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Effect of dietary conjugated linoleic acid on blood lipid and early atherogenesis in hamsters

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SUMMARY

Conjugated linoleic acid (CLA) is a term given to a group of positional and geometrical isomers of linoleic acid (18:2 n-6) in which the double bonds are conjugated (16, 18). The major dietary form of CLA isomer is the *cis*-9,*trans*-11 isomer, which is assumed to be the biologically active form. Data suggest that, in animals, a mixture of CLA isomers has potential benefits with regard to cardiovascular disease. The present study was designed to examine whether pure *cis*-9,*trans*-11 isomer has the same effect as a mixture of CLA isomers on early atherosclerosis and parameters related to its development. To this aim, 30 male Golden Syrian hamsters (n=10/group) were fed a mild atherogenic diet, and supplemented with either a mixture of CLA isomers, pure *cis*-9,*trans*-11-octadecadienoic acid or linoleic acid for 16 weeks.

Results indicate that the pure *cis*-9,*trans*-11-fed group had significantly lower total plasma cholesterol concentrations than the CLA and LA groups. In contrast, the LA group had significantly higher HDL-cholesterol than the CLA and pure *cis*-9,*trans*-11 isomer groups. There was no significant difference in plasma triglyceride levels among the groups. Furthermore, the CLA mixture or pure *cis*-9,*trans*-11 isomer did not modulate individual lipoprotein composition or in vivo free radical formation. The relative proportion of free cholesterol, cholesterol ester, triglyceride,

phospholipids and proteins within each lipoprotein class was not significantly different across the diet groups.

The data also showed that, under our experimental conditions, none of the dietary interventions (CLA or the pure *cis*-9,*trans*-11 isomer) appeared to have exerted an anti-atherogenic effect, compared to linoleic-acid-fed animals.

The present work does not support the view that a mixture of CLA isomers or pure *cis*-9,*trans*-11 isomer can protect against atherosclerosis.

RÉSUMÉ

L'acide linoléique conjugué (ALC) ou "Conjugated Linoleic Acid" (CLA) est une appellation désignant un groupe d'isomères géométriques et positionnels de l'acide linoléique possédant des doubles liaisons conjuguées. L'ALC est présent dans plusieurs aliments naturels et tout particulièrement dans ceux d'origine animale. Ceci est dû à un processus de bio-hydrogénation bactérien des acides gras alimentaires polyinsaturés chez les ruminants. La teneur en ALC dans les aliments est influencée par différents facteurs, *i.e.* les effets saisonniers, la durée et les conditions de stockage de même que le processus de transformation et de préparation. La principale forme alimentaire de L'ALC est l'isomère *cis*-9,*trans*-11 qui est présumé être l'isomère biologiquement actif.

De nombreux travaux scientifiques ont établi que l'ALC possède des propriétés physiologiques et biochimiques remarquables dont pourrait bénéficier l'humain et le protéger contre des maladies chroniques. Chez l'animal, il est démontré que l'ALC exerce un effet bénéfique au niveau du système cardio-vasculaire. Le but de cette étude est d'examiner si l'isomère pur *cis*-9,*trans*-11 a le même effet qu'un mélange d 'isomères de l'ALC sur le développement de l'athérosclérose.

Des hamsters mâles Syriens dorés ont été nourris d'un régime athérogène élaboré à partir de "Rodent Chow " enrichi de 10g/100g d'huile de noix de coco et de 0.05g/100g de cholestérol. Trois groupes de dix hamsters chacun ont été soumis à ce régime expérimental. Le groupe 1 a reçu 1% d'ALC (w/w) ajouté au régime. Au régime du groupe 2 on a ajouté 0.2% de l'isomère *cis*-9, *trans*-11 pur. Ce choix a été fait en fonction de la composition isomérique de l'ALC qui comprenait 20% de l'isomère *cis*-9,*trans*-11. Le groupe 3 a reçu pour sa part 0.2% d'AL, ce qui en fit le groupe contrôle du groupe 2.

Les trois diètes expérimentales sont administrées pendant 16 semaines et pendant la période d'alimentation on a mesuré la consommation alimentaire ainsi que le poids corporel. À la fin de l'expérience, on a mesuré le taux des lipides sanguins et l'étendue des lésions lipidiques dans l'aorte. Trois méthodes différentes ont été utilisées pour l'évaluation des lésions aortiques. Tout d'abord, l'étendue lipidique a été mesurée de façon quantitative par deux évaluateurs indépendants. Ensuite, les sections ont été photographiées et les images digitalisées pour évaluer la déposition des lipides par analyse morphometrique. Troisièmement, les dépôts lipidiques ont été déterminés par spectrophotométrie après coloration des coupes histologiques avec du "red oil O". On a mesuré aussi les lipides sanguines ainsi que la chemiluminescence du sang total et la synthèse des prostaglandines dans

les macrophages comme des indicateurs du flux des radicaux libres *in vivo*. De plus, la corrélation entre les déterminants biochimiques utilisés (le cholestérol total, le cholestérol HDL et les triglycérides) et l'athérosclérose a été établie. Les fractions des lipoprotéines VLDL, LDL, HDL_2 et HDL_3 ont été isolées par ultracentrifugation pour être analysées. Toutes les procédures expérimentales appliquées aux hamsters étaient conformes aux directives du Conseil Canadien de Protection des Animaux (CCPA) et furent approuvées par le Comité de déontologie de l'expérimentation sur les animaux de l'Université de Montréal.

Les résultats démontrent que tous les régimes alimentaires ont été bien acceptés par les animaux pendant les 16 semaines de l'expérience. À la fin de l'intervention, le groupe alimenté avec le *cis*-9,*trans*-11 avait un taux du cholestérol total plasmatique significativement plus bas que ceux des groupes nourris avec de l'ALC et de l'AL Par ailleurs, le groupe ayant consommé de l'AL avait un taux significativement plus élevé de cholestérol HDL que les deux autres. Les trois groupes n'ont montré aucune différence significative en ce qui concerne les triglycérides plasmatiques. Les proportions relatives de cholestérol libre, de cholestérol estérifié, de triglycérides, de phospholipides, et de protéines dans chaque classe de lipoprotéines n'étaient pas différentes dans les trois groupes examinés. L'évaluation des lésions d'athérosclérose par les trois méthodes révèle que l'étendue des dépôts lipidiques dans l'aorte ne présente aucune différence

significative entre les trois groupes. De plus, la formation des radicaux libres ne semble pas être modulée par l'ALC ou l'isomère examiné.

Notre étude n'appuie pas l'hypothèse que le mélange des isomères de l'ALC ou la forme *cis*-9,*trans*-11 protègent contre l'athérosclérose.

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LIST OF ABBREVIATIONS

BFM	body fat mass
BHT	butylated hydroxytoluene
C	cholesterol
CHD	coronary heart disease
cis-9,*trans*-11	*cis*-9,*trans*-11-octadecadienoic acid
CLA	conjugated linoleic acid
DTH	delayed-type hypersensitivity
DMBA	dimethylbenz[a]anthracene
EDTA	ethylenediaminetetraacetic acid
HDL	high-density lipoprotein
HDL_2	high-density lipoprotein subclass 2
HDL_3	high-density lipoprotein subclass 3
IQ	2-amino-3-methylimidazo[4,5-f]quinoline
LA	linoleic acid
LDL	low-density lipoprotein
ORO	oil red O
PGE_2	prostaglandin E_2
SCID	severe combined immunodeficient
TBARS	thiobarbituric acid-reactive substances
TC	total cholesterol
TG	triglyceride
TOSC	total oxyradical scavenging capacity
TPA	12-*O*-tetradecanoylphorbol-13-acetate
VLDL	very low-density lipoprotein

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I. INTRODUCTION

Excess dietary fat is a major contributing factor in several chronic diseases, such as coronary heart disease (CHD), stroke, diabetes mellitus, cancer, and obesity (1). Research using experimental animals as well as results from human population surveys (2) have shown that intake of a high proportion of dietary fat relative to other nutrients promotes the development of obesity. In turn, clinical observations demonstrate a connection between obesity and a variety of chronic diseases such as insulin resistance, hypertension, dyslipidemia, cardiovascular disease, non-insulin-dependent diabetes mellitus, gallstones and cholecystitis, respiratory dysfunction, and certain forms of cancer (3). Moreover, in epidemiological studies, a strong association has been demonstrated between obesity and mortality in populations (4-6). About half of all people enjoying a Western lifestyle will die of myocardial infarcts or strokes caused mainly by atherosclerotic disease (7). Large epidemiological studies have revealed that the main risk factors associated with development of atherosclerosis are smoking, hypertension, diabetes and dyslipidemias (8). Furthermore, lowering cholesterol levels of an individual reduces the probability that he will suffer a major cardiovascular event (9).

