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Involvement of superoxide radical in the impaired endothelium-dependent relaxation of cavernous smooth muscle in hypercholesterolemic rabbits

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Abstract Involvement of the superoxide radical in impaired relaxation of penile cavernous smooth muscle in hypercholesterolemia was investigated. New Zealand White rabbits ($n = 40$) were randomly divided into control and treatment groups. The control group ($n = 20$) received a regular diet while the treatment group ($n = 20$) was fed a diet of 2% cholesterol for 8 weeks. Blood level of cholesterol in the cholesterol-fed group was significantly higher than that of the control group. The contraction responses of cavernous tissues to norepinephrine were not significantly different in the two groups. The relaxation responses to endothelium-dependent agents (acetylcholine, bradykinin) were significantly reduced in the hypercholesterolemic group compared with the control group. However, the relaxation responses to endothelium-independent agents (papaverine, verapamil) were not significantly different in the two groups. The production of superoxide radicals was significantly higher in the hypercholesterolemic group than in the control group ($P < 0.01$). The activity of superoxide dismutase (total SOD, Mn-SOD, Cu,Zn-SOD) increased significantly in the hypercholesterolemic group compared with the control group ($P < 0.05$). The activities of catalase and glutathione peroxidase also increased in the hypercholesterolemic group, but were not significantly higher than those of the control group. Therefore, production of the superoxide radicals in rabbit cavernous tissues increases in the state of hypercholesterolemia, which may lead to functional impairment of cavernous smooth muscle relaxation in response to endothelium-mediated stimuli.

Key words Hypercholesterolemia · Superoxide radical · Superoxide dismutase · Catalase · Glutathione peroxidase · Cavernous smooth muscle

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Introduction

Hypercholesterolemia, a cardiovascular risk factor, has been reported to be closely related to vasculogenic impotence [23]. Previous research has focused primarily on investigation of hemodynamic alterations resulting from abnormal inflow. Recently, hypercholesterolemia has been reported to impair endothelium-mediated relaxation of blood vessels, in both experimental animals and humans. In 1992, Azadzoi and Goldstein [5] demonstrated the development of erectile dysfunction in 33% of rabbits fed a high cholesterol diet resulting in minimal occlusive lesions, and stated there might be factors other than luminal occlusion of large vessels that adversely influence erectile function. It has been suggested that the impairment of endothelium-dependent relaxation in hypercholesterolemia and atherosclerosis could be due to: (1) impairment of selective endothelium-dependent relaxation to acetylcholine [7], (2) endothelial cell damage by the atherosclerotic process [9], (3) impaired ability to synthesize or release endothelium-derived relaxing factor (EDRF) [28], (4) impaired diffusion and/or enhanced degradation of EDRF [26], (5) impairment of the ability of the smooth muscle cells of the atherosclerotic vessel to relax in response to the EDRF [21].

There have been many studies on the role of lipid peroxides in the development of atherosclerosis [8, 17].

Henriksson et al. [18] reported that a potent source of lipid peroxidization might be free radicals derived from oxygen. That is, if an increased release of free radicals exists in atherosclerotic vessel walls, this might lead to production of lipid peroxides. These free radicals would be harmful to the vessel wall. This might explain the impaired endothelium-mediated relaxation of blood vessels and the pathogenesis of erectile dysfunction in hypercholesterolemia with minimal occlusive lesions of vessels. However, there have been few studies on the relation of free radicals to hypercholesterolemia with erectile dysfunction.

The aim of this study was to find evidence of the possible generation of free radicals in hypercholesterolemic rabbits associated with impaired relaxation of penile cavernous smooth muscle. A possible method of studying this was by measuring the superoxide radical, and the activities of its scavengers superoxide dismutase (SOD), catalase, and glutathione peroxidase.

