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Silver fir (*Abies alba*) trunk extract protects guinea pig arteries from impaired functional responses and morphology due to an atherogenic diet

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ABSTRACT

Background: Diet, rich in plant polyphenols prevents atherogenesis that manifests as reduced vascular relaxation and formation of plaques.
Hypothesis: Atherosclerosis could be reduced by the intake of silver fir (*Abies alba*) extract (SFTE), rich in polyphenols.
Study design: Chronic, *in vivo* treatment animal study.
Methods: Guinea pigs (*Cavia porcellus*) were fed for 8 weeks with one of the following three diets: atherogenic, basic or atherogenic + SFTE. After isolation, we measured the relaxation and contractile responses of the thoracic aorta. Additionally, we measured the area of fatty plaques on the aortic walls.
Results: Compared to the basic diet, the atherogenic diet decreased the ability of the aorta to relax by 63% ($p < 0.001$). The addition of SFTE to the atherogenic diet improved the aorta relaxation response compared to that of the atherogenic diet without SFTE (the decrease relative to the basic diet was 26%, $p < 0.001$). The aorta contractility did not differ between the groups. The SFTE group generated significantly fewer atherosclerotic plaques than did the atherogenic group. The areas of atherosclerotic plaques were 7.4, 0.3 and 1.6% in the aortas of guinea pigs receiving atherogenic, basic or atherogenic + SFTE diets, respectively.
Conclusions: In a guinea pig model, prolonged treatment with antioxidative polyphenol-rich SFTE prevents aortic functional and morphological changes caused by an atherogenic diet.

Introduction

Atherosclerosis is the major precursor of cardiovascular disease and is a chronic inflammatory process in arterial walls that is caused by the accumulation of macrophages and low-density lipoproteins. The interaction between plasma cytokines, lipoproteins and artery-specific proteins influences lesion initiation and growth (Pelosi et al. 2014). Atherosclerosis is characterized by elevated total cholesterol and low-density lipoprotein cholesterol. The condition is a chronic disease that can remain asymptomatic for decades and that can be prevented by a healthy lifestyle. Strong evidence indicates that the inflammation of the blood vessel intima is caused by reactive oxygen species (ROS), which form upon oxidative stress. This response represents a state of imbalance between the production and elimination of free radicals that, in excessive quantities, damage tissues. In addition to pollution, smoking, exercise deficiency and stress, one of the major causative factors of atherosclerosis is a Western-type diet rich in saturated fats and poor in fiber and antioxidants (Miller et al. 2013).

Antioxidants are compounds or enzymes that are capable of counteracting the damaging effects of oxidation. Antioxidative and anti-inflammatory plant phenols have been demonstrated to reduce atherosclerosis and improve endothelial function (Stoclet et al. 2004). Most studies have focused on catechins from green tea, cocoa and maritime pine bark extract because they lower the intestinal absorption of lipids and decrease cholesterol synthesis (Moore et al. 2009; Salvamani et al. 2014). Polyphenols decrease oxidative stress, prevent inflammation, and reduce platelet aggregation and the

Abbreviations: SFTE, silver fir trunk extract; ROS, reactive oxygen species; EDTA, ethylenediaminetetraacetic acid; TCA, trichloroacetic acid; TBA, thiobarbituric acid; LDL, low-density lipoprotein; NO, nitric oxide; NOS, nitric oxide synthase; (MCP)-1, monocyte chemoattractant protein-1; VLDL, very low-density lipoprotein; BHT, butylated hydroxytoluene; PBMC, peripheral blood mononuclear cell; DPPH, 2,2-diphenyl-1-picrylhydrazyl; CVD, cardiovascular disease.

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proliferation of vascular smooth muscle cells (Curin and Andriantsitohaina 2005). Polyphenols also act as beneficial agents in cardiovascular disorders, diabetes mellitus, rheumatism, chronic venous insufficiency and other inflammatory diseases (Enseleit et al. 2012; Gulati 2014; Maimoona et al. 2011). Among other mechanisms, cardiovascular drugs also appear to act through scavenging effects (Marton et al. 2001).

