

Characterization of the Effects of Two Polyphenols-Rich Plant Extracts on Isolated Diabetic Human Mammary Arteries

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The beneficial effects of flavonoids on endothelial function in pathological conditions associated with oxidative stress, such as diabetes mellitus, represents an important research direction. The aim of the present study was to assess the vasodilator response of human internal mammary arteries (IMA) fragments isolated from diabetic patients undergoing coronary artery by-pass grafting, to different concentrations of two plant extracts obtained from hawthorn and green tea, rich in polyphenols. Arterial segments were harvested from 10 male patients with type 2 diabetes and ischemic heart disease and studied in organ bath in the presence vs. absence of plant extracts. We obtained a significant decrease in contractility and a dose-dependent improvement of relaxation after pre-treatment with extracts. We can conclude that hawthorn and green tea extracts significantly improved the endothelium-dependent relaxation in diabetic vessels, suggesting that polyphenols may improve endothelial function via the increase in nitric oxide bioavailability in a disease associated with significant cardiovascular impairment.

Keywords: endothelial dysfunction, diabetes, polyphenols

Endothelium is one of the most extensive tissues in the body with a crucial role in both maintaining the vessel wall integrity and regulating vascular reactivity via the release of several relaxing and contracting factors [1]. The most important endothelium-derived vasodilator is represented by nitric oxide (NO) that elicits smooth muscle relaxation via the increase in intracellular cyclic guanosine monophosphate (GMPc) [2]. A decrease in NO bioavailability is the most important cause in the development of endothelial dysfunction considered an early marker for atherosclerosis [3].

There is an increasing body of evidence that diabetes mellitus is associated with oxidative stress defined as an increase in reactive oxygen species (ROS) production and/or a decrease in the antioxidant defense both being responsible for endothelial dysfunction. In the past decades, World Health Organization (WHO) increasingly recommended the characterization of the effects of traditional plants devoid of side effects [4] and rich in compounds (polyphenols, glycosides, alkaloids, terpenes) with powerful antioxidant effects [5].

An increasing number of studies assessed the effects of flavonoids on the endothelial function; an increase in NOS activity in vascular endothelial cells by polyphenols has been reported [6].

Hawthorn extract (*Crataegus sp.*) is a source rich in polyphenols with cardioprotective properties [7]. Green tea is another source of polyphenols, with catechins as the

main component. Epidemiological evidence points to the lower incidence of chronic diseases such as coronary heart disease and stroke in countries with a high intake of tea [8].

This study was designed to assess the vasodilator response of human mammary arteries isolated from diabetic patients undergoing to coronary artery by-pass grafting (CABG) in the presence vs. the absence of different concentrations of two polyphenols-rich plant extracts (*Crataegus* and green tea extracts).

Experimental part

Materials and methods

Study group

Segments of internal mammary arteries (IMA) were harvested from 10 consecutive male patients aged between 48 to 68 years with type 2 diabetes mellitus and ischemic heart disease undergoing CABG. This study conforms to the principles outlined in the Declaration of Helsinki. The written informed consent was obtained from each subject. None of the patients had received Ca²⁺-channel antagonists or agonists of the β -adrenergic system such as dopamine or dobutamine within 7 days prior to surgery.

Internal Mammary Artery Dissection

Human IMA was dissected as a pedicle with its venae comitantes from the thoracic wall according to a no-touch technique leaving the vessels surrounded by internal

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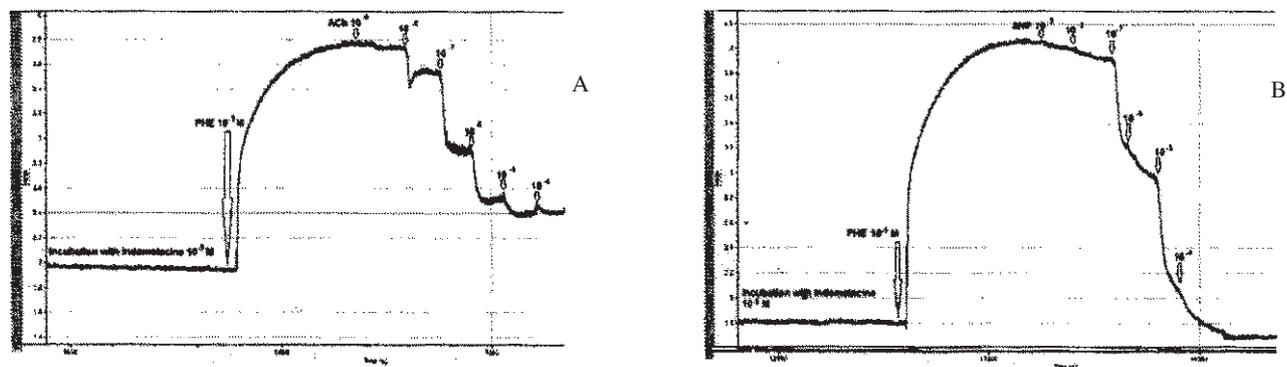


