Protective Effect of α-Mangostin Against Type-I Collagen Formation in Thioacetamide-Induced Cirrhotic Rat

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Objective: *To elucidate the protective effect of α-mangostin (α-MG) against increment of type-I collagen-positive hepatocytes in rat cirrhosis induced by thioacetamide (TAA).*
Material and Method: *Rats were separated into 4 groups. The first group was, the control, untreated with TAA. The cirrhotic rats, the second group, were induced by TAA injection (200 mg/kg), 3 times per week. Rats in the third group received treatment of TAA (200 mg/kg) alternating with α-MG (100 mg/kg) for every other day. Animals in the last group were treated only with α-MG (100 mg/kg), 3 times per week. The chemicals used each group were given intraperitoneally for 16 weeks. The type-I collagen and type-I collagen-positive hepatocytes were explored by using immunohistochemical technique.*
Results: *In cirrhotic livers type-I collagen was immunopositive in the connective tissue and a large number of hepatocytes. The number of type I collagen-positive-hepatocytes (414.00 ± 25.23) in TAA-induced cirrhosis group increased significantly when compared to those in the control group (131.40 ± 9.63). Interestingly, a significant decrease in the number of type-I collagen-positive-hepatocytes was observed in TAA-α-MG-prevention group (103.60 ± 36.55) and in α-MG-injected group (54.00 ± 5.30) compared to those in the control group and TAA-induced cirrhosis.*
Conclusion: *100 mg/kg of α-MG could lower the number of type-I collagen-positive-hepatocytes in TAA-induced cirrhosis. It is probable that α-MG helps to keep up more blood circulation to the liver cells through dilated sinusoids. This vascular adaptation enhances high oxygen blood to the hepatocytes which, in turn, reduces the damage of hepatocytes caused by TAA-derived reactive oxygen species.*

Keywords: *Type-I collagen, Hepatocytes, α-Mangostin, Thioacetamide, Cirrhosis*

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Chronic liver disease (CLDs) is represented by progressive accumulation of fibrous connective tissue especially type-I collagen and through the overgrowth and changes in the morphology of hepatic myofibroblast[1,2]. Moreover, oxidative stress in CLDs lead to an increase in the generation of reactive oxygen species (ROS) and other reactive intermediates, as well as a decrease in the efficiency of antioxidant defenses. This oxidative stress in CLDs may actively cause excessive tissue remodeling and fibrogenesis[3].

Thioacetamide (TAA), a thiono-sulfur containing compound, is a good inducer for hepatic cirrhosis in animal models since it carries out low mortality rate and mimics the pathogenesis observed in humans[4]. Moreover, thick bundles of type-I collagen fibers are found in TAA-induced cirrhotic livers[5].

Mangosteen (*Garcinia mangostana*) is a tropical tree which thrives in some Southeast Asian countries such as Indonesia, Malaysia, Sri Lanka, Philippines, and Thailand and is used popularly as a type of folk medicine. The fruit hulls of *G. mangostana* are used to treat skin infections, wounds, diarrhea, inflammation, and ulcers[6,7]. It is well known that α-mangostin (α-MG), a xanthone derivative of the fruit hull of mangosteen (*G. mangostana*), possess protective effects in models where increased oxidative stress and antioxidant deficit are major players[8]. Furthermore, α-MG shows antioxidant activity using the ferric thiocyanate method[9,10]. Recently, it has been reported that α-MG protects mitochondria from peroxidative damage[11].

However, there are currently no reports that

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emphasize the protective effect of α-MG, in preventing the production of type-I collagen in TAA-induced cirrhotic rat. In the present study, we aimed to examine the changes of type-I collagens in rat livers of the control, the TAA-induced cirrhosis, the TAA-α-MG-prevented and the α-MG-injected groups. The localization of collagen fibers was explored under a light microscope and the expression of type-I collagen was detected by immunohistochemical study.

