Cholesterol-induced stimulation of platelet aggregation is prevented by a hempseed-enriched diet[1]

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Abstract: Hypercholesterolemia indirectly increases the risk for myocardial infarction by enhancing the ability of platelets to aggregate. Diets enriched with polyunsaturated fatty acids (PUFAs) have been shown to reduce the detrimental effects of cholesterol on platelet aggregation. This study investigated whether dietary hempseed, a rich source of PUFAs, inhibits platelet aggregation under normal and hypercholesterolemic conditions. Male New Zealand white rabbits were fed one of 6 dietary interventions: regular control diet (RG); control diet + 10% hempseed (HP); control diet + 10% partially delipidated hempseed (DHP); control diet + 0.5% cholesterol (OL); control diet + 0.5% cholesterol + 10% hempseed (OLHP); control diet + 5% coconut oil (CO). After 8 weeks, blood was collected to measure ADP- and collagen-induced platelet aggregation and plasma levels of fatty acids, cholesterol, and triglycerides. The hempseed-fed animals (HP and OLHP) displayed elevated plasma levels of PUFAs and a prominent enhancement in 18:3n-6 (γ-linolenic acid, GLA) levels, a unique PUFA found in hempseed. The cholesterol-supplemented groups (OL and OLHP) had significantly elevated plasma levels of cholesterol and triglycerides, but platelet aggregation was significantly augmented only in the OL group. The addition of hempseed to this diet (OLHP) normalized aggregation. The direct addition of GLA to the OL platelet samples blocked the cholesterol-induced stimulation of platelet aggregation. The results of this study demonstrate that when hempseed is added to a cholesterol-enriched diet, cholesterol-induced platelet aggregation returns to control levels. This normalization is not due to a reduction in plasma cholesterol levels, but may be partly due to increased levels of plasma GLA.

Key words: hempseed, cholesterol, platelet, aggregation, gamma-linolenic acid.

Résumé : L'hypercholestérolémie augmente indirectement le risque d'infarctus du myocarde en en stimulant la capacité d'agrégation des plaquettes. Des travaux ont démontré que les diètes enrichies d'acides gras polyinsaturées (AGPI) diminuent les effets négatifs du cholestérol sur l'agrégation des plaquettes. La présente étude a examiné si le chènevis alimentaire, une riche source d'AGPI, peut inhiber l'agrégation des plaquettes, dans des conditions normales et hypercholestérolémiques. On a soumis des lapins blanc néo-zélandais à l'une des 6 diètes suivantes : diète témoin (DT); diète témoin + 10 % de chènevis (CH); diète témoin + 10 % de chènevis partiellement délipidé (DHP); diète témoin + 0,5 % de cholestérol (OL), diète témoin + 0,5 % de cholestérol + 10 % de chènevis (OLPH); diète témoin + 5 % d'huile de noix de coco (CO). Huit (8) semaines après chacune des diètes, on a fait des prélèvements sanguins pour mesurer l'agrégation plaquettaire induite par l'ADP et par le collagène, et pour mesurer les taux plasmatiques d'acides gras, de cholestérol et de triglycérides. Les animaux nourris avec du chènevis (HP et OLHP) ont présenté une augmentation des taux plasmatiques d'AGPI, et plus particulièrement des taux de 18:3n-6 (acide γ-linolénique, AGL), un AGPI particulier contenu dans le chènevis. Les groupes ayant reçu un supplément de cholestérol (OL et OLPH) ont montré une augmentation marquée des taux plasmatiques de cholestérol et de triglycérides. L'agrégation plaquettaire n'a augmenté significativement que chez le groupe OL uniquement, étant donné que l'ajout de chènevis à cette diète (OLPH) a normalisé l'agrégation. Plus directement, l'ajout d'AGL aux échantillons des plaquettes OL a bloqué la stimulation de l'agrégation plaquettaire induite par le cholestérol. Les résultats de la présente étude démontrent que l'ajout de chènevis à une diète enrichie de cholestérol ramène l'agrégation des plaquettes induite par le cholestérol aux valeurs témoins. Cet normalisation n'est pas dû à une diminution des taux de cholestérol plasmatique, mais pourrait être dû en partie à une augmentation des niveaux plasmatiques d'AGL.

