Acta Veterinaria (Beograd), Vol. 55, No. 2-3, 131-145, 2005.

UDK 619:612.015.6

THE EFFECT OF TOCOPHEROL ON SERUM IRON CONTENT IN EXPERIMENTAL ATHEROSCLEROSIS

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(Received 15. September 2004)

This paper deals with the effect of tocopherol on serum iron content in experimental atherosclerosis (ATS). Having in mind the importance of iron as a potent catalyst in some oxidative reactions, we examined the iron content in serum of Chinchilla rabbits with ATS induced by a hypercholesterolemic diet. Serum iron content was quantified by atomic absorption spectrophotometry. For this study six groups of rabbits were used: C – control group fed the usual diet for this species (n=10), O – control group fed an oil-containing diet (n=10), Ch – experimental group fed a hypercholesterolemic diet (n=10), T – experimental group received tocopherol intramuscularly (n=10), ChT – experimental group treated with cholesterol and tocopherol (n=11), and OT – experimental group which received oil and tocopherol (n=11). After two-months of treatment decrease of iron content was registrated in serum of T and OT group (p<0.05; p<0.01 respectively) compared to both control groups. In comparison with Ch group serum iron content was highly significantly (p<0.01) decreased in OT group and significantly (p<0.05) decreased in T group. Our findings indicate that tocopherol has an influence on serum iron content in rabbits suffering from ATS induced by a hypercholesterolemic diet.

Key words: experimental atherosclerosis, hypercholesterolemic diet, iron, rabbits, tocopherol

INTRODUCTION

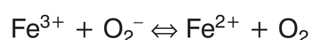
There is still insufficient knowledge of the pathogenesis of ATS. Many data attribute a pathogenic role to oxidative stress in ATS (Davies *et al*., 1982; Bridges *et al*., 1993; Dröge, 2002). Oxidative stress can be defined as an increased exposure to oxidants and/or a reduced defensive ability of the antioxidants (Bast *et al*, 1991; Rushmore *et al*, 1991; Mashima *et al*., 2001; Dröge, 2002; Fenster *et al*., 2003; Otterbein *et al*., 2003). The generation of reactive oxygen species (ROS) is an intrinsic characteristic of any living cell. ROS include oxygen free radicals and molecules that are strongly oxidizing, even more than molecular oxygen itself. These are the superoxide anion radical ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2) and the

hydroxyl radicals ($OH^{\cdot}$). The vast network of intracellular and extracellular antioxidant defenses point out that the level of ROS must be regulated for the survival of the cell. Thus, when ROS build up within a tissue and overwhelm the local antioxidant defense mechanisms, proteins, lipids, DNA and other components critical to normal tissue functions become oxidized, leading to loss of integrity and function and eventually to cell death (Buhl *et al*., 1994; Eichner *et al*., 1998; Moskovitz *et al*., 2002; Otterbein *et al*., 2003).

Iron appears to be an important factor which favors the development of ATS. Various metabolic processes are activated by iron. Iron itself is a prosthetic group of many enzymes and a constituent of the electron transport system. In aerobic systems many low-molecular weight iron chelates and free iron in particular, are very effective in generating ROS (Halliwell and Gutteridge, 1986; Stohs and Bagchi, 1995; Welch *et al*., 2002; Ghio *et al*., 2003). Iron is a catalyst of oxidative injury since $O_2^{-\cdot}$ and H_2O_2 produce $OH^{\cdot}$ in the presence of this metal:

$$Fe^{2+} + H_2O_2 \Leftrightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

This is the so-called Fenton reaction (Fenton, 1876; Fenton 1894; Wardman and Candeias, 1996; Henle and Linn, 1997), and Fe^{3+} in turn can be reduced to Fe^{2+} by $O_2^{-\cdot}$:



$$Fe^{3+} + O_2^{-} \Leftrightarrow Fe^{2+} + O_2$$

The sum of these reactions is known as the Haber-Weiss reaction (Haber and Weiss, 1934, Henle *et al*., 1999).

