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A nutraceutical formulation based on Annurca apple polyphenolic extract is effective on intestinal cholesterol absorption: A randomised, placebo-controlled, crossover study

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ABSTRACT

Complementary and/or alternative safe substances, able to correct impaired lipid profile in humans, are still in great demand. The objective of the present work was to evaluate the *in vitro* and clinical effects of a novel nutraceutical product (AMD), formulated with Annurca apple polyphenolic extracts, on the intestinal cholesterol micellar solubility. AMD was able to decrease *in vitro* cholesterol micellar solubility by about 85.7%, while Nuclear Magnetic Resonance experiments allowed to hypothesize dimeric procyanidins as potential responsible compounds for this effect. Then, a randomised, double blind, single centre, placebo-controlled, crossover study, was designed to evaluate the effect of AMD on the fecal cholesterol excretion. Clinical data indicated that fecal cholesterol excretion was significantly increased (about +35%) in the AMD period compared with placebo period ($P < 0.01$). AMD may be regarded as a novel complementary and/or alternative safe remedy with clinical relevance in the primary cardiovascular disease prevention.

1. Introduction

Cholesterol in the intestinal lumen typically consists of one third dietary cholesterol and two-thirds biliary cholesterol. The average daily diet contains 300–500 mg of cholesterol obtained from animal products. The bile provides an additional 800-1200 mg of cholesterol throughout each day as gallbladder contractions produce a flow of bile acids, cholesterol, and phospholipids to facilitate lipid digestion and absorption [1,2]. Dietary cholesterol is a mixture of free and esterified cholesterol whereas biliary cholesterol is non-esterified and is introduced into the small intestine as a cholesterol–bile salt–phospholipid water-soluble complex. Dietary cholesterol enters the small intestine solubilized in the oil phase of the stomach digest, whereas the biliary cholesterol enters in the micelle phase of the bile [1–3]. Experimental evidence indicates that biliary cholesterol and dietary cholesterol are absorbed equally; however, the pattern of exogenous and endogenous cholesterol absorption differs along the length of the intestinal lumen [1,3,4]. In the absence of bile secretion, or in the presence of bile acid-binding compounds, there is virtually no intestinal absorption of cholesterol [1,3,4].

Ezetimibe (Zetia® or Ezetrol®; Merck Sharp & Dohme Ltd, NJ, USA) is presently used to reduce absorption of dietary and biliary cholesterol, inhibiting its transport across the intestinal wall by binding to the duodenal Niemann-Pick C-like protein 1 L1 (NPC1L1) [5]. Actually, the enterocyte takes up both cholesterol and phytosterols from the intestinal lumen by NPC1L1 protein, which appears to be a common sterol transporter or permease in the brush border membrane. Nevertheless, the selectivity of this process accounts for the higher absorption rates of cholesterol (50–60%) compared to the phytosterols, which are very poorly absorbed [5,6]. Although ezetimibe reduces LDL-C concentrations, the data on reducing adverse cardiovascular outcomes remain equivocal, with the controversy provoked further by some clinical trials of inadequate design to assess relevant clinical questions [7,8]. In addition, very recent studies have pointed out the attention on potential adverse clinical effects [9].

It has been recently reported that green tea catechins are the most effective polyphenolic compounds in inhibiting the micellar cholesterol solubility in the small intestine. This effect may be the cause of the

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increased fecal excretion of cholesterol observed in experimental animals and hypocholesterolemic activity in experimental animals and humans [10–15]. Ogawa et al. (2016) have tried to clarify this mechanism, by performing a nuclear magnetic resonance (NMR) study to investigate the interaction between tea catechins and cholesterol micelles. Data indicated the ability of epigallocatechin gallate (EGCG) to lower the solubility of phosphatidylcholine (PC) and cholesterol in micellar solutions due to their elimination from the micelles by interaction between taurocholic acids and EGCG [16].

Annurca is the only apple cultivar native to Southern Italy, listed as a Protected Geographical Indication (PGI) product from the European Council [Commission Regulation (EC) No. 417/2006)]. Previous studies have extensively demonstrated that Annurca polyphenolic extract is able to positively influence cholesterol metabolism. Specifically, *in vitro* experiments have proved its capacity to enhance Apolipoprotein A1 expression, the main protein constituent of nascent discoidal high density lipoprotein cholesterol (HDL-C), and favor low density lipoprotein cholesterol (LDL-C) receptor binding activity, in human hepatocellular liver carcinoma cells (HepG2, HB-8065) [17,18]. Later, Annurca polyphenolic extract has been administered to mildly hypercholesterolemic healthy subjects under the form of a nutraceutical formulation, according to a randomised trial which has confirmed the previous *in vitro* results in terms of increase in plasma HDL-C and decrease in LDL-C levels [19]. These studies would indicate oligomeric procyanidins, mainly the dimeric procyanidin B2, as the major responsible for such effects on both *in vitro* and clinical HDL-C and LDL-C parameters. Nevertheless, the molecular mechanisms underlying these effects are still scarcely known.

Apple procyanidins are oligomeric compounds consisting of catechin monomeric units. It can be hypothesized that the lower molecular weight compounds (mainly dimers) may have a similar mechanism of action to that of monomeric catechins as regards their effects on micellar cholesterol solubility. To clarify this aspect, a first aim of the present work was to evaluate the *in vitro* effect of a nutraceutical product formulated by using Annurca water extract microencapsulated in maltodextrins (AMD) on micellar cholesterol solubility in a model reproducing the duodenal environment. Specifically, a main goal was to elucidate the molecular mechanism of action by performing an NMR study of the interaction between Annurca polyphenols and bile acids. Then, this formulation was tested for its potential effects on human fecal cholesterol excretion through a randomised clinical trial.

