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Dietary docosahexaenoic acid reverses nonalcoholic steatohepatitis and fibrosis caused by conjugated linoleic acid supplementation in mice

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ABSTRACT

It has been shown that docosahexaenoic acid (DHA) prevents nonalcoholic fatty liver disease (NAFLD) and insulin resistance (IR) caused by conjugated linoleic acid (CLA), a *trans* fatty acid (TFA). Here, we evaluated whether DHA will reverse existing CLA-induced NAFLD and IR in mice. DHA-specific effects on existing NAFLD involved significant ($P < 0.005$) lowering of hepatic weight and triacylglycerol content and expression of genes involved in fatty acid synthesis, enhancing expression of genes involved in fatty acid oxidation, and increasing serum adiponectin levels. Also, immunohistochemistry showed lower expression of hepatic CD163 (inflammation) and smooth muscle α-actin (fibrosis). Compared to the CLA diet, mice fed DHA and control diets had significantly ($P < 0.05$) lower serum insulin and ALT activity, but only DHA had lower ($P = 0.05$) expression of genes involved in fibrosis. DHA supplementation for 4 weeks reversed already existing hepatic steatosis, inflammation, and fibrosis caused by CLA.

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Abbreviations: *Acca*, acetyl co-A carboxylase A; *Acox1*, acyl coenzyme A oxidase 1; AT, adipose tissue; ALT, alanine aminotransferase; ARS, Agricultural Research Service; CLA, conjugated linoleic acid; *Col1a1*, procollagen type I alpha 1; CON, control; *Cpt1a*, carnitine palmitoyltransferase 1a-liver; DHA, docosahexaenoic acid; ECM, extracellular matrix; EPA, eicosapentaenoic acid; HOMA-IR, homeostasis model assessment of insulin resistance; H&E, haematoxylin and eosin; HSC, hepatic stellate cells; IR, insulin resistance; IL-6, interleukin-6; IL-8, interleukin-8; ALA, alpha-linolenic acid; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PPARG, peroxisome proliferator-activated receptor gamma; *Scd1*, stearoyl-coenzyme A desaturase 1; SMA, smooth muscle α-actin; T2DM, type 2 diabetes mellitus; TFA, *trans* fatty acid; TG, triglycerides; *Timp1*, tissue inhibitors of metalloproteinase-1; TNF-α, tumour necrosis factor alpha; USDA, United States Department of Agriculture

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1. Introduction

Prevalence of obesity has remained high in the US since 2003; more than one third of adults (~78 million people) and nearly one in five youths aged 2–19 years old are obese (Ogden, Carroll, Kit, & Flegal, 2014). Obesity remains to be a common and costly health condition in the US, costing $147 billion in 2008 (Finkelstein, Trogdon, Cohen, & Dietz, 2009). The social and medical impacts of being overweight and obese have prompted consumers to seek over-the-counter management solutions for weight loss or gain prevention. This is substantiated by the US weight loss market segment which was over $61 billion in 2012 (Marketdata Enterprises, 2013). A specific ingredient claimed to reduce body weight and fat loss is a mixture of conjugated *trans* fatty acid (TFA) isomers, i.e. *trans*-10, *cis*-12 and *cis*-9, *trans*-11 conjugated linoleic acid (CLA), which are available in pill-form supplements, foods, and beverages (Larsen, Toubro, & Astrup, 2003). Chemically speaking CLA is a TFA, but it is exempt from the mandatory nutrition labelling of TFA in foods that went into effect in 2006 because the US Food and Drug Administration defines TFAs to include only non-conjugated forms, i.e. isolated *trans* double bonds (FDA, 2003). However, the Institute of Medicine of the National Academy of Sciences defines TFAs to include both conjugated and non-conjugated forms. CLA isomers in dietary supplements are conjugated TFA. Although CLA isomers hold GRAS (generally regarded as safe) status from the FDA, the European Food Safety Authority panel concludes that the safety of CLA consumption longer than 6 months has not been established under its proposed use (EFSA, 2012).

Consumption of conjugated TFA, such as CLA, for weight loss has been increasing, and global sales of the CLA ingredient alone are expected to reach $200 million by 2017 (PRWeb.com, 2011). Consumer interest in CLA exists due to the marketing of its claimed anticarcinogenic, antiadipogenic, antiatherosclerotic, and delipidating effects (Blankson et al., 2000; Ip, Masso-Welch, & Ip, 2003; Stachowska et al., 2012). However, the side effects of CLA, particularly due to the t10, c12 CLA isomer, include hepatic steatosis (lipid accumulation), inflammation, and insulin resistance (IR) in animals and humans (Fedor, Adkins, Mackey, & Kelley, 2012; Martinez, Kennedy, & McIntosh, 2011; Poirier, Shapiro, Kim, & Lazar, 2006; Riserus, Arner, Brismar, & Vessby, 2002). IR is a characteristic of T2DM and is associated with metabolic disorders. The resistance of adipose tissue (AT) to the action of insulin results in unsuppressed lipolysis, thereby releasing free fatty acids for liver uptake, re-esterification, and triacylglycerol (TG) storage. Consequently, this steatosis makes the liver susceptible to inflammation due to cellular events involving oxidative stress, lipotoxicity, mitochondrial dysfunction, pro-inflammatory cytokines, and hepatic stellate cell (HSC) activation, which promotes fibrogenesis (Browning & Horton, 2004; Malhi & Gores, 2008). Collectively, liver inflammation and injury present in steatotic liver result in nonalcoholic steatohepatitis (NASH), a progressive stage of nonalcoholic fatty liver disease (NAFLD) that leads to end stage liver disease.

