Expressional Changes of Carbamoyl Phosphate Synthetase and Glutamine Synthetase in the Liver of Rat with Thioacetamide-Induced Cirrhosis

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Background: *In order to detoxify ammonia, mammalian livers use carbamoyl phosphate synthetase (CPS) and glutamine synthetase (GS) for conversion into respective non-toxic urea and glutamine. CPS is expressed in the periportal hepatocytes whereas GS is contained in the pericentral hepatocytes.*
Objective: *To examine the expressional changes of CPS and GS in the liver being induced to become cirrhotic by hepatotoxin thioacetamide (TAA).*
Material and Method: *Twenty-five male Wistar rats were divided into 5 groups of 5 animals each. Group 1 was for control. Groups 2 to 5 were treated with 200 mg/kg TAA intraperitoneally three times weekly for 1, 2, 3 and 4 months respectively. The immunohistochemical technique was employed in order to elucidate the expression of CPS and GS in each animal group.*
Results: *As centro-central fibrous bridging developed in the course of TAA treatment, expression of CPS declined dramatically and that of GS was no longer restricted to the pericentral hepatocytes. In month 4, CPS-positive hepatocytes were only found in some regenerative nodules, whereas GS expression became confined to the nodular periphery. Proper CPS staining required tissue fixation in a mixture of methanol, acetone and water (2:2:1 v/v) as opposed to 4% paraformaldehyde.*
Conclusion: *In response to the hepatotoxin TAA, the liver attempts to regenerate by means of conserving persistent CPS-positive hepatocytes and rearranging GS-positive hepatocytes in response to vascular obstruction.*

Keywords: *Cirrhosis, Fibrosis, Thioacetamide, Ammonia*

J Med Assoc Thai 2013; 96 (Suppl. 1): S71-S77
Full text. e-Journal: *http://jmat.mat.or.th*

One of the main functions of mammalian liver is the detoxification of ammonia[1] which is a waste product of protein and amino-acid catabolism. Despite ammonia plays central role in several biochemical pathways, its high concentration can promote acid-base imbalance[2] and induce hepatic encephalopathy[3-5]. Hepatocytes are well equipped in conversion of ammonia into nontoxic urea and glutamine molecules[6]. The periportal hepatocytes that express carbamoyl phosphate synthetase (CPS) are responsible for urea synthesis, whereas the pericentral hepatocytes that contain glutamine synthetase (GS) synthesize glutamine[7,8]. CPS determines the rate of urea synthesis[9], whereas the GS-positive pericentral hepatocytes scavenge ammonia to ensure complete ammonia detoxification[10,11]. CPS and GS are, therefore, crucial enzymes in a healthy liver. However, their expression pattern in the process during the liver becomes cirrhotic due to hepatotoxins or viral infections is not known. The sulfur-containing water-soluble compound thioacetamide (TAA) is a known specific hepatotoxin which causes hepatocyte necrosis and liver failure[12]. Thus far, changes of GS and CPS expression patterns of during TAA-induced cirrhosis were not studied. Therefore, the aim of the present study is to elucidate the temporal changes in expressions of the two ammonia-detoxifying enzymes in TAA-induced cirrhotic liver.

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Material and Method

Animal model

Twenty-five male Wistar rats weighing 120-150 grams were obtained from the Animal Center, Mahidol University, Thailand. The present study was

performed in accordance to the Thai guidelines for the handling of experimental animals and was approved by the Animal Ethics Committee of the Faculty of Medicine, Srinakharinwirot University (under license No. 6/2551). The rats were fed *ad libitum*. Five rats were designated as 'controls', while the remaining rats were assigned to four treatment groups of five rats each. The experimental groups were intraperitoneally injected three times weekly with 200 mg/kg thioacetamide for 1, 2, 3, or 4 months[13]. Animals were sacrificed after O_2/CO_2 gas inhalation by decapitation. Blood from the heart was collected and sent to a standard private laboratory (BRIA lab Co, Ltd.) for analysis of the liver function.

