Study of Curcumin on Microvasculature Characteristic in Diabetic Rat's Liver as Revealed by Vascular Corrosion Cast/Scanning Electron Microscope (SEM) Technique

Wipapan Khimmaktong MSc*,**, Hattaya Petpiboolthai MSc*,
Busaba Panyarachun PhD*, Vipavee Anupunpisit PhD*

** Department of Anatomy, Faculty of Medicine, Srinakharinwirot University, Bangkok, Thailand*
*** Department of Anatomy, Pramongkutklao Collage of Medicine, Bangkok, Thailand*

Objective: *To investigate the effect of curcumin on the structural change of microvasculature in STZ-induced diabetic rat's liver.*

Material and Method: *Diabetic rats were induced by streptozotocin (60 mg/kg BW). Male rats were divided into three groups, control (C), diabetic (DM) and diabetic rats treated with curcumin (DMC) (200 mg/kg BW). After 8 weeks of experiments, blood vessels of rat's liver were studied under conventional light microscope (LM) and vascular corrosion cast technique with scanning electron microscope (SEM).*

Results: *LM observation demonstrated that there were pathology and destruction of liver tissues and microvasculature in diabetic animals. The sinusoids around central veins were dilated and filled with red blood cells. There was an accumulation of lipid droplets in the cytoplasm of hepatocytes and hepatocyte nuclei showed pathological sign of pyknosis. Moreover, the inflammation change of liver tissues revealed the infiltration of lymphocytes and increasing of collagen deposition in the area of portal triad. In curcumin-treated rats, the distinguished recovery of liver tissues showed regained normal pattern of central veins, sinusoids, hepatocytes and portal triad, when compared with liver tissues of control group. By using vascular corrosion casting with SEM, the liver blood vessels of DM group revealed higher and expanded sizes, compared with control group; proximal parts of portal veins (C = 577.75 $\pm$ 126.23, DM = 892 $\pm$ 35.79, DMC = 469.5 $\pm$ 85.53 μm), distal parts of portal veins (C = 76.72 $\pm$ 1.48, DM = 200 $\pm$ 31.05, DMC = 76.38 $\pm$ 2.98 μm) and venules (C = 27.03 $\pm$ 0.55, DM = 45.15 $\pm$ 5.03, DMC = 28.38 $\pm$ 3.67 μm) and corresponding to increased blood volumes compared with control group; proximal parts of portal veins (C = 20.8 $\pm$ 1.28, DM = 62.2 $\pm$ 3.39, DMC = 14.9 $\pm$ 0.67 μm^3), distal parts of portal veins (C = 0.46 $\pm$ 0.03, DM = 3.81 $\pm$ 0.18, DMC = 0.41 $\pm$ 0.05 μm^3) and venules (C = 0.05 $\pm$ 0.05, DM = 0.24 $\pm$ 0.013, DMC = 0.05 $\pm$ 0.05 μm^3) respectively. Fascinatingly, liver microvasculature in curcumin treated group developed into regenerate and repair into healthy and normal characteristics.*

Conclusion: *Efficiency of curcumin treatment beneficially repaired and regenerated liver tissues of diabetic groups and also redeveloped the liver's microvascular complications. These results optimistically demonstrated the potential use of curcumin as a novel therapeutic agent in liver pathology of diabetic rats.*

Keywords: *Diabetes mellitus, Streptozotocin, Curcumin, Microvasculature, Vascular corrosion cast, SEM*

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Diabetes mellitus (DM) is a disorder of blood glucose metabolism that affects on the systemic ability in order to produce or respond to insulin, leading cause of major microvascular complications. Generally, insulin is a hormone that regulates the metabolism of carbohydrate and fat in the body. The DM are characterized by hyperglycemia, abnormal lipid and protein metabolisms along with specific long term complications, affecting on the retina, kidney, pancreas and nervous system[1]. DM condition seriously

Correspondence to:
Anupunpisit V, Department of Anatomy, Faculty of Medicine, Srinakharinwirot University, Sukhumvit 23, Wattana, Bangkok 10110, Thailand.
Phone: 0-2260-2234 ext. 4501, 4502, Fax: 0-2260-1532
E-mail: vipavee19@yahoo.com

conducts the destruction of important organs, systems including microvascular architectures such as in kidney and liver.

