

STUDY OF CURCUMIN EFFICIENCY FOR ANGIOGENESIS,  
EMPHASIZING ON VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)  
IN DIABETIC RATS' LIVER



PRESENTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE  
DEGREE OF DOCTOR OF PHILOSOPHY IN BIOMEDICAL SCIENCE  
AT SRINAKHARINWIROT UNIVERSITY

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To study the efficiency curcumin for angiogenesis, emphasizing on vascular endothelial growth factor (VEGF) in diabetic rat's liver, diabetic rats were induced by streptozotocin (60 mg/kg BW). Male rats were divided into three groups, control rats(C), diabetic rats (DM) and diabetic rats treated with curcumin (DMC) (200 mg/kg BW). At the end of 4, 8, and 12 weeks after STZ injection or STZ plus curcumin supplementation, blood sugar level, body weights, liver function test, and histopathology of liver tissue and blood vessel were investigated. Blood sugar levels of DM rats at 4, 8, and 12 weeks were elevated, but they were reduced after curcumin supplementation. In contrast, the body weights were lowered in DM rats at 8 and 12 weeks, but they were toward increased after curcumin supplementation. Concerning the liver function test, the Alkaline phosphatase (SAKP) level was increased in DM rats at 4, 8, and 12 weeks and it was reduced in DMC rats at 4, 8, and 12 weeks after curcumin administration. Light microscopic study revealed that there were histopathological lesions in liver tissues at 8 and 12 weeks of DM rats, including dilated blood sinusoids and central veins. Hepatocytes revealed edema, pyknosis nuclei, and lipids accumulation in cytoplasm. Moreover, the liver revealed inflammatory change within the portal triad in which infiltration of lymphocytes and collagen deposition were increased. Interestingly, administration of curcumin, which is a main yellow bioactive compound of turmeric rhizome, dramatically inhibited the severity of liver tissue and blood vessels of DMC rats compared with DM rats. Specifically, by vascular corrosion casting technique, the liver blood vessels showed the increasing of diameters and blood volumes of proximal and distal parts of portal veins together with venule branches in DM rats at 8 week. The distal portion of portal veins showed blind endings and shrinkage of portal venules. At 12 week DM experiments, the portal venules were seriously shrinkage. Their

diameters and blood volumes were decreased. In addition, the new development and neovascularization of new tortuous and shrinkage blood vessels have emerged and presented extensively from the proximal part of portal venules. In DMC rats at 8 and 12 weeks which were treated with curcumin, the healthy normal patterns of liver blood vessels have recovered and got restored. Moreover, the new development and neovascularization of new nourishing blood vessels have emerged and organized extensively. Therefore, the related vascular endothelial growth factor (VEGF) was interested and analyzed for its efficiency in diabetes. In general, VEGF is a key mediator of angiogenesis associated with various disorders, especially with inflammation. Concerning immunohistochemical staining for VEGF at the end of 4, 8 and 12 week experiments, VEGF was fundamentally recognized in the hepatocytes and endothelial cells of the central veins in all of three groups, evidenced by brownish color in their cytoplasm. The intensity and pattern of VEGF distribution varied, according to the durations of the experiments. VEGF intensity was strongly stained in DM rats at 12 week. After curcumin treatment, VEGF immunostaining scores were lowered in DMC rats at 8 and 12 weeks. Concerning Western blot analysis at 12 week DM experiment, the specific VEGF protein band from DM revealed the strongest intensity of VEGF expression when compared with control and DMC rats at the different durations. However, the expression of VEGF was reduced after treatment by curcumin in DMC rats when it was compared with DM rats. In conclusion, administration of curcumin successfully improved the destruction of the liver blood vessels and induced vascular remodeling associated with controlling VEGF expression. The potential beneficial effects of curcumin have been shown to exhibit anti-inflammatory, antioxidant and anti-diabetic activities. The efficiency and achievement of curcumin might be applied to be an alternative therapeutic agent in diabetic hepatic pathology.



การศึกษาผลของเคอร์คูมิน ที่มีต่อกระบวนการสร้างเส้นเลือดใหม่  
โดยเน้นที่การแสดงออกของสาร vascular endothelial growth factor (VEGF)

ในระดับหนูที่เป็นเบาหวาน



เสนอต่อบัณฑิตวิทยาลัย มหาวิทยาลัยศรีนครินทรวิโรฒ เพื่อเป็นส่วนหนึ่งของการศึกษา

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การศึกษาในครั้งนี้ เป็นการศึกษาผลของเคอร์คูมิน ที่มีต่อกระบวนการสร้างเส้นเลือดใหม่ โดยเน้นที่การแสดงออกของสาร vascular endothelial growth factor (VEGF) ในตับหนูที่เป็นเบาหวาน. ที่ถูกเหนี่ยวนำด้วยสาร streptozotocin (STZ) การทดลองได้แบ่งหนูออกเป็นสามกลุ่ม ได้แก่ กลุ่มควบคุม กลุ่มเบาหวาน (DM) และกลุ่มเบาหวานที่ได้รับเคอร์คูมินในปริมาณ 200 mg/kg BW (DMC) เมื่อสิ้นสุดการทดลองที่ระยะเวลา 4, 8 และ 12 สัปดาห์ ได้ทำการประเมินระดับน้ำตาลในเลือด น้ำหนัก การตรวจเลือดดูการทำงานของตับ (liver function test) รวมถึงลักษณะทางจุลกายวิภาคศาสตร์และพยาธิสภาพของเนื้อเยื่อตับและหลอดเลือด พบว่าระดับน้ำตาลในเลือดสูงขึ้นในหนูกลุ่ม DM ที่ 4, 8 และ 12 สัปดาห์ แต่ในหนูกลุ่ม DMC มีระดับน้ำตาลในเลือดลดลงเมื่อเปรียบเทียบกับระยะเวลาเท่ากัน หนูกลุ่ม DM มีน้ำหนักลดลงตามระยะเวลาของการเป็นเบาหวานและหนูกลุ่ม DMC กลับมีน้ำหนักเพิ่มขึ้นที่ระยะเวลาเดียวกัน การตรวจเลือดเพื่อดูการทำงานของตับพบว่า alkaline phosphatase (SAKP) มีค่าเพิ่มขึ้นในหนูกลุ่ม DM ที่ระยะเวลา 4, 8 และ 12 สัปดาห์ และมีค่าลดลงในหนูกลุ่ม DMC ที่ระยะเวลาเดียวกัน การศึกษาเนื้อเยื่อโดยใช้กล้องจุลทรรศน์แสงสว่าง ที่ระยะเวลา 4, 8 และ 12 สัปดาห์ของหนูกลุ่ม DM พบลักษณะทางพยาธิสภาพและความผิดปกติของตับโดยมีการทำลายโครงสร้างเซลล์ เนื้อเยื่อและโครงสร้างของหลอดเลือดของตับ ได้แก่ blood sinusoid และ central vein ที่มีการขยายขนาดกว้างขึ้น เกิดการบวมของเซลล์ hepatocyte และมีภาวะ pyknosis nucleus รวมถึงมีการคั่งสะสมของไขมันในไซโตพลาสซึมด้วย นอกจากนี้ยังพบภาวะอักเสบเกิดขึ้นที่บริเวณ portal triad โดยพบว่าการสะสมของ lymphocyte และ คอลลาเจนไฟเบอร์ที่เพิ่มขึ้นมาก การศึกษาให้สารเคอร์คูมินในหนูกลุ่ม DMC พบว่าโครงสร้างเซลล์และเนื้อเยื่อของอวัยวะตับและโครงสร้างของหลอดเลือดที่ตับมีการซ่อมแซมและฟื้นฟูสภาพเข้าสู่สภาวะปกติและใกล้เคียงกลุ่มควบคุม โดยเฉพาะที่ระยะเวลา 12 สัปดาห์หลอดเลือดมีการปรับสภาพโดยมีขนาดเข้าใกล้สภาวะปกติ การศึกษาโดยวิธี vascular corrosion cast ในหนูกลุ่ม DM ที่ระยะเวลา 8 สัปดาห์ พบว่าการขยายขนาดของหลอดเลือดและมีการเพิ่มปริมาณการไหลของเลือดใน portal vein ทั้งส่วนต้นและส่วนปลายรวมถึงส่วนของ portal venule บริเวณส่วนปลายของ portal vein มีลักษณะของหลอดเลือดปลายตัดและการเหี่ยวแฟบอย่างเด่นชัดที่ portal venule ระยะสัปดาห์ที่ 12 พบว่า portal venule เกิดการเหี่ยวแฟบเพิ่มมากขึ้น

รวมถึงหลอดเลือดมีขนาดเล็กลงและมีการตีบแคบของรูมากขึ้นเช่นกันนอกจากนั้นยังมีการงอกใหม่ของหลอดเลือดเล็กๆในลักษณะบิดงอและเหี่ยวแฟบออกมาจากส่วนต้นของ portal vein เกิดขึ้นอีกด้วย แต่เมื่อมีการให้สารเคอร์คูมินที่ระยะเวลา 8 และ 12 สัปดาห์ในหนูกลุ่ม DMC พบว่าโครงสร้างของหลอดเลือดที่ตีบมีการซ่อมแซมและฟื้นฟูสภาพเข้าสู่สภาวะปกติและใกล้เคียงกลุ่มควบคุม หลอดเลือดมีขนาดและมีปริมาณเลือดที่ไหลผ่านกลับเข้าใกล้สภาวะปกติ พบมีการงอกใหม่ของหลอดเลือดเล็กๆ ออกมาจากส่วนต้นของ portal vein ในลักษณะที่เต่งและสมบูรณ์ การวิจัยมุ่งสนใจที่สารโปรตีน Vascular endothelial growth factor (VEGF) ซึ่งเป็นสารสำคัญที่เกี่ยวข้องกับการเกิดการงอกใหม่ของหลอดเลือดจากหลอดเลือดต้นตอในสภาวะที่แตกต่างกันออกไปโดยเฉพาะในภาวะที่มีการอักเสบเกิดขึ้น ได้ทำการศึกษาดำเนินงานของเซลล์ที่สร้างสาร VEGF ในเนื้อเยื่อตับโดยเทคนิค Immunohistochemistry ด้วยแอนติบอดีที่จำเพาะเจาะจงเพื่อหาดำเนินงานของสาร VEGF ในหนูทั้งสามกลุ่มที่ระยะเวลา 4, 8 และ 12 สัปดาห์ พบว่า มีการแสดงการติดสีน้ำตาลในไซโตพลาสซึมของ hepatocyte และ endothelial cell ของ central vein ซึ่งพบว่าการแสดงออกของ VEGF อย่างมากที่สุดในหนู DM ที่ระยะเวลา 12 สัปดาห์ หลังจากให้สารเคอร์คูมินในหนูกลุ่ม DMC พบว่าการแสดงออกของ VEGF ลดลงในระยะเวลา 8 และ 12 สัปดาห์ สำหรับการศึกษาด้วยเทคนิค western blotting เพื่อหาปริมาณของสาร VEGF ในเนื้อเยื่อตับพบว่าหนูกลุ่ม DM ที่ระยะเวลา 12 สัปดาห์มีการแสดงออกของปริมาณของสาร VEGF มากที่สุด แต่หลังจากให้สารเคอร์คูมินในหนูกลุ่ม DMC พบว่าปริมาณของสาร VEGF ลดลงเมื่อเทียบกับหนูกลุ่ม DM การศึกษารั้งนี้พบว่า สาร curcumin ซึ่งเป็นสารที่มีอยู่ในขมิ้นชัน มีส่วนช่วยในการปรับปรุงและฟื้นฟูสภาพเนื้อเยื่อตับและหลอดเลือดจากสภาวะเบาหวานให้มีความรุนแรงลดลง มีความสัมพันธ์ในการควบคุมการแสดงออกของสาร VEGF ในหนูที่เป็นเบาหวาน โดยส่ง ผลที่เกี่ยวข้องในการรักษาภาวะการอักเสบ แอนติออกซิแดนต์ จึงช่วยคงสภาพและฟื้นฟูหลอดเลือดในตับที่เสื่อมสภาพจากสภาวะเบาหวาน มีผลที่ดีต่อการลดภาวะแทรกซ้อนของหลอดเลือดที่ตีบและน่าจะมีประโยชน์ในการนำมาประยุกต์ใช้เชิงการแพทย์ทางเลือกในการรักษาโรคเบาหวานต่อไป

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by  
Wipapan Khimmaktong

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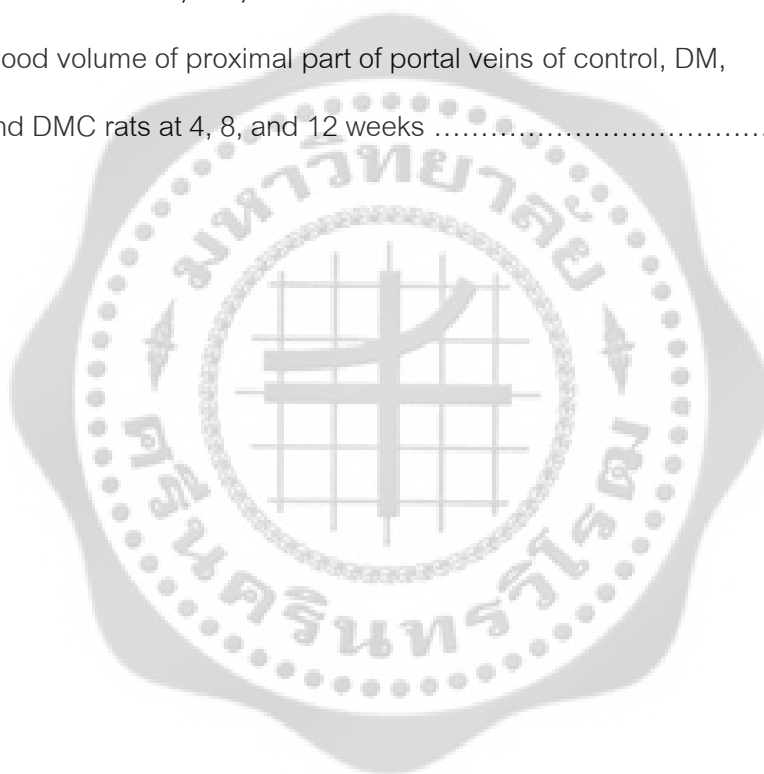
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## CHAPTER 1

### INTRODUCTION

Diabetic mellitus (DM) is a disorder of blood sugar or blood glucose metabolism that affects the body ability to produce or respond to insulin, which is a hormone that allows blood sugar to enter the cells of the body and be used for energy. DM is characterized by hyperglycemia, abnormal lipid and protein metabolism along with specific long term complication, affecting the retina, kidney and nervous system (1). DM condition seriously conducts to destruction of important organs, systems including microvascular architectures such as in kidney, pancreas and liver. Liver is the second-largest organ of the body (the largest is the skin) and also the largest gland of the body. The liver is the organ in which nutrients absorbed in the digestive tract are processed and stored for use by other parts of the body. It is thus an interface between the digestive system and the blood. Most of its blood (70-80%) comes from portal vein, the other smaller percentage is supplied by the hepatic artery. The position of liver in the circulatory system is optimal for gathering, transforming, and accumulating metabolites, for neutralizing and eliminating toxic substances. Liver also has the capability to store glucose from non-carbohydrate sources. This key function of liver makes it vulnerable to disease in subject with metabolic disorders, particularly diabetes (2).

Streptozotocin (STZ) is a widely used agent to induce insulin-dependent diabetic mellitus in experimental animals because of its toxic effects on islet beta cells (3). The pathological and biochemical features of this model are compatible to those of type 1 diabetes. It is a glucosamine-nitrosurea compound, as with other alkylating agents in the nitrosurea class. STZ is toxic to cells by causing damage to the DNA, though other mechanisms may also contribute. It also induces systemic microvascular alterations, as observed in retina and kidney (4,5).

Angiogenesis is the formation of new capillaries from the pre-existing vasculature by migration and proliferation of endothelial cells. Angiogenesis also plays a fundamental role in the growth, survival and function of normal and pathological tissues (26). The pathogenesis of diabetic complications may cause the abnormalities in endothelial function, such as increased endothelial permeability to macromolecules and endothelial proliferation. Vascular endothelial growth factor (VEGF) is the potent stimulator of angiogenesis with the capacity to promote endothelial cell proliferation, migration and tube formation. It is known that VEGF is very important to vascular development in both normal and pathological situations (145).

Curcumin, the principle of spice turmeric (*Curcuma longa*), has been used in traditional medicine as a household remedy for various diseases such as hepatic disorder and diabetic wound. Curcumin has been shown a wide spectrum of biological actions that have been reported to have hypolipidemic, hypoglycemic and antioxidant activities (6-8). Furthermore, curcumin is reported to attenuate diabetic complications such as nephropathy (9) and has ability to eliminate oxygen-derived free radicals from the peroxidation of cell lipids because of the presence of phenolic groups in the structure of curcumin (10).

In this study curcumin was used as daily supplement for investigation the improved and repaired structural changes of microvasculature in STZ- induced diabetic rat's liver.



Therefore, the aim of this study will be investigated as follow:

1. To determine the structural changes of liver tissues and blood vessels among control, STZ-induced diabetic rats (DM) and STZ-induced diabetic rats with curcumin supplementation (DMC) at 4, 8 and 12 weeks by using histological technique.
2. To localize VEGF in liver tissues among control, DM and DMC at 4, 8 and 12 weeks by using the immunohistochemical technique.
3. To study the expression of VEGF in liver among control, DM and DMC rats at 4, 8 and 12 weeks by Western blot analysis using antigen-antibody reaction.
4. To investigate structural changes of microvasculature in liver among control, DM and DMC rats at 4, 8 and 12 weeks by scanning electron microscope with vascular corrosion cast technique.

## CHAPTER 2

### REVIEW LITERATURE

#### Diabetes mellitus

The term diabetic mellitus describes a metabolic disorder of multiple aetiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in either insulin secretion or insulin action, or both. The effects of diabetes mellitus include long-term damage, dysfunction and failure of various organs.

The two major forms of diabetes are type 1, previously called insulin-dependent diabetes mellitus (IDDM) or juvenile onset diabetes and type 2, previously called non-insulin-dependent diabetes mellitus (NIDDM) or maturity onset diabetes. Type 1 diabetes is less common than type 2 diabetes and accounts for 5-10% of all diabetes cases.

#### Type 1 Diabetes

In type 1 diabetes, the pancreas does not produce insulin. Insulin is a hormone that is involved in regulating how the body converts glucose into energy. Onset is usually in childhood or adolescence. Type 1 diabetes is considered an autoimmune disorder that involves beta cells in the pancreas which are gradually destroyed. Eventually insulin deficiency is absolute. Without insulin to move glucose into cells, blood glucose levels become excessively high, a condition known as hyperglycemia. Because the body cannot utilize the sugar, Therefore, the sugar is spilled over into urine and is lost. Patients with type 1 diabetes need to take daily insulin for survival.

#### Type 2 Diabetes

Type 2 diabetes is the most common form of diabetes, accounting from 90-95% of cases. In type 2 diabetes, the body does not respond properly to insulin, a condition known as insulin resistance.

**TABLE 1** Comparison between type 1 and type 2 diabetes mellitus

|                         | Type 1                   | Type 2                          |
|-------------------------|--------------------------|---------------------------------|
| Age of onset            | Usually under 30 years   | Usually over 40 years           |
| Ketoacidosis            | Common                   | Rare                            |
| Body weight             | Thin                     | 80% are obese                   |
| Prevalence              | 0.5%                     | 2-4%                            |
| Genetics                | HLA associated in 40-50% | Familial but not HLA associated |
| Circulating islet cells | 50-85%                   | Less than 10%                   |
| Treatment with insulin  | Necessary                | Usually not require             |

#### **Associated diabetes to other conditions**

Conditions that damage or destroy the pancreas, such as pancreatitis, pancreatic surgery, or certain industrial chemicals can cause diabetes. Certain drugs can also cause temporary diabetes, including corticosteroids, beta blockers and phenytoin. Rare genetic disorders (Klinefelter syndrome, Huntington's chorea, Wolfram syndrome, leprechaunism, Rabson-Mendenhall syndrome, lipotrophic diabetes and others) and hormonal disorders (acromegary, Cushing syndrome, pheochromocytoma, hyperthyroidism, somatostatinoma, aldosteronoma) also increase the risk for diabetes.

#### **Risk factors**

Type 1 diabetes is much less common than type 2 diabetes, consisting of only 5-10% of all cases of diabetes. Nevertheless, like type 2 diabetes, the incidence of type 1 diabetes among children and adolescents has been rising over the past few decade. While type 2 diabetes has been increasing among African-American and Hispanic adolescents, the highest rates of type 1 diabetes are found among Caucasian youth. Type 1 diabetes can occur at any age but usually appears between infancy and the late 30 of age, most typically in childhood or adolescence. Male and female are equally at risk. The following study reports indicate risk factors for developing type 1 diabetes:

- Having a parent with type 1 diabetes (the risk is greater if a father has the condition)
- Having an older mother
- Having a mother who had preeclampsia during pregnancy
- Being ill in early infancy
- Having other autoimmune disorder such as Grave's disease, Hashimoto's thyroiditis (a form of hypothyroidism), Addison's disease, multiple sclerosis (MS) or pernicious anemia.

### Symptoms

The process that destroys the insulin-producing beta cells can be long and insidious. At the point when insulin production bottoms out, however, type 1 diabetes usually appears suddenly and is progressive quickly. Warning signs of type 1 diabetes include:

- Frequent urination (in children, a recurrence of bed-wetting after toilet training has been completed)
- Unusual thirst, especially for sweet, cold drinks
- Extreme hunger
- Sudden, sometimes dramatic, weight loss
- Weakness
- Extreme fatigue
- Blurred vision or other changes in eyesight
- Irritability
- Nausea and vomiting (acute symptoms)

Children with type 1 diabetes may also be restless, apathetic and have trouble functioning at school. In severe cases, diabetic coma may be the first sign of type 1 diabetes.

## Complications

Type 1 diabetes reduces the normal lifespan by about 5-8 Years. However, survival rates are improving in all ethnic groups and both genders. Longer survival rates are probably due to improvements in monitoring and tighter control of blood glucose. There are two important approaches to preventing complications from type 1 diabetes:

1. Intensive control of blood glucose and keeping glycosylated hemoglobin (HbA1c) levels below 7%. This approach can help prevent complications due to vascular (blood vessel) abnormalities and nerve damage (neuropathy) that can cause major damage to organ, including the eyes, kidneys and heart.
2. Managing risk factors for heart disease. Blood glucose control helps the heart, but it is also very important that people with diabetes control blood pressure, cholesterol levels and other factors associated with heart disease.

Type 1 diabetes can affect many major organs in your body, including heart, blood vessels, nerves, eyes and kidneys. Keeping the blood sugar level close to normal most of the time can dramatically reduce the risk of many complications.

### Heart and blood vessel disease.

Diabetes dramatically increases the risk of various cardiovascular problems, including coronary artery disease with chest pain (angina), heart attack, stroke, narrowing of the arteries (atherosclerosis) and high blood pressure. In fact, about 65 percent of people who have type 1 or type 2 diabetes die of some type of heart or blood vessel disease, according to the American Heart Association.

### Nerve damage (neuropathy).

Excess sugar can injure the walls of the tiny blood vessels (capillaries) that nourish the nerves, especially in the legs. This can cause tingling, numbness, burning or pain that usually begins at the tips of the toes or fingers and gradually spreads upward. Poorly controlled blood sugar could cause you to eventually lose all sense of feeling in the affected limbs. Damage to the nerves that affect the gastrointestinal tract can cause problems with nausea, vomiting, diarrhea or constipation. For men, erectile dysfunction may be an issue.

**Kidney damage (nephropathy).**

The kidneys contain millions of tiny blood vessel clusters that filter waste from the blood. Diabetes can damage this delicate filtering system. Severe damage can lead to kidney failure or irreversible end-stage kidney disease, requiring dialysis or a kidney transplant.

**Eye damage.**

Diabetes can damage the blood vessels of the retina (diabetic retinopathy), potentially leading to blindness. Diabetes also increases the risk of other serious vision conditions, such as cataracts and glaucoma.

**Foot damage.**

Nerve damage in the feet or poor blood flow to the feet increases the risk of various foot complications. Left untreated, cuts and blisters can become serious infections. Severe damage might require toe, foot or even leg amputation.

**Skin and mouth conditions.**

Diabetes may leave more susceptible to skin problems, including bacterial and fungal infections. Gum infections also may be a concern, especially if having a history of poor dental hygiene.

**Osteoporosis.**

Diabetes may lead to lower than normal bone mineral density, increasing the risk of osteoporosis.

**Pregnancy complications.**

High blood sugar levels can be dangerous for both the mother and the baby. The risk of miscarriage, stillbirth and birth defects are increased when diabetes isn't well controlled. For the mother, diabetes increases the risk of diabetic ketoacidosis, diabetic eye problems (retinopathy), pregnancy-induced high blood pressure and preeclampsia.

**Hearing problems.**

Hearing impairments occur more often in people with diabetes.

## Diagnosis

### Fasting Plasma Glucose Test

The fasting plasma glucose (FPG) test is the standard test for diagnosing diabetes. It is a simple blood test taken after 8 hours of fasting. FPG levels indicate conditions by blood glucose level:

- Normal.  $\leq 100$  mg/dL (or 5.5 mmol/L) or below.
- Pre-diabetes. (a risk factor for type 2 diabetes): Between 100-125 mg/dL (5.5-7.0 mmol/L)
- Diabetes.  $\geq 126$  mg/dL (7.0 mmol/dL) or higher.

### Glucose Tolerance Test

The oral glucose tolerance test (OGTT) is more complex than the FPG and may over diagnose diabetes in people who do not have it. Some doctor recommend it as a follow-up after FPG, if the latter test results are normal but the patient has symptoms or risk factors of diabetes. The test uses the following procedures that it first uses an FPG test and a blood test is then taken 2 hours later after drinking a special glucose solution. OGTT levels indicate conditions by blood glucose level:

- Normal.  $\leq 140$  mg/dL or below.
- Pre-diabetes. Between 140-199 mg/dL.
- Diabetes.  $\geq 200$  mg/dL or higher.
- People who have the FPG and OGTT tests must not eat for at least 8 hours prior to the test.

### Test for Glycosylate Hemoglobin

This test examines blood levels of glycosylate hemoglobin which is also known as hemoglobin A1c (HbA1c). The test is not affected by recent food intake so it can be taken anytime. In general, most adults with diabetes should aim for Hb1c level below 7%. Higher level indicate poor blood glucose control.

## Principle managements of diabetes patients

### 1. Non-pharmacological management for both type 1 and type 2 diabetes

#### 1.1 Diet

1.1.1 Diet should ideally contain no refined sugar and no diabetic foods.

1.1.2 Small frequent meals are better.

1.1.3 Calories, fat and salt intake should be limited.

1.1.4 Alcohol use should be moderated.

#### 1.2 Exercise

1.2.1 Exercise improves blood glucose control by improving insulin sensitivity

1.2.2 It can also reduce weight, blood pressure, dyslipidemic and the risk of cardiovascular disease.

### 2. Phamacological treatment

#### 2.1 For type 1 diabetes

Insulin is essential for strict control of blood glucose level in type 1 diabetes. Tight blood glucose control is the best way to prevent major complications in type 1 diabetes, including those that affect the kidneys, eyes, nerve pathways and blood vessels. Insulin treatment replaces or supplements the body's own insulin, restoring normal or near-normal blood sugar levels. Many different types of insulin treatment can successfully control blood sugar levels; the best option depends upon a variety of individual factors.

#### 2.2 For type 2 diabetes

2.2.1 Metformin is the drug of preference in the presence of obesity and insulin resistance. It reduces vascular risk in patients with diabetes. The mechanism of action is unclear, but it decreases gluconeogenesis and hence, hepatic glucose output and reduces fasting blood sugar. It may also increase insulin-stimulated glucose uptake in skeletal muscle.

2.2.2 Sulphonylureas are effective where there is insulin secretory deficiency rather than insulin resistance. They decrease both fasting blood glucose and postprandial hyperglycemia. Not all patients respond to sulphonylurea, and they should be



used with care in those with renal or liver disease and in the elderly, who are more susceptible to hypoglycemia.

2.2.3 Meglitinides act similarly to the sulphonylureas, increasing insulin secretion but their action is more rapid in onset and less sustained. They can be taken shortly before each meal, significantly decreasing postprandial hyperglycemia, but there is a risk of late postprandial hypoglycemia. Meglitinides are valuable in patients who are allergic or sensitive to sulphonylureas.

2.2.4 Thiazolidinediones (glitazones) increase insulin sensitivity in the peripheral tissues and are effective in improving glycemic control in patients with insulin resistance. They should be used cautiously in patients using insulin. They can cause in weight gain and fluid retention, even precipitating cardiac failure.

2.2.5 Alpha-glucosidase inhibitors reduce postprandial hyperglycemia by decreasing the rate of digestion of complex carbohydrates in the small intestine. They are of potential value in limiting progression from impaired glucose tolerance to diabetes.

### **Liver disorder in diabetes mellitus**

The liver helps maintain the regulation of carbohydrate homeostasis for normal blood glucose concentration and maintains in the fasting and postprandial states. Loss of insulin effect on the liver lead to glycogenolysis and an increase in hepatic glucose production. The liver is an insulin sensitivity tissue. Abnormality of triglyceride storage and lipolysis are an early manifestation of conditions characterized by insulin resistance and are detectable earlier than fasting hyperglycemia. The liver uses glucose as a fuel and also has ability to store it as glycogen and synthesize it from noncarbohydrate precursors (gluconeogenesis). A total hepatectomy in a dog results in death within a few hours from hypoglycemic shock (11). In type 2 diabetes, excessive hepatic glucose output contributes to the fasting hyperglycemia. Increasing of gluconeogenesis is the predominant mechanism responsible for this increased glucose output, while glycogenolysis has not been shown to be increased in patients with type 2 diabetes (12).

Diabetes liver disease includes simple steatosis and steatohepatitis with or without steatofibrosis. Most commonly takes the form of nonalcoholic fatty liver disease (NAFLD),

that usually affects patients with type 2 diabetes (21). A less common pattern of hepatic injury reported in patients with type 1 diabetes mellitus is in the form of glycogen accumulation and elevated liver enzyme levels with or without hepatomegaly (22).

#### **Glycogen deposition**

80% of diabetic patients are seen for the excess glycogen accumulation in the liver (13). The mechanism of glycogen deposition in hepatocytes is uncertain but is perhaps related to the large variations in glucose concentration and insulin dosing. No correlation between hepatic glycogen content and fasting blood glucose levels has been demonstrated. There is also no demonstrable association between the type of diabetes or the fat content of hepatocytes and the presence of glycogen. Patients showing solely excessive glycogen deposition may exhibit hepatomegaly and liver enzyme abnormalities and may have abdominal pain, nausea, vomiting and rarely ascites (14).

#### **Fatty liver, Steatohepatitis**

Hepatic fat accumulation is a well-recognized complication of diabetes with a reported frequency of 40-70%. Fat is stored in the form of triglyceride and may be a manifestation of increased fat transport to the liver, enhanced hepatic fat synthesis and decrease oxidation or removal of fat from the liver. The steatosis may be microvesicular or macrovesicular and may progress to fibrosis and cirrhosis. The degree of glycemic control does not correlate with the presence or absence of fat (15-16). Nonalcoholic steatohepatitis should be considered as a cause for chronically elevated liver enzymes in asymptomatic diabetes patients particularly if they are obese and hyperlipidemia (17). In type 2 diabetes patients with or without obesity, up to 30% have fat with inflammation, 25% have associated fibrosis and 1-8% have cirrhosis (18-19).

#### **Cirrhosis**

There is an increased incidence of cirrhosis in diabetes patients, at least 80% of patients with cirrhosis have glucose tolerance (20). The reported prevalence of cirrhosis in diabetes very widely. Diabetes increase the risk of steatohepatitis, which can progress to cirrhosis. Retrospective studies have shown that the diabetes increases the risk of complications of cirrhosis and reduces survival (29-30).

## **Laboratory tests for liver disease**

### **Liver function test**

Liver function test (LFTs) are commonly used in clinical practice to screen for liver disease. A number of LFT are available to test the proper function of liver. These tests detect for the presence of enzymes in blood that are normally most abundant in liver tissue, metabolites products that include the serum aminotransferases, alkaline phosphatase, bilirubin, albumin and prothrombin time.

### **Aminotransferases**

The aminotransferases such as alanine aminotransferase (ALT) or glutamate pyruvate transaminase (SGPT) and aspartate aminotransferase (AST) or glutamate oxaloacetate transaminase (SGOT) are both enzymes that are mainly found in the liver. If liver damage is present, the enzymes are released into the blood stream after hepatocytes injury. Abnormality of high concentrations of ALT and AST in the blood may indicate the damage of liver.

### **Alkaline phosphatase**

Alkaline phosphatase is usually found in the bile ducts. It is an enzyme that can become elevated when the bile ducts become blocked. Blockage occurs because liver disease interferes with the liver ability to maintain bile flow.

### **Bilirubin**

Bilirubin is the product of recycled red blood cells. Its levels may be elevated due to liver disease. The liver processes bilirubin in a series of complex steps. The bilirubin levels become elevated when the liver is no longer able to sustain the process.

### **Ammonia**

Ammonia is a toxin formed from the digestion of proteins. The liver normally remove the toxin but ammonia levels can become elevated when hepatic disease is present. High ammonia levels can have a severe effect to the brain in a condition known as hepatic encephalopathy.

### **Blood clotting times**

Liver disease can lead to prolong clotting time and bleeding disorder because it is responsible for synthesis of numerous blood clotting factors.

### **Gamma-Glutamyl Transferase**

Gamma-Glutamyl Transferase is commonly found in the liver. It is sensitive to hepatic changes and can become elevated in the presence of liver disease.

### **Albumin**

Albumin is the main protein that is produced by the liver. The levels of albumin may significantly drop when the liver function declines.

## **Pathophysiology of liver disease**

The pathophysiology of all forms of liver diseases represents failures of the numerous and complex hepatic metabolic functions. Although there is some variability in basic pathophysiology from one type of liver disease to another, all forms of liver diseases can reasonably be called hepatic failure. The signs and symptoms, the natural history, and the rationale of treatments for all forms of liver disease derive from certain relatively simple basic pathophysiologic concepts. The liver plays several complex roles in carbohydrate metabolism, amino acid metabolism, protein synthesis, and lipid metabolism. It is also the site of manufacture of a number of blood coagulation proteins. The basic derangements of hepatic failure will be discussed firstly. These will then be applied to the most common forms of liver disease, acute hepatitis and cirrhosis.

### **Defects in Carbohydrate Metabolism**

The hepatocyte actively stores glucose by converting it to the long chain starch, glycogen. Glycogen can then be later broken down to release glucose into the general circulation. The factors that control insulin, epinephrine, growth hormone, glucagon and the thyroid hormones tend to counterbalance each other so that the hepatocytes store glycogen as the blood sugar rises whereas break it back down into glucose as blood sugar level falls. Again, this is a critical function, an impairment of which produces some of the more serious manifestations of liver disease hyperglycemia and hypoglycemia. In fact, patients with severe liver disease often have glucose tolerance curves very much like those seen in diabetes mellitus. That is to say, with food ingestion, they tend to become hyperglycemic because the hepatocytes cannot store glycogen. As dietary intake is decreased or absent, the hepatocytes are not well able to mobilize glucose from what little stored glycogen, so

these patients have episodes of hypoglycemia. This phenomenon is sometimes called "hepatic diabetes."

#### **Defects in Amino Acid Metabolism**

The liver is the most important single organ involved in amino acid metabolism. Amino acids are delivered to the liver either from the gut or from the general circulation. In the liver, there are two major processes in the metabolism of amino acids: oxydative deamination and transamination. Amino acid metabolism is controlled primarily by the hepatocyte, one of the three types of cells in the liver. In widespread, advanced hepatic parenchymal disease, or in severe acute hepatic failure. This particular hepatocyte failure results in a severe derangement of amino acid metabolism and thus of ammonia and urea levels.

#### **Defects in Protein Synthesis**

Two of the liver's cell types, the hepatocyte and the Kupffer cell, are responsible for synthesizing many kinds of proteins. The hepatocyte synthesizes albumin and some immune globulins. The Kupffer cells, which line the hepatic sinusoids, and which are a part of the so called reticuloendothelial system, synthesize several kinds of immune globulins. Since the functions of both of these kinds of cells can be impaired in either chronic diffuse liver disease or in severe acute liver disease, a diminution of the levels of circulating albumin and of the immune globulins often appears in liver disease. Since impaired amino acid metabolism may also be found in liver disease, protein synthesis may be doubly impaired because of a decreased availability of amino acids and because of direct impairment of the synthetic processes themselves in the hepatocyte and in the Kupffer cell.

#### **Defects in Lipid Metabolism**

The liver has an extremely complex role in lipid metabolism. In the diseased liver, there are two prime manifestations of liver failure with regard to lipid metabolism. The first of these is the deposition of triglycerides within the organ itself. This is the basic mechanism of so called "fatty liver," which develops most often as a result of chronic alcoholism. The second prime feature of disordered hepatic lipid metabolism is a diminution in the rate of synthesis of cholesterol. In fact, a decrease below the normal level of serum cholesterol is often found in advanced diffuse liver disease or in severe acute liver disease.

The mechanisms by which diabetes might cause NAFLD are complex and have been studied in a fragmented manner mainly in isolated biological systems. It has been observed that the fatty liver, obesity and insulin resistance act as co-factors to cause liver damage (27, 28). Fatty liver is the result of an intracellular accumulation of triglycerides because of increased uptake of free fatty acids and *de novo* liponeogenesis in the hepatocytes. At the same time, there is a reduction in the hepatic secretion of very low density lipoproteins. The liver damage consists of cellular necrosis and inflammation, and these disorders result from an increase in mitochondrial oxidative stress on triglycerides with the consequential generation of free radicals and peroxisomes (23). The mitochondrial oxidative stress is increased also by the action of adipokines (cytokines produced by the adipocytes) such as leptin and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), which are produced in excess (24). The reduction of adiponectin, which is a regulatory adipokine, favors the activity of inflammatory adipokines (25). These chemical mediators, derived from inflammation and cell necrosis, as well as the adipokines activate the liver stellate cells and induce them to increase production of collagen, connective tissue growth factor and accumulation of extracellular matrix, in turn favoring fibrosis (26).

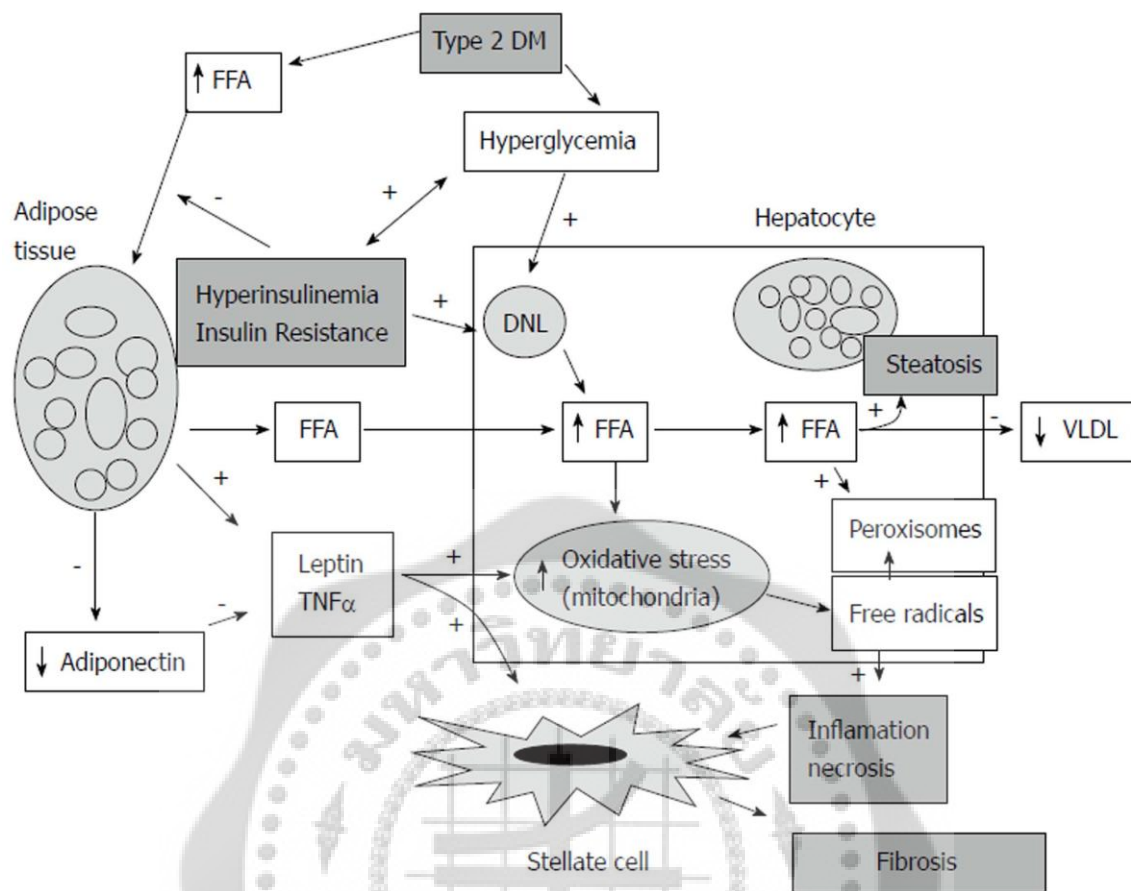


Figure 1 Liver damage caused by type 2 DM. Insulin resistance promotes release of free fatty acids (FFA) from adipose tissue. The FFAs are accumulated in the liver cells, and *de novo* liponeogenesis (DNL) contributes also. The reduced secretion of very low density lipoprotein (VLDL) by hepatic cells saturates hepatocytes producing steatosis. Mitochondrial oxidative stress is increased as a result of excess intracellular FFAs and the influence of adipokines (leptin and tumor necrosis factor alpha (TNF- $\alpha$ )). Excess of oxidative stress produces free radicals which in turn induces inflammation and cellular necrosis. Tissue inflammation stimulates the stellate cells to produce collagen (31).

(Diego GC, Joel OQ, Jose AG, Hector MG Liver cirrhosis and diabetes: Risk factors, pathophysiology, clinical implications and management. World J Gastroenterol 2009; 15(3): 281.)

## Angiogenesis in liver disease

Angiogenesis is the process of formation of new blood vessels from preexisting vessels, occurs in several organs during pathophysiological situations (38). Most chronic liver disease are characterized by fibrosis and inflammation. Moreover, an excessive amount of extracellular matrix is synthesized and accumulated during the fibrogenic process. Fibrotic tissue offers resistance to blood flow and to the delivery of oxygen, thus becoming hypoxic. Stimulation of hypoxia-inducible factors leads to an angiogenic switch to the upregulation of proangiogenic factors, and to formation of neovessels (55). However, mechanical stimulation of angiogenesis is not well characterized. There is a significant amount controversy with regard to shear stress acting on capillaries to cause angiogenesis. The mechanical of shear stress is considered the major stimulator of angiogenesis (33). Various different chemical stimulants have been identified, characterized and worked upon that they stimulate the process of angiogenesis. Many factors have been reported as inducing angiogenesis, such as hypoxia, inflammation and coagulation (35). Hypoxia and inflammation are the main stimuli for angiogenesis. Hypoxia promotes the angiogenesis by signaling through hypoxia inducible transcription factors (36). Vascular permeability is increased during inflammation. Moreover the monocytes, macrophages, platelets, mast cells and other leucocytes are recruited under the attraction of chemokines and able to produce angiogenesis cytokines and growth factors (37).

Regarding to cellular activity, most molecular mechanisms are common to the liver and other organs. The different mechanism might arise from the existence of two types of microvascular structures in the liver: 1) The production of unique proangiogenic factors and 2) The hepatic stellate cells. The existence of two types of microvascular structures in the liver such as hepatic arterioles, central and portal venules, which are lined by continuous endothelial cells and sinusoids which are lined by sinusoidal endothelial cells that are discontinuous and present fenestration. The production of unique proangiogenic factors, such as AN-GPTL3, the protein encoded by this gene is a member of the angiopoietin-like family of secreted factors. It is predominantly expressed in the liver, and has the characteristic structural of angiopoietins, consisting of a signal peptide, N-terminal coiled-coil domain and the C-terminal fibrinogen (FBN)-like domain (41). The hepatic stellate cells,



considered liver-resident pericytes, might contribute to angiogenesis through mechanisms different from those attribute to microcapillary pericytes. During liver regeneration, physiological hepatic angiogenesis might be leading the formation of new functional sinusoids, whereas pathological angiogenesis occurs in fibrosis and is characterized by the appearance of capillarized vascular structures (40). Angiogenesis occurs in almost all organs and tissues. It is a critical step in either physiological conditions or in tissue repair and growth in several pathophysiological conditions (38, 39), including chronic liver disease (CLD). Hepatocytes exposed to hypoxia during CLD progression are reasonably a source of vasoactive mediators as well as proangiogenic cytokine VEGF (40).

Vascular endothelial growth factor (VEGF, VEGF-A) has been demonstrated to be a major contributor to angiogenesis, increasing the number of capillaries in a given network. VEGF is biologically active as a homodimer of approximately 40 KDa, belonging to a family of secrete glycoproteins, VEGF-B, -C, -D, and placenta growth factor (PlGF). VEGF is a multifunctional protein that binds to two tyrosine kinase receptors: VEGFR1/Flt1 and VEGFR2/ KDR. VEGF was first described as a tumor derived factor with potent ability to induce endothelial cells permeability (42), proliferation and angiogenesis (43). In vitro studies clearly demonstrate that VEGF is a potent stimulator of angiogenesis because in the presence of this growth factor, plated endothelial cells will proliferate and migrate (34). Endothelial budding is facilitated by vasodilation and loosening of endothelial cells contacts. The preexisting vessels will have leakiness, which allow extravascular plasma protein and extracellular matrix component migrate out from endothelial cells. Moreover, nitric oxide (NO) is the main factor responsible for vasodilation (44), whereas VEGF increases vascular permeability. The basement membrane and extracellular matrix must be degraded to allow subsequent endothelial cells migration and proliferation. Next, the proteinases and plasminogen activator are performed. The proteolysis of extracellular matrix leads to the exposure of cryptic epitopes and release of extracellular matrix-embended factors that promote endothelial cells proliferation and migration (45).

According the angiogenesis in liver inflammation, endothelial cell proliferation is usually occurred. Endothelial cells proliferation response to growth factors secreted by endothelial cells or surrounding cells including hepatic stellate cells, leucocytes,

hepatocytes, and Kupffer cells. Endothelial proliferation may be also stimulated by other growth factors, such as acidic and basic fibroblast growth factor (aFGF and bFGf), Hepatocyte growth factor (HGF), and transforming growth factor (TGF) (51, 52). Moreover, Angiopoitin 1 (Ang-1) stabilizes neovessels by binding to the Tie-2 receptor, thereby affecting junctional molecules and facilitating communication between endothelial cells. However, an excess of Ang-1 makes vessels too tight and inhibits sprouting of blood vessels. In the absence of VEGF, Angiopoitin-2 (Ang-2) acts as an antagonist of Ang-1, destabilizes vessels and causes endothelial cells death. Blood vessels become regression and then they facilitate sprouting in the presence of VEGF (53).

Concerning acute liver damage or partial hepatectomy in rodent, hepatocytes proliferation starts with maximum activity in periportal areas. The reconstruction of sinusoids has begun by endothelial cells from these area (46). In periportal hepatocytes, VEGF is upregulated after partial hepatectomy (47) and VEGF involvement in liver regeneration associated with angiogenesis has been shown by investigated angiogenic factor in Hepatic cell carcinoma. Its expression increases gradually from low-grade dysplastic nodules to high-grade dysplastic nodules to early Hepatic cell carcinoma (48). The expression of VEGF and HGF are increase during chronic viral hepatitis, may contribute to enhance vascular permeability (49). Hepatic VEGF messenger RNA and protein levels are increased in chronic hepatitis C patients, suggesting a potential role in the induction of endothelial proliferation in this disease (49,50). Interestingly, treatment of Chronic hepatitis C patients with pegylated interferon plus ribavirin significant reduce VEGF and Ang-2 levels with respect to pretreatment values. This therapy can decrease VEGF levels that are significantly correlated with that in ALT and alkaline phosphatase serum levels of the patients. A similar correlation is found between the decrease in the serum levels of Ang-2 and ALT. These significant relationships suggest some degree of association between angiogenesis and the extent of liver damage (54,55).

## Liver function

The various functions of the liver are carried out by the hepatocytes. The liver regulates chemical levels in the blood and excretes a product called bile. The bile helps to break down fats, preparing for further digestion and absorption. All of the blood leaving the stomach and intestines passes through the liver. The liver processes this blood and breaks down the nutrients and drugs in the blood into forms that are easier to use for the rest of the body. The major liver functions include the following:

- A large part of amino acid synthesis.
- Performing several roles in carbohydrate metabolism:
  - Gluconeogenesis (the synthesis of glucose from certain amino acid, lactate or glycerol)
  - Glycogenolysis (the breakdown of glycogen into glucose)
  - Glycogenesis (the formation of glycogen from glucose)
- Mainstay of protein metabolism, synthesis as well as degradation.
- Performing several roles in lipid metabolism
- Cholesterol synthesis
- Lipogenesis, the production of triglycerides (fats).
- Producing coagulation factors I (fibrinogen), II (prothrombin), V, VII, IX, X and XI as well as protein C, protein S and antithrombin.
- Producing and excreting bile required for emulsifying fats. Some of the bile drains directly into the duodenum and some is stored in the gall bladder.
- Producing insulin-like growth factor 1 (IGF-1), a polypeptide protein hormone that plays an important role in childhood growth and continues to have anabolic effects in adults.
- In the first trimester fetus, the liver is the main site of red blood cells production. By the 32<sup>nd</sup> week of gestation, the bone marrow has almost completely taken over that task.
- Producing thrombopoietin that is a glycoprotein hormone concerning childhood growth and continues to have anabolic effects in adults.
- Breaking down insulin and other hormones.

- Liver glucoronidate bilirubin, facilitating its excretion into bile.
- Breaking down or modification toxic substances. (e.g., methylation) and most medicinal product in a process called drug metabolism.
- Converting ammonia to urea.

### Basic organization of the liver

The liver is a reddish brown organ that is located in the abdominal cavity, resting just below the diaphragm. The liver lies to the right of the stomach and overlies the gallbladder (Fig. 2). It is the organ in which nutrient absorbed in the digestive tract are possessed and stored for used by other parts of the body. It is connected to two large blood vessels, one called the hepatic artery and one called the portal vein. Most of its blood (80%) comes from portal vein and the smaller percentage is supplied by the hepatic artery. All the materials absorbed via the intestines reach the liver through the portal vein, except the complex lipids (chylomicrons), which are transported mainly by lymph vessels.

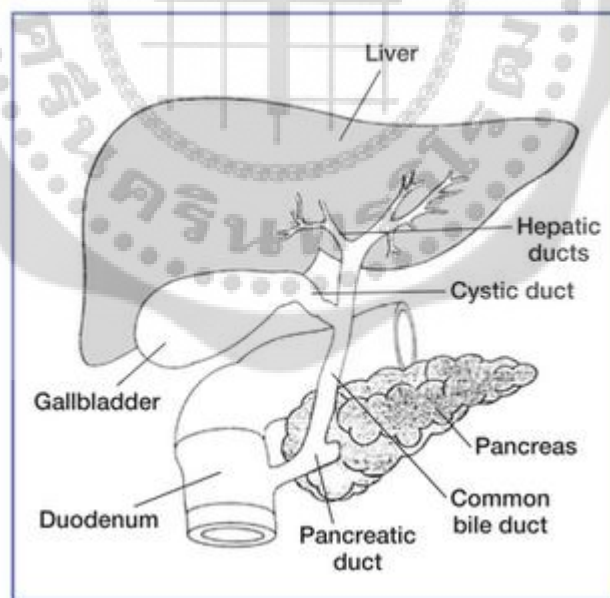


Figure 2 Normal liver and the biliary system (150)

(URL:[http://curezone.com/cleanse/liver/huldass\\_recipe.asp](http://curezone.com/cleanse/liver/huldass_recipe.asp))

### The liver lobule

The basic structural component of the liver is the liver cells, or hepatocyte. These epithelial cells are grouped in interconnected plates. In light microscope (LM) sections, structural units called liver lobules can be seen (Fig. 3).

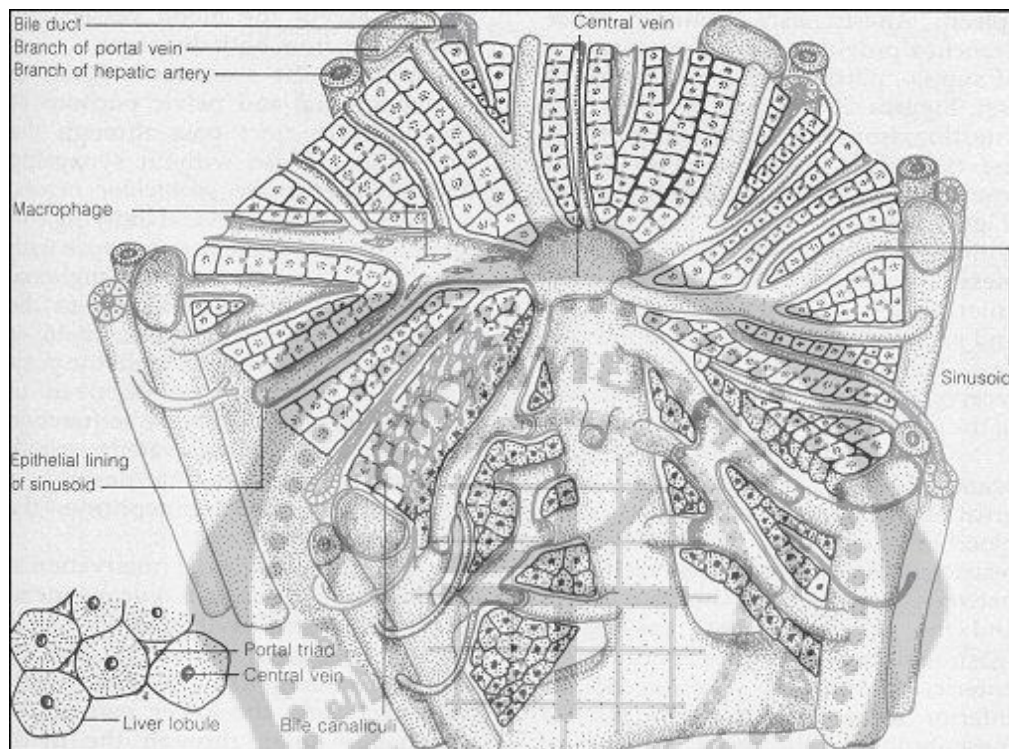


Figure 3 Liver lobule (151)

([URL:http://www.ener-chi.com/liver.htm](http://www.ener-chi.com/liver.htm))

The liver lobule is formed of a polygonal mass of tissues. In certain animals, the lobules are separated from each other by a layer of connective tissue. The peripheral regions of lobules are demarcated by connective tissues containing bile ducts, lymphatics, nerves and blood vessels. These regions, the portal triad (portal spaces) (Fig. 4) are present at a corners of the lobules. The portal triad contains a venule (a branch of portal vein), an arteriole (a branch of hepatic artery), a duct (part of the bile duct system) and lymphatic vessels.

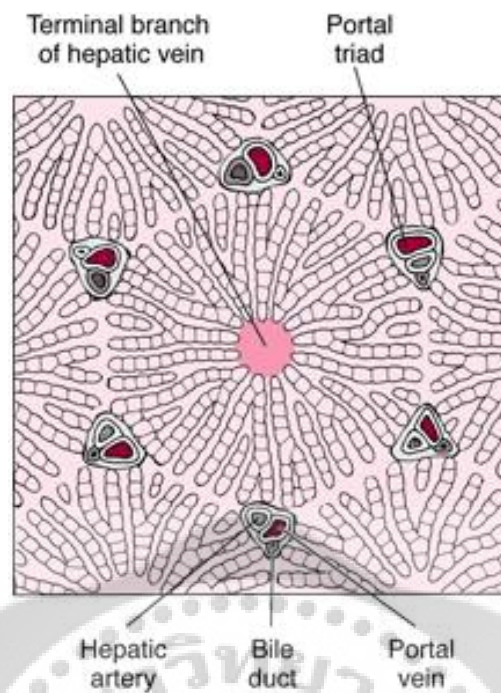


Figure 4 Portal triad (152)

(URL:<http://www.merckmanuals.com/professional/print/sec03/ch023/ch023a.html>)

### The Hepatocytes

Hepatocytes are polyhedral, with six or more surfaces, and have a diameter of 20-30  $\mu\text{m}$ . In LM section stained with Hematoxylin and Eosin, the cytoplasm is eosinophilic, mainly because of the large number of mitochondria and some smooth endoplasmic reticulum. The hepatocytes in the lobule are radially disposed and are arranged like the brick of a wall. These cellular plates are directed from the periphery of the lobule to its center and anastomose freely, forming a labyrinthine and spongelike structure. (Fig. 5). The space between these plates contains capillaries and, the liver sinusoids. Sinusoidal capillaries are irregularly dilated vessels composed solely of a discontinuous layer of fenestrated endothelial cells.



