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Virgin coconut oil enriched diet promotes reverse cholesterol transport by upregulating LXR α- ABCA1 pathway in macrophages - A comparative study

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Keywords

ABCA1.
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Abstract

Atherosclerotic cardiovascular disease is the leading cause of death globally. Reverse cholesterol transport (RCT) plays a major role in anti-atherogenesis. Dietary fats, particularly saturation level of dietary oils are reported to have an influence on RCT pathway. Here, we investigated the comparative effect of dietary oils, which vary in their degree of saturation *viz.* virgin coconut oil (VCO), olive oil (OO) and sunflower oil (SFO) in regulating RCT. ***Methods:*** Male Sprague–Dawley rats were fed test oils at 8% for 45 d along with a synthetic diet. Molecular mechanism behind fatty acid mediated RCT were studied in primary rat hepatocytes and macrophages. Cells were cultured with major fatty acids present in test oils viz. lauric acid, oleic acid and linoleic acid each at a dose of 500 µM for 12 hours. ***Results:*** Supplementation of VCO enhanced the hepatic mRNA expression of SRB1, ABCA1and its transcription factor LXRα when compared to other groups. Lauric acid upregulated the mRNA expression of these genes in cultured rat hepatocytes than cells treated with oleate and linoleate. Studies using LXR α agonist, T0901317 revealed that laurate stimulates ABCA1 expression partly via LXR α dependent pathways in macrophages. ***Conclusions:*** Results suggested that supplementation of VCO enhances LXR α-ABCA1 pathway involved in RCT compared to OO and SFO. Analysis of molecular mechanisms revealed that lauric acid, the major fatty acid present in VCO mediates RCT partly via PPAR α - LXR α - ABCA1 pathways in macrophages.

Introduction

Atherosclerotic cardiovascular disease (CVD) remains the leading cause of death worldwide (Guilbert, 2003). Epidemiological and clinical studies have consistently documented an inverse association between HDL cholesterol concentrations and CVD risk; but the risk increases progressively with higher levels of LDL cholesterol (Stein and Stein, 1999; Hatahet, 2003; Kannel *et al*., 1979). The macrophage-specific reverse cholesterol transport (RCT) is thought to be one of the most important HDL-mediated cardioprotective mechanisms (Rader *et al*., 2009). The HDL receptor- scavenger receptor B1 (SRB1), apolipoprotein A1 (apo A1), LCAT and ATP-binding cassette A1 (ABCA1) play crucial roles in mediating RCT (Santamarina-Fojo *et al*.,2001; Shetty *et al*., 2006; Ribalta *et al*.,1998). Based on the RCT model, apo A1 is an attractive target for therapeutic intervention (Brown and Chiacchia, 2008).

There are a number of factors that determine circulating HDL cholesterol and apo A1 levels, dietary fats alter HDL cholesterol concentrations, presumably through mechanisms related to RCT. It has been reported that saturation level of dietary fat have an influence on apo A1 mediated cholesterol efflux; and unsaturated fatty acids are known to inhibit the cholesterol efflux from macrophages (Uehara *et al*., 2002, 2007; Wang and Oram, 2002. Our earlier reports suggests that supplementation of virgin coconut oil (VCO) beneficially modulates hepatic lipid metabolism in rats, which is mostly reflected in its apolipoprotein levels compared to monounsaturated fatty acid rich olive oil (OO) and polyunsaturated rich sunflower oil (SFO) enriched diet in rats (Arunima and Rajamohan, 2012).

RCT and cholesterol efflux play a major role in anti-atherogenesis; modification of these processes may also provide new therapeutic approaches to CVD. Recent reports revealed that PPAR α activation promotes macrophage-specific RCT through a liver X receptor (LXR)-dependent pathway (Nakaya *et al.*, 2011). In addition, LXR and PPAR agonist are novel targets for improving *in vivo* macrophage-specific RCT (Julve *et al*., 2011).Our recent reports have suggests that VCO supplementation upregulates PPAR α expression and enhances fatty acid oxidation compared to OO and SFO fed rats (Arunima and Rajamohan, 2014). Further studies using fatty acids revealed that lauric acid, the major fatty acid present in oil extracted from coconut act as a natural ligand for PPAR α and mediate its effect via PPAR α dependent pathways in hepatocytes (Arunima and Rajamohan, unpublished). These novel findings opened new avenues of research on VCO and RCT pathway. Thus we conducted detailed studies on the effect of VCO in regulating expression of SRB1, ABCA1 and LXR α -the key mediators involved in RCT as compared to OO and SFO fed rats. Furthermore, the role of major fatty acids present in these test oils on RCT *in vitro* and fatty acid mediated activation of LXR α -ABCA1 pathway in macrophages were studied.

