

# The effect of Crocodile (*Crocodylus Siamensis*) Oil on Hepatic Energy Homeostasis Mechanism and Mitochondrial Function in Rats

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## Research Article

**Keywords:** crocodile oil, energy metabolism, liver, mitochondria, rats

**Posted Date:** August 18th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-816046/v1>

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# Abstract

**Background:** Fatty acid (FA) consumption can alter hepatic energy metabolism and liver mitochondrial functions. Crocodile oil (CO) is rich in both mono- and polyunsaturated fatty acids that contain natural anti-inflammatory and healing properties. We investigated the effect of CO on energy metabolism and mitochondrial morphology in rat liver.

**Methods:** Twenty-one male Sprague–Dawley rats were randomly divided into three groups. Group 1: rats treated with sterile water (RO); group 2: rats treated with CO (3% v/w); and group 3: rats treated with palm oil (PO) (3 % v/w). Rats were orally administered sterile water, CO, and PO once daily for 7 weeks. Body weight, food intake, liver weight, energy intake, blood lipid profiles, and liver-targeted metabolites were evaluated. Histopathological study of the liver, mitochondrial architecture of liver cell, and HDHD3 protein expression in liver mitochondria were determined.

**Results:** CO treatment had no effect on body weight, liver weight, liver index, dietary energy intake, and serum lipid profiles. The CO group exhibited significantly lower food intake than the RO group. The CO group also showed significantly higher oxaloacetate and malate levels, which encourage the TCA cycle imbalance, than the PO group. CO treatment significantly ameliorated hepatic steatosis as shown by a greater decrease of total surface area of lipid particles than that seen with PO treatment. CO administration maintained the liver's mitochondrial morphology by upregulating the energetic maintenance protein—HDHD3. Moreover, the chemical-protein interaction also showed that the main fatty acid composition of CO preserved liver metabolism via the AMPK signaling pathway.

**Conclusion:** Crocodile oil could support hepatic function through promoting the TCA cycle, maintaining hepatic mitochondrial architecture, and upregulating HDHD3.

## Introduction

The liver plays a major role in homeostasis in the human body and is considered as a metabolic biotransformation hub. The liver is responsible for multiple functions and physiological processes such as bile production, energy generation, and the metabolism of carbohydrates, proteins, and lipids [1]. Mitochondrial dysfunction is a general term, which includes alteration of different metabolic pathways and damage to mitochondrial components. In addition, these mitochondrial disturbances can have a variety of harmful consequences such as oxidative stress, energy deficiency, accumulation of triglycerides (steatosis), and cell death [2]. Liver steatosis results from increased adipose lipolysis that subsequently increases hepatic de novo lipogenesis (DNL), impaired synthesis and/or secretion of very low-density cholesterol, triacylglycerol (TAG) esterification dysfunction, or impaired mitochondrial  $\beta$ -oxidation [3]. Mitochondria are unique organelles that play a vital role in cells by metabolizing nutrients and are responsible energy metabolism, generation of free radicals and calcium homeostasis, cell survival, and cell death [4]. Hepatic mitochondria support many metabolic activities and contribute to the pathophysiology of insulin resistance and diabetes [5–6]. The main function of the mitochondria is to

synthesize ATP via oxidative phosphorylation (OXPHOS) in accordance with the oxidation of metabolites by the tricarboxylic acid (TCA) cycle and  $\beta$ -oxidation of fatty acids. A previous study reported that consumption of high-fat diet altered the energy metabolism of rat hepatocytes by inhibiting mitochondrial OXPHOS [7].

Over the last few decades, public concerns on the interaction between health and nutrition has been rapidly increasing. Fat is an essential macronutrient in common human diet, and vegetable oils represent now the most consumed fat in the world. Edible oils, which can be grouped into three classes of fatty acids—saturated fatty acids (SFAs), monounsaturated (MUFAs), and polyunsaturated fatty acid (PUFA)—are commonly used in industrial food manufacturing and home cooking worldwide. However, some of edible cooking oils, such as olive oil, soybean oil, and palm oil, are still more consumed than other oils of higher quality and properties. Palm oil contains higher ratios of SFA to MUFA and PUFA than other cooking oils [8]. Previously, Li et al. [9] studied the effects of palm oil diet compared with low-fat diet on the expression level of lipid breakdown-related genes and found that palm oil showed significantly downregulated hepatic PPAR- $\alpha$  expression levels. When PPAR $\alpha$  is inhibited by fatty acids, FA oxidation and the ensuing ATP production are diminished. Many previous studies found that SFA intake is associated with secondary diseases, such as glucose intolerance, insulin resistance, dyslipidemia, cardiovascular disease (CVD), and hepatic steatosis [10–12]. High intake of dietary fat is associated with increased ROS production, decreased respiration, respiratory uncoupling, and reduced ATP production [13–16].

*Crocodylus siamensis* is one of the native freshwater crocodile species of Southeast Asia [17]; these are now commercially bred on large scale in Thailand. Crocodile oil is extracted from the fatty tissues of the crocodile; it contains high concentrations of PUFA and MUFA [18] when compared to other type of animal oils. CO was reported to be very effective in the treatment of varied ailments, ranging from skin conditions to cancer, and has been used for centuries by traditional practitioners [19]. Many previous studies also reported that CO has been used to treat skin rashes and promote wound healing [19–20]. Another study in hamsters showed that a combination of low MUFA and low PUFA to SFA ratio induces weight gain and body fat accumulation, while a high MUFA and high PUFA/SFA ratio prevented white adipose tissue accumulation [21].

Therefore, the aim of this research was to investigate the effect of CO on hepatic energy metabolism and mitochondrial function in rats. We hypothesized that CO may be associated with alterations in mitochondrial protein expression of energy metabolic pathways in rat livers. This research may help clarify the action of CO in maintaining energy homeostasis in the liver and likely be a biomarker for metabolic liver disease.

## Materials And Methods

### Ethics statement

The research conducted adhered to the Guidelines for the Care and Use of Laboratory Animals. The ethics committee of Kasetsart University Research and Development Institute, Kasetsart University, Thailand, approved this study (Approval No. ACKU61-VET-088).