The dietary fat intake varies among various populations and in North America it ranges between 30% and 40% of total energy (1). Without a

doubt, a high intake of fat can promote obesity, thus, there is much effort directed towards developing strategies (e.g., proper nutrition, behavioral modification or pharmaceutical treatments) to modulate dietary fat intake in order to minimize the risk of chronic disease related to excess weight.

Recently, there has been much interest in a type of modified fatty acid found in milk fat that has potential benefit for humans with respect to risk of development of chronic diseases. This fatty acid, referred to as conjugated linoleic acid (CLA), is a mixture of positional and geometric isomers of octadecadienoic acid with conjugated double bonds (10). CLA has been shown to have a beneficial effect on blood lipids as well as early atherogenesis (11). It has been identified as a potent anticarcinogen in a number of model systems (12-15). The biologically active isomer has been assumed to be the *cis*-9,*trans*-11-octadecadienoic acid. This isomer is now commercially available, giving us the opportunity to test whether it is the biologically active form of CLA. In this study, we have examined whether pure *cis*-9,*trans*-11 isomer has the same effect as the mixture of CLA isomers in hamsters, with respect to growth rates, blood lipids and early atherogenesis.

II. REVIEW OF LITERATURE

Chemical characteristics of conjugated linoleic acid

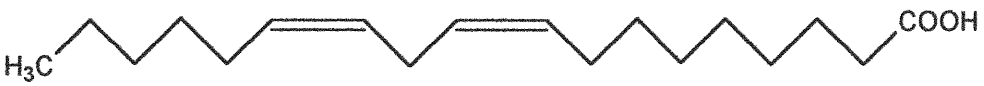
Definition

Conjugated linoleic acid (CLA) is a term given to a group of linoleic acid (18: 2 n-6) isomers in which the double bonds are conjugated, instead of being in the typical methylene interrupted configuration (16). The term originated in 1987 when Pariza and co-workers (12) reported anti-cancer activity associated with CLA isolated from grilled ground beef. CLA isomers have been reported to contain conjugated double bonds at positions 7,9, 8,10, 9,11, 10,12, 11,13 and 12,14 of octadecadienoic acid (17). Each group of positional isomers has *cis/cis*, *trans/trans*, *trans/cis* and *cis/trans* geometrical configurations (18), giving rise to 24 possible isomers of CLA. The *cis*-9,*trans*-11 isomer is the major dietary form of CLA isomer and accounts for more than 80% of total CLA in a variety of cheeses, and more than 75% in animal meat (19, 20). Moreover, it is the predominant isomer incorporated into membrane phospholipids of animals fed a mixture of CLA isomers (13, 21). Thus, the biologically active isomer has been assumed to be *cis*-9,*trans*-11 CLA isomer (Figure 1).

Figure 1

Configuration of Linoleic acid, *cis*-9,*trans*-11-octadecadienoic acid and *trans*-10,*cis*-12-octadecadienoic acid

linoleic acid



cis-9,*trans*-11-octadecadienoic acid

trans-10,*cis*-12-octadecadienoic acid

Natural Occurrence and Synthesis of Conjugated linoleic acid

CLA synthesis requires the presence of free linoleic acid, a protein, and a free radical-generating species. Moreover, the interaction requires

close molecular proximity between the protein reducing group, almost certainly a thiol residue, and the lipid free radical. These conditions occur in vivo and in vitro (22).

cis-Unsaturation is most common in natural lipids where the double bonds normally occupy specific positions in the carbon chain. *trans*-Unsaturation occurs naturally either as short-lived intermediates in biochemical pathways (e.g. during the biosynthesis of saturated fatty acids) or as stable end products (23). In ruminants, the process of biohydrogenation of dietary polyunsaturated fatty acids in the rumen also produces *trans*-unsaturated fatty acids. Kepler and co-workers identified the *cis*-9,*trans*-11 CLA isomer as the intermediate product of biohydrogenation of linoleic acid by *Butyrivibrio fibrisolvens*, which is one of the main rumen bacteria (24). During biohydrogenation, the *cis*-double bonds of the original all *cis*-polyunsaturated fatty acids are isomerized. This may involve a shift in position along the carbon chain (positional isomerization) or a change from *cis* to *trans* geometrical configuration or both (23).

Chin and co-workers (25) reported that the intestinal bacterial flora of rats is capable of converting free linoleic acid, but not linoleic acid esterified in triglycerides, to CLA isomers. This suggests that the intestinal microflora of nonruminants has limited ability to isomerize linoleic acid to CLA.

However germ–free rats are not able to produce CLA, indicating that intestinal bacteria are required for isomerization.

Food processing increases the isomerization of linoleic acid into CLA. For example, heat treatment (26, 27) increases the concentration of CLA in beef. In addition, food additives such as whey components increase the levels of CLA in processed cheese (28).

Sources of conjugated linoleic acid

CLA is present in many natural food sources, the main dietary sources being animal products (19, 29). Meat from ruminants contains more CLA than meat from nonruminants, except for turkey. Veal has the lowest (2.7mg CLA/g fat) and lamb has the highest (5.6mg CLA/g fat) CLA value. Dairy products also have high levels of CLA. Processed cheese has an average of 5.0mg CLA/g fat. Among different types of cheese, those which are aged or ripened more than 10 months have the lowest CLA content (19, 29). CLA concentration in fermented dairy products ranges from 3.82 to 4.66 mg/g of lipid, with cultured buttermilk having the highest content. The concentrations of CLA in bovine milk can be increased through a suitable dietary regimen (30). Plant oils contain less CLA than dairy fat, ranging from 0.1 mg CLA/g fat in coconut oil to 0.7 mg CLA/g fat in safflower oil. Seafood contains low amounts of CLA, ranging from 0.3 to 0.6 mg CLA/g fat (19,

29). Many factors affect the concentration of CLA in foods, i.e. seasonal effects, length and conditions of storage, processing, and preparation (19).

Biological effects of conjugated linoleic acid

Anti-atherogenesis

Nicolosi and co-workers reported in abstract form (31) the results of their investigation on the effect of CLA on plasma lipoproteins and atherogenesis. Five groups of 10 hamsters each were fed for 11 weeks hypercholesterolemic diets containing 0, 0.05, 0.1, and 1 % CLA or 1 % linoleate for comparative purposes. The hamsters fed 0.05 % CLA had significantly decreased plasma total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and triglyceride (TG) compared to the control diet (0% CLA). However, this diet had no effect on high-density lipoprotein cholesterol (HDL-C) compared to the control diet. Similar effects were observed in animals fed 0.1 or 1 % CLA. These authors also reported that CLA-fed animals had a significant decrease in aortic streak formation.

Lee et al. (11) reported that CLA had protective effects against atherosclerosis in rabbits. In their experimental design, rabbits were fed a semi-synthetic diet containing 14% fat and 0.1% cholesterol with 0.5g CLA/rabbit per day for 22 weeks. The control animals were fed a similar diet with 0.5g coconut oil/rabbit per day instead of CLA. By 12 weeks TC and LDL-C were significantly lower in the CLA-fed animals compared to the

control group. The ratio of LDL-C to HDL-C in the plasma of CLA-fed rabbits was also significantly lower than the control rabbits. There was no difference in hepatic cholesterol between the two groups. Examination of the aortas of CLA-fed rabbits showed 30% less cholesterol deposition, but the difference was not statistically significant. The results drawn from this study need to be interpreted with caution, as coconut oil is probably not a suitable control for CLA.

In another study by Nicolosi et al. (32) using hypercholesterolemic hamsters, no significant correlations were observed between plasma lipids, non-HDL-C and early atherosclerosis. Non-HDL-C is a combination of VLDL and LDL. The animals were fed a chow-based diet consisting of 10% coconut oil, 1% safflower oil, and 0.12% cholesterol (w/w) with 0 (control), 0.06, 0.11, and 1.1 % CLA or 1.1 % LA. All levels of CLA significantly reduced plasma TC and non-HDL-C concentrations with no effect on HDL-C, as compared to the control and LA group. The LA-fed animals had similar results to the CLA-fed groups, but plasma TC and non-HDL-C concentrations were not significantly different from the control group except for the plasma TG level. The LA supplement resulted in lower levels of plasma TC and non-HDL-C compared to the control group, whereas HDL-C was not affected. Morphometric analysis of fatty streak formation showed less early atherosclerosis in the CLA and LA-fed hamsters compared to the control group.

