*, 2002b). Artemether has been shown to be active against the juvenile stages of *Schistosoma spp.* in experimentally infected animals, while it is less effective on adult worms. The tegument has been identified as a key target of this drug. They performed a transmission electron microscope examination to assess the pattern and extent of ultrastructural alterations. Tegumental alterations were characterized by swelling, fusion of distal cytoplasma, focal lysis of the tegumental matrix and vacuolisation. Tubercles and sensory organelles frequently degenerated or collapsed. Typical features of subtegumental alterations, including muscle fibres, syncytium and parenchyma tissues, were focal or extensive lysis, vacuolisation and degeneration of mitochondria. Severe alterations were also observed in gut epithelial cells and vitelline cells of female worms.

Xiao and colleague studied the effect of praziquantel on *S. mansoni* infection in mice (Xiao *et al.*, 2000c). Praziquantel has been shown to be active against the adult worms. They compared effects of racemic praziquantel and its enantiomers, levo-praziquantel and dextro-praziquantel. The study thus clearly showed that levo-praziquantel was more active than dextro-praziquantel in causing tegumental damage.

Xiao and colleague studied the effect of praziquantel combine with artemether on *S. japonicum* infection in rabbits (Xiao *et al.*, 2000d). The results demonstrated that when rabbits infected with 7-14 day old schistosomules and 42 day old adult schistosomes were treated with praziquantel in combination with artermether, the

effects of the individual drugs could be increased significantly reduction of the mean total worm burden and female worm burden than was found for the groups treated with praziquantel or artemether alone.

Utzinger and colleague carried out experiments with mice, infected with juvenile or adult *S. mansoni*, and treated with artemether or artesunate at various doses (Utzinger *et al.*, 2002). Artemether administered to mice with juvenile and adult *S. mansoni* resulted in worm reductions of 88-97% and 46-51%, respectively, which was significantly higher than the 67-77% and 24-33%, respectively, obtained with artesunate. They conclude that artemether and artesunate are efficacious antischistosomal agents, with artemether displaying consistently higher activities.

Xiao and colleague conducted experiments *in vitro* to assess the effected of artemether in combination with hemin on adult *S. japonicum*, *S. mansoni* and *S. haematobium* (Xiao *et al.*, 2001b). They reported when schistosomes were maintained in a medium containing artemether at concentrations of 20μg/mL or less for 72h, no apparent effect on the schistosomes was seen. When the medium contained 50 or 100μg/mL hemin as well as artemether, the schistosomes showed decreased motor activity 2-24h after exposure, which was followed by the staining of the whole worm body a reddish-yellow color, dilatation of the intestine, and extensive vesiculation of the tegument. Some of the schistosomes died 24h after exposure, and almost all died within 48-72h. When schistosomes were exposed to the same concentrations of hemin alone, they were stained a light yellow color but there was no apparent effect on their survival. Their findings suggest that artemether interacts with hemin to exert a toxic effect on the worms, which might be of importance in the further elucidation of the mechanism of action of artemether on schistosomes.

CHAPTER IV

MATERIALS AND METHODS

1. Animals and drugs

1.1 Snail:

The freshwater snails, *N. aperta*, were collected at the Mekong River in Ubon Rajthani province, Thailand. They were maintained for the life cycle of *S. mekongi* in the Malacology Unit, Department of Biology, Faculty of Science, Mahidol University, Bangkok, Thailand (Figure 16).

Figure 16. The snails, *N. aperta*, were maintained in the Malacology Unit

1.2 Mice:

ICR male mice were bred at the National Laboratory Animal Center, Nakhon Pathom, Thailand. Mice, 6 weeks old, weighing 20-30g, were used in this experiment. The animals were housed in the shoe-box type cages (18x30x13 cm) containing sterile wood shaving bedding in a strictly hygienic conventional animal room at the Faculty of Science, Mahidol University bedding (Figure 17). Standard diet (Perfect companion Co. Ltd., Bangkok, Thailand) and tap water were available *ad libitum*. Room temperature was kept at 22-25°C with relative humidity of 60-70% and a 12:12 hour light-dark cycle

was maintained throughout. All experimental animals used in this study were approved following Guidelines for the Care and Use of Laboratory Animals, Mahidol University, authorized by the Animal Care and Use Committee, Faculty of Science, Mahidol University.

Figure 17. The mice were housed in the animal room

1.3 Drugs:

The drugs praziquantel (lot no. 000491) and artesunate (lot no. 040105) were the products of Atlantic Laboratories Corporation, Bangkok, Thailand. For intragastric administration, both drugs were suspended in 7% Tween-80 and 3% ethanol and had a final drug concentration of 30g/L. The volume of drug suspension given to mice was 10mL/kg.

2. Experimental design

Part I: Pathogenesis of *S. mekongi* infection in mice

Mice (n=180) were randomly assigned to three groups as shown in Table 1.

Group 1 (n=60) was the control group not infection with cercarias *S. mekongi*.

Group 2 (n=60) was the treated group infection with 10 cercarias *S. mekongi*.

Group 3 (n=60) was the treated group infection with 30 cercarias *S. mekongi*.

Mice were infected individually with cercaria shed from experimentally infected snails, after exposure to artificial light for at least 4h (Figure 18), by a looping method (Sommani *et al.*, 1973) (Figure 19).

Table 1. Pathogenesis of *S. mekongi* infection in mice

Treated time (day)	Mice		
	Group 1 (Control)	Group 2 (10 cercarias)	Group 3 (30 cercarias)
3	12	12	12
7	12	12	12
21	12	12	12
35	12	12	12
49	12	12	12

Figure 18. The infected snails were exposed to artificial light

Figure 19. Mice were infected by a looping method

At 3, 7, 21, 35, and 49 days postinfection (PI), 12 mice from each group were anesthetized, body weight (Figure 20 A) and sacrificed. Blood was collected from the heart, centrifuged at 12,000rpm for five minutes and serum was frozen. The organs (liver, lung and spleen) were removed, weighed (Figures 20 B, C, D) and fixed in suitable fixatives for microscopic study.

A. Body weight

B. Liver weight

C. Spleen weight

D. Lung weight

Figure 20. Body and organs weight

Part II: Comparison effects of praziquantel and artesunate in *S. mekongi* infected mice (*in vivo*)

Mice (n=45) were infected individually with 30cercaria shed from experimentally infected snails, after exposure to artificial light for at least 4h, by a looping method (Sornmani *et al.*, 1973). At 49days PI, mice were randomly assigned to seven groups as shown in Table 2.

Group 1 (n=15) was the control group received 7% Tween80+3% ethanol

Group 2 (n=15) was the treated group received 300mg/kg praziquentel

Group 3 (n=15) were the treated group received 300mg/kg artesunate

Table 2. Comparison effects of praziquantel and artesunate in *S. mekongi* infected mice (*in vivo*)

Time (days)	Mice		
	Group 1 Control	Group 2 300 mg/kg Praziquantel	Group 3 300 mg/kg Artersunate
1	5	5	5
3	5	5	5
7	5	5	5

At 1, 3, 7 days after treatment, 2 mice from each group were anesthetized and sacrificed. Blood was collected from the heart, centrifuged at 12,000rpm for five minutes and serum was frozen. The organs (liver, lung and spleen) were removed, weighed and fixed in suitable fixatives for microscopic study. Adult worms were collected from the remain 3 mice from each group by the perfusion method using 0.1M citrate in 0.15M NaCl solution (Sommani *et al.*, 1973) (Figure 21). The worms were washed several times with normal saline solution and fixed in suitable fixatives for microscopic study.

Figure 21. Adult worms were collected from the mice by the perfusion method

Part III: Comparison effects of praziquantel and artesunate in *S. mekongi* Tegument (*in vitro*)

Praziquantel and artesunate (Atlantic Lab., Thailand, lot no. 000491 and 040105, respectively) were initially prepared as a stock solution 10mg/mL which further diluted for use. Fresh hemin solution (Fluka, Switzerland, lot no. 448389/1) was prepared by dissolving 0.5mg of hemin in 0.1mL 0.1M NaOH and adding 0.395mL of Hanks' balanced salt solution (HBSS) followed by 0.005mL of 1M HCl to adjust the pH to 7.2-7.4. Mice (n=50) were infected individually with 30 cercaria shed from experimentally infected snails, after exposure to artificial light for at least 4h, by a looping method (Sornmani *et al.*, 1973). At 49 days PI, adult worms were collected from the mice by the perfusion method using 0.1M citrate in 0.15M NaCl solution (Sornmani *et al.*, 1973). After washing worms three times with HBSS containing penicillin 50IU/mL and streptomycin 50mg/mL, and selecting for the healthy worms with normal structure and good motility, and keeping in an ice bath at 4°C until incubation experiments began.

Ninety worms were separated into 3 groups with three replicates performed for each group. Group I (n=30) is the control group; group II (n=30) was treated with 80μg/ml praziquantel; and group III (n=30) was treated with 40μg/ml artesunate.

The medium was prepared with HBSS supplemented with 10% heat-inactivated calf serum, 50IU/mL penicillin and 50mg/mL streptomycin. The medium 1.9-2.0mL was added to 15 of the 24-well Falcon plate. Then the pair of worms was placed in each well. The plate was incubated at 37°C in 95%air plus 5%CO_2 for 20-30min before addition of praziquentel or artesunate at various concentrations as shown in Table 3 and 4. Control well contained worms and medium only. After worms, in group III, had been exposed to artesunate for 2h, 50μg/mL hemin were added in the wells. The total volume

for calculating the final concentrations in each well was 2.0mL. The plates were incubated continuously for 48h. At the end of 24h incubation, worms were washed three times and transferred to new wells with drugs as design.

After 3, 6, 12, 24, and 48h, motile activity, tegumental alterations and parasite survival were assessed by examination by the Olympus SZ-ST stereomicroscope (Tokyo, Japan). Worms were defined dead if they remained contracted, no motility during 2 min observation plus tegumental alteration including blebbing, collapse of vesicles or peeling.

