

## Acetyl-L-Carnitine Restores Abnormal Lipid Metabolism Induced by 2,3,7,8-Tetrachlorodibenzo-p-dioxin in Mice

SALAI B. MAGESH<sup>1</sup>, RASHMI RAJAPPA<sup>1</sup>, K. M. RAMKUMAR<sup>2</sup>,  
S. SURYANARAYANAN<sup>1</sup> and SUBBARAO V. MADHUNAPANTULA<sup>3\*\*</sup>

<sup>1</sup>Department of Water and Health, Faculty of Life Sciences,

Jagadguru Sri Shivarathreeswara University, Mysuru– 570015, Karnataka, India.

<sup>2</sup>SRM Research Institute, SRM University, Kattankulatur - 603203, Tamil Nadu, India.

<sup>3</sup>Centre of Excellence in Molecular Biology and Regenerative Medicine (CEMR), JSS Medical College, Jagadguru Sri Shivarathreeswara University, Mysuru -570 015, Karnataka, India.

\*Coressponding author E-mail: E-mail: mvsstsubbarao@jssuni.edu.in

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### ABSTRACT

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), an organic pollutant, is a potent toxin known to modulate lipid metabolism in mice. Prior studies have demonstrated the ability of Acetyl-L-Carnitine (ALC) to mitigate various diseases associated with abnormal lipid metabolism. Therefore, the efficacy of administration of ALC to protect mice from TCDD-induced abnormal lipid metabolism in mice was tested in this study. Experimentally, mice (n=5) were administered with 100ng/kg body weight TCDD for 30 days, followed by treatment with ALC (Oral, 50mg and 100mg/kg body weight) for additional 30 days. Compared to mice that received vehicle corn oil, mice that received TCDD showed elevated cholesterol, triglycerides, free fatty acids and phospholipids in liver and serum. In addition serum lipoproteins such as LDL and VLDL were also elevated in the TCDD treated mice. Oral administration of ALC (50mg and 100mg/kg b.w) showed a dose dependent health promoting effect against TCDD-induced abnormal lipid metabolism by restoring the lipids and lipoproteins to near normal level. In conclusion, ALC is a potent anti-hyperlipidemic compound, which helps in the treatment of disorders associated with lipid metabolism.

**Keywords:** TCDD, Triglycerides, ALC, Free fatty acids, hyperlipidemia.

### INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), a potent toxic environmental pollutant produced as a by-product of various industrial processes<sup>1</sup>. It is resistant to degradation and hence tends to accumulate in the environment, in animals, and even in human<sup>2</sup>. TCDD has a biological half-life of e<sup>7</sup>7 years in humans and is categorized as a Class-I carcinogen by the International Agency for Research on Cancer (IARC)<sup>3</sup>. In addition, TCDD is responsible for "Wasting Syndrome", a

disease that involves decrease in the body weight and adipose tissue<sup>4</sup>. Furthermore, a significant increase in serum lipids and lipid metabolites was also reported in Wasting Syndrome<sup>5</sup>. For example, TCDD affects the cholesterol metabolism and biosynthesis of bile acids<sup>6</sup>. In addition, TCDD also reduces the expression of peroxisome proliferator activated receptor (PPAR) and lipogenesis-related factors sterol regulatory element binding proteins (SREBP 1 & 2)<sup>7</sup>. Taken together, TCDD disrupts lipid metabolism causing wasting syndrome and dyslipidemia associated disorders<sup>8</sup>.

