Alpha-Mangostin Partially Preserves Expression of Ammonia-Metabolizing Enzymes in Thioacetamide-Induced Fibrotic and Cirrhotic Rats

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Background: *Ammonia metabolizing enzymes, carbamyol phosphate synthetase (CPS) and glutamine synthetase (GS), are expressed in the periportal and pericentral hepatocytes, respectively. CPS and GS function complementary to ensure complete ammonia detoxification. Immunohistochemical analysis confirmed the decline of both CPS and GS in cirrhotic rat liver induced by thioacetamide (TAA). Alpha-mangostin (AM), a major derivative of xanthone from mangosteen, has been reported to possess a wide range of pharmacological properties.*
Objective: *To examine the preventive effects of AM on CPS and GS expression in fibrotic and cirrhotic rats induced by TAA over sixteen weeks.*
Material and Method: *Twenty-four male Wistar rats were divided into 4 groups of 6 animals each. Group 1 was for control. Group 2 was for pure TAA treatment. Group 3 was for pure AM administration. Group 4, prevention group, was concurrently treated with TAA and AM. Immunohistochemical technique was employed in order to elucidate the expression of CPS and GS in each animal group.*
Results: *Immunohistochemical staining for CPS and GS showed an increasing decline from week eight to sixteen under pure-TAA condition. Fibrous bridgings, nodule formations, and regenerative nodules were detected. Pure-AM condition yielded strongly CPS and GS-stained hepatocytes in a fashion similar to the control. Results from the prevention group showed a decreasing decline of CPS and GS immuno-reactivity from week eight to sixteen as compared to pure-TAA condition. Fewer fibrous portal-caval bridgings were observed at week eight and CPS-positive hepatocytes were found in continuous rings.*
Conclusion: *Alpha-mangostin could partially preserve the normal expression of ammonia-metabolizing enzymes under TAA-induced fibrotic and cirrhotic conditions.*

Keywords: *Cirrhosis, Glutamine synthetase, Carbamoyl phosphate synthetase, Thioacetamide, Alpha-mangostin*

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Hyperammonemia, found in patients with chronic liver dysfunction, is related to the disturbances in its excretion and reutilization. The kidneys and intestines are central ammonia-producing organs, while the liver is essential to its detoxification by converting it into soluble urea and glutamine by the ammonia-metabolizing enzymes, carbamyol phosphate synthetase (CPS) and glutamine synthetase (GS), respectively[1].

The course of ammonia detoxification follows the theorized "metabolic zonation", with each zone participating in a distinct role in the process. The liver has three zonal subdivisions: periportal, midzonal and pericentral zones[2,3]. CPS enzyme in the periportal zone performs ureogenesis[4]. CPS is a mitochondrial enzyme found in 9-12 cell layers of hepatocytes that surround the terminal branches of portal veins and arteries[5]. It is an upstream enzyme, present in the first rows of hepatocytes to receive blood from the terminal branches of portal vein and hepatic arteries. It is considered a rate-limiting enzyme in the urea cycle since it catalyzes the conversion of ammonia into carbamoyl phosphate, an intermediate that feeds the Krebs cycle[6]. Ureogenesis occurs in up to 94% of periportal hepatocytes and follows a high-capacity but low-affinity system. This manner infers that occasionally, a small amount of ammonia molecules are 'missed' by CPS[2,3].

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In the pericentral zone, remaining ammonia is incorporated into glutamate for the synthesis of glutamine by GS, which is a cytosol enzyme found in last 1-2 cell layers of hepatocytes that surround the central efferent vein and is found in concentric, continuous rings[5]. It is localized in the pericentral hepatocytes of the most upstream tributaries of hepatic veins thereby, GS is designated to be a downstream enzyme with a scavenging role, ensuring complete detoxification of ammonia molecules missed by its upstream counterpart[7]. GS catalyzes ammonia into glutamine with a molecule of ATP and glutamate in a two-step process as follows: L-glutamate + NH_4^+ + ATP $\rightarrow$L-glutamine + ADP + Pi + H^+ and is present in 7-8% of hepatocyte population that surrounds terminal hepatic venules[8,9]. This reaction progresses when ammonia level does not reach a threshold adequate to propel urea synthesis as ascribed in the periportal zone. CPS and GS are complementary enzymes in detoxifying ammonia. The failure of both enzymes can lead to hepatic fibrosis and consequent cirrhosis[10]. Since the liver is scarred from drug and various toxins, protective or medicinal effects from nontoxic-metabolizing natural substances have been the target of research over the past decades. Such substance is found in some herbs and fruits, such as mangosteen.