Free iron plays an essential role in oxidative processes, so its delocalization from iron-binding proteins such as ferritin and transferrin is regarded as a key step in the onset of oxidative tissue damage (Corhay *et al*., 1992; Sigel and Sigel, 1999; Barbouti *et al*., 2001). Body iron status has been implicated in atherosclerotic cardiovascular disease. The main hypothesis was that high iron status was associated with increased oxidation of low density lipoproteins (LDL) (Iribarren *et al*., 1998; De Valk and Marx, 1999; Meyers, 2000; Niederau, 2000; Williams *et al*., 2002). In ATS patients oxidants and smoke are able to unbind iron from ferritin and thereby increase its potential for oxidative cell damage. Prior studies also indicate that the following iron loading macrophage population may release iron bound to ferritin and/or transferrin (Barnes, 1990). The presence of iron in a catalytic state in concentrations which exceed the available transferrin binding sites, or is released from ferritin, has been postulated to be a condition for $OH^{\cdot}$ tissue injury (Thompson *et al*., 1991; Chau, 2000; Howes *et al*., 2000). Hydroxyl radicals, the most potent of all the free radicals, exist only in a fraction of a microsecond, but they are capable of destroying vital enzymes and cause lipid peroxidation. The $O_2^{\cdot -}$ generated by arterial smooth muscle cells seems to be important in mediating both LDL modification and the facilitated uptake of the modified LDL by macrophages (Heinecke *et al*., 1986; Henle *et al*., 1999; Van Jaarsveld and Pool, 2002). There is indirect evidence for increased lipid peroxidation in aortic occlusive and aneurismal disease as demonstrated by an increase in iron concentration in these tissues (Piotrowski *et al*., 1990; Eichner *et al*., 1998; Shah

and Alam, 2003). Not only the magnitude of oxidative stress, but the fatty acid composition of esterified lipids present in the LDL particle, as well as the serum concentrations of divalent cations including iron, vitamin E and other antioxidants present in the LDL particle or in the aqueous phase of plasma may potentially influence the ability of LDL particles to undergo oxidative modification (Illingworth, 1993; Sloop, 1999; Kritchevsky *et al*., 2000; Steinberg and Witztum, 2002).

Vitamin E acts as a membrane-bound antioxidant, protecting both the cytosol and the membranes against ROS. This lipid soluble vitamin blocks electron transfer involved in the initiation and propagation of lipid peroxidation (Raij, 1993; Kamal-Eldin and Appelqvist 1996; Olson *et al*., 2000). It has been shown that vitamin E deficiency results in enhanced tissue susceptibility towards ROS and in an increased lipid peroxidation *in vivo* (Wojcicki *et al*., 1991; Dhalla *et al*., 2000; Urso and Clarkson, 2003). It has also been demonstrated that dietary intake of vitamin E suppressed elevated plasma concentrations of lipid peroxides both in patients with hyperlipoproteinemia and rabbits fed a cholesterol rich diet (Szczeklik *et al*., 1985; Wen *et al*., 1999; Upston *et al*., 2001). Since iron-mediated oxidative injury may be relevant to the pathogenesis of ATS, we directed our experimental goal into measuring the iron content in the serum of Chinchilla rabbits with experimental atherosclerosis. At the same time we examined the influence of tocopherol on serum iron content, having in mind the well-known antiatherogenic role of this vitamin.

MATERIAL AND METHODS

Experiments were performed on Chinchilla rabbits of both sexes whose initial weight was about 1600-2000 g. The investigated animals (n=62) were divided into six groups:

1. C – control group (n=10) fed a standard diet for this species,
2. O – control group (n=10) fed on oil - containing diet. These animals received 6 ml of edible oil through a gastric tube five times a week for two months,
3. Ch – experimental group (n=10) fed on a hypercholesterolemic diet. These animals received a 4% solution of crystalline cholesterol (ICN Galenika) in 6 ml of edible oil through a gastric tube five times a week for two months,
4. T – experimental group (n=10) received 100 mg of tocopherol intramuscularly (i.m.) per week, divided into three equal doses, for two months,
5. ChT – experimental group (n=11) fed on a hypercholesterolemic diet (4% solution of crystalline cholesterol /ICN Galenika/ in 6 ml of edible oil, orally given five times a week for two months) treated with tocopherol (100 mg per week, i.m. given in three equal doses, for two months), and
6. OT – experimental group (n=11) received oil (6 ml of edible oil, orally given five times a week for two months,) and tocopherol (100 mg per week i.m. given in three equal doses, for two months).