Since TCDD exposure to the body is mainly through diet, attempts have been made to identify effective compound(s) that can suppress TCDD-induced toxicity<sup>9</sup>. Acetyl-L-Carnitine (ALC) is an acetyl ester of the L-carnitine, produced in mammalian brain, liver and kidney by the enzyme, ALC-transferase<sup>10</sup>. Key functions of ALC include, accelerating the uptake of acetyl CoA, increasing acetyl CoA production and membrane phospholipid synthesis<sup>11</sup>. A wide range of clinical and preclinical applications of ALC have been reported and are primarily associated with neurologic disorders, in particular, Alzheimer's disease, Parkinsons disease, diabetic neuropathy, ischemia and reperfusion of the brain impairment<sup>12</sup>. Consumption of ALC for 4 weeks reversed the age related weakening of mitochondrial membrane potential in the hepatocytes of old rats<sup>13</sup>. In addition, ALC elevated the cardioliipin level and cellular oxygen utilization in the old rats to the level similar to young rats<sup>14</sup>. However, not much is known about its antihyperlipidemic activity<sup>15</sup>. Only few studies have demonstrated that administration of ALC effectively normalizes the concentration of plasma cholesterol and triglycerides in an experimental diabetic model and in hyper-cholesterolemic rabbits<sup>16</sup>. Hence in this study, the efficacy of ALC on lipid metabolism was investigated by measuring and comparing the changes in serum and hepatocyte lipids of control and TCDD administered mice.

## MATERIALS AND METHODS

Acetyl L Carnitine, TCDD, and silymarin were procured from Sigma Chemical Company (St Louis, MO). All the other chemicals used are of analytical grade.

### Animal experiments

JSS Medical College Animal Ethics Committee (JSSMC/IAEC/18/5675/DEC2013), Mysuru, India, approved the *in vivo* study protocol. Male Swiss albino mice weighing about 20-25g were procured from the Central Animal Facility, JSS Medical College. The mice were kept at standard laboratory conditions of controlled environment with the temperature 25±2°C and 12h light- dark cycle. The mice were allowed to eat commercially available standard diet and allowed free access of water.

Table 1: ALC administration mitigate the changes in serum lipids induced by TCDD administration in mice

Groups and treatment	Serum Lipid Profile						
	Cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)	Phospholipids (mg/dl)	FFA (mg/dl)
Control	69.9±3.7	42.3±2.4	41.4±0.9	18.8±0.8	8.4±0.4	68.6±1.6	3.2±0.3
C+ALC (L)	68.4±2.8	33±2.3	39.3±1.2	17.6±0.5	6.6±0.4	60.8±1.1	2.2±0.2
C+ALC (H)	68.2±2.4	31.3±2.3	39±1.8	19.9±0.6	6.2±0.4	63.5±3.1	2.9±0.2
TCDD	101.3±2.6*	92±3.2*	39.4±1.0	36±1.8*	18.4±0.6*	95.4±1.2*	6.8±0.3*
TCDD+C	85.9±2.3*	76.6±7.2*	44.8±1.8	28.5±2.2*	16±0.4*	83.8±2.5*	5.8±0.2*
TCDD+ALC (L)	75.9±1.5**	67.6±2.4**	49.0±1.1	21.7±1.2**	13.5±0.4**	71.8±1.2**	4.1±0.1**
TCDD+ALC (H)	71.2±1.6**	48±5.5**	44.2±1.5	18.9±1.5**	9.6±1.1**	70.3±1.2**	3.6±0.2**
TCDD+ALC (Co)	87.6±2.4**	73±4.6**	46.2±0.9	23.2±1.3**	14.6±0.9**	79.9±4.3**	4.2±0.3**
TCDD+SIL	66.2±3.3**	42.3±2.4**	47.4±1.1	18.5±1.1**	8.4±0.4**	61.5±1.4**	2.1±0.1**

### Experimental Design

Mice were divided into nine groups (n=5) and treated with respective compounds as mentioned below,

Group 1: Vehicle corn oil control; Control

Group 2: Normal mice treated with ALC (50mg/kg bw); C+ALC (L)

Group 3: Normal mice treated with ALC (100mg/kg bw); C+ALC (H)

Group 4: TCDD (100ng/kg bw) for 30 days; TCDD

Group 5: TCDD for 30 days + Saline for 30 days; TCDD+ C

Group 6: TCDD for 30 days + ALC (50mg/kg bw) for 30 days; TCDD+ALC (L)

Group 7: TCDD for 30 days + ALC (100mg/kg bw) for 30 days; TCDD+ALC (H)

Group 8: TCDD + ALC (100mg/kg bw) Co treated; TCDD+ALC (Co)

