

The efficacy of black tea in ameliorating endothelial function is equivalent to that of green tea

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Consumption of tea has been shown to improve endothelial function. It is assumed that catechins are the tea components responsible for these beneficial effects. In black tea, catechin concentrations are significantly lower than in green tea. The present study was designed to compare green and black tea with regard to amelioration of endothelial function. Endothelial function in response to both teas was assessed in bovine aortic endothelial cells (BAEC) and rat aortic rings. To elucidate whether these findings are also applicable to humans, flow-mediated dilation (FMD) and nitro-mediated dilation (NMD) were assessed by ultrasound in twenty-one healthy women before and 2 h after consumption of green and black tea (2 h of FMD and NMD), in comparison with water (control). In BAEC, green and black tea significantly increased endothelial NO synthase activity to the same extent. Similarly, both teas induced comparable endothelial-dependent vasodilation in rat aortic rings. In human subjects, ingestion of green and black tea led to significant increases in FMD: from 5.4 (SD 2.3) to 10.2 (SD 3) % (baseline-adjusted difference (BAD) for 2 h of FMD, green tea *v.* water: 5.0 (95 % CI 3.0, 7.0) %; $P < 0.001$) and from 5 (SD 2.6) to 9.1 (SD 3.6) % (BAD for 2 h of FMD, black tea *v.* water: 4.4 (95 % CI 2.3, 6.5) %; $P < 0.001$), respectively. The increase in FMD was not significantly different between the two tea preparations (BAD for 2 h of FMD, green tea *v.* black tea: 0.66 (95 % CI –0.76, 2.09) %; $P = 0.36$). NMD did not vary between any of the groups. In conclusion, green and black tea are equally effective in improving endothelial function.

Tea: Endothelial function: Flow-mediated dilation: Nitric oxide

Compelling evidence from epidemiological and clinical studies has established a positive correlation between the consumption of green and black tea and protection against atherosclerosis and CVD^{1–4}. In addition to the antioxidative, anti-inflammatory, anti-proliferative and anti-thrombotic properties of polyphenols contained in tea, favourable effects on endothelial function are the main underlying mechanisms suggested as being involved in the prevention of CHD by tea consumption^{5,6}. Evidence is accumulating that catechins, the main polyphenolic compounds in green tea, are the substances responsible for these beneficial effects. Accordingly, we and others have found that catechins, particularly epigallocatechin-3-gallate (EGCG), evoke endothelial-dependent vasodilation via rapid activation of endothelial NO synthase (eNOS)^{7,8}.

Both green and black teas contain catechins. In black tea, however, catechin concentrations are significantly lower than in green tea. This is ascribed to the manufacturing process, which either prevents or allows tea polyphenols in the leaves to be oxidised. Whereas in green tea the intention is to avoid oxidation of polyphenols, black tea is manufactured by promoting enzymic oxi-

dation (fermentation). During this process, catechins are oxidised to theaflavins and thearubigins. As a result, catechins constitute about 80–90 % of total flavonoids in green tea, whereas catechin content in black tea is only 20–50 % or even lower⁹. Tea is the major beverage after water consumed in the world. Since green tea is mainly consumed in East Asia and black tea is preferred in the Western world, a question of much interest is whether green tea is superior to black tea regarding beneficial effects on endothelial function¹⁰. The aim of the present study was therefore to compare the effects of green and black tea on the production of NO in endothelial cells, on vasorelaxation in rat aortic ring preparations, and on flow-mediated dilation (FMD) in human subjects.