After two months of treatment the respective groups of rabbits were sacrificed by air embolism (air injected intracardially). Tissue sections of the

thoracic aorta, obtained from each group of rabbits, were placed in a formalin solution to be subsequently molded and stained with haematoxylin eosin. Aorta tissue specimens were analysed histologically by light microscopy. The iron content in serum was determined by atomic absorption spectrophotometry (VARIAN AA-5).

Statistical evaluation of results was performed using Student's t-test. The values of the parameters for each individual animals were averaged and standard deviation (SD) was calculated. The significance of the differences between groups was calculated using two-tailed Student's t-test (appropriate type of Student's t-test for homogeneous samples with numerical variables). Data are expressed as mean ±SD, with $p<0.05$ being considered significant. Statistical analysis of data was carried out using a computer with the assistance of a statistical software package-SPSS 9.0 Professional Edition.

RESULTS

The mean iron content in rabbit's sera is presented in Table 1. The significance of the differences between groups is shown in Table 1, as well. As can be seen, a significant decrease in iron content was evaluated in serum of groups T and OT ($p<0.05$; $p<0.01$ respectively) compared to both control groups. In comparison with group Ch serum iron content was significantly ($p<0.01$) decreased in the OT group, and significantly ($p<0.05$) decreased in group T.

Table 1. Iron content in serum of the rabbits

Fe [μg/mL]	C n =10	O n =10	T n =10	Ch n =10	ChT n =11	OT n =11
$\bar{X}$ ± SD	5.01±0.99	5.33±0.91	3.30±2.04	5.34±1.47	3.65±1.84	3.62±0.60

*– $p<0.05$ **– $p<0.01$
C/T → $p<0.05$ C/OT → $p<0.01$
O/T → $p<0.05$ O/OT → $p<0.01$
Ch/T → $p<0.05$ Ch/OT → $p<0.01$

Figure 1-6 shows the thoracic aorta tissue of rabbits.

Thoracic aorta tissue of a control rabbit (C) is presented in Figure 1. The thoracic aorta tissue of the control group is without atherosclerotic changes.

Thoracic aorta tissue of a rabbit on oil – containing diet (O) is presented in Figure 2. An initial phase of atherosclerosis can be observed. Namely, the oil – containing diet in some manner leads to disturbance of iron metabolism and may augment the effects of this transition metal. It is well-known that iron in early atherosclerotic lesions is primarily localized to the lysosomes of foam cells (Meyers, 2000). Thus, considering the role of iron in enzyme catalyzed reactions, the immunogenic activity of edible oil may be involved with the findings presented in this study (Figure 2).

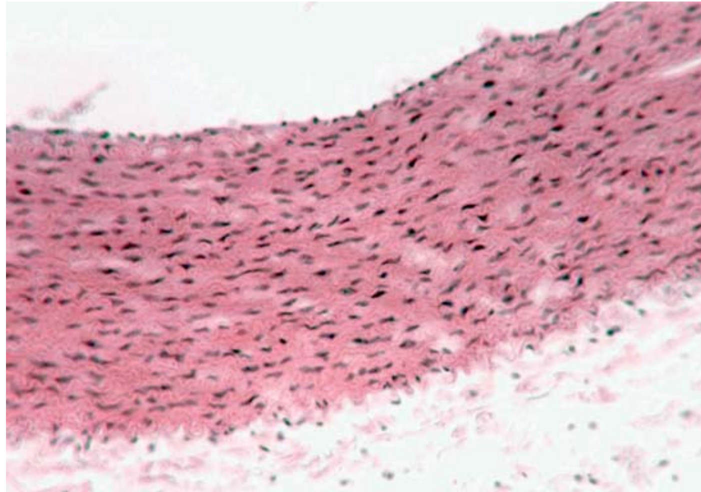


Figure 1. Thoracic aorta tissue of a control rabbit (C)