Mots-clés : chènevis, cholestérol, plaquette, agrégation, acide gamma-linolénique.

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Introduction

High cholesterol levels are a known risk factor for cardiovascular disease (Heart and Stroke Foundation of Canada 2003; Fletcher et al. 2005). Among its detrimental effects, cholesterol augments platelet aggregation (De La Cruz et al. 1997; De La Cruz et al. 2000; Naqvi et al. 1999, Renaud et al. 1986), which triggers the formation of blood clots and can lead to myocardial infarction and stroke. Given the prevalence of high blood cholesterol levels in the population (America Heart Association 2006) despite the availability of superior cholesterol-lowering agents, it is important to identify other ways of counteracting these detrimental effects.

Polyunsaturated fatty acid (PUFA)-containing foods have demonstrated the potential to reduce cardiovascular disease (Albert et al. 1998; Ander et al. 2004; Hu et al. 2002; Oomen et al. 2000; Yuan et al. 2001). While this is thought to occur via several mechanisms, a reduction in platelet aggregation may be key (Hirai et al. 1980; Li and Steiner 1990; Vas Dias et al. 1982). Diets rich in fatty fish have been clinically observed to reduce cardiovascular disease parameters (Albert et al. 1998; Hu et al. 2002; Oomen et al. 2000; Yuan et al. 2001), but consumption of fatty fish or fish oil supplements is not always well-tolerated. Therefore, plant-derived functional foods may present an advantage over fish or fish-derived oils as a source of PUFAs.

Varieties of hemp, *Cannabis sativa* L., are cultivated for oilseed and fibre in many areas of the world (Lozano 2001) and hempseeds have been used to treat various disorders in both Arabic (Lozano 2001) and traditional folk medicine (Grigoriev 2002). Recent clinical trials have demonstrated the ability of hempseed oil to treat ear, nose, and throat disorders (Grigoriev 2002) and atopic dermatitis (Callaway et al. 2005). The nutritional benefits of hempseed may be due to its complement of PUFAs. Hempseed possesses a ratio of omega-6 : omega-3 PUFAs of approximately 4:1. This ratio has been recommended by Health Canada as optimal to promote well-being (Holub 2002). In addition, hempseed provides a good source of protein (Tanelian 2001) and vitamin E (Hemp Oil Canada 2001).

While it is distinctly possible that the omega-3 fatty acid content of hempseed may inhibit cholesterol-induced platelet aggregation, the omega-3 fatty acids present in hempseed (α-linolenic (ALA) and stearidonic acids) are not the same as those found in fish and their physiological effects may be different. Furthermore, hempseed contains omega-6 fatty acids, some of which may actually increase the ability of platelets to aggregate by stimulating proaggregatory eicosanoid production (Burke et al. 2003). The goal of this study was to investigate the ability of dietary hempseed to reduce platelet aggregation under pathophysiological conditions (hypercholesterolemia) where platelet aggregation may be induced.

