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Original Article

Effect of Treatment with Conjugated Linoleic Acid on the Enzyme Carnitine Palmitoyl Transferase in Albino Mice

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ABSTRACT

Background: Conjugated Linoleic Acid is an omega-6 fatty acid with potential health benefits. The objective of the present study is to study the effect of Conjugated Linoleic Acid on the enzyme Carnitine Palmitoyl Transferase. **Methods:** Healthy Albino mice were divided into three groups *viz.*, first group of 10 animals given high fat diet, second group of high fat diet and Conjugated Linoleic Acid treated animals and third group of control animals given normal diet. After 22 days, animals were sacrificed and hepatic and adipose tissue separated. Carnitine palmitoyl transferase estimation was done by ELISA and western blot techniques. Serum samples collected on 7th, 15th and 22nd days and carnitine palmitoyl transferase assay done. **Results**: The results showed that there is an elevation of the levels of the enzyme Carnitine Palmitoyl Transferase in serum, hepatic and adipose tissues as measured by both ELISA and Western blot technique. **Conclusion:** Conjugated Linoleic Acid, acts via elevation of the enzyme Carnitine Palmitoyl Transferase when it is given in mice and causes weight reduction.

Key words: Omega-6 fatty acid, Albino mice, Elisa, Western blot technique.

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INTRODUCTION

Conjugated linoleic acid (CLA) is an omega-6 fatty acid with many health benefits and it is a mixture of different types of isomers of linoleic acid. These various isomers are derivatives of linoleic acid. CLA is mostly found in milk products and ruminant meats like beef and lamb.[1] CLA is important to human health. Many animal studies have shown that CLA has got a major role in the reduction of body weight and fat deposition. Animal studies have been conducted using CLA mixtures. The mixtures contained equal amounts of trans-10, cis-12 (t10c12) and cis9, trans-11 (c9t11) isomers. In 2008, USFDA declared CLA as Generally Recognized As Safe (GRAS). Results of various studies conducted on CLA suggest that CLA alters weight gain when animals are fed with a diet which contains low or medium levels of fat. CLA has got various potent physiological functions such as anti-carcinogenic, anti-obesity, anti-diabetic and antihypertensive properties. It was found through many studies that CLA can be effective to prevent lifestyle diseases or metabolic syndrome.[2] The weight lowering effect of CLA has been studied and Delany *et al*[3] observed that after feeding male mice with a high fat diet along with CLA, the body weight was reduced by day 22. The same effect continued for a period of 12 weeks.

The effects of conjugated linoleic acid on body composition were further studied. Hyperlipidemia or hypercholesterolemia in rats can be induced by supplementing cholesterol diet (sub-acute model). Devi S *et al*, in their study tested the anti hyperchoslestrolemic potential of fresh leaves of Vitis Vinifera.[4] ICR mice, which were fed on a diet containing CLA, showed that body fat was reduced and lean body mass increased in a study conducted by Park Y *et al*.[5]

Carnitine palmitoyl transferase I (CPT1) also known as carnitine acyltransferase 1 (CAT1) and is responsible for the formation of acyl carnitines by catalyzing the transfer of the acyl group to l-carnitine. The product is often Palmitoyl Carnitine, but other fatty acids may also be possible. Palmitoyl Carnitine belongs to a group of other enzymes called Carnitine Acyl Transferases which causes subsequent movement of the acyl carnitine from the cytosol into the mitochondrial membrane space.

CPT1 is an internal membrane protein that connects with the mitochondrial outer membrane through transmembrane regions in the peptide chain. Both the N- and C- terminals are exposed to the inner side of the membrane. Three isoforms of CPT 1 exist in the mammalian tissues. Except for skeletal muscle cells, the 1st isoform CPT 1-A or CPT1-L is found throughout the body on the mitochondria of all cells. The second isoform CPT1B is present in brown adipose cells and heart and skeletal muscle cells. The third isoform, present in the brain, CPT 1 C, was isolated in 2002. There is not much knowledge about CPT1 C. The CPT system is vital in the beta oxidation of long chain fatty acids. This system is essential, because even though the fatty acids are activated on the outer mitochondrial membrane, the activation of fatty acids should occur within the mitochondrial matrix. Long chain fatty acids require a shuttle system to be transported into the mitochondrial inner membrane.