Material and Method

Animal

Twenty male Wistar rats weighing between 150-200 g were used in the present study and were purchased from The National Laboratory Animal Centre (NLAC, Salaya, Thailand). The animals were kept in a room maintained at 25°C on a 12-hour light/dark cycle and fed *ad libitum* to water and food. The rats were divided into four groups: control group, TAA-induced cirrhosis group, TAA-α-MG-prevented group and α-MG-injected group, each containing 5 animals. The rats were administered with thioacetamide (TAA) for induction of cirrhosis via intraperitoneal injection (200 mg/kg) 3 times per week. In the TAA-α-MG-prevention group, the rats were injected with TAA (200 mg/kg) alternating with α-MG (100 mg/kg) for day by day. In α-MG-injected group, the rats were only injected with α-MG (100 mg/kg) for 3 times per week. The chemicals were injected intraperitoneally for 16 consecutive weeks. The rats were euthanized by ether inhalation and immediate decapitation. The rat livers were immediately removed through a midline abdominal wall incision. The present study was conducted according to The National Research Council (NRC) guide for care and use of laboratory animals and was approved by the Faculty of Medicine, Srinakharinwirot University Institutional Animal Care and Use Committee (IACUC).

Source of α-mangostin

Extract of α-MG powder used in the present study was received from the laboratory of Associate Professor Primchanien Moongkarndi, Faculty of Pharmacy, Mahidol University, Thailand and was proved as of 97-98% purity by HPLC[12].

Tissue preparation

After decapitation, the livers were immediately removed and fixed in 2.5% buffered formaldehyde, dehydrated in graded series of ethanol and embedded in paraplast. Serial sections of 5-7 μm were prepared and mounted on poly-L-lysine-coated slides. Liver samples from each group were histologically stained with Sirius red to visualize collagen fibers and the diameter of sinusoids. The remaining slides were stored at 4° C for immunohistochemical staining.

Immunohistochemistry

The sections were deparaffinized in xylene, rehydrated, washed in PBS, and autoclaved in 10 mM sodium citrate (pH 6.0) for 10 min to retrieve antigens and inactivate endogenous alkaline phosphatase. After cooling down at room temperature for 15 min and washing in PBS, the sections were blocked in TENG-T (10 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.25% gelatin, 0.05% Tween-20; pH 8.0), containing 10% normal goat, fetal calf or rabbit serum, in a moist incubation chamber for 30 min. The sections were then incubated overnight at room temperature with 1:500 rabbit anti-rat collagen type I (Chemicon) dissolved in the blocking solution[2]. Subsequently, the sections were washed 3 times in a mixture of PBS and 0.5 M sodium acetate, followed by incubation for 2 hours at room temperature with 1:200 alkaline phosphatase-conjugated goat anti-rabbit IgG (Dako Inc., Glostrup, Denmark). To reveal antibody binding, the sections were washed once and then incubated at room temperature with nitroblue tetrazoliumchloride/5-bromo-4-chloro-3-indolyl phosphate (toludine salt; Dako Inc.) diluted in 100 mM Tris (pH 9.5), 100 mM NaCl and 50 mM $MgCl_2$. After the reaction was stopped in bidistilled water, the sections were quickly dehydrated through ascending concentrations of graded ethanols, cleared in xylene, and mounted in Entellan (Merck, New Jersey, USA).

The evaluation of number of type-I collagen-positive-hepatocytes

Every fifth liver tissue sections stained with type-I collagen immunohistochemistry were chosen for evaluation of the number of type-I collagen-positive-hepatocytes. Light microscopy photos were taken at medium power fields of eyepiece (20X) and the number of type-I collagen-positive-hepatocytes were calculated using Adobe Photoshop program version 7.0.

The evaluation of the diameter of sinusoids

Every fifth liver tissue sections stained with Sirius red were chosen for evaluation of the diameter of sinusoids. The one hundred liver tissue sections per group were observed. The diameter of sinusoids in all groups was calculated using Panoramic viewer 1.14.50.