Silver fir trunk extract (SFTE) contains a complex mixture of bioactive polyphenols from the trunk of the silver fir tree (*Abies alba*), of which the main constituents are catechins, phenolic acids and lignans. SFTE exhibits strong *ex vivo* antioxidative activity when incubated with primary human peripheral blood mononuclear cells (Tavčar Benković et al. 2014); however, no scientific data have been published regarding SFTE's efficiency under physiological conditions. Other studies on *Abies alba* wood revealed the potency of ethyl acetate extract in scavenging free radicals and chelating ferrous ions (Vasincu et al. 2013). An ideal antioxidant should be readily absorbed by the body and should prevent or quench free radical formation or chelate redox metals at physiologically relevant levels (Poljsak et al. 2013).

As in humans, Western-type diets can induce atherosclerosis in certain rodent models. Among these models, guinea pigs exhibit a cholesterol profile that most closely resembles that of humans when challenged with a high-cholesterol diet. Therefore, guinea pig models are valuable for testing the efficiency of various therapies (Fernandez and Volek 2006).

In this study, we demonstrated that SFTE could prevent morphological and functional changes of the arterial wall of guinea pig aortas as a result of an 8-week atherogenic diet.

Material and methods

Silver fir trunk extraction

Silver fir trunk extract, characterized in our previous studies was prepared by the following two-step process, according to a modification of a previously published procedure (Štrukelj et al. 2012; Tavčar Benković et al. 2014): 5 kg of the ground trunk of the silver fir (*Abies alba* Mill., checked with www.theplantlist.org) was extracted with 25 l of water at 70°C for 2 h. The aqueous extract was then evaporated under vacuum to a volume of 5 l. In the second step, the concentrated aqueous extract was extracted with 3 × 3 l of ethyl acetate. Twenty-five milliliters of polyethylene glycol 400 was added to the ethyl acetate extract, and the ethyl acetate was then evaporated from the mixture. We obtained 50 ml of viscous, liquid SFTE. According to EMA guideline (European Medicines Agency (HMPC) 2010), the extract is "other herbal preparation" declared as: refined liquid extract from *Abies alba* Mill., truncus (DER = 100 : 1). Extraction solvent: water.

As recommended in EMA reflection paper (European Medicines Agency (HMPC) 2008), protocatehuic and p-coumaric acids were chosen as analytical markers since they are potentially connected to the biological activity of the extract and reference compounds are available for their quantification. Their specific analysis was carried out by a validated HPLC method (Tavčar Benković et al. 2014). The content of protocatechuic acid was 7.7 g/l and the content of p-coumaric acid was 3.7 g/l. The extract was further characterized by a HPLC fingerprint chromatogram (Fig. 1).

Determination of hydroxyl radical scavenging activity

The ability of the extracts to inhibit nonsite-specific hydroxyl radical-mediated peroxidation was carried out according to a previously described method (Hinneburg et al. 2006). The reaction mixture contained 200 μl of extract dissolved in phosphate buffer (0.2 M, pH 7.4), 200 μl of 1 mM $FeCl_3$ (dissolved in water), 100 μl of 1 mM ascorbic acid solution in phosphate buffer, 100 μl of 1 mM EDTA in phosphate buffer, 200 μl of 14 mM 2-deoxy-D-ribose in phosphate buffer and 100 μl of 10 mM H_2O_2 in phosphate buffer. Each tube was vortexed and incubated at 37°C for 60 min, after which 1 ml of 10% TCA with 0.5% TBA mixture was added. The samples were heated in a water bath at 85°C for 30 min, and the extent of oxidation was estimated based on the absorbance of the solution at 535 nm. The percentage inhibition values were calculated using the absorbances of the control (Ac) and sample (As); the controls contained all of the reaction reagents except for the extract or positive-control substance.