The liver is the second-largest organ of the body (the largest is the skin) and the largest gland. It works as an interface between the digestive system and the blood. Most of its blood (70-80%) comes from portal vein, while the other is supplied by the hepatic artery. The position of liver in the circulatory system is optimal for gathering, transforming, accumulating metabolites, neutralizing and eliminating toxic substances. Additionally, liver has the capability to store glucose from non-carbohydrate sources. This key function of liver makes it vulnerable to diseases in subjects with metabolic disorders, particularly diabetes[2].

Regarding the toxic effects on islet beta cells[3], streptozotocin (STZ) is a widely used agent to induce insulin-dependent diabetic mellitus in experimental animals. The pathological and biochemical features of this animal model are compatible to those of type 1 diabetes. It also potentially induces serious systemic microvascular alterations and complications, as observed in retina and kidney[4,5].

The vascular corrosion casting technique has become a standard technique to determine the three dimensional structures of the vascular bed[6,7]. The identification of structures in vascular cast not only presents for the description of the organ vasculature, but also helps to understand other mechanisms such as blood volume and blood flow regulation[8]. This is a great fundamental tool for understanding organ microvasculation in order to support further conditions in terms of physiological and pathological studies.

Curcumin, the principle of dietary spice turmeric (*Curcuma longa*), has been used in traditional medicine as a household herbal remedy for various diseases such as biliary disorders, anorexia, coryza, cough, rheumatism, sinusitis, hepatic disorders and diabetic wound[9]. Several studies have emphasized a wide spectrum of curcumin's biological actions in hypolipidemic, hypoglycemic and antioxidant activities[10-12]. Furthermore, curcumin is reported to attenuate diabetic complications such as nephropathy[13,14], islets of Langerhans neogenesis[15] and has ability to eliminate oxygen-derived free radicals for peroxidation of cell lipids, because of the presence of phenolic groups in the structure[16].

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

Material and Method

Induction of diabetes and experimental protocols

Thirty male Wistar rats (200-250 g) obtained from the National Laboratory Animal Center of Mahidol University. The procedure of the animal research was approved by Srinakarinwirot University Medical Center Animal Care Committee. Experimental diabetic rats were induced by intravenous injection in the lateral tail vein of streptozotocin (STZ) (Sigma, St. Louis, MO, USA) (60 mg/kg BW) dissolved in 0.9% normal saline. Control rats received injection with 0.9% normal saline alone. Rats with blood sugar level > 250 mg/dl were used as diabetic animals. Male rats were divided into three groups: control group (C) (n = 10), diabetic group (DM) (n = 10) and diabetic-treated with curcumin at a dose of 200 mg/kg BW (DMC) (n = 10). At the end of 8 weeks after STZ injection and plus curcumin supplementation, the liver tissues were studied under conventional light microscope and the liver microvasculature were investigated of both quality and quantity analyses by vascular corrosion cast technique with scanning electron microscope (SEM).

Histological study

The livers were fixed in Bouin's solution for overnight. Then, they were dehydrated in a 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 and liver tissues were then embedded in paraffin, cross sectioned at 5 μm thick and stained with hematoxylin and eosin (H&E). All sections were examined and photographed by Olympus light microscope (BX-50, Olympus, Japan).

Vascular corrosion cast technique

Each group of rats was perfused with 400 to 500 ml of 0.9% NaCl solution through the left ventricle to wash out the blood from the blood vessels. Then, the Batson's No. 17 plastic mixture was injected into blood circulation of rats. The animal with injected plastic was left at room temperature for 30 minutes and immersed in hot water (80°C) for 3 hours to assure polymerization of the plastic. After polymerization, the liver was removed from the abdomen and the tissue was corroded in 40% KOH solution at room temperature for 20-30 days. The liver vascular cast was rinsed in slow running tap and washed in several changes of distilled water for removal of any remaining tissues. It

was then air dried in room temperature, mounted on a metal stub with double glue tape and carbon paint before being coated with gold on sputtering apparatus. Finally, the cast was examined under SEM (JEOL JSM-5400) at accelerating voltage of 10 KV.