## **Crocodile oil preparation**

Crocodile oil extraction was performed according to the method described by Santativongchai et al. [22]. Abdominal fat samples were collected from slaughtered *C. siamensis* (age: 3–5 years) obtained from a crocodile farm in Nakhon Pathom province, Thailand.

The samples were pressed through two layers of filter cloth with distilled water at the proportion of 1:1 (w/v). Subsequently, the solution was left undisturbed until separation of the mixture was observed. The upper clear oil fraction was then collected, evaporated, and stored in a sealed container at room temperature.

## **Animal care, diets and experimental design**

Briefly, 21 Sprague–Dawley male rats (age: 7 weeks) were obtained from Nomura Siam International Co. Ltd., Samutprakan province, Thailand. The animals were individually housed under controlled environmental conditions ( $25 \pm 2^\circ\text{C}$  on a 12-h light/12-h dark cycle). Rats had ad libitum access to food and drinking water throughout the study. Rats were randomly divided into three groups ( $n = 7/\text{group}$ ). Rats in group 1 were treated with sterile water (RO), those in group 2 were treated with crocodile oil (3% v/w) (CO), and those in group 3 were treated with commercial palm oil (3% v/w) (PO). The animals were orally administered sterile water, CO, and PO once daily for 7 weeks.

## **Measurement of body weight, food intake, and energy intake of animals**

Food consumption was measured daily between 11:00 and 11:30 A.M. by weighting the rats. The food intake of each rat was measured by weighting the remaining chow. Food intake was also monitored daily to calculate the energy intake. The body weight was measured weekly throughout the experimental duration.

## **Sample collection**

At the end of experiment, all animals were sacrificed by a lethal dose of pentobarbital sodium. Blood samples were collected by cardiac puncture and were centrifuged at  $2,200\ g$  for 15 min at  $4^\circ\text{C}$ . The serum was stored at  $-20^\circ\text{C}$  until further analyses. The serum lipid profile included triglycerides, cholesterol, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) and were determined by Hitachi 7080 analyzer (Hitachi, Japan).

Liver specimens were immediately collected and weighed. The liver specimens were stored on ice-cold homogenate buffer (0.32 M sucrose, 1 mM EDTA, and 10 mM Tris-HCL; pH 7.4) when performing a standard protocol for mitochondrial extraction. Some of the liver tissue was also collected and separated

for histopathology analysis and preserved in 10% neutral buffer formalin. Liver samples were collected and homogenized in ice-cold PBS (20% w/v) and centrifuged at 2,000 *g* for 20 min at 4°C. The supernatants were stored at -80°C until further analysis.

## **Energy metabolism-related intermediates analysis**

The frozen supernatants were mixed with methanol in a ratio of 2:8 (v/v). After centrifugation (20,000 *g* for 20 min, 4°C), the supernatants were taken out and evaporated using a freeze dryer at -80°C. The metabolites were then re-dissolved in 500 µL HPLC buffer. Each 5 µL sample was subjected to HPLC analysis. Chromatography was performed as follows: the injection volume was set to 5 µL and the column was kept at 40°C. An InertSustain C18 (150×4.6 mm) measuring 5 µm was used to achieve separation of the mobile phase consisting of 8% 1 N sulfuric acid. The gradient elution was used at a flow rate of 1 mL/min.

## **Hepatic histopathology and fat accumulation analysis**

Fixed livers were stored in 10% formalin for 24-h at room temperature. The sample were embedded in paraffin and sectioned at 5-µm thickness, and stained with hematoxylin and eosin (H&E). Hepatic fat accumulation was determined by Oil Red O (ORO) staining. Liver cryosections (5 µm thick) were fixed with 10% NBF in PBS for 20 min and incubated in freshly prepared ORO solution for 10 min and then counterstained with hematoxylin for 20 sec. The sections were examined under a light microscope with a magnification 200×. The number and total surface area of lipid particles were analyzed.

## **Liver mitochondrial extraction**

The liver mitochondrial extraction was performed within 1–2 h to prevent cellular damage. Liver samples from each group were pooled, weighed, homogenized, and washed in homogenate buffer. Liver specimens were homogenized in a glass Potter–Elvehjem tissue grinder with an appropriate volume of the homogenate buffer (4 mL homogenate buffer/1 g of liver specimen). During this step, several up and down strokes were performed using a motor-driven Teflon pestle at 600 rpm. Next, homogenates were centrifuged at 1,000 *g* at 4°C for 5 min. The supernatants were collected and centrifuged at 15,000 *g* at 4°C for 2 min. The mitochondrial pellets were collected and washed several times in homogenate buffer. The pellets were resuspended in ice-cold final equilibrated buffer (250 mM sucrose, 5 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-HCl, and 2 mg/mL bovine serum albumin [BSA]; pH 7.2), and 200 µL of the resuspended pellet was then fixed in 2.5% glutaraldehyde in 0.1 M sucrose phosphate buffer (SPB) for electron microscopy analysis. The mitochondrial protein content was measured by protein assay (Bio-Rad®) using a spectrophotometer (NanoDrop-1000, Thermo Scientific).

## **Conventional electron microscopy**

Electron microscopy was used to examine the ultrastructure of liver mitochondria. Fixation of the liver specimens from each group was performed using 1% osmium tetroxide, dehydrated in graded ethanol, infiltrated in a series of LR white resin (EMS®, USA), embedded in pure LR white (EMS®, USA), polymerized at 60°C for 48 h, cut into 100-nm-thick sections, and stained with lead citrate and uranyl

acetate. The liver ultrastructure was examined under a transmission electron microscope (model HT7700, Hitachi, Japan). Intact mitochondria in hepatocytes were counted (50 cells per group) and compared with other treatment groups.

## **Metabolite-protein and protein-protein interaction**

We utilized STITCH V. 5.0 (<http://stitch.embl.de/>) to establish the interaction prediction between the expected metabolites and proteins that interact with the targeted mitochondrial energy-maintenance protein (haloacid dehalogenase-like hydrolase domain containing 3: HDHD3), such as the energy-related proteins (PRKAA, PRKAB, PRKAG, AKT, PPAR- $\alpha$ , PPAR- $\gamma$ ) and energy-related metabolites (lactate, pyruvate, oxaloacetate, citrate), with a required confidence threshold (score) of 0.40.