Tissue preparation and fixation

After the midline abdominal-wall incision from the pubic symphysis to the xyphoid process of the sternum was made, the livers were removed and fixed overnight by immersion in a mixture of methanol, acetone and water (MAW; 2:2:1 v/v) or in 4% paraformaldehyde at 4°C. All samples were dehydrated in a graded series of ethanols, embedded in paraplast, sectioned into 5-7 μm thick slices, and mounted on poly-L-lysine-coated slides. The slides were stored at 4°C.

Immunohistochemistry

Slides were deparaffinized in xylene, rehydrated in a descending series of graded ethanols, and washed in PBS for a minimum of 5 minutes. Sections were outlined using a "Pap" pen (Research product International Corp., USA), blocked in TENG-T (10 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.25% gelatin, 0.05% Tween-20; pH 8.0) with 10% fetal bovine serum (FBS) for 30 minutes in a moist incubation chamber. Without prior washing, TENG-T was replaced by primary antisera against GS (BD Transduction Laboratories, USA) and CPS[14]; a gift from Dr. W.H. Lamers, Amsterdam, the Netherlands, antibodies diluted 1:200 and 1:1,000, respectively, in the blocking solution. Sections were incubated overnight in a moist incubation chamber at room temperature. The following day, sections were washed 3 times with PBS prior to incubation for 2 hours at room temperature with alkaline phosphatase-conjugated goat anti-rabbit IgG (CPS) or goat anti-mouse IgG (Sigma, Germany; GS). Both secondary antibodies were dissolved at 1:100 in blocking solution. Then, the sections were washed thrice in PBS prior incubation at room temperature in nitroblue tetraliumchloride/5-bromo-4chloro-3-indolyl phosphate (NBT/BCIP, toluidine salt; Dako Inc. Glostrup, Denmark) diluted in 100 mM Tris pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$. Concentrations of antibody and staining times were chosen to assure a linear relationship between antibody binding and staining intensity[15]. The reaction was stopped in bidistilled water. Then, sections were quickly dehydrated through an ascending series of graded ethanols, cleared in three washes of xylene, and mounted in Permount (Merck, Germany).

Tissue analysis

The sections were examined under computerized light microscope and areas portraying prominent changes in CPS and GS expression were photographed at objective lens 40X.

Statistical analysis

To compare the values of liver function test among animal groups at various times of TAA treatment, one-way ANOVA analysis with Tukey's post-hoc test was utilized. A significant difference was set at $p < 0.05$.

Results

***Liver function test* (Table 1)**

The plasma liver function test obtained from the TAA-treated rats showed that there was first a

Table 1. Plasma AST, ALT and AP enzyme levels in control and experimental rats treated for 1-4 months

	AST	ALT	AP
Control	106 ± 17*	37 ± 1*	163 ± 18
Month 1	88 ± 12	31 ± 6	126 ± 8
Month 2	166 ± 56	36 ± 3	105 ± 14
Month 3	139 ± 14*	40 ± 1*	136 ± 11
Month 4	433 ± 86*	203 ± 44*	160 ± 13

Asterisks (*) refer to values significantly different from the control rats ($p < 0.05$)

decrease, then an increase in aspartate aminotranferase (AST) and alanine aminotransferase (ALT), while alkaline phosphatase (AP) changed much less and possible slower. AST in the control (106.27 ± 17.00) was higher than those in 1-month (88.5 ± 11.60). Then the levels in 2-months (166.83 ± 55.84) and 3-months (139.43 ± 14.46) were increased. AST level in 4-months (433.10 ± 85.98) was approximately four folds of the control. ALT level was significantly increased in the 4th month (203 ± 43.84) which was over five times of the control (36.97 ± 1.19). As for AP, there was much less change among the TAA-treated groups and control.

Effect of TAA

At 1-month of treatment by TAA, both the visceral surface and histological appearance of the liver could not be distinguished from those of the control. At 2-months, nodules were not yet readily visible but hepatocyte deaths were observed as indicated by a fainter staining of hematoxylin and eosin staining. However, after 3-months of TAA-treatment, diffusion of micronodules and macronodules could be observed. By 4-months, a mixed pattern of nodules coat the physical surface of the liver and the liver was reduced in size compared to untreated livers. Additionally, at this enhanced stage of cirrhosis, fissures of the lobes disappeared and liver seemed not possess any distinct lobes. On the histological level, distinct regenerative nodules and newly formed vessels which did not follow the expected portal triad organization and central veins were observed; collagen fibers surrounded newly formed nodules.

