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Inhibition of Mitochondrial Functions by Margosa Oil: Possible Implications in the Pathogenesis of Reye's Syndrome[1]

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ABSTRACT. Margosa oil (MO), a fatty acid-rich extract of the seeds of the neem tree and a reported cause of Reye's syndrome, has been used in the induction of an experimental model of Reye's syndrome in rats. It has been reported that MO causes a decrease in *in vivo* mitochondrial enzyme activity similar to that seen in Reye's syndrome. We have attempted to uncover some of the biochemical mechanisms of MO's toxicity by examining its effect *in vitro* on isolated rat liver mitochondria. Male rat liver mitochondria were isolated by centrifugation; oxygen uptake, reduced forms of cytochrome b, c + c_1, a + a_3, and flavoprotein, intramitochondrial concentrations of acetyl coA, acid-soluble coA, acid-insoluble coA, and ATP content were measured after incubation with and without MO. Our results reveal that MO is a mitochondrial uncoupler. State 4 respiration was increased while the respiratory control ratio was decreased. The intramitochondrial content of ATP was also decreased. There were substantial changes in the reduction of the respiratory chain components after incubation of mitochondria with MO. This decelerative effect on mitochondrial electron transport was alleviated by the addition of coenzyme Q and/or carnitine. These effects of MO on mitochondrial respiration may be due to changes in fatty acid metabolism caused by MO as MO caused a shift in the proportion of acid-soluble or acid-insoluble coA esters. Supplementary therapy with L-carnitine and coenzyme Q may be useful in the management of MO-induced Reye's syndrome. (*Pediatr Res* 22: 184–187, 1987)

Abbreviations

MO, margosa oil
RS, Reye's syndrome
CoQ, coenzyme Q
RCR, respiratory control ratio

MO, an extract of the seeds of the neem tree (Azadirachta indica A. Juss) distributed throughout the Indo-Malayan region, is used as a traditional remedy by the Hindu. This oil is known to cause RS in infants (1) and can induce symptoms similar to those of RS in rats (2). Several lines of morphological and biochemical evidence imply that in RS the mitochondrion is the major site of insult (3). Plasma from RS patients induce respiratory inhibition of isolated rat liver mitochondria (4). Similar effects have been reported with salicylates and salicyl compounds which are known to be associated with RS (5). Although there are several reports relating to the production of Reye-like hepatic mitochondrial enzyme deficits in the rat by MO there is no report on the *in vitro* effects of MO on the functions of isolated liver mitochondria (6, 7). Therefore, we designed this study to evaluate the effects of MO on isolated rat liver mitochondrial functions, and to determine if the effects of MO could be reversed by D,L-carnitine or coenzyme Q.

In view of the fact that major constituents of MO are long- and medium-chain fatty acids (8), it appears likely that these fatty acids may be involved in the mechanism of the toxicity of MO. Recently, secondary carnitine deficiency has been reported in several inherited metabolic diseases (9) and RS (10). Since L-carnitine serves not only as a vehicle of fatty acid transport into mitochondria but enhances regeneration of free CoA-SH in mitochondria (11), it may relieve the toxicity of MO on mitochondria if MO disrupts mitochondrial functions in a manner similar to that of fatty acids. On the other hand, MO also contains other mitochondrial toxins such as terpenoids (nimbin, nimbiol), which have a similar structure to CoQ, and have been used as insecticides. These compounds may act as electron acceptors which are competitive to CoQ. CoQ not only serves as a component of the mitochondrial respiratory chain, but it protects mitochondrial membranes against the injury induced by accumulated long-chain acyl CoA and long-chain acyl carnitine and lowers the production of intramitochondrial superoxide (12). Therefore, we tested whether or not the supplementation with coenzyme Q is effective in preventing the toxicity of MO on mitochondria *in vitro*.

MATERIALS AND METHODS

Mitochondria were isolated from the liver of rats using the method of Hogeboom *et al.* (13) with sight modification (13). Male Wistar rats (180–270 g) starved for 24 h were anesthetized with diethylether and killed by decapitation.