Table 3. Effects of praziquantel and artesunate in *S. mekongi* tegument (*in vitro*)

Incubation time (h)	Adult worms		
	Control	80 µg/ml Praziquantel	40 µg/ml Artesunate
3	6	6	6
6	6	6	6
12	6	6	6
24	6	6	6
48	6	6	6

3. Hematological studies

Hematological parameters such as hematocrit, hemoglobin, white blood cell count, white blood cell differential were determined as follows (Dacie and Lewis, 1991)

3.1 Hematocrit:

Hematocrit (packed cell volume) determination is the volume of erythrocytes expressed as a percentage of the volume of whole blood in a sample. Dried heparin, balanced oxalate, or EDTA is satisfactory as an anticoagulant. The microhematocrit method is as following:

1. Fill the capillary tube two-thirds with well-mixed, EDTA-blood
2. Seal one end of the tube with clay

3. Place the filled tube in the microhematocrit centrifuge, with the plugged end away from the center of the centrifuge
4. Centrifuge at 10,000 to 12,000rpm for 5 minutes
5. Place the tube in the microhematocrit reader. Read the hematocrit by following the manufacturer's instruction on the microhematocrit reading device.

3.2 Hemoglobin:

Hemoglobin was estimated by Drabkin's cyanmethemoglobin method. Drabkin's solution containing potassium ferricyanide, potassium cyanide and sodium bicarbonate was prepared. 20μL of blood was added to 5mL of Drabkin's solution. Readings were taken at 530nm in a spectrophotometer. Hemoglobin values were calculated from a hemoglobin curve prepared using hemoglobin standard.

3.3 White blood cell count:

The total white cell count determines the number of white cells per cubic millimeter of blood. The procedures are as following:

1. Draw well-mixed anticoagulated venous blood to the 0.5mark on the white cell pipette.
2. Draw diluting fluid (3% glacial acetic acid) to the 11.0mark on the white cell pipette. Shake the pipette for 3minutes.
3. Load in the hemocytometer counting chamber (Precicolor, HBG, Germany) (Figure 22), count the white cells with the low-power lens in each of the four large corner fields.
4. When all the cells in the four fields have been counted, multiply the count by 50 for the total white cell count.

Figure 22. The hemocytometer counting chamber.

3.4 White blood cell differential count:

Technique for making smears

Touch the drop of blood lightly with a clean, grease-free slide. Place the slide on a flat surface with the drop of blood up. Hold a second slide between thumb and forefinger and place the edge at a 45 degree angle against the top of the slide, holding the drop of blood. Back the second slide down until it touches the drop of blood. The blood will distribute itself along the edge of the slide in a formed angle. Push the second slide along the surface of the other slide, drawing the blood across the surface in a thin, even smear. If this is done with uniform rapidity and without wobbling the slide, a good smear will result. Try to keep the blood from reaching the extreme edges of the slides. Large cells have a tendency to stack up on the perimeter of the smear, and letting the smear reach the edges of the slide will aggravate this tendency. The smear should show no wavy lines or blank spots. Let the smear air-dry.

Technique for staining smears

Place the smear on a staining rack. Flood it with about 1mL of Wright's stain and allow it to stand for 2 minutes. Add an equal quantity of buffer. There should be no run-off of fluid from the slide. Mix the buffer and stain by blowing air through the rubber pipette tube and directing the current of air about the surface of the slide. Mix until a metallic (copper looking) film appears. Let stand for 3 minutes. Wash with tap

water, provided pH testing has shown the water to be neutral. If the tap water is not neutral, wash the slide with distilled water. The stain and the metallic film must be floated off to prevent streaking, so keep the slide flat and horizontal in the stream of water. If the slide is tilted, the metallic film will settle to the surface of the smear and remain there. Wipe the stain from the bottom of the slide. Let it air-dry. A good smear should be thin and evenly distributed, and it must be dry before staining.

Technique for differential count

Place the stained slide on the microscope with a drop of immersion oil and adjust the ocular lenses. Using the oil immersion lens, scan the fields for areas where the red cells just touch. In this area the blood smear is thinner, and consequently, the white cells are easier to identify. Count 100consecutive white cells, pressing the correct key on the cell counter for each type of white cell identified.

4. Biochemical studies

Serum was determined for the enzyme activity as follows:

4.1 Transaminase: (Reitman and Frankel, 1957)

4.1.1 Aspartate aminotransferase (AST): the enzymatic activity of AST was measured by the oxaloacetate formed in the reaction:

$$\text{alpha-ketoglutarate} + \text{L-aspartate} \xrightarrow{\text{AST}} \text{glutamate} + \text{oxaloacetate}$$

Oxaloacetate was measured colorimetrically as its hydrazone after the reaction with 2,4 – dinitrophenylhydrazine at 505nm.The procedures for the analysis of glutamic oxaloacetic transaminase activity were as follows:

1. Aspartate transaminase substrate 0.25mL were pipetted into a 12 x 75mm cuvette and warmed for 5min in a water bath at 37°C.

2. Serum 0.05mL was added, mixed and covered with parafilm.

3. The mixture was incubated at 37°C for 60min.

4. Color reagent 0.25mL was added and shaken gently.

5. The tube was allowed to stand at room temperature for 20min.

6. The 2.5mL of 0.4mol/L NaOH was added and mixed.

7. Exactly 5min later, the optical density of the unknown was read with water as a blank at 505nm.

8. The optical density for the value of oxaloacetic transaminase was referred to the calibration curve.

4.1.2 Alanine aminotransferase (ALT): the enzymatic activity of ALT was measured by the pyruvate produced in the reaction:

$$\text{alpha-ketoglutarate} + \text{L-alanine} \xrightarrow{\text{ALT}} \text{glutamate} + \text{pyruvate}$$

Pyruvate formed was measured colorimetrically as its hydrazone after the reaction with 2, 4-dinitrophenylhydrazine at 505nm. The procedures for the analysis of glutamic pyruvic transaminase activity were performed similar to those of oxaloacetic transaminase, substituting with the alanine transaminase substrate, and incubated at 37°C for 30min.

The procedures for calibration curve were as follows:

1. The solution was pipetted into cuvettes as indicated in Table 4:

Table 4. The calibration curve of AST and ALT

Tube No.	Calibration standard	Aspartate transaminase substrate (mL)	Water (mL)	Sigma-Frankel units	
				Aspartate substrate	Alanine substrate
1	0.0	1.0	0.2	0	0
2	0.1	0.9	0.2	20	23
3	0.2	0.8	0.2	55	50
4	0.3	0.7	0.2	95	83
5	0.4	0.6	0.2	148	125
6	0.5	0.5	0.2	216	-

2. Color reagent 1mL was added to all cuvettes and mixed. The tube was allowed to stand for 20min at room temperature. Ten mL of 0.4mol/L NaOH was added to all cuvettes and mixed.
3. Exactly 5min after adding NaOH, the optical density of all samples in 12 x 75mm cuvettes was read and recorded, using water as a blank at 505nm.
4. A calibration curve of optical density versus the corresponding units of transminase was plotted.

4.2 Alkaline phosphatase; (McComb and Bowers, 1972) It is the enzyme that hydrolyzes organic phosphates such as phenolphthalein monophosphate to phenolphthalein with a intense yellow color under an alkaline condition. The procedures for the analysis of this enzyme activity are indicated in Table 5.

Table 5.The procedures for alkaline phosphatase analysis

	Sample (mL)	Blank (mL)
Substrate (Phenolphthaline monophosphate)	1 drop	1 drop
Dist. water	1.0	1.0
Incubate at 37°C 5 min		
Serum	0.1	-
Incubate at 37°C 20 min		
Buffer solution (Na_3PO_4 + NaH_2PO_4)	5.0	5.0

The optical density was read at 550nm, and the value was read from calibration curve. The procedures for calibration curve: standard phenolphthalein concentrations of 25, 50, 75, and 100IU were used (by using standard 1.0mL + buffer 5.14 mL). The optical density was read at 550nm with water as a blank. A calibration curve of optical density and concentration was plotted. The curve was a straight line.

4.3 Total protein: Biuret method (Reinhold, 1953). Proteins form a purple colored complex with cupric ions in alkaline solution. The intensity of the purple color was measured at 540nm.

Table 6.The procedures for total protein analysis

	Sample (mL)	Standard (mL)	Blank (mL)
Color reagent	2	2	2
Serum	0.05	—	—
Standard solution	—	0.05	—
Mix and incubated 37°C 15 min			

The optical density was read at 540nm, and the value was calculated by

Total protein (g/dL) = (Abs. Sample / Abs. Stand.) x conc. Stand. (10g/dL).

4.4 Albumin: Bromocresol green method (Doumas *et al.*, 1971). Serum albumin binded selectively to the dye bromocresol green at pH 4.2. The increased in absorbance of the resulting albumin-dye complex, read at 630nm.The procedures for the analysis of this enzyme activity are indicated in Table 7.

Table 7.The procedures for albumin analysis

	Sample (mL)	Standard (mL)	Blank (mL)
Color reagent	2	2	2
Serum	0.01	—	—
Standard solution	—	0.01	—
Mix and incubated in room temperature 10 min			

The optical density was read at 630nm, and the value was calculated by

Albumin (g/dL) = (Abs. Sample / Abs. Stand.) x conc. Stand. (5g/dL).

5. Histopathological studies

5.1 Light microscopy

The organs were fixed in 10% buffered formaldehyde for 24h, washed with 70% ethanol, dehydrated with a series of ethanol and cleared with xylene solutions. They were embedded in paraffin, sectioned at 5μm thickness using a rotary microtome (HistoSTAT, Reichert, USA), stained with hematoxylin and eosin. They were examined for abnomalities using a Nikon E600 light microscope (Figure 23) and photographed by a Nikon DXM 1200digital camera (Tokyo, Japan) (Gretchen, 1972). The schedules are presented in Tables 8 and 9.

Figure 23. Nikon E600 light microscope

Table 8. Schedule for histological process

No	Method	Duration
1	Dissection	
2	Bouin's fluid	24 hours
3	70% alcohol	24 hours
4	80% alcohol	40 minutes
5	95% alcohol	40 minutes
6	95% alcohol	40 minutes
7	Absolute alcohol	40 minutes

First day: Removal and fixing tissues in 10% buffered formaldehyde 24 hours.

