Sains Malaysiana 52(6)(2023): 1821-1833
*http://doi.org/10.17576/jsm-2023-5206-17*

# Effect of Crocodile Oil (*Crocodylus siamensis*) on Brain Mitochondrial Protein Expression and Cognition in Male Rats

(Kesan Minyak Buaya (*Crocodylus siamensis*) terhadap Pengekspresan Protein Mitokondria Otak dan Kognisi pada Tikus Jantan)

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*Received: 7 September 2022/Accepted: 2 June 2023*

ABSTRACT

Crocodile oil (CO) is rich in polyunsaturated (PUFAs) fatty acids. Diets rich in PUFAs can maintain mitochondrial function, which is important in signal transduction and survival of neuronal cells. We investigated the effects of CO on brain mitochondrial protein expression and cognitive function in male rats. Twenty-one rats were randomly divided into three groups: (1) control, (2) treated with CO (3 mL/kg), and (3) treated with palm oil (PO; 3 mL/kg). Animals received oral gavage once-daily for seven weeks. The parameters that were measured were food intake, energy intake, body weight, serum lipid profiles, cognitive behavior, brain mitochondrial architecture, brain mitochondrial expression, and hippocampal structure. In CO and PO groups, food intake decreased significantly compared with that in the control group ($p<0.05$), but energy intake, body weight, and lipid profiles were not affected. Spatial learning in the PO group decreased significantly compared with that in control and CO groups ($p<0.05$). Crocodile oil significantly decreased the percentage of abnormal mitochondria ($p<0.05$) and the expression of apoptotic marker ($p<0.05$) compared with those in the PO treatment but also increased energy production marker ($p<0.05$) compared with those in the control and PO treatment. Moreover, percentage of intact hippocampal cells was not different between CO and control groups, but neuronal cells were lost in the PO group ($p<0.05$). This study suggest that CO could enhance the brain energy production and maintain cognitive function. CO can be an alternative dietary oil for treating brain energy disorder in the future.

Keywords: Cognition; crocodile oil; energy production; mitochondrial function; palm oil

ABSTRAK

Minyak buaya (CO) kaya dengan asid lemak tak tepu (PUFA). Diet yang kaya dengan PUFA boleh mengekalkan fungsi mitokondria, penting dalam transduksi isyarat dan kemandirian sel neuron. Kami mengkaji kesan CO pada pengekspresan protein mitokondria otak dan fungsi kognitif pada tikus jantan. Dua puluh satu tikus dibahagikan secara rawak kepada tiga kumpulan: (1) kawalan, (2) dirawat dengan CO (3 mL/kg) dan (3) dirawat dengan minyak sawit (PO; 3 mL/kg). Haiwan menerima gavage oral sekali sehari selama tujuh minggu. Parameter yang diukur ialah pengambilan makanan, pengambilan tenaga, berat badan, profil lipid serum, tingkah laku kognitif, seni bina mitokondria otak, pengekspresan mitokondria otak dan struktur hipokampus. Dalam kumpulan CO dan PO, pengambilan makanan menurun dengan ketara berbanding dengan kumpulan kawalan ($p<0.05$), tetapi pengambilan tenaga, berat badan dan profil lipid tidak terjejas. Pembelajaran ruang dalam kumpulan PO menurun dengan ketara berbanding

dengan kumpulan kawalan dan CO ($p<0.05$). Minyak buaya menurunkan peratusan mitokondria yang tidak normal ($p<0.05$) dan pengekspresan penanda apoptosis dengan ketara ($p<0.05$) berbanding dengan rawatan PO tetapi juga meningkatkan penanda pengeluaran tenaga ($p<0.05$) berbanding dalam kawalan dan rawatan PO. Selain itu, peratusan sel hipokampus utuh tidak berbeza antara CO dan kumpulan kawalan, tetapi sel neuron hilang dalam kumpulan PO ($p<0.05$). Kajian ini mencadangkan bahawa CO boleh meningkatkan pengeluaran tenaga otak dan mengekalkan fungsi kognitif. CO boleh menjadi minyak pemakanan alternatif untuk merawat gangguan tenaga otak pada masa hadapan.

Kata kunci: Fungsi mitokondria; kognisi; minyak buaya; minyak sawit; pengeluaran tenaga

## Introduction

Learning and memory are the most important processes in the brain because they allow organisms to use experience and adapt to new conditions (Reshetnikov et al. 2020). The brain is highly rich in lipids, which account for approximately 50% of its dry weight (Hamilton et al. 2007). Lipid and lipid intermediates are essential components that support structural, biochemical, and cell signaling functions of the brain (Bruce, Zsombok & Eckel 2017).