Statistical analysis

One-way ANOVA with the Tukey's test was used to analyze statistical significance. The data were expressed as mean ± standard error (SE). The value of $p < 0.05$ was considered statistical significance.

Results

Light microscopy

The distribution of collagen fibers in the control, TAA-induced cirrhosis, TAA-α-MG-prevented and α-MG-injected rat livers were studied using a Sirius red staining dye. The collagen fibers were indicated by the dark red labeling of Sirius red as being generally restricted around the vascular walls and portal tract in the control rat liver (Fig. 1A). The fibers appeared thick surrounding the vascular walls and portal tract. Some septa connecting portal tract and central vein were referred to as porto-central septa, while those linking one portal tract to another were porto-portal septa. There were regenerating nodules surrounded by thicken broad fibrous septa comprising a mixed type of micro- and macronodular cirrhosis in the TAA-treated rat liver (Fig. 1B). In the TAA-α-MG-prevention group, only thin collagen fibers surrounding the vascular walls and portal tracts were observed. Dense and thick collagen fibers embracing hepatic nodule could not be found. However, it could be only seen less of collagen fibers (Fig. 1C). It is noteworthy that, under the same magnification, the blood vessels in the α-MG-injected liver was higher in number than in the normal liver (Fig. 1D). The diameters of hepatic sinusoids in the TAA-α-MG-prevention and α-MG-injected groups were greater than that in the control and TAA-induced groups (Figs. 2A-D) as shown in Table 1.

Immunohistochemistry

Type-I collagen in the normal, TAA-induced cirrhosis, TAA-α-MG-prevented and α-MG-injected rat livers was examined by type-I collagen immunohistochemistry. It was found that a small amount of hepatocytes with dark blue or pale blue cytoplasmic labeling of type-I collagen were presented around the vascular walls and portal tracts in the control group (Fig 3A). In the TAA-induced group, a great number of type-I collagen-positive-hepatocytes was found surrounding the vascular wall and nearby broad fibrous septa. Moreover, fibers were present in hepatic nodules between the central vein and portal tracts. A great number of hepatocytes with dark blue or pale blue cytoplasmic labeling of type-I collagen were also discovered in regenerated nodules (Fig. 3B). The TAA-α-MG-prevented rat liver displayed minor amounts of

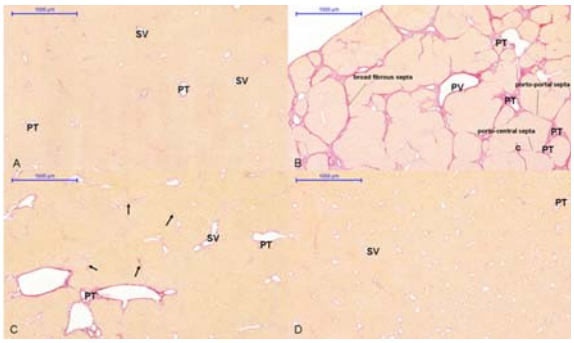

Fig. 1 Light micrographs of control (A), TAA-induced cirrhosis (B), TAA-α-MG-prevented (C) and α-MG-injected (D) rat livers stained with Sirius red. c, central vein; PT, portal tract; PV, portal vein; SV, sublobular vein
A. The control rat liver showed normal collagen fibers with dark red labeling of Sirius red situating in the vascular wall and portal tract
B. Extensive accumulation of broad fibers embracing the regenerating hepatic nodule. Note the porto-portal septa and porto-central septa
C. The thin line collagen fibers (black arrows) surrounding hepatic lobule
D. At the same magnification to control rat liver shows that the numbers of blood vessels increased extensively

