# IMMUNOPEROXIDASE LOCALIZATION OF THE LEPTOSPIRAL ANTIGEN IN THE KIDNEY, SKELETAL MUSCLES AND HEART OF HAMSTERS INFECTED WITH *LEPTOSPIRA INTERROGANS* SEROVAR PYROGENES

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## ABSTRACT

This study is designed to characterize the expression and distribution of leptospiral antigen of *Leptospira interrogans* serovar pyrogenes in kidney, skeletal muscles (Hamstring and Gastrocnemius muscles) and heart of infected hamsters. Six control hamsters and 24 infected hamsters were used and sacrificed at different times of infection. The experimental organs of the sacrificed animals were removed and processed microscopically using the indirect immunoperoxidase staining method. The infected kidneys revealed the positive golden-brown coloration in the cytoplasm of epithelial lining cells of all types of the renal tubule, homogeneous hyalinic material within the renal tubular lumen, the renal corpuscles as well as the red blood cells, and some homogeneous hyalinic materials in the urinary space. The stains were of various intensities depending on the post infection time. This observation may explain the renal histopathology and haematuria occurring in leptospirosis patients. The skeletal muscle of all infected groups showed the positive stain in the muscle fibers, supporting connective tissues and nerve fibers with various intensities. The leptospiral antigens depositing within the skeletal muscle tissue might explain the necrosis and the causation of neuropathy and myalgia in previous studies of leptospirosis. In the infected heart, positive stain was observed in all layers of the heart wall. Evidence of this antigen throughout the cardiac tissue is likely to explain the degenerative changes of cardiac muscle cells observed in infected patients Nevertheless, all infected experimental organs also displayed the leptospiral antigen in the cytoplasm of inflammatory cells. These inflammatory cells might engulf the leptospires themselves or the leptospiral antigens and retain them in their cytoplasm causing a positive stain. This finding might be because the pathogenic leptospires located and multiplied in the blood stream of the infected animals and then invaded many organs of the body,which corresponds to the leptospiral antigen found in the vessel wall of all infected organs. Therefore, the depositing leptospiral antigens might be a primary cause of the histopathology of all the infected organs.

KEYWORDS: LEPTOSPIROSIS / IMMUNOPEROXIDASE / KIDNEY / SKELETAL MUSCLE / HEART

149 pp.

การศึกษาหาตำแหน่ง แอนติเจน ของเชื้อเลปโตสไปร่าในไต กล้ามเนื้อลาย และ หัวใจของหนูแฮมสเตอร์ที่ติดเชื้อ *Leptospira interrogans* ซีโรวาร์ Pyrogenes ด้วยวิธีการทางอิมมูโนเปอร์ออกซิเดส

(IMMUNOPEROXIDASE LOCALIZATION OF THE LEPTOSPIRAL ANTIGEN IN THE KIDNEY, SKELETAL MUSCLES AND HEART OF HAMSTERS INFECTED WITH *LEPTOSPIRA INTERROGANS* SEROVAR PYROGENES)

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บทคัดย่อ

งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาลักษณะ และการกระจายของแอนติเจนของเชื้อเลปโตสไปร่าในไต กล้ามเนื้อลาย (Hamstring และ Gastrocnemius) และหัวใจของหนูแฮมสเตอร์ที่ติดเชื้อ *Leptospira interrogans* ซีโรวาร์ pyrogenes โดยการแบ่งหนูแฮมสเตอร์เป็น 2 กลุ่ม กลุ่มควบคุม 6 ตัว และกลุ่มทดลอง 24 ตัว จากนั้นฆ่าหนูในระยะเวลาต่างๆ กันหลังจากการติดเชื้อนำอวัยวะดังกล่าวมาเข้าสู่กระบวนการทางจุลกายวิภาคศาสตร์เพื่อย้อมด้วยวิธี indirect immunoperoxidase พบว่าไตของหนูที่ติดเชื้อจะพบบริเวณที่ติดสีน้ำตาลทอง ซึ่งเป็นตำแหน่งแอนติเจนของเชื้อเลปโตสไปร่าภายใน cytoplasm ของเซลล์ที่บุท่อไตทุกชนิด สารในท่อไต หน่วยไต เม็ดเลือดแดง และสารใน urinary space ซึ่งความเข้มข้นของสีน้ำตาลทองที่พบในบริเวณต่างๆ จะขึ้นอยู่กับระยะเวลาหลังการติดเชื้อ ตำแหน่งแอนติเจนที่พบนี้จะสอดคล้องกับภาวะปัสสาวะเป็นเลือดและพยาธิสภาพที่พบภายในไตที่เกิดขึ้นในผู้ป่วยโรคฉี่หนู ส่วนกล้ามเนื้อลายจะพบแอนติเจนของเชื้อเลปโตสไปร่าภายในเซลล์กล้ามเนื้อ เนื้อเยื่อเกี่ยวพัน และในเส้นประสาทที่มาเลี้ยงกล้ามเนื้อนั้น ซึ่งการพบแอนติเจนของเชื้อเลปโตสไปร่าในกล้ามเนื้อลายนี้ น่าจะเป็นสาเหตุทำให้เกิดการตายของเซลล์กล้ามเนื้อ ความบกพร่องของเส้นประสาท และอาการปวดกล้ามเนื้อในผู้ป่วยโรคฉี่หนูได้ ส่วนของหัวใจจะพบแอนติเจนของเชื้อเลปโตสไปร่านี้ในทุกชั้นของผนังหัวใจ ซึ่งเป็นสาเหตุที่ทำให้เกิดการเสื่อมของเนื้อเยื่อหัวใจ นอกจากนี้ทุกอวัยวะที่ทำการทดลองในครั้งนี้ยังพบแอนติเจนของเชื้อเลปโตสไปร่าใน cytoplasm ของ inflammatory cells ซึ่งเซลล์เหล่านี้น่าจะมาทำหน้าที่กำจัดเชื้อเลปโตสไปร่าให้หมดไปจากบริเวณที่มีการติดเชื้อ จึงทำให้พบแอนติเจนของเชื้อเลปโตสไปร่าภายใน cytoplasm ของ inflammatory cells ได้ จากการศึกษาที่พบนี้อาจเนื่องมาจากเชื้อเลปโตสไปร่าจะแบ่งตัว เพิ่มจำนวน และอาศัยอยู่ภายในหลอดโลหิตของสัตว์ที่ติดเชื้อ หลังจากนั้นจึงกระจายเข้าสู่อวัยวะหลายอวัยวะภายในร่างกายโดยผ่านทางกระแสโลหิต จึงสอดคล้องกับการพบแอนติเจนของเชื้อเลปโตสไปร่าภายในท่อ และผนังของหลอดโลหิตของอวัยวะทุกอวัยวะที่เกี่ยวข้องในการทดลอง ดังนั้นแอนติเจนเหล่านี้น่าจะเป็นสาเหตุของการเกิดพยาธิสภาพในอวัยวะต่างๆ ที่ติดเชื้อ

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# CHAPTER I
# INTRODUCTION

Leptospirosis is a worldwide zoonotic disease which affects humans and animals. It is an acute febrile illness caused by bacteria of the genus *Leptospira* (Triampo, 2007). In humans it causes a wide range of symptoms, and some of infected persons may have no symptoms at all. Symptoms of leptospirosis including high fever, severe headache, chills, muscle aches, vomiting and may include jaundice, red eyes, abdominal pain, diarrhea, or a rash. If the disease is not treated, the patient could develop renal failure, meningitis, liver failure, and respiratory distress. In uncommon cases death occurs (Leptospirosis, 2005).

**History of Leptospirosis**

Leptospirosis has been around for millions of years, but the bacterial cause of illness was only identified recently. Their strains and the exact way they cause illness and immunity is still in progress. (The history of leptospirosis and Weil's disease leptospires, 2004).

Leptospirosis was first indentified as an occupational disease of sewer workers in 1883. In 1886, a German physician, Adolf Weil described the clinical manifestations in four men who had severe jaundice, fever and hemorrhage with renal involvement (Leptospirosis, 2006). Spirochetes were discovered to cause a wide range of illnesses in the latter half of the 19th Century, but the first credited account of a leptospire segregated from a patient was by Stimpson in 1907. Stimpson named the bacteria *'Spirocheta interrogans'* due to their stained appearrance looking like question marks, and the name has remained (The history of leptospirosis and Weil's disease leptospires, 2004). In 1915, Ryukichi Inada and co-workers in Japan detected both spirochaete bacteria and specific antibodies in the blood of Japanese miners with infectious jaundice. They named the new organism *Spirochaeta icterohaemorrhagiae*. The genus was renamed *Leptospira* in 1917 (Leptospirosis, 2006).

## Epidemiology of Leptospirosis

Leptospirosis occurs in all mammals, such as pets, livestock, wild animals and humans, although in some species it's rare. Crustaceans and fish observed uncommonly. In birds seem to be resistant to infection in nature except when they are very young. Reptiles and amphibians can be infected but not as commonly as mammals. Insects do not involve in being hosts except where they take blood of other animals (Leptospirosis, 2004). Leptospira organisms can also be found in cattle, pigs, horses, dogs, rodents, and wild animals. Humans become infected through the contact with water, food, or soil containing urine from these infected animals. This may occur by swallowing contaminated food or water or through skin contact, especially with mucosal surfaces, such as the eyes or nose, or with skin rupture. The disease is not known to be transfered from person to person (Leptospirosis, 2005). Most outbreaks tend to be seasonal in nature and are often linked to environment factors, to animals and to agricultural and occupation cycles like cultivating rice in marshy land. Outbreaks of leptospirosis occur mainly after flooding, leading to an occupational hazard for sanitary and agricultural workers as well as being recreational hazard for human (Triampo, 2007).

### 1. Worldwide epidemiology of leptospirosis

Leptospirosis is considered to be a major public health problem worldwide (Triampo, 2007). First case was observed in India in the early 20$^{th}$ century. Leptospirosis is endemic in several countries with sporadic seasonal outbreaks. Its event is more frequent in the Andaman Islands (Hasnain, 2004).

A great number of pulmonary hemorrhage due to leptospirosis was first described in China in 1965 and was later seen in Korea in 1984 and 1985 (Trevejo, 1998). In the Korean epidemic of 1987, the primary cause of death was acute respiratory failure due to overwhelming pulmonary hemorrhage, while no patient was reported to have renal failure severe enough to cause death or require dialysis. In the early 1980s, Brazilians reported leptospirosis associated with acute respiratory distress, hypoxia and hemoptysis also found without jaundice or renal failure. In 1988,

there were only 3 reported fatalities from heavy pulmonary hemorrhage, but pulmonary disease is now one of the dominant causes of *Leptospira*-associated mortality in some regions of Brazil (Plank, 2000).

*Leptospira* have also been observed to be responsible for yearly outbreaks since 1988 of pulmonary disease, including hemorrhage, on the Andaman and Nicobar Islands. These were the first recognized cases of pulmonary leptospirosis in India, but pulmonary and cardiac complications are now the leading cause of leptospiral deaths in many parts of that region (Kuriakose, 1997).

In 1995, a dominant outbreak of leptospirosis in Nicaragua was involved by pulmonary hemorrhage. Of the 2,259 patients with non-malarial febrile illnesses, there were 40 fatalities attributed to leptospirosis. All of these fatalities were due to pulmonary hemorrhage and acute respiratory distress syndrome with no evidence of jaundice or renal involvement (Trevejo, 1998). Life-threatening pulmonary involvement has also been reported recently in Australia and on the French territory of Réunion Island (Paganin, 1996).

In July 1998, athletes from 44 states of the United States and seven countries participated in triathlons in Springfield, Ill. The Illinois Department of Health, the U.S. Department of Agriculture, and the Center for Disease Control investigated an outbreak of acute febrile illness in which 110 of 1194 (9.2%) participant experienced chills, headache, myalgia, diarrhea, eye pain or conjunctivitis. The investigation indicated that *Leptospira* bacteria caused illness in some of the athletes exposed to Lake Springfield, where the Illinois triathlon was held (Update: leptospirosis and unexplained acute febrile illness among athletes participating in triathlons--Illinois and Wisconsin, 1998).

## 2. Leptospirosis in Thailand

In Thailand, leptospirosis is becoming a major concern of the public health officials and control strategies are being developed. It occurs mainly in the rainy season, with an increase in cases beginning in August, reaching a peak in October, and beginning to fall in November (Triampo, 2007).

Leptospirosis was first recorded in Thailand 62 years ago. The incidence had dramatically increased since 1996, with a peak in 2000. During this period, leptospirosis showed the same seasonal variations and affected the same high risk groups, mostly agricultural workers. The root and elicit factors of the epidemic are still unknown. The most probable views for the widespread are:

1) Climatologically and ecological conditions in the Northeast, favor the transmission of the disease during the rainy season.
2) The changing ecology and epidemiology of domestic animals and changes in the agricultural practices in the Northeast.
3) Increase in rodent density resulting in an increased risk of infection.
4) Physicians and villagers have increased their awareness and ability to recognize the disease and seek health care, and an increase in the availability of laboratory testing.
5) The increase may be due to an associated shift in the predominant infecting serovars.

A number of interventions were implemented in order to reduce the incidence of leptospirosis among rice farmers in the Northeast. This included advising the use of boots and gloves during farming work, and large rat extermination campaigns. Boots have been provided to local rice farmers for barrier protection (Tangkanakul, 2005).

**Risk Occupations**

The main occupational groups at risk today include farm and agricultural workers, veterinary surgeons, pet shop workers, plumbers, abattoir workers and meat handlers, coal miners, workers in the fishing industry, sewer workers and the military. Other groups at high risk of contacting leptospirosis include the survivors of natural disasters (e.g. flooding) and the increasing number of people engaging in recreational water sports (Leptospirosis, 2006).

**Taxonomy of *Leptospira interrogans***

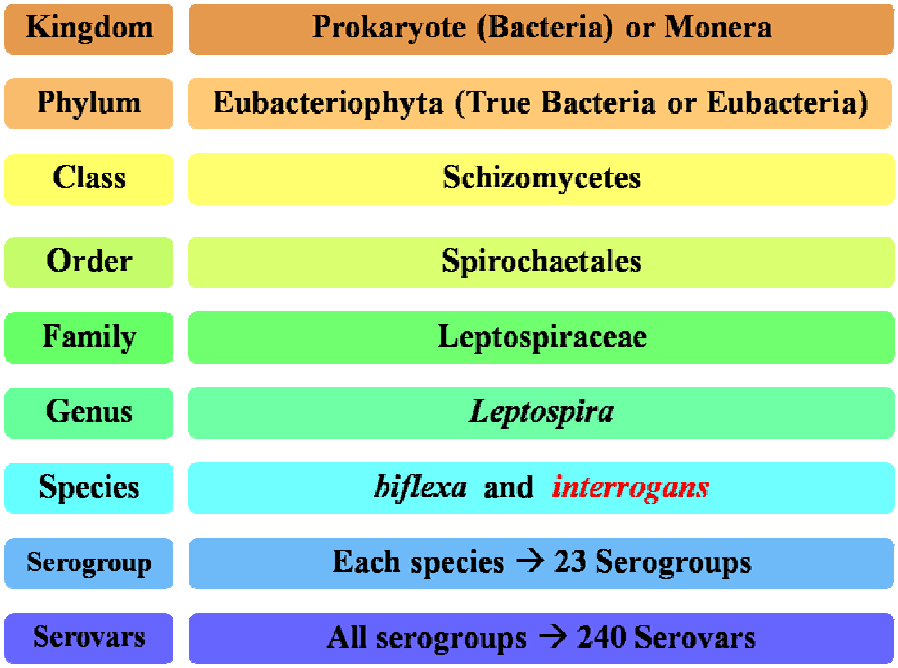



**Diagram 1** Taxonomy of *Leptospira interrogans*

The *Leptospira interrogans* is in the phylum eubacteriophyta, class schizomycetes and in order spirochaetales. The family Leptospiraceae contains only three genera: *Leptonema, Turneria* and *Leptospira*. The genus *Leptospira* comprises 10 genomospecies, of which the most important are the pathogenic *L.interrogans* and the non–pathogenic *L.biflexa*. Each genomospecies is subdivided into about 23 serogroups, into which are placed individual serovars. To date, about 240 serovars have been characterized (Diagram 1).

Prior to the development of DNA analysis, classification was by serological cross-testing (using serum antibodies to identify similar and different types of bacteria) (Leptospirosis, 2006).

Lipopolysaccharide (LPS) is the major antigen involved in serological classification. Structural heterogeneity in the carbohydrate component of LPS moieties derived from differences in the genes involved in LPS biosynthesis appears to be the basis for the large degree of antigenic variation observed among serovars (Peña - Mactezuma, 1999).

## Spirochaete Bacteria

Order Spirochaetales, the term spirochetes will be used to encompass this group of flexous, thin, gram-negative, chemoheterotrophic, helical-shaped organisms, which differ morphologically from other prokaryotes by the presence of an axial fibril, known also as an endoflagellum, axistyle, or axial filament (Holt, 1978). As in Gram-positive bacteria, the cytoplasmic membrane of spirochaetes is nearly associated with the peptidoglycan cell wall. Spirochaetes also have an outer membrane which contributes a barrier defending intrinsic antigens, such as the endoflagella, from the external environment (Haake, 2000). By current definition, any prokaryotic cell with an axial fibril is considered a spirochete (Holt, 1978).

## Biology of Leptospires

*Leptospira* morphology is uncommon among spirochetes as it has individuality hooked ends. They are helical form (Plank, 2000) which is tightly coiled spirochetes, usually 0.1–0.3 μm in width and 5–25 μm in length. The cells have sharp ends, either or both of which are regularly curved into a distinctive hook. Two axial filaments (periplasmic flagella) with polar insertion (insertion pore) are located in the periplasmic space. Leptospires have a typical cell envelop in common with other spirochetes, in which the cytoplasmic membrane and peptidoglycan cell wall are closely associated and are overlain by an outer sheath (Fig 1) (Haake, 2000). Leptospiral lipopolysaccharide has a composition similar to that of other gram-negative bacteria, but has lower endotoxic activity (Levett, 2001).

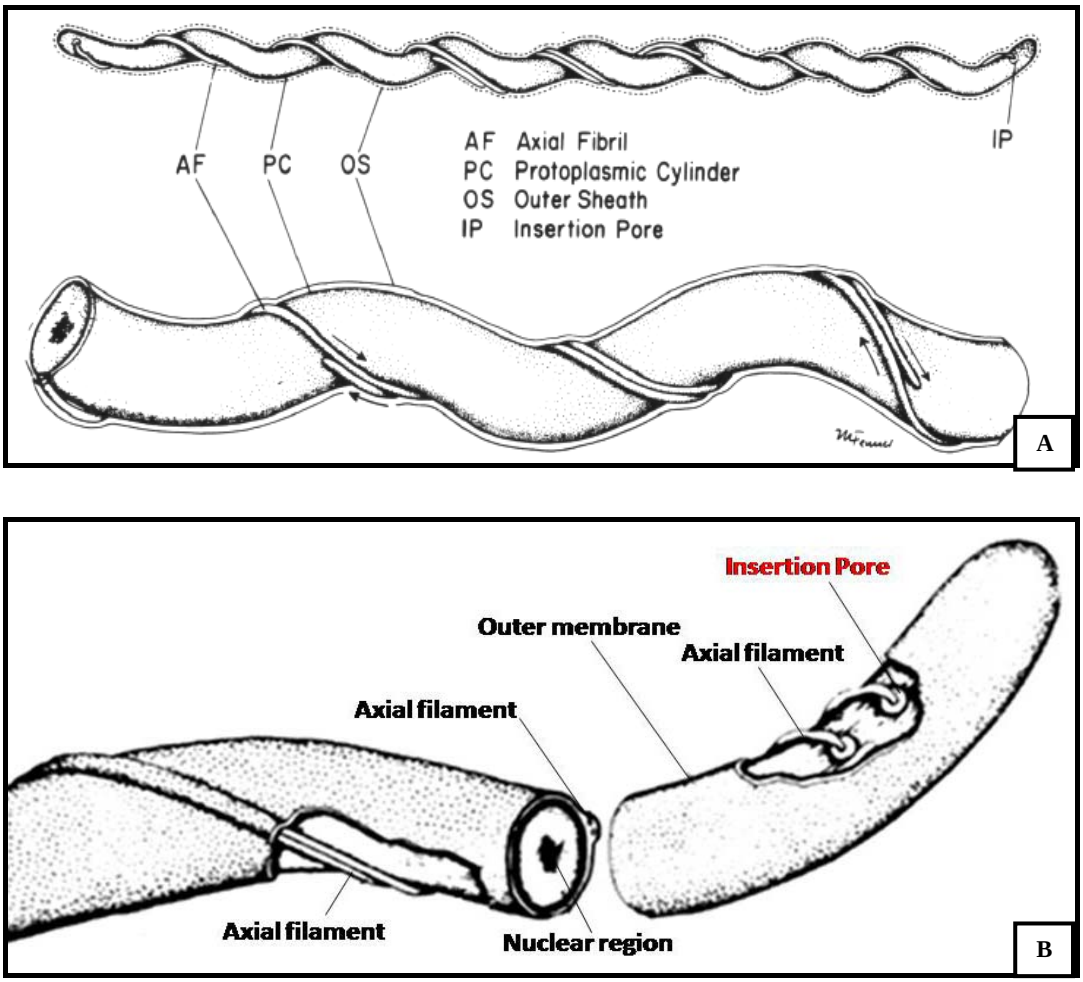



**Figure 1** Diagrammatic representation of a typical leptospire as interpreted from electron micrographs shows (A) the basic anatomical components of leptospires. (B) An outer sheath covers the cell. The axial fibrils are between the outer sheath and the layers of the protoplasmic cylinder and insert into the cylinder by way of an insertion pore.

**1. Outer sheath (Outer membrane)** (Figure 1 – 3)

The outer sheath is seen as the most external layer of the cell (Holt, 1978), which provides a barrier shielding underlying antigens from the environment (Haake, 2000). In negatively stained preparations, the outer sheath of most spirochetes appeared to be composed of polygonal structural subunits with a diameter of 0.8 nm separated from each other by 0.2 nm wide partitions. (Figure 2) In the center of each

unit, a central core approximately 0.25 nm in diameter was frequently found (Listgartan, 1964).

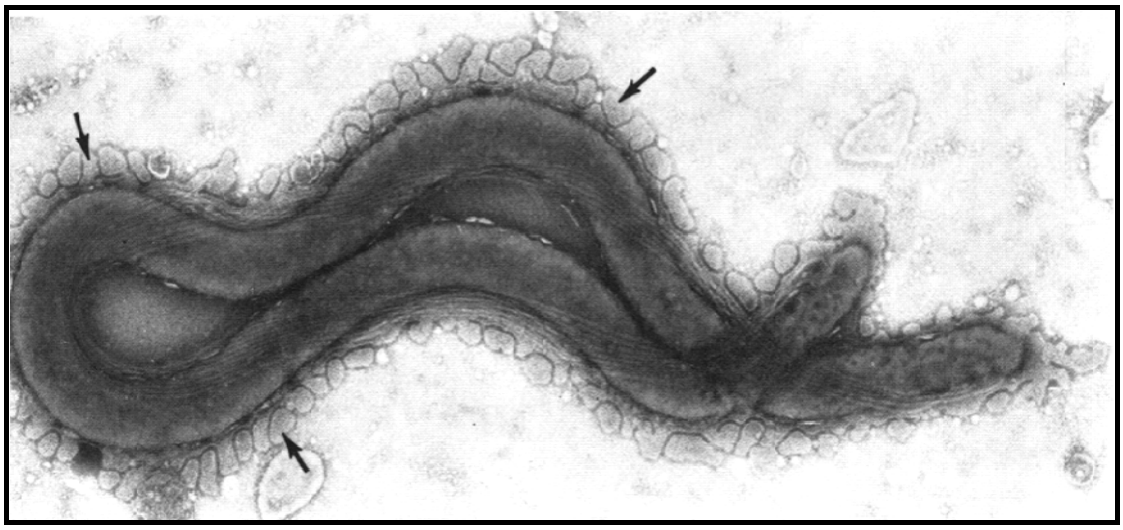


**Figure 2** Photomicrograph shows the polygonal structural subunits of outer sheath. Negative contrast ×29,900.

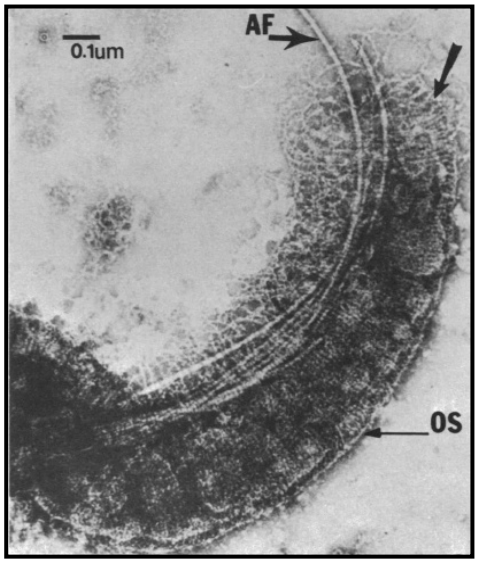



**Figure 3** Photomicrograph demonstrated the spirochete with a striated or fibrillar outer sheath (arrow)

These layer of leptospires contain fibrillar element that run transversely across the cell (Fig 3) which known as ‘transverse striation’. Fine, transverse striation, have a periodicity of 45 nm, in the outer sheath of *Leptospira interrogans* pomona.

**Chemical composition of the outer sheath** (Holt, 1978)

The isolated materials from the outer sheath of *L.interrogans* pomona contains protein, lipid and carbohydrate and are similar to the outer membranes of gram–negative bacteria. Essentially all lipid found in the outer sheath of *L. interrogans* is phopholipid with almost 98% of it occurring as phophatidylethanolamine. The outer sheath is the outermost layer of spirochetes; it may interact with host defense mechanisms.

Lipopolysaccharide (LPS)

Several reports in the literature indicated the presence of a lipopolysaccharide (LPS) layer in outer sheath of spirochetes. The outer sheath contains a variety of lipoproteins and transmembrane protein (Cullen, 2002). Isolated outer sheath of *L.interrogans* pomona, which is assumed to contain the LPS, is known to contain hexose , pentoses , hexosamine and 6-deoxyhexose (Holt, 1978). Several leptospiral outer membrane proteins may be important for adhesion of *Leptospira* to host tissues and in resisting complement, respectively (Barbosa, 2006; Choy, 2007; Verna, 2006).

The outer sheath of *Leptospira*, like those of most Gram-negative bacteria, contains lipopolysaccharide (LPS). Variation of the highly immunogenic LPS structure regards for the numerous serovars of leptospires (Levett, 2001). Furthermore, leptospiral LPS has low endotoxic activity (*Leptospira*, 2008).

**2. Protoplasmic cylinder** (Figure 4)

The general contour of the protoplasmic cylinder extends various distances beyond the wall of the cylinder. Cross section indicate that the substance within the cylinder is less electron dense than its wall (Miller, 1962). The protoplasmic cylinder of these organisms contained alanine, glutamic acid, glucosamine and muramic acid (Holt, 1978).

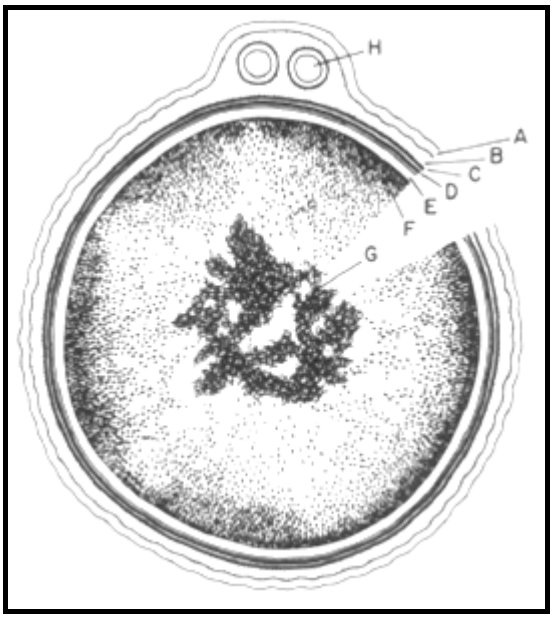



**Figure 4** Interpretative drawing revealing the morphology of leptospires. The outer sheath (A), envelopes the cell. Situated in the electron-opaque periplasmic region (B) are the axial filaments (H). The layer of the protoplasmic cylinder consists of the outermost peptidoglycan layer (D), and internal to the peptidoglycan which is the innermost layer of the cell is the cytoplasmic membrane (E). The cytoplasmic region contains ribosome (F) and a centrally disposed nuclear region (G).

**3. Cytoplasmic membrane** (Figure 4)

The innermost triple-layered membrane bounded the cytoplasm giving a tubular appearance (Ritchie, 1965). The membrane was of unit dimension, approximately 6 – 8 nm in thickness. The observation showed that this cytoplasmic membrane in *L.interrogans* pomona was asymmetric—the outer leaflet was thicker and more electron dense than the inner leaflet (Holt, 1978).

**4. Axial filament (fibril or periplasmic flagella)** (Figure 5A)

*Leptospira* have two axial filaments, one attached subterminally at each end, that extend toward the cell's center without overlapping (Faine, 1999). Each fibril seemed to be inserted at one end of the organism only, the free end of each axial

filament extending toward the opposite end for a distance which varied from cell to cell (Listgartan, 1964).

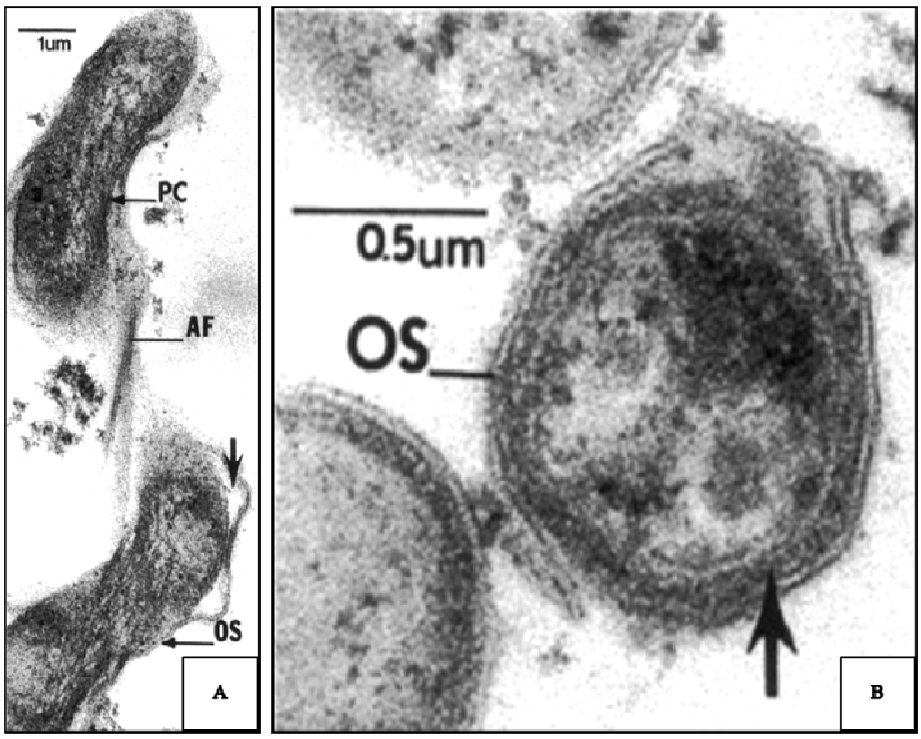



**Figure 5** Electron micrographs of thin sections of chemically fixed cells of *L.interrogans.* It has an empty or electron-transpatent periplasmic space (A, arrow) and display a tight-fitting outer sheath (B) and an entrapped electron-dense periplasmic space (B, arrow).

**Life cycle of pathogenic leptospires** (Diagram 2)

**1. Survival of leptospires in the environment**

*Leptospira* exists in two groups, the pathogenic parasitic types and the free-living saprophytes. All require the same basic to survive (water, oxygen, stable pH and temperature) but their life cycles and food requirements are different. It was formerly thought that saprophytic leptospires could transform into pathogens after they entered a host, but this is now known to be untrue (Survival of leptospires in the environment, 2004).

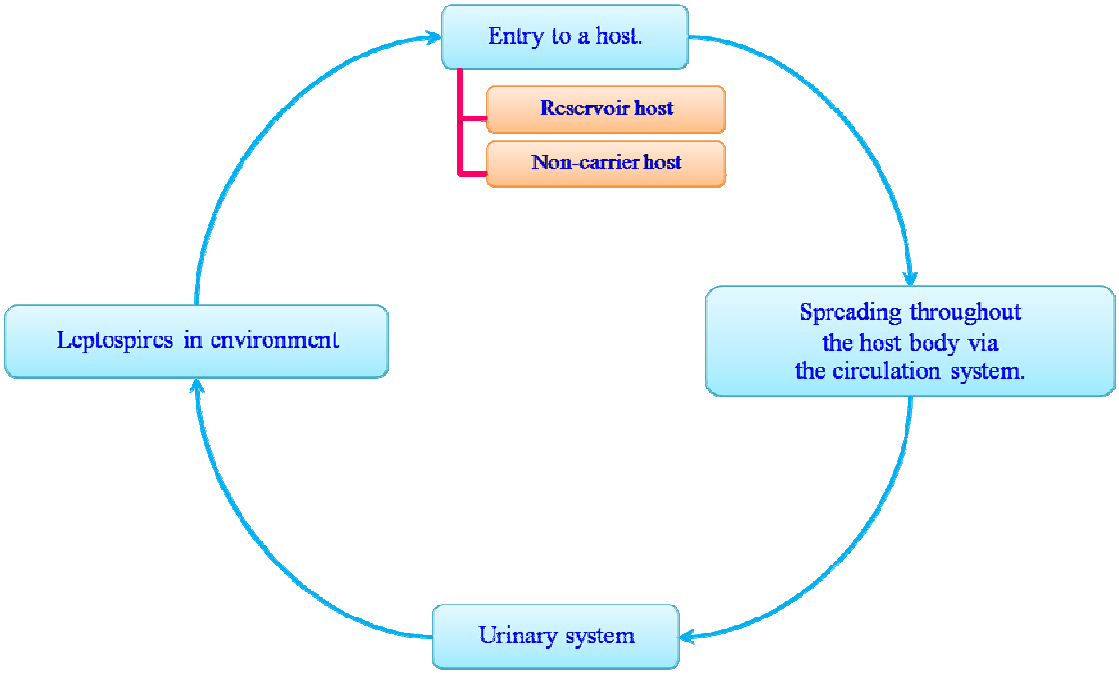



**Diagram 2** Life cycle of pathogenic leptospirosis.

Saprophytic leptospires

They live as free-swimming bacteria in the water and do not use an animal host for their life cycle. They feed from microscopic degenerate organic matter in the water and will survive and reproduce anywhere with suitable water condition and supply of organic materials. They cannot cause significant infection and their only interest, apart from being some of the most numerous creatures on the planet, is that they can because positive reactions in some laboratory tests, making analysis of the 'safety' of water samples more difficult. The presence of saphrophytic leptospires in a body of water is not a sign that it is unclean or contaminated, in fact the bacteria prefer clean water without chemical pollution. It also has no bearing on the likely presence of pathogenic strains, however there is a theory that their widespread presence could explain why many fish and amphibians are immune to infection from the pathogenic strains - these species may have developed antibodies because of constant exposure to the saphrophytes even though they were not made ill by them (Survival of leptospires in the environment, 2004).

**Pathogenic parasitic leptospires**

They require a host in order to complete their life cycle, and whilst they can be cultivated in prepared serum they do not support stable colonies outside of the host. Survival and duplication within the host animal is of course determined by the host's immune response and species, what is of concern here is the survivability of the bacteria outside the host. Pathogenic *Leptospira* causes illness in their host, although of course they do not intend to do so - in fact many researchers argue that illness is a bad idea for *Leptospira*, since a host that stays alive will be able to shed more of the bacteria in their urine. However the bacteria clearly haven't worked that out yet. (Survival of leptospires in the environment, 2004)

Outside a host animal, or the lab, it is believed that pathogenic leptospires have a close to zero reproductive output - in some cases limited binary fission has been seen, possibly caused by long-term absence of a host species, but in general terms the bacteria do not multiply. Individuals can however survive for extended periods if the conditions are suitable, so whilst a colony in a pond or lake may not increase over time, it is also unlikely to decrease particularly fast either. Where carrier hosts regularly add fresh urine to an environment the pathogenic colonies can maintain an almost continuous presence (Survival of leptospires in the environment, 2004).