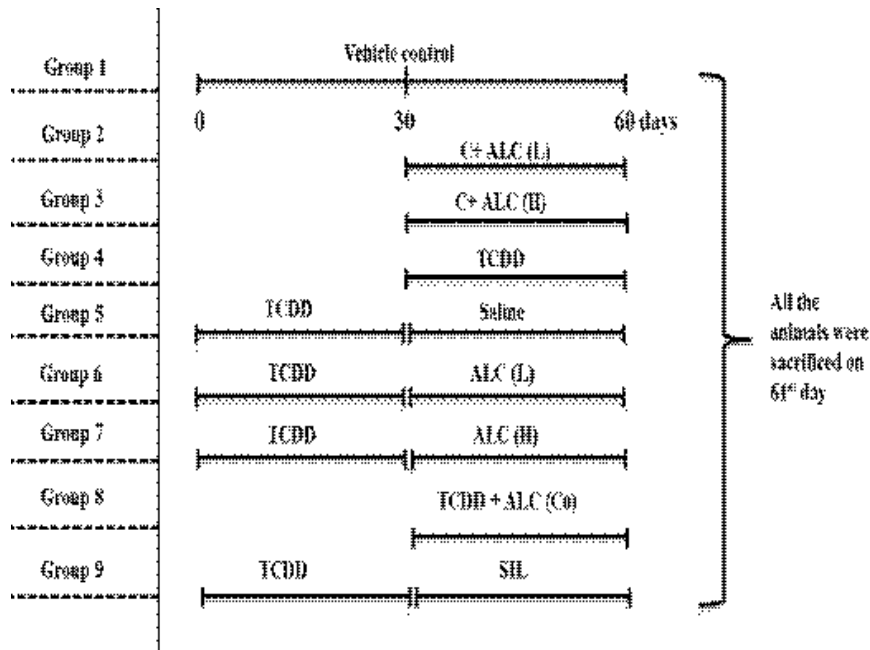
Group 9: TCDD + Silymarin (50mg/kg bw); TCDD+SIL

TCDD (100 ng/kg bw) was dissolved in 0.15ml corn oil and administered intra-peritoneally<sup>17</sup>. ALC was dissolved in the aqueous suspension (50 and 100mg/kg bw) and administered orally<sup>18</sup>.

Corn oil alone is administered to the control group. After treatment period, mice were deprived of food overnight, anesthetized and sacrificed<sup>19</sup>. Serum was separated from the collected blood by centrifugation and stored at -20°C. Liver was quickly excised from the experimental animals and rinsed in ice-cold phosphate buffered saline, blotted and stored at -80°C. The extraction of lipids from liver tissues is done by the method of Folch *et al.*, 1957<sup>20</sup>. Figure 1 show the schematic representation of experimental design.

### Hematological and Biochemical Analysis

Blood collected from the control and experimental animals were used for the analysis of serum lipids. Total Cholesterol, Triglycerides and High density lipoprotein-cholesterol (HDL) were determined using kits from Coral Clinical System, India. Phospholipids and Free fatty acid (FFA) levels were assessed as described by Zilversmit *et al.*, 1950<sup>21</sup> and Falholt *et al.*, 1973<sup>22</sup>. Very low density lipoprotein-cholesterol (VLDL) and Low-density lipoprotein-cholesterol (LDL) were calculated



**Fig. 1:** Schematic representation of experimental design. TCDD (100 ng/kg bw) was administered intra-peritoneally. ALC was dissolved in the aqueous suspension (50 and 100mg/kg bw) and administered orally. Control group was administered with the corn oil alone. The treatment period of the study is 60 days. On 61<sup>st</sup> day mice were deprived of food for overnight, anesthetized and sacrificed. Blood and liver tissues were collected to determine the lipid profile.

by Friedwald formula:  $VLDL = TG/5$ ;  $LDL = TC - (HDL + VLDL)$ .