NAFLD is regarded as the liver component of metabolic syndrome and is the most common form of chronic liver disease in developed countries, and it affects more than 30% of the US population (Browning et al., 2004). Its prevalence is even greater in high risk groups; NAFLD is present in 70% of adult T2DM patients and over 90% in those who are obese with associated IR, and 20% of NAFLD may develop to NASH, which requires medical attention (Henao-Mejia et al., 2012). More concerning is the rapid increase in NAFLD in the paediatric population worldwide over the last decade and its prevalence is increased to 85% in obese children (Welsh, Karpen, & Vos, 2013). Simple steatosis is considered to be a benign condition; however, NASH-associated end stage liver disease will be the main cause for liver transplants in the US in the next two decades. Between 26% and 37% of NASH patients develop fibrosis within 6 years, and 9% of these patients develop cirrhosis within 10 years; incidence of NASH-associated hepatocellular carcinoma is predicted to increase in the US in the coming decades (Starley, Calcagno, & Harrison, 2010).

Lifestyle intervention is the first line of defence to reduce hepatic steatosis, but reports on improvements in other histological aspects of the disease are lacking (Thoma, Day, & Trenell, 2012). Limited medicinal intervention is available for NASH in adults and none exists for children. Insulin sensitisers such as thiazolidinediones have been suggested as therapeutic candidates for improving steatosis and inflammation; however, its long-term safety has been questioned (Lincoff, Wolski, Nicholls, & Nissen, 2007). Nutritional interventions such as vitamin E and n-3 PUFAs offer evidence for their use but are not recommended for all populations, and interpretations of the results from human studies have been limited due to a small sample size, duration of treatment, and dosage administered (Chalasani et al., 2012).

Previous studies using fish oil, which contains a mixture of eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), prevented the CLA-induced NAFLD and IR (Ide, 2005; Winzell, Pacini, & Ahren, 2006). To understand the role of EPA and DHA individually, we compared their efficacy in preventing CLA-induced IR and NAFLD (Vemuri, Kelley, Mackey, Rasooly, & Bartolini, 2007). We found that both EPA and DHA prevented the CLA-induced NAFLD while only DHA prevented the increase in IR, and it also partially prevented the decrease in circulating adiponectin. Our choice to use DHA in the current study was based on the results of our previous study (Vemuri et al., 2007). DHA possesses several metabolic and health promoting effects (Adkins & Kelley, 2010) and is present in a variety of natural and supplemented food products. The popularity of CLA as a weight-loss supplement comes from the marketing of its ability to reduce body fat mass without disclosure of its adverse effects, including those on NAFLD (Iwata et al., 2007; Ramos, Mascarenhas, Duarte, Vicente, & Casteleiro, 2009). The current study examined the capability of DHA to reverse existing CLA-induced (1) hepatic steatosis through the suppression of hepatic lipogenesis and activation of fatty acid oxidation, (2) hepatic inflammation and fibrosis by inhibiting tissue macrophage and HSC activation and serum alanine aminotransferase (ALT) activity, and (3) IR and low adiponectin levels by restoring AT mass.

2. Materials and methods

2.1. Experimental animals and diets

The experimental protocol was approved by the Institutional Committee for the Animal Use and Care of University of California, Davis (Animal Protocol #15018). Eight-week old C57BL/6N female mice (Charles River, Raleigh, NC) were maintained in the Genome and Biomedical Sciences Facility by the Teaching & Research Animal Care Services. The strain and sex of the animals, experimental conditions, and diet preparation were based on our previous studies (Fedor et al., 2012; Kelley et al., 2009; Warren et al., 2003). Diets were prepared by constant mixing and flushed with nitrogen gas to prevent fatty acid oxidation, packaged into 20 g aliquots, flushed with nitrogen gas for storage, and kept at –20 °C. Fresh diet (20 g/cage) was served daily and the food left in the jars from the previous day was weighed and recorded. Body weight was recorded every 7 d. All experiments were in compliance with the guidelines of the National Institutes of Health for experimental animals. All mice were fed Picolab Rodent Diet 20 (LabDiet, St. Louis, MO, USA) providing 24, 13, and 62% energy from protein, fat, and carbohydrate, respectively, for a 1 wk acclimatisation period before they were randomly assigned to 1 of 5 experimental diet groups for 8 wk (8 animals per group and 4 mice per cage). A modified AIN93G diet (5% total fat content; 50 g fat/kg diet) was used as the base diet, in which soybean oil was replaced with corn oil to decrease ALA content to control for the endogenous conversion of ALA to DHA that could potentially blunt the CLA effects on hepatic steatosis (Table S1). Three groups of mice were fed diets containing 0.5% (w/w) CLA for 4 weeks to establish NAFLD and IR, then their diets were replaced with the control (CLA→CON), CLA+1.5% DHA (CLA→CLA + DHA), or 1.5% DHA (CLA→DHA) diets for another 4 weeks. Two additional groups of mice were fed either CON or CLA for 8 weeks to verify the effects of a CLA-enriched diet (Fig. S1). To confirm the occurrence of NAFLD, hyperinsulinaemia, and loss of AT mass in the CLA diet group before the switch to the replacement diets, separate mice were fed either the CON or CLA diets for 4 weeks (Table S2). The DHA and CLA (Larodan Fine Chemicals, Malmo, Sweden) used in the diets were free fatty acids and were greater than 90% pure. Fatty acid composition of the diets was determined as previously described (Fedor, Adkins, Newman, Mackey, & Kelley, 2013).

2.2. Tissue collection and liver immunohistochemistry and triacylglycerol analysis

Animals were terminated by CO_2 asphyxiation after withholding food for 10–12 hours. Blood was collected by cardiac puncture and centrifuged in serum separator tubes (BD Microtainer SST, Franklin Lakes, NJ, USA). Tissues were collected, weighed, and snap-frozen in liquid nitrogen and stored at –80 °C for future analysis. A portion of the liver was fixed with 10% neutralised formalin, embedded in paraffin and sectioned in 4 μm thickness (Anatomic Pathology Services, Veterinary Medical Teaching Hospital, UC Davis, CA, USA). Liver sections from paraffin-embedded tissues were stained with haematoxylin and eosin (H&E) and probed with antibodies against CD163 and smooth muscle α-actin (SMA) (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) (Adkins et al., 2013). Morphological analysis of liver tissues was performed using a Keyence fluorescence microscope (Elmwood Park, NJ, USA) at the UC Davis Institute for Regenerative Cures (Sacramento, CA, USA).