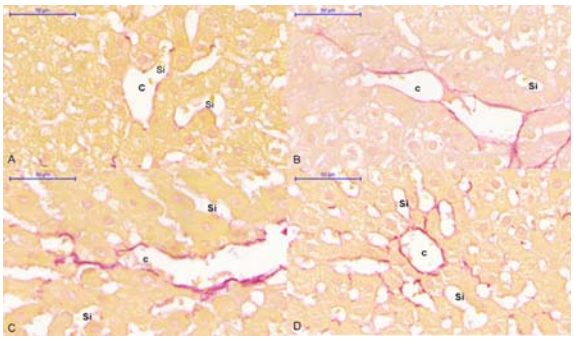

Fig. 2 Light micrographs of rat liver tissues staining with Sirius red illustrating the blood sinusoids in the control (A), the TAA-induced cirrhosis (B), the TAA-α-MG-prevented (C) and the α-MG-injected groups (D). c, central vein; Si, sinusoid

Table 1. Diameter of sinusoids

Groups	Diameter (μm)
Control	7.89 ± 1.70
TAA-induced cirrhosis	7.62 ± 2.3
TAA-α-MG-prevented	10.22 ± 2.86
α-MG-injected	9.12 ± 2.34

hepatocytes expressing type-I collagen but showed dominance around large blood vessels and only in certain areas of liver tissue (Fig. 3C). Only some areas of liver injected with α-MG showed a great number of hepatocytes expressing type-I collagen (Fig. 3D).

In order to evaluate the synthesis of type-I collagen from hepatocytes, the number of type-I collagen-positive-hepatocytes per medium power field was explored. The numbers of type-I collagen-positive-hepatocytes (414.00 ± 25.23) in the TAA-induced cirrhosis group was significantly higher than those of the control group (131.40 ± 9.63). Interestingly, there was a significant decrease in the number of type-I collagen-positive-hepatocytes seen in the TAA-α-MG-prevented group (103.60 ± 36.55) and α-MG-injected group (54.00 ± 5.30) when compared to the control and TAA-induced cirrhosis groups (Fig. 4).

Discussion

The present study showed that type-I collagen-immunopositive hepatocytes were localized in both normal liver and TAA-treated liver. A great number of hepatocytes in the TAA-induced group expressing type-I collagen was found surrounding the vascular wall and nearby broad fibrous septa. This finding could support that pro-fibrogenic cells may originate from hepatocytes through a process of epithelial-to-mesenchymal transition[3]. It could be probable that type-I collagen around the hepatic sinusoids, the walls of both portal and hepatic veins (in normal liver), and broad fibrous septa of regenerative nodules (in cirrhotic liver) was partly synthesized by those hepatocytes.

It is probable that TAA administration triggers the formation of reactive oxygen species (ROS) which in turn leads to hepatocyte damage[14] through multiple mechanisms such as lipid peroxidation, glutathione depletion and reduction in the SH-thiol groups[13]. The alive hepatocytes situating in the regenerative nodules probably also get signal from generated ROS to produce type-I collagen surrounding the nodules as well as forming porto-central septa and porto-portal septa as seen in the present study. This collagen formation is probably one of natural defense mechanism to keep the hepatocytes alive and apart from the toxic TAA flowing in the bloodstream. That is why, in the present study, much more number of type-I collagen-positive-hepatocytes were observed in TAA-treated group

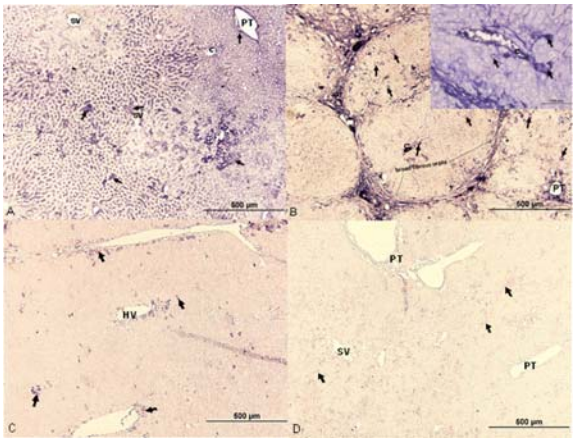

Fig. 3 Immunostaining of type-I collagen in control (A), TAA-induced cirrhosis (B), TAA-α-MG-prevented (C) and α-MG-injected (D) rat livers. c, central vein; HV, hepatic vein; PT, portal tract; SV, sublobular vein