Carbamoyl phosphate synthetase (CPS)

CPS was detected in ten or more hepatocyte layers surrounding the portal veins of the control (Fig. 1) and 1-month of TAA-treated livers (Fig. 2). At this stage, the liver possessed a homogenous and evenly distributed pattern of expression (Fig. 2). When the application of TAA progressed from 2- to 3-months, immunostaining for CPS was partially positive in individual hepatocytes (Fig. 2). At TAA-treatment for 4-months, expression of CPS declined heterogeneously among regenerative nodules. Some nodules contained a pool of CPS whereas other nodules did not (Fig. 2).

Glutamine synthetase (GS)

The control and 1-month of TAA treated groups shared the same expression pattern of GS which was presented to surround the central veins in one to two complete concentric rings (Fig. 1, 2). However, its staining intensity faded accordingly to the progression of TAA application (Fig. 2). As the histological feature of the liver began to alter due to the formation of bridging fibrotic septa, GS was no longer found around central veins but instead, around the incomplete rims of regenerative nodules (Fig. 2).

Discussion

The liver enters cirrhosis once it becomes hyperammonemia via both the degeneration and change in zonation of CPS- and GS-positive hepatocytes originally expressed in the periportal and pericentral zones respectively. Furthermore, the change in its vascular pattern and formation of fibrous bridgings, which, as observed in Fig. 2, eventually encloses new nodules, can be understood as a way the liver attempts to block and fend off incoming ammonia. Immuno-histochemical analysis reveals a change in the zonation of both CPS and GS enzymes, which can also be

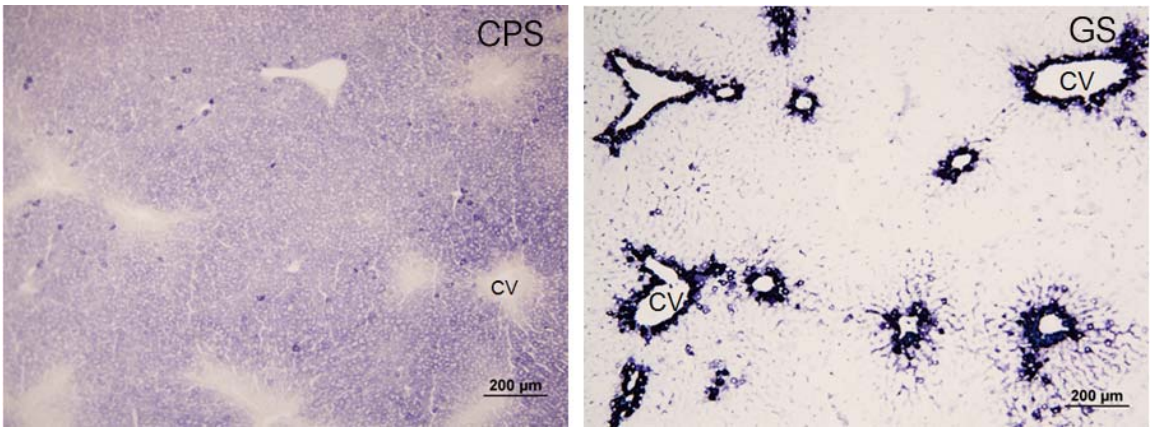

Fig. 1 Immunostaining pattern for the presence of CPS and GS in the control rat liver CV, central vein