Munday and co-workers (33) investigated the effect of dietary CLA on early atherogenesis in C57BL/6 mice. Three groups of twenty mice were fed atherogenic diets containing 1% cholesterol, 14.5% triacylglycerol, and 0.5% cholic acid with 0.5% CLA, 0.25% CLA + 0.25% LA or 0.5% LA (control) for 15 weeks. In contrast to previous animal studies, the data showed mice fed diets containing 0.25% CLA, but not 0.5% CLA, developed a significantly greater area of fatty streaks than the controls. There were no significant differences in serum TC, or in serum HDL-C among the dietary groups. Serum TG was significantly lower in mice receiving the 0.5% CLA diet than in the controls. In their study, the fatty streak area was not correlated with the serum TC, HDL-C, or TG or with either the HDL-C: TC ratio, or body weight.

Recently, Kritchevsky et al. (34) reported the effects of CLA in the establishment and regression of experimentally induced atherosclerosis in rabbits. For the establishment of atherosclerosis study, the animals were fed a semipurified atherogenic diet containing 0.2% cholesterol with 0 (control) or 1% CLA (w/w) for 90 days. Serum cholesterol levels were significantly higher in the CLA-fed group compared to the control group. Rabbits fed 1% CLA had 31% less severe atherosclerosis in the aortic arch and 40% less severe atherosclerosis in the thoracic aorta compared to the control group. However, the differences were not statistically significant. They also tested the effect of different levels of dietary CLA. Rabbits were

fed the atherogenic diet with 0% (control), 0.1%, 0.5%, or 1.0% CLA. The severity of atherosclerosis in the aortic arch and thoracic aorta was significantly reduced in rabbits fed all levels of CLA compared to the control group. To measure the effect of dietary CLA on the progression of atherosclerosis, animals with established atherosclerosis were fed a cholesterol-free semipurified diet with 0 (control) or 1% CLA (w/w) for 90 days. As with the first part of the feeding study, serum cholesterol levels were significantly higher in the CLA-fed group compared to the control group. However, in the control group, serum cholesterol levels had fallen by 83% and HDL-C had risen by 121% at the end of the feeding period when compared to the establishment of the atherosclerosis study. Similarly, in the CLA group, where the initial feeding diet was supplemented with cholesterol, serum cholesterol had fallen by 67% and HDL-C had risen by 74% at the end of the feeding period. The animals fed CLA had 31% less severe atherosclerosis in the aortic arch and 30% less severe atherosclerosis in the thoracic aorta compared to the control group. The differences in serum cholesterol and HDL-C levels were not statistically significant. Regarding different levels of dietary CLA, the aorta atherogenic lesion data showed that there was no statistically significant difference in the rabbits fed 0.1% or 0.5% CLA compared to the 0% CLA group. However, 1% CLA showed the ability to reduce the progression of established lesions.

The reports concerning the anti-atherogenic effect of CLA remain contradictory. This could be due to the use of different animal models and various experimental protocols to test the hypothesis that CLA prevents or reduces the establishment and progression of atherosclerosis. Nevertheless, a majority of the studies seem to support the hypothesis that CLA inhibits atherosclerosis. Thus it is important to continue to study the effect of CLA on this disease, and whether the beneficial effect, if any, can be attributed to a particular isomer or a group of isomers in concert.

Anticarcinogenesis

Linoleic acid (18:2 n-6)-rich dietary fat usually correlates with enhanced tumorigenesis in various organs (35-37). In contrast, CLA has been established as a potent skin cancer inhibitor with apparent antioxidant activity in animal models (12). In light of these results, it has been proposed that CLA might have anticarcinogenic effects in internal organs as well.

Ha and co-workers (13) showed that synthetic CLA inhibits the initiation of mouse forestomach tumorigenesis by benzo(a)pyrene, a known carcinogen. The mice were given by gavage 0.1ml of CLA + 0.1ml of olive oil, 0.1ml of LA + 0.1ml of olive oil or 0.1ml of olive oil (control) twice weekly for 4 weeks. The CLA treatment group developed only about half the neoplasms compared to the control group, whereas LA had no effect.

Liew et al. (38) reported that CLA protects against early stages of colon cancer induced by a dietary heterocyclic amine. In their experiments, CLA was administered to male F344 rats by gavage on alternating days for 4 weeks. The heterocyclic amine, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) was given by gavage every other day in weeks 3 and 4 (100 mg/kg body wt) of the feeding study. The dose of CLA given by gavage was calculated to provide an average daily intake equivalent to that received by rats consuming a diet containing 0.5% CLA. IQ induces DNA adducts in the colon which are known to promote colon cancer. The results show that CLA inhibits the formation of IQ-DNA adduct in the colon compared to rats given IQ alone, a mechanism that may account for the protection against colon cancer.

Cesano et al. (15) determined the effect of CLA on the growth and progression of human prostatic cancer in immunodeficient (SCID) mice. They have established a human prostatic adenocarcinoma (DU-145) model in SCID mice characterized by a rapid growth of the tumor cells and metastatic spread to the lungs. SCID mice were pretreated with etoposide (an antineoplastic drug) in order to inhibit their innate immunity. After pretreatment, DU-145 cells were injected in the left flank region of the animal. The animals were fed diets with 1.7% LA (control) or 1.7% LA + 1% CLA (w/w) for 14 weeks, starting 2 weeks before tumor inoculation. Mice fed the CLA-supplemented diet displayed significantly smaller tumors than

the control group ($p<0.001$). The CLA-supplemented group also showed a drastic reduction in lung metastases. Metastatic spread to the lungs was observed in 80% of mice fed the control diet. In contrast, only 10% of the animals receiving CLA-supplemented diet had lung metastases.

Ip et al. (14) studied the effect of dietary CLA on mammary tumor development. Tumor formation was initiated by dimethylbenz[a]anthracene (DMBA). Rats treated with a single dose of 10 mg of DMBA at 50 days of age were given 1% CLA (w/w) in the diet for 4 weeks, 8 weeks or continuously until the end of the experiment. The control rats were not given CLA at any time. Supplementation with CLA was started 4 days after carcinogen administration. No cancer protection was observed in the 4 or 8 week-CLA treatment groups. In contrast, marked tumor inhibition was observed in rats that were given CLA for the entire duration of the experiment (20 weeks).

Belury et al. (39) determined the role of increasing levels of dietary CLA in animal models with chemically induced carcinogenesis. Mice fed diets containing 0.0%, 0.5%, 1.0% or 1.5% (w/w) CLA, were treated with the skin tumor promotion agent 12-O-tetradecanoylphorbol-13-acetate (TPA). Twenty-four weeks after tumor promotion was begun, diets containing 1.0% and 1.5% CLA inhibited tumor yield compared to the

control without CLA. A dietary level of 0.5% CLA (w/w) had no effect on the tumor yield.

In summary, the studies cited above point to an anticarcinogenic effect of dietary CLA in several models and under appropriate experimental conditions. The exact mechanism of action of CLA is the focus of current investigations in several laboratories.

Bone Metabolism

Watkins et al. (40) reported that male broiler chicks given butter fat, a source of natural CLA isomers, demonstrated an increased rate of bone formation. They also showed that dietary butter fat lowered ex vivo bone prostaglandin E_2 production. This is relevant in that excess production of prostaglandin E_2 is linked to osteoporosis and arthritis and is associated with bone and proteoglycan loss. Li et al. (41) investigated the effects of CLA on bone remodeling in male rats. The rats fed 1% CLA (w/w)-supplemented diet had the highest amounts of CLA in their bone periosteum. The *t*-9,*t*-11 CLA isomer was only detected in bone marrow and periosteum with rats fed the CLA. The *c*-9,*t*-11/*t*-9,*c*-11 and *t*-10,*c*-12 CLA isomers were incorporated into all tissues of rats fed the CLA-supplemented diet. Similar to the Watkins et al. study (40), Li et al. (41) found that CLA lowered ex vivo prostaglandin E_2 production in bone organ cultures. These

findings suggest that CLA has the potential to influence bone formation and resorption.