## **Immunogold labelling technique**

The immunogold labelling technique was used to compare the expression of HDHD3 (mitochondrial energy marker), and its localization on mitochondria among the groups. Rabbit polyclonal anti-HDHD3 was used as the primary antibody marker (MyBioSource, USA).

The mitochondrial pellet from the pooled liver extract in each group was secondary fixed, and tissue processing was performed as described previously. The tissue sections were blocked using 50 mM glycine in phosphate-buffered saline (PBS) followed by 5% BSA (EMS, USA) in PBS for 30 min each. Then, they were incubated with 1:50 diluted primary antibodies for 1 h prior to applying goat anti-rabbit IgG conjugated with 10-nm gold particles (EMS®, USA). Between each step, sections were washed several times using 0.1% BSA in PBS. To improve contrast of the gold particle labelling, a silver enhancement kit (Aurion R-Gent SE-EM kit, EMS, USA) was used after rigorously washing the tissue sections with distilled water. Finally, the sections were stained with lead citrate and uranyl acetate prior to transmission electron microscopy. The number of labelled gold particles was counted for the intact stage of liver mitochondria (50 mitochondria/group were evaluated).

## **Data analysis and statistical methods**

The data are expressed as means  $\pm$  SD. Statistical analysis was performed by analysis of variance (one-way ANOVA) followed by Tukey's post-hoc test in R project statistical computing package (R core team, 2019). For all analyses,  $p < 0.05$  was considered to indicate statistical significance.

## **Results**

### **Effect of dietary CO on final body weight, body weight gain, food intake and energy intake**

As shown in Table 1, CO- and PO-treated rats showed a significant decrease in food intake when compared to the RO group. However, there were no significant differences on final body weight, body weight gain and energy intake between each group.

Table 1

Effect of dietary crocodile oil on final body weight, body weight gain, liver weight, liver index, average food intake and average energy intake of rats for 7 weeks.

Group	Final body weight (g)	Body weight gain (g)	Liver weight (g)	Liver index	Food intake (g/day)	Energy intake (kcal/g)
RO	511.37 ± 9.57	26.17 ± 6.52	12.45 ± 1.84	0.024 ± 0.0015	18.82 ± 0.68 <sup>a</sup>	57.21 ± 2.05
CO	520.84 ± 13.95	39.33 ± 13.76	13.67 ± 1.9	0.026 ± 0.0034	15.84 ± 0.75 <sup>b</sup>	60.16 ± 2.28
PO	515.31 ± 10.909	34.67 ± 11.11	13.34 ± 1.27	0.026 ± 0.0019	14.98 ± 0.83 <sup>b</sup>	58.53 ± 2.51

Data are expressed as the mean ± SD. Different letters indicate statistically significant differences between groups ( $P < 0.05$ ).

## Effect of dietary CO on blood lipid profiles liver energy metabolism-related metabolites

The serum lipid profile is presented in Fig. 1. The results showed no significant difference among the three groups. Interestingly, compared to the PO group, both CO and PO groups showed a decreasing trend of triglyceride levels.

As show in Fig. 2, the CO-treated group indicated significantly increased oxaloacetate and malate levels in the liver. However, CO administration had no effect on hepatic lactate, pyruvate, citrate, and alpha-ketoglutarate level (Fig. 2).

## Effect of dietary CO on hepatic lipid accumulation

The liver H&E histologic examination revealed microvesicular fat depositions in CO- and PO-treated rats, however the size of fat droplets in CO was obviously smaller than PO. Furthermore, macrovesicular steatosis were found in PO, while the macrovesicular in CO was almost absent (Fig. 3). The effect of CO supplementation on intracellular lipid levels in hepatocytes was also visualized by Oil Red O staining (Fig. 3). The CO group showed a significant lower total surface area of lipid droplets in the liver than the PO group. However, the total lipid particle area seen in CO-treated rats were significantly higher than in RO-treated rats.

## Effect of CO on mitochondrial architecture and expression of HDHD3 in liver mitochondria

Electron microscopy analysis was conducted after administration of CO for 7 weeks. The CO treated rats showed a higher percentage of intact mitochondria than the PO-treated rats (Fig. 4). The expression of energy maintenance protein, HDHD3, was chosen to verify their activity in the intact stage of

mitochondria. The expression of mitochondrial HDHD3 was significantly upregulated in the CO group compared to the PO group. Meanwhile, the expression of HDHD3 protein was significantly upregulated in the RO group compared with the CO and PO groups (Fig. 5).

## The chemical-protein and protein-protein interaction analysis

As shown in Fig. 6, the interaction network showed that the HDHD3 protein was linked with the energy homeostatic pathway in the liver. HDHD3 showed functional interaction with only one protein in the AMPK signaling pathway (PRKAG1). Furthermore, HDHD3 also showed functional interactions with energy related-metabolites and was associated with ATP metabolic processes. Meanwhile, three main fatty acids of CO (linoleic acid, oleic acid and, palmitic acid) well interacted with other chemicals in hepatic energy metabolism and proteins associated with the AMPK signaling pathway. Thus, CO can improve the activity of energy metabolism in the liver by up-regulating the mitochondrial protein, HDHD3.

## Discussion

In this study, by using metabolomics and analyzing the ultrastructure of mitochondria, we investigated the different effects between CO and PO diets on hepatic metabolism and mitochondrial function. Our results showed that compared to PO, CO, an alternative source of PUFA and MUFA, could increase some key metabolites in the TCA cycle, decrease hepatic steatosis, improve liver mitochondrial architecture, and upregulate HDHD3 protein expression. Crocodile oil administration was useful to treat clinical liver metabolic damage.

