

Red wine polyphenols correct vascular function injured by chronic carbon tetrachloride intoxication

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Abstract. The aim of the study was to evaluate the effect of red wine polyphenols extract ProvinolsTM on the development of cardiovascular injury in the model of carbon tetrachloride (CCl₄) intoxication. We followed the thoracic aorta vasoactivity and left ventricle nitric oxide (NO) synthase activity in male Wistar rats. In the preventive experiment lasting for 12 weeks the control group, the group receiving CCl₄ (0.5 ml/kg) two times a week subcutaneously, the group receiving ProvinolsTM (30 mg/kg/day) in drinking water and the group receiving CCl₄+ProvinolsTM was used. In the recovery experiment, the initial 12 weeks of CCl₄ treatment were followed by 3 weeks of spontaneous recovery or recovery with ProvinolsTM. CCl₄-intoxication resulted in the injury of vasoactivity which was demonstrated by the inhibition of acetylcholine-induced relaxation as well as noradrenaline-induced contraction. In the preventive as well as recovery experiment administration of polyphenols refreshed endothelium-dependent relaxant response and normalized inhibited contraction to adrenergic stimuli. ProvinolsTM treatment significantly increased NO-synthase activity in all groups. The results revealed beneficial effects of red wine polyphenols on vascular function injured by chronic CCl₄ intoxication. The correction of endothelial function seems to be attributed to the activation of NO pathway by polyphenols.

Key words: Red wine polyphenols — Carbon tetrachloride — Rat aorta — Vasoactivity — NO-synthase

Introduction

Consumption of diets rich in polyphenols, including those obtained in vegetables, fruit and red wine, has been shown to play a role in the maintenance of health and diseases prevention. Many epidemiological studies have suggested that polyphenolic compounds are able to reduce the risk of cardiovascular diseases and to provide protective effect on cardiovascular system (for review, see Zenebe et al. 2001). Moreover, polyphenols help to maintain equilibrium between endothelium-derived vasoconstrictor and vasodilator factors

in stress (Púzserová et al. 2006). The beneficial effects of these compounds might be explained by wide range of biological pathways. Major activities of plant polyphenols represent the antioxidant properties (Zenebe et al. 2001; Pourahmad et al. 2010). Moreover, polyphenols act on different targets involved in the metabolism of mammalian cells, including nitric oxide (NO) (Stocklet et al. 1999). Our previous experiments using the model of experimental hypertension demonstrated protective effects of red wine polyphenols on increased blood pressure, myocardial fibrosis, vascular wall remodeling and impaired arterial reactivity. Improved vascular function and structure in this model was associated with reduced oxidant status as well as with the increased NO production after activation of NO-synthase (Bernátová et al. 2002; Pecháňová et al. 2004).

Liver cirrhosis is associated with complex cardiovascular changes and patients with liver cirrhosis due to complex modifications of circulation and neurohormonal changes

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revealed the presence of several potentially important cardiovascular risk predictors for sudden cardiac death and hemodynamic failure (Milovanovic et al. 2009). Moreover, several experimental studies revealed that changes in NO production and NO level in intra-hepatic and splanchnic circulation seems to be associated with altered liver function and pathogenic processes such as cirrhosis (Wiest and Groszmann 2000). Carbon tetrachloride (CCl₄), a potent hepatotoxin, nephrotoxin and carcinogen, has been frequently applied for experimental hepatic cirrhosis development (Morales-Ruiz et al. 1996; Thrall et al. 2000; Ogeturk et al. 2005). A mutual relation between hepatic cirrhosis induced by CCl₄ and structural changes of blood vessels has been documented by Fernández-Varo et al. (2007) who showed that conductive vessels of cirrhotic rats undergo an intense process of vascular remodeling. CCl₄ intoxication is also associated with chronic modifications in hemodynamics and changes in vascular function (Castro et al. 1994; Mathie et al. 1996a,b; Atucha et al. 2000; Babál et al. 2006). The process of intoxication with this toxic substance includes free radical formation that results in lipoperoxidation and oxidative stress (Abraham et al. 1999). Several natural products having the antioxidant properties, including ginkgo biloba, propolis component and black tea extracts have been reported to reduce CCl₄-induced nephrotoxicity and hepatotoxicity (Bahcecioglu et al. 1999; Fadhel and Amran 2002; Ogeturk et al. 2005).