Methods

Diet and feeding

All experiments conformed to the guidelines of the Canadian Council on Animal Care (Olfert et al. 1993). Male New Zealand White (NZW) rabbits were housed on a 12 h light : 12 h dark cycle in individual cages at constant room temperature (20 °C). Six interventional diets were made as follows: (*i*) regular control (RG), (*ii*) control diet supplemented with 10% (*w*/*w*) hempseed (HP), (*iii*) control diet supplemented with 10% (*w*/*w*) partially delipidated hempseed (DHP), (*iv*) control diet supplemented with 0.5% (*w*/*w*) cholesterol (OL), (*v*) control diet supplemented with 10% hempseed and 0.5% cholesterol (OLHP), and (*vi*) control diet supplemented with 5% (*w*/*w*) coconut oil (CO). The concentration of hempseed used in this study (10%) is similar to that used in other animal studies to show health-related benefits of flaxseed (Dupasquier et al. 2007; Dupasquier et al. 2006; Ander et al. 2004). Hempseed was ground, water was added to the partially delipidated hempseed, and the coconut oil was heated to 25 °C to facilitate homogenous distribution with the control diet. During mixing, approximately equal quantities of water were added to each interventional diet. Once mixed, the interventional diets were ground to resemble rabbit chow, spread in a thin layer, and air-dried over a period of 2–3 days. The diets were stored at 4 °C until used. The specific fatty acid composition of the diets is shown in Prociuk et al. (2006). Each rabbit was fed 125 g/day of diet for 8 weeks. This amount was determined on the basis of the daily food consumed in a previous study and the nutritional requirements of the rabbits (Ander et al. 2004).

Platelet aggregation analysis

Blood was collected from the jugular vein of each animal into sodium citrate tubes. The tubes were gently inverted and then stored at a room temperature of 20 °C for 30 min. The platelet-rich plasma was obtained by centrifugation of the samples at 100*g* for 15 min. Collection of the top layer from this step followed by subsequent centrifugation of the remaining sample at 2400*g* for 15 min provided the platelet-poor plasma. Equal volumes of the platelet-rich and platelet-poor plasma fractions were aliquoted into separate cuvettes. Either adenosine diphosphate (ADP) or collagen was then added to the platelet-rich plasma such that the final concentrations were 7.5 μmol/L and 4 μg/mL, respectively. Upon addition of the ADP or collagen aggregate, the sample was immediately measured in a Chrono-log aggregometer. The platelet-poor plasma served as the blank and was treated in an identical fashion. Both percentage of maximal aggregation and rate of aggregation were obtained by using the Aggro/Link (v. 4.75) software. In a separate experiment, 25 μmol/L γ-linolenic acid (GLA) was added to the samples collected from OL rabbits to determine its effect on platelet aggregation.

Plasma collection and lipid analyses

Blood collected with EDTA was centrifuged at 1800*g* for 10 min at 4 °C. The erythrocyte-free plasma was removed, aliquoted, and snap-frozen in liquid nitrogen. Plasma was stored at –80 °C. Plasma cholesterol ester and triglycerides were determined enzymatically as described previously (Prociuk et al. 2006).

Fatty acid extraction and analysis

Fatty acids were extracted from the interventional diets by using an adaptation of the Folch method (Folch et al. 1957). The interventional diet was ground, weighed to 1 g in a

glass beaker, and transferred to a separatory funnel. From 40 mL of a mixture of 2:1 (*v*/*v*) $CHCl_3$:CH_3OH, an aliquot was used to rinse out the beaker and the washing was transferred into the separatory funnel. This was repeated twice. The remaining solvent was then added to the funnel such that the total volume of solvent was 40 mL. Next, 8.4 mL of 0.73% (*w*/*v*) NaCl was added and the funnel was shaken vigorously. After separation of the organic and aqueous phases (approximately 16 h), the bottom $CHCl_3$ layer was collected and then the aqueous layer was once again extracted with 40 mL of the $CHCl_3$:CH_3OH mixture and 8.4 mL of the NaCl solution. The organic layer was collected and combined with the first extraction. The lipid solution was dried over Na_2S0_4 and filtered, and the solvent removed via rotary evaporation (40 °C). The exposed lipids were then dried under nitrogen gas. Samples were stored in 3 mL of $CHCl_3$ at –20 °C.