### Analysis of lipid profile in liver tissue

#### Estimation of Total Cholesterol

The total cholesterol levels were assessed by the method of Zlatkis *et al.*, 1953<sup>23</sup>. Cholesterol was first oxidized by Ferric chloride – acetic acid solution and, next, the color formed measured at 560 nm. Briefly, to 0.1 ml of lipid extract 9.9ml of ferric chloride – acetic acid reagent was added and the reaction mixture was permitted to stand for 15 minutes. Next, the reaction mixture was centrifuged, and to 5.0ml supernatant 3.0ml of sulfuric acid added. The contents were kept at 37° C for 20 min and the color developed measured at 560nm. Results were expressed as mg total cholesterol/100g tissue.

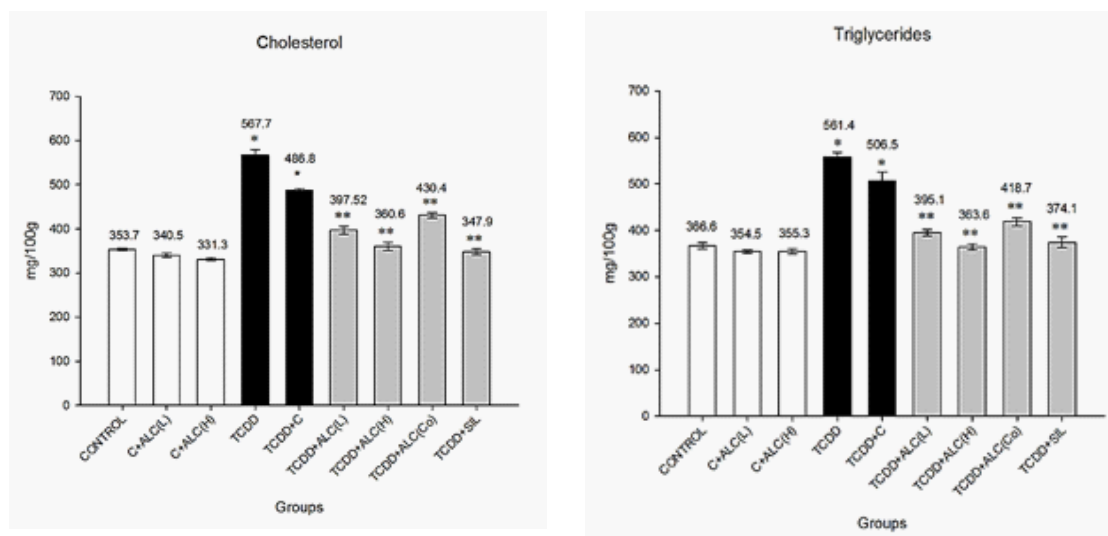
#### Estimation of Triglycerides

Triglycerides were determined by the method of Foster and Dunn<sup>24</sup>. First, the glycerol

moiety is oxidized to formaldehyde. Next, the oxidized glycerol was allowed to condense with acetyl acetone to yield 3,5-Dicarbethoxy-1,4-dihydrocollidine which is read at 405nm. Briefly, 0.1ml of lipid extract was vaporized to dryness. Then 0.1ml of methanol, 4 ml of isoproponal and 0.4ml of neutral alumina were added. The tubes are well shaken for 20 min, centrifuged and the supernatant collected. To 2.0ml of supernatant added 600µl of saponification reagent and incubated at 65°C for 20.0 min and allowed to cool. Then, added 1.0 and 0.5ml of sodium metaperiodate and acetyl acetone reagent, respectively. The reaction mixture was heated at 65°C for 15.0 min using water bath, cooled and yellow color formed was read at 405 nm. The levels of triglyceride is expressed as mg/100g tissue.