Mangosteens are cultivated in tropical rainforests of some Southeast Asia nations. Despite the dark, reddish purple and hard outer shell, the mangosteen fruit has a white, soft and juicy edible pulp within; it has a slight acidic and sweet flavor. Also known as the "queen of fruits", it is one of the best tasting tropical fruits with a pericarp that exhibit medicinal properties. For centuries, the mangosteen pericarp has been used as a medicinal agent in Southeast Asian countries for the treatment of skin infections and wounds. It is an Ayuvedic medicine also for the treatment of diarrhea and dysentery. Phyto-chemical studies on the pericarps of such fruit reveals that it contains a variety of secondary metabolites of both oxygenated and prenylated derivatives known as xanthones. Xanthones are chemical compounds with a dibenzo-γ-pyrone structure[11].

Currently, over 1,000 different xanthones have been described and isolated from the pericarp, the whole fruit, bark and leaves. Previous studies show that the fruit possess properties including anti-inflammation, anti-bacterial, anti-microbial, antiviral, and antifungal activities[12]. Alpha-mangostin (AM), the major constituent, attenuates lipid peroxidation and protects the antioxidant tissue, defense system in isoproterenol-induced myocardial infection[13]. Several other studies show that it possesses antioxidant activity by scavenging radicals, decreases basal lipid peroxidation and prevents disrupted mitochondrial function in the brain synaptosomal fractions and prevents the increasing of reactive oxygen species (ROS) generation induced by Cisplatin[14-16]. Furthermore, it also possesses high anti-inflammatory activity by decreasing LPS-induced inflammatory gene expression in myofibroblasts and inhibits carrageenan-induced paw edema in mice[12,17]. Thereby, the derivative alpha-mangostin is a potential therapeutic agent to the conditions of fibrosis and cirrhosis, but can it protect the liver against toxins?

This study investigates into whether AM has a protective effect of thioacetamide-induced fibrosis and cirrhosis. Thioacetamide (TAA) is first discovered as a potential hepatotoxin when a single-dose injection causes hepatic centriolobular necrosis with a subsequent regenerative response in animals. However, chronic administration of TAA leads to fibrosis, cirrhosis and hepatocarcinoma[18]. Currently, TAA is used as a toxin for hepatic researches in animal models as opposed to carbon tetrachloride (CCl_4), which was the earlier alternative since it has a high specificity for the liver as a target organ and a particular regio-specificity for the perivenous region[19]. TAA has also been recommended for experimental models of cirrhosis in rats[20].

Material and Method

Animal model

Twenty-four male Wistar rats, about four to five weeks old weighing between 120-150 grams, were obtained from the Animal Center, Mahidol University, Thailand. The 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. 110/2553). The rats were fed ad libitum. The animals were divided into 4 groups of 6 rats each. Group 1 was control receiving no treatment. Group 2 was assigned to receive only 200 mg/kg of TAA intraperitoneally thrice a week for 8, 12 and 16 weeks, 2 rats per period. Group 3 was treated only with 100 mg/kg of AM intraperitoneally twice a week in the same fashion as group 2. Group 4 was designed to test prevention by the administration of TAA and AM concurrently for 16 weeks in a similar fashion to the groups 2 and 3. Purified 96% alpha-mangostin assessed by HPLC was obtained from

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Tissue preparation and fixation

Animals from each group were sacrificed via immediate decapitation under an O_2/CO_2 gas inhalation. An abdominal incision was immediately made to remove the liver. Thereafter, the liver tissues were left to fix overnight via immersion in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature. All samples were dehydrated in a graded series of ethanol and embedded in paraplast. All fixed tissue samples were sectioned into 5 µm thickness. The tissue sections were then mounted on poly-L-lysine-coated slides. The slides were stored at 4°C upon immunohistochemical analysis.