### 1.1. Temperature

Pathogenic leptospires reproduce best at body temperature, but can survive over a wide range. They appear to be unable to tolerate temperatures over about 42°C, which can explain why bacterial growth is reduced or reversed in patients with very high fevers, with no reports of survivial when heated to over 55°C. Cold is easier to tolerate, and they can be frozen (in ice or liquid nitrogen. Leptospires have been isolated from kidneys that have been deep-frozen as part of the food distribution chain. Their level of activity and ability to reproduce drops when cooled below 10°C (Survival of leptospires in the environment, 2004).

### 1.2. Moisture

As leptospires do not have a waterproof membrane they must remain immersed in water to survive, and so are killed immediately when their environment dries out. The inability to survive out of water is the single most important control factor in the natural environment, as it means they are unable to create infection risks from dry surfaces (Survival of leptospires in the environment, 2004).

### 1.3. Chemicals

Pathogenic leptospires are extremely sensitive to chemicals of all kinds and so are quite easy to kill. Detergents, acids and heavy metals are all lethal at very low concentrations and this means that pathogenic colonies find it difficult to survive in very polluted water - so the worst industrial effluent is (in terms of leptospirosis at least) safer than a pure mountain lake (Survival of leptospires in the environment, 2004).

## 2. Entry to a host

The bacteria entry the host via portals such as damaged skin, certain mucous membranes, the lungs and conjunctival membranes. They are not thought to penetrate the undamaged skin except where it has been exposed to water and has distended significantly. Transpose to the portal requires the bacteria to be covered in water and so normally associates direct contact with urine or water containing the bacteria in suspension. Entry via the lungs requires breathing of aerosol droplets and not the bacteria alone. Leptospires cannot exist as spores or reactivate once desiccated in the natural environment (Life cycle of pathogenic leptospires, 2004).

Formerly within the host tissues, pathogenic strains can duplicate as they are optimized for metabolism at body temperatures. Their survival depends on the lack of an effective host immune response, but they do not seem to cause an inflammatory reaction and so in a host without adapted immunity the chances of being able to establish a positive growth curve are high. In virulent strains the bacteria are encountering from the innate immune system and so can multiply rapidly, until the

adaptive system has a change to select and replicate a cognate antibody. Saphrophytes, and the pathogens that are less harmful, seem to be easily targeted by the innate immune system and so are dislodge (Life cycle of pathogenic leptospires, 2004).

Reservoir hosts

Many species in the environment will maintain the bacteria as a viable population, via urinary shedding. Reservoir hosts are carrier-state species and thus are regularly not clinically fever, or have a suitable short natural life that the infection has no bearing on host populations. Rats are the main reservoir host but other rodents, marsupials and wild mammals are recognized to perform the function. Humans can not become the carrier-state and so are accidental to the bacterial life cycle. Reservoir hosts can cross-infect each other by urine contraction. Sexual transmission is known to occur in many species (Survival of leptospires in the environment, 2004).

Non-carrier hosts

These hosts are infected either directly from carrier-state hosts through contact with urine or via the environment where urine from infected hosts has been able to remain viable in water or soil. Non-carrier hosts are a omission in the bacterial ecosystem as for genetic dissemination the bacteria of progress prefer a host that will survive for as long as possible and shed as many bacteria as possible-non-carrier hosts often die from their disease and tend to be less competent urinary shedders while they are still alive. However leptospires do not recognize which hosts they are infecting and so many species pay the price simply by being in the wrong place at the wrong time (Survival of leptospires in the environment, 2004).

**3. Growth**

Leptospires that survive entry and the immediate innate response will rapidly migrate to the bloodstream and lymphatic system, so spreading throughout the host body within a very short time. A virulent strain are rapidly removed by the immune system, but the speed of spread is far higher than other bacterial infections and a host can show leptospira within blood samples in a matter of minutes from exposure.

Pathogenic bacteria duplicate by binary fission, so the colony increases exponentially, and the typical duplicate time is 8 hours. The growth continues unverified until the adaptive immune response generates or the host dies. Virulence is directly associated to pathogenicity - the host permits illness because of the sheer number of leptospires in their body (Survival of leptospires in the environment, 2004).

## 4. Transmission (Figure 6)

Leptospirosis is spread through contact with water, soil, vegetation, or any part of a moist environment contaminated by urine or tissue of infected animals or humans. These bacteria can be inactivated by drying but can survive in a moist environment for weeks or months. There are two forms of transmission, direct and indirect transmission. Humans and animals can become infected through direct contact with infected urine or other body tissues (MacAllister, n.d.).

### 4.1. Direct transmission

Direct transmission occurs when blood or body fluids containing leptorspires pass directly from an acutely infected animal or from a renal carrier animal's urine to another susceptible animal. Modes of direct transmission include transplacental or haematogenous (congenital) infection, sexual contact and suckling milk from an infected mother. Carriage of leptospires in the genital tract and other tissues of some animals have been demonstrated and presumably play an important part in the venereal transmission of leptospires within a single breeding species. This is the usual route for transmission of leptospires in a maintenance host species. Transmission can occur with semen used for artificial insemination and with embryos used for *in vitro* fertilization. Direct transmission of leptspirosis from animals to humans also occurs, as an occupational disease of handlers of animals or animals products, or when acquired from pet animals (Faine, 1999).

The presence of leptspires in brain and eye may be considered unimportant for general transmission, except in the connection between humans and animals which they hunt or husband for food. People can be infected from them while dressing or processing carcasses for meat and meat products (Faine, 1999).

### 4.2. Indirect transmission

Indirect transmission occurs when an animal acquires leptosirosis from environmental leptospires originating from the urine of excretor or carrier animal. Particularly, surface waters (ponds, lakes, rivers and streams), sewage, slaughterhouse drainage fluids, drain water, mud and soil harbor leptospires for variable durations. Indirect transmission may be facilitated by water birds, whose wet feet and feathers can transfer leptosires from one environment to another, or by arthropods (ticks or fleas). The question of the maintenance of pathogenic leptosires and their growth in the environment in soils or surface waters is dealt with elsewhere. The various animal species that can be affected by indirect transmission may be reservior hosts or non-carrier host (Faine, 1999).

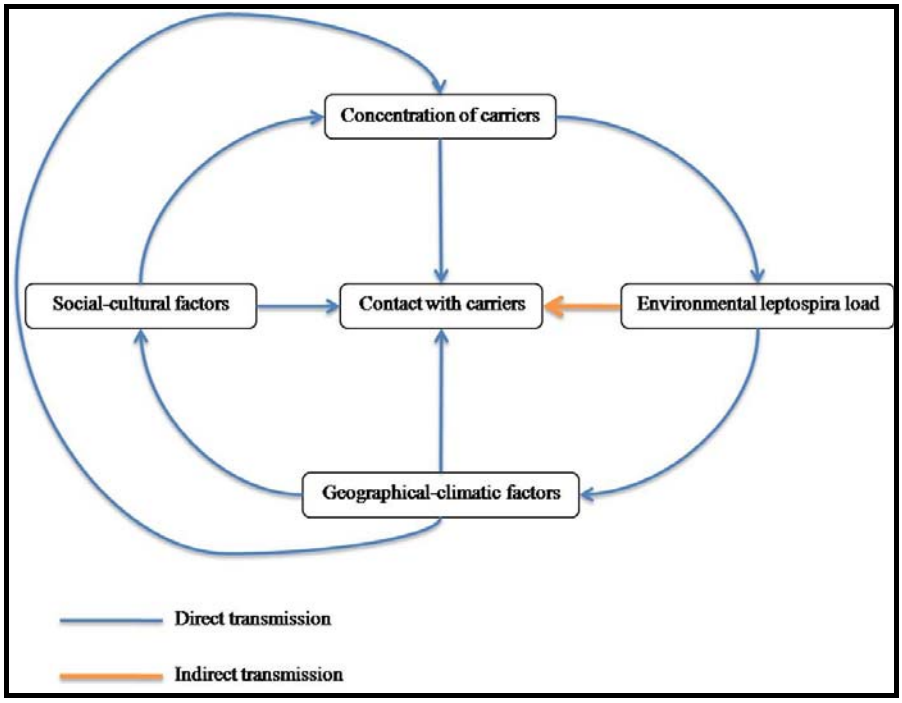



**Figure 6** Factors affecting epidemiology of leptospirosis. Contact with carriers is central, influenced in turn by the other factors. The environmental leptosiral load affects only indirect transmission. (Modified from S. Faine. *et al.*, 1999)

**Clinical Menifestation** (Rubin, 2005)

The symptoms of leptospirosis began 4 days to 3 weeks after exposure to *L.interrogans.* In most cases, the disease resolved within a week without sequelae. In more severe infections, leptospirosis was a biphasic disease including leptospiremic and immune phases.

1. **The leptospiremic phase wa**s characterized by the presence of leptospires in the blood circulation and cerebrospinal fluid (CSF). There was an abrupt onset of fever, shaking chills, headache and myalgias. After 1 to 2 weeks, the symptoms abated as the leptospires disappeared from the blood and body fluid.
2. **The immune phase** which began within 3 days of the end of the leptospiremic phase was accompanied by the production of IgM antibodies. The earlier recurrent symtoms and indications of meningeal irritation become apparent. At this time, the cerebrospinal fluid reveals a prominent pleiocytosis. In severe cases, jaundice arises and may be followed by hepatic and renal failure and the appearance of pervasive hemorrhages and shock. This severe form of leptospirosis has documentally been referred to as “Weil disease”

Untreated Weil disease holds up a mortality degree of 5 to 30%. At autopsy the tissues of the patients of Weil disease are hemorrhages in several organs. Microscopically, the chief lesion is a diffuse vasculitis with capillary injury. The liver shows disorganization of the hepatic cord, erythrophagocytosis by Kuffer cell, minimal necrosis of hepatocytes, neutrophils in the hepatic sinusoids and a mixed inflammatory cell infiltration in the portal triad. The kidneys show swollen and necrotic tubules. Organisms are numerous in the lumina of the renal tubule (Rubin, 2005).

**Pathology of leptospirosis**

Grossly, jaundice is almost always present in fatal cases. Hemorrhages are apparent in many organs and tissues such as skeletal muscles, lungs, kidneys and liver etc (Damjanov, 1996). Historically, icterus has been considered the criterion for

distinguishing mild forms of the disease from Weil's syndrome, which is characterized by a combination of hepatic and renal impairment, hemorrhage, and vascular collapse, which can be rapidly fatal (Plank, 2000).

**1. liver**

Jaundice is mostly a product of liver disorganization more than necrosis of hepatocytes. *Leptospira* scatter to the sinusoids of the liver, the space of Disse, and parenchymal cells. They are also able to attack the space between parenchymal cells, disorganizing the caniculi and releasing bile into the systemic circulation, with subsequent hyperbilirubinemia usually without concurrent liver enzyme elevation. Sinusoids might be disrupted or obliterated. Kupffer cells are usually swollen, and may carry *Leptospira* and erythrocytes. Focal hemorrhage, interstitial edema, petechiae, and necrotic foci are also documented (Arean, 1962). As in the study of Pilakasiri *et.al.,* 1999 which studied in organs of hamsters infected with *L.interrogans* serovar pyrogenes, the liver showed the disorganization of the hepatic cord, cloudy swelling of the hepatocytes, congestion of the hepatic blood vessels and hepatic sinusoid, prominent Kupffer cells, focal necrosis of the hepatocyte, inflammatory cell infiltration in the hepatic parenchyma and hepatic sinusoids as well as the portal area (Pilakasiri, 1999).

*Leptospira* could induce hepatocyte apoptosis, which could limit the inflammatory response, allowing microbial proliferation in that organ. If so, this multiplication might direct to the increase bacterial numbers and greater systemic morbidity, perhaps partially explaining why severe disease has been traditionally associated with jaundice (Merien, 1998). Icterus is no longer an accurate marker of disease severity, as clinical forms of leptospirosis are more frequently manifesting without it. In a current series from India, only 20/52 patients who died were icteric (Kuriakose, 1997). Among survivors, liver function documentedly restored to baseline (Plank, 2000).

**Kidney**

Renal involvement is usual, historically accounting for the largest ratio of deaths from leptospirosis. Pathologic findings are according to the hypoxic nephrosis, although the paucity of lesions together with renal function impairment proposes that there is toxin-mediated intracellular damage. Acute vasculitis and hemorrhage followed by interstitial edema, tubular epithelial necrosis, and basement membrane disorganization (Arean, 1962). These changes are considered by abnormal urinalyses, frequently displaying proteinuria, sterile pyuria, hematuria, and hyaline and granular casts. Serum urea and creatinine also tend to be increased

In infected kidney showed numerous histological changes including degenerative changes of the renal tubular cells and the glomerular tuft, congestion of the renal blood vessels, hemorrhage and inflammatory cell infiltration. The renal tubular cells showed vacuolar degeneration. They were enlarged and contained lightly stained vacuolated cytoplasm. The nuclei were still uninjuried with a vesicular appearance. The boundary of each cell was hard to distinguish (Pilakasiri, 1999)

**2. Lung**

The incidences of pulmonary disease are observed in leptospirosis patient.The pathologic findings in patients with pulmonary disease are predominantly hemorrhagic rather than inflammatory, although many of the patients with hemorrhage do not have hemoptysis. The findings reveal diffuse petechiae and hemorrhage throughout the respiratory tract including the trachea, alveoli and interstitium. Pulmonary vessels show blebs, endothelial sloughing, platelet and leukocyte thrombi as well as suggesting the hemorrhagic infarction. The hypoxia may worsen vascular damage and exacerbate hemorrhage. Pulmonary edema is the most distinct in cases presenting with myocarditis. Hyaline membranes are observed in alveoli and bronchioles, and seldom, leptospiral fragments and whole organisms (Arean, 1962). Not surprisingly, respiratory degeneration can be rapid (Plank, 2000).

Histopathologically, the infected lung sections displayed a small number of alveoli filled with numerous red blood cells and inflammatory cells. The alveolar and

interalveolar capillaries were distended and engorged with red blood cells. The infiltration included predominantly of neutrophils. The alveolar septum was slightly thickened by a predominantly interstitial infiltration (Pilakasiri, 1999).

**3. Skeletal muscle**

Musculoskeletal injuries are common and not well localized, while myalgias have a predilection for the lower extremities. Muscle biopsies have revealed leptospiral organisms as well as focal hemorrhages and sarcoplasmic degeneration (Arean, 1962). Rhabdomyolysis has occurred in a number of cases (Plank, 2000).

4. **Heart**

Cardiac involvement is usual in severe disease and can be as myocarditis, congestive heart failure. The infected heart showed numerous histological changes including degenerative changes of the cardiac muscle cells, congestion of the cardiac blood vessels, hemorrhage and inflammatory cell infiltration (Plank, 2000).

The cardiac muscle cells displayed vacuolar degeneration. They were expanded and contained a lightly stained vacuolated cytoplasm. The nuclei were still normal with a vesicular appearance. The boundary of each cell was hardly distinguishable (Muensoongnoen, 2006).

**5. Blood vessel**

Pathologists' findings support the theory that diffuses vasculitis is responsible for many signs and symptoms of leptospirosis. Autopsies of humans and other animals indicated pervasive hemorrhage, consistent with epistaxis, hemoptysis, hematemesis, melena, conjunctival suffusion, skin rashes and other bleeding diatheses often seen with leptospirosis. Bleeding occurs in the skeletal muscles, cardiac muscle, lungs, pleural space, peritoneum, adrenal gland, kidneys, liver, and subarachnoid space. No evidence of propagated intravascular coagulation has been documented. Although thrombocytopenia is common, hemorrhagic disease occurs regularly even in its absence (Nicodemo, 1990). Clotting times can be increased but corrected with vitamin K (Plank, 2000).

Vasculitis appears to affect mostly the capillaries, according to the fact that lesions are most severe in filter organs such as the liver, lungs, and kidneys. The endoplasmic reticulums of endothelial cells are usually dilated and mitochondria tend to be enlarged. The endothelial junctions can be separated and fenestrate expanded with extravasation of inflammatory and red blood cells, as well as *Leptospira* along with necrosis of endothelial cells (De Brito, 1979).

## Pathogenesis of leptospirosis

The process by which leptospires cause disease is not well understood. A number of putative virulence factors have been suggested including toxin production, attachment, immune mechanism, surface protein and immunity but with few exceptions their role in pathogenesis remains unclear (Levett, 2001).

### 1. Toxin Production

The production of toxin by pathogenic leptospires *in vivo* was inferred by Arean (1962). Endotoxic activity had been reported in several serovars. Leptospiral LPS preparations exhibited activity in biological assays for endotoxin, but at much lower potencies (Levett, 2001).

Serovar pomona was notable for the production of hemolytic disease in cattle, while serovar ballum produces similar symptoms in hamsters. Hemolysins from various serovars had been described. The hemolysins of serovars ballum, hardjo, pomona, and tarassovi were sphingomyelinases (Bernheimer, 1986; Real, 1989). Virulent strains exhibited chemotaxis towards hemoglobin (Yuri, 1993). Plasma had been arised to prevent hemolysis. Phospholipase C activity had been reported in serovar canicola. A hemolysin from serovar lai was not associated with sphingomyelinase or phospholipase activity and was thought to be a poreforming protein (Lee, 2000).

Strains of serovars pomona and copenhageni elaborate a protein cytotoxin, and cytotoxic activity had been detected in the plasma of infected animals (Knight, 1973).

*In vivo*, this toxin elicited a typical histopathology effect with infiltration of macrophages and polymorphonuclear cells. A glycolipoprotein portion with cytotoxic activity was improved from serovar copenhageni. A similar section from serovar canicola suppressed $Na^{+}$-$K^{+}$ ATPase. Inhibitory activity was associated with unsaturated fatty acids, especially palmitic and oleic acids. However, balanced activity was illustrated in *L. biflexa* serovar patoc (Burth, 1997), implying that other virulence factors might be of greater significance.

**2. Attachment**

Leptospires had been shown to attach to epithelial cells. Virulent leptospires adhered to renal epithelial cells *in vitro*, and adhesion was enhanced by subagglutinating concentrations of homologous antibody. Leptospires are phagocytosed by macrophages in the presence of specific antibody. Inhibition of macrophage action increased reponsiveness to infection (Levett, 2001). Virulent leptospires became associated with neutrophils, but are not killed (Wang, 1984). Phagocytosis was occured only in the presence of serum and complement, suggesting that the outer envelope of leptospires possesses an antiphagocytic component. Leptospiral LPS stimulated adherence of neutrophils to endothelial cells (Dobrina, 1995) and platelets, causing aggregation and suggesting a role in the development of thrombocytopenia (Levett, 2001).

**3. Immune Mechanisms**

The second stage of acute leptospirosis was also referred to as the immune phase, in which the disappearance of the organism from the bloodstream coincided with the appearance of antibodies. The clinical severity of the disease often appeared to be out of proportion to the histopathological findings. Immune mediated disease had been proposed as one factor influencing the severity of the symptoms (Levett, 2001).

The production of immune complexes resulting to inflammation in the central nervous system had been postulated. Levels of circulating immune complexes were correlated with severity of symptoms, and in patients who survived, circulating

immune complex levels fell concurrently with clinical improvement. However, in experimental infections in guinea pigs, leptospiral antigen was localized in the kidney interstitium, while immunoglobulin G (IgG) and C3 were deposited in the glomeruli and in the walls of small blood vessels (Levett, 2001).

The pathogenesis of equine recurrent uveitis appeared to involve the production of antibodies against a leptospiral antigen which cross-reacted with ocular tissues. Retinal damage in horses with uveitis was related to the presence of B lymphocytes in the retina (Levett, 2001). Antiplatelet antibodies had been demonstrated in human leptospirosis (Davenport, 1989). In leptospirosis and septicemia, such antibodies were directed against cryptantigens exposed on damaged platelets and do not play a causal role in the development of thrombocytopenia. Other autoantibodies had been detected in acute illness, including IgG anticardiolipin antibodies (Rugman, 1991) and antineutrophil cytoplasmic antibodies. Nevertheless, the significance of antineutrophil cytoplasmic antibodies in the pathogenesis of vascular injury in leptospirosis had been quested.

Virulent leptospires induced apoptosis *in vivo* and *in vitro* (Merien, 2000, Merien, 1998). In mice, apoptosis of lymphocytes was elicited by LPS via induction of tumor necrosis factor alpha (TNF-a). Elevated levels of inflammatory cytokines such as TNF-a had been reported in patients with leptospirosis (Levett, 2001).

## 4. Surface Proteins

The outer membrane of leptospires contained LPS and several lipoproteins (outer membrane proteins [OMPs]) (Haake, 2000). The LPS was highly immunogenic and was responsible for serovar specificity (Peña - Mactezuma, 1999). A converse relation between expression of transmembrane OMPs and virulence was observed in serovar grippotyphosa (Haake, 1991). Outer membrane lipoprotein (LipL36) was down–regulated *in vivo* (Barnett, 1999) and was not recognized by the humoral immune response to host-adapted leptospirosis in hamsters (Haake, 1998). Other OMPs were also down – regulated *in vivo* (Nicholson, 1993). Outer membrane elements may play important role in the pathogenesis of interstitial nephritis (Barnett,

1999; Haake, 2000). A fibronectinbinding protein produced only by virulent strains was described recently (Merien, 2000).

## 5. Immunity

Immunity to leptospirosis was largely humoral in nature (Adler, 1977). Passive immunity could be consulted by antibodies alone. A serovar-specific antigen (F4) extracted from LPS lacked endotoxic activity and induced protective immunity in rabbits, guinea pigs, and mice (Levett, 2001). A similar antigen (TM), which inhibited agglutination by homologous antisera (Adachi, 1977), was shown to be distinct from F4 (Adler, 1980) but had a common epitope. Sodium dodecyl sulfate extracts of entire cells brought to production of protective antibody, which was also attaching and complement fixing. Immunity was strongly restricted to the homologous serovar or closely related serovars. Serovar specificity was conferred by the LPS antigens (Kalambaheti, 1999; Midwinter, 1994). Broadly reactive genus-specific antigens have also been described (Myers, 1878, Palit, 1973; Sonrier, 2001).

Several of the leptospiral OMPs were highly conserved (Haake, 2000; Shang, 1996), and the potential for subunit vaccines which could generate broadly cross-protective immunity had been suggested by recent studies using OmpL1 and LipL41 (Haake, 1999), which induced synergistic protection.

## Normal Histology of experimental organs

**Kidney** (Young *et al.*, 2000)

The basic organization of the kidney parenchyma is composed of the nephron (secretory part) and the collecting system (excretory part) (Figure 7). The nephron is the functional unit of the kidney and consists of two major components including the renal corpuscle and the renal tubule.

### Renal Corpuscle

Renal corpuscle is responsible for the filtration of plasma and is a combination of two structures which are the Bowmann’s capsule and the glomerulus. Bowmann’s capsule consists of a single layer of flattened cell resting on a basement membrane; it

is derived from the distended blind end of the renal tubule. The glomerulus is a globular network of anastomosing capillaries which invaginates Bowmann's capsule. Within the capsule, the glomerulus is invested by a layer of epithelial cells called podocytes which constitute the visceral layer of Bowmann's capsule. The visceral layer is reflected around the vascular stalk of the glomerulus to become continuous with the parietal layer which constitutes Bowmann's capsule proper. The space between the visceral and parietal layers is known as Bowmann's space or unrinary space and is continuous with the lumen of the renal tubule. The parietal epithelium of Bowmann's capsule is continuous with the epithelium lining the proximal tubule. In the renal corpuscle, water and low molecular weight constituents of plasma are filtered from the glomerular capillaries into Bowmann's space to form the glomerular ultrafiltrate, which then passes into the renal tubule. Thus the filtration barrier between the capillary lumen and Bowmann's space consists of the capillary endothelium, the podocyte layer and their common basement membrane known as the glomerular basement membrane.

The afferent arteriole which supplies the glomerulus, and the efferent arteriole which drains it, enter and leave the corpuscle at the vascular pole which is situated opposite the entrance to the renal tubule, the urinary pole. The relatively wide diameter afferent arteriole enters Bowmann's capsule at the vascular pole of the renal corpuscle and then branches to form an anastomosing network of glomerular capillaries, each major branch giving rise to a lobule. The glomerulus is thus suspended in Bowmann's space from the vascular pole. The spaces between the capillary loops in each glomerular lobule are filled by basement membrane-like material called mesangium which contains mesangial cells.

**Renal Tubule**

Renal tubule extends from Bowmann's capsule to its junction with a collecting duct. The renal tubule is up to 55 mm long in humans and is lined by a single layer of epithelial cells. The primary function of the renal tubule is the selective reabsorption of water, inorganic ions and other molecules from the glomerular filtrate. The renal tubule has a convoluted shape and has four distinct histophysiological zones,

including proximal convoluted tubule, loop of Henle, distal convoluted tubule and collecting duct, each of which has a different role in tubular function.

The proximal convoluted tubule is the longest section of the nephron. The convoluted part of proximal tubules coils close to the glomerulus in the cortex. Their walls are formed by a low columnar or simple cuboidal epithelium which has a prominent blue stained brush border of tall microvilli. This increases the surface area of plasma membrane. The cytoplasm of proximal tubular epithelial cells stains intensely. The nuclei are stained pale blue.

The loop of Henle includes the distal straight part of proximal tubule called the pars recta, the thin descending and ascending limbs and the thick ascending limb. The thin segments of the loop of Henle dip down into the medulla where they form a hairpin bend. The limbs of the loop of Henle are closely associated with parallel wide capillary loops, the vasa recta, which arise from the efferent arterioles of glomeruli located near the cortico – medullary junction.

The distal convoluted tubule is a continuation of the thick limb of the loop of Henle. Microanatomically, distal convoluted tubules may be differentiated from proximal convoluted tubules by the absence of a brush border that makes a larger more clearly defined lumen.

**Collecting System**

The excretory part, the collecting tubules, descend through the cortex in parallel bundles called medullary ray, progressively merging in the medulla to form the large duct of Bellini or papillary duct which open at the tips of the renal papillae. Duct of Bellini is lined by the tall columnar epithelium while the collecting duct is lined by the low columnar epithelium. Both of those epithelia are poorly stained, well defined cellular outline with clearly perinuclear region and have no brush border.

### Juxtaglomerular Apparatus

The juxtaglomerular apparatus is specialization of the glomerular afferent arteriole and the distal convoluted tubule of the same nephron and is made up of three components: the macula densa of the distal convoluted tubule, rennin-secreting juxtaglomerular cells of the afferent arteriole and extraglomerular mesangial cells.

Mecula densa is lying in the angle between the afferent and efferent arterioles at the vascular pole of the glomerulus. The macula densa is an area of closely packed, specialized cells lining the distal convoluted tubule where it adjoins the glomerular vascular pole. Compared with other distal convoluted tubular lining cells, the cells of the macula densa are taller and have larger more prominent nuclei which are situated towards the luminal surface.

Juxtaglomerular cells are specialized smooth muscle cells of the wall of the afferent arteriole forming a cluster just before it enters the glomerulus.

Extraglomerular mesangial cells form a conical mass the apex of which is continuous with the mesagium of glomerulus. It is bounded by the afferent and efferent arterioles; laterally and its base connect the macula densa. These cells are flattened and elongated with extensive fine cytoplasmic processes extending from their ends and surrounded by a network of mesangial material.

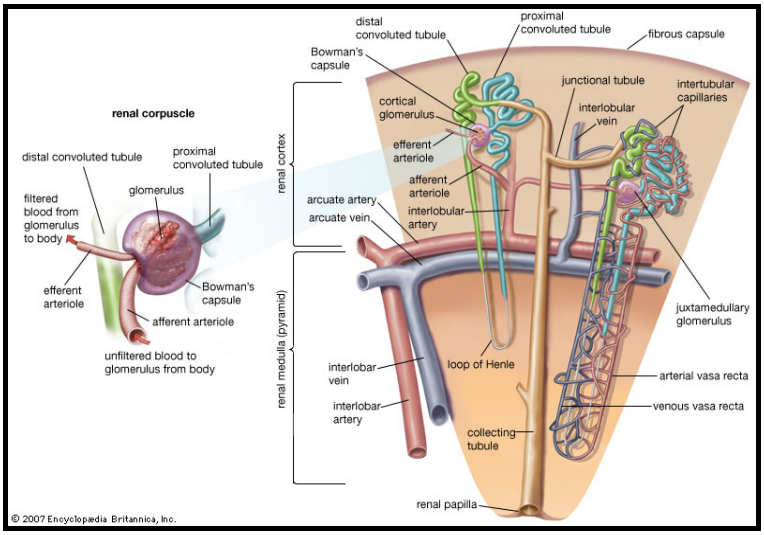



**Figure 7** Schematic drawing illustrating the nephron and the collecting system of the kidney (http://www.britannica.com, 2008).

**Skeletal muscle**

The muscular tissue is responsible for body movement. Each muscle cell is called a muscle fiber (Ross, 2003). It consists of the cylindrical muscle cells that are characterized by the presence of great numbers of contractile cytoplasmic filament (Junqueira, 1980). The highly developed function of the cytoplasmic organelles of muscle cells has led to the use of a special terminology for some muscle cell components: plasma membrane, sacrolemma; cytoplasm, sacroplasm; endoplasmic reticulum, sacroplasmic reticulum (Young, 2000). The individual muscle fibers are grouped together into elongated fasciculus and each fasciculus is packed together forming the muscle bundle. The connective tissue associated with the muscle is named according to its relationship with the muscle fiber (Ross, 2003) (Figure 8). The delicate supporting tissue called 'endomysium' occupying the space between individual muscle fibers (Young, 2000). The 'perimysium' is a thicker connective tissue layer that surrounds a group of fibers to form a 'fasciculus'. Epimysium is the sheath of dense connective tissue that surrounds a collection of fascicles, muscle bundle (Ross, 2003).

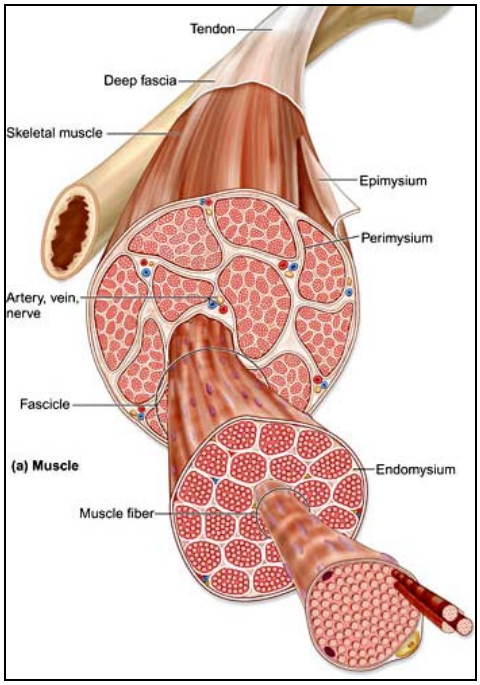



**Figure 8** Schematic drawing illustrating the skeletal muscle together with the supporting connective tissue (http://academic.kellogg.cc.mi.us, 2008).

The muscle fiber is filled with longitudinally arrayed subunit called myofibril which consists of the thick filament of myosin and the thin filament of actin. As observed with the light microscope, longitudinally sectioned muscle cell shows the cross-striations being composed of light and dark bands. The darker bands are called A band, the lighter bands are called I band. Each I band is bisected by a dark transverse line, the Z line (Junqueira, 1980). The functional unit of the myofibril is the sacromere, which is the basic contractile unit of the muscle. It is the portion of a myofibril between two adjacent Z line (Ross, 2003).

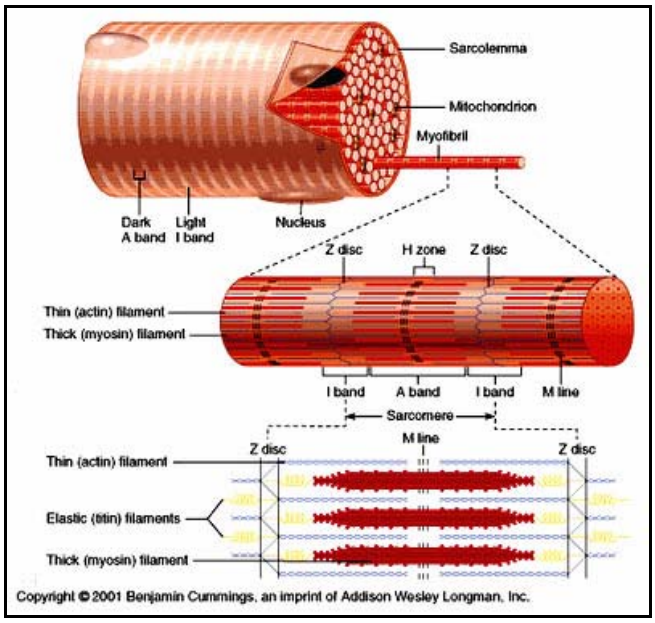



**Figure 9** Schematic drawing illustrating the structure of the sacromere of the skeletal muscle (http://katie-humanbio.blogspot.com/2008/04/skeletal-muscle-fiber-contraction.html, 2008).

Electron microscopic studies reveals the pattern of sacromere consisting two types of filament, thick and thin filaments, as describe above. The central portion of a sacromere is A band which are mainly composed of thick filaments and the portions of the thin filaments which overlap the thick filament. The I band consists of the portion of thin filaments that do not overlap the thick filament (Junqueira, 1980). The dark A band is bisected by a less dense or light region called the H band (Ross, 2003). The H band is portion of the A band that consists of only thick filament (Junqueira, 1980). Bisecting the light H band is a narrow dense line called the M line (Ross, 2003).

**Heart**

The heart is a folded endothelial tube which the wall is thickened. It is the major determinant of the systemic blood pressure (Kierzenbaum, 2002). The heart contracts rhythmically to pump the blood into the circulatory system (Junqueira, 1980).The wall of heart consists of three layers: endocardium, myocardium and epicardium

Endocardium is the inner layer and consists of an innermost endothelial layer, subendothelial layer and the outermost subendocardial layer. The endothelium is continuous with the endothelial layer of the blood vessel. The endothelium rests on a thin subendothelial layer of loose connective tissue (Junqueira, 1980). The connective tissue of the subendocardial layer contains the impulse-conducting system, veins and nerves (Ross, 2003).

Myocardium is a middle layer of a functional syncytium of striated cardiac muscle fibers (Kierzenbaum, 2002) which arranged in layers that cover the heart chambers in a complex spiral manner (Junqueira, 1980). The cardiac muscle cells are branched cylinder with one or at most two nuclei, centrally located within the cell (Kierzenbaum, 2002). The end of fibers splits longitudinally into a small number of branches (Young, 2000). The end of each cell exhibit densely staining cross-bands, called ‘intercalated disk’ which are specialized attachment sites between adjacent cells.

Epicardium consisting of a layer of mesothelial cells on the outer surface of the heart and its underlying connective tissue which are the middle submesothelial and the inner subepicardial layers (Ross, 2003). The submesothelial layer consists of supporting connective tissue associated with elastic fibers. A subepicardial layer consisting of loose connective tissue contains veins, nerves and nerve ganglia. The adipose tissue that generally surrounds the heart accumulates in this layer (Junqueira, 1980).

