Experimental Study

Erectile dysfunction medication induced-changes in plasma levels of homocysteine and antioxidant enzyme activities as risk factors for cardiovascular disease

H.H. Baghdadi, PhD\textsuperscript{a}, A. Allam, MD\textsuperscript{b} and S.A. Sheweita, PhD\textsuperscript{c,*}

\textsuperscript{a} Department of Clinical Biochemistry, College of Medicine, Taibah University, Almadinah Almunawwarah, Kingdom of Saudi Arabia
\textsuperscript{b} Department of Medicine, College of Medicine, Taibah University, Almadinah Almunawwarah, Kingdom of Saudi Arabia
\textsuperscript{c} Department of Biotechnology, Institute of Graduate Studies and Research, Alexandria University, Egypt

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Abstract

Objective: Drugs for erectile dysfunction (ED) act by vasodilatation. Hyperhomocysteinaemia is an independent risk factor for premature atherosclerosis, venous thrombosis and other cardiovascular diseases in both men and women. In addition, oxidative stress has long been regarded as a key pathophysiological mediator that eventually leads to cardiovascular disease, whereas oxidative stress is alleviated by antioxidant enzymes such as superoxide dismutase (SOD) and catalase. The aim of the present study was...
to determine the levels of plasma homocysteine and antioxidant enzymes in male rats given ED drugs.

**Methods:** Male rats were given a daily dose of 1.48 mg/kg body weight sildenafil citrate (Viagra®), 0.285 mg/kg vardenafil (Levitra®) or 0.285 mg/kg tadalafil (Cialis®) for 3 weeks, and plasma levels of homocysteine, SOD and catalase were measured; high- and low-density lipoproteins, total cholesterol and triglycerides were also determined.

**Results:** The level of homocysteine was increased by 93% and 67% in plasma of sildenafil- and vardenafil-treated rats, respectively, whereas tadalafil did not change the level significantly. Sildenafil and vardenafil also increased SOD activity by 35% and 46%, respectively; and sildenafil, vardenafil and tadalafil increased the activity of catalase by 33%, 50% and 43%, respectively. A nonsignificant increase in the level of total cholesterol was seen after treatment with all the drugs, and sildenafil, vardenafil and tadalafil increased the levels of high-density lipids by 25%, 41%, and 25%, respectively.

**Conclusion:** The ED medications sildenafil and vardenafil increase the levels of homocysteine and antioxidant enzyme activities. Tadalafil appeared to be safer than the other two drugs, as it did not change the level of homocysteine. Patients taking sildenafil or vardenafil should therefore be advised to take vitamin B12 and folic acid in order to reduce the level of homocysteine, as these vitamins play an important role in biotransformation of homocysteine into methionine.

**Keywords:** Catalase; Hyperhomocysteinemia; Sildenafil citrate; Superoxide dismutase; Tadalafil; Vardenafil

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**Introduction**

The inability to attain or maintain penile erection sufficient for satisfactory sexual performance is defined as erectile dysfunction (ED). A class of vasodilator drugs, phosphodiesterase type-5 (PDE-5) inhibitors, have been developed for treatment of ED and are used by millions of men around the world. These drugs inhibit PDE-5, which is found in most vascular beds as well as in cardiac myocytes by increasing cyclic GMP levels in response to nitric oxide, thereby augmenting the relaxation of smooth muscle tissues. The first PDE-5 inhibitor used for the treatment of ED was sildenafil citrate (Viagra®). Two additional drugs, vardenafil (Levitra®) and tadalafil (Cialis®) are also used. Vardenafil was shown in a rabbit model to increase intracavernosal pressure more quickly and to a greater extent than sildenafil. The duration of action is similar, but vardenafil is more potent and more selective biochemically. Tadalafil is long-acting, being effective for up to 36 h. A large body of evidence shows that PDE-5 inhibitors are generally safe, with the risk for vascular disease. 5