The livers were removed, weighed, and rapidly homogenized in 10 volumes of ice-cold medium containing 70 mM sucrose, 210 mM mannitol, 0.1 mM EDTA, 10 mM Tris-HCl, pH 7.4, in Potter-Elvehjem type glass-Teflon homogenizer (Pyrex Iwaki Glass Co., Tokyo, Japan). The homogenate was centrifuged for 10 min at 700 × *g*, and the supernatant was centrifuged for 10 min at 8000 × *g*. The sediment was suspended in the same medium and centrifuged for 5 min at 700 × *g* to remove remaining cell debris and the supernatant was recentrifuged for 10 min at 9000 × *g*. The mitochondrial sediment was resus-

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pended in the incubation medium containing 250 mM mannitol, 10 mM potassium phosphate, 2 mM $MgCl_2$, 0.2 mM EDTA, 10 mM KCl, 10 mM Tris-HCl, pH 7.4, in a volume numerically equal (in ml) to the original weight (in g) of the liver. Thus 1.0 ml of this suspension was derived from 1.0 g of liver.

The protein contents of the primary mitochondrial suspensions varied from 7.2 to 11.8 mg/ml. The morphological purity of the preparations was checked by electron microscopy.

Oxygen uptake by the mitochondria was measured polarographically with a Clark-type oxygen electrode (Yanaco model PO 100-A, Yanagimoto Co., Tokyo, Japan), according to the method of Chance and Williams (14). Incubation was carried out at 30° C in the incubation medium with a pH of 7.4, containing 250 mM mannitol, 10 mM potassium phosphate, 2 mM $MgCl_2$, 0.2 mM EDTA, 10 mM KCl, 10 mM Tris-HCl, and 19.8 μg rotenone, with and without additions of MO at concentrations of 2.5 or 25 μl/ml of the incubation medium, respectively. Then 3.3 μmol succinate was added, followed by 1.98 μmol ADP in a total volume of 3.3 ml. ATP content in the incubation medium was measured enzymatically according to the method of Bücher (15), using an ATP-kit (Boehringer-Mannheim Co., Mannheim, West Germany). Protein content was measured by the method of Lowry *et al.* (16). Quantitative analysis of cytochromes b, c + c_1, and a + a_3, and flavoprotein was carried out according to the method of Chance and Hagihara (17) by measuring differences in spectra between the anaerobic and aerobic states at room temperature using a Shimadzu UV-3000 split-beam spectrophotometer (Shimadzu, Kyoto, Japan).

The incubation conditions were similar to that used for the study of mitochondrial respiration, except that the final incubation volume was 1.5 ml, rotenone was not added, and the final protein concentration was 2.9 mg per incubation. The mitochondrial suspensions in both sample and reference cuvettes were first made aerobic by shaking with air and then, one of the following substrates was added to the sample cuvette: 1.5 μmol succinate, 1.5 μmol pyruvate, or 3.0 μmol D,L-isocitrate, to give final concentrations of 1.0, 1.0, and 2.0 mM, respectively. The reference cuvette receives the same volume of the incubation medium. The mitochondria were allowed to become anaerobic by the endogenous respiration after the addition of substrate. In some experiments, D,L-carnitine and/or coenzyme Q were added at final concentrations of 50 and 0.1 mM, respectively. The measurements of ATP concentration were carried out using the same aliquots which were incubated for 30 min at 30° C. Concentration of acetyl-CoA, acid-soluble CoA, and acid-insoluble CoA were measured by the enzymatic method (18) with the other aliquots after they were fractionated according to the procedure of Ingebresten *et al.* (19).

RESULTS

When succinate was used as a substrate for mitochondrial respiration, mean values of RCR ADP/O ratio, the ATP content in control incubations were 5.05 ± 0.22, 1.90 ± 0.05, and 1.31 ± 0.21 μmol/mg of mitochondrial protein, respectively (Table 1). MO at a concentration of 2.5 μl/ml stimulated state 4 respiration, inhibited state 3 respiration, and caused a decrease in RCR to 34.9% of the control values. At a concentration of 25 μl/ml MO caused a profound uncoupling of oxidative phosphorylation and decreased the ATP content to 45.0% of the control mean value.