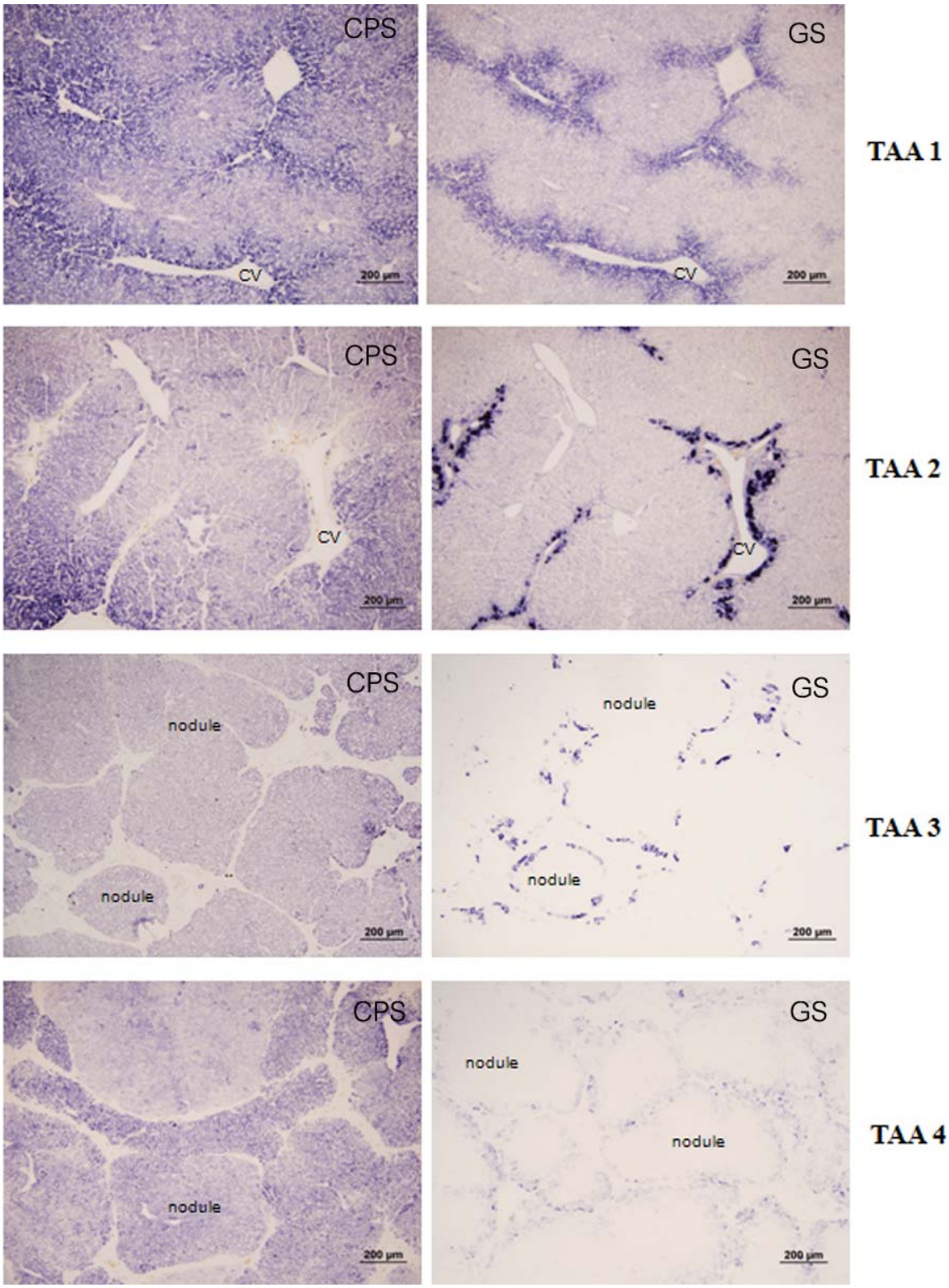

Fig. 2 Immunoexpression of the CPS (left column pictures) and GS (right column pictures) in the livers after treatment with TAA for 1-month (TAA1), 2-months (TAA2), 3-months (TAA3), and 4-months (TAA4). Note that CPS and GS were stained on adjacent sections. CV, central vein; nodule, hepatic regenerative nodule

explained as the effort of the liver during regeneration.