Statistical analysis

The results were expressed as mean ± standard error of the mean (SE). Statistical analysis was performed by using ANOVA. The value of $p < 0.01$ was considered to indicate statistical significance.

Results

The results from LM observation demonstrated that there were pathology and destructions of liver cells and tissues, especially liver microvasculature in diabetic animals when compared with the control group (Fig. 1A, 1B). Liver sections of DM rats were shown that sinusoids around the central veins were dilated and filled with lots of red blood cells (Fig. 2A). There was an accumulation of lipid droplets in the cytoplasm of hepatocytes and pyknosis of hepatocyte nuclei was evident (Fig. 2B). Moreover, the rat's liver of DM showed the inflammatory changes of liver tissues that was revealed by infiltration of lymphocytes (Fig. 3A, 3B and 3D) and increasing of thick bundles of collagen deposition in the area of portal triad (Fig. 3C). In curcumin-treated rats, the liver tissue showed closely normal pattern of healthy central veins, sinusoids, hepatocytes (Fig. 4A) and portal triad (Fig. 4B), when compared with liver tissues of control rats (Fig. 1A, 1B). Interestingly, the curcumin-treated liver tissues demonstrated recovered typical arrangement of radiating plates of hepatocytes and sinusoids together with central veins.

SEM study of corrosion casts revealed clearly

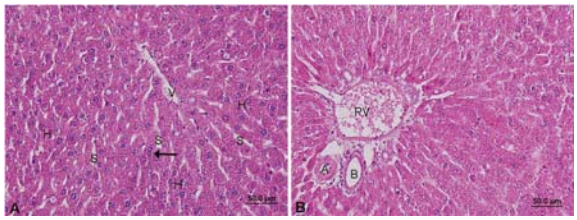

Fig. 1 Light micrographs of control rat's liver at 8 weeks (H & E). A: showing the hepatic plates of hepatocytes (H) that radiate from the central vein (V). Hepatocytes are large polyhedral cells with large round nuclei. Some hepatocytes are binucleate cells (an arrow). The space between hepatic plate is the liver sinusoid (S). B: showing a portal triad consisting of a branch of portal vein (PV), hepatic artery (A) and bile duct (B). x40

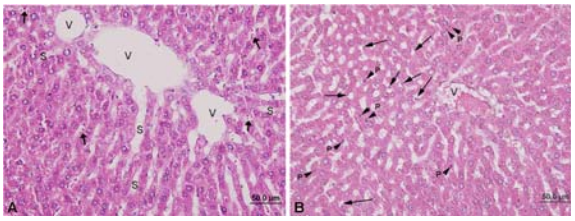

Fig. 2 Light micrographs of DM rat's liver at 8 weeks (H & E). A: showing dilated liver blood sinusoids (S) and central veins (V). The sinusoids are also filled with red blood cells (arrows). B: showing the accumulation of lipid droplets in the hepatocytes (arrows). Many hepatocytes had pyknotic nuclei (P) that appeared small and condense, V = central vein. x40

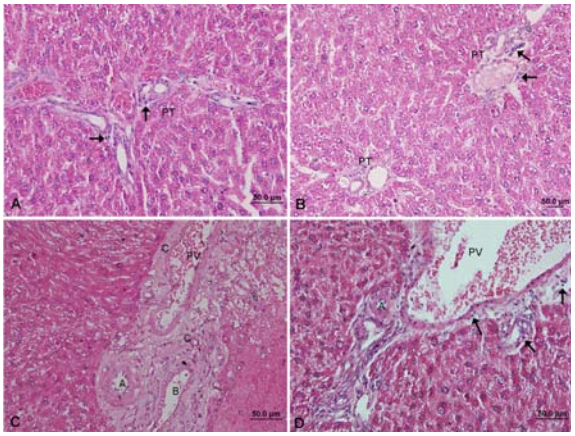

Fig. 3 Light micrographs of DM rat's liver at 8 weeks (H & E). A, B and D: showing the inflammation of the portal triad (PT) that demonstrated some lymphocytes (arrows) nearly portal vein (PV). C: showing collagen deposition (C) filled in area of portal triad (PT), A = hepatic artery, B = Bile duct. x40