TG was extracted from frozen liver (Fedor et al., 2012). Briefly, ~25 mg of frozen liver was powderised and lipids were extracted with 2:1 (v/v) chloroform/methanol and centrifuged at 10,000 × g. The supernatant was transferred to a clean glass vial and the residual precipitate was re-extracted with chloroform/methanol. The lipid emulsion was broken by adding 1M NaCl, centrifuged, and the lipid phase collected, and dried. Total lipids were reconstituted using 1 mL of 60% butanol and 40% Triton-114/methanol (2:1). Liver TG content was measured using the Wako L-type TG M kit (Wako Diagnostics, Richmond, VA, USA).

2.3. Quantitative real-time PCR analysis (qRT-PCR)

Total RNA was isolated from the liver using the PureLink RNA Mini Kit according to manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). RNA quality and integrity were verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Purified total RNA (500 ng) from each sample was used for first strand synthesis (Superscript III First Strand Synthesis System; Life Technologies, Grand Island, NY, USA). RNA abundance for hepatic acetyl-CoA carboxylase A (*Acca*), stearoyl-coenzyme A desaturase 1 (*Scd1*), acyl coenzyme A oxidase (*Acox1*), carnitine palmitoyltransferase 1a-liver (*Cpt1a*), tissue inhibitor of metalloproteinase 1 (*Timp1*), and procollagen type I, alpha 1 (*Col1a1*) was determined by using gene-specific Taqman primers and 6-carboxyfluorescein-dihydrocyclopyrroloindaole minor groove binder (FAM-MGB)-labelled probes per the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Approximately 15 ng of cDNA was air dried overnight in each well of a 384-well plate prior to adding the PCR reagents (4 μL of 2× Taqman Gene Expression Master Mix, 0.4 μL specific primer-probe assay, and 3.6 μL water). PCR reactions were conducted in triplicate and measured in real-time using an ABI 7900 HT Fast Real-Time PCR System and documented by SDS software v.2.2.2 (Applied Biosystems). Percent of control gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, which normalises against the β-actin endogenous control gene and the control group average delta cycle threshold (ΔCt) (Adkins et al., 2013).

2.4. Analyses of serum adiponectin, glucose, and alanine aminotransferase activity levels

Enzyme-linked immunosorbent assays were used to measure serum adiponectin (Millipore, Billerica, MA, USA) and insulin (Mercodia, Winston Salem, NC, USA) levels. Glucose was measured using an enzymatic glucose assay (Sigma-Aldrich, St. Louis, MO, USA), and alanine aminotransferase (ALT) activity was determined by monitoring the rate of NADH oxidation to NAD^+ employing lactate dehydrogenase (Cayman Chemicals, Ann Arbor, MI, USA).

2.5. Statistics

SAS version 9.4 statistical software was used for all analyses (SAS, 2013). The Kenward–Roger method was used for estimating the degrees of freedom in the denominator of F tests for the fixed effects: diet, week, and the interaction. The random effects are cage within diet, week × cage within diet, and animal within cage and diet. The latter nested effect defines the experimental units for incorporating a first order, autoregressive covariance structure among the repeated measures. A mixed model was also used for other variables not involving weeks, with diet as the fixed effect and cage within diet as the random effect. Single degree of freedom contrasts were used to make the four comparisons to the CLA diet group, i.e. 8 week CON vs CLA diet group and each of the 3 replacement diet groups vs CLA diet group. Log transformation was used for all gene expression variables as well as the three AT weight variables. When transformation did not satisfy the heterogeneity of variance problem, the heterogeneity was incorporated in the model. All data shown are means ± SEM. Differences were considered significant at $P \leq 0.05$.

3. Results

The current study examined whether DHA attenuated existing CLA-induced pathologies; thus, comparisons were made between mice fed each of the 3 replacement diets (CLA→CON, CLA→CLA + DHA, and CLA→DHA) to mice fed the CLA diet. For all endpoint variables, mice fed the CLA diet for 8 weeks were significantly ($P < 0.05$) different from mice fed the CON diet for 8 weeks except serum glucose levels (215 ± 12 vs. 207 ± 9 mg/dL). No difference in food intake or body weight was observed between mice fed the replacement diets compared to that of CLA-fed mice (data not shown); all groups consumed 95–100% of their food.

3.1. DHA reverses CLA-induced hepatic steatosis

Livers of mice fed the 3 replacement diets weighed significantly ($P < 0.001$) less than that of the CLA-fed group (Fig. 1A). Similarly, the hepatic TG contents of mice fed the replacement diets were significantly ($P < 0.05$) lower than those in the CLA-fed mice (Fig. 1B); the CLA→CLA + DHA and CLA→DHA diet-fed mice had lower liver TG content (64 and 76% reduction, respectively; $P < 0.005$) compared to the CLA-fed mice. Replacing the diet with CLA→CON also resulted in lower TG content (47% reduction, $P < 0.05$) than the CLA-fed mice. Serum ALT activity was measured to assess liver injury (Fig. 1C). Mice fed the CLA→CON and CLA→DHA diets had significantly ($P < 0.05$) lower ALT activity than mice consuming the CLA diet, indicating reduced liver injury in these two groups.

The examination of H&E stained liver sections for steatosis corroborated the chemically determined liver TG concentrations (Fig. 2A–E). Mice fed the replacement diets exhibited less steatosis compared to the CLA-fed diet; these diets attenuated existing steatosis, and livers of mice fed the CLA→DHA diet appeared to be free from steatosis.