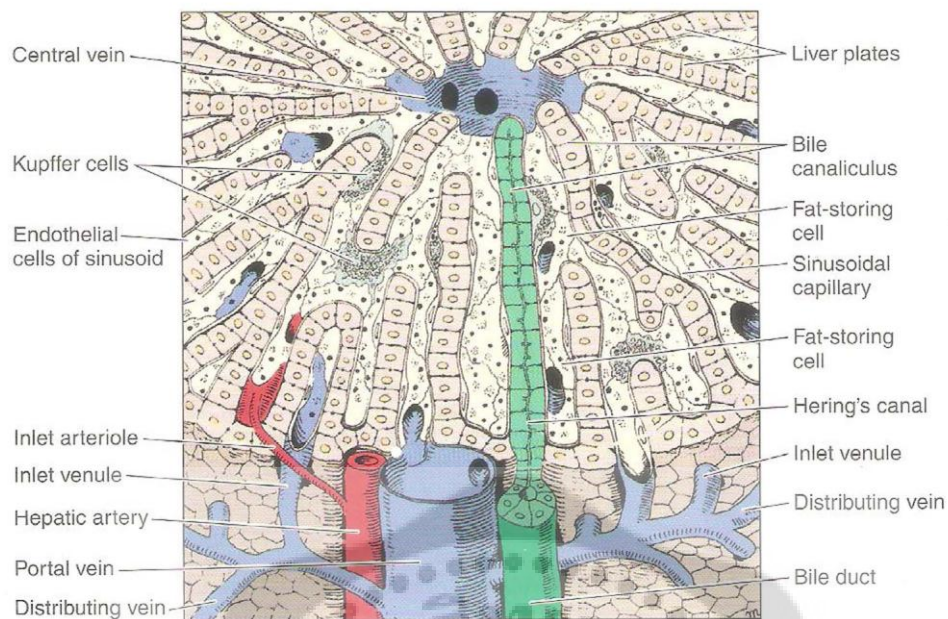


Figure 5 Organization of the liver (153)

(URL:<http://anatomytopics.wordpress.com/tag/lesser-sac/>)

The endothelial cells are separated from the underlying hepatocytes by a discontinuous basal lamina and a subendothelial space known as the space of Disse, which contains microvilli of the hepatocyte. Blood fluids readily percolate through the endothelial wall and make intimate contact with the surface of the hepatocytes, permitting an exchange of macromolecules from the sinusoidal lumen to the hepatocytes and vice versa. This exchanges is physiologically important not only because of the large number of macromolecules (lipoproteins, albumin, fibrinogen) secreted into the blood by hepatocyte but also because the liver takes up the catabolizes many of these large molecules.

#### **Kupffer cells**

Kupffer cells are macrophages, which are found on the luminal surface of the endothelial cells. Their main functions are to metabolize aged erythrocytes, digest hemoglobin, secrete proteins related to immunologic processes and destroy bacteria that eventually enter the portal blood through the large intestine. Kupffer cells are account for 15% of the liver cells population. Most of them are located in the peripheral region of the liver lobule, where they are very active in phagocytosis.

## Blood supply of liver

The liver receives blood supply from two sources, 80% of the blood derives from the portal vein, which carries oxygen-poor and nutrient-rich blood from the abdominal viscera and 20% derives from the hepatic artery, which supplies only oxygen-rich blood.

### Portal vein system

The portal vein is normally formed posterior to the union of the splenic vein and superior mesenteric vein, branches repeatedly and sends small portal venules to the portal spaces. The portal venules branch into the distributing veins that run around the periphery of the lobule. From the distributing veins, small venules empty into the sinusoids. The sinusoids run radially and converge in the center of the lobule to form the centrilobular vein. It receives more sinusoids and gradually increases in diameter and leaves the lobule with the sublobular vein. The sublobular veins converge and fuse to form the hepatic veins that empty into inferior vena cava.

### Arterial system

Arterial blood reaches the liver through the hepatic artery, normally a branch of the celiac artery. The hepatic artery branches to form the interlobular arteries. The interlobular arteries irrigate the structures of portal canals and others end directly in the sinusoids, the space that provide a mixture of arterial and portal venous blood together.

## Curcumin

Turmeric or *Curcuma longa* is a tropical plant native to southern and southeastern tropical Asia. A perennial herb belongs to the ginger family. The parts, which are commonly used are the rhizomes. The most active component in turmeric is curcumin, which is a diferuloylmethane presenting in extracts of the plant. The structure of curcumin ( $C_{12}H_{20}O_6$ ) was first isolated in 1815 by Vogel and Pellatier and its chemical structure was determined in 1973 (56). It has a melting point at 176-177°C; forms a reddish-brown salt with alkali and is soluble in ethanol, alkali, ketone, acetic acid and chloroform.

An active component of turmeric (*Curcuma longa*) is used as a spice and as an Ayurvedic medicine for centuries on the Indian subcontinent. The pure orange-yellow,



crystalline powder is insoluble in water. Turmeric was described its taxonomic position is as follow:

|          |               |
|----------|---------------|
| Class    | Liliopsida    |
| Subclass | Commelinids   |
| Order    | Zingiberales  |
| Family   | Zingiberaceae |
| Genus    | Curcuma       |
| Species  | Curcuma longa |

. Turmeric contains three different analogues of curcumin (diferuloylmethane, also called curcumin, demethoxycurcumin, and bisdemethoxycurcumin). Whether all three analogues exhibit equal activity is not clear. Although in most systems, curcumin is found to be most potent (57) but bisdemethoxycurcumin is found to exhibit higher activity in some systems (58). There are also suggestions that the mixture of all three is more potent than either one alone (59). When administered orally, curcumin is metabolized into curcumin glucuronide and curcumin sulfonate (60). However, concerning systemically or intraperitoneal administration, it is metabolized into tetrahydrocurcumin, hexhydrocurcumin, and hexhydrocurcuminol. Tetrahydrocurcumin has been shown to be active in some systems (61-63) and not in others (64-65). Whether other metabolites of curcumin exhibit biological activity is not known.

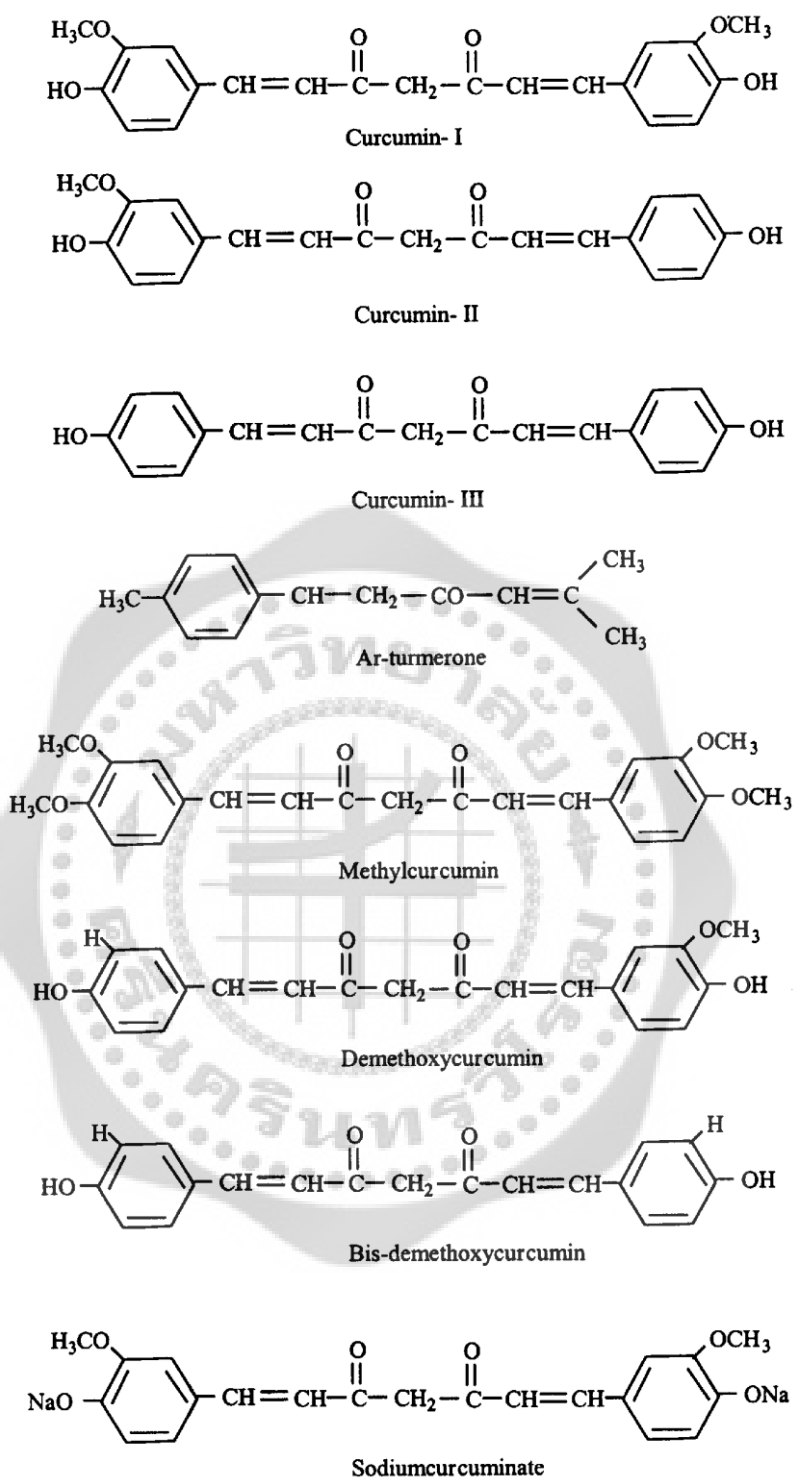


Figure 6 Chemical structures of curcumin and its analogues. (154)

(Chattopadhyay I, Biswas K, Bandyopadhyay U, Banerjee RK. Turmeric and curcumin: Biological actions and medicinal applications. Current Science 2004; 87 (1): 45)

The use of turmeric for health purposes is not formally reported. As a folklore medicine, its use has been documented in both Indian and Chinese cultures. The long list of uses include antiseptic, analgesic, anti-inflammatory, antioxidant, antimalarial, insectrepellant, and other activities associated to turmeric (66). Perhaps one of the most often prescribed uses is for wound-healing (67). This activity is well known to people from the Indian subcontinent area. Modern research has provided considerable evidences, and the mechanism by which curcumin could accelerate wound-healing has been described (68-72). Turmeric has been traditionally described as an anti-inflammatory agent. Recent scientific evidence has indeed demonstrated that turmeric and curcumin in particular, exhibit potent anti-inflammatory activities as determined by a wide variety of systems (73-76). Therefore, it is not too surprising that turmeric displays activities against a variety of diseases. Though curcumin also exhibits potent antioxidant activity, whether the anti-inflammatory activity of curcumin is mediated through its antioxidant mechanism is not clear. Since most well-characterized antioxidants do not exhibit antiinflammatory activity, it is unlikely that the anti-inflammatory activity of curcumin is due to its antioxidant activity (77-78).

### Effects of Curcumin

The effects of curcumin have revealed that curcumin can modulate several different transcriptional factors (79-85), cytokines (86-90), growth factors (91-93), kinases (94-97), and other enzymes (98-100). Although most diseases are caused by dysregulated inflammation, the strategy to find a safe and efficacious anti-inflammatory agent is a real challenge in modern medicine. Steroids are perhaps the best known anti-inflammatory agents, however, there are numerous side effects associated with them. In addition to steroids, numerous nonsteroidal antiinflammatory drugs (NSAIDs) have been discovered within the last century, and these include salicylates, ibuprofen, sulindac, phenylbutazone, naproxen, diclofenac, indomethacin, and coxibs (101). Experience over the years has indicated that most of these NSAIDs are associated with a constellation of side effects. Perhaps the best example is the cardiovascular system-related side effects which is recently identified with most coxibs (102-103). Although the intake of such anti-inflammatory

agents can be justified for chronic conditions, they are not appropriate as chemopreventive agents under normal conditions because the purpose requires long periods of time. Thus, there is a great need to discover safe and efficacious anti-inflammatory agents. Numerous lines of evidence suggest that curcumin is a potent anti-inflammatory agent. First, curcumin suppresses the activation of the transcription factor nuclear factor kappa B (NF- $\kappa$ B), which regulates the expression of pro-inflammatory gene products (104-107). Second, curcumin downregulates the expression of COX-2, an enzyme linked with most types of inflammations (107-109). Third, curcumin inhibits the expression of another pro-inflammatory enzyme: 5-LOX.<sup>177</sup> Additionally, curcumin has been shown to bind to the active site of 5-LOX and inhibit its activity (111). Fourth, curcumin downregulates the expression of various cell surface adhesion molecules that have been linked with inflammation (112). Fifth, curcumin downregulates the expression of various inflammatory cytokines, including TNF, IL-1, IL-6, IL-8, and chemokines (113-115). Sixth, curcumin has been shown to inhibit the action of TNF, one of the most pro-inflammatory of the cytokines (116). Seventh, curcumin is a potent antioxidant, which might contribute to its anti-inflammatory action. (63, 117-119). All of this recent evidence confirms the anti-inflammatory action of curcumin, gradually known for thousands of years. Its pharmacological safety combined with its anti-inflammatory action, makes it an ideal agent to explore for preventive and therapeutic situations. Whereas pro-oxidants are considered as mediators of numerous diseases, antioxidants are generally believed to delay or halt the disease. However, this paradigm is not always valid, as most cytokines mediate their effects through pro-oxidant mechanisms.

Reactive oxygen species (ROS) also play an important role in cell-mediated cytotoxicity (CMC) of the immune system. Numerous reports indicate that curcumin could mediate both pro-oxidant and antioxidant roles. First, curcumin could induce the expression of ROS, which plays an important role in the antiproliferative effects of this molecule (120). Second, curcumin binds thioredoxin reductase (TR) and converts this enzyme to NADPH oxidase, thus leading to the production of ROS (121). Third, curcumin suppresses lipid peroxidation (122-123). Fourth, curcumin increases the expression of intracellular glutathione (124-125). All of these combined reports suggest the ability of curcumin to modulate the redox status of the cells. Moreover, curcumin can also modulate the cellular

action of various growth factors and cytokines. First, curcumin has been shown to downregulate the effect of epidermal growth factor (EGF) through down regulation of expression and activity of EGF receptors (EGFR) (126). Second, curcumin has been shown to downregulate the activity of human EGFR-2 (called HER2/neu) (127), a growth factor receptor closely linked with cancer of the breast, lung, kidney, and prostate. Third, curcumin suppresses the action of interleukin (IL)-6 through the downregulation of STAT3 activation (128). Fourth, curcumin modulates the action of TNF, a growth factor for tumor cells (129). Fifth, curcumin negatively regulates the action of IL-2 (130), a growth factor for T cells.

Angiogenesis is a process of vascularization of the tissue, which is critical for the growth of solid tumors. Numerous molecules have been linked with angiogenesis. These include vascular endothelial growth factor (VEGF), COX-2, fibroblast growth factor (FGF), and TNF. Evidence suggests that curcumin could suppress angiogenesis (131,132). Curcumin includes its ability to downregulate the expression of VEGF. Likewise, it downregulates FGF-mediated angiogenesis. Curcumin was found to negatively regulate the expression of COX-2 (133) and suppresses both the expression and action of TNF (119). Curcumin suppresses the angiogenesis of tumors through the suppression of angiogenic cytokines. The anti-inflammatory effects of curcumin contribute to its antitumor activity as well (75,76). Curcumin has also been shown to play a role in diabetes mellitus type II, in which the patient develops a resistance to insulin (134-135). Both NF- $\kappa$ B and TNF have been linked with the induction of resistance to insulin. Because curcumin can downregulate the activation of NF- $\kappa$ B and downregulate TNF expression and TNF signaling (119), it can be exploited in diabetic patients.

### **Clinical experience with curcumin**

Curcumin has enormous potential for a variety of diseases. There are, however, still several unanswered questions. First, phase I clinical trials have indicated that as high as 12 g of curcumin per day for over 3 months is well tolerated in humans (136). What the optimum dose of curcumin is for the treatment of a given disease is not clear. Serum levels of curcumin tend to be low (136), which might be responsible for its pharmacological safety, Curcumin was given to both sexes of Wistar rats, guinea pigs and monkeys at adose of 300

mg/body weight. No pathological behavioral abnormalities or lethality were observed (137) on both growth and the level of erythrocytes, leukocytes, blood constituents such as hemoglobin, total serum protein, and alkaline phosphatase (138). Human clinical trials also indicate that curcumin has no toxicity when is administered at doses of 1-8 g/day and 10 g/day (139). These data have led to the notion that curcumin has low bioavailability. Second, the tissue concentration of curcumin and how it compares to what is seen in cell culture conditions are not known. There are studies, however, that suggest that agents such as piperine (a component of black pepper) can enhance the bioavailability of curcumin through suppression of its glucuronidation occurring primarily in the liver and in the intestine (140). Third, whether there are components of turmeric other than curcumin that have beneficial effects either alone or in combination with curcumin needs to be determined. Fourth, what effect do other spices have on the pharmacology and the biology of curcumin needs to be determined. Fifth, structural analogues of curcumin that are more bioavailable and efficacious are needed, however, this might compromise the safety of curcumin.

Streptozotocin

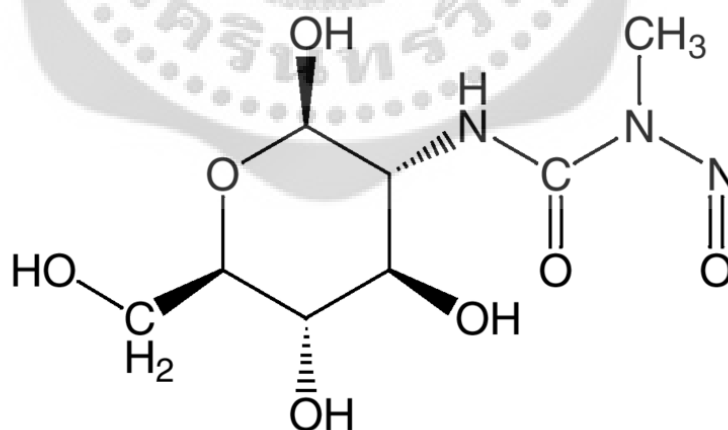


Figure 7 Structure of Streptozotocin (141)

([URL:http://common.wikimedia.org/wiki/File:Streptozotocin\\_Structure\\_NTP\\_png.](http://common.wikimedia.org/wiki/File:Streptozotocin_Structure_NTP_png.))

Streptozotocin (STZ, 2-deoxy-2(3-methyl-3-nitrosoureido)-D-glucopyranose) is a broad-spectrum antibiotic with oncolytic, oncogenic, and diabetogenic properties (142-143). The diabetogenic action is mediated by selective destruction of pancreatic beta cells. STZ has been widely utilized as a method for inducing diabetes mellitus in experimental animals and for treatment of beta cell tumors and other neoplasms in humans.

Streptozotocin is a mixture of  $\alpha$ - and  $\beta$ -stereoisomers that appear as a pale yellow or off-white crystalline powder. STZ is very soluble in water, ketones, and lower alcohols and only slightly soluble in polar organic solvents. Streptozotocin functions as a DNA synthesis inhibitor in both bacterial and mammalian cells (144). In bacterial cells, a specific interaction with cytosine moieties leads to the degradation of the bacterial DNA (145). In mammalian cells, the mechanism of action that results in cell death has not been fully identified, but is thought to be a result of DNA and chromosomal damages by mechanisms involving free radical generation during STZ metabolism (146).

Studies over the past 30 years have demonstrated that xenobiotics including STZ can cause a critical reduction in insulin secreting cells. STZ is diabetogenic because it selectively destroys the insulin producing beta cells by inducing necrosis. It is postulated that the selective beta-cell toxicity of STZ is related to the glucose moiety in its chemical structure, which enables STZ to enter the cell via the low affinity glucose transporter GLUT2 in the plasma membrane (146). It is hypothesized that the diabetogenic action of STZ in animals is mediated through a reduction of nicotinamide adenine dinucleotide (NAD) in pancreatic cells (147). The DNA damage caused by STZ mediated alkylation is repaired by an excision repair process, which requires the activation of the NAD dependent enzyme poly (ADP-ribose) synthetase (148). It is postulated that in the beta cell this enzyme is continuously activated, thus depleting the cell of NAD. The critical loss of NAD leads to a cessation of cellular function and eventually cell death (148). Cytotoxic effects of STZ are dependent upon DNA alkylation by site specific action with DNA bases and by free-radical generation during STZ metabolism (149). Types of DNA lesions are formed by STZ include monoadducts, single and double stranded breaks, and alkali-labile sites. Severe DNA damage by STZ results in cell death by apoptosis or necrosis. Diabetic individuals and experimental animals exhibit high oxidative stress

To produce diabetes, STZ is conventionally administered as a single injection. STZ is cleared from the blood stream rapidly (serum half-life, 15 min). Beta cells necrosis can be detected by electron microscope within hours after STZ injection. Elevated blood glucose levels are demonstrable within 1-2 days, and dissolution and phagocytosis of necrotic cells are observed histologically after 3 days (143).

#### **Vascular corrosion cast**

The arrangement of microvasculature has been studied by light microscopy of serial sections or tissues injected with various dyes, contrast agents and resins (155). However, light microscopy cannot reveal the three-dimensional organization with adequate resolution. Therefore, the currently used method in the routine examination of the vascular system is scanning electron microscopy (SEM) of corrosion casts, also referred to as microvascular corrosion casting/SEM method. It is a standard method, which allows three dimensional visualization with good resolution of the microvessels including fine capillaries (156). SEM of vascular corrosion casts can obtain qualitative as well as quantitative informations important to anatomists, pathologists and clinicians.



## CHAPTER 3

### MATERIALS AND METHODS

#### 3.1 Animal

Six-week-old Wistar rat (weight 200-250 g) obtained from National laboratory Animal Center of Mahidol University were used in all experiment. The procedure of the animal research was approved by Srinakarinwirot University Medical Center Animal Care Committee. Rats were maintained in room temperature environment on 12-h light/dark periods ( $20 \pm 2^{\circ}\text{C}$ ) and had access ad libitum to water and standard rat chow.

#### 3.2 Induction and assessment of diabetes

Experimental diabetic rats were induced by intravenous in lateral tail vein of streptozotocin (STZ) (Sigma, St, Louis, MO, USA) (50 mg/kg BW) dissolved in 0.9% NaCl. Control rats received injection with 0.9% NaCl alone. Blood samples were obtained in both STZ injected and control animals. Blood sugar level was measured and analyzed by one-touch glucometer. Rats with blood sugar level  $> 250$  mg/dl were used as diabetic animals.

#### 3.3 Treatment schedule

After the last STZ injection, control and diabetic rats were randomly selected and divided into three groups.

Group I = control rats (C rats)

Group II = diabetic rats were fed only corn oil diet 3 ml/kg BW by intragastric feeding (DM rats)

Group III = diabetic rats were fed corn oil diet 3 ml/kg BW containing curcumin 200 mg/kg BW by intragastric feeding (DM rats) (curcumin 99.99% pure, sigma, St, Louis, MO, USA).

All animals were clinically observed and weighted weekly. At the end of 4, 8 and 12 weeks after STZ injection or curcumin supplementation, the rats of each groups were sacrificed and blood was drawn from the heart into sample tubes for screening of liver function test. Each animals, the liver was removed, dissect and immediately fixed in Bouin's solution (Sigma, St, Louis, MO, USA) in preparation for histological study and further fixed in 4% paraformaldehyde for immunohistochemical study under light microscope. The other liver was snap-frozen in liquid nitrogen, store at  $-80^{\circ}\text{C}$  until analysis for Western blotting study. For vascular corrosion casting technique, the rats of each groups were used for studying the microvasculature of the liver by methyl methacrylate injection (Batson's no. 17) and corroded with 40% KOH solution combined with scanning electron microscopic (JEOL JSM-5400 SEM) observation.

### 3.4 Histological preparation

The livers were fixed in Bouin's solution for overnight. Then they were dehydrated in graded series of ethanol through 70, 80, 90, 95 and 100% with two changes for 1 hour each. Three changes of xylene with 30 minutes each were used as clearing reagent before filtration, embedded in paraffin, cross sectioned at 5  $\mu\text{m}$  thick and stained with hematoxylin and eosin (H&E). All sections were examined and photographed by Olympus light microscope (BX-50, Olympus, Japan).

### 3.5 Immunoperoxidase method for detection of VEGF

The livers were fixed in 4% paraformaldehyde for 24 hour. Then they were dehydrated in graded series of ethanol through 70, 80, 90, 95 and 100% two times for 1 hour each. Three changes of xylene with 30 minutes each were used as clearing reagent before filtration, embedded in paraffin and cross sectioned at 5  $\mu\text{m}$  thick. The sections were placed onto gelatin

coated slide, rehydrated in xylene and series of grade ethanols, then slides were immersed in 3% H<sub>2</sub>O<sub>2</sub> in methanol for 10 minutes to block endogenous peroxidase, following by incubation in serum blocking solution (reagent A: Zymed Laboratories, USA).

The primary antibody, monoclonal vascular endothelial growth factor (VEGF) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was added at a 1:100 dilution for 24 hours at 4 °C. Negative control sections were stained under identical condition with the buffer solution substituting for the primary antibody. After washing by phosphate buffer saline (PBS), tissue sections were incubated with biotinated secondary antibody IgG (H+L) (Reagent B: Zymed Laboratories, USA) for 45 minutes, wash continuously in PBS. Next, tissue sections were incubated with avidin-biotin complex (Reagent C: Zymed Laboratories, USA) Peroxidase conjugated were subsequently localized by 3, 3' diaminobenzidine tetrahydrochloride (DAB) (34065, Peirce, USA) as a chromogen.

The tissue sections were counter stained with hematoxylin, washed with several deionized water, dehydrated through a series of ethanol and xylene with two changes for 10 minutes each, covered with a glass cover slipped, examined and photographed by Olympus light microscope (BX-50, Olympus, Japan).

### **3.6 Sample preparation for SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis and Western blotting**

Each amount of 0.05 g snap-frozen liver from control, DM and DMC rats of 4, 8 and 12 weeks respectively, were homogenized in 975 µl PBS containing 10 µl protease inhibitor cocktail (Bio Basic Inc., Canada). After centrifugation at 12,000 rpm for 15 minutes at 4 °C, supernatant were collected and stored at -80 °C until use.

### 3.6.1 Protein assay

In order to measure protein concentration, the method of Bradford was used for determining concentration of solubilized protein. Protein samples and protein standards (bovine serum albumin or BSA) are processed in the same manner by mixing them with assay protein determination reagent (USB corporation 2000). Using a spectrophotometer (Amersham Novaspec III) to measure the absorbances at 595 nm. Comparison to a standard curve provides a relative measurement of protein concentration.

### 3.6.2 SDS-PAGE analysis

For protein separation in VEGF and actin protein in rats by sodium dodecyl sulfate polyacrylamide gel, 12% Polyacrylamide gel was suitable. Therefore the 12% separating gel solution (acrylamide solution, 1.5 M Tris-Cl pH 8.8, 10% SDS, deionized water, 10% APS and TEMED) (TABLE 3), and 4% stacking gel (acrylamide solution, 0.5 M Tris-Cl pH 6.8, deionized water, 10% APS and TEMED) (TABLE 4) were performed. ECL DualVue Western Blotting Markers (RPN810, Amersham Bioscience) was used as a molecular weight marker, identified at 15-150 kDa. The electrophoretic condition was set to 100 V for 2 hours by using Mini-PROTEIN II, BioRad, USA. The gels were stained with Ponceau s solution (0,1 % Ponceau S in 5 % acetic acid) that used to prepare a stain for rapid reversible detection of protein bands on nitrocellulose or PVDF membranes and analyzed for total proteins.

TABLE 2      Casting the 12% separating gel

| 12% Separating gel                                |         |
|---------------------------------------------------|---------|
| - Deionized water                                 | 1.6 ml  |
| - 1.5 M Tris pH 8.8 (Sigma)                       | 1.3 ml  |
| - 30% Acrylamide solution (GE Healthcare)         | 2.0 ml  |
| - 10% SDS                                         | 50 µl   |
| - 10% (W/V) ammonium persulfate (10% APS) (Serva) | 50 µl   |
| - TEMED (Serva)                                   | 10 µl   |
| - Total monomer                                   | 5.01 ml |

TABLE 3 Casting the 4% stacking gel

| 4% Stacking gel                                   |             |
|---------------------------------------------------|-------------|
| - Deionized water                                 | 1.2 ml      |
| - 0.5 M Tris pH 6.8 (Sigma)                       | 50 $\mu$ l  |
| - 30% Acrylamide solution (GE Healthcare)         | 264 $\mu$ l |
| - 10% SDS                                         | 20 $\mu$ l  |
| - 10% (W/V) ammonium persulfate (10% APS) (Serva) | 20 $\mu$ l  |
| - TEMED (Serva)                                   | 4 $\mu$ l   |
| - Total monomer                                   | 2.008 ml    |

### 3.6.3 Western blot analysis for VEGF

Each amount of 10  $\mu$ g protein sample of liver from control, DM and DMC rats of 4, 8 and 12 weeks respectively, were diluted 1:2 in 2x treatment buffer (0.125 M Tris Cl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.2% bromophenol blue) and boiled for 5 minutes. Protein samples were subjected to polyacrylamide gel electrophoresis and transferred (conditions to 100 V, 0.35 A and 300 W for 1.30 hour) onto nitrocellulose membranes (Hybond, Amersham pharmacia, USA). The membrane was blocked non-specifically bind protein with 5% nonfat dry milk in 0.1% Tween phosphate buffered saline (PBS-T) for 1 hour. Nitrocellulose Blot was then probed with monoclonal antibodies, anti-VEGF (1:500, STCSC-53462, Santa cruz, USA) for 24 hours at 4°C. After incubation with primary antibody, the blot was washed three times in PBS-T, followed by incubation for 2 hours at room temperature with 1: 5,000 of goat anti-mouse horseradish peroxidase IgG (SC 2005, Santa Cruz, USA) that were used as secondary antibodies. Finally, membranes were exposed to film (for ECL) for VEGF detection by Enhanced chemiluminescence (ECL) method.

### 3.6.4 Detection of actin protein

Stripping is used when more than one protein was investigated on the same blot, or the same protein with different antibodies. After detection of VEGF protein, the same membrane was washed three times in PBS. Stripping procedure could be performed by stripping the membrane in warm stripping buffer (50°C) for 3 minutes. The blot was washed three times in PBS, followed by reblocking with 5% nonfat dry milk in 0.1% Tween phosphate buffered saline (PBS-T) for 1 hour. Nitrocellulose Blot was then probed with monoclonal antibodies, anti-actin (1:1,000, SC-56459, Santa Cruz, USA) for 24 hours at 4°C. After incubation with primary antibody, the blot was washed three times in PBS-T, followed by incubation for 2 hours at room temperature with 1: 5,000 of goat anti-mouse horseradish peroxidase IgG (SC 2005, Santa Cruz, USA) that were used as secondary antibodies. Finally, membranes were exposed to film (for ECL) for actin detection by Enhanced chemiluminescence (ECL) method.

### 3.6.5 Molecular weight and Quantitative analysis of VEGF protein expression

The bands of VEGF proteins on nitrocellulose membrane in control, DM and DMC groups of rats from 4, 8 and 12 weeks on ECL film from ECL method were calculated for molecular weight by using gel documentation system (G:BOX) bioimaging System, Syngene, UK). The band density of VEGF protein were evaluated by using the image-analysis software Scion image program (National Institutes of health, Bethesda, MD).

## 3.7 Vascular corrosion cast technique

### 3.7.1 Animal preparation

Each animals of every group was anaesthetized with diethyl ether, laid down on the metal mesh placing in the stainless tray. The thoracic cavity was opened by subcostal incision

to create a chest flap that was closed clamped and retracted to expose the heart. Heparin, 0.5 ml (Leo, 5,000 iu/ml) was suddenly injected into the left ventricle and allowed to circulate for 1 to 2 minutes. A blunt no. 18 gauge needle was inserted into the left ventricle and its tip was directed into the lumen of the ascending aorta. A needle was held in place with arterial clamp. The right atrium was cut and open being the outlet part of blood and injected fluid. Approximately 400-500 ml of 0.9% NaCl solution was infused through the cannula until the effluent was clear.

### 3.7.2 Batson's No. 17 Plastic Mixture Injection

Approximately 50 ml of freshly prepared in the ice bath of Batson's No. 17 plastic mixture was immediately injected into the cannula, following the perfusion with NaCl solution through the ascending aorta. The plastic mixture should flow out from the right atrium when all vascular bed were completely filled with the mixture. Each animal was left at room temperature for 30 minutes to allow setting of the casting medium. The liver was removed and immersed in warm water (80°C) for 3 hours to assure polymerization of the plastic. The liver was then immersed in 40% KOH for 30-60 days to corrode away the animal tissues. The specimens were rinsed in slow running tap water and washed in several changes of distilled water. It was then dissected and trimmed under stereomicroscope and air dried. The vascular cast of liver was placed on a metal stub with double glue tape and carbon paint before being coated with gold on sputtering apparatus. Finally, the cast were examined under the scanning electron microscope (JEOL JSM-5400) at accelerating voltage of 10 KV.

## 3.8 Statistical analysis

The results were expressed as mean  $\pm$  Standard error of the mean (SE). Statistical analysis was performed by using ANOVA followed by Bonferroni posttest. The value of P less than 0.05 was considered to indicate statistical significant.

## CHAPTER 4

### RESULTS

#### 4.1 Effect of curcumin on blood sugar, bodyweight, and liver function test in experimental diabetic rats

##### 4.1.1 Blood sugar level

Effect of curcumin supplementation on blood sugar levels in diabetic rats were presented in TABLE 4 and Figure 8. The blood sugar levels of control, DM, and DMC rats were compared at 4, 8, and 12 week experiments. Blood sugar levels were significantly elevated in all DM and DMC rats at 4, 8, and 12 weeks ( $p<0.0001$ ) as compared with control rats until termination of the study at 12 weeks. The blood sugar levels of DMC rats at 4, 8, and 12 weeks were reduced when compared with DM rats respectively, but there were no significantly different in blood sugar levels between DM and DMC rats.



TABLE 4 Blood sugar levels (mg/dl) of control, DM, and DMC rats at 4, 8, and 12 weeks

| Weeks | Control          | DM                 | DMC                |
|-------|------------------|--------------------|--------------------|
| 4     | 92.50 $\pm$ 3.30 | 436.0 $\pm$ 18.20  | 390.50 $\pm$ 29.16 |
| 8     | 95.25 $\pm$ 4.27 | 477.50 $\pm$ 47.52 | 434.25 $\pm$ 31.87 |
| 12    | 102.0 $\pm$ 3.24 | 420.75 $\pm$ 16.78 | 365.25 $\pm$ 55.79 |

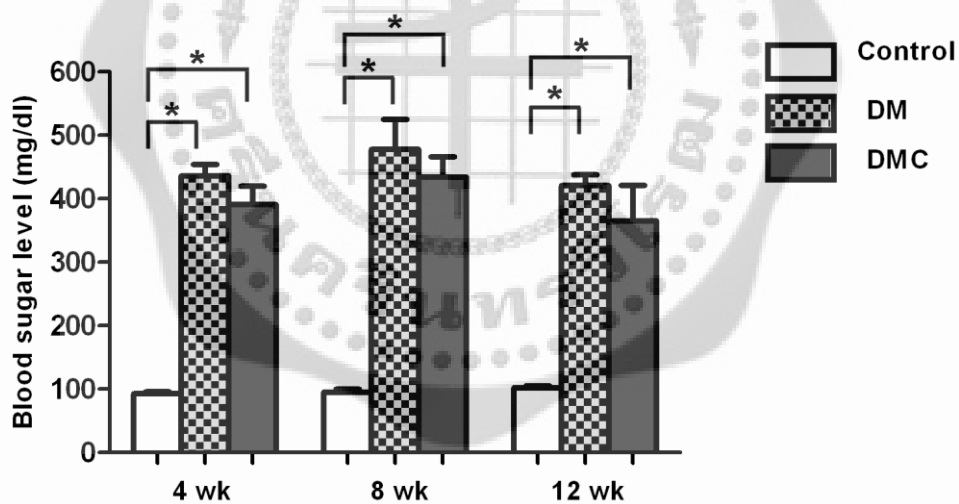


Figure 8 Diagram of blood sugar levels of control, DM, and DMC rats at 4, 8, and 12 weeks. Values are means  $\pm$  SEM. \*  $p < 0.0001$

Moreover, blood sugar levels were compared at different durations of either control, DM, and DMC rats. There were no significantly changes in blood sugar levels of control, DM, and DMC rats at 4, 8, and 12 weeks. (TABLE 5 and Figure 9).

TABLE 5 Blood sugar levels (mg/dl) of 4, 8, and 12 weeks of control, DM, and DMC rats

| Groups  | 4 wk         | 8 wk         | 12 wk        |
|---------|--------------|--------------|--------------|
| Control | 92.50±3.30   | 95.25±4.27   | 102.50±3.24  |
| DM      | 436.0±18.20  | 477.50±47.52 | 420.75±16.78 |
| DMC     | 390.50±29.16 | 434.25±31.87 | 365.25±55.79 |

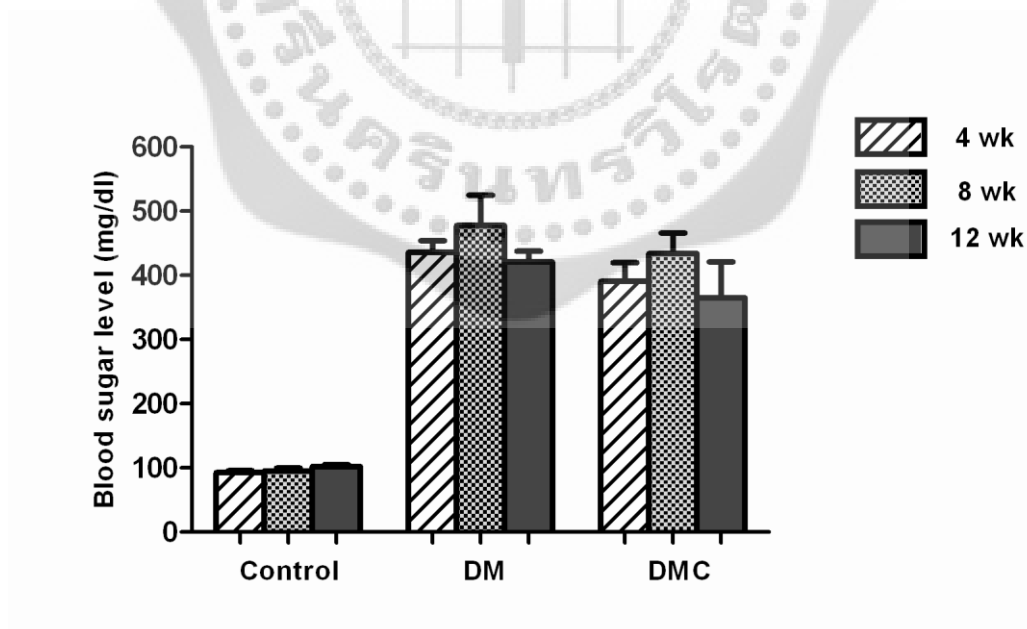


Figure 9 Diagram of blood sugar levels at 4, 8, and 12 weeks of control, DM, and DMC rats.

#### 4.1.2 Body weight

TABLE 6 and Figure 10 information showed the body weights in control, DM,DMC rats at 4, 8, and 12 weeks. Body weight were reduced in DM and DMC rats as compared with the control rats at 8 and 12 weeks. They were significantly decreased in DM ( $p<0.0001$ ) and DMC ( $p<0.05$ ) rats when compared with control rats. The body weight of DMC rats at 8 and 12 weeks were significantly increased when compared with DM rats respectively ( $p<0.05$ ).

TABLE 6 Body weights (gram) at 4, 8, and 12 weeks of control, DM, and DMC rats

| Weeks | Control           | DM                 | DMC                |
|-------|-------------------|--------------------|--------------------|
| 4     | 319.0 $\pm$ 8.22  | 263.75 $\pm$ 15.44 | 273.63 $\pm$ 16.27 |
| 8     | 391.25 $\pm$ 9.20 | 240.38 $\pm$ 16.37 | 315.13 $\pm$ 22.18 |
| 12    | 435.13 $\pm$ 6.38 | 224.75 $\pm$ 5.58  | 363.22 $\pm$ 22.80 |

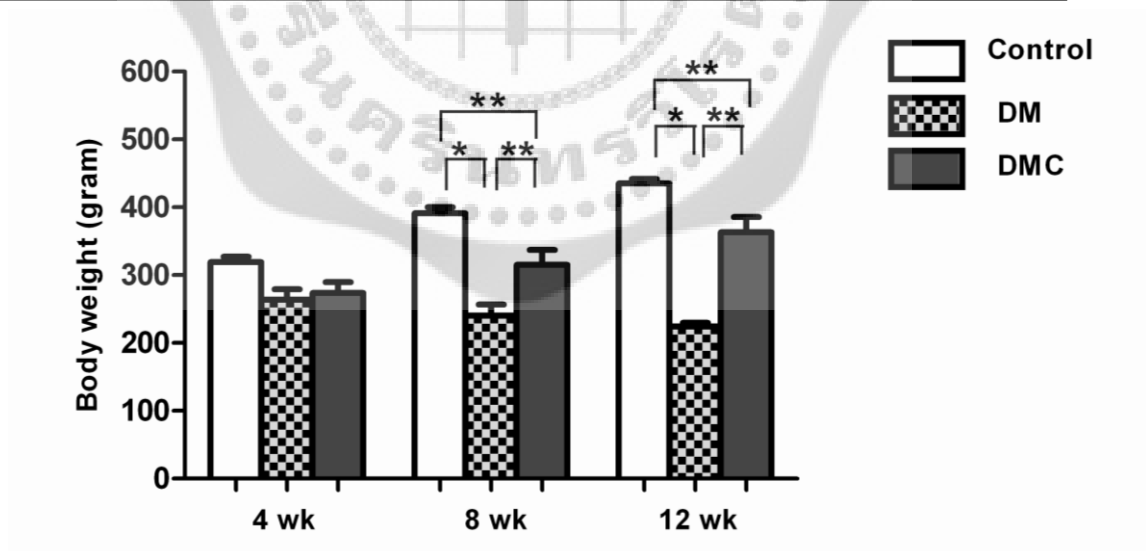


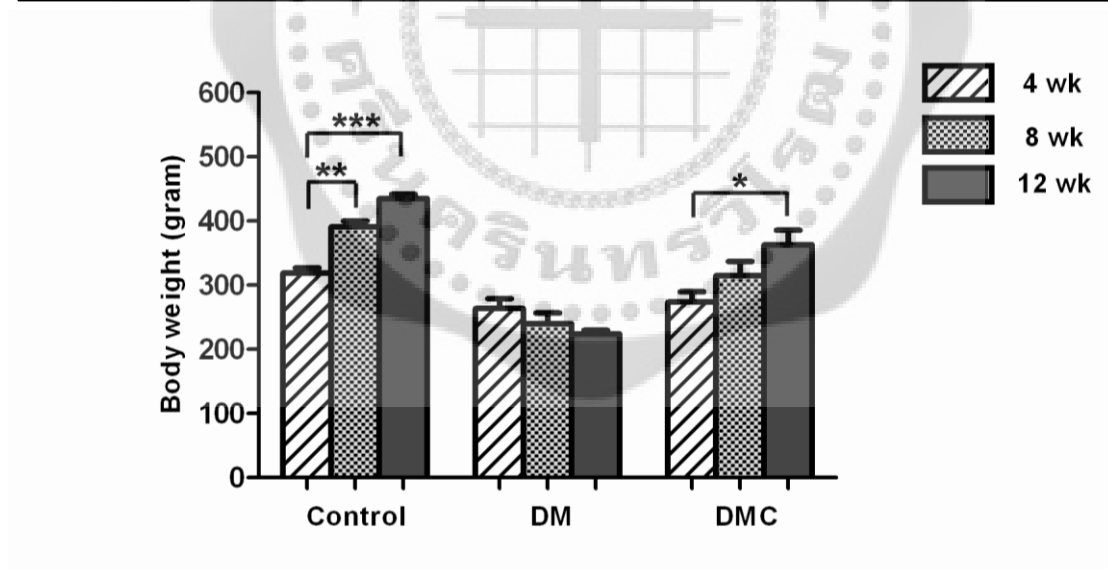
Figure 10 Diagram of the body weight at 4, 8, and 12 weeks of control, DM, and DMC rats.

Values are means  $\pm$  SEM. \*  $p<0.0001$ , \*\*  $p<0.05$

The body weight levels were compared at different durations of either control, DM, or DMC rats. The body weight were elevated in control rats at 4, 8, and 12 weeks. They were significantly increased between 4 and 8 weeks ( $p<0.05$ ) and 4 and 12 weeks ( $p<0.0001$ ) of control rats. There were no significantly changes in body weight of DM rats at 4, 8, and 12 weeks. According to the body weight in DMC rats, they were significantly increased between 4 and 12 weeks ( $p<0.01$ ) (TABLE 7 and Figure 11)

**TABLE 7** Body weights (gram) of Control, DM, and DMC mice at 4, 8, and 12 weeks

| Groups  | 4 wk               | 8 wk               | 12 wk              |
|---------|--------------------|--------------------|--------------------|
| Control | 319.0 $\pm$ 8.22   | 391.25 $\pm$ 9.20  | 435.13 $\pm$ 6.38  |
| DM      | 263.75 $\pm$ 15.44 | 240.38 $\pm$ 16.37 | 224.75 $\pm$ 5.58  |
| DMC     | 273.63 $\pm$ 16.27 | 315.13 $\pm$ 22.18 | 363.22 $\pm$ 22.80 |



**Figure 11** Diagram of the body weights of control, DM, and DMC rats at 4, 8, and 12 weeks. Values are means  $\pm$  SEM. \*  $p<0.01$ , \*\*  $p<0.05$ , \*\*\*  $p<0.0001$

#### 4.1.3 The liver function test

The liver function test of blood sample in control, DM, and DMC rats at 4, 8, and 12 week experiments were shown in Table 8. The SGOT (Glutamate oxaloacetate transaminase), SGPT (Glutamate pyruvate transaminase) levels in three groups of rats were increased than normal levels (The SGOT and SGPT are normally < 30-40 unit/L) but there were not different significantly when compared among three groups of rats and different durations. In contrast, the SAKP (Alkaline phosphatase) levels was increased in DM rats at 4, 8, and 12 weeks ( $204.5 \pm 54.43$ ,  $554.0 \pm 59.65$ , and  $313.50 \pm 40.37$  respectively) when compared with control rats at 4, 8 and 12 weeks ( $120.25 \pm 7.33$ ,  $114.5 \pm 10.23$ , and  $86.25 \pm 11.96$  respectively). The SAKP levels were significantly increased in DM rats when compared with control rats at 8 and 12 weeks ( $p < 0.001$ ,  $p < 0.05$ ). After administration of curcumin, the SAKP levels was reduced in DMC rats at 4, 8, and 12 weeks ( $208.5 \pm 51.49$ ,  $355.25 \pm 74.34$ , and  $195.50 \pm 55.54$  respectively) when compared with DM rats at 4, 8 and 12 weeks. The SAKP levels of DMC rats at 8 weeks also showed significantly reduced when compared to DM rats at 8 week ( $p < 0.05$ ).

| Groups  | 4 week       |             |             | 8 week       |            |                 | 12 week      |            |                |
|---------|--------------|-------------|-------------|--------------|------------|-----------------|--------------|------------|----------------|
|         | SGOT(U/L)    | SGPT(U/L)   | SAKP(U/L)   | SGOT(U/L)    | SGPT(U/L)  | SAKP(U/L)       | SGOT(U/L)    | SGPT(U/L)  | SAKP(U/L)      |
| Control | 125.50±10.32 | 54.75±3.66  | 120.25±7.33 | 120.25±6.71  | 39.25±3.47 | 114.5±10.23     | 90.25±5.28   | 34.50±4.17 | 86.25±11.96    |
| DM      | 116.0±13.88  | 51.50±6.74  | 204.5±54.43 | 148.25±16.16 | 60.75±6.29 | 554.0±59.65*    | 108.75±20.77 | 42.75±8.19 | 313.50±40.37** |
| DMC     | 153.25±31.54 | 78.75±14.87 | 208.5±51.49 | 177.0±49.18  | 37.50±2.25 | 355.25±74.34*** | 106.5±12.25  | 36.75±4.33 | 195.50±55.54   |

**TABLE 8** The liver function test of three group of rats in different durations, Values were expressed as mean±SE. \*  $p<0.001$ , \*\*  $p<0.01$  when compared to control rats. \*\*\*  $p<0.05$  when compare to DM rats. SGOT: Glutamate oxaloacetate transaminase; SGPT: Glutamate pyruvate transaminase; SAKP: Alkaline phosphatase.

## 4.2 Histological examination

### 4.2.1 General structure of normal liver tissue

About 80% of liver tissue was parenchyma consisting of hepatocytes arranged as a labyrinth of cellular plates. The remaining 20% was stroma, a delicate supportive framework of connective tissue that formed the outer capsule (Figure 12A). At the porta hepatis, this capsule was continuous with the arborization of connective tissue that accompanied the branching pattern of the entering hepatic artery, and portal vein and the emerging bile duct. Connective tissue in the liver indistinctly divided hepatic parenchyma into classic liver lobules (Figure 12B), which were the structural units. In rats, the lobules were poorly defined and the amount of connective tissue between lobules were scanty. Branches of these three structures were organized to be portal triads (PT) (Figure 12E). Their sizes progressively decreased to the terminal ramifications. Portal triads surrounded by small amounts of interlobular connective tissue sit at corner of each lobule in area called portal tracts, around which were limiting plates of hepatocytes (H). In transverse section, each lobule consisted of plates of hepatocytes, one or two cell layers in thickness, which were separated by hepatic sinusoids (S) and appeared to radiate out from a small central vein (V) (Figure 12D).

Hepatocyte arrangement resembled that of a sponge with sinusoids presented by the spaces. Each triad consisted of a branch of the bile duct (B), portal vein (PV), and hepatic artery (A), which divided into smaller branches. Small lymphatic vessels (L) often accompanied them. The hepatic arteriole consisted of one to three layers of smooth muscle cells and a relatively small lumen. The portal venule had a larger, often collapsed lumen with a more attenuated wall. The bile ductile was lined by simple cuboidal to columnar epithelium. Concerning central veins, they were thin-walled venules with an attenuated endothelium. They normally lacked significant investment of connective tissue stroma. The lumen of each central vein had numerous openings, which allowed several hepatic sinusoids to drain freely into.

The liver acinus was an oval to diamond-shaped area of hepatic parenchyma refined in relation to blood supply from terminal branches of the portal vein and hepatic artery. It was smaller and harder to see but it was useful functionally and clinically because it was the best for describing metabolic and pathologic changes in relation to many disease. Its short axis ran along the border of two classic hepatic lobules where as the long axis was an imaginary line between two central veins closest to the short axis (Figure 12C). Hepatocytes in the acinus were arranged in three concentric, elliptical zones around the short axis (Figure 12F). In relation to their proximity to the distributing veins (DV), cells in the hepatic acinus could be subdivided into 3 zones. Zone 1(1), most central, was closest to the terminal distributing branches of the portal venule and hepatic arteriole. This zone firstly received oxygen, hormones, and nutrients from the blood stream, and most glycogen and plasma protein synthesis by hepatocytes occurred here. Zone 3 (3) was furthest from the distributing vessels, between Zone 1 and 3 was the intermediate Zone 2 (2). A gradient of metabolic activity existed for many hepatic enzymes in the three zones. Zone 3 was poorly oxygenated, was the first are to show ischemic necrosis and fat accumulation if metabolism was altered. Moreover zone 3 was the site of most drug and alcohol detoxification. The classic hepatic lobule and liver acinus were not contradictory concepts of lobulation but rather complement each other.

#### **4.2.2 The effect of curcumin treatment on histological changes in liver tissue of control, DM, and DMC rats at 4, 6, and 12 week experiment**

At light microscopic level, the liver cells, blood vessels of liver such as branches of portal vein, hepatic artery and bile duct in portal triad, central veins and sinusoids were observed and compared from control, DM, and DMC rats among 4, 8, and 12 week experiments.



### **Histological observation in the liver tissue of control rats at 4, 8, and 12 weeks**

The liver cells and blood vessels in control rats among 4, 8, and 12 weeks had the normal pattern. The hepatocytes were radially disposed in the liver lobule. They formed a plate which contained one or two cell layers similar to the bricks of a wall. These cellular plates were directed from the periphery of the lobule to its center and anastomosed freely, forming a labyrinthine and spongy like structure. The space between these plates contained capillaries and sinusoids which were irregularly dilated vessels. It arised in periphery of the lobule, fed by the inlet venules, terminal branches of portal veins, and hepatic arteriole. The sinusoids ran in the direction of the center, where they drained into the central vein (Figure 13, 16, 23A and C). The portal triad contained a portal venule (a branch of portal vein), an arteriole (a branch of hepatic artery), a bile duct (part of the bile duct system) and lymphatic vessels (Figure 13, 16, and 23B). Three zones of the hepatic lobule based upon oxygen supply. Zone 1 encircled the portal triad (Figure 13, 16, and 23D) where the oxygenated blood from the hepatic artery entered. Zone 3 was located around the central vein, where oxygenation was poor. Zone 2 was located in between the two zones.

According to blood vessels, the diameters of central veins and sinusoid had the normal pattern. No significant changes were observed in those diameters of central veins (The data was shown in TABLE 8 and 9) and sinusoid (The data was shown in TABLE 10 and 11) in control rats at 4, 8, and 12 weeks.

### **Histological observation in the liver tissue of DM rats at 4, 8, and 12 weeks**

Concerning the examination of the liver of diabetic rats at 4 week experiment, the liver cells and blood vessels were likely to the normal pattern of the control group. Liver tissues were exhibited the anastomosing plates of the hepatocytes that radiated from the central vein (Figure 14C and D). Blood sinusoids was located between plates of hepatocytes. Hepatocytes were large polyhedral cells with large round nuclei. Occasionally, binucleate cells were seen in some hepatocytes (Figure 14 A). In generally the portal triad contained a portal venule, an arteriole, a bile duct and lymphatic vessels

According to 8 week condition of DM rats, the microvascular changes were indicated by the appearance of congestion in zone 3 around the central vein (Figure 17 D). The blood sinusoids around the central vein (TABLE 8 and 9) and central veins were dilated (TABLE 10 and 11) and filled with clotted red blood cells (Figure 17 A and C). Few sinusoids were opened into the central vein. Edema appeared in the hepatocytes. Pyknosis of the hepatocytes nuclei was evident (Figure 17 B and D). In addition, some hepatocytes were containing small dense nuclei with eosinophilic cytoplasm. Another finding was that of the liver fat change. Lipids accumulated in the hepatocytes and formed non-membrane bound vacuoles with peripheral nuclei (Figure 17 B). In addition, most of the hepatocytes nuclei appeared pyknotic which were indicated by the condensed basophilic chromatin pattern. Moreover, DM rats liver revealed inflammatory change within the portal triad that the lymphocytes infiltrated in this area (Figure 18 A, B, D, 19, and 20) and collagen fiber deposition was increased in the portal triad (Figure 17 C and 21).

The diameter of blood sinusoids around the central vein (The data was shown in TABLE 8 and 9) and central veins (TABLE 10 and 11) at 12 weeks were seriously increased and filled with clotted red blood cells (Figure 24 A and B). The degenerative and distorted liver cells and microvasculature were revealed that the edema (Figure 18 D) and pyknosis (Figure 18 A, C, and D) of hepatocytes, lipids accumulated in the hepatocytes and formed non-membrane bound vacuoles with peripheral nuclei (Figure 18 C). DM rats at 12 weeks revealed seriously inflammatory change within the portal triad, demonstrated the lymphocytes infiltration (Figure 25 A, B, C, D, and 26) and collagen fiber deposition in this area (Figure 27).

#### **Histological observation in the liver tissue after supplementation of curcumin in DMC rats at 4, 8, and 12 weeks**

The hepatocytes radiating from the central vein were seem to be normal pattern after curcumin supplementation at 4 week. Blood sinusoids was located between plates of hepatocytes. Hepatocytes are large polyhedral cells with large round nuclei (Figure 15A, C, and D). The portal triad contained a portal venule, an arteriole, a bile duct and lymphatic vessels (Figure 15B) which were similar in normal portal triad of control rats at 4, 8, and 12 weeks.