The brain has high energy requirements, and therefore, is dependent on energy production by mitochondria (Chapa-Dubocq, Makarov & Javadov 2018). Hundreds to thousands of mitochondria are contained in a single neuron (Rango & Bresolin 2018). Dietary fats and oils have a variety of functions, including providing energy and maintaining body temperature and levels of nutrients such as lipid-soluble vitamins (Ly et al. 2018). The brain is sensitive to changes in dietary fat intake, especially the hippocampus, which is involved in learning and memory formation (Horman et al. 2020). As a consequence, dietary fat intake affects mitochondrial proteins and brain functions, such as cognition, behavior, and synaptic plasticity (Chianese et al. 2018; Ortiz-Avila et al. 2015). Currently, palm oil (PO) extracted from fruit of palms (*Elaeis guineensis*) is one of the most highly produced and consumed edible oils worldwide (Edem, Eka & Umoh 2002). However, PO is composed of 50% palmitic acid (PA), 40% oleic acid (OA), and 10% linoleic acid (LA) (Lv et al. 2018). Palmitic acid is closely correlated with cognitive impairment in association with inflammation, abnormal redox activity, and especially mitochondrial dysfunction in both *in vitro* and *in vivo* studies (González-Giraldo et al. 2018; Hosseinzadeh, Moazedi & Chinipardez 2007; Melo et al. 2020). Metabolic and health effects strongly depend on the type of fat (Pellizzon et al. 2002). Therefore, the type of fatty acids in dietary fat is a key determinant in maintaining healthy brain function.

Crocodile oil (CO) has been used in traditional medicine for centuries and has proven very effective in treating ailments ranging from skin conditions to cancer (Makaba, Tingginehe & Ruru 2021). Crocodile oil has antimicrobial (inhibiting both Gram-positive and Gram-negative bacteria) and anti-inflammatory properties (Buthelezi et al. 2012). It can accelerate wound healing and promote skin regeneration via down regulation of the p38 mitogen-activated protein kinase (MAPK) signaling pathway (Li et al. 2012; Li et al. 2021). Li et al. (2012) found that CO from Siamese crocodiles (*Crocodylus siamensis*) is rich in monounsaturated (MUFAs) and polyunsaturated (PUFAs) fatty acids. Our previous study found OA (41.07%), LA (21.08%), and PA (19.92%) were highest content in CO (Srisuksai et al. 2023). Amounts of OA and LA in CO are 4 time higher than those in fish oil (Santativongchai et al. 2020). In addition, oral administration of high dose of CO (2,000 mg/kg body weight) not cause acute toxicity in rats (Praduptong et al. 2018). Intake of OA can protect against cognitive decline during aging and improve cognitive function, compared with controls (Sakurai et al. 2021). Linoleic acid is an essential precursor of arachidonic acid (AA), and when AA is administered, spatial cognition increases and memory performance improves (Okaichi et al. 2005). Moreover, LA intake is the main source of oxidized linoleic acid metabolites, which regulate neuronal morphology and hippocampal neurotransmission (Hennebelle et al. 2020). However, effects of CO on brain mitochondrial proteins expression and cognitive function in rats have not been investigated to date. Therefore, the objective of this study was to investigate the effects of CO on brain mitochondrial protein expression and cognitive function in male rats.

## Materials and Methods

### Crocodile oil preparation

Crocodile oil was extracted as described by Santatiwongchai et al. (2020). Abdominal fat was obtained as a by-product from slaughtered *C. siamensis* (3-5 years) collected from a crocodile farm in Nakorn Pathom Province, Thailand. The samples were pressed through two layers of filter cloth with distilled water at the proportion of 1:1 (w/v). The solution was left until the separation of the mixture was observed and the clear oil fraction was then collected, evaporated, and stored in a sealed container at room temperature.

### Animals

Twenty-one male Wistar rats (7 weeks old) were obtained from Nomura Siam International, Bangkok, Thailand. Animals were acclimated to the laboratory environment for 1 week before initiating the experiment. Animals were kept under controlled conditions with a 12 h:12 h light:dark light cycle at room temperature (25 ± 2 °C) and 60% to 70% relative humidity and had free access to water and rat chow. All procedures were approved by the animal use and care committee of Kasetsart University Research and Development Institute, Kasetsart University, Thailand (Approval no. ACKU61-VET-088).

### Experimental design

According to Naphatthalung et al. (2018), the animals were treated with CO or PO at dose 3 mL/kg bodyweight. Healthy rats were randomly divided into three treatment groups (n = 7 per group): (1) sterile water (control), (2) crocodile oil (CO), and (3) palm oil (PO). Sterile water, crocodile oil (3 mL/kg body weight), or palm oil (commercial grade, Samut Prakarn, Thailand, 3 mL/kg body weight) was administered via oral gavage daily for 7 weeks.

### Food intake, energy intake, and body weight

Daily food intake of each animal was measured by weighing the remaining chow, with food spillage accounted for in the intake measurement. Daily energy intake per animal was calculated as food intake × Et, where Et is total energy of the chow diet, which was 3,427.5 kcal/kg of diet. Energy content was approximately 12 kcal/1.5 mL of CO or PO. Body weights of all animals were monitored once a week during the experiment. Body mass index (BMI) was calculated as final body weight / (body length)$^2$.