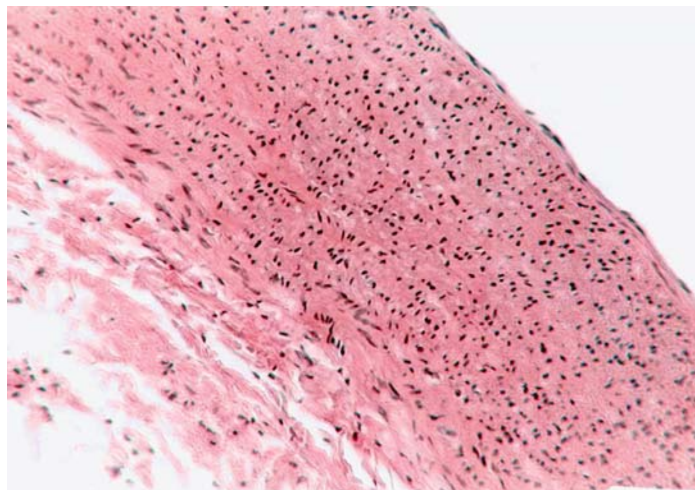


Figure 2. Thoracic aorta tissue of a rabbit fed the oil – containing diet (O). An initial phase of atherosclerosis can be observed

Thoracic aorta tissue of a rabbit fed the hypercholesterolemic diet (Ch) with evident atherosclerosis is presented in Figure 3. Striking thickening of the intima can be noticed. Derangement of internal elastic lamina, which is locally damaged, can be also observed. Lipid laden cells appeared between the endothel and subendothelial tissue. After two months of treatment the thoracic aorta tissue of

hypercholesterolemic rabbits accumulated large amounts of lipid. Cholesterol and cholesteryl ester atheromatously degenerated the wall of the thoracic aorta, this is not observed in any other investigated group of rabbits. As shown in Table 1, a significant decrease in iron content was evaluated in the serum of groups T and OT ($p<0.05$; $p<0.01$ respectively) compared to group Ch. The amount of iron deposition in the aorta has been directly associated with severity of the atherosclerosis (Meyers, 2000). In this model the cholesterol immunostimulation capacity is related to iron content and the pathohistological findings are also observed in the thoracic aorta tissue of group Ch, (Figure 3).

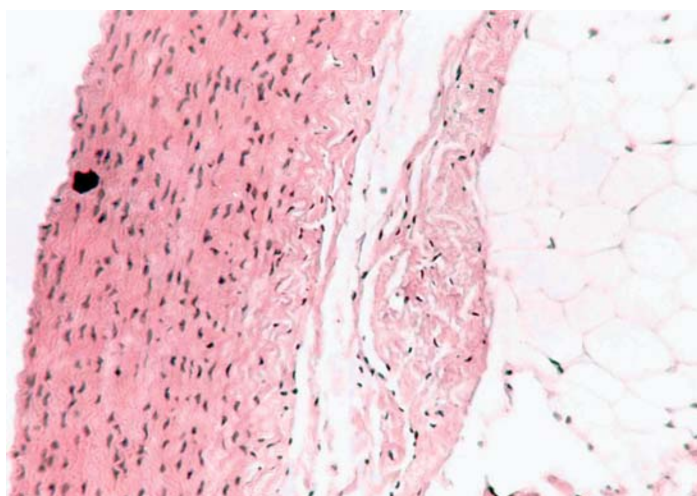


Figure 3. Thoracic aorta tissue of a rabbit fed the hypercholesterolemic diet (Ch) with evident atherosclerosis. Striking thickening of the intima can be noticed. Derangement of internal elastic lamina, that is locally damaged, can also be observed, too. Lipid-laden cells appeared between endothel and subendothelial tissue

Thoracic aorta tissue of a rabbit treated with tocopherol without significant pathomorphological changes (T) is presented in Figure 4. A slight thickening of the intima and minor derangement of the internal elastic lamina can be noticed. In comparison with group Ch serum iron content was significantly ($p<0.05$) decreased in group T (Table 1). This serum iron depletion could be associated with tocopherol antioxidant effects (Figure 4).