Fig. 1. Representative work graphs: vascular relaxation to cumulative doses of acetylcholine (10^{-9} - 10^{-4} M) in (A) and sodium-nitroprusside (10^{-9} - 10^{-4} M) in (B)

thoracic fascia. No vasodilators were administered systematically or topically during the harvest period. After anticoagulation with heparin, IMAs were checked for optimal flow. If satisfactory, the distal end (1 to 2 cm) was divided and placed in cold (4°C) physiologic Krebs - Henseleit solution. The vessel segments were immediately transferred to the laboratory.

Vascular Rings Preparation

Vascular segments were washed in Krebs-Henseleit buffer and cut in 2 - 4 mm rings after the removal of the adherent connective tissue. The rings were further carefully suspended between two parallel stainless steel hooks in two 10 mL organ bath chambers containing Krebs - Henseleit buffer with the following composition (in mmol/L): NaCl 118; KCl 2.8; NaHCO_3 25; CaCl_2 2.5; MgSO_4 1.2; KH_2PO_4 1.2; glucose 11; and EDTA 0.03. The buffer was gassed with a mixture of CO_2 (5%) and O_2 (95%), and maintained at 37°C and pH 7.4. The upper hook was connected to a force transducer for isometric tension recording. The amplified analog signal from the force transducer was digitized using a data acquisition system (Digidata 1200B, Axoscope 10 software, Molecular Devices Ltd).

Experimental Protocol in Organ Bath

The rings were equilibrated for 60 min at 37°C under 1.75 cN passive tension and the buffer was replaced every 15 min. To confirm the viability of vascular smooth muscle, vessel rings were contracted twice with KCl. A relaxation response to acetylcholine, an endothelium-dependent vasodilator, of 15% or more from the stable tension induced by KCl was considered as functional endothelium. After washout and return to baseline the tissues were pre-contracted with 10^{-5} M phenylephrine (PE). Cumulative concentration-response curves (10^{-9} to 10^{-4} M) for acetylcholine (ACh) (fig. 1A), as the endothelial-dependent response, and for sodium-nitroprusside (SNP) (fig. 1B), as the endothelial-independent response, were recorded in the absence vs. the presence of *Crataegus* and green tea (60 min incubation, 2 concentrations). Indometacine (10^{-5} M), as inhibitor of cyclooxygenase, was present in the organ bath throughout the experiments in order to eliminate the influence of prostaglandine synthesis on the vasodilator response. Relaxation response of each vascular ring was assessed by measuring the reduction in vascular tone at cumulative dose of the vasodilator agent and expressed as percentage change from the stable tension produced by PE.

Preparation of the Plant Extracts

The quality of the raw material was investigated by macro- and microscopic methods that confirmed the

botanical identity. Dried *Crataegus* flowers (*Crataegus monogyna* Jacq., 12.5 g) were extracted with 200 mL boiling water and stored in the dark at 20°C for 24 h. The extract was then filtered and concentrated in a rotaevaporator in low pressure conditions. Similar quantities and the same method were used to obtain from *Camelia sinensis* leaves the green tea extract. Finally, the drug extract rapport (DER) used in this study was 1:3 for hawthorn, respectively 1:2 for green tea sample. Samples for the bath organ tests consisted of fluid extracts soluted in water. *Crataegus* extract represented 37.8 mL concentrate in 62.2 mL water, whereas green tea extract was 26 mL concentrate plus 74 mL water. Quality and quantity of total phenols in the final extracts were assessed by the Folin-Ciocalteu method. The results showed that *Crataegus* sample contained $336\mu\text{g}$ catechin/mL whereas the green tea sampled contained $824\mu\text{g}$ catechin/mL. For both extracts, 2 concentrations (100μ and $200\mu\text{g/mL}$) for organ bath studies were used.