### Morris water maze

After 7 weeks, the Morris water maze (MWM) was used to test spatial learning and memory. The method of Vorhees and Williams (2006) was used, with modifications. To perform the MWM test, a dark-blue 200-cm diameter pool was filled to a depth of 35 cm with clear water maintained at 26 ± 1 °C. The pool was in a large and quiet test room surrounded by several different cues that were visible from the pool and could be used by animals for spatial orientation. Cue positions remained unchanged throughout the study. Testing was conducted over 6 days, with the first day used to screen for locomotor activity. The test consisted of acquisition and probe phases. The acquisition phase was performed for training on 4 consecutive days with four trials per day. A clear platform (15-cm diameter) was submerged 2 cm beneath the water surface in a designated target quadrant. Animals had 120 s to locate the hidden platform. If an animal did not find the platform within 120 s, then it was guided to the platform. The time taken to reach the platform was recorded. The probe test was performed 24 h after the last trial of the acquisition phase. In the probe test, the platform was removed and each animal was allowed to swim for 60 s. The time spent in the target quadrant was recorded. The swimming activity of each animal was tracked via a video camera positioned directly above the center of the pool. Average swimming speed, latency time to find the platform, time spent in the target quadrant, and path length were analyzed using a computerized video tracking system (SMART program version 3.0.04, Panlab, Barcelona, Spain).

### Sample collection

All animals were euthanized with pentobarbital (60 mg/kg) after fasting for at least 6 h. Blood was collected from the left ventricle to measure serum lipid profiles. Whole blood was centrifuged at 2,000 × g for 10 min at 4 °C. Serum was stored at -20 °C. Serum lipid profiles including cholesterol, triglyceride, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) levels were determined using a Hitachi 7080 analyzer (Hitachi, Tokyo, Japan).

After collecting blood, animals were immediately decapitated, and whole brain samples were rinsed in

ice-cold saline (0.9% NaCl). Brain of the seven animals in each group were divided into two parts. One part was immediately placed in homogenate buffer (0.32 M sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA) and 10 mM Tris-HCl, pH 7.4) and used to extract mitochondria. The other part was fixed in 10% neutral buffer formalin for 48 h and used for histological study.

### MITOCHONDRIAL EXTRACTION

To examine mitochondrial architecture and functions, mitochondria were extracted as described by Ampawong, Isarangkul and Aramwit (2017). Brains from each group were pooled, weighed, chopped, and washed in homogenate buffer. Brains (1 g/4 mL of homogenate buffer) were homogenized in a glass Potter Elvehjem tissue grinder using a motor-driven Teflon pestle at 600 rpm in an ice-cooled condition. Homogenates were centrifuged at 1,000 × g at 4 °C for 5 min. Supernatants were collected and centrifuged at 15,000 × g at 4 °C for 2 min. Mitochondrial pellets were resuspended in ice-cold, final equilibrated buffer (250 mM sucrose, 5 mM $KH_2PO_4$, 10 mM Tris-HCI, 2 mg/mL bovine serum albumin (BSA), pH 7.2), and 200 µL of resuspended pellet was fixed in 2.5% glutaraldehyde in 0.1 M sucrose phosphate buffer. Fixed specimens were secondarily fixed in 1% osmium tetroxide, dehydrated in graded ethanol, infiltrated in a series of LR white resin (EMS, Houston, TX, USA), embedded in pure LR white (EMS), polymerized at 60 °C for 48 h, and cut into 100-nm-thick sections.

### IMMUNOGOLD LABELING TECHNIQUE

Immunoelectron microscopic analysis was performed to confirm the function of mitochondria on energy production, apoptosis, and antioxidative environment as characterized by expression of extracellular signal-regulated kinase mitogen-activated protein kinase (ERK/MAPK), haloacid dehalogenase-like hydrolase domain containing protein 3 (HDHD3), caspase-9, Sirtuin 1 (SIRT1), and nuclear factor erythroid 2-related factor 2 (NRF2).

Polyclonal rabbit antibodies against ERK/MAPK (MyBioSource, San Diego, CA, USA, MBS9600093), HDHD3 (MyBioSource, MBS7002116), caspase-9 (MyBioSource, MBS841177), SIRT1 (MyBioSource, MBS3200904), and NRF2 (MyBioSource, MBS9610368) were used as primary antibodies. Sections were blocked using 50 mM glycine in phosphate-buffer saline (PBS), followed by 5% BSA in PBS for 30 min. Sections were incubated with 1:50 dilution of primary antibody for 1 h before applying goat anti-rabbit IgG conjugated with 10-nm gold particles (EMS). Between each step, sections were washed several times using 0.1% BSA in PBS. To improve the contrast of gold particle labeling, a silver enhancement kit (Aurion R-Gent SE-EM kit, EMS) was use after rigorously washing the sections with distilled water. Last, sections were stained with lead citrate and uranyl acetate in preparation for transmission electron microscopy (HT7700, Hitachi, Tokyo, Japan). Percent of abnormal mitochondria was determined, and labeled gold particles in mitochondria were counted. At least 50 mitochondria were evaluated per group.

### HISTOLOGICAL STUDY

After fixation, the brain was dehydrated, infiltrated, embedded, sectioned, and stained with hematoxylin and eosin (H&E). Histological change was evaluated by counting the number of intact cells per 1 mm in CA1, CA3, and DG regions in the hippocampus.

### STATISTICAL ANALYSES

Statistical analyses were performed using GraphPad Prism version 9.4.1 (San Dieago, CA, USA). Data are expressed as the mean ± standard error of the mean (SEM). Data from the MWM test were analyzed using one-way analysis of variance (ANOVA) and repeated measures of ANOVA followed by Turkey's post hoc test. Other data were analyzed by one-way ANOVA followed by Turkey's post hoc test. The association between two parameters was analyzed via simple linear regression. Statistical significance was accepted at $P < 0.05$.