Fatty acids were esterified by using the Lepage and Roy (1986) method. For plasma fatty acid esterification, samples of plasma were added to 2 mL of a solution of 4:1 (*v*/*v*) CH_3OH:C_6H_6, after which 200 µL of C_2H_3ClO was added and the mixture vortexed. For extracted diet esterification, an aliquot of the extract in $CHCl_3$ was placed under a stream of nitrogen to evaporate the solvent, after which 3:2 (*v*/*v*) CH_3OH:C_6H_6 and 5:100 (*v*/*v*) C_2H_3ClO:CH_3OH were added to tubes containing the nitrogen-evaporated extracts and vortexed. From this point, both plasma and diet extracts followed an identical protocol. The tubes were weighed to obtain a weight before the esterification reaction. Next, they were heated for 1 h at 95 °C to promote methanolysis during which they were vortexed every 15 min. Once cool, they were reweighed to evaluate the possibility of sample evaporation. Samples having a difference in weight greater than 1% were rejected. Neutralization of the reaction occurred upon addition of 6% K_2CO_3. Liberation of the organic solvent containing the fatty acid methyl esters (FAMEs) resulted after centrifugation for 5 min at 4500*g* at 22 °C. An aliquot of the upper C_6H_6 layer was removed and analyzed with gas chromatography (GC) on a Varian GC/MS/MS instrument equipped with a CP-3800 GC, a CP-8400 autosampler, and a flame ionization detector (FID).

Statistical analyses

Data were analyzed by using one-way analysis of variance (ANOVA). Values of p less than 0.05 were considered statistically significant and parametrically analyzed with the Student–Newman–Keuls test.

Results

Diet composition

Each diet was sampled and extracted.Compared with the control (RG) diet, the hempseed (HP) diet was significantly enriched in several fatty acids, including 18:2n-6 (linoleic acid, LA), 18:3n-6 (γ-linolenic acid, GLA), and 18:3n-3 (α-linolenic, ALA). The partially delipidated hempseed (DHP) diet contained approximately half the amounts of LA, GLA, and ALA compared with the HP diet. The CO diet was significantly enriched in many saturated fats compared with the RG diet. The composition of the 6 diets is reported in detail elsewhere (Prociuk et al. 2006).

Body weight

Animals were weighed before the start of the diet and at the end of 8 weeks. All animals included in the calculation of mean body weight completed the assigned diet for 56 ± 3 days. One animal in the OLHP group ceased feeding and was removed from the study at 50 days. This animal was removed from the data analysis. Animals gained approximately 1 kg in body weight over the course of the study. No statistically significant differences in animal weights were observed among the groups before the start of the diet or at the end of the study.

Plasma levels of cholesterol esters, triglycerides, and fatty acids

Plasma was analyzed for cholesterol ester, triglyceride, and fatty acid content. The OL and OLHP groups had significantly higher levels of plasma cholesterol esters and triglycerides than all other groups (Fig. 1). As well, the OLHP group had a significantly elevated plasma cholesterol ester level compared with that of the OL group.

Levels of LA and ALA were significantly elevated in the OL and OLHP groups compared with all other groups. As well, the OLHP levels of these fatty acids were significantly elevated over the OL levels. Levels of GLA were significantly elevated in the HP and OLHP groups compared with all other groups, and the OLHP levels of this fatty acid were significantly elevated over the HP levels. The plasma fatty acid content has been reported elsewhere (Prociuk et al. 2006) as a function of these dietary interventions.

Platelet aggregation

Total platelet aggregation (Fig. 2) and the rate of platelet aggregation (Fig. 3) were measured after stimulation by either ADP or collagen. The OL group had significantly increased ADP-induced aggregation (Fig. 2) and rate of aggregation (Fig. 3) compared with all groups except DHP. The addition of hempseed to the cholesterol-supplemented diet normalized ADP-induced aggregation. Similar trends between these groups were also observed with collagen-induced platelet aggregation. The OL group had a significantly increased rate of collagen-induced aggregation (Fig. 3) compared with all other groups. The addition of hempseed to the cholesterol diet normalized this effect. All groups, with the exception of the OL group, exhibited rates of aggregation that were similar to control values.