Materials and methods

Materials

Forty male New Zealand White rabbits weighing 1.7–2.0 kg were randomly assigned to control ($n = 20$) and treatment ($n = 20$) groups. Ten rabbits from each group were used to study the relaxation responses of cavernous smooth muscle in an organ bath. The other 10 rabbits from each group were used for measuring the activities of the superoxide radical and its scavengers. The control group received a standard diet and the treatment group was fed a 2% diet of cholesterol (Solvey Duphar, Netherlands) for 8 weeks. The cholesterol was dissolved in vegetable cooking oil and then mixed with the standard diet. All animals were weighed at 4 and 8 weeks. Blood samples for measurement of plasma cholesterol were obtained from an ear vein before initiation of the assigned diet and at 8 weeks. Subsequent to obtaining blood samples at 8 weeks, the rabbits were killed and the entire penis extracted. The corpus cavernosum was excised from the penis and dissected free from the tunica albuginea and surrounding connective tissue. The cavernous tissues taken from 10 rabbits in each group were stained with hematoxylin-eosin, elastin, and Masson's trichrome stain. This study was approved by the ethical committee for the protection of persons and animals in biochemical research of the Institute of Medical Science, Chung-Ang University, Seoul, Korea.

Methods

Relaxation responses of cavernous strips

The excised cavernous tissue was immediately placed in oxygen-saturated HEPES-buffered physiological salt solution (NaCl, 140 mM; KCl, 5 mM; $CaCl_2$, 2 mM; $MgCl_2$, 1 mM; HEPES, 5 mM; glucose, 11 mM, pH titrated with 1 N NaOH to 7.4) and studied within 1 h. Strips of cavernous smooth muscle were trimmed to a size of $0.2 \times 0.2 \times 1.0$ cm and mounted. To record isometric tension, the strips were attached by a silk tie to a fixed support at one end and to a wire connected to a force tranducer (52-9545, Harvard, USA) and polygraph (50-8630, Harvard, USA) at the other end. The tissue was placed in a 30-ml organ chamber and bathed in physiological salt solution of the following composition (mM): NaCl, 116; KCl, 5; $CaCl_2$, 2; $NaHCO_3$, 24; $MgSO_4$, 1; glucose, 11. This solution was bubbled with a mixture of 95% O_2 and 5% CO_2 and maintained at 37°C, pH 7.4. The resting tension was adjusted for each strip to the optimal isometric tension at which contraction by norepinephrine was maximal, and the developed tension was recorded.

Relaxation responses induced by the endothelium-independent vasodilators papaverine hydrochloride (Aldrich, USA) and verapamil (Sigma, USA), and those induced by the endothelium-dependent vasodilators acetylcholine (Sigma, USA), bradykinin (Sigma, USA) and adenosine (Sigma, USA) were then studied in the cavernous strips in which tone had been elicited with norepinephrine (10^{-6} M). Concentration-response curves were determined by adding successive logarithmic increments of the vasodilator agents from 10^{-9} to 10^{-4} M (except bradykinin: 10^{-10} to 10^{-5} M) to the chamber.

Contractile tension was expressed in grams. Relaxation was expressed as a percentile of the maximal relaxation (tension at maximal relaxation induced by the vasodilator/tension at maximal contraction induced by norepinephrine 10^{-6}M). The concentration-response curves in the hypercholesterolemic group were compared with that of the control group using Student's unpaired t-test.

Measurement of superoxide radical production, and activities of SOD, catalase, and glutathione peroxidase in the cavernous tissues

Homogenates of rabbit cavernous tissues were prepared mechanically (Janke and Kunkel, T25-S1, Germany) at a ratio of 1 : 5 (w/v) in 50 mM phosphate-buffered solution (pH 7.8) containing 0.1 mM EDTA [12]. The homogenates were centrifuged at 600 g for 10 min (Centrikon T124, Kontron, Italy). The supernatants were used for measurement of superoxide radical. For the measurement of the activities of Cu,Zn-SOD, Mn-SOD, catalase, and glutathione peroxidase, mitochondria in the supernatants were disrupted by ultrasound (Vibra Cell Sonic Materials, USA) for 15 min at 50 W [30]. All procedures were carried out at 0–4°C.

Assay of superoxide radical production. Superoxide production was measured by monitoring the SOD-inhibitable reduction of ferricytochrome c [6]. Briefly, a reaction solution containing 420 μl of 50 mM potassium phosphate buffer (pH 7.8), with 0.1 mM EDTA, and 50 μM cyanide was incubated at 37°C. The solution was mixed with 10 μl of homogenates and 50 μl of 0.1 mM cytochrome c, and the enzyme activity was measured by recording the absorbance change using a spectrophotometer at 550 nm for 2 min. The reaction solution was mixed with 3 μg of SOD, then inhibition of the reaction was assayed. An extinction coefficient ($E^M_{cm} = 195\,000$) was used for measurement of reduced cytochrome c.