## RESULTS

### EFFECTS OF CROCODILE OIL ON FOOD AND ENERGY INTAKE, BODY WEIGHT, AND BODY MASS INDEX

Food intake, energy intake, and BMI of rats after 7 weeks of treatment are shown in Table 1. Food intake decreased significantly in both CO and PO groups compared with that in the control group. However, there were no significant differences in energy intake, or BMI among the groups. In addition, Body weight of rat was not significantly different among the groups throughout the study (Figure 1).

### EFFECTS OF CROCODILE OIL ON SERUM LIPID PROFILES

After 7 weeks of treatment, levels of cholesterol, triglycerides, HDL-C, and LDL-C were not significantly different among the groups (Table 2). Nevertheless, triglyceride levels tended to decrease in CO and PO groups compared with that in the control group.

### EFFECTS OF CROCODILE OIL ON SPATIAL LEARNING AND MEMORY

Spatial learning and memory were measured using the MWM test. Latency time to the platform in the CO group was not significantly different compared with that in the control group, but in the PO group, it was significantly longer on day 4 of the acquisition phase (Figure 2(A) and 2(D)). However, time spent in the target quadrant and average swimming speed were not significantly different among the groups (Figure 2(B) and 2(C)). These results strongly suggested that PO reduced the spatial learning of rats but not their locomotor activity.

### EFFECTS OF CROCODILE OIL ON MITOCHONDRIAL ARCHITECTURE AND EXPRESSION OF MITOCHONDRIAL PROTEIN MARKERS ERK/MAPK, HDHD3, CASPASE-9, SIRT1, AND NRF2

In this study, the percentage of abnormal mitochondria was not different between control and CO groups but increased significantly in the PO group (Figure 3(A)). Quantitative gold labeling indicated that in the PO group, expression of HDHD3 decreased and that of caspase-9 increased significantly compared with expression in the control group (Figure 3(C) and 3(D)). In addition, mitochondrial expression of ERK/MAPK decreased significantly in the PO group compared with that in CO group (Figure 3(B)). However, expression of HDHD3 increased significantly in the CO group compared with that in the control group (Figure 3(C)). Expression of SIRT1 was not significantly different between groups (Figure 3(E)), whereas expression of NRF2 increased significantly in both CO and PO groups compared with that in the control group (Figure 3(F)).

### EFFECTS OF CROCODILE OIL ON MORPHOLOGY AND QUANTITY OF HIPPOCAMPAL CELLS

As shown by H&E staining, hippocampal CA1, CA3, and DG neurons in control and CO groups were large and neatly arranged with distinct nuclear membrane and nuclei. In addition, neuronal cytoplasm was lightly stained. Therefore, the neurons in this group were considered to represent intact neurons. In the PO group, some neuron lost normal morphology and showed disordered arrangement, darkly stained cytoplasm, and smaller cell bodies (Figure 4(A). As shown in Figure 4(B), intact cell on hippocampal CA1 and DG regions in control and CO groups was significantly higher than the PO group.

TABLE 1. Effects of crocodile oil on food and energy intake, and body mass index

| Parameter | Group | | |
|---|---|---|---|
| | Control | Crocodile oil | Palm oil |
| Food intake (g/rat/day) | 18.59 ± 0.49 | 15.84 ± 0.18* | 15.10 ± 0.53* |
| Energy intake (kcal/rat/day) | 63.72 ± 1.67 | 66.30 ± 0.30 | 64.76 ± 1.80 |
| Body mass index (g/cm$^2$) | 0.73 ± 0.03 | 0.71 ± 0.01 | 0.69 ± 0.01 |

Values are the mean ± SEM. *$P < 0.05$, vs the control group

TABLE 2. Effects of crocodile oil on serum lipid profiles

| Serum lipid profile | Group | | |
|---|---|---|---|
| | Control | Crocodile oil | Palm oil |
| Cholesterol (mg/dL) | 73.13 ± 9.51 | 63.82 ± 4.02 | 66.10 ± 8.23 |
| Triglycerides (mg/dL) | 138.25 ± 30.14 | 90.40 ± 15.14 | 83.68 ± 6.44 |
| HDL-C (mg/dL) | 53.58 ± 9.28 | 46.70 ± 3.42 | 46.40 ± 7.16 |
| LDL-C (md/dL) | 11.68 ± 1.10 | 8.70 ± 1.17 | 9.18 ± 1.64 |

Values are the mean ± SEM. HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol

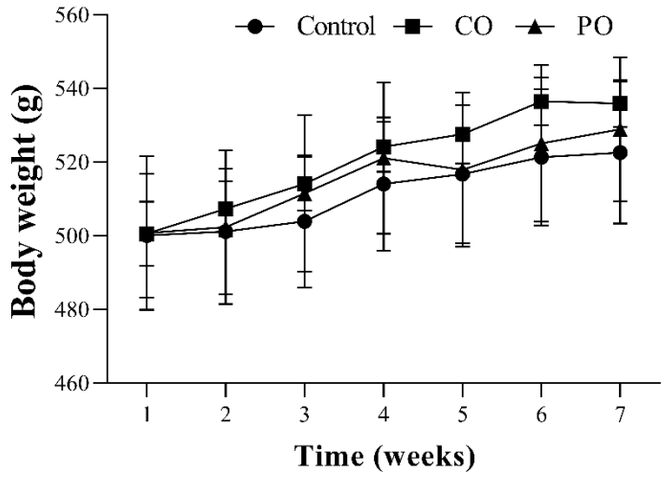


FIGURE 1. Bodyweight in rats on the 7-weeks treatment. Values are the mean ± SEM

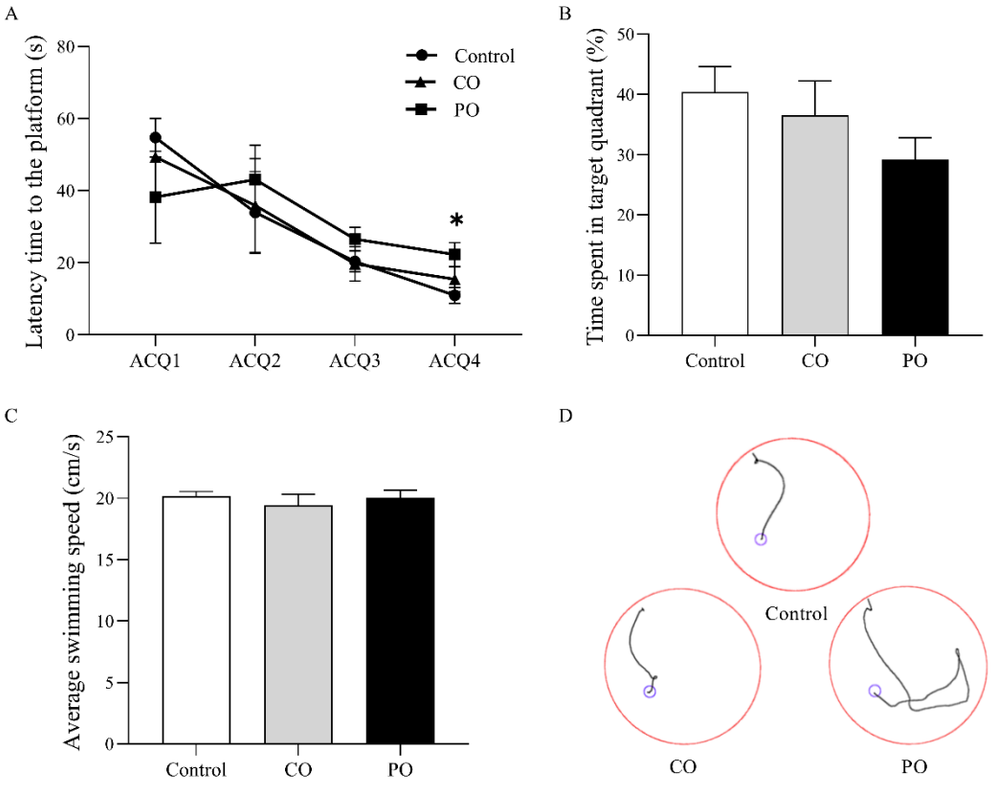


FIGURE 2. Spatial learning and memory assessed by the Morris water maze test. (A) Latency time to the platform. (B) Percentage of time spent in target quadrant. (C) Average swimming speed. (D) Path length in the last acquisition phase. Values are the mean ± SEM. *$P < 0.05$, between groups