The CPT IA form causes Carnitine Palmitoyl Transferase 1 deficiency. This rare disorder presents a risk of hepatic encephalopathy, hypoketotic hypoglycaemia, and sudden death in infancy. CPT 1 is associated with type 2 diabetes Melletus and increase of insulin resistance. CPT1 deficiency cause free fatty acid (FFA) levels in humans to become elevated, fat to accumulate in skeletal muscle, and oxidation of free fatty acids is decreased. CPT1 has been thought to be responsible for these symptoms. The decrease in CPT 1, causes a further decrease in the transport of long chain fatty acids into the mitochondria of heart and muscle, decreasing

fatty acid oxidation in their cells. The shunting of long chain fatty acids away from mitochondria causes deposition of fat. Hence CPT1 is considered essential in the future treatment of many metabolic disorders.

MATERIALS AND METHODS

Raising polyclonal antibody for human Carnitine Palmitoyl Transferase

By using recombinant human carnitine palmitoyl transferase protein, antihuman CPT rabbit polyclonal antibody was generated. Approximately 300 micrograms of protein was emulsified with complete Freund's adjuvant (sigma) and the rabbit was immunized at multiple sites subcutaneously. Three booster doses of 150 micrograms purified protein were given with incomplete Freund's adjuvant (Sigma) at three-week intervals. After the third booster dose, the animal was bled; serum was separated and stored at -20°C. 1:1000 dilutions were used for western blotting and ELISA.

In vivo Animal studies

Albino mice (*Musmusculus*) which were healthy and ten to twelve (10-12) weeks old, thirty in number, weighing 21 to 25 gms were housed in a clean room. The mice were maintained at standard temperature of 22°C (±3°C) with 50% to 70% relative humidity, light and dark (12h) was maintained. The feed was commercially available pellets from Hindustan lever limited, Mumbai and autoclaved Milli Q water given to the animals.

First set of 10 animals was given a high fat diet, the second set of 10 animals was given a high fat diet plus conjugated linoleic acid and third set of 10 animals were control and fed normal feed and water. A 200µl of Blood was collected from each animal on the 7th day, 15thday- and on 22nd day through retro-orbital bleeding.[6] Serum was collected by centrifucation at 3000 RPM for 20 min. Animals were sacrificed and 500 mg of liver and adipose tissue was rapidly excised separately. Tissues were rinsed with cold phosphate buffer and homogenized by polytron (Brinkman) in homogenization buffer (PBS containing 0.05% sodium azide, 0.5% Triton X-100, and a protease inhibitor cocktail, pH 7.2, 4°C) and then sonicated for 5 min. Homogenates were centrifuged. Carnitine Palmitoyl Transferase amounts in the supernatants were separated and measured by means of ELISA.

Carnitine Palmitoyl Transferase Assay by ELISA

Liver and adipose tissue homogenate (100µl per well) were placed in ELISA plate and the samples incubated at room temperature (20-23°C) for 2 h and the ELISA plate washed 4 times with 300-350µl/well of 1x TBST (Tris Buffered Saline with 0.05% TWEEN 20). 100µl/well Biotinylated anti- Carnitine Palmitoyl Transferase antibody was added and incubated for 2 h. ELISA plate was washed 4 times with 300-350ml/well of 1x TBST and added 100µl /well of Streptavidin-horseradish peroxidase solution. Plates were incubated at room temperature for 30 min. ELISA plate was washed 4 times with 300-350µl/well of 1x TBST and 100 µl/well of substrate solution Tetra Methyl Benzidine (TMB) added. ELISA plates were incubated at room temperature for 20-30 min. The reaction was terminated by adding 100µl/well of Stop Solution (10mM EDTA in 1XTBS). The plate was read at 450nm and results were calculated.