Materials and Methods

Reagents

TRI reagent, oligonucleotides, primers for RT-PCR, Anti rabbit IgG, Eagle's minimum essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS), penicillin, streptomycin, type IV collagenase, lauric acid, oleic acid, linoleic acid, MTT and T0901317 were purchased from Sigma Aldrich Co.(St Louis, MO,USA). Antibodies against apo A1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). cDNA synthesis and PCR reactions were done using kit purchased from Fermentas, Thermo Fisher Scientific Inc., Canada and all other chemicals used were of highest analytical grade.

Extraction of virgin coconut oil

Coconut palm (*Cocos nucifera* L.) grown at the Kerala University campus were used for the extraction of VCO and CO. Extraction of oils as follows: For extracting VCO, solid endosperm of mature coconut (West coast tall variety) was crushed, made in to viscous slurry and squeezed through cheese cloth to obtain coconut milk which was refrigerated for 48 h, then subjected to mild heating (50°C) in a thermostat oven. The obtained VCO filtered through cheese-cloth was used for the present study (Nevin and Rajamohan, 2004).

Olive oil and sunflower oil

Olive oil (Pietro Coricelli brand) and sunflower oil (Sundrop brand) were purchased from local market.

Animals and diets

Male Sprague - Dawley rats (100-120 g) bred in our department animal house was used for the study.

Animals were individually housed under hygienic conditions in polypropylene cages in a room maintained at an ambient temperature of 25 ± 1°C with12:12-h light-dark cycle. Each rat was given 12 g synthetic diet containing 8% dietary oils daily for 45 days (Table 1). Experimental groups were as follows; Group I rats given 8% VCO, Group II rats given 8% OO and Group III rats given 8% SFO. The fatty acid compositions of test oils were estimated by GC-MS and reported previously (Table 2) (Arunima and Rajamohan, 2013). Entire protocol was approved by Animal Ethics Committee, University of Kerala. Food intakes of rats were noted daily and the body weight was determined weekly. After 45 days, animals were fasted overnight and sacrificed by sodium pentathone injection; tissues were collected for various estimations.

Table 1: formulation of synthetic diet

Ingredients [a]	Group I	Group II	Group III
Corn starch	71	71	71
Casein	16	16	16
Virgin coconut oil	8	--	--
Olive oil	--	8	--
Sunflower oil	--	--	8
Salt mixture	4	4	4
Vitamin mixture	1	1	1

[a] g per 100 g wet weight.

Table 2: Fatty acid analysis of test oils by GC-MS [a]

Fatty acid (%)	VCO	OO	SFO
C6:0	0.804	--	--
C8:0	7.464	--	--
C10:0	7.697	--	--
C 12:0	46.400	--	--
C14:0	16.904	--	--
C16:0	8.509	22. 489	16.957
C16:1	--	2.942	--
C18:0	4.197	10.124	11.553
C18:1	6.619	62.258	20.848
C18:2	1.406	--	47.142
C20:0	--	2.188	1.44
C 22:0	--	--	2.06

[a] Values are mean of 3 estimations

Preparation and maintenance of hepatocytes

Hepatocytes were isolated from normal rats (Sprague-Dawley strain) by collagenase perfusion (Selgen, 1976) as described before (sudhakaran *et al*., 1986). Hepatocytes ($0.65x10^6$ cells/mL) in Eagle's MEM supplemented with 10% FBS, non essential amino acids and antibiotics were seeded into 35mm petridishes. After 4 hours, the unattached cells were removed and the medium was changed to serum free medium supplemented with 500 µM BSA (fatty acid free) bound fatty acids (laurate, oleate and linoleate) for 12 hours at a fatty acid/BSA ratio of 2:1.Untreated cells received an equal amount of BSA equal to that brought with the BSA-fatty acid complex.

Preparation and maintenance of macrophages

Thioglycolate-recruited macrophages were isolated from 6 to 8 week-old male mice by peritoneal lavage 3 days after intraperitoneal injection of 1.5 mL 3% sterile thioglycolate medium (Zhang *et al*., 2008). Macrophages in DMEM supplemented with 10% FBS and antibiotics were seeded onto 35mm petridishes. The unattached cells were removed after 4 hours, then the medium was changed to serum free medium supplemented with 500µM BSA (fatty acid free) bound fatty acids (laurate, oleate and linoleate) in the presence or absence of LXR α agonist for 12 hours at a fatty acid/BSA ratio of 2:1.Untreated cells received an equal amount of BSA equal to that brought with the BSA-fatty acid complex.