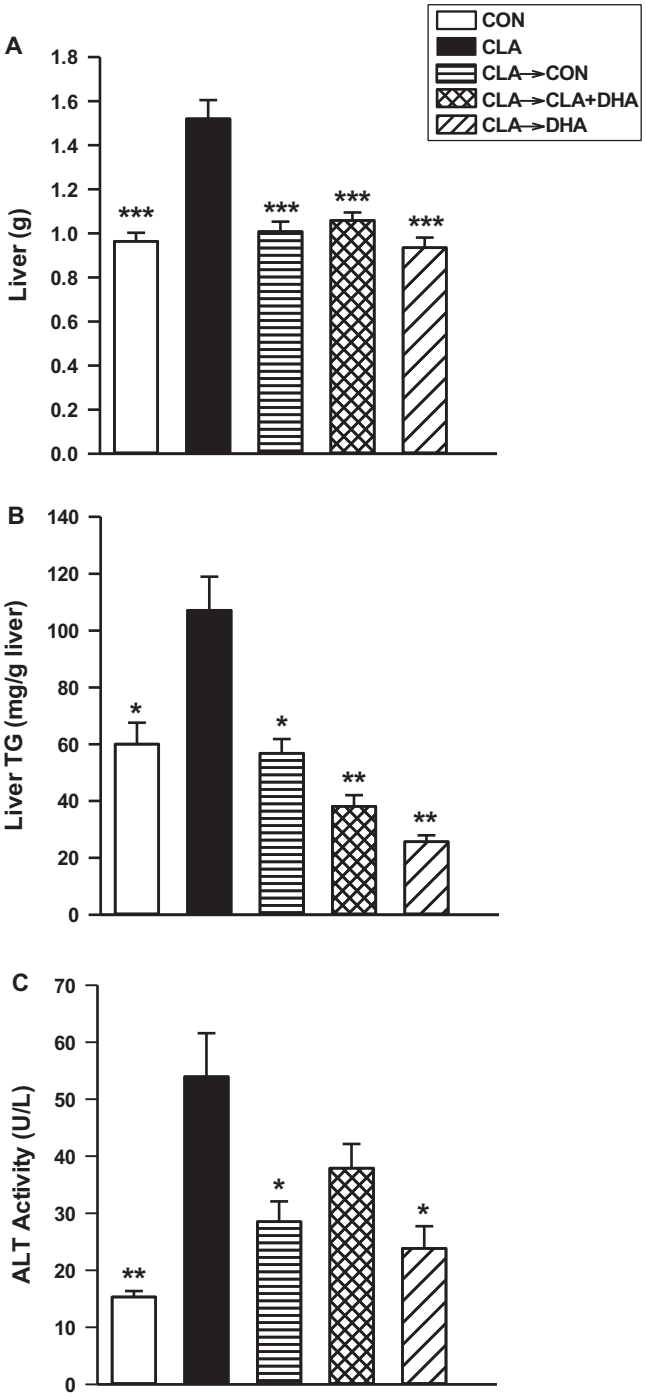

Fig. 1 – Attenuation of CLA effects on (A) liver weight, (B) liver triacylglycerol, and (C) serum alanine aminotransferase (ALT) activity by mice fed a CLA diet for 4 weeks followed by diets replaced with control (CLA→CON), CLA + DHA (CLA→CLA + DHA), or DHA (CLA→DHA) for another 4 weeks. Data are expressed as mean ± SEM, N = 7–8 per group, except ALT is N = 6. *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$ compared with CLA-fed mice. CLA, 0.5% t10,c12 conjugated linoleic acid; DHA, 1.5% docosahexaenoic acid.

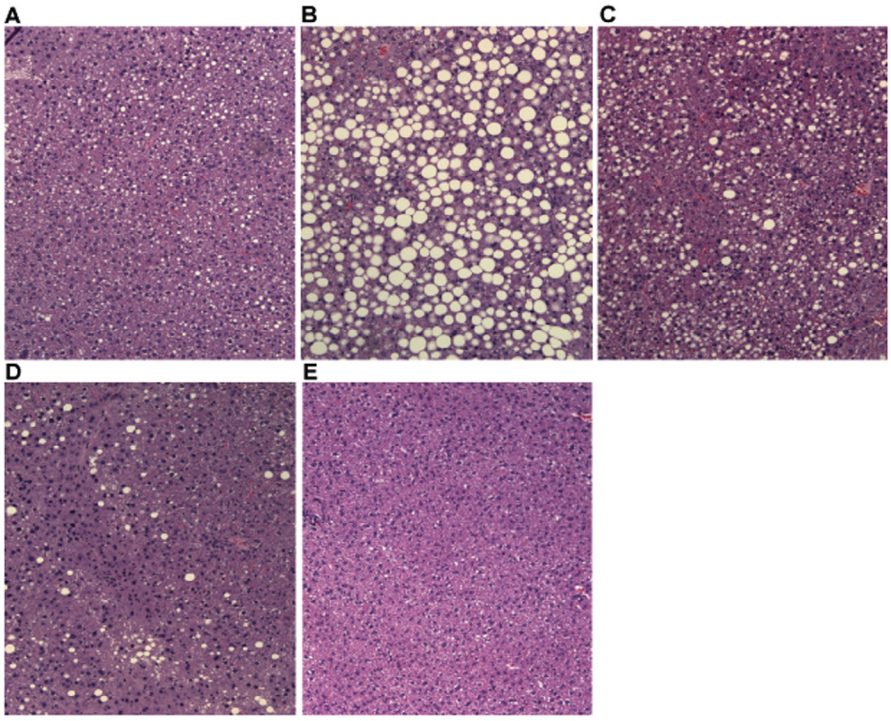

Fig. 2 – Representative photomicrographs of haematoxylin and eosin stained livers (×200) of mice fed a (A) control diet (CON) or (B) CLA diet for 8 weeks, or a CLA diet for 4 weeks followed by diets replaced with (C) control (CLA→CON), (D) CLA + DHA (CLA→CLA + DHA), or (E) DHA (CLA→DHA) for another 4 weeks. CLA, 0.5% t10,c12 conjugated linoleic acid; DHA, 1.5% docosahexaenoic acid.