Second day: Washing out the fixative

Third day: Dehydration

8	Absolute alcohol	40 minutes
9	Dioxane	1 hour
10	Dioxane	1 hour
11	Paraffin	40 minutes
12	Paraffin	40 minutes
13	Embedding	
14	Sectioning	24 hours
15	Staining	
16	Examination	

Fourth day: Sectioning, spreading on glass slides, drying overnight

Fifth day: Staining, mounting, and examination

Table 9. Schedule for staining sections

No	Method	Duration
1	Xylene (I)	3 minutes
2	Xylene (II)	3 minutes
3	Xylene (III)	3 minutes
4	Absolute alcohol	3 minutes
5	95% alcohol	3 minutes
6	70% alcohol	3 minutes
7	Running water	3 minutes
8	Hematoxylin	1 minute
9	Running water	3 minutes
10	Eosin	1 minute
11	70% alcohol	3 minutes
12	95% alcohol (I)	3 minutes
13	95% alcohol (II)	3 minutes
14	Absolute alcohol (I)	3 minutes
15	Absolute alcohol (II)	3 minutes
16	Xylene (I)	3 minutes
17	Xylene (II)	3 minutes
18	Examination	

5.2 Transmission electron microscopy

Small pieces of tissue were fixed in 4% glutaraldehyde-phosphate buffer (0.1mol/L, pH 7.4) at 4°C for 24h and postfixed in 1% osmium tetroxide for 1h. They were dehydrated through a graded series of alcohol, cleared in propylene oxide and embedded in polyethylene beam capsule containing Epon 812 resin. Ultrathin sections were cut on an ultramicrotome, stained with uranyl acetate and lead citrate, and examined by a Hitachi H-7000 transmission electron microscope (Figure 24) at 75kV (Gretchen, 1972). The schedule is presented in Table 10.

Figure 24. Hitachi H-7000 transmission electron microscope

5.2.1 Semithin section staining

Semithin sections were cut using glass knives on a LKB ultramicrotome, stained with 1% toluidine blue in 1% borax for 20min. They were washed in distilled water, dried, mounted, and examined by a light microscope for orientation (Gretchen, 1972).

5.2.2 Ultrathin section staining

Ultrathin sections were cut using glass knives, collected on naked copper grids and stained with uranyl acetate and lead citrate. They were examined and viewed with a Hitachi H-7000 electron microscope operated at 75kV (Gretchen, 1972).

Staining with uranyl acetate and lead citrate: The uranyl acetate was spun for 5min.Grids on blobs of the stain were stained for 30min in the dark room. Grids were washed in distilled water five times and blotted dried. Grids were floated on blobs of lead citrate with a piece of sodium hydroxide for 30min.Grids were washed in boiled-distilled water five times twice and blotted dried.

Table 10. Schedule for transmission electron microscope procedures

No	Method	Duration
1	0.1M cacodylate pH 7.4	Prefix 2 h at 4° C
2	1% osmium tetroxide in 0.1M cacodylate	Wash 3 times at 4°C
3	Filtered water (cold)	Postfix 1 h at 4°C
4	50% alcohol	Wash 3 times at 4°C
5		Wash 2 times x 15 min at 4°C
6	70% alcohol	Wash 2 times x 15 min at 4°C
7	80% alcohol	Wash 2 times x 15 min at 4°C
8	90% alcohol	Wash 2 times x 15 min at 4°C
9	95% alcohol	Wash 2 times x 15 min at 4°C
10	100% alcohol	Wash 3 times x 15 min at 4°C
11	100% alcohol	Wash 3 times x 15 min at RT
12	Propylene oxide	Wash 2 times x 15 min at RT
13	Propylene oxide : plastic (2:1)	1h at room temp
14	Propylene oxide : plastic (1:2)	Overnight at room temp
15	Embedding in capsule beam	Overnight at room temp
16	Hot oven 45°C	48h
17	Hot oven 60°C	48h
18	Sectioning	
19	Staining	
20	Examination	

5.3 Scanning electron microscopy

Small pieces of tissue were fixed in 4% glutaraldehyde-phosphate buffer (0.1mol/L, pH 7.4) at 4°C for 24h and postfixed in 1% osmium tetroxide for 1h. They were dehydrated through a graded series of alcohol, dried in a Hitachi HCP-2 critical point dryer machine using liquid carbon dioxide as a transitional medium. After drying, they were mounted on aluminium stubs and coated with platinum and paladium in an ion-sputtering apparatus, Hitachi E-102, at 10-15mA for 6min. They were examined and photographed in a Hitachi scanning electron microscope S-2500 (Figure 25), operating at 15kV (Gretchen, 1972). The schedule is presented in Table 11.

Figure 25. Hitachi scanning electron microscope S-2500

Table 11. Schedule for scanning electron microscope procedures

No	Method	Duration
1	4% glutaraldehyde in 0.1M cacodylate pH 7.4	Prefix 2 h at 4°C
2	0.1M cacodylate pH 7.4	Wash 3 times at 4°C
3	1% osmium tetroxide in 0.1M cacodylate	Postfix 1 h at 4°C
4	Filtered water (cold)	Wash 3 times at 4°C
5	50% alcohol	Wash 2 times x 15 min at 4°C
6	70% alcohol	Wash 2 times x 15 min at 4°C
7	80% alcohol	Wash 2 times x 15 min at 4°C
8	90% alcohol	Wash 2 times x 15 min at 4°C
9	95% alcohol	Wash 2 times x 15 min at 4°C
10	100% alcohol	Wash 3 times x 15 min at 4°C
11	100% alcohol	Wash 3 times x 15 min at RT
12	Critical point drying	
13	Mounted and coated	
14	Examination	

6. Statistical analysis

Body and organ weight, hematological data, and enzymatic activities were analyzed using SPSS 12.0 for Windows software (SPSS, Chicago, IL). All data were expressed as mean values $\pm$ S.D. A two-way analysis of variance (ANOVA) was performed separately for each time, and also separately tested in each group. Scheffe post hoc test for multiple comparisons was used for determination of significant differences between the control and treated groups. The level of statistical significance is set at the probability level of 0.05.

CHAPTER V

RESULTS

Part I: Pathogenesis of *Schistosoma mekongi* infection in mice

1. Body and organ weights

Changes in body and organ weight in mice infected with *S. mekongi* cercarias in each time are presented in Table 12. The lung, liver and spleen indices were calculated as follows:

organ index = organ weight of mice (g)/body weight of mice (g).

Table 12 shows the mean ± S.D. of these measurements for forty-nine days. An analysis of variance was performed separately for each time, and also separately tested in each group. The data were tested at an $\alpha = 0.05$ for growth differences among experimental groups (Figure 26).

1.1 Body weight

The body weight of the control group was significantly decreased from 33.47 g to 32.07, 31.31, 30.94 g in the infected groups with 10 cercarias (3A, 4A and 5A, respectively) and to 31.78, 30.92, 29.99, 29.45 g in the infected groups with 30 cercarias (2B, 3B, 4B and 5B, respectively) ($p \leq 0.05$).

1.2 Liver weight and liver index

The liver weight of the control group was significantly increased from 1.44 g to 1.65, 2.04 g in the infected groups with 10 cercarias (4A and 5A, respectively) and to 1.72, 2.25 g in the infected groups with 30 cercarias (4B and 5B, respectively) ($p \leq 0.05$).

The liver index of the control group was significantly increased from 4.32% to 5.26, 6.61% in the infected groups with 10 cercarias (4A and 5A, respectively) and to

4.96, 5.74, 7.66% in the infected groups with 30 cercarias (3B, 4B and 5B, respectively) ($p \leq 0.05$).

1.3 Lung weight and lung index

The lung weight of the control group was significantly increased from 0.217 g to 0.236 g in the infected groups with 10 cercarias (5A) and to 0.253, 0.276 g in the infected groups with 30 cercarias (4B and 5B, respectively) ($p \leq 0.05$).

The lung index of the control group was significantly increased from 0.648% to 0.719, 0.762% in the infected groups with 10 cercarias (4A and 5A, respectively) and to 0.707, 0.723, 0.843, 0.936% in the infected groups with 30 cercarias (2B, 3B, 4B and 5B, respectively) ($p \leq 0.05$).

1.4 Spleen weight and spleen index

The spleen weight of the control group was significantly increased from 0.084 g to 0.169 g in the infected groups with 10 cercarias (5A) and to 0.137, 0.204 g in the infected groups with 30 cercarias (4B and 5B, respectively) ($p \leq 0.05$).

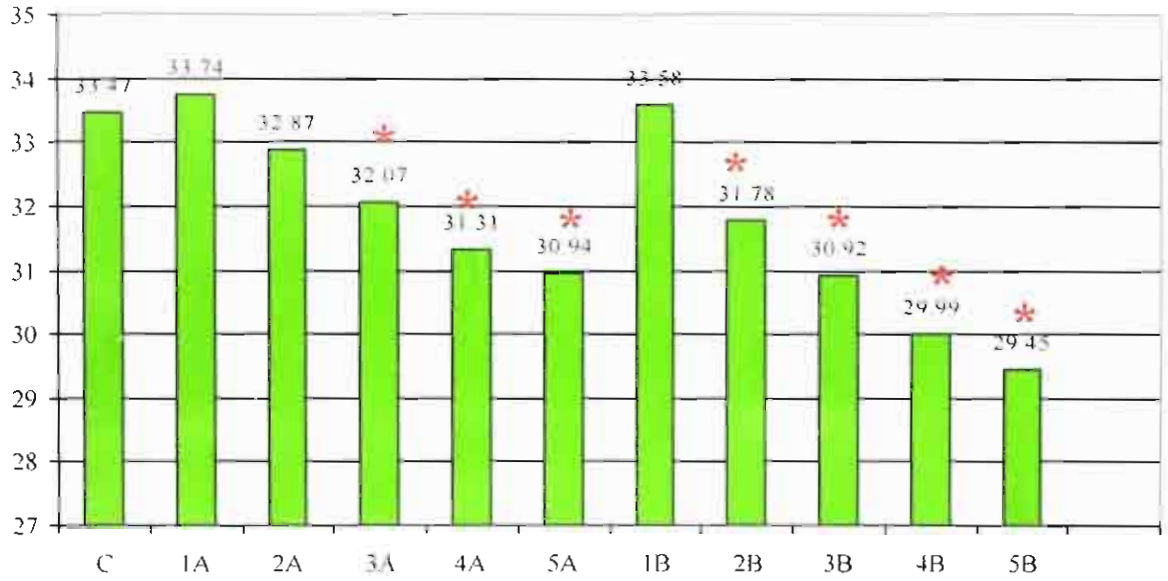
The spleen index of the control group was significantly increased from 0.253% to 0.546% in the infected groups with 10 cercarias (5A) and to 0.456, 0.691% in the infected groups with 30 cercarias (4B and 5B, respectively) ($p \leq 0.05$).