The aim of this work was to investigate the effect of red wine polyphenols extract ProvinolsTM on cardiovascular system affected by CCl₄ chronic intoxication. The study was focused on the reactivity of thoracic aorta and on the left ventricle NO-synthase activity in rats.

Materials and Methods

Experimental animals and treatments

Procedures used were performed in accordance with institutional guidelines and were approved by State veterinary and food administration of the Slovak Republic and by Ethical committee of Slovak Academy of Sciences. All rats were housed under a 12 h light/12 h darkness cycle, at a constant humidity and temperature, with free access to standard laboratory rat chow and drinking water. The Institute of Normal and Pathological Physiology provided veterinary care.

Male Wistar rats, 3 month old, were divided into 6 groups, 8 animals in each. The preventive experiment lasting for 12 weeks consisted of four groups: the control group receiving olive oil only, the group receiving CCl₄ 0.5 ml/kg of body weight twice a week subcutaneously in a 1 : 1 solution with olive oil, the group receiving dried red wine extract

ProvinolsTM (40 mg/kg/day) in drinking water and the group receiving ProvinolsTM+CCl₄. In the recovery experiment, the initial 12 weeks of CCl₄ treatment were followed by 3 weeks of spontaneous recovery in the first, and recovery with ProvinolsTM administration in the second group of animals. To make sure that each animal received the complete dose of ProvinolsTM, calculated amount of ProvinolsTM was given to each rat in the appropriate volume of water. Daily water consumption was estimated individually for every animal one week before the experiment. During the experiment, water consumption was controlled and ProvinolsTM concentration in the drinking fluid was adjusted, if necessary. The red wine extract dry powder ProvinolsTM was kindly provided by Mr. D. Ageron (Société Française de Distillerie, Vallont Pont d'Arc, France). The following ProvinolsTM polyphenols content (in mg/g of dry powder) has already been reported: proanthocyanidins 480, total anthocyanins 61, free anthocyanins 19, catechin 38, hydroxycinnamic acid 18, flavonols 14 (Diebolt et al. 2001).

In vitro study

For functional study *in vitro* the thoracic aorta was isolated, cleaned of connective tissue and cut into rings (3–4 mm in length). The rings were vertically fixed between two stainless steel wires – triangles in 20 ml incubation organ bath with Krebs solutions of the following millimolar composition: NaCl 118; KCl 5; NaHCO₃ 25; MgSO₄ 1.2; KH₂PO₄ 1.2; CaCl₂ 2.5; glucose 11; ascorbic acid 1.1; CaNa₂EDTA 0.032. The solution was oxygenated with 95% oxygen and 5% carbon dioxide and kept at 37°C. Electromechanical transducer Sanborn FT 10 and potentiometric recorder (Labora) were used for recording of changes in isometric tension. The resting tension was adjusted to 10 mN and applied to each ring. The relaxant responses were followed on the rings pre-contracted with sub maximal dose of phenylephrine (10⁻⁵ mol/l) to produce a stable plateau of contraction. The rings were then exposed to cumulative doses of acetylcholine (1 × 10⁻⁸–3 × 10⁻⁵ mol/l). The extent of relaxation of arterial rings was expressed as a percentage of the phenylephrine-induced contraction. Contractile responses were induced by increasing concentrations of noradrenaline (1 × 10⁻⁹–3 × 10⁻⁵ mol/l) in a cumulative manner and expressed as the developed tension per cross-sectional area of tissue (mN/mm²).

NO synthase activity

NO synthase activity was determined in crude homogenates of the left ventricles by measuring the formation of L-[³H]citrulline (L-Cit) from L-[³H]arginine (Amersham) as previously described by Púzserová et al. (2007). NO synthase activity was expressed as picokatal (pkat) per gram of protein.

Statistical analysis

The data were expressed as means \pm S.E.M. For the statistical evaluation of differences between groups, one-way analysis of variance (ANOVA) was used and followed by Bonferroni *post-hoc* test. The differences of means were considered to be significant at $p < 0.05$.