3.2. *DHA supplementation reverses CLA-induced changes in hepatic gene expression*

The expression of hepatic genes involved in fatty acid synthesis, *Acca* and *Scd1*, and β-oxidation, *Acox1* and *Cpt1a*, was determined to explore possible mechanisms by which DHA reduces TG accumulation. Mice fed the 3 replacement diets had significantly ($P < 0.0001$) lower expression of *Acca* and *Scd1* compared to the CLA-fed mice (Fig. 3A and B). Mice fed the 3 replacement diets had significantly ($P < 0.01$) higher mRNA expression of *Acox1* when compared to mice fed the CLA diet; mice fed the CLA→CLA + DHA and CLA→DHA diet exhibited a 4.6- and 9.8-fold increase in *Acox1* expression, respectively, suggesting a DHA specific effect (Fig. 3C). Compared to mice fed the CLA diet, the expression of *Cpt1a* was greater in mice fed the CLA→CON (4.1-fold; $P < 0.001$) and CLA→CLA + DHA diets (4.7-fold; $P < 0.001$), but not the CLA→DHA diet because of the large variance within this group. (Fig. 3D).

3.3. *DHA resolves hepatic inflammation and mild fibrosis*

Hepatic fibrosis reflects a process resulting from decreased degradation and increased synthesis of the extracellular matrix (ECM) components. Matrix metalloproteinases are involved in the degradation of ECM, which is an important feature in tissue repair. *Timp1*, a valued indicator of hepatic fibrogenesis, decreases the activity of metalloproteinases and thus the clearance of ECM. Thus, overall accumulation of ECM depends upon its synthesis and the activities of metalloproteinases and their inhibitors. Mice fed the CLA→DHA diet had significantly lower (62% reduction; $P < 0.05$) *Timp1* mRNA expression compared to CLA-fed mice, indicating a DHA-specific anti-fibrotic response in steatotic liver (Fig. 3E). *Col1a1* is a marker for ECM synthesis and mice fed the CLA→DHA diet showed lower (46% reduction; $P = 0.05$) *Col1a1* expression when compared to the CLA-fed mice (Fig. 3F).

CD163 staining of the livers was used to determine whether or not Kupffer cells were activated in CLA-induced NAFLD (Fig. 4A–E). CD163 positive cells were detected with high frequency in mice fed the CLA diet compared to the CON diet. No CD163 staining was observed in livers of mice fed the CLA→CLA + DHA diet, suggesting a strong anti-inflammatory effect of DHA over CLA. SMA is a marker for activated HSC, which produces ECM proteins in fibrosis in response to hepatic injury. Abundant positive SMA staining was observed in the sinusoids of the livers of mice fed the CLA diet compared to mice fed the CON diet (Fig. 4F–J), suggesting activated HSC and a response to cellular changes.

3.4. *DHA does not attenuate elevated serum insulin but restores adiponectin levels*

Mice fed the CLA→CON and CLA→DHA diets both had significantly ($P < 0.05$) lower insulin levels than mice consuming the CLA diet, indicating a DHA independent effect (Fig. 5A), but differences were not observed between mice fed the CLA and CLA→CLA + DHA diet. No difference was observed in the HOMA-IR index between mice fed the CLA→CON and CLA→DHA diets versus mice fed the CLA diet; however, the CLA→CLA + DHA fed mice had a higher (1.6-fold; $P < 0.05$) HOMA-IR index compared to the CLA-fed mice (Fig. 5B). This may be explained by

