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Effects of dark chocolate on endothelial function in patients with non-alcoholic steatohepatitis

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Cocoa;
Polyphenols

Abstract *Background and Aim:* Oxidative stress plays a pivotal role in inducing endothelial dysfunction and progression from simple fatty liver steatosis (FLD) to non-alcoholic steatohepatitis (NASH). Polyphenols could reduce oxidative stress and restore endothelial function by inhibiting the nicotinamide-adenine-dinucleotide-phosphate (NADPH) oxidase isoform Nox2.

The aim of this study was to assess endothelial function and oxidative stress in a population affected by simple FLD and NASH. Furthermore, we analysed the effect of high vs low content of cocoa polyphenols on endothelial function and oxidative stress in patients with NASH.

Methods: In a cross-sectional study we analysed endothelial function, as assessed by flow-mediated dilation (FMD), and oxidative stress, as assessed by Nox2 activation, serum isoprostanes and nitric oxide bioavailability (NOx), in patients with NASH (n = 19), FLD (n = 19) and controls (n = 19). Then, we performed a randomized, cross-over study in 19 subjects with NASH comparing the effect of 14-days administration of 40 g of chocolate at high (dark chocolate, cocoa >85%) versus low content (milk chocolate, cocoa <35%) of polyphenols on FMD and oxidative stress.

Compared to controls, NASH and FLD patients had higher Nox2 activity and isoprostanes levels and lower FMD and NOx, with a significant gradient between FLD and NASH. The interventional study showed that, compared to baseline, FMD and NOx increased (from 2.9 ± 2.4 to 7.2 ± 3.0% $p < 0.001$ and from 15.9 ± 3.6 to 20.6 ± 4.9 μM, $p < 0.001$, respectively) in subjects given dark but not in those given milk chocolate. A simple linear regression analysis showed that Δ (expressed by difference of values between before and after 14 days of chocolate assumption) of FMD was associated with Δ of Nox2 activity (Rs = −0.323; $p = 0.04$), serum isoprostanes (Rs: −0.553; $p < 0.001$) and NOx (Rs: 0.557; $p < 0.001$).

Conclusions: Cocoa polyphenols improve endothelial function via Nox2 down-regulation in NASH patients.

Abbreviations: nicotinamide adenine dinucleotide phosphate, (NADPH); non-alcoholic steatohepatitis, (NASH); soluble Nox2, (sNox2-dp); simple fatty liver disease, (FLD); nitric oxide bioavailability, (NOx).

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Introduction

Non-alcoholic fatty liver disease (NAFLD) represents the most common hepatic disease in western world [1]. NAFLD includes different conditions ranging from simple fatty liver to non-alcoholic steatohepatitis (NASH). The clinical burden of NAFLD is characterized not only by liver complications, such as cirrhosis and hepatocellular carcinoma but also by an increased risk of cardiovascular disease and mortality [2]. Cardiovascular mortality overtakes liver-related mortality in patients with NAFLD. It has been reported that patients with NAFLD have signs of early atherosclerosis, assessed by surrogate markers such as intima-media thickness (IMT) and flow-mediated dilation (FMD) [3,4]. In particular, compared to controls, NAFLD patients have higher values of IMT and lowered vasodilation as assessed by FMD [3–5].

Oxidative stress plays a pivotal role to determine endothelial dysfunction and progression from simple steatosis to NASH [6–8]. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is the major cellular source of reactive oxygen species in humans [9,10]; NADPH oxidase activation has been associated with several cardiovascular [9] and liver [11] diseases. Antioxidant agents such as polyphenols can reduce oxidative stress [12,13]. Cocoa, that has high content of polyphenols, reduces oxidative stress via lowering activation of NADPH oxidase [14–16] and induce NO-mediated vascular relaxation [17]. Recent human and animal studies suggest that cocoa might help avert alcoholic fatty liver disease development by oxidative stress reduction [12,18]. Experimental studies showed that NADPH oxidase plays a pivotal role in the development of NASH [11] and that cocoa supplementation reduces liver damage by Nox inhibition [19]. Furthermore, we demonstrated that dark chocolate enhances artery dilation via lowering activation of NOX2 [14,15] and that reduces NOX2 activation in patients with NASH [12]. Interestingly, prior studies showed the ability of cocoa to improve insulin sensitivity [20,21] and a minor prevalence of diabetes in populations with high daily cocoa consumption [22].