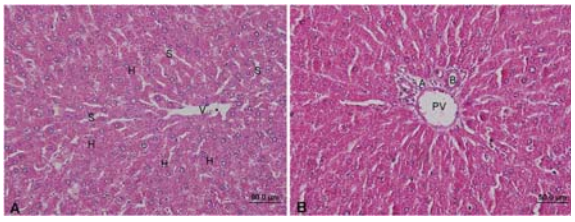

Fig. 4 Light micrographs of DMC rat's liver at 8 weeks (H & E). A: showing normal pattern with radiating anastomosing plates of hepatocytes (H), central vein (V) and space of sinusoid (S). B: showing the portal triad consisting of a branch of portal vein (PV), hepatic artery (H) and bile duct (B). x40

high magnification and detection of microvascular changes. In the normal condition of the control group, numerous normal even blood vessel architectures of proximal and distal parts of portal veins together with 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 (Fig. 5 A-D).

In comparison to the control group, liver vascular changes of the DM group demonstrated the event of liver blood vessel hypertrophy, which presented unhealthy destroyed characteristics of blood vessel morphology (Fig. 6 A-B). The noticeable lesions of diabetic pathology consisted of the expansion of proximal and distal parts of portal veins together with venule branches. Moreover, distal portions of portal veins showed signs of abnormal structures of blind endings and shrinkage of venules (Fig. 6 C, D). On the contrary, the DMC group, which was treated with curcumin, the healthy normal patterns of hepatic vessels were recovered and restored. Portal veins in the curcumin-treated group (Fig. 7) became regenerated and repaired into healthy and normal characteristics as in the control group. Moreover, the new development and neovascularization of new nourishing blood vessels were emerged and presented extensively.

Interestingly, liver blood vessels in the DM group revealed significantly higher and expanded sizes of proximal and distal parts of portal veins together with venules when compared with the control (Table. 1). Concerning to the sizes of proximal and distal parts of portal veins and venules, the average diameters of normal, DM and DMC were measured and compared as the followings; proximal parts of portal veins (C = 577.75 $\pm$ 126.23, DM = 892 $\pm$ 35.79, DMC = 469.5 $\pm$ 85.53 μm), distal parts of portal veins (C = 76.72 $\pm$ 1.48, DM = 200 $\pm$ 31.05, DMC = 76.38 $\pm$ 2.98 μm) and venules (C = 27.03 $\pm$ 0.55, DM = 45.15 $\pm$ 5.03, DMC = 28.38 $\pm$ 3.67 μm), respectively.

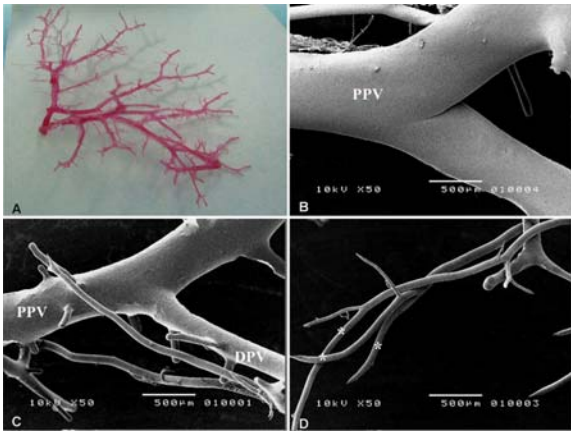

Fig. 5 SEM micrographs of liver vascular casts of control group, A: showing the whole of blood vessels of medial lobe of liver. B, C and D: showing the proximal parts of portal vein (PPV), distal part of portal vein (DPV) giving branches to venules (*). Bar = 500 μm