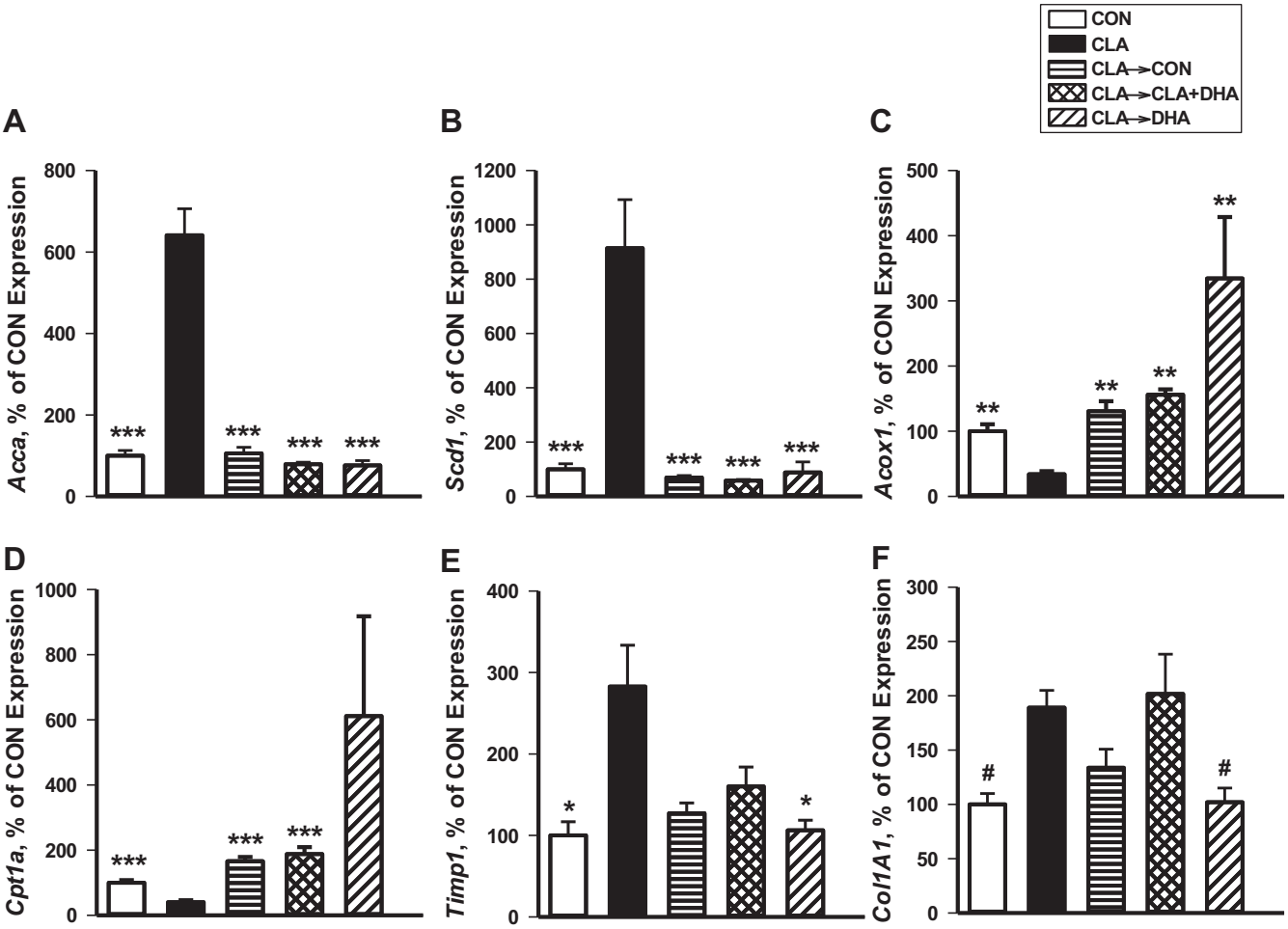

Fig. 3 – Attenuation of CLA effects on mRNA expression of (A) acetyl-CoA carboxylase A (*Acca*), (B) stearoyl-coenzyme A desaturase 1 (*Scd1*), (C) acyl coenzyme A oxidase (*Acox1*), (D) carnitine palmitoyltransferase 1a-liver, (*Cpt1a*), (E) tissue inhibitor of metalloproteinase 1 (*Timp1*), and (F) procollagen type 1, alpha 1 (*Col1a1*) by mice fed a CLA diet for 4 weeks followed by diets replaced with control (CLA→CON), CLA + DHA (CLA→CLA + DHA), or DHA (CLA→DHA) for another 4 weeks. Results are expressed as mean ± SEM, N = 7 per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#}P = 0.05$ compared with CLA-fed mice. CLA, 0.5% t10,c12 conjugated linoleic acid; DHA, 1.5% docosahexaenoic acid.

the higher fasting serum glucose concentration in the CLA→CLA + DHA group (1.2-fold; $P < 0.01$) compared to the mice in the CLA diet group (Fig. 5C). Adiponectin is a cytokine expressed by AT and sensitises the body to insulin. Mice fed the replacement diets had significantly ($P < 0.01$) higher adiponectin concentration compared to the CLA-fed mice (Fig. 5D). Adiponectin concentration in mice fed the CLA→CLA + DHA and CLA→DHA diets were 1.4-fold and 3.1-fold higher, respectively, compared to the CLA-fed mice, indicating that the increase is attributable to DHA.

3.5. *Eliminating CLA from the diet counteracts CLA-induced delipidating effects in adipose tissue*

For all AT depots studied, the retroperitoneal, periovarian, and periuterine, mice fed the CLA→CON and CLA→DHA diets had significantly higher AT mass than CLA-fed mice (Fig. S2). No differences were observed between AT mass of mice fed the CLA and CLA→CLA + DHA diets, indicating a strong CLA effect over DHA.

4. Discussion

This work demonstrated that feeding mice a CLA-enriched diet for 8 weeks resulted in higher liver weight and TG content, insulin concentration, HOMA-IR, and hepatic lipogenic and fibrogenic enzyme gene expression, and lower AT mass, hepatic β-oxidation gene expression, and adiponectin concentration, compared to mice fed the CON diet for 8 weeks. We also verified the presence of NAFLD and IR in CLA-fed mice at 4 weeks before switching over to their replacement diets. The principal findings in the current study are that DHA reversed existing CLA-induced NAFLD through the attenuation of hepatic lipid accumulation, inflammation, and fibrogenesis in mice. The single concentration of DHA used in this study was successful in attenuating some pathophysiologic alterations of CLA-induced NAFLD, while other variables were normalised by the removal of CLA from the diet. The largest impact that DHA had in our outcomes was its ability to lower the existing CLA-induced increase in liver TG by the CLA→CLA + DHA and

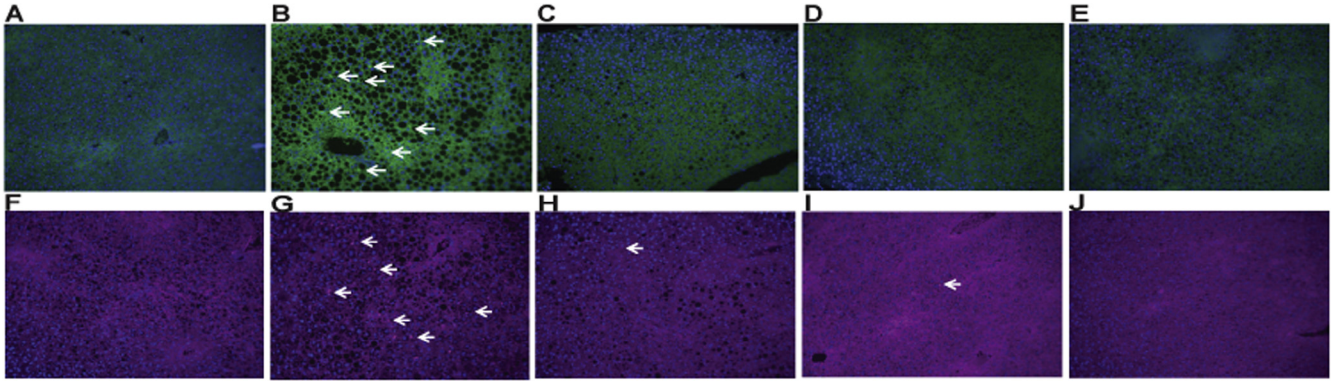

Fig. 4 – Attenuation of CLA effects on (A–E) Kupffer cell activation visible after immunohistochemical staining with antibodies against CD163 and (F–J) activated hepatic stellate cells visible after staining with antibodies against smooth muscle α-actin in liver sections of mice fed a CLA diet for 4 weeks followed by diets replaced with control (CLA→CON; Panels C and H), CLA + DHA (CLA→CLA + DHA; Panels D and I), or DHA (CLA→DHA; Panels E and J) for another 4 weeks. Panels A and F = control-fed mice (CON) for 8 weeks and Panels B and G = CLA-fed mice for 8 weeks. Liver sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for nuclei (×200). CLA, 0.5% t10,c12 conjugated linoleic acid; DHA, 1.5% docosahexaenoic acid.