Immunohistochemistry

Slides were deparaffinized in xylene, rehydrated in a descending graded series of ethanol, and washed in PBS prior antigen retrieval. Thereby, slides were autoclaved in 10 mM sodium citrate with pH 6.0 for 10 minutes at 120°C. After being left to cool down to room temperature, slides were washed in PBS. Sections were outlined using a Pap-pen then 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 dissolved in the blocking solution, which includes GS and CPS antibodies. The ratio of antibody to blocking solution or TENG-T with 10% FBS follows: 1:1,000 for CPS and 1:200 for GS. The sections were incubated overnight in a moist incubation chamber at room temperature. The following day, sections underwent three washes of PBS prior incubation for 2 hours at room temperature by secondary antibodies, which are alkaline phosphatase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (Sigma). The ratio of both antibodies to TENG-T with 10% FBS blocking solution was 1:100. Upon completion of incubation time, sections underwent three rewashes of PBS. For viewing of antibody binding, sections were incubated with nitroblue tetralium chloride/5-bromo-4chloro-3-indolyl phosphate (toluidine salt; Dako Inc. Glostrup, Denmark) diluted in 100 mM Tris pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$ at room temperature, in such order. Concentrations of antibody and staining times were chosen to assure a linear relationship between antibody binding and staining intensity. Once a preferred intensity of staining was assured by light microscope screening, reaction was stopped in bidistilled water. Then, sections were quickly dehydrated through an ascending graded series of ethanol, cleared in three washes of xylene, and mounted in Entellan (Merck).

Tissue analysis

The sections were examined under computerized light microscope, and areas portraying prominent changes in CPS and GS expression were photographed at 40X, 100X and 400X magnification.

Results

Controls

The normal histology of the liver in the controlled conditions appeared tightly packed with hepatocytes as seen by the staining with H&E (not shown). The staining of CPS was prominent around the periportal veins (Fig. 3A) and GS-positive hepatocytes are distinct, as they outline the contours of central veins in 1-2 cell layers (Fig. 4A).

Treatment with thioacetamide

Upon eight weeks of treatment with TAA, hepatocyte deaths were observed. The formation of fibrotic septa emerged as early as week 8 (Fig. 1A) and became distinct regenerative nodules with newly formed perinodular vascular plexus by week 16 (Fig. 1C). Immunohistochemical staining of GS no longer contoured individual central veins, but became immunopositive around the edges of newly formed vessels (Fig. 4B). Into 16 weeks of treatment, CPS staining intensity was less immunopositive in the regenerative nodules (Fig. 3B).

Treatment with alpha-mangostin

Application of AM for 8 to 16 weeks resulted in a pool of high density-stained hepatocytes with widened sinusoids (Fig. 2A-2C). Immunoreaction for CPS was present around periportal veins and continued to be of high density until it reached a few layers that surrounded central veins, where GS was distinctly prominent (Fig. 3C). The density of GS-positive hepatocytes lessened as compared to the control; however, concentric rings of GS-positive hepatocytes remained continuous (Fig. 4C).

Treatment for preventive conditions

Eight weeks into the conditions designed for preventive effects of alpha-mangostin (TAA + AM), tissue damage was observed. H&E staining exemplifies