SOD activity. SOD activity was assayed by the inhibition of cytochrome c reduction using a xanthine/xanthine oxidase system as described by McCord and Fridovich [25] and Crapo et al. [10]. The reaction mixture consisted of 2.3 ml of 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 0.3 ml of 0.1 mM ferricytochrome c, 0.3 ml of 0.5 mM xanthine, and 0.1 ml of 0.05 mM potassium cyanide. Enzyme activity was measured by the change in absorbance using a spectrophotometer at 550 nm (Caryze UV-Vis spectrometer, Varian, Australia). One unit of SOD was defined as the amount of enzyme that causes 50% inhibition of cytochrome c reduction. Specific activity was defined as the units of activity per milligram of protein.

Assay of catalase activity. Catalase activity was measured as described by Aebi [1]. For this assay, the reaction mixture contained 2.0 ml of 0.05 M potassium phosphate buffer (pH 7.0), 1.0 ml of 0.01 M H_2O_2, and 20 μl of sample. Enzyme activity was measured at 240 nm for 2 min. One unit of enzyme activity was defined as the

amount of enzyme that degrades 1 micromole of H_2O_2 per minute under the given conditions.

Assay of glutathione peroxidase activity. Glutathione peroxidase activity was measured as described by Flohe and Gunzler [14]. The reaction mixture consisted of 0.5 ml of 0.1 M potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 0.1 ml of glutathione peroxidase (0.24 units), 0.1 ml of 0.5 mM glutathione (GSH) and 0.1 ml of sample. Enzyme activity was measured at 340 nm for 5 min. One unit of enzyme activity was defined as the amount of enzyme that oxidizes 1 micromole of NADPH per minute under the given conditions.

Statistical analysis

Data were expressed as mean ± standard error of the mean with n representing the number of specimens. Results were considered statistically significant when $P < 0.05$.

Results

Blood level of cholesterol

At the start of the experiment the body weights of the rabbits were similar in the cholesterol-fed group (1.78 ± 0.05 kg, $n = 10$) and the control group (1.89 ± 0.05 kg, $n = 10$). After 4 weeks of cholesterol diet the body weight of cholesterol-fed group (2.35 ± 0.07 kg, $n = 10$) was slightly higher than that of the control group (2.08 ± 0.04 kg, $n = 10$). However, after 8 weeks of the cholesterol diet, the cholesterol-fed group showed no significant difference in body weight compared with the control group.

After 8 weeks of the experimental diet, blood levels of cholesterol were significantly higher ($P < 0.01$) in the treatment group (2620 ± 187.07 mg/dl, $n = 9$) than in the control group (113.7 ± 32.09 mg/dl, $n = 10$) (Table 1). On histological examination of the cavernous tissues, minimally occlusive lesions of the arterioles due to intimal thickening were found in all the cholesterol-fed rabbits. The ratio of the diameter of the arteriolar lumen to the thickness of the arteriolar wall was 1 : 1 in the control group but up to 1 : 4 in the hypercholesterolemic group.

Relaxation responses of cavernous strips

Smooth muscle contractions induced by norepinephrine were similar in the control and treatment groups. The more potent cavernous smooth muscle relaxants were papaverine, verapamil, and acetylcholine when tissues from both groups were compared at same concentration of each drug. Bradykinin and adenosine were found to be less potent than these three agents.

Acetylcholine (10^{-5}, 10^{-4} M) induced or bradykinin (10^{-10} to 10^{-5} M) induced relaxation of the cavernous

Table 1 Plasma cholesterol levels after 8 weeks of experimental diet for cholesterol-fed and control rabbits

	Plasma cholesterol level (mg/dl)	
	Control group	Cholesterol-fed group
Baseline	44.8 ± 2.00 ($n = 10$)	44.8 ± 2.00 ($n = 10$)
8 weeks	113.7 ± 32.09 ($n = 10$)*	2620 ± 187.07 ($n = 9$)**