#### Estimation of Free fatty acid (FFA)

The FFA levels were assessed by the method of Falhot *et al.*, 1968<sup>22</sup>. Free fatty acids react with copper reagent and form a complex. The complex thus formed was made soluble in



**Fig. 2:** The effect of ALC on cholesterol and triglycerides levels. The total cholesterol was estimated by the method of Zlatkis *et al.*, 1953. Cholesterol was oxidized by Ferric chloride – acetic acid solution. The colour thus formed was measured at 560nm. Triglycerides were estimated by the method of Foster and Dunn, 1973. The glycerol moiety was oxidized to formaldehyde and further condensed by acetyl acetone to yield 3,5-Dicarbethoxy-1,4-dihydrocollidine which was read at 405nm. A significant 1 fold increase in liver cholesterol and triglycerides levels was observed upon TCDD administration. However, oral treatment of ALC (50 mg and 100 mg/ kg body weight) and silymarin for 30 days significantly reduced the total cholesterol and triglycerides content to near normal.

chloroform, and allowed to react with diphenyl carbazide (a color developer). The developed color was read at 550nm. Briefly, to 0.1 ml lipid extract, 6.0ml chloroform: heptane: methanol mixture (5:5:1), 2.5 ml copper reagent and 200mg of activated silicic acid were added. Mixed well, centrifuged and copper layer collected. To 3.0ml of copper layer added 0.5ml of diphenyl carbazide and the color developed was read at 550nm. The results were expressed as mg/100g tissue.

### Estimation of phospholipids

The inorganic phosphorus, phosphomolybdic acid reacts with ANSA solution to form a blue color which is measured at 660 nm<sup>21</sup>. Briefly, to 0.1 ml of lipid extract, was added 1.0ml of 5N H<sub>2</sub>SO<sub>4</sub> and 2-3 drops of conc. nitric acid. Then, added 2.5ml of 3% ammonium molybdate and 0.1 ml of ANSA solution, mixed well and heated in water bath for 6.0min. The blue color developed was read

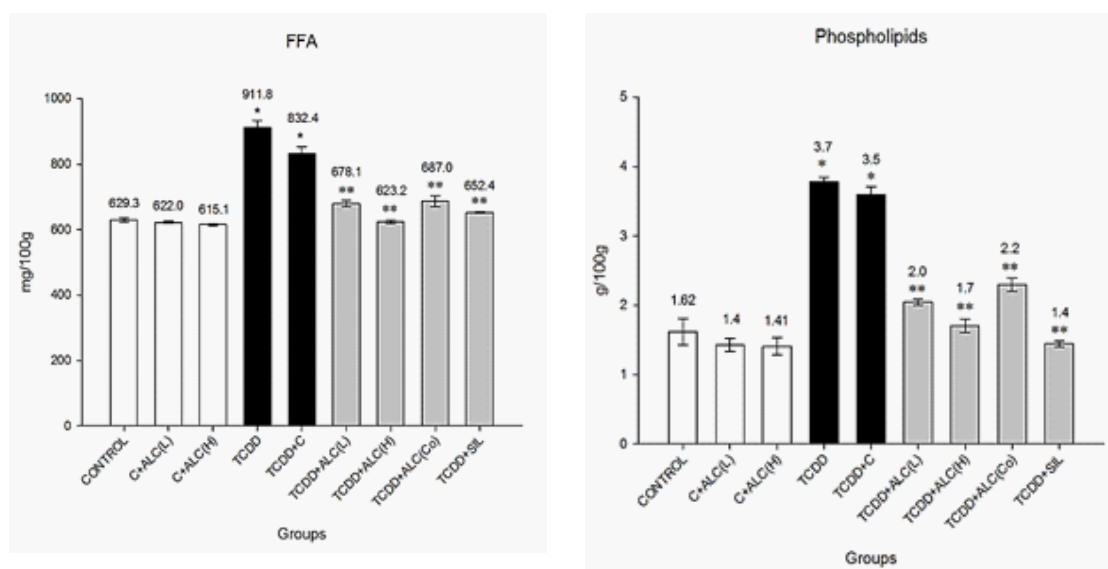
at 660 nm and the inorganic phosphorous content expressed as g/100g tissue.

### Statistical analysis

The statistical significance among nine groups was evaluated by one way ANOVA using SPSS version 20 followed by Tukey post hoc test. P<0.05 was considered significant.