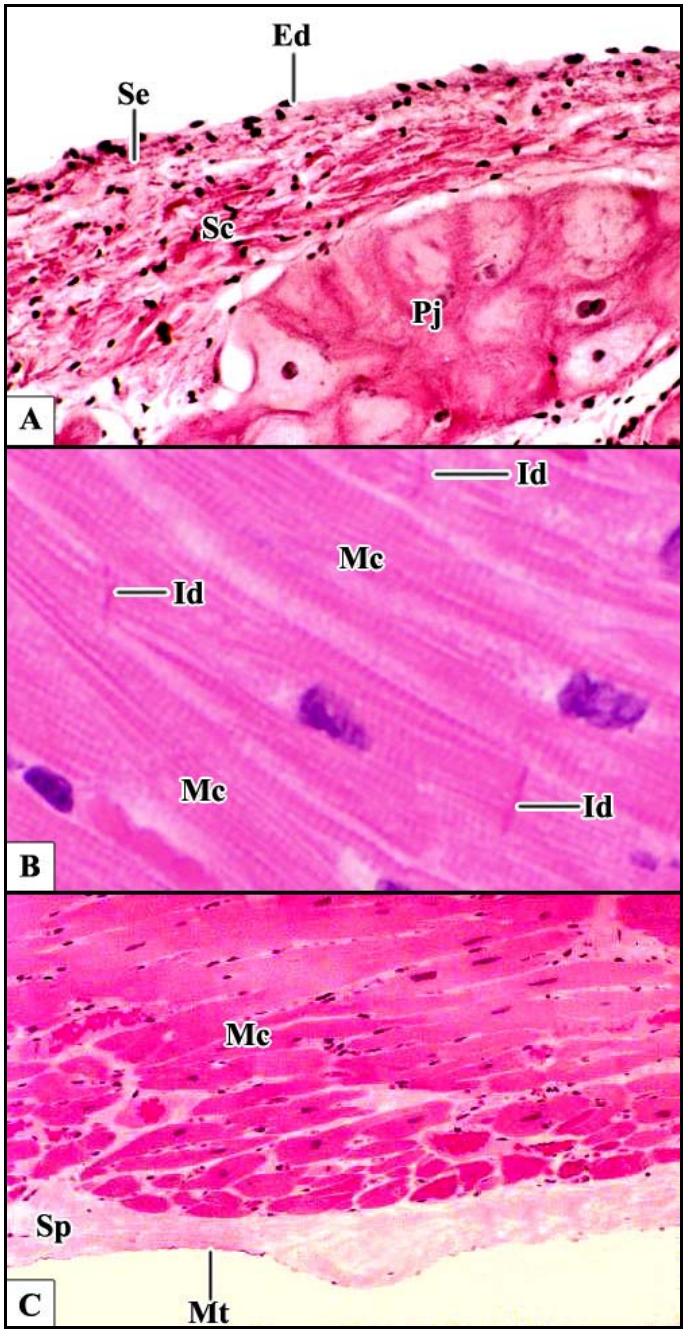



**Figure 10** Photomicrograph illustrating normal histological feature of the heart wall. The endocardium is shown in A, the myocardium (Mc) in B , and the epicardium in C. (Obj 20x in A and C; Obj 100x in B) (http://www.education.edu, 2008)

Abbreviation: Ed, Endothelial lining cell; Id, Intercalated disk; Mt, Mesothelial layer; Pj, Perkinje's fiber; Sc, Subendocardial layer; Se, Subendothelial layer; Sp, Subepicardial layer.

# CHAPTER II
# OBJECTIVES

This study is intended to characterize the expression and distribution of the leptospiral antigen of *Leptospira interrogans* serovar pyrogenes in kidney, heart and skeletal muscles (Hamstring and Gastrocnemius muscles) of the infected hamsters at different periods of the infection. The results from this study may clarify

1. the route of disease in the infected oragans (kidney, heart and skeletal muscles) from the expression and distribution of the leptospiral antigen of *Leptospira interrogans* serovar pyrogenes in the infected tissues at different infectious duration.
2. the understanding whether the development of the disease related to progression of the pathogen or not.
3. the understanding whether pathogenesis occurs according to the pathways of blood circulation.

# CHAPTER III
# LITERATURE REVIEWS

Nicodemo *et al.* (1997) localized the leptospiral antigen distribution. Lung fragment from 12 patients were collected immediately after death and studied by immunohistochemistry. Leptospiral antibody was a polyclonal rabbit antiserum against *Leptospira interrogans* serogroup icterohaemorrhagiae, serovar monymusky. Leptospiral antigen was detected in eight cases as positive granular material on the luminal surface of the endothelium and in the cytoplasm of the endothelial cells of septal capillaries and also in the filamentous form attaching to the endothelium of the septal capillaries.

Barnett *et al.* (1999) characterized the expression and distribution of leptospiral outer membrane antigens during renal infection by immunohistochemical technique. The antibody for the outer membrane antigen included F71C2, OmpL1, LipL41 and LipL36. Leptospires within the proximal tubules of kidney sections obtained at 10 days post-infection with culture-derived *L.kirschneri* stained positively for antibody F71C2 (specific for LPS), OmpL1 and LipL41. There was separate staining of intraluminal colonies scattered throughtout the cortex. LipL36 antisera did not stain the same location at concentrations that were positive for staining smears of culture-derived *L.kirschneri.* Prominent fine granular staining happened within the cytoplasm of the proximal convoluted tubular epithelial cells surrounding the luminal colonies when sections were stained with antibody F71C2. Kidney sections gained at 28 days after infection were positive for the represent of leptospiral antigen within tubules and in certain areas, in the interstitium and at points of interstitial inflammatory cellular aggregates. Antibodies to LPS, LipL41 and OmpL1 stained leptospiral colonies within tubules in the renal cortex. LPS reactivity was observed in the interstitium and in regions of interstitial cellular inflammation as coarse or fine granular staining. In some cases, the LPS and OmpL1 antigens were localized obviously within phagocytic cells. Interstiral OmpL1 reactivity was less prominent

than that observed with LPS. LipL41 reactivity was observed only within the renal tubules. LipL36 was not revealed during leptospiral infection of the kidney.

Yener and Keles (2001) investigated the role of leptospires in interstitial nephritis. Sixty-eight white-spotted kidneys and 30 grossly normal kidneys from slaughtered cattle were examined immunohistochemically for the presence of *Leptospira interrogans* antigens. The presence of *L.interrogans* antigen was found in 21 of 68 white-spottled kidneys and in 4 of 30 grossly normal kidneys. Immunoperoxidase staining showed deposits of leptospiral antigens. They appeared as either granular or in thread-like filaments, located especially at the lumen of tubules and at the lumial surface of tubular cell, the interstitium of the cortex and outer medulla. The antigen deposits were also extended in the medulla. Linear and focal deposits were localized on the endothelial lining of capillaries, small vessels and arcuate ateries. Phagocytosed antigen was also seen in the cytoplasm of syncytial and multinucleated giant cells, in the cytoplasm of hyperplastic and degenerative tubular epithelium, macrophages and the epithelium of calyx renalis.

Da Silva *et al.* (2002) reported that leptospiral antigen was detected by using immunoperoxidase staining. All four patients lived and worked in São Gonçalo, a vast and poverty stricken urban settlement near Niterói, Rio de Janeiro State, Brazil. This prompted the post-morten detection of leptospiral antigen in lung tissue. Monoclonal antibodies against *Leptospira interrogans* serovar copenhageni were used. Finely granular positive reactions corresponding to *leptospira* engulfed by macrophage were found in septa and alveoli.

Wild *et al.* (2002) compared the immunoreactivity in canine renal tissues with chronic interstitial nephritis suspected or proven to have leptospirosis stained with antisera specific for three leptospiral antigens. Antibodies consisted of a monoclonal antibody to *Leptospira kirschneri* serovar grippotyphosa lipopolysaccharide, F71C2-1, and two polygonal antibodies to outer membrane protein, including OmpL1, and LipL41. The murine monoclonal antisera against LPS were positive in the extracellular granular debris in intertubular areas, debris in macrophages and cytoplasmic granules in tubular epithelial cells. Antisera with specificity for the outer membrane proteins OmpL1 and LipL41 were detected only in the intact organisms

attached to microvillous surface of proximal convoluted tubule. The immunoreactivity was confined in the tubular lumen.

Colegrove *et al.* (2005) studied the development of leptospirosis in the natural environment and in a rehabilitation setting by using immunohistochemical staining. Leptospirosis was identified in six northern elephant seals *(Mirounga angustirostris)* that were stranded along the coast of California (USA) in 1995. Histological lesions in all seal included tubulointerstitial nephritis with tubular degeneration and necrosis. One affected seal had an eminent titer to *Leptospira interrogans* serovar pomona. Four of the six seals developed leptospirosis during rehabilitation and two seal had evidence of exposure in the wild. Immunohistochemical staining was accomplished using an established streptavidin-biotin staining protocol. The *Leptospira*–specific polyclonal antibody was directed against *L.interrogans* serovar bratislava, canicola, hardjo, icterohaemorrhagiae and pomona and *L.kirschneri* serovar grippotyphosa. Variable numbers of spirochetes were observed within the lumens of renal tubules in all six seals by using immunohistochemistry. Positive granular staining of antigen was illustrated within numerous tubular epithelial cells and within intertubular inflammatory cells. The staining of antigen in all samples was most extended in sites of interstitial inflammation. These results indicated that northern elephant seals were susceptible to leptospirosis and could develop the disease both in the natural environment and in a rehabilitation setting.

Da Silva *et al.* (2005) demonstrated leptospires in fragments of kidney, liver and uterus of 96 cows with unknown sanitary status randomly chosen at slaughter in Paraná, Brazil. Sections of kidney, liver and uterus were stained by indirect immunoperoxidase with hyperimmune serum against serovar Hardjo and serovar Canicola. Indirect immunoperoxidase assay performed by using hyperimmune serum against serovar Canicola showed positive results in the kidney of one animal characterized by the visualization of leptospires stained in intense brown in the lumen of renal tubules. However, the liver and uterus of these animals did not express any positive area.

Szeredi and Haake (2006) examined the utility of immunohistochemistry (IHC) in the diagnosis of leptospiral equine abortion. Ninety-six fetuses from 57 farms examined revealed evidence of leptospiral infection. Tissue sections of liver, kidney,

lung, spleen, heart, brain and allantochorion were examined by IHC. This technique was performed by using two different leptospiral antisera to detect leptospiral antigen in each case, including a multivalent whole-cell rabbit antiserum and a rabbit antiserum specific for the leptospiral major outer membrane protein, LipL32. IHC displayed a moderate to large number of leptospiral antigens in nearly all of the tissue samples. Leptospiral antigens were most clearly observed in the liver followed by heart, lung and kidney. Typical leptosprial antigens were wavy forms, small cocci and large granules in the cytoplasm of macrophages, endothelial cells, tubular epithelial cells, hepatocytes, cardiac muscle cells. Typical leptospiral wavy forms and aggregates were found extracellularly in the connective tissues and in the lumen of renal tubules, the alveoli of the lung, the sinusoid of the liver, spleen and the lumen of blood vessels in various fetal organs and the placenta.

Yang *et al.* (2006) detected the pathologic changes by using immunehistochemical staining. The leptospirosis model of guinea pig was organized by intraperitoneal injection of *Leptospira interrogans* strain Lai. Forty guinea pigs of either sex were ordered to five groups including the uninfected negative control. They were injected by intraperitoneally with 1 ml of the leptospiral culture ($5\times10^8$). Guinea pigs were scacrified at 24, 48, 72 and 96 hours after infection. Livers, kidneys and lungs were collected from normal and infected animals. The rabbit antiserum against to *L.interrogans* strain Lai was provided using a modified procedure. Immunohistochemical staining was established as described. Sections were incubated in primary rabbit antibody (1:6,000 dilutions) specific for *L.interrogans* strain Lai. Pathologic changes were first found in the infected liver tissue at 48 hours after infection. At 24 hours post–inoculation, spiral form of bacteria similar to the intact leptospires began to appear in the liver by immunohistochemical staining. Starting from 48 hour after infection, leptospires were seen in granular forms. In kidneys, large number of intact leptospires was seen by immunohistochemical staining within the renal tubules, the interstitium and the glomeruli at 72 hours after infection. Degenerated granular leptospires were revealed in the interstitium and tubules at 96 hours after infection. Besides in lungs, leptospires were detected inside septal capillaries and in interstitium at 48 hours after infection. Leptospires in spiral form were firstly seen. Starting from 72 hours after infection, leptospires were appeared in

granular forms. There were less leptospires in the lung as contrast to the liver and kidney at the identical period.

De Brito *et al.* (2006) used an immunohistochemical assay for detection of the leptospiral antigen in the liver and kidney of human fatal cases of leptospirosis. This study was performed to exact the appearance of leptospiral antigens. Two primary sera were performed for the localization of leptospiral antigen. One serum was obtained by hyper–immunizing the sheep with sequential intravenous doeses of 1, 2, 3 and 4 ml of *L.interrogans* serogroup icterohaemorrhagiae. The secondary immune serum was accomplished in rabbits. They found that, in the liver of the experimental group leptospiral antigen was usually detected as dots and rarely as deposits in the cytoplasm of macrophages at the inflammatory infiltrated portal area and more frequently in the cytoplasm of macrophages along the sinusoidal lining (Kupffer cells) and circulating macrophages. Focal leptospiral antigen deposits were detected on the cell membrane of adjacent hepatocytes. In the kidney, leptospiral antigen deposits were observed also in the cytoplasm of macrophages associated with the focal interstitial infiltrate. Leptospiral antigen deposits were seldom on the luminal side of tubular cells.

# CHAPTER IV
# MATERIALS AND METHODS

## 1. Animal Preparation

Thirty adult female hamsters *(Mesocricetus auratus),* age 4–6 weeks and weight 80–120 grams were used in this study. The experimental animals were fed on standard diet and kept under control conditioned room, temperature at 25–28 °c with 12 hours light per day throughout the experimental period. A total of 30 adult female hamsters were raised in the animal room of the Department of Laboratoy Animals, Armed Forces Research Institute of Medical Sciences (AFRIMS) Bangkok, Thailand.

## 2. Preparation of bacteria for sample collection

*Leptospira interrogans* serovar pyrogenes was isolated from blood of a febrile patient with clinical leptospirosis in Buri–Ram Province. The patient acquired the disease by contact with contaminated water in a ruin pond in Buri–Ram Province, October 1999. One to two drops of blood were added to 5.0 ml of protein supplement semisolid Ellinghausen–McCullough–Johnson–Harris (EMJH) media containing 100 µg of 5–fluorouracil per ml and incubated at 30 °c. Culture was examined by dark field microscope every week to confirm the presence of viable leptospires and the absence of contamination. The culture was maintained in screw-cap test tubes containing 5 ml a semisolid EMJH media. Subculture was made by inoculating 0.5 ml of culture from last transfer into a fresh semisolid media every month. Half millimeter of *leptospira* insolated from stock culture was inoculated into 5 ml of liquid EMJH and incubated at 30 °c. The leptospira isolate grown in the culture for 5 days was centrifuged for 10 minutes at 5000 r.p.m. The supernatant was decanted and the leptospira isolated cells were washed 2 times in phosphate buffer saline (PBS). The isolated leptospires were resuspended in PBS and turbidity adjusted to the

McFarland number 0.5 which corresponded to approximate density of $10^8$ leptospires/ml.

### 3. Sample Collection

Six control hamsters injected intraperitoneally with 0.5 ml PBS were served as the two control groups. Another 24 animals were divided for the experimental groups. They were injected intraperitoneally with 0.5 ml of PBS containing $1 \times 10^8$ leptospires/ml.

All animals were killed by first anaesthetizing with $CO_2$ and then were put into the $CO_2$ chamber, laid down on plate and the thoracic cavity was opened by subcostal incision.

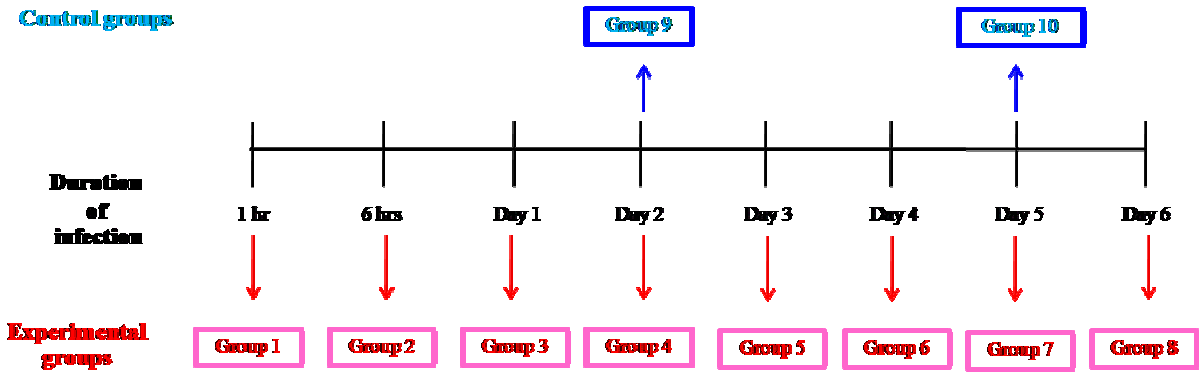



**Diagram 3** Diagram revealing the sample collection.

Three of the control hamsters were sacrificed on the second day and the three on the fifth day after injection with 0.5 ml PBS. The experimental animals were divided into eight groups of three each depending on the time they were sacrificed. Three infected hamsters were sacrificed at various time including; 1 hour, 6 hours and on days 1, 2, 3, 4, 5 and 6 after injection with 0.5 ml of PBS containing $1 \times 10^8$ leptospires/ml. The kidney, heart and skeletal muscles (gastrocnemius and hamstring muscles) of all the sacrificed animals were removed and processed for conventional light microscopy.

**4. Preparation of specimen for light microscopy**

All the dissected organs were fixed with Bouin's solution for 2 days. The fixed organs were washed in 50% ethanol, followed by 70% ethanol until all the yellow of Bouin's solution was removed. Then the fixed organs were processed by routine conventional technique for light microscopy (Appendix B). After processing, the specimens were cut to a thickness of 3 µm with a rotary microtome and placed on the coated glass slide with 3 - aminopropyltriethoxysilane (Appendix C) and then dried at 60°c

**5. Indirect immunoperoxidase staining technique**

These sections were then transferred to xylene to remove the paraffin from the section before being stained with immunoperoxidase technique by using rabbit primary antibody to *Leptospira interrogans,* serovar Pyrogenes (batch#303 from Royal Tropical Institute Kit Biomedical Research, the Netherlands) and rabbit ABC staining system kit (from Santa Cruz Biotechnology, Inc). Finally the slides were mounted with Resin solution.

**Fundamental of indirect immunoperoxidase staining technique** (Diagram 4 and Appendix D)

Indirect immunoperoxidase method utilizes the peroxidase enzyme–conjugated anti–immunoglobulin secondary antibody via avidin–biotin peroxidase complex system (ABC staining system) to detect the reaction of the primary antibody to the antigen (Wikipedia, 2008). In this study the antigens are the leptospires themselves or the leptospiral antigen within the infected tissue sections.

Indirect staining method enhances the sensitivity of antigen detection. The indirect methods designed for greater amplification of the visible signal produced by the binding of primary antibodies to the antigen within the tissue sections (Haines, 1991).

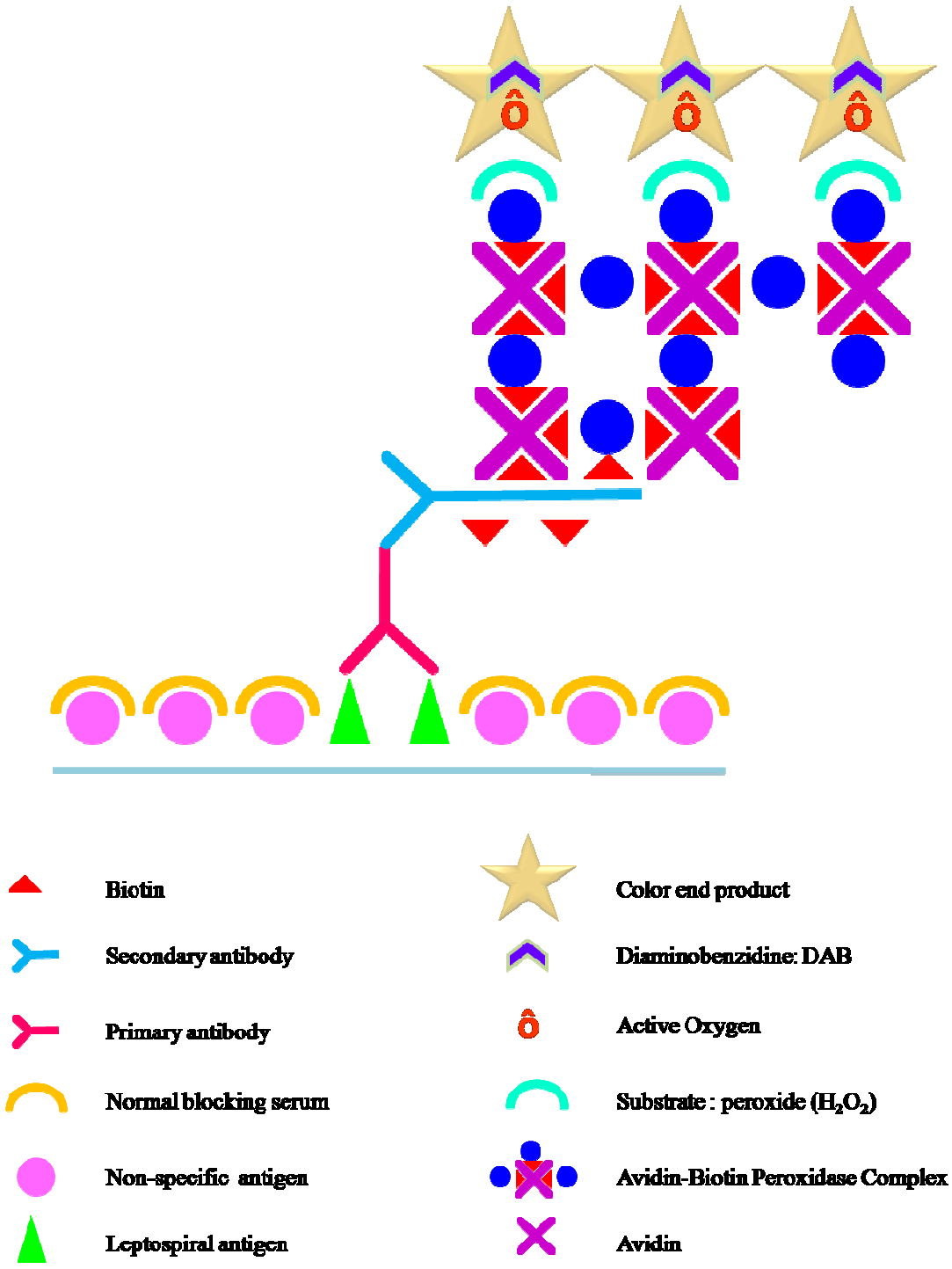



**Diagram 4** Diagram illustrating indirect avidin–biotin immunoperoxidase staining technique.

This system applies the high affinity of B-vitamin, biotin, for the egg-white glycoprotein, avidin (Haines, 1991). Each avidin has four binding sites for biotin. Antigen within the infected tissue sections are localized by an unlabeled primary antibody and then followed by a biotin conjugated secondary antibody that would bind with the Fc fragment of the primary antibody. Then the avidin–biotin peroxidase complex was applied. The hourseradish peroxidase is commonly used as an enzyme labels. Peroxidase activity in the system is the presence of electron donor first results in the formation of an enzyme – substrate complex (peroxidase – peroxide complex). The electron donor provides the driving force in continuing catalysis of $H_2O_2$ (hydrogen peroxide). When the $H_2O_2$ is immediately absence the enzyme-substrate reaction stopped. The product of this reaction is the active oxygen, which oxidizes the chomogen (DAB) to get the golden – brown precipitation. Finally, the expected antigen which is marked by the indirect immunoperoxidase staining will show the golden – brown end product of 3, 3'-diaminobenzidine tetrahydrochloride (DAB) (Naish, 1989).

Regarding to the negative staining control, the primary antibody was omitted in the staining procedure of the infected groups.

**6. Analysis of the tissue sections**

All stained sections of each both negative experimental control and the infected groups were examined under the light microscope. The coloration of each organ was photographed by the attached digital camera.

# CHAPTER V
# RESULTS

**Kidney**

The renal tissue of the control group and the experimental negative staining control of the infected groups showed no golden–brown coloration in the sections including the renal tubules, renal corpuscle, inflammatory cells and blood vessels. These sections were expressed as the blue stain of the Carrazi's haematoxylin solution (Figure 11)

The leptospiral antigens in the organs of the infected hamsters were characterized immunohistochemically by using the indirect immunoperoxidase staining technique. The areas where the antigen was depositing should display the positive golden–brown coloration. Concerning the renal tissue of different experimental groups, it was found that some of the epithelia lining all kind of the renal tubules, renal corpuscles, inflammatory cells as well as the wall and lumen of the vessels showed the positive golden–brown staining of different intensity. The lumen of the renal tubules showed the positive coloration only in the late experimental groups.

**Proximal tubule** (Figure 12)

The cytoplasm of the epithelial lining cells of the proximal tubules demonstrated the positive stain of various intensities in all infected groups from the first hour to six days groups post infection. Those of the one hour to the one day groups showed the moderately stained cytoplasm (Figure 12A–C) while the mild stain was seen in the groups of two days to five days after infection (Figure 12D–G). The nuclear shrinkage of the epithelia was shown in the proximal tubules of the five days post infection group. Besides those of the six days group which was the last day of the experiment revealed the intensive positive stain together with the vacuolar

degenerative cytoplasm while the lumen of the tubules also showed the mildly–stained red blood cells with homogeneous hyalinic material (Figure 12H).

**Distal tubule** (Figure 13)

The cytoplasm of the distal tubular epithelial lining cells was golden–brown coloration with various intensities in all of the infected groups. The group of first hour post infection appeared as mild stain (Figure 13A) together with the moderate stain in the later period from six hours to four days (Figure 13B–F) and became mild in those of five days which also displayed the nuclear shrinkage (Figure 13G). Finally, the moderately–stained cytoplasm came again in the group of six days meanwhile the intensively–stained red blood cells and homogeneous hyalinic material were demonstrated in the lumen (Figure 13H).

**Collecting duct** (Figure 14)

As the proximal and distal tubules, the epithelia of the collecting duct showed as positive stain of different strength in all of the infected groups. The mild stain was observed in the group of one and six hours (Figure 14A–B), moderate in those of one day to three days (Figure 14C–E), intensive in those of four days (Figure 14F) however they became mild and moderate again in those of five days (Figure 14G) and six days (Figure 14H) respectively. In addition, the interstitium of the four days group appeared as large intensive golden–brown extracellular clump (Figure 14F) while the vacuolar degeneration of the renal parenchyma and nuclear shrinkage were located in the group of five days (Figure 14G). Furthermore, the lumen of the ducts in the six days group illustrated the mildly–stained red blood cells and homogeneous hyalinic material (Figure 14H).

**Renal corpuscle** (Figure 15)

The golden–brown area was observed in the glomerulus of all experimental groups except only in the one hour group (Figure 15A). The positive stained area usually located in the capillary loop represented by the podocytes and some in either parietal or visceral layer of the Bowmann’s capsule. The podocytes of the six hours, one day, two days and five days groups demonstrated the mildly–stained cytoplasm

(Figure 15B–D and G) while those of three days, four days and six days revealed moderately, negatively and intensively stained cytoplasm respectively (Figure 15E–F and H). Concerning the urinary space, all of the experimental groups showed the negatively–stained empty space (Figure 15A–F) except the groups of five and six days that were occupied by the moderately and intensively stained red blood cells repectively (Figure 15G–H). In addition, the urinary space of the five days group was occupied by the negatively–stained homogeneous hyalinic material (Figure 15G).

**Inflammatory cells** (Figure 16)

The inflammatory cells that were mostly neutrophils and infiltrated throughout the renal parenchyma also displayed the positively–stained cytoplasm. However, the positive coloration intensity performed different degree in various experimental groups. Those in the early groups of one hour, six hours and one day expressed the mild stain (Figure 16A–C) and then intensive in the two to five days groups (Figure 16D–G) after all they became mild again in the groups of six days (Figure 16H). In addition, not only the neutrophils but the plasma cells and lymphocytes were also being found. Some plasma cells showed the positive mildly–stained cytoplasm (Figure 16C) while the lymphocytes were hardly observed the coloration since they contained only few cytoplasm (Figure 16A–B, F and G). There were many locations where the inflammatory cells were observed, including the hemorrhagic area of the renal parenchyma (Figure 16A–B) the capillary loop of glomerulus (Figure 15C), scattered around the renal corpuscle (Figure 15F) and the interstitium (Figure 16D and G)

**Renal blood vessels** (Figure 17)

The renal blood vessels displayed positive golden–brown stain in all infected group. However, the pattern of the positive stained area was different, including the content in the lumen and the component of the vessel wall. Furthermore the intensity of these stained areas was varied. The luminal content which was supposed to be mainly the red blood cells revealed the mild stain in the group of six hours (Figure 17B), four days (Figure 17E) and six days (Figure 17H), moderate stain in one hour (Figure 17A) and one day (Figure 17C), intensive stain in two days (Figure 17D), three days (Figure 17E) and five days (Figure 17G). The inflammatory cells were also

clearly seen in the lumen of these blood vessels (Figure 17G). Concerning the vessel wall, the staining was performed only in the internal elastic lamina and tunica adventitia layer. The internal elastic lamina was detected as golden–brown membrane of different intensity. Those were mild stain in the groups of one hour (Figure 17A), six hours (Figure 17B), three to five days (Figure 17E–G); moderate stain in one day (Figure 17C), two days (Figure 17D). After all the internal elastic lamina of the six days groups performed the negative stained coloration (Figure 17H). The tunica adventitia layer was classified only two intensity; mild and intensive. The mild stain was located in the groups of six hours (Figure 17B), three days (Figure 17E) and six days (Figure 17H) intensive stain in one day (Figure 17C), two days (Figure 17D), four days (Figure 17F) and five days (Figure 17G). Those in the group of one hour showed the negative stained internal elastic lamina (Figure 17A).

**Table 1** Immunoperoxidase staining of the renal tissue of hamsters infected with *Leptospira interrogans,* serovar pyrogenes.

| Renal tissue | Durations of post infected hamster | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **1 hr.** | **6 hrs.** | **1 Day** | **2 Days** | **3 Days** | **4 Days** | **5 Days** | **6 Days** |
| **Proximal tubules** | | | | | | | | |
| • Epithelial lining cells | ++ | ++ | ++ | + | + | + | + | +++ |
| • Lumen | - | - | - | - | - | - | - | + |
| **Distal tubules** | | | | | | | | |
| • Epithelial lining cells | + | ++ | ++ | ++ | ++ | ++ | + | ++ |
| • Lumen | - | - | - | - | - | - | - | +++ |
| **Collecting ducts** | | | | | | | | |
| • Epithelial lining cells | + | + | ++ | ++ | ++ | +++ | + | ++ |
| • Lumen | - | - | - | - | - | - | - | + |
| **Renal corpuscle** | | | | | | | | |
| • Podocytes | - | + | + | + | ++ | - | + | +++ |
| • Urinary space | - | - | - | - | - | - | ++ | +++ |
| **Inflammatory cells** | | | | | | | | |
| • Neutrophils | + | + | + | +++ | +++ | +++ | +++ | + |
| **Renal blood vessels** | | | | | | | | |
| • Lumen | ++ | + | ++ | +++ | +++ | + | +++ | + |
| • Internal elastic lamina | + | + | ++ | ++ | + | + | + | - |
| • Tunica adventitia | - | + | +++ | +++ | + | +++ | +++ | + |

Evaluation of results using a four–grade, semi–quantitative scale: negative (-), mild (+), moderate (++) and intensive (+++)

**Figure 11** Photomicrographs illustrating the experimental negative control of the renal tissue in the groups of one hour (A and B), three days (C and D), and six days (E and F) post infected hamsters with *Leptospira interrogans,* serovar pyrogenes. The proximal tubules (Pt) are shown in A, the collecting ducts (Cd) in B, the distal tubules (Dt) in (C), the glomerulus (Gm) in D, the inflammatory cell infiltration in E and the blood vessel in F. No any golden brown stained area is seen in the sections. (A – D, Obj 40x and E – F , Obj 100x)

Abbreviation: Ie, Internal elastic lamina; Lu, Lumen of blood vessel; Np, Neutrophil; Sm, Smooth muscle cells; Us, Urinary space.

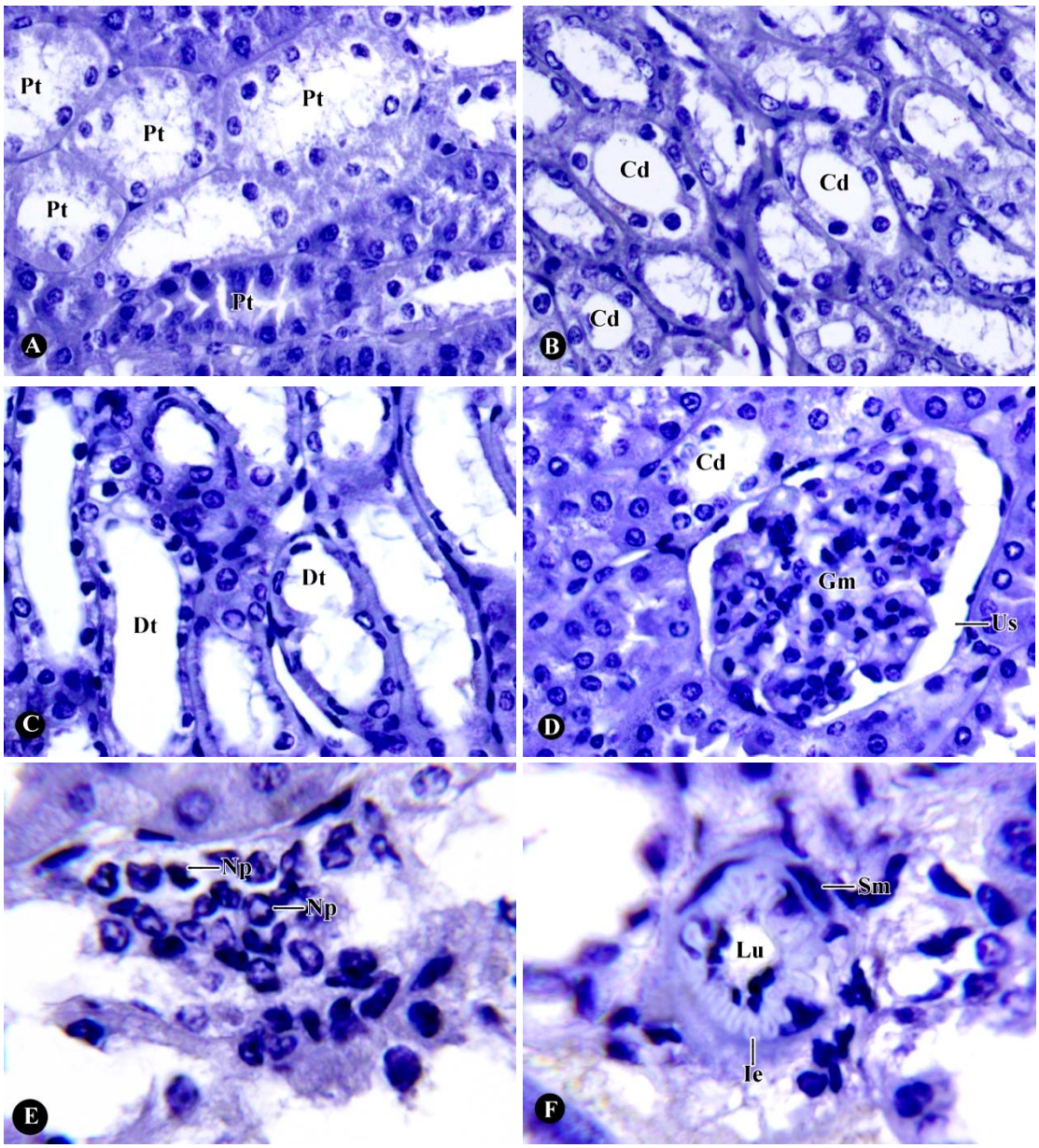



**Figure 11**

**Figure 12** Photomicrographs of the kidney illustrating the proximal tubule (Pt) in the groups of one hour (A), six hours (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans*, serovar pyrogenes. (Obj 40x)

A – C) The renal tissue of the infected kidney illustrates the proximal epithelial lining cells (Ep) which appear as moderate golden – brown coloration.

D – G) The infected kidney tissue showing the proximal epithelial lining cells (Ep) which appear as mild golden – brown precipitation in their cytoplasm.

H) The proximal tubules in the cortex of infected hamster are lined by vacuolar (Vc) cytoplasmic epithelium which the cytoplasm shows the intensive golden – brown precipitation while the lumen of proximal tubule reveals the mildly – stained red blood cells.

