

INVESTIGACIÓN

The oleic acid esterification of policosanol increases its bioavailability and hypocholesterolemic action in rats

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RESUMEN

La esterificación de ácido oleico en policosanoles aumenta su biodisponibilidad y el efecto hipocolesterolémico en ratas

Los Policosanoles están formados por una mezcla de alcoholes alifáticos de cadena larga y se obtienen de las ceras de la caña de azúcar. Más de cincuenta estudios indican que los policosanoles reducen el colesterol sérico, mientras que otros no logran reproducir este efecto. El objetivo de esta investigación fue evaluar la biodisponibilidad de policosanoles esterificados y no esterificados (NEP), en relación con sus efectos hipocolesterolémicos. Para ello, a ratas Sprague Dawley se les administró una dosis oral diaria de 100 mg kg⁻¹ de NEP, 117 mg kg⁻¹ de policosanoles esterificados con ácido butírico (BAEP), ó 164 mg kg⁻¹ de policosanoles esterificados con ácido oleico (OAEP). La absorción de los policosanoles se evaluó en el plasma entre 0 y 3 horas después de la ingestión. Para evaluar los cambios en el colesterol total, colesterol-LDL, colesterol-HDL y triglicéridos en el plasma y en la fosforilación de la hígado 3-hidroxi-3-metilglutaril coenzima A reductasa (HMG-CoA red), la ingesta de las ratas fue suplementada con policosanoles no esterificados o esterificados durante 5 semanas. Los resultados indicaron que la absorción de los policosanoles fue significativamente mayor en las ratas tratadas con los OAEP que en las sometidas a los NEP o las que se les administró BAEP. Los OAEP redujeron significativamente el colesterol plasmático total y el colesterol-LDL de las ratas, además de aumentar 5.6 veces ($P < 0,05$) sobre los valores control, en el contenido hepático de la HMG-CoA fosforilada red. En conclusión, la esterificación de policosanoles con ácido oleico aumenta la biodisponibilidad de los policosanoles, y mejora significativamente el perfil de lípidos séricos en ratas normocolesterolémicas en asociación con la inactivación de la colesterogénesis control HMG-CoA red.

PALABRAS-CLAVE: Absorción de Policosanoles – Biodisponibilidad – Efecto reductor de colesterol – Octacosanol – Policosanol – Triacontanol – Ratas.

SUMMARY

The oleic acid esterification of policosanol increases its bioavailability and hypocholesterolemic action in rats

Policosanol comprises a mixture of long-chain aliphatic alcohols from sugarcane wax. More than 50 studies indicate that policosanol decreases serum cholesterol, while others failed to reproduce this effect. The objective of this investigation was to assess the bioavailability of esterified policosanol and non-esterified policosanol (NEP), in relation to their hypocholesterolemic effects. Sprague Dawley rats were given a daily oral dose of 100 mg/kg of NEP, 117 mg kg⁻¹ of butyric acid esterified policosanol (BAEP), or 164 mg kg⁻¹ of oleic acid esterified policosanol (OAEP). Policosanol absorption was evaluated in plasma between 0 and 3 hours after ingestion. To assess changes in total cholesterol, LDL-cholesterol, HDL-cholesterol and triacylglycerols in plasma and liver 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA red) phosphorylation, the rats were supplemented with non-esterified or esterified policosanol for 5 weeks. The results indicate that policosanol absorption was significantly greater in OAEP-treated rats than in those subjected to NEP or BAEP administration. OAEP significantly reduced plasma total and LDL-cholesterol in rats, in addition to a 5.6-fold increase ($P < 0.05$) in the hepatic content of phosphorylated HMG-CoA red over the control values. In conclusion, esterification of policosanol with oleic acid enhances policosanol bioavailability, and significantly improves the serum lipid profile in normocholesterolemic rats in association with the inactivation of HMG-CoA red controlling cholesterologenesis.

KEY-WORDS: Bioavailability – Cholesterol lowering effect – Octacosanol – Policosanol – Policosanol absorption – Rats – Triacontanol.

1. INTRODUCTION

Coronary heart disease is a leading cause of death in industrialized nations, with elevated levels of total serum cholesterol, low density lipoprotein

(LDL)-cholesterol, and triglycerides, as important cardiovascular risk factors (Singh and Mehta, 2002). Policosanol, a mixture of primary aliphatic alcohols originally derived from sugarcane wax (*Saccharum officinarum*) (Janikula, 2002), in which octacosanol (63%), triacontanol (13%) and hexacosanol (6%) prevail (Gouni-Berthold *et al.*, 2002), effectively improves serum lipid profiles. This effect was shown in animal models (Menéndez *et al.*, 1996; Arruzazabala *et al.*, 2000), healthy human subjects (Hernández *et al.*, 1992), and patients with either type II hypercholesterolemia (Pons *et al.*, 1992; Batista *et al.*, 1996; Mas *et al.*, 1999; Castaño *et al.*, 2001), type II diabetes (Torres *et al.*, 1995; Crespo *et al.*, 1999), or postmenopausal women (Castaño *et al.*, 2000). Interestingly, 10 mg of this mixture had similar efficacy to an equivalent dose of statins in lowering the serum LDL concentrations (Castaño *et al.*, 1999; Prat *et al.*, 1999), and its supplementation caused a significant increase in the fecal excretion of neutral and acidic sterols in hamsters (Ng *et al.*, 2005), whereas octacosanol administration to humans decreased bile acid concentration in feces (Keller and Gimmler, 2008). Various studies have suggested an inhibition of the enzyme which limits the cholesterologenic route, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA red) (Singh *et al.*, 2006; Menendez *et al.*, 2001; Oliaro-Bosso *et al.*, 2009), as the mechanism of action of policosanol hypocholesterolemic activity. However, recent clinical trials failed to show significant effects. In fact, no changes in lipid variables in subjects receiving policosanol were reported (Greyling *et al.*, 2006; Berthold *et al.*, 2006; Dulin *et al.*, 2006; Kassis and Jones, 2006) and neither primary hypercholesterolemic subjects (Francini-Pesenti *et al.*, 2008a) nor diet-resistant hypercholesterolemic patients (Francini-Pesenti *et al.*, 2008b) were affected by policosanol treatment for 8 weeks.