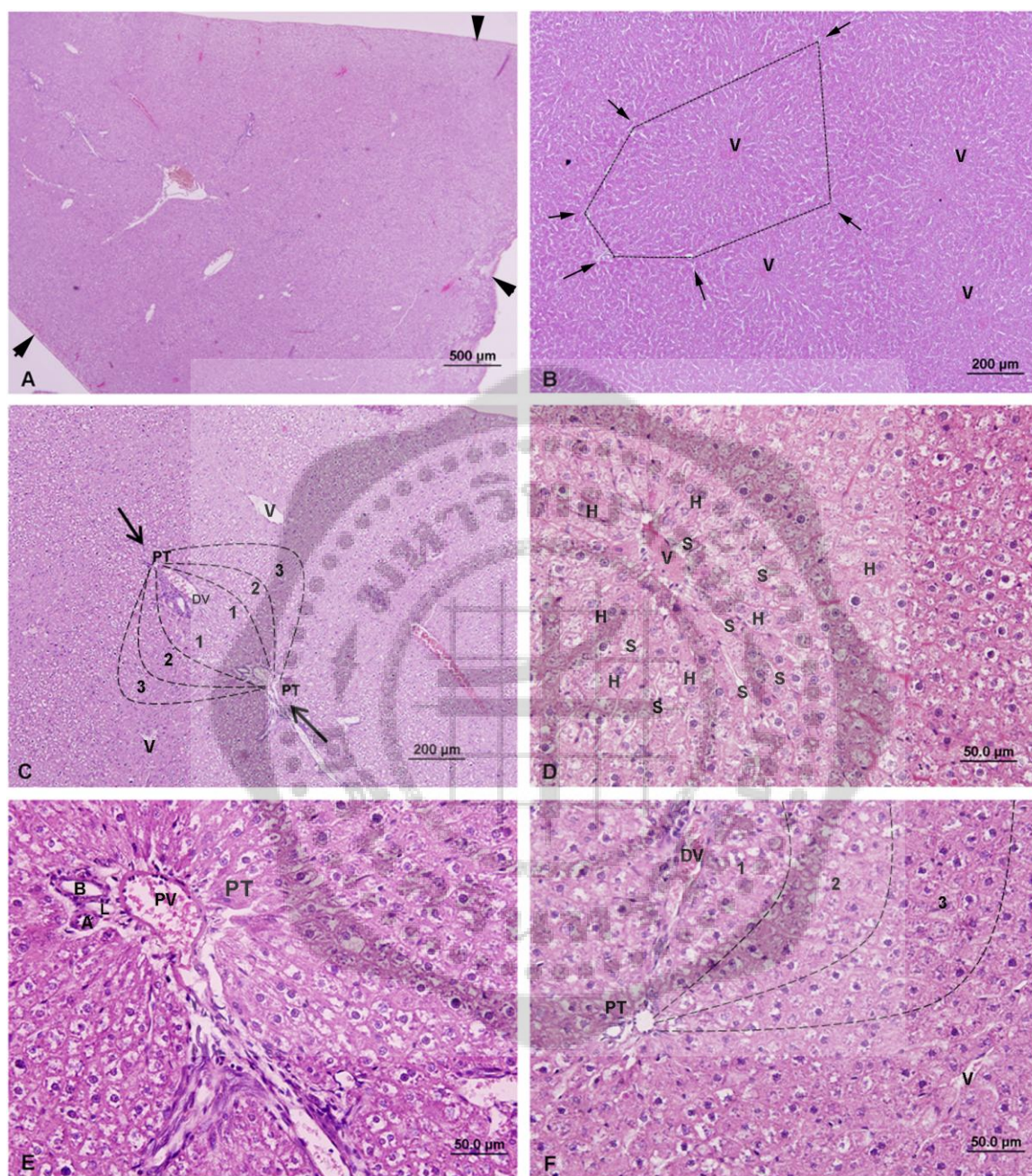
The diameters of the central vein (TABLE 8 and 9) and sinusoids (TABLE 10 and 11) at 8 weeks were increased as compared with control rats. Damages of liver cells and microvessels, central vein and sinusoids, in DMC rats were less as compared with DM rats at 8 weeks. Administration of curcumin in DMC rats inhibited the increasing of microvessels dilatation, lipid accumulation, pyknosis nuclei of hepatocytes, and inflammation in the area of portal triad (Figure 22B). Reducing of inflammation was presented by the decreasing of lymphocytes, collagen fiber deposition in portal triad and reducing of clotted red blood cells in central veins and sinusoids (Figure 22A, C, and D).

Liver tissues injury associated at 12 weeks was characterized by widened central veins and sinusoids, lipid accumulation of hepatocytes and inflammation in the area of portal triad. After treatment of curcumin supplementation, the healthy and nourishing microvessels and hepatocytes (Figure 28 A, B, C, and D) were observed in the liver tissue of DMC rats but the pyknosis of hepatocytes were still observed (Figure 28A, C, and D). The diameters of the central vein (TABLE 8 and 9) and sinusoids (The data was shown in TABLE 10 and 11) at 12 weeks were less as compared with DM rats at 8 weeks. Therefore, curcumin attenuated the severity of liver damaged from STZ-induced diabetes. These results also indicated that STZ induced histological changed associated with central vein and sinusoids dilatation, lipid accumulation and pyknosis of hepatocytes, and inflammation in the area of portal triad.

**Figure 12** Light micrographs of general structure of normal liver tissue stained with hematoxylin & eosin (H&E)

- A Low magnification of the liver tissue, illustrating the liver capsule (arrowheads). 10 x
- B Low magnification of liver tissue, showing the classic liver lobule (dotted line). Portal triads (arrow) sit at corner of the lobule and central vein (V) was in the center of the lobule (V). 20 x
- C Low magnification of the distributing veins (DV) and central vein (V) between two classic liver lobules, Zone 1(1), most central, was closest to the terminal distributing branches of the portal venule and hepatic arteriole in portal triad area (arrows). Zone 3 (3) was furthest from the distributing vessels, between Zone 1 and 3 was Zone 2 (2). 20 x
- D High magnification of the liver lobule, showing the cellular plate of hepatocytes (H) The space between these plates contained capillaries and liver sinusoids (S). The sinusoids ran in the direction towards the center, where they drained into the central vein (V). 40 x
- E High magnification of portal triad (PT), showing a portal venule (PV), an hepatic arteriole ( A ), a bile duct (B) and lymphatic vessels(L). 40 x
- F High magnification of the divided zone between two classic liver lobules, zone 1(1), Zone 2 (2), and Zone 3 (3) by distributing veins (DV), central vein (V), and portal triad (PT). 40 x

Figure 12



**Figure 13** Light micrographs of liver tissue stained with H&E from control rats at 4 week (4 wk, control)

- A Micrograph of the liver tissue, illustrating the hepatocytes (H), central veins (V), and sinusoids (S). 40 x
- B Micrograph of the liver tissue, illustrating the portal triad (PT), portal venule (PV), hepatic arteriole (A), a bile duct (B) and lymphatic vessels (L). 40 x
- C Micrograph of the liver tissue, showing the hepatocytes (H), central veins (V), and sinusoids (S). 40 x
- D Micrograph of the divided zone between two classic liver lobules, zone 1(1), Zone 2 (2), and Zone 3 (3) , central vein (V), and portal triad (PT). 40 x

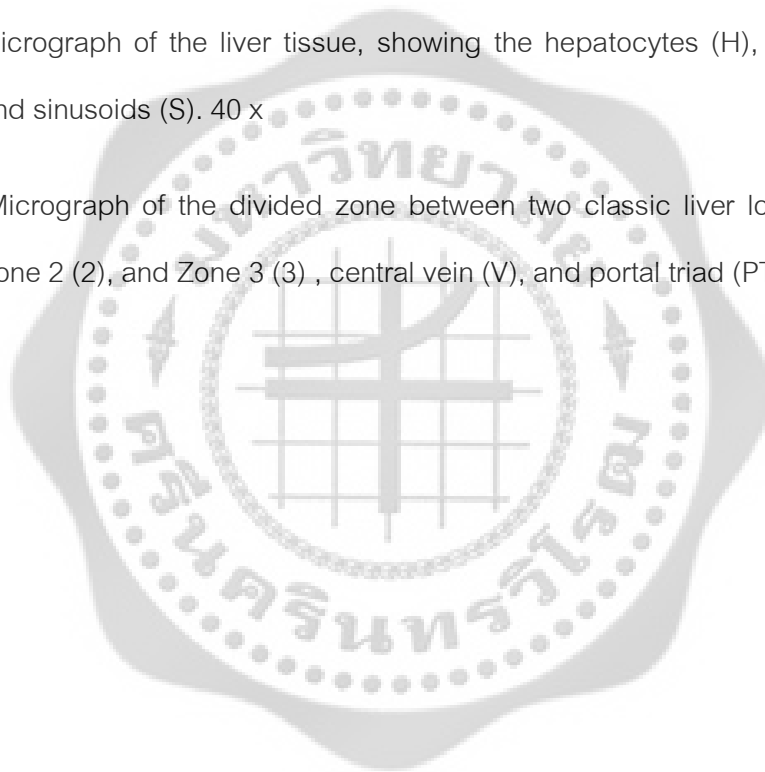
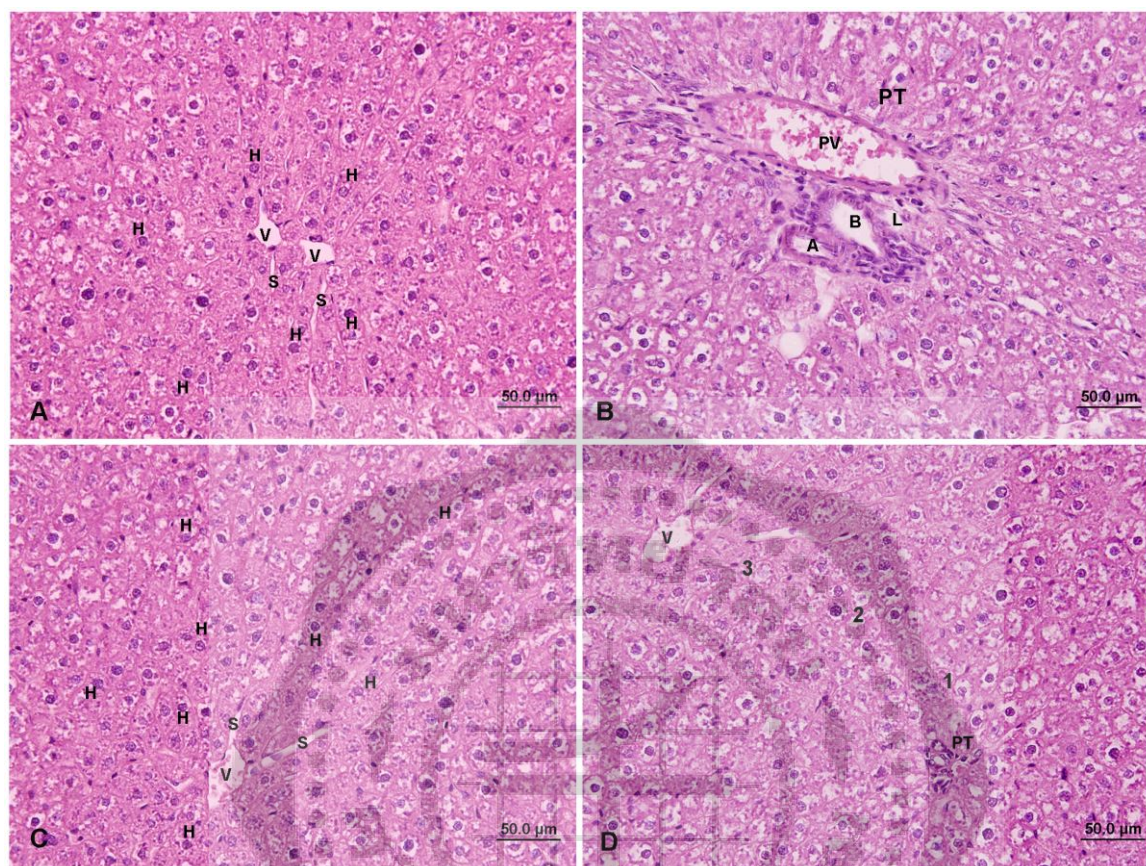




Figure 13



4 wk (Control)

**Figure 14** Light micrographs of liver tissue stained with H&E from DM rats at 4 week (4 wk, DM)

- A Micrograph of the liver tissue, illustrating the hepatocytes (H), central veins (V), sinusoids (S), and binucleated hepatocytes (arrows). 40 x
- B Micrograph of the liver tissue, illustrating the portal triad (PT), portal venule (PV), hepatic arteriole (A), a bile duct (B) and lymphatic vessels (L). 40 x
- C Micrograph of the liver tissue, showing the hepatocytes (H), central veins (V), and sinusoids (S). 40 x
- D Micrograph of the divided zone between two classic liver lobules, zone 1(1), Zone 2 (2), and Zone 3 (3) , central vein (V), and portal triad (PT). 40 x

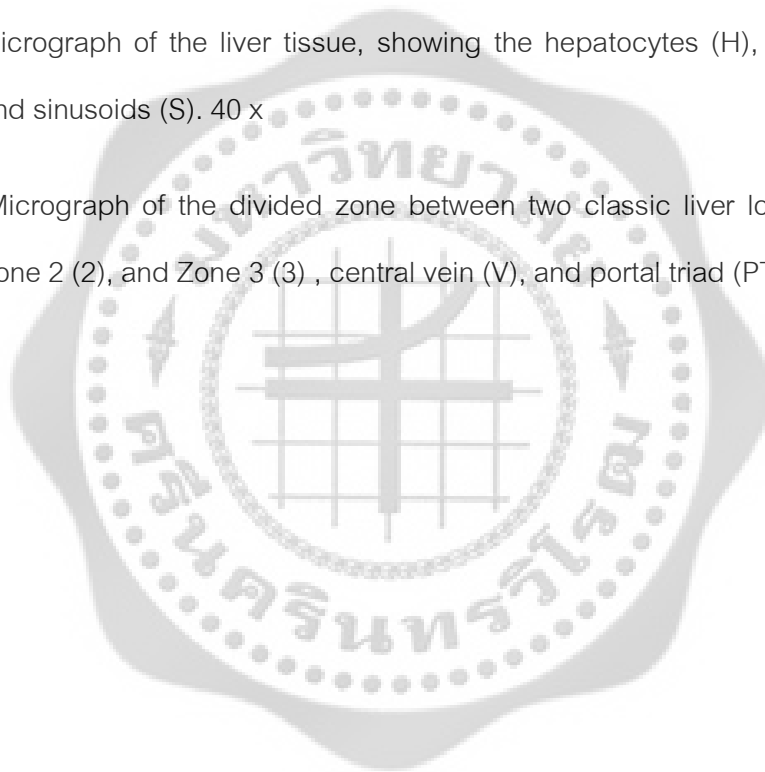
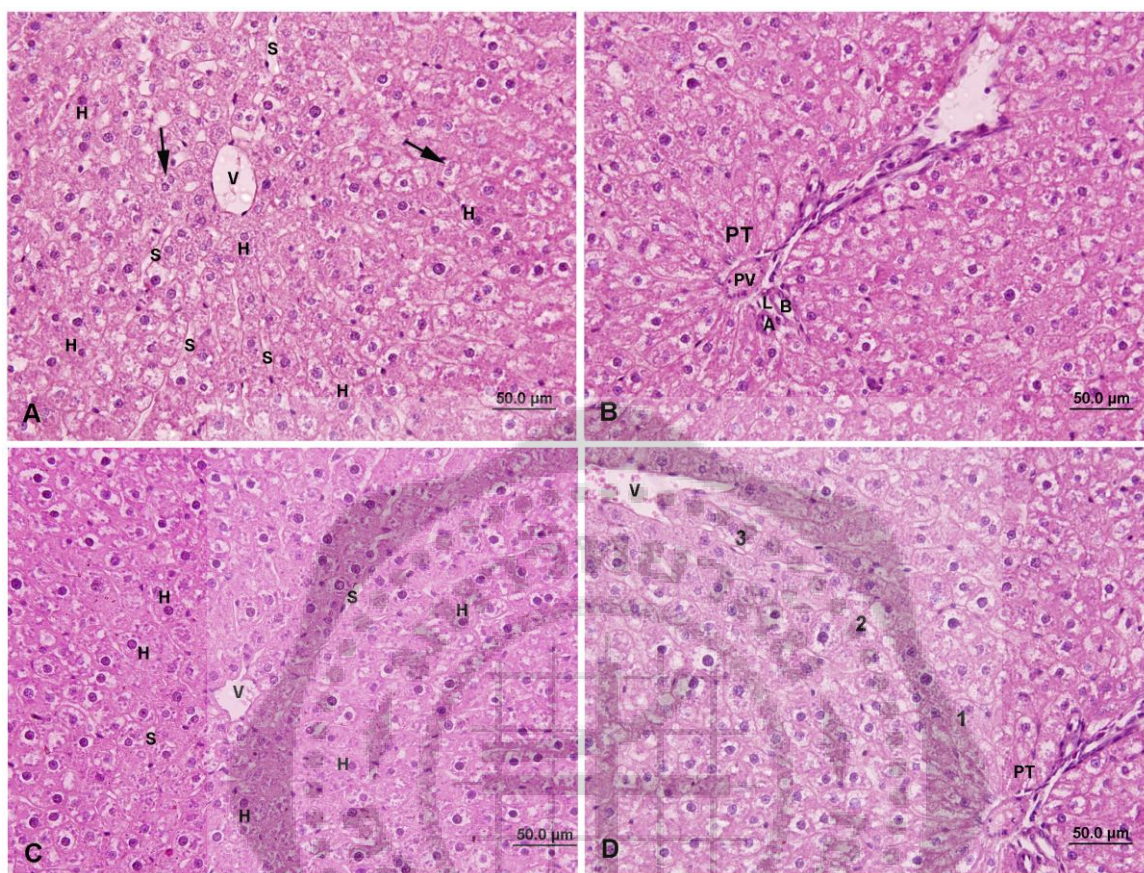




Figure 14



4 wk (DM)

**Figure 15** Light micrographs of liver tissue stained with H&E from DMC rats at 4 week (4 wk, DMC)

- A Micrograph of the liver tissue, illustrating the hepatocytes (H), central veins (V), and sinusoids (S). 40 x
- B Micrograph of the liver tissue, illustrating the portal triad (PT), portal venule (PV), hepatic arteriole (A), a bile duct (B) and lymphatic vessels(L). 40 x
- C Micrograph of the liver tissue, showing the hepatocytes (H), central veins (V), and sinusoids (S). 40 x
- D Micrograph of the divided zone between two classic liver lobules, zone 1(1), Zone 2 (2), and Zone 3 (3) , central vein (V), and portal triad (PT). 40 x

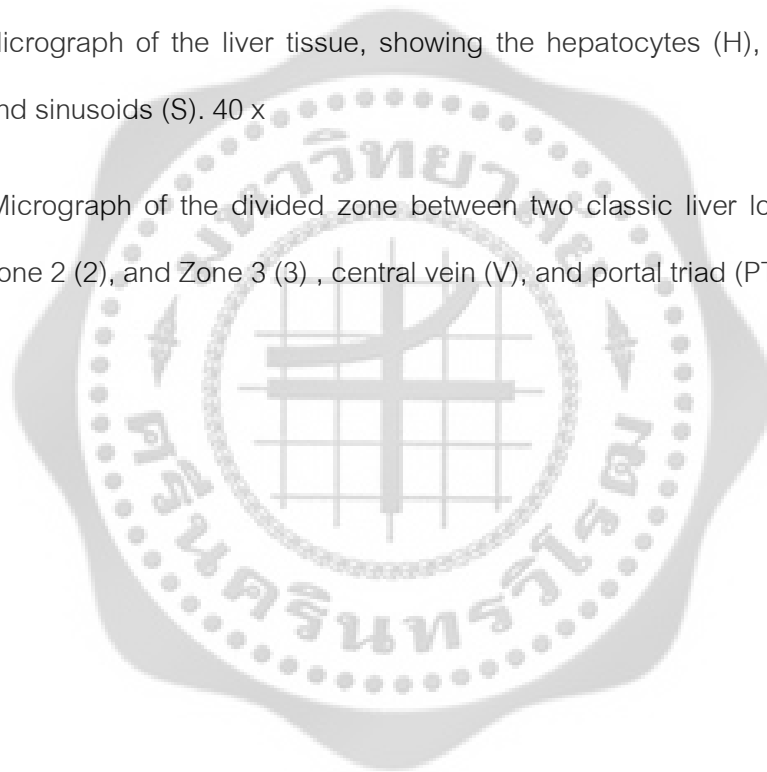
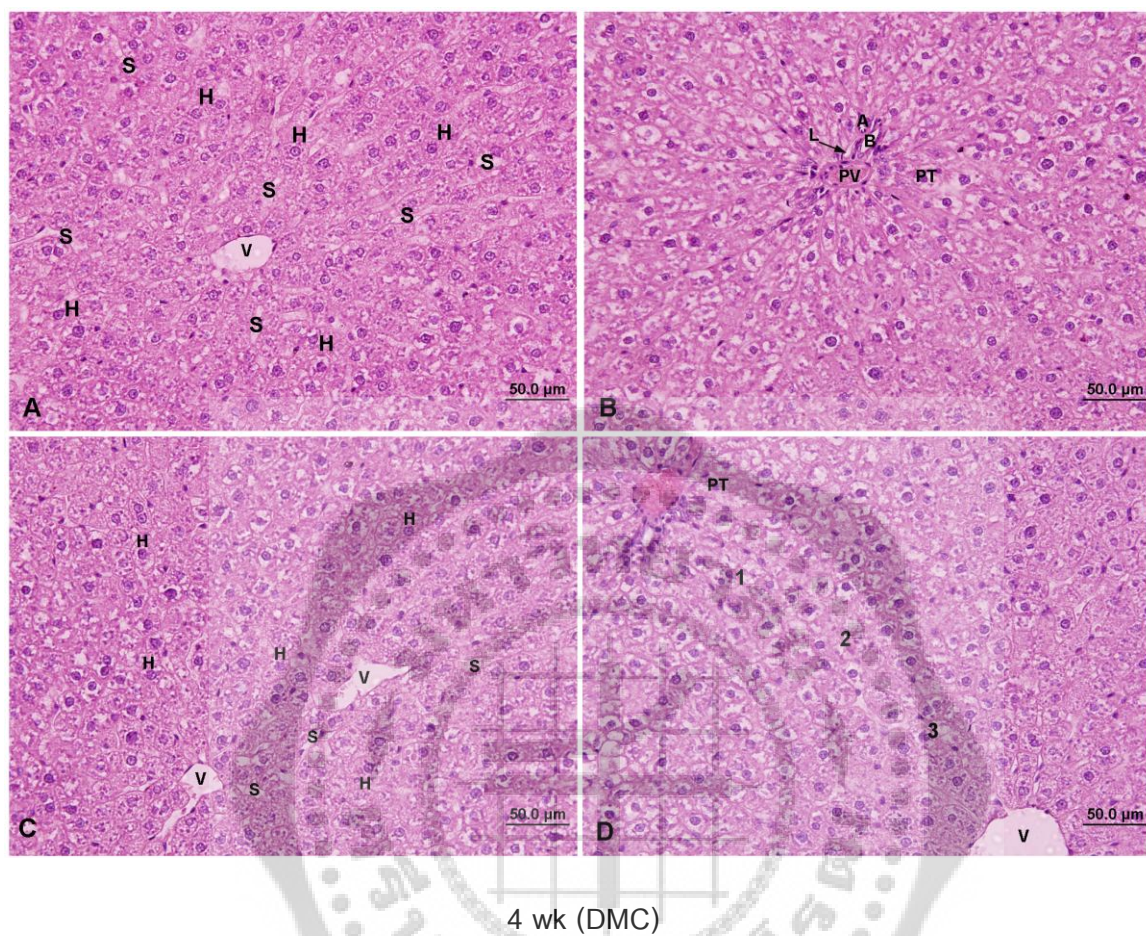




Figure 15



**Figure 16** Light micrographs of liver tissue stained with H&E from control rats at 8 week (8 wk, control)

- A Micrograph of the liver tissue, illustrating the hepatocytes (H), central veins (V), sinusoids, and binucleated hepatocytes (arrows) (S). 40 x
- B Micrograph of the liver tissue, illustrating the portal triad (PT), portal venule (PV), hepatic arteriole (A), a bile duct (B) and lymphatic vessels(L). 40 x
- C Micrograph of the liver tissue, showing the hepatocytes (H), central veins (V), and sinusoids (S). 40 x
- D Micrograph of the divided zone between two classic liver lobules, zone 1(1), Zone 2 (2), and Zone 3 (3) , central vein (V), and portal triad (PT). 40 x

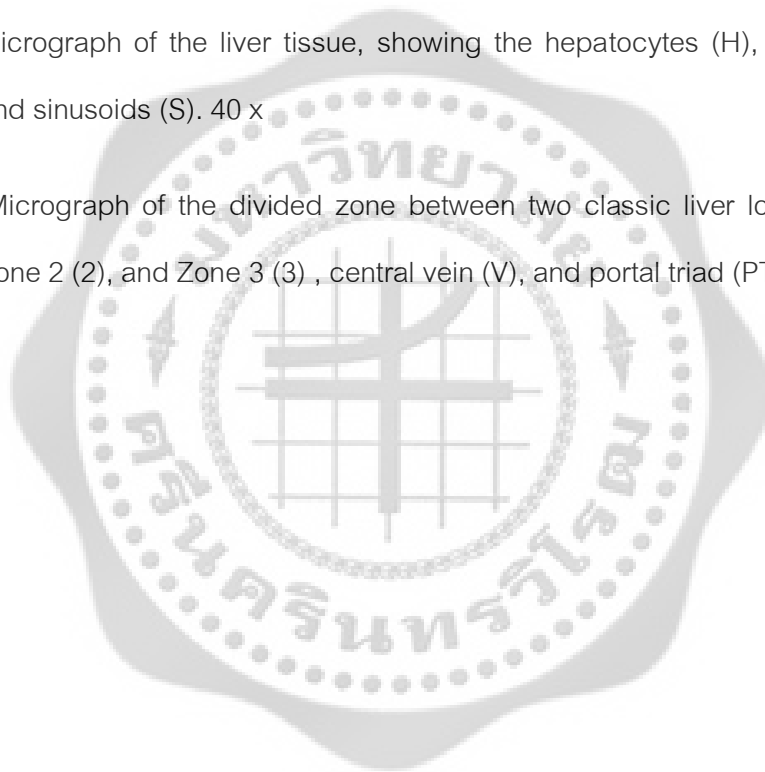
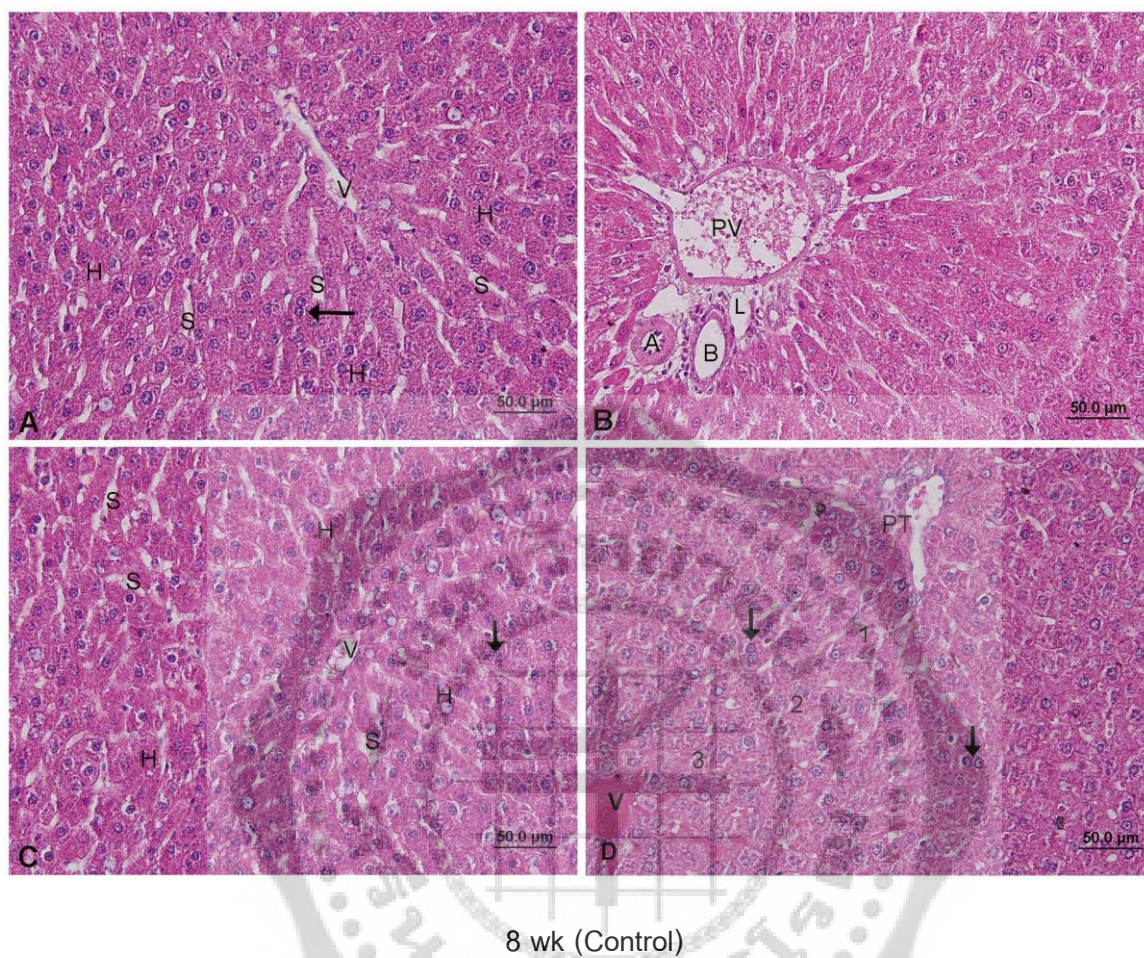




Figure 16

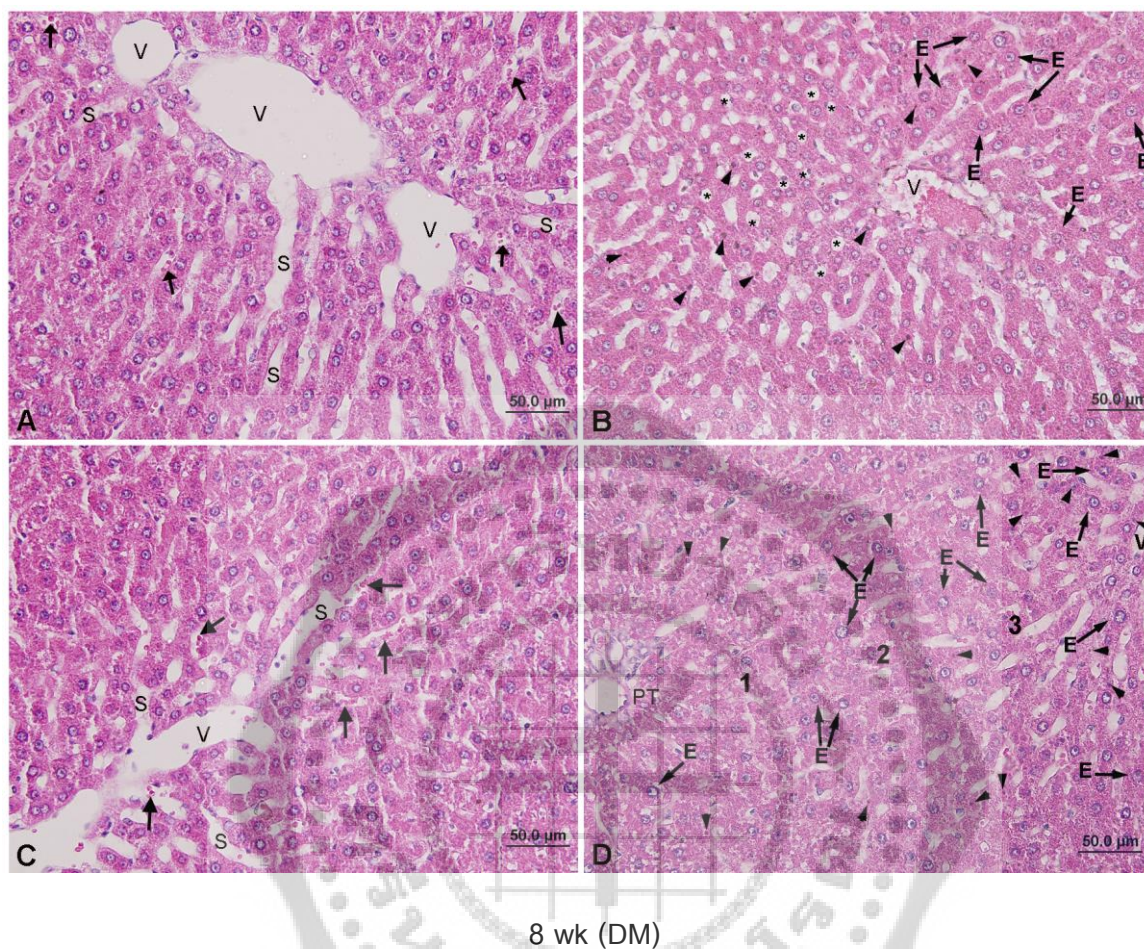


**Figure 17** Light micrographs of liver tissue stained with H&E from DM rats at 8 week (8 wk, DM)

- A Micrograph of zone 3 around the central vein of liver tissue, showing the blood sinusoids (S) and central veins (V) which were dilated and filled with clotted red blood cells (arrows). 40 x
- B Micrograph of zone 3 around the central vein (v) of liver tissue, illustrating the edematous hepatocytes (E), pyknosis of the hepatocytes nuclei (arrowhead), and lipid accumulated in hepatocytes (\*). 40 x
- C Micrograph of zone 3 around the central vein of liver tissue, showing the blood sinusoids (S) and central veins (V) which were dilated and filled with clotted red blood cells (arrows). 40 x
- D Micrograph of the divided zone between two classic liver lobules, illustrating the edematous hepatocytes, especially at zone 3 was around the central vein (v), the edematous hepatocytes (E), pyknosis of the hepatocytes nuclei (arrowhead). 40 x



Figure 17



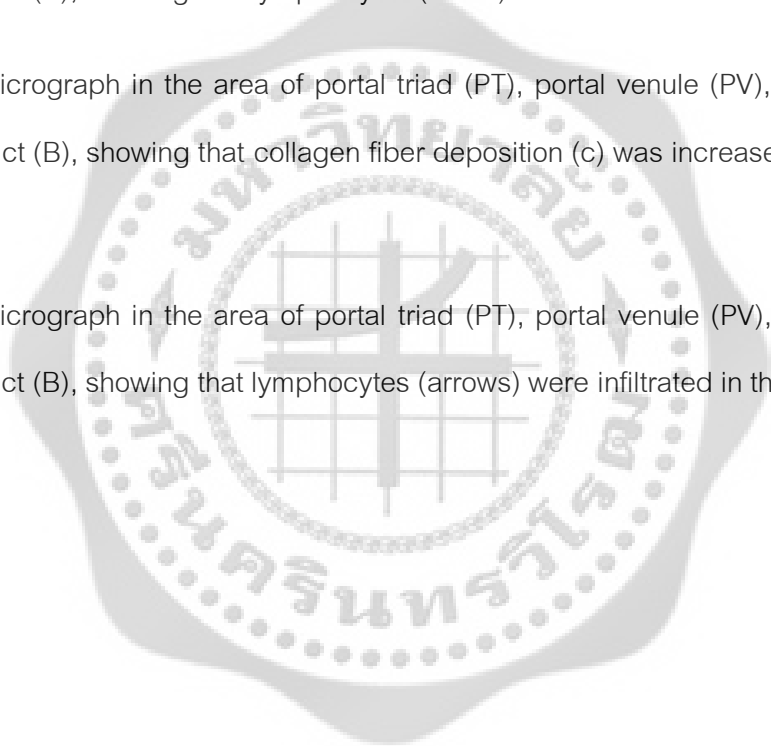
**Figure 18** Light micrographs of liver tissue stained with H&E from DM rats at 8 week (8 wk, DM)

A Micrograph in the area of portal triad (PT), portal venule (PV), hepatic arteriole (A), and a bile duct (B), showing the inflammatory change within the portal triad that lymphocytes (arrows) were infiltrated in this area. 40 x

B Micrograph in the area of portal triad (PT), portal venule (PV), hepatic arteriole (A), and a bile duct (B), showing that lymphocytes (arrow) were infiltrated in this area. 40 x

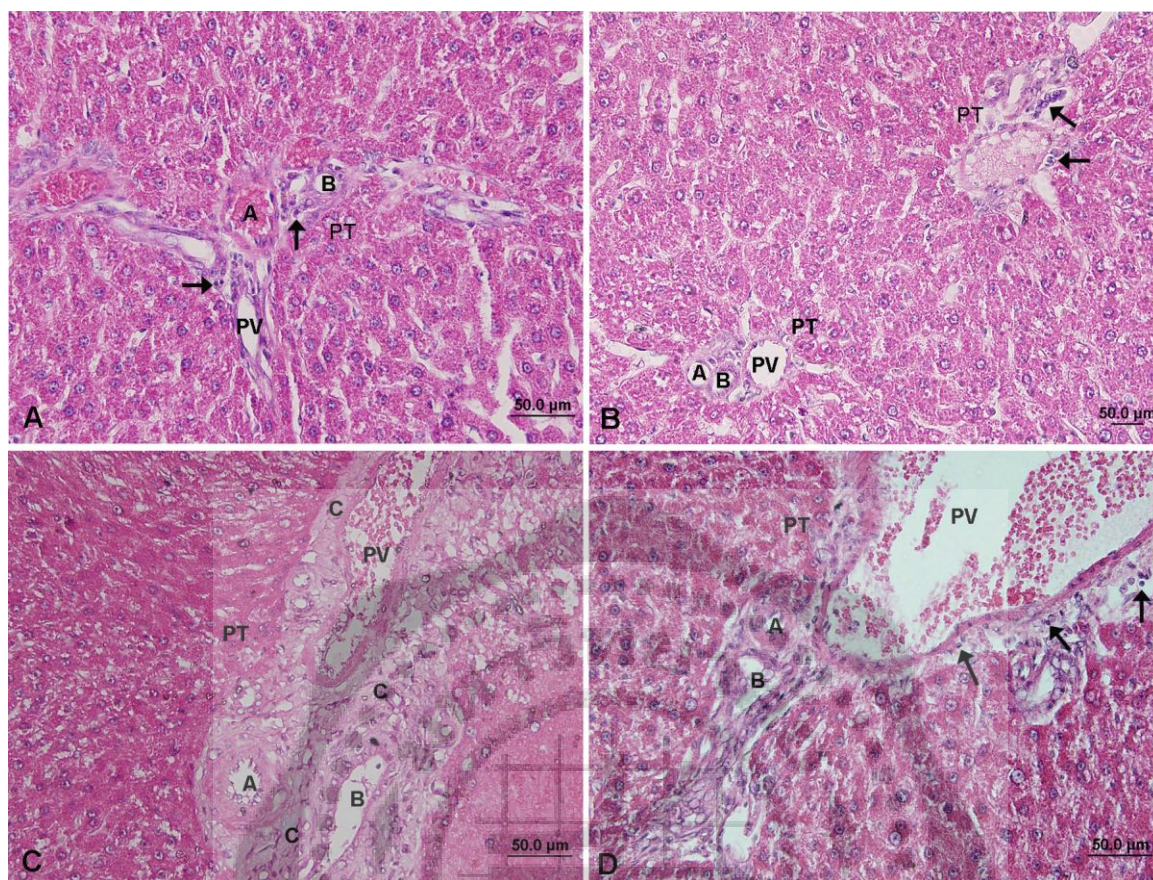
C Micrograph in the area of portal triad (PT), portal venule (PV), hepatic arteriole (A), and a bile duct (B), showing that collagen fiber deposition (c) was increased in this area. 40 x

D Micrograph in the area of portal triad (PT), portal venule (PV), hepatic arteriole (A), and a bile duct (B), showing that lymphocytes (arrows) were infiltrated in this area. 40 x



**Figure 18**



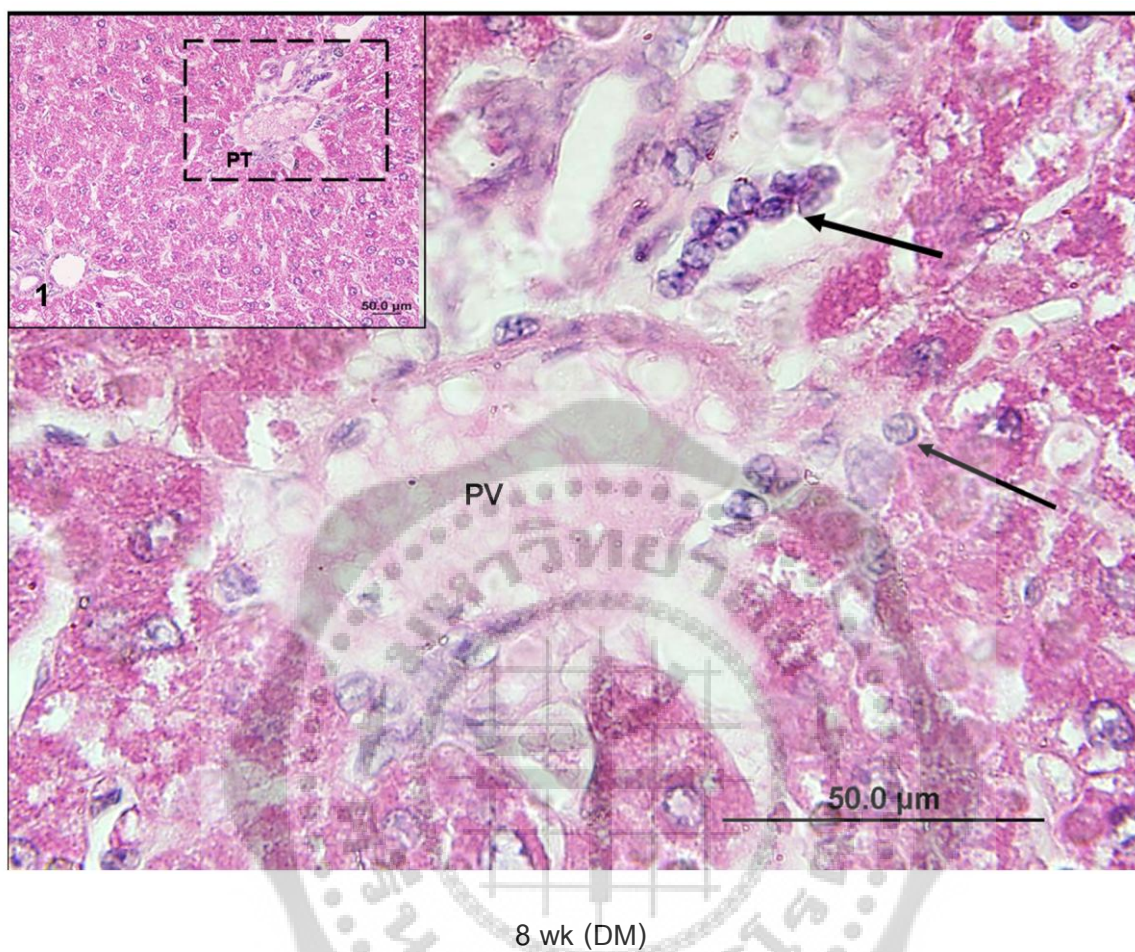


8 wk (DM)

**Figure 19** Light micrographs of the portal triad (PT) of liver tissue stained with H&E from DM rats at 8 week (8 wk, DM), the high magnification of inset 1, showing that lymphocytes (arrows) were infiltrated around the portal vein (PV) 100 x



Figure 19

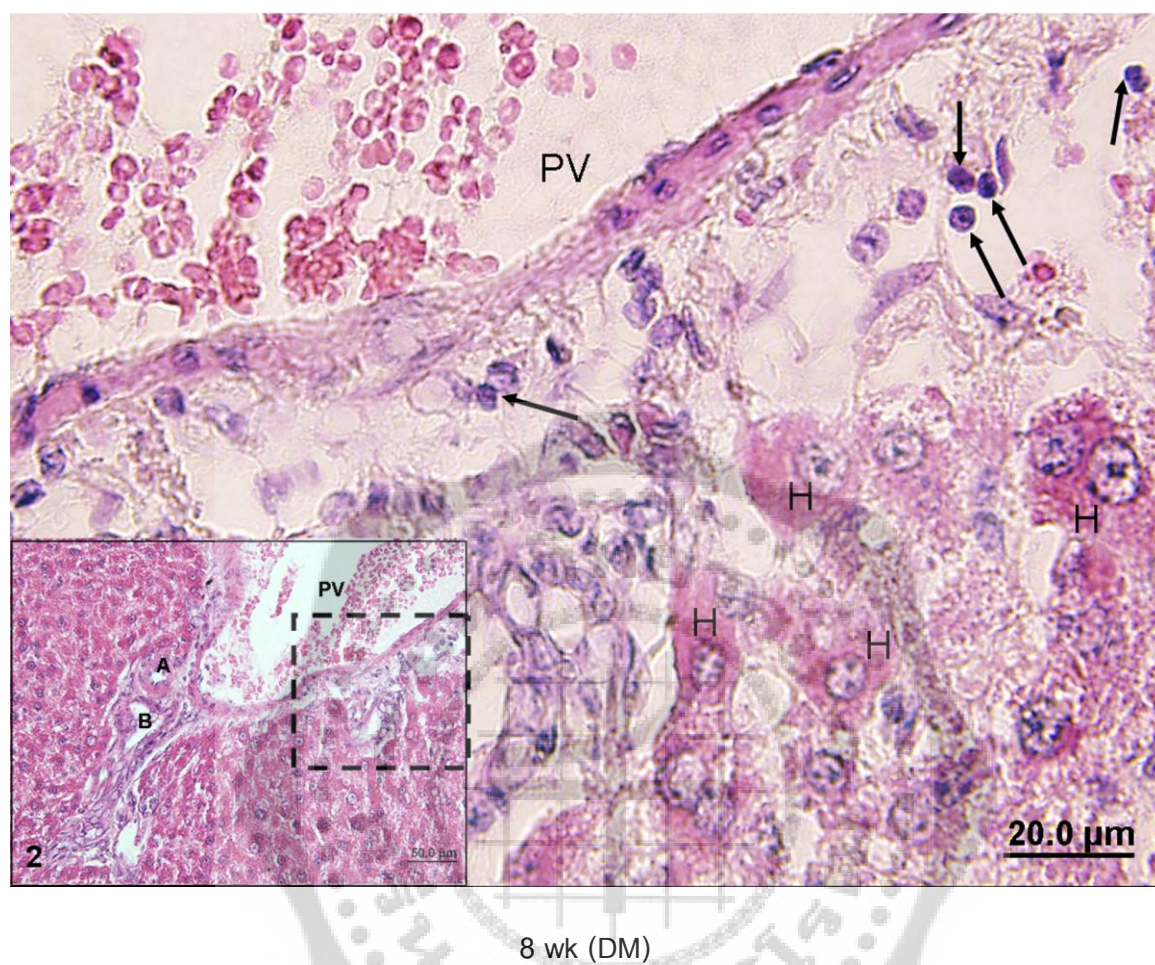




**Figure 20** Light micrograph of the portal triad (PT) and hepatocytes (H) of liver tissue stained with H&E from DM rats at 8 week (8 wk, DM), the high magnification of inset 2, showing that lymphocytes (arrows) were infiltrated around the portal vein (PV).100 x



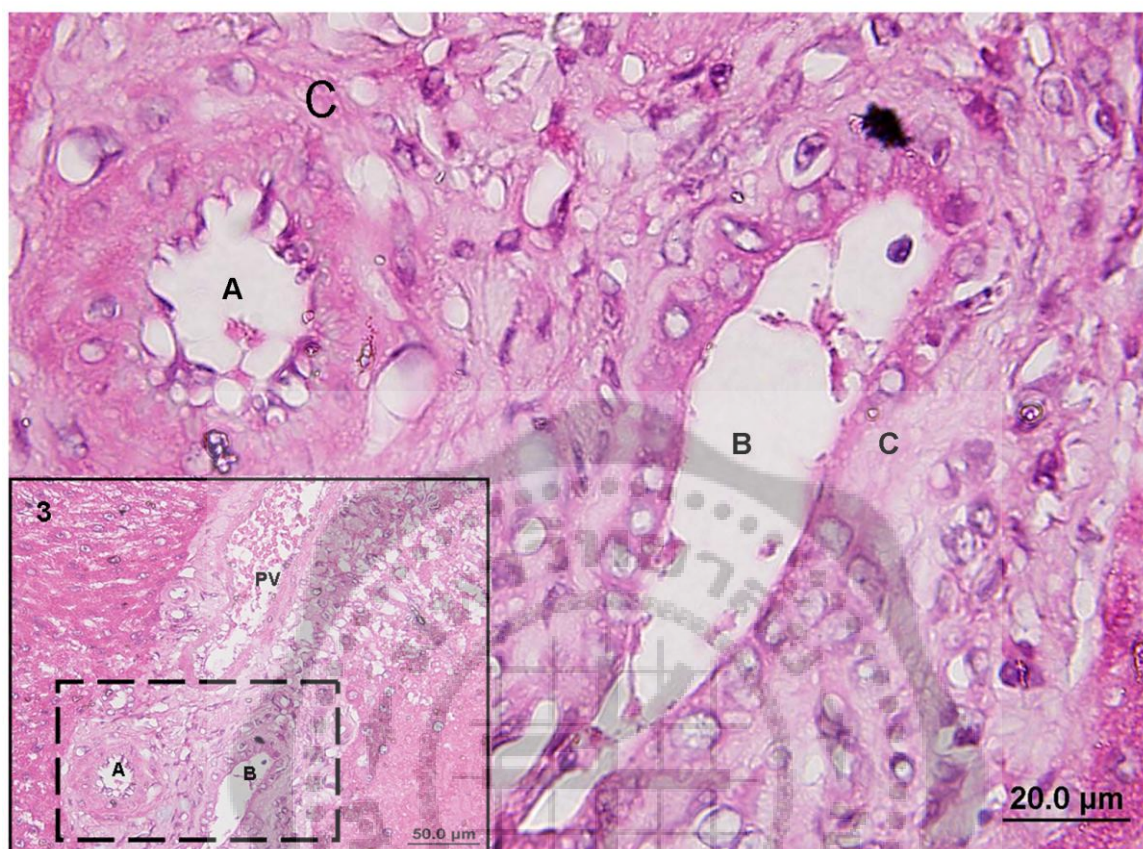
Figure 20



**Figure 21** Light micrograph of the portal triad (PT) of liver tissue stained with H&E from DM rats at 8 week (8 wk, DM), the high magnification of inset 3, showing that collagen fiber deposition (C) were infiltrated around the portal vein (PV), hepatic arteriole (A), and a bile duct (B).100 x



Figure 21



8 wk (DM)

**Figure 22** Light micrographs of liver tissue stained with H&E from DMC rats at 8 week (8 wk, DMC)

- A Micrograph of the liver tissue, illustrating the hepatocytes (H), central veins (V), and sinusoids (S). 40 x
- B Micrograph of the liver tissue, illustrating the portal triad (PT), portal venule (PV), hepatic arteriole (A), a bile duct (B) and lymphatic vessels(L). 40 x
- C Micrograph of the liver tissue, showing the hepatocytes (H), central veins (V), and sinusoids (S). 40 x
- D Micrograph of the divided zone between two classic liver lobules, zone 1(1), Zone 2 (2), and Zone 3 (3) , central vein (V), and portal triad (PT). 40 x

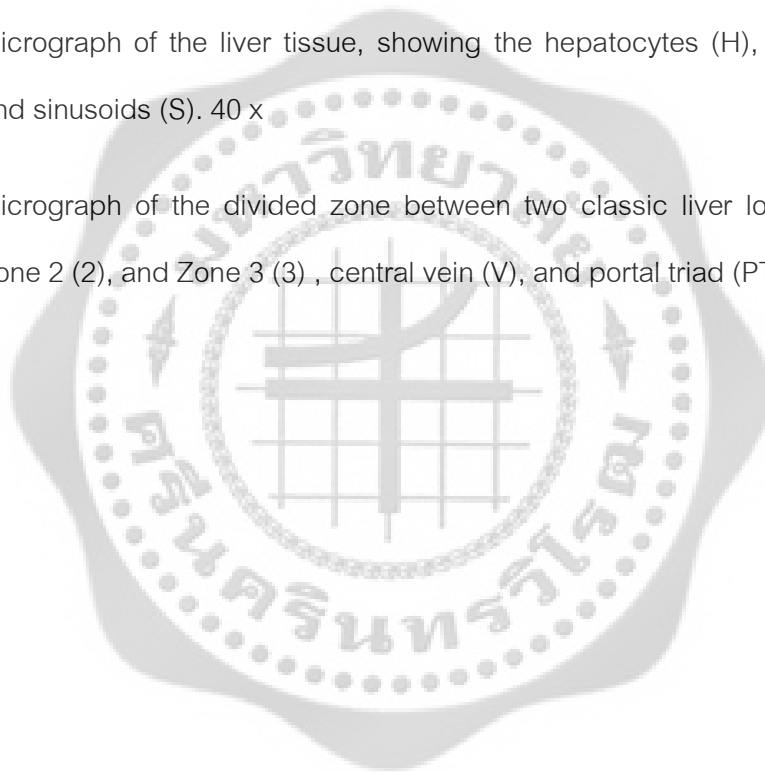
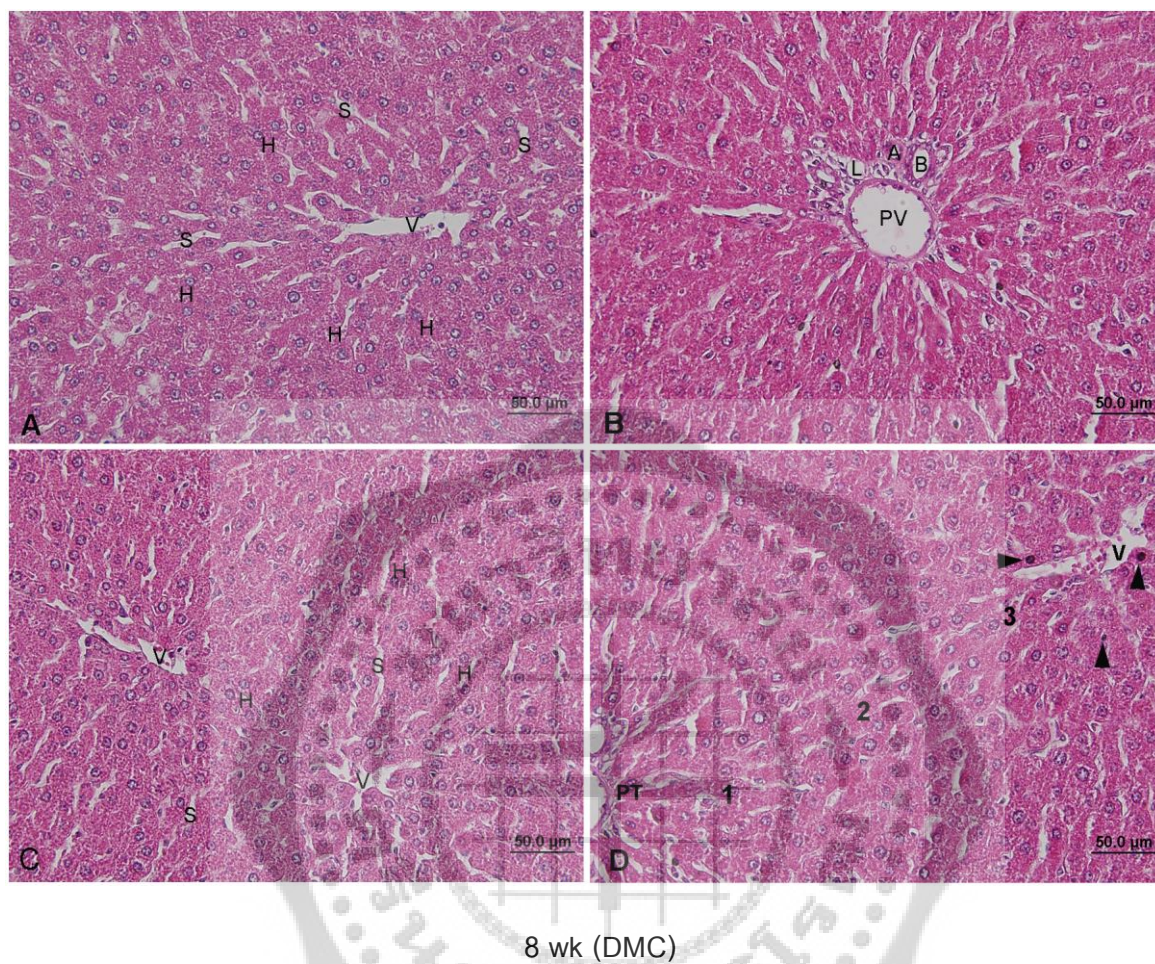