The thoracic aorta tissue of a rabbit treated with edible oil and tocopherol (OT) is presented in Figure 5. Pathological changes of the intima and the internal elastic lamina are similar to those observed in rabbits treated with tocopherol. Lipid infiltrations are present between the endothel and subendothelial tissue. We assume that there are no significant atherosclerotic changes in the thoracic aorta tissue in this group due to antioxidative mechanisms of tocopherol (Figure 5). In comparison with group Ch serum iron content was significantly ($p<0.01$)

decreased in group OT as a result of a possible protective function of tocopherol (Table 1).

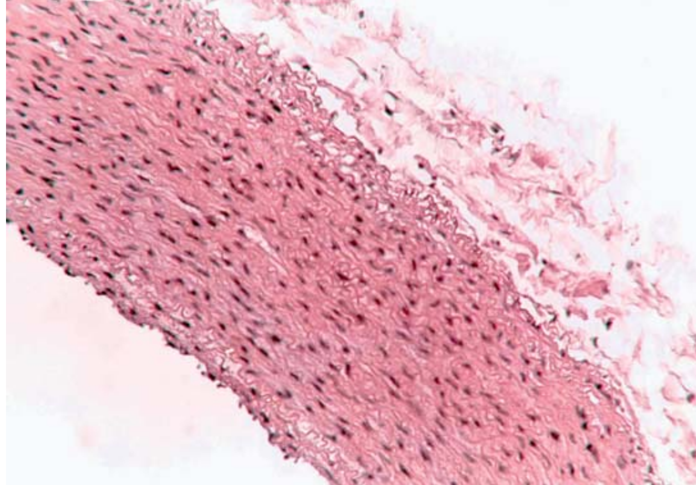


Figure 4. Thoracic aorta tissue of a rabbit treated with tocopherol (T). Small thickening of the intima and minor derangement of internal elastic lamina can be noticed

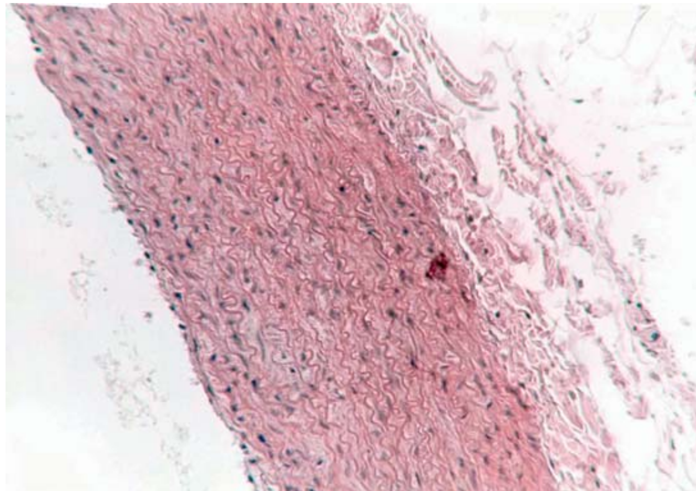


Figure 5. Thoracic aorta tissue of a rabbit treated with edible oil and tocopherol (OT). Pathological changes of the intima and internal elastic lamina are similar to those observed in rabbits treated with tocopherol. Lipid infiltrations are present between endothel and subendothelial tissue

The thoracic aorta tissue of a rabbit treated with cholesterol and tocopherol (ChT) is shown in Figure 6. Alterations of the intima and internal elastic lamina are similar to those observed in group Ch, but they are not so extensive. Accumulation of cells with lipid droplets between the endothel and subendothelial tissue is markedly smaller than in rabbits fed hypercholesterolemic diet alone. In this animal model in rabbits treated with cholesterol and tocopherol (ChT), tocopherol exerts considerable antiatherogenic activity (Figure 6).