In the context of bioavailability of policosanols, few investigations have been performed. In nature, fatty alcohols comprising policosanol are esterified (wax), but the extraction process releases the fatty alcohols from the wax (Haim and Videla, 2008). These forms of non-esterified policosanol (NEP) were used in all the reported studies, including those of bioavailability assessment (Marrero and González-Bravo, 2001; Menéndez *et al.*, 2005). These two reports involve validation of an analytical method using a maximum octacosanol plasma concentration of 14 ng mL⁻¹ after a single oral dose of 40 mg policosanol kg⁻¹ (Marrero and Gonzalez-Bravo, 2001) and an *in vivo* study of octacosanol metabolism detecting 30 ng octacosanol mL⁻¹ plasma after a 60 mg/kg dose (Menéndez *et al.*, 2005), thus revealing a rather low absorption of policosanol given as non-esterified compounds. In view of these considerations, we have hypothesized that a potential explanation for the discrepancies reported in the field may be related to the insufficient absorption of NEP. Consequently, the aims of this study were to determine the role of

policosanol esterification in the bioavailability and hypocholesterolemic effect of these compounds in rats. For these purposes, first, pharmacokinetic studies of policosanol administered as (i) NEP, (ii) butyric acid esterified policosanol (BAEP), and (iii) oleic acid esterified policosanol (OAEP) were assessed by quantifying plasma octacosanol, triacontanol, and their main metabolites *in vivo* (octacosanoic acid and triacontanoic acid) after a single oral dose. Then, total cholesterol, LDL-cholesterol, and HDL-cholesterol concentrations in plasma were determined in rats supplemented with non-esterified and esterified policosanol for 5 weeks, in addition to the hepatic content of total and phosphorylated HMG-CoA red controlling cholesterologenesis.

2. MATERIALS AND METHODS

2.2. Synthesis of esterified policosanol

Preparation of BAEP and OAEP. A policosanol mixture of 96% purity (62% octacosanol, 19% triacontanol) was purchased from Pharmapia International (Shanghai, China). Fatty acids, butyric acid (99%) and oleic acid (90%), were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). The preparations of BAEP and OAEP were made by mixing policosanol with the respective fatty acid (20% excess related to policosanol) in the presence of 0.5% sulfuric acid (J.T. Baker, Phillipsburg, NJ, USA) at 150 °C. The concentration of NEP in the products of the reaction was less than 2% (detected by gas chromatography (model 7890A, Agilent Technologies, California, USA) coupled with an inert mass spectrometry detector (model 5975C, Agilent Technologies, California, USA)).

Purification of esterified policosanol. The products of the esterification reaction were subjected to neutralization with KOH (J.T. Baker, Phillipsburg, NJ, USA) dissolved in ethanol/distilled water (1/1) for 1 h at 30 °C (ethanol was purchased from Merck KGaA, Darmstadt, Germany). Then the sample was subjected to liquid-liquid (distilled water-heptane) separation, removing the organic phase which contained the esterified policosanol (heptane was purchased from Merck KGaA, Darmstadt, Germany). Qualitative and quantitative analyses were applied to esterified policosanols, verifying the structure and proportionality of the alcohols of the mixture in the esterified product.

Preparation of the policosanol dispersions. For oral administration to rats, 3 dispersions in water at 10% p/v were prepared: a) NEP, b) BAEP, and c) OAEP. The surfactants used were Tween-20 and Span-85 at 5% based aqueous solution. Both surfactants were purchased from Sigma Aldrich Co. (St. Louis, MO, USA).

2.3. In vivo studies

Animal treatments. Male Sprague Dawley rats (Bioterio Central, Faculty of Medicine, University of

Chile, Santiago, Chile) were kept in a temperature and light controlled room (20°C and a 12-h light-dark cycle) and fed with a standard solid diet (Alimentos Champion, Santiago, Chile) and tap water *ad libitum*. Rats employed for bioavailability assessment weighing 150-180 g and fasted overnight (12 h), were given a single oral dose of Tween-20/Span-85 water vehicle suspensions of NEP (100 mg kg⁻¹ body weight), BAEP (117 mg kg⁻¹), or OAEP (164 mg kg⁻¹). The animals used in the chronic treatment with an initial body weight of 50-70 g, received either daily oral isovolumetric amounts of Tween-20/Span-85 water vehicle suspension alone (controls) or containing NEP (100 mg kg⁻¹) or OAEP (164 mg kg⁻¹) for 5 weeks.

Blood and liver samples. Blood samples for pharmacokinetic studies and assessment of lipid parameters were taken under anesthesia (Zoletil, 1 mL kg⁻¹ body weight, Virbac Laboratory, Carros, Francia) by exsanguinations through cardiac puncture using heparinized tubes, and plasma was obtained by centrifugation (800 g for 10 min at 4°C) and stored at -80°C in aliquots of 20 µL until analysis. Liver samples were taken, frozen in liquid nitrogen, and kept at -80°C for HMG-CoA red assessment. The study protocol was approved by the Bioethics Committee of the Faculty of Medicine, University of Chile (CBA #0325 FMUCH).

2.4. Bioavailability of policosanol and long-chain fatty acids

Determination of plasmatic NEP. The presence of non-esterified octacosanol and non-esterified triacontanol in plasma was investigated. The determination was carried out by adding the internal standard betulin (99% GC purity, Sigma Aldrich Co., St. Louis, MO, USA) to 100 µL of plasma, followed by sample desolventization under a nitrogen stream. After exhaustive evaporation, the residue was reconstituted in 100 µL of the derivatizing agent MSTFA (Sigma Aldrich Co, St. Louis, MO, USA) for 20 min at 80°C. Finally 0.5 µL were injected automatically into the gas chromatography (model 7890A, Agilent Technologies, California, USA) coupled with an inert mass spectrometry detector (model 5975C, Agilent Technologies, California, USA) for quantitative analysis. The chromatographic conditions were described by Haim *et al.* (2009).