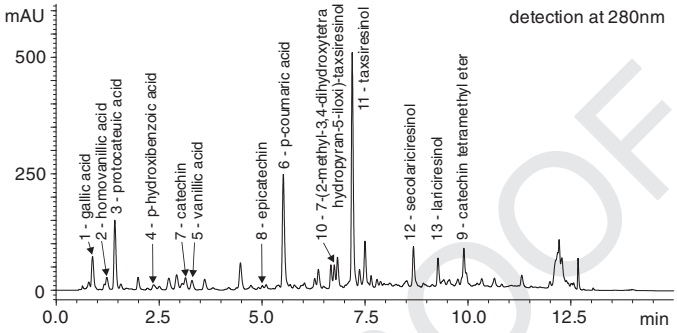

Fig. 1. Fingerprint chromatogram of SFTE.

Animal studies

The experiments were conducted in accordance with the guidelines of the Veterinary Administration of the Republic of Slovenia (Permit No. 34401-23/2009/3), which conform to the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Animal Research, National Research Council, Washington D.C. (National Academy Press, 1996).

Eighteen Dunkin Hartley guinea pigs (*Cavia porcellus* L.) of both sexes, aged between five and eight months, were housed at a constant ambient temperature (24 ± 1°C) and under a regular 12:12 h circadian cycle. The male and female subjects were randomly assigned to one of three experimental groups, and 2–4 animals were kept in each cage. Each animal had unlimited access to water and to one of the following feeds: the atherogenic diet (2 males, 3 females) (38.5% Altromin 3123 (Lage, Germany) guinea pig maintenance diet pellets, 38.5% Altromin 3113 (Lage, Germany) guinea pig breeding diet pellets, 8.6% yolk (Mercator, Slovenia), 5% lard (Mercator, Slovenia), 8.4% fructose (KEFO, Ljubljana, Slovenia), 1% cholesterol (Acros Organics, Belgium)); the basic diet (3 males, 3 females) (100% Altromin 3123 (Lage, Germany) guinea pig maintenance diet pellets); or the atherogenic diet (3 male, 4 female) (0.02% SFTE, thoroughly mixed with feed). At an average feed consumption of 50 g/kg of body weight, the extract intake corresponded to 10 mg of SFTE per kg of body weight.

After 8 weeks, the animals were sacrificed using CO_2 after a prior injection of 8500 I.U. of heparin per animal (Krka, Novo Mesto, Slovenia). The thoracic aortas were isolated, rinsed of blood, dissected, and cleansed of fat and connective tissue. The tissue was cut transversally into 8 cylindrical rings (5 mm in length), using caution to preserve the endothelium. The aortic rings were immediately mounted in standard organ baths filled with a K-H solution of the following composition (in mM): NaCl 118.8, $NaHCO_3$ 23.8, KCl 4.7, $MgSO_4$ 1.2 (Merck, Darmstadt, Germany), KH_2PO_4 1.2, $CaCl_2$ 2.5 and glucose 11 (Kemika, Zagreb, Croatia). The abdominal aorta was isolated, rinsed of blood, dissected, cleansed of fat and connective tissue, and preserved in 10% formalin in buffered saline for morphological examination.

Vascular reactivity studies

After mounting, the rings were equilibrated at 20 mN resting tension for 90 min, with periodic adjustment of the ring to the desired

level, and the K–H solution was exchanged every 15 min. The rings were then contracted with 60 mM of KCl until stable contraction was obtained, after approximately 30 min, and then rinsed with K–H solution for 10 min. The KCl-induced contraction and rinsing was repeated one time. Phenylephrine and acetylcholine (Sigma–Aldrich Chemie, Steinheim, Germany) were dissolved in distilled water. For the relaxation measurements, the rings were precontracted with 100 μmol/lof phenylephrine for 5 min until the contraction reached a plateau, and then the rings were relaxed by incubation with five cumulative final concentrations of acetylcholine (10^{-8}-10^{-4} mol/l) for 2 min intervals. The tension was recorded after each interval. Finally, the rings were equilibrated with the K–H solution for 10 min.

For the contraction measurements, the aortic rings were sequentially contracted six times with phenylephrine (final concentrations of 10^{-9}-10^{-4} mol/l) for 2 min intervals, and the tension was recorded after each interval.