Figure 22



**Figure 23** Light micrographs of liver tissue stained with H&E from control rats at 12 week (12 wk, control)

- A Micrograph of the liver tissue, illustrating the hepatocytes (H), central veins (V), sinusoids, and binucleated hepatocytes (arrows) (S). 40 x
- B Micrograph of the liver tissue, illustrating the portal triad (PT), portal venule (PV), hepatic arteriole (A), and a bile duct (B). 40 x
- C Micrograph of the liver tissue, showing the hepatocytes (H), central veins (V), and sinusoids (S). 40 x
- D Micrograph of the divided zone between two classic liver lobules, zone 1(1), Zone 2 (2), and Zone 3 (3) , central vein (V), and portal triad (PT). 40 x

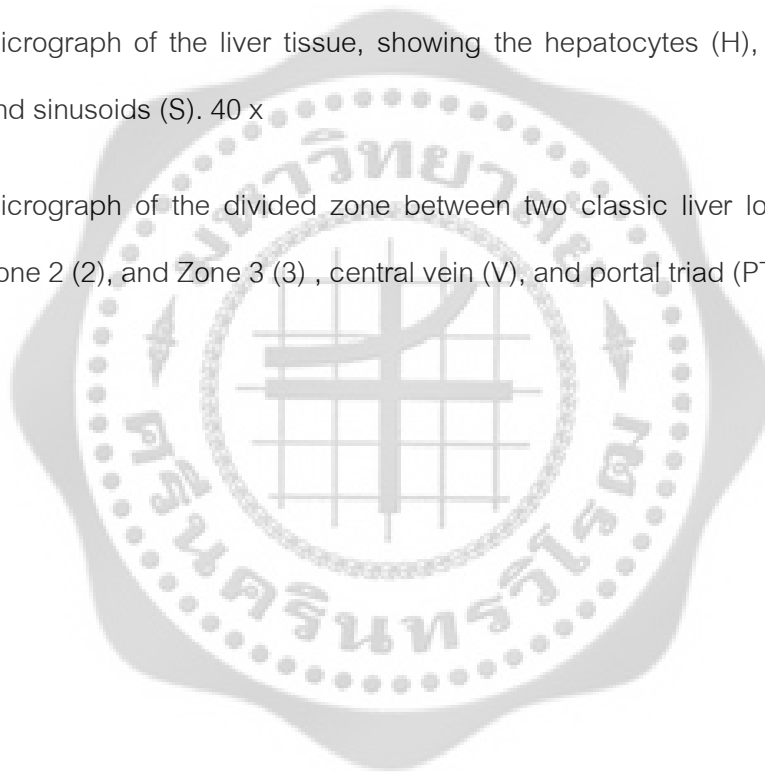
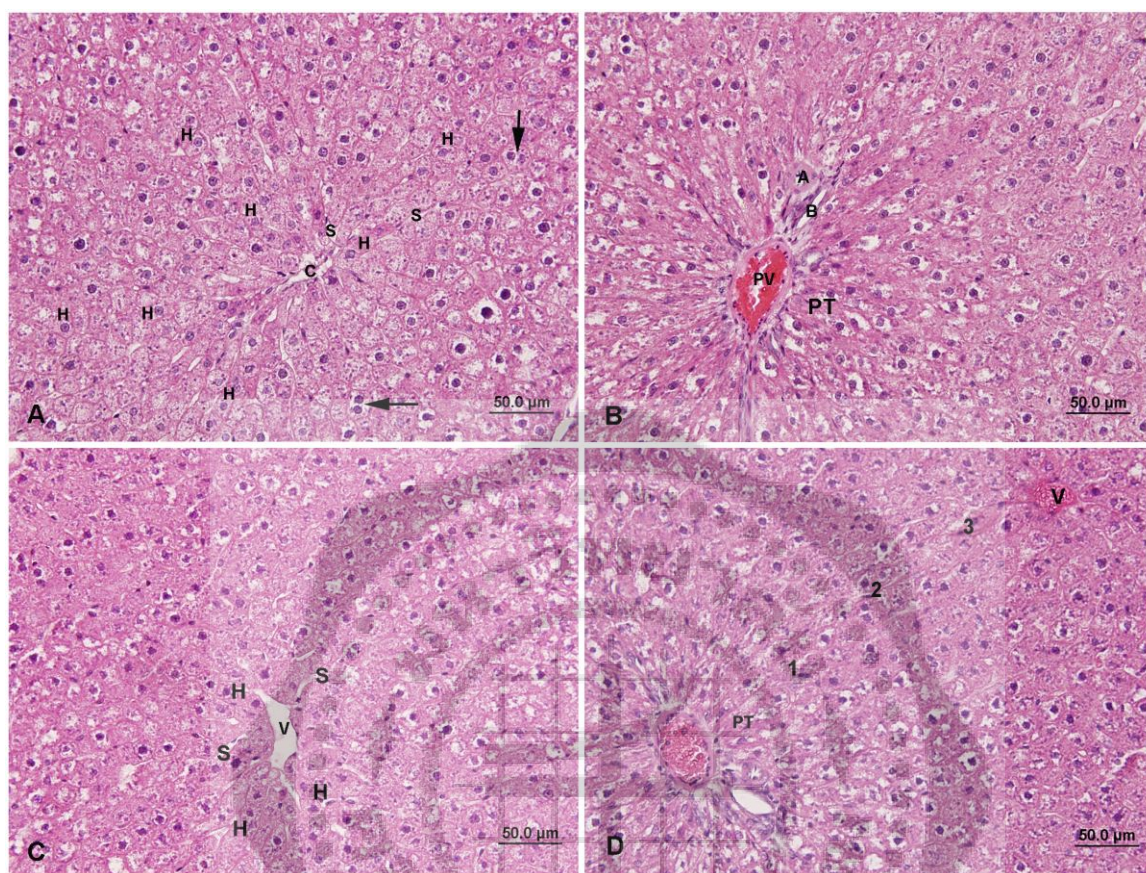




Figure 23



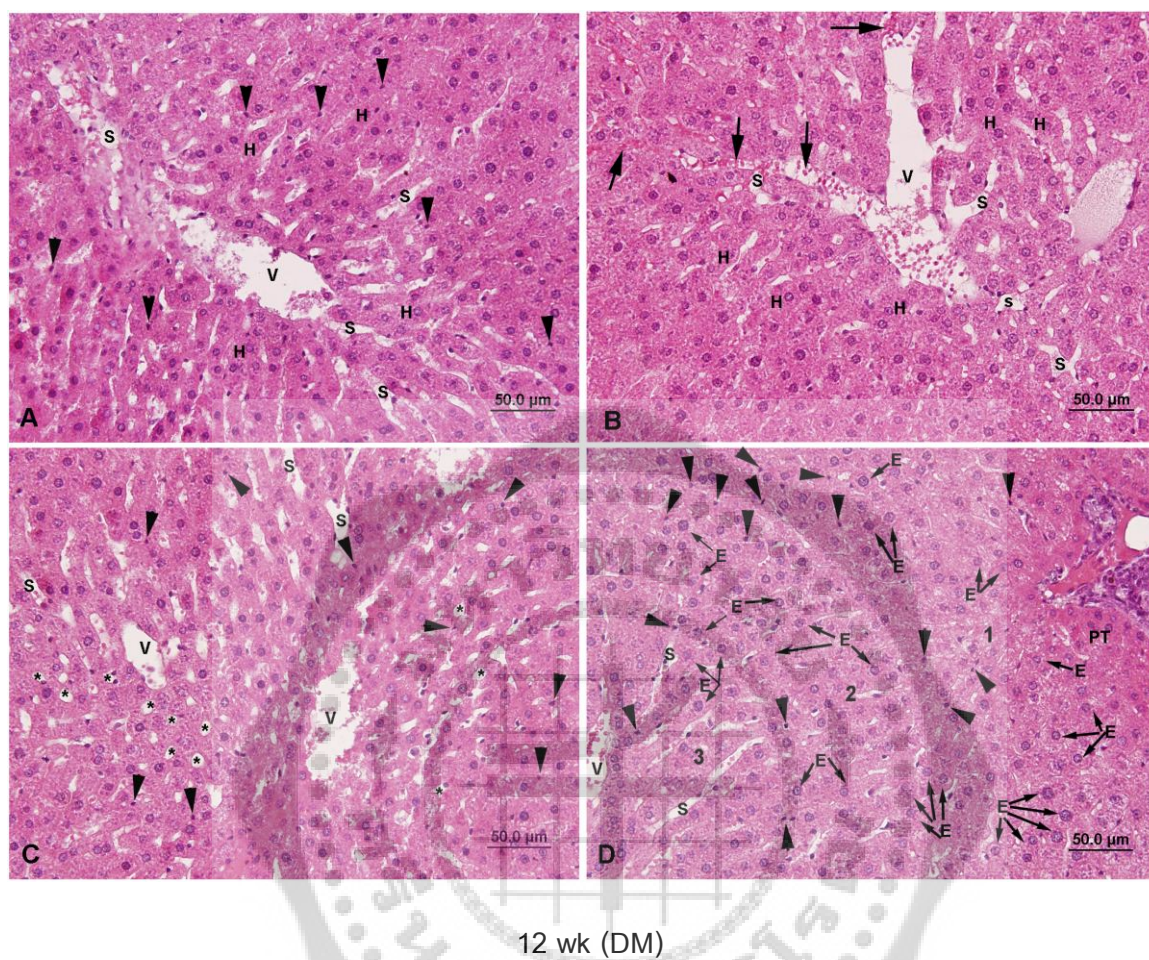
12 wk (Control)

**Figure 24** Light micrographs of liver tissue stained with H&E from DM rats at 12 week (12 wk, DM)

- A Micrograph of zone 3 around the central vein of liver tissue, showing that blood sinusoids (S) and central veins (V) were seriously dilated, some hepatocytes (H) showed pyknosis nuclei (arrows). 40 x
- B Micrograph of zone 3 around the central vein of liver tissue, showing hepatocytes (H), the blood sinusoids (S), and central veins (V) that were seriously dilated and filled with clotted red blood cells (arrows). 40 x
- C Micrograph of zone 3 around the central vein of liver tissue, showing that blood sinusoids (S) and central veins (V) were seriously dilated, some hepatocytes showed pyknosis nuclei and had lipid accumulation (\*). 40 x
- D Micrograph of the divided zone between two classic liver lobules in the area of portal triad (PT), central vein (v) and blood sinusoid (S), illustrating the edematous hepatocytes (E) in zone 1, 2, and 3 around the central vein (v), pyknosis of the hepatocyte nuclei (arrowhead). 40 x



Figure 24



**Figure 25** Light micrographs of liver tissue stained with H&E from DM rats at 12 week (12 wk, DM)

A Micrograph in the area of portal triad (PT), portal venule (PV), hepatic arteriole (A), and a bile duct (B), showing the serious inflammatory change within the portal triad that the lymphocytes (arrows) were infiltrated in this area. 40 x

B Micrograph in the area of portal triad (PT), portal venule (PV), hepatic arteriole (A), and a bile duct (B), showing that lymphocytes (arrows) were infiltrated in this area. 40 x

C Micrograph in the area of portal triad (PT), portal venule (PV) and a bile duct (B), showing that lymphocytes (arrows) were infiltrated and collagen fiber deposition (c) was increased in this area. 40 x

D Micrograph in the area of portal triad (PT), portal venule (PV), hepatic arteriole (A), and a bile duct (B), showing that lymphocytes (arrows) were infiltrated in this area. 40 x

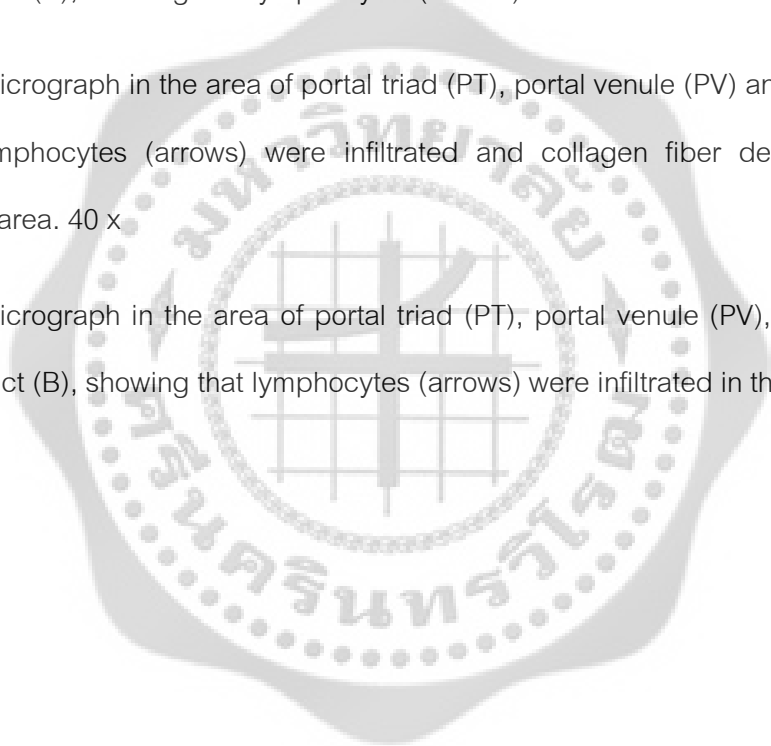
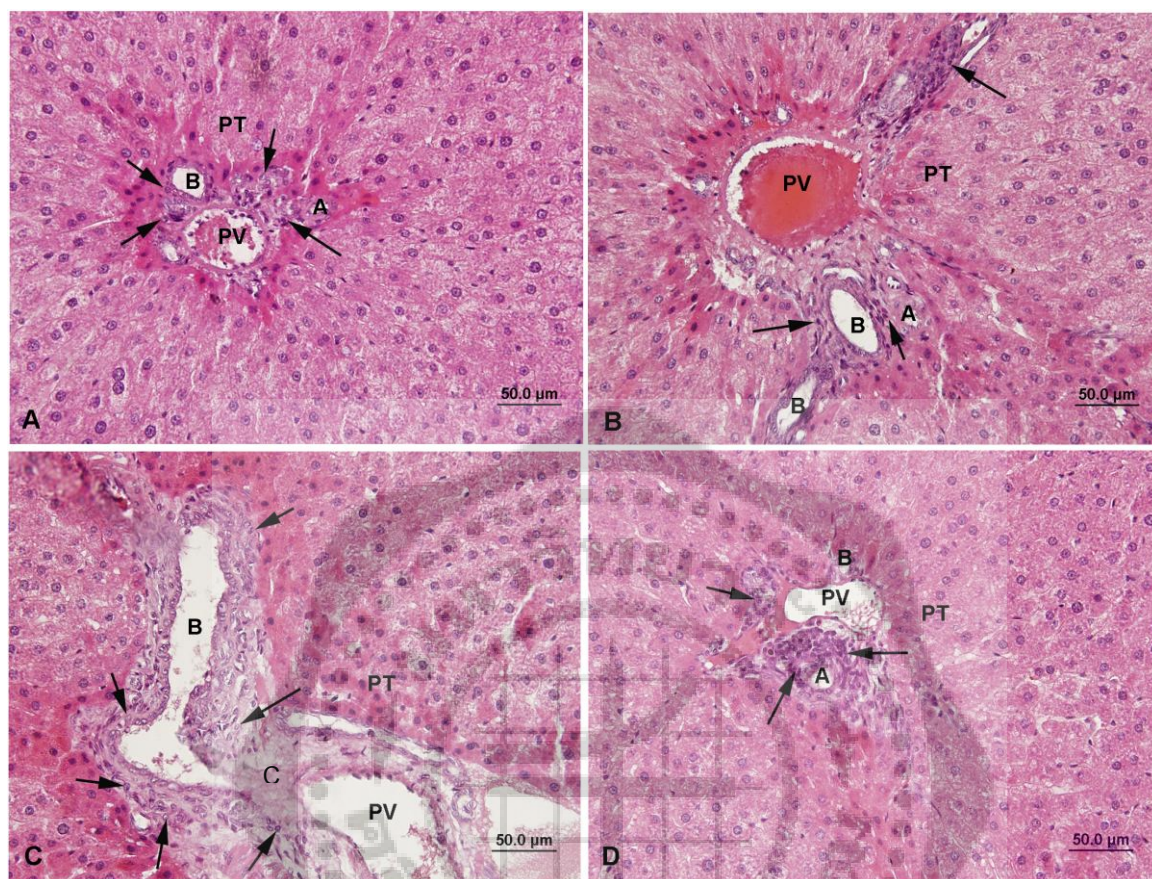




Figure 25



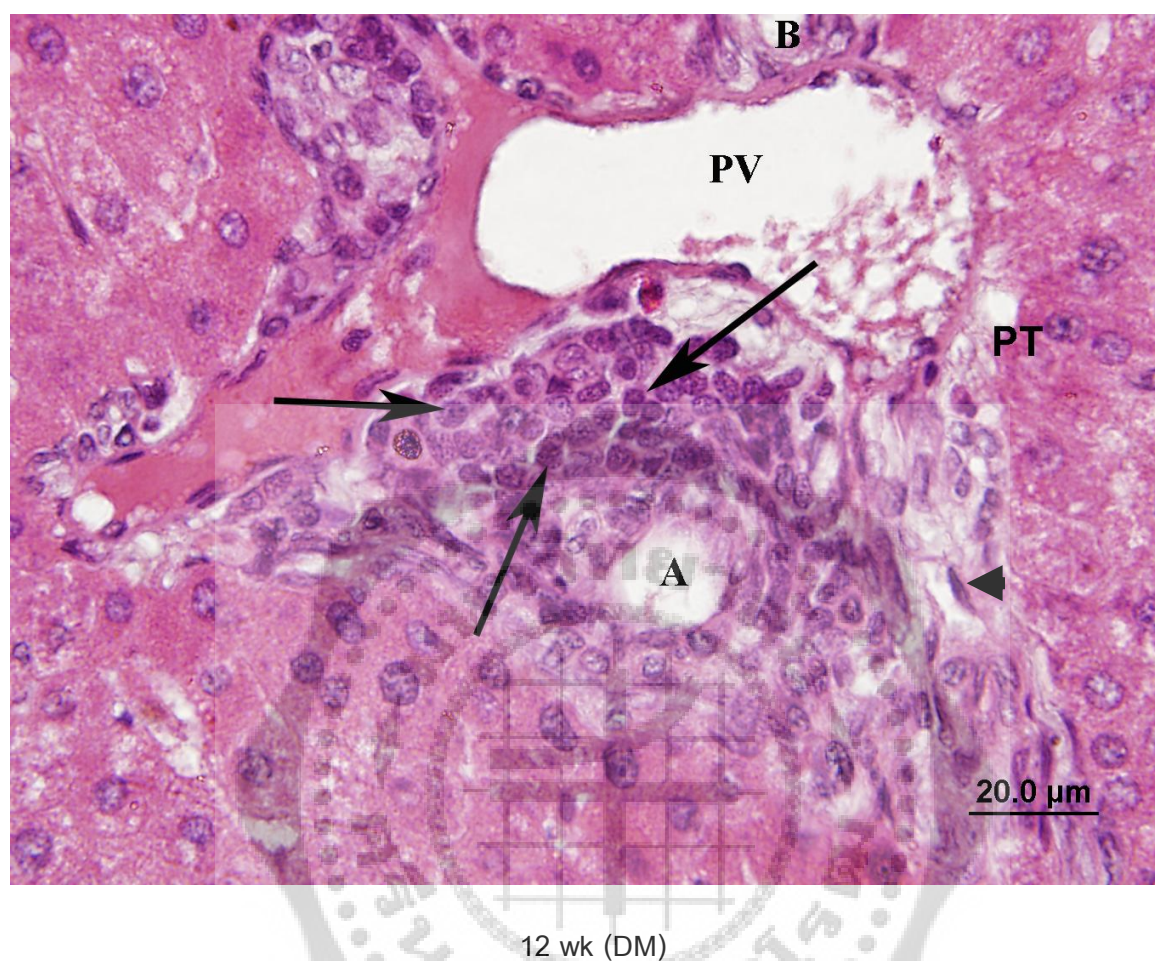
12 wk (DM)

**Figure 26** Light micrographs of the portal triad (PT) of liver tissue stained with H&E from DM rats at 12 week (12 wk, DM), showing that the lymphocytes (arrows) were infiltrated around the portal vein (PV), hepatic arteriole (A), and a bile duct (B) and showing hepatic stellate cells (arrowhead).100 x





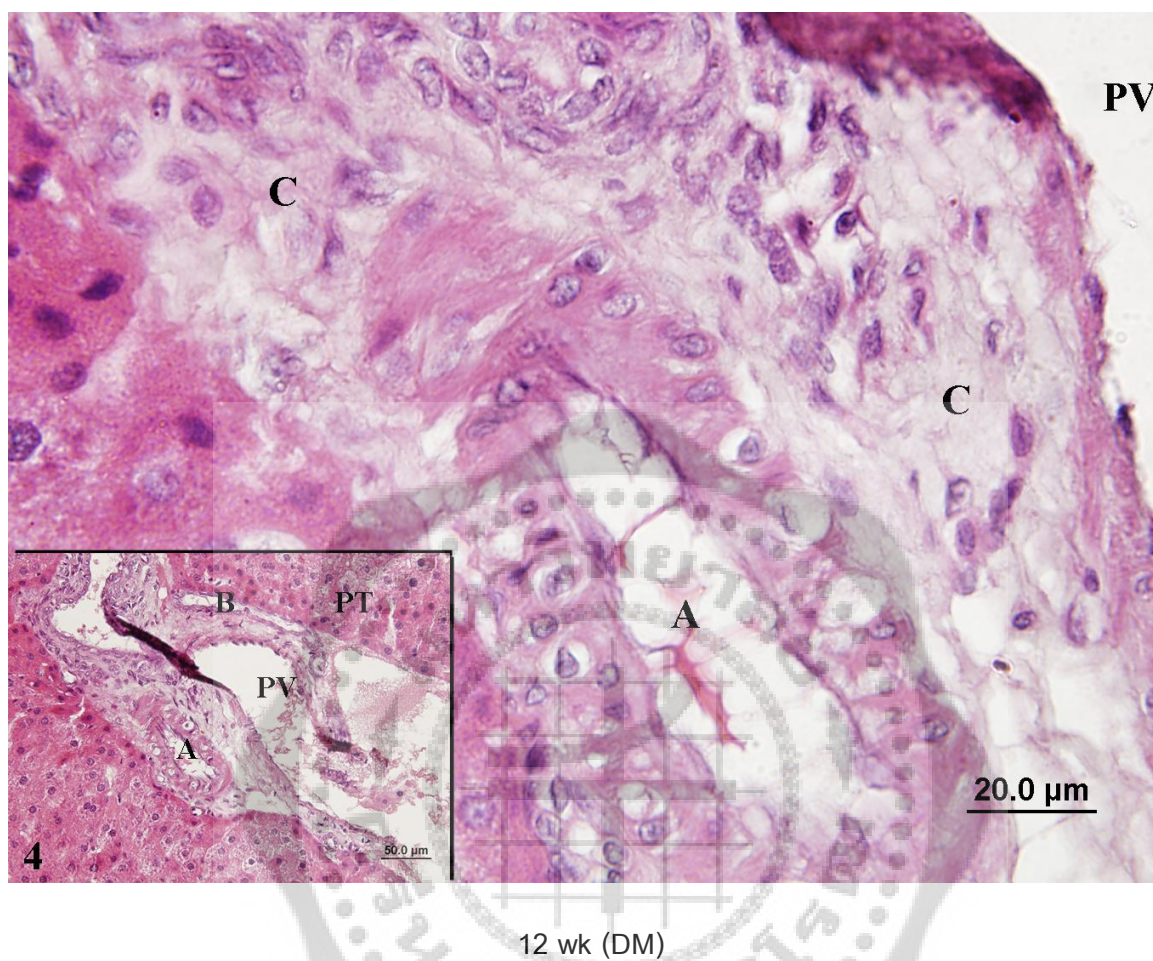
Figure 26



**Figure 27** Light micrographs of the portal triad (PT) of liver tissue stained with H&E from DM rats at 12 week (12 wk, DM), the high magnification of inset 4, showing that collagen fiber deposition (C) were infiltrated around the portal vein (PV), hepatic arteriole (A), and a bile duct (B).100 x



Figure 27

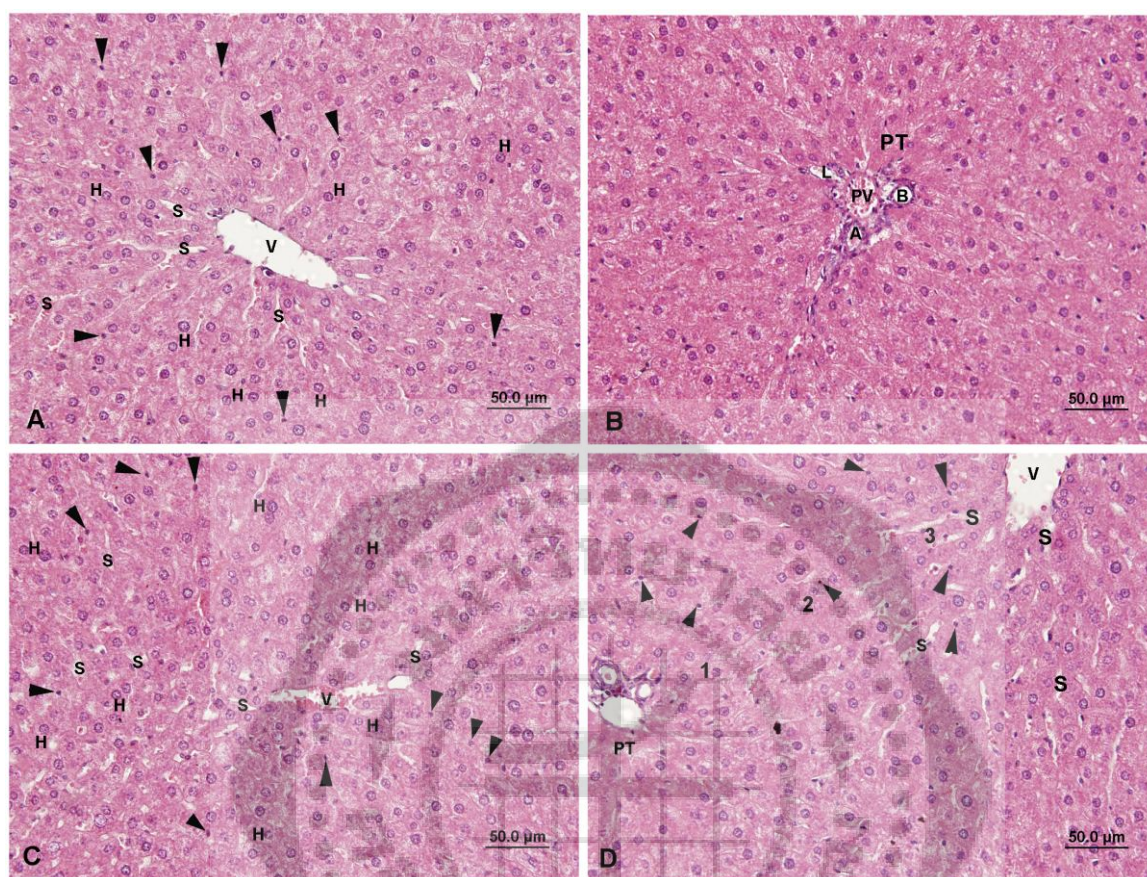


**Figure 28** Light micrographs of liver tissue stained with H&E from DMC rats at 12 week (12 wk, DMC)

- A Micrograph of the liver tissue, illustrating the hepatocytes (H), central veins (V), sinusoids (S), and pyknosis nuclei of hepatocytes (arrowheads). 40 x
- B Micrograph of the liver tissue, illustrating the portal triad (PT), portal venule (PV), hepatic arteriole (A), a bile duct (B) and lymphatic vessels (L). 40 x
- C Micrograph of the liver tissue, illustrating the hepatocytes (H), central veins (V), sinusoids (S), and pyknosis nuclei of hepatocytes (arrowheads). 40 x
- D Micrograph of the divided zone between two classic liver lobules, zone 1(1), Zone 2 (2), and Zone 3 (3) , central vein (V), portal triad (PT), blood sinusoids (S), and pyknosis nuclei of hepatocytes (arrowheads). 40 x



Figure 28



12 wk (DMC)

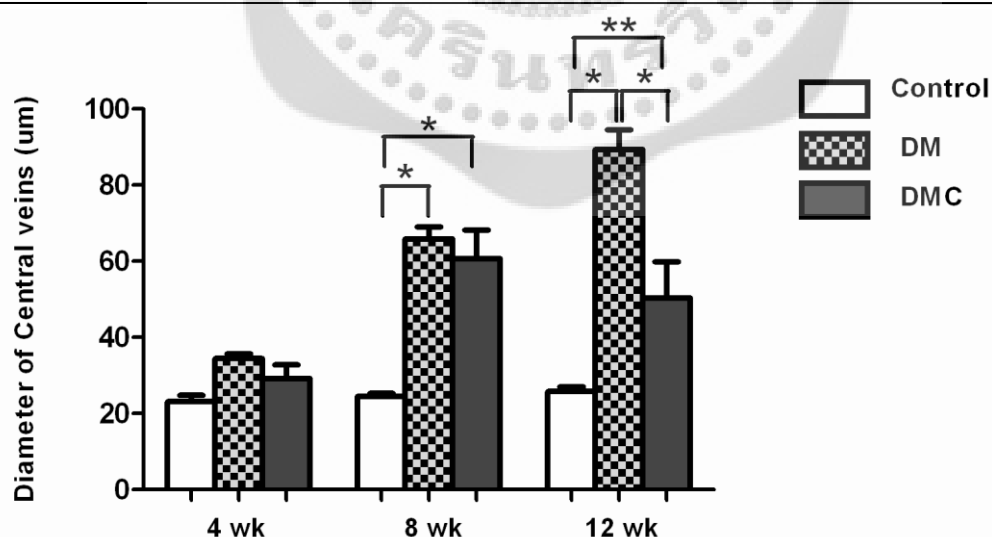
### 4.3 Quantitative analysis of diameter of central veins and sinusoids

#### 4.3.1 Diameter of central veins quantitative analysis

The diameter of central veins at Light microscopic levels were score quantitatively in a blind fashion in three groups of rats at different durations. The data were shown that the DM rats had significantly increased diameter of central veins indices when they were compared to control rats ( $p<0.0001$ ). These increased diameter of central veins indices in DM rats were significantly reduced by treatment with curcumin in DMC rats at 12 week ( $p<0.0001$ ) (TABLE 9 and Figure 29).

**TABLE 9** The diameter of central veins at light microscopic levels of control, DM, and DMC rats at 4, 8, and 12 weeks

| Weeks | Control          | DM               | DMC              |
|-------|------------------|------------------|------------------|
| 4     | 23.10 $\pm$ 1.70 | 34.43 $\pm$ 1.30 | 29.19 $\pm$ 3.63 |
| 8     | 24.54 $\pm$ 0.74 | 65.90 $\pm$ 3.18 | 60.71 $\pm$ 7.52 |
| 12    | 25.8 $\pm$ 1.26  | 89.36 $\pm$ 5.19 | 50.41 $\pm$ 9.47 |



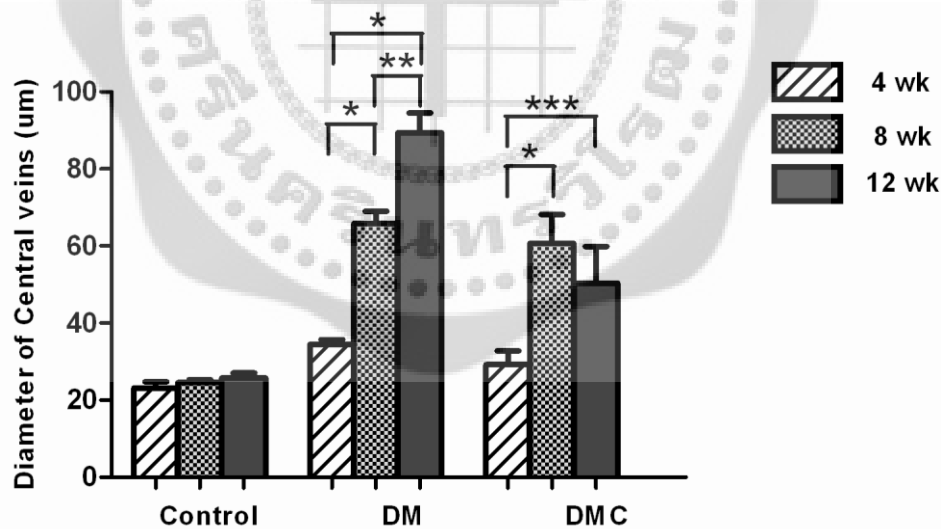
**Figure 29** Diagram of the diameter of central veins of control, DM, and DMC rats at 4, 8,

and 12 weeks. Values are means  $\pm$  SEM. \*  $p<0.0001$ , \*\*  $p<0.01$

TABLE 10 and Figure 30 information showed the diameter of central veins at light microscopic levels in three groups of rats at different durations in the same conditions. There was increased correlation with time dependent at 4, 8, and 12 weeks in DM rats. There were no significant changes at 4, 8, and 12 weeks of control rats. However, the diameters of DMC rats at 8 week was no significantly decreased when compared with DMC rats 12 week.

**TABLE 10** The diameter of central veins at light microscopic levels at 4, 8, and 12 weeks of control, DM, and DMC rats

| Groups  | 4 wk       | 8 wk       | 12 wk      |
|---------|------------|------------|------------|
| Control | 23.10±1.70 | 24.54±0.74 | 25.8±1.30  |
| DM      | 34.43±1.30 | 65.90±3.18 | 89.36±5.19 |
| DMC     | 29.19±3.63 | 60.71±7.52 | 50.41±9.47 |



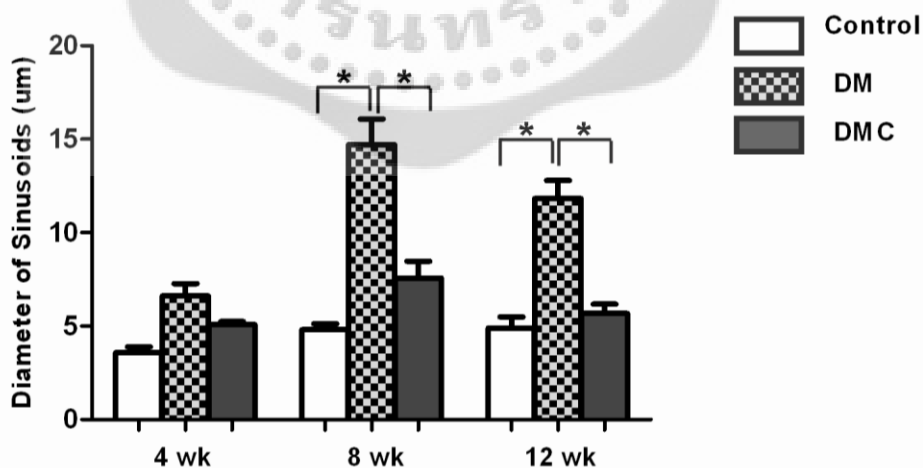
**Figure 30** Diagram of the diameter of central veins at 4, 8, and 12 weeks of control, DM, and DMC rats. Values are means  $\pm$  SEM. \*  $p<0.001$ , \*\*  $p<0.01$ , \*\*\*  $p<0.05$

#### 4.3.2 Diameter of sinusoids: quantitative analysis

The diameter of sinusoids at light microscopic levels were scored quantitatively in a blind fashion in three groups of rats at different durations. The data were shown that the DM rats had significantly increased in the diameter of sinusoids indices when they compared to control rats at 8 and 12 weeks ( $p<0.0001$ ). These increased diameter of sinusoids indices in DM rats were significantly reduced by treatment with curcumin in DMC rats at 8 and 12 week ( $p<0.0001$ ) (TABLE 11 and Figure 31).

**TABLE 11** The diameter of sinusoids at light microscopic levels of control, DM, and DMC rats at 4, 8, and 12 weeks

| Weeks | Control   | DM         | DMC       |
|-------|-----------|------------|-----------|
| 4     | 3.58±0.31 | 6.61±0.66  | 5.08±0.16 |
| 8     | 4.8±0.33  | 14.70±1.37 | 7.57±0.91 |
| 12    | 4.89±0.60 | 11.83±0.97 | 5.68±0.50 |



**Figure 31** Diagram of the diameter of sinusoids of control, DM, and DMC rats at 4, 8, and

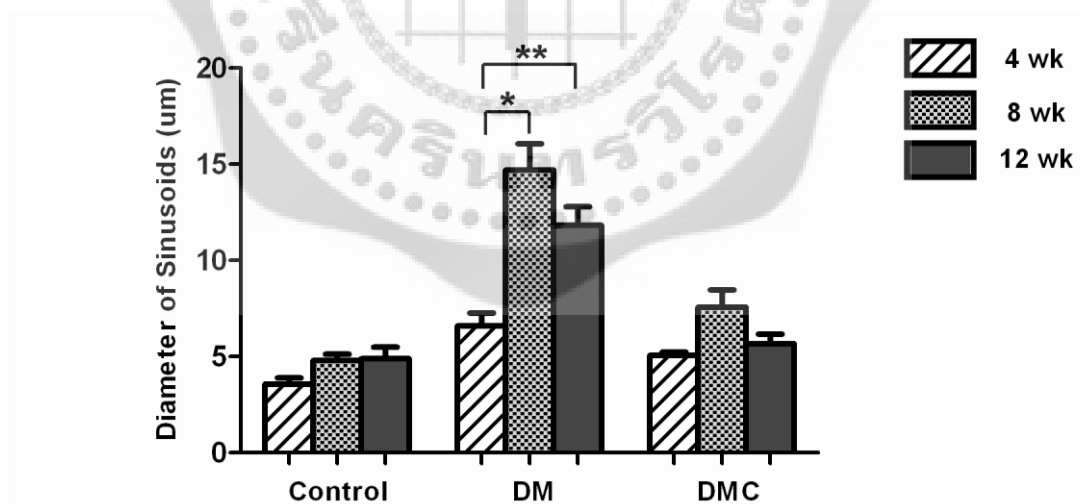
12 weeks. Values are means  $\pm$  SEM. \*  $p<0.0001$



TABLE 12 and Figure 32 information showed the diameter of sinusoids at light microscopic levels in three groups of rats at different durations in the same conditions. The diameter of sinusoids in DM rats at 8 week was significantly increased when compared with DM rats 4 week ( $p<0.0001$ ), however, there was no significantly reduction at 12 week when compared with DM rats 8 week ( $p<0.001$ ). There were no significantly changes at 4, 8, and 12 weeks of control and DMC rats.

**TABLE 12** The diameter of sinusoids at light microscopic levels at 4, 8, and 12 weeks of control, DM, and DMC rats

| Groups  | 4 wk      | 8 wk       | 12 wk      |
|---------|-----------|------------|------------|
| Control | 3.58±0.31 | 4.8±0.33   | 4.89±0.60  |
| DM      | 6.61±0.56 | 14.70±1.37 | 11.83±0.97 |
| DMC     | 5.08±0.16 | 7.57±0.91  | 5.68±0.50  |



**Figure 32** Diagram of the diameter of sinusoids at 4, 8, and 12 weeks of control, DM, and DMC rats. Values are means  $\pm$  SEM. \*  $p<0.0001$ , \*\*  $p<0.001$

#### 4.4 Effect of curcumin on VEGF immunoperoxidase staining in the liver of experimental diabetic rats

Immunohistochemical staining for VEGF demonstrated that VEGF was fundamentally recognized in the hepatocytes and endothelial cells of the central veins. In general, the localized VEGF was evidenced by brownish color in the cytoplasm and was shown to less extent in the area of portal triad. The intensity and pattern of this VEGF distribution and localization varied according to the duration of the experiments. Immunoreactivity cells with brownish color in the cytoplasm of hepatocytes and endothelial cells of the central veins were evaluated and compared from the liver tissue of control, DM, and DMC rats at 4, 8, and 12 weeks experiments.

##### 4.4.1 VEGF immunoreactivity in the liver tissue at 4 week

The VEGF immunoreactivity did not show brownish color of negative control in the liver tissue of control, DM, and DMC rats (Figure 33A-35A, respectively), whereas it was presented in positive reaction in the section that was incubated with monoclonal anti-VEGF (Figure 33B-35B, respectively). VEGF immunoreactivity in the liver tissue of control rats revealed mild intensity in the cytoplasm of hepatocytes and endothelial cells of central veins (Figure 33C-E) whereas weak immunoreactivity was found in the portal triad (Figure 33F).

In DM rats at 4 week, VEGF immunoreactivity in the liver tissue of DM rats also revealed mild intensity in the cytoplasm of hepatocytes and endothelial cells of central veins (Figure 34C-E) whereas weak immunoreactivity was found in the portal triad (Figure 34F).

After curcumin supplementation in DMC rats at 4 week, the intensity of VEGF with brownish color was diminished in cytoplasm of hepatocytes and endothelial cells of central veins (Figure 35C-E) whereas weak immunoreactivity was found in the portal triad (Figure 35F).

#### 4.4.2 VEGF immunoreactivity in the liver tissue at 8 week

In addition, the VEGF immunoreactivity did not show brownish color of negative control in the liver tissue of control, DM, and DMC rats at 8 week, the (Figure 36A-36A, respectively), whereas it was presented in positive reaction in the section that incubated with monoclonal anti-VEGF (Figure 36B-36B, respectively). VEGF immunoreactivity in the liver tissue of control rats revealed mild intensity in the cytoplasm of hepatocytes and endothelial cells of central veins (Figure 36C-E) whereas weak immunoreactivity was found in the portal triad (Figure 36F).

In the liver tissue of DM rats at 8 week showed strong intensity of VEGF immunostaining in the cytoplasm of hepatocytes and endothelial cells of central veins (Figure 37C-E) whereas mild immunoreactivity was found in the endothelial cells of portal venule of portal triad(Figure 37F).

Supplementation of curcumin in DMC rats at 8 week, the intensity of brownish color was decreased in the cytoplasm of hepatocytes and endothelial cells of central veins (Figure 38C-E) whereas weak immunoreactivity was found in the portal triad(Figure 38F).

#### 4.4.3 VEGF immunoreactivity in the liver tissue at 12 week

According to 12 week condition, the VEGF immunoreactivity did not show brownish color of negative control in the liver tissue of control, DM, and DMC rats (Figure 39A-41A, respectively), whereas it was presented in positive reaction in the section that was incubated with monoclonal anti-VEGF (Figure 39B-41B, respectively). VEGF immunoreactivity in the liver tissue of control rats revealed mild intensity in the cytoplasm of hepatocytes and endothelial cells of central veins (Figure 39C-E) whereas weak immunoreactivity was found in the portal triad (Figure 39F).

In the liver tissue of DM rats at 12 week showed more strong intensity of VEGF immunostaining in the cytoplasm of hepatocytes and endothelial cells of central veins (Figure 40C-E) whereas mild immunoreactivity was found in the endothelial cells of portal venule of portal triad(Figure 40F).

Supplementation of curcumin in DMC rats at 12 week, the intensity of brownish color was decreased in the cytoplasm of hepatocytes and endothelial cells of central veins (Figure 41C-E) whereas week immunoreactivity was found in the portal triad(Figure 41F).

#### 4.4.4 Comparison of VEGF immunoreactivity staining in the cytoplasm of hepatocytes and endothelial cells of central veins of control, DM, and DMC rats from 4, 8, and 12 week experiments

VEGF immunoreactivity of hepatocytes with brownish color in the cytoplasm and endothelial cells of central veins were compared from the liver tissues of control, DM, and DMC rats at 4, 8, and 12 weeks. It was demonstrated that VEGF staining in the cytoplasm of hepatocytes and endothelial cells of central veins of control rats at 4, 8, and 12 weeks revealed mild intensity (Figure 42A, D, and G respectively). Concerning the results of 4 week, VEGF immunoreactivity of DM (Figure 42B) and DMC rats (Figure 42C) revealed the mild intensity. VEGF intensity of DM and DMC rats showed no difference when compared with control rats. VEGF intensity of hepatocytes and endothelial cells of central veins at 4 week were  $162.33 \pm 0.53$  in control,  $163.91 \pm 0.63$  in DM, and  $162.85 \pm 0.61$  in DMC rats.

In contrast, the stronger immunoreactivity of VEGF in the hepatocytes and endothelial cells of central veins were found in DM rats at 8 week (Figure 42E). After curcumin treatment in DMC rats, the intensity of VEGF in the hepatocytes and endothelial cells of central veins were lowered (Figure 42F). VEGF intensity of the hepatocytes and endothelial cells of

central veins at 8 week were  $159.32 \pm 0.48$  in control,  $178.45 \pm 1.19$  in DM, and  $168.72 \pm 0.86$  in DMC rats.

Moreover, the intensity of VEGF in DM rats at 4 week showed marked increased staining in the hepatocytes and endothelial cells of central veins (Figure 42H), when compared with control and DMC rats. Administration of curcumin in DMC rats reduced the immunoreactivity of VEGF in the hepatocytes and endothelial cells of central veins (Figure 42I), VEGF intensity of the hepatocytes and endothelial cells of central veins at 12 week were  $161.25 \pm 0.60$  in control,  $185.16 \pm 1.20$  in DM, and  $175.95 \pm 0.67$  in DMC rats.

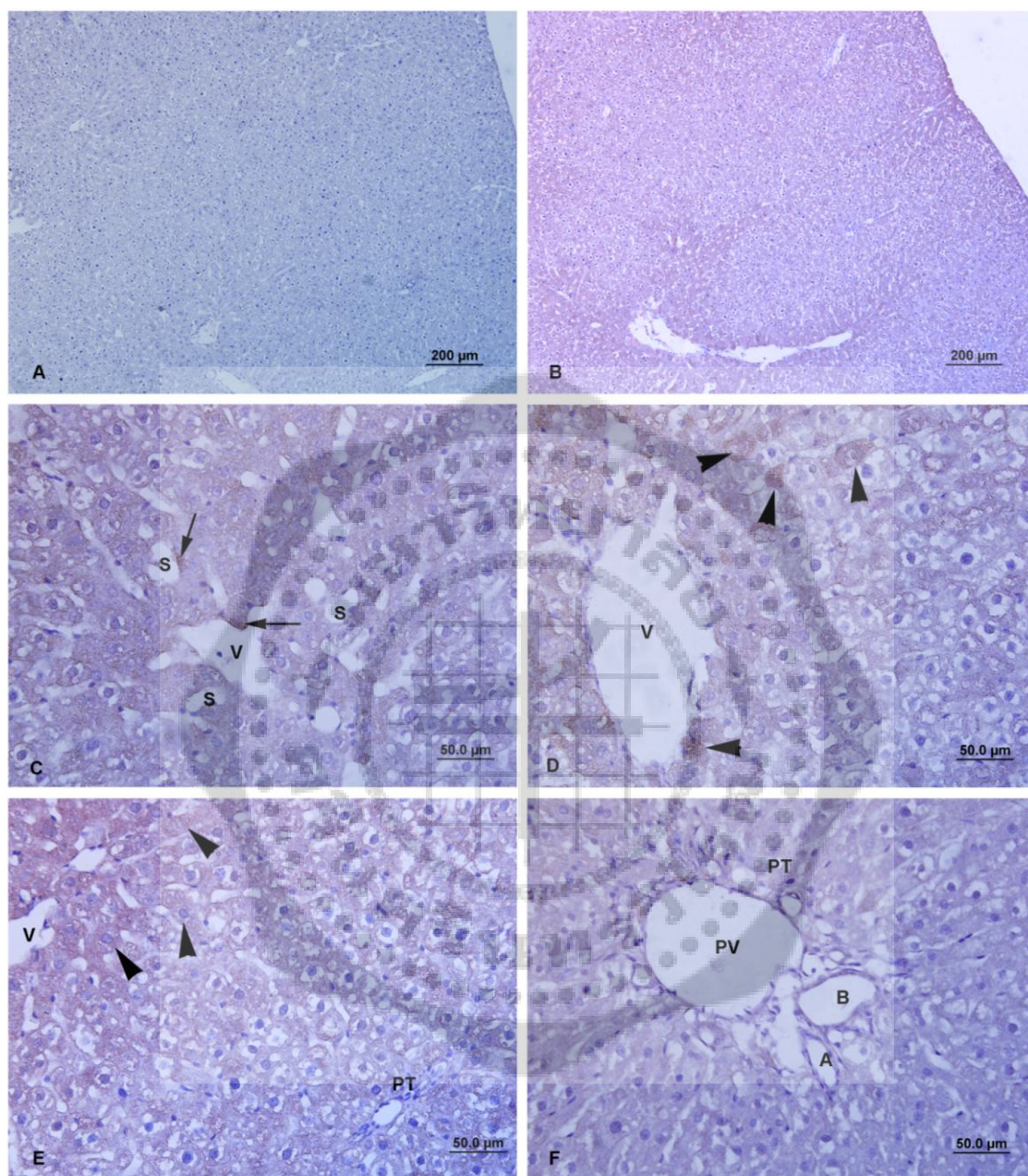
VEGF intensity in the hepatocytes and endothelial cells of central veins of DM, and DMC rats among 4, 8, and 12 weeks were compared. It was demonstrated that the VEGF intensity was strongly stained in DM rats at 12 week as compared with DM rats at 4 and 8 weeks. Moreover, the intensity of VEGF of the hepatocytes and endothelial cells of central veins of DMC 12 week were higher than DMC rats at 4 and 8 weeks.