Immune System

Hayek et al. (42) examined the influence of dietary 1% CLA (w/w) on the immune response of young (4 months) and old (22 months) mice. CLA increased splenocyte blastogenesis in cell cultures. However, CLA supplementation did not influence the delayed-type hypersensitivity (DTH) skin reaction and natural killer cell activity. The immunostimulatory effect was more pronounced in young than in old mice and was not mediated through a change in PGE_2 (prostaglandin E_2) or IL-1 production.

Miller et al. (43) studied the ability of CLA to prevent endotoxin-induced growth suppression. Dietary CLA at a level of 0.5%(w/w) prevented anorexia from endotoxin injection in mice. Under these conditions, splenocyte blastogenesis was increased by CLA.

Growth and Body composition

Chin et al. (44) examined the effect of dietary CLA on rat growth and development. Feeding diets supplemented with 0.25% and 0.5% CLA during gestation and lactation improved the postnatal body weight gain of pups. Pups that continued to receive the CLA-supplemented diet after weaning had significantly greater body weight gain and improved feed

efficiency (gram of body weight gain per gram of food intake) relative to control animals. Belury et al. (45) also found that the feed efficiency increased between 1.5 and 2.0-fold in mice fed 0.5-1.5 % dietary CLA compared to those fed diets without CLA. In a study using other animal species, Bee et al. (46) evaluated the effects of CLA on growth performance in piglets reared on sows fed diets supplemented with 2% CLA (w/w) or 2% LA (w/w). The piglets reared on sows fed CLA during pregnancy and lactation grew significantly faster ($p<0.01$) than piglets reared on sows fed LA in the postweaning period, irrespective of the starter diet.

Yamasaki et al. (47) reported the effect of dietary 2% CLA on the body weight of 4-wk-old male rats. The animals were fed diets containing 8% safflower oil (control) or 2% CLA (w/w) + 6% safflower oil for 12 weeks. There was no significant difference in food intake and body weight between dietary groups throughout the feeding period. Similarly, Sugano et al. (48) reported that CLA did not influence growth and food intake in rats. In their experiments, 4-wk-old Sprague-Dawley male rats were fed for 3 weeks diets with 0.0%, 0.5% or 1.0% CLA (w/w). There was no difference in food intake or growth rate of rats for 3 weeks among the groups. The feed efficiency also was comparable among the groups.

The contradictory results reported by the investigators cited above have not yet been resolved. Further investigation is required in order to clarify the CLA's biological activity relating to growth rate.

Aside from effects on growth rate, CLA has also been shown to affect body composition. In 6-wk-old ICR mice, Park and co-workers (49) reported the effects of dietary CLA on body composition after feeding for 32 days an experimental diet containing 5.5% corn oil (control), or 5% corn oil plus 0.5% CLA (w/w). The percentage body fat in CLA-fed mice was significantly reduced by 57% in male and by 60% in female animals relative to controls. In contrast, the percentages of whole body protein and carcass water were significantly higher. DeLany and co-workers (50) also reported that AKR/J mice fed 1%, 0.75% or 0.5% CLA accumulated less body fat and more protein in the carcass. The effects of CLA on body composition were observed as early as 2 weeks after treatment and persisted throughout 12 weeks of CLA feeding without any major effects on food intake. Santora et al. (51) showed that C57BL/6 mice fed purified diets containing 1% CLA for 2 weeks exhibited decreased food intake and body fat compared to mice fed diets containing 1% stearic, pure *trans*-11-octadecenoic or elaidic acid, but did not show changes in body protein.

Dugan et al (52) demonstrated that pigs fed CLA deposited 6.8% less subcutaneoous fat and gained 2.3% more lean body mass than pigs

fed sunflower oil. The experimental diets were cereal-based containing 2% CLA (w/w) or 2% sunflower oil. Sunflower oil is a rich source of α-linoleic acid. In another study using pigs (53), Ostrowska et al. reported that dietary CLA increased lean tissue deposition and decreased fat deposition. Pigs were fed one of six experimental diets for 8 weeks. The diets contained 0, 0.125%, 0.25%, 0.5%, 0.75% or 1% CLA (w/w). The carcass composition revealed that the rate of lean tissue deposition reached a plateau at a CLA level of 0.5%, whereas the depression in fat deposition was linear up to at least 1% CLA. At the highest level of CLA supplementation, carcass fat deposition was reduced by 31% and lean tissue deposition (as defined as the sum of water and protein) was increased by 17%.

A strong fat-to-lean repartitioning effect was observed by Stangl (54) in Sprague-Dawley rats fed a diet based on the AIN-93 formulation and containing 0 (control) or 3% CLA (w/w) mixture. The percentage carcass fat of CLA-fed animals was reduced by 27% relative to control animals. In contrast, the percentage carcass protein was enhanced by 11%, giving a 45% higher protein/fat ratio in CLA-fed rats compared with rats fed no CLA. It was further observed that mobilizations of fat deposits were enhanced with CLA feeding (55). In a study using the same species, fat pad weight was reduced within 7 days in animals fed 0.5% CLA compared to the 0% CLA group.

Park et al. (56) investigated the effects of CLA preparations enriched in the *cis*-9,*trans*-11 CLA-isomer or the *trans*-10,*cis*-12 CLA-isomer, on body composition in weanling ICR mice. They observed that body composition changes were brought about by a diet rich in *trans*-10,*cis*-12 CLA isomer rather than *cis*-9,*trans*-11 CLA isomer. The results indicate that contrary to earlier beliefs, the *trans*-10,*cis*-12 isomer and not the *cis*-9,*trans*-11 isomer may be the biologically active form.

In contrast to animal studies, an experimental diet containing CLA at 1% of total calories had no significant effect on body composition in healthy women after 64 days of intervention (57). However, a study on overweight or obese human subjects (58) showed that CLA compared with placebo resulted in significant reduction in body fat mass (BFM). The placebo group received 9g olive oil daily for 12 weeks. The treatment groups were given 1.7, 3.4, 5.1 or 6.8g CLA per day for 12 weeks, respectively. The reduction of body fat was significant in groups given CLA at 3.4 and 6.8g per day. There were no significant differences in lean body mass, body mass index, or blood lipids.

Anti-oxidant property

Initially, Ha et al. showed (13) that synthetically prepared CLA mixture was an effective antioxidant in vitro using the thiocyanate method. CLA was most effective at the lowest tested (0.375 μmol) level. At higher

concentrations, CLA was less efficient as an antioxidant. Under the conditions of the test, CLA was more potent than α-tocopherol and almost as effective as butylated hydroxytoluene (BHT). Ip et al. (21) found that dietary CLA was an effective antioxidant in mammary tissue but not liver tissue in the rat.

Van den Berg et al. (59) reinvestigated the antioxidant properties of CLA. In contrast to previous studies, their results showed that CLA was not effective as an antioxidant. They tested the antioxidant activities of CLA, vitamin E (α-tocopherol), and BHT using unsaturated phospholipid model membranes (PLPC vesicles). When oxidation of PLPC (1.0mM) was initiated by the lipid-soluble 2,2'-azobis (2,4-dimethylvaleronitrile) or the water-soluble 2,2'-azobis (2-amidinopropane) hydrochloride, vitamin E and BHT at 0.75μM efficiently inhibited PLPC oxidation. In contrast, 0.75μM CLA did not have any significant effect on PLPC oxidation. Livisay et al. (60) also showed that dietary CLA did not inhibit lipid oxidation in rat liver microsomes and skeletal muscle homogenates. Interestingly, using the total oxyradical scavenging capacity (TOSC) assay (61), *trans*-10,*cis*-12 CLA isomer was shown to be a stronger antioxidant than *cis*-9,*trans*-11 CLA. At high concentration (200μM) *cis*-9,*trans*-11 CLA isomer exhibited pro-oxidant activity, whereas *trans*-10,*cis*-12 CLA isomer acted as a strong antioxidant at all concentrations (2-200μM). Leung et al. (61) commented that the contradictory results of previous studies on the antioxidant properties of

CLA, were done using mixtures of CLA isomers (mainly 43% *cis*-9,*trans*-11/*trans*-9,11 CLA isomer and 45% *trans*-10,*cis*-12 CLA isomer). They proposed that the effectiveness of CLA as an antioxidant depends on the actual proportion of *trans*-10,*cis*-12 to *cis*-9,*trans*-11 isomers in the mix.