Determination of plasmatic total policosanol and long-chain fatty acids (non-esterified + esterified analyte). Total concentrations in plasma were determined for octacosanol, triacontanol, octacosanoic acid, and triacontanoic acid. The protocol included saponification and acidification of the sample, stages that hydrolyze the esters and reconstitute the fatty acids, respectively. Therefore, the technique allowed for the measuring of esterified and non-esterified analytes present in the plasma. The detailed methodology and validation of the technique were reported by our group (Haim *et al.*, 2009).

2.5. Lipid analysis

Fasting plasma samples were drawn from control rats and animals subjected to NEP or OAEP supplementation for 5 weeks. Total cholesterol, LDL-cholesterol, HDL-cholesterol and triglyceride concentrations in plasma were measured with enzymatic kits and standardized reagents (Valtek Diagnostics, Santiago, Chile), and results were expressed as mg dL⁻¹.

2.6. Western blot analysis of HMG-CoA reductase

Liver samples (100-500 mg) frozen in liquid nitrogen were homogenized and suspended in a buffer solution pH 7.9 containing 10 mM HEPES, 1 mM EDTA, 0.6% Nonidet P-40, 150 mM NaCl, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 µg mL⁻¹ aprotinin, 1 µg mL⁻¹ leupeptin, and 1 mM orthovanadate). Soluble protein fractions (60 µg) were separated on 12% polyacrylamide gels using SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose membranes (Towbin *et al.*, 1979), which were blocked for 1 hour at room temperature with TBS-containing 5% bovine serum albumin. The blots were washed with TBS-containing 0.1% Tween 20 and hybridized with either rabbit polyclonal antibodies for HMG-CoA red, phospho-HMG-CoA red (Ser 872) (Millipore, MA, USA), or mouse monoclonal antibody for β-actin (ICN Biomedicals, Inc., Aurora, OH, USA). After extensive washing, the antigen-antibody complexes were detected using horseradish peroxidase goat anti-rabbit IgG or goat anti-mouse IgG and a SuperSignal West Pico Chemiluminescence kit detection system (Pierce, Rockford, IL).

2.7. Statistical analysis

The values shown represent the mean ± standard error of the mean (SEM) for the number of separate experiments indicated. One-way or two-way ANOVA followed by Newman-Keuls post-test assessed the statistical significance of difference among values. *P* values < 0.05 were considered significant. All statistical analyses were performed using GraphPad Prism software (version 4.0, GraphPad Software Inc., San Diego, CA, USA).

3. RESULTS

3.1. The effect of NEP administration on non-esterified policosanol and total policosanol (non-esterified + esterified) concentrations in plasma

The administration of NEP (100 mg kg⁻¹) to rats did not increase the plasmatic concentrations of non-esterified alcohols, ranging from 3.79 ± 0.40 to 4.70 ± 0.81 ng mL⁻¹ for octacosanol (Fig. 1A) and from 1.34 ± 0.04 to 2.13 ± 0.21 ng mL⁻¹ for

triacontanol (Fig. 1B), respectively. Conversely, the total policosanol in plasma significantly increased at min 60 compared with time zero (496 ± 25 compared with 170 ± 9 ng mL⁻¹ for octacosanol (Fig. 1A) and 130 ± 11 compared with 27 ± 5 ng mL⁻¹ for triacontanol (Fig. 1B), respectively). Esterified policosanol concentrations were calculated as the subtraction between total and non-esterified policosanol values. At time zero (fasting plasma samples without policosanol administration), 2.3% of total circulating octacosanol was detected as non-esterified alcohol, whereas the remaining fraction (97.7%) represented esterified octacosanol (Fig. 1A, inset). As expected, the proportions were similar for triacontanol (5% non-esterified and 95% esterified alcohol) (Fig. 1B, inset). Consequently, policosanol circulates predominantly in its esterified form.

3.2. Comparison of plasma total policosanol concentrations in rats subjected to a single dose of NEP, BAEP, and OAEP

Fasting plasma samples were obtained after the administration of a single, oral dose of NEP, BAEP, or OAEP, and the kinetics of octacosanol (Fig. 2A),

octacosanoic acid (Fig. 2B), triacontanol (Fig. 2C) and triacontanoic acid (Fig. 2D) were determined between 0 and 180 min. According to the data presented in Fig. 1, only total policosanol concentrations were evaluated in the plasma. Analysis of the data presented in Fig. 2 shows post-treatment plasma policosanol and long-chain fatty acid response, expressed as the area under the curves at 0-3h after the ingestion of the policosanol enriched dispersion (Figs. 3A and 3B). The results indicate a significantly higher response in the group given OAEP (43690 ± 5142 ng octacosanol min mL⁻¹ for OAEP compared with 30455 ± 2870 for NEP and 7978 ± 118 for BAEP) (Fig. 3A), which is similar to that obtained for triacontanol (Fig. 3B). Also, the group treated with OAEP elicited a significantly higher peak plasma octacosanol concentration at min 60 compared with NEP and BAEP (654 ± 88 ng mL⁻¹ for OAEP compared with 325 ± 25 for NEP and 65 ± 18 for BAEP) (Fig. 3C). Comparable results were obtained for triacontanol (Fig. 3D). Furthermore, the peak plasma concentration for long-chain fatty acids in the OAEP group were detected at min 60, with octacosanoic acid being higher ($P < 0.05$) than NEP and BAEP (158 ± 19 ng mL⁻¹ for OAEP, 81 ± 7