Palm oil treatment led to lower malate and oxaloacetate levels and induced liver fat accumulation and mitochondrial dysmorphology, which contributed to the impairment of energy metabolism. Palm oil is rich in SFAs, especially palmitic acid, unlike most other vegetable oils that are richest in unsaturated fatty acids [23]. Palm oil is currently the most used edible oil in many food industries, even though the associated health risks are unclear. Results of the Oil red O staining showed that the liver of rats fed with PO for 7 weeks showed significantly increased hepatic steatosis compared with the RO and PO group. A similar finding was noted in a previous study which reported that PO altered hepatic metabolism and caused lipid accumulation via disturbed hepatocyte transcription [24]. A further study by Wen et al. [25] showed that SFAs like palmitic acid can switch on the NOD-like receptor family pyrin containing 3 (NLRP3) inflammasome via an AMP-activated protein kinase-autophagy pathway that activates macrophage NLRP3 inflammasomes that are associated with the development of liver steatosis [26]. Previous studies have found that abnormalities of mitochondrial morphology and function may be associated with fatty liver accumulation [27–28]. Meanwhile, Li et al. [9] compared the effect of PO and low-fat diet on the expression level of lipid breakdown-related genes and found that the PPAR- $\alpha$  expression levels of the PO-treated group were significantly downregulated. The nuclear receptor protein—PPARs—are fundamentally important for energy homeostasis. PPAR $\alpha$  activates genes encoding enzymes

involved in fatty acid oxidation (FAO), fatty acid transport proteins, and their derivatives to enter into the  $\beta$ -oxidation pathway [29]. Our study indicated the potential unhealthy effects of PO in the diet: PO appeared to induce hepatic lipid accumulation by down-regulating the transcriptional level of energy homeostatic genes and cause liver damage by impairing the mitochondrial function.

Energy metabolism related-metabolite flow reflects the energy production in the liver. Compared to PO administration, 7 weeks of CO administration led to a significant increase in oxaloacetate and malate levels in the TCA cycle. The TCA cycle is the central metabolic hub of the cell and also an important source of precursors for energy supply. Crocodile oil from *C. siamensis* is rich in MUFA and PUFA, which are the main constituents of oleic acid and linoleic acid [30]. Although the effects of CO on energy metabolism are still not entirely clear, we found that linoleic acid (LA) and  $\alpha$ -linolenic acid (ALA) are essential fatty acids for good health and the precursors of long-chain PUFA for the omega-6 group of arachidonic acid and the omega-3 group of Eicosapentaenoic acid (EPA) and Docosahexanoic acid (DHA) [31]. Belury et al. [32] found that conjugated LA administration activated PPAR- $\alpha$  in mice liver. Moreover, previous data reported that conjugated LA could induce anti-inflammatory macrophages in a PPAR- $\gamma$ -dependent manner [33]. Evidence from animal and clinical studies from previous review suggests that macrophage targeting may improve the liver fatty disease progress and related metabolic disorders [34]. Moreover, Liu et al. [35] studied the mechanism by which omega-3 PUFAs influence TCA cycle under conditions of obesity and found that PUFAs may alleviate obesity by affecting mitochondrial function and restoring TCA cycle homeostasis, especially the transcription and translation of TCA cycle enzymes such as citrate synthase (CS), isocitrate dehydrogenase 1 (IDH1), isocitrate dehydrogenase 2 (IDH2), succinate dehydrogenase subunits A (SDHA), succinate dehydrogenase subunits B (SDHB), fumarate hydratase (FH), and malate dehydrogenase 2 (MDH2) in HepG2 cells. Thus, fat diet-enriched of linoleic feeding could induce energy metabolic activity in rats by triggering the PPAR in TCA intermediate production.

To explore the possible mechanisms of CO for the improvement in liver energy metabolism, we observed the liver mitochondrial morphology and expression level of the protein related to energy homeostasis. A previous study reported that fat diet rich in SFAs elicited hepatic fat accumulation and insulin resistance, which was consistent with impaired mitochondrial function, increased reactive oxygen species production, and a dysregulated expression profile of mitochondrial dynamics proteins [36]. Moreover, high-fat diet was associated with decreased energy expenditure and expression of genes controlling lipid metabolism and mitochondrial function [37]. Ampawong et al. [38] showed that dysregulated lipid metabolism can lead to mitochondrial alteration and high levels of apoptosis in addition to defective energy production. Our study indicated that CO-treated rats showed significantly higher percentage of intact mitochondria than the PO group. Zhang et al. [39] indicated that PUFAs may significantly increase mitochondrial fusion by up-regulating mitofusin 2 (Mfn2) expression in steatotic hepatocytes. Moreover, PUFAs may also increase ATP levels and reduce ROS production through Mfn2 activity. Our results also confirmed the protective effect of CO on energy metabolism, and we found that CO not only maintains mitochondrial morphology but also enhances energy production via upregulating mitochondrial HDHD3 protein in the liver. Our results also showed the downregulation of HDHD3 in PO-treated rats. The

interaction network between the HDHD3 and other proteins in STITCH database such as AKT, PPAR $\alpha$ , PPAR $\gamma$ , PRKA $\alpha$ , and PRKA $\gamma$  were used to investigate the effects of CO on hepatic energy metabolism (Fig. 5). The HDHD3 proteins were found to be associated with the protein kinases AMP-activated non-catalytic subunit gamma 1 (PPKAG1) and ATP production. The proteins encoded by genes are catalytic subunits of AMP-activated protein kinase (AMPK) [40–42]. The AMPK is an important energy-sensing enzyme that monitors the cellular energy status which regulates the transcription of many genes involved in mitochondrial energy metabolism and the oxidation of glucose and fatty acids [43]. Moreover, activation of AMPK was shown to reduce liver lipid content in many preclinical studies [44] by modulating *de novo* lipogenesis (DNL), fatty acid oxidation (FAO), and fatty acid release from the adipose tissue [45]. The major catabolic pathway of FAO could resolve hepatic lipid storage and convert fatty acids to acetyl-CoA that could enter the TCA cycle and then be utilized by the mitochondria to produce ATP.

The consumption of oil rich in PUFA could enhance the hepatic AMPK activity and influence the regulation of hepatic lipid metabolism and gene expression [46]. Similar to the previous results of marked effects of krill oil (enriched with PUFA) on liver function, the results showed that the oil regulates genes and pathways involved in hepatic energy metabolism [47]. Polyunsaturated fatty acids have a unique ability towards metabolic associated fatty liver disease. AMPK also directly activates PPAR $\gamma$  co-activator 1 $\alpha$  to induce mitochondrial biogenesis [48–50]. Mitochondrial biogenesis occurs in response to liver mitochondrial dysfunction in order to maintain cellular homeostasis against oxidative stress and injury by forming new mitochondria [51]. Therefore, the CO group could help to maintain the mitochondrial number and function. This study revealed that CO could improve liver energy metabolic dysfunction and mitochondrial contents via activating the mitochondrial HDHD3 protein.