The reports concerning CLA as an antioxidant are conflicting and also, the knowledge of effect on antioxidation is still limited.

The hamster as a model for atherosclerosis

The hamster has been described as an appropriate animal model for investigating the regulation of plasma lipid and lipoprotein metabolism. Spady et al. (62) studied cholesterol synthesis in vivo in the major organs of five different animal species. Their results showed the rates of sterol synthesis in the monkeys, hamsters, rabbits, and guinea pigs were remarkably similar and varied from 2.9 to 4.6 µmol/hr per 100g body weight. In contrast, sterol synthesis in the rat was 16.1 µmol/hr per 100g body weight. The rat has a high capacity for sterol synthesis compared to other species, especially man who synthesizes about 1.3 µmol/hr per 100g body weight. Bravo et al. (63) characterized lipid composition of plasma lipoproteins in male golden Syrian hamsters by discontinuous gradient ultracentrifugation. They also studied the differences with male Wistar rats and the analogies with human plasma lipoproteins. The animals were fed a commercial rodent diet containing 18.5% raw protein, 3% fat and 6%

cellulose for at least 2 weeks. Plasma lipoproteins of hamsters comprised significant amounts of VLDL, LDL and HDL. The distribution of cholesterol between free and esterified forms and the distribution of phospholipid classes in the different lipoprotein fractions were more similar to those found in man than to those in the Wistar rat. They confirmed the close resemblance between cholesterol metabolism in hamster and man. Arbeeny and co-workers (64) found that the VLDL that is secreted by the perfused hamster liver contains only apoB-100, as is the case in humans. Moreover, the hamster is a small animal capable of developing similar atherosclerotic lesions in response to a hyperlipidemic diet as humans (65). Therefore, recently, the hamster has become a widely used animal model for atherogenesis, which prompted us to choose it as our experimental model for studies on CLA.

III. HYPOTHESIS AND OBJECTIVES

Hypothesis

1. a) CLA improves blood lipid indicators of atherosclerosis.

   b) CLA decreases early atherosclerosis.

Objectives

Measure the effect of CLA isomers and pure *cis*-9,*trans*-11 isomer on:

1. blood lipid indicators of atherosclerosis.
2. Fatty streak development in early atherosclerosis in hamsters.

IV. METHODOLOGY

We chose to examine and compare the effects of a mixture of CLA isomers and pure *cis*-9,*trans*-11 CLA-isomer on the development of early atherogenesis in Male Golden Syrian hamsters. All experimental procedures using hamsters were approved by the Animal Care Committee of the Université de Montréal in accordance with the guidelines of the Canadian Council on Animal Care.

We followed the dietary protocol described by Kowala et al. (66) and Otto et al. (67) to induce atherosclerosis in hamsters. The animals were fed a mild atherogenic diet, consisting of Rodent Chow supplemented with 10g/100g coconut oil and 0.05g/100g cholesterol. Three groups of 10 hamsters each were given free access to the experimental diets. The diet in group 1 was the mild atherogenic diet plus 1% CLA (w/w). The group 2 diet was the mild atherogenic diet plus 0.2% pure *cis*-9,*trans*-11 isomer. This level was chosen because in the CLA isomer mixture, the *cis*-9,*trans*-11 isomer contribution was 20%. The group 3 diet was the mild atherogenic diet plus 0.2% LA. Thus, group 3 served as the control for group 2.

The measures taken during the feeding period were food intake, growth curve and blood lipids. At the end of the experiment, blood lipids,

whole blood chemiluminescence, macrophage prostaglandin synthesis and extent of aortic fatty streak lesions were determined.

Blood lipids

Non-fasted blood was collected via the retro-orbital sinus into EDTA blood collection tubes at 2 and 6 weeks of the experiment. Fasted blood was collected at sacrifice at the end of the 117 day feeding period. Plasma TC, TG, HDL-C were measured using commercial assay kits. In addition, lipoprotein fractions were isolated by ultracentrifugation for lipid composition analysis (68).

Measurement of aortic fatty streak lesions

Three different methods were used to evaluate fatty streak formation. The heart was perfused with 4% phosphate-buffered formalin solution. The perfused heart, including the thoracic aorta, was placed in a vial containing 4% phosphate-buffered formalin until analysis. The section of the aorta between the third neck vessel and 1mm above the aortic valve was examined for fatty streak lesion development as follows: first, the aortas were rinsed in 60% isopropanol, immersed in oil red O (ORO) stain solution for 30 minutes, blotted dry, and rinsed with Na cacodylate buffer for 1 minute. The aortas were then opened longitudinally and mounted with the luminal surface facing upward on a glass cover slip, using aqueous mounting medium. All segments were photographed, then, the amount of

lipid deposits was scored independently by two evaluators using a scale of 1 to 4 as follows: 1, lesions covering 0-25% of the section; 2, lesions covering 26-50% of the section; 3, lesions covering 51-75% of the section; 4, lesions covering 76-100% of the section. In the second evaluation, the photomicrographs were digitized and subjected to digital image analysis using the public domain software ImageJ (National Institutes of Health, U.S.A.). The total areas of fatty streak lesions were measured digitally in each photomicrograph. Finally, the quantity of aortic neutral lipid was determined by the amount of ORO staining using the method of Nunnari et al. (69). An area of each stained aorta was measured and cut, and ORO extracted with chloroform/methanol (2:1). The concentration of stain was determined from absorbance, which was applied to a standard curve. The concentration of ORO was divided by the area of the aorta to give intimal ORO $\mu g/mm^2$.

Statistical Analysis

Statistical routines available in SPSS (SPSS Inc., Chicago IL, USA) were used to detect differences among groups. The data were analyzed by ANOVA and the Tukey-b test was applied *a posteriori*. Significance was declared at $P < 0.05$.

V. ORIGINAL ARTICLE

Conjugated Linoleic Acid, Pure cis-9,trans-11-Octadecadienoic Acid and Linoleic Acid have Different Effects on Plasma Total Cholesterol and HDL-Cholesterol but Do Not Affect Aortic Fatty Streak Formation[1]

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Short Title: CLA, hamster serum lipids and atherogenesis

Abstract

In order to test the hypothesis that dietary conjugated linoleic acid (CLA) protects against atherogenesis, 30 male hamsters were divided equally into 3 groups and fed for 16 weeks a diet with added hydrogenated coconut oil and cholesterol, and supplemented with either a mixture of CLA isomers (CLA group), pure *cis*-9,*trans*-11-octadecadienoic acid (C9T11 group) or linoleic acid (LA group). Early atherogenesis was assessed by intimal Oil Red-O staining of the aortic arch. End-point determinations of biochemical correlates of atherogenesis were plasma total cholesterol, HDL-cholesterol and total triglycerides. Lipoproteins VLDL, LDL, HDL_2 and HDL_3 were isolated by ultracentrifugation for compositional analysis. Furthermore, whole-blood chemiluminescence and resident macrophage PGE_2 production were determined as indices of *in vivo* free radical flux. At the end of the feeding period, the C9T11 group had significantly lower total plasma cholesterol than the CLA and LA groups. In contrast, the LA group had significantly higher HDL-cholesterol than the CLA and C9T11 groups. There were no differences in the degree of atherogenesis among any of the groups, and there were no differences in individual lipoprotein composition, release of macrophage PGE_2 and whole blood chemiluminescence. In this experiment, the results do not support the hypothesis that CLA protects against atherogenesis. This may be related to its inability to modulate *in vivo* free radical flux, nor to affect individual lipoprotein composition.

Key words: hamster, conjugated linoleic acid, cholesterol, serum lipids, lipoproteins, aortic fatty streaks, atherogenesis

Conjugated linoleic acid (CLA[3]) is a term applied to a mixture of isomers where the normal methylene-interrupted double bond structure of the parent linoleic acid has been transformed into a conjugated double bond system and where one or both of the double bonds have been transformed from the *cis* to the *trans* configuration. Dietary CLA has consistently been shown to be a modulator of serum lipid levels in different laboratory animals under different experimental protocols (1-7). In addition, there have been reports that CLA may have antioxidant properties (8,9). Because of the link between lipemia and atherosclerosis (10) and the role of lipid peroxidation in cardio vascular disease (11,12), a number of laboratories have undertaken studies on potential benefits of dietary CLA against atherosclerosis (3,5,6,13). However, the presence of different isomers in CLA preparations used in these experiments makes interpretation difficult with respect to the identity of the biologically active form of CLA.