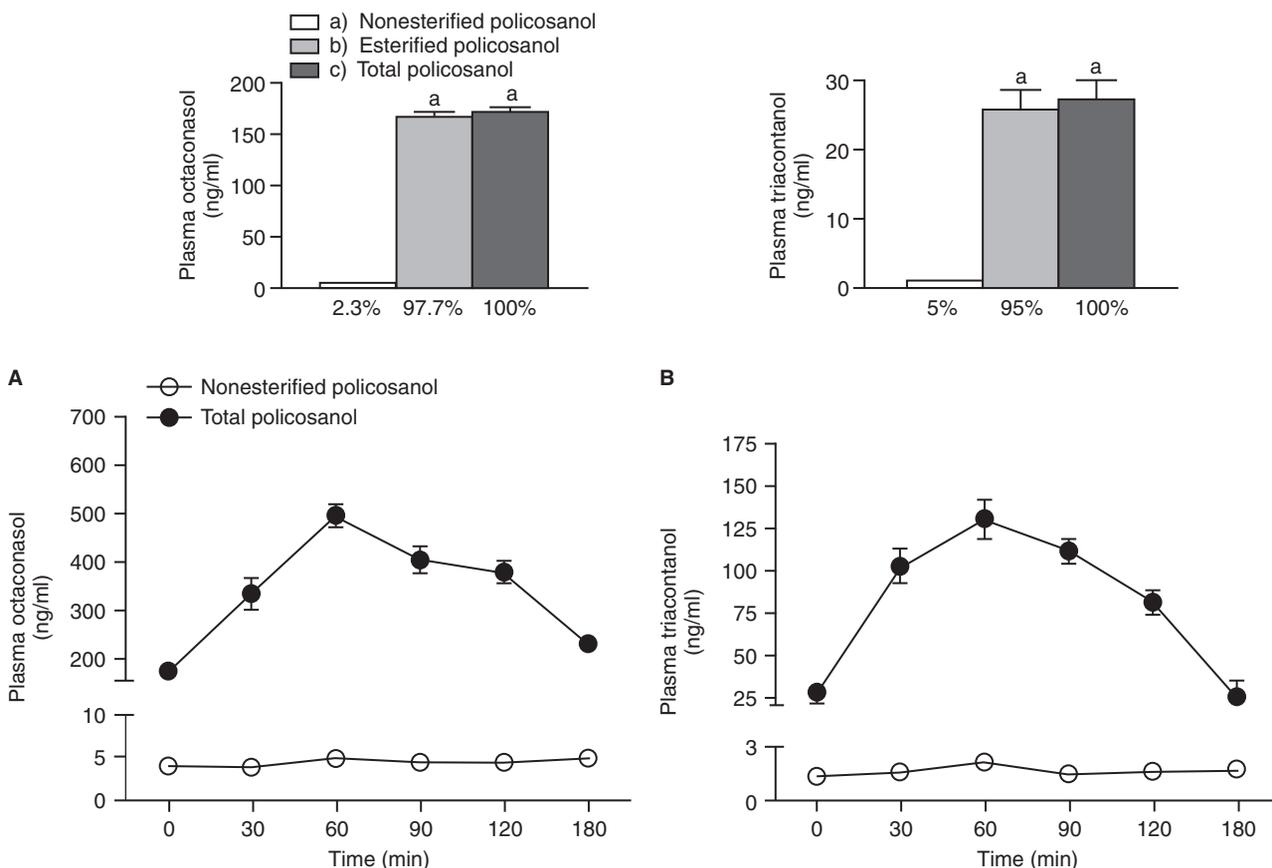


Figure 1

Plasma concentration-time profile of total and non-esterified octacosanol (A) and triacontanol (B) in rats after a single oral dose of non-esterified policosanol (100 mg/kg) given at time zero. Insets A and B correspond to non-esterified, esterified, and total octacosanol and triacontanol concentrations obtained at time zero, respectively. Data are means \pm SEM for three to five separate experiments per point. Statistical significance in insets A and B ($P < 0.05$) is shown by the letters identifying each experimental group, assessed by one-way ANOVA followed by Newman-Keuls' post-test.

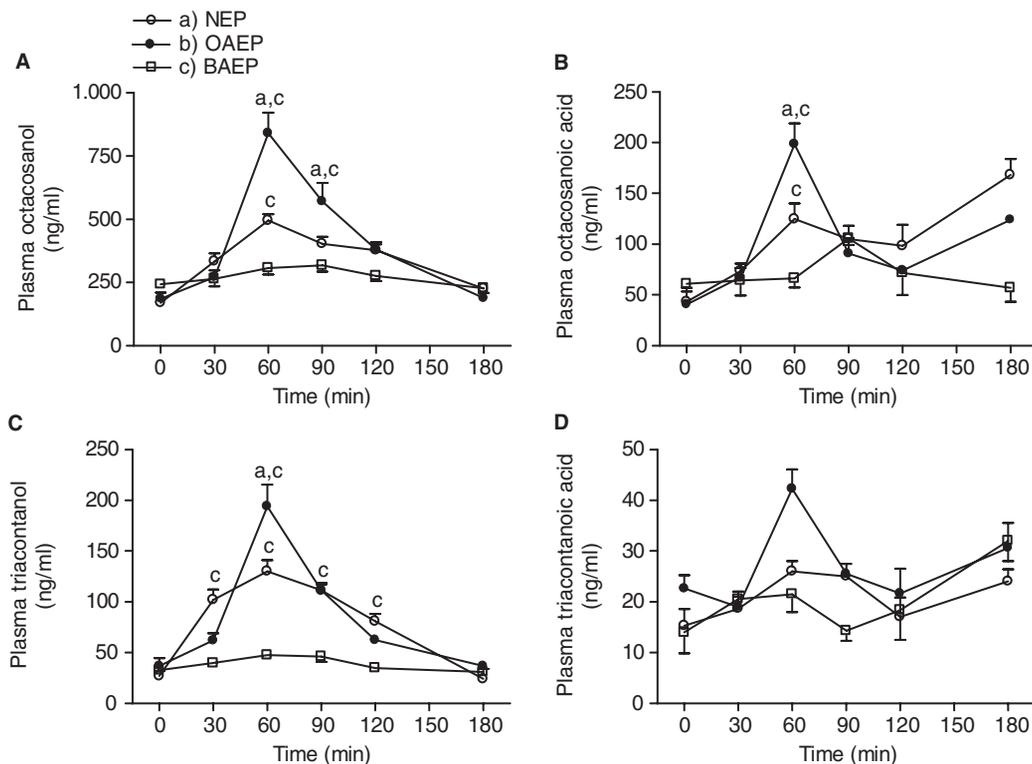


Figure 2

Plasma concentration-time profile of total octacosanol (A), octacosanoic acid (B), triacontanol (C), and triacontanoic acid (D) in rats after a single oral dose of either non-esterified policosanol (NEP; 100 mg/kg), oleic acid esterified policosanol (OAEP; 164 mg/kg), or butyric acid esterified policosanol (BAEP; 117 mg/kg) given at time zero. Data are means \pm SEM for three to five separate experiments per point. Statistical significance ($P < 0.05$) is shown by the letters identifying each experimental group, assessed by two-way ANOVA followed by Newman-Keuls' post-test.

for NEP, and 5 ± 5 for BAEP) (Fig. 3C), whereas that of triacontanoic acid was comparable to NEP and higher than BAEP ($P < 0.05$) (Fig. 3D). Nevertheless, the variations in very long-chain fatty acid concentrations in the plasma were small in relation to the kinetics of their precursors.