The concentrations of reduced forms of flavoprotein and cytochromes in control incubations are presented in Table 2. By 5 min after the addition of succinate, all reduced forms of the cytochromes were recognizable. The concentrations of all the reduced components were calculated at 5 and 10 min after the addition of succinate to evaluate the effects of MO. The levels of the reduced cytochromes were significantly decreased 5 and 10 min after the addition of MO at the concentration of 2.5 μl/ml. The decreases in the levels of the reduced cytochromes which were caused by MO were partially alleviated by the additions of D,L-carnitine, and they recovered to the levels similar to those of control values by the additions of CoQ or both. The content of ATP in the incubation solutions was significantly decreases by the addition of MO at 2.5 μl/ml per incubation (Table 2). The ATP levels recovered to 82.1% of control values following the addition of CoQ.

Incubation of isolated rat liver mitochondria with 2.5 μl/ml MO resulted in decreased intramitochondrial levels of acetyl CoA and acid-soluble CoA, and increased levels of acid-insoluble CoA (Table 3). These effects of MO seemed to be dose dependent.

DISCUSSION

The clinicopathological findings in RS implicate the mitochondrion as the primary site of involvement (3, 20). It is, therefore, possible for mitochondrial toxins to induce RS. It has recently been reported that MO can trigger RS in infants (1) and that it can mimic symptoms similar to those in RS in animal model *in vivo* (2). Therefore, we designed this study to investigate the effects of MO on some mitochondrial functions. The major components of MO are long-chain, saturated and unsaturated fatty acids (mainly stearic, oleic, palmitic, linoleic, and smaller amounts of myristic, arachidic, and behenic) (8), and medium-chain fatty acids in smaller quantities. It also contains small amounts of nimbin and nimbiol. These compounds have a terpenoid skeleton which is shared by CoQ. They have been used as insecticides such as rotenone. These compounds act as electron acceptors which are competitive to CoQ. Since long- and medium-chain fatty acids have detergent effect, it is possible that MO exerts a destructive effect on mitochondrial membranes. Moreover, the depletion of the intramitochondrial levels of acetyl CoA and acid-soluble CoA may inhibit several mitochondrial enzyme activities (3). Trauner (21) reported that a continuous infusion of octanoate can induce pathologic abnormalities in the rabbit similar to those observed in patients with RS. Octanoate is a potent uncoupler of oxidative phosphorylation (22, 23). Ogburn *et al.* (24) found increases in serum levels of long-chain

Table 1. *Effects of MO on state 3 and state 4 rates of O_2 consumption, RCR, ADP/O ratio, and intramitochondrial ATP content**

Incubations	State 3	State 4	RCR	ADP/O	ATP
Control (10)	188.4 ± 36.5	37.3 ± 7.15	5.05 ± 0.22	1.90 ± 0.05	1.31 ± 0.21
Margosa + 2.5 μl/ml (7)	138.1 ± 12.0†	77.5 ± 6.71†	1.76 ± 0.12†	1.81 ± 0.04†	1.15 ± 0.22
+25 μl/ml (7)	‡	‡	‡	‡	0.59 ± 0.15†

* The values of oxygen consumption represent mean ± SD natom oxygen per min per mg protein. The values of ATP concentration represent mean ± SD μmol per 10 min per mg protein. The numbers in parentheses represent numbers of experiments.

† Values were significantly less ($p < 0.01$) with a one-tailed t tested for independent mean probability.

‡ MO at a concentration of 25 μl/ml showed linear line from the point of state 2 in oxogroph. We cannot recognize the distinction between state 4 and state 3 oxygen consumption. The state 4 oxygen consumption rate was closer to the state 3 rate. This indicates as a profound uncoupling effect. When substrate is present, ADP induces an increased rate of oxygen consumption (state 3). The slower state 4 rate is resumed when all the ADP is phosphorylated. ADP/O ratio are calculated by dividing the known amount of added ADP by the measured oxygen consumption during state 3.