We previously reported (2) that, in hamsters, a commercially available mixture of conjugated linoleic acid isomers, but not pure *cis*-9,*trans*-11-octadecadienoic acid (C9T11), is able to lower serum total cholesterol and serum triglyceride over a 6-week feeding period. We extended the feeding experiment on the same groups of animals for another 10 weeks and now present terminal data on serum lipids and aortic fatty streak formation. In addition, we measured whole blood

chemiluminescence and macrophage prostaglandin E_2 synthesis to determine whether dietary CLA intervenes in either of these two oxygen radical-dependent pathways.

MATERIALS AND METHODS

Animals and diets. This is the extension of a previous feeding experiment, the results of which have been previously published (2). Thirty male Golden Syrian hamsters (110-110 g) purchased from Charles River Breeding Laboratories (St. Constant, Quebec, Canada) were divided into three groups of ten, and subjected to a housing and feeding regimen as described in the previous report (2). Briefly, the animals were housed five to a cage and fed a mild atherogenic diet consisting of Rodent Chow 5001 (Agribrands-Purina Canada, Strathroy, ON, Canada) supplemented with 10g/100g hydrogenated coconut oil (ICN, Mississauga ON, Canada) and 0.05g/100g cholesterol (ICN). The use of a commercial feed instead of a defined diet potentiates the atherogenic effect as discussed in the previous report (2). The three groups of animals were fed the following: CLA group, mild atherogenic diet plus CLA (Nu-Chek, Elysian MN, USA) at 1g/100g diet; C9T11 group, mild atherogenic diet plus C9T11 (Matreya, Pleasant Gap PA, USA) at 0.2g/100g diet; LA group, mild atherogenic diet plus LA (Nu-Chek) at 0.2g/100g. As explained previously, (2), the dietary level of C9T11 was chosen to match the proportion of this particular isomer in the commercial preparation of CLA and the LA group served as its control. The diets were freshly prepared every 2 weeks and stored at –20°C until use. Animals had free access to food, which was renewed twice weekly. All experimental procedures using hamsters were approved by the Animal

Care Committee of the Université de Montréal in accordance with the guidelines of the Canadian Council on Animal Care.

Collection of animal tissues. Food was removed from the hamster cages 24 h prior to tissue sample collection. Animals were anaesthetized with ketamine and resident peritoneal macrophages were harvested before blood collection by washing the peritoneal cavity with 30 mL sterile phosphate-buffered saline. Animals were then sacrificed by exsanguination through cardiac puncture. Blood was collected into EDTA-containing tubes. The heart was immediately perfused with cold 4% phosphate-buffered formalin. The perfused heart including the thoracic aorta were dissected and stored in 4% phosphate-buffered formalin pending analysis.

Measurement of aortic fatty streak lesions. Aortas were rinsed with 60% isopropanol, immersed in oil red O staining solution for 30 min, blotted dry then rinsed with sodium cacodylate buffer for 1 min. Stained aortas were then opened longitudinally and mounted on a glass slide luminal surface up and covered with aqueous mounting medium and a glass cover slip. The section of aorta between the third neck vessel to 1 mm above the aortic valve, measuring approximately 5 mm in length, was examined for lesion development (14,15). Three separate methods were used to evaluate lesion development. First, the extent of lipid staining was scored

independently by two evaluators using a scale of 1 to 4 as follows: 1, lesions covering 0 to 25% of the section; 2, lesions covering 26 to 50% of the section; 3, lesions covering 51 to 75% of the section; 4, lesions covering 76 to 100% of the section. Second, the sections were photographed and the images digitized. The total area of lesions covering the aortic section was measured using ImageJ, a public domain software originally written at the National Institutes of Health. Third, the amount of oil red O stain on each aortic section was determined directly by spectrophotometry as described by Nunnari et al (16). Briefly, a defined section of the aorta was dissected, its total area measured and then extracted with solvent consisting of $CHCl_3/CH_3OH$ (2:1, v/v). The quantity of oil red O extracted was calculated by comparing its absorbance to a standard curve generated separately. The result was expressed as $\mu g/mm^2$ intimal oil red O.

Lipoprotein fractionation and analysis. Blood samples from 3 animals within a diet group were pooled and centrifuged for 10 min at 4000 rpm to collect the plasma. The different lipoprotein fractions were collected by successive centrifugation according to the method described by Levy et al. (17). Triglyceride in the various fractions was measured colorimetrically using a Peridochrom Triglycerides GPO-PAP kit (Boehringer Mannheim, Montreal QC Canada). Total cholesterol was measured colorimetrically

using a Sigma Diagnostics Cholesterol kit (Sigma, Oakville ON Canada). Phospholipid phosphate was measured according to Bartlett (18).

Chemiluminescence. Phorbol myristate acetate-induced superoxide release by phagocytic cells in whole blood was measured by chemiluminescence as described by Allen and Pruit (19). Lucigenin (Sigma) was used as the chemiluminescence probe. Chemiluminescence intensity was measured for 30 s at 10-min intervals using a Packard Tri-Carb 1500 scintillation counter (Canberra Packard, Mississauga ON Canada) operated in the out-of-coincidence mode. Data were analyzed as described by Allen et al (20).

PGE_2 secretion. Resident peritoneal macrophages were washed and plated as described by Whelan et al (21). Briefly, the cells were plated in 25-cm^2 culture flasks at a density of 8×10^4 cells/cm^2 and allowed to adhere to the culture surface for 1 h in a CO_2-incubator at 37°C. Non-adherent cells were removed by washing the culture flasks twice with phosphate-buffered saline. The adherent cells were treated with the calcium ionophore A23187 (Sigma) for 30 min to stimulate prostaglandin release. PGE_2 released into the culture medium was measured by enzyme-linked immunoassay using the Biotrak system (Amersham Pharmacia Biotek, NJ, USA).

Statistical analyses. Analysis of variance and post-hoc tests were performed using routines available in SPSS.

RESULTS

All diets were well accepted by the animals over the 16-wk feeding period. The plasma lipid levels are shown in Table 1. No significant differences in plasma triglycerides were observed at 16 wks for any of the diet groups. C9T11 had significantly lower plasma total cholesterol, HDL cholesterol and non-HDL cholesterol compared to the 2 other diet groups. In contrast, the only significant effect of CLA was to lower HDL-cholesterol level relative to the LA group. Table 2 shows the composition of lipoprotein classes. The relative proportion of free (unesterified) cholesterol, cholesterol ester, triglyceride, phospholipids and proteins within each lipoprotein class was not significantly different across the diet groups. Evaluation of early atherogenesis by different methods revealed that there were no significant differences in the extent of formation of aortic fatty streak lesions among the groups (Table 3).

Phorbol myristate acetate was able to induce superoxide release by phagocytes in whole blood as indicated by production of superoxide-induced chemiluminescence of lucigenin (Table 4). Neither CLA nor C9T11 were able to significantly modulate superoxide-induced chemiluminescence relative to LA. Table 4 also shows that calcium ionophore induces resident macrophages to release PGE_2 into culture medium. As in the results of

chemiluminescence, neither CLA nor C9T11 significantly influenced this oxygen radical-dependent pathway with respect to the LA group.

Table 1

Effect of dietary conjugated linoleic acid (CLA), *cis*-9,*trans*-11-octadecadienoic acid (C9T11) and linoleic acid (LA) on plasma total triglycerides, total cholesterol, HDL-cholesterol and non-HDL-cholesterol[1]

Plasma lipids	Diet group		
	CLA	c9t11	LA
	n=10	n=9	n=9
	mmol/L		
Triglycerides	2.45 ± 0.62	2.16 ± 0.51	2.55 ± 0.90
Total cholesterol	3.46[a] ± 0.59	2.76[b] ± 0.45	3.92[a] ± 0.74
HDL-cholesterol	1.25[a] ± 0.25	1.11[a] ± 0.29	1.60[b] ± 0.28
non-HDL-cholesterol	2.21[a] ± 0.50	1.65[b] ± 0.34	2.31[a] ± 0.53

[1]The animals were fed the diets for 16 weeks. Values are means ± SD from the indicated number (n) of 24-h fasted animals. Values in a row with different letter superscripts differ, $P < 0.05$.