CLA→DHA diets. DHA and other n-3 PUFAs have been studied in humans to reduce hepatic fat content in NAFLD patients (Capanni et al., 2006; Dasarathy et al., 2014; Nobili et al., 2013; Scorletti et al., 2014; Spadaro et al., 2008), and were effective in some but not all studies.

Hepatic steatosis arises from a combination of an imbalance of hepatic fatty acid uptake, *de novo* lipogenesis, fatty acid oxidation, and TG elimination via VLDL assembly and secretion. Hepatic lipogenesis is initiated by the enzyme, ACCA, which converts acetyl Co-A to malonyl-CoA, which is then used as a substrate for fatty acid synthase to form palmitic acid (C16:0). Palmitic acid can then be desaturated by SCD1 to palmitoleic acid (C16:1) or elongated to stearic acid (C18:0) and further desaturated to oleic acid (C18:1), which can then be used to form TG. TG accumulation in the liver can also be attributed to a decrease in fatty acid oxidation via the increase in malonyl-CoA, which in turn inhibits CPT1A, a transferase that transports fatty acyl-CoA into the mitochondria for β-oxidation. ACOX1 is the first and rate-limiting enzyme that catalyses peroxisomal β-oxidation of long chain fatty acids, and a disruption of the ACOX1 gene results in the development of severe microvesicular steatohepatitis (Fan et al., 1996). Results from this study indicate that the increase in the expression of *Acca* and *Scd1* genes and decrease in the expression of *Acox1* and *Cpt1a* genes caused by the CLA diet contributed to hepatic TG deposition and that DHA counteracted this CLA-induced response. It is also possible that an imbalance of increased hepatic fatty acid uptake and decreased TG removal could contribute to the CLA-induced hepatic steatosis; however, this aspect was not monitored in this study.

The mechanisms by which CLA induces hepatic steatosis may not only be limited to actions directly on the liver, but also on the AT. We observed a CLA-induced reduction of AT mass was accompanied by hepatomegaly with steatosis. CLA has been shown to decrease TG content in AT by increasing lipolysis caused by the differential expression and localisation of lipid droplet-associated proteins such as perilipin (lipolysis modulation) and adipose differentiation-related protein (lipid storage) (Chung, Brown, Sandberg, & McIntosh, 2005; den Hartigh, Han, Wang, Omer, & Chait, 2013). A decrease in the expression of perilipin, the outer surface lipid droplet protein that serves to protect lipids from hydrolysis by lipases in AT, would increase basal lipolytic activity and decrease fat mass (Tansey et al., 2001). Furthermore, CLA has been shown to increase proinflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interleukins 6 (IL-6) and 8 (IL-8) that can induce adipocyte delipidation (Brown et al., 2004; LaRosa et al., 2006; Tsuboyama-Kasaoka et al., 2000); TNF-α has the ability to terminate perilipin gene expression, thus leading to lipolysis (Souza et al., 1998), and IL-6 and IL-8 can activate mitogen-activated protein kinase/extracellular signal-related kinase signalling, which can induce delipidation (Brown et al., 2004). CLA has also been shown to decrease the expression of AT peroxisome proliferator-activated receptor gamma (PPARG), a transcription factor that regulates adipocyte differentiation, and its downstream target genes related to glucose and fatty acid uptake and utilisation (Brown et al., 2004; Fedor et al., 2013). In all, the resultant nonesterified fatty acids that are released into circulation can be taken up by non-lipid storing peripheral tissues, such as the liver and muscle.

Adiponectin is an AT derived hormone that regulates hepatic insulin sensitivity and plays a role in reducing lipogenic activity and the induction of fatty acid oxidation and anti-inflammatory and anti-fibrotic actions by acting on Kupffer cells and HSC, respectively (Finelli & Tarantino, 2013). Serum adiponectin levels in this study negatively correlated with hepatic TG content in the CLA diet group. All 3 replacement diet groups had significantly higher adiponectin levels compared to the CLA diet group level; DHA appeared to have a specific effect in counteracting CLA-induced reduction of adiponectin as observed by the CLA→CLA + DHA diet group. DHA has been shown to increase adiponectin mRNA expression and adiponectin production in 3T3-L1 adipocytes through a PPARG-dependent mechanism (Oster, Tishinsky, Yuan, & Robinson, 2010). PPARG is a key transcription factor for the adiponectin gene. It is possible that mice fed the