Results

In vitro vascular reactivity

In control Wistar rats the application of acetylcholine (10^{-8} – 10^{-5} mol/l) induced concentration-dependent relaxation of thoracic aorta. The chronic treatment with CCl_4 significantly inhibited acetylcholine-induced relaxation (Fig. 1). The chronic treatment with ProvinolsTM did not affect the relaxant response of thoracic aorta to acetylcholine. On the other hand ProvinolsTM treatment prevented the reduction of endothelium-dependent relaxation to

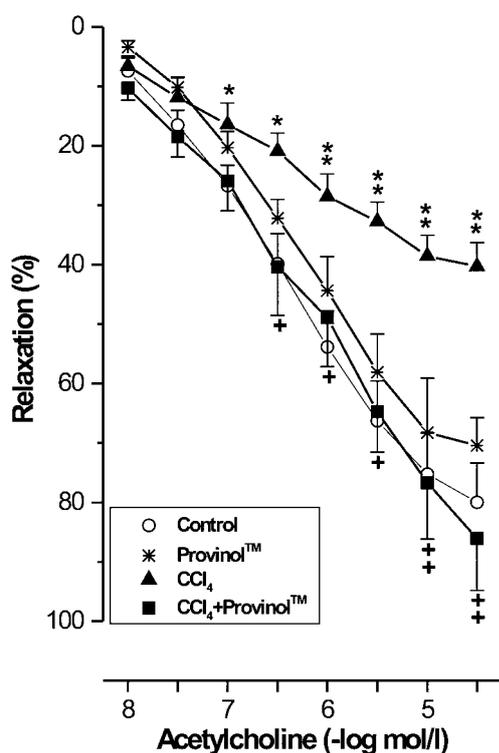


Figure 1. Effect of chronic treatment with red wine polyphenols (ProvinolTM), carbon tetrachloride (CCl_4) and simultaneous administration of carbon tetrachloride and provinols (CCl_4 + ProvinolTM) on acetylcholine-induced relaxation of the rat thoracic aorta in Wistar rats. Values are means \pm SEM. * $p < 0.05$, ** $p < 0.01$ compared with Control group; + $p < 0.05$, ++ $p < 0.01$ compared with CCl_4 group.

acetylcholine induced by CCl_4 treatment and the maximal relaxation was comparable to control (Fig. 1). The response to acetylcholine obtained in the spontaneous recovery group was not significantly different from that of the CCl_4 -treated group but it was significantly reduced compared with the relaxation of control group (Fig. 2). In contrast, the response to acetylcholine was significantly improved in rings from rats treated with ProvinolsTM during recovery period and maximal relaxation was not significantly different from that in control group (Fig. 2).

Noradrenaline induced concentration-dependent contraction in all tested groups. In the CCl_4 group, the contractile response was significantly attenuated compared with that in control rats (Fig. 3). ProvinolsTM treatment did not modify the concentration-response curve to noradrenaline, but it affects the decreased reactivity of the aorta to noradrenaline induced by CCl_4 treatment. There was observed

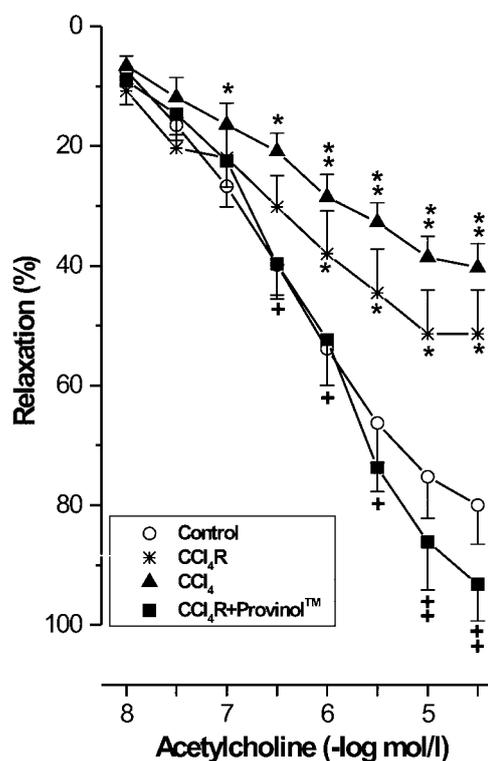


Figure 2. Effect of chronic treatment with red wine polyphenols on relaxant response of the rat thoracic aorta in Wistar rats during recovery period. Concentration-response curves for acetylcholine-induced relaxation of rings taken from control, carbon tetrachloride-treated (CCl_4), carbon tetrachloride-treated rats followed by 3-weeks of spontaneous recovery (CCl_4 R), and carbon tetrachloride-treated rats followed by 3-weeks treatment with red wine provinols (CCl_4 R + ProvinolTM) are shown. Values are means \pm SEM. * $p < 0.05$, ** $p < 0.01$ compared with Control group; + $p < 0.05$, ++ $p < 0.01$ compared with CCl_4 group.