## Conclusions

Our research provides new insights to understand the chronic effect of CO consumption on hepatic energy metabolism and mitochondrial function. The CO treatment resulted in lowered food intake and increased some key hepatic energy metabolite levels. It also prevents hepatic steatosis, improves mitochondrial morphology, and plays a significant role in maintaining energy metabolism by upregulating HDHD3 expression. To our knowledge, this is the first report on the effect of CO on energy metabolism via hepatic function. Our results may have clinical and therapeutic application through the expression of protein-associated metabolic homeostasis. Crocodile oil can be a potential essential oil substitute and a good choice for an economical therapeutic agent to treat metabolic energy disorders in the future.

## Abbreviations

HDHD3, haloacid dehalogenase like hydrolase domain containing 3; TCA, tricarboxylic acid; AMPK; AMP-activated protein kinase; CS, citrate synthase; IDH1, isocitrate dehydrogenase 1; IDH2, isocitrate dehydrogenase 2; SDHA, succinate dehydrogenase subunits A; SDHB, succinate dehydrogenase subunits B; FH, fumarate hydratase; MDH2, malate dehydrogenase 2; PRKA $\alpha$ , protein kinase AMP-activated-alpha; PRKA $\beta$ , protein kinase AMP-activated-beta; PRKA $\gamma$ , protein kinase AMP-activated-gamma; Akt,

serine/threonine protein kinase; PPAR $\alpha$ , Peroxisome Proliferator Activated Receptor Alpha; PPAR $\gamma$ , peroxisome proliferator activated receptor gamma.

## Declarations

### Acknowledgments

This research was supported by the Department of Zoology, Faculty of Science and partially supported by the Faculty of Veterinary Medicine, Kasetsart University, Thailand and the Science Achievement Scholarship of Thailand (SAST).

**Conflict of interests:** The authors disclose no conflicts of interest

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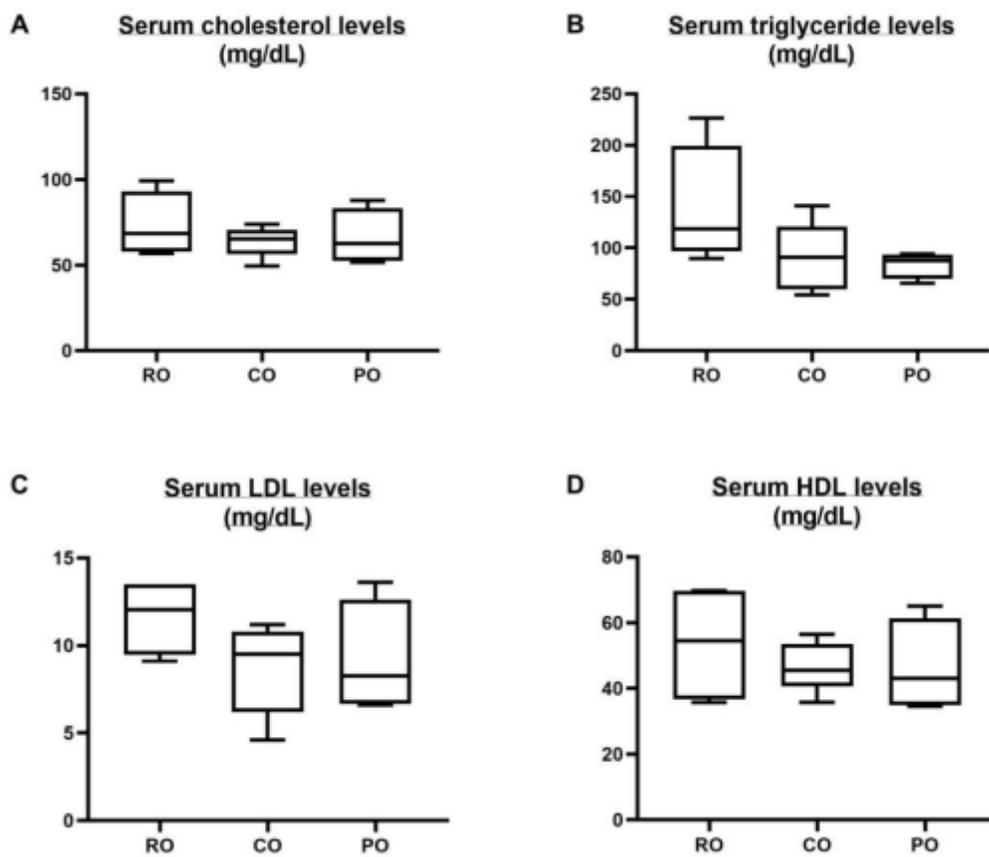
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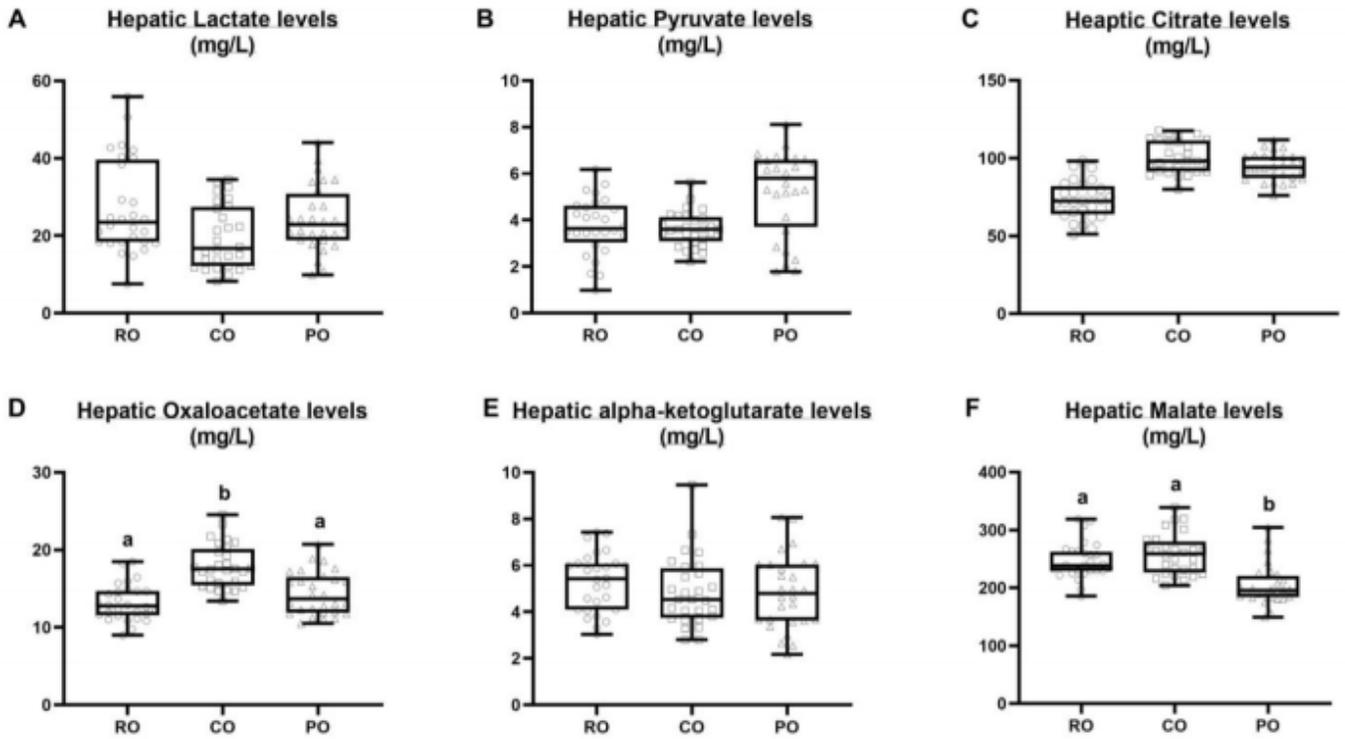
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## Figures