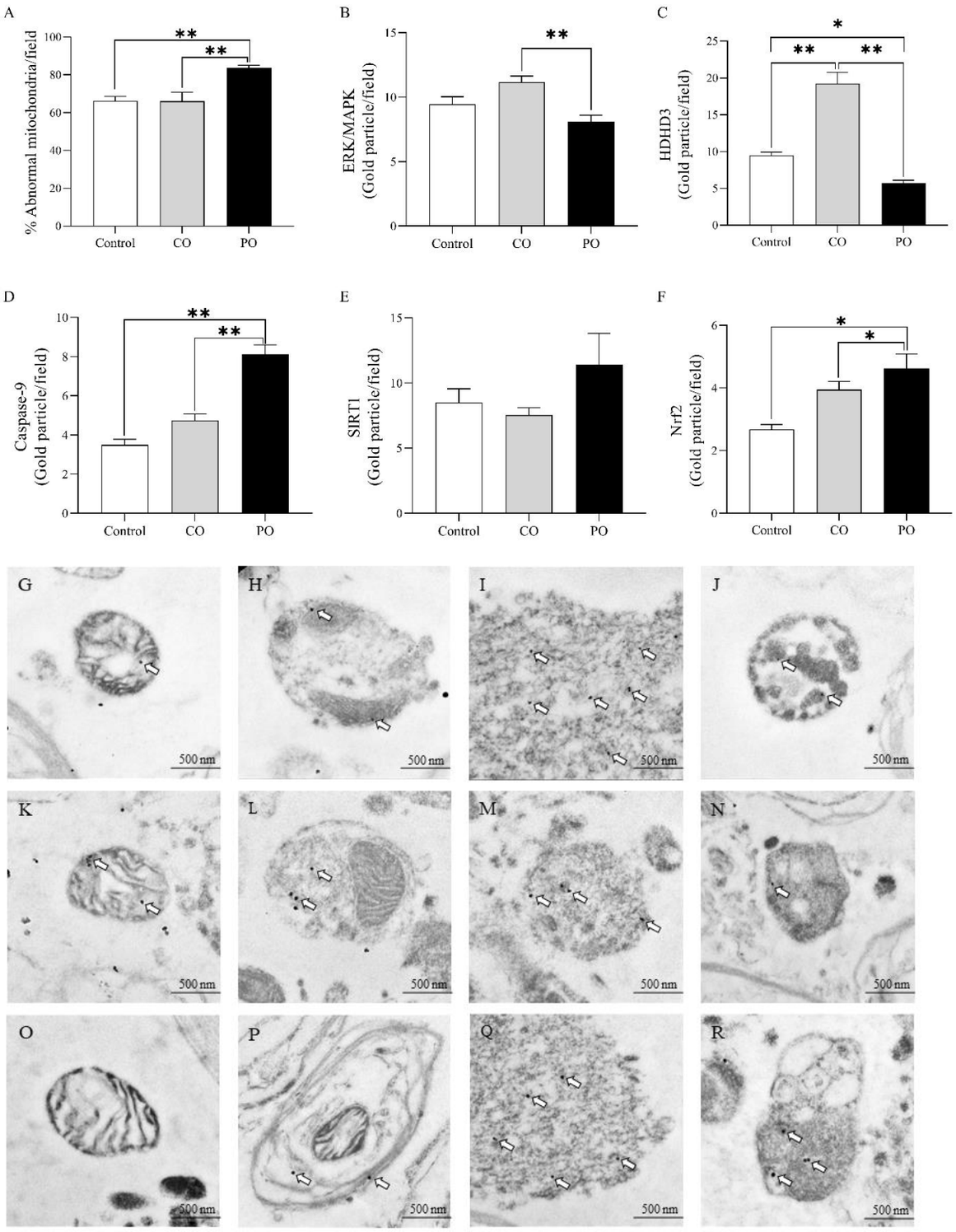


FIGURE 3. Mitochondria architecture and immunogold labeling of ERK/MAPK, HDHD3, caspase-9, SIRT1, and NRF2 in rats. (A) Percentage of abnormal mitochondria in control rats and those treated with crocodile oil (CO) or palm oil (PO). Expression of (B) ERK/MAPK, (C) HDHD3, (D) caspase-9, (E) SIRT1, and (F) NRF2 in the different groups. Electron micrographs of mitochondria labeled with (G–J) ERK/MAPK, (K–N) HDHD3, and (O–R) caspase-9 in (G, K, and O) intact; (H, L, and P) autophagy; (I, M, and Q) ghost; and (J, N, and R) clumping mitochondria. Arrows indicate gold particles. Values are the mean ± SEM. *$P$ < 0.05 and **$P$ < 0.01, between groups