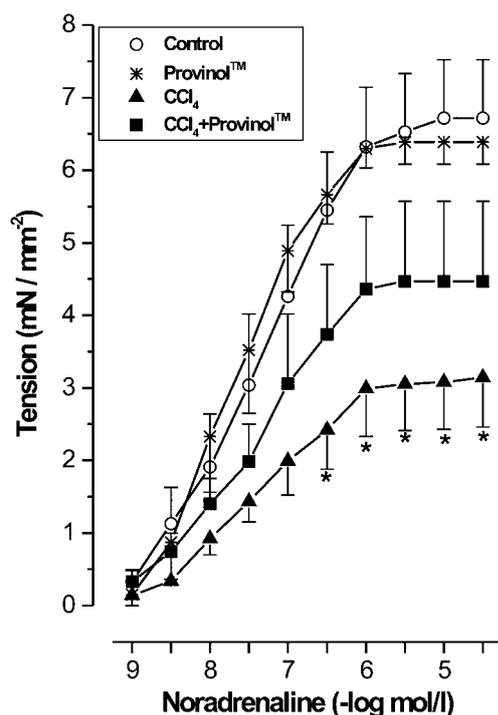


Figure 3. Effect of chronic treatment with red wine polyphenols (ProvinolTM), carbon tetrachloride (CCl₄) and simultaneous administration of carbon tetrachloride and provinols (CCl₄ + ProvinolTM) on noradrenaline-induced contraction of the rat thoracic aorta in Wistar rats. Values are means \pm SEM. * $p < 0.05$ with Control group.

no significant difference between contractile responses in control group compared to group contemporary treated with CCl₄ and ProvinolsTM (Fig. 3). Three weeks of spontaneous recovery did not modify the decreased response to noradrenaline induced by CCl₄ treatment (Fig. 4). However, ProvinolsTM treatment during recovery period restored the decreased reactivity of the thoracic aorta to noradrenaline induced by CCl₄ treatment (Fig. 4).

NO synthase activity

The results of NO-synthase activity are summarized in Fig. 5. In left ventricles taken from control group, NO-synthase activity was 5.21 ± 0.22 pkat/g protein. This parameter was not significantly affected by the treatment with CCl₄, activity of NO-synthase represents 4.88 ± 0.25 pkat/g protein. However, the treatment with ProvinolsTM significantly increased activity of NO-synthase in all groups. After ProvinolsTM administration the activity of NO-synthase in left ventricle was significantly increased by 33% and by 42% compared to control group ($p < 0.01$) and CCl₄ group ($p < 0.01$), respectively. Similarly, the treatment with ProvinolsTM during recovery

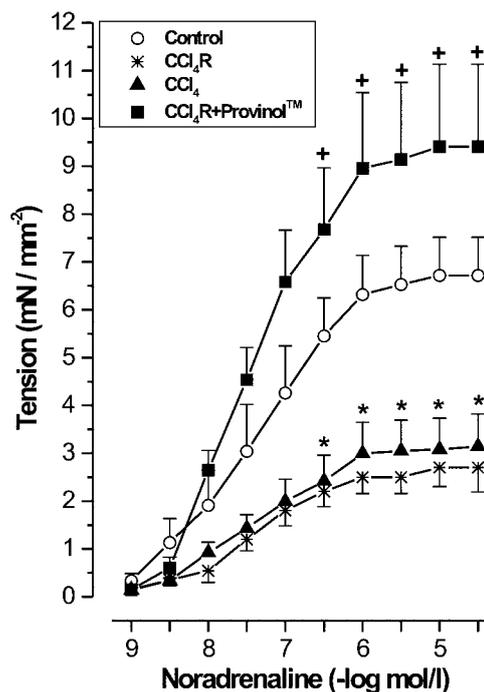


Figure 4. Effect of chronic treatment with red wine polyphenols on contractile response of the rat thoracic aorta in Wistar rats during recovery period. Concentration-response curves for noradrenaline-induced contraction of rings taken from control, carbon tetrachloride-treated (CCl₄), carbon tetrachloride-treated rats followed by 3-weeks of spontaneous recovery (CCl₄R), and carbon tetrachloride-treated rats followed by 3-weeks treatment with red wine provinols (CCl₄R + ProvinolTM) are shown. Values are means \pm SEM. * $p < 0.05$ compared with Control group; † $p < 0.05$ compared with CCl₄ group.

period significantly increased NO-synthase activity by 35% and by 44% compared to control ($p < 0.01$) and CCl₄ group ($p < 0.01$), respectively. Simultaneous treatment with CCl₄ and ProvinolsTM led to the increase in NO-synthase activity by 34% only with comparison to CCl₄ group ($p < 0.01$).