A. Distribution of type-I collagen-positive-hepatocytes around some areas of the vascular walls

B. Note the hepatocytes with dark blue or pale blue immunolabeling of type-I collagen (see inset; black arrows) in regenerative nodules and between portal tract and central vein or between portal tract and portal tract

C. The type-I collagen-positive-hepatocytes (black arrows) surrounding large of blood vessels

D. Hepatocytes express type-I collagen immunohistochemistry (black arrows) demonstrated within the liver parenchyma

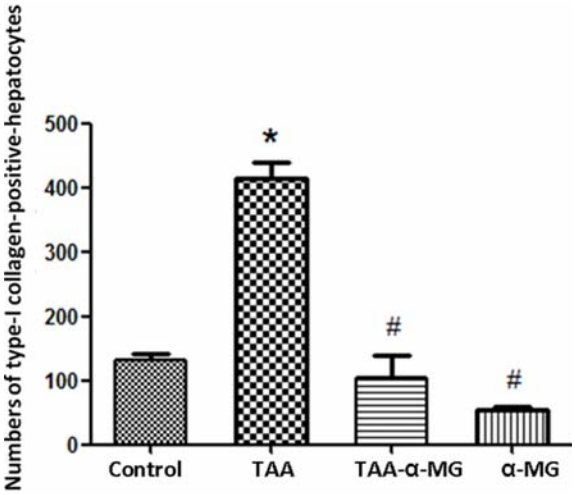

Fig. 4 The numbers of type-I collagen-positive-hepatocytes in control, TAA-induced cirrhosis, TAA-α-MG-prevented and α-MG-injected rat livers at 16 weeks. Data are expressed as means ± SE, *p < 0.05 compared with control group, #p < 0.05 compared with TAA group

compared to the normal.

α-Mangostin, one of the most abundant xanthones in mangosteen (*Garcinia mangostana*), is known as an antioxidant[15]. In addition, the extract of *G. mangostana* significantly reduced ROS production comprised of polymorphonuclear leucocytes (PML) with 77.8% of superoxide anion (O_2) inhibition ratio (62.6%, 44.9% and 35.18% for *H. cordata*, *E. odoratum*, and *S. alata*, respectively)[16]. Recently, it has been reported that α-MG significantly inhibited hepatic stellate cells *in vitro* assay system[17]. This postulates that α-MG is more effective in reducing the synthesis of type-I collagen from hepatocytes. The present study demonstrated that the diameter of sinusoids was wider in the α-MG-treated liver. It could, therefore, be that α-MG helps to bring high oxygen blood and nutrients to hepatocytes via dilated sinusoids in order to lower the hepatocyte damage caused by TAA-generated ROS.

The authors showed, in the present study, that the number of blood vessels in the α-MG-injected rat liver was higher compared to that in control. In α-MG-injected group, the distribution of type-I collagen surrounding vascular wall significantly decreased except in some areas. This finding suggests that intraperitoneal administration of α-MG at 100 mg/kg 3 times per week, which does not harm to the hepatic parenchyma, is an effective experimental protocol to lower the collagen synthesis.

In the present study, it could be concluded that the number of type-I collagen-immunopositive hepatocytes in TAA-α-MG-prevented group was about 3 folds less than that in TAA-treated group. It could be that α-MG exerts a protective effect against type-I collagen formation in cirrhotic rat model.

Acknowledgement

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Potential conflicts of interest

None.