**Figure 33** Immunoreactivity staining of VEGF in control rat livers at 4 week

(4 wk, Control)

- A-B Negative control (A) and positive reaction (B) of VEGF immunoreactivity in the liver tissue
- C Micrograph of the central vein (V) and blood sinusoids (S) of liver tissue, normally showing mild immunoreactivity staining of VEGF with brownish color in the endothelial cell of central veins (arrows). 40 x
- D Micrograph of the central vein of liver tissue, showing mild immunoreactivity staining of VEGF with brownish color in the hepatocytes nearly a central vein (arrowheads). 40 x
- E Micrograph of the area of zone 3 around a central vein (V) of liver tissue and portal triad (PT), showing mild immunoreactivity staining of VEGF with brownish color in the hepatocytes nearly a central vein (arrowheads). 40 x
- F Micrograph in the area of portal triad (PT), portal venule (PV), hepatic arteriole (A), and a bile duct (B), showing very mild immunoreactivity staining of VEGF with brownish color within the portal triad. 40 x

Figure 33



4 wk (Control)

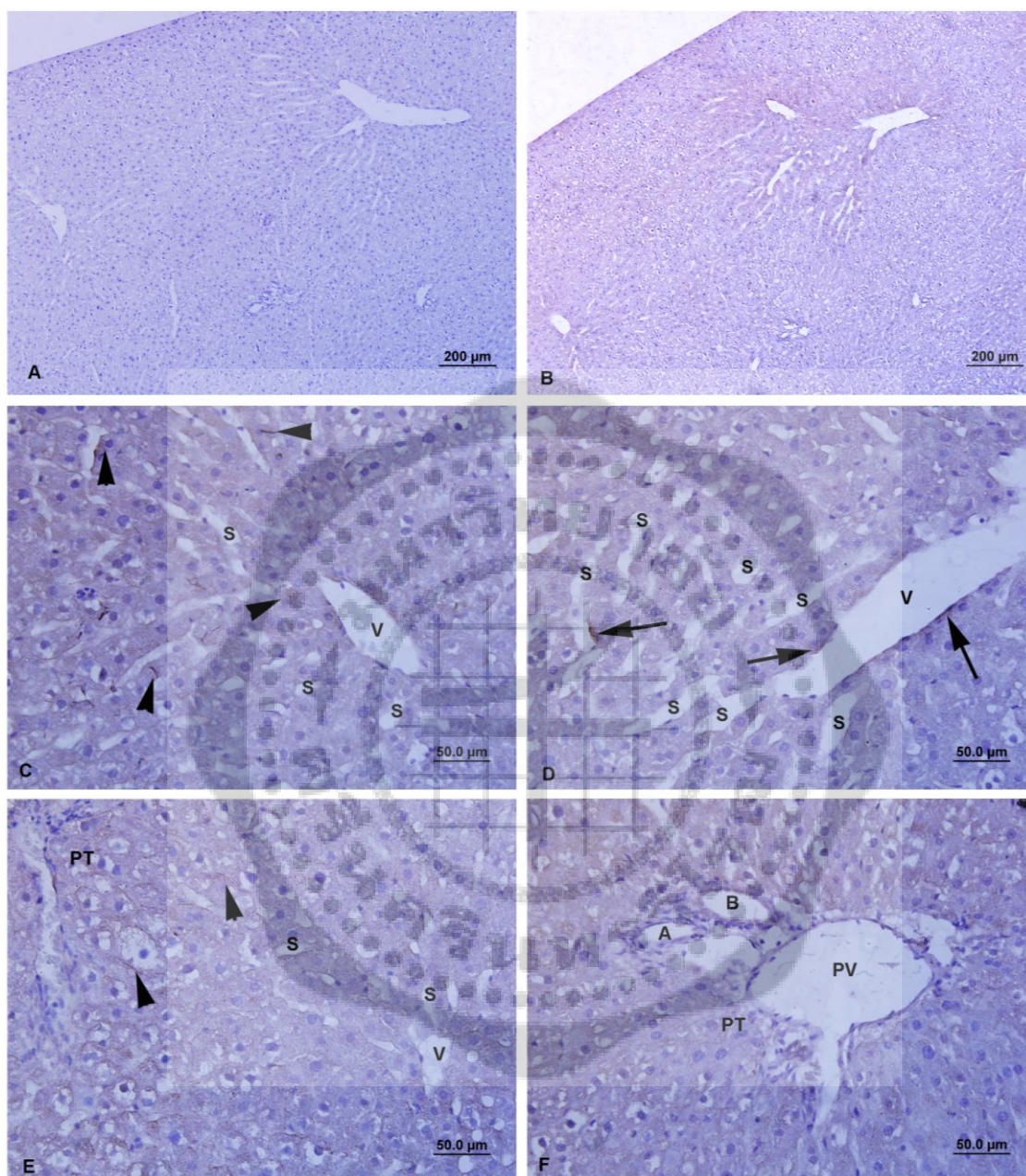
**Figure 34** Immunoreactivity staining of VEGF in DM rat livers at 4 week

(4 wk, DM)

- A-B Negative control (A) and positive reaction (B) of VEGF immunoreactivity in the liver tissue
- C Micrograph of the central vein and blood sinusoids (S) of liver tissue, normally showing mild immunoreactivity staining of VEGF with brownish color in the hepatocytes nearly a central vein (arrowheads). 40 x
- D Micrograph of the central vein (V) and blood sinusoids (S) of liver tissue, showing mild immunoreactivity staining of VEGF with brownish color in the endothelial cell of central veins (arrows). 40 x
- E Micrograph of the area of zone 3 around a central vein of liver tissue and portal triad (PT), showing mild immunoreactivity staining of VEGF with brownish color in the hepatocytes nearly a central vein (arrowheads). 40 x
- F Micrograph in the area of portal triad (PT), portal venule (PV), hepatic arteriole (A), and a bile duct (B), showing mild immunoreactivity staining of VEGF with brownish color within the portal triad. 40 x



Figure 34



4 wk (DM)

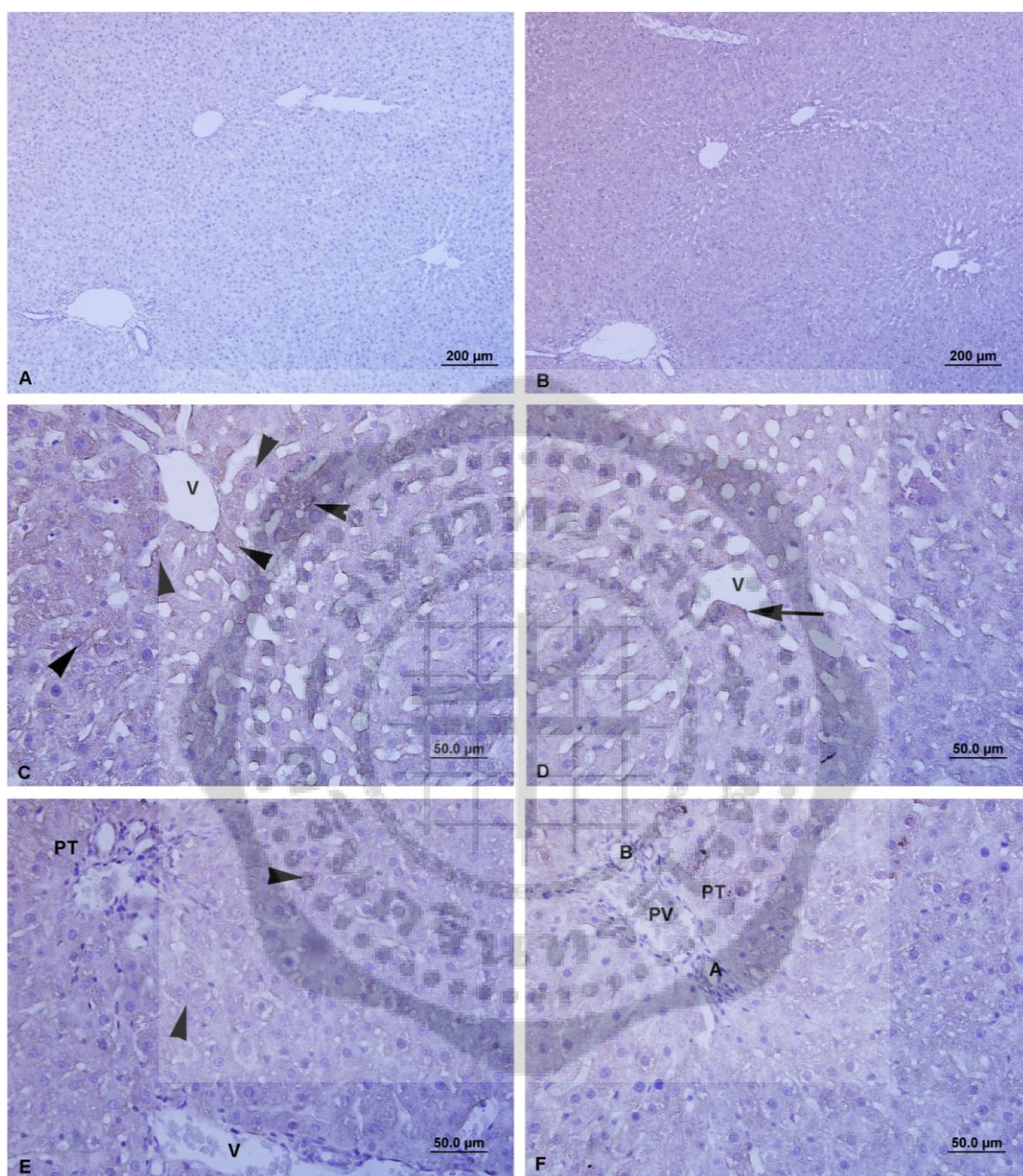
**Figure 35** Immunoreactivity staining of VEGF in DMC rat livers at 4 week

(4 wk, DMC)

- A-B Negative control (A) and positive reaction (B) of VEGF immunoreactivity in the liver tissue
- C Micrograph of the central vein and blood sinusoids (S) of liver tissue, normally showing mild immunoreactivity staining of VEGF with brownish color in the hepatocytes nearly a central vein (arrowheads). 40 x
- D Micrograph of the central vein (V) of liver tissue, showing mild immunoreactivity staining of VEGF with brownish color in the endothelial cell of central vein (arrow). 40 x
- E Micrograph of the area of zone 3 around a central vein of liver tissue and portal triad (PT), showing mild immunoreactivity staining of VEGF with brownish color in the hepatocytes nearly a central vein (arrowheads). 40 x
- F Micrograph in the area of portal triad (PT), portal venule (PV), hepatic arteriole (A), and a bile duct (B), showing mild immunoreactivity staining of VEGF with brownish color within the portal triad. 40 x



Figure 35



4 wk (DMC)

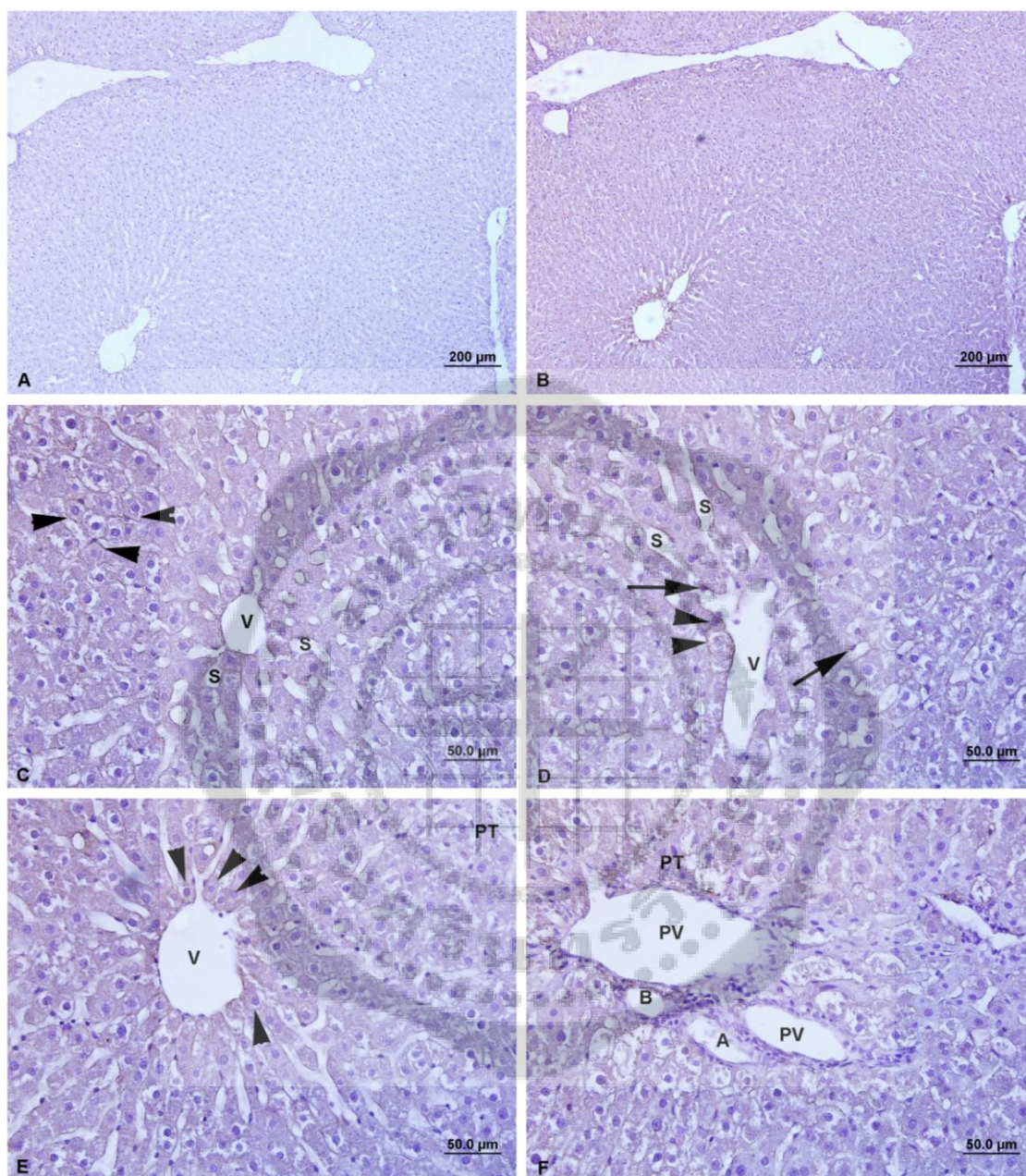
**Figure 36** Immunoreactivity staining of VEGF in control rat livers at 8 week

(8 wk, Control)

- A-B Negative control (A) and positive reaction (B) of VEGF immunoreactivity in the liver tissue
- C Micrograph of the central vein (V) and blood sinusoids (S) of liver tissue, normally showing mild immunoreactivity staining of VEGF with brownish color in the hepatocytes around a central veins (arrows). 40 x
- D Micrograph of the central vein of liver tissue, showing mild immunoreactivity staining of VEGF with brownish color in the hepatocytes nearly a central vein (V) (arrows) and the endothelial cells of central vein and sinusoid(S) (arrowheads). 40 x
- E Micrograph of the area of zone 3 around a central vein (V) of liver tissue, showing mild immunoreactivity staining of VEGF with brownish color in the hepatocytes nearly a central vein (arrowheads). 40 x
- F Micrograph in the area of portal triad (PT), portal venule (PV), hepatic arteriole (A), and a bile duct (B), showing very mild immunoreactivity staining of VEGF with brownish color within the portal triad. 40 x



Figure 36



8 wk (Control)

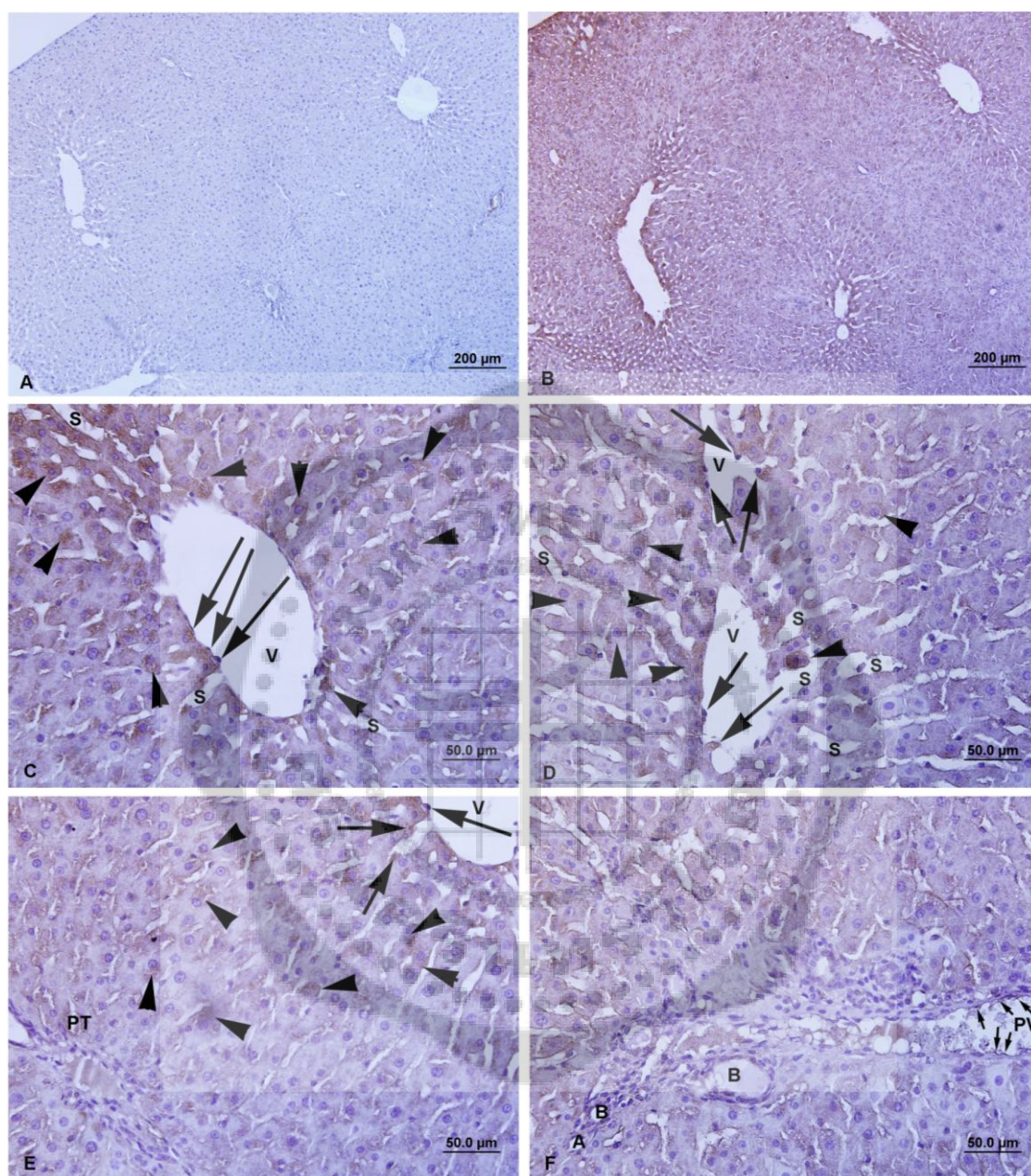
**Figure 37** Immunoreactivity staining of VEGF in DM rat livers at 8 week

(8 wk, DM)

- A-B Negative control (A) and positive reaction (B) of VEGF immunoreactivity in the liver tissue
- C Micrograph of the central vein (V) and blood sinusoids (S) of liver tissue, showing strong immunoreactivity staining of VEGF with brownish color in the hepatocytes nearly a central vein (arrowheads) and endothelial cells of central veins (arrows). 40 x
- D Micrograph of the central vein (V) and blood sinusoids (S) of liver tissue, showing strong immunoreactivity staining of VEGF with brownish color in the hepatocytes nearly a central vein (arrowheads) and endothelial cells of central veins (arrows). 40 x
- E Micrograph of the area of zone 3, 2 and 1 around a central vein of liver tissue and portal triad (PT), showing strong immunoreactivity staining of VEGF with brownish color in the hepatocytes nearly a central vein (arrowheads) and endothelial cells of central vein and sinusoid (arrows). 40 x
- F Micrograph in the area of portal triad (PT), portal venule (PV), hepatic arteriole (A), and a bile duct (B), showing mild immunoreactivity staining of VEGF with brownish color within the portal triad and endothelial cells of portal venule. 40 x



Figure 37



8 wk (DM)

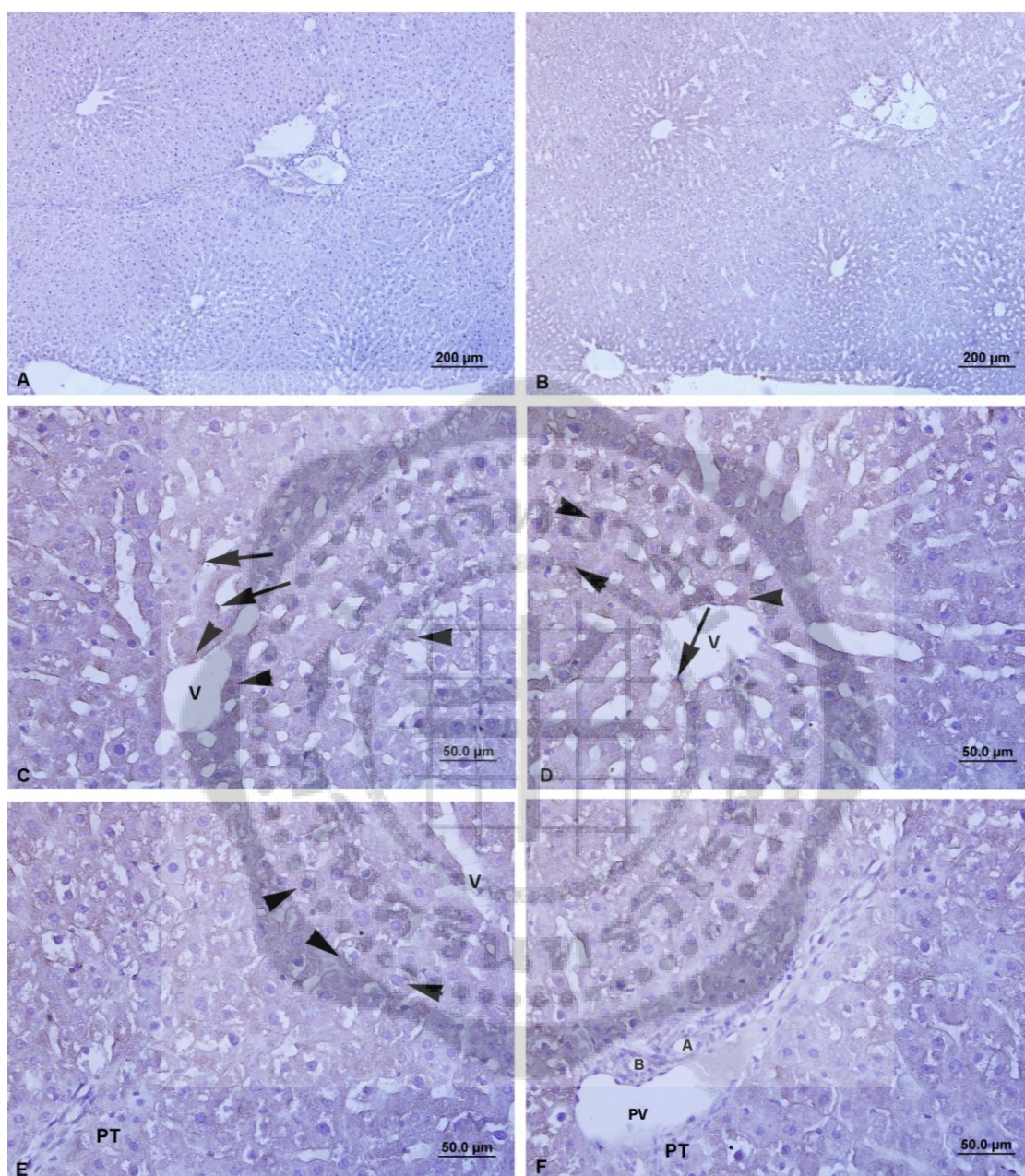
**Figure 38** Immunoreactivity staining of VEGF in DMC rat livers at 8 week

(8 wk, DMC)

- A-B Negative control (A) and positive reaction (B) of VEGF immunoreactivity in the liver tissue
- C Micrograph of the central vein (V) and blood sinusoids (S) of liver tissue, showing mild immunoreactivity staining of VEGF with brownish color in the hepatocytes nearly a central vein (arrowheads) and endothelial cells of sinusoids (arrows). 40 x
- D Micrograph of the central vein (V) of liver tissue, showing mild immunoreactivity staining of VEGF with brownish color in the hepatocytes nearly a central vein (arrowheads) and endothelial cell of central vein (arrow). 40 x
- E Micrograph of the area of zone 3 around a central vein of liver tissue and portal triad (PT), showing mild immunoreactivity staining of VEGF with brownish color in the hepatocytes nearly a central vein (arrowheads). 40 x
- F Micrograph in the area of portal triad (PT), portal venule (PV), hepatic arteriole (A), and a bile duct (B), showing mild immunoreactivity staining of VEGF with brownish color within the portal triad. 40 x



Figure 38



8 wk (DMC)

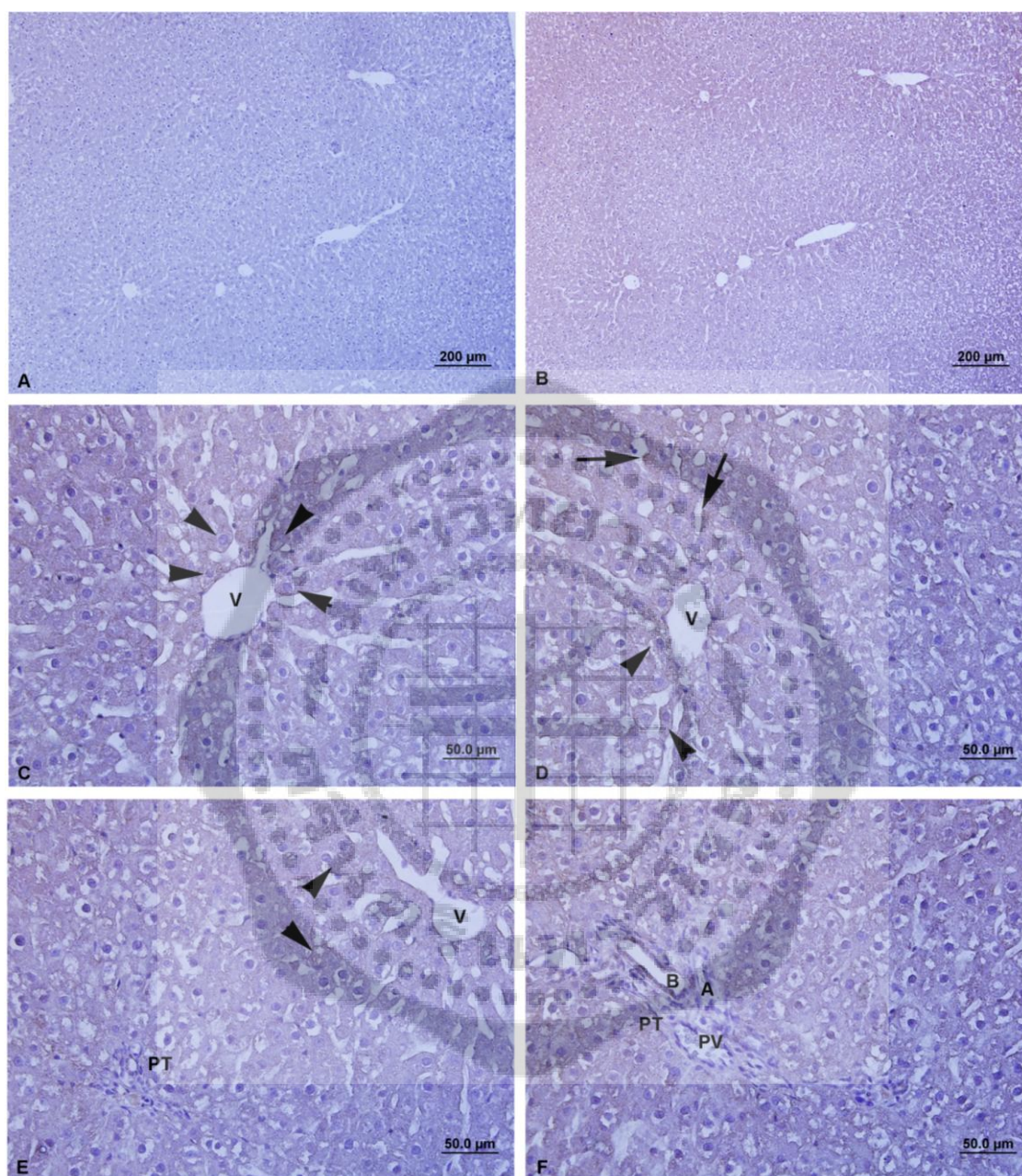
**Figure 39** Immunoreactivity staining of VEGF in control rat livers at 12 week

(12 wk, Control)

- A-B Negative control (A) and positive reaction (B) of VEGF immunoreactivity in the liver tissue
- C Micrograph of the central vein (V) and blood sinusoids of liver tissue, normally showing mild immunoreactivity staining of VEGF with brownish color in the hepatocytes around a central veins (arrows). 40 x
- D Micrograph of the central vein (V) of liver tissue, showing mild immunoreactivity staining of VEGF with brownish color in the hepatocytes nearly a central vein (arrowheads) and the endothelial cells of sinusoids (S) (arrows). 40 x
- E Micrograph of the area of zone 3 around a central vein (V) of liver tissue, showing mild immunoreactivity staining of VEGF with brownish color in the hepatocytes nearly a central vein (arrowheads). 40 x
- F Micrograph in the area of portal triad (PT), portal venule (PV), hepatic arteriole (A), and a bile duct (B), showing very mild immunoreactivity staining of VEGF with brownish color within the portal triad. 40 x



Figure 39



12 wk (Control)

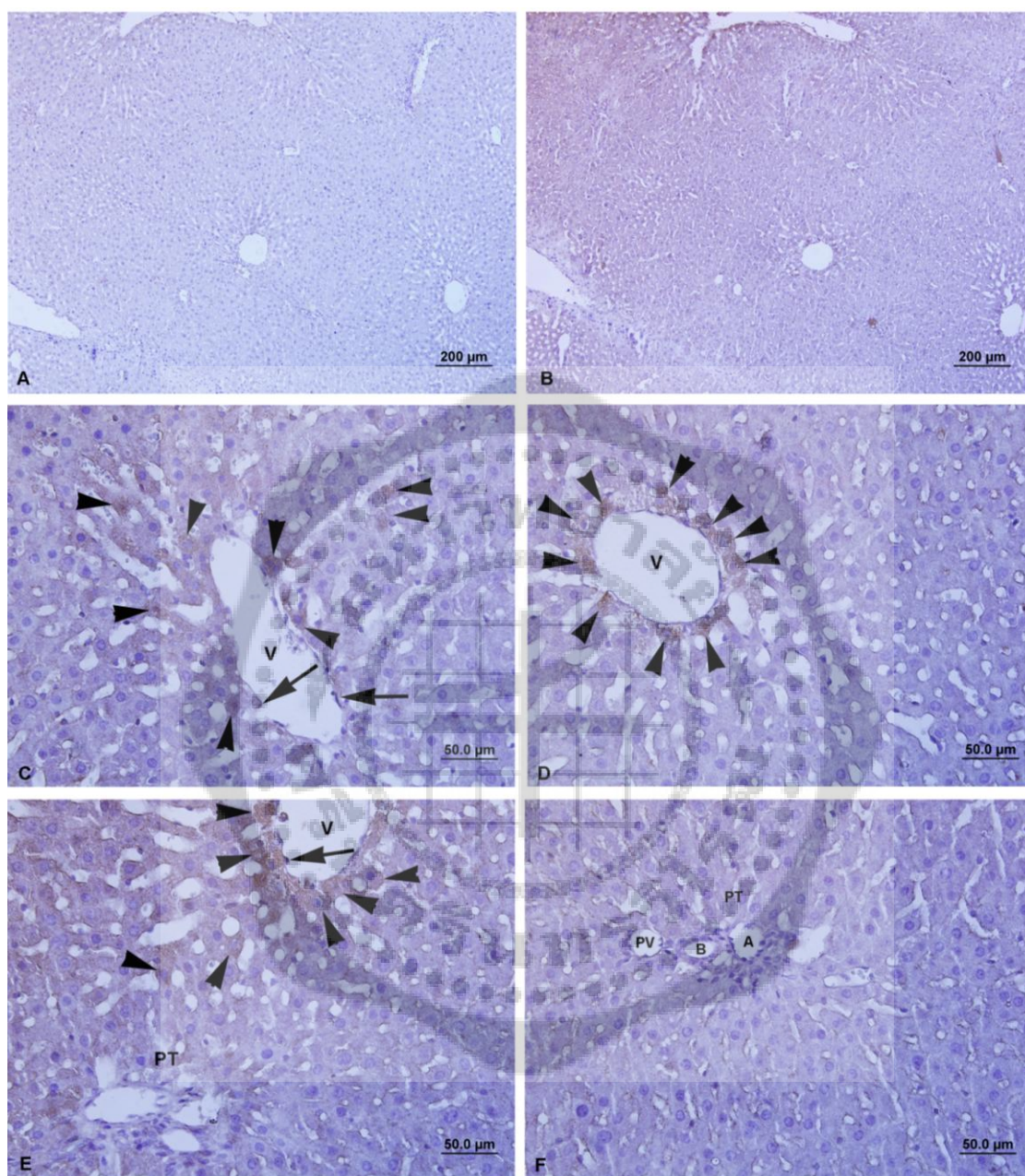
**Figure 40** Immunoreactivity staining of VEGF in DM rat livers at 12 week

(12 wk, DM)

- A-B Negative control (A) and positive reaction (B) of VEGF immunoreactivity in the liver tissue
- C Micrograph of the central vein (V) and blood sinusoids (S) of liver tissue, showing very strong immunoreactivity staining of VEGF with dark brownish color in the hepatocytes nearly a central vein (arrowheads) and endothelial cells of central veins (arrows). 40 x
- D Micrograph of the central vein (V) and blood sinusoids of liver tissue, showing very strong immunoreactivity staining of VEGF with dark brownish color in the hepatocytes around a central vein (arrowheads). 40 x
- E Micrograph of the area of zone 3 , 2 and 1 around a central vein of liver tissue and portal triad (PT), showing very strong immunoreactivity staining of VEGF with brownish color in the hepatocytes nearly a central vein (arrowheads) and endothelial cell of central vein (arrows). 40 x
- F Micrograph in the area of portal triad (PT), portal venule (PV), hepatic arteriole (A), and a bile duct (B), showing mild immunoreactivity staining of VEGF with brownish color within the portal triad and endothelial cells of portal venule. 40 x



Figure 40



12 wk (DM)

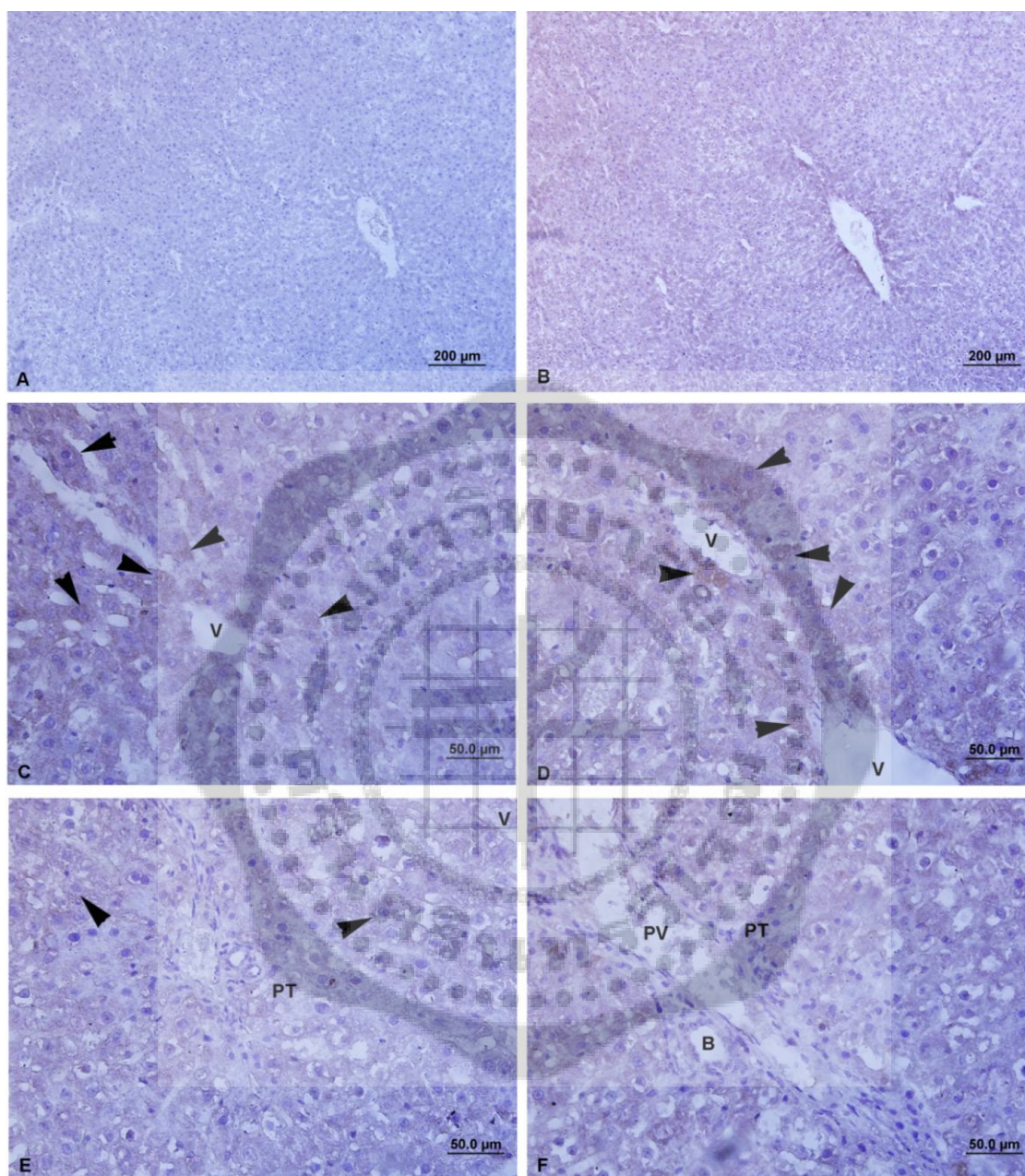
**Figure 41** Immunoreactivity staining of VEGF in DMC rat livers at 12 week

(12 wk, DMC)

- A-B Negative control (A) and positive reaction (B) of VEGF immunoreactivity in the liver tissue
- C Micrograph of the central vein (V) and blood sinusoids (S) of liver tissue, showing mild immunoreactivity staining of VEGF with brownish color in the hepatocytes nearly a central vein (arrowheads). 40 x
- D Micrograph of the central vein (V) and blood sinusoids (S) of liver tissue, showing mild immunoreactivity staining of VEGF with brownish color in the hepatocytes nearly a central vein (arrowheads). 40 x
- E Micrograph of the area of zone 3 around a central vein of liver tissue and portal triad (PT), showing mild immunoreactivity staining of VEGF with brownish color in the hepatocytes nearly a central vein (arrowheads). 40 x
- F Micrograph in the area of portal triad (PT), portal venule (PV) and a bile duct (B), showing mild immunoreactivity staining of VEGF with brownish color within the portal triad. 40 x



Figure 41



12 wk (DMC)



**Figure 42** Morphologic comparison of the VEGF Immunoreactivity staining in the area of central veins (v) among three groups of rats

A-C Immunostaining of the area of central veins from control, DM, and DMC rats at 4 week, respectively

D-F Immunostaining of the area of central veins from control, DM, and DMC rats at 8 week, respectively

G-I Immunostaining of the area of central veins from control, DM, and DMC rats at 12 week, respectively

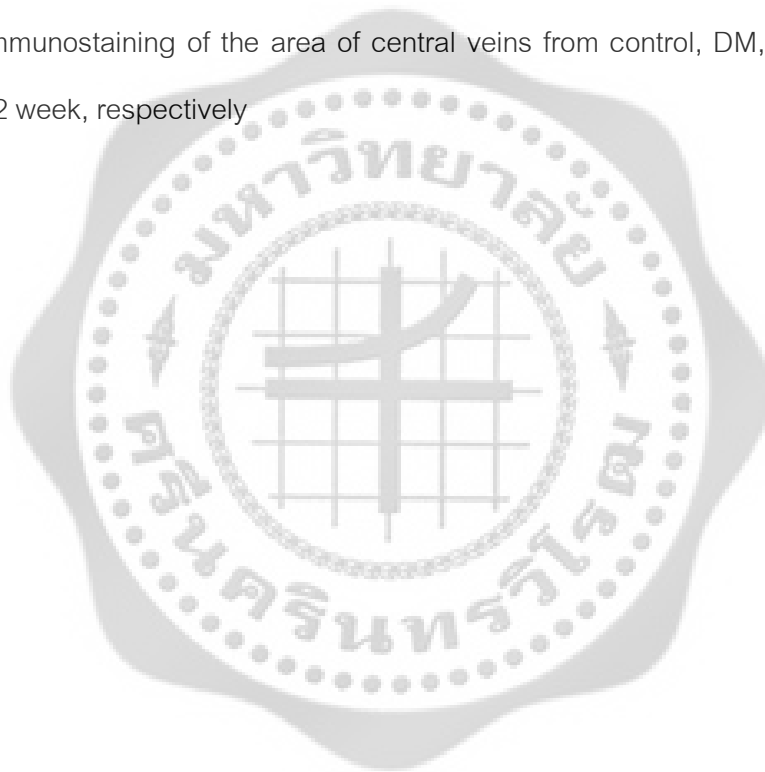
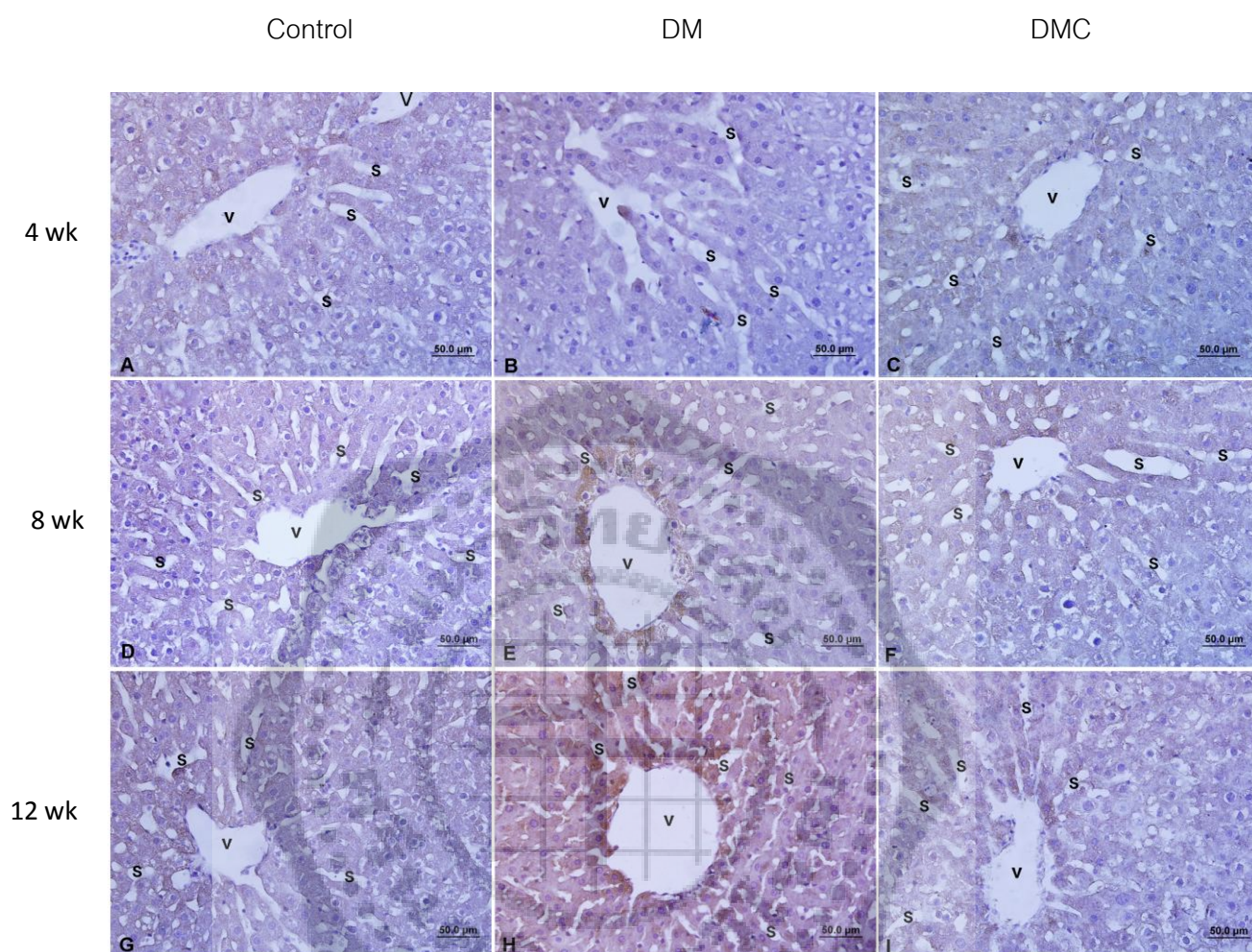
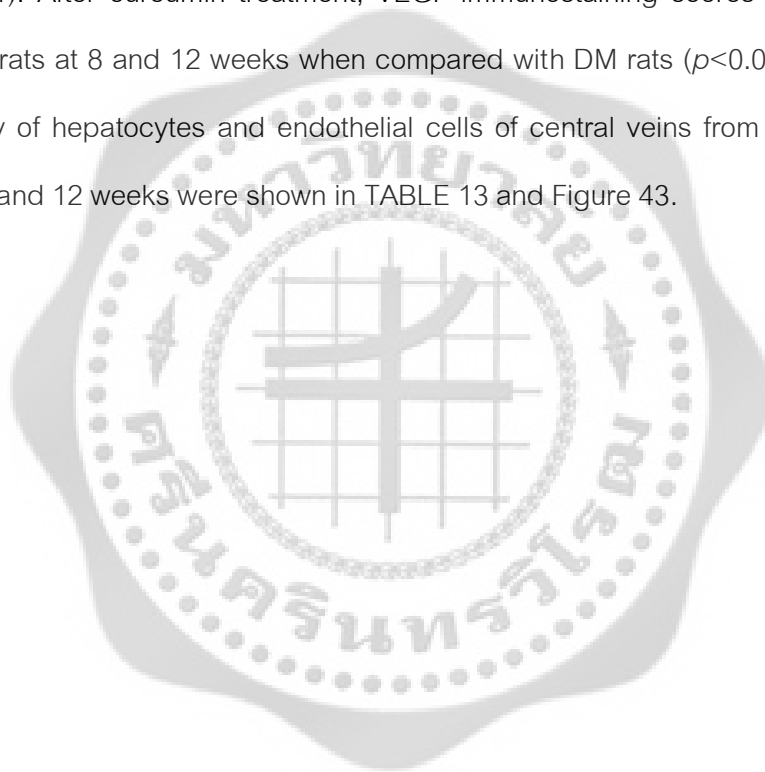


Figure 42



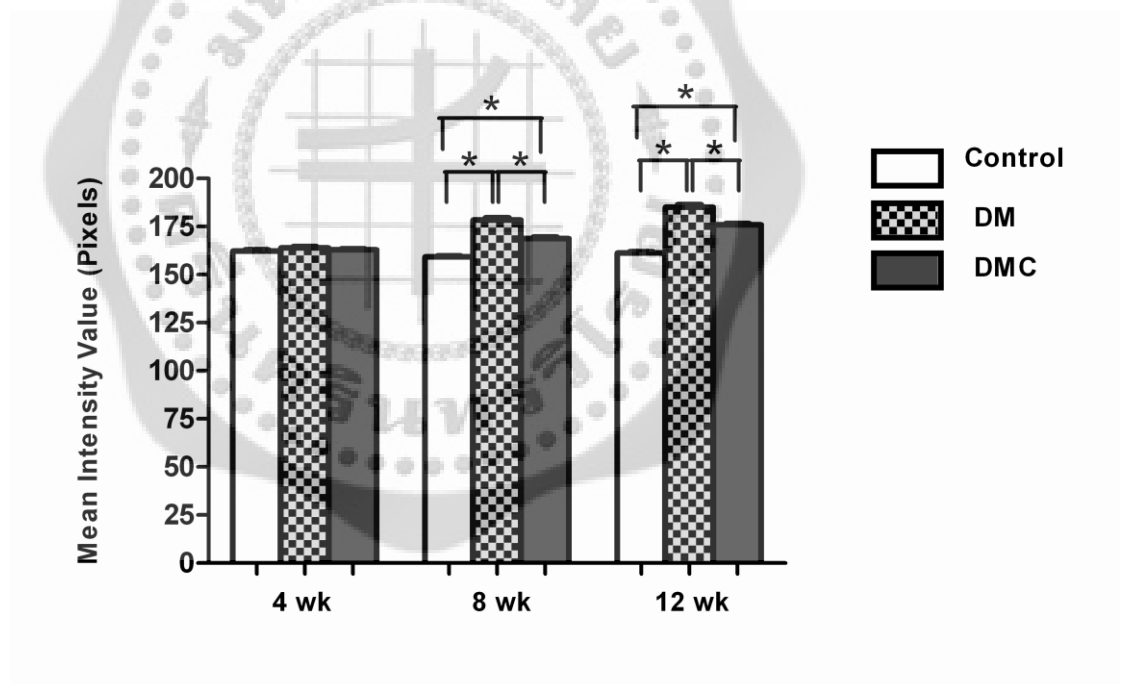
#### 4.5 Quantitative analysis for VEGF immunostaining in the cytoplasm of hepatocytes and endothelial cells of central veins of control, DM, and DMC rats from 4, 8, and 12 week experiments

By Quantitative scoring, there were no different in VEGF intensity among control, DM, and DMC rats at 4 week. The significantly elevated VEGF stainings of hepatocytes and endothelial cells of central veins were observed in all DM compared with control rats at 8 and 12 weeks ( $p<0.0001$ ). After curcumin treatment, VEGF immunostaining scores were significantly lowered in DMC rats at 8 and 12 weeks when compared with DM rats ( $p<0.0001$ ). The scoring of VEGF intensity of hepatocytes and endothelial cells of central veins from control, DM, and DMC rats at 4, 8, and 12 weeks were shown in TABLE 13 and Figure 43.



**TABLE 13** Mean intensity value of the VEGF immunostaining of hepatocytes and endothelial cells of central veins at 4, 8, and 12 weeks of control, DM, and DMC rats

| Weeks | Control           | DM                | DMC               |
|-------|-------------------|-------------------|-------------------|
| 4     | 162.33 $\pm$ 0.53 | 163.91 $\pm$ 0.63 | 162.85 $\pm$ 0.61 |
| 8     | 159.32 $\pm$ 0.48 | 178.45 $\pm$ 1.19 | 168.72 $\pm$ 0.86 |
| 12    | 161.25 $\pm$ 0.60 | 185.16 $\pm$ 1.20 | 175.95 $\pm$ 0.67 |



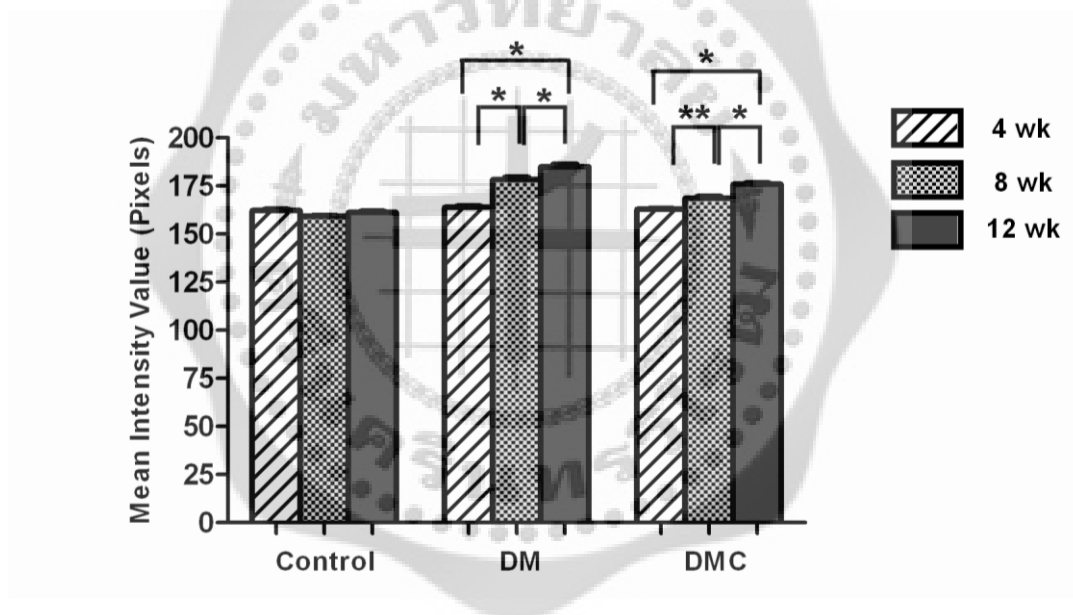
**Figure 43** Mean intensity value of the VEGF immunostaining of area of central veins at 4, 8, and 12 weeks of control, DM, and DMC rats. Values are means  $\pm$  SEM. \*  $p < 0.0001$

VEGF immunostaining score was not significantly changed in control at different timing points. VEGF immunostaining score was significantly increased in DM rats at 12 week when compared with 4 and 8 weeks ( $p<0.001$ ). VEGF intensity of hepatocytes and endothelial cells of central veins in DMC rats were more intense with time duration. The score of DMC rats at 8 and 12 weeks were shown significantly different from 4 week ( $p<0.001$ ,  $p<0.0001$  respectively). Data was shown in TABLE 14 and Figure 44.



**TABLE 14** Mean intensity value of the VEGF immunostaining of hepatocytes and endothelial cells of central veins from control, DM, and DMC rats at 4, 8, and 12 weeks

| Groups  | 4 wk        | 8 wk        | 12 wk       |
|---------|-------------|-------------|-------------|
| Control | 162.33±0.53 | 159.32±0.48 | 161.25±0.60 |
| DM      | 163.91±0.63 | 178.45±1.19 | 185.16±1.20 |
| DMC     | 162.85±0.61 | 168.72±0.86 | 175.95±0.67 |



**Figure 44** Mean intensity value of the VEGF immunostaining of area of central veins from control, DM, and DMC rats at 4, 8, and 12 weeks. Values are means  $\pm$  SEM. \*  $p < 0.0001$ , \*\*  $p < 0.001$

## 4.6 SDS-PAGE and Western blot analysis

SDS-PAGE and Western blot analysis were used as molecular techniques for characterization of VEGF protein in the livers of control, DM, and DMC rats at 4, 8, and 12 weeks. In SDS-PAGE analysis, the 10 µg of extracted liver protein in each group from Bradford protein assay were separately electrophoresis on 12% SDS-PAGE. The nitrocellulose membranes were stained by Ponceau's solution and showed many bands of total protein samples. Western blot analysis established the specific protein on membrane by using monoclonal anti-VEGF antibody and goat-anti mouse horseradish peroxidase IgG secondary antibody as specific antibody. In negative control, monoclonal anti-VEGF was used as primary negative control and goat-anti mouse horseradish peroxidase IgG secondary antibody was used as secondary negative control to confirm the results of this study respectively.

### 4.6.1 Characteristic of VEGF protein in the liver at 4 week

#### SDS-PAGE analysis of VEGF protein

The protein extracted from livers of control, DM, and DMC rats at 4 week were separated on 12% SDS-PAGE as following sequences: 6 µl of ECL marker (ECLM) (Lane 1), In order to measure protein concentration, the method of Bradford was used for determining concentration of solubilized protein, 10 µg of protein extracted from control (Lane 2), 10 µg of protein extracted from DM (Lane 3), and 10 µg of protein extracted from DMC (Lane 4)

#### Western blot analysis of VEGF protein

The interested VEGF protein band was observed and demonstrated by using monoclonal anti-VEGF antibody. Western blot analysis established the specific VEGF protein band at molecular weight 23 kDa on film by Enhanced chemiluminescence (ECL) method in control, DM, and DMC samples from rats liver at 4 week (Figure 45A). This specific protein represented VEGF protein. In addition, the positive control expression and characterization of



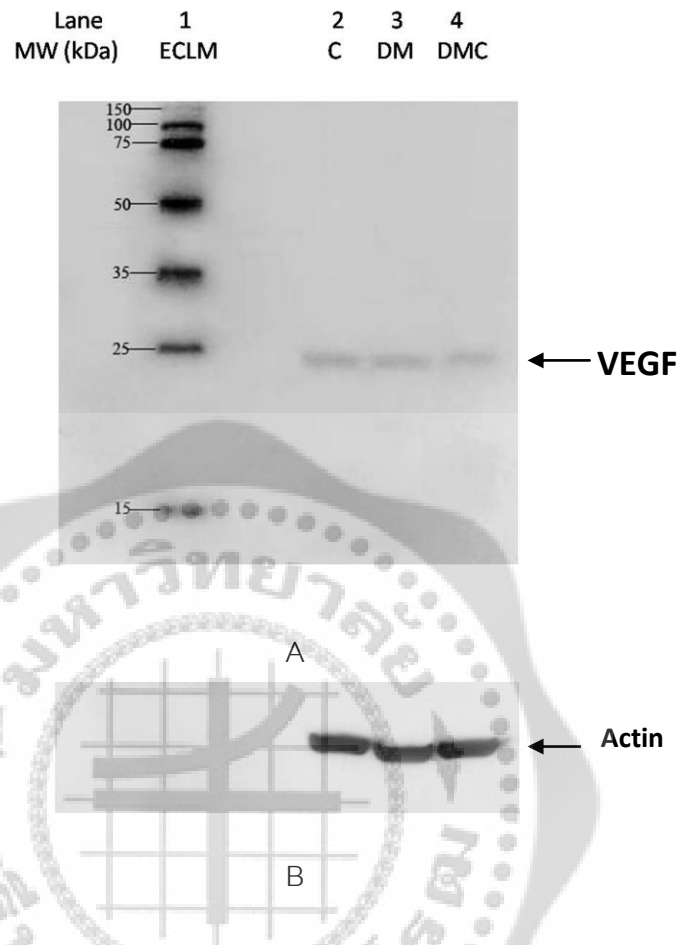
actin protein, house keeping protein, was examined and revealed at molecular weight 43 kDa. The actin protein was further used for protein analysis (Figure 45B).

The intensity protein bands on the film of DM and DMC rats at 4 weeks were no different in relating to control ( $96.40 \pm 0.61$  %,  $90.61 \pm 1.36$  % of the control rats).

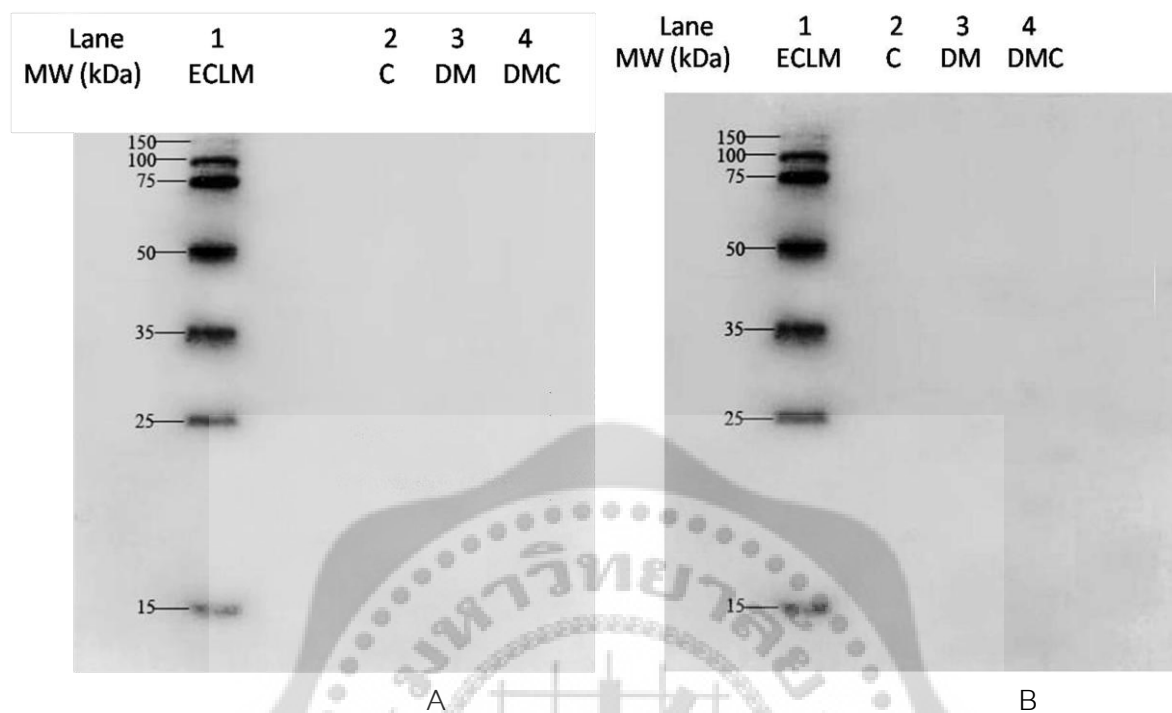
#### **Western blot analysis of negative control of primary and secondary antibodies**

The Monoclonal anti-VEGF antibody was used as primary negative control in this experiment. The primary antibody dilution of 1:500 was used as primary antibody and membranes were exposed to film (for ECL) for VEGF detection by Enhanced chemiluminescence (ECL) method. There was no any protein bands representing on ECL film (Figure 46A ). Western blot analysis verified the primary negative control of anti-VEGF antibody in rats liver at 4 weeks as the following: 6  $\mu$ l of ECL marker (ECLM) (Lane 1), 10  $\mu$ g of protein extracted from control (Lane 2), 10  $\mu$ g of protein extracted from DM (Lane 3), and 10  $\mu$ g of protein extracted from DMC (Lane 4).

Goat anti-mouse IgG, horseradish peroxidase conjugated secondary antibody was used as secondary negative control to confirm the result of western blot analysis. There was no any protein bands representing on ECL film (Figure 46B). Western blot analysis verified the secondary negative control of secondary antibody in rats liver at 4 weeks as the following: 6  $\mu$ l of ECL marker (ECLM) (Lane 1), 10  $\mu$ g of protein extracted from control (Lane 2), 10  $\mu$ g of protein extracted from DM (Lane 3), and 10  $\mu$ g of protein extracted from DMC (Lane 4).



**Figure 45** A: Western blot analysis demonstrated VEGF protein, B: Western blot analysis demonstrated actin protein from control (C), DM, and DMC rats liver at 4 week as the following : 6  $\mu$ l of ECL marker (ECLM) (Lane 1), 10  $\mu$ g of protein extracted from control (Lane 2), 10  $\mu$ g of protein extracted from DM (Lane 3), and 10  $\mu$ g of protein extracted from DMC (Lane 4) rats livers. Specific protein bands at molecular weight 23 kDa represent VEGF protein (Lane 2, 3, and 4). Specific protein bands at molecular weight 43 kDa represent actin protein (Lane 2, 3, and 4).



**Figure 46** Western blot analysis of primary negative control of anti-VEGF antibody (A) and secondary negative control of goat anti-mouse IgG antibody (B); 6  $\mu$ l of ECL marker (ECLM) (Lane 1), 10  $\mu$ g of protein extracted from the livers of control (Lane 2), 10  $\mu$ g of protein extracted from DM (Lane 3), and 10  $\mu$ g of protein extracted from DMC (Lane 4) at 4 week.