3.3. Comparison of plasma lipid concentrations in rats subjected to chronic NEP and OAEP administration

OAEP significantly decreased both total plasma cholesterol (Fig. 4A) and LDL-cholesterol (Fig. 4B) (40 ± 2 ($n = 8$) mg dL⁻¹ and 7 ± 1 ($n = 8$)) compared to NEP (52 ± 2 and 13 ± 2) and the control group (51 ± 1 and 14 ± 2), respectively. Conversely, plasmatic HDL-cholesterol (Fig. 4C) and triglyceride (Fig. 4D) concentrations were not modified by NEP or OAEP supplementation. The total cholesterol/HDL-cholesterol ratio (Fig. 5A) and LDL-cholesterol/HDL-cholesterol ratio (Fig. 5B) were significantly ($P < 0.05$) decreased by OAEP compared to the controls and the NEP-treated rats.

3.4. Comparison of the hepatic protein content of HMG-CoA red in rats subjected to chronic NEP and OAEP administration

The evaluation of total HMG-CoA red (phosphorylated + non-phosphorylated) in rat liver

revealed comparable values in rats subjected to NEP and OAEP administration, and compared to control values (Fig. 6A). Nevertheless, phosphorylated HMG-CoA red content significantly increased in the OAEP group (2.16 ± 0.3 ($n = 8$)) versus the NEP group (0.90 ± 0.11 ($n = 8$)) and the controls (0.38 ± 0.08 ($n = 6$)) (Fig. 6B).

4. DISCUSSION

The present investigation was focused on the possible reasons regarding the controversy over policosanol efficacy as a hypocholesterolemic agent. Differences between the length of the study period, typical dietary habits, or ethnic diversity of the participants of clinical trials seem unlikely to be the cause of the large variation in the cholesterol lowering effects of this supplement. Nevertheless, there is agreement among the authors that a critical point that needs to be addressed is the availability of the alcohol components in the policosanol mixture, due to their poor absorption in the gut (Marrero and González-Bravo, 2001; Menéndez *et al.*, 2005). Because the form of administration of a treatment often plays a role in determining its efficacy, the objective of this study was to evaluate policosanol chemical modifications for their absorption capacity. Considering the fact that policosanol is esterified in nature (Haim and Videla,

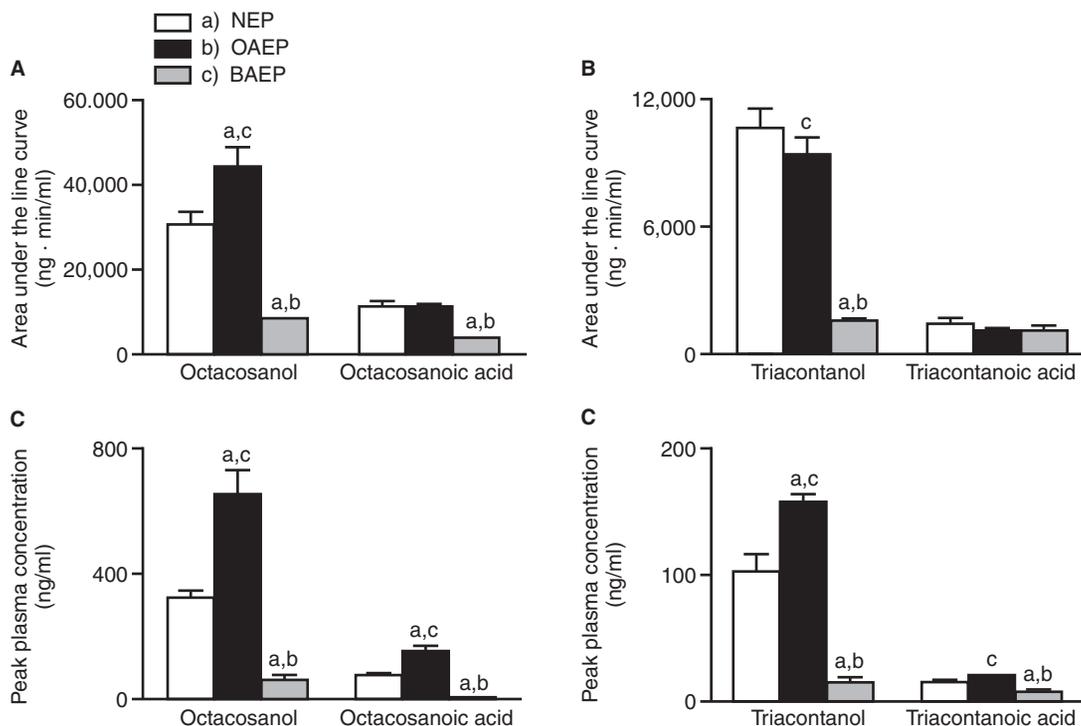


Figure 3

Area under the time curve for octacosanol and octacosanoic acid (A) and triacontanol and triacontanoic acid (B) and the respective peak plasma concentrations at 60 min (C and D), corrected for values at time zero, in rats after a single oral dose of either non-esterified policosanol (NEP; 100 mg/kg), oleic acid esterified policosanol (OAEP; 164 mg/kg), or butyric acid esterified policosanol (BAEP; 117 mg/kg) given at time zero (for details, see Fig. 2). Data are means \pm SEM for three to five separate experiments per point. Statistical significance ($P < 0.05$) is shown by the letters identifying each experimental group, assessed by one-way ANOVA followed by Newman-Keuls' post-test.