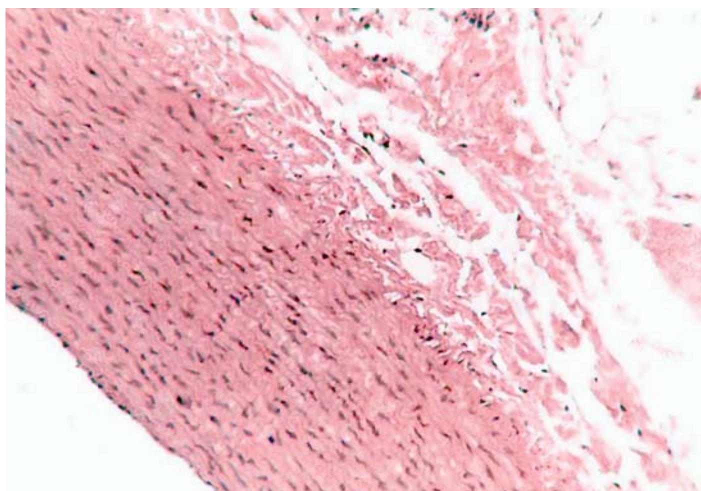


Figure 6. Thoracic aorta tissue of a rabbit treated with cholesterol and tocopherol (ChT). Alterations of the intima and internal elastic lamina are similar to those observed in group Ch, but they are not so extensive. Accumulation of cells with lipid droplets between endothel and subendothelial tissue is markedly smaller than in rabbits fed on hypercholesterolemic diet alone

Dietary supplementation with tocopherol (T, OT and ChT group) appears to have potential in the prevention of experimental atherosclerosis (Figure 4, 5 and 6).

DISCUSSION

In comparison with group C iron content in serum was not significantly increased in group O and Ch (Table 1). The association between atherogenesis and hypercholesterolemia has been documented in animals and humans (Ross, 1986; Ross and Agius, 1992; Sloop, 1999; Gaut and Heinecke 2001; Berliner, 2002). Because of the extreme sensitivity of rabbits to dietary cholesterol, this experimental protocol causes massive increases of serum cholesterol content in groups Ch and O. Since cholesterol is an extremely immunogenic molecule, massive hypercholesterolemia induced in rabbits by special diets may increase

the local lymphoproliferative response (Clarkson *et al*., 1974; Alving and Wassef, 1999; Berliner, 2002; Wick *et al*., 2004). Increased numbers of immune and inflammatory effector cells in areas adjacent to endothelial injury could alter the oxidant/antioxidant balance in the arterial wall (Heinecke *et al*., 1986; Piotrowski *et al*., 1990; Repine *et al*., 1997; Mashima *et al*., 2001; Dröge, 2002; Fenster *et al*., 2003). It seems that in this model the increased number and dysfunction of immune and inflammatory effector cells, as well as cholesterol immunostimulation capacity, are also related to iron content and the pathohistological findings observed in the thoracic aorta tissue in groups Ch and O (Figure 2 and 3). Additionally, the oil – containing diet in some way disturbes iron metabolism. Thus, considering the role of iron in enzyme catalyzed reactions, the immunogenic activity of edible oil may be involved with findings presented in this study.