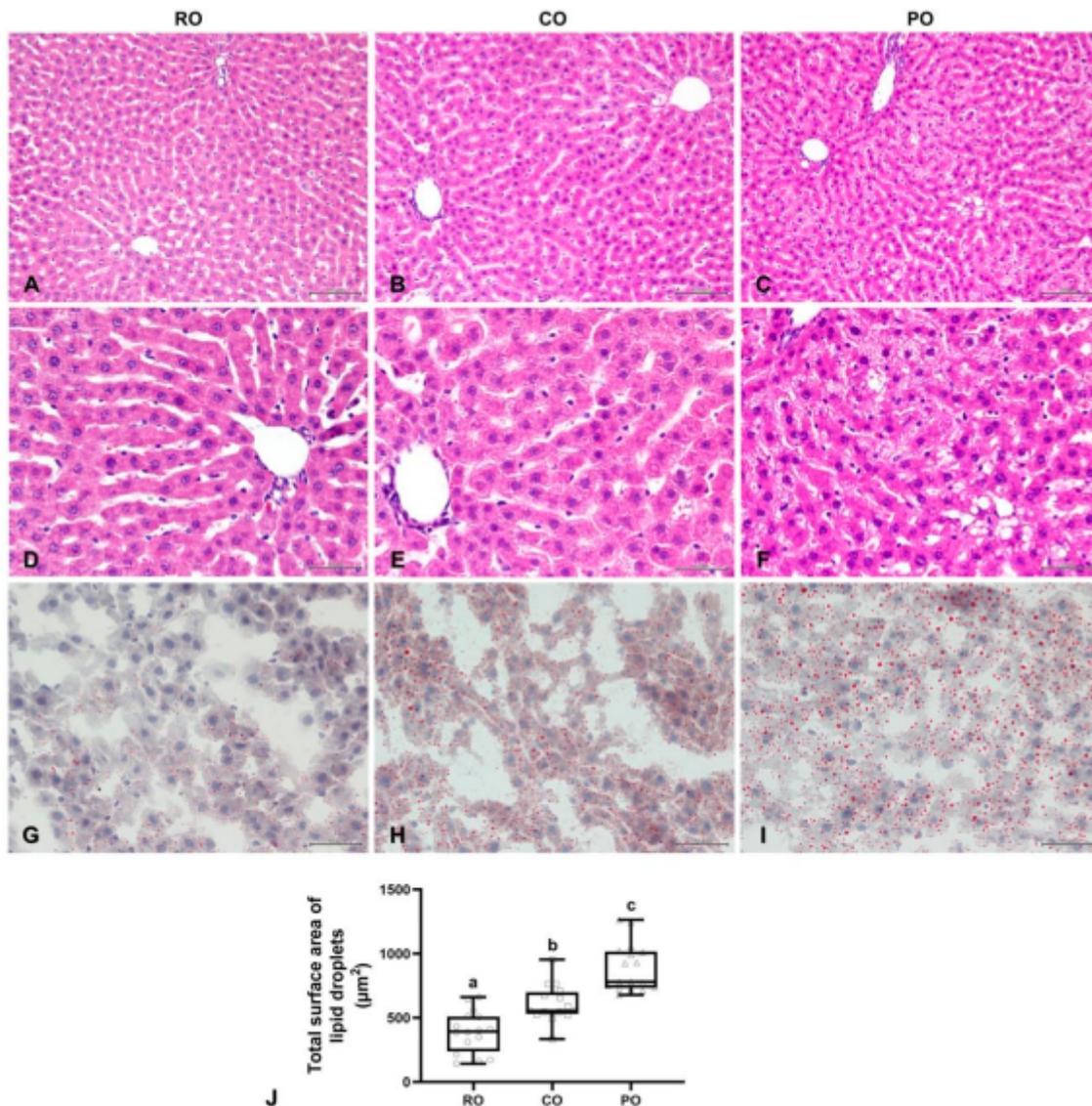
**Figure 1**

Effect of dietary crocodile oil on serum (A) cholesterol, (B) triglyceride, (C) low-density cholesterol (LDL), and (D) high-density cholesterol (HDL) levels of rats for 7 weeks. Data are expressed as the mean $\pm$ SD.