Preparation of albumin-bound fatty acids

A 10mM stock solution of each fatty acids were prepared by diluting the free fatty acid in ethanol and precipitating it with the addition of NaOH (final concentration of 0.25M). Excess ethanol was evaporated under N_2 gas, and the precipitated sodium salt was reconstituted with 0.9% NaCl and stirred at room temperature for 10 minutes with defatted BSA (final concentration at 10% in 0.15M NaCl). Each solution was adjusted to pH 7.4 with NaOH and stored in multiple aliquots at −20°C protected from light and tubes were evacuated under N_2 gas (Hannah *et al*., 2001).

MTT assay

MTT assay was done in both hepatocytes and macrophages according to the method described by Mosmann, which is directly correlated to cell viability; cell viability was calculated as a percentage from the viability of the control (untreated) cells.

Isolation of total genomic RNA

Total genomic RNA was isolated from the heart using TRI Reagent by the method described by Chomczynski and Mackey.

cDNA synthesis and Reverse Transcriptase (RT) -PCR

cDNA synthesis and RT- PCR reactions were performed in a thermocycler (Eppendorf AG, Germany) as per manufacturer's instructions using kit purchased from Fermentas. The primers used are: APO A1 (forward) 5'-GAAATGGAAAGAGGATGTGGAG-3' and (reverse) 5'-GTTCAAGGTAGGGTTGCTCTTG-3',SRB1(forward) 5'-GTCAGCACCTGCAGGTTTGG-3'and SR B1(reverse) 5'-TTTCTCCTGCTGCGCAGTTG-3',ABCA1(forward) 5' GGGTGGAGGACAGAATGACATC-3' and ABCA1 (reverse) 5'CCCAGTTTTCGAATTGCCC-3', LXRα (forward) 5'-GAGAAGCTGGTGGTGGCTGCCCA-3'and LXRα (reverse) 5'-AGCTGTAGGAAGCCAGGGAG-3', GAPDH (forward) 5' -CCT TCA TTG ACC TCA ACT AC-3' and (reverse) 5'-GGA AGG CCA TGC CAG TGA GC 3'.

The PCR products were electrophoresed on 1.5% agarose gel and visualized by staining with ethidium bromide. The gels were subjected to densitometric scanning (Bio-Rad Gel Doc, California, USA) to determine the optical density of each band and then normalized against an internal control, GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) using Quantity One imaging software.

Statistical analysis

Data are presented as means with their standard errors. Statistical analysis was performed by One-way ANOVA followed by Duncan's multiple range tests using the SPSS/PC (version 17.0; SPSS) software package program. *p* value <0.05 were considered significant.

Results

mRNA expression of SRB1, ABCA1 and LXR α in liver

Hepatic mRNA expression of SRB1 was increased significantly in VCO fed rats when compared to rats fed with OO and SFO. While there was no significant difference in SRB1 expression among rats fed OO and SFO. The mRNA expression of ABCA1 and its transcripton factor LXR α were also found to be upregulated in VCO fed rats when compared to those fed OO and SFO. But supplementation of OO downregulated the ABCA1 expression than SFO fed rats (Figure.1).