2008), and that phytosterols,— which are hypocholesterolemic phytochemical agents (Wu *et al.*, 2009; Clifton, 2009) that exhibit some similar physical and chemical properties to policosanol, have demonstrated a cholesterol lowering effect when they are administered in their esterified form (Nissinen *et al.*, 2006), we postulated that the esterification of policosanol could improve its bioavailability in relation to the non-esterified form, such as the commercial product. For this purpose, two policosanol ester families, BAEP and OAEP were synthesized, and the policosanol kinetics in plasma after a single oral dose of these esterified policosanol versus the non-esterified administrated group were compared. In addition, this work represents the first study incorporating the quantification of non-esterified, esterified, and total (esterified + non-esterified) policosanol in the blood.

Interestingly, a fraction higher than 95% of policosanol circulates as esterified alcohol in blood plasma, while 5% or less is found as NEP, proportions that were calculated from fasting rat plasma without the administration of policosanol. This difference becomes even greater after the administration of the policosanol dose. In addition, plasma concentrations of non-esterified octacosanol and triacontanol did not change after the oral dose of policosanol, regardless of the administration mode (NEP or OAEP). Therefore, only esterified policosanol concentrations increased in the

blood after supplementation. The pharmacokinetic studies previously reported (Marrero and González-Bravo, 2001; Menéndez *et al.*, 2005, Gonzalez-Bravo *et al.*, 1996) did not include a saponification step of plasma extracts. For this reason, the concentrations observed represent only non-esterified circulating policosanol, which explains the low values previously reported (Marrero and González-Bravo, 2001; Menendez *et al.*, 2005, Gonzalez-Bravo *et al.*, 1996). Furthermore, several studies have described the hydrolysis of wax as a necessary step for the absorption of fatty alcohols and fatty acids (Hargrove *et al.*, 2004; Place, 1992). Based on this contention, our results suggest that a major fraction of policosanol is re-esterified inside the enterocyte, in agreement with the absorption of dietary lipids such as cholesterol, in which case esterification is produced by the catalytic action of the enzyme acyl-CoA cholesterol acyltransferase (Buhman *et al.*, 2000).

The administration of OAEP demonstrated a major bioavailability in relation to NEP, evaluated as octacosanol and triacontanol plasma concentrations at 60 min (maximal concentration) and the respective areas under the time-curves. This novel and important finding indicates that the esterification of policosanol with the medium-chain monounsaturated fatty acid oleic acid improves absorption in the gastrointestinal tract with respect to the available market product (NEP), and might represent an alternative way of supplementing policosanol.

Additionally, BAEP did not alter the kinetics of octacosanol or triacontanol after the ingestion of a single oral dose, demonstrating that this administration form was not bioavailable in the animal model studied. It is postulated that the difference observed between the relatively high absorption of OAEP over that of BAEP may be attributed to the high stereospecificity of pancreatic esterases in the small intestine for long/medium-chain fatty acids esters (Brockerhoff, 1970), rather than for short-chain fatty acid esters, which is the case of BAEP.

Despite the fact that the active components of a policosanol mixture are fatty alcohols, D-003, a product also derived from sugarcane wax, has been reported to exert a cholesterol lowering effect in normocholesterolemic rabbits (Mendoza *et al.*, 2001) and in patients with hypercholesterolemia type II (Castaño *et al.*, 2003). D-003 is constituted by long-chain fatty acids, predominantly octacosanoic acid, components that are the main metabolites produced *in vivo* from policosanol (Menéndez *et al.*, 2005). Consequently, the biologically active form of policosanol may include long-chain fatty alcohols and long-chain fatty acids. These fatty acids are derived from the partial oxidation of their precursors. Taking into account these antecedents, our study considered the kinetics not only of the more

abundant alcohols of the policosanol mixture (octacosanol and triacontanol), but also of the respective fatty acids produced from their metabolism (octacosanoic acid and triacontanoic acid). The long-chain octacosanoic and triacontanoic fatty acids maintained low plasma concentrations in the time period assessed, showing a tendency to increase as their precursors disappeared from circulation, results that are consistent with the metabolic pathway proposed for policosanol (Haim and Videla, 2008; Menéndez *et al.*, 2005; Hargrove *et al.*, 2004). Nevertheless, a proportion lower than 33% of policosanol disappearance can be explained by its metabolic conversion into the respective long-chain fatty acids. The remaining fraction of policosanol in circulation is likely to be taken up by different tissues for energetic purposes, an explanation based on the evidence of radioactively marked CO₂ expiration as a terminal metabolite of octacosanol beta-oxidation in rats (Kabir and Kimura, 1993).

The data presented for the NEP group are consistent with recent studies showing the lack of policosanol effect in modifying total or LDL-cholesterol (Greyling *et al.*, 2006; Berthold *et al.*, 2006; Dulin *et al.*, 2006; Kassis and Jones, 2006; Francini-Pesenti *et al.*, 2008a; Francini-Pesenti *et al.*, 2008b; Kassis *et al.*, 2007; Murphy *et al.*, 2008),

