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Full Length Research Paper

Reduction of small arteries contractility with improving the relaxation properties by *Ginkgo biloba* extract

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The effect of ethanolic *Ginkgo biloba* L. leaf extract (GE) on vascular smooth muscle contractility and endothelium-dependent relaxation were investigated in this study. Direct applications on isolated vessels from Wistar rats as well as animal feeding with the extract (dosage of 0.32 ml/kg for 10 days) were used. Vascular contractility of small isolated mesenteric arteries of the animals was examined using wire myography. The extract significantly suppressed the vascular contraction to KCl in dose-dependent mode (4×10^{-3} mg/ml of phenolic compounds (PC) – up to 82%; 4×10^{-2} mg/ml of PC – 37%, $p < 0.01$; and 0.4 mg/ml of PC – 4%, $p < 0.001$). Ten (10) days of administration of the extract for the animals in drinking water resulted in slight decrease of contractility to KCl (95%) and phenylephrine (88%). Both the endothelium-dependent relaxation to acetylcholine and endothelium-independent relaxation to sodium nitroprusside (SNP) were slightly improved by shifting the dose–response curve leftward with the significantly improved maximum relaxation (86.39 ± 3.61 to 60.84 ± 12.45%, $p < 0.05$ with 1 mM acetylcholine and 82.86 ± 8 to 55.86 ± 10.85%; $p < 0.05$, with 1 mM SNP). These results suggested that GE improved and revealed the vasorelaxant effects mainly attributed to smooth muscle involving mechanisms without impairment of the endothelium-depended relaxation.

Key words: *Ginkgo biloba*, myography, contractility, endothelium, vascular smooth muscles.

INTRODUCTION

The endothelium maintains the balance between vasodilation and vasoconstriction; vascular tone is a result of crosstalk between smooth muscles and endothelium (Higashi et al., 2006). The vascular endothelium and the impairment of endothelium- dependent relaxation may lead to increased contractility of blood vessels and development of hypertension (Angus et al., 2000; Arzumanian et al., 2003). Herbal preparations may play an important role in preventing the damage of mitochondria and vascular endothelial cells (Addabbo et al., 2009).

It is known since old times that the active ingredients of *Ginkgo biloba* L. effectively improve the blood circulation in the brain and that is why *G. biloba* preparations are widely used for prevention of dementia, macula degeneration, tinnitus and winter depression (Gertz and Kiefer, 2004; Janssens et al., 1999; Masteikova et al., 2008). These pharmacological effects are predetermined by the active ingredients of the plant: flavonglycosides, terpenoids etc. Recently, the effect of different active ingredients of *G. biloba* and the biological effect of its tinctures and extracts has been intensively studied not

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Abbreviations: GE, *Ginkgo biloba* leaf extract; **NO,** nitric oxide; **NOS,** nitric oxide synthase; **SNP,** sodium nitroprusside.

only on the brain, but also on the cardiovascular and other systems. DeFeudis et al. (2003) and Jang et al. (2012) found that flavonglycosides have the strongest free radical-scavenging effect, and affect on hemo-dynamic disorders, vasomotor functions of ischemia zones, permeability of capillaries, ion regulation of cellular homeostasis, and some intracellular enzymes - phospholipases, phosphodiesterases and protein kinases. Oyama et al. (1996) found that proantho-cyanidins in *Ginkgo* leaves participate in releasing vasorelaxant substances - especially nitric oxide (NO) - from the endothelium. Terpenoids and flavonoids are also reported to possess vasorelaxant properties, improving cerebral blood flow (Kleijnen and Knipschild, 1992). Trumbeckaite with coworkers showed that *Ginkgo biloba* leaf extract (GE) (1:5) could possess uncoupling activity on heart mitochondria *in vitro* (Trumbeckaite et al., 2007; Janssens et al., 2000; Korshunov et al., 1997; Shen and Zhou, 1995).

These findings have led us to consider the possibility that GE might have protective effects in cardiovascular disease as well as antihypertensive effects. However, few reports have clarified the effect of EGB 761 on system components regulating blood pressure (Kubota et al., 2006a; Satoh et al., 2004; Starkov, 1997).

Therefore, the aim of this study was to investigate the effects of GE on vascular contractility *in vitro* as well as the effect of the use of the extract *per os* on the contractility, evaluating the endothelium-dependent and independent relaxation.