Statistical analysis

Data were analysed using GraphPad Prism 5 and Microsoft Office Excel 2003 software (Microsoft Corporation). Central tendencies of the variables obtained from n different rings were expressed as mean (M) and the dispersion as the standard deviation (SD). Statistical comparison between two groups of averages was performed using the Student *t* test.

Results and discussions

Ex vivo incubation with *Crataegus* attenuates endothelium-dependent relaxation in diabetic mammary artery segments

Vascular reactivity of the mammary artery segments in organ bath system in the presence vs. absence of the *Crataegus* extract (200 or $100\mu\text{g}$ polyphenol/mL) was studied. We observed a significant dose-dependent reduction in the vascular segments contractility (fig. 2A). Endothelium-dependent relaxation of vascular segments in response to acetylcholine was significantly improved after pre-treatment with *Crataegus* (fig. 2B). No changes of endothelium-independent relaxation in response to sodium-nitroprusside were reported (fig. 2C).

(A) Vascular contractility in response to phenylephrine (10^{-3} M) in arterial segments with (*CRATAEGUS* 200 and *CRATAEGUS* 100) and without (CONTROL) preincubation with *Crataegus* (200 and $100\mu\text{g}$ polyphenols/mL), n= 8. (B) Acetylcholine-induced relaxation in phenylephrine-precontracted mouse aortic segments with (*CRATAEGUS* 200 and *CRATAEGUS* 100) and without (CONTROL) preincubation with *Crataegus* (200 and $100\mu\text{g}$ polyphenols/mL), n=8. (C) Sodium-nitroprusside induced

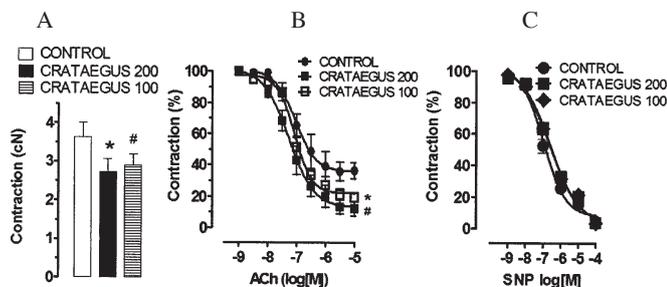


Fig. 2. The effects of *ex vivo* incubation with *Crataegus* on vascular function

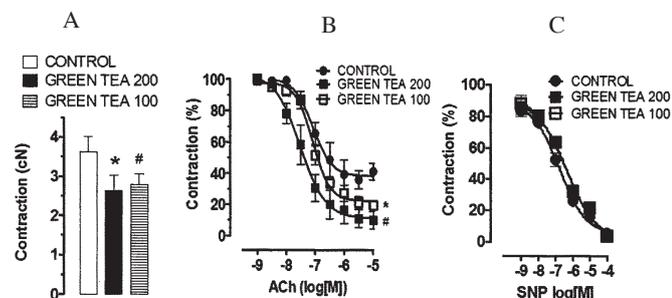


Fig. 3. The effects of *ex vivo* incubation with green tea on vascular function

relaxation in phenylephrine-precontracted mouse aortic segments with (*CRATAEGUS* 200 and *CRATAEGUS* 100) and without (CONTROL) preincubation with *Crataegus* (200 and 100 μg polyphenols/mL), $n=8$. * $p<0.05$ *CRATAEGUS* 200/ vs. CTL, # $p<0.05$ *CRATAEGUS* 100 vs. CTL

Ex vivo incubation with green tea attenuates endothelium-dependent relaxation in diabetic mammary artery segments

Similar results were reported in the presence of the second extract in the organ bath, green tea (200 or 100 μg polyphenol/mL), namely: (i) a significant reduction in the contractility of the vascular segments (fig. 3A), (ii) a significant improvement of endothelium-dependent relaxation in response to acetylcholine of vascular segments was significantly improved after pre-treatment with Green Tea (fig. 3B), and (iii) no changes in the endothelium-independent relaxation in response to sodium-nitroprusside (fig. 3C).