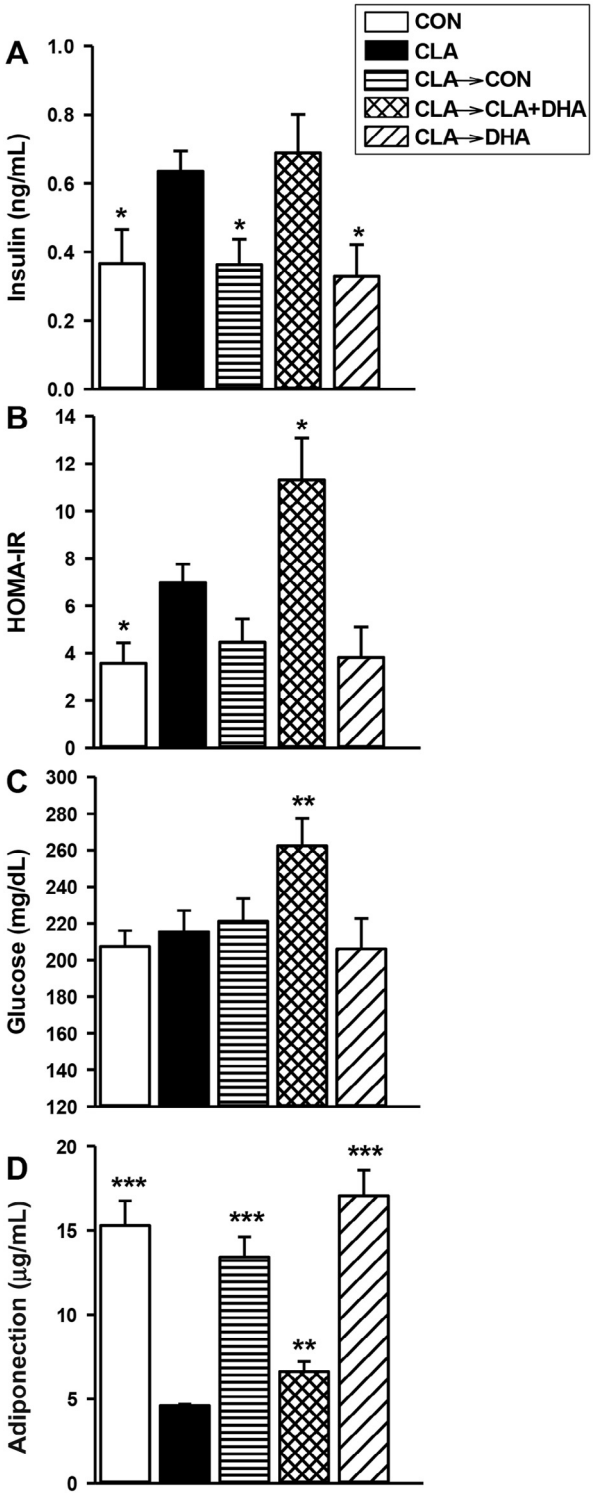

Fig. 5 – Modulation of CLA effects on (A) serum insulin, (B) homeostasis model assessment of insulin resistance (HOMA-IR), (C) serum glucose, and (D) serum adiponectin by mice fed a CLA diet for 4 weeks followed by diets replaced control (CLA→CON), CLA + DHA (CLA→CLA + DHA), or DHA (CLA→DHA) for another 4 weeks. Results are expressed as mean ± SEM, N = 7–8 per group, except adiponectin is N = 6. *P < 0.05, and **P < 0.01, ***P < 0.001 compared with CLA-fed mice. CLA, 0.5% t10,c12 conjugated linoleic acid; DHA, 1.5% docosahexaenoic acid.

CLA→CLA + DHA diet longer than 4 weeks or using a higher dose of DHA may have further increased adiponectin levels in this group.

Insulin resistance is often associated with NAFLD and is associated with high liver TG. IR worsened in the CLA→CLA + DHA diet group possibly due to the higher circulating glucose concentration than the CLA group, which may have resulted from the inability of insulin to suppress hepatic glucose production. The hyperinsulinaemia observed in the CLA→CLA + DHA diet group has also been demonstrated in other studies involving concurrent supplementation of n-3 PUFA or fish oil and CLA (Ide, 2005; Winzell et al., 2006), but it was alleviated by increasing the concentrations of the n-3 PUFA (Ide, 2005). It is possible that insulin sensitivity can be restored with a longer duration or higher dose of DHA supplementation.

The up-regulation of *Timp1* mRNA levels observed in the CLA diet group by myofibroblastic HSC suggests an imbalance in matrix metabolism leading to fibrosis through a reduction in ECM degradation by matrix metalloproteinases. The significant down-regulation of hepatic *Timp1* expression only in the CLA→DHA diet group suggests that DHA normalised this CLA-induced imbalance of matrix metabolism. We did not monitor matrix metalloproteinase mRNA expression to observe whether or not this gene was equally up-regulated by CLA. However, the overall evidence towards development of hepatic fibrosis in the CLA group is supported by the up-regulation of *Col1a1* expression and greater number of SMA-positive myofibroblasts in the CLA group compared with the CON group. DHA demonstrated an anti-fibrotic effect through the down-regulation of CLA-induced increase in the markers of fibrosis used in this study. An elevation of ALT activity, as seen in the CLA group, is an indication of liver damage and may suggest inflammation. It is highly possible that DHA protects against liver fibrogenesis by attenuating inflammation and injury. It is well recognised that DHA exerts anti-inflammatory actions by regulating transcription factors and producing three- and five-series eicosanoids and inflammation-resolving lipid mediators (Adkins & Kelley, 2010). The reason as to why DHA, when supplemented together with CLA, as in the CLA→CLA + DHA group, was less effective in suppressing *Col1a1* expression than *Timp1* could be attributed to the constant activation of the oxidant-response element in the procollagen promoter in hepatocytes with severe steatosis (Greenwel, Dominguez-Rosales, Mavi, Rivas-Estilla, & Rojkind, 2000) in which the overwhelmed oxidant stress is not sufficiently counteracted by the antioxidant properties of DHA (Richard, Kefi, Barbe, Bausero, & Visioli, 2008).

In conclusion, our results show that a 4-week DHA supplementation completely reversed existing hepatic steatosis, inflammation, and fibrosis, and restored circulating adiponectin levels and DHA also partially restored the fully depleted AT mass, but did not restore insulin, glucose, and IR. Thus, within the 4 weeks on the replacement diets, the liver was more responsive to DHA than AT or IR. More studies are needed to determine if a longer duration of DHA supplementation could reverse existing CLA-induced effects on AT mass and IR. CLA is a popular weight-loss supplement; however, in recognising the health consequences of its consumption, the long-term safety of CLA supplements should be thoroughly investigated. Adding the right dosage of DHA to CLA dietary supplements may dampen or eliminate the adverse effects in the liver.

Conflict of interest

Authors have no conflict of interest to declare. Reference to a company, commercial product, or trade name in this publication is solely for the purpose of providing specific information and does not imply approval, endorsement, or recommendation by the U.S. Department of Agriculture to the exclusion of others that may be suitable. USDA is an equal opportunity employer and provider.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jff.2015.11.028.

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