Abbreviation: Dt, Distal tubule; If, Inflammatory infiltrated cell; Th, Thin segment of Henle's loop

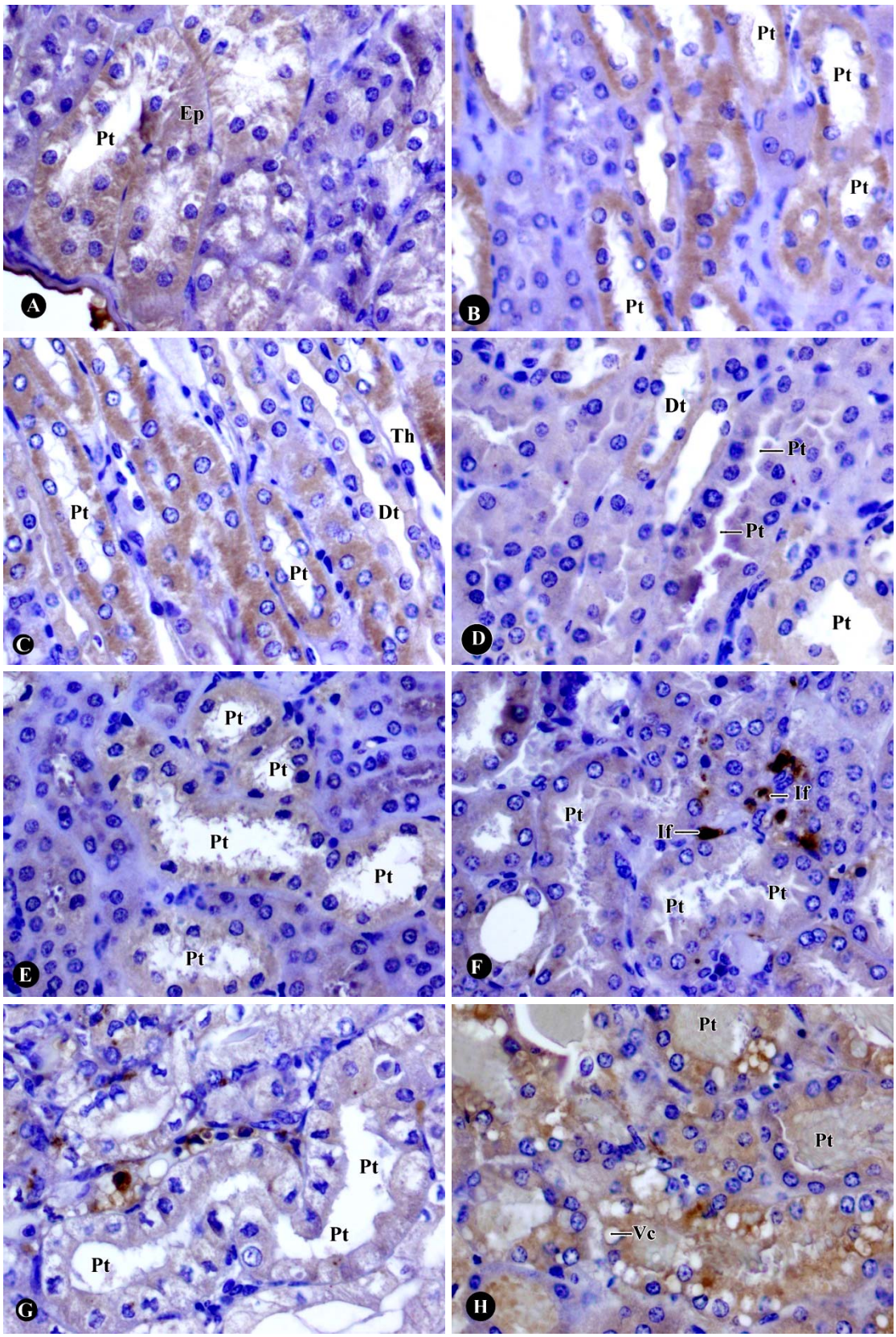

A
Ep
Pt
B
Pt
Pt
Pt
Pt
C
Th
Pt
Dt
Pt
D
Dt
Pt
Pt
Pt
E
Pt
Pt
Pt
Pt
Pt
F
If
Pt
If
Pt
Pt
G
Pt
Pt
Pt
H
Pt
Pt
Vc

**Figure 12**

**Figure 13** Photomicrographs of the kidney showing the distal tubules (Dt) in the groups of one hour (A), six hours (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans,* serovar pyrogenes.

A) Some of the distal tubular epithelial lining cells in the inner medulla of renal tissue show the mildly – stained cytoplasm. (Obj 20x)

B – D) The epithelium lining the distal tubules in the renal medulla shows the moderately – stained cytoplasm. Some congested areas in the renal parenchyma are seen in figure D. (Obj 40x)

E) The epithelial lining cells of the distal tubule illustrate the vacuolar degenerate cytoplasm which appears as clear vacuolar cytoplasm (Vc) on their luminal surface as well as the moderately – stained cytoplasm. (Obj 40x)

F) The cytoplasm of distal tubular epithelial lining cells are moderately– stained which is more intensive than those of the proximal tubular epithelial lining cells. (Obj 40x)

G) The nuclear shrinkage (Ns) of various renal tubular epithelial lining cells is revealed while the epithelial lining cells of the distal tubules perform the mildly – stained cytoplasm. (Obj 40x)

H) The lumen of the distal tubules are congested by intensive golden–brown material. The cytoplasm of the distal tubular epithelial lining cells is moderate golden – brown coloration. (Obj 40x)

Abbreviation: Cd, Collecting duct; Gm, Glomerulus; If, Inflammatory infiltrated cell; Ns, Nuclear shrinkage; Pt, Proximal tubule; Rb, Red blood cell; Th, Thin segment of Henle’s loop; Us, Urinary space.

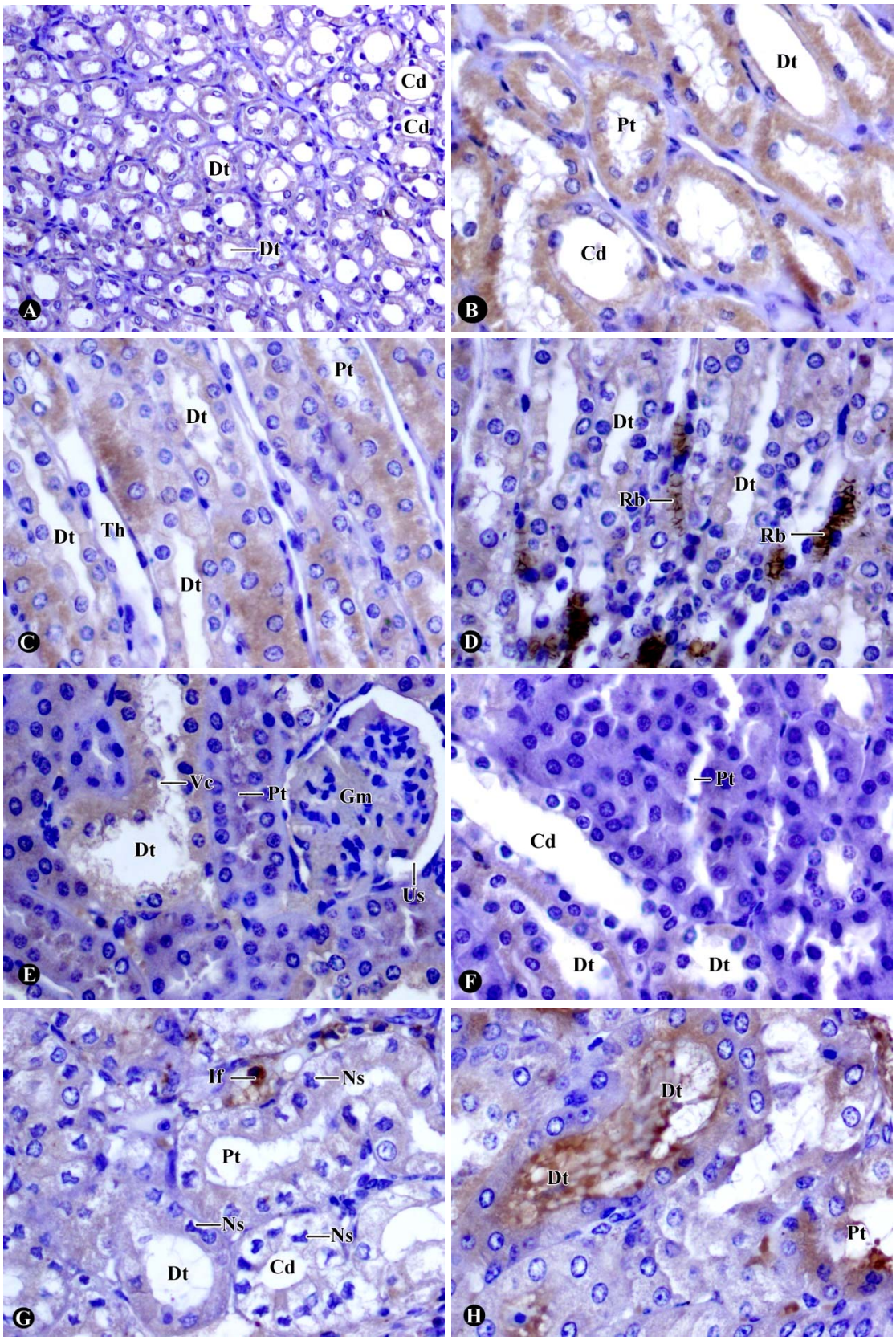

Cd
Cd
Dt
Dt
A
Dt
Pt
Cd
B
Pt
Dt
Dt
Th
Dt
C
Dt
Dt
Rb
Dt
Rb
D
Vc
Pt
Gm
Dt
Us
E
Pt
Cd
Dt
Dt
F
If
Ns
Pt
Ns
Ns
Dt
Cd
G
Dt
Dt
Pt
H

**Figure 13**

**Figure 14** Photomicrographs of the renal tissue illustrating the collecting duct (Cd) in the groups of one hour (A), six hours (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans*, serovar pyrogenes. (Obj 40x)

A –B) The epithelial cells lining the collecting duct display the mildly – stained cytoplasm with vacuolar degeneration (Vc).

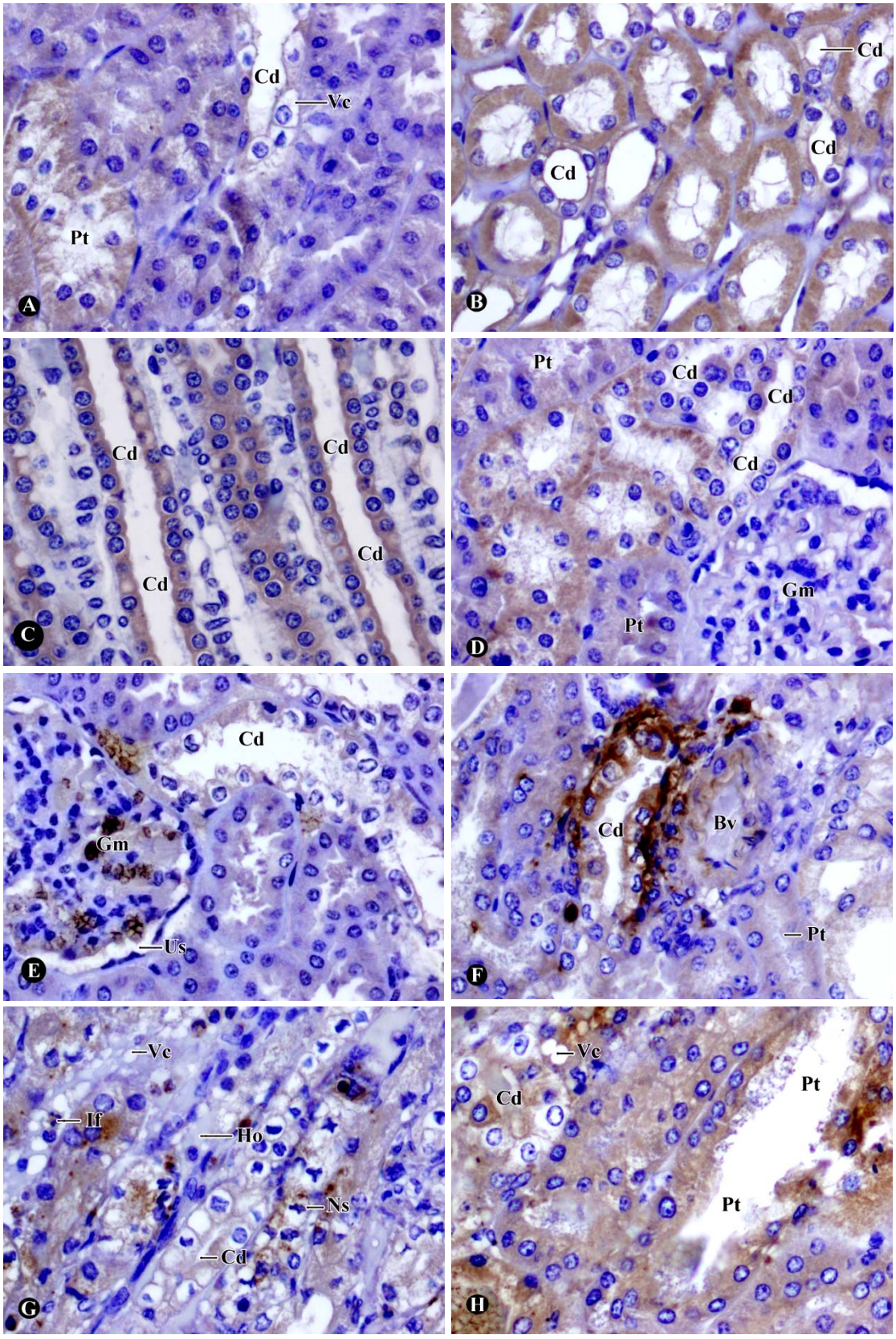
C – E) The moderately – stained cytoplasm of the epithelial cells lining the collecting duct are demonstrated.

F) The intensive golden – brown coloration is shown in the cytoplasm of the epithelial lining cells of the collecting duct.

G) The degenerative change of the renal tissue which consists of the vacuolar degeneration of the renal parenchyma, nuclear shrinkage (Ns), homogeneous hyalinic material (Ho) in the interstitium and inflammatory cells infiltration (If). The epithelial lining cells display mildly – stained cytoplasm.

H) The moderately – stained cytoplasm of the epithelial lining cells of collecting duct is demonstrated.

Abbreviation: Gm, Glomerulus; Pt, Proximal tubule; Us, Urinary space.


Cd
Vc
Pt
A
Cd
Cd
Cd
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Cd
Cd
Cd
Cd
C
Pt
Cd
Cd
Cd
Gm
Pt
D
Cd
Gm
Us
E
Cd
Bv
Pt
F
Vc
If
Ho
Ns
Cd
G
Vc
Cd
Pt
Pt
H

**Figure 14**

**Figure 15** Photomicrographs of the kidney revealing the renal corpuscle in the groups of one hour (A), six hours (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans*, serovar pyrogenes. (Obj 40x, inset 100x)

A) The glomerulus (Gm) shows negatively – stained area.

B) Mild positive stain is clearly observed in the cytoplasm of podocytic cells (arrow head) of the capillary loops in the glomerulus.

C) The inflammatory cell (If) in the capillary loop shows the intensively – stained cytoplasm. The podocytic cells in the glomerulus reveal the mildly – stained cytoplasm (arrow head).

D) The positive stain on the parietal layer of the Bowmann's capsule (arrow) displays the intensively – stained coloration. The podocytic cells display the mildly – stained cytoplasm (arrow head).

E) The moderate positive stain is deposited in the cytoplasm of podocytic cells (arrow head) and intensive one in the vascular stalk (arrow) of the capillary loop.

F) The intensively – stained cytoplasm of inflammartory cells (If) around the renal corpuscle is shown while the podocytes show the negatively – stained cytoplasm (arrow head).

G) Urinary space (Us) is occuplied by moderately – stained red blood cells (Rb) and negative stain homogeneous hyalinic material. The podocyte shows mildly – stained cytoplasm (arrow head).

H) The intensively – stained red blood cells (Rb) are occuplied in the urinary space. The podocytes are moderately – stained cytoplasm (arrow head).

Abbreviation: Cd, Collecting duct; Dt, Distal tubule; Md, Macula densa cells; Pt, Proximal tubule.

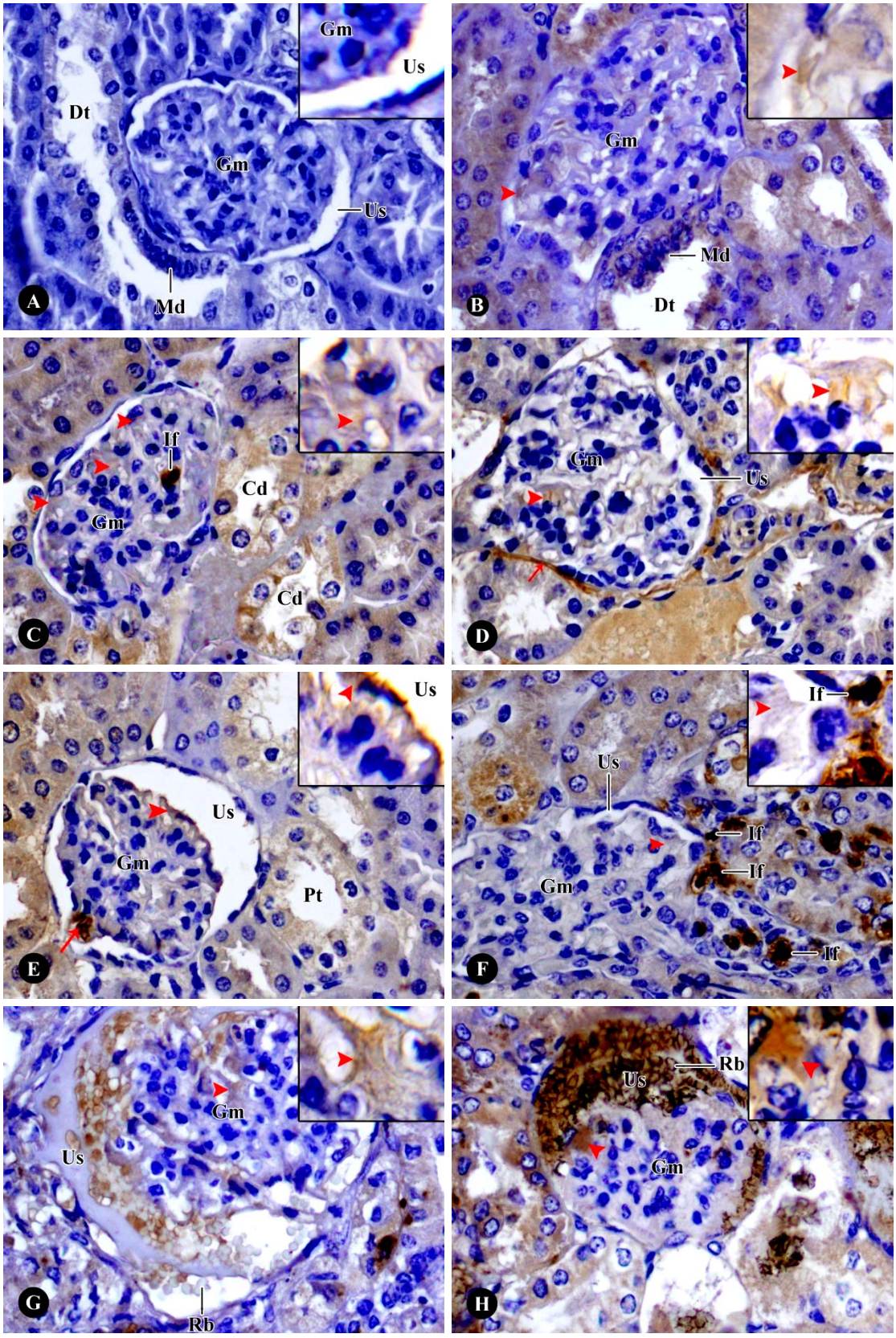

Gm
Us
Dt
Gm
Us
Md
A
Gm
Md
Dt
B
If
Cd
Gm
Cd
C
Gm
Us
D
Us
Us
Gm
Pt
E
If
Us
If
Gm
If
If
F
Gm
Us
Rb
G
Rb
Us
Gm
H

**Figure 15**

**Figure 16** Photomicrographs of the kidney showing the inflammatory cell infiltration in the groups of one hour (A), six hours (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans*, serovar pyrogenes. (Obj 40x, inset 100x)

A) The neutrophils (Np) and lymphocyte (Lm) infiltrate in the hemorrhagic area. The mild positive stain is also deposited in the area where the neutrophils and lymphocytes demonstrated the mildly and negatively – stained cytoplasm, respectively.

B) The neutrophil (Np) expresses the mild golden – brown coloration.

C) The mild golden – brown coloration is deposited in the area where the plasma cells (Pm) are aggregated and appear mildly – stained cytoplasm.

D) The leptospiral antigen is displayed by the intensively – stained cytoplasm of the neutrophil (Np).

E) The cytoplasm of the neutrophils (Np) shows intensive positive stain.

F) The inflammatory cells which mostly are the neutrophils (Np) show intensively – stained cytoplasm.

G) The moderately – stained interstitium (arrow) which is infiltrated by mostly the lymphocytes is shown.

H) The neutrophils which infiltrate in the degenerated area reveal mildly – stained cytoplasm.

Abbreviation: Pt, Proximal tubule.

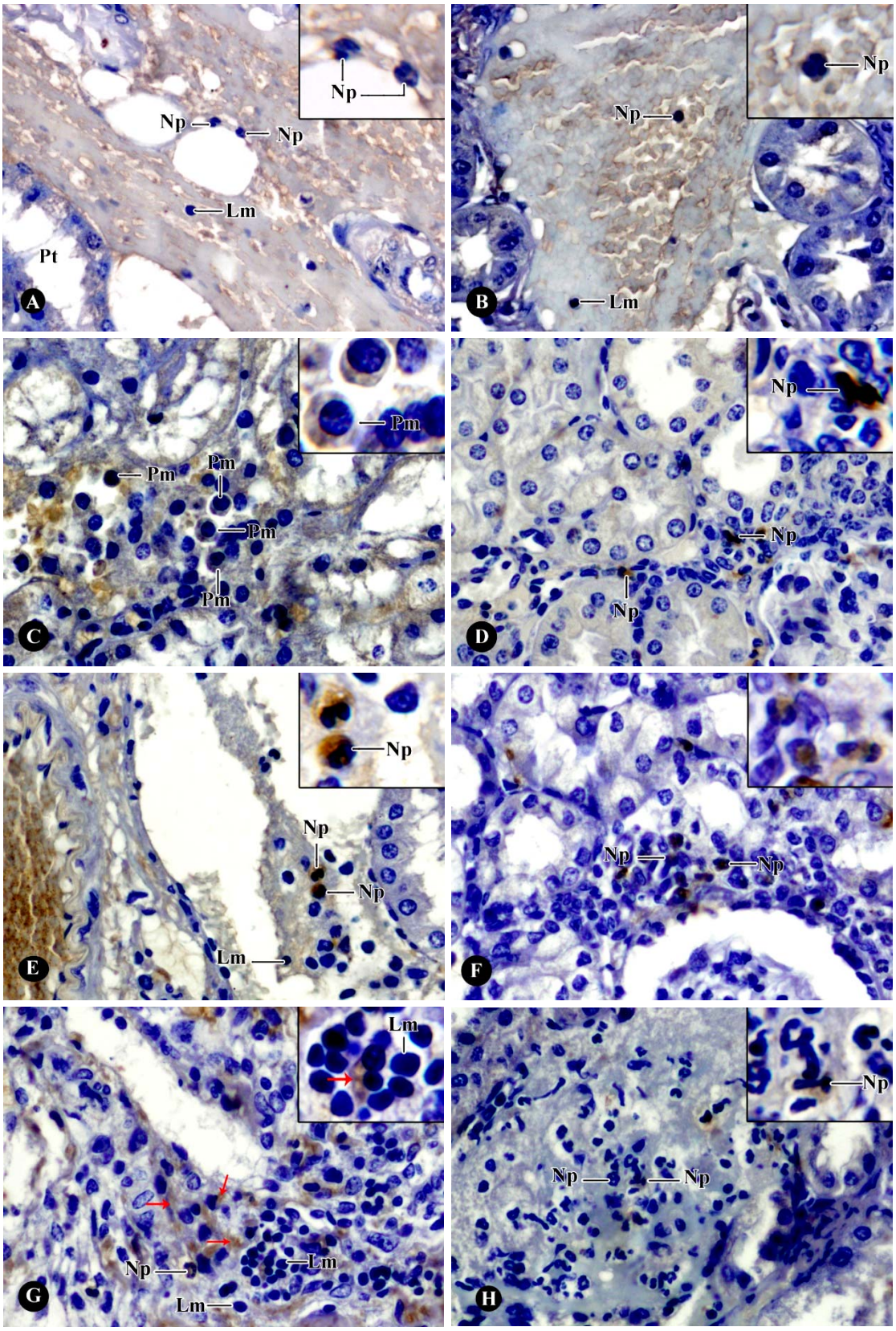

Np
Np
Np
Lm
Pt
A
Np
Np
Lm
B
Pm
Pm
Pm
Pm
Pm
C
Np
Np
Np
D
Np
Np
Np
Lm
E
Np
Np
Np
F
Lm
Np
Lm
Lm
G
Np
Np
Np
H

**Figure 16**

**Figure 17** Photomicrographs of the kidney illustrating the blood vessels in the groups of one hour (A), six hours (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans*, serovar pyrogenes. (Obj 40x)

A) The moderately – stained area in the lumen (Lu) of the blood vessel and mildly – stained area on the internal elastic lamina (Ie) are shown.

B) The mildly – stained internal elastic lamina, tunica adventitia (Av) and luminal content (Lu) are performed.

C) The moderately – stained area in the lumen of blood vessel and on the internal elastic lamina as well as intensively – stained tunica adventitia are displayed.

D) The intensively – stained area are demonstrated in the lumen of the blood vessel and tunica adventitia while the internal elastic lamina is moderately – stained.

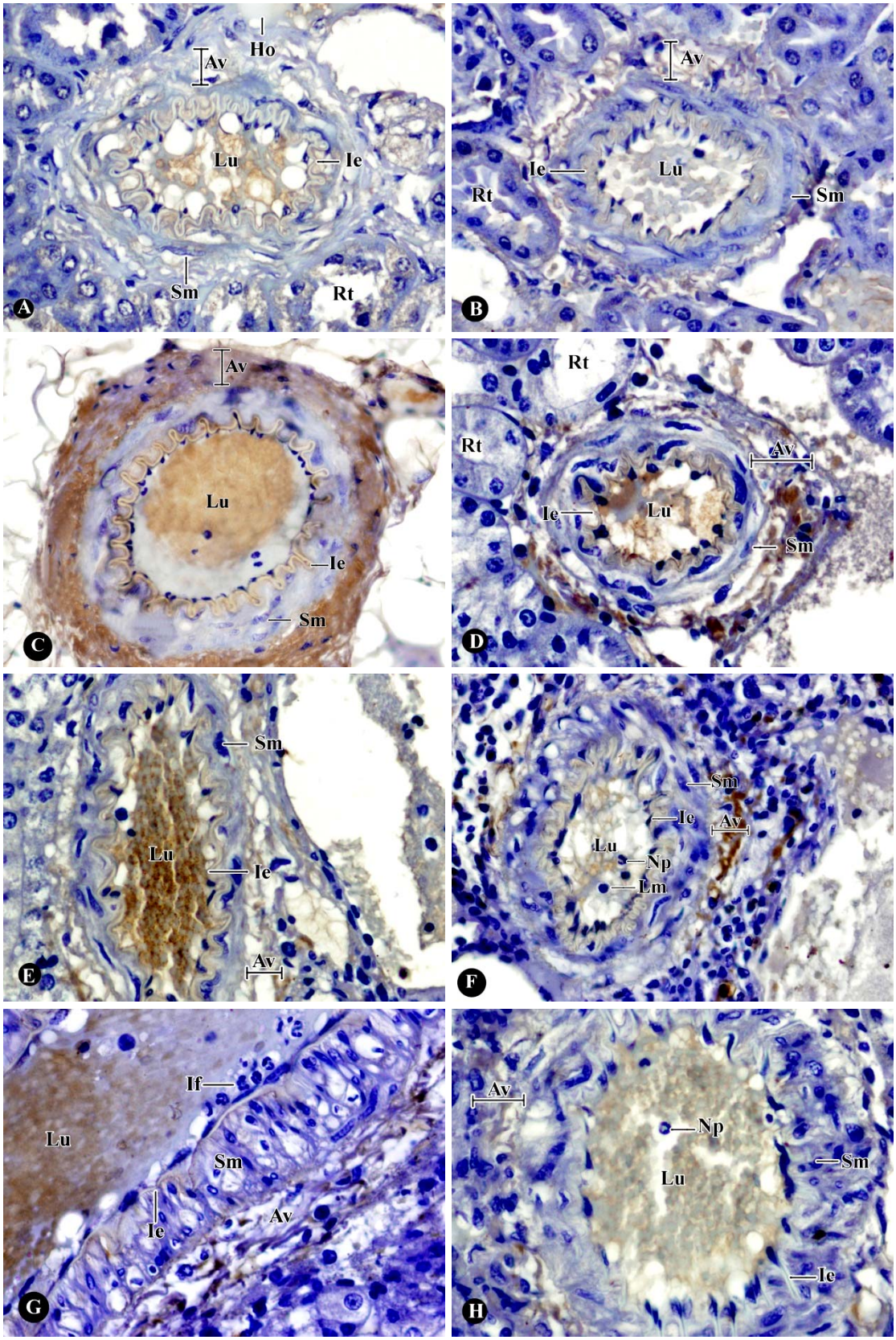
E) The intensively – stained area is found in the lumen of the blood vessel, while the mildly – stained one is deposited on the internal elastic lamina) and tunica adventitia layer.

F) The mildly – stained areas are found in the lumen of the blood vessel and on the internal elastic lamina, while the tunica adventitia shows the intensively – stained coloration.

G) The intensively – stained area is seen in the lumen and the tunica adventitia, while the internal elastic lamina is mild stain.

H) The mildly – stained coloration is shown in the lumen of the blood vessel and tunica adventitia layer. It is not performed in the internal elastic lamina.

Abbreviation: Lm, Lymphocyte; Np, Neutrophil; Rt, Renal tubule; Sm, Smooth muscle cells.


Ho
Av
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Sm
B
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Ie
Sm
C
Rt
Rt
Av
Ie
Lu
Sm
D
Sm
Lu
Ie
Av
E
Sm
Ie
Av
Lu
Np
Lm
F
If
Lu
Sm
Ie
Av
G
Av
Np
Lu
Sm
Ie
H

**Figure** **17**

**Skeletal muscles**

The tissue of hamstring and gastrocnemius muscles of the control group and the experimental negative staining control of the infected groups showed no golden–brown coloration in the tissue sections including the inflammatory cells, muscle fiber, blood vessels, connective tissue supporting muscle bundle and nerve supplying muscle bundle. These sections were seen as the blue stain of the Carrazi's haematoxylin solution. (Figure 18 and 24)

**1. Hamstring muscle** (Table 2)

Some structure in the hamstring muscle of different experimental groups, including inflammatory cells, muscle fibers, blood vessels, connective tissue and nerve fibers showed the positive golden–brown coloration of different intensity.

**Inflammatory cells** (Figure 19)

The inflammatory cells were mainly the neutrophils which scattered throughout the muscular tissue sections from the one hour to the four days groups displayed the negatively–stained cytoplasm (Figure 19A–F). The group of five days post infection, the neutrophils showed the intensively–stained cytoplasm which occupied within the moderately–stained hemorrhagic area (Figure 19G). Anyhow, the cytoplasm of the neutrophils became negative stain again in the group of six days post infection (Figure 19H).These inflammatory cells were mostly found in the hemorrhagic area like in the groups of one hour (Figure 19A), six hours (Figure 19B), one day (Figure 19C) and five days (Figure 19G) post infection. In addition, these cells could be also demonstrated in the homogeneous hyalinic material (Figure 19D), in the lumen of the vessels (Figure 19E) as well as in the connective tissue supporting muscle bundle (Figure 19F and H)

**Muscle fibers** (Figure 20)

The muscle fiber of hamstring muscle demonstrated the positive stain of various intensities in all infected groups from the first hour to six days post infection. Those of one hour (Figure 20A), one day (Figure 20C), two days (Figure 20D) and six days (Figure 20H) groups expressed the mild golden–brown coloration in the muscle

cell while the group of six hours (Figure 20B) post infection expressed moderate golden–brown coloration. Besides those of the three days to five days post infection groups (Figure 20E–G) revealed the intensive positive stain in their skeletal muscle fibers.

**Blood vessels** (Figure 21)

The blood vessels supplying the hamstring muscle displayed the positive golden–brown stain in all infected groups. However, the pattern of the positive–stained area was different, including the content in the lumen and the component of the vessel wall. Additionally, the intensity of these stained areas varied. The one hour to two days post infection groups showed the same staining pattern (Figure 21A–D) which expressed the negative–stain in the lumen, endothelial cell and smooth muscle cells in muscular layer. Besides the tunica adventitia revealed the moderate golden–brown coloration. The contents in the lumen which were supposed mainly to be the red blood cells were positively stained of different density from the group of three days onward to the group of six days. Those in the group of three days expressed the moderate stain while in the group of four day to six days showed the mild stain (Figure 21E–H). Concerning the vascular wall, the positive stain was located in the endothelium, smooth muscle cell and tunica adventitia at different intensity in each experimental groups from three days onward. The cytoplasm of the endothelium showed the positive stain of moderate intensity only in the groups of four days (Figure 21F). The smooth muscle cells in the muscular layer were stained mildly in the groups of three and five days (Figure 21E and G) and moderately in the group of four days (Figure 21F). The tunica adventitia from the three days onward performed the intensive stain except those of the four days group revealed the mild stain (Figure 21E–H).

**Connective tissue** (Figure 22)

The connective tissue supporting the muscle bundle demonstrated the positive stain with various intensities in all experimental groups except only in the one hour group that revealed the negative stain (Figure 22A). The connective tissue of six hours

post infection demonstrated the mildly–stained area (figure 22B) while those of one to six days revealed intensively–stained areas (Figure 22C–H).

**Nerve fibers** (Figure 23)

The golden–brown area was observed in the nerve fibers supplying the hamstring muscle of all experimental groups. The positive stain was illustrated in various intensities. The mild stain appeared in the groups of one day (Figure 22C), four days (Figure 22F) and six days (Figure 22H); moderate stain in one and six hours (Figure 22A and B) as well as intensive stain in two days (Figure 22D), three days (Figure 22E) and five days (Figure 22G) post infected groups.

**Table 2** Immunoperoxidase staining of the hamstring muscle of hamsters infected with *Leptospira interrogans*, serovar pyrogenes.

| Hamstring muscle | Durations of post infected hamster | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **1 hr.** | **6 hrs.** | **1 Day** | **2 Days** | **3 Days** | **4 Days** | **5 Days** | **6 Days** |
| **Inflammatory cells** | | | | | | | | |
| • Neutrophils | - | - | - | - | - | - | +++ | - |
| **Muscle fibers** | + | ++ | + | + | +++ | +++ | +++ | + |
| **Blood vessels** | | | | | | | | |
| • Lumen | - | - | - | - | ++ | + | + | + |
| • Endothelium | - | - | - | - | - | ++ | - | - |
| • Muscular layer | - | - | - | - | + | ++ | + | - |
| • Tunica adventitia | ++ | ++ | ++ | ++ | +++ | + | +++ | +++ |
| **Connective tissue** | - | + | +++ | +++ | +++ | +++ | +++ | +++ |
| **Nerve** | ++ | ++ | + | +++ | +++ | + | +++ | + |

Evaluation of results using a four–grade, semi–quantitative scale: negative (-), mild (+), moderate (++) and intensive (+++)

**Gastrocnemius muscle** (Table 3)

The various degrees of the positive golden–brown coloration were displayed in the gastrocnemius muscular tissue sections, including inflammatory cells, muscle fiber, blood vessels, connective tissue and nerve fiber.