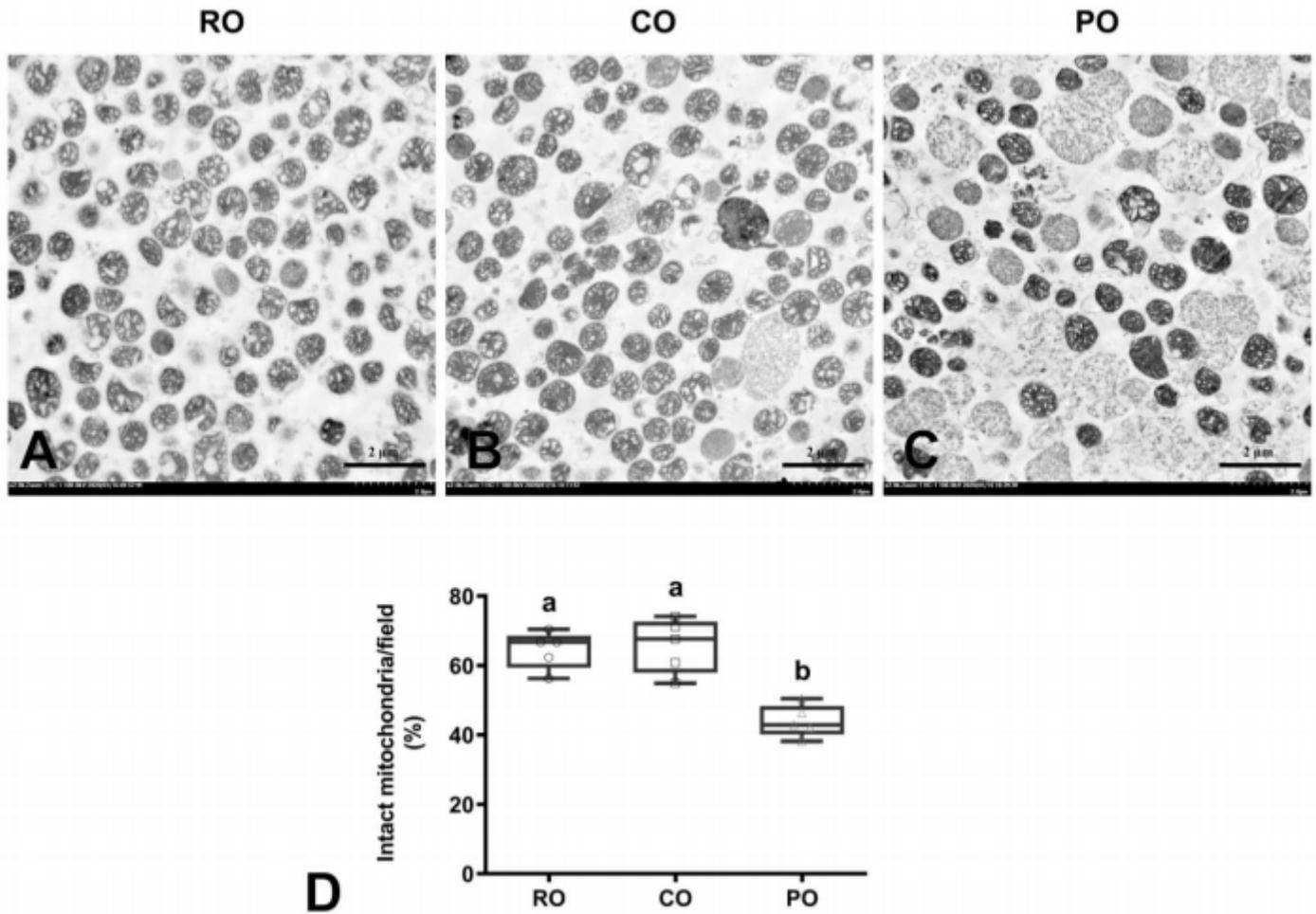
**Figure 2**

Effect of dietary crocodile oil on liver energy metabolism related-metabolites (A) lactate, (B) pyruvate, (C) citrate, (D) oxaloacetate, (E) alpha-ketoglutarate, and (F) malate levels. Data are expressed as the mean $\pm$ SE. Different letters indicate statistically significant differences between groups ( $P < 0.05$ ).