MATERIALS AND METHODS

Preparation of *Ginkgo biloba* extract (GE)

Dried *G. biloba* L. leaves (Poland, Acorus Calamus) were extracted using 70% ethanol (1:5); particle size in the extract range from 2 to 3 mm, the production method was percolation, and the flow speed of extract was 0.5 ml/min (Bernatoniene et al., 2002). GE contained 3.4 ± 0.08 mg/ml phenolic compounds (PC). Several classes of flavonoids: quercetin - 8.8 ± 2 µg/ml, hyperoside - 41.2 ± 4 µg/ml, rutin - 227.5 ± 8 µg/ml, and quercetrin - 93.1 ± 7 µg/ml were identified in this extract by high performance liquid chromatography (HPLC) analysis (Trumbeckaite et al., 2007).

Animals

Wistar rats were given GE or ethanol (solvent control) at a dosage of 0.32 ml/kg in drinking water for 10 days, control animals received untreated drinking water. The animals were handled according to the rules defined by the European Convention for the protection of Vertebrate Animals Used for Experimental and Other Purposes; the experiments were approved by Lithuanian State Food and Veterinary service (License No.0006).

Measurements of the force of vascular isometric contractility

We studied small mesenteric arteries (diameter: 350 to 450 µm) in rats. Measurements of the force of isometric contractility were performed using a small blood vessel myograph (*version 410, JP Trading, Denmark*); the recordings were registered using computer software *MyoSight 4.1*.

The animals were anesthetized using halothane, the abdominal cavity was opened, and a part of the jejunum with mesenterium was removed. Isolated organs were immediately placed into a modified Ringer's solution with the following composition (mM/l): NaCl – 139.3; KCl – 3.5; $CaCl_2$ – 2.3; $MgCl_2$ – 1.3; NaH_2PO_4 – 0.58; Na_2HPO_4 – 2.1; $NaHCO_3$ – until pH = 7.4; and glucose – 11.1. Under optical microscope (*Olympus, Japan*), arterial segments (ca. 1 mm in length) were separated from the surrounding tissues and fixed in the myograph bath to the force transducer and a micrometer by inserting into the lumen two steel wires 40 µm in diameter. Constant temperature of the solution (37°C), was maintained in the bath, and the solution was continuously aired with a mixture of oxygen and carbon dioxide (95% O_2 with 5% CO_2).

Normalization of the tension of the blood vessels and maintenance of their viability were performed as described previously (Laukeviciene et al., 2006).

Investigation of GE effects on vascular contractility *in vitro* and *in vivo*

To determine the effect of GE *in vitro,* GE was added into Ringer's solution in the bath until the final selected concentration was reached (1/1000, 1/100 or 1/10). With the addition of the extract, the blood vessels were incubated for 30 min, after what their contractility was tested using 80 mM KCl Ringer's solution. The investigation of contractility was performed at least three times, rinsing the samples with Ringer's solution between the tests.

To determine the effect of GE *in vivo,* we used small mesenteric arteries of rats that received *Ginkgo* extract supplement for 10 days (Group 1). Contractility was investigated using 80 mM KCl Ringer's solution and 30 µM phenylephrine. Relaxation was investigated using 1 µM to 10 mM acetylcholine and 1 µM to 10 mM sodium nitroprusside (SNP), adding them into the bath until the required concentration in a cumulative manner.

Statistics

Statistical analysis was performed using Prism 3.0 program (GraphPad Software Inc., La Jolla, CA, USA). The results are presented as means ± SE. of 5 independent experiments. The data were analysed with repeated measures analysis of variance (ANOVA) followed by Tukey's multiple comparison test. $p < 0.05$ was taken as the level of significance.

RESULTS

First, we performed solvent control test and found that rat treatment *per os* with ethanol for 10 days using the same concentration as in studied GE, 0.32 ml/kg, did not influence respiration rates of isolated heart mitochondrial oxidizing all substrates used in our study (Data not shown).

In the next series of studies, we investigated the effect of GE on vascular contraction *in vitro* (Figure 1). The contraction force of blood vessels to 80 mM of KCl decreased in dose dependent mode after 30 min incubation with *Ginkgo* extract supplement. Following the incubation with GE at $4x10^{-3}$ mg/ml of PC, the force decreased up to 82% (6.41 ± 1.39 to 5.25 ± 0.84 mN),