#### 4.6.2 Characteristic of VEGF protein in the liver at 8 week

##### SDS-PAGE of VEGF protein

The protein extracted from livers of control, DM, and DMC rats at 8 week were separated on 12 % SDS-PAGE. The nitrocellulose membrane was stained by Ponceau s solution and showed many bands of total protein samples. Total protein samples from rat livers at 8 week were separated on 12 % SDS-PAGE as the following: 6  $\mu$ l of ECL marker (ECLM) (Lane 1), 10  $\mu$ g of protein extracted from the livers of control (Lane 2), 10  $\mu$ g of protein extracted from DM (Lane 3), and 10  $\mu$ g of protein extracted from DMC (Lane 4).

##### Western blot analysis of VEGF protein

The interested VEGF protein band was observed and demonstrated by using monoclonal anti-VEGF antibody. Western blot analysis established the specific VEGF protein band at molecular weight 23 kDa on film by Enhanced chemiluminescence (ECL) method in control, DM, and DMC samples from rats liver at 8 week (Figure 47A). This specific protein represented VEGF protein. The bands from DM revealed more intensity of VEGF expression than control and DMC rats on ECL film. In addition, the positive control expression and characterization of actin protein, house keeping protein, was examined and revealed at molecular weight 43 kDa. The actin protein was further used for protein analysis (Figure 47B).

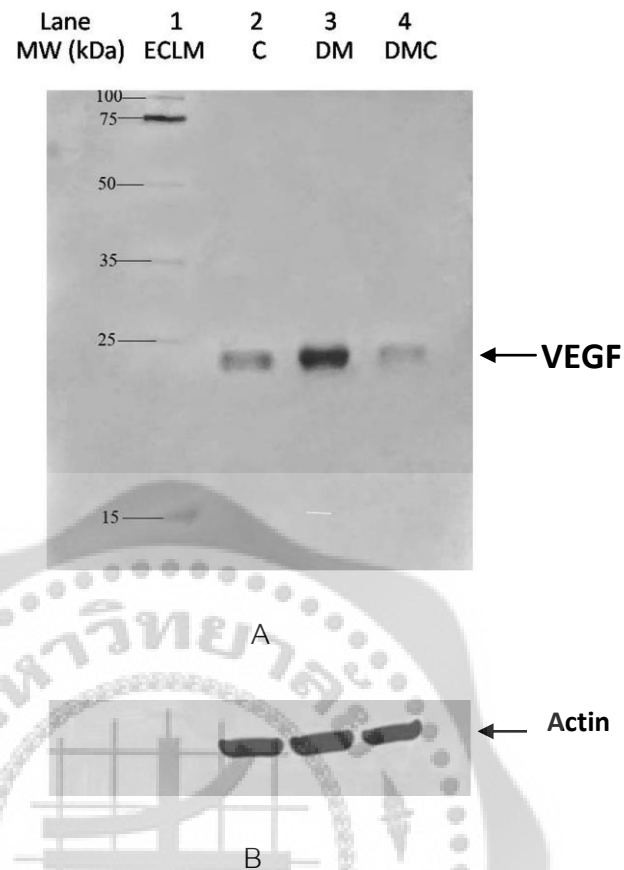
The expression of VEGF was reduced after treatment by curcumin in DMC rats when it was compared with DM rats. The amount of VEGF protein in DM rats was significantly increased in relating to control ( $248.95 \pm 1.60\%$  of the control). In DMC rats, the quantity of VEGF significantly increased in relating to control ( $123.75 \pm 0.54\%$  of the control). However, the amount of VEGF in DMC was significantly decreased in relating to the amount of VEGF in DM rats (70.30%).

Western blot analysis demonstrated the expected VEGF protein from control, DM, and DMC rat livers by using anti-VEGF antibody as the following: 6  $\mu$ l of ECL marker (ECLM) (Lane 1), 10  $\mu$ g of protein extracted from control (Lane 2), 10  $\mu$ g of protein extracted from DM (Lane 3), and 10  $\mu$ g of protein extracted from DMC (Lane 4) (Figure 47A).

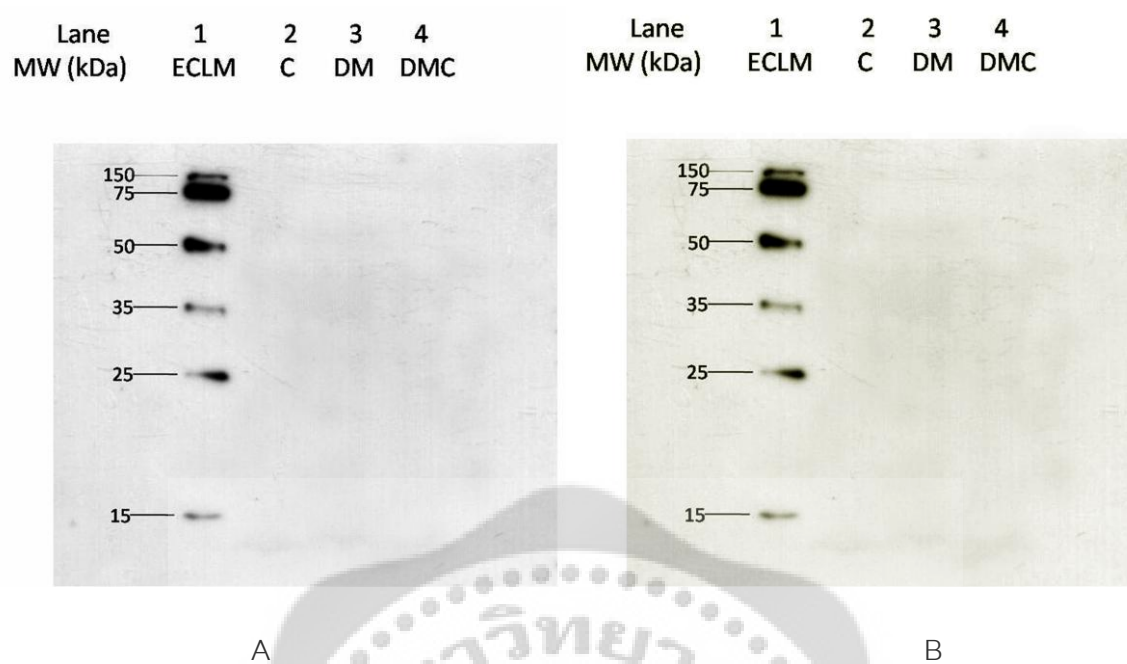
#### **Western blot analysis of negative control of primary and secondary antibodies**

The Monoclonal anti-VEGF antibody was used as primary negative control in this experiment. The primary antibody dilution of 1:500 was used as primary antibody and membranes were exposed to film (for ECL) for VEGF detection by Enhanced chemiluminescence (ECL) method. There was no any protein bands representing on ECL film (Figure 48A). Western blot analysis verified the primary negative control of anti-VEGF antibody in rats liver at 4 weeks as the following: 6  $\mu$ l of ECL marker (ECLM) (Lane 1), 10  $\mu$ g of protein extracted from control (Lane 2), 10  $\mu$ g of protein extracted from DM (Lane 3), and 10  $\mu$ g of protein extracted from DMC (Lane 4).

In addition, goat anti-mouse IgG, horseradish peroxidase conjugated secondary antibody was used as secondary negative control to confirm the result of western blot analysis. There was no any protein bands representing on ECL film when there were exposed to film (Figure 48B). Western blot analysis verified the secondary negative control of secondary antibody in rats liver at 8 weeks as the following: 6  $\mu$ l of ECL marker (ECLM) (Lane 1), 10  $\mu$ g of protein extracted from control (Lane 2), 10  $\mu$ g of protein extracted from DM (Lane 3), and 10  $\mu$ g of protein extracted from DMC (Lane 4).



**Figure 47** A: Western blot analysis demonstrated VEGF protein, B: Western blot analysis demonstrated actin protein from control (C), DM, and DMC rats liver at 8 week as the following : 6  $\mu$ l of ECL marker (ECLM) (Lane 1), 10  $\mu$ g of protein extracted from control (Lane 2), 10  $\mu$ g of protein extracted from DM (Lane 3), and 10  $\mu$ g of protein extracted from DMC (Lane 4) rats livers. Specific protein bands at molecular weight 23 kDa represent VEGF protein (Lane 2, 3, and 4). Specific protein bands at molecular weight 43 kDa represent actin protein (Lane 2, 3, and 4).



**Figure 48** Western blot analysis of primary negative control of anti- VEGF antibody (A) and secondary negative control of goat anti-mouse IgG antibody (B); 6  $\mu$ l of ECL marker (ECLM) (Lane 1), 10  $\mu$ g of protein extracted from the livers of control (Lane 2), 10  $\mu$ g of protein extracted from DM (Lane 3), and 10  $\mu$ g of protein extracted from DMC (Lane 4) at 8 week.



#### 4.6.3 Characteristic of VEGF protein in the liver at 12 week

##### SDS-PAGE of VEGF protein

The protein extracted from livers of control, DM, and DMC rats at 12 week were separated on 12 % SDS-PAGE. The nitrocellulose membrane was stained by Ponceau s solution and showed many bands of total protein samples. Total protein samples from rat livers at 12 week were separated on 12 % SDS-PAGE as the following: 6 µl of ECL marker (ECLM) (Lane 1), 10 µg of protein extracted from the livers of control (Lane 2), 10 µg of protein extracted from DM (Lane 3), and 10 µg of protein extracted from DMC (Lane 4).

##### Western blot analysis of VEGF protein

The interested VEGF protein band was observed and demonstrated by using monoclonal anti-VEGF antibody. Western blot analysis established the specific VEGF protein band at molecular weight 23 kDa on film by Enhanced chemiluminescence (ECL) method in control, DM, and DMC samples from rats liver at 8 week (Figure 49A). This specific protein represented VEGF protein. The bands from DM revealed more strong intensity of VEGF expression than control and DMC rats on ECL film. In addition, the positive control expression and characterization of actin protein, house keeping protein, was examined and revealed at molecular weight 43 kDa. The actin protein was further used for protein analysis (Figure 49B).

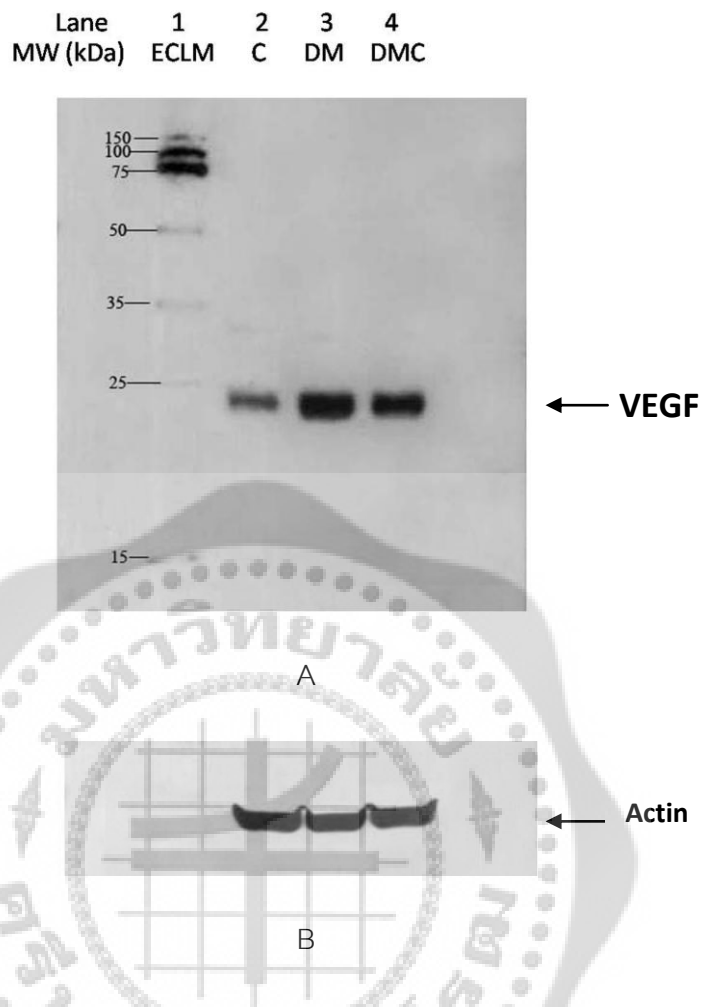
The specific protein represented VEGF protein. The band of VEGF expression in DM rats revealed the most strongest band as compared with control and DMC rats. Moreover, the amount of VEGF protein in DM rats was significantly increased in relating to control ( $285.25 \pm 1.48\%$  of the control). In DMC rats, the quantity of VEGF significantly increased in relating to control ( $230.40 \pm 0.10\%$  of the control). However, the amount of VEGF in DMC was significantly decreased in relating to the amount of VEGF in DM rats (81.45%).

Western blot analysis demonstrated the expected VEGF protein from control, DM, and DMC rat livers by using anti-VEGF antibody as the following: 6  $\mu$ l of ECL marker (ECLM) (Lane 1), 10  $\mu$ g of protein extracted from control (Lane 2), 10  $\mu$ g of protein extracted from DM (Lane 3), and 10  $\mu$ g of protein extracted from DMC (Lane 4) (Figure 49A).

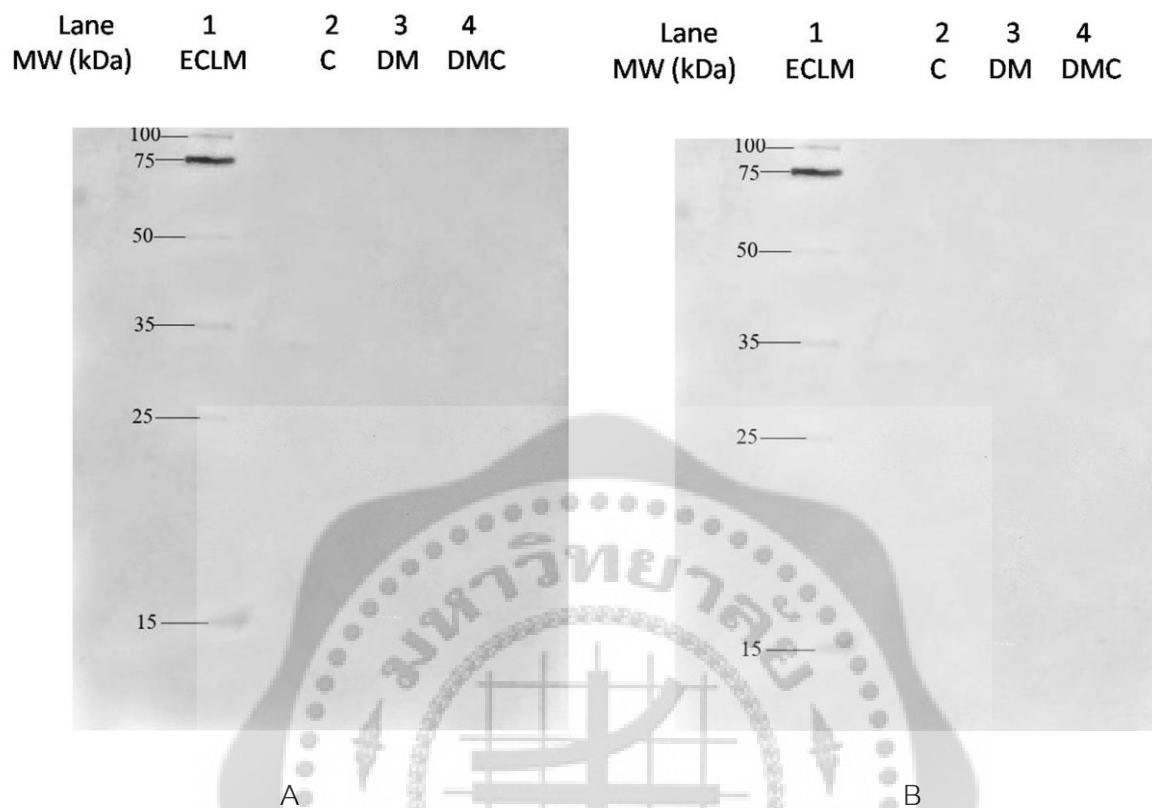
#### **Western blot analysis of negative control of primary and secondary antibodies**

The Monoclonal anti-VEGF antibody was used as primary negative control in this experiment. The primary antibody dilution of 1:500 was used as primary antibody and membranes were exposed to film (for ECL) for VEGF detection by Enhanced chemiluminescence (ECL) method. There was no any protein bands representing on ECL film (Figure 50A). Western blot analysis verified the primary negative control of anti-VEGF antibody in rats liver at 4 weeks as the following: 6  $\mu$ l of ECL marker (ECLM) (Lane 1), 10  $\mu$ g of protein extracted from control (Lane 2), 10  $\mu$ g of protein extracted from DM (Lane 3), and 10  $\mu$ g of protein extracted from DMC (Lane 4).

In addition, goat anti-mouse IgG, horseradish peroxidase conjugated secondary antibody was used as secondary negative control to confirm the result of western blot analysis. There was no any protein bands representing on ECL film when there were exposed to film (Figure 50B). Western blot analysis verified the secondary negative control of secondary antibody in rats liver at 12 weeks as the following: 6  $\mu$ l of ECL marker (ECLM) (Lane 1), 10  $\mu$ g of protein extracted from control (Lane 2), 10  $\mu$ g of protein extracted from DM (Lane 3), and 10  $\mu$ g of protein extracted from DMC (Lane 4).



**Figure 49** A: Western blot analysis demonstrated VEGF protein, B: Western blot analysis demonstrated actin protein from control (C), DM, and DMC rats liver at 12 week as the following : 6  $\mu$ l of ECL marker (ECLM) (Lane 1), 10  $\mu$ g of protein extracted from control (Lane 2), 10  $\mu$ g of protein extracted from DM (Lane 3), and 10  $\mu$ g of protein extracted from DMC (Lane 4) rats livers. Specific protein bands at molecular weight 23 kDa represent VEGF protein (Lane 2, 3, and 4). Specific protein bands at molecular weight 43 kDa represent actin protein (Lane 2, 3, and 4).



**Figure 50** Western blot analysis of primary negative control of anti- VEGF antibody (A) and secondary negative control of goat anti-mouse IgG antibody (B): 6  $\mu$ l of ECL marker (ECLM) (Lane 1), 10  $\mu$ g of protein extracted from the livers of control (Lane 2), 10  $\mu$ g of protein extracted from DM (Lane 3), and 10  $\mu$ g of protein extracted from DMC (Lane 4) at 12 week.

## 4.7 Quantitative analysis of VEGF protein expression in the liver

### 4.7.1 At 4 week

Quantitative analysis of VEGF protein expression were no different among control, DM, and DMC rats. The intensity protein bands on the film of DM and DMC rats at 4 weeks were no different in relating to control ( $96.40 \pm 0.61$  %,  $90.61 \pm 1.36$  % of the control rats) (Table 15 and Figure 51).

### 4.7.2 At 8 week

Quantitative analysis of VEGF protein expression were significantly different among control, DM, and DMC rats. The amount of VEGF protein in DM rats was significantly increased in relating to control ( $248.95 \pm 1.60$ % of the control) ( $p < 0.0001$ ). In DMC rats, the quantity of VEGF significantly increased in relating to control ( $123.75 \pm 0.54$ % of the control) ( $p < 0.001$ ). However, the amount of VEGF in DMC was significantly decreased in relating to the amount of VEGF in DM rats ( $p < 0.0001$ ) (Table 15 and Figure 51).

### 4.7.3 At 12 week

Quantitative analysis of VEGF protein expression of VEGF expression in DM rats was significantly increased in relating to control ( $285.25 \pm 1.48$ % of the control) ( $p < 0.0001$ ). In DMC rats, the quantity of VEGF significantly increased in relating to control ( $230.40 \pm 0.10$ % of the control) ( $p < 0.0001$ ). It was interested that the amount of VEGF in DMC was significantly decreased when compared with the amount of VEGF in DM rats ( $p < 0.0001$ ) (Table 15 and Figure 51).

TABLE 15 VEGF/actin expression of control, DM, and DMC rats at 4, 8, and 12 weeks

| Weeks | Control | DM          | DMC         |
|-------|---------|-------------|-------------|
| 4     | 100     | 96.40±0.61  | 90.61±1.36  |
| 8     | 100     | 248.95±1.60 | 123.75±0.54 |
| 12    | 100     | 285.25±1.48 | 230.40±0.10 |

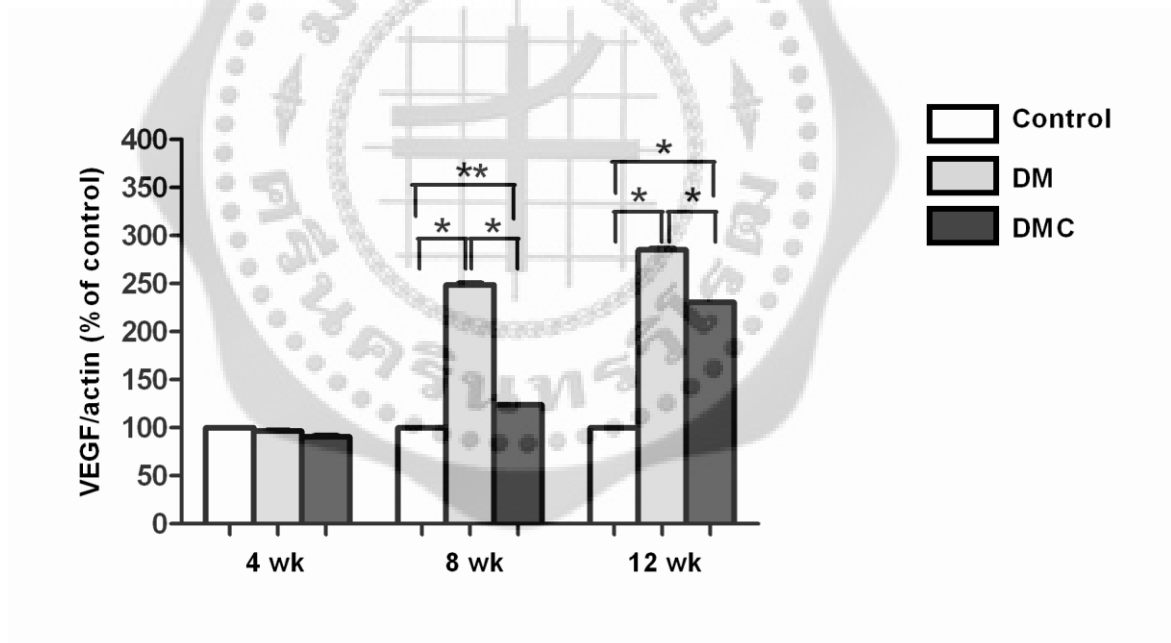


Figure 51 VEGF/actin expression of control, DM, and DMC rats at 4, 8, and 12 weeks.

Values are means  $\pm$  SEM. \*  $p < 0.0001$ , \*\*  $p < 0.001$

Moreover, the effect of curcumin on VEGF expression was compared at different durations in the same conditions. It was found that VEGF protein expression was significantly elevated with duration of experiments in DM and DMC rats. VEGF expression was higher at 12 week than 4 and 8 weeks experiments in DM and DMC rats. Also, VEGF protein expression of DM rats at 12 week was significantly higher than DM rats at 4 and 8 weeks ( $p<0.0001$ ). (Table 16 and Figure 52).

TABLE 16 VEGF/actin expression of 4, 8, and 12 weeks of control, DM, and DMC rats

| Groups  | 4 wk             | 8 wk              | 12 wk             |
|---------|------------------|-------------------|-------------------|
| Control | 100              | 100               | 100               |
| DM      | 96.40 $\pm$ 0.61 | 248.95 $\pm$ 1.60 | 285.25 $\pm$ 1.48 |
| DMC     | 90.61 $\pm$ 1.36 | 123.75 $\pm$ 0.54 | 230.40 $\pm$ 0.10 |

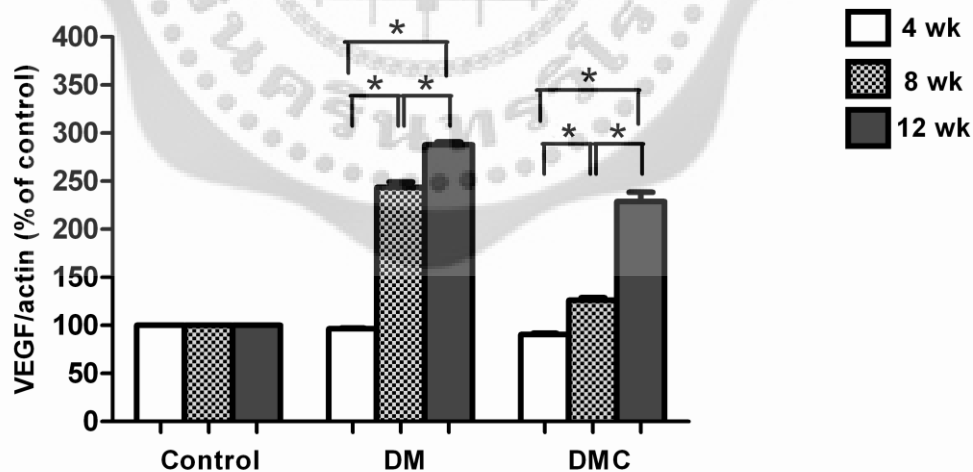


Figure 52 VEGF/actin expression at 4, 8, and 12 weeks of control, DM, and DMC rats.

Values are means  $\pm$  SEM. \*  $p<0.0001$



#### **4.8 Vascular corrosion cast technique with scanning electron microscope of liver microvasculature**

Vascular corrosion cast of liver microvasculature at the levels of proximal and distal part of portal veins together with portal venule branches in control, DM, and DMC rats at 4, 8, and 12 weeks were studied. The proximal and distal part of portal veins together with portal venule branches in control, DM, and DMC rats at 4, 8, and 12 weeks were investigated of both quality and quantity analyses corresponding to the diameters and the blood volumes.

##### **4.8.1 Vascular corrosion cast technique with scanning electron microscope of liver microvasculature of control rats at 4, 8, and 12 weeks**

Scanning electron micrographs revealed clearly high magnification and detection of microvascular changes in control rats at 4, 8, and 12 weeks. In normal condition of control group, numerous normal even blood vessels architectures of proximal and distal part of portal veins together with portal venule branches were conspicuously observed. Presentation of prominent microvascular network was characterized for healthy normal sizes together with normal shape and standard pattern of liver vasculature organization (Figure 53, 56, and 61). The data of diameter and blood volume of proximal and distal part of portal veins together with portal venule branches were not different significantly of diameter and blood volume among control rats at 4, 8, and 12 weeks.

##### **4.8.2 Vascular corrosion cast technique with scanning electron microscope of liver microvasculature of DM rats at 4, 8, and 12 weeks**

In comparison to control rats, liver vascular changes of DM rats at 4 week experiment especially demonstrated the event of liver blood vessels hypertrophy which presented mild unhealthy destroyed characteristics blood vessel morphology (Figure 54). The portal venules showed signs of mild blind endings and shrinkage.

The noticeable lesions of diabetic pathology consisted of the expansion of proximal and distal parts of portal veins together with venule branches in DM rats at 8 week. The data of diameter and blood volume of proximal and distal part of portal veins together with portal venule branches were different significantly of diameter and blood volume when compared with control and DMC rats at 8 weeks. It demonstrated the event of liver blood vessels hypertrophy which presented unhealthy destroyed characteristics blood vessel morphology (Figure 57). The distal portion of portal veins showed signs of abnormal structures of blind endings and shrinkage of portal venules. Moreover, the new development and neovascularization of new shrinkage blood vessels have emerged and presented extensively from the proximal part of portal vein (Figure 58).

At 12 week experiments, the lesion of diabetic pathology consisted of seriously destruction of blood vessels and presented unhealthy destroyed characteristics blood vessel morphology in DM rats liver (Figure 62). It demonstrated the event of liver blood vessels especially in portal venules, were seriously shrinkage and their diameter were decreased when compared with DM rats at 4 and 8 week. The data were shown in TABLE 17 and 18, Figure 66 and 67. The distal portion of portal veins also showed signs of abnormal structures of blind endings and shrinkage of portal venules. Moreover, the new development and neovascularization of new tortuous and shrinkage blood vessels have emerged and presented extensively from the proximal part of portal vein (Figure 63).

#### **4.8.3 Vascular corrosion cast technique with scanning electron microscope of liver microvasculature of DMC rats at 4, 8, and 12 weeks**

Scanning electron microscopic technique of vascular corrosion casts of proximal and distal part of portal veins together with portal venule branches in DMC rats at 4 weeks were shown in figures 55. They revealed high magnification and detection of blood vessels architectures of proximal and distal part of portal veins together with portal venule branches. The portal venules showed signs of mild blind endings and shrinkage looked like DM rats at 4

week. The data of diameter and blood volume of distal part of portal veins and portal venule branches were not different significantly of diameter and blood volume when compared with control and DM rats at 4 weeks. The data were shown in TABLE 17 and 18, Figure 66 and 67.

On the contrary, DMC rats at 8 week which was treated with curcumin, the healthy normal patterns of liver blood vessels have recovered and got restored. Portal veins in curcumin-treated group (Figure 59) became regenerated and repaired in to healthy and normal characteristics. Moreover, the new development and neovascularization of new nourishing blood vessels have emerged and presented extensively from the proximal part of portal vein (Figure 60).

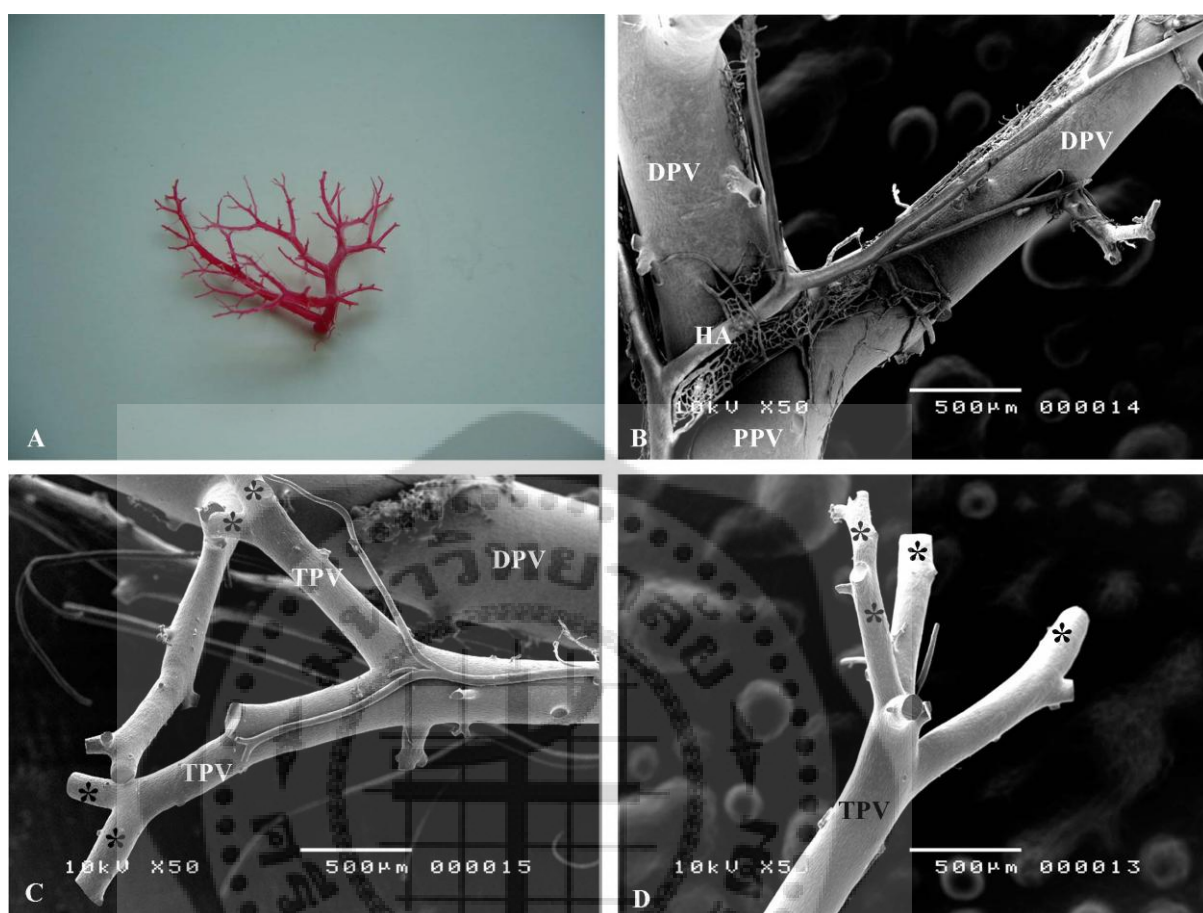
At 12 week experiments, the more healthy normal patterns of hepatic vessels have recovered and got restored when compared with DMC rats at 8 week. The proximal, distal of portal veins, and portal venule branches in curcumin-treated group (Figure 64) became regenerated and repaired in to healthy and normal characteristics look like in control group. The new development and neovascularization of new nourishing blood vessels still have emerged and presented extensively from the proximal part of portal vein (Figure 65).

**Figure 53** Photomicrograph of vascular cast in control rats liver at 4 week

(4 wk, Control)

- A Photomicrograph of the whole vascular cast in control rats liver at 4 week
- B SEM micrograph of liver vascular cast of control rat, showing the blood vessels of medial lobe of liver. The proximal part of portal vein (PPV), giving branches to the two distal part of portal veins (DPV) corresponding with a branch of hepatic artery (HA). Bar = 500  $\mu$ m.
- C SEM micrograph of liver vascular cast of control rat, showing the blood vessels of medial lobe of liver at level of distal part of portal vein (DPV), the terminal portal vein (TPV) that giving branches to the portal venules (\*). Bar = 500  $\mu$ m.
- D SEM micrograph of liver vascular cast of control rat, showing the blood vessels of medial lobe of liver. The terminal portal vein (TPV) giving branches to the portal venules (\*). Bar = 500  $\mu$ m.

Figure 53



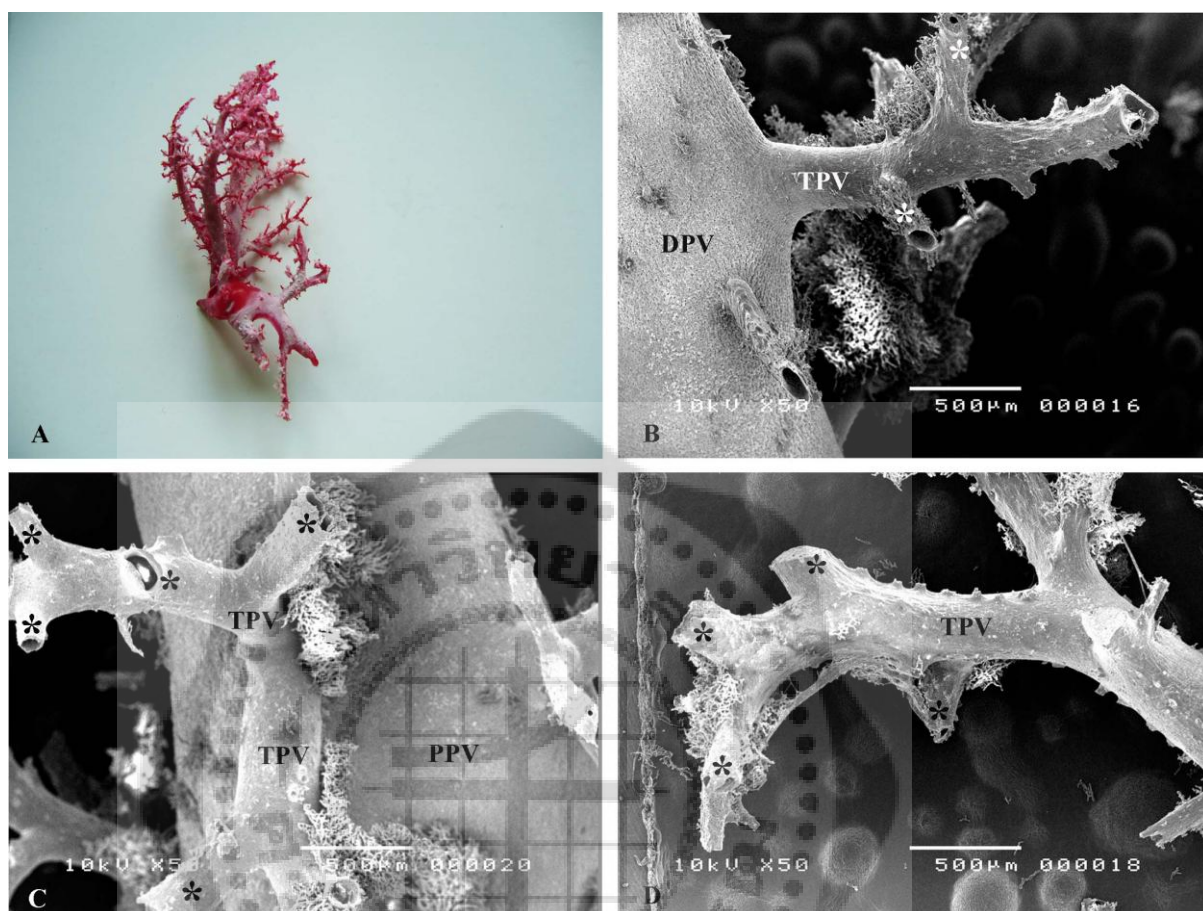
4 wk (Control)

**Figure 54** Photomicrograph of vascular cast in DM rat livers at 4 week

(4 wk, DM)

- A Photomicrograph of the whole vascular cast in DM rat livers at 4 week
- B SEM micrograph of liver vascular cast of control rat, showing the blood vessels of medial lobe of liver at level of distal part of portal vein (DPV), the terminal portal vein (TPV) that gave branches to blind ending and mild shrinkage portal venules (\*). Bar = 500  $\mu$ m.
- C SEM micrograph of liver vascular cast of DM rat, showing the blood vessels of medial lobe of liver at level of proximal part of portal vein (PPV), the terminal portal vein (TPV) that gave branches to the portal venules (\*). Bar = 500  $\mu$ m
- D SEM micrograph of liver vascular cast of control rat, showing the blood vessels of medial lobe of liver. The terminal portal vein (TPV) distributed branches to blind ending and mild shrinkage portal venules (\*). Bar = 500  $\mu$ m.

Figure 54



4 wk (DM)

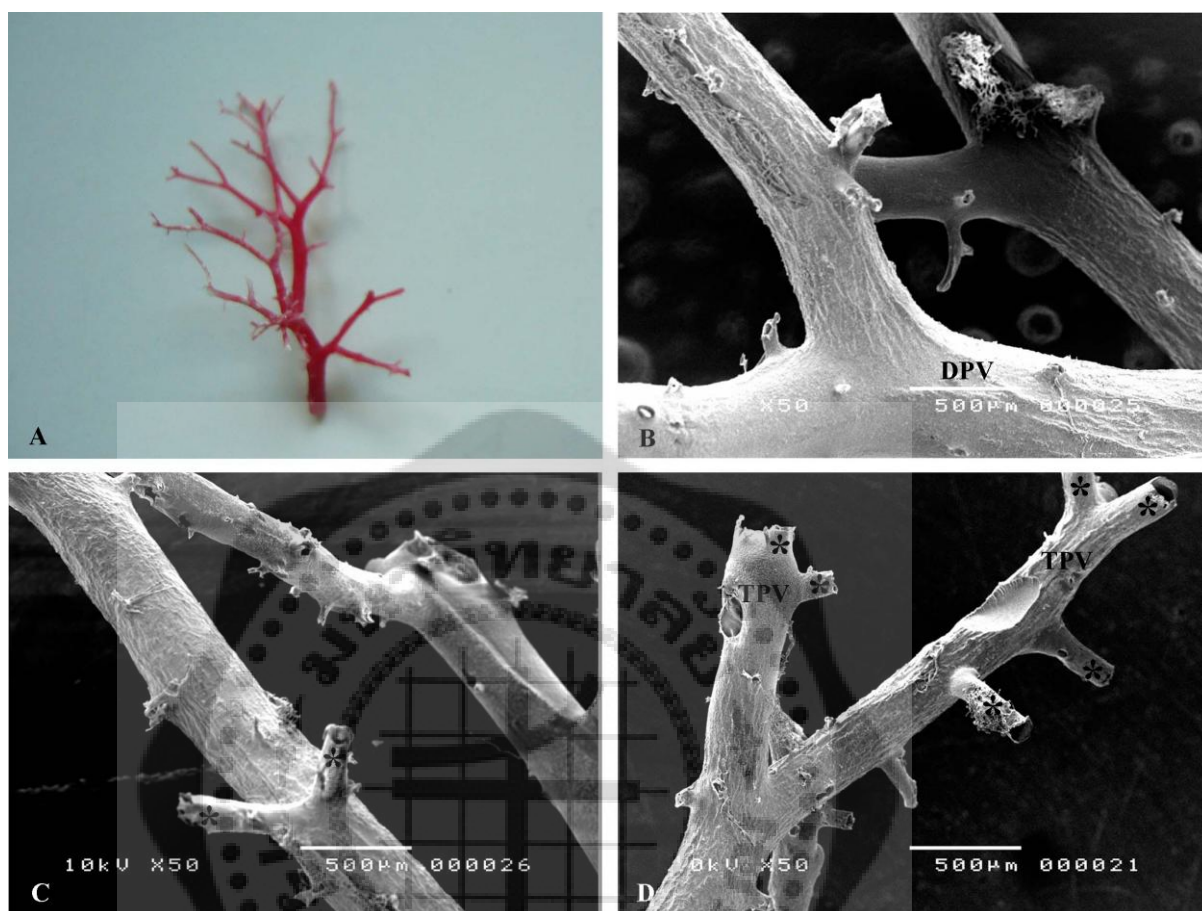


**Figure 55** Photomicrograph of vascular cast in DMC rat livers at 4 week

(4 wk, DMC)

- A Photomicrograph of the whole vascular cast in DM rat livers at 4 week
- B SEM micrograph of liver vascular cast of control rat, showing the blood vessels of medial lobe of liver at level of distal part of portal vein (DPV), the terminal portal vein (TPV) that gave branches to blind ending and mild shrinkage portal venules (\*). Bar = 500  $\mu$ m.
- C SEM micrograph of liver vascular cast of DM rat, showing the blood vessels of medial lobe of liver at level of proximal part of portal vein (PPV), the terminal portal vein (TPV) that gave branches to blind ending and mild shrinkage portal venules (\*). Bar = 500  $\mu$ m
- D SEM micrograph of liver vascular cast of control rat, showing the blood vessels of medial lobe of liver. The terminal portal vein (TPV) distributed branches to blind ending and mild shrinkage portal venules (\*). Bar = 500  $\mu$ m.

Figure 55



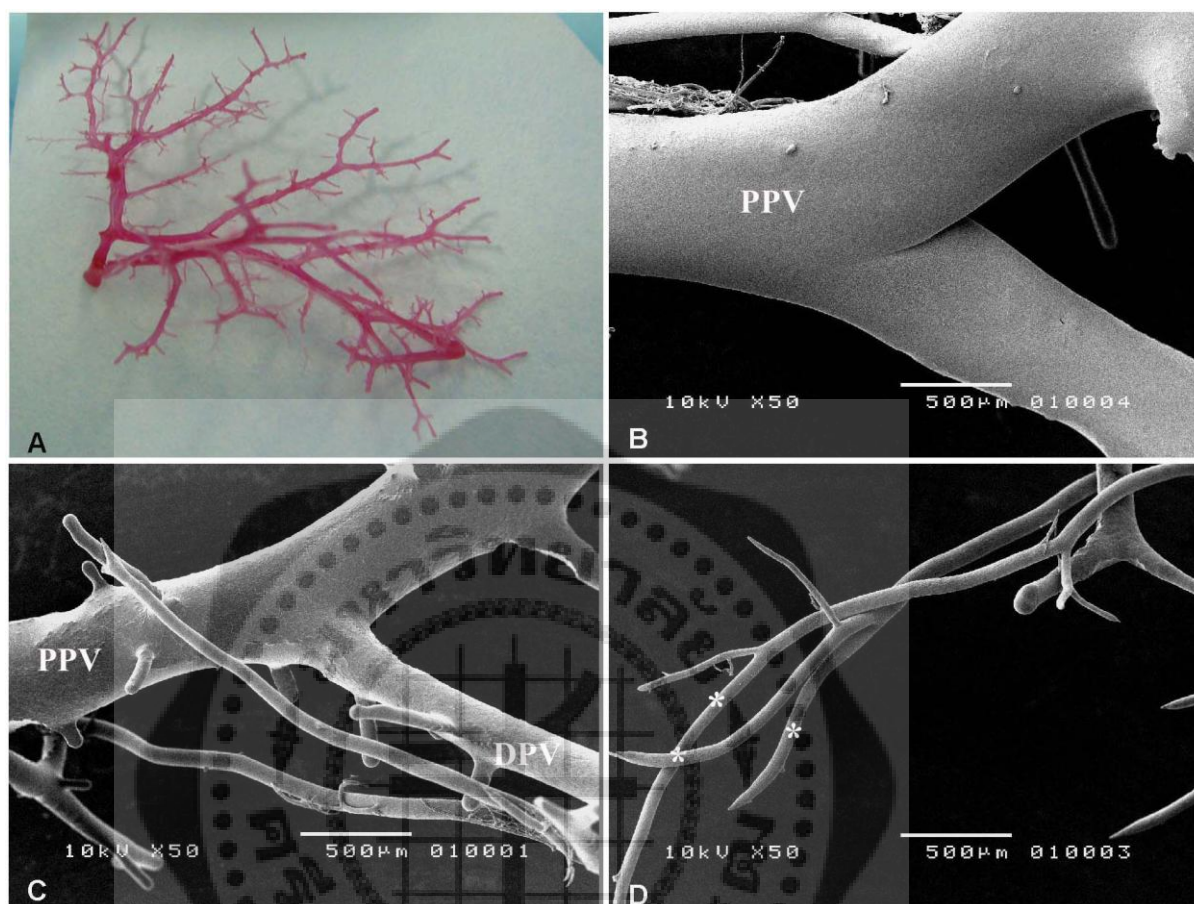
4 wk (DMC)

**Figure 56** Photomicrograph of vascular cast in control rat livers at 8 week

(8 wk, Control)

- A Photomicrograph of the whole vascular cast in control rat livers at 8 week
- B SEM micrograph of liver vascular cast of control rat, showing the blood vessels of medial lobe of liver. The proximal part of portal vein (PPV), gave branches to the distal part of portal veins. Bar = 500  $\mu$ m.
- C SEM micrograph of liver vascular cast of control rat, showing the blood vessels of medial lobe of liver. The proximal part of portal vein (PPV), gave branches to the two distal part of portal veins (DPV). Bar = 500  $\mu$ m.
- D SEM micrograph of liver vascular cast of control rat, showing the the numerous branches of the portal venules (\*) of medial lobe of liver. Bar = 500  $\mu$ m.

Figure 56



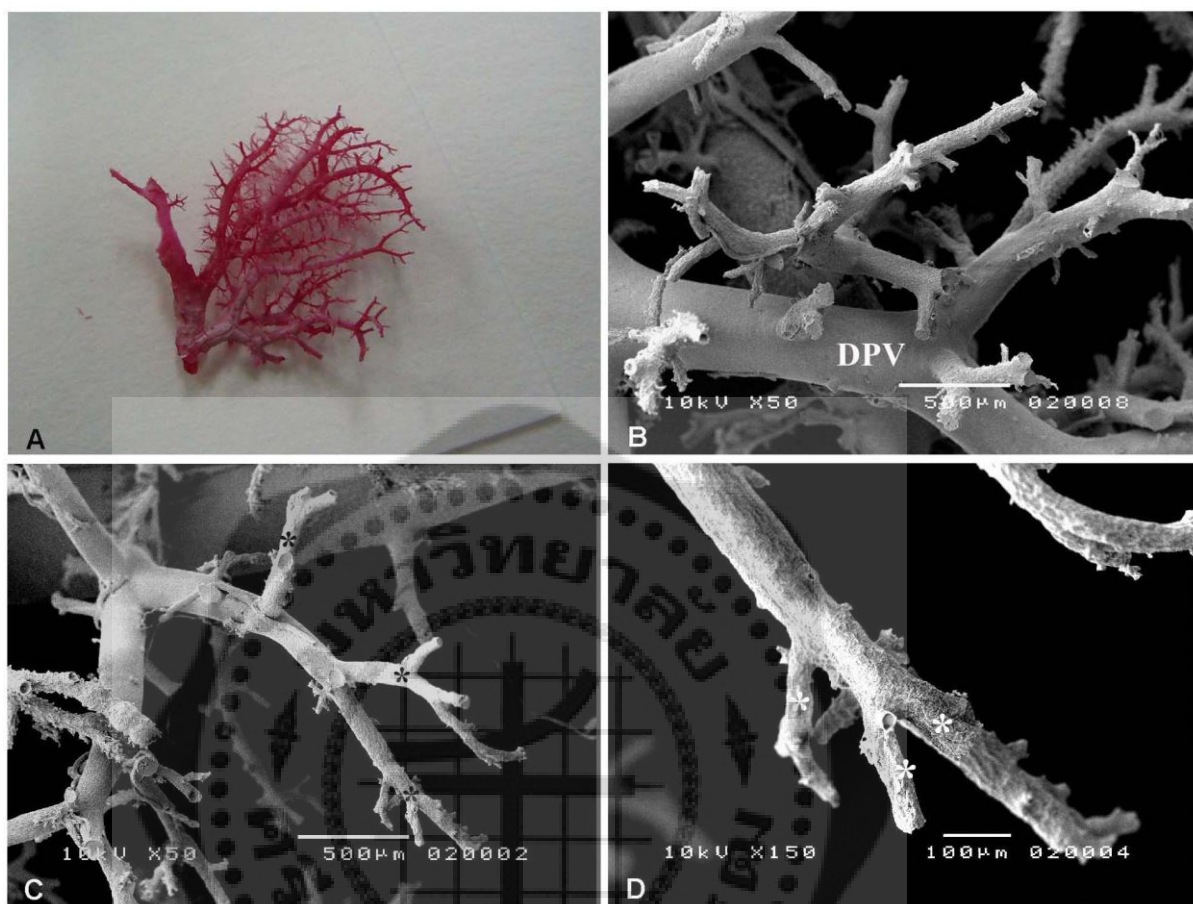
8 wk (Control)

**Figure 57** Photomicrograph of vascular cast in DM rat livers at 8 week

(8 wk, DM)

- A Photomicrograph of the whole vascular cast in DM rat livers at 4 week
- B SEM micrograph of liver vascular cast of DM rat, showing the distal part of portal vein (DPV) of medial lobe of liver and branches to be blind ending and shrinkage portal venules. Bar = 500  $\mu$ m.
- C SEM micrograph of liver vascular cast of DM rat, showing the blind ending and shrinkage portal venules (\*) of medial lobe of liver. Bar = 500  $\mu$ m.
- D SEM micrograph of liver vascular cast of DM rat, at high magnification, showing the blood vessels of medial lobe of liver. The terminal portal vein (TPV) gave branches to be blind ending and mild shrinkage portal venules (\*). Bar = 100  $\mu$ m.

Figure 57



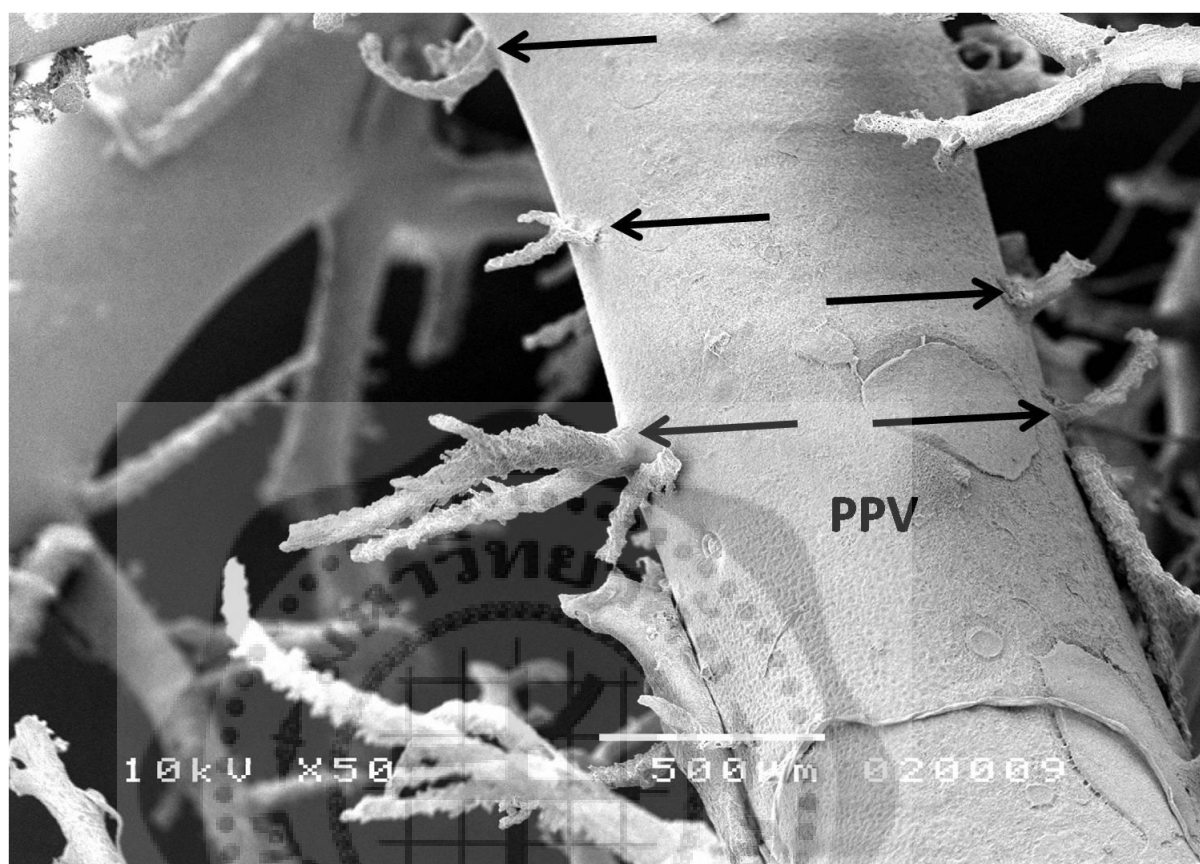
8 wk (DM)

**Figure 58** SEM micrograph of liver vascular cast of DM rat liver at 8 week (8 wk, DM), showing the proximal part of portal vein (PPV) gave branches to the many branches of the new development and neovascularization of new shrinkage blood vessels have emerged and presented extensively from the proximal part of portal veins (arrows). The Bar = 500  $\mu$ m.





Figure 58



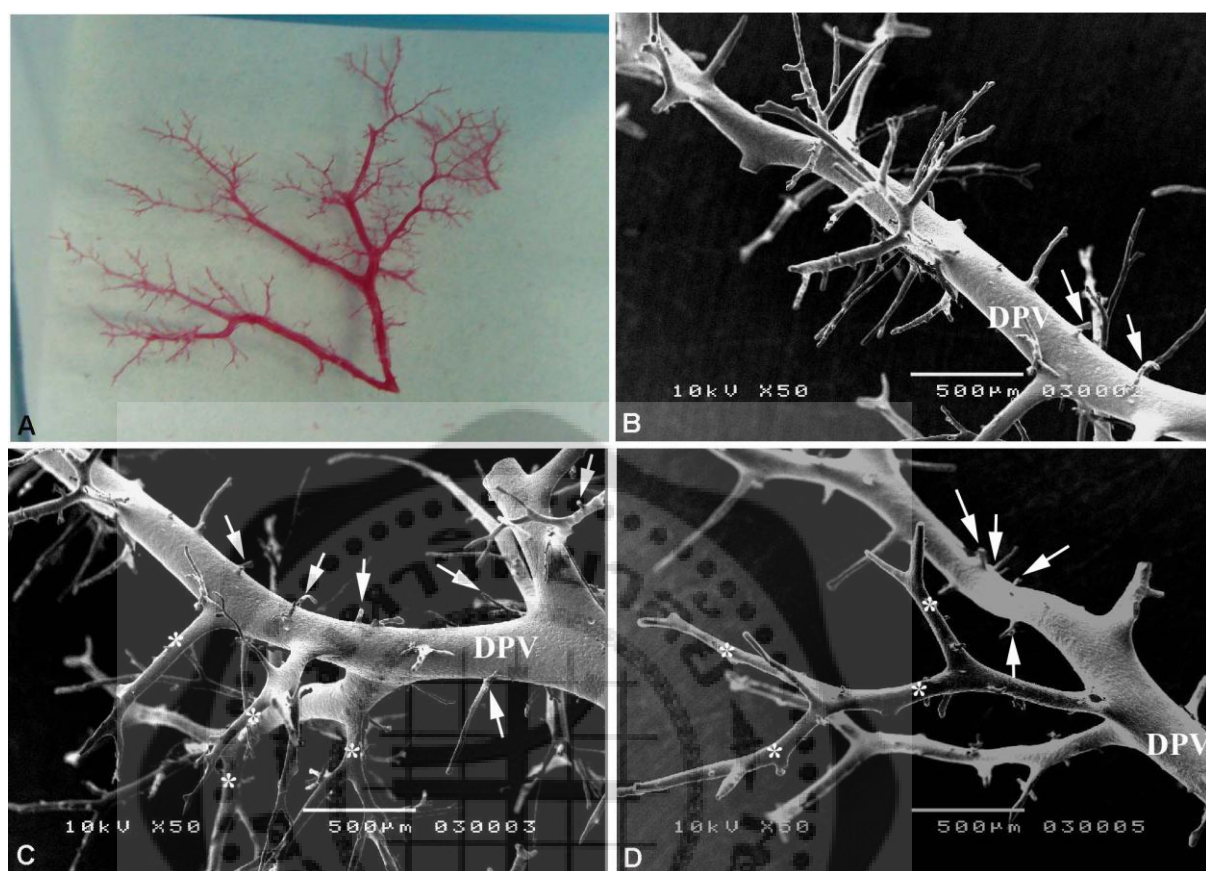
8 wk (DM)

**Figure 59** Photomicrograph of vascular cast in DMC rat livers at 8 week

(8 wk, DMC)

- A Photomicrograph of the whole vascular cast in DMC rat livers at 8 week
- B SEM micrograph of liver vascular cast of DMC rat, showing the distal part of portal vein (DPV) gave branches to the many branches of portal venules and new development of new nourishing blood vessels have emerged and presented extensively from the distal part of portal vein (arrows). The Bar = 500  $\mu$ m.
- C SEM micrograph of liver vascular cast of DMC rat, showing the distal part of portal vein (DPV) gave branches to the many branches of portal venules (\*) and new development of new nourishing blood vessels have emerged and presented extensively from the distal part of portal vein (arrows). The Bar = 500  $\mu$ m.
- D SEM micrograph of liver vascular cast of DMC rat, showing the distal part of portal vein (DPV) giving branches to the many branches of portal venules (\*) and new development of new nourishing blood vessels have emerged and presented extensively from the distal part of portal vein (arrows). The Bar = 500  $\mu$ m.