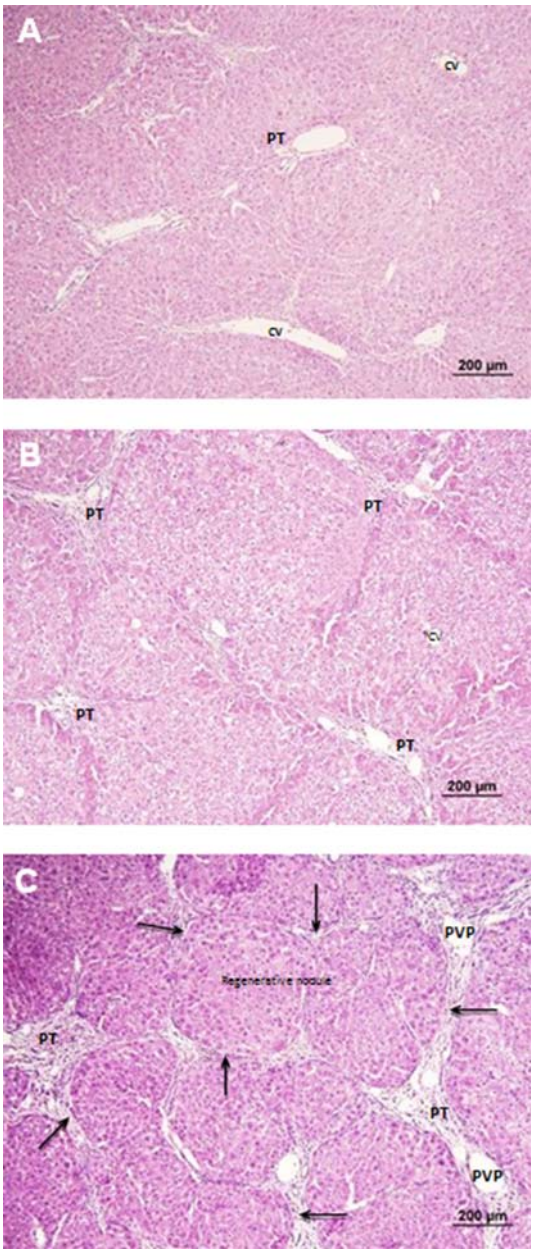

Fig. 1 Liver histology treated under TAA for 8 (A), 12 (B) and 16 (C) weeks stained with hematoxylin and eosin. Progressively, fibrotic septa became distinct regenerative nodules (arrows). CV, central vein; PT, portal tract, PVP, perinodular vascular plexus.

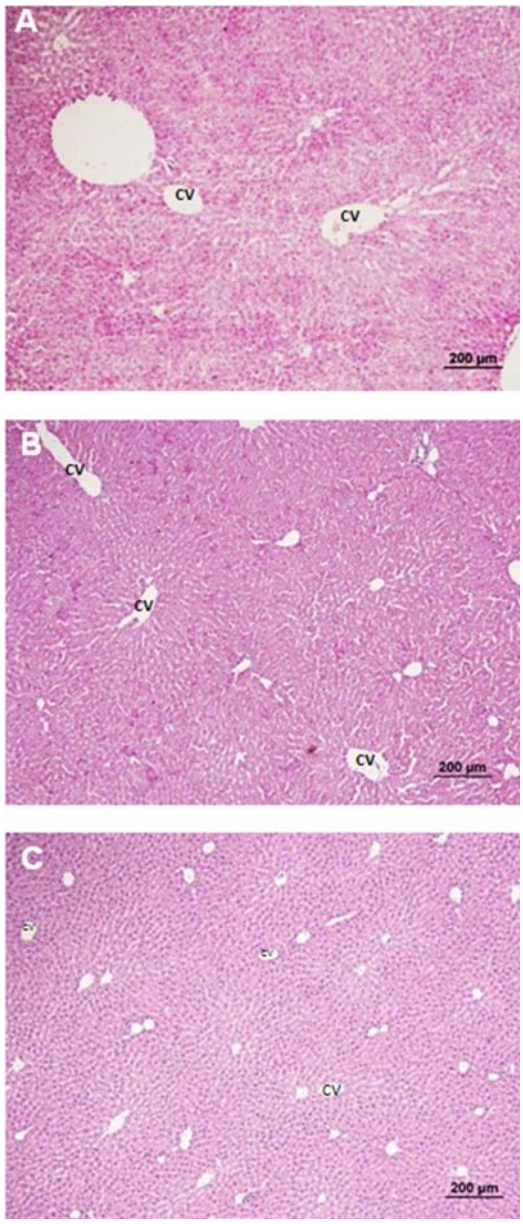

Fig. 2 The effect of alpha-mangostin on liver histology from treatment for 8 (A), 12 (B) to 16 (C) weeks (H&E). Progressive treatment displays widened sinusoids particularly at the pericentral area. CV, central vein.

hepatocyte death, widened sinusoids and presence of fibrous tissues (not shown). Immunostaining for GS no longer followed a continuous rings pattern but appeared to be darker in some cells but fainter in others (Fig. 4D). Remarkably, sixteen weeks into preventive condition, liver cells appeared to regenerate as they were expressed as tight pool of cells observed in the H&E staining with narrower sinusoids (not shown). The immuno-reactivity of CPS and GS positive hepatocytes, on the other hand, appeared to be fewer in numbers as compared to the same condition treated for 8 weeks (Fig. 3D, 4D). In sixteen weeks of treatment,