Table 12. Changes in body and organ weights (mean ± S.D., n=12) in mice infected with *S. mekongi* cercarias in each time

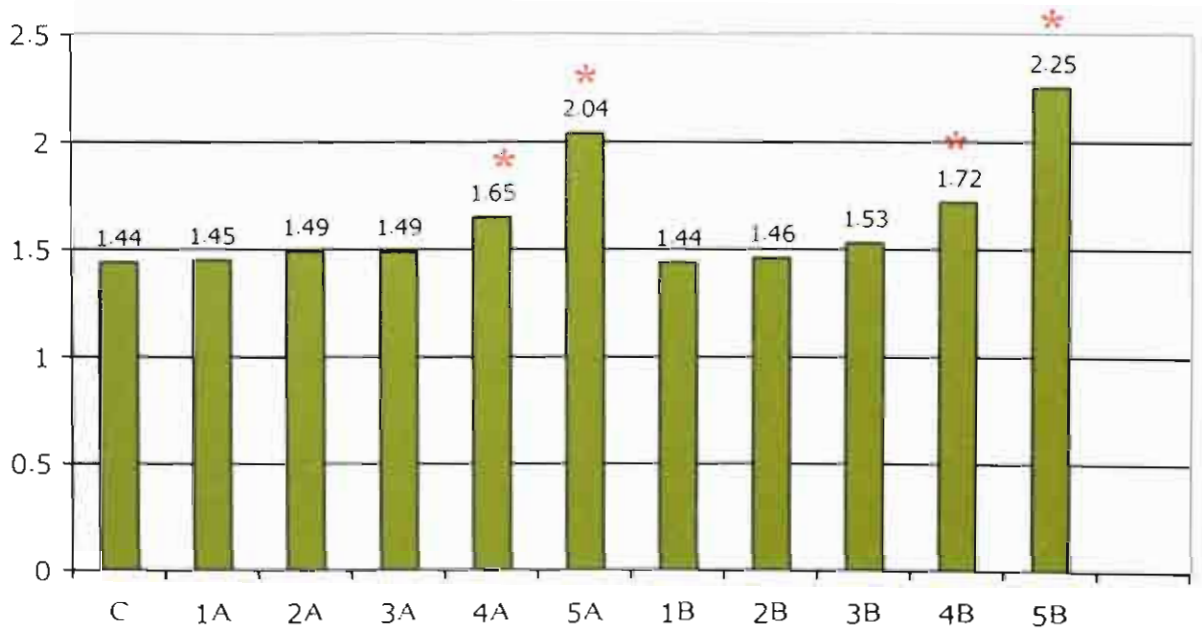
Parameters	Cont.	Infected groups with 10 cercarias					Infected groups with 30 cercarias				
		1A	2A	3A	4A	5A	1B	2B	3B	4B	5B
BW (g)	33.47 ±1.01	33.74 ±0.86	32.87 ±1.64	32.07* ±1.03	31.31* ±0.83	30.94* ±0.86	33.58 ±1.47	31.78* ±0.10	30.92* ±1.12	29.99* ±1.02	29.45* ±1.13
Liver (g)	1.44 ±0.09	1.45 ±0.10	1.49 ±0.06	1.49 ±0.07	1.65* ±0.09	2.04* ±0.23	1.44 ±0.08	1.46 ±0.10	1.53 ±0.05	1.72* ±0.11	2.25* ±0.27
Liver index (%)	4.32 ±0.29	4.31 ±0.29	4.53 ±0.24	4.65 ±0.27	5.26* ±0.32	6.61* ±0.79	4.30 ±0.25	4.61 ±0.37	4.96* ±0.29	5.74* ±0.49	7.66* ±1.02
Lung (g)	0.217 ±0.016	0.217 ±0.024	0.218 ±0.021	0.223 ±0.012	0.225 ±0.012	0.236* ±0.030	0.222 ±0.012	0.224 ±0.012	0.223 ±0.012	0.253* ±0.022	0.276* ±0.038
Lung index (%)	0.648 ±0.053	0.643 ±0.076	0.662 ±0.066	0.694 ±0.050	0.719* ±0.046	0.762* ±0.100	0.661 ±0.046	0.707* ±0.046	0.723* ±0.047	0.843* ±0.080	0.936* ±0.108
Spleen (g)	0.084 ±0.005	0.080 ±0.006	0.090 ±0.008	0.091 ±0.008	0.101 ±0.018	0.169* ±0.060	0.086 ±0.004	0.090 ±0.013	0.089 ±0.008	0.137* ±0.067	0.204* ±0.094
Spleen Index (%)	0.253 ±0.020	0.237 ±0.019	0.275 ±0.024	0.285 ±0.027	0.324 ±0.053	0.546* ±0.191	0.257 ±0.018	0.285 ±0.042	0.289 ±0.026	0.456* ±0.217	0.691* ±0.319

Note *The mean difference was significant when compared to the control group at 0.05 level
Control = no infected with cercarias
1A = infected with 10 cercarias in 3 days
2A = infected with 10 cercarias in 7 days
3A = infected with 10 cercarias in 21 days
4A = infected with 10 cercarias in 35 days
5A = infected with 10 cercarias in 49 days
1B = infected with 30 cercarias in 3 days
2B = infected with 30 cercarias in 7 days
3B = infected with 30 cercarias in 21 days
4B = infected with 30 cercarias in 35 days
5B = infected with 30 cercarias in 49 days

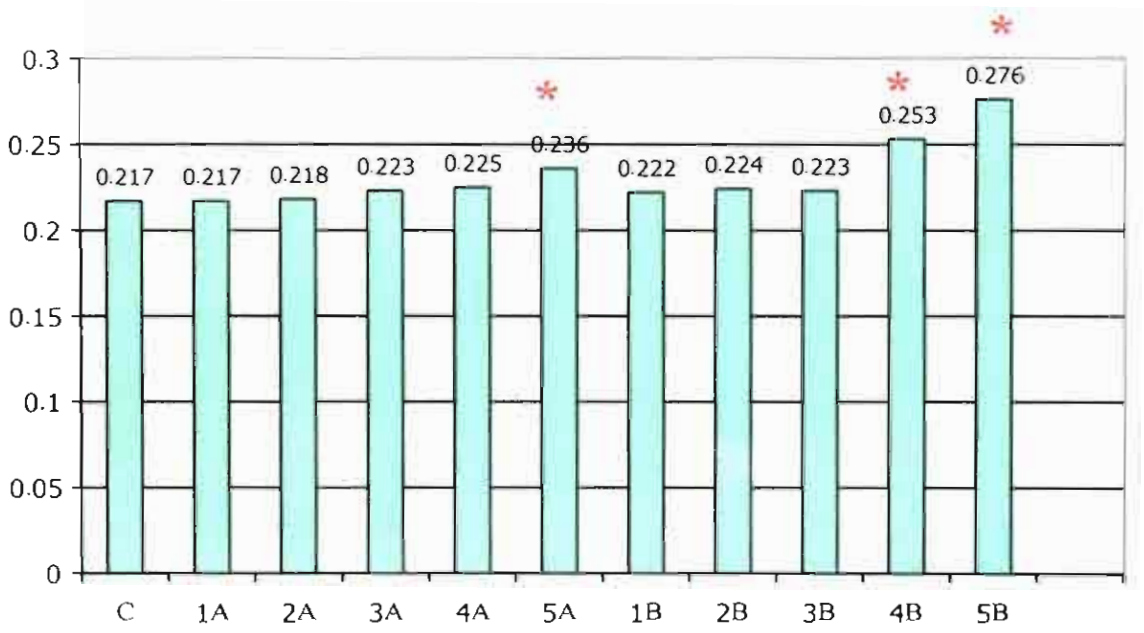
A. BODY WEIGHT (g)



B. LIVER WEIGHT (g)



C. LUNG WEIGHT (g)



D. SPLEEN WEIGHT (g)

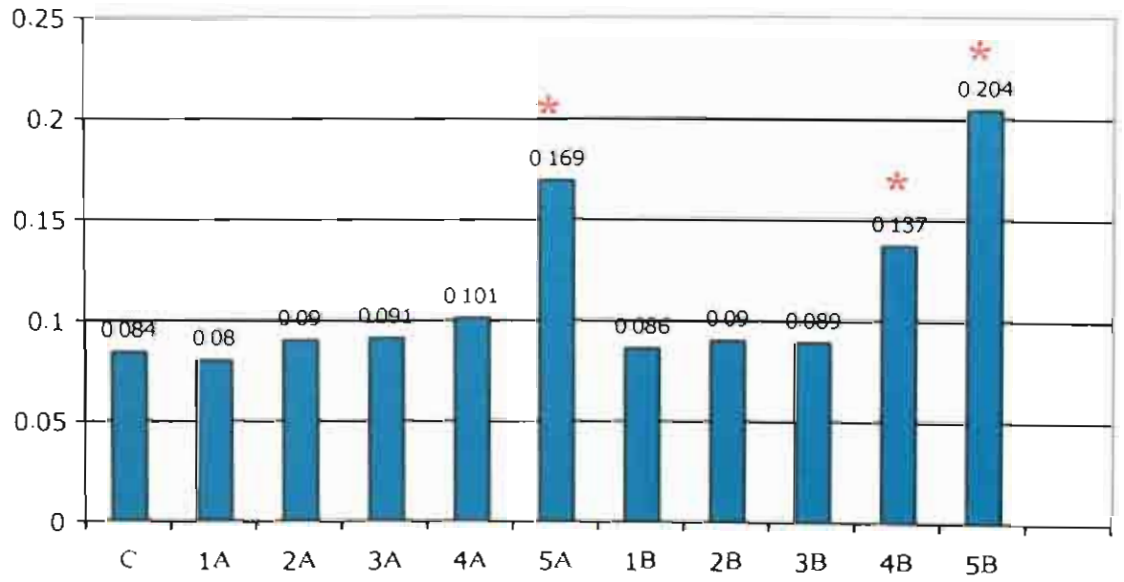


Figure 26. Changes in body and organ weights (mean ± S.D., n=12) in mice infected with *S. mekongi* cercarias in each time.