Methods

Measurement of endothelial nitric oxide synthase activity and phosphorylation

Bovine aortic endothelial cells were maintained and incubated as recently described¹¹. Subsequently, bovine aortic endothelial

cells were treated with the indicated doses of green or black tea for 15 min. eNOS activity was assessed by the formation of L-[³H]citrulline from L-[³H]arginine after separation of the amino acids by cation exchange chromatography as described previously⁸. Briefly, stimulation was initiated by addition of the tea preparations, 10 μM-cold L-arginine, and L-[³H]arginine (111 GBq (3 μCi)/ml). After 15 min, the reaction was terminated with ice-cold stop solution containing 5 mM-L-arginine and 4 mM-EDTA. Cells were denatured with 96 % ethanol and, after evaporation, the soluble cellular components were extracted with 20 mM-HEPES-Na (pH 5.5). L-[³H]citrulline was separated from L-[³H]arginine by Dowex chromatography, and L-[³H]citrulline formation was quantified by liquid scintillation counting. For Western blots, cells were treated with the two types of tea, or water as control, washed twice with PBS, and lysed in buffer containing (mM): HEPES (pH 7.9), 20; NaCl, 100; Na₃VO₄, 1; sodium pyrophosphate, 4; EDTA, 10; phenylmethylsulfonyl fluoride, 1; NaF, 10; okadaic acid, 0.1; Triton X-100, 1 %. Total protein (15 μg per lane) was subjected to SDS-PAGE, and membranes were probed with anti-eNOS from BD Transduction Laboratories (Lexington, KY, USA) and with anti-phospho-eNOS (Ser1179) from Cell Signaling Technologies (Beverly, MA, USA). Bands were visualised by using either BCIP (5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt) and nitro blue tetrazolium (Sigma, Deisenhofen, Germany), or the enhanced chemiluminescence detection system (Amersham, Freiburg, Germany).

Vasorelaxation studies

Thoracic aortas from male Wistar rats were rapidly excised, cleaned of connective tissue, and cut into rings 2 to 3 mm in length for organ-chamber experiments. The rings were prepared as recently described⁸. Following equilibration and submaximal precontraction with phenylephrine (0.05 μM), relaxation to cumulative doses (5–50 μl) of black and green tea was performed. Selected studies were conducted in rings treated with the NOS inhibitor *N*-nitro-L-arginine methyl ester (1 mM) before phenylephrine exposure. Vasorelaxation is expressed as percentage of precontraction with phenylephrine.

Endothelial function in human subjects

Endothelial function was measured by high-resolution vascular ultrasound (Sonoline Antares; Siemens, Erlangen, Germany) as recently described¹¹. Briefly, endothelium-dependent FMD was assessed by measuring the change in brachial artery diameter after reactive hyperaemia for 2 min, according to established guidelines^{12,13}. Endothelium-independent nitro-mediated dilation (NMD) was measured after sublingual application of nitroglycerine spray (0.4 mg) for 6 min. FMD and NMD were defined as the maximum percentage change in brachial artery diameter compared with baseline measurement. Analyses of diameter changes were conducted offline (Tom Tec Imaging Systems, Unterschleissheim, Germany) by two different investigators blinded to subject treatment.

Study design

As part of our research, we recently showed that addition of milk to black tea inhibits the vascular effects of black tea

alone¹¹. In the present analysis we investigated the effects of green and black tea on FMD in human subjects in a direct comparison of the two types of tea. Study subjects were recruited by press advertisements. None of the participants had taken medication for at least 3 months before entering the study. Subjects with cardiovascular risk factors such as high blood cholesterol, diabetes, arterial hypertension and obesity were excluded. The participants were asked not to drink tea 4 weeks before and during the study. Study subjects were required to make three clinical visits, at least 3 d apart, and at the same time of the day after fasting overnight. Each subject consumed either 500 ml boiled water, freshly brewed black tea or green tea in a cross-over study design. A quantity of 5 g tea leaves (Darjeeling black or Darjeeling green tea; King's Teagarden, Berlin, Germany) were brewed for 3 min with 500 ml boiled water. FMD and NMD were measured before and 2 h after (i.e. 2 h of FMD and NMD) consumption of the beverages. The participants had a standardised breakfast during consumption of the beverage. The study was approved by the Charité University Hospital Ethics Committee, and participants provided their written informed consent.