**Inflammatory cells** (Figure 25)

The inflammatory cells were mainly the neutrophils scattering throughout the muscular tissue sections in all experimental groups. These neutrophils displayed the negatively–stained cytoplasm in all experimental groups (Figure 25A–G) except those in the six days groups that showed the intensively–stained cytoplasm (Figure 25H). The inflammatory cells occupied in various locations. Those were connective tissue (Figure 25A), lumen of the blood vessels (Figure 25B–C and D–F) and homogeneous hyalinic material (Figure 25G–H).

**Muscle fibers** (Figure 26)

The muscle fibers of gastrocnemius muscle showed the positive stain in different intensities of all infected groups. However, the pattern of the positive stained area varied. Those of two days (Figure 26D) and three days (Figure 26E) groups revealed the mild golden–brown coloration in the muscle cell while the groups of one and six days (Figure 26C and H) groups expressed moderate stain. Besides those of the one hour (Figure 26A), six hours (Figure 26B), four days (Figure 26F) and five days (Figure 26G) post infection groups demonstrated the intensive golden–brown coloration.

**Blood vessels** (Figure 27)

The vessels supplying the gastrocnemius muscle showed the positive golden–brown stain in some of the infected groups including one day (Figure 27C), two days (Figure 27D), three days (Figure 27E), five days (Figure 27G) and six days (Figure 27H) after infection. The pattern of the positive stained area was similar. The positive stain of different intensity was found only in the tunica adventitia. The other component of the vessel wall and the lumen performed the negative stain. The mild stain was localized in the groups of two and six days (Figure 27D and H), moderate in

three days (Figure 27E)and intensive in one day (Figure 27C) and five days (Figure 27G) post infection groups.

**Connective tissue** (Figure 28)

The positive golden–brown area was observed in the connective tissue supporting the muscle bundle of all the infected groups. However, the positive coloration intensity demonstrated different degree in various experimental groups. Those in the first hour group showed the mild stain (Figure 28A) and then moderate in the six hours (Figure 28B) and one day (Figure 28C) then they became intensive in the groups of two days to six days post infection (Figure 26D–H).

**Nerve fibers** (Figure 29)

The nerve fibers supplying the muscle bundle demonstrated both negative and positive stain. The positive stained nerve fibers were revealed in various degrees. The mild stain was seen in the groups of six hours (Figure 29B), three days (Figure 29E) and four days (Figure 29F), moderate stain in two days (Figure 29D) and intensive stain in one hour (Figure 29A), one days (Figure 29C) and five days (Figure 29G). Those in the group of six days expressed the negative stained nerve fibers (Figure 29H).

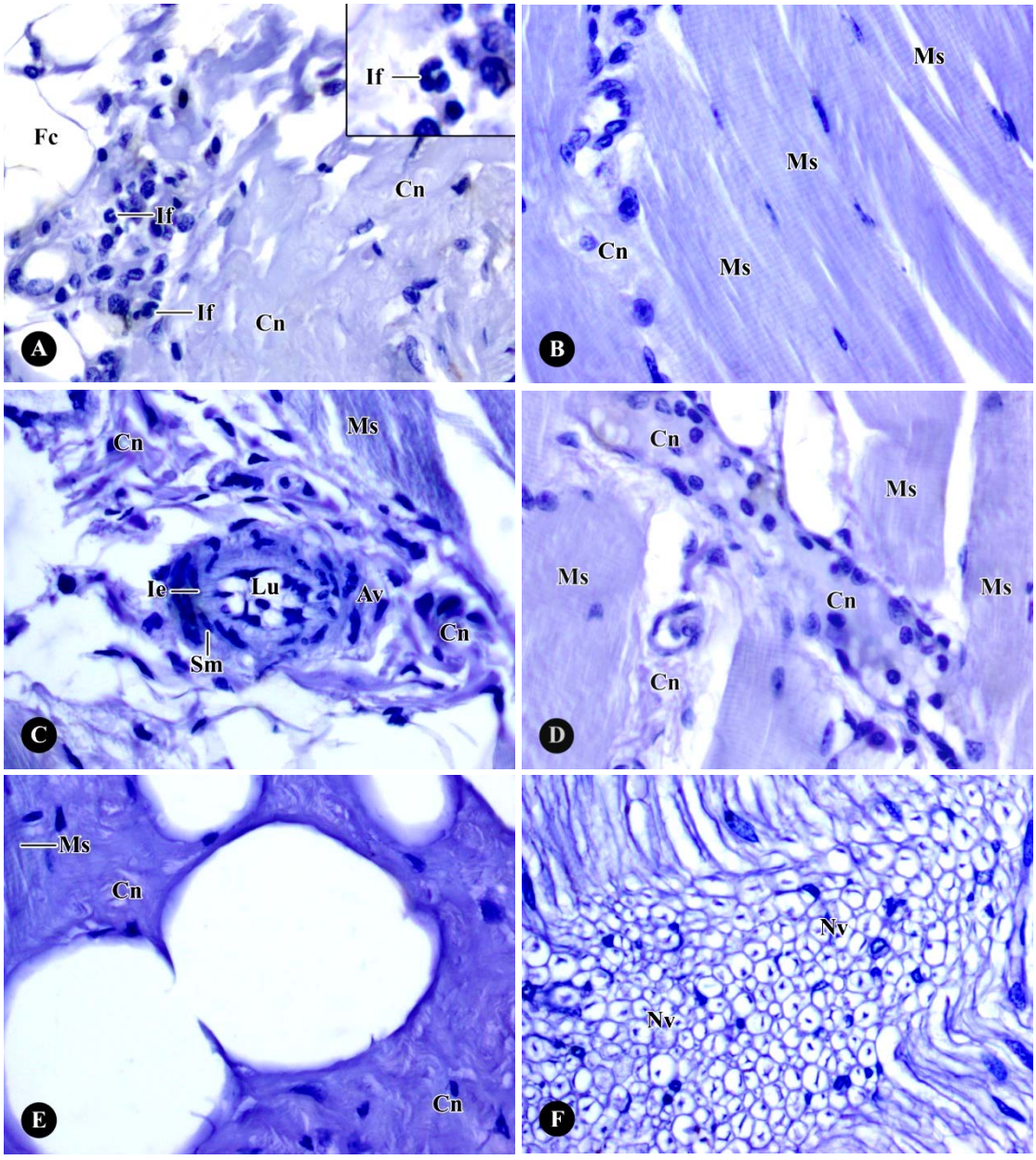
**Table 3** Immunoperoxidase staining of the gastrocnemius muscle of hamsters infected with *Leptospira interrogans*, serovar pyrogenes.

| Gastrocnemius muscle | Durations of post infected hamster | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **1 hr.** | **6 hrs.** | **1 Day** | **2 Days** | **3 Days** | **4 Days** | **5 Days** | **6 Days** |
| **Inflammatory cells** | | | | | | | | |
| • Neutrophils | - | - | - | - | - | - | - | +++ |
| **Muscle fibers** | +++ | +++ | ++ | + | + | +++ | +++ | ++ |
| **Blood vessels** | | | | | | | | |
| • Lumen | - | - | - | - | - | - | - | - |
| • Endothelium | - | - | - | - | - | - | - | - |
| • Muscular layer | - | - | - | - | - | - | - | - |
| • Tunica adventitia | - | - | +++ | + | ++ | - | +++ | + |
| **Connective tissue** | + | ++ | ++ | +++ | +++ | +++ | +++ | +++ |
| **Nerve** | +++ | + | +++ | ++ | + | + | +++ | - |

Evaluation of results using a four–grade, semi–quantitative scale: negative (-), mild (+), moderate (++) and intensive (+++)

**Figure 18** Photomicrographs illustrating the experimental negative control of the hamstring muscle in the groups of three days (A), five days (B – E) and six days (F) post infected hamsters with *Leptospira interrogans*, serovar pyrogenes. The inflammatory cell infiltration (If) are shown in A, the skeletal muscle fiber (Ms) in B, the blood vessel supplying the muscle in C, the connective tissue supporting muscle bundle (Cn) in D and E and the nerve supplying muscle bundle (Nv) in F. No any golden–brown stained area is seen in the tissue sections. (Obj 40x; inset 100x)

Abbreviation: Av, Tunica adventitia layer; Fc, Fat cell; Ie, Internal elastic lamina; Lu, Lumen of blood vessel; Sm, Smooth muscle in muscular layer of blood vessel.


A
Fc
If
Cn
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If
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Av
Cn
Sm
D
Cn
Ms
Ms
Cn
Ms
Cn
E
Ms
Cn
Cn
F
Nv
Nv

**Figure 18**

**Figure 19** Photomicrographs of the hamstring muscle showing the inflammatory cell infiltration (If) in the groups of one hour (A), six hours (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamster with *Leptospira interrogans*, serovar pyrogenes.(Obj 40x)

A – F) The neutrophils (Np) perform the negatively – stained cytoplasm in the various area including the negatively – stained hemorrhagic area (A and B), mildly – stained hemorrhagic area (C), negatively – stained homogeneous hyalinic material (Ho) (D), the lumen of blood vessel (E) and the mildly – stained connective tissue (F).

G) The neutrophils show the intensive golden – brown cytoplasm in the moderately – stained hemorrhagic area.

H) The negatively – stained cytoplasm of neutrophils are seen in the intensively – stained connective tissue (Cn).

Abbreviation: Bv, Blood vessel; Ms, Muscle fiber; Rb, Red blood cell.

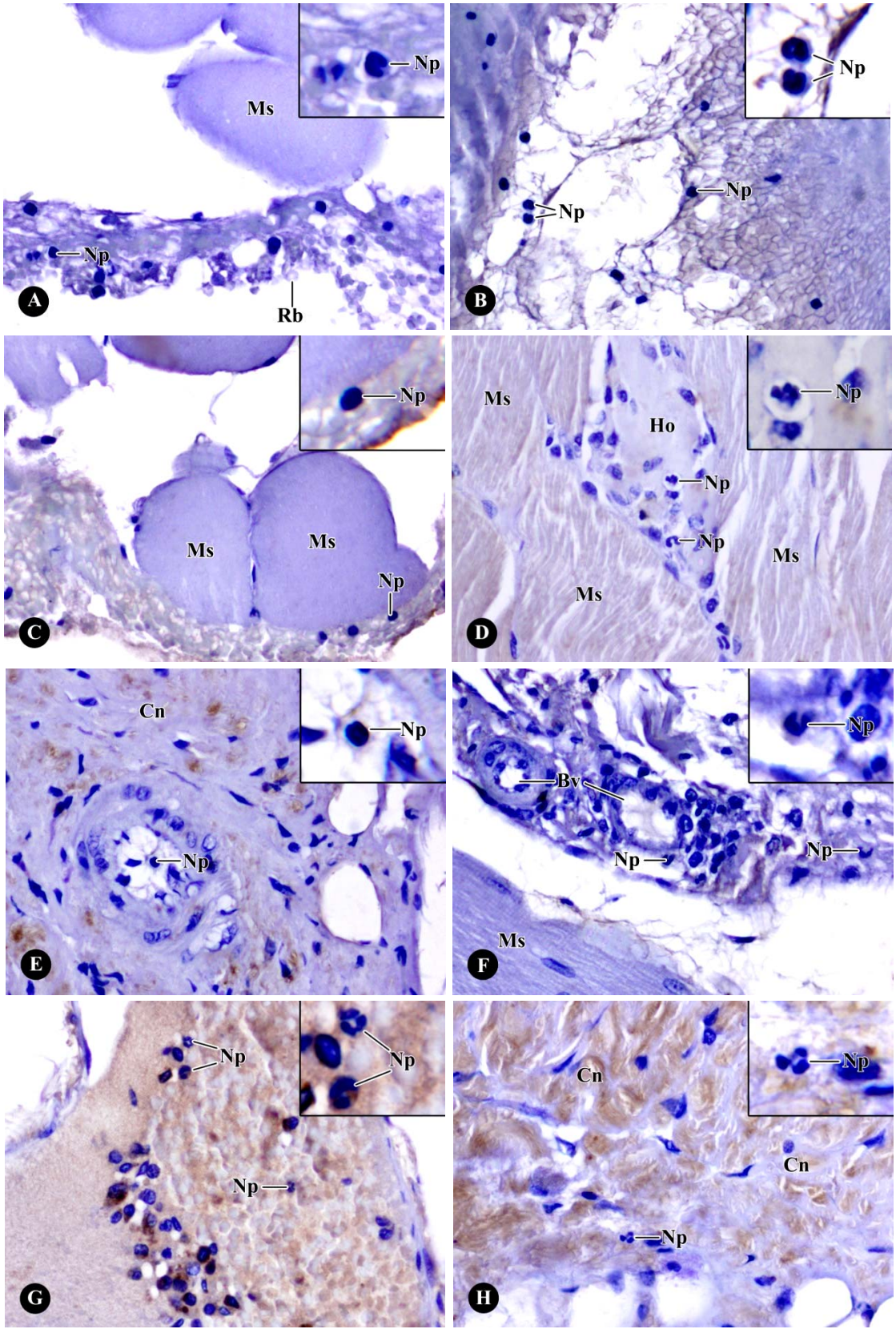



**Figure 19**

**Figure 20** Photomicrographs of the hamstring muscle illustrating the muscle fiber (Ms) in the groups of one hour (A), six hours (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamster with *Leptospira interrogans*, serovar pyrogenes. (Obj 40x)

A) The mild gold – brown coloration are scattered throughout within the muscle fiber.

B) The moderately – stained muscle fiber are shown and spread nearby the connective tissue.

C) The mild golden – brown coloration are localized within the muscle fiber (arrow).

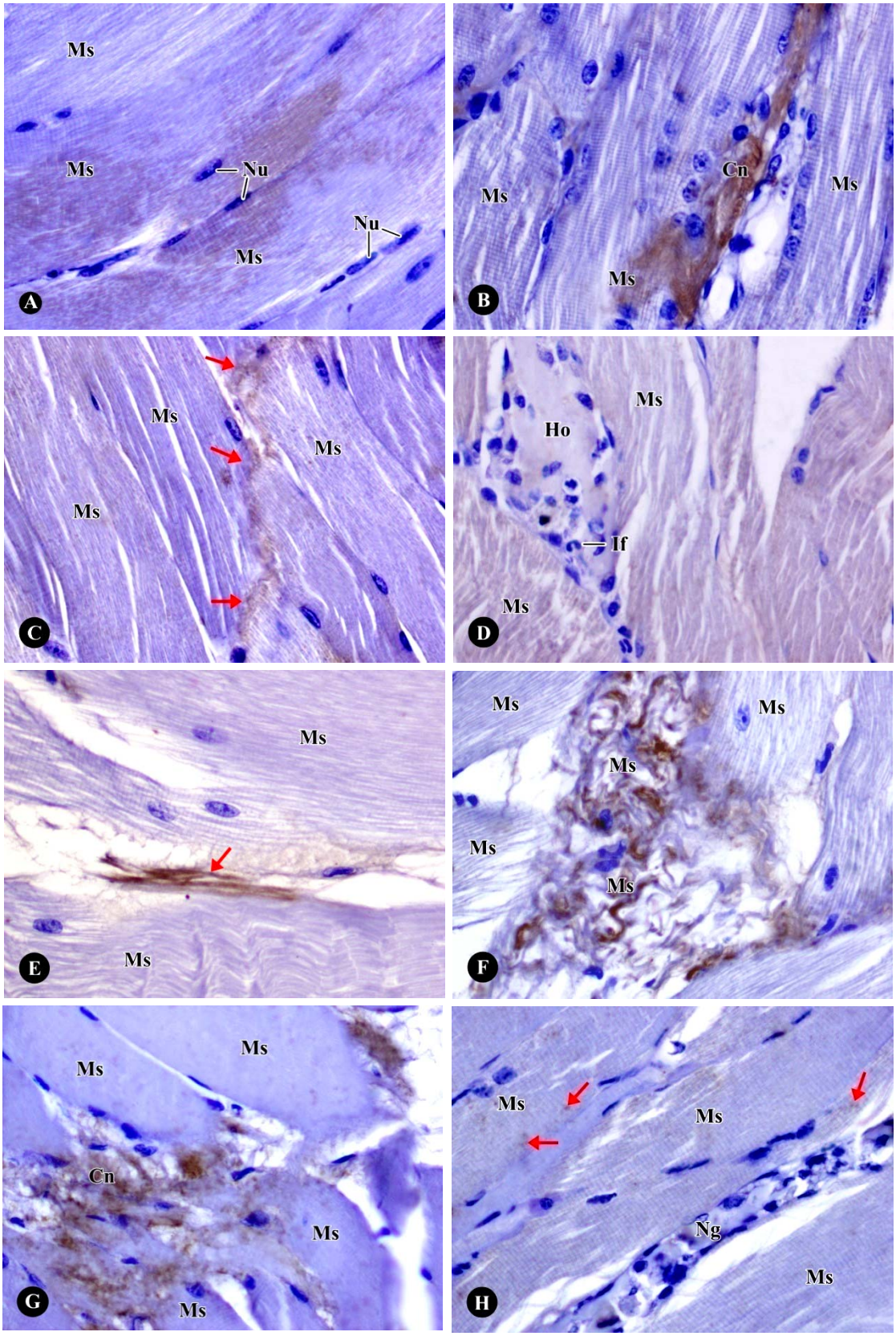
D) The mild golden – brown coloration are seen within the muscle fiber.

E) The intensive golden – brown coloration are deposited on the peripheral area of muscle fiber (arrow).

F) The intensive golden – brown coloration are observed at the end of the degenerative muscle fiber.

G) The intensive golden – brown coloration are scattered both in the connective tissue and muscle fiber.

H) The muscle fibers show the mild golden – brown spot in their sacroplasm.


Ms
Ms
Nu
Nu
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A
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Cn
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B
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Ms
C
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Ho
If
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Ms
Ms
E
Ms
Ms
Ms
Ms
Ms
F
Ms
Ms
Cn
Ms
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H

**Figure 20**

**Figure 21** Photomicrographs of hamstring muscle revealing the blood vessels supplying the muscle bundle in the groups of one hour (A), six hours (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamster with *Leptospira interrogans,* serovar pyrogenes.(Obj 40x)

A – D) The moderately – stained layer in the tunica adventitia (Av) of the blood vessel supplying the muscle bundle are shown.

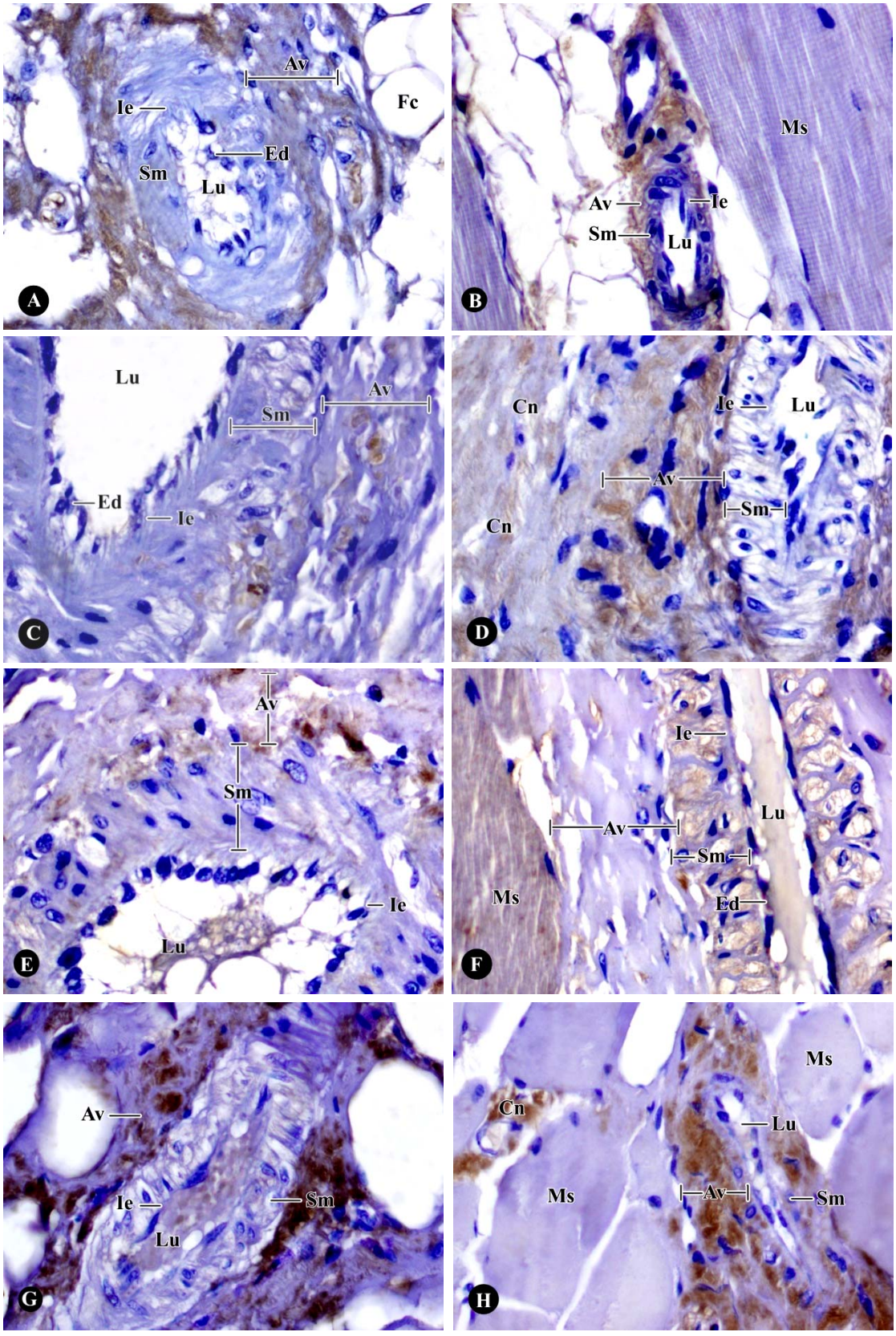
E) The mildly – stained areas are demonstrated in the cytoplasm of smooth muscle cells (Sm) in muscular layer, moderately in the lumen and intensively in the tunica adventitia.

F) The moderate golden – brown coloration are observed in the cytoplasm of endothelial lining cell (Ed) and smooth muscle cells of muscular layer while in the lumen and tunica adventitia layer are expressed the mild golden – brown coloration.

G) The mildly – stained area is found in the lumen of the blood vessel and the smooth muscle cells while the tunica adventitia shows the intensively – stained layer.

H) The mildly – stained area is observed in the lumen of the blood vessel while the tunica adventitia expresses the intensively – stained area.

Abbreviation: Cn, Connective tissue; Fc, Fat cell; Ie, Internal elastic lamina; Ms, Muscle fiber.


Av
Ie
Fc
Ed
Sm
Lu
A
Ms
Av
Ie
Sm
Lu
B
Lu
Av
Sm
Ed
Ie
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Cn
Ie
Lu
Av
Sm
Cn
D
Av
Sm
Ie
Lu
E
Ie
Lu
Av
Sm
Ed
Ms
F
Av
Ie
Sm
Lu
G
Ms
Cn
Lu
Ms
Av
Sm
H

**Figure 21**

**Figure 22** Photomicrographs of the hamstring muscle illustrating the connective tissue (Cn) supporting the muscle bundle in the groups of one hour (A), six hours (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamster with *Leptospira interrogans,* serovar pyrogenes.

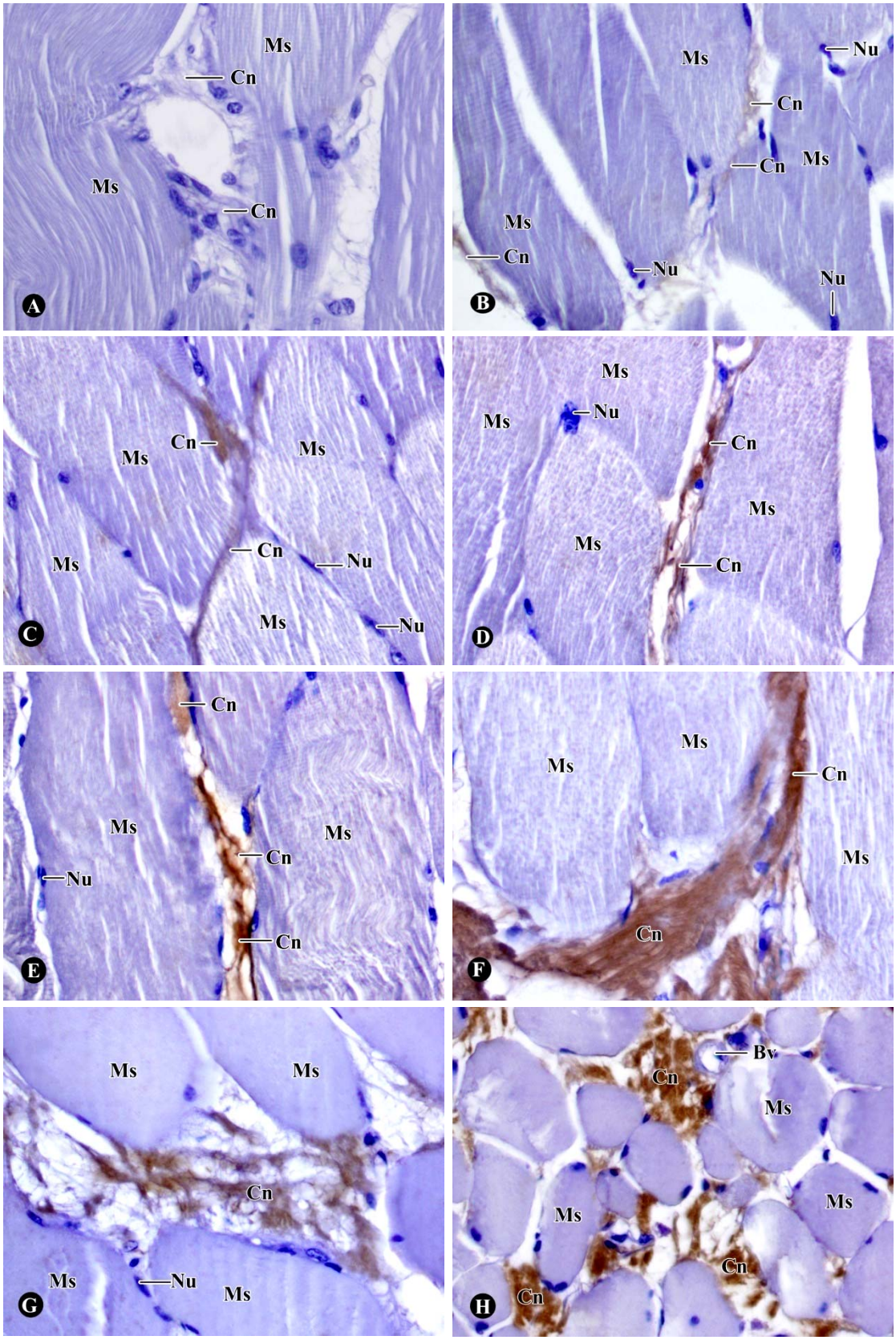
A) The negatively–stained connective tissue is shown. (Obj 40x)

B) The connective tissue between muscle fibers (Ms) is mild–stain. (Obj 40x)

C – G) The intensively–stained area is shown in the connective tissue supporting muscle fiber. (Obj 40x)

H) The intensively–stained connective tissue are scattered between skeletal muscle cells throughout the muscle belly. (Obj 20x)

Abbreviation: Bv, Blood vessel; Nu, Nucleus of muscle fiber.


A
Ms
Cn
Ms
Cn
B
Ms
Nu
Cn
Cn
Ms
Ms
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Nu
Nu
C
Cn
Ms
Ms
Ms
Cn
Nu
Ms
Nu
D
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Ms
Nu
Cn
Ms
Ms
Cn
E
Cn
Ms
Ms
Nu
Cn
Cn
F
Ms
Ms
Cn
Ms
Cn
G
Ms
Ms
Cn
Ms
Nu
Ms
H
Bv
Cn
Ms
Ms
Ms
Cn
Cn

**Figure 22**

**Figure 23** Photomicrographs of hamstring muscle revealing the nerve (Nv) supplying the muscle fiber (Ms) in the groups of one hour (A), six hours (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamster with *Leptospira interrogans*, serovar pyrogenes.(Obj 40x)

A) The moderate golden – brown coloration is found within the nerve fiber supplying the muscle cell.

B) The moderate golden – brown coloration is seen in the nerve fiber.

C) The mild – stain is seen in the nerve supplying the hamstring muscle.

D) The nerve fibers reveal the intensive golden – brown coloration.

E) The intensively – stained nerve fibers are shown.

F) The mild golden – brown coloration are seen in the nerve supplying the muscle fiber.

G) The nerve fibers display the intensive golden – brown coloration.

H) The mild golden – brown coloration is seen in the nerve supplying the muscle bundle.

Abbreviation: Bv, Blood vessel; Cn, Connective tissue; Fc, Fat cell; Np, Neutrophil.

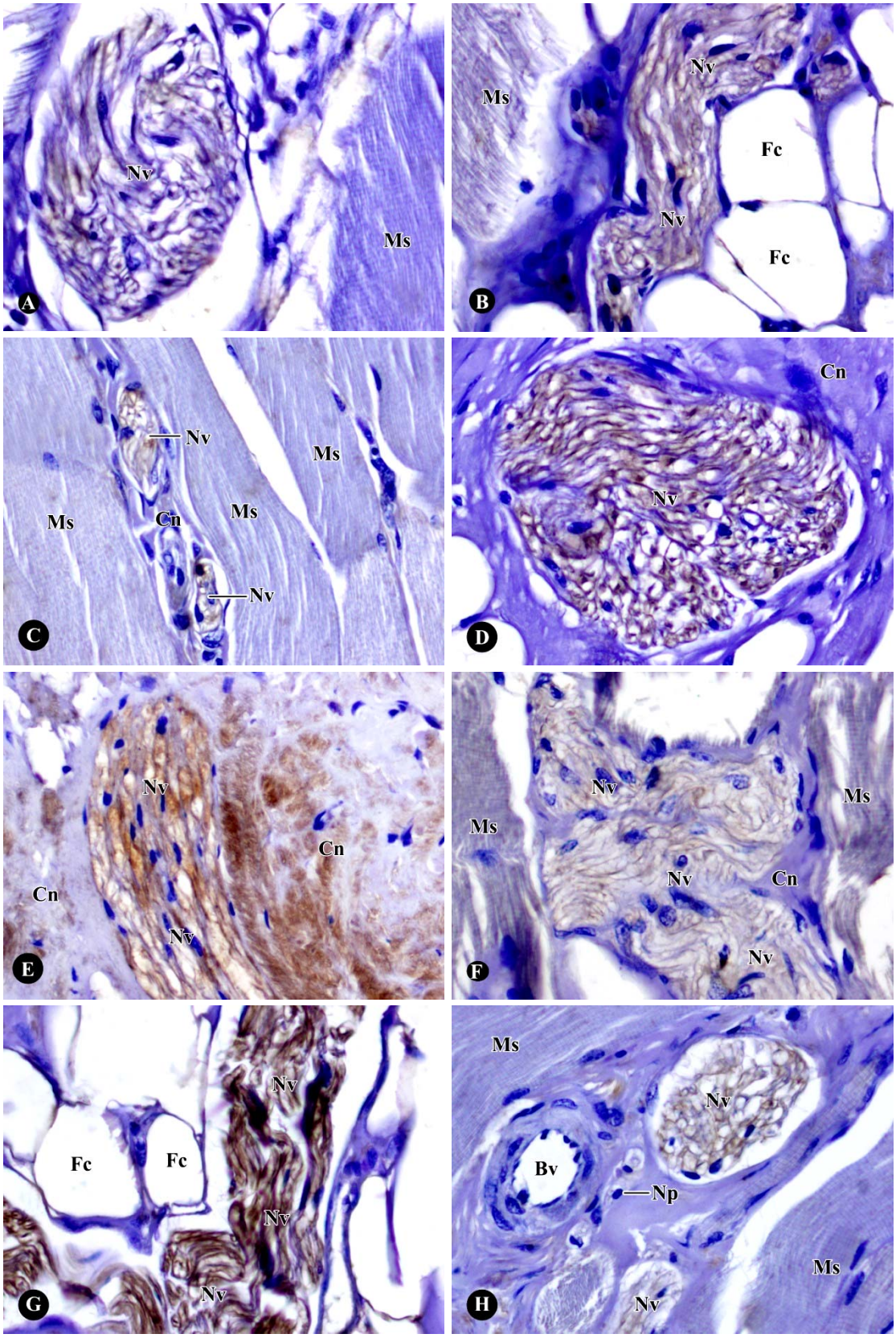
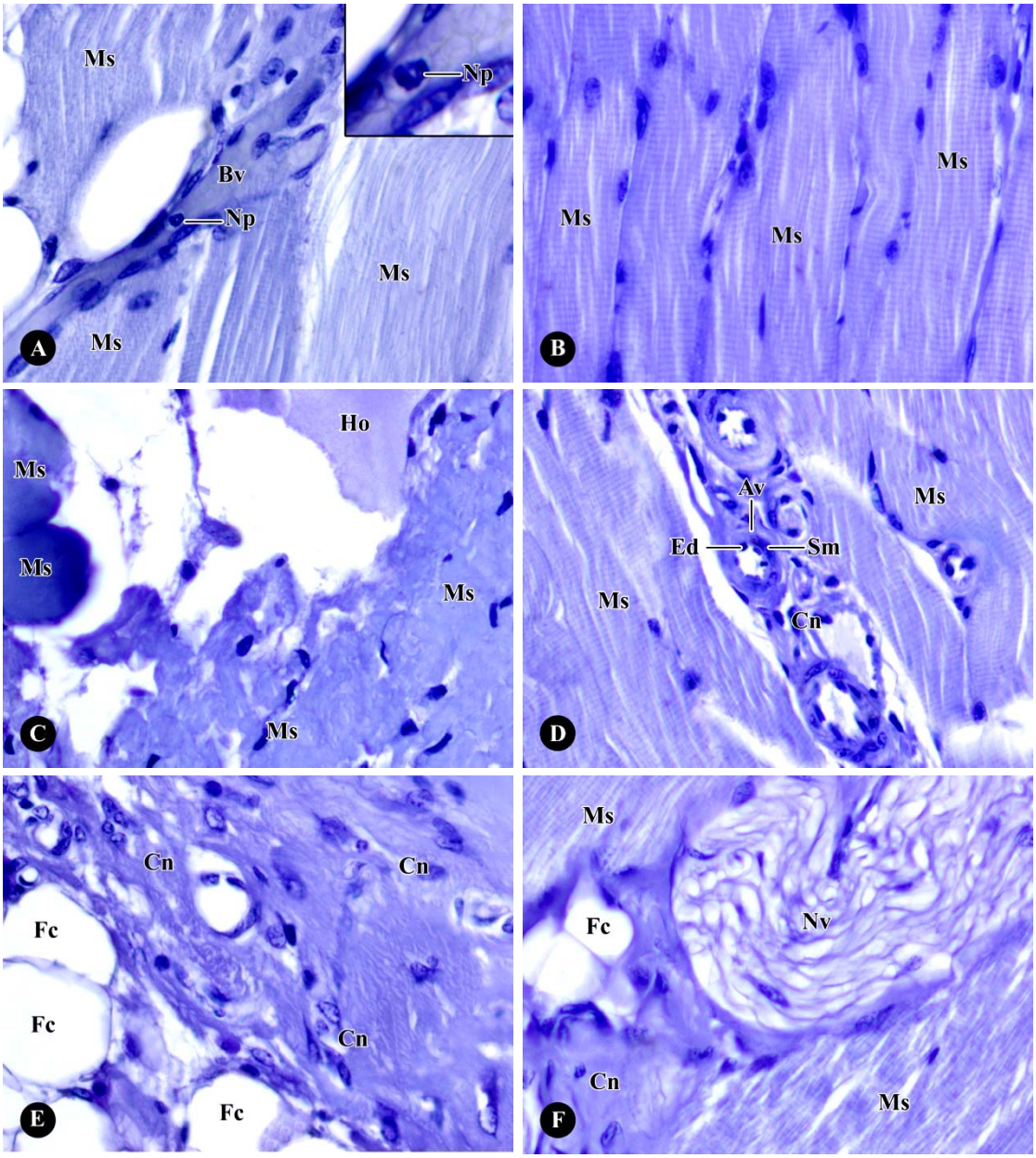

A
Nv
Ms
B
Ms
Nv
Fc
Nv
Fc
C
Nv
Ms
Ms
Cn
Ms
Nv
D
Cn
Nv
E
Nv
Cn
Cn
Nv
F
Nv
Ms
Ms
Nv
Cn
Nv
G
Fc
Fc
Nv
Nv
Nv
H
Ms
Nv
Bv
Np
Ms
Nv

**Figure 23**

**Figure 24** Photomicrographs revealing the experimental negative control of the gastrocnemius muscle in the groups of six hours (A and B), one day (C), three days (D) and six days (E and F) post infected hamsters with *Leptospira interrogans*, serovar pyrogenes. The inflammatory cell infiltration (If) are shown in A, the skeletal muscle fiber (Ms) in B and C, the blood vessel supplying the muscle in D, the connective tissue supporting muscle bundle (Cn) in E and the nerve supplying muscle bundle (Nv) in F. No any golden – brown stained area is seen in the tissue sections. (Obj 40x; inset 100x)

Abbreviation: Av, Tunica adventitia layer; Ed, Endothetial lining cell; Fc, Fat cell; Ho, Homogeneous hyalinic material; Np, Neutrophil; Sm, Smooth muscle in muscular layer of blood vessel.