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ผลของสารแอลฟาแมงโกสตินต่อการป้องกันการก่อรูปของคอลลาเจนชนิดที่ 1 ในหนูที่ถูกชักนำให้เกิดตับแข็งด้วยสารไธโออะเซตาไมด์

รักษวรรณ พูนคำ, รมิดา วัฒนโภคาสิน, วิสุทธิ์ ประดิษฐ์อาชีพ

วัตถุประสงค์*: เพื่อศึกษาผลของสารแอลฟาแมงโกสตินต่อการป้องกันการเพิ่มขึ้นของเซลล์ตับที่ย้อมติดคอลลาเจนชนิดที่ 1 ในหนูที่ถูกชักนำให้เป็นตับแข็งด้วยสารไธโออะเซตาไมด์*

วัสดุและวิธีการ*: แบ่งหนูทดลองเป็น 4 กลุ่ม โดยกลุ่มที่ 1 เป็นกลุ่มควบคุม (control group) กลุ่มที่ 2 เป็นหนูที่ถูกชักนำให้เกิดภาวะตับแข็งด้วยการฉีดสารไธโออะเซตาไมด์ 200 มิลลิกรัมต่อกิโลกรัม เป็นเวลาสามครั้งต่อสัปดาห์ ต่อเนื่อง 16 สัปดาห์ (TAA-induced cirrhosis group) กลุ่มที่ 3 คือหนูที่ได้รับสารไธโออะเซตาไมด์ 200 มิลลิกรัมต่อกิโลกรัม สลับกับสาร α-mangostin 100 มิลลิกรัมต่อกิโลกรัม (TAA-α-MG-prevention group) เป็นเวลา วันเว้นวัน ต่อเนื่อง 16 สัปดาห์ และกลุ่มสุดท้ายเป็นหนูที่ได้รับเพียงสารแอลฟาแมงโกสติน 100 มิลลิกรัมต่อกิโลกรัมเท่านั้น เป็นเวลาสามซรั้งต่อสัปดาห์ ต่อเนื่อง 16 สัปดาห์ (α-MG-injected group) โดยทั้ง 4 กลุ่มถูกเลี้ยงทั้งหมด 16 สัปดาห์ คอลลาเจนชนิดที่ 1 และเซลล์ตับที่ย้อมติดคอลลาเจนชนิดที่ 1 ถูกศึกษาด้วยวิธีการย้อมทางอิมมูนโนฮิสโตเคมี*

ผลการศึกษา*: ในตับแข็งมีการกระจายของเส้นใยคอลลาเจนชนิดที่ 1 และเซลล์ตับที่ย้อมติดคอลลาเจนชนิดที่ 1 เป็นจำนวนมาก จำนวนของเซลล์ตับที่ย้อมติดคอลลาเจนชนิดที่ 1 ในกลุ่ม TAA-induced cirrhosis เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ คิดเป็น 414.00 ± 25.23 เทียบกับกลุ่ม control ซึ่งมีจำนวน 131.40 ± 9.63 เป็นที่น่าสนใจว่ามีการลดลงอย่างมีนัยสำคัญทางสถิติของจำนวนเซลล์ตับที่ย้อมติดคอลลาเจนชนิดที่ 1 ในกลุ่ม TAA-α-MG-prevention ซึ่งคิดเป็น 103.60 ± 36.55 และในกลุ่ม α-MG-injected คิดเป็น 54.00 ± 5.30 เทียบกับกลุ่ม control และ กลุ่ม TAA-induced cirrhosis*

สรุป*: สารแอลฟาแมงโกสตินขนาด 100 มิลลิกรัมต่อกิโลกรัม มีผลให้จำนวนเซลล์ตับที่ย้อมติดคอลลาเจนชนิดที่ 1 น้อยลง ทั้งนี้อาจเนื่องมาจากสารแอลฟาแมงโกสตินชักนำให้มีหลอดเลือดมาเลี้ยงตับมาก และหลอดเลือดฝอยใกล้เซลล์ตับขยายใหญ่ ส่งผลให้ออกซิเจนมาที่เซลล์ตับเพิ่มขึ้น เป็นการลดการอักเสบของเซลล์ตับ จากปฏิกิริยา Reactive oxygen species (ROS) ที่เกิดจากสารไธโออะเซตาไมด์*