Between the two ammonia-metabolizing enzymes, CPS is one with higher expression[15]. Its large population in the liver tissue is required since, in view of ammonia detoxification, it functions are in a high-capacity/low-affinity manner[8]. In healthy livers, CPS is found in 9-12 hepatocyte layers thick around the afferent portal vein and in the center of the liver and maintains a homogenous distribution throughout each acinus lobulation. It is rarely expressed around the central vein[16], but once cirrhosis is induced and destruction of the zonation pattern occurs, CPS can also be found in the pericentral zone shown in the present study. By the third month of continuous TAA treatment, CPS expression is no longer shown around the hepatic portal veins and arteries but instead, resides in certain regenerative nodules. The shift in the liver histology can be understood as an attempt to protect the small amount of surviving CPS-positive hepatocytes. Ammonia will not be able to enter the nodules since they are protected by collagen fibers and other extracellular matrix components.

As opposed to the 94% of CPS-positive hepatocytes, GS is only positive in 7-8% of the hepatocytes[8]. GS is found in 2-3 hepatocyte layers thick but often found in continuous, distinct concentric circles surrounding the central veins or larger branches of the hepatic vein in the parenchyma. However, smaller population of GS is not a subordinate one since it executes in a low-capacity/high-affinity manner in detoxifying ammonia[16]. GS employs the role of a "scavenger", thereby ensures complete ammonia detoxification. The complementary functions of CPS and GS are of extreme importance to a healthy liver[17]. The third month of TAA treatment marks the fibrosis-turn-cirrhosis stage of liver disease as fibrous matter accumulates and encircles newly formed and enclosed nodules. The pattern of GS, which originally surrounds the central veins, alters to follow encircling fibers. Having a competitive, rummaging nature, GS works faster and more efficiently than would CPS in critical situations like a hyperammonemic one. Hence, GS is observed along the contours of regenerative nodules to pick-up incoming ammonia. Another hypothesis involves in the direction of perfusion. The change in vasculature may result in a change of blood perfusion from antegrade (portal to caval vein) flow to a retrograde (caval to portal vein) flow hence the change in the pattern of distribution of GS[8].

TAA has been selected to be the toxin for this hepatological study as opposed to intragastric administration of carbon tetrachloride (CCl_4), which often results in high mortality rates, a low portal venous pressure, and high susceptibility to necrosis[18]. Previous studies[18-20] have shown that injury induced by CCl_4 causes severe necrosis of perivenous GS-positive hepatocytes while those induced by TAA gives rise to slowly developing cirrhosis which is observed in the present study. Comparatively, CCL_4 induces micronodular cirrhosis, 27% mortality rate, more necrosis and cellular swelling[18], which is not the case observed in our use of TAA. In conclusion, the use of TAA in the present study also gives support for more prominent regenerative nodules and fibrosis in a fashion that well defines and reflects major features in the human disease.

It could be concluded that the changes of CPS and GS leading to four months of continuous TAA treatment gives rise to the possibility of liver regeneration despite withdrawal of toxins as would be in the case of viral infections. The rearrangement and decline of CPS and GS patterns demonstrates a new target in which liver cirrhosis could be preventable or how liver regeneration could be induced.

Acknowledgement

The present study was granted by the National Research Council of Thailand and research fund through Srinakharinwirot University under the contract No. 220/2551.

Potential conflicts of interest

None.

References

1. Campbell NA, Reece JB. Biology. 8th ed. San Francisco, CA: Pearson Benjamin Cummings; 2009.
2. Felipo V, Butterworth RF. Neurobiology of ammonia. Prog Neurobiol 2002; 67: 259-79.
3. Guy J, Yee HF Jr. Health disparities in liver disease: Time to take notice and take action. Hepatology 2009; 50: 309-13.
4. Monfort P, Cauli O, Montoliu C, Rodrigo R, Llansola M, Piedrafita B, et al. Mechanisms of cognitive alterations in hyperammonemia and hepatic encephalopathy: therapeutical implications. Neurochem Int 2009; 55: 106-12.
5. Tso P. The physiology of the liver. In: Rodney AR, David RB, editors. Medical physiology: principles for clinical medicine. Philadelphia: Wolters Kluwer/ Lippincott Williams & Wilkins; 2009: 530-40.
6. Kaiser S, Gerok W, Haussinger D. Ammonia and