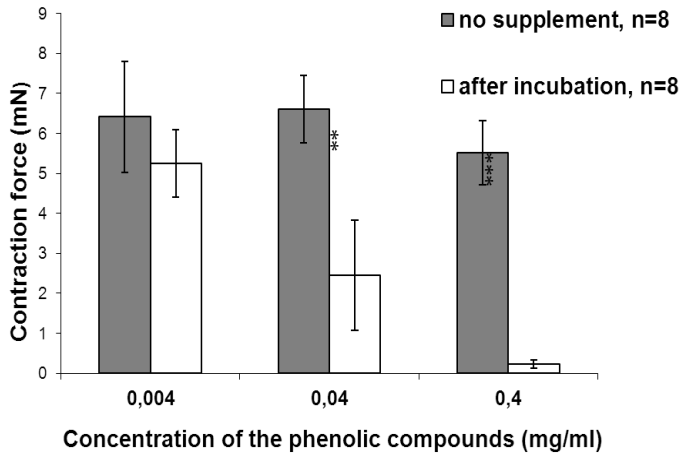

Figure 1. Reduction in the absolute contraction force caused by 80 mM KCl in small (350 to 450 μm in diameter) mesenteric arteries following incubation *in vitro* with *Ginkgo* extract supplement. ***, $p < 0.001$, **, $p < 0.01$.

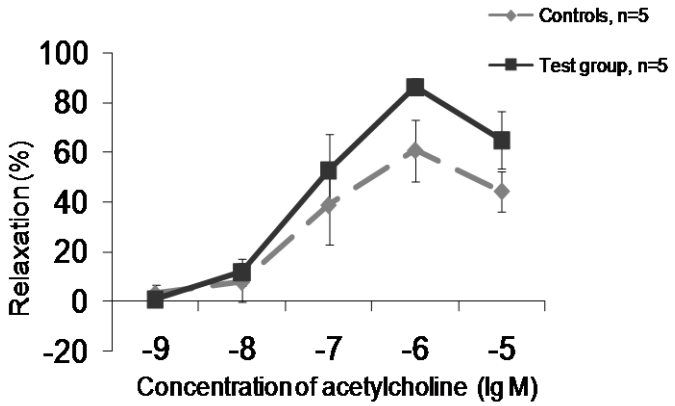

Figure 2. Endothelium-dependent relaxation of small mesenteric arteries in test group rats and controls under exposure to acetylcholine (Ach) (1 nM to 10 μM) on phenylephrine precontraction (30 μM). Phenylephrine-induced contraction was considered to be 100%. *, $p < 0.05$.

after incubation with GE at 4×10^{-2} mg/ml of PC – up to 37% (6.61 ± 0.81 to 2.45 ± 1.38 mN; $p < 0.01$), and after incubation with GE at 0.38 mg/ml – up to 4% (5.51 ± 0.79 to 0.22 ± 0.1 mN; $p < 0.001$).

To determine the effect of GE *in vivo*, we compared the contractility and relaxation properties of isolated blood vessels between controls and GE treated rats. Vascular contraction to 80 mM KCl in the test group was weaker than in control (7.22 ± 0.73 and 6.89 ± 0.54 VmN, respectively) as well as contraction to 30 μM phenylephrine (11.5 ± 1.02 and 10.16 ± 0.8 VmN, respectively). Vascular relaxation induced by NO in the test group was improved in both cases, that is, when treating the blood vessels with acetylcholine, causes the release of NO from the endothelium (endogenous NO)

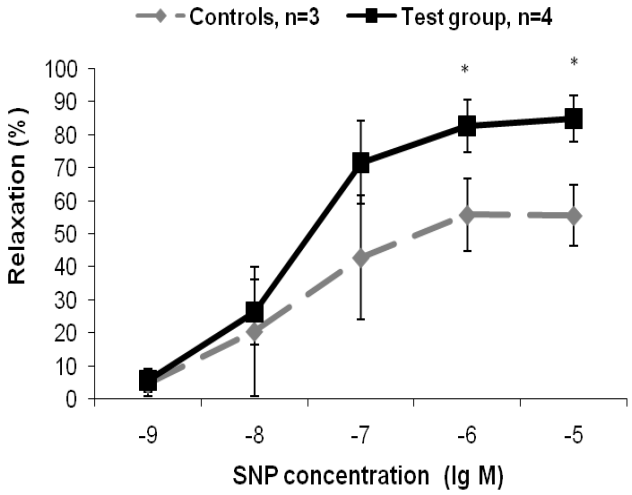

Figure 3. Exogenous NO-induced relaxation of small mesenteric arteries in test group rats and controls under exposure to SNP (1 nM to 10 μM) on phenylephrine precontraction (30 μM). Phenylephrine-induced contraction was considered to be 100%. *, $p < 0.05$.

and with SNP, releasing exogenous NO. When exposed to 1 mM acetylcholine, blood vessels of the test group relaxed statistically reliably better than those in the control rats (86.39 ± 3.61 and 60.84 ± 12.45 %, $p < 0.05$) (Figure 2). Exogenous NO also had a better vasorelaxing effect in rats that received GE (1 mM SNP, respectively, 82.86 ± 8 and 55.86 ± 10.85%; $p < 0.05$, while 10 mM SNP – respectively, 85.1 ± 7.02 and 55.62 ± 9.26%, $p < 0.05$) (Figure 3).