Figure 59



**Figure 60** SEM micrograph of liver vascular cast of DMC rat livers at 8 week (8 wk, DMC), showing the new nourishing blood vessels (arrows) have emerged and presented extensively from the proximal part of portal vein (PPV) The Bar = 100  $\mu$ m.



Figure 60



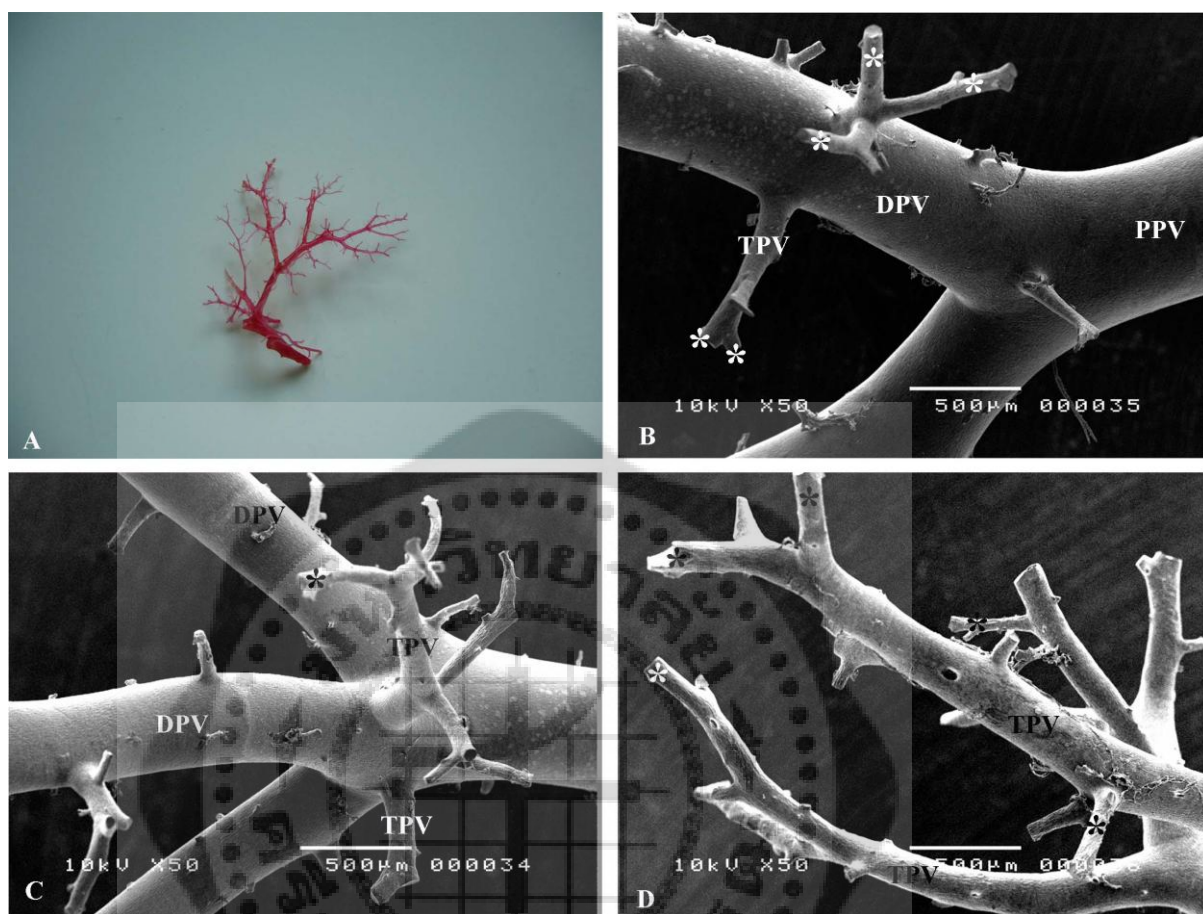
8 wk (DMC)

**Figure 61** Photomicrograph of vascular cast in control rat livers at 12 week

(12 wk, Control)

- A Photomicrograph of the whole vascular cast in control rats livers at 12 week
- B SEM micrograph of liver vascular cast of control rat, showing the blood vessels of medial lobe of liver. The proximal part of portal vein (PPV), gave branches to the two distal part of portal veins (DPV) and the terminal portal vein (TPV) gave branches to the portal venules (\*). Bar = 500  $\mu$ m.
- C SEM micrograph of liver vascular cast of control rat, showing the distal part of portal vein (DPV) of medial lobe of liver and the terminal portal vein (TPV) gave branches to the portal venules (\*). Bar = 500  $\mu$ m.
- D SEM micrograph of liver vascular cast of control rat, showing the blood vessels of medial lobe of liver. The terminal portal vein (TPV) gave branches to the portal venules (\*). Bar = 500  $\mu$ m.

Figure 61



12 wk (Control)

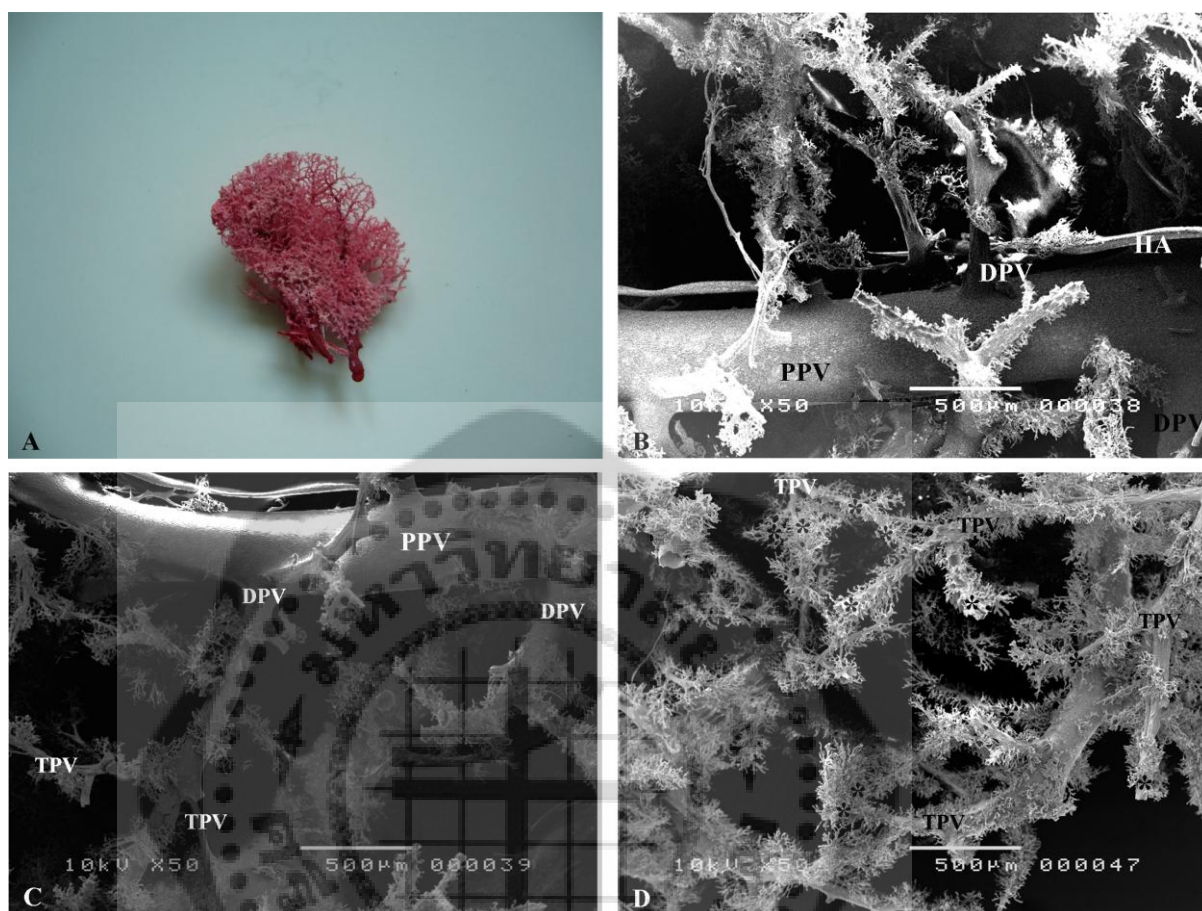


**Figure 62** Photomicrograph of vascular cast in DM rat livers at 12 week

(12 wk, DM)

- A Photomicrograph of the whole vascular cast in DM rat livers at 12 week
- B SEM micrograph of liver vascular cast of control rat, showing the proximal part of portal vein (PPV) corresponding with a branch of hepatic artery (HA), the shrinkage distal part of portal vein (DPV) of medial lobe of liver and branches to seriously shrinkage portal venules. Bar = 500  $\mu$ m.
- C SEM micrograph of liver vascular cast of control rat, showing the proximal part of portal vein (PPV), the shrinkage distal part of portal vein (DPV) of medial lobe of liver and terminal portal vein (TPV) gave branches to seriously shrinkage portal venules. Bar = 500  $\mu$ m.
- D SEM micrograph of liver vascular cast of control rat, at high magnification, showing the blood vessels of medial lobe of liver. The terminal portal vein (TPV) gave branches to blind ending and seriously shrinkage portal venules (\*). Bar = 500  $\mu$ m.

Figure 62

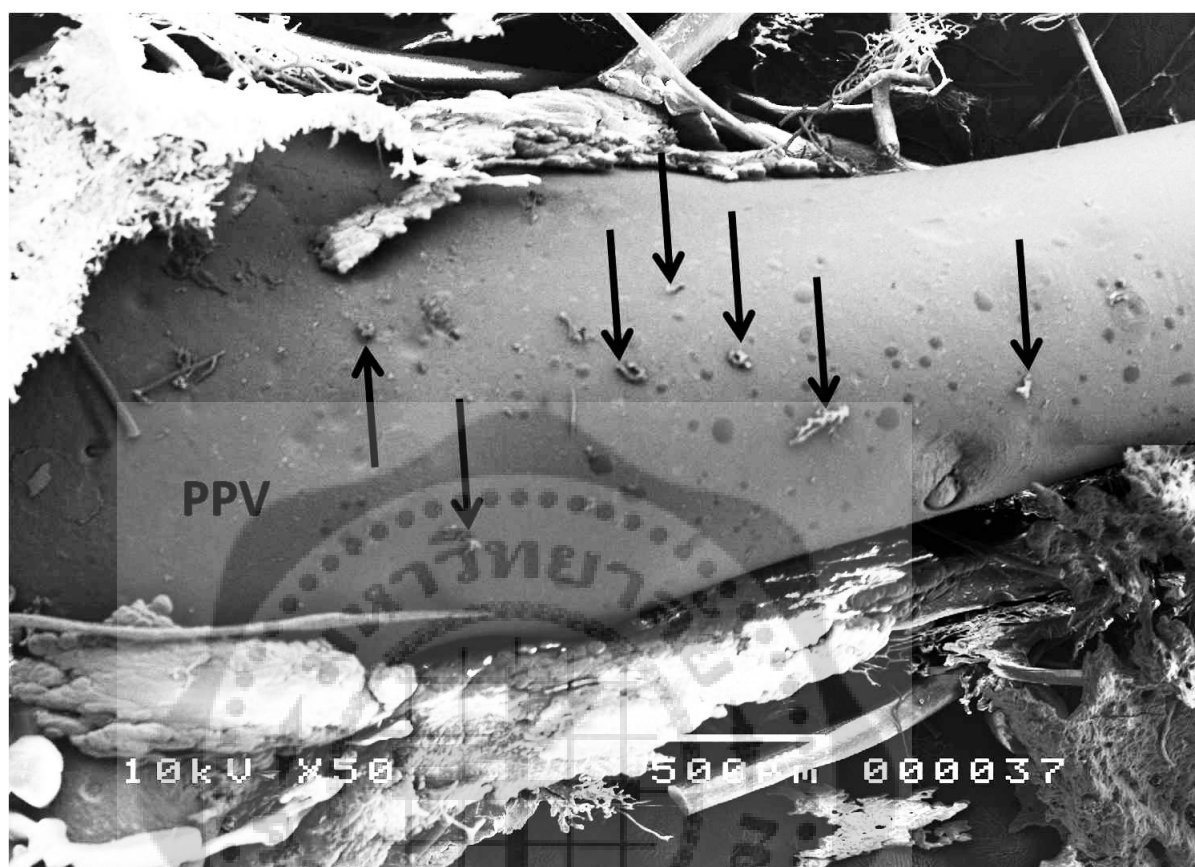


12 wk (DM)

**Figure 63** SEM micrograph of liver vascular cast of DM rat liver at 12 week (12 wk, DM), showing the proximal part of portal vein (PPV) gave to the many branches of tortuous and shrinkage new development of blood vessels (arrows). The Bar = 500  $\mu$ m.



Figure 63



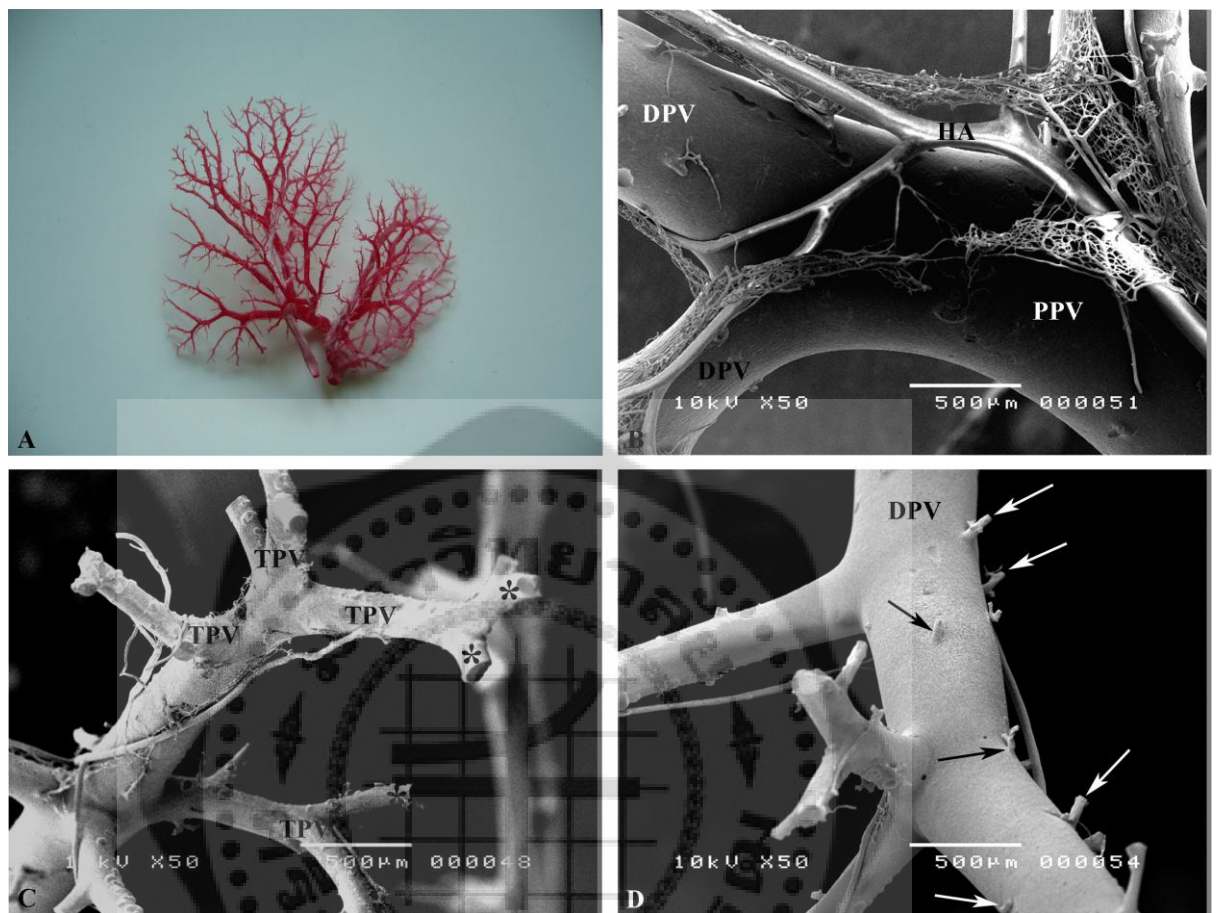
12 wk (DM)

**Figure 64** Photomicrograph of vascular cast in DMC rat livers at 12 week

(12 wk, DMC)

- A Photomicrograph of the whole vascular cast in DMC rat livers at 12 week
- B SEM micrograph of liver vascular cast of DMC rat, showing the blood vessels of medial lobe of liver. The proximal part of portal vein (PPV), gave branches to the two distal part of portal veins (DPV) corresponding with a branch of hepatic artery (HA). Bar = 500  $\mu$ m.
- C SEM micrograph of liver vascular cast of DMC rat, showing the terminal portal vein (TPV) gave to many branches of portal venules (\*). The Bar = 500  $\mu$ m.
- D SEM micrograph of liver vascular cast of DMC rat, showing the distal part of portal vein (DPV) gave to new nourishing of blood vessels (arrows). The Bar = 500  $\mu$ m.

Figure 64



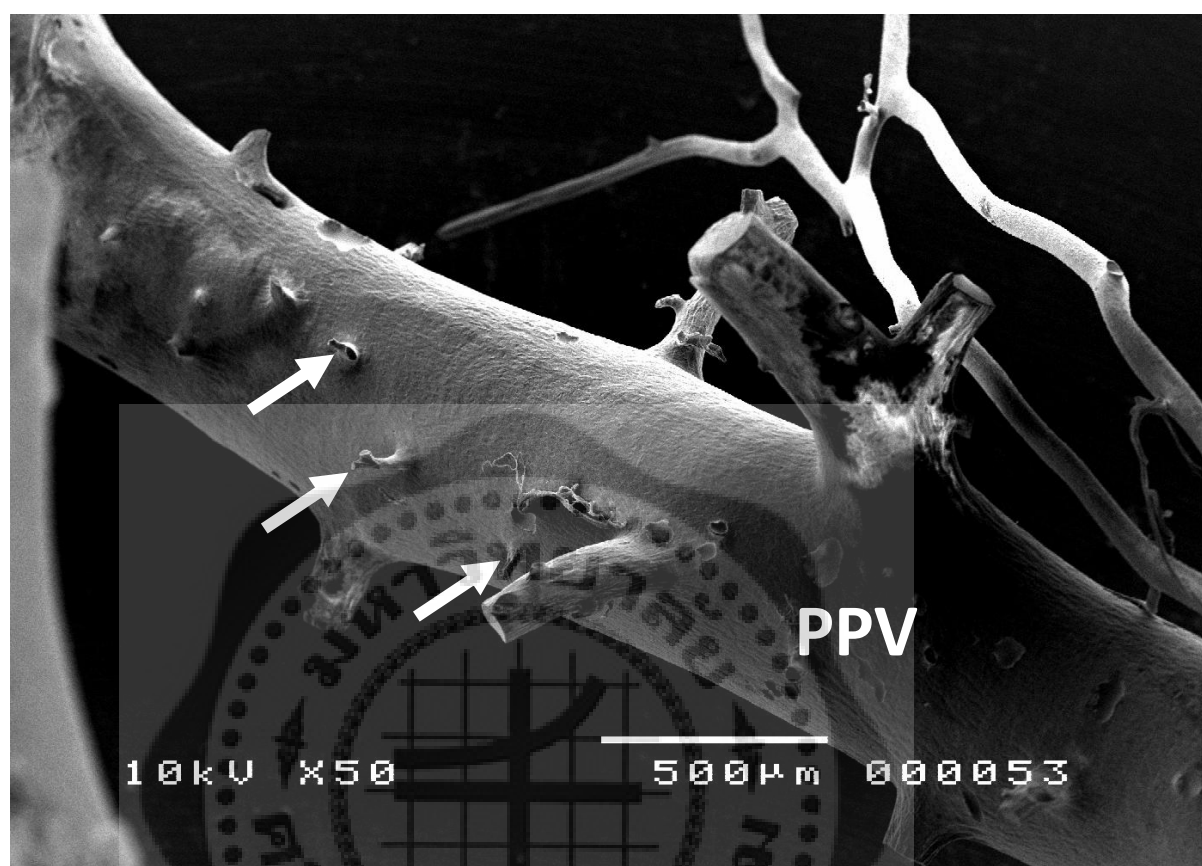
12 wk (DMC)

**Figure 65** SEM micrograph of liver vascular cast of DMC rat livers at 12 week (12 wk, DMC), showing the new nourishing blood vessels (arrows) have emerged and presented extensively from the proximal part of portal vein (PPV) The Bar = 100  $\mu$ m.





Figure 65



12 wk (DMC)

#### 4.8.4 Quantitative analysis of diameter of portal venule branches by vascular corrosion cast technique with scanning electron microscope

The diameters of portal venule branches of control, DM, and DMC rats from 4, 8, and 12 week experiments were scored quantitatively in a blind fashion. The data were shown that the DM rats had significant increase in the diameter of portal venule branches at 8 weeks indices when they were compared to control rats ( $p<0.001$ ). In contrast, DM rats had significant decrease in the diameter of portal venule branches at 12 weeks indices when they compared to control rats ( $p<0.001$ ). These were increased diameter of portal venule branches indices in DM rats were significantly increased when they were compared with DMC rats at 8 week ( $p<0.001$ ). In contrast, the diameters of portal venule branches were reduced by treatment with curcumin in DMC rats at 12 week ( $p<0.001$ ) (TABLE 17 and Figure 66).

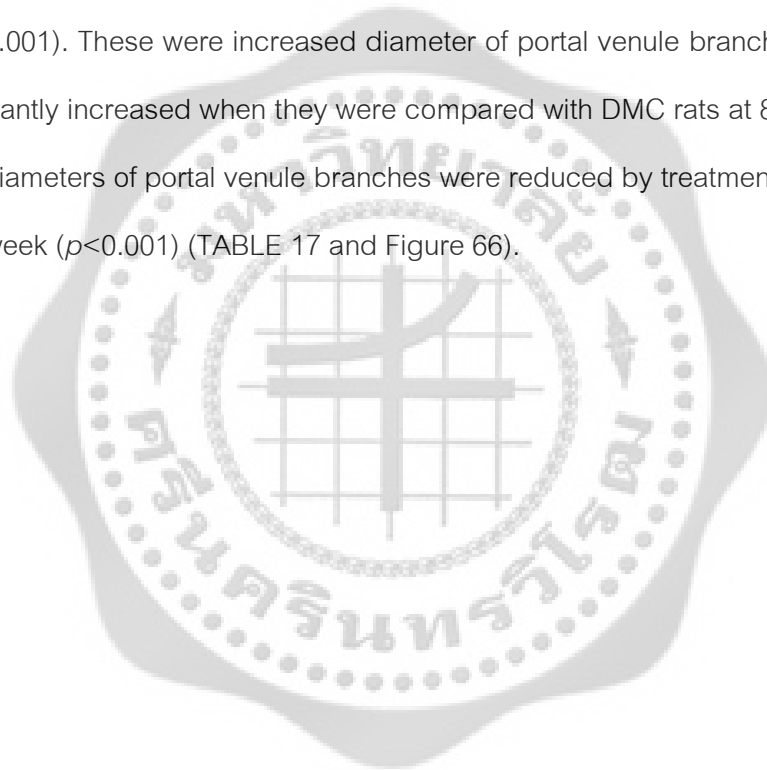


TABLE 17 Diameters of portal venules at 4, 8, and 12 weeks of control, DM, and DMC rats

| Weeks | Control           | DM                | DMC               |
|-------|-------------------|-------------------|-------------------|
| 4     | 150.25 $\pm$ 7.50 | 175.25 $\pm$ 5.75 | 168.5 $\pm$ 7.93  |
| 8     | 142.75 $\pm$ 8.11 | 190.25 $\pm$ 5.74 | 139.75 $\pm$ 6.32 |
| 12    | 141.0 $\pm$ 5.99  | 82.56 $\pm$ 11.39 | 127.5 $\pm$ 8.51  |

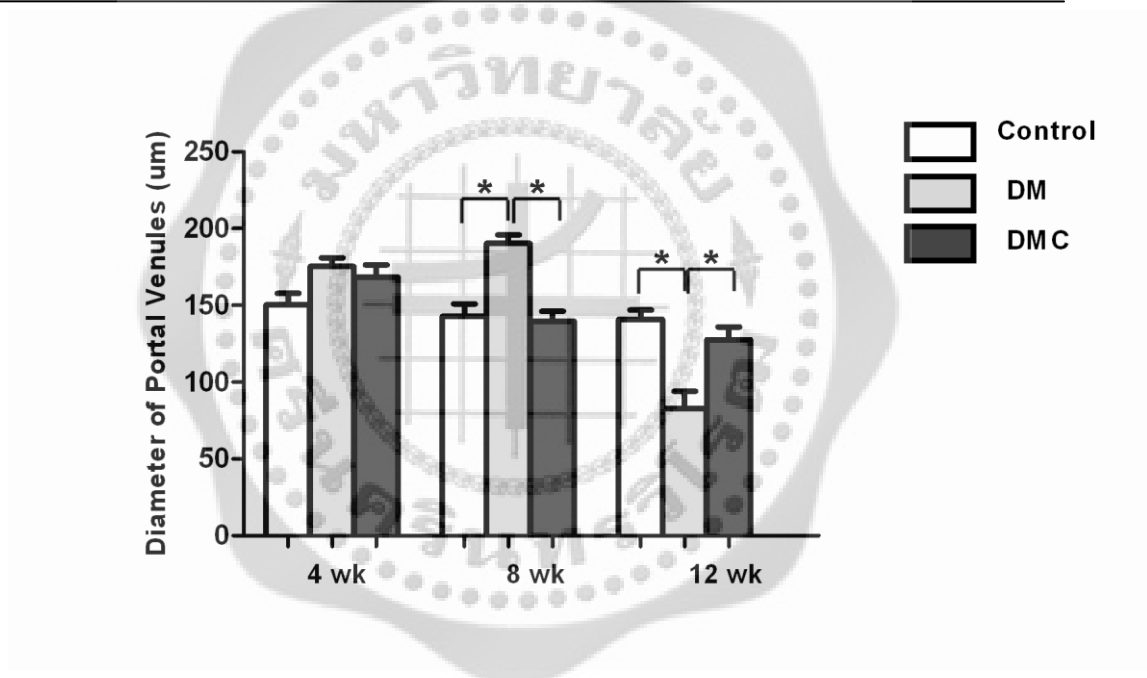
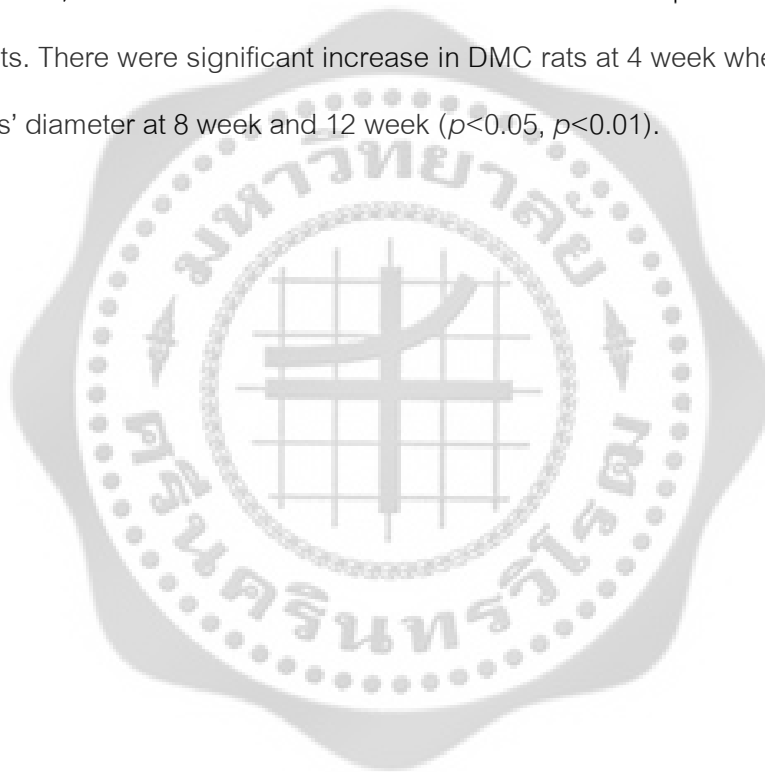


Figure 66 Diameters of portal venules at 4, 8, and 12 weeks of control, DM, and DMC rats.

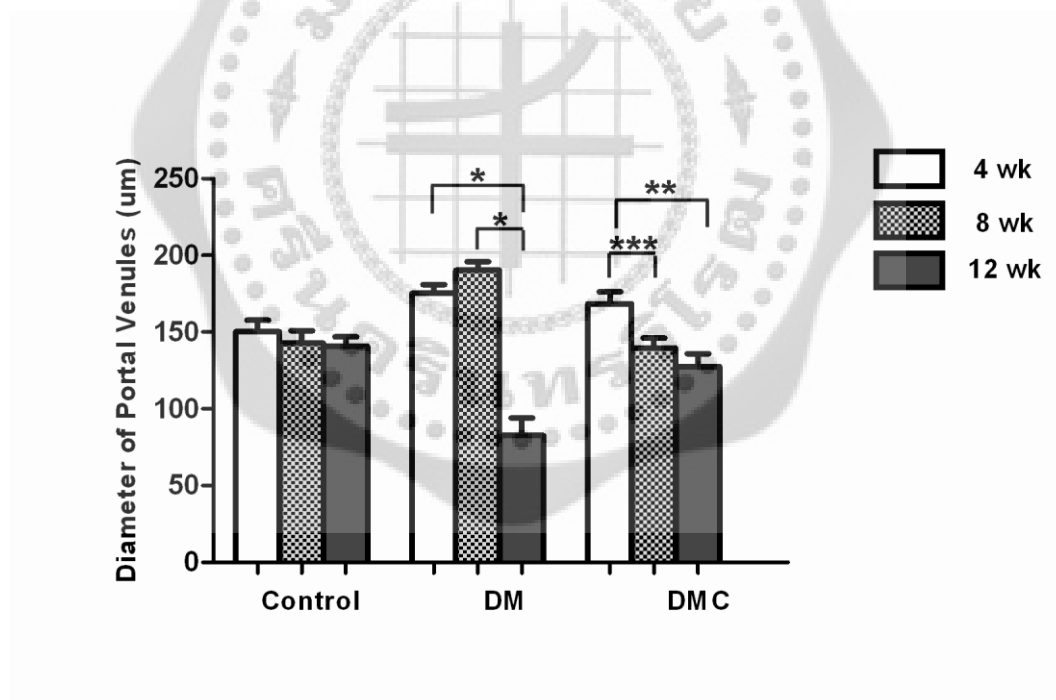
Values are means  $\pm$  SE \*  $p < 0.001$

TABLE 18 and Figure 67 The information form showed the diameters of portal venule branches by vascular corrosion cast with scanning electron microscopic levels in three groups of rats (control, DM, and DMC) at different durations in the same conditions. There was significant decrease in DM rats' diameters at 4 week when compared with DM rats at 8 and 12 week ( $p<0.001$ ). There were no significantly changes in diameters at 4, 8, and 12 weeks of control rats. However, there was decreased correlation with time dependent at 4, 8, and 12 weeks in DMC rats. There were significant increase in DMC rats at 4 week when compared with those of DMC rats' diameter at 8 week and 12 week ( $p<0.05$ ,  $p<0.01$ ).



**TABLE 18** Diameters of portal venules from control, DM, and DMC rats at 4, 8, and 12 weeks

| Groups  | 4 wk              | 8 wk              | 12 wk             |
|---------|-------------------|-------------------|-------------------|
| Control | 150.25 $\pm$ 7.50 | 142.75 $\pm$ 8.11 | 141.0 $\pm$ 5.99  |
| DM      | 175.25 $\pm$ 5.75 | 190.25 $\pm$ 5.74 | 82.56 $\pm$ 11.39 |
| DMC     | 168.5 $\pm$ 7.93  | 139.75 $\pm$ 6.32 | 127.5 $\pm$ 8.51  |



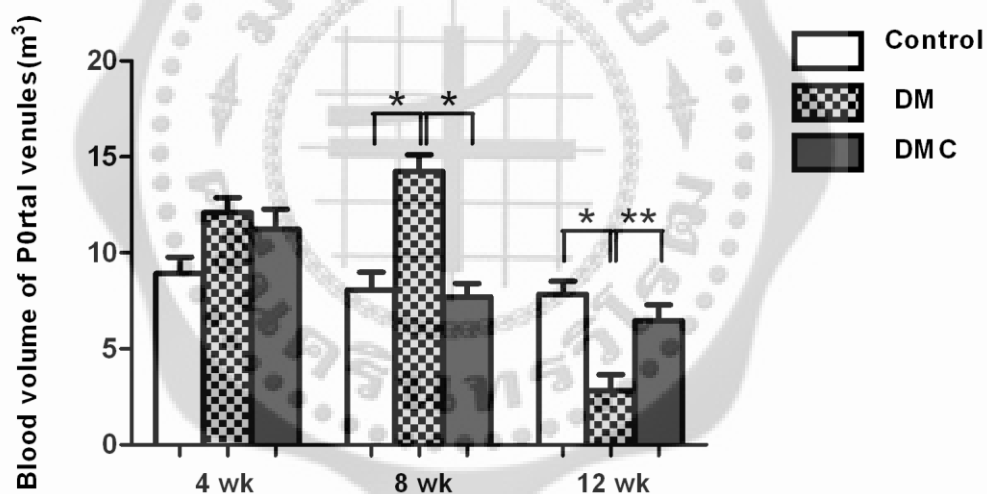
**Figure 67** Diameters of portal venules from control, DM, and DMC rats at 4, 8, and 12 weeks. Values are means  $\pm$  SE \*  $p<0.001$ , \*\*  $p<0.01$ , \*\*\*  $p<0.05$

#### 4.8.5 Quantitative analysis of blood volume of portal venule branches by vascular corrosion cast technique with scanning electron microscope

The blood volume of portal venule branches of control, DM, and DMC rats from 4, 8, and 12 week experiments were scored quantitatively in a blind fashion in three groups of rats at different durations. The data were shown that the DM rats had significantly increased in the blood volume of portal venule branches at 8 weeks indices when they were compared to control rats ( $p<0.001$ ). In contrast, there was significant decrease in the blood volumes of portal venule branches at 12 weeks indices when they were compared to control rats ( $p<0.001$ ). However, the blood volume of portal venule branches were reduced by treatment with curcumin in DMC rats at 8 week ( $p<0.001$ ). In contrast, The increased blood volume of portal venule branches indices in DMC rats were significantly increased when they were compared with DM rats at 12 week ( $p<0.05$ ) (TABLE 19 and Figure 68).

**TABLE 19** Blood volume of portal venules at 4, 8, and 12 weeks of control, DM, and DMC rats

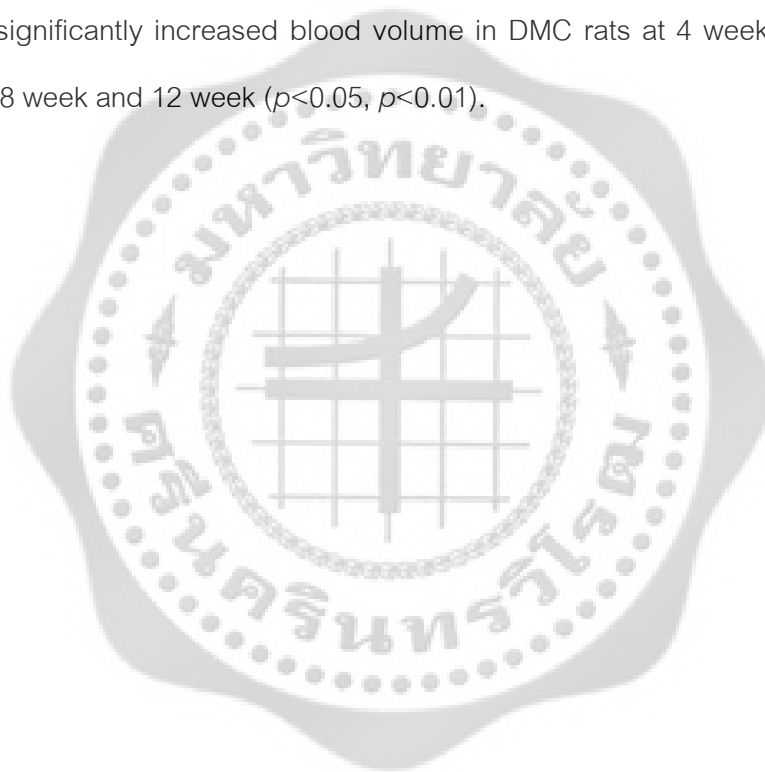
| Weeks | Control   | DM         | DMC        |
|-------|-----------|------------|------------|
| 4     | 8.93±0.85 | 12.09±0.78 | 11.22±1.05 |
| 8     | 8.08±0.91 | 14.25±0.85 | 7.71±0.69  |
| 12    | 7.85±0.68 | 2.83±0.83  | 6.47±0.83  |



**Figure 68** Blood volume of portal venules at 4, 8, and 12 weeks of control, DM, and DMC rats. Values are means ± SE \*  $p < 0.001$ , \*\*  $p < 0.05$

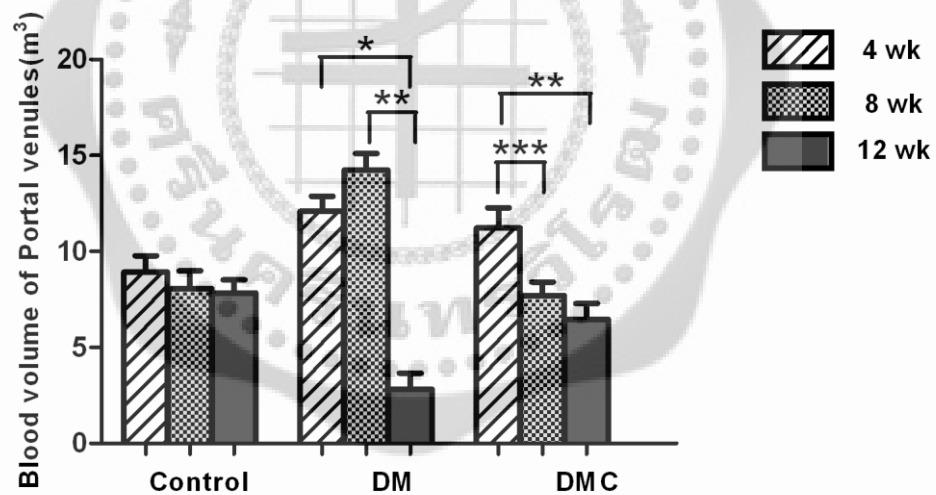


TABLE 20 and Figure 69 information showed the blood volume of portal venule branches of vascular corrosion cast with scanning electron microscopic levels in three groups of rats at different times intervals in the same conditions. There was significant decrease in DM rats' blood volume at 4 week when compared with DM rats at 12 week ( $p<0.001$ ) and was significantly decreased in DM rats at 8 week when compared with DM rats at 12 week ( $p<0.01$ ). There were no significantly changes in blood volume at 4, 8, and 12 weeks of control rats. However, there was decreased correlation with time dependent at 4, 8, and 12 weeks in DMC rats. There was significantly increased blood volume in DMC rats at 4 week when compared with DMC rats at 8 week and 12 week ( $p<0.05$ ,  $p<0.01$ ).



**TABLE 20** Blood volume of portal venules from control, DM, and DMC rats at 4, 8, and 12 weeks

| Groups  | 4 wk       | 8 wk       | 12 wk     |
|---------|------------|------------|-----------|
| Control | 8.93±0.85  | 8.08±0.91  | 7.85±0.68 |
| DM      | 12.09±0.78 | 14.25±0.85 | 2.83±0.83 |
| DMC     | 11.22±1.05 | 7.71±0.69  | 6.47±0.83 |



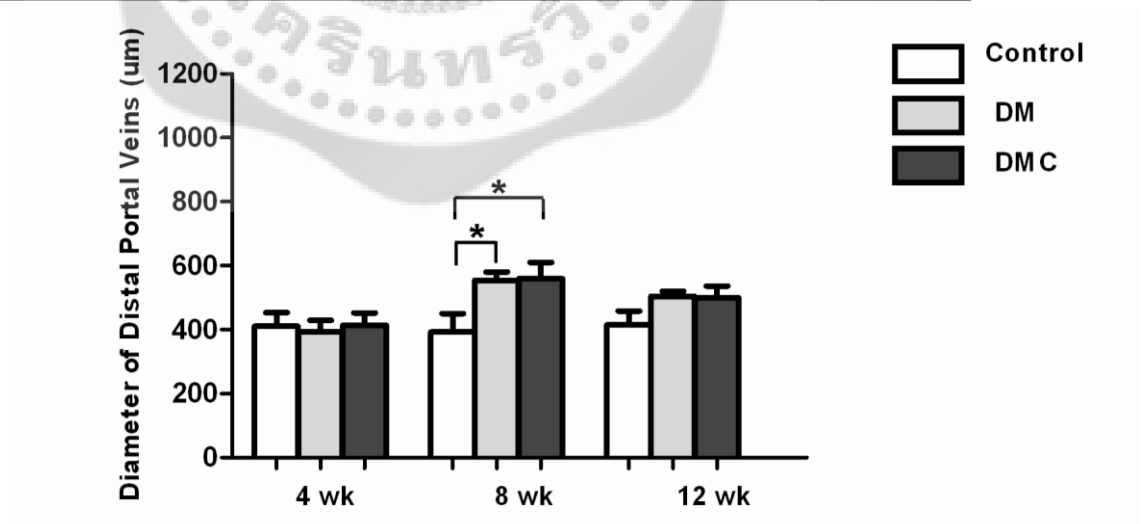
**Figure 69** Blood volume of portal venules from control, DM, and DMC rats at 4, 8, and 12 weeks. Values are means  $\pm$  SE \*  $p < 0.001$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.05$

#### 4.8.6 Quantitative analysis of diameters of distal part of portal veins branches by vascular corrosion cast technique with scanning electron microscope

The diameters of distal part of portal veins were scored quantitatively in a blind fashion in three groups of rats at different time intervals. The data were shown that the DM and DMC rats presented were significantly increased diameters of distal part of portal veins indices when they were compared to control rats at 8 week ( $p<0.05$ ) (TABLE 21 and Figure 70).

**TABLE 21** Diameters of distal part of portal veins at 4, 8, and 12 weeks of control, DM, and DMC rats

| Weeks | Control      | DM           | DMC          |
|-------|--------------|--------------|--------------|
| 4     | 410.25±43.42 | 393.25±36.64 | 413.25±39.35 |
| 8     | 392.25±58.69 | 554.5±25.95  | 559.25±51.04 |
| 12    | 415.25±43.08 | 504.75±15.42 | 499.75±36.91 |

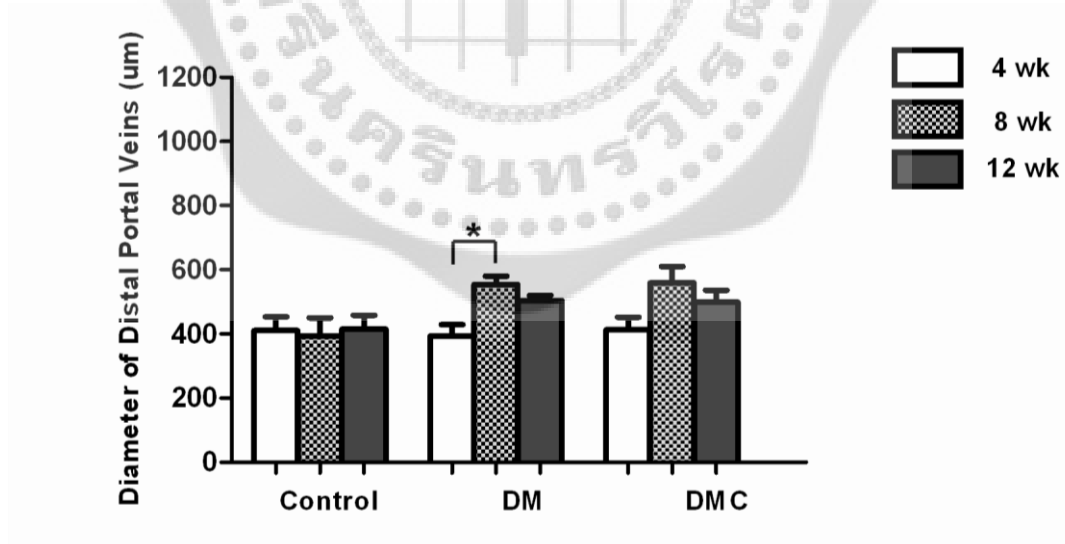


**Figure 70** Diameters of distal part of portal veins at 4, 8, and 12 weeks of control, DM, and DMC rats. Values are means  $\pm$  SE \*  $p<0.05$

TABLE 22 and Figure 71. The information form showed the blood volume of portal venule branches of vascular corrosion cast with scanning electron microscopic levels in three groups of rats at different durations in the same conditions. There was significantly increased blood volume only in DM rats at 4 week when compared with DM rats at 8 week ( $p<0.05$ ).

**TABLE 22** Diameters of distal part of portal veins from control, DM, and DMC rats at 4, 8, and 12 weeks

| Groups  | 4 wk         | 8 wk         | 12 wk        |
|---------|--------------|--------------|--------------|
| Control | 410.25±43.42 | 392.25±58.69 | 415.25±43.08 |
| DM      | 393.25±36.64 | 554.5±25.95  | 504.75±15.42 |
| DMC     | 413.25±39.35 | 559.25±51.04 | 499.75±36.91 |



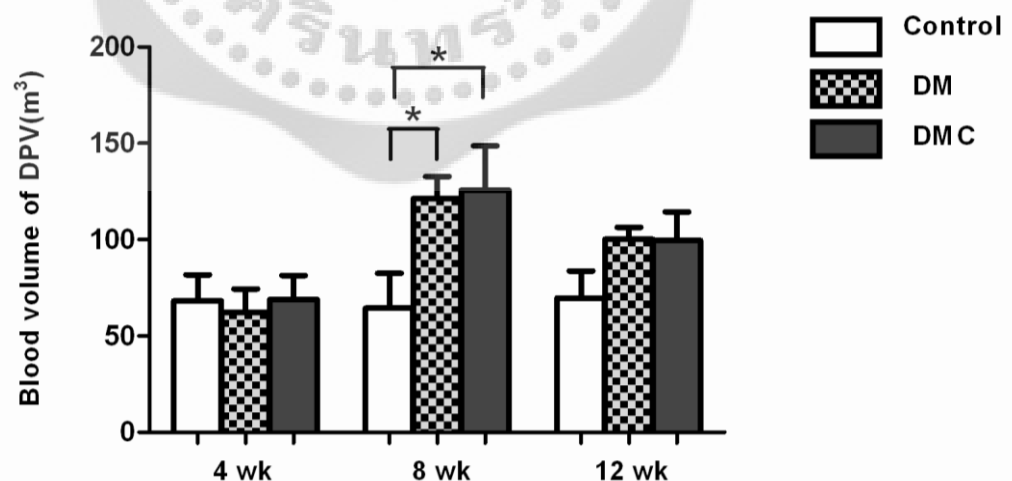
**Figure 71** Diameters of distal part of portal veins from control, DM, and DMC rats at 4, 8, and 12 weeks. Values are means  $\pm$  SE \*  $p<0.05$

#### 4.8.7 Quantitative analysis of blood volume of distal part of portal veins by vascular corrosion cast technique with scanning electron microscope

The blood volume of distal part of portal veins of control, DM, and DMC rats from 4, 8, and 12 week experiments were scored quantitatively in a blind fashion in three groups of rats at different durations. The data were shown that the DM and DMC rats revealed significantly increased in blood volume of distal part of portal veins at 8 weeks indices when they were compared to control rats ( $p<0.05$ ) (TABLE 23 and Figure 72).

**TABLE 23** Blood volume of distal part of portal veins at 4, 8, and 12 weeks of control, DM, and DMC rats

| Weeks | Control     | DM           | DMC         |
|-------|-------------|--------------|-------------|
| 4     | 68.28±13.40 | 64.45±18.12  | 69.78±14.0  |
| 8     | 62.28±12.08 | 121.48±11.25 | 100.28±6.09 |
| 12    | 68.85±12.65 | 125.83±22.92 | 99.63±14.84 |

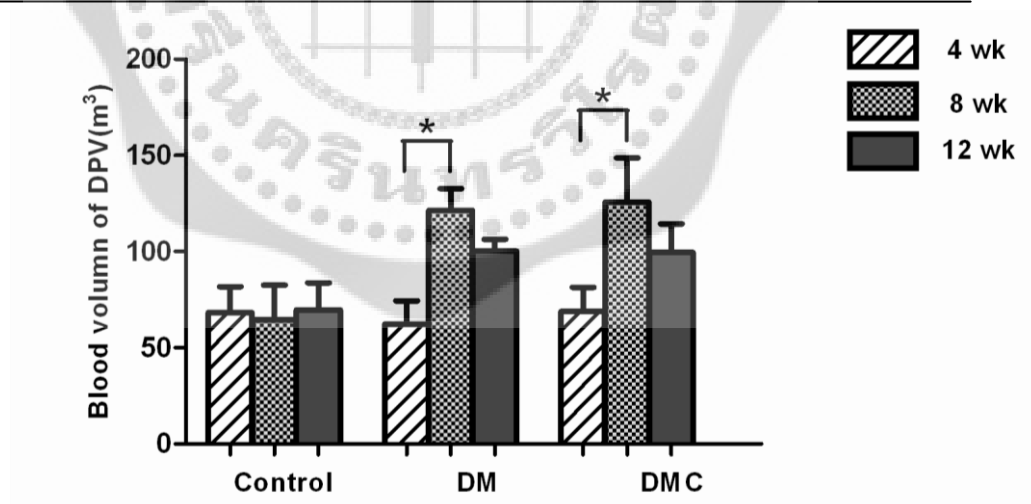


**Figure 72** Blood volume of distal part of portal veins at 4, 8, and 12 weeks of control, DM, and DMC rats. Values are means  $\pm$  SE \*  $p<0.05$

TABLE 24 and Figure 73. The information form showed the blood volume of distal part of portal veins of vascular corrosion cast with scanning electron microscopic levels in three groups of rats at different time intervals in the same conditions. There was significantly increased in DM and DMC rats at 4 week when compared with DM and DMC rats at 8 week ( $p<0.05$ ).

**TABLE 24** Blood volume of distal part of portal veins from control, DM, and DMC rats at 4, 8, and 12 weeks

| Groups  | 4 wk        | 8 wk         | 12 wk        |
|---------|-------------|--------------|--------------|
| Control | 68.28±13.40 | 62.28±12.08  | 68.85±12.65  |
| DM      | 64.45±18.12 | 121.48±11.25 | 125.83±22.92 |
| DMC     | 69.78±14.0  | 100.28±6.09  | 99.63±14.84  |



**Figure 73** Blood volume of distal part of portal veins at 4, 8, and 12 weeks of control, DM, and DMC rats. Values are means  $\pm$  SE \*  $p<0.05$

#### 4.8.8 Quantitative analysis of diameters of proximal part of portal veins branches by vascular corrosion cast technique with scanning electron microscope

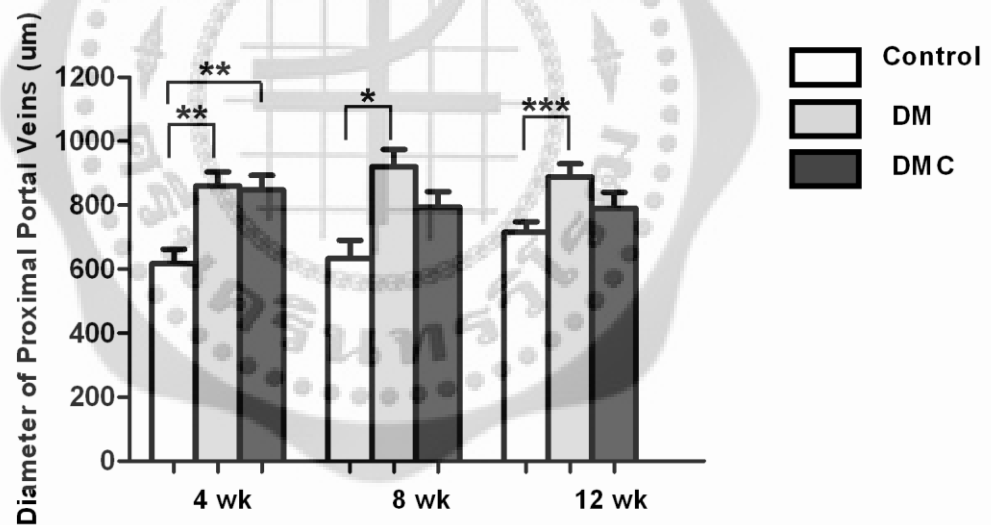
The diameters of proximal part of portal veins were score quantitatively in a blind fashion in three groups of rats at different time intervals. The data were shown that the DM and DMC rats revealed significantly increased diameters of proximal part of portal veins indices when they were compared to control rats at 4 week ( $p<0.01$ ). Moreover, the diameter of proximal part of portal veins of DM rats were significantly increased when compared to control rats at 8 and 12 weeks ( $p<0.001$ ,  $p<0.05$ ) (TABLE 25 and Figure 74).





**TABLE 25** Diameters of proximal part of portal veins at 4, 8, and 12 weeks of control, DM, and DMC rats

| Weeks | Control      | DM           | DMC          |
|-------|--------------|--------------|--------------|
| 4     | 617.25±45.71 | 860.5±44.08  | 847.25±46.65 |
| 8     | 633.25±57.24 | 920.5±54.34  | 794.5±48.07  |
| 12    | 715.25±33.18 | 888.25±40.82 | 790.0±50.53  |

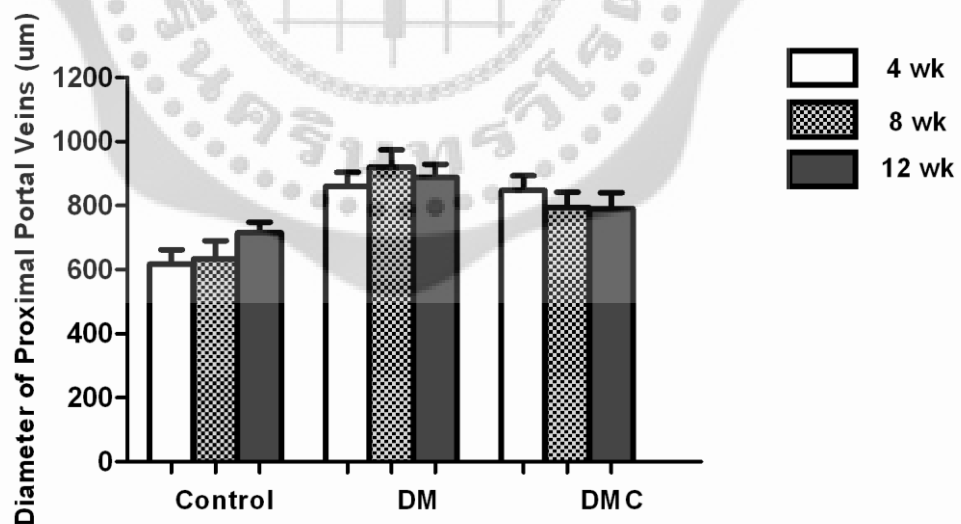


**Figure 74** Diameters of proximal part of portal veins at 4, 8, and 12 weeks of control, DM, and DMC rats. Values are means  $\pm$  SE \*  $p < 0.001$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.05$

TABLE 26 and Figure 75. The information form showed the diameters of proximal part of portal veins of vascular corrosion cast with scanning electron microscopic levels in three groups of rats at different time intervals in the same conditions. There were no significantly different among three groups of rats at different timing.