Sodium Dodecyl Sulphate Poly Acramide Gel Electrophoresis (SDS- PAGE) of proteins

A very common method for separating proteins by electrophoresis. It uses a discontinuous polyacramide gel as a support medium and sodium dodecyl sulphate to denature the proteins. This method is called Sodium Dodecyl Sulphate Poly Acramide Gel Electrophoresis. (SDS- PAGE).

SDS-PAGE was carried out by the method described by Laemmli, 1970 in a discontinuous buffer system.

Staining of SDS-PAGE with Coomassie Brilliant Blue R 250

The gels stained with 0.5% Coomassie Brilliant Blue R 250 in 100 ml of methanol : acetic acid : water (45 :10 :45) for 2-3 h and destained in 200 ml of methanol : acetic acid : water (45 :10 :45) for 3-4 hrs on a shaker, with several changes of the destaining solution. Then the proteins were transferred from SDS-PAGE to PVDF membrane.

Western blot analysis of proteins Immunodetection by colour reaction (ALP)

The blot was thoroughly washed in PBS- phosphate buffer solution to remove Ponceau and blocked with 5% Blotto in TBST (Tris Buffered Saline containing 0.1% Tween 20) for one hour at room temperature. The blot was rinsed with TBST and incubated with the primary antibody and rabbit CLP antibody (1 :1000 dilution) diluted appropriately in TBST for 1-2 hrs at room temperature, washed 3 times for 5-10 min each with TBST, followed by incubation with alkaline phosphatase-conjugated antibody diluted in TBST for 45 min at room temperature. The blot was then washed 3 times for 5-10 min each with TBST. The blot was developed in 100 mM tris. HCl pH9.0, 10mM MgCl2, 100mM NaCl, with 100 mg/ml NBT and 50 mg/ml BCIP as substrate for alkaline phosphatase- conjugated secondary antibodies.

RESULTS

Statistical analysis was performed by using SPSS software version 20.0 and MS Excel 2007. Descriptive statistical data was presented as mean + standard deviation and percentages. Independent student-*t* test was performed to compare the means of two different groups of continuous variables. Anova was used to compare the mean of different continuous groups. For statistical analysis $P<0.05$ was considered as statistically significant.

According to the results, it was noted that Carnitine palmitoyl transferase enzyme was elevated on the day 22 when serum was tested and the p value came as significant. When the animals were sacrificed and the hepatic tissue tested on 22nd day the p value is again significant. Similarly, when the adipose tissue was tested on day 22nd the p value came significant. To compare between the groups, Student- *t* test was employed and One Way Anova was used to compare within the groups.

Group A is the test group

Group B is the control group for all the tests.

When Student- *t* test was employed to compare between the groups on the 7th day the p-value was 0.85 and is not significant and on 15th and 22nd day the p-value is 0.00, which is highly significant.

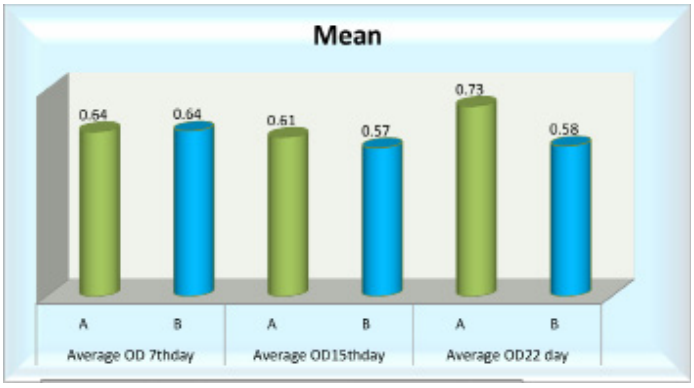
When the hepatic and adipose tissue values on 22nd day were analysed, it was observed that p value is 0.00 and came as significant.

