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Red Wine Polyphenols Prevent Endothelial Damage Induced by CCl_4 Administration

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Summary

It became evident in the present study that carbon tetrachloride (CCl_4), in addition to its known liver and renal toxicity, causes serious damage to endothelial cells. The preventive effect of red wine on cardiovascular diseases has been documented in a number of human population studies as well as in animal experimental models. In this study, the endothelium protective effect of polyphenolic compounds isolated from red wine was studied in rats administered 0.5 ml of CCl_4/kg body weight intraperitoneally twice a week for 8 weeks. Endothelemia (endothelial cells/10 µl of plasma) was used as the marker of endothelial cell injury *in vivo*. Chronic CCl_4 treatment for 8 weeks lead to a 3-fold increase of free endothelial cells circulating in the blood when compared to the baseline values (2.5±0.3). Parallel oral administration of polyphenols 40 mg/kg/day significantly decreased the endothelemia. Polyphenolic compounds alone did not produce significant changes. Three weeks of spontaneous recovery after the 8-week treatment with CCl_4 did not lead to a marked decrease of endothelemia, but the administration of red wine polyphenols during the 3-week period significantly decreased free endothelial cells in the blood. It can be concluded that long-term administration of CCl_4 may serve as a useful experimental model of endothelial damage. The red wine polyphenolic compounds exert a powerful protective effect on endothelial cells from the injury caused by CCl_4. This effect was documented by decreased endothelemia that corresponded to diminished endothelial cell swelling and detachment evaluated by histology of the vascular intima. The endothelium protective effect may be one of the key factors that contribute to the preventive action of red wine on cardiovascular diseases.

Key words

Red wine • Polyphenols • Endothelium damage • Endothelemia • CCl_4

Introduction

Despite intense research, cardiovascular diseases belong to processes with not yet completely understood etiology and pathogenesis. Endothelial cell distress stays in the center of most of the pathological changes in the cardiovascular system. An increase in the number of free endothelial cells circulating in blood is a phenomenon

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accompanying vascular endothelium injury and enhanced endothelemia may occur in various pathological conditions (Hladovec 1989). High counts of circulating endothelial cells were documented in ischemic or valvular heart diseases, in claudicatio intermittens (Hladovec 1978, Šimko *et al.* 1998), in hypercholesterolemic patients (Castano *et al.* 1999) and some other diseases (Sochorová *et al.* 2000).

Oxidative stress is one of the principal mechanisms leading to endothelial cell damage. Metabolism of CCl_4 is accompanied by massive production of free radicals (Michalopoulos and DeFrances 1997). Thus, chronic administration of this compound produces oxidative stress followed by damage in many tissues, especially in the liver (Ryoo and Buschmann 1983, Turkdogan *et al*. 2001). Functional changes of blood vessels were also documented after CCl_4 administration (Niederberger *et al.* 1996, Petrowsky *et al.* 1999, Titos *et al.* 2000). Damage of endothelial cells is supposed to be responsible for some of the changes in both systemic and pulmonary circulation (Hollinger 1982, Maignan and Gulati 1986, Raymond *et al.* 2003).

Various experimental models showed that augmentation of the antioxidant system or application of compounds decreasing oxidative stress reduced liver injury in CCl_4-treated rats (Deulofeu *et al*. 2000, Peres *et al.* 2000). Positive effects of antioxidants on liver damage have also been proved *in vitro* in liver cell culture systems (Svegliati-Baroni *et al.* 1999) as well as in clinical studies (Feher *et al.* 1998, Watson *et al.* 1999). Similarly, endothelium-protective activity of several scavengers of oxygen-derived free radicals has been demonstrated (Hladovec 1986).

Beneficial effects of dietary flavonoids on the cardiovascular system have been largely reviewed (Mojžišová and Kuchta 2001, Zenebe *et al.* 2001). In the present study, endothelemia and vascular endothelium histology were evaluated for the detection of endothelial cell damage after long-term administration of CCl_4. Application of red wine extract (Provinols™) proved the positive influence of polyphenols on cardiovascular system through the protective effect on the vascular endothelium.