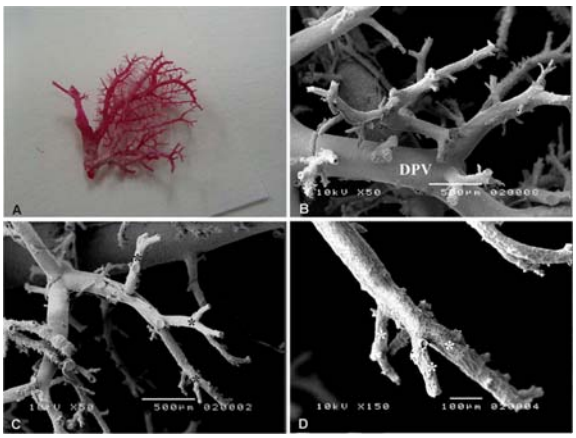

Fig. 6 SEM micrographs of liver vascular casts of DM group, A: showing the whole of blood vessels of medial lobe of liver. B, C and D: showing the distal of portal vein (DPV) giving branches to blind ending and shrink venules (*). Bar = 500 μm

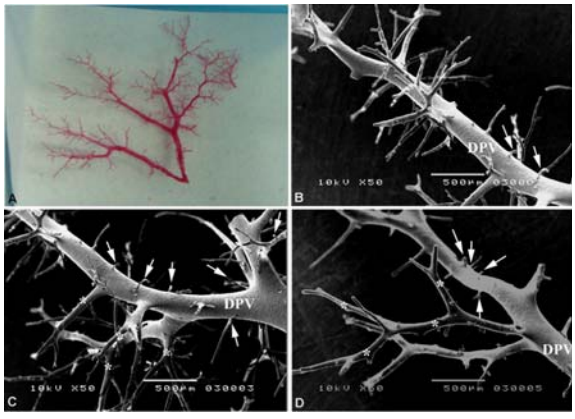

Fig. 7 SEM micrographs of liver vascular casts of DMC group, A: showing the whole of blood vessels of medial lobe of liver. B, C and D: showing the distal of portal vein (DPV) giving branches to many venules (*) and new development of blood vessels (white arrows). Bar = 500 μm

Table 1. The average diameters of proximal and distal parts of portal veins, and venules in control (C), diabetes (DM), and diabetes treated with curcumin (DMC)

Group	Diameters of blood vessels (μm), mean ± SE		
	Proximal parts of portal v.	Distal parts of portal v.	Venules
Control	577.75 ± 126.23	76.72 ± 1.48	27.03 ± 0.55
DM	892 ± 35.79	200 ± 31.05	45.15 ± 5.03
DMC	469.5 ± 85.53	76.38 ± 2.98	28.38 ± 3.67
p-value	0.001	< 0.001	< 0.001

SE = Standard error of mean

Table 2. The average blood volumes of proximal and distal parts of portal veins, and venules in control (C), diabetes (DM), and diabetes treated with curcumin (DMC)

Group	Blood volumes of blood vessels (μm^3) mean ± SE		
	Proximal parts of portal v.	Distal parts of portal v.	venules
Control	20.8 ± 1.28	0.46 ± 0.03	0.05 ± 0.05
DM	62.2 ± 3.39	3.81 ± 0.18	0.24 ± 0.013
DMC	14.9 ± 0.67	0.41 ± 0.05	0.05 ± 0.05
p-value	< 0.001	< 0.001	< 0.001

SE = Standard error of mean

Fascinatingly, liver microvasculature in the curcumin-treated group recovered and reconstructed in good physical shape conditions among three groups of proximal, distal parts of portal veins and venules (Table. 2). The blood volumes of DM hepatic blood vessels were also increased, when compared with the control, whereas the hepatic blood volumes of DMC were decreased, when compared with DM. The average values of blood volumes of proximal and distal parts of portal veins, and venules among three groups (C, DM, DMC) were presented as the followings; proximal parts of portal veins (C = 20.8 ± 1.28, DM = 62.2 ± 3.39, DMC = 14.9 ± 0.67 μm^3), distal parts of portal veins (C = 0.46 ± 0.03, DM = 3.81 ± 0.18, DMC = 0.41 ± 0.05 μm^3) and venules (C = 0.05 ± 0.05, DM = 0.24 ± 0.013, DMC = 0.05 ± 0.05 μm^3) respectively. These findings confirmed previous studies[14,15] that curcumin might have efficiency to help or improve a diabetic condition in terms of recovery of blood vessels.

Discussion

The diabetogenic action from STZ is mediated by selective destruction of pancreatic beta cells in islets of Langerhans. The important studies have been 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, Poly ADP-ribose polymerase-1[17]. 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.