Discussion

Intoxication by CCl₄ which represents a suitable model of system toxicity leading to oxidative damage of different organs is also associated with cardiovascular changes. In this study the chronic treatment with CCl₄ induced the significant impairment of endothelial function which was documented by diminished vasorelaxation to acetylcholine in isolated thoracic aorta. This is in a good consensus with previous study which revealed a significant injury of vascular endothelium caused by CCl₄. The results documented the significant increase of circulating free endothelial cells

(endothelium) and histology of blood vessels showed serious damage to the endothelial cells (Babál et al. 2006) due to oxidative stress (International Programme on Chemical Safety, 1999). Diminished vasoconstriction shown in this study is in an agreement with other studies which referred hyporesponsiveness to different endogenous vasoconstrictors, such as angiotensin II, methoxamine, and noradrenaline in CCl_4 -induced model of cirrhosis have been claimed in different vascular areas (Castro et al. 1994; Clária et al. 1994; Mathie et al. 1996a,b). Although hyperactivity of vasodilating substances, especially associated with NO overproduction, belongs to the most often hypothesized explanations (Morales-Ruiz et al. 1996; Atucha et al. 2000) none adequate mechanism underlying this phenomenon has been clearly demonstrated. Our experiments revealed that the activity of NO-synthase in the left ventricle was not affected by the treatment with CCl_4 and relaxant response to acetylcholine, an activator of NO-synthase was inhibited. Similarly, Mathie et al. (1996a,b) revealed that endothelial NO was unchanged or, if anything, diminished in experimental cirrhosis induced by CCl_4 intoxication and did not participate in the decreased response to contractile agonists in mesenteric arterial bed. We suppose that in CCl_4 -induced model of cirrhosis the release of endothelial NO is inhibited due to endothelium injury so the increase of NO level is not involved in the decreased response of thoracic aorta to noradrenaline and very probably did not play the role in the arterial hyporesponsiveness. Hlavačková et al. (2009) reported that administration of CCl_4 led to changes in content of collagen types

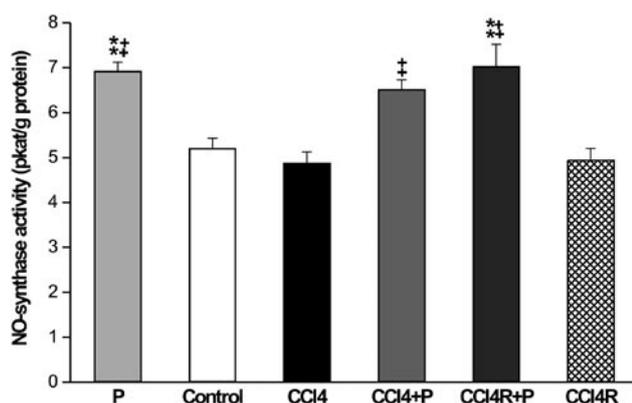


Figure 5. Nitric oxide (NO) synthase activity measured by L-[3H] citrulline formation in the left ventricle in the control group (Control), red wine polyphenols-treated rats (P), carbon tetrachloride-treated rats (CCl_4), in rats treated with carbon tetrachloride and red wine polyphenols simultaneously (CCl_4 + P), in rats after 3 week spontaneous recovery period (CCl_4 R), and in rats treated with polyphenols during recovery period (CCl_4 R + P). ** $p < 0.01$ compared with Control group; + $p < 0.01$ compared with CCl_4 group.

in the aorta and Fitch et al. (2005) showed that increased wall collagen content resulted in the increase in the aortic stiffness which was accompanied by decreased contractile efficiency to different vasoconstrictor agents. We suppose that the decreased contractility of thoracic aorta may be the consequence of arterial wall structural remodeling (such as imbalance in components of extracellular matrix and arterial wall stiffness) due to CCl_4 intoxication.