glutamine metabolism in human liver slices: new aspects on the pathogenesis of hyperammonaemia in chronic liver disease. Eur J Clin Invest 1988; 18: 535-42.
7. Gebhardt R, Mecke D. Heterogeneous distribution of glutamine synthetase among rat liver parenchymal cells in situ and in primary culture. EMBO J 1983; 2: 567-70.
8. Haussinger D. Hepatocyte heterogeneity in glutamine and ammonia metabolism and the role of an intercellular glutamine cycle during ureogenesis in perfused rat liver. Eur J Biochem 1983; 133: 269-75.
9. Miura Y, Miyamoto K, Raze JM, Urabe H. Immobilization of urea cycle enzymes. I. Characterization of immobilized carbamoylphosphate synthetase and ornithine carbamoyltransferase. J Biomed Mater Res 1977; 11: 743-54.
10. Haussinger D. Regulation of hepatic ammonia metabolism: the intercellular glutamine cycle. Adv Enzyme Regul 1986; 25: 159-80.
11. Eisenberg D, Gill HS, Pfluegl GM, Rotstein SH. Structure-function relationships of glutamine synthetases. Biochim Biophys Acta 2000; 1477: 122-45.
12. Al Bader A, Mathew TC, Abul H, Al Sayer H, Singal PK, Dashti HM. Cholangiocarcinoma and liver cirrhosis in relation to changes due to thioacetamide. Mol Cell Biochem 2000; 208: 1-10.
13. Lee TF, Lin YL, Huang YT. Protective effects of kaerophyllin against liver fibrogenesis in rats. Eur J Clin Invest 2012; 42: 607-16.
14. Charles R, De Graaf A, Lamers WH, Moorman AF. Control of the changes in rat-liver carbamoylphosphate synthase (ammonia) protein levels during ontogenesis: evidence for a perinatal change in immunoreactivity of the enzyme. Mech Ageing Dev 1983; 22: 193-203.
15. van Straaten HW, He Y, van Duist MM, Labruyere WT, Vermeulen JL, van Dijk PJ, et al. Cellular concentrations of glutamine synthetase in murine organs. Biochem Cell Biol 2006; 84: 215-31.
16. de Groot CJ, ten Voorde GH, van Andel RE, te Kortschot A, Gaasbeek Janzen JW, Wilson RH, et al. Reciprocal regulation of glutamine synthetase and carbamoylphosphate synthetase levels in rat liver. Biochim Biophys Acta 1987; 908: 231-40.
17. Gebhardt R, Baldysiak-Figiel A, Krugel V, Ueberham E, Gaunitz F. Hepatocellular expression of glutamine synthetase: an indicator of morphogen actions as master regulators of zonation in adult liver. Prog Histochem Cytochem 2007; 41: 201-66.
18. Stankova P, Kucera O, Lotkova H, Rousar T, Endlicher R, Cervinkova Z. The toxic effect of thioacetamide on rat liver in vitro. Toxicol In Vitro 2010; 24: 2097-103.
19. Lee VM, Cameron RG, Archer MC. Zonal location of compensatory hepatocyte proliferation following chemically induced hepatotoxicity in rats and humans. Toxicol Pathol 1998; 26: 621-7.
20. Kiso K, Ueno S, Fukuda M, Ichi I, Kobayashi K, Sakai T, et al. The role of Kupffer cells in carbon tetrachloride intoxication in mice. Biol Pharm Bull 2012; 35: 980-3.

การเปลี่ยนแปลงของเอนไซม์คาร์บาโมอิลฟอสเฟสซินทิเทสและกลูตามีนซินทิเทสที่ตับของหนูทดลองที่ถูกชักนำให้เกิดภาวะตับแข็ง ด้วยสารไธโออะเซตาไมด์

มณฑินี คุณวิโรจน์พานิช, สมเกียรติ วัฒนศิริชัยกุล, วิสุทธิ์ ประดิษฐ์อาชีพ

***ภูมิหลัง**: ตับใช้เอนไซม์คาร์บาโมอิลฟอสเฟสซินทิเทส (CPS) และกลูตามีนซินทิเทส (GS) ทำการเปลี่ยนแอมโมเนียซึ่งเป็นพิษให้เป็นยูเรีย และกลูตามีนซึ่งไม่เป็นพิษตามลำดับ โดยในตับปกติ CPS มีการแสดงออกอยู่ในเซลล์ตับที่อยู่ล้อมรอบ portal vein ส่วน GS มีการแสดงออกอยู่ในเซลล์ตับที่อยู่ล้อมรอบ central vein*