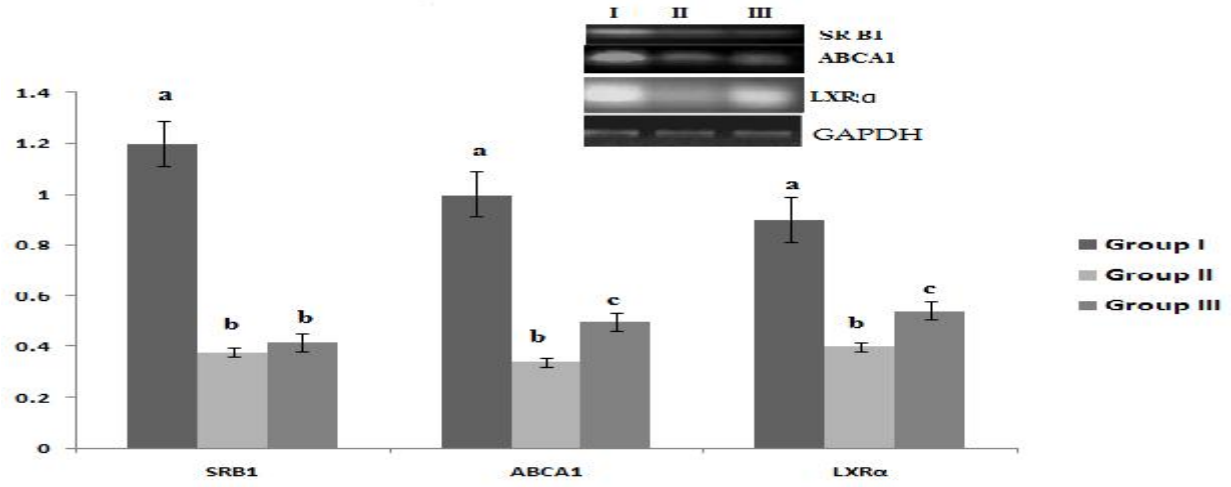

Figure 1: Effect on the mRNA expression of SRB1, ABCA1 and LXR α in liver

Values are mean ± SEM of six rats per group.Values not sharing a common superscript differs significantly at *p* <0.05. Group I, 8% virgin coconut oil-fed rats; group II, 8% olive oil-fed rats; group III, 8% sunflower oil-fed rats.

Effect of major fatty acids present in test oils on the mRNA expression of SRB1 in hepatocytes

Results were described in Figure.2. Lauric acid, the major fatty acid present in VCO increased the mRNA expression of SR B1 in hepatocytes when compared to untreated control and cells treated with oleate and linoleate. But there was a downregulation in SR B1 with oleate compared to other groups. Presence of linoleate upregulated the SRB1 expression than oleate treatment in hepatocytes.

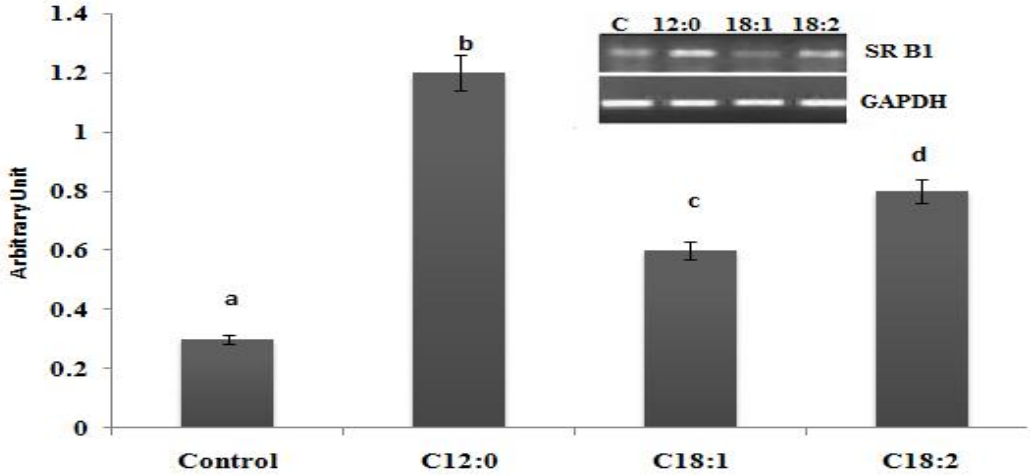

Figure 2: Effect of major fatty acids present in test oils on the mRNA expression of SRB1 in hepatocytes

Values are mean ± SEM of three separate experiments in triplicates. Values not sharing a common superscript differs significantly at *p* <0.05. Group I-control; Group II- 500 µM laurate, Group III-500 µM oleate; Group IV- 500 µM linoleate.

Effect of major fatty acids present in test oils on the mRNA expression of ABCA1 and LXR α in hepatocytes

Laurate upregulated the mRNA expression of both ABCA1 and its transcription factor- LXRα in hepatocytes when compared to untreated control and those treated with oleate and linoleate (Figure. 3). But cells treated with oleate downregulated the expression of ABCA1 and LXRα in hepatocytes when compared to cells treated with laurate and linoleate. Cells treated with linoleate also upregulated the expression of these genes in hepatocytes compared to oleate.