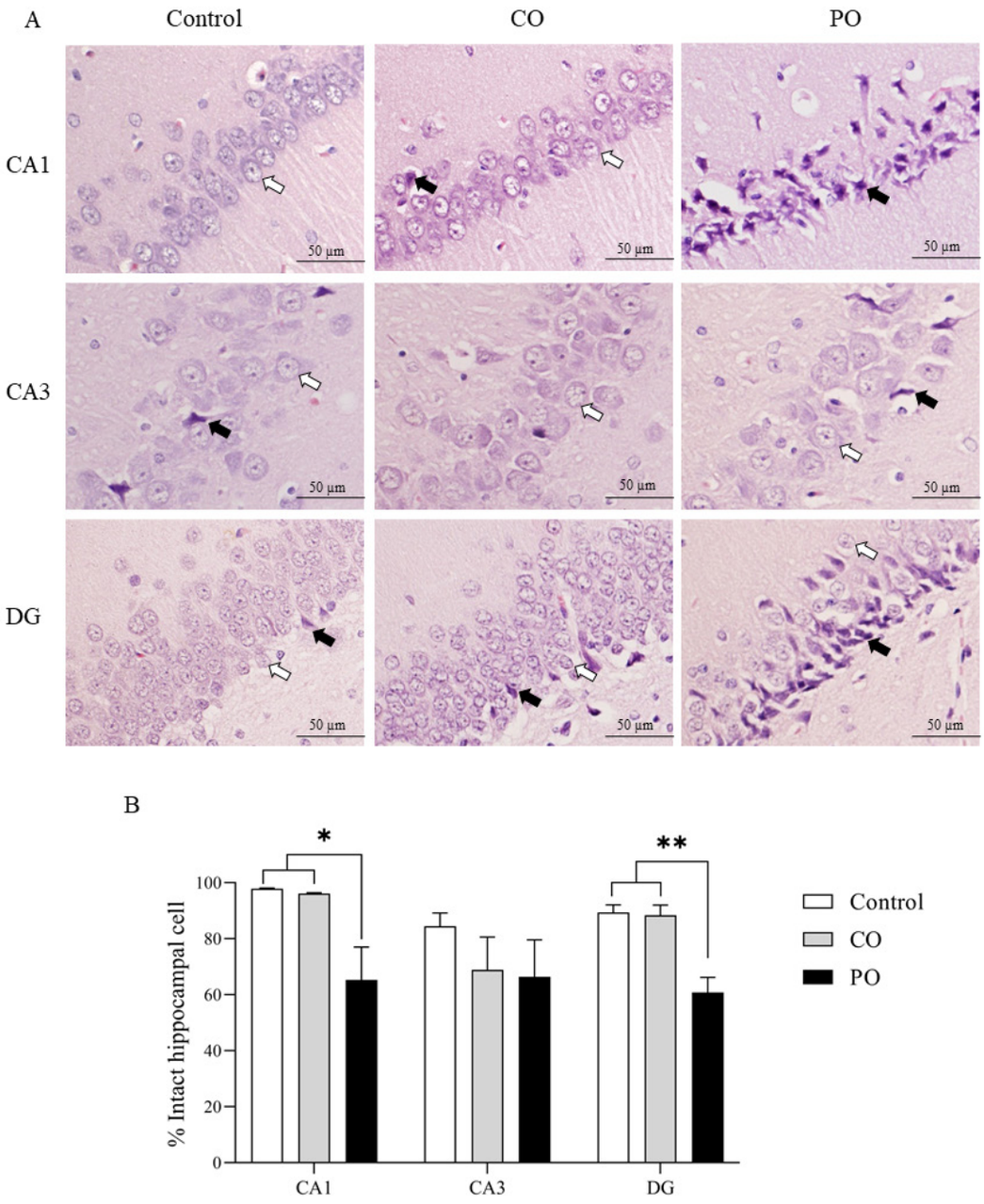


FIGURE 4. Hematoxylin and eosin (H&E) staining in the hippocampus of rats. (A) Micrograph of neuronal cells in control, CO, and PO-treated rats in CA1, CA3, and DG regions. Black arrows indicate abnormal neurons, and white arrows indicate intact neurons. (B) Percentage of intact neuronal cells in three regions of the hippocampus in the different groups. Values are the mean ± SEM. *$P < 0.05$ and **$P < 0.01$, between groups

## DISCUSSION

In recent decades, many studies have focused on potential unhealthy effects of PO in diets because of its high PA (saturated fatty acids (SFAs)) content (Mancini et al. 2015). Evidence is increasing that rates of cognitive declines may increases with relatively high intake of SFAs. By contrast, relatively high intake of either MUFAs or PUFAs leads to decreases in rates of cognitive decline (Morris & Tangney 2014). CO is rich in MUFAs and PUFAs and has PA content two times lower and LA content two times higher than those in PO (Edem, Eka & Umoh 2002; Santativongchai et al. 2020). Therefore, this study investigated the effects of CO on mitochondrial protein expression and cognitive function in relation to hippocampal cell architecture in male rats.

In both CO and PO groups, food intake decreased significantly, but energy intake was not affected. Diet rich in SFAs and PUFAs could promote satiety which will help the body to establish energy balance (Kozimor, Chang & Cooper 2013). The absence of effects of dietary fatty acids on caloric intake in all groups is consistent with previous findings (Boon et al. 2013; Pellizzon et al. 2002). Effects of dietary fat on body weight depend on fatty acid composition (Rolland et al. 2002). Although a diet high in SFAs can increase body weight, 10% and 15% PO-supplemented diets result in normal growth rates in rats (Boon et al. 2013; Edem, Eka & Umoh 2002). In addition, PUFAs do not induce weight gain in rats (Salama, Amin & Hassan 2023). Consistent with those previous results, growth rates were normal in both CO and PO groups, with body weights and BMIs comparable with those in the control group.

Epidemiological studies indicate that the composition of dietary oils may affect serum lipid profiles (Schwingshackl et al. 2018). In this study, no significant differences were observed in serum lipid profiles after CO and PO treatment. Similar to this study, a previous study found a normal range of cholesterol levels with 8 weeks of dietary PO (Ly et al. 2018). In addition, these findings are consistent with those of Boon et al. (2013) who compared control and 15% PO-supplemented diets and found no significant differences in cholesterol, triglycerides, HDL-C, and LDL-C.

The MWM is a well-established test of spatial learning and long-term memory (Reshetnikov et al. 2020). The acquisition phase is used to test spatial learning and short-term memory abilities, whereas the probe phase is used to test long-term spatial memory (Wichai et al. 2019). In this study, latency time to find the platform on day 4 in the PO group was significantly longer than control and CO groups (Figure 2(A) and 2(D)). These results indicated that intake of CO maintained spatial learning and memory; whereas intake of PO reduced spatial learning. Several studies in human and animal models link SFAs, especially PA, to impaired learning and memory (Hosseinzadeh, Moazedi & Chinipardez 2007; Melo et al. 2020).