Methods

Animals

All procedures and experimental protocols were approved by the Ethical Committee of the Institute of Normal and Pathological Physiology SAS, and conform to the European Convention on Animal Protection and Guidelines on Research Animal Use.

Male Wistar rats, 3 months old, were divided into six groups, 8 animals in each. The preventive experiment lasting for 8 weeks consisted of four groups: the control group, the group receiving CCl_4 0.5 ml/kg of body weight twice a week intraperitoneally in a 1:1 solution with olive oil, the group receiving Provinols™ (40 mg/kg/day) in drinking water and the group receiving Provinols™ + CCl_4. In the recovery experiment, the initial 8 weeks of CCl_4 treatment were followed by three weeks of spontaneous recovery in the first group and recovery with Provinols™ administration in the second group of animals. To make sure that each animal received the complete dose of Provinols™, calculated amount of Provinols™ 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 Provinols™ concentration in the drinking fluid was adjusted, if necessary. All animals were housed at a temperature of 22-24 °C and fed with a regular pellet diet *ad libitum*.

The red wine extract dry powder Provinols™ was kindly provided by Mr. D. Ageron (Société Francaise de Distillerie, Vallont Pont d´Arc, France). The content of polyphenols in Provinols™ has already been reported (Diebolt *et al.* 2001) and it was (in mg/g of dry powder): proanthocyanidins 480, total anthocyanins 61, free anthocyanins 19, catechin 38, hydroxycinnamic acid 18, flavonols 14.

Endothelemia evaluation

At the end of the experiment, animals were sacrificed by exsanguination from the heart under ether narcosis. Heparinized blood was collected from the right ventricle and concentration of endothelial cells was calculated according to the modified method of Hladovec (1978). Briefly, free endothelial cells were isolated from heparinized platelet-rich plasma. Platelets were removed by centrifugation after ADP addition and the cells were counted in Bürker's chamber and expressed in terms of cell counts in 10 μl of plasma.

The endothelial cell phenotype of isolated cells was confirmed by immunocytochemical staining of smear preparations with anti-von Willebrand factor (FVIII-RAg) antibodies (DAKO, Glostrup, Denmark) following

the procedure described elsewhere (Babál *et al.* 2000). Briefly, the cell suspension was spread on poly-L-lysine coated slides, dried in air, fixed for 10 min in 70 % ethanol. After 5 min rehydration in PBS (phosphate buffered saline, pH 7.4) with 0.5 % H_2O_2 for endogenous peroxidases extinction and following 5 min wash in PBS, the slides were incubated for 20 min at room temperature with primary anti-von Willebrand factor (FVIII-RAg) rabbit antibodies (DAKO) diluted 1:200 in PBS, washed in PBS for 10 min, incubated 15 min with anti-rabbit IgG peroxidase complex (EnVision® kit, DAKO), washed in PBS for 10 min and color reaction was developed with diaminobenzidine (DAKO).

Histology

The thoracic aorta, carotid, pulmonary and renal arteries were fixed for 24 h in 10 % formalin, routinely processed in paraffin and 5 μm thick slices were cut perpendicularly to the vessel axis and stained with hematoxylin and eosin. The slides were evaluated in a Leica light microscope.

Statistics

The results were expressed as mean ± SD and statistically analyzed by one-way ANOVA with the Keuls-Neumann test.

Results

The animals tolerated well the experimental procedure. Isolated endothelial cells were identified as large flat cells (Fig. 1), providing cytoplasmic positivity in the isolated cells after immunocytochemical staining with anti-von Willebrand factor (FVIII-RAg) antibodies.