Since the production of ROS depends on iron as a catalyst, increase of iron content in serum of groups Ch and O may be of importance in the development of pathomorphological changes in the thoracic aorta tissue of these animals (Figure 2 and 3). Increased availability of catalytically active metals has been associated with oxidative injury. H_2O_2 is a major ROS produced by arterial wall cells during atherogenesis, and it is converted under oxidative stress into a more potent ROS leading to LDL oxidation (Wilkins and Leae, 1994; Hazen *et al*., 1996; Meyers, 2000; Shah and Alam, 2003). The presence of even a small amount of peroxides in the lipoprotein can significantly contribute to its subsequent oxidation in the presence of transition metal ions (Lynch and Frei, 1993;Dabbagh *et al*., 1997; Chau, 2000; Howes *et al*., 2000). The release of iron from ferritin by the action of $O_2^{\cdot -}$, generated by membrane-bound NADPH oxidase from NADPH localized in the cell membrane, could contribute to oxidative damage by making iron available for the site-specific Haber-Weiss reaction (Piotrowski *et al*., 1990; Eichner *et al*., 1998; Iribarren *et al*., 1998; Welch *et al*., 2002; Williams *et al*., 2002). An association of $O_2^{\cdot -}$ with connective tissue injury is suggested by the fact that $O_2^{\cdot -}$ generated by xanthine oxidase inhibits collagen gelation (Greenwald and Moy, 1979) and depolymerizes purified hyaluronic acid (McCord, 1974). It is possible, therefore, that$O_2^{\cdot -}$ released by stimulated endothelial cells may injure the matrix of the microvascular, as well as the perivascular tissue (Matsubara and Ziff, 1986; Galis *et al*., 1994; Heinecke, 1998; Libby, 2002; Wick *et al*., 2004). Once endothelial injury is present, ROS may contribute to the acceleration of the atherosclerotic process by enhancing leukocyte chemotaxis, activation and platelet deposition (Piotrowski *et al*., 1990; Hanson *et al*., 2002; Heinecke, 2002; Wick *et al*., 2004). Furthermore, oxidation of polyunsaturated fatty acids, important components of atherosclerotic plaque, can result in the formation of lipid peroxides which may damage components of the arterial wall such as protein and mucopolysaccharides, or result in the generation of additional peroxides. Lipid peroxides and OH are also potent inhibitors of prostacyclin synthesis and therefore modulate neutrophil stimulation, $O_2^{\cdot -}$ production, and platelet inhibition. As a result, endothelial injury and subsequent thrombus formation may be facilitated (Piotrowski *et al*., 1990; Tousoulis *et al*., 2002; Bonetti *et al*., 2003).

Decrease of the iron content in serum of groups T and OT ($p<0.05$; $p<0.01$ respectively) compared to both control groups could be explained by an efficient antioxidant activity of tocopherol (Figure 4-6). In comparison with group Ch the serum iron content was significantly ($p<0.01$) decreased in group OT, and significantly ($p<0.05$) decreased in group T (Table 1). These findings may also be explained by the protective action of tocopherol (Figure 6 and 4). The antioxidant activity of the vitamin E compounds (tocopherols and tocotrienols grouped as chromanols) is mainly due to their ability to donate their phenolic hydrogens to lipid free radicals. The lipophilicity of the molecule (as determined by the number of methyl substituents in the chroman ring and the structure and stereochemistry of the phytyl tail) is an important feature for the biological activity of the tocopherols since it determines the kinetics of their transport and retention within the membranes. One tocopherol molecule can protect about 10^3-10^8 polyunsaturated fatty acid molecules at low peroxide levels. It has been shown that a small α-tocopherol/ polyunsaturated fatty acids ratio in biomembranes (e.g., 1:500 α-tocopherol/arachidonic acid molecules in the erythrocyte membrane) is enough to interrupt the free radical chain reactions. At low temperatures, favoring hydrogen-binding, tocopherol molecules can donate hydrogen atoms to lipid hydroperoxides or to the 8a-peroxy-α-tocopherones decomposing them to stable lipid alkoxides plus alkoxy radicals or to 8a-oxy-α -tocopherone radicals, respectively. The 8a-oxy-α-tocopherone radical is expected to be unstable and to abstract a hydrogen atom from any available α-tocopherol and to finally rearrange to α-tocopherolquinone, which was reported to be formed during ferric iron-catalyzed reactions between α-tocopherol and methyllinoleate hydroperoxides (Grunger and Tappel, 1970; Igarashi *et al*., 1976; Diplock, 1994).