Analysis of tea components

Tea was prepared as described in the Study design. The concentrations of individual tea substances in brewed tea were determined as described, with slight modifications¹⁴. In brief, tea samples were diluted with 10 % acetonitrile containing EDTA (500 μg/ml) and ascorbic acid. The samples were analysed by HPLC. The HPLC detection system consisted of an Agilent 1100 (Agilent Technology, San Diego, CA, USA) with a binary pump, a thermostated autosampler, a column oven, a photodiode array detector, and a data system with Agilent 1100 ChemStation software. The column was eluted at 35°C with a binary gradient of 100 % solution A (9 % acetonitrile, 2 % acetic acid, containing EDTA (20 μg/ml)) for 10 min, 68 % solution A and 32 % of solution B (80 % acetonitrile, 2 % acetic acid, containing EDTA (20 μg/ml)) for 10 min at a flow rate of 1.0 ml/min. The eluent was monitored at 278 nm. The signals were verified by using UV spectra (diode array detector) and comparisons of the retention times with reference compounds. Quantification was carried out using the relative response factor concept of ISO 14505-2¹⁴.

Statistical analysis

Goodness of fit for normal distribution was examined by determining skewness and kurtosis. A general linear model was applied to compare the three beverages (water, black tea, and green tea). Baseline was included as a continuous covariate, and the beverages and time (as a nuisance factor) were coded by dummy variables. In cases of overall significance, pairwise tests were performed without further adjustment for multiple testing (closed testing procedure)¹⁵. Adjustment of the correlation of measurements from the same subjects took place by application of generalised estimating equations¹⁶. No time effects or time *v.* treatment interactions were found. All statistical tests were two-sided (level of significance = 0.05). We performed statistical analysis by using SPSS (release 12.0.1; SPSS Inc., Chicago, IL, USA), except for generalised estimating equations analysis, in

which we applied SAS (release 9.1; SAS Institute Inc., Cary, NC, USA).

Results

Analysis of tea components

We measured the concentrations of various tea compounds, including the catechins, in both green tea and black tea preparations. The concentrations of single tea compounds are shown in Table 1. The overall catechin concentration in black tea was about half that in green tea. The concentrations of EGCG – one of the most potent catechins – were 30% lower in black tea than in green tea.

Measurement of endothelial nitric oxide synthase activity in endothelial cells

To clarify whether eNOS activation is one of the underlying mechanisms by which both teas enhance endothelial function, we incubated bovine aortic endothelial cells with increasing concentrations of green or black tea, and measured eNOS activity in intact cells. As shown in Fig. 1(a), incubation of cells with varying amounts of either green or black tea activated eNOS dose dependently. There was no difference in the magnitude of the increase in eNOS activity after treatment of cells with green or black tea. The final concentration of total catechins after addition of 100 μ l of green and black tea to cells was 63.2 and 35 μ M, respectively. Likewise, both green and black tea preparations induced comparable levels of eNOS phosphorylation (Fig. 1(b)).

Tea-induced vasorelaxation of rat aortic rings

To compare the effects of green and black tea on vasoreactivity, we exposed phenylephrine-precontracted rat aortic rings to cumulative doses of the respective tea preparation. Both green and black tea (n 18) induced pronounced, dose-dependent vasorelaxation in the aortic rings (Fig. 1(c)). In aortic rings, the final concentration of total catechins after addition of 10 μ l of green and black tea was 1 and 0.56 μ M, respectively. The degree of vasodilation evoked by green and black tea was virtually identical. Pretreatment of rat aortic rings with the NOS-inhibitor N-nitro-L-arginine methyl ester completely prevented tea-induced vasodilation, indicating that relaxations of rat aortic rings induced by green or black tea are due to