To determine if the elevated plasma GLA levels were responsible for the normalization of platelet aggregation in the OLHP group, GLA was directly added to the platelet samples obtained from cholesterol-supplemented rabbits or rabbits fed a regular diet. The GLA was added to the samples immediately before the initiation of platelet aggregation with ADP. GLA did not alter platelet aggregation in samples obtained from RG animals. However, GLA significantly inhibited ($p < 0.05$) maximal platelet aggregation (71.9% ± 4.5% of the OL activity) and the rate of aggregation (76.5% ± 5.0% of OL) in the OL samples.

Discussion

Three basic hypotheses were evaluated in this study. First, that dietary supplementation with cholesterol would signifi-

Fig. 1. Plasma cholesterol ester and triglyceride levels as a function of 6 dietary interventions in rabbits. RG, regular diet; HP, 10% hempseed diet; DHP, 10% partially delipidated hempseed diet; OL, 0.5% cholesterol diet; OLHP, 0.5% cholesterol + 10% hempseed diet; CO, 5% coconut oil diet. Mean ± SE values (n = 4 for all groups) are shown for cholesterol ester (A) and triglyceride (B) concentrations. Significant at *, $p < 0.05$ versus RG, HP, DHP, and CO groups; †, $p < 0.05$ versus CH group.

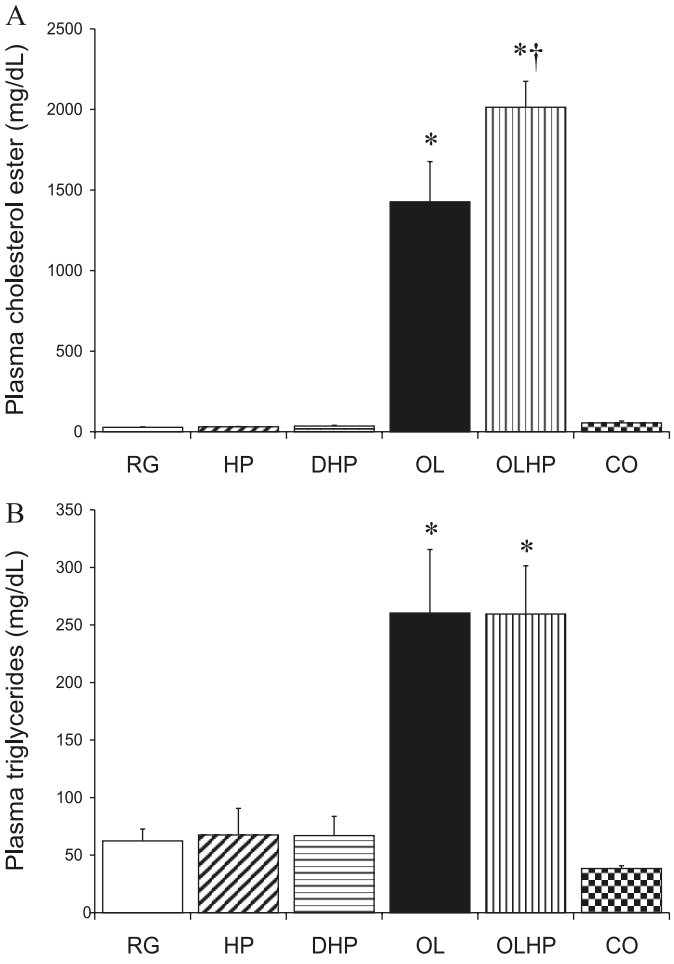
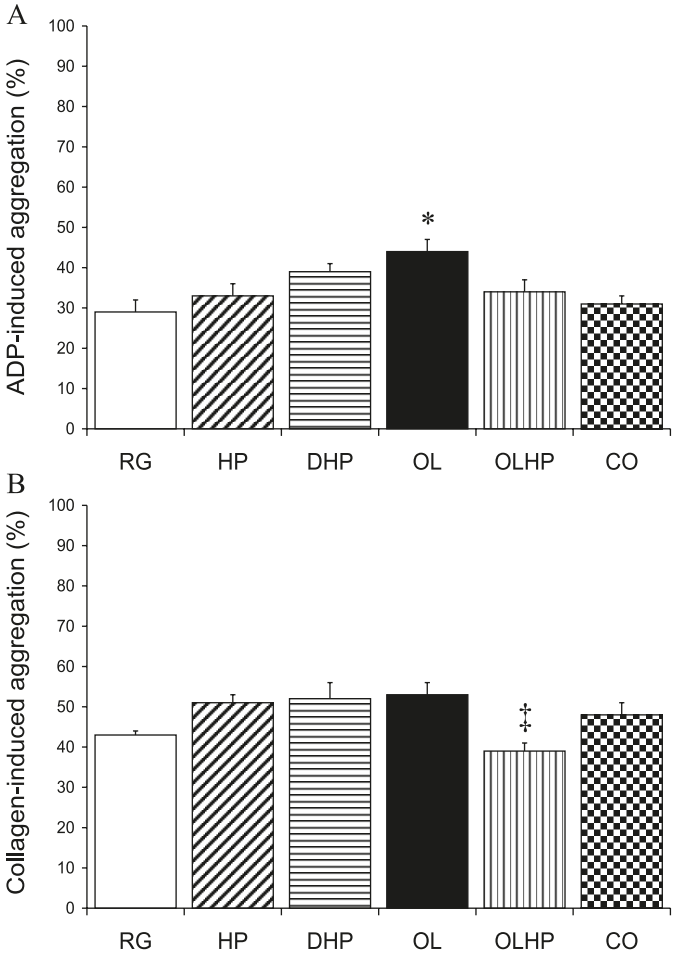