Under certain conditions, where ROS levels are raised beyond the capacity of the protective mechanisms, or when these mechanisms are faulty, iron and tocopherol can act as synergists (Acworth and Bailey, 1995; Hallberg, 1995; Kamal-Eldin and Appelqvist, 1996; Olson *et al*., 2000). This finding is compatible with a nonsignificant increase of iron content in serum of groups ChT and OT compared to group T (Table 1, Figure 5 and 6). A study performed in recent year has shown that α-tocopherol in the presence of iron may act either as an antioxidant or as a prooxidant depending on experimental conditions (Lynch, 1997; Yamamoto and Niki, 1998; Wen *et al*., 1999; Olson *et al*., 2000; Djahansouzi *et al*., 2001; Upston *et al*., 2001; Padayatty *et al*., 2003).

Vitamin E may also play important roles in other biological processes, which do not necessarily involve its antioxidant function. These include structural roles in the maintenance of cell membrane integrity and anti-inflammatory effects, by direct and regulatory interaction with the prostaglandin synthetase complex of enzymes which participate in the metabolism of arachidonic acid (Olson *et al*., 2000; Neuzil *et al*., 2001).

Vitamin E is important in the regulation of intercellular signaling and cell proliferation through modulation of protein kinase C (Olson *et al*., 2000; Neuzil *et al*., 2001; Theriault *et al*., 2002; Yoshida *et al*., 2002).

Atherosclerosis is a disease involving both oxidative modifications and disbalance of the immune system. Vitamin E in the form of α-tocopferol is quantitatively the most important lipophilic redox-active, low-molecular-weight component in the human circulation and vascularization, and has thus received a lot of attention as a possible modulator of atherogenesis (Burton and Ingold, 1986; Neuzil *et al*., 2001).

Results of the present study indicate the effect of tocopherol on serum iron content in experimental atherosclerosis.The changes of serum iron content could be of importance in the pathogenesis of this disease.

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DEJSTVO TOKOFEROLA NA SADRŽAJ GVOŽĐA U EKSPERIMENTALNOJ ATEROSKLEROZI

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SADRŽAJ

Cilj ovog rada je bio da se ispita dejstvo tokoferola na sadržaj gvožđa u serumu u ekserimentalnoj aterosklerozi (ATS). S obzirom na ulogu gvožđa kao katalizatora u reakcijama nastanka slobodnih radikala važnih u patogenezi ATS, određivan je sadržaj ovog oligoelementa u serumu kunića sa ATS izazvanom hiperholesterolskom dijetom. Ispitivane životinje su podeljene u šest grupa: C – kontrolna grupa na dvomesečnoj ishrani uobičajenoj za ovu životinjsku vrstu

(n=10), O – kontrolna grupa na dvomesečnoj uljanoj dijeti (n=10), Ch – eksperimentalna grupa na dvomesečnoj hiperholesterolskoj dijeti (n=10), T – eksperimentalna grupa koja je u toku dva meseca dobijala tokoferol (n=10), ChT – eksperimentalna grupa koja je u toku dvomesečne hiperholesterolske dijete tretirana tokoferolom (n=11), i OT – eksperimentalna grupa koja je u toku dvomesečne uljane dijete tretirana tokoferolom (n=11). Sadržaj gvožđa u serumu je određivan metodom atomske apsorpcione spektrofotometrije. Sadržaj gvožđa u serumu T i OT grupe je statistički značajno snižen ($p<0,05$; $p<0,01$) u odnosu na obe kontrolne grupe. U poređenju sa Ch grupom, visoko statistički značajno smanjenje ($p<0,01$) sadržaja gvožđa nađeno je u serumu OT grupe, dok je smanjenje sadržaja gvožđa u serumu T grupe na nivou statističke značajnosti ($p<0,05$). Naši rezultati ukazuju da tokoferol utiče na sadržaj gvožđa u serumu kunića sa ATS izazvanom hiperholesterolskom dijetom.