Ms
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Bv
Np
Ms
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Ms
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Ms
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Ms
C
Av
Ms
Ed
Sm
Ms
Cn
D
Cn
Cn
Fc
Fc
Cn
Fc
E
Ms
Fc
Nv
Cn
Ms
F

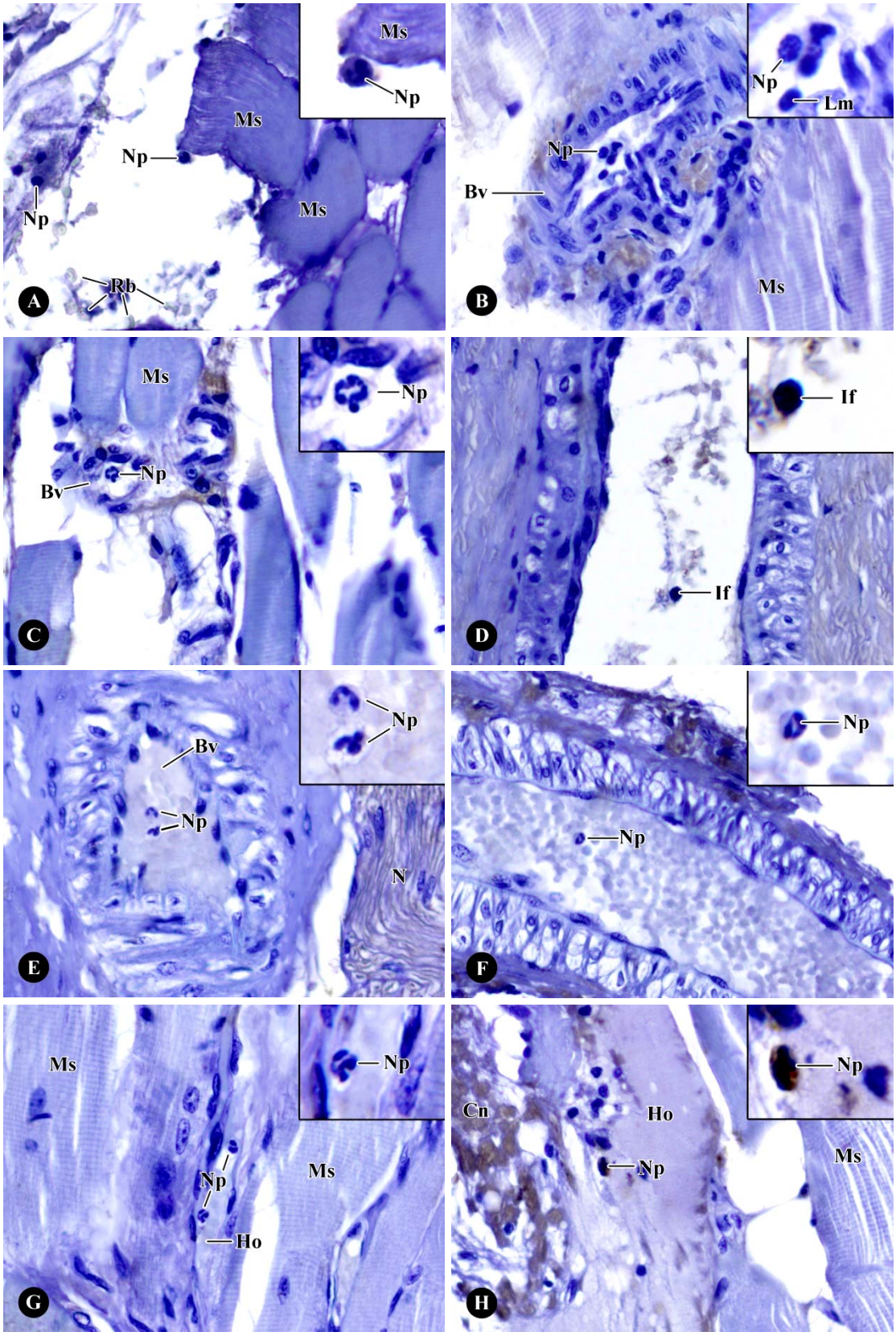
**Figure 24**

**Figure 25** Photomicrpgraphs of gastrocnemius muscle revealing the inflammatory cells (If) in the groups of one hour (A), six hour (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans*, serovar pyrogenes. (Obj 40x; inset 100x)

A – G) The neutrophils (Np) in various area of the muscular tissue of all the experimental groups except those of the six days group demonstrate the negative stain. The neutrophils locating in the connective tissue (A), lumen of the blood vessel (B – C and E – F) and homogeneous hyalinic material (Ho) (G) are shown. Some unidentified inflammatory cells are displayed in the lumen of the blood vessel (D).

H) The intensive golden – brown coloration is expressed in the cytoplasm of the neutrophil situating in the positive mild golden–brown homogeneous hyalinic material.

Abbreviation: Ms, Muscle cell; Rb, Red blood cell.


Ms
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C
If
If
D
Np
Bv
Np
N
E
Np
Np
F
Ms
Np
Np
Ms
Ho
G
Cn
Ho
Np
Np
Ms
H

**Figure 25**

**Figure 26** Photomicrpgraphs of gastrocnemius muscle revealing the muscle fiber (Ms) in the groups of one hour (A), six hour (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans*, serovar pyrogenes.

A – B) The intensive golden – brown coloration are located within the muscle cell (arrow). (Obj 100x)

C) The moderately – stained area are shown within the muscle cell (arrow) adjacent to the connective tissue. (Obj 100x)

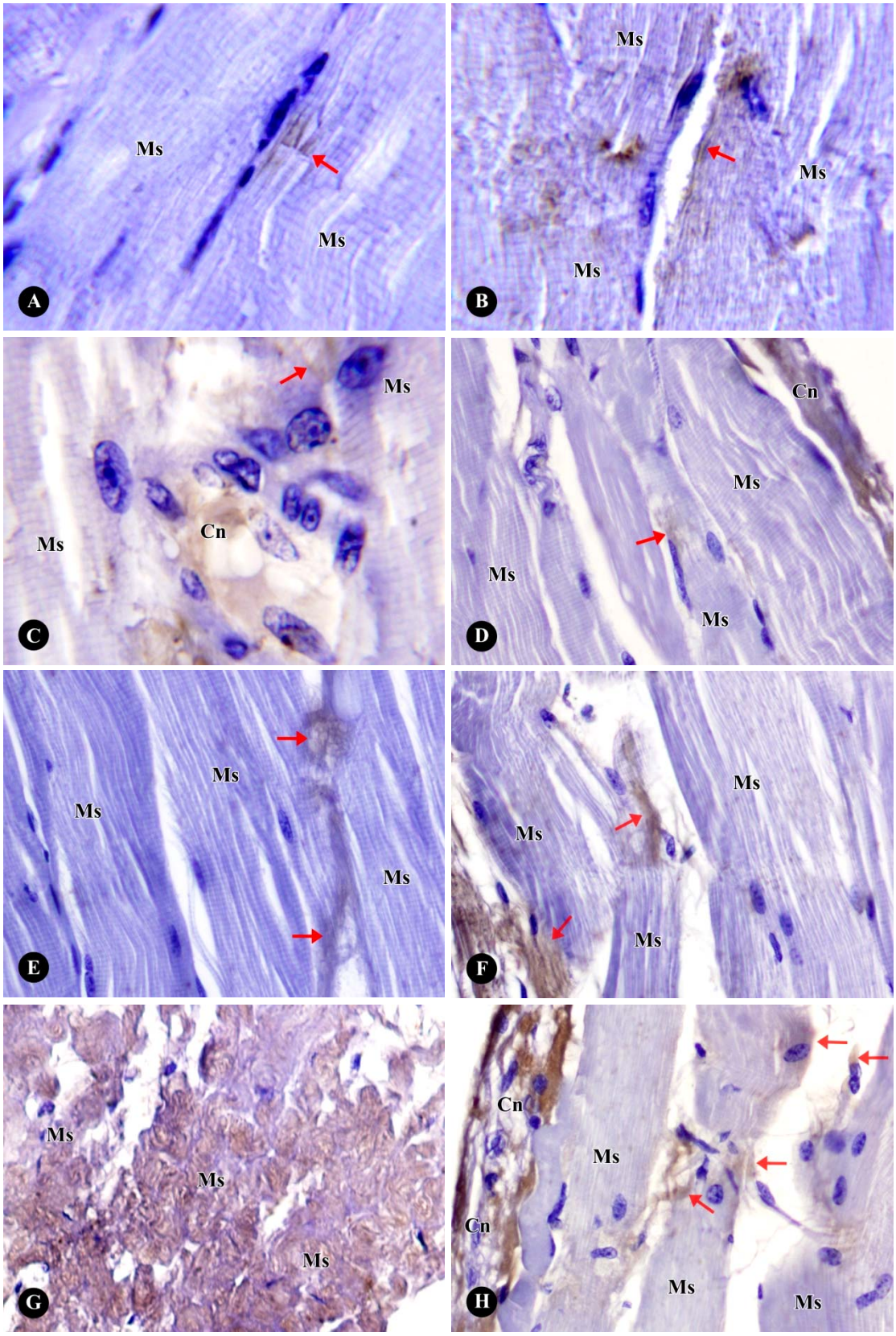
D – E) The mild golden – brown coloration are localized within the muscle fibers (arrow). (Obj 40x)

F) The intensive golden – brown coloration are deposited on the peripheral area of muscle fibers (arrow). (Obj 40x)

G) The intensive golden – brown coloration are scattered throughout the degenerated muscle fibers. (Obj 40x)

H) The muscle fibers reveal the moderate golden – brown area within the muscle fibers (arrow). (Obj 40x)

Abbreviation: Cn, Connective tissue.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F
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Ms
Ms
G
Ms
Ms
Ms
H
Cn
Ms
Cn
Ms
Ms

**Figure 26**

**Figure 27** Photomicrpgraphs of gastrocnemius muscle revealing the blood vessels supplying the muscle bundle in the groups of one hour (A), six hour (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans,* serovar pyrogenes. (Obj 40x)

A – B) The negative – stain are shown both in the lumen (Lu) and the vessel wall.

C) The intensive golden – brown coloration are seen in the tunica adventitia (Av) which are continuous with adjacent connective tissue supporting the muscle bundle. The endothelial lining cells (Ed) and smooth muscle cells in muscular layer (Sm) show the negative golden–brown stain.

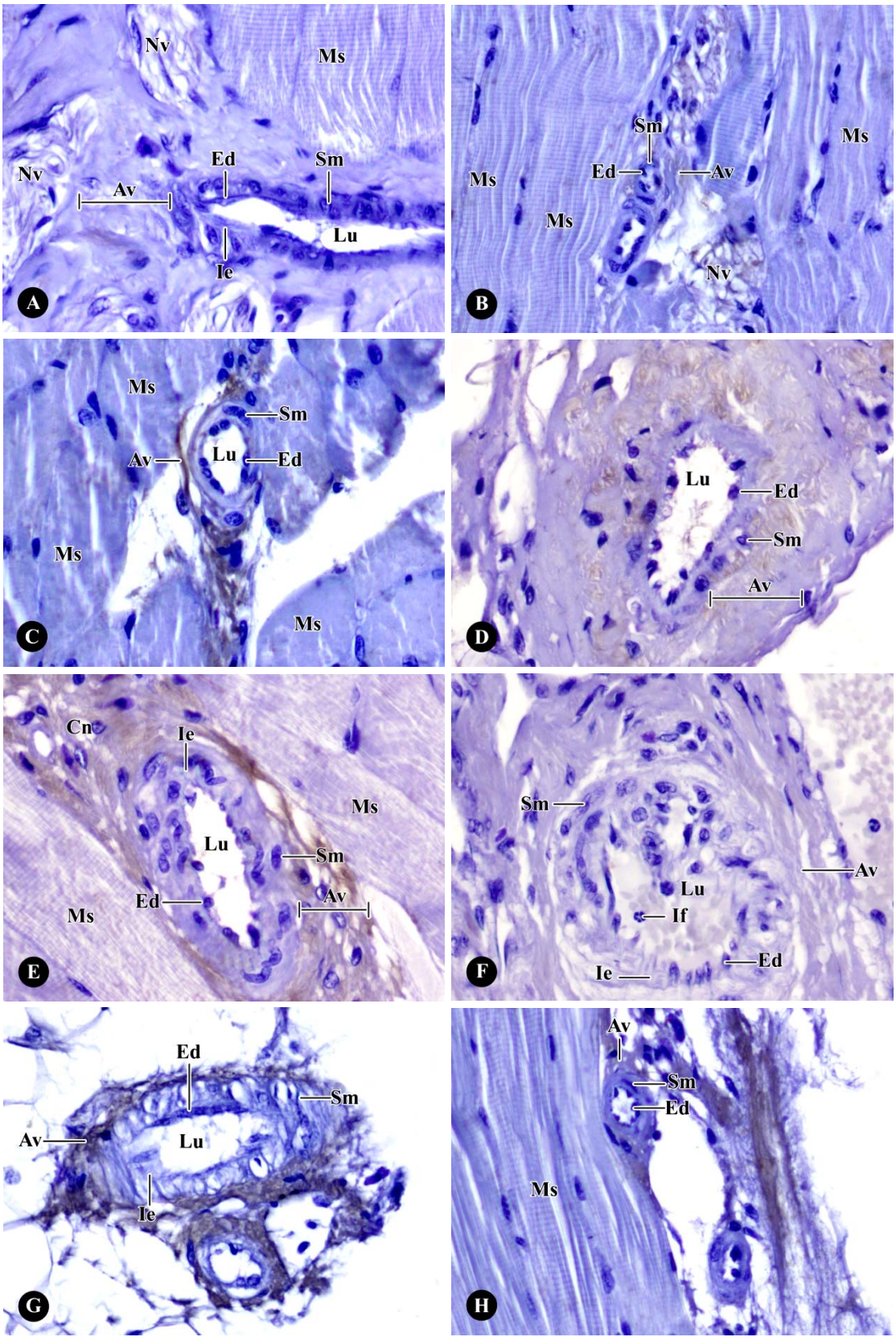
D – E) The mildly (D) and moderately (E) stained area are demonstrated in the tunica adventitia while the endothelial lining cells and muscular layer express the negative – stained areas.

F) The negatively – stained areas are observed both in the lumen and the vessel wall. The lumen is occupied by the negatively – stained red blood cells and the inflammatory cells.

G) The intensively – stained areas are expressed in the tunica adventitia while the endothelial lining cells and muscular layer illustrate the negative golden – brown stain.

H) The negatively-stained areas are displayed in the endothelial lining cells and muscular layer while the tunica adventitia shows the mild golden–brown coloration which is continuous with the nearby connective tissue.

Abbreviation: Cn, Connective tissue; Ie, Internal elastic lamina; Ms, Muscle fibers; Nv, Nerve fibers supporting the muscle bundle.


Nv
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Nv
Ed
Sm
Av
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Lu
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Ms
Lu
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Ed
Av
Ms
E
Sm
Av
Lu
If
Ie
Ed
F
Ed
Sm
Av
Lu
Ie
G
Av
Sm
Ed
Ms
H

**Figure 27**

**Figure 28** Photomicrpgraphs of gastrocnemius muscle illustrating the connective tissue supporting the muscle bundle (Cn) in the groups of one hour (A), six hour (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans*, serovar pyrogenes.

A) The mildly – stained connective tissue are shown. (Obj 40x)

B – C) The connective tissue between muscle fibers (Ms) is moderate – stain. (Obj 40x)

D) The intensively – stained connective tissue is shown between the muscle fibers. (Obj 40x)

E) The intensive golden – brown coloration is found in the connective tissue supporting muscle bundle. (Obj 100x)

F) The connective tissue shows the intensive golden – brown coloration. (Obj 40x)

G) The intensively – stained connective tissue are revealed. (Obj 40x)

H) The intensive – stained connective tissue are split off from the muscle bundle. (Obj 20x)

Abbreviation: Bv, Blood vessel; Ha, Hemorrhagic area; Nv, Nerve fibers supplying the muscle bundle.

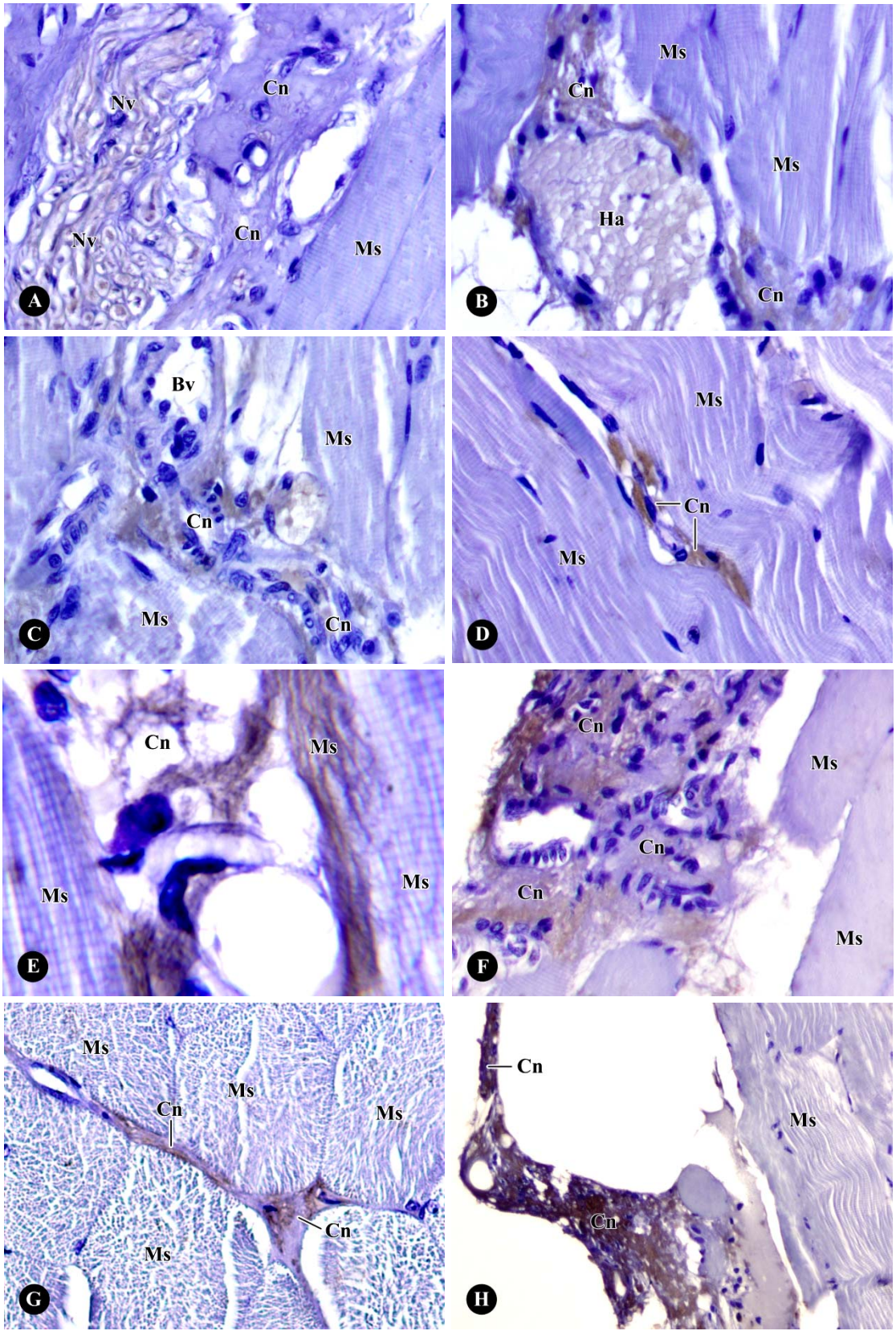

Nv
Cn
Nv
Cn
Ms
A
Ms
Cn
Ms
Ha
Cn
B
Bv
Ms
Cn
Ms
Cn
C
Ms
Cn
Ms
D
Cn
Ms
Ms
Ms
E
Cn
Ms
Cn
Cn
Ms
F
Ms
Cn
Ms
Ms
Cn
Ms
G
Cn
Ms
Cn
Ms
H

**Figure 28**

**Figure 29** Photomicrpgraphs of gastrocnemius muscle illustrating the nerve fibers supplying the muscle bundle (Nv) in the groups of one hour (A), six hour (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans*, serovar pyrogenes.

A) The intensive golden – brown coloration is found within the nerve fibers supplying muscle cell. (Obj 100x)

B) The mildly – stained nerve fibers are shown. (Obj 100x)

C) The intensive golden – brown coloration are seen in the nerve fibers. (Obj 40x)

D) The moderate golden – brown coloration are demonstrated in the nerve supplying the muscle bundle. (Obj 40x)

E – F) The mild – stain are observed in the nerve fibers supplying the gastrocnemius muscle. (Obj 40x)

G) The nerve fibers display the intensive golden – brown coloration. (Obj 40x)

H) The negative golden – brown coloration are seen in the nerve fibers. (Obj 40x)

Abbreviation: Cn, Connective tissue.

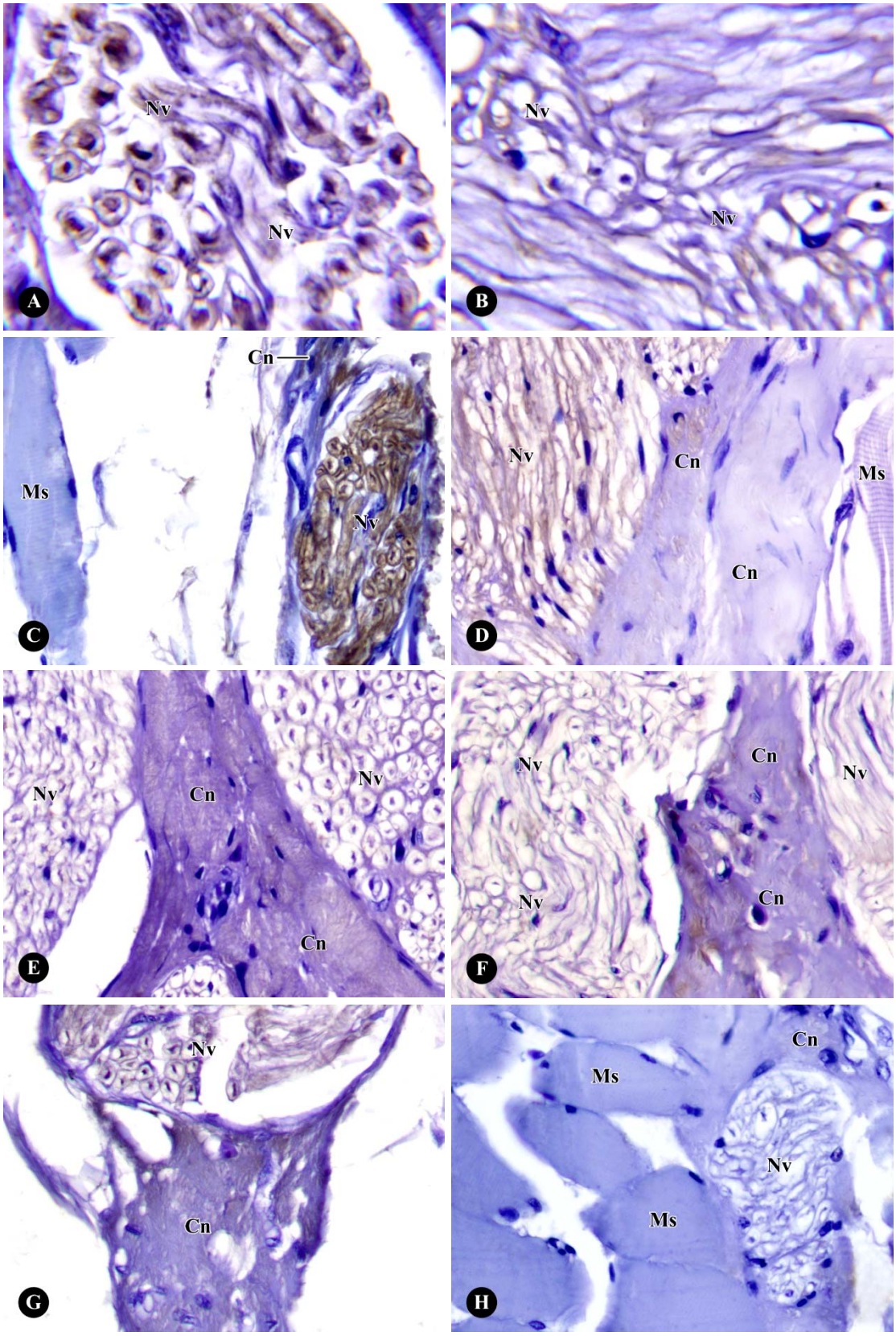

A
Nv
Nv
B
Nv
Nv
C
Cn
Ms
Nv
D
Nv
Cn
Ms
Cn
E
Nv
Cn
Nv
Cn
F
Nv
Cn
Nv
Nv
Cn
G
Nv
Cn
H
Cn
Ms
Nv
Ms

**Figure 29**

**Heart** (Table 4)

The heart tissue of control groups and the experimental negative staining control of the infected groups expressed no golden–brown coloration in the sections, including the endocardium, myocardium, epicardium, inflammatory cells and blood vessels. These sections were expressed as the blue stain of the Carrazi's haematoxylin solution. (Figure 30)

Some of the heart tissue of different experimental groups, such as the endocardium, myocardium, epicardium, inflammatory cells as well as the lumen and the wall of the vessels displayed the positive golden–brown staining of various intensities.

**Endocardium** (Figure 31)

The endocardium of heart demonstrated only the intensively positive stain in the subendocardial layer in the groups of one day (Figure 31C), three days (Figure 31E), four days (Figure 31F), five days (Figure 31G) and six days (Figure 31H). The negatively–stained areas in the subendocardial layer expressed in the groups of one hour (Figure 31A), six hours (Figure 31B) and two days (Figure 31D) after infection. Additionally, the negatively–stained areas were displayed in the endothelial lining and subendothelial layer in all infected groups from the groups of first hour to six days post infection. Besides, those of the four days group revealed the vacuolarization within the cytoplasm of endothelial lining wall of heart. (Figure 31F)

**Myocardium** (Figure 32)

The cardiac muscle cells of different experimental groups demonstrated the positive stain of different intensity in all infected groups from the first hour to six days post infection. Those of one hour (Figure 32A) groups expressed the mild golden–brown coloration while the groups of six hours to six days (Figure 32B–H) post infection revealed the intensive golden–brown coloration.

**Epicardium** (Figure 33)

The submesothelial and mesothelial layer of the epicardium of all infected groups displayed the positive golden–brown coloration with various intensities while the subepicardial layer revealed no positive stain. In the submesothelial layer, the moderate stain was seen in the group of one hour post infection (Figure 33A) while the groups of six hours to six days post infection expressed the intensive golden–brown coloration (Figure 33B–H). The mesothelial layer showed two different degrees of golden–brown coloration. The mild golden–brown coloration demonstrated in the groups of one hour (Figure 33A) and three to six days (Figure 33E–H) after infection while the groups of six hours to two days post infection revealed the moderate golden–brown coloration (Figure 33B–D).

**Inflammatory cells** (Figure 34)

The inflammatory cells which infiltrated throughout the heart tissue were mostly the neutrophils. The neutrophils in the groups of one hour, one day and six days showed the positive coloration in different intensity. Those in the early groups of one hour (Figure 34A) and one day (Figure 34C) expressed the mild stain while the intensive golden–brown coloration was displayed in six days post infection. (Figure 34H) Additionally, not only the neutrophils but the plasma cells were also discovered. Some plasma cells showed the positive mildly–stained cytoplasm in the group of one day (Figure 34C) while the group of three days displayed the negatively–stained cytoplasm of plasma cells. (Figure 34E) The inflammatory cells located in many locations, such as the heart chambers (Figure 34A, B, D, F–H), lumen of large vessel (Figure 34C) and submesothelial layer of epicardium (Figure 34E).

**Blood vessels** (Figure 35)

The blood vessels supplying the heart showed the positive golden–brown stain in all infected groups. However, the pattern of the positive stained areas was different, consisting of the content within the lumen and the composition of the vessel wall. Moreover, the intensities of these stained areas were varied. The luminal content which was assumed to be mainly red blood cells demonstrated the mild stain in the

groups of one hour (Figure 35A), one day (Figure 35C) and three to four days (Figure 35E– F). The inflammatory cells were distinctly observed in the lumen of blood vessel in the groups of one day after infection (Figure 35C). Concerning the vessel wall, the positive staining was performed in the endothelial lining cells and tunica adventitia. The endothelial lining cells were positive mild stain only in the group of four days after infection (Figure 35F). In addition, in the groups of two days (Figure 35D and four days (Figure 35F) after infection the endothelial lining cells were splitted out from the vessel wall. The positive coloration in the tunica adventitia was classified into two different intensities; moderate and intensive. The moderate stain was localized in the group of two days (Figure 35D) while intensive stain was shown in six hours (Figure 35B), one day (Figure 35C) and four days (Figure 35F). Those in the groups of one hour (Figure 35A), three days (Figure 35E), five days (Figure 35G) and six days (Figure 35H) revealed the negatively–stained tunica adventitia.

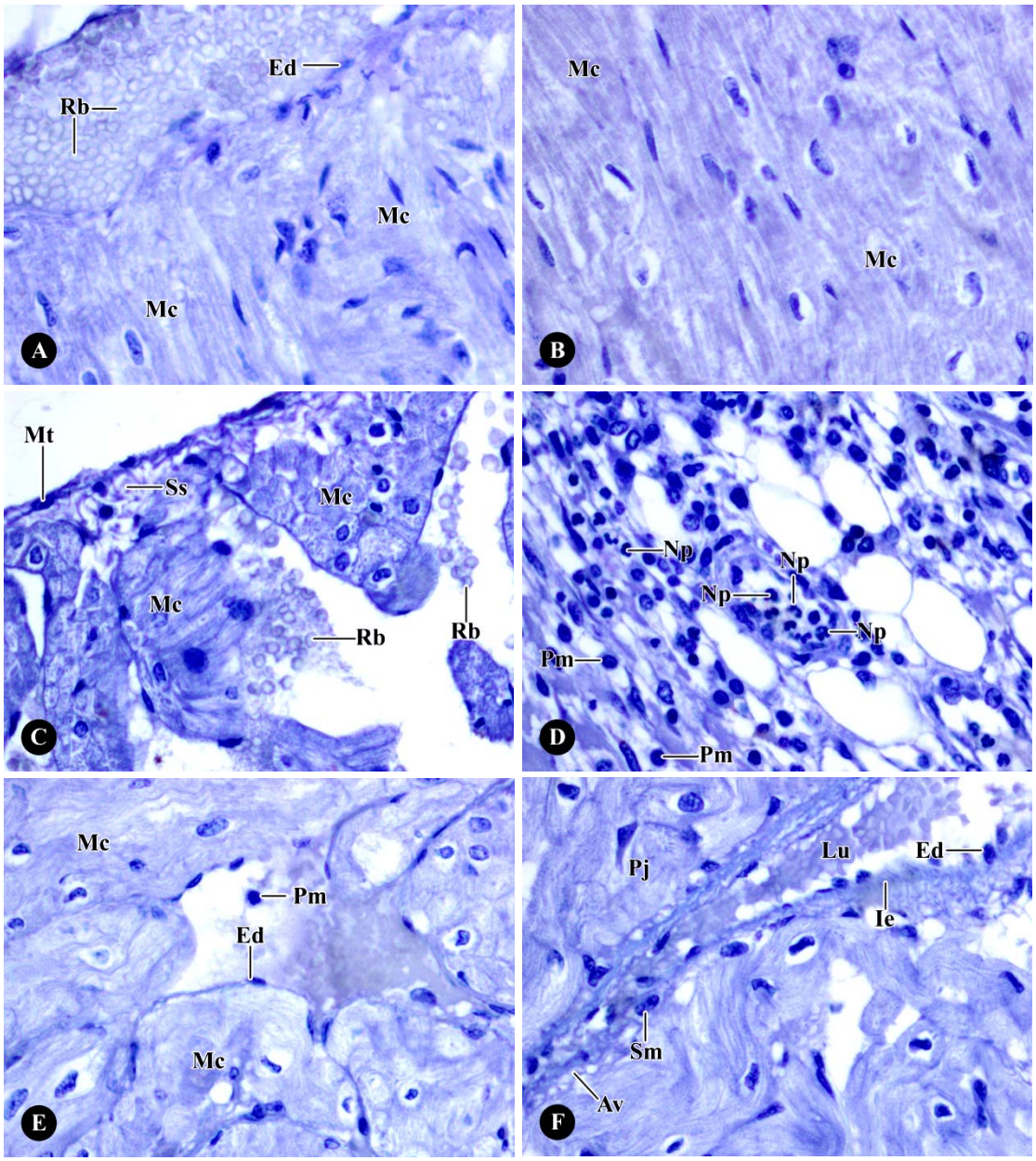
**Table 4** Immunoperoxidase staining of the heart of hamsters infected with *Leptospira interrogans,* serovar pyrogenes.

| Heart | Durations of post infected hamster | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **1 hr.** | **6 hrs.** | **1 Day** | **2 Days** | **3 Days** | **4 Days** | **5 Days** | **6 Days** |
| **Endocardium** | | | | | | | | |
| • Endothelial lining cell | - | - | - | - | - | - | - | - |
| • Subendothelial layer | - | - | - | - | - | - | - | - |
| • Subendocardial layer | - | - | +++ | - | +++ | +++ | +++ | +++ |
| **Myocardium** | + | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| **Epicardium** | | | | | | | | |
| • Subepicardial layer | - | - | - | - | - | - | - | - |
| • Submesothelial layer | ++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| • Mesothelial layer | + | ++ | ++ | ++ | + | + | + | + |
| **Inflammatory cells** | | | | | | | | |
| • Neutrophils | + | - | + | - | - | - | - | +++ |
| **Blood vessels** | | | | | | | | |
| • Lumen | + | - | + | - | + | + | ++ | ++ |
| • Endothelium | - | - | - | - | - | + | - | - |
| • Muscular layer | - | - | - | - | - | - | - | - |
| • Tunica adventitia | - | +++ | +++ | ++ | - | +++ | - | - |

Evaluation of results using a four–grade, semi–quantitative scale: negative (-), mild (+), moderate (++) and intensive (+++)

**Figure 30** Photomicrographs illustrating the experimental negative control of the heart in the groups of one hour (A and B), three days (C and D) and four days (E and F) post infected hamsters with *Leptospira interrogans*, serovar pyrogenes. The endocardium is shown in A, the myocardium (Mc) in B, the epicardium in C, the inflammatory cell infiltration in D and E, and the blood vessel supplying the heart in F. No any golden – brown stained area is seen in the sections. (Obj 40x)

Abbreviation: Av, Tunica adventitia; Ed, Endothelial lining cell; Ie, Internal elastic lamina; Lu, Lumen of blood vessel; Mt, Mesothelial layer; Np, Neutrophil; Pj, Perkinje's fiber; Pm, Plasma cell; Rb, Red blood cell; Sm, Smooth muscle cell in muscular layer of blood vessel; Ss, Submesothelial layer of epicardium.


Ed
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Pm
Pm
D
Mc
Pm
Ed
Mc
E
Pj
Lu
Ed
Ie
Sm
Av
F

**Figure 30**

**Figure 31** Photomicrographs of the heart illustrating the endocardium in the groups of one hour (A), six hour (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans*, serovar pyrogenes.