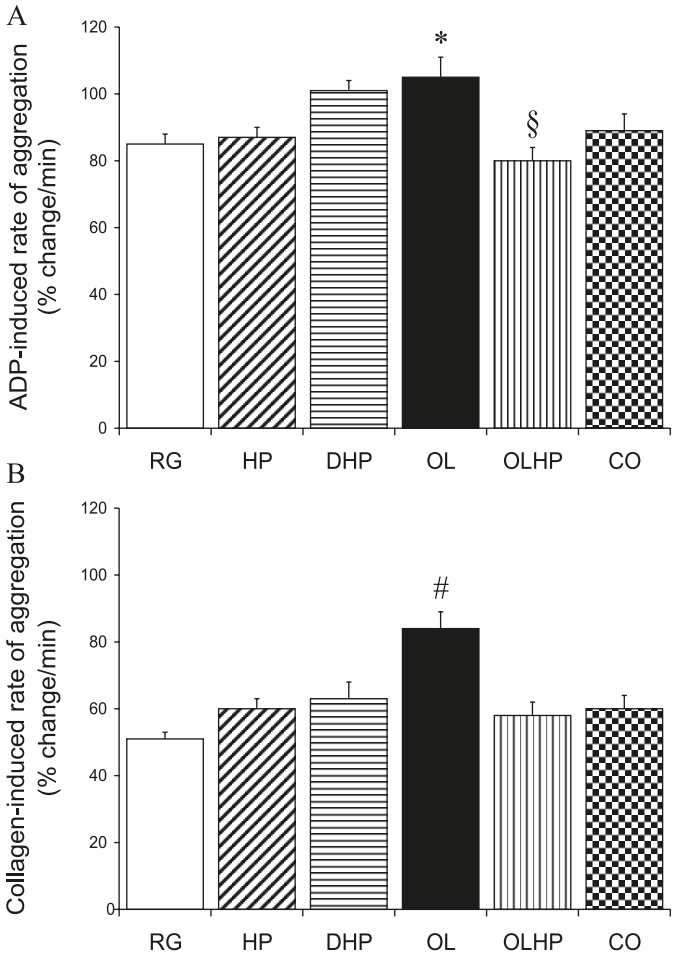
Fig. 2. Total platelet aggregation as a function of 6 dietary interventions in rabbits. RG, regular diet; HP, 10% hempseed diet; DHP, 10% partially delipidated hempseed diet; OL, 0.5% cholesterol diet; OLHP, 0.5% cholesterol + 10% hempseed diet; CO, 5% coconut oil diet. Mean ± SE values (n = 7–10) are shown for each group for either ADP-induced (A) or collagen-induced (B) platelet aggregation. Significant at *, $p < 0.05$ versus RG, HP, OLHP, and CO groups; ‡, $p < 0.05$ versus HP, DHP, OL, and CO groups.