The vascular responses were processed and recorded on a Dewetron acquisition system (Dewetron, Graz, Austria) after analogue-digital conversion (NI PCI-6013; National Instruments, Austin, TX, USA) on the hard disk of a personal computer using the DeweSoft 6.1 software (Dewetron, Trbovlje, Slovenia).

Atherosclerotic plaque area measurement

The abdominal aortas were cut longitudinally and dyed for 15 min with Oil red O solution (210 mg Oil Red O (Sigma-Aldrich O-0625, St. Louis, ZDA), 60 ml of isopropanol (Merck, Darmstadt, Germany) and 40 ml of water). The tissues were then flushed with water, mounted between two glass plates and scanned. The red atherosclerotic area was identified manually on the computer screen as a percentage of the total arterial wall inner surface area with the ImageJ (Image Processing and Analysis in Java, National Institute of Health, ZDA) software.

Data analysis

The statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). The values are expressed as the mean ± SEM for the *n* observations, where n represents the number of animals and *m* represents the number of aortic rings used in each of the studied groups. The relaxation responses of the arterial rings are expressed as a percentage relative to the phenylephrine precontracted aortic rings. The isolated thoracic aorta results were fitted and plotted using sigmoidal concentration-response curves. A two-way analysis of variance (ANOVA) with a Bonferroni's post hoc test was used to perform the intergroup comparisons. A value of $p < 0.05$ was considered significant.

Results

Determination of hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of SFTE (IC50 = 2.9 μg/ml) was better than that of resveratrol (IC50 = 5.8 μg/ml), Butylated hydroxytoluene (BHT) (IC50 = 9.2 μg/ml), vitamin E (IC50 = 10.1 μg/ml) or epigallocatechin gallate (IC50 = 18.3 μg/ml).

Animal studies

The average final body weight of the animals after the experiment was 1049 g for the atherogenic diet group, 923 g for the basic diet group and 923 g for the atherogenic + SFTE diet group. The differences were statistically significant ($p < 0.05$).

The endothelium-dependent relaxation of the aortic rings precontracted with phenylephrine was measured by vasorelaxation tests with acetylcholine. The relaxation of the thoracic aorta rings from the three groups of animals strengthened with increasing concentrations of acetylcholine (Fig. 2). The extent of relaxation was dramatically reduced in the aortic rings of the animals fed an atherogenic diet and was highly improved with the inclusion of SFTE in the diet. The differences between the groups were significant at the three highest acetylcholine concentrations.

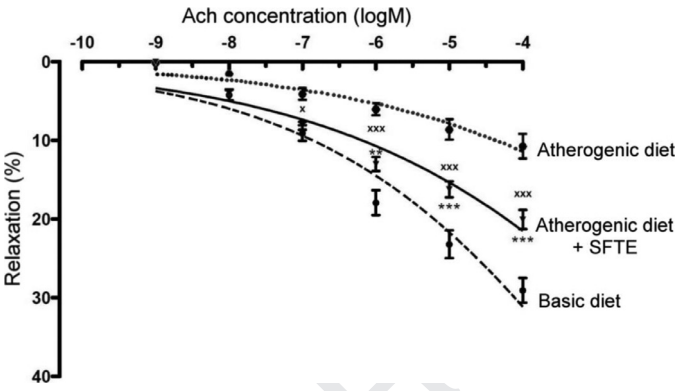

Fig. 2. Relaxation of pre-contracted thoracic aorta rings with the cumulative addition of increasing concentrations of acetylcholine. Each point on the curve represents the mean ± SEM. Asterisks and crosses represent significant differences (two-way ANOVA with Bonferroni post-hoc test): xxx for $p < 0.001$, xx for $p < 0.01$, x for $p < 0.05$ when comparing an atherogenic diet to atherogenic diet + SFTE; *** for $p < 0.001$, ** for $p < 0.01$, * for $p < 0.05$ when comparing an atherogenic diet + SFTE to a basic diet.