***วัตถุประสงค์**: เพื่อศึกษารูปแบบการแสดงออกของเอนไซม์ที่ใช้ในการกำจัดแอมโมเนีย: CPS และ GS ในตับของหนูทดลองที่ถูกชักนำให้เกิดภาวะตับแข็งด้วย สารไธโออะเซตาไมด์ (TAA) เป็นเวลาต่างๆ กัน*

***วัสดุและวิธีการ**: ใช้หนูแรท 25 ตัวแบ่งออกเป็น 5 กลุ่ม ๆ ละ 5 ตัว กลุ่มที่ 1 เป็นหนูกลุ่มควบคุม กลุ่มที่ 2-5 เป็นหนูที่ถูกชักนำด้วยสารไธโออะเซตาไมด์ขนาด 200 มิลลิกรัม/กิโลกรัม โดยการฉีดเข้าในเยื่อบุช่องท้อง จำนวน 3 ครั้ง/สัปดาห์เป็นเวลา 1, 2, 3 และ 4 เดือน ตามลำดับใช้เทคนิคทางอิมมูโนฮิสโตเคมิสทรี เพื่อศึกษาการแสดงออกของเอนไซม์ CPS และ GS ในหนูแต่ละกลุ่ม*

***ผลการศึกษา**: ในทุกช่วงเวลาของการชักนำด้วยสาร TAA พบว่าจำนวนเซลล์ตับที่มีการแสดงออกของ CPS ลดลงอย่างเห็นได้ชัด และไม่พบการแสดงออกของ GS ในเซลล์ตับที่อยู่ล้อมรอบ central veins เหมือนกับที่พบในเซลล์ตับของหนูกลุ่มควบคุม แต่กลับพบว่า GS มีการแสดงออกในเซลล์ตับที่อยู่ตรงรอยเชื่อมต่อระหว่าง central veins ที่อยู่ติดกันแทน และในหนูกลุ่มที่ถูกชักนำด้วย TAA เป็นเวลา 4 เดือน พบการกระจายตัวและการแสดงออกของ CPS อยู่เฉพาะในเซลล์ตับบางเซลล์ที่อยู่ในก้อนเนื้อตับแข็งบางก้อนเท่านั้น ส่วน GS พบว่ามีการแสดงออกอยู่เพียงในเซลล์ตับที่อยู่ล้อมรอบขอบก้อนเนื้อตับแข็ง การศึกษาครั้งนี้ยังพบว่าการตรึงสภาพของเนื้อเยื่อตับด้วยส่วนผสมของเมทานอล:อะซิโทน:น้ำ ในอัตราส่วน 2:2:1 สามารถช่วยทำให้เกิดปฏิกิริยาการแสดงออกของ CPS บนเนื้อเยื่อได้ดีกว่าการตรึงสภาพด้วยสารพาราฟอมาลดีไฮด์*

***สรุป**: หนูทดลองที่ได้รับสาร TAA เพื่อชักนำให้เกิดภาวะตับแข็ง มีการตอบสนองด้านการแสดงออกของเอนไซม์ที่ทำหน้าที่กำจัดแอมโมเนียโดยพยายามคงรูปแบบการแสดงออกของ CPS ในเซลล์ตับเอาไว้ให้เหมือนกับตับที่ไม่ได้รับสาร TAA เพียงแต่ปริมาณเซลล์ที่แสดงออกมีจำนวนน้อยลง ส่วนรูปแบบการกระจายตัวของ GS มีการปรับเปลี่ยนไปจากตับปกติ โดยมีการแสดงออกเฉพาะในเซลล์ตับที่อยู่ที่ขอบ ๆ ของก้อนเนื้อตับแข็ง ที่ฟื้นคืนสภาพซึ่งอยู่ใกล้หลอดเลือดที่ตีบลง*