cantly augment platelet aggregation. Second, that dietary supplementation with ground hempseed would inhibit platelet aggregation. Third, that dietary supplementation with ground hempseed would reduce the detrimental effects of hypercholesterolemia on platelet aggregation. Specifically, we hypothesized that the unique composition of fatty acids contained in hempseed would provide these beneficial effects. Moreover, it was inherent in these hypotheses that if hempseed were to exert any effect on platelet aggregation, the hempseed-derived fatty acids would have to be efficiently absorbed after 8 weeks of dietary supplementation.

Hempseed-only (HP) supplementation of the diet increased the plasma levels of the fatty acid GLA. Supplementation of the diet with partially delipidated hempseed resulted in plasma levels of fatty acids similar to those of the control (RG), with the expected decrease in levels of GLA compared with the HP plasma. Hempseed and cholesterol (OLHP) cosupplementation significantly elevated cholesterol and triglyceride levels and induced a nonselective, significant elevation in plasma levels of all fatty acids. Cholesterol alone (OL) supplementation produced a significant elevation in the level of plasma cholesterol and triglycerides, which was accompanied by a significant elevation in levels of all plasma fatty acids except GLA. This was not surprising as this fatty acid was only present in the hempseed-supplemented diets (HP, OLHP, and DHP). Our data, therefore, demonstrate that cholesterol stimulates nonselective fatty acid absorption of all fatty acids present in the diet. This finding is comparable with the work of Thomson et al. (1987) and Ander et al. (2004), which demonstrated that transient feeding of a high-cholesterol diet (2%) increased fatty acid levels in the blood, potentially through a stimulation of jejunal permeability to fatty acids (Thomson et al. 1987). Cosupplementation of the cholesterol-enriched diet with a rich source of fatty acids (i.e., hempseed) resulted in a significant augmentation of this effect beyond that found with cholesterol supplementation alone. This indicates that there is an additive effect of cosupplementation of hempseed and cholesterol that enhances absorption of hempseed-derived fatty acids.

Fig. 3. Rate of platelet aggregation as a function of 6 dietary interventions in rabbits. RG, regular diet (n = 8); HP, 10% hempseed diet (n = 8); DHP, 10% partially delipidated hempseed diet (n = 7); OL, 0.5% cholesterol diet (n = 8); OLHP, 0.5% cholesterol + 10% hempseed diet (n = 9); CO, 5% coconut oil diet (n = 10). Mean ± SE values are shown for each group for either ADP-induced (A) or collagen-induced (B) platelet aggregation. Significant at *, $p < 0.05$ versus RG, HP, and CO groups; §, $p < 0.05$ versus DHP and OL groups; #, $p < 0.05$ versus RG, HP, DHP, OLHP, and CO groups.