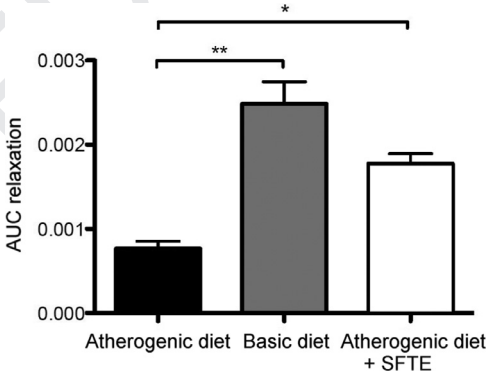

Fig. 3. Areas under the curves of relaxation, as a function of acetylcholine concentration. One-way ANOVA with Bonferroni post-hoc test: * $p < 0.05$ when comparing an atherogenic diet to an atherogenic diet + SFTE, ** $p < 0.01$ when comparing an atherogenic diet to a basic diet.

Fig. 3. presents the areas under the relaxation curves from Fig. 2. The thoracic aorta of the guinea pigs fed an atherogenic diet for 8 weeks demonstrated a 63% decrease ($p < 0.001$) in the relaxation response, compared to the animals that were fed the basic diet. The addition of SFTE to the atherogenic diet significantly improved the relaxation response. The aortic relaxation of the atherogenic diet + SFTE group decreased by 26% ($p < 0.001$) in comparison to the basic diet group.

The ability of the aortas to contract did not differ significantly between the three groups receiving different diets (Fig. 4). We observed that some contraction values increased above 100% for the basic group. We postulated that the extent of the phenylephrine-stimulated contraction was greater than that of the KCl-stimulated contraction in the basic group. The extent of the aortic ring contraction was identical for the atherosclerotic diet + SFTE group.

The inner surface of the abdominal aortas from guinea pigs that received an atherogenic diet for 8 weeks exhibited drastically increased areas of atherosclerotic plaques in comparison to the animals fed a basic diet (Fig. 5). The addition of SFTE to the atherogenic diet

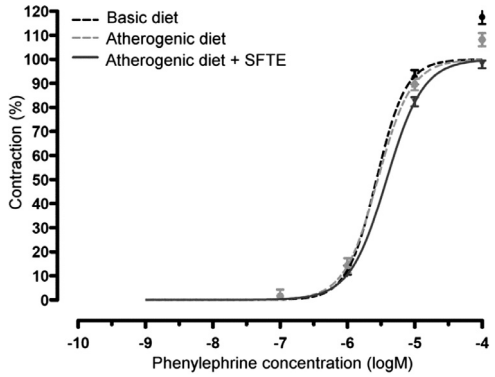

Fig. 4. Contraction of thoracic aorta rings, with cumulative addition of increasing concentrations of phenylephrine. Two-way ANOVA with Bonferroni post-hoc test. Each point on the curve represents a mean ± SEM.

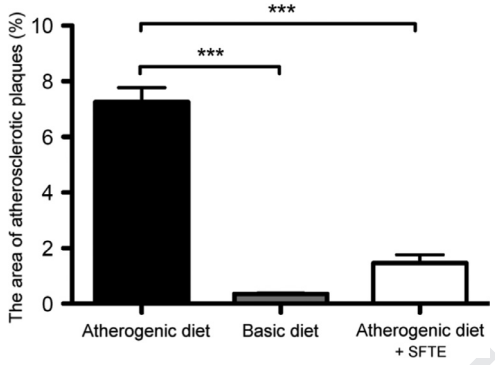

Fig. 5. The area of atherosclerotic plaques, shown as the percent of the total area of the inner surface of the arterial wall. One-way ANOVA with Bonferroni post-hoc test: *** $p < 0.001$. The atherosclerotic plaque areas were 7.4, 0.3 and 1.6% in the aortas of guinea pigs receiving the atherogenic, basic and atherogenic + SFTE diets, respectively. The group that received SFTE in addition to the atherosclerotic diet generated significantly fewer atherosclerotic plaques than did the group receiving the atherogenic feed alone (typical walls of the aortas are shown in Fig. 6).

significantly decreased the atherosclerotic process, compared to that in the animals receiving an atherogenic diet without SFTE.