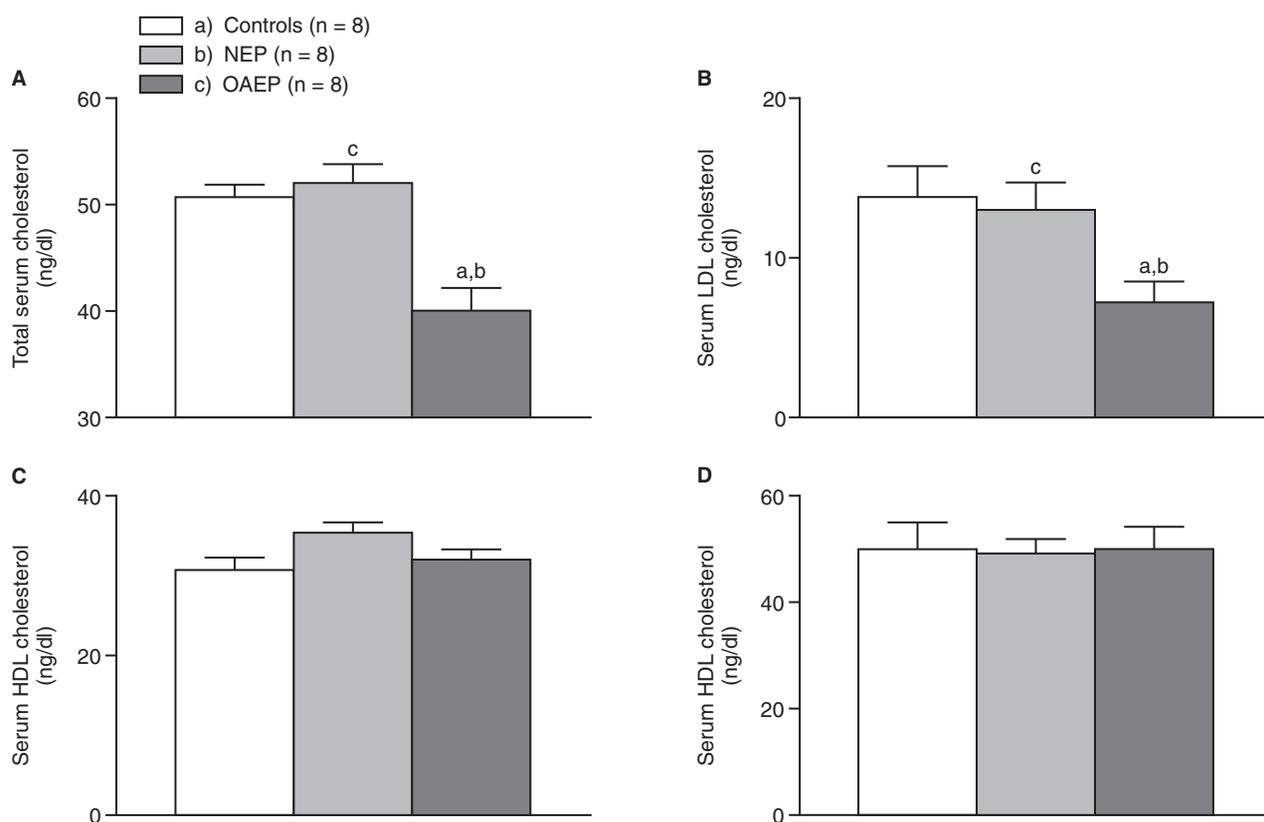


Figure 4

Plasma concentrations of total cholesterol (A), LDL-cholesterol (B), HDL-cholesterol (C), and triglycerides (D) in rats after daily administration of either vehicle for policosanol dispersion (control), non-esterified policosanol (NEP; 100 mg/kg), or oleic acid esterified policosanol (OAEP; 164 mg/kg) for five weeks. Data are means \pm SEM for the number of animals indicated in parentheses. Statistical significance ($P < 0.05$) is shown by the letters identifying each experimental group, assessed by one-way ANOVA followed by Newman-Keuls' post-test.

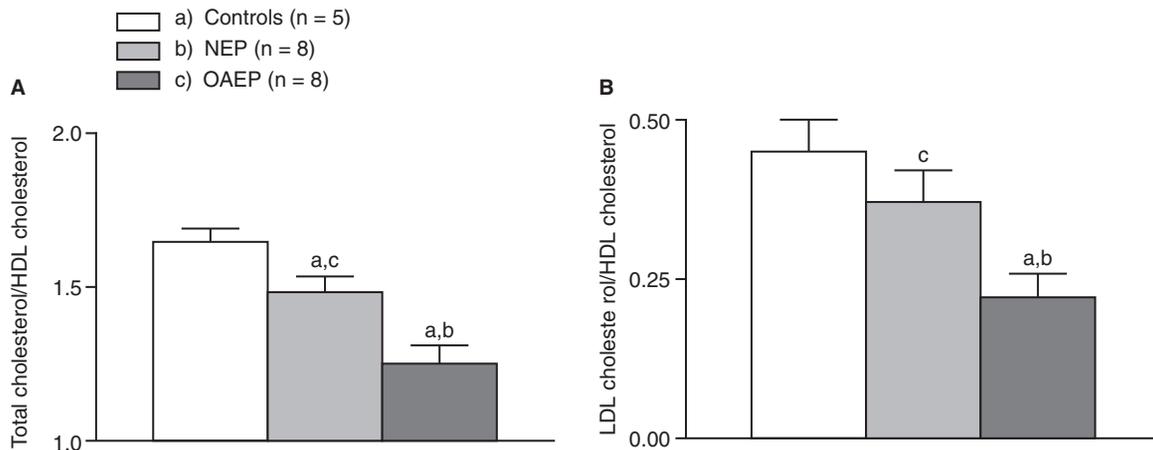


Figure 5 Total cholesterol/HDL-cholesterol and LDL-cholesterol/HDL-cholesterol ratios in rats after daily administration of either vehicle for policosanol dispersion (control), non-esterified policosanol (NEP; 100 mg/kg), or oleic acid esterified policosanol (OAEP; 164 mg/kg) for five weeks. Data are means \pm SEM for the number of animals indicated in parentheses. Statistical significance ($P < 0.05$) is shown by the letters identifying each experimental group, assessed by one-way ANOVA followed by Newman-Keuls' post-test.

considering that the supplementation of rats with 100 mg/kg body weight of NEP, the same form of administration in previously reported investigations, did not alter lipid concentrations when compared to the control group. Nevertheless, the OAEP group exhibited significantly lower concentrations of plasmatic LDL-cholesterol, as the primary endpoint, and total cholesterol, as the secondary endpoint, in normocholesterolemic rats after 5 weeks of

treatment. Furthermore, OAEP administration achieved significant reductions in LDL-cholesterol/HDL-cholesterol and total cholesterol/HDL-cholesterol ratios, relationships that are of major relevance for human cholesterol homeostasis. Considering that BAEP has no effect on plasma total cholesterol and LDL-cholesterol compared to OAEP, the presence of oleic acid from OAEP after hydrolysis and absorption may play a role in lowering plasma cholesterol levels.