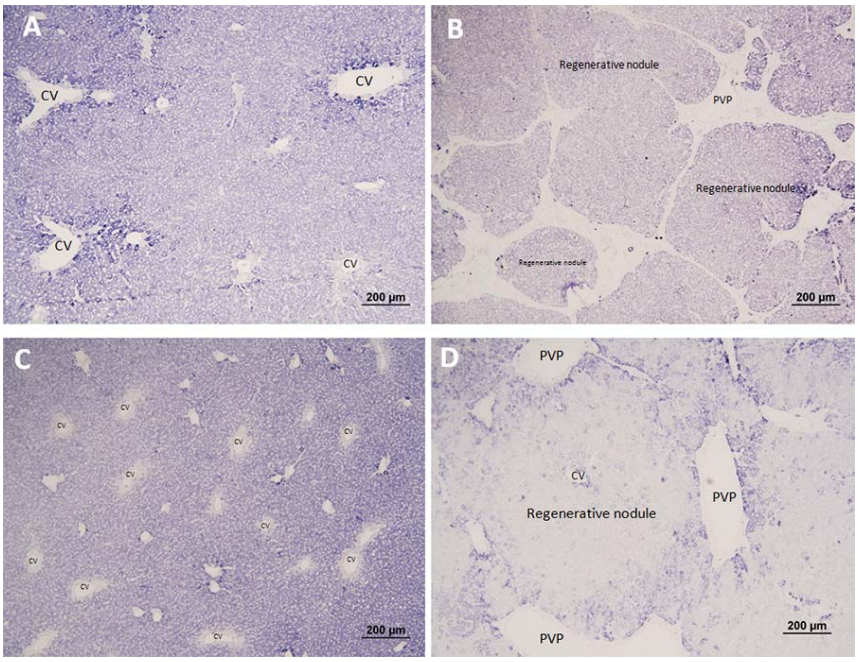

Fig. 3 Comparing immunohistochemical staining for CPS at 16 weeks after treatment under the conditions (A) control, (B) TAA, (C) AM, and (D) TAA + AM. The CPS immunointensity is less in the TAA-treated group. Note repositioning of the CPS-stained hepatocytes at the rims of regenerative nodules closed to the perinodular vascular plexus (PVP) in the prevention group (TAA + AM). CV, central vein.

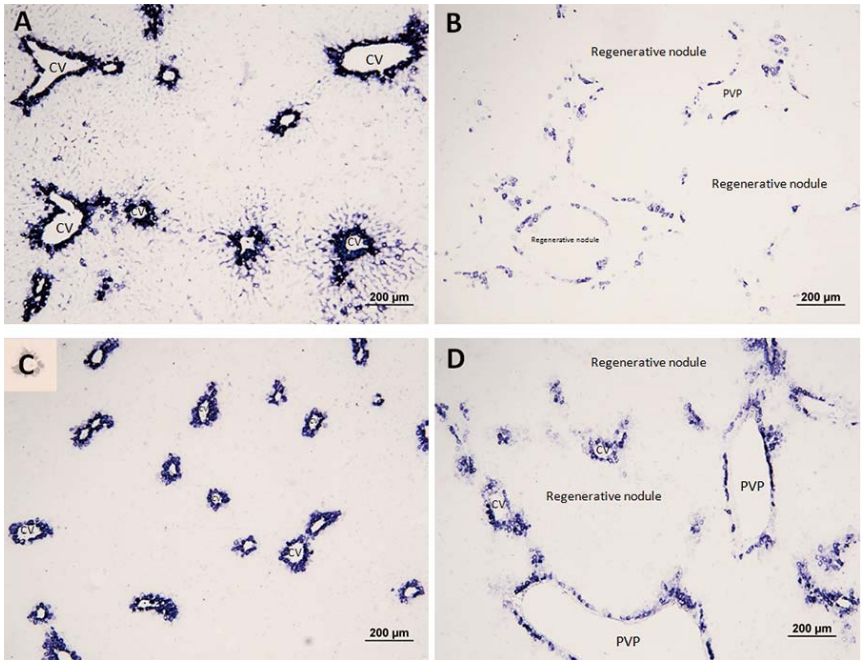

Fig. 4 Comparing immunohistochemical staining for GS at week 16 after treatment under the conditions (A) control, (B) TAA, (C) AM, and (D) TAA + AM. The GS, in the prevention group, is expressed in the hepatocytes of regenerative nodules residing closed to the central vein (CV) and perinodular vascular plexus (PVP). Note the tissue sections of 4A to 4D are adjacent to those of 3A to 3D.

generation of fibrous tissue was observed but did not result in distinct regenerative nodules; shapes of blood vessels also did not appear to have undergone massive alterations.