A. Body weight B. Liver weight C. Lung weight D. Spleen weight

As shown in Table 12, cercarias infection showed a trend of lightening the mouse body weight. Significant differences increase ($p<0.05$) in liver, lung and spleen weight and organ index were observed in the infected groups when compare with the control group. The results showed that the longer exposure time and higher cercarias infection resulted in decreased body weight but increased in organs weight and index.

2. Hematological Studies

Changes in hematological measurements in mice infected with *S. mekongi* cercarias in each time are presented in Table 13. It shows the mean $\pm$ S.D. of these measurements for forty-nine days. An analysis of variance was performed separately for each time, and also separately tested in each group. The data were tested at an $\alpha = 0.05$ for growth differences among experimental groups (Figure 27).

2.1 Hemoglobin

Hemoglobin of the control group was significantly decreased from 13.63 g/L to 12.38, 11.98 g/L in the infected groups with 10 cercarias (4A and 5A, respectively) and to 12.27, 10.28 g/L in the infected groups with 30 cercarias (4B and 5B, respectively) ($p\leq0.05$).

2.2 Hematocrit

Hematocrit of the control group was significantly decreased from 41.29% to 34.58, 29.96% in the infected groups with 10 cercarias (4A and 5A, respectively) and to 33.96, 26.58% in the infected groups with 30 cercarias (4B and 5B, respectively) ($p\leq0.05$).

2.3 White blood cell count

White blood cell count of the control group was significantly increased from 3183 x10^9/l to 3496, 3738 x10^9/l in the infected groups with 10 cercarias (4A and 5A, respectively) and to 3533, 4029 x10^9/l in the infected groups with 30 cercarias (4B and 5B, respectively) ($p \leq 0.05$).

Compared with those of control group, the hematological parameters, including Hb and Hct of the infected group decreased significantly ($p < 0.05$), whereas white blood cell count was increased significantly ($p < 0.05$). These results revealed that the longer exposure time and higher cercarias infection resulted in hematological parameters.

Table 13. Changes in hematological parameters (mean ± S.D., n=12) in mice infected with *S. mekongi* cercarias in each time

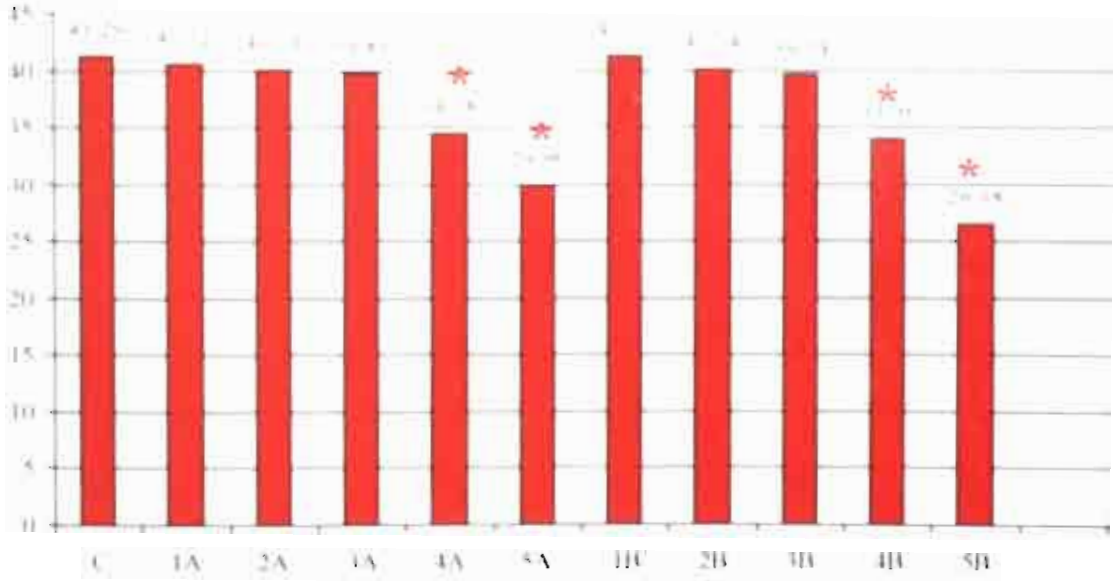
Parameters	Control	Infected groups with 10 cercarias					Infected groups with 30 cercarias				
		1A	2A	3A	4A	5A	1B	2B	3B	4B	5B
Hb (g/L)	13.63 ±0.52	13.53 ±0.42	13.43 ±0.32	13.57 ±0.43	12.38* ±0.44	11.98* 0.84	13.60 ±0.50	13.57 ±0.58	13.30 ±0.47	12.27* ±0.68	10.28* ±0.87
Hct (%)	41.29 ±1.57	40.52 ±1.74	40.13 ±1.68	39.83 ±1.64	34.58* ±2.12	29.96* ±3.22	41.21 ±1.64	40.04 ±1.89	39.54 ±1.78	33.96* ±3.37	26.58* ±2.52
Wbc (x10^9/l)	3183 ±167	3155 ±95	3208 ±79	3175 ±108	3496* ±210	3738* ±322	3196 ±92	3213 ±149	3350 ±165	3533* ±237	4029* ±630

Note *The mean difference was significant when compared to the control group at 0.05 level
Control = no infected with cercarias
1A = infected with 10 cercarias in 3 days
2A = infected with 10 cercarias in 7 days
3A = infected with 10 cercarias in 21 days
4A = infected with 10 cercarias in 35 days
5A = infected with 10 cercarias in 49 days
1B = infected with 30 cercarias in 3 days
2B = infected with 30 cercarias in 7 days
3B = infected with 30 cercarias in 21 days
4B = infected with 30 cercarias in 35 days
5B = infected with 30 cercarias in 49 days

A. Hb (g/dl)


16
14
12
10
8
6
4
2
0
12.27
10.28
C
1A
2A
3A
4A
5A
1B
2B
3B
4B
5B

B. Hct (%)


40
30
20
10
0
C
1A
2A
3A
4A
5A
1B
2B
3B
4B
5B

C. WBC ($\times 10^3$/l)

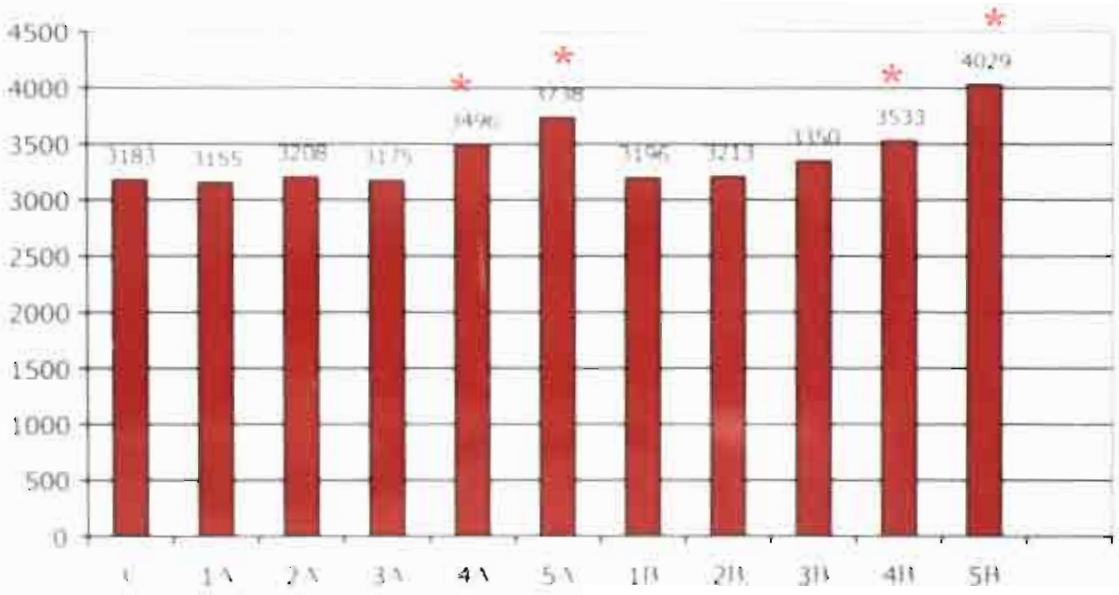


Figure 27 Changes in hematological parameters (mean ± S.D., n = 12) in mice infected with *S. mekongi* cercarias in each time.

A. Hemoglobin B. Hematocrit C. White blood cell count

3. Biochemical Studies

Changes in enzyme activity measurements in mice infected with *S. mekongi* cercarias in each time are presented in Table A-3 in Appendix. Table 14 shows the mean ± S.D. of these measurements for forty-nine days. An analysis of variance was performed separately for each time, and also separately tested in each group. The data were tested at an $\alpha = 0.05$ for growth differences among experimental groups (Figure 28).

3.1 Aspartate aminotransferase activities

AST of the control group was significantly increased from 89.67 U/L to 122.50, 153.60 U/L in the infected groups with 10 cercarias (4A and 5A, respectively) and to

111.80, 137.50, 173.20 U/L in the infected groups with 30 cercarias (3B, 4B and 5B, respectively) ($p \leq 0.05$).

3.2 Alanine aminotransferase activities

ALT of the control group was significantly increased from 32.75 U/L to 62.08, 64.50 U/L in the infected groups with 10 cercarias (4A and 5A, respectively) and to 65.92, 74.67 U/L in the infected groups with 30 cercarias (4B and 5B, respectively) ($p \leq 0.05$).

3.3 Alkaline phosphatase

ALP of the control group was significantly increased from 25.08 U/L to 37.50, 67.75 U/L in the infected groups with 10 cercarias (4A and 5A, respectively) and to 49.33, 69.83 U/L in the infected groups with 30 cercarias (4B and 5B, respectively) ($p \leq 0.05$).

3.4 Albumin

ALB of the control group was significantly increased from 4.00 g% to 4.69, 5.35 g% in the infected groups with 10 cercarias (4A and 5A, respectively) and to 4.77, 4.81, 5.74, 6.08 g% in the infected groups with 30 cercarias (2B, 3B, 4B and 5B, respectively) ($p \leq 0.05$).