## RESULTS AND DISCUSSION

Hyperlipidemia is one of the known consequences of TCDD<sup>25</sup>. Exposure to TCDD has been shown to promote the accumulation of lipids, which leads to wasting syndrome and diabetes like symptoms<sup>26</sup>. TCDD induced hyperlipidemia is associated with decreased expression of peroxisome proliferator activated receptor gamma (PPAR- $\gamma$ ) and lipogenesis factors such as sterol regulatory element binding proteins (SREBP)<sup>27</sup>. Earlier



**Fig. 3A & B:** The effect of ALC on free fatty acid and phospholipids. Free fatty acids were estimated by the method of Falhot *et al.*, 1968. Free fatty acids react with copper reagent, and form a colored complex with cupric ions. The complex thus formed was made soluble in chloroform, and a color developing agent diphenyl carbazide added. The colour developed was read at 550 nm. Phospholipid content was estimated by the method of Zilversmit *et al.*, 1950. The inorganic phosphorus, phosphomolybdic acid reaction with ANSA solution forms a blue color which was measured at 660nm. A significant elevation in liver free fatty acids and phospholipids levels (~ 1 fold) was observed when mice were exposed to TCDD. However, oral treatment with ALC (50 mg and 100 mg/ kg body weight) and silymarin for 30 days significantly reduced the values to near normal.

studies carried out with ALC showed a significant anti-hyperlipidemic effect in experimental models<sup>28, 29</sup>. In the present study, the effect of ALC on lipid metabolism in TCDD exposed mice was evaluated. Mice exposed to TCDD showed an about one fold increase in the serum cholesterol, LDL, VLDL, triglycerides, free fatty acids and phospholipids compared to vehicle control group (Table 1). However, surprisingly, no significant change in the HDL content was noticed in the TCDD treated group compared to the vehicle control. In addition, level of cholesterol in liver tissues was significantly higher in TCDD induced mice compared to vehicle control (Figure 2A). Increase in the concentration of serum lipids with TCDD exposure was attributed to an increase in the mobilization of FFA from the peripheral fat depots due to the action of hormone-sensitive lipases<sup>30</sup>. Further the observed increase in the cholesterol in TCDD induced mice could be due to high HMG CoA reductase (a rate-limiting enzyme involved in cholesterol biosynthesis) activity<sup>31</sup>. Oral administration of ALC (50 and 100 mg/kg bw) and SIL significantly reduced these elevated lipids in a dose dependent manner (Table 1 and Figure 2A).

TCDD exposed animal models are susceptible to hypertriglyceridemia, which might be due to the elevated VLDL secretion or decreased VLDL clearance. Elevated VLDL and LDL in the serum of TCDD injected mice (Table 1) was related to the reduction in the activity of lipoprotein lipases as evidenced by Brewster *et al.*, 1984<sup>32</sup>. Oral administration of ALC restored the VLDL and LDL suggesting that ALC promotes the release and triggers the lipoprotein lipases thus

resulting in the reduced lipid content<sup>33</sup>. In addition, lipoprotein lipases play a key role in the hydrolysis of triglycerides and uptake of FFA. Deficiency/reduced activity of lipoprotein lipases enhances the release of FFA from adipose tissue<sup>34</sup>. Excess free fatty acids in the plasma promotes the conversion in to phospholipids and cholesterol in the liver<sup>35</sup>. In the current study, concentration of triglycerides in serum and liver tissues of TCDD induced mice was increased (~ 1 fold) compared to the vehicle control (Table 1 and Figure 2B). Oral administration of ALC significantly decreased the levels of triglycerides in serum and liver tissues (Table 1 and Figure 2B). In addition, TCDD induced mice showed higher levels of phospholipids and free fatty acids in serum and liver tissue (Table 1 and Figure 3A& B) which is in agreement with the earlier studies<sup>36</sup>. However, ALC treatment significantly decreased the levels of free fatty acids and phospholipids in the serum and tissues thus improving lipid metabolism in TCDD induced mice.

## CONCLUSION

In conclusion, oral administration of ALC is an effective lipid lowering agent to protect mice from TCDD induced toxicity, hence, can be a potential therapeutic agent in managing hyperlipidemia related diseases.

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