*$P < 0.05$, **$P < 0.01$

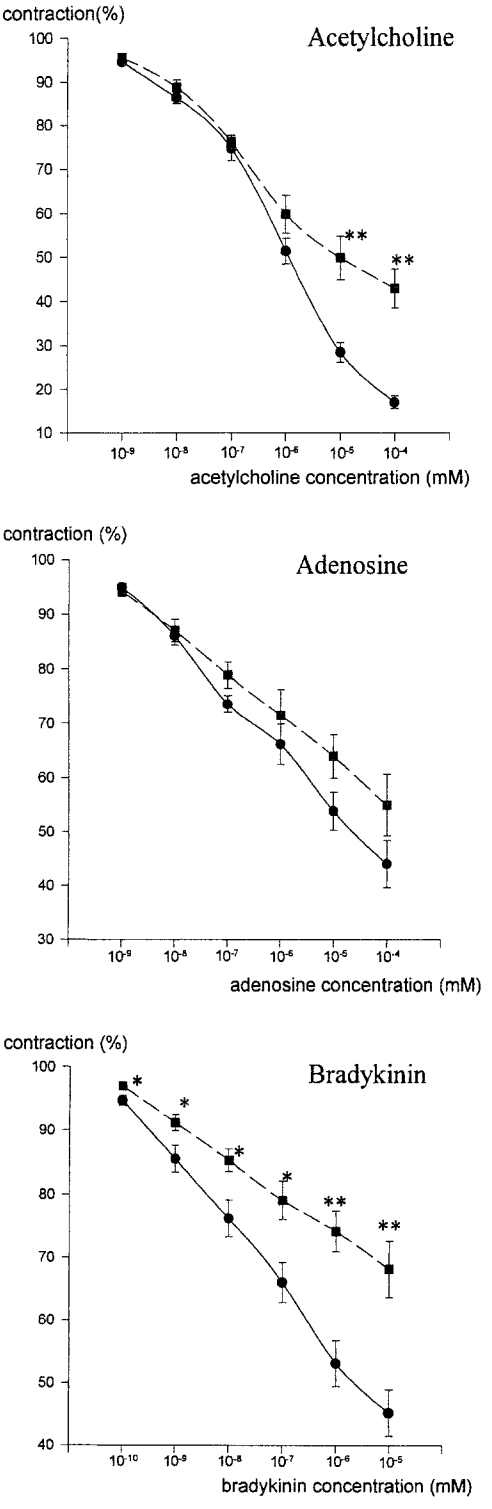

Fig. 1 Relaxation response of strips of corpus cavernosum from control (*circles*) and hypercholesterolemic (*squares*) rabbits, induced by endothelial-dependent vasodilators (*$P < 0.05$, **$P < 0.01$)

strips was significantly less pronounced in tissue from the treatment group than in tissue from the control group ($P < 0.05, P < 0.01$) (Fig. 1). Adenosine-induced relaxation of the cavernous strips, which was of a lesser magnitude than the acetylcholine-induced relaxation, was also less pronounced in tissue from the treatment group than in that from the control group, but this difference was not significant ($P > 0.05$) (Fig. 1). There was no significant difference in the relaxation response of the cavernous strips to papaverine (10^{-9} to 10^{-4} M) and verapamil (10^{-9} to 10^{-4} M) between the treatment group and the control group ($P > 0.05$) (Fig. 2).

Production of superoxide radical, and activities of SOD, catalase, and glutathione peroxidase

The production of superoxide radicals was significantly higher in the treatment group than in the control group ($P < 0.01$) (Table 2). The activities of total SOD, Mn-SOD and Cu,Zn-SOD were significantly higher in the treatment group than in the control group ($P < 0.05$ in each) (Table 3). The activities of catalase and glutathione peroxidase were higher in the treatment group than in the control group but the difference were not statistically significant ($P > 0.05$ in each) (Table 3).