Table 2

Effect of dietary conjugated linoleic acid (CLA), *cis*-9,*trans*-11-octadecadienoic acid (C9T11) and linoleic acid (LA) on plasma VLDL, LDL, HDL_2 and HDL_3 composition in hamsters[1]

	Diet group		
	CLA	C9T11	LA
	Weight percent[2]		
VLDL			
Free cholesterol	1.17 ± 0.47	1.32 ± 0.51	1.52 ± 0.51
Cholesterol ester	4.6 ± 1.5	4.05 ± 0.91	4.6 ± 1.0
Triglyceride	49.8 ± 7.5	52.3 ± 5.5	51.1 ± .09
Phospholipids	11.6 ± 1.1	9.8 ± 1.7	11.2 ± 1.1
Protein	33 ± 8	33 ± 4	31.6 ± 1.5
	100.2	100.5	100.0
LDL			
Free cholesterol	2.22 ± 0.42	2.26 ± 0.07	2.14 ± 0.19
Cholesterol ester	25.9 ± 1.3	23.0 ± 3.2	24.9 ± 3.6
Triglyceride	8.1 ± 3.4	5.1 ± 1.1	4.1 ± 0.5
Phospholipids	17.1 ± 1.8	15.8 ± 4.3	17.4 ± 1.0
Protein	46.7 ± 5.6	53.9 ± 3.1	51.4 ± 2.1
	100.0	100.1	99.9

HDL_2			
Free cholesterol	1.7 ± 0.53	1.18 ± 0.40	2.1 ± 0.45
Cholesterol ester	16 ± 3	18.0 ± 1.6	14.5 ± 2.4
Triglyceride	3.2 ± 2.3	4.7 ± 2.9	2.9 ± 1.5
Phospholipids	19 ± 8	20.9 ± 3.8	24.9 ± 2.1
Protein	60 ± 9	55.3 ± 0.7	55.7 ± 2.6
	99.9	100.1	100.1
HDL_3			
Free cholesterol	0.89 ± 0.16	0.87 ± 0.24	0.99 ± 0.17
Cholesterol ester	15.5 ± 1.3	13.2 ± 0.89	13.9 ± 0.3
Triglyceride	2.0 ± 1.7	0.9 ± 0.4	0.9 ± 0.1
Phospholipids	30 ± 3	28.9 ± 1.0	29.8 ± 1.3
Protein	52.1 ± 1.9	56.1 ± 1.9	54.5 ± 0.9
	100.5	100	100.1

[1]The animals were fed the diets for 16 weeks. Lipoproteins were isolated by preparative ultracentrifugation.

[2]Values are percentages by weight (mean ± SD, n=3 pooled blood samples)

Table 3

Effect of dietary conjugated linoleic acid (CLA), *cis*-9,*trans*-11-octadecadienoic acid (C9T11) and linoleic acid (LA) on formation of aortic fatty streaks in hamsters[1]

Measurement method		Diet group		
		CLA	C9T11	LA
		Scale of 1 to 4		
Semi-quantitive[2]	A	1.7 ± 0.6	1.7 ± 0.8	1.6 ± 0.6
	B	1.8 ± 0.8	1.8 ± 1.0	1.8 ± 0.6
		ng dye/mm^2 of aorta		
Bound Oil red-O dye[3]		30.4 ± 4.3	32.1 ± 4.8	28.6 ± 4.5
		mm^2 lesion area/10 mm^2 aorta		
Computer-aided image analysis[4]		0.67 ± 0.44	0.82 ± 0.34	0.88 ± 0.62

[1]The animals were fed the diets for 16 weeks. Values are means ± SD, n=6. The section of formalin-fixed aorta between the aortic valve and the third neck vessel was assessed for fatty streak formation by three different methods. There were no significant differences among the means.

[2]Aortic sections were scored by two separate evaluators using a scale of 1 to 4 as follows: 1, lesions from 0 to 25% of the section; 2, lesions on 26 to

50% of the section; 3, lesions on 51 to 75% of the section; 4, lesions on 76 to 100% of the section.

[3]Sections stained with dye were extracted with organic solvent, the absorbance of the extract measured and compared with a standard curve generated from known concentrations of Oil Red-O.

[4]Sections where photographed and the images digitized for image analysis. Total area of fatty streak lesions were measured using ImageJ, a public domain software originally written at the National Institutes of Health.

Table 4

Effect of dietary conjugated linoleic acid (CLA), *cis*-9,*trans*-11-octadecadienoic acid (C9T11) and linoleic acid (LA) on prostaglandin production and whole-blood chemiluminescence in hamsters[1]

Indicator	Diet group		
	CLA	C9T11	LA
	Total luminescence x 10^{-6}		
Whole-blood chemiluminescence[2]	7.26 ± 3.34	5.87 ± 1.46	5.75 ± 1.81
	Ng/flask		
Macrophage prostaglandin E_2 synthesis[3]	1.86 ± 0.33	2.59 ± 0.37	2.14 ± 0.57

[1]The animals were fed the diets for 16 weeks. Values are means ± SD, n=4 except for chemiluminescence of LA group where n=3.

[2]Total luminescence was calculated as the area under the curve of luminescence intensity over time. Luminescence intensity was measured as cpm using a liquid scintillation counter in non-coincident mode. There were no significant differences among the means at α=0.05.

[3]Washed resident peritoneal macrophages seeded at 2×10^2 cells and attached to 25 cm^2 culture flasks were treated with calcium ionophore A23187 for 30 min. Prostaglandin released into the culture media was

measured by enzyme-linked immunoassay. There were no significant differences among the means at α=0.05.

DISCUSSION

It is generally believed that elevated LDL-cholesterol and VLDL-triglyceride are risk factors for atherosclerosis while HDL-cholesterol is considered to be protective against this disease (10). Since CLA modulates one or more of these lipoproteins under certain conditions, a possible protective effect of CLA on early experimentally induced atherogenesis has been explored in the past (3,5,6,13). An early report on rabbits (3) showed a significant protective effect of CLA on aortic fatty streak formation and collagen build-up with a tendency for CLA-induced lowering of serum triglyceride and LDL-cholesterol in this model. Subsequently, Nicolosi et al. (6) reported that dietary CLA diminished fatty streak formation in the aortic arch of hamsters. However, these investigators found that linoleic acid alone at comparable levels afforded the same protection as CLA relative to the control group showing that the protective effect was not specific to CLA. Furthermore, they found no correlation between fatty streak formation and serum cholesterol and triglyceride levels. Munday et al. (5) reported that in an atherosclerosis-susceptible mouse model, dietary CLA induced higher serum HDL-cholesterol and lower serum triglycerides but paradoxically caused an increase in aortic fatty streak formation. Recently, Kritchevsky et al. (13) extended their earlier study (3) and confirmed that dietary CLA reduces atherogenesis in the aortic arch and thoracic aorta of rabbits. Furthermore, they found that CLA is able to induce regression of pre-established atherosclerotic lesions. In contrast to Munday et al. (5),

Kritchevsky et al (13) found that under their experimental conditions, CLA increased total serum cholesterol and triglycerides but lowered the proportion of HDL-cholesterol in these animals.

Clearly, the CLA effect is sensitive not only to the animal model used but also to the different experimental conditions to which the same animal model might be exposed. Therefore, it is important that more data be accumulated comprising different experimental protocols in order to gain a better understanding of the biological and physiological context in which CLA exerts potentially beneficial effects, including possibly anti-atherogenic activity.

Our data on hamster blood lipoproteins at 16 weeks of feeding, presented in Table 1, are different from the data we reported on the same animals when they were still at 2 and 6 weeks of feeding (2). In the younger animals, the CLA group had significantly lower blood triglyceride and blood total cholesterol than either the C9T11 or LA groups. In contrast, by16 weeks of feeding, there were no longer any differences in triglyceride level among the groups. Furthermore, at 16 weeks of feeding the C9T11 group, instead of the CLA group had significantly lower blood total cholesterol than the two other groups. The younger animals had displayed similar blood HDL-cholesterol in all diet groups (2). This, too, changed at

16 weeks feeding in that the HDL-cholesterol of the LA group was now significantly higher than either of the CLA or C9T11 groups. The data suggests age-dependent effects and/or adaptive mechanisms in the way CLA affects turnover of blood lipoproteins. This merits further investigation.