Polyphenolic compounds exert beneficial effect on the cardiovascular system which is mediated mainly by an increase of NO bioavailability and oxygen-free radical scavenging properties in cardiovascular system (Stocklet et al. 1999; Lodovici et al. 2001; Zenebe et al. 2001). Moreover, endothelium protective properties of red wine polyphenols were manifested when decreased endothelium and histopathological changes in the endothelial lining produced by CCl_4 were prevented and reduced in according to the treatment with polyphenols extract (Babál et al. 2006). Red wine polyphenols increase NO production, that exerts an anti-apoptotic effect, and it could be responsible for the decrease of endothelium (Dimmeler and Zeiher 1999). Our functional experiment on the thoracic aorta revealed that the red wine polyphenols were able to restore the relaxation to acetylcholine which was blunted after CCl_4 treatment. This indicates that polyphenols really activate endothelial NO-synthase since acetylcholine-induced relaxation of rat thoracic aorta has earlier been demonstrated to be mediated by NO and acetylcholine has been shown as an activator of NO production in conduit artery (Török and Kristek 2001). In addition, we showed that refreshed endothelium-dependent vasorelaxation was accompanied by the increased activity of NO-synthase in the left ventricle after the treatment with ProvinolsTM. Our results are in a good consensus with observations in the model of NO-deficient hypertension where the potentiation of the inhibited thoracic aorta vasorelaxation has been attributed to the activation of NO pathway by polyphenols (Bernátová et al. 2002; Pechánová et al. 2004). Moreover, the increased production of NO due to stimulation of NO-synthase activity in arterial wall (also in aorta) due to the action of polyphenolic compounds has been confirmed by observations of our as well as other laboratories (Stocklet et al. 1999; Zenebe et al. 2001; Benito et al. 2002). Finally, as it is mentioned above, the treatment with CCl_4 was associated with destruction of endothelial integrity and the capacity of functional endothelium was decreased so we can suggest that the release of NO was decreased. Moreover, the injury of endothelial cell lining was reported to be associated with an increased production of oxygen-free radicals (Azevedo et al. 2000; Souza et al. 2000) which react with NO and decrease its bioavailability. Polyphenols as scavengers of oxygen free-radicals could play a crucial role in prevention of further NO degradation. The results suggest that the endothelium protective properties and the

increased activation of NO-synthase activity and/or NO bioavailability in the cardiovascular system may be involved in the beneficial effect of the red wine polyphenols in the model of CCl₄ intoxication.

The administration of red wine polyphenols in this study also improved the thoracic aorta contractility blunted by CCl₄ intoxication. Polyphenols are compounds which readily penetrate into layer of the vessel wall and are easily adsorbed onto the extracellular protein fibers, which organize the vessel layers and so lead to reduced deterioration of the vasculature and the prevention of oxidative damages to cellular membrane (Han et al. 2004). Authors also showed that red wine polyphenols were able to influence the collagen-types content ratio in the wall of the aorta and so positively affect the imbalance in components of extracellular matrix. Moreover, Han et al. (2005) documented that green tea polyphenols prevented appreciable inferiority in such mechanical properties as failure strength, elastic modulus and compliance. Similarly, Mizutani et al. (1999) showed that extract of wine polyphenolics improved aortic fragility and elasticity in stroke-prone spontaneously hypertensive rats. Although the augmentation of blunted contraction after CCl₄ intoxication could also be associated with increased production of vasoconstrictor products such as thromboxane A₂ induced by polyphenols (Diebolt et al. 2001), the improved vessel contractility after red wine polyphenols administration issued very probably from positive remodeling process in the vascular wall.

In conclusion, subcutaneous application of CCl₄ induced injury of vasoactivity in rat thoracic aorta. Red wine polyphenolic compounds revealed protective effects on vascular function in rats intoxicated with CCl₄ – the improvement of relaxant as well as contractile properties of arterial wall. The correction of endothelial function after CCl₄ intoxication seems to be attributed to the activation of NO pathway by polyphenols.

Acknowledgment. We thank L. Kosnacova for excellent technical assistance. The presented work was supported by Slovak VEGA 2/0178/09, VEGA 2/0111/11, VEGA 1/0524/08, APVV-0538-07 grant agencies and with the support by the Framework Programme for Research and Technology Development, project: Creation of Centre of Excellency for Sudden Cerebral Vascular Events, Comenius University Faculty of Medicine in Bratislava (ITMS: 26240120015), cofinanced by European Regional Development Fund.

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Received: October 1, 2010

Final version accepted: December 30, 2010