Baseline endothelemia in the controls was 2.47±0.28 cells/10 μl of plasma (from blood collected from the right ventricle). Administration of CCl_4 caused almost threefold increase of the endothelial cell concentration (7.32±1.12) (Fig. 2). Treatment with polyphenols significantly decreased the CCl_4-induced endothelemia to 4.87±0.66, although polyphenols alone did not cause significant changes (3.10±0.53).

Spontaneous recovery period of 3 weeks did not lead to a significant change in endothelemia (6.88±0.98) induced by previous 8-week CCl_4 treatment. Administration of polyphenols from red wine significantly lowered the presence of free endothelial cells circulating in the blood stream by one third (4.61±0.36).

Histology of blood vessels showed cytoplasmic vacuolization and blebbing of the endothelial cells, sometimes with identifiable subendothelial edema formation and detachment of the cells. The changes were the most pronounced in the CCl_4 group, the three-week spontaneous recovery did not lead to a remarkable improvement of the morphological changes. On the other hand, the application of polyphenols in the preventive as well as in the recovery experiment resulted in apparent preservation of endothelial cell morphology (Fig. 3).

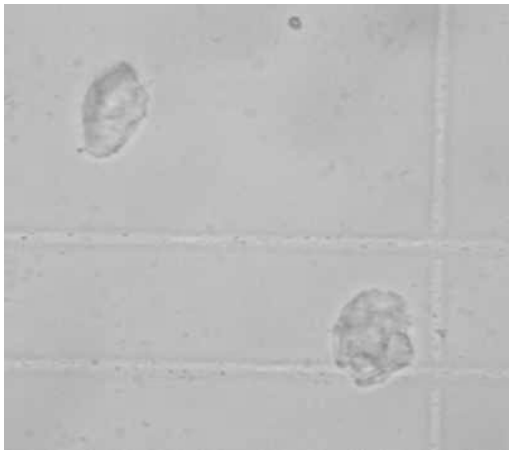

Fig. 1. Endothelial cells isolated from blood of animal administered CCl_4. Phase contrast, 400 x.

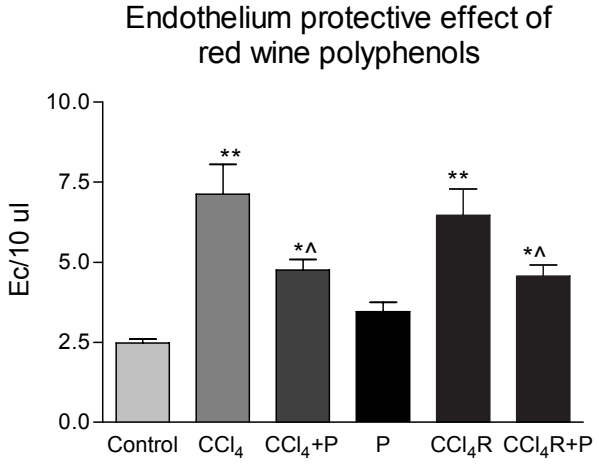

Fig. 2. Changes of endothelemia in animals treated with CCl_4 and the effect of red wine polyphenols administration. Control; CCl_4 – animals administered carbon tetrachloride for 8 weeks; P – polyphenols; CCl_4P – carbon tetrachloride and polyphenols; CCl_4R – carbon tetrachloride treated animals after 3 weeks of spontaneous recovery; CCl_4RP – carbon tetrachloride treated animals after 3 weeks of recovery with red wine polyphenols administration. * $p<0.05$, ** $p<0.001$ versus control; ^ $p<0.05$ versus CCl_4 or CCl_4R, respectively.