In this study we wanted to extend the results of our previous study [12] analysing the relation among NOX2, oxidative stress and FMD in patients with NAFLD. First, in a cross-sectional study we analysed FMD and NOX2 activation in patients with NASH, simple fatty liver disease (FLD) and controls. Then, we performed a randomized cross-over study, in a population affected by NASH, to assess the effect of chocolate (milk vs dark), given daily for 14 days, on artery dilation and NOX2.

Methods

The study was performed in patients referred to the Internal medicine and metabolic disease outpatient clinic of Sapienza University for suspected metabolic syndrome, who had a liver ultrasonography scanning (US) as part of routine clinical examination. In the first phase, we performed a cross sectional study to compare oxidative stress, as assessed by blood levels of isoprostanes and soluble Nox2 (sNox2-dp), a marker of Nox2 activation, in 19 biopsy proven NASH patients, 19 patients with FLD and 19 matched controls without evidence of steatosis. Frequency matching procedures were applied to select controls. Matched controls were selected such that the distribution of the age and of the relevant characteristics in this group was similar to the distribution in the cases. Nineteen out of 122 patients visited who underwent a regular health check-up at our outpatient clinic for metabolic diseases between March 2015 and January 2016 were selected.

In the second phase of the study we performed an interventional trial in NASH patients to investigate the effect of daily consumption of 40 g of chocolate (20 g every 12 h, dark vs milk chocolate), for 14 days, on FMD and oxidative stress, as assessed by blood levels of isoprostanes and sNOX2-dp serum levels. The study was performed between January 2015 and March 2016.

NASH patients were randomly allocated to a treatment sequence with 40 g/day of commercially available dark chocolate (cocoa solids >85%, cocoa mass, fat reduced cocoa, cocoa butter, sugar, vanilla) and 40 g commercially available milk chocolate (cocoa solids <35% cocoa, sugar, cocoa butter, milk powder, cocoa mass, milk fat, lactose, skimmed milk powder, soya lecithin, vanilla, milk solids <20%) for two weeks, in a cross-over, single-blind design.

The content of total polyphenols was 799 mg/L GAE for dark chocolate and 296 ml/L GAE for milk chocolate.

There was at least 1 week washout between the two phases of the study. FMD, oxidative stress, serum levels of CK-18 and metabolites of chocolate were assessed at baseline, after a 24 h abstinence from food rich in polyphenols, and at 14th day, 12 h after ingestion of chocolate.

During the study, participants were required to follow a diet adjusted according to their anthropometric and clinical characteristics and to the amount of calories coming from chocolate intake; furthermore participants avoided foods high in polyphenols and additional chocolate. Blood samples (to analyse oxidative stress and epicatechin levels) were collected in the morning (between 8.00 and 9.00 a.m.) after a fasting period of 8 h at baseline and 14 days after the period of daily chocolate ingestion.

All subjects underwent a full medical history, and ultrasonography and physical examination. Subjects were excluded from the study if they had liver insufficiency, serious renal disorders (serum creatinine >2.8 mg/dL), acute cerebrovascular disease, acute myocardial infarction, or if they were current smokers or taking antioxidants. All the participants in the study received a questionnaire to evaluate their fruit and vegetable intake [23].

The number of NASH patients initially assessed for inclusion into the interventional study was 22; after initial assessments, 2 patients were excluded from the study for current smoking. One dropout (for lack of adherence to the diet) has been observed during the study; these patients were excluded from the study.

Informed written consent was obtained from all subjects: the study was conformed to the ethical guidelines of

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