(A) Vascular contractility in response to phenylephrine (10^{-5}M) in arterial segments with (GREEN TEA 200 and GREEN TEA 100) and without (CONTROL) preincubation with Green Tea (200 and 100 μg polyphenols/mL), $n=8$. (B) Acetylcholine-induced relaxation in phenylephrine-precontracted mouse aortic segments with (GREEN TEA 200 and GREEN TEA 100) and without (CONTROL) preincubation with Green Tea (200 and 100 μg polyphenols/mL), $n=8$. (C) Sodium-nitroprusside induced relaxation in phenylephrine-precontracted mouse aortic segments with (GREEN TEA 200 and GREEN TEA 100) and without (CONTROL) preincubation with Green Tea (200 and 100 μg polyphenols/mL), $n=8$. * $p<0.05$ GREEN TEA 200 vs. CTL, # $p<0.05$ GREEN TEA 100 vs. CTL.

Our study investigated the vasodilatory properties of extracts from *Crataegus* flowers and green tea in isolated human internal mammary artery harvested from diabetic patients undergoing CABG. We report a significant decrease in contractility with a strong improvement of relaxation in the presence of both extracts.

Polyphenols have been demonstrated to increase NOS activity in vascular endothelial cells [9]. After the pioneering studies performed in rat aortic rings with various grape products [10], similar beneficial effects have been reported in numerous animal and human isolated vessels studies using plant polyphenols from various sources, i.e., tea, hawthorn, wine, chamomile and cocoa [11-13]. In all these studies, plant polyphenols significantly increased endothelium-dependent vasodilation.

It has been previously shown that *Crataegus* extracts induces an endothelium-dependent relaxation in both rat and human arterial vessels with similar potency and effectiveness. Accordingly, our results are in the line with previous reports on the vasodilator properties of hawthorn extracts in rat aorta [14] and mesenteric artery [15], respectively. However, the vasodilator properties of the *Crataegus* extract are strongly dependent on the presence of intact endothelium. This is an important observation that may explain the lack of efficiency of the *Crataegus* extracts in patients with coronary artery disease with an impaired endothelial layer. It has been also reported that extracts from *Crataegus* are able to induce endothelium-dependent relaxation in isolated blood vessels through the activation of endothelial NO synthase (eNOS) leading to formation of NO and preventing the progression of atherosclerosis [16, 17].

With respect to the tea extracts, it has been reported that activation of eNOS by black tea polyphenols was mediated via the estrogen receptors (ERs). Estrogens are able to induce vasorelaxation by activating ERs through the Src/PI3-kinase/Akt pathway leading to the activation of eNOS [18]. An increase in endothelial NOS activity and NO bioavailability in cultured vascular endothelial cells has also been reported in association with black tea consumption [19]. Importantly, several *in vivo* studies have shown a significant increase in brachial artery flow-mediated (i.e. the NO-mediated dilation, FMD) after green and black tea consumption [20]. The observed increments in FMD were not different between the black and green tea preparations.

Several lines of evidence indicate that ROS act as key intracellular mediators activating redox-sensitive protein kinases to induce biological responses such as cell growth, survival, and apoptosis. Therefore, the role of the redox-sensitive PI3-kinase/Akt pathway on the endothelial formation of NO in response to a *Crataegus* special extract (WS1442) was studied [17]. These authors reported that WS1442 caused the activation of the PI3-kinase/Akt pathway in a redox-sensitive manner, as indicated by the time-dependent phosphorylation of Akt at Ser473, and its prevention by ROS scavengers, and by PI3-kinase inhibitors in endothelial cells, respectively. Moreover, the PI3-kinase/Akt pathway also mediated eNOS activation since this response was abolished by wortmannin.

Altogether, the majority of available data support the evidence that tea and flavonoids from tea are able to improve NO bioavailability and thereby, to increase the endothelium-dependent relaxation in healthy subjects as well as in various pathological conditions.

Given that tea and *Crataegus* extracts contain an assortment of polyphenolic compounds, including quercetin and catechin it is difficult to ascertain if the improvement in endothelial function was due to a single dominant ingredient, or due to the synergistic action of combined polyphenolic compounds. The clinical relevance of the endothelium-dependent effects of plant polyphenols is likely dependent on their systemic availability. Thus, the

effects reported *in vitro* should always be recapitulated by the *in vivo* experiments.

Conclusions

Crataegus and green tea extracts induced a significant, dose-dependent NO-mediated relaxation in isolated vessel rings harvested from diabetic patients. By increasing NO bioavailability and improving endothelial function, administration of dietary flavonoids will become part of the life style changes in diabetic patients in order to prevent cardiovascular complications.

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