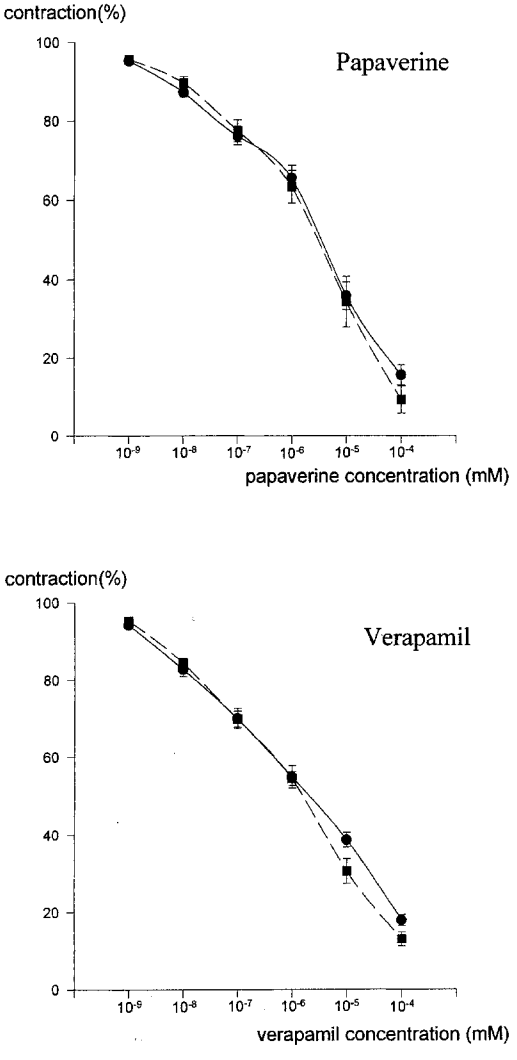

Fig. 2 Relaxation response of strips of corpus cavernosum from control (*circles*) and hypercholesterolemic (*squares*) rabbits, induced by endothelial-independent vasodilators

Table 2 Production of superoxide radical in the corpus cavernosum of the control and hypercholesterolemic groups

	Superoxide radical (μmol/min per g tissue)	
	Control group	Hypercholesterolemic group
Superoxide radical	0.20 ± 0.15 ($n = 10$)	12.91 ± 1.58 ($n = 9$)*

*$P < 0.01$

Table 3 Activities of superoxide dismutase(*SOD*), catalase, and glutathione peroxidase in the corpus cavernosum of the control and hypercholesterolemic groups

	Activity (units/mg protein)	
	Control group	Hypercholesterolemic group
Superoxide dismutase		
Total SOD	28.13 ± 1.85 ($n = 9$)	40.21 ± 3.79 ($n = 8$)*
Mn-SOD	12.97 ± 0.82 ($n = 8$)	16.36 ± 1.23 ($n = 8$)*
Cu,Zn-SOD	12.66 ± 1.87 ($n = 7$)	20.77 ± 2.86 ($n = 8$)*
Catalase	17.03 ± 1.87 ($n = 8$)	24.05 ± 3.40 ($n = 8$)
Glutathione peroxidase	10.13 ± 1.00 ($n = 8$)	10.90 ± 1.41 ($n = 8$)

*$P < 0.05$

Discussion

In 1957, Duff et al. [13] demonstrated that increasing the dietary intake of cholesterol in rabbits produces hypercholesterolemia and eventually a form of atherosclerosis. In 1981, Klimov et al. [22] reported that in this model of experimental atherosclerosis, damage to endothelial cells developed, and these changes could be expected to interfere with endothelium-dependent vascular relaxation responses. Jayakody et al. [19] reported that the endothelium-dependent relaxation to acetylcholine was impaired in aorta from rabbits fed a cholesterol-containing diet.

There is no standard proper dosage nor period of cholesterol treatment for the induction of hypercholesterolemia. The rabbits in this study were fed a 2% cholesterol diet for 8 weeks, resulting in a serum cholesterol level which was significantly higher than that of the control group. We found no significant degenerative changes of the cavernous smooth muscles but did note minimal luminal occlusion of the cavernous arterioles due to intimal thickening in the cholesterol-fed rabbits.

In 1994, Kim et al. [21] reported that contraction responses of cavernous smooth muscle to norepinephrine increased in hypercholesterolemic rabbits compared with a control group. But in this study, as in others [4], the contraction response to norepinephrine was similar in the two groups.