**TABLE 26** Diameters of proximal part of portal veins from control, DM, and DMC rats at 4, 8, and 12 weeks

| Groups  | 4 wk          | 8 wk         | 12 wk        |
|---------|---------------|--------------|--------------|
| Control | 617.25±45.71  | 633.25±57.24 | 715.25±33.18 |
| DM      | 860.5±44.08   | 920.5±54.34  | 888.25±40.82 |
| DMC     | 847.25±46.653 | 794.5±48.07  | 790.0±50.53  |



**Figure 75** Diameter of proximal part of portal veins from control, DM, and DMC rats at 4, 8, and 12 weeks.

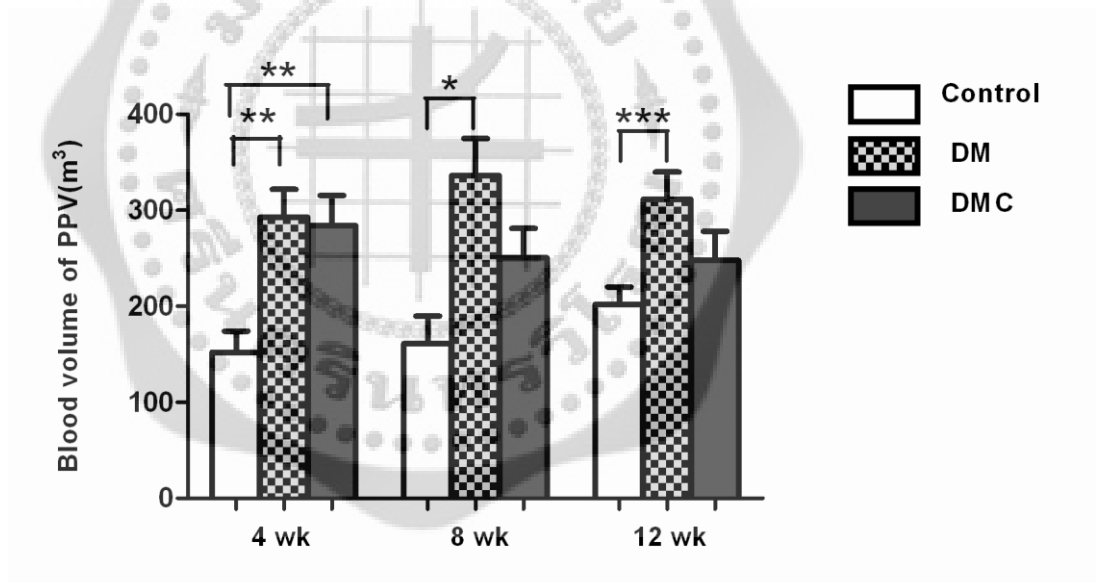
#### 4.8.9 Quantitative analysis of blood volume of proximal part of portal veins by vascular corrosion cast technique with scanning electron microscope

The blood volume of proximal part of portal veins of control, DM, and DMC rats from 4, 8, and 12 week experiments were score quantitatively in a blind fashion in three groups of rats at different time intervals. The data were shown that the DM rats presented significantly increased in the blood volume of proximal part of portal veins at 8 and 12 weeks indices when they were compared to control rats ( $p<0.001$ ,  $p<0.05$ ). (TABLE 27 and Figure 76).



**TABLE 27** Blood volume of proximal part of portal veins at 4, 8, and 12 weeks of control, DM, and DMC rats

| Weeks | Control     | DM           | DMC         |
|-------|-------------|--------------|-------------|
| 4     | 68.28±13.40 | 64.45±18.12  | 69.78±14.0  |
| 8     | 62.28±12.08 | 121.48±11.25 | 100.28±6.09 |
| 12    | 68.85±12.65 | 125.83±22.92 | 99.63±14.84 |

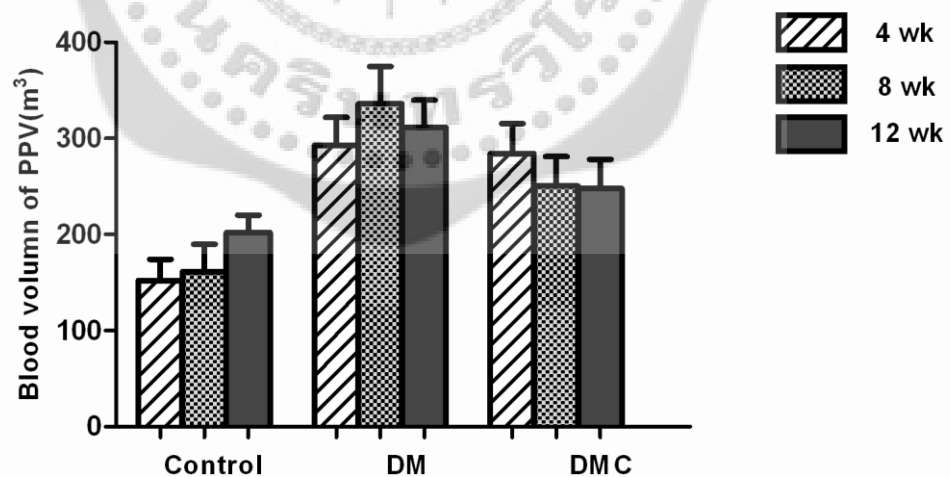


**Figure 76** Blood volume of proximal part of portal veins at 4, 8, and 12 weeks of control, DM, and DMC rats. Values are means  $\pm$  SE \*  $p < 0.001$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.05$

TABLE 28 and Figure 77. The information form showed the blood volume of proximal part of portal veins of vascular corrosion cast with scanning electron microscopic levels in three groups of rats at different timings in the same conditions. There were no significantly different among three groups of rats at different timing.

**TABLE 28** Blood volume of proximal part of portal veins from control, DM, and DMC rats at 4, 8, and 12 weeks

| Groups  | 4 wk        | 8 wk         | 12 wk        |
|---------|-------------|--------------|--------------|
| Control | 68.28±13.40 | 62.28±12.08  | 68.85±12.65  |
| DM      | 64.45±18.12 | 121.48±11.25 | 125.83±22.92 |
| DMC     | 69.78±14.0  | 100.28±6.09  | 99.63±14.84  |



**Figure 77** Blood volume of proximal part of portal veins at 4, 8, and 12 weeks of control, DM, and DMC rats.

## CHAPTER 5

### DISCUSSIONS

#### 5.1 The effect of curcumin on blood sugar level, body weight and liver function test

The diabetogenic action from STZ is mediated by selective destruction of pancreatic beta cells in islets of Langerhans. The important studies have shown that STZ causes increased generation of reactive oxygen species, DNA damage and extensive DNA repair leading to depletion of intracellular nicotinamide adenine dinucleotide (NAD) level. As a result, it further causes severe depletion of ATP pool, activation of DNA repair enzyme and poly ADP-ribose polymerase-1 (157). Poly ADP-ribose polymerase-1 overactivation and depletion of free radical scavenging potential caused by STZ lead to damage beta cells in Islets of Langerhans. Moreover, reactive oxygen species also play an important role in the pathogenesis and development of complications of diabetes mellitus (158). Specially, the principal pathological feature of diabetes is the morphological and functional alteration of microvessels. DM has been further reported as an important risk factor for coronary artery disease, atherosclerosis and stroke (159). Generally, an increase in intima-media complex thickness has been demonstrated in DM patients (160, 161).

In this study, an animal model for diabetes mellitus was developed by the injection of STZ into male rats. At the end of 4, 8, and 12 weeks experiments respectively, the blood samples were collected from the lateral tail veins of the 12 hours fasted rats. Fasting blood sugar was determined by one-touch glucometer. It was revealed that STZ injected rats produced a significantly increased blood sugar levels in DM rats at 4, 8, and 12 weeks corresponding with time intervals. Supplementation of curcumin (200 mg/kg BW) by intragastric feeding in DMC rats for 4, 8, and 12 weeks revealed that the blood sugar levels were toward

lowered, but there were no significantly different levels compared to the DM rats at the same ages. These findings agree with several workers who have reported that STZ-induced diabetes mellitus and insulin deficiency lead to increase of blood glucose and reveal association between specific diabetic complications and disturbances in various tissues, such as diabetic nephropathy and cardiovascular diseases (162). The increase of blood sugar levels further connect to stimulate the sorbital pathway and Protein kinase C (PKC), then, all of which may lead to induce growth factor production and cytokines. Direct high glucose concentration which induces mitogen-activated protein kinase (MAPK) and PKC activation also causes increased production of oxidative stress in the diabetic kidney (163-164).

Regarding curcumin, it potentially inhibits lipid peroxidation in rat liver microsome (133). Along with being an inhibitor of lipid peroxidation, It is known that curcumin has an inhibitory on PKC activity. Activation of PKC by glucose has been implicated in the regulation and activation of membrane-associated NAD(P)H-dependent oxidases and subsequent production of superoxide anion (165). Curcumin's protective function against peroxidative damage of biomembranes, known to be a free-radical-mediated chain reaction, has mainly been attributed to the scavenging of the reactive free radicals involved in peroxidation. Curcumin supplementation has also reduced the oxidative stress encountered by the diabetic rats, as demonstrated by lower levels of thiobarbituric acid reactive substance (TBARS), which may have been due to decrease influx of glucose into the polyol pathway, leading to an increased NADPH/NADP ratio and elevated activity of the potent antioxidant enzyme glutathione peroxidase (GPx). Moreover, the activity of sorbital dehydrogenase, which catalyzes the conversion of sorbital to fructose, is lowered significantly by treatment with curcumin (166). When curcumin is given to laboratory animals with diabetes, it is shown that the blood sugar levels shows general decrease.

In this study, the results demonstrated that the body weights of STZ-induced diabetic rats significantly decreased as compared to control rats at 8 and 12 weeks experiments. After curcumin supplementation, the body weights of DMC rats were significantly increased when

compared with DM rats at 8 and 12 weeks. Moreover, weight gain was noticed in control rats at time course of studies while DM and DMC had weight loss. According to the results, hyperglycemia did not help to gain body weight in DM and DMC rats. The reduction of body weight probably led to decrease gastrointestinal hormones relief that participated in the stimulation of beta cells proliferation. Thus, if diabetic rats are treated with curcumin in longer time as more than 12 weeks, it will be expected that body weight might tend to recover as normal levels.

Liver function test are commonly used in clinical practice to screen for liver disease. In present study, it was designed to observe the effects of STZ-induced diabetes on the liver and liver enzymes at various time intervals after STZ treatment. According to the functional test, the levels of SAKP were significantly increased in the livers of DM rats when compared with control rats at 8 and 12 weeks. After curcumin supplementation, the levels of SAKP of DMC rats were decreased when compared with DM rats at 8 and 12 weeks. SAKP is a membrane bound enzyme glycoprotein enzyme. It is an enzyme that become elevated when the bile ducts become blocked. Blockage occurs because liver disease interferes with the liver's ability to maintain bile flow. It is present in highest concentration in the sinusoids and in the endothelium of the central and portal veins; smaller concentration occur in the biliary canaliculi (167). Increase in the levels of SAKP in STZ-induced diabetic rats were also reported by many researchers (168-169). SGOT and SGPT are both enzymes that are mainly found in mitochondria of the liver. If liver damage is present, the enzymes are released into the bloodstream after liver cell death. Abnormally high concentrations of SGOT and SGPT in the blood may indicated damage to the liver. SGOT is normally present in liver, heart, skeletal muscle, kidneys, brain, and red blood cells. The blood SGOT are thus elevated with liver damage or with an insult to the heart ( for example, from heart attack). Elevated SGOT levels are not specific for liver damage, and SGOT has also been used as a cardiac marker. By contrast, SGPT is normally found largely in the liver. Therefore the SGPT is a much more specific indicator of liver dysfunction.



The previous studies have shown that subtle membrane changes are sufficient to allow passage of intracellular enzymes to the extracellular space. Very large concentration gradient between the hepatocytes and the sinusoidal space usually exists for enzymes. Cell damage increases permeability causing cytosolic isoenzymes to spill into the sinusoids and from further into the peripheral blood (170). Moreover, the researchers have showed that STZ-induced in rats produced alterations in hepatic functions as described in poorly controlled diabetics (167). Rajesh et al. (171) reported that the assessment of liver functioning after supplementation of soluble curcumin SGOT, SGPT, SAKP was decreased in the activities of SGOT, SGPT and SAKP in serum evidenced the positive effect of curcumin on liver. Pari et al. (172) also reported about the administration of 80 mg/kg body weight tetrahydrocurcumin is an antioxidative substance, derived from curcumin, for 8 days in female wistar rats treated with hepatotoxic compound chloroquine. Concerning their results, SGOT, SGPT, SAKP and total bilirubin levels were found to be significantly decreased in rats after supplemented with curcumin.

## **5.2 The effect of curcumin on histological study of the liver tissue and blood vessels**

In this work, an animal model for diabetic mellitus was developed by the injection of streptozotocin into the DM rats and treated with curcumin in DMC rats. At the end of 4, 8 and 12 week experiments, the liver tissues and blood vessels were studied by LM observation. The results demonstrated that the characteristics of liver tissues and blood vessels of STZ-induced diabetic rats revealed pathology and destructions. Interestingly, healthy liver tissues and blood vessels became recovered and redeveloped in curcumin-treated diabetic animals.

The results from LM observation of liver sections of DM rats at 8 and 12 weeks were shown that sinusoids around the central veins in area of zone 3 (the area of poorly oxygenated and it was the first sign to show ischemic necrosis and fat accumulation if metabolism was altered) and central veins were dilated and filled with lots of red blood cells. After treatment of curcumin supplementation, the healthy and nourishing microvessels and hepatocytes were

observed in the liver tissue of DMC rats. The diameters of sinusoids and central veins indices of DM rats had significantly increased when they were compared to control rats at 8 and 12 weeks. After curcumin supplementation, the diameters of sinusoids and central veins of DM rats were significantly reduced when compared with DMC rats at 8 and 12 week. These findings of present study are in agreement with the reports (173-174) that the histopathological characteristic of liver has the lesions, causing by STZ injection. The rat liver sections showed the dilated portal vessels, sinusoids and veins. In mice kidney at 4 and 8 weeks after injection with STZ revealed glomerular and renal tubular hypertrophy, and glomerular capillary dilatation in all DM mice. After treatment by curcumin, kidney tissues in DMC mice dramatically reduced glomerular and renal tubular hypertrophy and glomerular capillary dilatation as compared with DM mice (175).

Hepatic sinusoids are special capillaries that are limited by fenestrated endothelial cells without a genuine basement membrane. There were surrounded by perisinusoidal cells storing vitamin A, Kupffer cells as well as pit cells, resident macrophages and large granular lymphocytes (176). Hepatic sinusoidal capillarization includes a series of pathological changes, in which sinusoidal endothelial cells lose fenestrae, basement membrane develops and sinusoidal outlets collapse. All these pathological changes disable the normal sinusoid intrinsic functions. Capillarization of the sinusoid may cause a disturbance in the exchange of many bioactive substances between the sinusoidal blood and hepatocytes across the Disse space, contributing to the pathogenesis of liver fibrosis (177).

According the results, the presented pyknosis of hepatocyte nuclei and edematous hepatocytes were characterized by perpetuation of cell injury and cell death of hepatocytes. An accumulation of lipid droplets in the cytoplasm of hepatocytes was markedly evident. According to the accumulation of lipid droplets in the cytoplasm of hepatocytes, this characteristic was reminiscent to the formation of fatty liver. It could be due to the increased influx of fatty acids

into the liver, which was induced by hypoinsulinemia and the low capacity of excretion of lipoprotein secretion from liver resulting from a deficiency of apoprotein B synthesis (178). Generally, fatty liver is the result of an intracellular accumulation of triglycerides because of increased uptake of free fatty acids and *de novo* liponeogenesis in the hepatocytes. At the same time, there is a reduction in the hepatic secretion of very low density lipoproteins. The liver damage consists of cellular necrosis and inflammation, and these disorders result from an increase in mitochondrial oxidative stress on triglycerides with the consequential generation of free radicals and peroxisomes (23). The mitochondrial oxidative stress is also increased by the action of adipokines (cytokines produced by the adipocytes) such as leptin and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), which are produced in excess (24). The reduction of adiponectin, which is a regulatory adipokine, favors the activity of inflammatory adipokines (25). These chemical mediators, derived from inflammation and cell necrosis, as well as the adipokines activate the liver stellate cells and induce them to increase production of collagen, connective tissue growth factor and accumulation of extracellular matrix, in turn favoring fibrosis (26). The glycogen content of the hepatocytes in the liver of STZ-treated animals was markedly decreased. It could be due to the displacement of glycogen in the cytoplasm of hepatocytes as a consequence of accumulation of lipid droplets (179).

Moreover, the rat's liver of DM showed the inflammatory changes of liver tissues that were revealed by infiltration of lymphocytes and increasing of thick bundles of collagen deposition in the area of portal triad. In curcumin-treated rats, the liver tissue showed closely normal pattern of healthy central veins, sinusoids, hepatocytes and portal triad, when compared with liver tissues of control rats. Interestingly, the curcumin-treated liver tissues demonstrated recovered typical arrangement of radiating plates of hepatocytes and sinusoids together with central veins. These findings of present study are in agreement with the reports that the pancreatic islets of diabetic mice at 8 week after injected with STZ were enlarged and

lymphocytes infiltration involved the islets and adjacent ducts and veins. In the pancreas of diabetic mice fed with curcumin showed small islets and aggregation of lymphocytes adjacent to interlobular ducts (180).

Most chronic liver diseases are characterized by fibrosis and inflammation. The adipokines activate the hepatic stellate cells and induce them to increase production of collagen, connective tissue growth factor and accumulation of extracellular matrix, in turn favoring fibrosis. Hepatic stellate cells, also known as perisinusoidal cells or Ito cells (earlier lipocytes or fat-storing cells), are pericytes found in the perisinusoidal space (a small area between the sinusoids and hepatocytes) of the liver also known as the space of Disse. Changes in the space of Disse microenvironment, which are caused by activated HSCs, play an important role in facilitating the progression of liver fibrosis (229). Migration of resident HSCs within the space of Disse and potentially in other compartments is considered important for progression of liver fibrosis because of the increased number of activated HSCs in areas of inflammation (230). The stellate cell is the major cell type involved in liver fibrosis, which is the formation of scar tissue in response to liver damage. When the liver is damaged, stellate cells can change into an activated state. The activated stellate cell is characterized by proliferation, contractility, and chemotaxis. The amount of stored vitamin A decreases progressively in liver injury. The activated stellate cell is also responsible for secreting collagen scar tissue, which can lead to cirrhosis (231).

Hepatic fat accumulation is a well-recognized complication of diabetes with a reported frequency of 40-70%. Fat is stored in the form of triglyceride and may be a manifestation of increased fat transport to the liver, enhanced hepatic fat synthesis and decreased oxidation or removal of fat from the liver. The steatosis may be microvesicular or macrovesicular and may progress to fibrosis and cirrhosis. Moreover, an excessive amount of extracellular matrix is synthesized and accumulated during the fibrogenic process. Fibrotic tissue offers resistance to blood flow and to the delivery of oxygen, thus becoming hypoxic. Stimulation of hypoxia-

inducible factors leads to an angiogenic switch to the upregulation of proangiogenic factors, and to the formation of neovessels (55).

Liver inflammation is initiated by Kupffer cells, the resident macrophages of the liver. Moreover, this cells involves the formation and release of many inflammatory mediators thereby recruiting neutrophils, lymphocytes, and other inflammatory cells into damaged regions (181). It is a complex and multifactorial process in which hepatocytes, other nonparenchymal cells (endothelial cells and hepatic stellate cells (HSC), and infiltrating inflammatory cells) interact with one another through soluble mediators, surface receptors, and adhesion molecule expression (182). The main pro-inflammatory cytokines released by Kupffer cells in response to hepatocellular stress, interleukin-1 (IL-1), interleukin-6 (IL-6), and TNF- $\alpha$ , stimulate hepatocytes and other nonparenchymal cells in a paracrine manner. TNF- $\alpha$  released from LPS-stimulated rat Kupffer cells requires internalization of LPS, acidification of endosomes, elevation of intracellular calcium, PKC activation, and protein tyrosine kinase activation. Stimulated sinusoidal endothelial cells express cell adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and mediate immigration of neutrophils and blood monocytes supported by chemokines which are released by HSCs (183). Most of the hepatic antioxidant defences are essentially confined to parenchymal cells, Kupffer cells, HSCs, and endothelial cells which are potentially more exposed or sensitive to oxidative stress-related molecules. Activated neutrophils, macrophages and Kupffer cells are major source of ROS during inflammation.

Various compounds may inhibit the formation of pro-inflammatory cytokines thus offering the means for modulation of the process and possibly avoiding extensive damage (inflammation) of the surrounding tissue by invading neutrophils and lymphocytes. Curcumin downregulates the expression of various cell surface adhesion molecules that have been linked with inflammation (112), downregulates the expression of various inflammatory cytokines, including TNF- $\alpha$ , IL-1, IL-6, IL-8, and chemokines (113-115). Curcumin has been shown to inhibit the action of TNF- $\alpha$ , one of the most pro-inflammatory of the cytokines (116). Moreover, curcumin is a potent antioxidant, which might contribute to its anti-inflammatory action. (63, 117-119).

### 5.3 The effect of curcumin on VEGF expression of the liver tissue and blood vessels by immunohistochemical study and Western blotting

Immunohistochemical staining for VEGF in animal model for diabetic mellitus was developed by the injection of streptozotocin into the DM group and treated with curcumin in DMC group. At the end of 4, 8 and 12 week experiments, there demonstrated that VEGF was fundamentally recognized in the hepatocytes and endothelial cells of the central veins generally, evidenced by brownish color in the cytoplasm and to less extent in the area of portal triad of three groups of rats. The intensity and pattern of this distribution varied, according to the durations of the experiments. VEGF intensity was strongly stained in DM rats at 12 week as compared with DM rats at 4 and 8 weeks. After curcumin treatment, VEGF immunostaining scores were significantly lowered in DMC rats at 8 and 12 weeks when compared with DM rats.

In agreement of previous reported, VEGF immunostaining was found to be localized in hepatocellular carcinoma and surrounding cirrhotic liver tissues (197) and after administration of expression vector encoding for vascular endothelial growth factor D in normal and ischemic rat livers (198). Most of the liver conditions were proved to be involved in the morphological and pathological changes of intrahepatic vessels. Previous study demonstrated that, when livers were under the states of chronic inflammations, chronic necrosis and fibrosis, the density of microvessels were presumed to reduce probably due to the decreased expression of angiogenesis related growth factors such as VEGF, insulin-like growth factor, and over expression of transforming growth factor beta, platelet-derived growth factor (199).

By, Western blotting method, the interested VEGF protein band was observed and demonstrated by using monoclonal anti-VEGF antibody. Western blot analysis established the specific VEGF protein band at molecular weight 23 kDa on film by Enhanced chemiluminescence (ECL) method in control, DM, and DMC samples from rats liver at 4, 8 and 12 week experiments. This specific protein represented VEGF protein. The bands from DM

revealed more intensity of VEGF expression than control and DMC rats on ECL film, especially in 12 week experiment. The expression of VEGF was reduced after treatment by curcumin in DMC rats when it was compared with DM rats.

Concerning immunohistochemical study and Western blotting, the results could be implied that curcumin could improve liver tissue and blood vessels destruction from STZ by anti-inflammation activity resulting the improving of liver tissue and blood vessels. This event might be related to the structures in which increasing of VEGF was required for tissues repair and remodeling. In this study, the change of VEGF protein expression levels in liver tissue of three groups of rats in different durations by Western blotting were correlated with the localization of VEGF by immunohistochemical method. The increasing of VEGF protein level in liver tissue of DM at 12 weeks suggested that chronic hyperglycemia might stimulate inflammatory situation to hypoxia and expression of VEGF protein for maintenance of permeable endothelium blood vessels of the liver. The neovascularization could further occur occurred from angiogenesis.

Strong evidence supports the hypothesis that VEGF is a key mediator of angiogenesis associated with various disorders (200). Oxygen tension is a key regulator of VEGF gene expression both in vitro and in vivo. VEGF as a hypoxia-inducible angiogenic factor has been extensively described in recent years (201,202). Both human and experimental studies have also outlined that several peptide mediators other than VEGF, Ang-1 and HGF are likely to be involved in hepatic angiogenesis associated with the fibrogenic progression process in CLDs. Unequivocal data have been provided for the pro-angiogenic action of PDGF (204). as well as for leptin. In addition, leptin is an adipocytokine that has been suggested to exert a pro-fibrogenic effect in promoting the development from non-alcoholic fatty liver disease (NAFLD) to nonalcoholic steatohepatitis (NASH) (205).

Hypoxia is well known to be one of the strongest stimulants for angiogenesis and HIF-1 $\alpha$  has been well recognized to play an essential role in hypoxic adaptation during mammalian development (219). Gopfert et al. (220) showed that moderate hypoxia enhanced HIF-1 $\alpha$  expression in rat hepatocytes whereas Maeno et al. (221) found that HIF-1 $\alpha$  expression was preceded by VEGF and fms-like tyrosine kinase-1 (flt-1) expressions. It was hypothesized that

HIF-1a was related to sinusoidal endothelial reconstruction. In the previous study, VEGF mRNA expression was observed to occur after HIF-1a mRNA expression in all analyzed organs, and we therefore would like to propose that elevation of the portal venous pressure after PHx, which arises through shear stress, causes portal venous stasis. Then this event results in hypoxia in the viscera around the portal vein, and consequently elevates the HIF-1a level, thereby leading to VEGF production.

The VEGF growth factors are a family of homodimeric glycoproteins that are encoded by 4 VEGF genes (A–D), and a related growth factor, placental growth factor (PLGF) is encoded by placenta growth factor gene (PIGF). It is well known that VEGF-A expression is regulated by hypoxia, glucose concentration, pH and several oncogenes. In general, all VEGF family members elicit their biological functions by their bindings to cell-surface VEGF receptors (VEGFRs). The binding initiates a cellular response via activation of the intracellular tyrosine kinase domain of the receptor. VEGF-R2 (also called KDR) is the key receptor that induces both angiogenesis and vascular permeability (206). While extensive research has shown that VEGF is crucial for developmental, physiologic and pathologic angiogenesis, whether it is required in the adult is unclear. Early studies of genetic VEGF targeting and neutralization studies in tumorigenic mice (39) suggested that VEGF was not necessary required in the adult as these adult mice showed no obvious phenotype, making VEGF as an attractive target in many diseases. As a result, a wealth of pharmacologic agents have been designed to target either VEGF or its receptors. Though beneficial effects have been observed, the presence of consistent significant side effects suggests that VEGF has been important in the maintenance of quiescent vasculature and non-vascular tissues.

In addition, the highest density of VEGF expression has been found in tissues with fenestrated vasculature and was localized to the epithelium in contact with fenestrated vessels. Tissues, whose microvasculature are fenestrated, have secretory and/or filtration functions, for example the glomerulus (urine filtrate), choroid plexus (cerebrospinal fluid production), pancreas (exocrine and endocrine secretions) and liver (filtration and secretion). Thus, VEGF in these tissues may mediate both survival and maintenance of the fenestrated state of the



endothelial cells. Though endothelial cells are the main target of VEGF, they do not generally express VEGF (212), but instead respond to VEGF secreted in a paracrine manner. It is speculated that expression of both the receptor and ligand in EC would lead to a dangerous positive feedback, resulting in overproliferation, such as in hemangiomas and in tumorigenesis (213).

VEGF, like other growth factors (e.g. hepatocyte growth factor and fibroblast growth factor), is named because of initial observations demonstrating endothelial cells as its target. However, VEGF may act on other cell types based on the presence of either VEGFR1 or VEGFR2 on those cells. In addition, several reports have demonstrated the action of VEGF on monocytes, macrophages, mast cells, eosinophils, dendritic cells, megakaryocytes, lymphocytes, hematopoietic stem cells/bone marrow-derived circulating cells, type II lung alveolar epithelial cells and lens epithelium (222). VEGF has been shown to mediate the survival of hematopoietic stem cells, neuronal cells, neuronal stem cells and lymphocytes; the differentiation of megakaryocytes and dendritic cells; and to mobilize bone marrow precursor cells. According to its direct effects on endothelial cells during angiogenesis, VEGF also influences angiogenesis via recruitment of bone marrow endothelial cell precursors and inflammatory cells.

VEGF expression has been demonstrated at the highest amount in HCC and minimal in cirrhosis, which has strongly correlated with the degree of vascularization. Indeed, HCC has been characterized by an increased expression of VEGF receptors, demonstrated by both mRNA and protein expression levels (207). Deli et al (208) and Mathonnet et al (209) reported earlier that cirrhotic liver, surrounding HCC, expressed higher levels of VEGF compared to that of the tumor. In the previous study we not only provided the quantitative analysis of VEGF expression, but also divided cirrhotic liver into two components: 1) cirrhotic nodules where the levels of vascularization and VEGF expression were decreased, and 2) fibrotic stroma, surrounding these nodules, where both the number of vessels and VEGF expression were significantly increased. It has been previously demonstrated that the expression of VEGF in HCC is significantly associated with a higher proliferative index of tumor cells. Also, VEGF is

expressed in the fibrovascular stroma and pericellular matrix of HCC (210). The expression of VEGF by hepatocytes in the cirrhotic liver has recently been demonstrated. One of the sources of VEGF in the fibromuscular stroma may be fibroblasts, since the ability of these cells to produce VEGF was demonstrated earlier (211).

Several researchers have reported that VEGF secreted by proliferating hepatocytes represents an important stimulator of sinusoidal endothelial cell proliferation (214). Previous studies have reported that VEGF mRNA expression is markedly increased in the regenerating liver between 48 and 120 hr after hepatectomy, and gradually decreased thereafter (215). Taniguchi et al. (216) have demonstrated that VEGF mRNA expression is mainly detected in periportal hepatocytes by immunohistochemistry and in situ hybridization and suggested that VEGF promotes hepatocyte proliferation through the reconstruction of liver sinusoids via proliferation of sinusoidal endothelial cells. Shimizu et al. (214) have also demonstrated that VEGF secreted by replicating hepatocytes induces sinusoidal endothelial cell proliferation during regeneration after Partial hepatectomy (PHx) in rats. The results have revealed that not only the systemic VEGF level, but also the portal VEGF levels gradually increased during the first 168 h after PHx. In addition, the portal venous VEGF levels were significantly higher than the systemic VEGF level for 72 to 168 h after PHx (217). These results suggest that portal venous VEGF plays an important role in liver regeneration after PHx. On the other hand, Bockhorn et al. (218) have recently reported that VEGF is important during liver regeneration, however exogenous VEGF administration does not improve liver regeneration and survival liver regeneration after 90% PHx in rats. For the next step, liver regeneration under VEGF administration from portal vein should be analyzed.

Curcumin has been described a multitude of therapeutic activities and has been associated with suppression of inflammation, angiogenesis, tumorigenesis, and diabetes, and with therapeutic effects in diseases of the cardiovascular, pulmonary and neurological systems and of skin and liver. In general, most of these effects can be attributed to the antioxidant, anti-inflammatory and anticancer activities of curcumin. Curcumin is an effective scavenger of reactive oxygen species and reactive nitrogen species (166). Curcumin's protective function is

against peroxidative damage of biomembranes, known to be a free-radical-mediated chain reaction. Its function has mainly been attributed to the scavenging of the reactive free radicals involved in peroxidation. These scavenging properties of curcumin have also been considered to be responsible for its protective role against oxidative damage of DNA and proteins which are believed to be associated with a variety of chronic diseases such as cancer, atherosclerosis, neurodegenerative diseases and aging. In addition to its direct antioxidant activity, curcumin may function indirectly as an antioxidant by inhibiting the activity of inflammatory enzymes or by enhancing the synthesis of glutathione. The anti-inflammatory activity of curcumin seems to be comparable to steroidal drugs and non-steroidal drugs such as indomethacin and phenylbutazone. Many studies of curcumin's anti-inflammatory properties point to its ability to suppress prostaglandin synthesis by its effect on cyclooxygenase (COX) (165). COX is a key enzyme responsible for the conversion of arachidonic acid to prostaglandins. Curcumin has also been shown to suppress the production of cytokines such as interferon- $\gamma$  (IFN- $\gamma$ ), interleukins and tumor necrosis factor (TNF); to inhibit the inducible nitric oxide synthase (iNOS); and to suppress the activation of NF- $\kappa$ B. These pathways affected by curcumin not only play an important role in inflammation; they have also been associated with the development and progression of cancer. Curcumin has promising chemopreventive and therapeutic potential for various cancers including leukemia (223).

Few patients with hepatocellular carcinoma are diagnosed early and treatment options are very limited. Therefore surgery is currently the most effective treatment. However, recurrence rates are high and long-term survival is poor. Use of radiation and chemotherapy is limited and identifying novel chemopreventive and chemotherapeutic agents are imperative. Several studies have investigated that curcumin could inhibit the hypoxia that induced angiogenesis through down-regulation of hypoxia-inducible factor HIF-1(224) and it could blocked the transactivation of the c-met promoter by activated protein 1 (AP-1). Curcumin alone or in combination with cisplatin or doxorubicin exerted cell growth inhibitory and apoptotic effects, in part due to changes in NF- $\kappa$ B levels (225). Curcumin exhibited anticancer properties in several *in vivo* models of hepatocellular carcinoma. In mice treated with N-bis (2-hydroxypropyl) nitrosamine (DHPN), curcumin significantly inhibited liver adenoma formation

and growth. Concerning mice with diethylnitrosamine (DEN)-induced hepatocarcinogenesis, curcumin reduced tumor multiplicity and incidence (226). In an orthotopic implantation model of hepatocellular carcinoma CBO140C12, cells curcumin suppressed intrahepatic metastasis but did not affect the growth of the implanted tumor itself (227). In N-nitrosodiethylamine (DENA)-initiated and phenobarbital-promoted hepatocarcinogenesis in Wistar rats, curcumin showed beneficial effects against oxidative tissue damage during chemically induced hepatocarcinogenesis (228) suggesting that curcumin may exhibit potential as a therapeutic in liver cancer.

#### **5.4 The effect of curcumin of liver microvasculature on vascular corrosion cast technique with scanning electron microscope**

In this work, an animal model for diabetic mellitus was developed by the injection of streptozotocin into the DM group and by the treatment with curcumin in DMC group. At the end of 4, 8 and 12 week experiments, the blood vessels were studied by vascular corrosion cast technique with SEM. The results demonstrated that the characteristics of proximal and distal part of portal veins together with portal venule branches of STZ-induced diabetic rats revealed pathology and destructions. Interestingly, healthy and blood vessels became recovered and redeveloped in curcumin-treated diabetic animals.

Liver vascular changes of DM rats at 4 week experiment especially demonstrated the event of liver blood vessels hypertrophy which presented mild unhealthy destroyed characteristics of blood vessel morphology. The portal venules showed signs of mild blind endings and shrinkage. Moreover, the noticeable lesions of diabetic pathology consisted of the expansion of proximal and distal parts of portal veins together with venule branches in DM rats at 8 week. The data of diameters and blood volume of proximal and distal parts of portal veins together with portal venule branches were different significantly of diameter and blood volume when compared with control and DMC rats at 8 weeks. It demonstrated the event of liver blood vessels hypertrophy which presented unhealthy destroyed characteristics blood vessel morphology. Moreover, distal portion of portal veins showed signs of abnormal structures of

blind endings and shrinkage of portal venules. At 12 week experiments, the lesion of diabetic pathology consisted of seriously destruction of blood vessels and presented unhealthy destroyed characteristics blood vessel morphology in DM rats liver. It demonstrated the event of liver blood vessels, especially portal venules, were seriously shrinkage and their diameter were decreased when compared with DM rats at 4 and 8 week. Moreover, distal portion of portal veins also showed signs of abnormal structures of blind endings and shrinkage of portal venules.

The research of vascular corrosion cast in the liver of diabetes models, almost have no reports in the previous studies. However, the 17-month-old Otsuka Long-Evans Tokushima Fatty rat (spontaneous diabetic rats) which was used as a new experiment model of diabetic retinopathy, have been reported that they had some potential problems, including incomplete corrosion, incomplete filling, and extravasation of injected resin. Occasionally, there was a leakage of plastic material or incomplete filling of retinal capillaries, perhaps due to differences in physiological pressure between vessels and capillaries in the retinal blood vessels. A microaneurysm was presented in retinal vascular corrosion cast in their studies (184). Recently, Tee et al. (185) reported the transgenic mouse model of diabetic retinopathy and preparation of choroidal corrosion casts for quantitative analysis using scanning electron microscopy. The damage was worst in the smallest vessels, that was the capillaries of the choriocapillaris, and was remarkably similar to a study using choroidal casts in human diabetes in which microaneurysms, constrictions, shrinkage, blind endings and capillary dropout were observed. The intermediate-sized vessels, namely the arterioles and venules, showed a different damage profile. These findings agree with the report of Tee et al. that the damage was worst in the smaller vessels, portal venule, than the distal and proximal portal veins in which shrinkage and blind endings were demonstrated.

Glucose is the main cause for microvascular complications of diabetes like retinopathy, nephropathy and neuropathy. The negative role of hyperglycemia on endothelial functions and pathological changes occurring in diabetes are well established. There are four major molecular signaling mechanisms activated by hyperglycemia in endothelial cells (186). These include:

activation of PKC, increased hexosamine pathway flux, increased advanced glycation end product formation, and increased polyol pathway flux.

Protein kinase C (PKC) is a family of serine-threonine kinases that plays an important role in signal transduction mechanisms (187). The PKC pathway is further activated in diabetes as a result of hyperglycemia. In this pathway, PKC is activated by the increased amounts of diacylglycerol (DAG), which is synthesized directly from glycolytic intermediates such as dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (188-189). PKC appears to be activated in a range of diabetic tissues including the retina, kidney, heart, and aorta. An activation of PKC has been implicated in many processes relevant to diabetic complications, including regulation of vascular permeability and flow, increased production of cytokines, and increased synthesis of basement membranes (190). In diabetes microvascular complications, for example, PKC affects the activation of a number of growth factors and changes the function of vasoactive factors. These vasoactive factors include vasodilators such as nitric oxide (NO) as well as vasoconstrictors such as angiotensin II and endothelin-1 (191).

Only a small proportion of glucose is metabolized to sorbitol during normoglycemia, while in hyperglycemia the enzyme aldose reductase (AR) is activated, leading to an accumulation of intracellular sorbitol and fructose that increase the flux through the polyol pathway (192). Sorbitols and other polyols accumulate intracellularly, leading to osmotic damage and swelling. Aldose reductase (AR) is the first and rate-limiting enzyme of the polyol pathway, which converts monosaccharides (glucose) to their polyols or sugar alcohols (sorbitol). This enzyme is widely distributed throughout the body, including those tissues that are susceptible to chronic diabetic complications (retina, lens, cornea, glomerulus, nervous system and the blood vessels). In fact, alterations in sorbitol and fructose metabolism are implicated as factors contributing to vascular complications in diabetes mellitus (193). Non-enzymatic glycation has recently attracted increasing interest as a crucial pathophysiologic event behind hyperglycemia-related alterations and in the pathophysiology of the development of diabetic complications. Proteins and lipids exposed to aldose sugars go through reactions, which ultimately lead to the formation of advanced glycation end products (AGEs), a

heterogeneous mixture of complex structures (194). Although the series of reactions producing AGEs occur at a very slow rate under normal circumstances, in diabetes their formation is accelerated to an extent related to the level and duration of hyperglycemia (195). The potential pathophysiological significance of AGEs is associated with their accumulations in plasma, cells and tissues. Moreover, their contributions are related to the formation of cross-links, generation of reactive oxygen intermediates, and interactions with particular receptors on cellular surfaces (196).

After curcumin supplementation, the healthy normal patterns of liver blood vessels have recovered and got restored at 8 and 12 week respectively. Liver blood vessels in curcumin-treated group became regenerated and repaired in to healthy and normal characteristics. Moreover, the new development and neovascularization of new nourishing blood vessels have emerged and presented extensively. Angiogenesis is the process of formation of new blood vessels from preexisting vessels, occurs in several organs during pathophysiological situations (38). Most chronic liver disease are characterized by fibrosis and inflammation. Moreover, an excessive amount of extracellular matrix is synthesized and accumulated during the fibrogenic process. Fibrotic tissue offers resistance to blood flow and to the delivery of oxygen, thus becoming hypoxic. Stimulation of hypoxia-inducible factors leads to an angiogenic switch to the upregulation of proangiogenic factors, and to formation of neovessels (55).

However, mechanical stimulation of angiogenesis is not well characterized. There is a significant amount controversy with regard to shear stress acting on capillaries in order to cause angiogenesis. The mechanical of shear stress is considered the major stimulator of angiogenesis (33). Various different chemical stimulants have been identified, characterized and worked upon that they stimulate the process of angiogenesis. Many factors have been reported as inducing angiogenesis, such as hypoxia, inflammation and coagulation (35). Hypoxia and inflammation are the main stimuli for angiogenesis. Hypoxia promotes the angiogenesis by signaling through hypoxia inducible transcription factors (36). Vascular permeability is increased during inflammation. Moreover the monocytes, macrophages, platelets, mast cells and other leucocytes are recruited under the attraction of chemokines and

able to produce angiogenesis cytokines and growth factors (37). Angiogenesis can be envisaged as a dynamic, hypoxia stimulated and growth factor-dependent ubiquitous process leading to the formation of new vessels from pre-existing blood vessels (203). Angiogenesis should be distinguished from other terms that, although related to vessel formation and growth, are defined significantly different processes such as vasculogenesis, arteriogenesis and collateral vessel growth. Angiogenesis occurs virtually in almost all organs and tissues and is considered a critical step in either physiological conditions or in tissue repair and growth in several pathophysiological conditions, including chronic liver diseases (CLDs) (32, 35).

Pathological angiogenesis, irrespective of the aetiology, has been indeed extensively described in chronic liver diseases characterized by an extensive and prolonged necro-inflammatory and fibrogenic process, including chronic HBV, HCV, autoimmune hepatitis, and primary biliary cirrhosis (32). The formation of new vessels, which is closely associated with the pattern of typical fibrosis development of the different Chronic liver disease, leads to the progressive formation of the abnormal angio-architecture distinctive of cirrhosis, i.e. the common end-point of fibrogenic CLDs. Specifically, the association of fibrogenesis and angiogenesis should be regarded as crucial in the modern evaluation of disease progression and in the search for therapeutic targets. In addition, depending on the different patterns of fibrogenic evolution (i.e. post-necrotic, biliary, centrolobular, pericellular/perisinusoidal), the extent of neo-angiogenesis may have profound consequences on the rate of disease progression to cirrhosis and represent a key determinant affecting reversibility of fibrosis.

Numerous lines of evidence suggest that curcumin is a potent anti-inflammatory agent. Firstly, curcumin suppresses the activation of the transcription factor nuclear factor kappa B (NF- $\kappa$ B), which regulates the expression of pro-inflammatory gene products (104-107). Secondly, curcumin downregulates the expression of COX-2, an enzyme linked with most types of inflammations (107-109). Thirdly, curcumin inhibits the expression of another pro-inflammatory enzyme: 5-LOX (177). Additionally, curcumin has been shown to bind to the active site of 5-LOX and inhibit its activity (111). Finally, curcumin downregulates the expression of



various cell surface adhesion molecules that have been linked with inflammation. All of these recent evidences confirm the anti-inflammatory action of curcumin, gradually known for hundreds of years. Its pharmacological safety combined with its anti-inflammatory action, make it as an ideal agent to explore for preventive and therapeutic situations. Whereas pro-oxidants are considered as mediators of numerous diseases, antioxidants are generally believed to delay or halt the diseases.

Extensive liver injury of various etiologies, which are manifested as hepatocyte damage and associated with inflammatory and immune response, is the starting point for events resulting in fibrosis. Generally, liver inflammation is initiated by Kupffer cells which are the resident macrophage of the liver. This inflammation involves the formation and release of many inflammatory mediators and other inflammatory cells into damage regions. The sinusoid endothelial lines the sinusoidal space and forms a barrier, which serves to divide the liver into functional compartments. However, Kupffer cells are able to traverse this barrier and pass in and out of the hepatic space, facilitating their signaling functions. This signaling role is the ability of Kupffer cells to respond to local changes by the release of cytokines and other signaling molecules such as reactive oxygen species, a potent antioxidant agent, and free radical scavenger.

It is possibly proposed that curcumin could improve the destruction of the liver blood vessels because the potential beneficial effects of curcumin have been shown to exhibit anti-inflammatory<sup>3</sup>, antioxidant and anti-diabetic activities. Potential treatment with curcumin in diabetes has been demonstrated meaningfully about the therapeutic consequence in improvement and recovery of liver tissues and blood vessels. The efficiency and achievement of curcumin might be applied to be an alternative therapeutic agent in diabetic hepatic pathology.

## 5.5 Conclusion

The results in the presented study can be concluded as the followings:

1. Blood sugar levels were elevated in all DM and DMC rats at 4, 8, and 12 weeks as compared with control rats. The blood sugar levels of DMC rats at 4, 8, and 12 weeks were reduced when compared with DM rats respectively.
2. Body weight were reduced in DM and DMC rats as compared with the control rats at 8 and 12 weeks. The body weight of DMC rats at 8 and 12 weeks were significantly increased when compared with DM rats respectively.
3. The liver function test of blood samples in control, DM, and DMC rats revealed that SGOT and SGPT levels in three groups of rats were increased than normal levels. In contrast, the SAKP levels was increased in DM rats when compared with control rats at 4, 8 and 12 weeks. After administration of curcumin, the SAKP levels was reduced in DMC rats when compared with DM rats at 4, 8 and 12 weeks.
4. Histopathological study revealed that there were inflammation in liver tissue at 8 week of DM rats, the blood sinusoids around the central vein and central veins were dilated and filled with clotted red blood cells. Edema appeared in the hepatocytes and pyknosis nuclei was evident. Lipids accumulated in the hepatocytes which revealed peripheral nuclei, and formed non-membrane bound vacuoles. Moreover, the liver revealed inflammatory change within the portal triad that the white blood cells infiltrated in this area and collagen deposition was increased in the portal triad. The blood sinusoids around the central vein and central veins at 12 weeks were seriously dilated and filled with clotted red blood cells. The degenerative and distorted liver cells and microvasculature were revealed as that the edema of hepatocytes and lipids accumulation. DM rats at 12 weeks revealed seriously inflammatory change within the portal triad revealing the lymphocytes infiltration and collagen fiber deposition in this area. Administration of curcumin in DMC rats at 8

week inhibited the increasing of microvessels dilatation, lipid accumulation, pyknosis nuclei of hepatocytes, and inflammation in the liver tissue. Reducing of inflammation was presented by the less numbers of lymphocytes, collagen fiber deposition, and white blood cells in portal triad and by reducing of clotted red blood cells in central veins and sinusoids. Moreover, the healthy and nourishing microvessels and hepatocytes were observed in the liver tissue of DMC rats at 12 weeks.

5. By vascular corrosion casting technique, the noticeable lesions of diabetic pathology consisted of the expansion of proximal and distal parts of portal veins together with venule branches in DM rats at 8 week. It presented unhealthy destroyed characteristics blood vessel morphology. The distal portion of portal veins showed signs of abnormal structures of blind endings and shrinkage of portal venules. Moreover, the new development and neovascularization of new shrinkage blood vessels have emerged and presented extensively from the proximal part of portal venule. At 12 week experiments, the lesion of diabetic pathology consisted of seriously destruction of blood vessels and presented unhealthy destroyed characteristics blood vessel morphology in DM rats liver. The portal venules were seriously shrinkage and their diameter were decreased. Moreover, the new development and neovascularization of new tortuous and shrinkage blood vessels have emerged and presented extensively from the proximal part of portal venules. In DMC rats at 8 and 12 week which were treated with curcumin, the healthy normal patterns of liver blood vessels have recovered and got restored. The portal veins became regenerated and repaired in to healthy and normal characteristics. Moreover, the new development and neovascularization of new nourishing blood vessels have emerged and presented extensively.
6. The liver tissue of DM rats at 8 week showed strong intensity of VEGF immunostaining in the cytoplasm of hepatocytes and endothelial cells of central veins. In the liver tissue of DM rats at 12 week showed more strong intensity of VEGF immunostaining than DM rats at 8 week whereas mild immunoreactivity was found in

the endothelial cells of portal venule of portal triad. Supplementation of curcumin in DMC rats at 8 and 12 week, the intensity of brownish color was decreased in the cytoplasm of hepatocytes and endothelial cells of central veins whereas week immunoreactivity was found in the portal triad. By Quantitative scoring, elevated VEGF staining of hepatocytes and endothelial cells of central veins were observed in all DM compared with control rats at 8 and 12 weeks. After curcumin treatment, VEGF immunostaining scores were lowered in DMC rats at 8 and 12 weeks when compared with DM rats.

7. Western blot analysis established the specific VEGF protein band at molecular weight 23 kDa on film by Enhanced chemiluminescence (ECL) method in control, DM, and DMC samples from rats liver at 4, 8 and 12 week. This specific protein represented VEGF protein. The VEGF bands from DM revealed more intensity of VEGF expression than control and DMC rats at 4, 8 and 12 week. The amount of VEGF protein expression were no different among control, DM, and DMC rats at 4 week. At 8 week, the amount of VEGF protein in DM rats was increased when compared to control and DMC rats. The amount of VEGF protein in DM rats at 12 week was increased more than the amount of VEGF protein in DM rats at 8 week and other groups. However, the amount of VEGF in DMC was decreased in relating to the amount of VEGF in DM rats at 8 and 12 week.

In order to present the whole integrated comparative characteristics, the liver morphology and function among control, DM, and DMC experiments were simply concluded and compared at 4, 8, and 12 weeks respectively. All results are presented in TABLE 29-32

**TABLE 29** Summary of the liver tissue and blood vessels evidence in Control (C), DM and DMC in different duration (4, 8 and 12 weeks) from the present study

| Characteristic      | 4 weeks |    |     | 8 weeks |    |     | 12 weeks |    |     |
|---------------------|---------|----|-----|---------|----|-----|----------|----|-----|
|                     | C       | DM | DMC | C       | DM | DMC | C        | DM | DMC |
| Blood sugar level   | N       | ↑  | ↑   | N       | ↑  | ↑   | N        | ↑  | ↑   |
| Body weight         | N       | ↓  | ↓   | N       | ↓  | ↓   | N        | ↓  | ↓   |
| Liver function test |         |    |     |         |    |     |          |    |     |
| SGOT                | N       | ↓  | ↑   | N       | ↑  | ↑   | N        | ↑  | ↑   |
| Liver function test |         |    |     |         |    |     |          |    |     |
| SGPT                | N       | ■  | ■   | N       | ■  | ■   | N        | ■  | ■   |
| Liver function test |         |    |     |         |    |     |          |    |     |
| SAKP                | N       | ↑  | ↑   | N       | ↑  | ↑   | N        | ↑  | ↑   |

N.....: Representing the normal evidence of each characteristic

.....■.....: Representing the similar evidence with the control group

**TABLE 30** Summary of the liver tissue and blood vessels evidence in Control (C), DM and DMC in different duration (4, 8 and 12 weeks) by histological examination from the present study

| Characteristic                 | 4 weeks |    |     | 8 weeks |    |     | 12 weeks |    |     |
|--------------------------------|---------|----|-----|---------|----|-----|----------|----|-----|
|                                | C       | DM | DMC | C       | DM | DMC | C        | DM | DMC |
| Histological observation       | N       | ↑  | ↑   | N       | ↑  | ↑   | N        | ↑  | ↑   |
| (* Liver tissue inflammation)  |         |    |     |         |    |     |          |    |     |
| Diameter of central veins (LM) | N       | ↑  | ↑   | N       | ↑  | ↑   | N        | ↑  | ↑   |
| Diameter of sinusoids(LM)      | N       | ■  | ■   | N       | ↑  | ■   | N        | ↑  | ■   |

N.....: Representing the normal evidence of each characteristic

.....■.....: Representing the similar evidence with the control group

**TABLE 31** Summary of the localization and expression of VEGF protein in liver tissue evidence in Control (C), DM and DMC in different duration (4, 8 and 12 weeks) from the present study

| Characteristic                           | 4 weeks |             |             | 8 weeks |             |             | 12 weeks |             |             |
|------------------------------------------|---------|-------------|-------------|---------|-------------|-------------|----------|-------------|-------------|
|                                          | C       | DM          | DMC         | C       | DM          | DMC         | C        | DM          | DMC         |
| VEGF intensity<br>(Immunohistochemistry) | N       | .....■..... | .....■..... | N       | .....↑..... | .....↑..... | N        | .....↑..... | .....↑..... |
| VEGF expression<br>(Western blotting)    | N       | .....■..... | .....■..... | N       | .....↑..... | .....↑..... | N        | .....↑..... | .....↑..... |

N .....: Representing the normal evidence of each characteristic

.....■.....: Representing the similar evidence with the control group

**TABLE 32** Summary of the characteristics of blood vessels evidence in Control (C), DM and DMC in different duration (4, 8 and 12 weeks) by vascular corrosion cast technique from the present study

| Characteristic                                          | 4 weeks |    |     | 8 weeks |    |     | 12 weeks |    |     |
|---------------------------------------------------------|---------|----|-----|---------|----|-----|----------|----|-----|
|                                                         | C       | DM | DMC | C       | DM | DMC | C        | DM | DMC |
| Vascular casting                                        |         |    |     |         |    |     |          |    |     |
| Observation                                             |         |    |     |         |    |     |          |    |     |
| ( ** Microvascular pathology: shrinkage, blind ending ) |         |    |     |         |    |     |          |    |     |
| Vascular casting                                        |         |    |     |         |    |     |          |    |     |
| Observation                                             |         |    |     |         |    |     |          |    |     |
| (*** New development of blood vessels)                  |         |    |     |         |    |     |          |    |     |
| Diameter and blood volume of proximal portal veins      |         |    |     |         |    |     |          |    |     |
|                                                         |         |    |     |         |    |     |          |    |     |
| Diameter and blood volume of distal portal veins        |         |    |     |         |    |     |          |    |     |
|                                                         |         |    |     |         |    |     |          |    |     |
| Diameter and blood volume of portal venules             |         |    |     |         |    |     |          |    |     |
|                                                         |         |    |     |         |    |     |          |    |     |

N .....: Representing the normal evidence of each characteristic

..........: Representing the similar evidence with the control group



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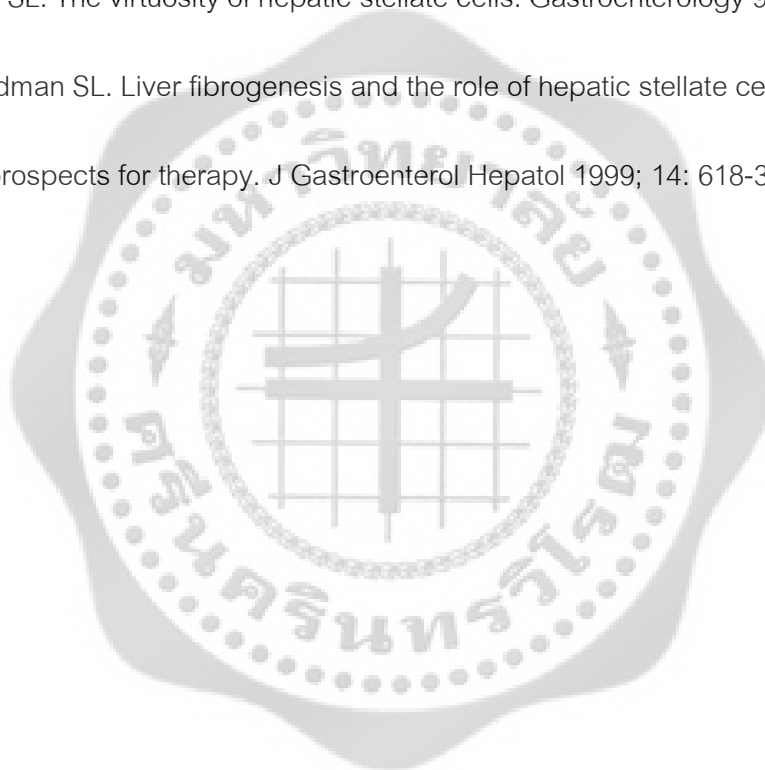
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