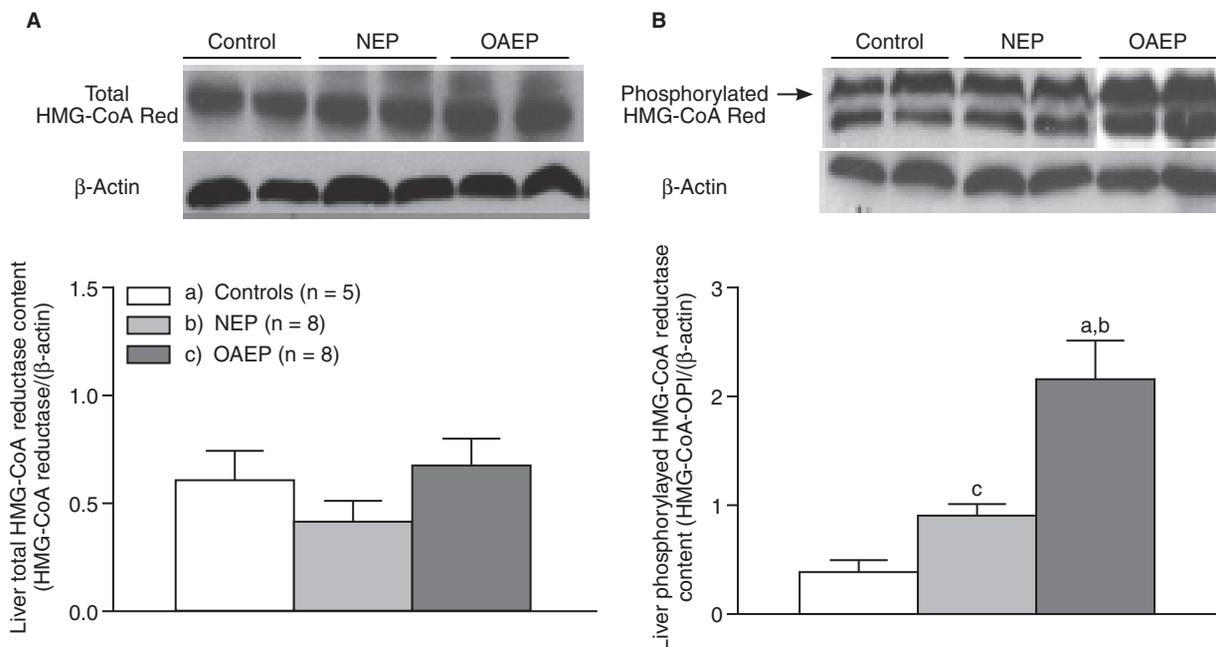


Figure 6 Liver protein expression of total (A) and phosphorylated (B) 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA red) in rats after daily administration of either vehicle for policosanol dispersion (control), non-esterified policosanol (NEP; 100 mg/kg), or oleic acid esterified policosanol (OAEP; 164 mg/kg) for five weeks. Upper panels show representative blots of total HMG-CoA red (97 kDa) and β -actin (43 kDa) (A) and phosphorylated HMG-CoA red (98 kDa) and β -actin (B) expression using 60 μ g of soluble protein. Lower panels show the respective densitometric quantification expressed as total HMG-CoA red/ β -actin (A) and phosphorylated HMG-CoA red/ β -actin (B) ratios to compare lane-to-lane equivalence in total protein content. Data are means \pm SEM for the number of animals indicated in parentheses. Statistical significance ($P < 0.05$) is shown by the letters identifying each experimental group, assessed by one-way ANOVA followed by Newman-Keuls' post-test.

However, previous studies have reported (i) the presence of comparable levels of plasma cholesterol in postmenopausal women before and after consumption of high oleic acid sunflower oil for 28 days (Sánchez-Muniz *et al.*, 2002); and (ii) the lack of effects of the partial exchange of palmitic acid for oleic acid in healthy, normocholesterolemic humans (Ng *et al.*, 1992; Choudhury *et al.*, 1995) and cebus monkeys (Hayes and Khosla, 1992), suggesting that oleic acid is neutral with respect to plasma cholesterol homeostasis.

Regarding the mechanisms underlying the hypocholesterolemic action of policosanol, Singh *et al.* (Singh *et al.*, 2006) found increased amounts of phosphorylated AMP-kinase after the incubation of hepatoma cells with policosanol. These authors postulated that policosanol inactivates HMG-CoA red by promoting its phosphorylation by AMP-kinase, in agreement with data reported by Oliaro-Bosso *et al.* in HUVEC and HepG2 cells (Oliaro-Bosso *et al.*, 2009). Despite the evidence that phosphorylated AMP-kinase decreases HMG-CoA red activity, this kinase plays a central role in the regulation of energy homeostasis, regulating several enzymes involved in glucose and lipid metabolism (Wong *et al.*, 2009). Therefore, we decided to quantify directly the phosphorylated HMG-CoA red, instead of AMP-kinase, in order to establish a possible relationship between policosanol administration and HMG-CoA red activity. The data presented indicate that supplementation with NEP or OAEP did not significantly alter the amount of total HMG-CoA red protein compared to control values, whereas OAEP increased the phosphorylated form of the enzyme, in agreement with the reduction in the total and LDL-cholesterol concentrations in serum, an effect that is not observed for NEP.

5. CONCLUSION

The present study shows that OAEP is more bioavailable and effective than NEP in reducing plasma total and LDL-cholesterol in normocholesterolemic rats, in association with HMG-CoA red inactivation through OAEP-induced phosphorylation. Alternative mechanisms for the cholesterol-lowering action of OAEP, namely, enhancement in both bile acid excretions due to 7- α -hydroxylase up-regulation and/or clearance of LDL-cholesterol due to higher cell-membrane LDL receptor availability, are currently under study in our laboratory. Considering that this is the first investigation using an esterified policosanol in replacement of the conventional mixture (NEP), additional studies are needed to fully understand their digestion, absorption, and metabolism in the body.

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