3.5 Total protein

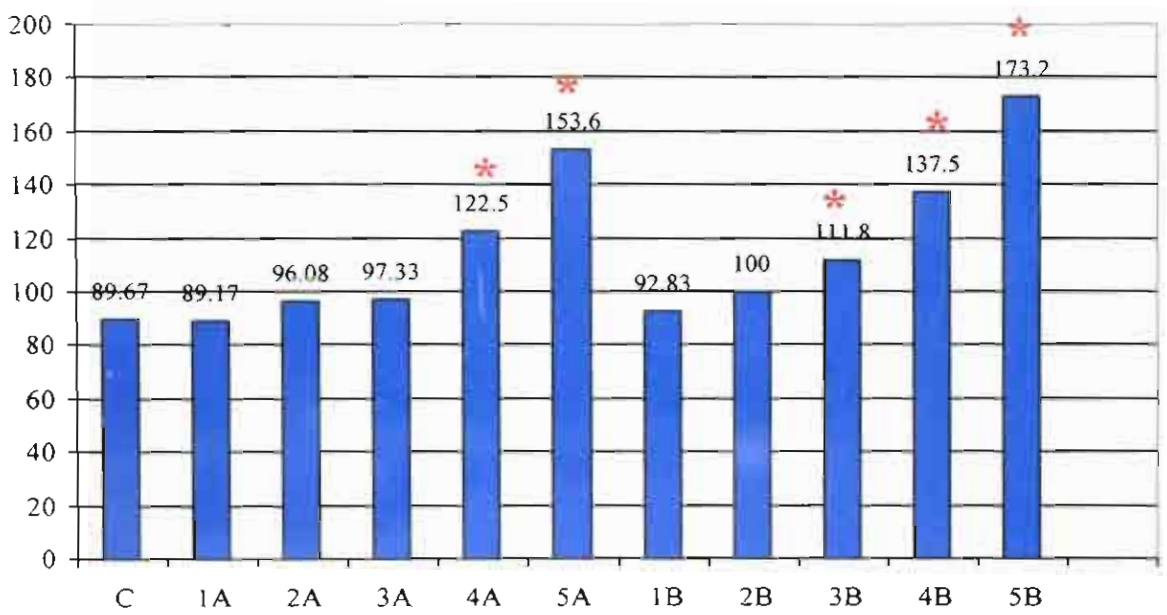
There were no statistically different between control and infected groups.

Table 14. Changes in enzyme activity measurements (mean ± S.D., n=12) in mice infected with *S. mekongi* cercarias in each time

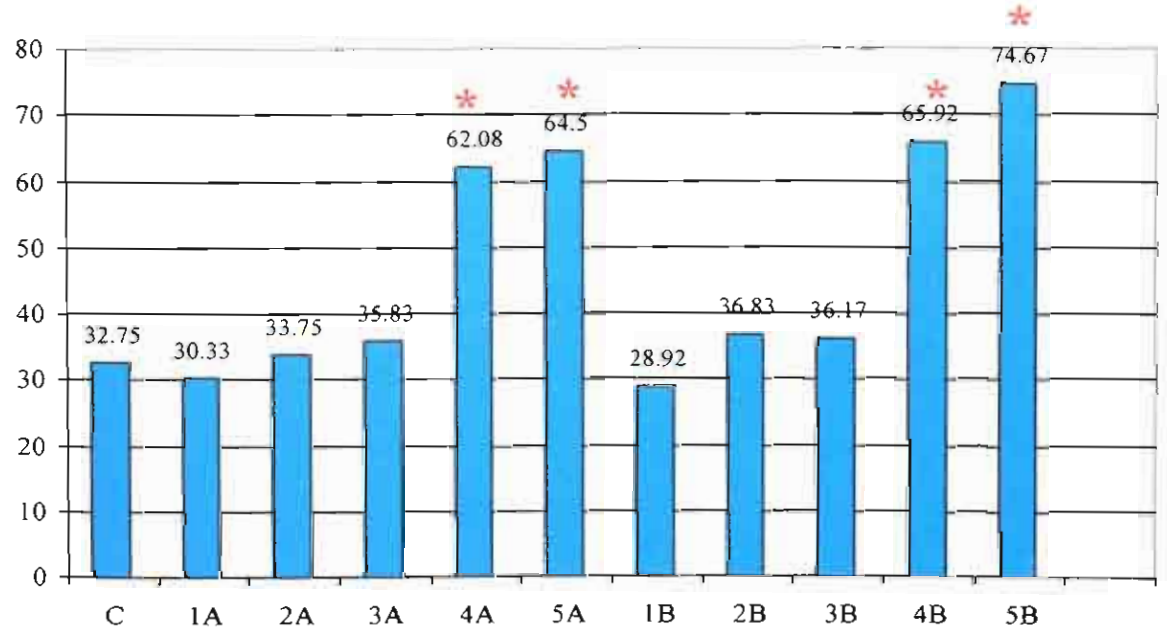
Parame ters	Control	Infected groups with 10 cercarias					Infected groups with 30 cercarias				
		1A	2A	3A	4A	5A	1B	2B	3B	4B	5B
AST (U/L)	89.67 ±6.26	89.17 ±5.36	96.08 ±5.95	97.33 ±6.60	122.5* ±17.3	153.6* ±14.2	92.83 ±4.04	100.1 ±12.0	111.8* ±11.6	137.5* ±15.4	173.2* ±33.7
ALT (U/L)	32.75 ±3.08	30.33 ±2.71	33.75 ±4.31	35.83 ±3.33	62.08* ±7.42	64.50* ±8.62	28.92 ±3.18	36.83 ±4.55	36.17 ±6.22	65.92* ±8.53	74.67* ±7.14
ALP (U/L)	25.08 ±4.85	24.58 ±3.09	24.67 ±2.84	26.25 ±3.62	37.50* ±7.38	67.75* ±7.44	23.67 ±3.26	29.92 ±2.35	28.83 ±1.34	49.33* ±4.21	69.83* ±6.44
ALB (g%)	4.00 ±0.52	4.06 ±0.53	4.12 ±0.60	4.38 ±0.56	4.69* ±0.32	5.35* ±0.60	4.10 ±0.57	4.77* ±0.66	4.81* ±0.66	5.74* ±0.56	6.08* ±0.32
TP (g%)	6.44 ±0.43	6.41 ±0.36	6.43 ±0.57	6.46 ±0.42	6.66 ±0.36	6.74 ±0.36	6.41 ±0.38	6.42 ±0.39	6.55 ±0.59	6.73 ±0.41	6.75 ±0.32

Note *The mean difference was significant when compared to the control group at 0.05 level
Control = no infected with cercarias
1A = infected with 10 cercarias in 3 days
2A = infected with 10 cercarias in 7 days
3A = infected with 10 cercarias in 21 days
4A = infected with 10 cercarias in 35 days
5A = infected with 10 cercarias in 49 days
1B = infected with 30 cercarias in 3 days
2B = infected with 30 cercarias in 7 days
3B = infected with 30 cercarias in 21 days
4B = infected with 30 cercarias in 35 days
5B = infected with 30 cercarias in 49 days

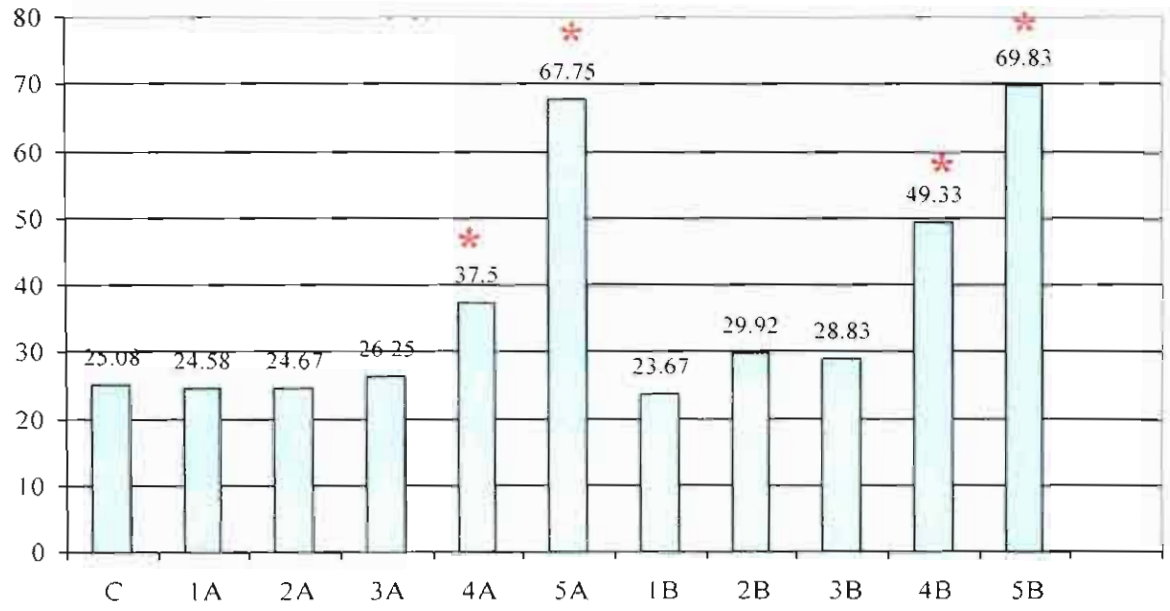
A. AST (U/L)