Reactive oxygen species play an important role in the pathogenesis and development of complications of DM[18]. The principal pathological feature of diabetes is the morphological and functional alterations of microvessels. DM has been reported as an important risk factor for coronary artery disease, arthrosclerosis and stroke[19]. An increase in intima-media complex thickness has been demonstrated in the DM patients[20,21].

Extensive liver injury of various etiologies, which manifested as hepatocyte damage and associated with inflammatory and immune response, is the starting point for resulting in fibrosis. Liver inflammation is

initiated by Kupffer cells, the resident macrophage of the liver. This inflammation involves the formation and release of many inflammatory mediators and other inflammatory cells into damage regions[22]. The sinusoid endothelial lines the sinusoidal space and form 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 spaces, facilitating their signaling function. This signaling role is the ability of Kuffer's cells to respond to local changes by the release of cytokines and other signaling molecules such as reactive oxygen species[23] and a potent antioxidant agent and free radical scavenger[24].

Regarding to curcumin, it potentially inhibits lipid peroxidation in rat liver microsome[11]. Along with being an inhibitor of lipid peroxidation, the efficacy of curcumin has been widely studied in decreasing various diabetic complications such as diabetic retinopathy[25], wound healing[26] diabetic nephropathy[27] and hepatotoxicity[28]. Its potential as a hypoglycemic agent has also been observed in humans[29] and animals[33].

The present study, an animal model for diabetic mellitus was developed by the injection of streptozotocin into the DM group and treated with curcumin in the DMC group. At the end of the 8 week experiment, the liver tissues and blood vessels were studied by LM observation and vascular corrosion cast technique with SEM. The results demonstrated that the characteristics of liver tissues and blood vessels of STZ-induced diabetic rats revealed pathology and destruction. Interestingly, healthy liver tissues and blood vessels recovered and redeveloped in curcumin-treated diabetic animals. Regarding the diameters and blood volumes of liver blood vessels in the DM group were significantly increased compared to the control group at the 8 week experiments. After rats had curcumin supplementation, the diameters and blood volumes of liver blood vessels of DMC the group were toward decreased significantly, when compared with the DM group. These findings of the present study are in agreement with the reports 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[30,31]. The pancreatic islets of diabetic mice at 8 weeks after being 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[15]. In mice kidneys at 4 and 8 weeks after being injected 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[14].

It was possible that curcumin could improve the destruction of the liver blood vessels that the potential beneficial effects of curcumin have been shown to exhibit anti-inflammatory[32], antioxidant[24] and anti-diabetic activities[33,34]. Potential treatment with curcumin in diabetes 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.

Potential conflicts of interest

Phramongkutklao Hospital's Foundation under Her Royal Highness Princess Maha Chakri Sirindhorn's Patronage.

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การศึกษาของ curcumin ต่อการเปลี่ยนแปลงของหลอดเลือดตับในหนูที่เป็นเบาหวานโดยวิธี Vascular corrosion cast ด้วยกล้องจุลทรรศน์อิเลคตรอนแบบส่องกราด

วิภาพรรณ ขิมมากทอง, หัทยา เพชรพิบูลไทย, บุษบา ปันยารชุน, วิภาวี อนุพันธ์พิศิษฐ์

วัตถุประสงค์: *เพื่อศึกษาโครงสร้างของหลอดเลือดตับในสัตว์ที่ป่วยเป็นเบาหวานที่ถูกกระตุ้นให้เป็นเบาหวาน (โดยสาร streptozotocin) และเป็นเบาหวานที่ได้รับการรักษาแล้วด้วยสาร curcumin ในระดับจุลทรรศน์แสงสว่าง (LM) และจุลทรรศน์อิเล็กตรอนชนิดส่องกราด (SEM) ร่วมกับ vascular corrosion cast*