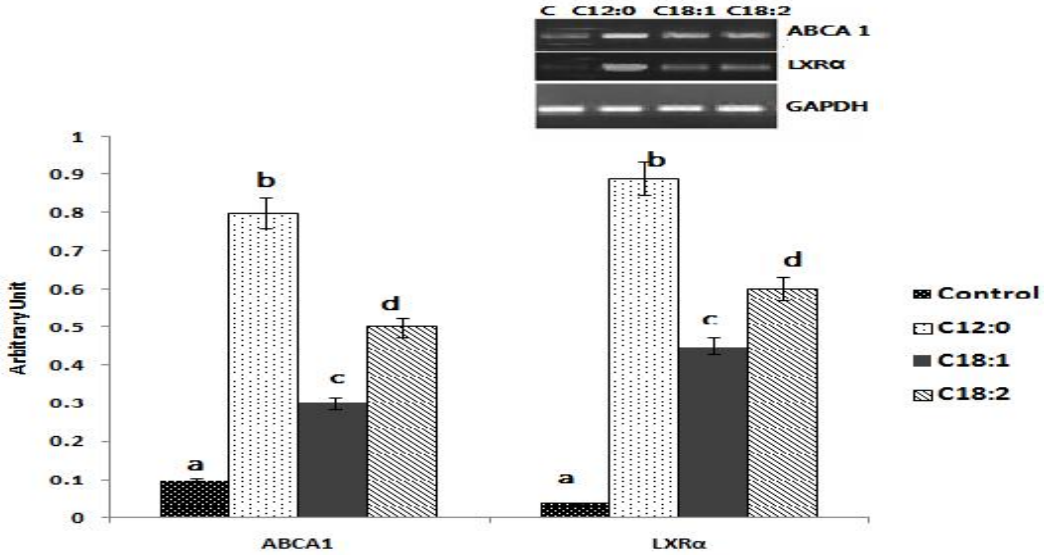

Figure 3: Effect of major fatty acids present in test oils on the mRNA expression of ABCA1 and LXR α in hepatocytes

Values are mean ± SEM of three separate experiments in triplicates. Values not sharing a common superscript differs significantly at p <0.05. Group I-control; Group II- 500 µM laurate, Group III-500 µM oleate; Group IV- 500 µM linoleate.

Effect of exogenous fatty acids and LXR α agonist on cell viability

MTT assay showed that fatty acids and agonist treated cells had the same viability as control cells during the experimental period (Figure. 4).

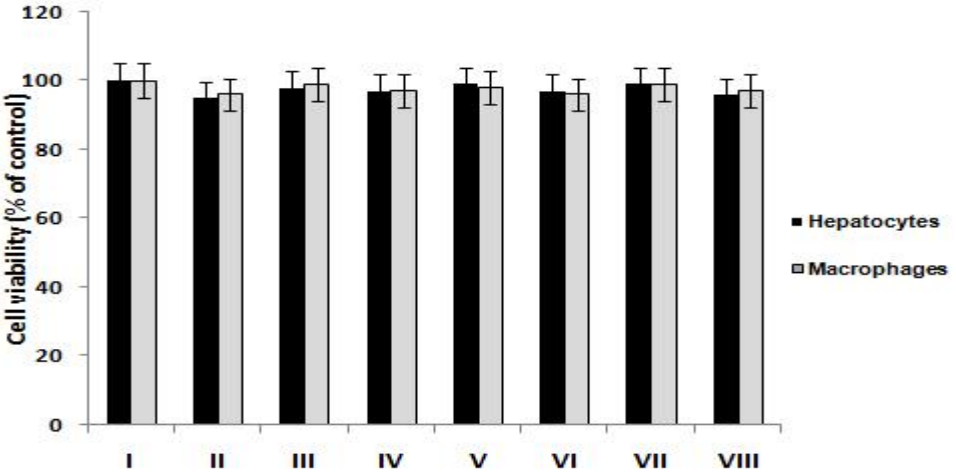

Figure 4: Effect of exogenous fatty acids and LXR α agonist on cell viability

Values are means ± SEM of three independent experiments.
I-control; II-T0901317 (1µM); III-laurate (500µM); IV- laurate (500 µM) + T0901317 (1µM); V-oleate (500µM); VI-oleate (500µM) + T0901317 (1µM); VII- linoleate (500µM); VIII-linoleate (500µM) +T0901317 (1µM).

Interacting effects of LXR α agonist and fatty acids on the mRNA expression of ABCA1 in hepatocytes and macrophages in culture

To study the role of LXRα –ABCA1 signaling pathways on fatty acid mediated cholesterol efflux, isolated hepatocytes and macrophages were incubated for 18 h with serum-free medium in the presence of different fatty acids (500µM) in the presence or absence of LXR α agonist, T0901317(1µM) .