2. Materials and methods

2.1. Reagents and standards

All chemicals and reagents used were either analytical-reagent or HPLC grade. The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA) before use. The standards used for the identification and quantification of phenolic acids and flavonoids were: chlorogenic acid, [+]-catechin, [-]-epicatechin, isorhamnetin, myricetin, phloretin, phloridzin (phloretin-2-*O*-glucoside), procyanidin B_2, quercetin, quercitrin (quercetin-3-*O*-rhamnoside), rutin (quercetin-3-*O*-rutinoside), isoquercitrin (quercetin-3-*O*-glucoside), hyperin (quercetin-3-*O*-galactoside) and cyanidin-3-*O*-galactoside chloride (Sigma Chemical Co., St. Louis, MO). Acetonitrile and methyl alcohol were of HPLC grade (Carlo Erba, Milano, Italy). Sodium taurocholate, phosphatidylcholine, cholesterol, NaCl, and sodium phosphate, were purchased from Sigma Chemical Co.

2.2. Fruit collection and sample preparation

Annurca (*M. pumila* Miller cv Annurca) apple fruits were collected in Valle di Maddaloni (Caserta, Italy) in October 2016 when fruits had just be harvested (green peel). Fruits were reddened, following the typical treatment for about 30 days, and then analysed [20]. Other two apple varieties analysed in this study, Pink Lady (PL) (*M. pumila* Miller cv Pink Lady), and Golden Delicious (GD) (*M. pumila* Miller cv Golden Delicious), were acquired in a local supermarket. Lyophilised apples (10 *g*) were treated with 100 mL of 80% methanol (0.5% formic acid) for 24 *h* at 4 °C to extract phenolic compounds. After centrifugation, the supernatant was slowly filtered through an Amberlite XAD-2 column packed as follows: resin (10 *g*; pore size 9 nm; particle size 0.3–1.2 mm; Supelco, Bellefonte, PA, USA) was soaked in methanol, stirred for 10 min and then packed into a glass column (10 x 2 cm). The column was washed with 100 mL of acidified water (pH 2) and 50 mL of deionised water for sugar and other polar compound removal. The adsorbed phenolic compounds were extracted from the resin by elution with 100 mL of methanol, which was evaporated by flushing with nitrogen.

2.3. Industrial preparation of Annurca nutraceutical product (AMD)

AMD consisted of Annurca apple extract microencapsulated in maltodextrins. Large-scale production of AMD was accomplished by MB-Med Company (Turin, Italy). Apples were extracted with water at 35 °C. After centrifugation, the extract was spray-dried in combination with maltodextrins, obtaining a fine powder with a maltodextrins/extract ratio 4:1.

2.4. HPLC-DAD/ESI-MS analysis

Extracts from the three different apple varieties and AMD were solubilized with 1% formic acid. Analyses were run on a Jasco Extrema LC-4000 system (Jasco Inc., Easton, MD) provided with photodiode array detector (DAD). The column selected was a Kinetex® C18 column (250 mm × 4.6 mm, 5 µm; Phenomenex, Torrance, CA). The analyses were performed at a flow rate of 1 mL/min, with solvent A (2% acetic acid) and solvent B (0.5% acetic acid in acetonitrile and water 50:50, v/v). After a 5 min hold at 10% solvent B, elution was performed according to the following conditions: from 10% (B) to 55% (B) in 50 min and to 95% (B) in 10 min, followed by 5 min of maintenance. Flavonols, procyanidins, dihydrochalcones, flavanols and hydroxycinnamic acids were monitored at 280 nm and anthocyanins at 520 nm. For quantitative analysis, standard curves for each polyphenol standard were prepared over a concentration range of 0.1–1.0 µg/µL with six different concentration levels and duplicate injections at each level. The identity of polyphenols was confirmed by LC-ESI/MS experiments and data were compared to those of commercial standards. The same chromatographic apparatus and conditions (HPLC system, gradient elution, column, temperature) was coupled to an Advion Expression mass spectrometer (Advion Inc., Ithaca, NY) equipped with an Electrospray (ESI) source. Mass spectra were recorded from m/z = 50 to 1200, both in negative and in positive ionization mode. The capillary voltage was set at −28 V, the spray voltage was at 3 kV and the tube lens offset was at −10 V in negative ion mode, while the capillary voltage was set at 34 V, the spray voltage was at 3.5 kV and the tube lens offset was at 55 V in positive ion mode. The capillary temperature was 275 °C. Data were acquired in full scan and SIM modes.

2.5. GC–MS analysis

The effects of purified apple polyphenolic extracts and AMD on the micellar solubility of cholesterol were examined as described by previous authors [21]. A bile salt micellar solution containing 6.6 mmol/L sodium taurocholate, 0.6 mmol/L PC, 0.5 mmol/L cholesterol, 132 mmol/L NaCl, and 15 mmol/L sodium phosphate (pH 6.8) was prepared by sonication and stored at 37 °C for at least 24 h. Aliquots (100 µL) of apple polyphenolic extract solutions and AMD (100 µg/mL) in deionized water stored at 37 °C were added to the 3 mL micellar solutions. The mixture was incubated for 1 h at 37 °C. The solution reveals an evident opalescence due to cholesterol precipitation. The