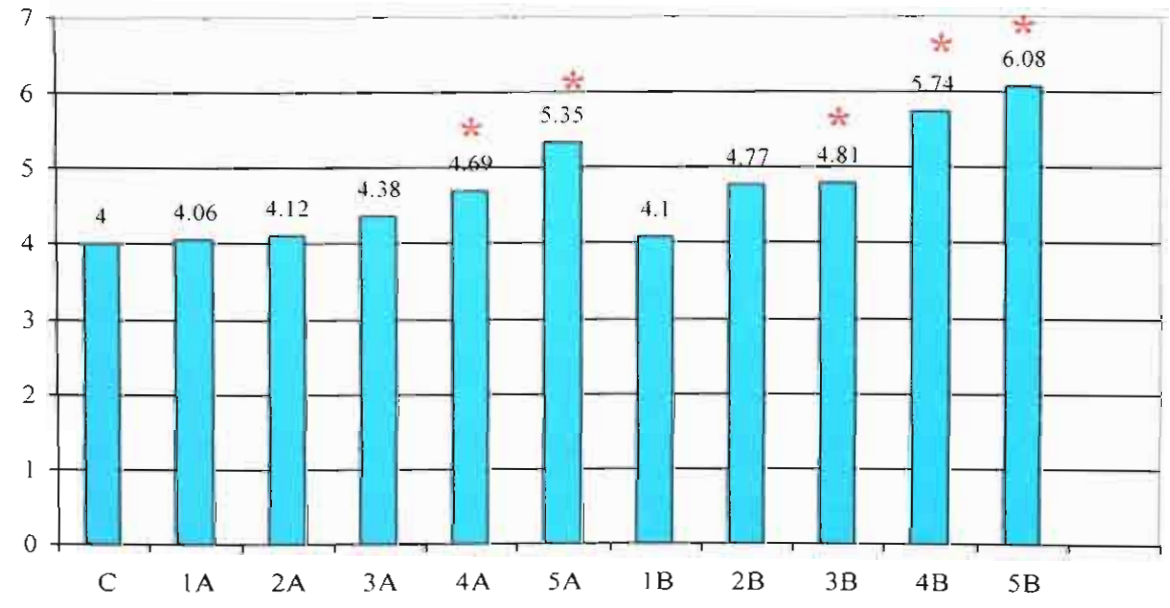
B. ALT (U/L)



C. ALP (U/L)



D. ALB (g%)



E. TP (g%)

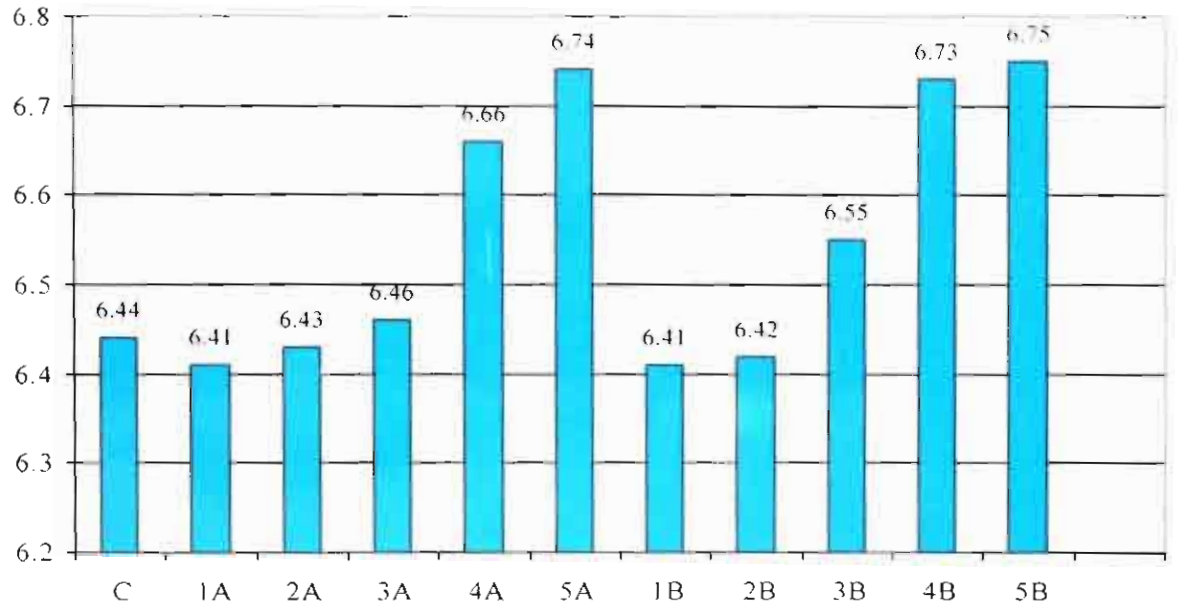


Figure 28. Changes in enzyme activity measurements (mean ± S.D., n=12) in mice infected with *S. mekongi* cercarias in each time.

A. AST B. ALT C. ALP D. ALB E. TP

Compared with those of control group, the biochemical parameters, including aminotransferase activities of aspartate and alanine, and alkaline phosphatase, of the infected group increased significantly ($p < 0.05$). Serum total protein levels were unaffected while serum albumin levels increased significantly. The results showed that the longer exposure time and higher cercarias resulted in the higher level of enzyme activities. In the forty-nine days of exposure, mice infected with 30 cercarias had the highest level of enzyme activities.

4. Histopathological Studies

4.1 Lung

Control group: The lung of control mice revealed typical parenchymatous appearance (Figure 29).

The respiratory bronchiole branched to form alveolar ducts. These thin-walled, fibroelastic tubes were lined with a squamous epithelium and possessed alveoli that appear as out-pocket of the main wall. The main wall of the duct between alveoli contained smooth muscle. The terminal portion of the respiratory duct gave rise to the alveolar sacs, composed of a variable number of alveoli that appear as small compartments opening into the alveolar sac. The alveoli were the smallest and most numerous subdivisions of the respiratory system. The interalveolar septum often contained 10 to 15 μm openings between neighboring alveoli that function to equalize air pressure in adjoining alveoli.

The wall of the alveoli was formed by a thin sheet (~2μm) of tissue separating two neighbouring alveoli. This sheet was formed by epithelial cells and intervened connective tissue. Collagenous, reticular, and elastic fibres were present. Between the connective tissue fibres, there were found a dense, anastomosing network of pulmonary capillaries. The walls of the capillaries were in direct contact with the epithelial lining of the alveoli. The basal laminae of the epithelium and endothelium may actually fuse. Neighbouring alveoli may be connected to each other by small alveolar pores. The epithelium of the alveoli was formed by two cell types. Alveolar type I cells (small alveolar cells or type I pneumocytes) were extremely flattened, as thin as 0.05 μm, and formed the bulk (95%) of the surface of the alveolar walls. The shape of the cells was very complex, and they might actually form part of the epithelium on both faces of the

alveolar wall. Alveolar type II cells (large alveolar cells or type II pneumocytes) were irregularly or cuboidal shaped. They formed small bulges on the alveolar walls. Type II alveolar cells were contained large numbers of granules called cytosomes or multilamellar bodies, which consisted of precursors to pulmonary surfactant, the mixture of phospholipids, which keep surface tension in the alveoli low.

Treated groups: Light microscopic study of the lung of mice infected *with S. mekongi* cercarias showed several pathological changes, and their frequencies increased with increasing dose and time of exposure.

Infected with 10 cercarias: At 3 days PI, the lung revealed no pathological changes. The structure of the lung was essentially well preserved (Figures 30 A-B). At 7-35 days PI, the lungs revealed variable degrees of congestion, thickening of alveolar sac, and acute inflammation consisting of polymorphonuclear leukocytes that infiltrated the interstitium around small bronchioles and adjacent alveoli. Blood congestion was observed in the capillaries (Figures 31 A-B, 32 A-B, and 33 A-B, respectively). At 49 days PI, the lung revealed normal appearance similar as those of control group (Figures 34 A-B).

Infected with 30 cercarias: At 3 days PI, the lung revealed no pathological changes. The structure of the lung was essentially well preserved (Figures 30 C-D). At 7, 21, 35, and 49 days PI, the lungs revealed similar pathological changes as those of infected with 10 cercarias but they were more severe. (Figures 31 C-D, 32 C-D, 33 C-D, and 34 C-D, respectively).

Figure 29. Light micrographs of a transverse section of lung of control mice.

A-B. Low and high magnification showing the normal appearance of lung tissue (bar = 200 and 50 μm, respectively).

C. Higher magnification showing the normal appearance of alveoli that are single sac-like air space (bar = 20 μm).

D. Highest magnification showing alveolar type I which are the squamous epithelial cells that form part of the alveolar wall, alveolar type II cells which can be seen as cells that bulge into the alveolus, and alveolar capillaries (C) which are identified by erythrocytes in the lumen (bar = 10 μm).