Discussion

Upon cirrhosis, resident hepatocytes are destroyed and the extracellular matrix give rise to fatty and fibrous tissues, which border the intrahepatic blood vessels and biliary ducts-a condition labeled cholangiocarcinoma[21]. In the present study, we have observed the effect of TAA on liver histology. As aforementioned, it was chosen because of its natural mimic of the progression of cirrhosis[20]. At eight weeks of treatment with TAA, changes in the vasculature were prominent and fibrous bridgings were observed. By the sixteenth week, the once healthy liver histology was comprised with distinct regenerative nodules, marking its entry into the cirrhotic state. The rearrangement of the liver histology exemplified changes in the resident hepatocytes and the ammonia-metabolizing enzymes, which are valuable players in the detoxification process. CPS-positive hepatocytes were once found in 9-12 cell layers around portal veins have faded and GS, once found in distinct concentric circles around the efferent central veins, scattered around newly formed regenerative nodules. The high-affinity nature of GS enzyme may cause them to migrate toward newly formed vessels as part of the regeneration process. This shift, however, unquestionably disrupts the process of detoxifying ammonia from first CPS, in a high-capacity but low-affinity manner, then to scavenging GS, which facilitates in a low-capacity but high-affinity manner thus an abundant pool of ammonia remains[8].

Upon withdrawal of causative agents either due to treatment or natural defense mechanisms, the liver will advance into a regenerative stage[22,23]. However, since the removal of some hepatotoxins (hepatitis B and C viruses or the taking of drugs to treat other diseases) is impossible, this study attempted a step in the prevention of chronic hepatic diseases. The medicinal hero investigated was alpha-mangostin, a major derivative of xanthones present in the pericarp of the mangosteen fruit. Additionally, AM is also a mild hepato-protective agent[12]. Application of pure AM showed remarkable results in coloring CPS-positive hepatocytes dark and highlighting the concentric rings of GS-positive hepatocytes around the central veins. However, there is little to no difference in the densities of AM application from weeks 8 to 16. Specifically, the application of AM had the highest effect at week 8 on GS, with the thickest cell layers and most prominent continuous ring stained, as opposed to continuous treatment to week 16. This may be due to the limited effect of AM on the liver.

To test for the 'preventive' effects of AM against chronic liver diseases, AM was given concurrently with TAA. Eight weeks into the preventive condition, the histology of the liver and immunohistochemical staining of both CPS and GS appeared to mimic the control or the pure AM condition, but with a much fainter density of staining. Several trials were repeated and eliminated the possibility of different staining time and other errors. Into week 16 of concurrent treatment, the vasculature did not alter drastically but did so only with minor changes such that the density of stain and the number of CPS and GS positive hepatocytes have decreased. It may be inferred then, that the liver in the 'normal' state, cannot fully recover upon the application of AM alone but attempts to maintain the damage that has occurred. Yet, if perhaps the hepatotoxin were removed, AM could help to enhance the reversal of fibrosis and prevent regression into cirrhosis.

Conclusion

This study has shown that alpha-mangostin performs a positive effect in maintaining the liver's health as it progresses towards end-stage cirrhosis; however, its effect is limited. When the liver becomes fibrotic and cirrhotic, expression of ammonia-metabolizing enzymes namely CPS and GS is altered. Even though alpha-mangostin is unable to reverse the normal expression of CPS and GS completely as predicted, it could partially preserve the normal expression pattern of the two ammonia-metabolizing enzymes under TAA-induced fibrotic and cirrhotic conditions.

What is already known on this topic ?

The ammonia-metabolizing enzymes, carbamyol phosphate synthetase (CPS) and glutamine synthetase (GS), in normal rat liver, are expressed in periportal and pericentral hepatocyte zones, respectively.

In fibrotic and cirrhotic livers, the expression of CPS and GS changed and did not follow the zonation pattern found in normal liver.

What this study adds ?

Alpha-mangostin could partially preserve the

normal immuno-expression pattern of the two ammonia-metabolizing enzymes (CPS and GS) under TAA-induced fibrotic and cirrhotic conditions.

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Potential conflicts of interest

None.