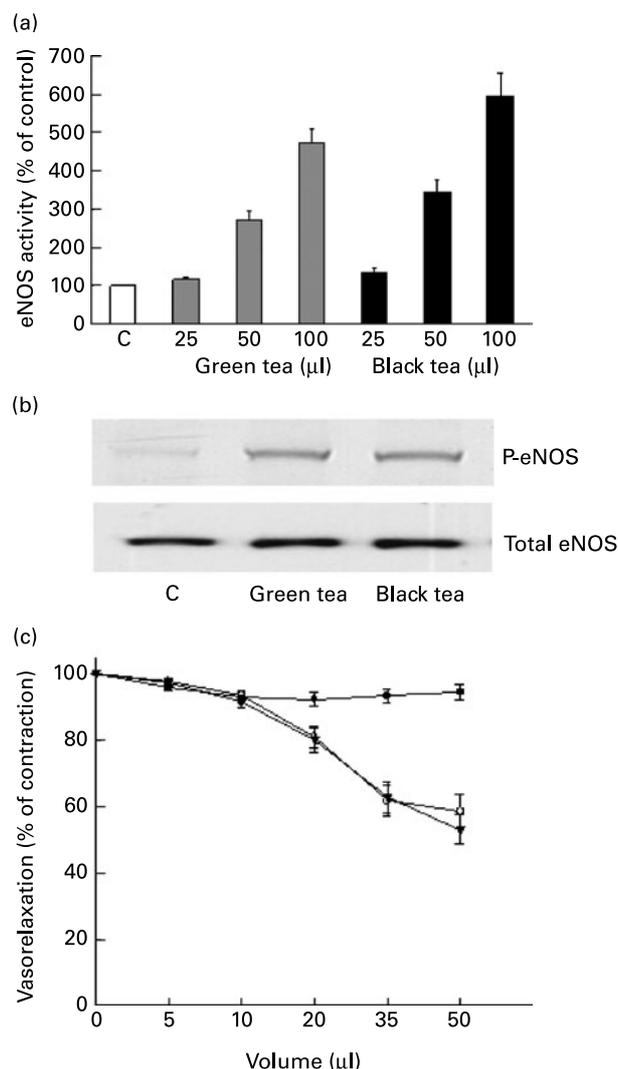


Fig. 1. Impact of green and black tea preparations on NO production in endothelial cells and on vasorelaxation in aortic rings. (a) Cells were treated for 15 min with the indicated amounts of green or black tea, or with water (control; C), and endothelial NO synthase (eNOS) activity was measured. Values are means (n 5), with standard errors represented by vertical bars. (b) Endothelial cells were treated for 15 min with 100 μ l of the indicated tea preparations. The Western blot showing the phosphorylation of eNOS (P-eNOS) was performed with an antibody specific to the eNOS Ser¹¹⁷⁹ phosphorylation site. Total eNOS served as the control for equal loading. The Western blot is representative of three independent experiments. (c) Precontracted rat aortic rings were treated with cumulative doses of either water (control; \circ –; n 16), green tea (\bullet –; n 18) or black tea (\blacktriangledown –; n 18), and vasorelaxation was determined. Vasorelaxation is expressed as percentage of contraction. Values are means, with standard errors represented by vertical bars.

Table 1. Concentrations of individual tea components (μ M)

	Darjeeling green	Darjeeling black
Caffeine	814	1034
Gallic acid	30	91
Catechin	30	0
Gallocatechin	52	0
Epicatechin	79	50
Epigallocatechin	257	70
Epicatechin gallate	130	116
Epigallocatechin-3-gallate	464	324
Total catechins	1012	560

generation of NO, and are hence endothelial dependent. The vasodilating effect of tea occurred rapidly, within minutes.

Endothelial function in human subjects

A total of twenty-one healthy postmenopausal women completed the study. Baseline characteristics of the study group are shown in Table 2. Endothelial function was assessed by measuring FMD of the forearm brachial artery, before and 2 h after ingestion either of 500 ml water (control), black

Table 2. Baseline characteristics of the study population (Mean values and standard deviations for twenty-one subjects)