DISCUSSION

Our findings demonstrated that GE possesses activity to reduce the vascular contractility. The correlation between the degree of inhibition of vasoconstriction and the concentration of the extract used in the study proved biological activity of the preparation, that is, the preparation weakened contraction of smooth vascular muscles, thus, reducing vascular contractility *in vitro* in a concentration-dependent manner. These results corresponded with the findings obtained by other researchers who studied standard commercial ginkgo extract EGB 761 (Kotil et al., 2008; Kubota et al., 2006a; Nishida, 2003; Satoh, 2004), which indicates that the effect of our GE preparation is similar to that of the commercially available preparation.

Investigating the effect of GE feeding on blood vessels contractility, we applied two different contractors with different pathways of contraction – KCl and phenylephrine (Laukeviciene et al., 2006). Exposure of blood vessels to high KCl induces depolarization of the sarcolemma, resulting in the influx of Ca^{2+} ions through potential-regulated Ca^{2+} channels into the cell, causing

the reaction of these Ca^{2+} ions with the sarcoplasmatic ryanodine receptors, which, in turn, leads to further increase in intra-sarcoplasmic Ca^{2+} concentration and muscular contraction. Meanwhile, phenylephrine causes the increase in the intracellular Ca^{2+} concentration and muscle contraction by affecting membrane α-adrenergic receptors. Despite the different pathways, the muscle contraction was weaker to both contractors after the treatment. These results indicate that extract possibly reduce release of Ca^{2+} ions from intracellular storage places or affected further course of muscular contraction, that is, sensitivity of the contractile apparatus to calcium ions or energy supply (Corriu et al., 1996; Fleming and Busse, 1999). Studies with EGB 761 showed that muscle contraction decreased due to inhibition of Ca^{2+} influx through the Ca^{2+} channel and might be in part due to the inhibitions of Ca^{2+} -activated K^{+} current in rat aorta (Nishida, 2003) or vasodilation, mainly due to the inhibitions of Ca^{2+} influx through the Ca^{2+} channel (Satoh, 2004). GE use *per os* resulted in weaker contraction of blood vessels in experimental rats. The obtained results suggest that GE may have vasospasm-alleviating and antihypertensive properties. This assumption is corroborated by the findings indicating that administration of *Ginkgo* significantly decreased systolic blood pressure in spontaneously hypertensive rats (Kubota et al., 2006a) and was effective against vasospasm in a dose-dependent mode (Kotil et al., 2008). The majority of reports focused on the effect of *Ginkgo* on conductive blood vessels (aorta), while we studied small blood vessels whose tone significantly affects blood pressure. This suggests that the studied GE had antihypertensive properties by the analogy with the antihypertensive effect of *G. biloba* extract (Kubota et al., 2006b; Furchgott and Zawadzki, 1980).

The relaxant effect of the tincture was also confirmed by the findings of our study on vasorelaxation. Vasorelaxation as a response to exposure to sodium monoxide (NO) in the tested rats did not decrease, but actually increased in both cases – when exposed to acetylcholine that causes the release of NO from the endothelium (endogenous NO) and when exposed to SNP that, when breaking down, releases NO (exogenous NO). A part of other authors also documented that EGB 761 improved endothelium-dependent relaxation (Kubota et al., 2006a; Nishida, 2003), but there are contradicting findings, that the extract decreases internal calcium in human endothelial cells (Campos-Toimil et al., 2000), which would reduce the release of NO from the endothelium. According to some authors, EGB 761 causes the release of NO from the endothelium, but has no effect on relaxation caused by exogenous NO (Kubota et al., 2006b).

It is noteworthy that the cardiovascular effect of EGB 761 is dose-dependent. In rats that received EGB for prolonged periods, the indexes of peripheral blood circulation were worse than in control rats (Tada et al., 2008).

Conclusion

Ethanolic extract of *G. biloba* L. reduces smooth muscle contractility in small mesenteric blood vessels of rats, and improves both endothelium dependent and independent relaxation, that is, has a vasorelaxant effect.

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