The objective of this study was to determine whether dietary hempseed causes an effect on platelet aggregation. Given the results of previous studies demonstrating the role of PUFAs in reducing platelet aggregation (Allman et al. 1995; De La Cruz et al. 1997; Guivernau et al. 1994; Hirai et al. 1980; Renaud et al. 1982; Smith et al. 1989; Vas Dias et al. 1982), we hypothesized that the fatty acids present in hempseed are responsible for any beneficial effect observed on platelet aggregation. As we observed, however, 3 of our treatment groups (HP, DHP, CO) did not demonstrate a significantly altered profile for many plasma fatty acids, nor did these groups have significantly elevated plasma cholesterol levels over those of control. Therefore, we did not expect, nor did we observe, a difference in platelet aggregation in these 3 groups compared with the control. In contrast, we found that cholesterol supplementation (OL), and the resulting hypercholesterolemia, led to a significant stimulation of platelet aggregation. This finding agrees with a previous study that demonstrated a positive correlation between platelet cholesterol and ADP-induced platelet aggregation (Renaud et al. 1986) and with other studies that demonstrated an increase in platelet aggregation in hyperlipidemic rabbits (De La Cruz et al. 2000; De La Cruz et al. 1997) and hypercholesterolemic patients (Naqvi et al. 1999). Importantly, cosupplementation of dietary cholesterol and dietary hempseed in the present study returned platelet aggregation activity to control levels. We suggest that this was not likely due to a change in the concentration of circulating haemostatic factors. In a recent clinical study, hempseed oil supplementation was found to have no effect on circulating levels of haemostatic factors, but aggregation was not measured (Schwab et al. 2006). Instead, the effects observed in our study may be the result of the complement of fatty acids found in the OLHP plasma where the most striking change was an approximately 12-fold increase in plasma GLA levels in the OLHP group compared with those in the OL group. It is possible, therefore, that hempseed-derived GLA may inhibit the cholesterol-induced stimulation of platelet aggregation. Consistent with this hypothesis, evening primrose oil, a source of GLA, significantly reduced thrombin-induced platelet aggregation in butter-fed rabbits (Renaud et al. 1982). Because there was a significant reduction in plasma total cholesterol and triglycerides (Renaud et al. 1982) in these animals, however, it was not possible to determine whether these inhibitory effects of evening primrose oil were translated through a

direct action on platelet aggregation or through a reduction in the inciting stimulus: cholesterol. Similarly, hyperlipidemic patients exhibited a significantly reduced level of platelet aggregation after EPO supplementation (Guivernau et al. 1994), but the EPO treatment also induced a concurrent reduction in serum lipids (Guivernau et al. 1994) again making it impossible to identify the mechanism for the observed antiaggregatory effect. In our study, hempseed cosupplementation did not inhibit or reduce the elevation in plasma cholesterol levels observed with cholesterol supplementation. This allowed us to identify, for the first time, that GLA had the capacity in vitro to directly inhibit the cholesterol-induced stimulation of platelet aggregation. We conclude that the dietary hempseed-induced increase in plasma GLA is the most plausible mechanism to explain the inhibitory effect of dietary hempseed on the proaggregatory action of cholesterol. Furthermore, we observed that GLA did not induce a similar inhibitory effect in RG samples, suggesting that it must directly inhibit the proaggregatory action of cholesterol.

The present investigation has demonstrated the capacity of dietary hempseed to normalize platelet aggregation in hypercholesterolemic rabbits. This effect was observed despite significantly elevated plasma cholesterol and triglyceride levels. This effect was observed in conjunction with an elevation in plasma PUFA content and was shown to specifically reflect an action of GLA. Platelet aggregation is recognized to be associated with acute coronary syndromes (Massberg et al. 2003) and aggregation is augmented in hypercholesterolemic individuals (De La Cruz et al. 1997; De La Cruz et al. 2000; Naqvi et al. 1999; Renaud et al. 1986). These results, which show the inhibitory effect of dietary hempseed on platelet aggregation even in the presence of high circulating cholesterol levels, may have significant potential for reducing the incidence of cardiovascular disease.

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