In hepatocytes, presence of LXRα agonist T0901317 stimulated the mRNA expression of ABCA1. Treatment of laurate also increased the ABCA1 expression and co-incubation of laurate enhanced the stimulatory effect of agonist compared to untreated control and cells treated with oleate or linoleate. While treatment of oleate showed a significant downregulation in ABCA 1 expression and inhibited the stimulatory effect of agonist when compared to laurate and linoleate. Presence of linoleate also decreased the ABCA1 expression and inhibited the stimulatory effect of agonist compared to laurate in hepatocytes (Figure. 5).

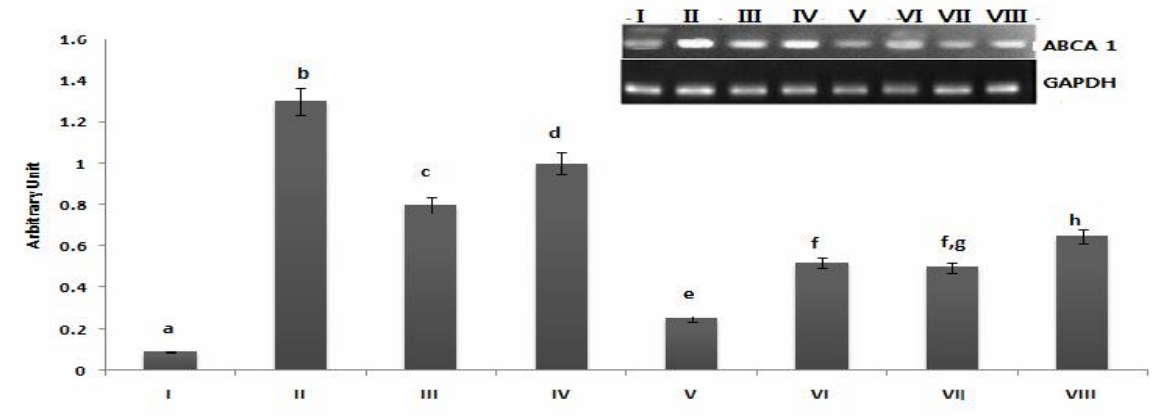

Figure 5: Interacting effects of LXR α agonist and fatty acids on the mRNA expression of ABCA1 in hepatocytes

Values are mean ± SEM of three separate experiments in triplicates. Values not sharing a common superscript differs significantly at p <0.05.I-control; II-T0901317 (1µM); III-laurate (500µM); IV- laurate (500 µM) + T0901317 (1µM); V-oleate (500µM); VI-oleate (500µM) + T0901317 (1µM); VII- linoleate (500µM); VIII-linoleate (500µM) + T0901317 (1µM).

In macrophages, laurate increased the ABCA1 expression and further enhanced the stimulatory effect of the agonist when compared to cells treated with oleate and linoleate. While the unsaturated fatty acids viz. oleate and linoleate decreased the ABCA1 expression in macrophages. The maximum inhibitory effect was observed with oleate followed by linoleate (Figure. 6).

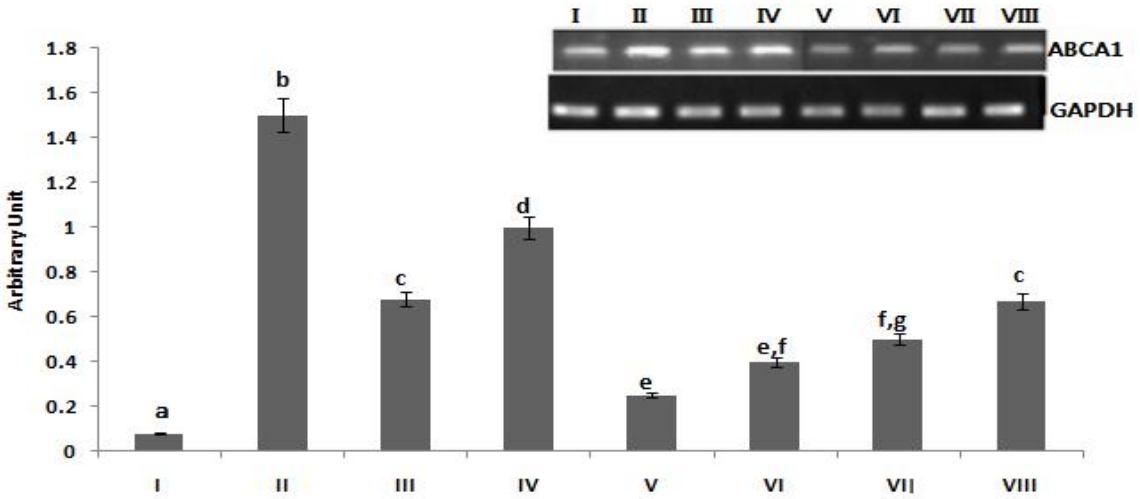

Figure 6: Interacting effects of LXR α agonist and fatty acids on the mRNA expression of ABCA1 in macrophages