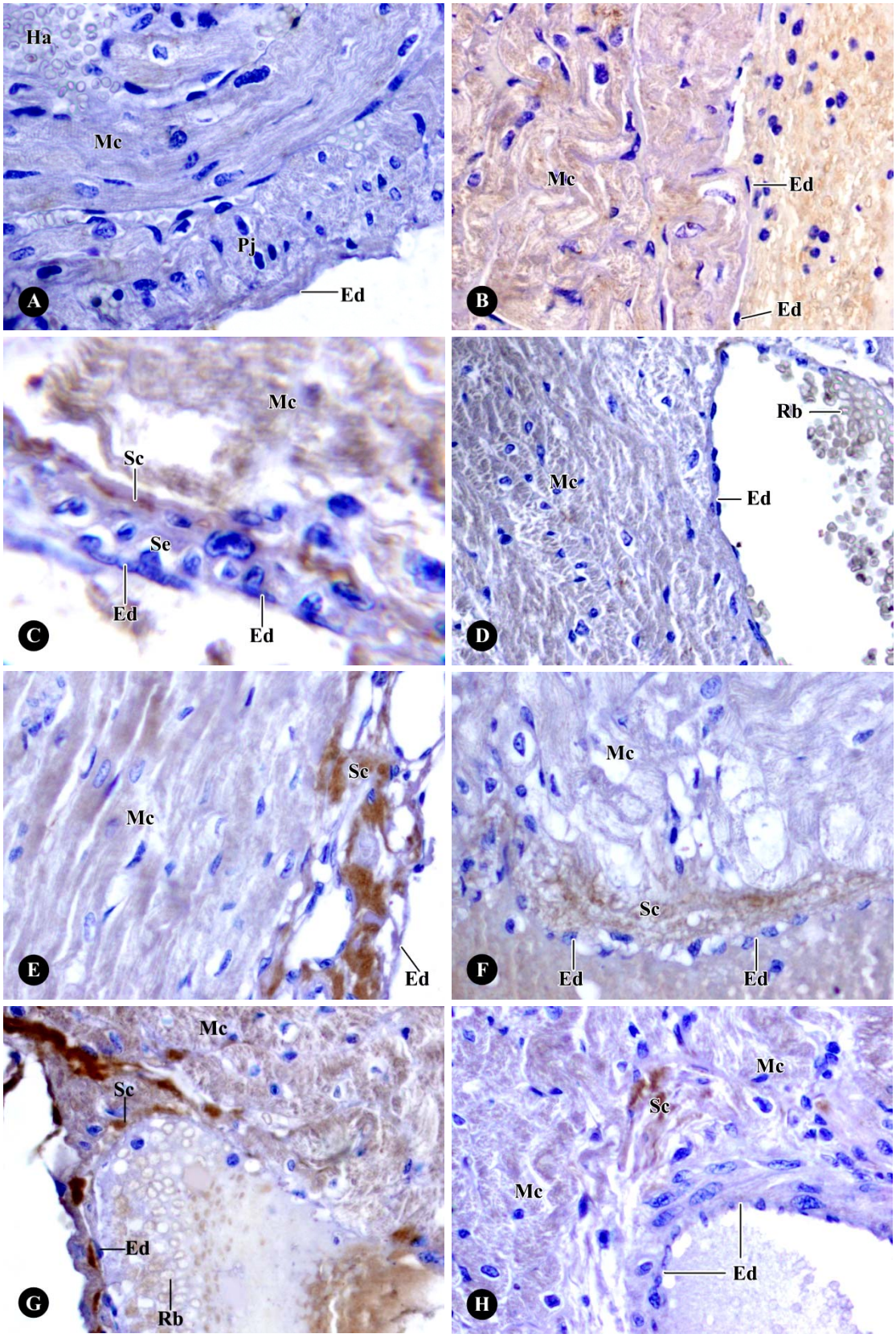
A – B) The infected heart tissue showing the endocardium which is composed of the endothelial lining cells (Ed), subendothelial and subendocardial layer which appear as negative golden–brown coloration. (Obj 40x)

C) The endocardium of the infected heart illustrates the negative golden–brown coloration in the endothelial lining cells and subendothelial layer (Se) while the subendocardial layer (Sc) shows the intensively–stained area. (Obj 100x)

D) The endocardium shows the negatively – stained layer. (Obj 40x)

E – H) The endothelial lining cells and subendothelial layer show the negatively – stained while the subendocardial layer shows the intensive golden – brown coloration. (Obj 40x)

Abbreviation: Ha, Hemorrhagic area; If, Inflammatory cell; Mc, Myocardium; Pj, Perkinje's fiber; Rb, Red blood cell.


Ha
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Sc
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Rb
G
Mc
Sc
Mc
Ed
H

**Figure 31**

**Figure 32** Photomicrographs of the heart revealing the myocardium (Mc) in the groups of one hour (A), six hour (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans,* serovar pyrogenes. (Obj 40x)

A) The myocardium appears as the mildly – stained area.

B) The intensive golden – brown precipitation is expressed in the cardiac muscle cells within the myocardium.

C) Some of the cardiac muscle cells show the intensively – stained areas (arrow) whereas the overall of the tissue expresses the moderate golden–brown coloration.

D) The intensive golden – brown coloration deposit on the cardiac muscle cells.

E) The intensively – stained areas are shown in the cardiac muscle cells.

F) Some of the cardiac muscle cells express the intensively – stained areas while other areas are deposited the mild golden – brown coloration.

G) The intensive golden – brown coloration displays within the cardiac muscle cell (arrow).

H) The intensive golden – brown coloration expresses in the hemorrhagic area (Ha) while some of cardiac muscle cells show the intensively–stained area (arrow).

Abbreviation: Ed, Endothelial lining cell; Ha, Hemorrhagic area; Ho, Homogeneous hyalinic material; Rb, Red blood cell.

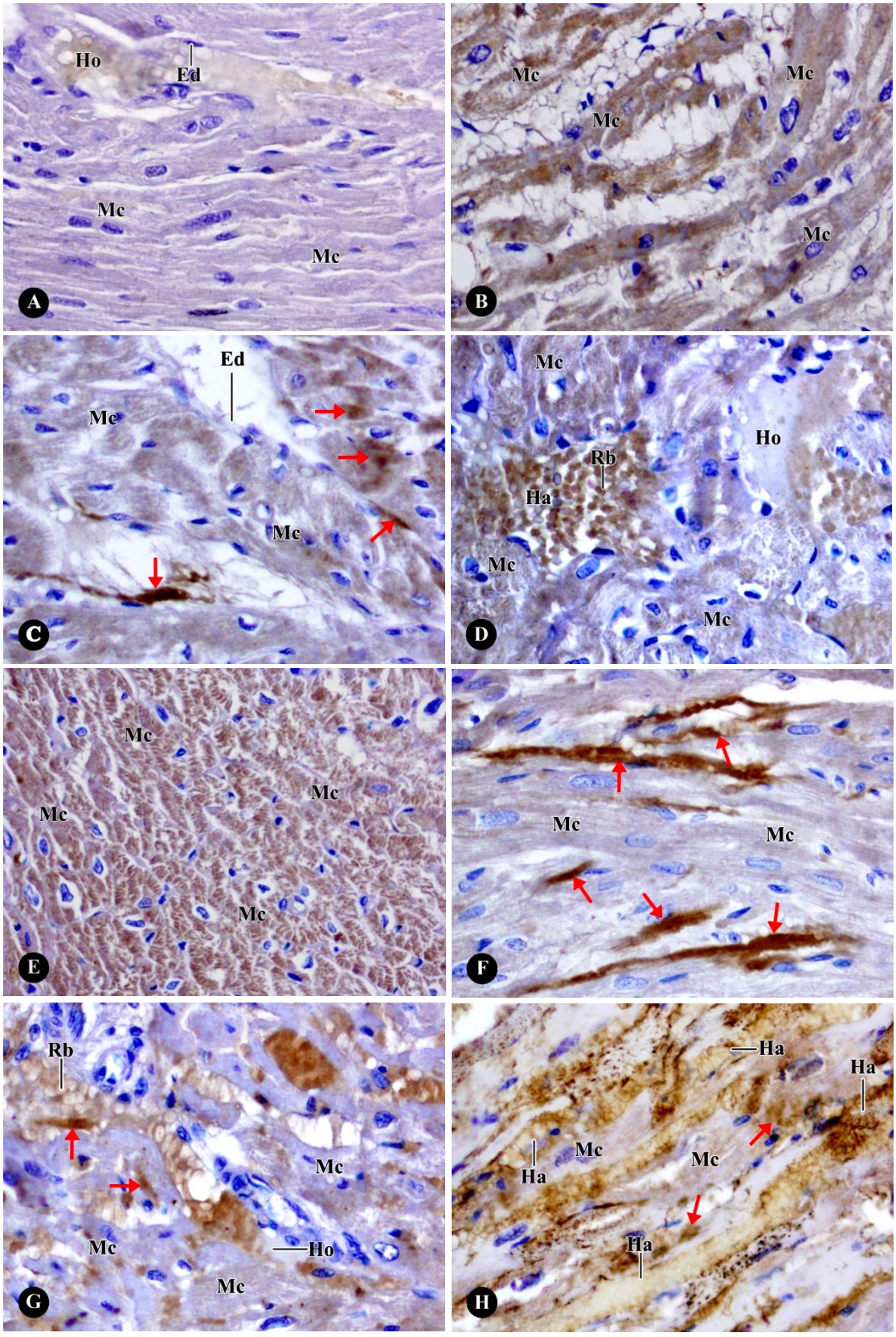
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**Figure 32**

**Figure 33** Photomicrographs of the heart showing the epicardium in the groups of one hour (A), six hour (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans*, serovar pyrogenes.

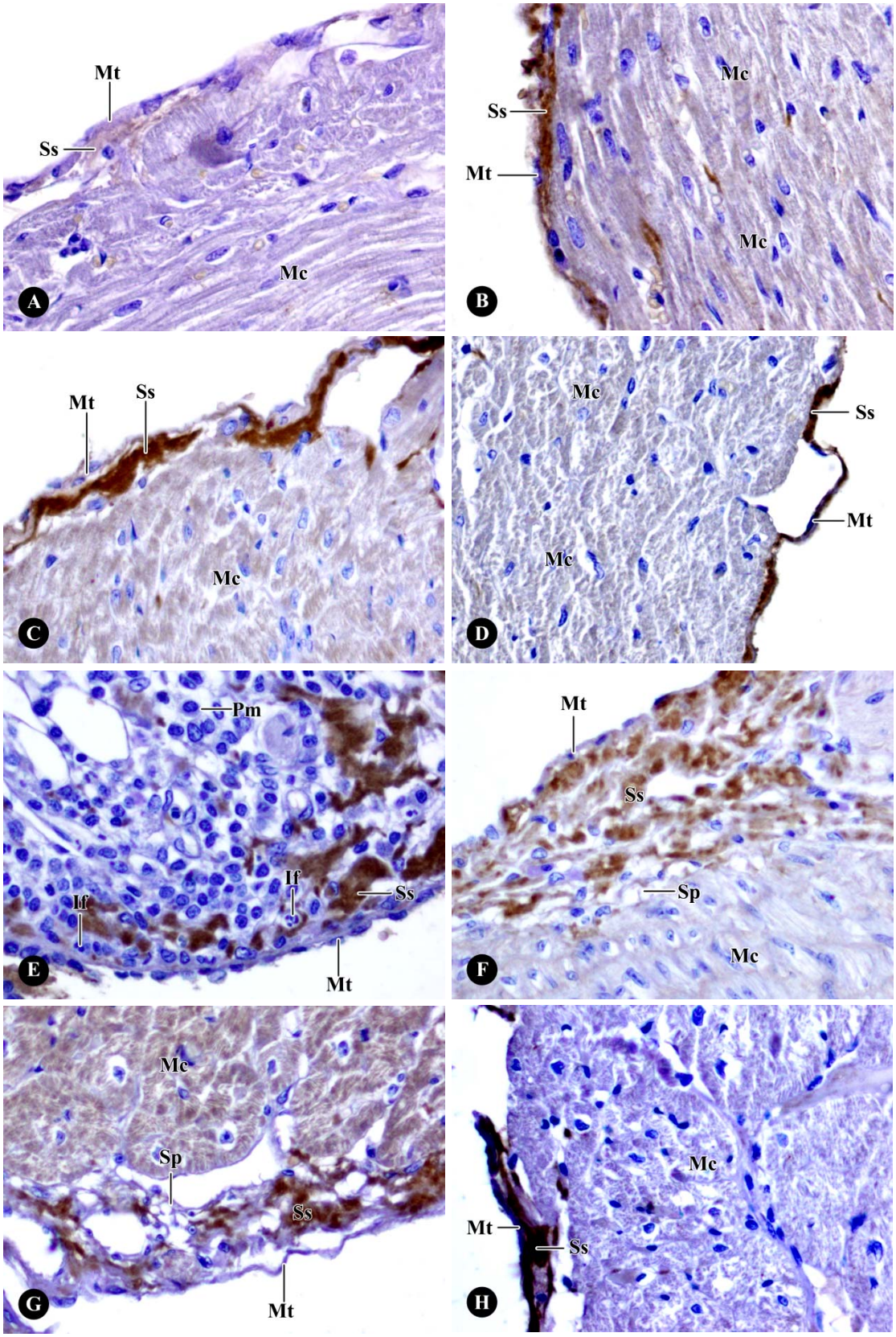
A) The moderately–stained areas are demonstrated within the cytoplasm of cells lining the mesothelial layer (Mt) while submesothelial layer (Ss) reveals the moderate stain. (Obj 40x)

B – D) The intensive golden–brown coloration expresses in the submesothelial layer while the cells lining mesothelial layer display the moderately–stained cytoplasm. (B and C, Obj 40x; D, Obj 20x)

E) The intensively–stained areas deposit within the submesothelial layer which is infiltrated by the inflammatory cells (If) together with plasma cell (Pm). (Obj 40x)

F – H) The intensive golden – brown coloration is expressed within the submesothelial layer while the cells lining mesothelial layer express the mildly – stained cytoplasm. (Obj 40x)

Abbreviation: Sp, Subepicardial layer.


Mt
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E
Mt
Mt
Ss
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Ss
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**Figure 33**

**Figure 34** Photomicrographs of the heart tissue revealing the inflammatory cells in the groups of one hour (A), six hour (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans*, serovar pyrogenes. (Obj 40x; inset 100x)

A) The mildly–stained neutrophil (Np) infiltrates in the congested blood vessel. The mild positive stain is also shown in this area.

B) The neutrophils and plasma cells express the negatively–stained cytoplasm.

C) The mild golden–brown coloration is deposited in the cytoplasm of plasma cells.

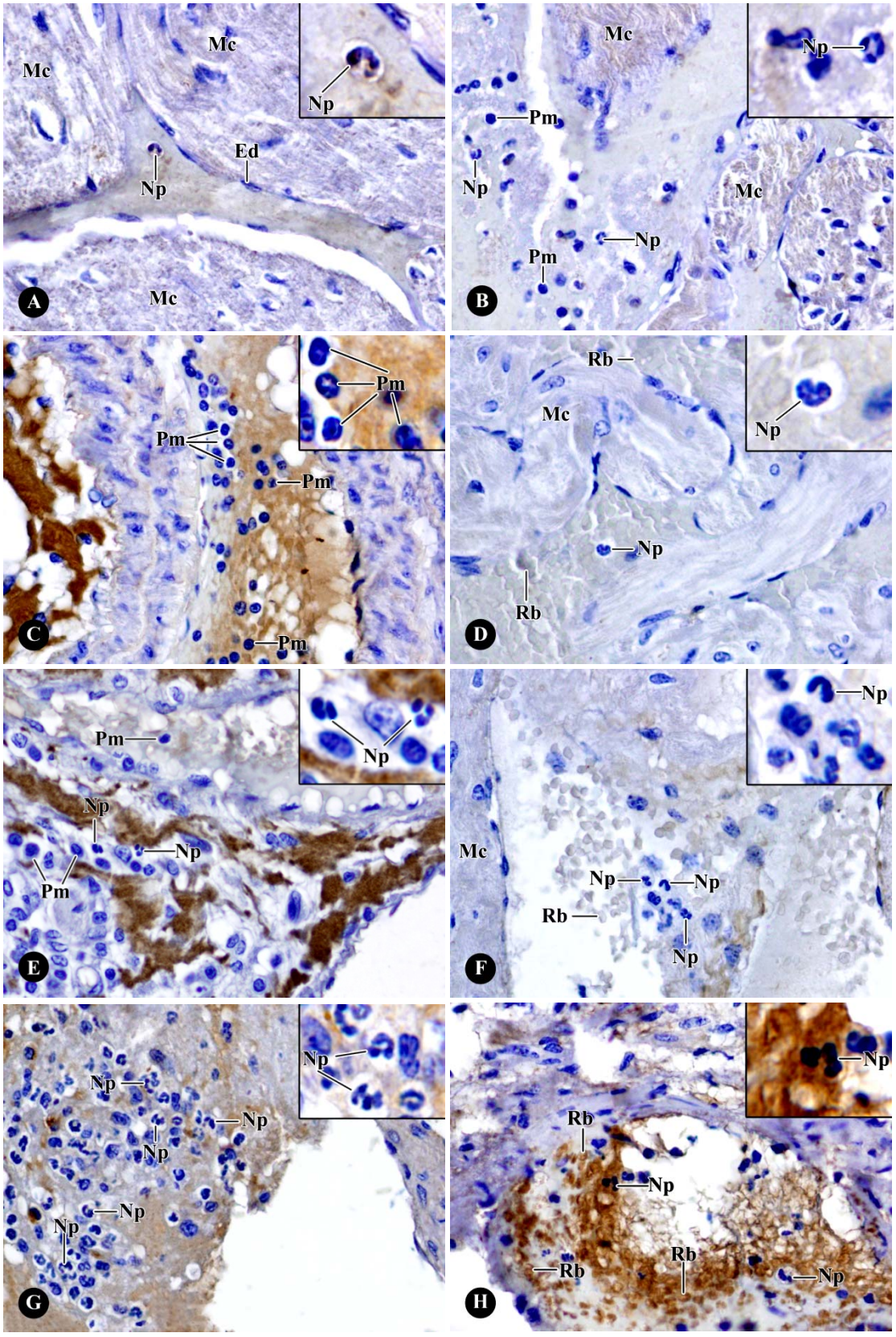
D) The neutrophil which infiltrates in the hemorrhagic area reveals the negatively – stained cytoplasm.

E) The negatively – stained cytoplasm are expressed in the neutrophils and plasma cells which infiltrate in the submesothelial layer of mesothelium.

F – G) The cytoplasm of the neutrophils shows negative stain.

H) The leptospiral antigens are displayed in the neutrophils by the intensively–stained cytoplasm.

Abbreviation: Ed, Endothelial lining cell; Mc, Myocardium; Rb, Red blood cell.


Mc
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Np
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**Figure 34**

**Figure 35** Photomicrographs of the heart displaying the blood vessels in the groups of one hour (A), six hour (B), one day (C), two days (D), three days (E), four days (F), five days (G) and six days (H) post infected hamsters with *Leptospira interrogans*, serovar pyrogenes. (Obj 40x)

A) The mildly–stained contents in the lumen (Lu) of the blood vessel and negatively–stained area in the cytoplasm of endothelial lining cells (Ed), smooth muscle cells (Sm) in muscular layer and tunica adventitia (Av) are displayed.

B) The intensively–stained tunica adventitia is performed.

C) The mildly–stained contents in the lumen of large size vessel as well as intensively–stained tunica adventitia are demonstrated.

D) The negatively–stained area is demonstrated in the lumen of the blood vessel while the tunica adventitia shows moderate–stain.

E) The mildly–stained contents are found in the lumen of the blood vessel while the vessel wall shows negatively–stained area.

F) The endothelial lining cells display the mild stain and the tunica adventitia shows the intensive golden–brown coloration.

G – H) The moderately–stained contents are shown only in the lumen of the blood vessel.

Abbreviation: Ie, Internal elastic lamina; Mc, Myocardium.

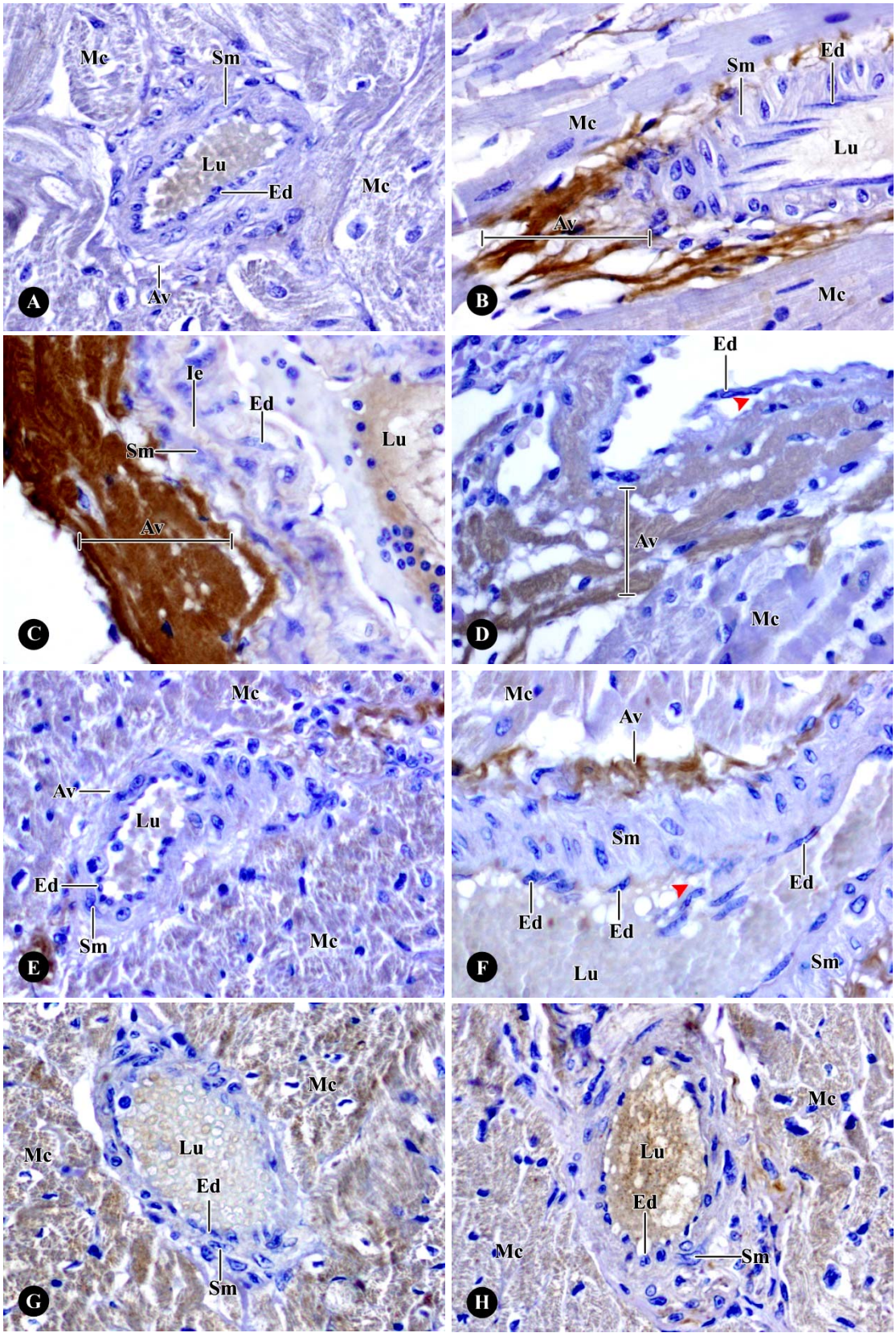
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**Figure 35**

# CHAPTER VI
# DISCUSSION

## Kidney

All organs of various infected hamsters studied including the kidney, cardiac muscle, gastrocnemius muscle and hamstring muscle demonstrated the positive golden–brown stained area in the sections. The golden–brown precipitations were presumed to be the leptospiral antigen that was localized in the infected tissue sections.

The cytoplasm of the epithelium lining the proximal tubules in all infected groups showed the positive stain of various densities. This finding reflected that the leptospiral antigen or even the leptospire itself could penetrate the cell lining of the proximal tubules as early as one hour after infection and then went on until six days which was the last day of the experiment. The leptospiral antigen located in those proximal tubular cells might explain why the degenerative changes were observed mostly in the proximal tubules as in the study of Pilakasiri (2001). The study of Pilakasiri (2001) which studied in the hamsters infected with *Leptospira interrogans* serovar pyrogenes reported that the epithelial cells lining most of the proximal tubules were swollen and contained various degrees of degenerative nuclei. These observations were a sign of tubular degenerate and could either be attributed to the migration of leptospires themselves into the proximal tubular cells or to a leptospiral toxin. In laboratory infected hamsters with serotype *pomona*, Miller and Willson (1967) pointed out that during acute leptospirosis there was some actual penetration of the leptospires into the proximal and distal tubular cells. De Arriaga (1982) studied the guinea pigs infected with *L.icterrohaemorrhagiae,* reported that the proximal tubules lined either by flattened or edematous cells. Sitprija (1980) expressed that the intact leptospires were detected in increasing numbers in the proximal tubular epithelial cells at nine hours after inoculation with *L.bataviae* in

hamsters. Twigg (1976) studied in the 12 rodents infected with leptospires by using the silver – staining technique of the Levaditi or Warthin–Starry. It had been reported that the leptospires were embedded in the wall of the tubule in the position closely related to the cell walls separating adjacent epithelial cells. Occasionally the attachment might penetrate as far as the base of the epithelial cell of one point or even at two points.

However, in this study the cytoplasm of the proximal tubular cells revealed the positive stain of different intensity which was varied from mild, moderate and intensive depending on the post infection time. The moderately–stained cytoplasm was observed in the earlier period, mild in the following period and intensive in the latest day of infection. This finding might be clarified that the pathogenic leptospires located and multiplied themselves in the blood stream of the infected animal and then invaded many organs of the body. Therefore in the early infection with moderate stain found in the study might due to the leptospires or their antigens in the blood stream went into the renal tissue by the way of the renal artery supplying the renal parenchyma in the earlier period. Then in the following period expressing the mild stain should be because of the self repairing process to recover the injury of the renal tissue. The self repairing process might somehow get rid of some leptospiral antigens in the cells which leaded to produce less intensity than those of the early groups. As Yener and Keles (2001) had reported that the regeneration and proliferation of the tubular epithelium were detected in the kidney of infected cattle. De Brito (2006) reported that in the infected kidney of human patient, leptospiral antigen were observed also in the cytoplasm of macrophages associated with the focal interstitial infiltration. The leptospiral antigen was initially localized to the cell membranes but was later found in lysosomes and finally degraded. From this report the macrophage and inflammatory cell might engulf some kind of leptospiral antigen in order to wipe out the antigens in the infected tissue.

Moreover, those in the six days group the cytoplasm became the intensive stain which might be the effect of the injury of the capillary loops in the glomeruli. This circumstance eased the leptospires, leptospiral antigen and red blood cells covered by leptospiral antigen coming out into the urinary spaces and somehow this antigen might

invade the proximal tubular cells adding with the preexisting antigen in the cell cytoplasm. It has been known for time that leptospires multiply in the blood stream, invade many organs and finally localized in the proximal convoluted tubules (Hartley, 1952; De Brito, 1965 and 1967; Marchall, 1974). As in Yang (2007) reported that the early observational studies had indicated that the *leptospira* spreaded through hematogenous route to the kidney, then circulated to the glomeruli, peritubular capillary and then migrated to the interstitium, renal tubule and finally remained in the proximal tubular lumen. In addition, Ballard (1986) reported that the virulent leptospires adhered to renal epithelial cells *in vitro*. Miller and Wilson (1962) and Pilakasiri (2001) noted that numerous lysosomal vacuoles were seen in the infected tubular epithelial cells. Michna and Campbell (1969) gave a reason that the organisms migrated from the interstitial space to the tubular lumen inside such vacuoles. All of these evidences obviously were the explanation of why the lumen and the epithelium of the proximal tubules in the six days groups showed the moderate and intensive stain respectively.

Concerning the distal tubules and collecting duct, it had been demonstrated that the cytoplasm of their epithelia were stained mildly in early period, moderately and intensively in the following period and became mildly or moderately again in the late period. This pattern of intensity might be the results of the renal blood supply and the damage of the renal tissue as followed. Renal parenchyma received blood supply from the renal blood vessels via peritubular arteries which supplied the blood throughout the renal tissue. Therefore in first hour post infection group, the epithelial lining cells received the leptospiral antigen from the blood in low concentration rate, making the mild stained appearance. After that the cells had moderately or intensively–stained cytoplasm which should be that they had been received more leptospiral antigen from the blood supplying the renal tissue in the high concentration rate. Then they were able to repair themselves resulting to be mild stain again. Finally, the cells became the moderately–stain again which could happen by receiving the high concentration of leptospiral antigen from the damaged capillary loop of glomerulus and released the blood associated with the leptospiral antigen to urinary space and renal tubular lumen. In addition, the renal tubular epithelial lining cells loss of

function which an effected on the severity of renal tissue damage in the latest period of infection. These appearances corresponded to the work of Pilakasiri (2001) that reported there were some changes of the distal tubules including, dilatation of the tubular lumen, flattening of the epithelium and also necrosis of these epithelia. Twigg (1976) reported that the leptospires were rarely confined in the collecting ducts. These sometimes might be that they lacked points of attachment and were presumably moving in the urine flow. Presently, no any reports describe obviously the pathogenesis of distal tubules and collecting ducts. However, as described above, the leptospiral antigens were revealed in the cytoplasm of epithelial lining cells of distal tubules and collecting duct.

Regarding to the renal corpuscle, the capillary loops which were represented by the podocytes and some either parietal or visceral layer of the Bowmann's capsule revealed the positive golden–brown areas. It appeared earliest in the groups of six hours to six days post infection. Nevertheless, those in the one hour group performed the negative staining. This observation might be discussed in the way that the low concentration of leptosprial antigen in the afferent arteriole to the glomerulus in the earliest post infection group, one hour group, was the major reason. In the later groups more leptospiral antigen circulating in the blood stream made the glomerulus mild positive stain. Besides, the groups of five and six days post infection showed the positive stained red blood cells and some homogeneous hyalinic materials in the urinary space. This might because of the injury of the capillary loops that allowed the blood content in the vessels leaked out to the urinary space causing the haematuria. According to Sitprija (1980) reported that the cytotoxin was present in the blood circulation in the course of the disease which was known to have an injurious effect to the vascular endothelium and it could further contribute to fluid leakage from the vascular space to the urinary space of Bowmann's capsule. De Brito (1966) described that the definite glomerular lesion in experimental leptospirosis was characterized by focal areas fusion of endothelial cells foot process which was the morphological basis of proteinuria. Moreover, Pilakasiri (2001) expressed that the damage to the glomerulus might alter the permeability of the capillary wall allowing the leakage of abnormal quantities of plasma proteins into the urine giving rise to proteinuria.

Therefore, the damaged glomerulus might allow the passage of red blood cells across the capillary basement membrane causing haematuria.

The positive color was performed in the inflammatory cells which were mainly neutrophils. The interstitial inflammatory infiltrations were mentioned as a sign of interstitial nephritis (Pilakasiri *et al.*, 2001). The coloration pattern was mild, then moderate and intensive, and returned to mild again in the last period. The pattern of the coloration was corresponded to those of epithelial lining cells of distal tubules and collecting ducts. Cameron (2008) reported that, the positive samples showed leptospiral antigen within the cytoplasm of peritubular inflammatory cells in the renal tissue of marine mammals and directly correlated with the sea lions that exhibited tubulointerstitial nephritis. Macfarland (2000) reported that neutrophils ingested the debris and the foreign particles being similar to the feeding process of the amoeba. In addition, in some periods of infection the plasma cells were presented which were responsibility to produce the antibody against the leptospires themselves and leptospiral antigen. The plasma cells were differentiated from the B–lymphocyte, and were responsible to antigen activation of leptospiral antigen (Young *et al.*, 2000).

The renal blood vessels also showed the positive stain in the lumen and the component of the vessel wall including, the internal elastic lamina and the tunica adventitia. Pilakasiri (2001) found densely packed red blood cells in the blood vessels of the cortex and medulla of infected kidney of hamsters. These appearances were likely to be because it was clearly known that the leptospires or their antigens circulated to organs by the blood vessels. Therefore, these antigens showed are located in the blood vessels as mentioned. According to Szeredi and Haake (2006), the moderate to large numbers of leptospires were detected by the Warthin–Starry staining and immunohistochemical staining. They reported the body of the leptospires showing the typical wavy forms in the lumen of blood vessels in various organs. De Brito (1966) described that the interstitial capillaries of the kidney showed chiefly endothelial cell tumefaction and areas of disjunction thus pointing out to a vascular lesion in leptospirosis which could contribute to the hemorrhagic lesion. Corresponding to Pilakasiri (2001), the primary lesion in all forms of leptospirosis in all animals was the damage to the endothelial cell membranes of small blood vessels,

caused by leptospiral toxin. The immediate effect was to loosen the junction between endothelial cells, allowing both fluid, leptospires and leptospiral antigen to leaked out into extravascular space, followed by erythrocytes where the damage was severe and prolonged (De Brito *et al.*, 1979).

**Skeletal muscles**

The skeletal muscle of both the hamstring and gastrocnemius in all infected groups showed the positive stain of various intensities. This finding reflected that the leptospiral antigen or even the leptospires themselves could come inside the muscle fibers of the skeletal muscle as early as one hour to six days post infection. The leptospiral antigen deposited within the muscle fibers might give the explanation of the necrosis demonstrated in the leptospiral infected skeletal muscle in the report of Pilakasiri (2001). Pilakasiri (2001) studied in the hamster infected with *L.interrogans,* serovar pyrogenes and it had been reported that the tissue sections of hamstring muscle showed the necrosis area in some muscle fibers together with the inflammatory cell infiltrations. This necrotic area performed a homogeneous eosinophilic material replacing the degenerated muscle fibers. Pereira (2005) studied the skeletal muscles in marmoset monkeys *(Callithrix jacchus)* infected with *L.interrogans,* serovar copenhageni showed the hemorrhagic necrosis of skeletal muscle. Furthermore Laurain (1954) studied the skeletal muscle of hamsters infected with *L.icterohaemorrhagiae* are reported the focal hyalinization of muscle fibers. More severely damaged fibers were markedly fragmented and the sacrolemma collapsed.

The inflammatory cells which were mainly neutrophils were located in all experimental groups. However, the intensive positive coloration of the cytoplasm of the neutrophils was demonstrated in the late period. The hamstring muscle performed the positive stain in the five days group and the gastrocnemius muscle in the six days groups. The pattern of intensity might be the result of the increasing concentration of the leptospiral antigen in the blood circulation. This antigen invaded the muscular tissue via the blood supply. In addition, the injury of blood vessels showed allow the blood contents together with the leptospiral component in the vessels leak out to the

muscular tissue. Meanwhile the neutrophils in the muscular tissue tried to get rid of this antigen by engulfing them making their cytoplasm show the intensive positive stain. Macfarland (2000) reported that neutrophils ingested the debris and the foreign particles being similar to the feeding process of the amoeba.

The different components of the blood vessels showed the positive stain of various intensities. The hamstring muscle demonstrated golden–brown coloration in the lumen, cytoplasm of the endothelium, smooth muscle cells in the muscular layer and the tunica adventitia while the gastrocnemius muscle expressed the positive stain only in the tunica adventitia. According to Pilakasiri (2001) found the dilatation and congestion of blood vessels within the infected skeletal muscle. These phenomena are normally known as in hyperemia. A vascular event is the increase in the permeability of the venules and capillaries. Prolonged hyperemia caused by inflammation may cause diffusion of red blood cells into interstitial tissue via the vessel wall (Damjanov, 1996). These finding might be the explanation of the positive stain in the various component of the vessels wall. However, Park *et al.* (1989) studied 96 patients with leptospirosis and reported that the calf muscle showed the interstitial hemorrhage and a few mononuclear inflammatory cells were seen around the dilated vessels within the connective tissue.