Characteristic	Mean	SD
Age (years)	58.7	4.5
Systolic blood pressure (mmHg)	118.5	13.7
Diastolic blood pressure (mmHg)	72.1	8.0
BMI (kg/m ²)	23.1	1.7
HbA _{1c} (%)	5.8	0.9
Homocysteine (μmol/l)	10.2	2.1
C-reactive protein (mg/l)	1	1
Total cholesterol (mmol/l)	5.3	0.9
HDL-cholesterol (mmol/l)	1.9	0.3
LDL-cholesterol (mmol/l)	3.1	0.9
TAG (mmol/l)	0.84	0.30
Lipoprotein(a) (mg/l)	100	87

tea, or green tea in a cross-over study. All results of the intervention study are presented in detail in Tables 3 and 4. Briefly, ingestion of green and black tea led to significant increase of FMD: from 5.4 (SD 2.3) to 10.2 (SD 3) % (baseline-adjusted difference for 2 h of FMD, green tea *v.* water: 5.0 (95 % CI 3.0, 7.0) %; $P < 0.001$), and from 5 (SD 2.6) to 9.1 (SD 3.6) % (baseline-adjusted difference for 2 h of FMD, black tea *v.* water: 4.4 (95 % CI 2.3, 6.5) %; $P < 0.001$), respectively (Fig. 2). The increase in FMD was not significantly different between the two tea preparations (baseline-adjusted difference for 2 h of FMD, green tea *v.* black tea: 0.66 (95 % CI -0.76, 2.09) %; $P = 0.36$). Water showed no effect on FMD. Endothelial-independent NMD was not significantly different after consumption of the three beverages.

Discussion

The present study is the first to compare the effects of green and black tea on endothelial function *in vitro* and *in vivo*. In both rat aortic rings and in healthy women, green and black tea significantly enhanced endothelial function in a comparable manner. The amount of tea-induced increase of eNOS activity in endothelial cells was furthermore not different after incubation of the cells with green or black tea. In the present study, we used freshly brewed tea (rather than tea extracts) for our *in vivo* as well as *in vitro* studies, to mimic real-life conditions. Although, as expected, the catechin

content was much lower in black tea (Table 1), the efficacy of this fermented tea preparation in terms of amelioration of endothelial function was identical to green tea.

Several clinical studies have described beneficial effects of both green and black tea on endothelial function^{11,17-19}. We recently showed that addition of milk to black tea completely prevented the improvement of endothelial function by black tea alone in human subjects. In addition to these *in vivo* results, we were able to demonstrate a selective decrease in concentrations of catechins in tea with milk compared with tea without milk – findings that suggest an important role of catechins in black tea for amelioration of endothelial function¹¹.

The underlying molecular mechanisms for favourable tea-induced effects on endothelial function are multifactorial; they involve direct effects on the NO system, as well as anti-oxidative effects associated with an increase in NO availability. Although information is limited concerning the biological activities of theaflavins, and especially of thearubigins – the heterogeneous oxidation products that are the main polyphenols in black tea – there is increasing evidence that they are effective antioxidants^{20,21}. However, data are not uniform with regard to the antioxidative capacity of theaflavins as compared with catechins. Lee *et al.* reported that the antioxidative capacity per serving of green tea is much higher than that of black tea in commercial tea products²². Some authors found that the antioxidant effects of theaflavins in black tea are equal to that of catechins in green tea²³. Yoshida *et al.* have shown that theaflavin digallate had a higher inhibitory effect on LDL oxidation in macrophages *in vitro* when compared with EGCG. The authors postulated that the higher antioxidative capacity of theaflavins is due to their greater number of hydroxyl (OH) groups, since theaflavins represent polymers of catechins²⁰. It is assumed that the degree of hydroxylation is positively correlated with their antioxidant efficiency²⁴. Antioxidative activities of black tea thearubigins on lipid peroxidation have likewise been reported²⁵. In conclusion, these data suggest that the antioxidative effects of green and black tea may differ depending on the polyphenol composition.

Beyond the antioxidant properties of catechins and their oxidised products theaflavins and thearubigins, direct effects on the NO system may also contribute to improvement of endothelial function after green and black tea consumption. We have recently shown that EGCG, the main catechin in

Table 3. Flow-mediated dilation (FMD) and nitro-mediated dilation (NMD) before and 2 h after consumption of water, green tea and black tea (Mean values and standard deviations for twenty-one subjects)