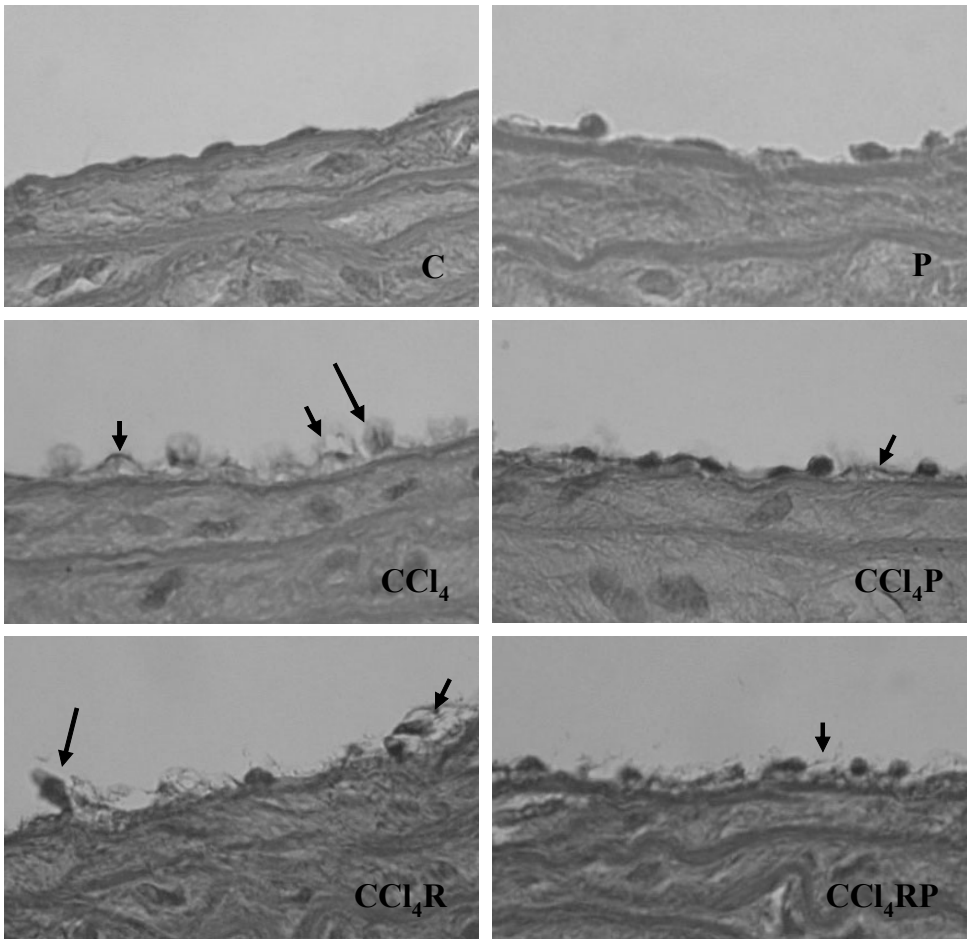

Fig. 3. Histological changes of vascular endothelium in animals treated with CCl_4 and the effect of red wine polyphenols administration. Polyphenols (P) did not produce any changes when compared to control (C); animals administered carbon tetrachloride for 14 weeks (CCl_4) developed cytoplasmic vacuolization of endothelial cells, subendothelial edema (arrowhead) and detachment of endothelial cells (arrow) that did not change after 3 week recovery (CCl_4R); administration of polyphenols with carbon tetrachloride (CCl_4P) or during the recovery period (CCl_4RP) lead to significant improvement of vascular endothelium morphology. Hematoxylin and eosin, 250 x.

Discussion

The peripheral blood endothelial cell count has previously been established as a sensitive and specific marker of vascular diseases (Dignat-George and Sampol 2000). The principal finding in the present report was the significant increase of circulating free endothelial cells after long-term administration of CCl_4. Raised endothelemia had been reported in different diseases, especially in ischemic heart or limb muscle vascular disease (Hladovec 1989, Šimko *et al.* 1998). Increased endothelemia also accompanies allogenic transplantation and may represent a signal of immunity-mediated endothelial damage and become a predictor of chronic allograft nephropathy and long-term allograft rejection (Woywodt *et al.* 2003). With the exception of cyclosporine (Woywodt *et al.* 2003), increased endothelemia as a result of drug toxicity to endothelial cells has not been reported. The mechanism of toxicity of cyclosporine and other calcineurin-inhibitors has not been established (Raymond *et al.* 2003). Our results provide new data documenting CCl_4 toxicity to the vascular endothelium followed by increased number of circulating free endothelial cells. It has been shown that CCl_4 is not only hepatotoxic but exerts systemic toxicity (Weber *et al.* 2003). Administration of CCl_4 may represent a useful model of oxidative damage to the endothelium with increased endothelemia.