The connective tissue supporting the muscle bundle of both hamstring and gastrocnemius displayed the positive stain in all experimental groups. The degrees of the positive stain were mostly intensive. This should be presumed that the leptospiral antigen or the leptospires themselves preferred to locate in the connective tissue supporting the organs and invaded the area via the blood supply.

The nerve supplying the skeletal muscle fibers also showed the clearly positive stain in various intensities in all infected groups in the hamstring muscle and almost all infected groups in the gastrocnemius muscle. Various neurological disturbances had been reported in cases of leptospirosis. Heath (1965) reported that numerous neurological manifestations had been reported in the immune phase. This phenomenon might be the causation of neuropathy which was an uncommon manifestation of leptospirosis. Souza *et al.* (2006) described an unusual case of leptospirosis in a 54

years old man presenting peripheral nerve palsy. The electrophysiological results of their studies showed that no response could be obtained from the right fibular nerve. At seven months after the initiation of treatment for leptospirosis, additional electrophysiological studies and a neurological examination showed. In a neuromuscular biopsy study of Azouvi (1989) found signs of Wallerian degeneration and perivascular inflammatory infiltration of epinural vessel in case of leptospirosis. The neurological pathology in case of leptospirosis of such a condition is unclear. However, the pathological pathway of nerve fiber in case of leptospirosis might be that, after invading the blood circulation, the spirochetes played a role in the pathogenesis of neuropathies by generating systemic vasculitis and by including circulating immune complex (Levett, 2001; Yaqoob and Ahmad, 1989). Furthermore, the nerve fiber supplying the infected muscular tissue revealed the positive stain. This should be presumed the leptospiral antigen invaded the nerve fiber and interfered the physiological function of the nerve.

**Heart**

The subendocardial layer of the endocardium of heart in almost infected groups revealed the intensive positive stain. The positive coloration expressed in one day and three to the last day of the experiment. This staining pattern reflected that the leptospiral antigen or even the leptospires themselves could come inside the endocardium, especially the subendocardial layer. This result corresponding to Chakurka (2008) which studied the cardiovascular lesions in leptospirosis from autosy specimens by using conventional histopathological examination and found that the inflammation was predominantly observed in the subendocardial region, especially with patchy distribution where a predominant neutrophilic infiltrate was present.

Regarding to the myocardium, it had been demonstrated that the sacroplasm of cardiac muscle cells were stained mildly in the first hour and intensive in the following period. This staining pattern might be the result of the leptospiral antigen in the blood vessel supplying the cardiac tissue. Heart tissue received blood supply from blood vessel which supplied the blood throughout the heart tissue. Therefore in the first hour after infection group, the cardiac muscle cells received the leptospiral

antigen in low concentration by the normal blood supply, causing the mild stained appearance. After that the cardiac muscle cells had intensive stain which might be that they had been received more leptospiral antigen from both normal blood supplying the cardiac muscle together with those that were released from the damaged vessel. According to the studied of Muensoongnoen (2006), it was reported that most cardiac muscle cells showed various degrees of degenerative changes. The focal myocardial cell degeneration was found in fatal case with leptospiral infection (Park, 1989). Chakurkar (2008) reported that the predominant feature on histopathological examination was the presence of interstitial myocarditis. The inflammation was accompanied by interstitial edema with separation of the myofibers and varying form of myocardial injury. This phenomenon was a principle cause of hypotension. These effects have been attributed to focal myocarditis (Dixon, 1991). Levett (2001) suggested that the pathological feature in the heart infected with *Leptospira* consisted of interstitial myocarditis with infiltration. From the supporting researches as described above reflected that the pathogens which were the leptospiral antigen or even the leptospires might be present within the positive stained areas corresponding to the histopathological change as having been reported.

Concerning the epicardium, the submesothelial layer presented the moderate stain in the group of one hour while the groups of six hours to six days post infection presented the positive intensive stain. This observation might be discussed in the way that in the earliest group acquired the low concentration of leptospiral antigen from the blood supply, and then in the following groups the leptospiral antigen in blood circulation increased. Besides, the cytoplasm of the mesothelial cells in all infected groups showed the positive stain. The leptospiral antigen in the cytoplasm of the mesothelial cells displayed the mild stain in the groups of one hour and three to six days after infection while other groups presented the positive moderate stain. As described in the renal tissue, Sitprija (1980) reported that the cytotoxin was found in the blood stream. The toxin had an injurious effect to the endothelial lining cells of vessels and contributes to fluid leakage from the lumen of vessels to the extravascular regions which included the epicardial layer. Chakurkar (2008) reported that the

inflammatory cells also infiltrated into the epicardium. The involvement was also present in the epicardium of the infected tissue.

Some inflammatory cells which were chiefly neutrophils infiltrated in the infected heart, those in the groups of one hour, one day and six days performed the positive staining. Furthermore, in some periods of infection the plasma cells were presented which were responsible to work against the pathogens which should be the leptospires or leptospiral antigen as mention already. Faine (1999) described that some cases of leptospiral infection died from toxic myocarditis with localized foci of inflammatory cells infiltration. Muensoongnoen (2006) reported that the necrotic cardiac muscle cells were surrounded by numerous inflammatory cells. Chakurkar (2008) described that the types of inflammatory cells were mainly plasma cells, lymphocytes and macrophages which predominated in subendocardial and perivascular location. Arean (1962) reported that foci of necrosis were observed in most case with leptospirosis and were associated with intense infiltration by neutrophils. The positive coloration seen in the cytoplasm of the neutrophils should be presumed that these cells engulf the pathogens in order to destroy them.

The blood vessels also showed positive stain both in the lumen and some component of the vessel wall consisting of the endothelial lining cells and tunica adventitia. However, the smooth muscle in the muscular layer showed the negative stain. In addition, in some periods of infection the endothelial lining cells were spitted out from the vessel wall. According to the research of De Brito (1987) which reported that the antigen deposits were detected in focal areas beneath the endothelial lining cells in human with leptospirosis. The opened endothelial junctions were observed with leakage of carbon particles. The abnormal permeability was shown by carbon leakage and passage of red blood cells and leptospire through the opened inter-endothelial junctions. It was possible that toxins acted directly upon the junctions. The focal distribution of the lesions itself suggested a relationship to impairment of the blood flow. The borders of the opened junctions were sometimes straight but mostly they seemed very irregular with many projections, showing the “defective zip-like pattern”. The endothelial damage might cause by leptospires or an inflammatory response excited by the leptospiral toxin. The primary lesion in animals with

leptospirosis was the damage to the endothelial lining cells of blood vessels caused by leptospiral toxin. The immediate effect was to loosen the junction between endothelial cells, followed by the releasing of the fluid and leptospires together with leptospiral antigen into the extravascular region (De Brito, 1979). According to Dorigatti (2005) which suggested that the primary lesion was the damage to the endothelial cells of the vessels which were prompted by the toxin component of the leptospiral wall. Muensoongnoen (2006) found that the vessels in the infected heart were dilated and congested.

However, this study found the leptospiral antigen in several areas of the infected organs which was presented by the positive golden–brown staining coloration. These positive areas corresponded to the lesion sites of the infected organs of several researches as described above. Therefore, the detected leptospiral antigens might be the primary cause of all the lesions which occurred within the infected organs of the patients and the experimental animals associated with leptospirosis.

# CHAPTER VII
# CONCLUSION

Leptospirosis can occur in human and animals which are infected by the bacteria *Leptospira interrogans* of many serovars. The infected individual may lead to multiorgan dysfunction, such as the kidney, lung, liver, spleen, heart and skeletal muscle. Leptospiral antigen serovar icterrohaemorrhagiae was revealed immunohistochemically in the macrophages of infected human liver and kidney (Brito, 2006). Furthermore the leptospiral antigen was expressed on hepatic membrane, as well as the luminal surface of tubular cell in the kidney. However, leptospiral antigens serovar pyrogenes causing the outbreak in Northeast of Thailand have not been localized in the infected organs. The attempt to reveal these antigens in the kidney, skeletal muscles (Hamstring and Gastrocnemius muscles) was performed in this study.

*Leptospira interrogans* serovar pyrogenes was isolated from a febrile patient with clinical leptospirosis in Buri-Ram Province. These pathogens were cultured and use in this study. Six control hamsters injected intraperitoneally with 0.5 ml PBS were served as the control group. Another 24 animals were the experimental groups. They were injected intraperitoneally with 0.5 ml of PBS containing $1 \times 10^8$ leptospires/ml. Three of the control hamsters were sacrificed on the $2^{nd}$ day and the last three on the $5^{th}$ day after injection with 0.5 ml PBS. Three infected hamsters were sacrificed at a time at 1 hour, 6 hours and on days 1, 2, 3, 4, 5 and 6 after injection. The kidney, skeletal muscles (hamstring and gastrocnemius muscles) and heart of all the sacrificed animals were removed and processed for immunohistochemical study of the tissue sections by the indirect immunoperoxidase staining technique. Rabbit primary antibody to *Leptospira interrogans*, serovar Pyrogenes (batch#303 from Royal Tropical Institute Kit Biomedical Research, the Netherlands) and rabbit ABC (Avidin–Biotin–peroxidase complex) staining system kit (from Santa Cruz Biotechnology, Inc) were applied.

All infected organs demonstrated the positive golden–brown stained areas which were presumed to be the leptospiral antigen in the sections. The cytoplasm of the epithelium lining the proximal tubules in all infected groups showed the positive stain of various densities which was varied from mild, moderate and intensive depending on the post infection time. The moderately–stained cytoplasm was observed in the earlier period, mild in the following period and intensive in the latest day of infection. This finding might be clarified that the pathogenic leptospires located and multiplied themselves in the blood stream of the infected animal and then invaded many organs of the body. Therefore in the early infection with moderate stain found in the study might due to the leptospires or their antigens in the blood stream went into the renal tissue by the way of the renal artery supplying the renal parenchyma in the earlier period. Then in the following period expressing the mild stain should be because of the self repairing process to recover the injury of the renal tissue. The self repairing process might somehow get rid of some leptospiral antigens in the cells which leaded to produce less intensity than those of the early groups. From this report the positive golden–brown macrophages and inflammatory cells might engulf some kind of leptospiral antigen in order to wipe out the antigens in the infected tissue. Moreover, in the latest day group the cytoplasm became intensive stain which might be the effect of the injury of the capillary loops in the glomeruli. This circumstance eased the leptospires or the leptospiral antigen and red blood cells covered by leptospiral antigen coming out into the urinary spaces and somehow these antigens might invade the proximal tubular cells adding with the preexisting antigen in the cell cytoplasm and made the cells show intensive stain.

Concerning the distal tubules and collecting duct, it had been demonstrated that the cytoplasm of their epithelia were stained mildly in early period, moderately and intensively in the following period and became mildly or moderately again in the late period. This pattern of intensity might be the results of the renal blood supply and the damage of the renal tissue as followed. Renal parenchyma received blood supply from the renal blood vessels via peritubular arteries which supplied the blood throughout the renal tissue. Therefore in first hour post infection group, the epithelial lining cells received the leptospiral antigen from the blood in low concentration rate,

making the mild stained appearance. After that the cells had moderately or intensively–stained cytoplasm which should be that they had been received more leptospiral antigen from the blood supplying the renal tissue in the high concentration rate. Then they were able to repair themselves resulting to be mild stain again. Finally, the cells became the moderately–stain again which could happen by receiving the high concentration of leptospiral antigen from the damaged capillary loop of glomerulus and released the blood associated with the leptospiral antigens to urinary space and renal tubular lumen. In addition, the renal tubular epithelial lining cells loss of function which effected on the severity of renal tissue damage in the latest period of infection.

Regarding to the renal corpuscle, the capillary loops which were represented by the podocytes and some either parietal or visceral layer of the Bowmann's capsule revealed the positive golden–brown areas. Those in the one hour group performed the negative staining. This observation might be discussed in the way that the low concentration of leptosprial antigen in the afferent arteriole to the glomerulus in the earliest post infection group. In the later groups more leptospiral antigen circulating in the blood stream made the glomerulus mild positive stain. Besides, the group of last period of infection showed the positive stained red blood cells and some homogeneous hyalinic materials in the urinary space. This might because of the injury of the capillary loops that allowed the blood contents in the vessels leaked out to the urinary space causing the haematuria.

The skeletal muscle of both the hamstring and gastrocnemius in all infected groups showed the positive stain of various intensities. The leptospiral antigen deposited within the muscle fibers might give the explanation of the necrosis demonstrated in the leptospiral infected skeletal muscle. The connective tissue supporting the muscle bundle displayed the positive stain in all experimental groups. The degrees of the positive stain were mostly intensive. This should be presumed that the leptospiral antigen or the leptospires themselves preferred to locate in the connective tissue supporting the organs and invaded the area via the blood supply. The nerve supplying the skeletal muscle fibers also showed the clearly positive stain in various intensities in all infected groups in the hamstring muscle and almost all

infected groups in the gastrocnemius muscle. Various neurological disturbances had been reported in cases of leptospirosis. This phenomenon might be the causation of neuropathy which was an uncommon manifestation of leptospirosis. Furthermore, the nerve fiber supplying the infected muscular tissue revealed the positive stain. This should be presumed the leptospiral antigen invaded the nerve fiber and interfered the physiological function of the nerve.

The subendocardial layer of the endocardium of heart in almost infected groups revealed the intensive positive stain. The positive coloration expressed in one day and three to the last day of the experiment. This staining pattern reflected that the leptospiral antigen or even the leptospires themselves could come inside the endocardium, especially the subendocardial layer. Regarding to the myocardium, it had been demonstrated that the sacroplasm of cardiac muscle cells were stained mildly in the first hour and intensive in the following period. This staining pattern might be the result of the leptospiral antigen in the blood vessel supplying the cardiac tissue. Cardiac muscle cells received blood supply from blood vessel which supplied the blood throughout the heart tissue. Concerning the epicardium, the submesothelial layer presented the moderate stain in the group of one hour while the groups of six hours to six days post infection presented the positive intensive stain. This observation might be discussed in the way that in the earliest group acquired the low concentration of leptospiral antigen from the blood supply, and then in the following groups the leptospiral antigen in blood circulation increased.

Additionally in all of experimental organs, the leptospiral antigens were also observed in the inflammatory cells and blood vessels. The positive color was performed in the inflammatory cells being mainly neutrophils which had ingested the debris and the foreign particles. Meanwhile the neutrophils in the infected tissues tried to get rid of these antigens by engulfing them making their cytoplasm show the intensive positive stain. In addition, in some periods of infection the plasma cells were presented which had the responsibility to produce the antibody against the leptospires themselves or the leptospiral antigen.

The blood vessels also showed the positive stain in the lumen and the component of the vessel wall. These appearances were likely to be because it was clearly known that the leptospires or their antigens circulated to organs by the blood vessels. The primary lesion in all forms of leptospirosis in all animals was the damage to the endothelial cell membranes of small blood vessels, caused by leptospiral toxin. The immediate effect was to loosen the junction between endothelial cells, allowing fluid, leptospires or leptospiral antigen to leak out into extravascular space, followed by erythrocytes where the damage was severe and prolonged.

However, this study found the leptospiral antigen in several areas of the infected organs which was presented by the positive golden–brown staining coloration. These positive areas corresponded to the lesion sites of the infected organs of several researches as described above. Therefore, the detected leptospiral antigens might be the primary cause of all the lesions which occurred within the infected organs of the patients and the experimental animals associated with leptospirosis.

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# APPENDIX

# APPENDIX A

**Bouin's solution** (Bancroft, 1996)

Composition:

| | | |
|---|---|---|
| Saturated aqueous picric acid solution | 75 | ml. |
| 40% formaldehyde | 25 | ml. |
| Glacial acetic acid | 5 | ml. |

Bouin's solution was prepared by mixing the saturated aqeous picric acid solution with 40% formaldehyde. The solution was mixed well, and then added glacial acetic acid into the prepared solution. When the solution was mixed very well, it will be immediately used.

# APPENDIX B

## Conventional Paraffin technique for light microscopic study

### Conventional light microscope

1. **Washing of tissue**

The pieces of the animal tissue were washed in several changes of normal saline until the blood was completely removed.

2. **Fixation**

The removed pieces of tissue were fixed in Bouin's solution for 48 hours at room temperature.

3. **Washing**

All of tissues were washed with many changes of 50% and 70% ethanol until the yellow color of picric acid of the Bouin's solution disappeared.

4. **Processing**

Dehydration

This process was the first step of the processing which aqueous tissue fluid had been removed from the tissues by graded series of ethanol. The tissues were immersed first in 80% ethanol then progressed through 90%, 95% and finally to 100% ethanol. (Diagram 5) In this way all the aqueous tissue fluids were removed with little disruption to the tissue due to diffusion currents.

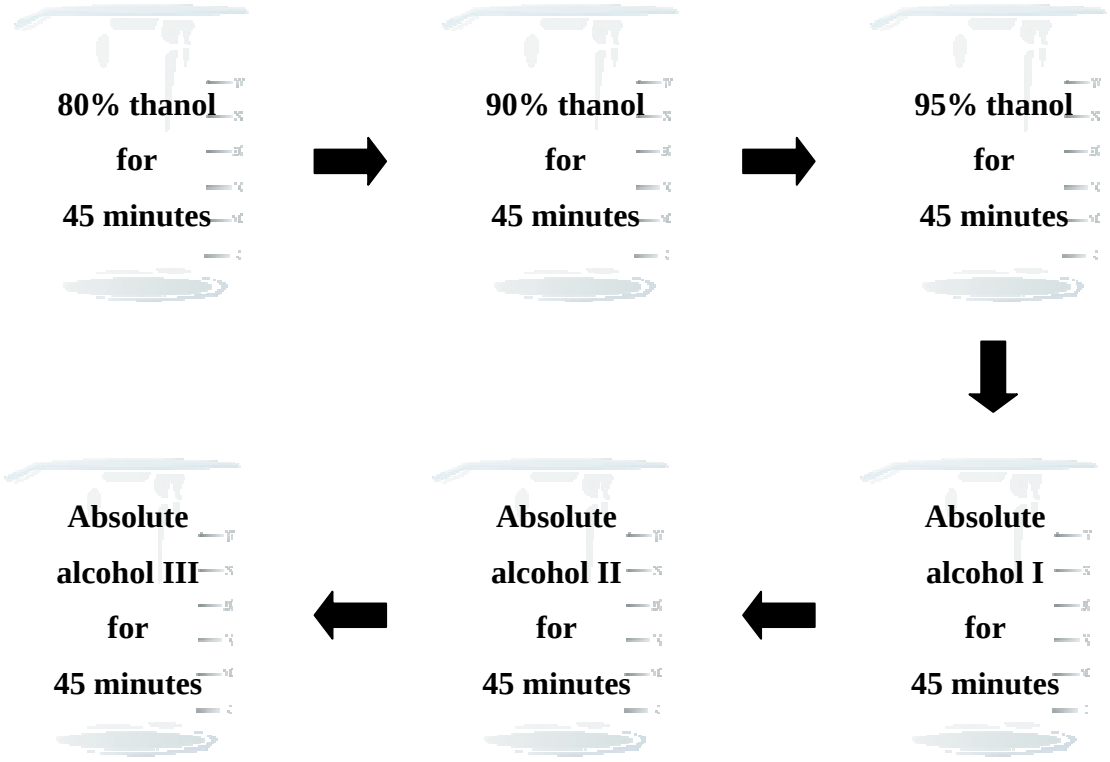


**Diagram 5** Dehydration processes.

Clearing

The pieces of tissue were now filled with dehydrant, absolute ethanol. This dehydrant were removed from the tissue by clearing agent, xylene.The xylene is appropriate for routine histology schedules of less than 24 hours. Immersion in xylene must not be prolonged. After clearing the tissue became translucent. (Diagram 6)

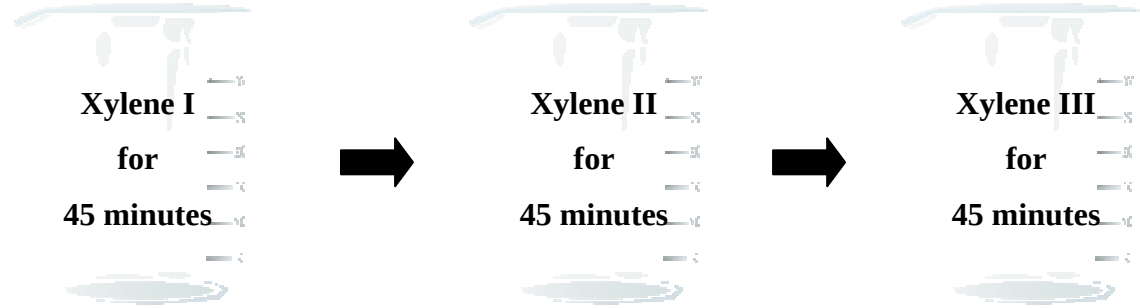


**Diagram 6** Clearing processes.

Infiltration

The cleared tissues were placed in the melting paraplast in order to replace the clearing agent with the embedding medium. For this study paraplast had been used as the embedding medium. (Diagram 7)

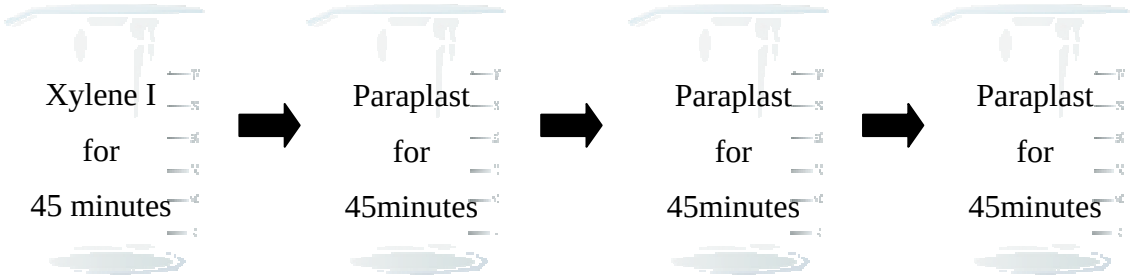


**Diagram 7** Infiltration processes

**5. Embedding**

The infiltrated tissues were placed in the melting paraplast which occupied in the base of the embedding mold. Other melting paraplast was added immediately over the embedded tissue until the mold was full. Then, the mold was placed on the cool plate and after the paraplast had solidified, the embedded tissue block was removed and ready for sectioning.

**6. Sectioning with microtome**

The embedded tissue blocks were then cut to a thickness of 3 µm with a rotary microtome. (Figure 36) The tissue sections were placed on the glass slides which were coated with 3 – aminopropyltriethoxysilane (Appendix C) and then dried at 60 °c for 30 minutes on the glass slide warmer.

**Figure 36** Microtome (Leica RM 2135)

# APPENDIX C

## Coating glass slides with 3 – aminopropyltriethoxysilane (silane)

Silane reagents are frequently used to strengthen the bonding of glass fiber to the tissue sections, to promote the adhesion of tissue section to glass substrates.

Coating glass slides protocol (Diagram 8)

1. Wash glass slides in acetone for 10 seconds.
2. Immerse slides in a freshly prepared 3% solution of 3–amino-propyltriethoxysilane in dry acetone for 20 seconds.
3. Wash glass slides in acetone for 10 seconds.
4. Wash glass slides in running tap water for 1 minutes
5. Wash briefly in distilled water twice.
6. Dry overnight at 60 °c and store at room temperature.

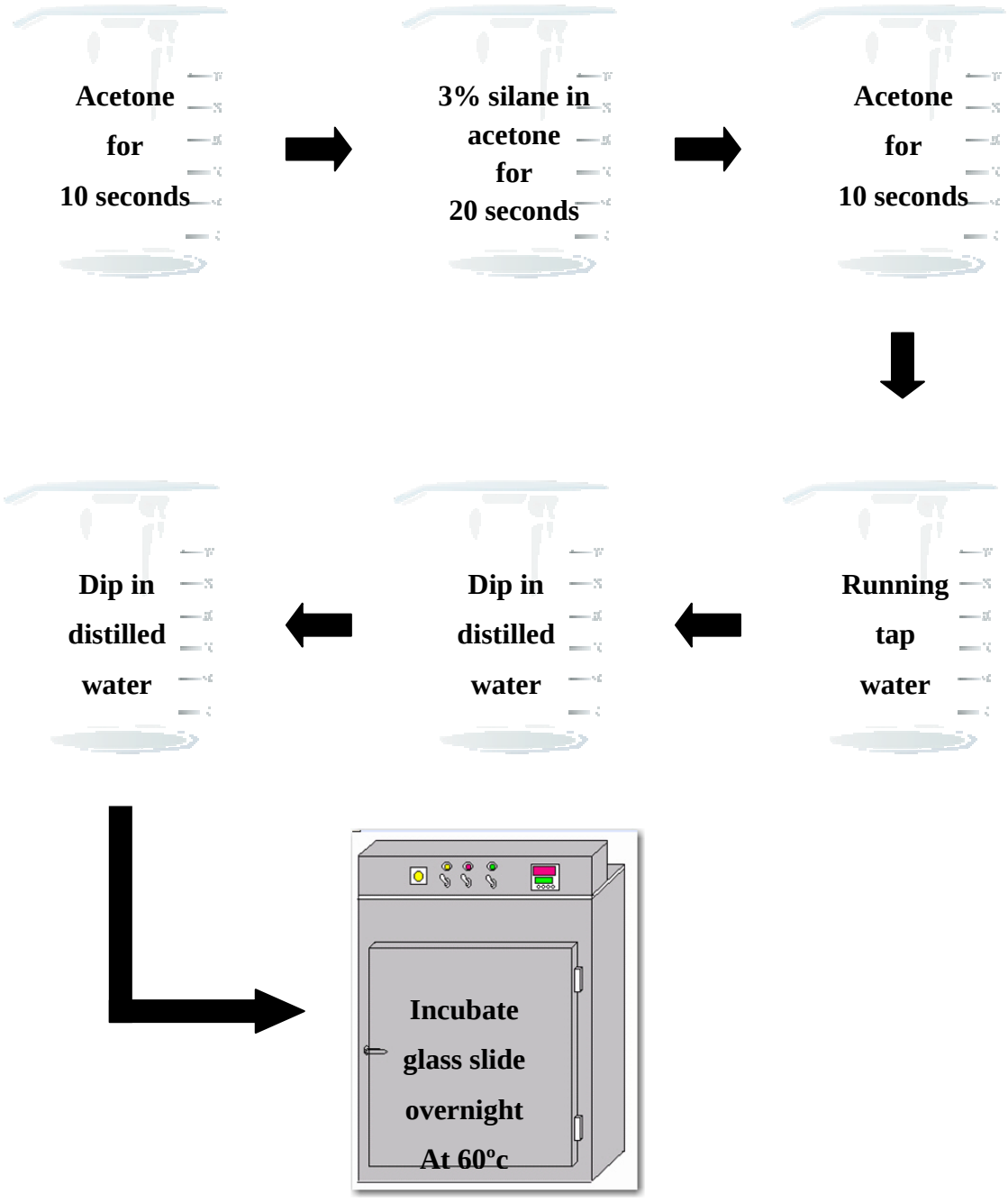


**Diagram 8** Coating glass slide processes.

# APPENDIX D

## Indirect immunoperoxidase staining technique

### Preparation of ABC Staining System working solutions

Use only freshly prepared buffers. Prepare all working solutions in the mixing bottles provided. After preparation, insert the drop dispenser top (supplied in inverted position) into the cap in correct orientation. Place the entire unit on the bottle and twist the cap. The drop dispenser will snap into place. To remove the drop dispenser for refilling, press laterally with thumb until the top snaps off. To prevent evaporation, secure the caps on bottles when not in use. After completion of the staining procedure, working solutions should be discarded and mixing bottles were washed with distilled $H_2O$.

- Blocking serum: In mixing bottle 1 (Blue cap)
  Mix;
  - 75 µl of normal blocking serum stock
  - 5 ml of PBS
- Biotinylated secondary antibody: In mixing bottle 2 (Green cap)
  Mix;
  - 75 µl of normal blocking serum stock
  - 5 ml of PBS
  - 25 µl of biotinylated secondary antibody stock
- AB enzyme reagent: In AB mixing bottle (Purple cap)
  Mix;
  - 50 µl of reagent A (Avidin)
  - 50 µl of reagent B (biotinylated HRP)
  - 2.5 ml of PBS

  Mix and let stand for approximately 30 minutes.

- Peroxidase substrate: In substrate mixing bottle (Yellow cap)

  Mix;

  - 1.6 ml of distilled $H_2O$
  - 5 drops of 10x substrate buffer
  - 1 drop of 50x DAB chromogen
  - 1 drop of 50x peroxidase substrate

**Immunoperoxidase staining procedure** (Diagram 9)

All steps are carried out at room temperature in a humidified chamber. Staining dishes or coplin jars may also be used. Apply sufficient volumes of dropping reagents (approximately 50 ml) to cover the sections completely; avoid drying of specimens between each step.

- Deparaffinizing the paraplast-embedded tissue sections by immerging in the solutions as followed:
  - Xylene for 3 minutes. (2 times)
  - Absolute alcohol for 3 minutes. (3 times)
  - 95% ethanol for 3 minutes. (2 times)
  - 70% ethanol for 3 minutes
- After deparaffinizing, the tissue slides were stained by dropping the reagents as following procedure:
  - 8 minutes in 3% hydrogen peroxide diluted in PBS to quench endogenous peroxidase activity.
  - 5 minutes in deionized $H_2O$.
  - Washed in PBS twice for 5 minutes each.
  - Incubated the tissue sections for one hour in 1.5% blocking serum in PBS (Mixing bottle 1).
  - Incubated the sections with primary antibody (dilution; 1:1000) for 1 hour at room temperature in the humidified chamber.
  - Washed the tissue sections with three changes of PBS for 5 minutes each.
  - Incubated the sections for 1 hour with biotinylated secondary antibody as prepared in mixing bottle 2

- Washed with three changes of PBS for 5 minutes each.
- Incubated for 30 minutes with AB enzyme reagent (AB mixing bottle).
- Washed with three changes of PBS for 5 minutes each.
- Incubated in 1 – 3 drops of peroxidase substrate (substrate mixing bottle) for 4 minutes to develop golden – brown precipitation.
- Washed in distilled $H_2O$ for 5 minutes.
- Counterstained in Carazzi's haemotoxylin solution for 3 minutes and 30 seconds.
- Immediately washed with several changes of distilled $H_2O$.
- Washed with running tap water for 5 minutes.
- Dipped the sections in distilled $H_2O$.

- The stained tissue sections were dehydrated by immerging in the solution as follows:
  - Immerged in 95% ethanol twice for 5 minutes each.
  - Immerged in absolute alcohol twice for 5 minutes each.
  - Immerged in xylene twice for 5 minutes each.
- Wiped off excess xylene.
- Immediately added 1 – 2 drops of permanent mounting medium (Resin solution) and covered with a glass coverslip.

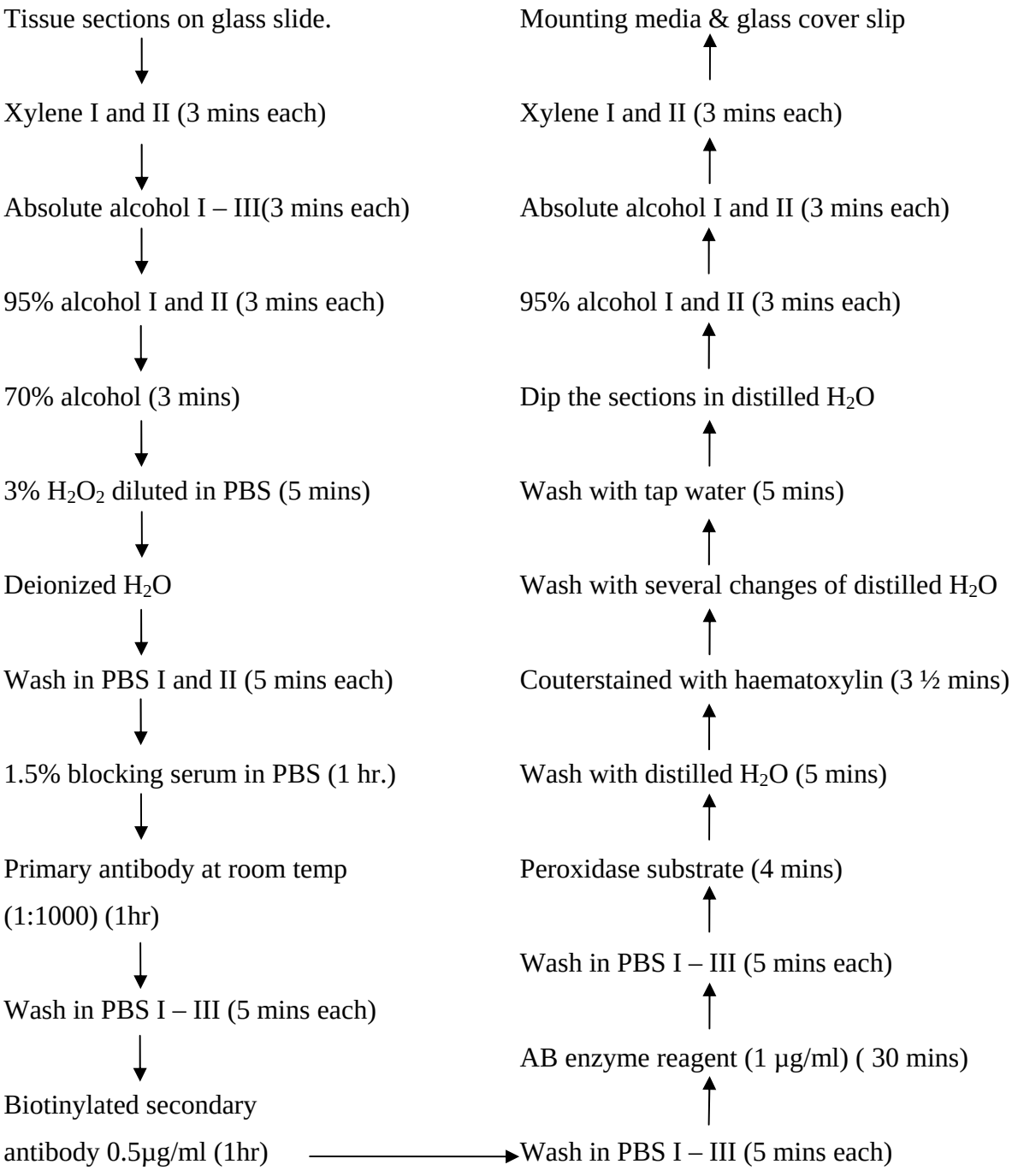


**Diagram 9** Immunoperoxidase staining procedure.

# APPENDIX E

**Carazzi's haematoxylin solution** (Bancroft, 1996)

This is an alum haematoxylin which is chemically ripened by using potassium iodate.

*Stock A*: Composition

| | | | |
|---|---|---|---|
| - | Haematoxylin | 5.0 | gm. |
| - | Glycerol | 100 | ml. |

*Stock B*: Composition

| | | | |
|---|---|---|---|
| - | Potassium alum | 25.0 | gm. |
| - | Distilled water | 400 | ml. |

Carazzi's haematoxylin solution was a mixture between stock A and B as the haematoxylin-alum-glycerol solution. And then, added potassium iodate for 0.1 gm into the haematoxylin-alum-glycerol solution. The staining solution was mixed well and was ready to use; it remains usable for about 6 months. Care might be taken in preparing the haematoxylin solution to avoid overoxidation by not using heat to dissolve the reagents. Finally, filter before use.

# APPENDIX F

**Phosphate Buffer Saline solution; PBS pH 7.2** (Bancroft, 1996)

Preparation of stock solutions

*Stock A*: 3.12 gm of 0.2 M sodium dihydrogen orthophosphate (mw 156) dissolved in 100 $cm^3$ of distilled water.

*Stock B*: 2.83 gm of 0.2 M disodium hydrogen orthophosphate (mw 142) dissolved in 100 $cm^3$ of distilled water.

Phosphate Buffer Saline pH 7.2 was a mixture between stock A and B. For preparing the PBS 100 $cm^3$, the 14.0 $cm^3$ of stock A was mixed with 36.0 $cm^3$ of stock B, and then made up to 100 $cm^3$ with distilled water. Finally, the digital pH meter was used to check the pH of the solution and pH 7.2 was adjusted by 0.2 M of sodium dihydrogen orthophosphate in distilled water and 0.2 M disodium hydrogen orthophosphate in distilled water.

# BIOGRAPHY


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