วัสดุและวิธีการ: *หนูเพศผู้ถูกแบ่งออกเป็น 3 กลุ่ม ได้แก่ หนูกลุ่มควบคุม และหนูที่ถูกเหนี่ยวนำให้เป็นเบาหวานโดยสาร STZ (60 mg/kg BW) และหนูที่ถูกเหนี่ยวนำให้เป็นเบาหวานที่ได้รับขมิ้นชัน (200 mg/kg BW) โดยทำการทดลองเป็นเวลา 8 สัปดาห์ เมื่อครบกำหนดได้นำตับไปศึกษาดูลักษณะทางเนื้อเยื่อวิทยาโดยกล้องจุลทรรศน์แสงสว่าง และโครงของหลอดเลือดโดยจุลทรรศน์อิเล็กตรอนชนิดส่องกราดร่วมกับ vascular corrosion cast แล้วทำการเปรียบเทียบวิเคราะห์ทั้งด้านคุณภาพและปริมาณของเลือด*

ผลการศึกษา: *ผลการศึกษาโดยกล้องจุลทรรศน์แสงสว่าง พบว่าหนูกลุ่มที่ถูกกระตุ้นให้เป็นเบาหวานมีความผิดปกติของตับโดยมีการทำลายโครงสร้างเซลล์และเนื้อเยื่อและโครงสร้างของหลอดเลือดของตับมีการขยายขนาดของ central vein และ sinusoid มีการสะสมของไขมันใน cytoplasm ของ hepatocyte บางเซลล์มี pyknotic nucleus ซึ่งแสดงถึงการถูกทำลายของเนื้อเยื่อตับ และยังพบว่ามีกระบวนการอักเสบจากการมี lymphocyte เกาะกลุ่มอยู่รอบ portal vein รวมถึงการเพิ่มมากขึ้นของ collagen fiber บริเวณ portal triad จากการศึกษาโดยจุลทรรศน์อิเล็กตรอนชนิดส่องกราดร่วมกับ vascular corrosion cast พบว่าหลอดเลือดในตับกลุ่มเบาหวานมีการขยายขนาดใหญ่มากขึ้นเมื่อเทียบกับกลุ่มควบคุมที่ระดับ proximal part of portal vein (C = 577.75 $\pm$ 126.23, DM = 892 $\pm$ 35.79, DMC = 469.5 $\pm$ 85.53 μm), distal part of portal vein (μm), และระดับ venule (C = 27.03 $\pm$ 0.55, DM = 45.15 $\pm$ 5.03, DMC = 28.38 $\pm$ 3.67 μm) พร้อมทั้งมีปริมาณเลือดที่ไหลผ่านสูงมากเมื่อเทียบกับกลุ่มควบคุมระดับ proximal part of portal vein (C = 20.8 $\pm$ 1.28, DM = 62.2 $\pm$ 3.39, DMC = 14.9 $\pm$ 0.67 μm^3) distal part of portal vein (C = 0.46 $\pm$ 0.03, DM = 3.81 $\pm$ 0.18, DMC = 0.41 $\pm$ 0.05 μm^3), และระดับ venules (C = 0.05 $\pm$ 0.05, DM = 0.24 $\pm$ 0.013, DMC = 0.05 $\pm$ 0.05μm^3) ตามลำดับแต่ในสัตว์ที่ป่วยเป็นเบาหวานและได้รับการรักษาด้วยสาร curcumin พบว่าโครงสร้างเซลล์ และเนื้อเยื่อของอวัยวะตับ และโครงสร้างของหลอดเลือดที่ตับมีการซ่อมแซมและฟื้นฟูสภาพเข้าสู่สภาวะปกติและใกล้เคียงกลุ่มควบคุม หลอดเลือดมีขนาดและมีปริมาณเลือดที่ไหลผ่านกลับเข้าใกล้สภาวะปกติ*

สรุป: *สาร curcumin ซึ่งเป็นสารที่มีอยู่ในขมิ้นชันมีผลในการรักษา ช่วยคงสภาพและฟื้นฟูหลอดเลือดในตับที่เสื่อมสภาพจากสภาวะเบาหวานในระยะเวลา 8 สัปดาห์ มีผลที่ดีต่อการลดภาวะแทรกซ้อนของหลอดเลือดที่ตับ และน่าจะมีประโยชน์ในการนำมาประยุกต์ใช้เชิงการแพทย์ทางเลือกในการรักษาโรคเบาหวาน*