Table 2. *Effects of MO on mitochondrial respiratory chain components and intramitochondrial ATP content**

Additions	5 min				10 min				30 min
	Fp	Cyt b	Cyt c+ c_1	Cyt a + a_3	Fp	Cyt b	Cyt c + c_1	Cyt a + a_3	ATP
Control (10)	0.49 ±0.05	0.12 ±0.02	0.17 ±0.03	0.12 ±0.02	0.61 ±0.07	0.11 ±0.02	0.19 ±0.03	0.11 ±0.03	1.06 ±0.12
+D,L-carnitine 50 μM (7)	0.60 ±0.21†	0.10 ±0.02	0.18 ±0.03	0.11 ±0.01	0.70 ±0.23	0.11 ±0.02	0.17 ±0.03	0.09 ±0.02	0.98 ±0.08
+Coenzyme Q 0.1 mM (7)	0.59 ±0.10	0.10 ±0.02	0.16 ±0.02	0.12 ±0.03	0.69 ±0.10	0.10 ±0.01	0.18 ±0.03	0.10 ±0.02	0.90 ±0.21
+D,L-carnitine 50 μM Coenzyme Q 0.1 mM (7)	0.53 ±0.16	0.10 ±0.02	0.18 ±0.03	0.13 ±0.04	0.62 ±0.20	0.10 ±0.02	0.17 ±0.03	0.11 ±0.02	0.95 ±0.17
MO 2.5 μl/ml	0.48 ±0.31	0.04 ±0.03‡	0.08 ±0.04‡	0.03 ±0.03‡	0.61 ±0.20	0.09 ±0.03	0.13 ±0.02‡	0.07 ±0.01‡	0.27 ±0.05‡
+D,L-carnitine 50 μM (7)	0.51 ±0.18	0.09 ±0.02†	0.13 ±0.05	0.09 ±0.04†	0.58 ±0.15	0.10 ±0.01	0.17 ±0.03	0.09 ±0.02	0.35 ±0.06‡
+Coenzyme Q 0.1 mM (7)	0.53 ±0.16	0.10 ±0.02	0.16 ±0.03	0.10 ±0.03	0.61 ±0.17	0.10 ±0.02	0.18 ±0.03	0.08 ±0.03	0.87 ±0.23†
+D,L-carnitine 50 μM Coenzyme Q 0.1 mM (7)	0.55 ±0.15	0.09 ±0.02†	0.16 ±0.03	0.11 ±0.02	0.61 ±0.20	0.10 ±0.01	0.16 ±0.04	0.10 ±0.02	0.89 ±0.19†

* The values of respiratory chain components represent mean ± SD nmol/mg protein. The values of ATP concentration represent mean ± SD μmol/mg protein per 30 min. The numbers in parentheses represent numbers of experiments.

† Values were significantly less ($p < 0.05$) using a one-tailed t tested for independent mean probability.

‡ Values were significantly less ($p < 0.01$) using a one-tailed t tested for independent mean probability.

Table 3. *Effects of MO on intramitochondrial CoA esters**

Incubations	Acetyl CoA	Acid-soluble CoA	Acid-insoluble CoA	Total CoA
Control (6)	0.52 ± 0.05	1.06 ± 0.05	0.88 ± 0.09	1.93 ± 0.11
Margosa +2.5 μl/ml (6)	0.38 ± 0.02†	0.72 ± 0.02†	1.64 ± 0.04‡	2.06 ± 0.05
+25 μl/ml (6)	0.29 ± 0.02‡	0.60 ± 0.04†	1.80 ± 0.06‡	2.15 ± 0.11

* The values of coenzyme A components represent mean ± SD nmol/mg protein. The numbers in parentheses represent numbers of experiments. Acid-soluble CoA includes free CoA-SH, soluble acyl derivatives (acetyl CoA, succinyl CoA etc.), and acid-insoluble CoA includes long chain acyl CoA derivatives (C_{10}–C_{12} or higher).