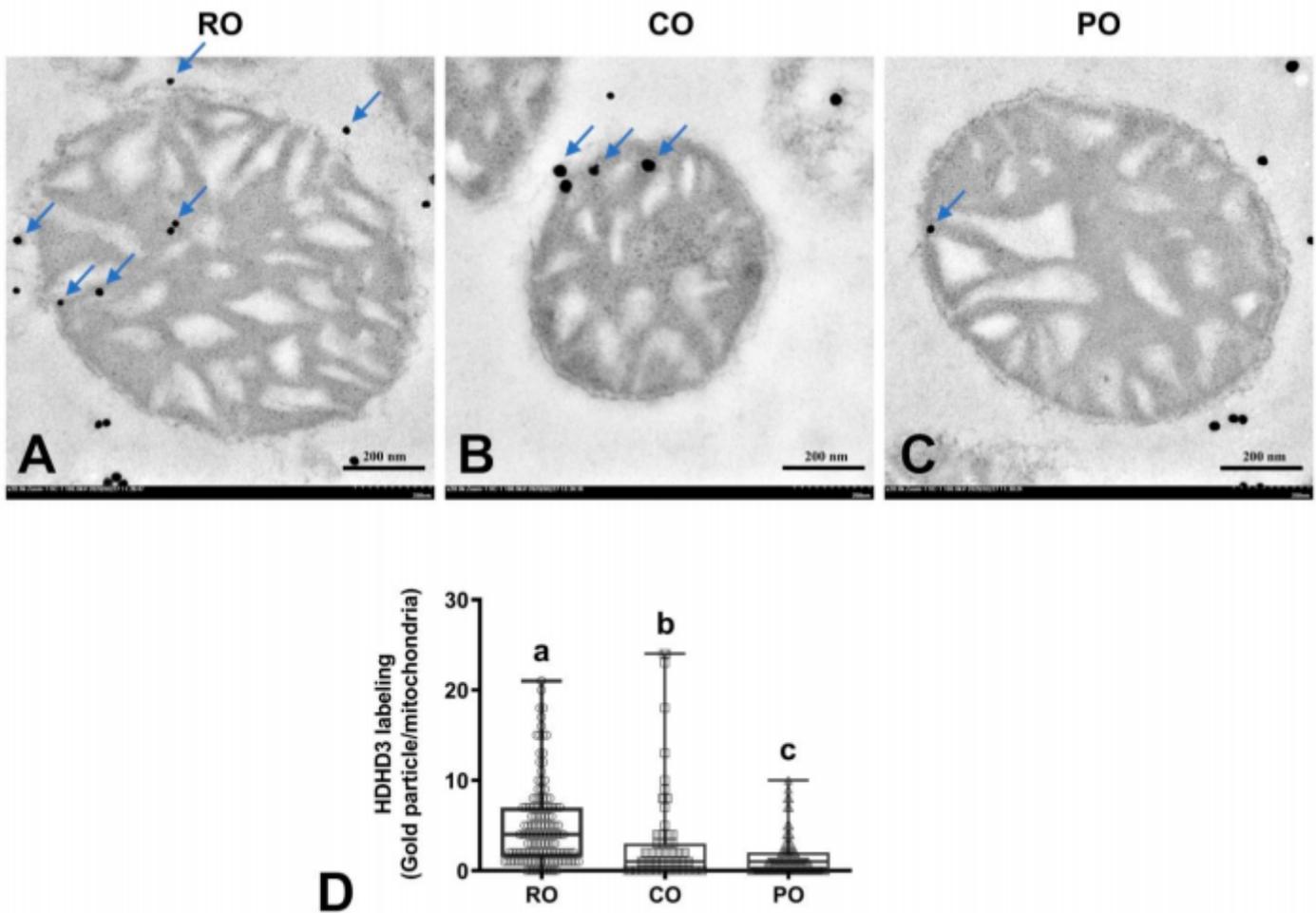
**Figure 3**

Histological analysis of liver lipid accumulation after 7 weeks of crocodile oil administration. (A-C) Representative photos of H&E staining (200x). (D-F) Representative photos of H&E staining (400x) with morphological forms of hepatic steatosis; CO showed microvesicular steatosis, whereas PO showed micro and macrovesicular steatosis. (G-I) Representative photos of H&E (400x). The bar graph indicates the total surface area of the lipid droplets (J); data is represented by mean±SD. Different letters indicate statistically significant differences between groups (P<0.05).



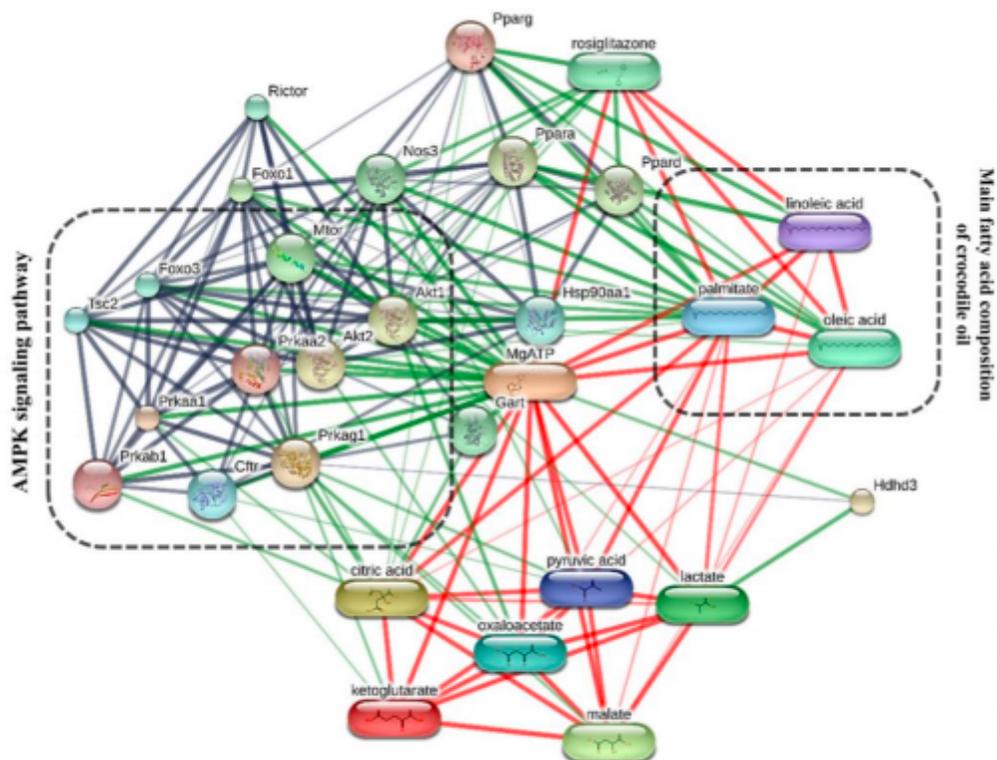
**Figure 4**

Comparison of electron micrograph of the hepatic mitochondria conformation among the RO (A), CO (B), and PO (C) groups. The bar graph indicates the percentage of Intact mitochondria (D), data is represented by mean  $\pm$  SD. Different letters indicate statistically significant differences between groups ( $P < 0.05$ ). Scale bars represent 2  $\mu\text{m}$ .



**Figure 5**

Comparison of the HDHD3 immunogold labelling at the intact stage of hepatic mitochondria among the RO (A), CO (B) and PO (C) groups. The bar graph indicates the level of gold particles in HDHD3 labelling (D); data is represented by mean $\pm$ SD. Different letters indicate statistically significant differences between groups ( $P < 0.05$ ). Scale bars represent 200 nm. Blue arrow indicates a number of HDHD3-labeled gold particle.



**Figure 6**

The chemical-protein and protein-protein interaction network of HDHD3 and three main fatty acids of CO on the energy metabolic pathway in the rat livers, analyzed by STITCH v 5.0.