Values are mean ± SEM of three separate experiments in triplicates. Values not sharing a common superscript differs significantly at p <0.05. I-control; II-T0901317 (1µM); III-laurate (500µM); IV- laurate (500 µM) + T0901317 (1µM); V-oleate (500µM); VI-oleate (500µM) + T0901317 (1µM); VII- linoleate (500µM); VIII-linoleate (500µM) + T0901317 (1µM).

Discussion

Present study indicates that supplementation of VCO stimulated RCT when compared to rats fed OO and SFO. Our preliminary studies using these test oils has reported that supplementation of VCO decreased the levels of total cholesterol in serum; but increased HDL cholesterol and apo A1 secretion in rats when compared to those fed other oils (Arunima and Rajamohan, 2012). As described before, HDL cholesterol in combination with apo A1 and LCAT plays a key role in mediating RCT (Gary *et al*., 2005; Tall, 1990). Increased levels of plasma high density lipoproteins protect against the development of atherosclerosis in rodents and humans possibly as a result of enhanced RCT i.e., transfer of cholesterol from the arterial wall to the liver, followed by excretion into bile (Benoit *et al*., 1999; Gordon *et al*., 1989). A major route for transport of HDL cholesteryl esters (CE) involves the selective uptake of CE in liver and steroidogenic tissues without the degradation of HDL protein (Rinninger *et al*., 1993; Glass *et al*., 1993). Recently, a member of the CD36 gene family, the scavenger receptor BI (SR-BI), encoded by 'SCAR B1' gene was shown to function as a receptor for HDL (Krause and Auerbach, 2001). SR B1 is an integral protein found in numerous cell types/tissues including liver and adrenal and which facilitate the selective uptake of cholesterol esters from HDLs in the liver (Kozarsky *et al*., 1997; Varban *et al*., 1998).

In the present study, an upregulation in the hepatic mRNA expression of SR B1 was observed in VCO fed rats. Experimental evidences indicate that SR B1 expression protects against atherosclerosis by mediating the hepatic uptake and biliary secretion of HDL cholesterol (Rigotti *et al*., 2003; Mardones *et al*., 2001).Moreover, the SRB1 expression in macrophage foam cells in atherosclerotic plaques may affect the flux of cholesterol between cells and HDL (Jian *et al*., 1998; Covey *et al.*, 2003). There are reports that dietary fats differentially modulate the hepatic expression of SR B1 (Hatahet, 2003). To date, few studies have focused on the role of individual fatty acids and expression of SR B1 (Acton *et al*., 1996). Reports demonstrated that supplementation of unsaturated fatty acids increases hepatic expression of SR B1 compared to saturated fatty acids (SFAs) (Hatahe *et al*., 2003; Spady *et al*., 1999). In contrast, Loison and colleagues (2002) suggested that SFAs increase HDL-cholesterol concentration and hepatic SR B1 expression in hamsters. In our present study an increased mRNA expression of SRB1 was observed in hepatocytes when exposed to laurate, which can be correlated with the increased SR B1 expression in VCO fed rats. Recent studies showed that, apart from fatty acids, polyphenols present in dietary oils have a role in enhancing the SRB1 expression (Marta Farràs *et al*., 2013). Chemical analysis of the test oils revealed that compared to other oils, VCO contain higher amounts of polyphenols (Arunima and Rajamohan,2013), which may enhance the SRB1 expression in hepatocytes.