	Water (basal)		Water (2 h)		Green tea (basal)		Green tea (2 h)		Black tea (basal)		Black tea (2 h)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
FMD _{base} (mm)	3.07	0.26	3.10	0.28	3.10	0.25	3.10	0.25	3.07	0.28	3.08	0.29
FMD _{max} (mm)	3.24	0.28	3.30	0.29	3.26	0.24	3.42	0.27	3.22	0.27	3.35	0.29
FMD _{max} (%)	5.5	3.0	6.4	2.8	5.4	2.3	10.2	3.0	5.0	2.6	9.1	3.6
NMD _{base} (mm)	3.10	0.28	3.12	0.27	3.12	0.26	3.12	0.26	3.10	0.27	3.11	0.29
NMD _{max} (mm)	3.71	0.28	3.78	0.31	3.72	0.31	3.80	0.32	3.72	0.30	3.78	0.33
NMD _{max} (%)	20.2	4.5	21.0	4.6	19.1	5.2	21.4	4.1	20.2	5.8	21.7	4.1

FMD_{base}, diameter of brachial artery before hyperaemic stimulus; FMD_{max}, maximum dilation of brachial artery after hyperaemic stimulus, shown in mm and as maximum percentage change in brachial artery diameter compared with FMD_{base}; NMD_{base}, diameter of brachial artery before application of 0.4 mg nitroglycerine; NMD_{max}, maximum dilation of brachial artery after application of 0.4 mg nitroglycerine, shown in mm and as maximum percentage change in brachial artery diameter compared with NMD_{base}.

Table 4. Baseline-adjusted differences for 2 h of flow-mediated dilation (FMD) 2 h after consumption of green tea and black tea (Percentages and 95% confidence intervals for twenty-one subjects)

	Baseline-adjusted difference (%)	
	FMD	95% CI
Green tea v. water	5.0*	3.0, 7.0
Black tea v. water	4.4*	2.3, 6.5
Green tea v. black tea	0.66	-0.76, 2.09

* $P < 0.001$.

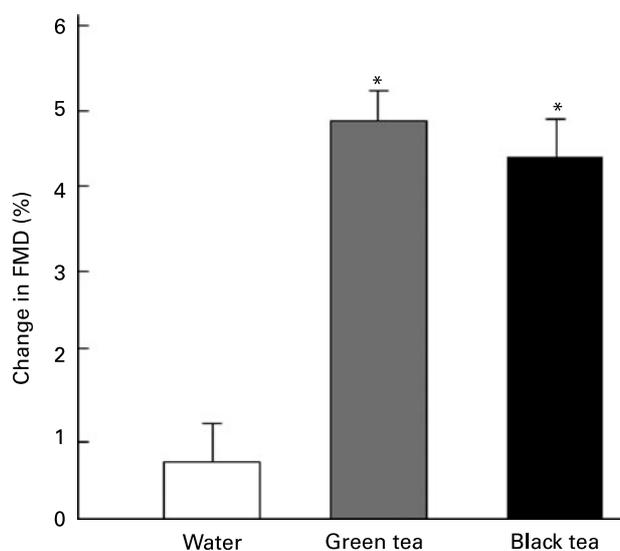


Fig. 2. Changes in flow-mediated dilation (FMD) (%) after consumption of tea preparations. Volunteers consumed either 500 ml of water, green tea, or black tea, and changes in FMD were measured. Values are means for twenty-one subjects, with standard errors represented by vertical bars. * Mean value was significantly higher than that for water ($P < 0.001$).

green tea, acts as a natural eNOS activator by directly enhancing eNOS activity⁸. In a similar manner, black tea polyphenols stimulate eNOS activity through activation of oestrogen receptor α in vascular endothelial cells²⁶. In corroboration of these data, we found a comparable increase in eNOS activity by green and black tea in the present study.

At present, there are few data directly comparing the efficacy of green and black tea on cardiovascular effects. Here we show for the first time that green and black teas are equally effective in ameliorating endothelial function. Accordingly, drinking black tea may result in beneficial cardiovascular health effects similar to those of green tea. These results suggest that other polyphenolic compounds, such as theaflavins and thearubigins generated during oxidation of catechins in the process of black tea manufacture, may compensate for the reduced catechin content in black tea.

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