The ultrastructure of mitochondria is different depending on physiological and pathological conditions. Normal mitochondria contain normal cristae and a complete membrane boundary, whereas in abnormal mitochondria, there is loss of cristae and an incomplete membrane boundary or there is fine granular material (Ampawong, Isarangkul & Aramwit 2017). In this study, mitochondrial expression of HDHD3 decreased significantly, but mitochondrial expression of caspase-9 and percentage of abnormal mitochondria increased significantly in the PO group compared with that in control group (Figure 3(A), 3(C), and 3(D)). In addition, mitochondrial expression of ERK/MAPK decreased significantly in the PO group compared with that in CO group (Figure 3(B)). ERK is a MAPK member that contributes to cell survival (Monick et al. 2008), and HDHD3 is a protein marker indicating mitochondrial energy production (Rujimongkon et al. 2022). In this study, correlation analysis showed a significant positive correlation between ERK/MAPK and HDHD3 expression ($r = 0.223$; $P < 0.01$). Inhibition of ERK activity leads to rapid loss of mitochondrial membrane potential (MMP) and decreases in ATP levels (Monick et al. 2008). Consistent with results of this study, a previous study reports that PA (main fatty acid in PO) reduces MMP in astrocytic cells (González-Giraldo et al. 2018), and reduction in MMP results in ATP depletion (Zorova et al. 2018). The permeability transition pore can open when ATP is depleted, leading to water influx and ultimately swelling of mitochondria, which is consistent with observation in this study (Chapa-Dubocq, Makarov & Javadov 2018). Mitochondrial swelling is the major factor leading to mitochondria-mediated cell death through apoptosis and necrosis pathways (Chapa-Dubocq, Makarov & Javadov 2018). Correlation analysis in this study showed a significant negative correlation between HDHD3 and caspase-9 expression ($r = -0.272$; $P < 0.01$). Caspase-9 is the initiator caspase associated with the intrinsic or mitochondrial pathway of apoptosis (Allan & Clarke 2009). In several studies, PA induces neuronal cell death via caspase activation (Ulloth, Casiano & De Leon 2003; Yuan et al. 2013). Consistent with previous reports, percentages of intact hippocampal

cells in the PO group were significantly lower than control and CO groups (Figure 4).

Notably, HDHD3 expression increased significantly in the CO group compared with that in control and PO groups (Figure 3(C)). Linoleic acid, the PUFA with the highest content in CO, is a major component of cardiolipin (Chicco & Sparagna 2007; Snoke et al. 2023). Cardiolipin is an important phospholipid in mitochondrial oxidative phosphorylation and is required for maximal cytochrome c oxidase (COX) activity (Fajardo et al. 2015). COX is a proton-pumping respiratory complex that maintains MMP and therefore ATP production (Li et al. 2006). In several studies, dietary oils deficient in LA led to reductions in COX activity and MMP and impaired ATP production (Fajardo et al. 2015; Yamaoka, Urade & Kito 1990). Therefore, increased consumption of LA can increase ATP production via COX activation (Mulligan et al. 2012). Cardiolipin also anchors cytochrome c to the inner mitochondrial membrane (Chicco & Sparagna 2007). Thus, loss of cardiolipin or reduction in LA content initiates release of cytochrome c, which can trigger activation of caspase-9 and initiation of apoptosis (Fiorucci et al. 2022). Moreover, LA prevents cell death and maintains mitochondrial structure and increases mitochondrial biogenesis in streptozotocin-treated cells (Jeng et al. 2009). The results in this study are showed percentages of intact hippocampal cells in a CO group were not significantly different compared with those in the control group (Figure 4).

Although mitochondrial SIRT1 expression was not significantly different among groups, SIRT1 expression tended to increase in the PO group compared with that in control and CO groups (Figure 3(E)). SIRT1 is an NAD-dependent histone deacetylase that has crucial roles in multiple biological processes, including aging, stress response, apoptosis, and the cell cycle, among others (Chou et al. 2013). SIRT1 deacetylates MAPK phosphatase 1 to increase direct dephosphorylation to ERK, which results in the suppression of cellular survival (Kwon et al. 2020). Inhibition of ERK induced cell death via activation of caspase activity (Monick et al. 2008), which is consistent with results in this study. In this study, mitochondrial expression of NRF2 increased significantly in both CO and PO groups compared with that in the control group (Figure 3(F)). NRF2 apparently regulates cellular resistance to oxidative stress (Ma 2013). Oxidative stress occurs when production of free radicals exceeds ability of cells to defend against them, and free radicals have key roles in the loss of neurons and the development of degenerative diseases (Poon et al. 2004). Despite the loss of carotenoids, flavonoids, and phenolic acids during refining, PO or palm olein retains high concentrations of vitamin E (a tocotrienol), which is a powerful scavenger of free radical (Ismail et al. 2020). Studies show that PO or the PO tocotrienol-rich fraction can improve antioxidant properties in animals (Djohan et al. 2021; Taib et al. 2015). Previous studies found that CO contains vitamin E and has antioxidant properties (Makaba, Tingginehe & Ruru 2021; Srisuksai et al. 2023). Therefore, this study provides the first evidence of CO effects on mitochondrial antioxidant markers. The mitochondrial antioxidant marker increased in both CO and PO groups, but hippocampal cell loss and cognitive impairment was only observed in the PO group. Therefore, antioxidant properties may not the main factor in maintaining cognitive function.

## Conclusion

This study suggest that CO could enhance the brain energy production and maintain cognitive function. CO can be an alternative dietary oil for treating brain energy disorder e.g., depression, bipolar disorder, schizophrenia, autism, and Alzheimer's disease in the future.

## ACKNOWLEDGEMENTS

The authors thank Miss Thanaporn Thanikakornkul and Mr. Rawirot Luansi for their care of animals. The authors also thank Miss Tapanee Kanjanapruthipong for her advice regarding immunolabelling technique.

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