Discussion

In this study, the prophylactic effects, i.e., cardiovascular protective effects, of SFTE on the functional and morphologic changes in the aorta that arise from a chronic atherogenic diet were studied. The effects of three different diets on the contraction and relaxation responses of thoracic aortas and on the development of atherosclerotic plaques in the abdominal aortas of guinea pigs were tested. The guinea pigs were divided into three groups and fed for 8 weeks with an atherogenic diet, basic diet or atherogenic diet + SFTE (Fig. 6).

The guinea pigs were shown to be one of the best models for evaluating atherosclerotic damage (Janić et al. 2014). Similar to humans, these animals store most of their blood cholesterol in LDL form, possess similar enzymes associated with cholesterol synthesis and are highly sensitive to dietary lipid intake (Fernandez and Volek 2006). A high-cholesterol diet successfully induced experimental atherogenesis and caused significant changes in the guinea pig aortas after 8 weeks, with an average of 50 g of feed consumed per animal per day.

We demonstrated that prolonged simultaneous feeding of an atherogenic + SFTE diet, which is rich in antioxidative polyphenols, prevents the functional and morphological changes caused by an atherogenic diet in the aortas of guinea pigs. Based on the literature, the observed effect can be achieved by one or more of the following mechanisms: nonspecific effect of caloric restriction, influence on smooth muscle function (directly or via NO), influence on the inflammation process (directly on macrophages or via cytokines) and prevention of lipoprotein formation and oxidation. Each of these possible mechanisms will be discussed in the following chapters.

On average, the animals in all three groups consumed equal amounts of feed (approximately 50 g per animal per day), but their final body weights were significantly different. The reduced body weight might have contributed to the observed prevention of functional and morphological changes.

The functionality of the aortic rings depends on their amount of smooth muscle, endothelial functionality and size, and pathological changes in these vessels lower their functional responses. The SFTE-rich diet significantly improved the relaxation response of the aortas. In our previous study, SFTE was shown to increase the relaxation ability of rat aorta via a NO-dependent mechanism, as this effect was prevented by a NOS inhibitor (Drevenšek et al. 2015). Similar results were obtained in studies using polyphenol-rich extracts from other plant species, which improved endothelial function through increased NO synthesis (de Pascual-Teresa et al. 2010; Maimoona et al. 2011). The phenols from strawberry leaves, kaempferol and rutin, were shown to be direct, endothelium-dependent vasodilators that were mediated by NO and cyclooxygenase products (Mudnic et al. 2009).

In contrast, neither an atherogenic diet nor SFTE-rich diet influenced the phenylephrine-initiated contraction of the aortas. Phenylephrine is an α_1 agonist that induces the vasoconstriction of smooth muscle cells through mechanisms in the endothelium and vascular smooth muscle cells. We postulate that SFTE does not directly affect smooth muscle cells; however, it can act through several other mechanisms.

We observed that SFTE reduced atherosclerotic plaque formation by 80%. In previous studies, various plant polyphenols reduced vessel wall inflammation and prevented the formation of atherosclerotic plaques through different pathways (Leifert and Abeywardena 2008). In general, they reduced cholesterol absorption and accumulation in macrophages (Curin and Andriantsitohaina 2005). For