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การคงไว้บางส่วนถึงการแสดงออกของเอนไซม์ที่ใช้ในการกำจัดแอมโมเนีย ด้วยสารแอลฟาแมงโกสตินในหนูที่เกิดภาวะพังผืดในตับและภาวะตับแข็งโดยการชักนำด้วยสารไธโออะเซตาไมด์

มณฑินี คุณวิโรจน์พานิช, อุดมศรี โชว์พิทธพรชัย, ปริ่มเฉนียน มุ่งการดี, วิสุทธิ์ ประดิษฐ์อาชีพ

ภูมิหลัง: *คาร์บาโมอิลฟอสเฟสซินเทส (CPS) และกลูตามีนซินทิเทส (GS) เป็นเอนไซม์ที่ใช้ในการกำจัดแอมโมเนียโดยแสดงออกที่เซลล์ตับซึ่งล้อมรอบ portal vein และล้อมรอบ central vein ตามลำดับ ทั้งสองเอนไซม์ทำหน้าที่เปลี่ยนแอมโมเนียซึ่งเป็นพิษให้เป็นยูเรียและกลูตามีน ซึ่งไม่เป็นพิษตามลำดับ จากการศึกษาด้วยอิมมูโนฮีสโตเคมิสทรีในหนูทดลองที่ถูกกระตุ้นให้เกิดภาวะตับแข็งด้วยสารไธโออะเซตาไมด์ (TAA) พบการแสดงออกของเอนไซม์ CPS และ GS ลดลง อีกทั้งมีรายงานถึงฤทธิ์ทางด้านเภสัชวิทยาอย่างกว้างขวางของสารแอลฟาแมงโกสติน (AM) ซึ่งเป็นอนุพันธ์ของแซนโทนจากมังคุด*

วัตถุประสงค์: *เพื่อศึกษาผลของ AM ต่อการป้องกันการเปลี่ยนแปลงการแสดงออกของ CPS และ GS ในหนูที่ถูกชักนำให้เป็นตับแข็งด้วย TAA มาแล้วเป็นเวลา 16 สัปดาห์*

วัสดุและวิธีการ: *หนูแรท 24 ตัว แบ่งออกเป็น 4 กลุ่ม กลุ่มละ 6 ตัว กลุ่มที่ 1 เป็นหนูกลุ่มควบคุม กลุ่มที่ 2 ถูกชักนำด้วย TAA กลุ่มที่ 3 ถูกชักนำด้วย AM ส่วนกลุ่มที่ 4 เป็นกลุ่มที่ชักนำด้วย TAA พร้อมกันไปกับการป้องกันด้วยสาร AM โดยศึกษาการแสดงออกของ CPS และ GS ของหนูแต่ละกลุ่ม ด้วยเทคนิคทางอิมมูโนฮีสโตเคมิสทรี*

ผลการศึกษา: *ในหนูกลุ่มที่ถูกชักนำด้วย TAA เป็นเวลาตั้งแต่ 8-16 สัปดาห์ พบการแสดงออกของCPS และ GS ลดลง และมีการปรากฏของ fibrous bridging และ regenerative nodule ส่วนหนูกลุ่มที่ถูกชักนำด้วย AM อย่างเดียวพบรูปแบบการแสดงออกของ CPS และ GS ในเซลล์ตับเหมือนกลุ่มควบคุม การแสดงออกของ CPS และ GS ในหนูกลุ่มป้องกันพบว่าลดลงตามลำดับตั้งแต่ 8-16 สัปดาห์ เมื่อเทียบกับกลุ่มควบคุม แต่อย่างไรก็ตามการแสดงออกของทั้งสองเอนไซม์นี้มีมากและเด่นชัดกว่ากลุ่มที่ถูกกระตุ้นด้วย TAA อย่างเดียว นอกจากนี้ยังพบ fibrous portal caval bridging จำนวนเล็กน้อยที่ 8 สัปดาห์ และ CPS มีการแสดงออกอย่างต่อเนื่องเป็นวงของเซลล์ตับ*

สรุป: *แอลฟาแมงโกสตินมีบทบาทสำคัญในการคงไว้บางส่วนถึงการแสดงออกของเอนไซม์ที่ใช้ในการกำจัดแอมโมเนียในหนูที่ถูกชักนำให้เกิดภาวะพังผืดและตับแข็งด้วย TAA*