In view of the differences in plasma total cholesterol and HDL-cholesterol among the diet groups, and based on the current paradigm concerning serum cholesterol and atherogenesis, we expected to see differences in the extent of early atherogenesis in the aortic arch of the animals. However, under our experimental conditions, none of the dietary groups offered any advantage over each other with respect to fatty streak lesion development (Table 2). While both CLA and C9T11 were able to modulate blood lipids in certain ways, the lipoprotein compositional data show that neither one of them affect lipoprotein metabolic conversions to any significant extent relative to the LA group (Table 3). This indicates that the mechanisms involving cholesterol delivery and recovery from peripheral tissue are either unaffected, or all affected in the same way, by CLA, C9T11 and LA. Since atherogenesis may be related to disturbances in cholesterol transport, the inability of the different diets to affect fatty streak lesion development may be due in part to the inability of these diets to affect lipoprotein composition differentially.

Another mechanism by which CLA may exert beneficial effects is its reported antioxidant activity. There is strong evidence that point to peroxidative damage to LDL and the initiation and progression of atherosclerotic lesions (22). Thus, the reported anti-atherogenic effect of CLA may be related to its possible antioxidant properties. However, the reports concerning CLA as an antioxidant are conflicting (8,9,23-26). Part of the problem may be due to differences in the antioxidant properties of the different CLA isomers. Leung et al. (27) reported that the trans-10,cis-12 isomer but not C9T11 has antioxidant activity. They therefore proposed that the total antioxidant of CLA will depend on the proportions of the different isomers present in that mixture. Our results indicate that relative to the LA group, neither CLA nor C9T11 are able to modulate two mutually independent pathways that involve free radicals, namely, phagocytic superoxide release and the prostaglandin pathway (Table 4). This observation may therefore also explain why, under the present experimental conditions, CLA or C9T11 were unable to influence fatty streak lesion development relative to the LA group.

In conclusion, in our experimental model, we did not observe any differences in the way CLA, C9T11, or LA influenced aortic fatty streak lesion development in the hamster. This may be related to the inability of these compounds to influence lipoprotein interconversions differently from

each other, nor to display antioxidant activity in pathways involving free radical intermediates.

Footnotes

[1]Supported in part by the Dairy Farmers of Canada

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[3]Abbreviations used: CLA, conjugated linoleic acid; LA, linoleic acid; C9T11, *cis*-9,*trans*-11-octadecadienoic acid

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VI. DISCUSSION

Conjugated linoleic acid (CLA) is a term given to a group of positional and geometrical isomers of linoleic acid (18:2 n-6) in which the double bonds are conjugated (16, 18). The major dietary form of CLA isomer is the *cis*-9,*trans*-11 isomer, which is assumed to be the biologically active isomer. In animals, CLA has been shown to have potential benefits in cardiovascular health. The present study was designed to examine whether pure *cis*-9,*trans*-11 isomer has the same effect as the mixture of CLA isomers on early atherosclerosis.

It is generally accepted that LDL-cholesterol and VLDL-triglyceride are risk factors for atherosclerosis while HDL-cholesterol is believed to be protective against this disease (70). In an early study, Lee et al. (11) reported that CLA had protective effects against atherosclerosis in rabbits and there was a correlation between atherosclerosis and plasma lipids. By contrast, Nicolosi et al. (32) showed no statistically significant correlation between fatty streak formation and serum cholesterol and triglyceride levels. Their results indicated that CLA protected against the development of early arterial lipid accumulation. But, they also found that linoleic acid alone at comparable levels afforded the same protection as CLA relative to the control group showing that the protective effect was not specific to CLA. Furthermore, Munday et al. (33) observed that fatty streak development

was not correlated with serum lipid ratio or with any other measured variable. They reported that in the atherosclerosis-susceptible mouse model, dietary CLA induced higher serum HDL-cholesterol and lower serum triglycerides. Moreover, the aortic fatty streak area was significantly higher, suggesting that CLA promoted athersclerosis. Recently, Kritchevsky et al. (34) extended their earlier study (11) and reported that dietary CLA reduces atherogenesis in the aortic arch and thoracic aorta of rabbits. The effects on atherogenesis were observed even though plasma lipids were unaffected.

Our data on hamster plasma lipids at 16 weeks of feeding is presented in Table 1. It should be pointed out that plasma lipid analysis on the same animals when they were still at 2 and 6 weeks of feeding (71), showed that triglycerides were significantly decreased in the CLA-fed group, but not in the pure *cis*-9,*trans*-11-fed group and LA-fed group. However, by 16 weeks of feeding, there were no longer any differences among the groups. Plasma total cholesterol levels were also significantly decreased in the CLA-fed group compared to the other groups at 2 and 6 weeks of feeding. Interestingly, at 16 weeks of feeding the pure *cis*-9,*trans*-11 fed group had significantly lower blood total cholesterol than the two other groups. The animals had displayed similar plasma HDL-cholesterol in all diet groups at 2 and 6 weeks. This changed at 16 weeks feeding in that the HDL-cholesterol of the LA-fed group was significantly higher than either of the CLA or pure *cis*-9,*trans*-11-fed groups. Our data suggests that

additional investigation is needed to verify whether age-dependent effects and/or adaptive mechanisms in the way CLA affects turnover of blood lipoproteins.

In view of the differences in plasma total cholesterol and HDL-cholesterol among the diet groups, and based on the current paradigm concerning serum cholesterol and atherogenesis, we expected to see differences in the extent of early atherogenesis in the aortic arch of the animals. However, under our experimental conditions, the data indicated that none of the dietary groups appeared to have an anti-atherogenic effect (Table 3). While both CLA mixture and pure *cis*-9,*trans*-11 isomer were able to modulate plasma lipid levels in certain ways, the lipoprotein compositional data show that neither one of them affect lipoprotein metabolic conversions to any significant extent relative to the LA group (Table 2). Since atherogenesis may be related to disturbances in cholesterol transport, the inability of the different diets to affect fatty streak lesion development may be due in part to the inability of these diets to affect lipoprotein composition differentially. de Deckere et al. (72) reported interesting findings, in that the CLA mixture and the *trans*-10,*cis*-12 isomer decreased LDL-cholesterol, VLDL triglyceride and HDL-cholesterol, whereas the *cis*-9,*trans*-11 isomer had no such effect. Therefore, the data seem to suggest that the *trans*-10,*cis*-12 isomer plays a key role that affects lipoprotein composition.

Increasing evidence shows that oxidative processes may contribute to the pathogenesis of atherosclerosis (73-75). Therefore, the alleged anti-atherogenic effect of CLA may be related to its possible antioxidant properties. Previous studies (13, 21) reported that CLA was an effective antioxidant. In contrast, van den Berg et al. (59) observed that CLA did not act as an efficient radical scavenger comparable to vitamin E or butylated hydroxytoluene (BHT). Their results did not show any clear antioxidant properties of CLA under a variety of experimental conditions. Lee et al. (11) measured lipid peroxidation in plasma using TBARS assay. They reported that CLA-fed rabbits had similar results in plasma lipid peroxidation compared with the control group. But, the CLA-fed rabbits exhibited less histological evidence of atherogenesis as assessed by vascular lipid deposition. Thus, the reports concerning CLA as an antioxidant are conflicting. Part of the problem may be due to differences in the antioxidant properties of the different CLA isomers. Interestingly, Leung et al. (61) showed that the *trans*-10,*cis*-12 isomer acted as a strong antioxidant but not the *cis*-9,*trans*-11 isomer. These investigators commented that the contradictory results of previous studies on the antioxidant properties of CLA, were obtained with mixtures of CLA isomers. Our results indicate that relative to the LA group, neither CLA nor pure *cis*-9,*trans*-11 isomer group are able to modulate or interfere with two mutually independent pathways that involve free radicals, namely, phagocytic superoxide release and the prostaglandin pathway (Table 4). The data indicated that CLA does not

appear to have an anti-atherogenic effect under our experimental conditions.

VII. CONCLUSION

We investigated the effect of dietary CLA and pure *cis*-9,*trans*-11 isomer on blood lipids and early atherogenesis in male Golden Syrian hamsters fed a mild atherogenic diet for 117 days.

Under our experimental conditions, the aortic fatty streak lesions were of similar intensity in groups fed a mixture of CLA isomers, pure *cis*-9,*trans*-11 isomer or LA. Furthermore, the CLA mixture or pure *cis*-9,*trans*-11 isomer did not modulate individual lipoprotein composition or in vivo free radical formation.

The present work does not support the view that a mixture of CLA isomers or the pure *cis*-9,*trans*-11 isomer can protect against atherosclerosis.

In the future, it will be important to investigate the properties of the various CLA isomers, individually and in combination. Each isomer may have its own characteristic activity.

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