When Anova was employed to compare within the group for 7th, 15th and 22 nd days, the p value 0.00 and came as highly significant.

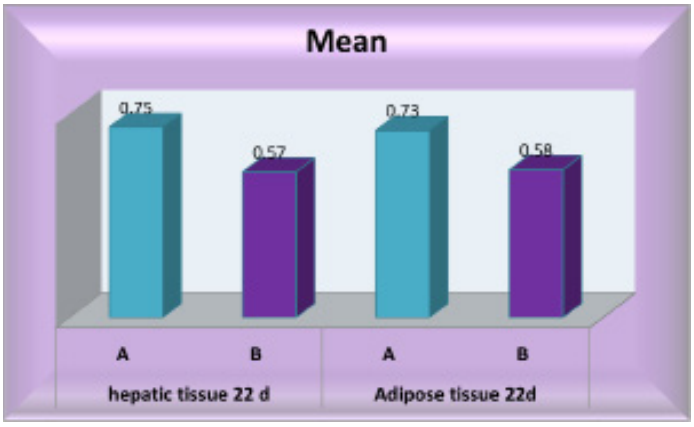
DISCUSSION

The present study is conducted to see the effect of Conjugated Linoleic Acid, the test drug on the enzyme Carnitine Palmitoyl Transferase which is involved in the fatty acid oxidation thereby causing lipolysis. The study was conducted for a period of 22 days and in one group of 10 animals, high fat diet was given as 5 mg per kg body weight. In the second group of 10 animals, high fat diet was given and also conjugated linoleic acid

t Test				
		Mean	Std. Deviation	p-value
Average OD 7th day	A	0.64	0.01	0.85
	B	0.64	0.02	
Average OD 15th day	A	0.61	0.01	0.00
	B	0.57	0.01	
Average OD 22nd day	A	0.73	0.01	0.00
	B	0.58	0.00	



Group Statistics				
		Mean	Std. Deviation	p-value
hepatic tissue 22 d	A	0.75	0.01	0.00
	B	0.57	0.01	
Adipose tissue 22d	A	0.73	0.01	0.00
	B	0.58	0.00	



ANOVA WITHIN GROUP				
		Mean	Std. Deviation Lower Bound	95% Confidence Interval for Mean Upper Bound
average OD within group	7.00	0.64	0.01	0.62
	15.00	0.61	0.01	0.60
	22.00	0.73	0.01	0.72

in the same dose of 5mg/kg. Third group of 10 animals kept as control. The CPT assay was done on 7th, 15th and 22nd day in serum. On the 22nd day CPT assay was also done in adipose and hepatic tissue also. From the tests, it was evident that there is a rise in the enzyme CPT more on 15th and 22nd day in the serum, on the 22nd day, there is elevation of CPT in the adipose and hepatic tissue also.

In 1987, Ha YL *et al* initially discovered CLA and identified anticarcinogenic property.[7] Subsequently, CLA was shown to exhibit anti-obesity properties also. Due to the increase in the prevalence of obesity over the past 30 years, the utility of CLA as weight loss medication is also on an increase. Treatment with CLA mainly t10c12 isomer, decreases body fat mass in many studies. Of the two major isomers of CLA, t10 c12 isomer has been proved to have the main anti-obesity effect. Park *et al* first demonstrated that CLA modulated body composition. In this study, both male and female mice had less BFM (Body Fat Mass) when compared to controls. Subsequently, it was demonstrated through other studies that CLA supplementation reduces body fat mass in mice and rats. But the results are less consistent in humans. Other studies show that CLA decreases BFM at the same time increasing lean body mass (LBM). Supplementation of a CLA mixture in overweight and obese people (3–4 g/day for 24 weeks) decreased BFM and increased lean body mass (LBM).[8]