The CCl_4 administration also caused morphological changes documenting distress of the vascular endothelium. Histological endothelial changes similar to those caused by CCl_4 were reported after prolonged ischemia as well as after ischemia and reperfusion that represents another form of damage produced by increased oxidative stress (Chetham *et al.* 1999). A remarkable but anticipated finding was the fact that maximum morphological damage observed in the CCl_4 groups matched the maximum in endothelemia. Cyclosporine administration, despite the increased endothelemia, did not produce any specific vascular morphological changes (Woywodt *et al.* 2003). It is likely that the occurrence of increased numbers of circulating free endothelial cells after cyclosporine treatment will involve mechanisms different from those resulting from

CCl_4 administration.

Increased endothelemia after chronic CCl_4 administration could be anticipated. Surprisingly, the number of circulating free endothelial cells did not decrease during the spontaneous three-week recovery period. This implies, that in parallel to the direct toxic effect of CCl_4 on endothelial cells, a permanent damage develops that does not recede during the three weeks of recovery. In contrast to the high endothelemia and morphological signs of cellular damage, no defects in endothelial lining were observed indicating unaffected replication capability. One may speculate that the persisting endothelemia could be the result of permanent damage to mitochondrial DNA (Grishko *et al.* 2001), the repair of which takes longer than three weeks or it will remain permanent. This phenomenon will require more detailed analysis to elucidate the principles of endothelial cell damage evoked by CCl_4.

There are several lines of evidence that polyphenolic compounds contained in fruits and vegetables have beneficial effect on cardiovascular system function (Zenebe *et al.* 2001). This effect is mediated mainly by the oxygen free radical scavenging properties and stimulation of nitric oxide production in vascular endothelium by the polyphenols. The polyphenols isolated from red wine were shown to improve vascular functions and morphology in the rat experimental model of NO-deficient hypertension (Bernátová *et al.* 2002, Pecháňová *et al.* 2004). Our present results document highly effective endothelium protective properties of red wine polyphenols that are manifested by a significant decrease of endothelemia produced by CCl_4 administration. It is likely that the endothelium protection is responsible, at least in part, for the beneficial effects of polyphenols on the cardiovascular system. In experimental hypertension, the administration of polyphenols decreased the histologically manifesting arterial remodeling (Babál *et al.* 1997, Pecháňová *et al.* 2004). In this work, in congruence with decreased endothelemia, histopathological changes in the endothelial lining produced by CCl_4 were reduced or even prevented, which implies an effective endothelium protection by the red wine polyphenols.

Special attention should be paid to the effect of red wine polyphenols on the reduction of persisting endothelemia after the three-week recovery period. If there is damage to the mitochondrial DNA that results in permanent production of oxygen radicals, the polyphenols play a crucial role in prevention of further endothelial cell damage. The stimulation of endothelial NO synthase by the polyphenols is another possibility to be considered for the protective effect (Pecháňová *et al.* 2004). Increased NO production, which has an anti-apoptotic effect, could be responsible for the decrease of endothelemia (Dimmeler and Zeiher 1999).

In summary, we have shown that administration of CCl_4 to the experimental animals is associated with serious damage to the vascular endothelium and an increase in circulating free endothelial cells. These circulating endothelial cells reflect the vascular endothelium damage by CCl_4 and represent a marker of oxidative stress injury to the endothelial cells. The red wine polyphenols were highly effective in preventing endothelium damage in this study and provided data supporting the beneficial effect on cardiovascular system through protection of endothelial cells.

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