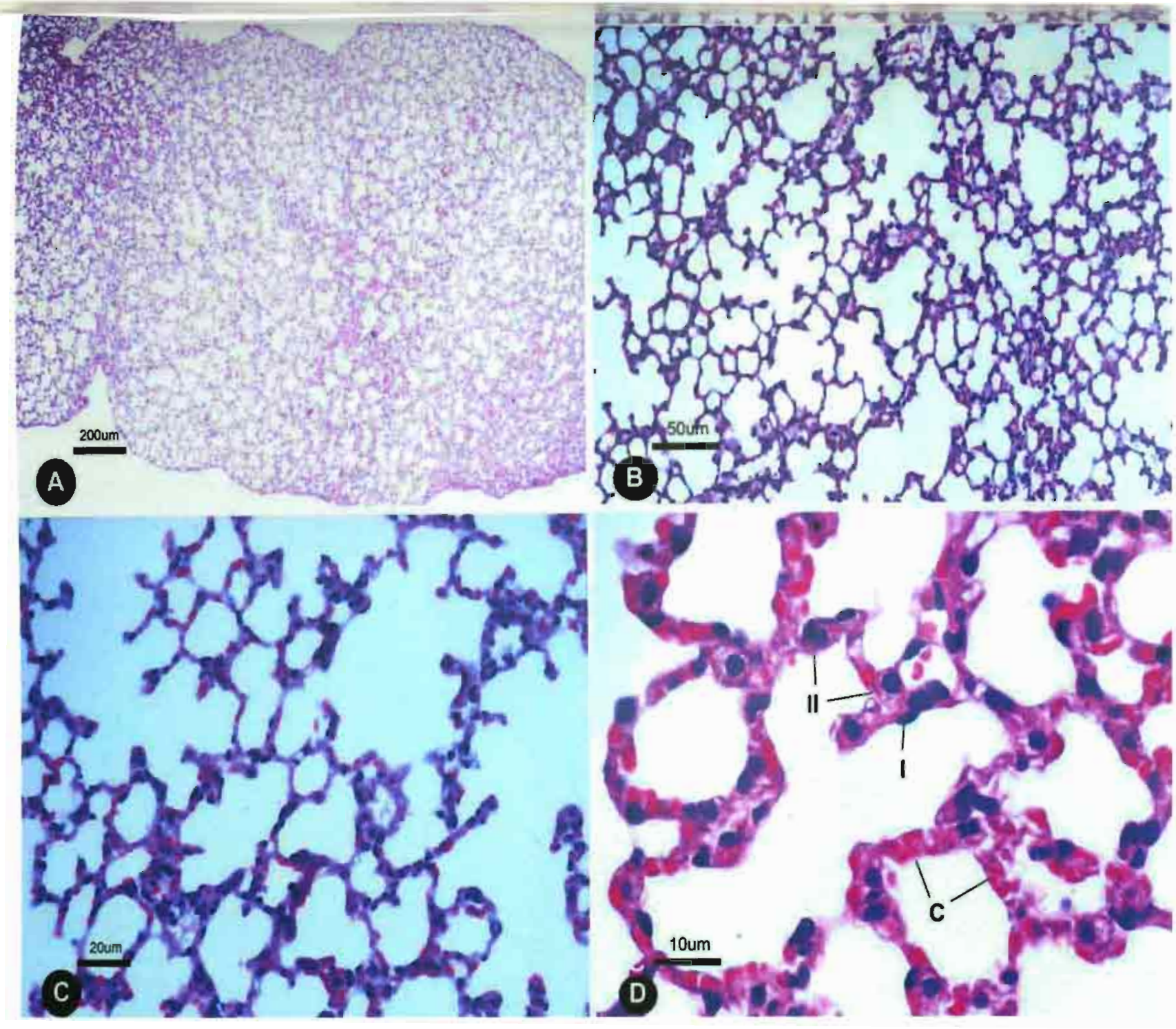

200um
A
50um
B
20um
C
II
I
C
10um
D

Figure 30. Light micrographs of a transverse section of lung of mice after 3 day infected with *S. mekongi* cercarias.

A. Infected with 10 cercarias, lower magnification showing no pathological changes, appearance of normal lung tissue (bar = 200 μm)

B. Infected with 10 cercarias, higher magnification showing the normal appearance of lung tissue (bar = 10 μm)

C. Infected with 30 cercarias, lower magnification showing no pathological changes, appearance of normal lung tissue (bar = 200 μm)

D. Infected with 30 cercarias, higher magnification showing the normal appearance of lung tissue (bar = 10 μm)

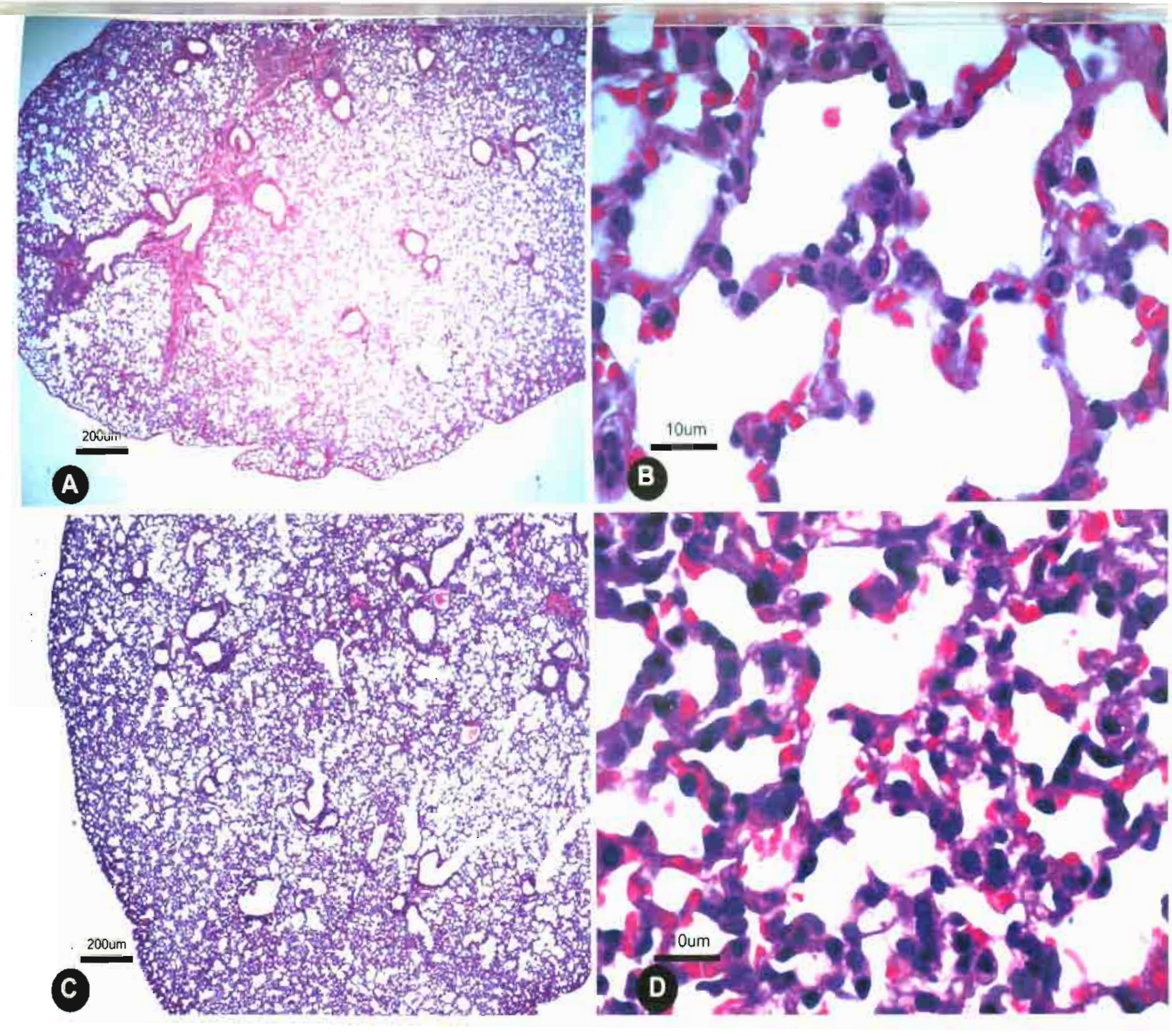

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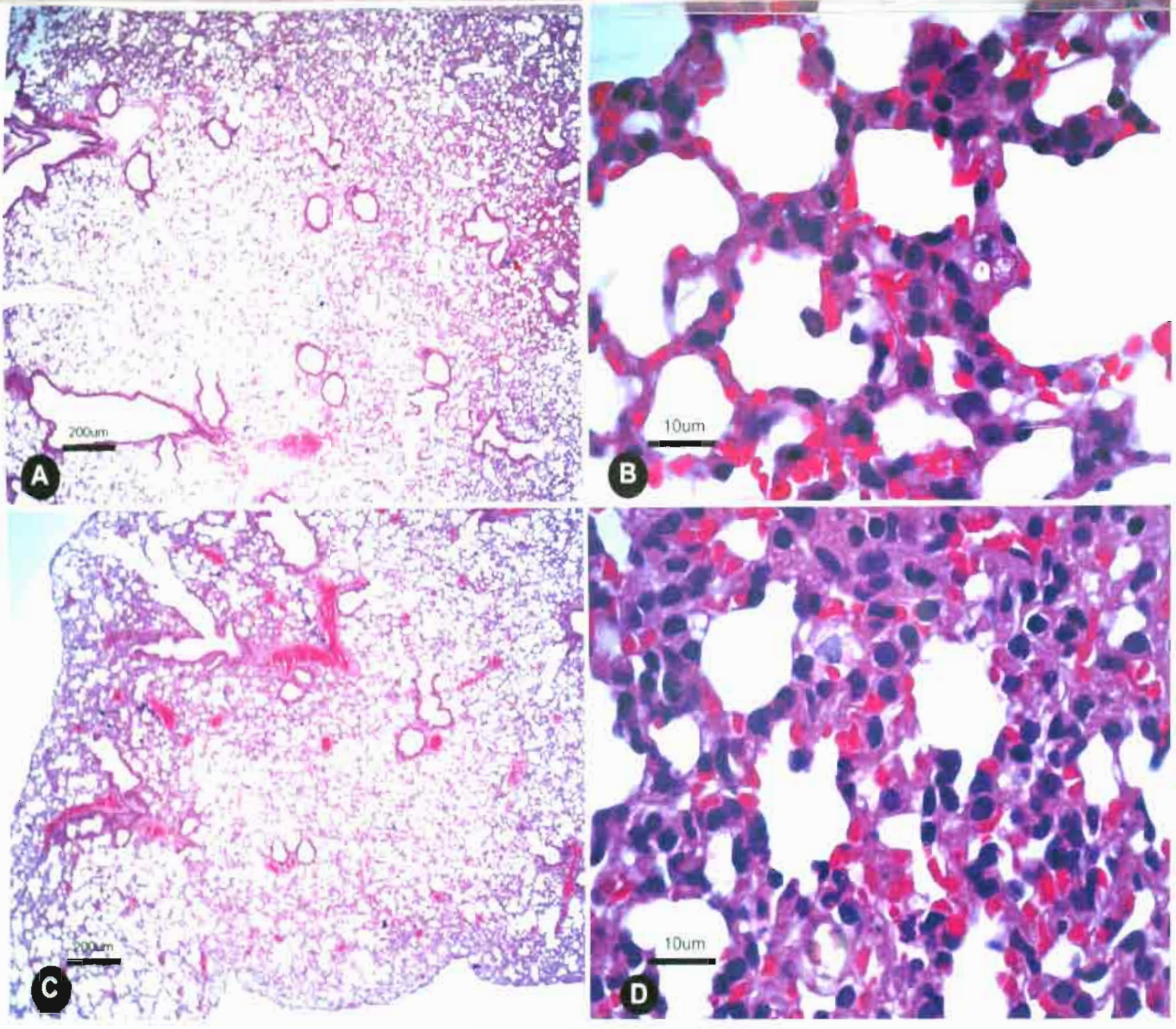
Figure 31. Light micrographs of a transverse section of lung of mice after 7day infected with *S. mekongi* cercarias.

A. Infected with 10 cercarias, low magnification (bar = 200 μm).

B. Infected with 10 cercarias, higher magnification showing mild congestion (bar = 10 μm).

C. Infected with 30 cercarias, low magnification (bar = 200 μm).

D. Infected with 30 cercarias, higher magnification showing moderate congestion (bar = 10 μm).


200um
A
10um
B
200um
C
10um
D