ATP binding cassette transporter A1 (ABCA1) is recognized as the principal molecule involved in cholesterol efflux from macrophage foam cells (Oram *et al*., 2000). It is expressed in a variety of cell types, including hepatocytes and macrophages and is highly upregulated upon lipid loading through the activation of the nuclear liver X receptors (LXR) (Costet *et al*., 2000; Schwartz *et al*., 2000). Results indicate that supplementation of VCO showed a significant increase in the hepatic ABCA 1 mRNA expression compared to those fed OO and SFO. There are reports that LXR activation markedly increases ABCA1 mRNA and protein levels (Jiang *et al*., 2006). In the present study, supplementation of VCO also increased the mRNA expression of LXRα compared to those fed OO and SFO. LXR α is a member of the ligand activated transcription factor of nuclear receptor super family, whose activation leads to modulation in the expression of genes involved in cholesterol homeostasis including ABCA1, which plays a crucial role in HDL cholesterol remodeling (Brunham *et al*., 2006). Hence, the observed LXR activation in VCO supplementation may stimulate the ABCA1 expression and mediate cholesterol efflux in VCO fed rats.

ABCA1 expression is under tight regulation; cholesterol loading of macrophages dramatically increases ABCA1 mRNA and protein levels (Lawn *et al*., 1999; Langmann *et al.*, 1999). This regulation is mediated by nuclear LXRs (LXRα or LXRβ) and retinoid X receptor (RXR) (Schwartz *et al*., 2000; Costet *et al*., 2000), which form heterodimers that are activated by oxysterols and retinoic acid, respectively (Repa and Mangelsdorf, 1999). Because of their robust transcriptional regulation of ABCA1, LXRs have become attractive new targets for drug development. There is evidence that fatty acids may appear to regulate LXR α activation as well as ABCA1 transcription (Mark Bouwens *et al*., 2010).

For studying the effect of major fatty acids present in test oils on LXRα-ABCA1 pathway in detail, hepatocytes were incubated with different fatty acids (500µM). Our observations indicate that laurate, the major fatty acid found in oil extracted from coconut upregulated the LXRα expression and further stimulated ABCA1 expression in hepatocytes compared to cells treated with unsaturated fatty acids *viz*. oleate and linoleate. There are reports that SFAs can upregulate ABCA1 gene expression compared to unsaturated fatty acids (Uehara *et al.*, 2007). In addition, these unsaturated fatty acids are known to suppress ABCA1 gene expression dose dependently and can reduce apoA1 mediated cholesterol efflux by destabilizing ABCA1 protein in macrophages (Yutong Wang *et al*., 2004; Uehara *et al*., 2002). This may be due to the fact that unsaturated fatty acids may compete for oxysterol binding to LXR and thus antagonize the activation of LXR by oxysterols (Ou *et al*., 2001). This might be one of the reasons for decreased ABCA1 expression in oleate and linoleate treated cells. Another possibility is that, activators of peroxisome proliferator-activated receptor alpha (PPAR α) can also increase the ABCA1 expression (Jiang *et al*., 2006). There are evidence that PPARα binds with LXR and

the PPARα-LXR heterodimerization enhances the ABCA1 expression (Costet *et al*.,2000;Repa *et al*.,2000; Chen *et al.*, 2001;Venkateswaran *et al*.,2000). Analysis of the molecular mechanism has revealed that PPAR α agonist, GW7647 stimulates cellular cholesterol efflux and correspondingly the RCT pathway by upregulation of ABCA1 in macrophages via a PPARα-LXR-dependent pathway (Nakaya *et al*., 2011). From our earlier studies, it was observed that laurate can stimulate the PPARα expression and enhanced the apo A1 secretion in hepatocytes via PPARα dependenent pathways in hepatocytes (Arunima and Rajamohan, unpublished). Since laurate act as a PPARα ligand, which may upregulate the ABCA1expression via PPARα-LXR-dependent pathways.

Furthermore, the molecular aspects of LXRα-ABCA1 pathways in fatty acid mediated RCT was studied using LXRα agonist T0901317. Results revealed that laurate stimulates the ABCA1 expression and enhanced the stimulatory effect of the agonist both in hepatocytes and macrophages. While the unsaturated fatty acids viz.oleate and linoleate downregulated the ABCA1 expression and inhibited the stimulatory effect of the agonist in both cell types. Hence, it is clear that laurate mediates cholesterol efflux partly via LXRα-ABCA1 pathway in macrophages. Other reports also indicate that unsaturated fatty acid decreases ABCA1 mRNA expression in macrophages by a mechanism consistent with antagonism (Uehara *et al*., 2002).

From the above observations, it can be concluded that supplementation of VCO enhances LXR α-ABCA1 pathway involved in reverse cholesterol transport compared to OO and SFO. Furthermore, laurate mediates reverse cholesterol transport partly *via* PPAR α - LXR α - ABCA1 pathways in macrophages.

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Conflict of Interest

The study authors declare no conflicts of interest.

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