Ahn and Saenz de Tejada [2] reported that endothelium-dependent relaxation was not impaired in rabbits with diet-induced hypercholesterolemia, but our study, as well as another study [4], found that the relaxation responses of cavernous tissues to endothelium-dependent vasodilators were significantly reduced, whereas the relaxation responses to endothelium-independent vasodilators showed no significant difference between the two groups. In contrast, there are some reports that hypercholesterolemia induces impairment of endothelium-independent as well as endothelium-dependent relaxation. Kim et al. [21] reported that since the relaxation response to an endothelium-independent vasodilator (papaverine) was reduced significantly in the hypercholesterolemic group compared with the control group, impotent men with hypercholesterolemia may not respond to or may require higher doses of papaverine. Jünemann et al. [20] found significant degeneration of the cavernous smooth muscle with loss of intercellular contacts in experimental hypercholesterolemia. On the basis of these histological findings, it was suggested that the impairment of endothelium-dependent relaxation develops at an early stage of hypercholesterolemia, and that if the hypercholesterolemia persists long term, it might cause smooth muscle cell degeneration.

Many reports have shown the role lipid peroxides play in the pathogenesis of aortic atherosclerosis. Low density lipoprotein (LDL), which is the major carrier of cholesterol in blood, was oxidized by oxygen free radicals from arterial endothelium and smooth muscle cells, and the oxidized LDL was toxic to endothelia. LDL peroxidation participates in the early stage of atherosclerosis, and injuries to the endothelial cells have a principal role in the progression of atherosclerotic lesions [18]. Recent studies have shown that oxidized LDL inhibits endothelium-dependent relaxation and enhances contraction of the vascular smooth muscle [3, 24, 27].

It was recently reported that endothelial and smooth muscle cells, neutrophils and monocytes or platelets might be a source of these oxygen free radicals, which are known to interact with polyunsaturated fatty acids of biological membranes, thus leading to lipid peroxidation [29]. Oxygen free radicals such as the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (HO·) and singlet oxygen ($^1O_2$) are generated by a partial reduction of oxygen during biological processes [15]. Among the free radical species, superoxide radical had the main role in oxygen toxicity. These free radicals are generated in the univalent pathway of reduction of molecular oxygen to water. Toxic effects of the superoxide radical were inhibition of catalase activity, peroxidation of polyunsaturated fatty acids in biological membranes, destruction of polysaccharides, mutation and ulceration. Antioxidant enzymes such as SOD, catalase, and glutathione peroxidase catalyzed the removal of reactive oxygen radicals [15]. The first line of defense against free radicals is SOD, which scavenges the superoxide radical. The next line of defense consists of glutathione peroxidase and catalase. The two kinds of SOD are copper- and zinc-containing SOD (Cu, Zn-SOD) and manganese-containing SOD (Mn-SOD). It has been reported that SOD activity is generally increased in accordance with the activity of superoxide radical, but little information has been reported about changes in catalase and glutathione peroxidase activity.

In 1985, Henriksson et al. [18] found evidence of the generation of free radicals in hyperlipidemia and atherosclerosis, and elucidated the relationships between the superoxide free radical and its scavengers, SOD and catalase. In this study, SOD in atherosclerotic aorta was increased by feeding a high cholesterol diet. This might be a defense mechanism against an increased level of superoxide radicals in the vessel walls. If increased release of superoxide radicals exists in atherosclerotic vessel walls, then this might lead to production of lipid peroxides, inhibited prostacyclin synthetase and then eventually decreased production of the vasodilator, prostacyclin.

Gryglewski et al. [16] reported that superoxide radicals contribute significantly to the destruction of EDRF, and that EDRF was protected from breakdown by SOD and Ca^{2+}, but not by catalase. Del Boccio et al. [11] reported that changes in aortic antioxidant defense mechanisms and lipid peroxidation precede the massive vascular lipid infiltration in cholesterol-fed rabbits. They also found that some antioxidant mechanisms were increased (SOD, glutathione peroxidase, and total thiol compounds) whereas others were depressed (catalase, glutathione reductase, and glutathione transferase), thus potentially reducing or increasing vascular susceptibility to oxidative injury.

In our study, the production of superoxide radicals and the activities of total SOD, Mn-SOD, and Cu, Zn-SOD increased significantly in the hypercholesterolemic group compared with the control group. The activities of catalase and glutathione peroxidase also increased in the hypercholesterolemic group, but were not significantly higher than in the control group. This suggests that production of superoxide radicals in cavernous tissues increases in the state of hypercholesterolemia, which may lead to functional impairment of cavernous smooth muscle relaxation in response to endothelium-mediated stimuli. SOD seems to play a major role as scavenger against the superoxide radical in cavernous tissue.

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