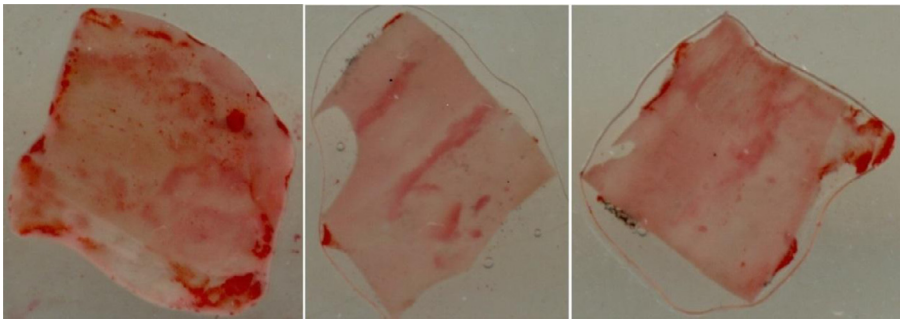

Fig. 6. Typical views of open abdominal aortas with visible atherosclerotic plaque areas from animals on the different diets. 1. Atherogenic 2. Basic 3. Atherogenic + SFTE.

example, a mixture of vitamins and minerals lowered LDL plasma levels, macrophage-positive areas and plaques in rabbits (Leborgne et al. 2005). In mice, quercetin inhibited monocyte chemoattractant protein (MCP)-1, a critical contributor to the initiation and development of atherosclerotic lesions that directly promotes the migration of inflammatory cells to the vascular wall. Quercetin also decreased plasma levels of interleukin 17, which acts as a potent mediator by increasing chemokine production in various tissues to recruit monocytes and neutrophils to the site of inflammation (Garelnabi et al. 2014). In ovariectomized guinea-pigs, polyphenol-rich grape intake altered hepatic cholesterol metabolism by lowering plasma triglycerides and VLDL cholesterol (Zern et al. 2003). Catechin supplementation of apoE-deficient mice also reduced the mean atherosclerotic lesion area. These authors confirmed the atheroprotective effect of catechin through the down-regulation of certain genes involved in energy metabolism, lipid metabolism and lipid trafficking (Auclair et al. 2009). Polyphenols also regulate various mechanisms involved in inflammation (Tangney and Rasmussen 2013).

One of the explanations for SFTE efficiency is its strong antioxidant activity. *In vitro* tests revealed that SFTE is a better hydroxyl radical scavenger than resveratrol, BHT, vitamin E or epigallocatechin gallate. Our previous study evaluated the high ability of SFTE to scavenge free radicals in PBMC cells (Tavčar Benković et al. 2014), and those findings were supported by a study by Vasincu et al., who showed that ethyl acetate bark extract effectively scavenged free radicals (DPPH, superoxide anions and hydroxyl radicals) and chelated ferrous ions (Vasincu et al. 2013). In general, antioxidants reduce the formation of free radicals and other reactive oxygen species that can directly injure the arterial endothelium and cause lipid peroxidation of LDL particles, which then enter the blood vessel wall.

Western-type diets are typical for some developed countries. High intake of saturated fats, sugar and low intake of fibers and antioxidants lead to elevated incidences of disease such as cardiovascular diseases (CVDs), diabetes, obesity and cancer (Everitt et al. 2006). In guinea pigs, we demonstrated that SFTE extract could prevent the damage caused by such diet. In accordance, many epidemiological and experimental studies have shown the beneficial role of antioxidants in the diets of humans as prophylaxis against cardiovascular disease (Bertelli and Das 2009).

After the strong *in vitro* potential for individual antioxidants such as α-tocopherol, β-carotene and ascorbic acid was demonstrated, many clinical trials have reported disappointing results because an excessive number of single agents exhibited pro-oxidative activity (Lonn 2001). Our results indicate that complex, antioxidant-rich, natural extracts possess several pharmacological activities and potential therapeutic advantages and might provide numerous benefits for human health.

Conclusion

SFTE added to an atherogenic diet of guinea pigs improved the relaxation response and prevented the development of atherosclerotic plaques in aortas. The treatment did not influence aortic contractility. SFTE was shown to be a potential agent in the prophylaxis and treatment of atherosclerotic changes.

Conflict of interest

We wish to draw the attention of the editor to the following facts which may be considered as potential conflicts of interest. We received no financial contributions to this work. However, in the past 3 years, our department received financial support from following companies: Krka, d.d., Novo mesto; Lek d.d.; Medis, d.o.o.; ABIES LABS razvoj in proizvodnja d.o.o.

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