It was postulated that CLA increases resting metabolism, which is a function of basal metabolic rate (BMR), thermogenesis, and physical exercise. CLA has been proposed to reduce body fat by elevating energy expenditure via increased BMR, thermogenesis, or lipid oxidation in animals. Studies showed that CLA supplementation increased lean body mass by increasing energy consumption. For example, CLA supplementation (6.4 g/day for 12 weeks) increased LBM by a minimum of 0.64 kg in healthy obese humans when compared to controls.[9]

Supplementation with t 10, c12 CLA in rodents has been shown to induce uncoupling protein 2 transcription in white adipose tissue (WAT).[10] CLA also caused increased expression of Carnitine Palmitoyl Transferase1 (CPT1) in white adipose tissue (WAT) of mice, which are treated with t 10 c 12 CLA.[11,12] CPT1 is involved in fatty acid uptake by mitochondria and is responsible for the rate-limiting step of fatty acid oxidation as already discussed before.

It is supposed that CLA may increase PPARγ activity due to its pro-inflammatory effects in adipocytes thereby reducing lipid formation. According to previous studies, treatment with 10,12 CLA has been shown to increase the secretion of interleukins 6 and 8 from rodents[13] and human[14] adipocyte cultures, and TNF-α thereby suppressing insulin sensitivity[15,16]

The potential mechanism responsible for anti-obesity effect of CLA is the ability of CLA to increase fatty acid oxidation in the liver and adipose tissues. Many studies have confirmed the ability of CLA to increase fatty acid oxidation. In a similar study conducted by Sharma G *et al*, Pre-adipocyte differentiation assay (3T3-L1) was used for studying anti-adipogenic activity of the extract of Prunus Persica.[17] According to the study conducted by Martin *et al*, they concluded that hepatic and adipose Carnitine Palmitoyl Transferase (CPT) activity, was increased in rats when they are fed a diet containing t10c12 CLA for 6 weeks. CPT is a rate-limiting enzyme for fatty acid oxidation. Moreover, rats fed with mixed CLA isomers produced lower respiratory quotients, which indicates increased oxidation in the body. These results, together with other studies, suggest that CLA has got the ability to increase fat oxidation.[18,19]

In a study conducted by Rahmani A. H. *et al*, they observed that the extract of Pomegranate (Punica granulatum) has got potential for health management[20] in a similar way to CLA. Similarly, Iram Nazis *et al* conducted a study on the anti-obesity effect of *Zingiber officinale* (Ginger)[21] Finally from the present study conducted and many previous studies in animals

and some studies in humans we can conclude that CLA acts by elevating the enzyme Carnitine Palmitoyl Transferase which acts by means of increasing fatty acid oxidation and causing lipolysis and reduction of weight.

CONCLUSION

From this study, it is observed that there is an elevation of the enzyme Carnitine Palmitoyl Transferase when Conjugated linoleic acid, the test drug is given in albino mice. Finally, it is inferred that CLA acts by elevating the enzyme CPT which acts by causing fatty acid beta-oxidation and thereby lipolysis and weight reduction. In the present scenario, CLA could be recommended as a healthy food supplement for weight reduction in the place of conventional medicines to reduce the adverse effects. However, more studies on animal models with CLA should be conducted to explore further health benefits.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

CLA: Conjugated Linoleic Acid; **CPT:** Carnitine Palmitoyl Transferase; **CPT1:** Carnitine Palmitoyl Transferase 1; **CA 1:** Carnitine Acyl Transferase 1; **TBST:** Tris Buffered Saline with Tween 20; **SDS:** PAGE-Sodium Dodecyl Sulphate Poly Acramide Gel Electrophoresis of proteins; **PBS:** Phosphate Buffer Solution; **BFM:** Body Fat Mass; **LBM:** Lean Body Mass; **BMR:** Basal Metabolic Rate; **WAT:** White Adipose Tissue.

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