† Values were significantly less ($p < 0.05$) using a one-tailed t tested for independent mean probability.

‡ Values were significantly less ($p < 0.01$) using a one-tailed t tested for independent mean probability.

polyunsaturated fatty acids, some of which are constituents of MO, in patients with RS.

Our results show that MO uncouples mitochondrial oxidative phosphorylation, inhibits the respiratory chain at a site between FP_2 and cytochrome b, decreases intramitochondrial levels of acetyl CoA and acid-soluble CoA esters, and reduces the content of ATP in mitochondria. However, Greene *et al.* (25) reported no reduction of ATP content in the liver from patients with RS. Kang *et al.* (26) reported concentrations of the acid-soluble CoA esters were higher in Reye's liver samples than in samples from controls. It has been shown that fatty acids uncouple oxidative phosphorylation (27, 28) and that they enhance the activity of ATPase (29). The MO-induced decline in the level of ATP and retardation of reduction in cytochromes *in vitro* are very likely to be linked with these properties of fatty acids. The presence of fatty acids in excessive amounts would produce a decline in the mitochondrial levels of ATP and CoA-SH through acylation. These mechanisms probably partly account for the MO-induced decline in the ATP level (Table 2) and for the decrease in acetyl CoA which was associated with a concomitant increase in the acid-insoluble CoA fraction (Table 3).

The decreases in the reduced forms of cytochromes induced by the addition of MO were alleviated partly by the inclusion of D,L-carnitine (Table 2). This alleviation is presumably caused by the decrease in fatty acyl-CoAs by the removal of nonesterified fatty acids through acylation with carnitine. Carnitine serves not only as a vehicle of fatty acid transport into mitochondria, but it enhances regeneration of free CoA-SH in mitochondria via formation of acylcarnitines from acyl-CoA derivations (11, 30). The availability of carnitine is closely related to nutritional status (31). Therefore, the toxicity of MO may be amplified when it is given to malnourished children. The decreased levels of ATP content were alleviated by the addition of coenzyme Q or coenzyme Q plus carnitine. CoQ has an ATP-conserving effect, protects the membrane structure, and lowers the generation of superoxide (12).

Our results suggest that MO is a potent mitochondrial toxin. It uncouples mitochondrial oxidative phosphorylation, inhibits the respiratory chain, decreases intramitochondrial acetyl CoA and free CoA-SH, and finally causes a mitochondrial energy crisis. These *in vitro* observations lead us to speculate that MO causes RS in some nutritionally compromised children. Moreover, the alleviation of MO-induced mitochondrial dysfunction by the addition of carnitine and coenzyme Q *in vitro* raises the possibility that several metabolic abnormalities in MO-induced RS are partly correctable by coenzyme Q and carnitine.

In RS, the occurrence of lactic acidosis, hyperammonemia, hypoglycemia, and fatty liver is well documented (20), as is the case with in the MO-induced Reye-like syndrome (1). In view of the fact that acetyl-CoA serves as substrate, together with oxaloacetate, for citrate synthesis, and that acetyl-CoA is an obligatory positive effector on pyruvate carboxylase (32), the MO-induced decline in acetyl-CoA level is likely to diminish influx of citrate into citric acid cycle. This seems to be partly related to

lactic acidosis. Gluconeogenesis from lactate and pyruvate would be inhibited due to the depletions of ATP and acetyl CoA which are induced by MO. Since the priming reaction of the β-oxidation of fatty acids requires ATP and CoA-SH, the decrease in the intramitochondrial free-CoA pool and low ATP conditions may inhibit β-oxidation of fatty acids. This would partially lead to the fatty liver seen in both RS- and MO-induced RS. Since acetyl-CoA is a substrate for synthesis of N-acetylglutamate, the depletion of acetyl-CoA may inhibit the synthesis of N-acetylglutamate (33). This may result in an insufficient nitrogen disposal leading to hyperammonemia. These mechanisms may partly account for the clinical and biochemical manifestations of RS induced by MO. Supplementative therapy with L-carnitine and CoQ may be effective in dealing with MO-induced RS.

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