**Homocysteine and ED**

Homocysteine is a sulfur-containing amino acid, the metabolism of which depends mainly on two pathways: re-methylation to methionine, which requires folate and vitamin B12, and trans-sulfuration to cystathionine, which requires pyridoxal-5-phosphate. Hyperhomocysteinemia, a condition that arises from disrupted homocysteine metabolism, was shown in epidemiological studies to be associated with an increased risk for vascular disease. Elevated plasma homocysteine concentrations in people with homocystinuria were responsible for the development of premature occlusive vascular disease, and homocysteine–cysteine mixed disulfide was significantly higher after a methionine load in patients with coronary artery disease than in controls. This finding provided the basis for subsequent studies, culminating in a meta-analysis, published in 1995 of 27 studies involving more than 4000 patients with occlusive (cardiovascular, peripheral and cerebrovascular) vascular disease and the same numbers of controls. The results showed that homocysteine was an independent, graded risk factor for atherosclerotic disease in the coronary, cerebral and peripheral vessels. A 5-μM increment in total plasma homocysteine was associated with increased risks for coronary heart disease of 60% for men and 80% for women. The meta-analysis concluded that the risk for arteriosclerotic vascular disease increased with increasing homocysteine level, regardless of whether cholesterol was normal or elevated. Experiments on arterial segments and in experimental animals as well as observations of hyperhomocysteinemic patients have shown that homocysteine can contribute to several processes that lead to atherosclerosis.

There is growing interest among researchers about the role of oxidative stress in the pathophysiological mechanism of ED. Oxidative stress occurs when there is an imbalance between prooxidants and the ability of antioxidants to scavenge excess reactive oxygen species. The role of oxidative stress and reactive oxygen species in the pathophysiological mechanisms of male and female infertility has been evaluated extensively, however, its role in ED has not been investigated comprehensively. Both in vitro and in vivo studies have shown significant associations between the production of reactive oxygen species and ED, especially in diabetic animal models, and oxidative stress was shown to be involved in ED. Superoxide dismutase (SOD), an antioxidant enzyme that catalyzes the conversion of superoxide anion to hydrogen peroxide and molecular oxygen, is a promising therapeutic target for ED.

To our knowledge, there have been no studies of the association between treatment with ED medications such as sildenafil, vardenafil and tadalafil and plasma homocysteine levels as an independent risk factor for cardiovascular disease. Therefore, the present study was carried out to determine the levels of homocysteine and also of antioxidant enzymes and other biochemical parameters, including low-density lipoproteins (LDL), high-density lipoproteins (HDL), triglycerides and total cholesterol, in the plasma of rats given ED drugs for 3 weeks.
Materials and Methods

Animals

Fifty male Sprague–Dawley rats weighing 200–220 g were obtained from the animal house of the Faculty of Medicine, Alexandria University, Alexandria, Egypt. The rats were housed in standard cages and given food and water ad libitum. The local ethics committee of the Institute of Graduate Studies and Research, Alexandria University, approved the design of the experiments, and the protocol conformed to the guidelines of the United States National Institutes of Health. After a period of acclimatization, the animals were divided into four groups. One group of 11 rats was used as control and received ddH2O as vehicle, and groups of 13 rats were given daily doses of sildenafil (1.48 mg/kg), tadalafil (0.2 mg/kg) or vardenafil (0.2 mg/kg) for 3 weeks. Sildenafil (Pfizer), tadalafil (Lilly) and vardenafil (Schering-Plough) were obtained from local pharmacies in Saudi Arabia. At the end of the experimental period, the rats were sacrificed by cervical decapitation after administration of diethyl ether as anesthetic, and fasting blood samples were collected from the sacrificed animals in heparinized tubes. Plasma samples were obtained by centrifugation at 4000 rpm for 20 min and stored at –80 °C until use.

Homocysteine determination

Most homocysteine in plasma is bound to proteins by disulfide bonds with thiol-containing residues; oxidized homocysteine and homocysteine–cysteine mixed disulfide are also present. Total homocysteine was measured after reduction of disulfide bonds and detection of released homocysteine by use of dithiothreitol. Homocysteine in test samples is converted to S-adenosyl-l-homocysteine (SAH) by use of S-adenosyl-l-homocysteine hydroxylase and excess adenosine. The subsequent solid-phase enzyme immunoassay is based on competition between SAH in the sample and immobilized SAH bound to the walls of the microtitre plate for binding sites on a monoclonal anti-SAH antibody. After removal of anti-SA antibody not bound to the plate, a secondary rabbit anti-mouse antibody labeled with horseradish peroxidase is added. Peroxidase activity is measured spectrophotometrically after addition of the substrate, and the absorbance is inversely related to the concentration of total homocysteine in the sample.33 The full procedure for homocysteine determination was followed according to the instructions for the homocysteine enzyme immunoassay kit (Axis-Shield, Germany).

SOD activity

SOD was measured according to the method of Sun et al.,34 with a kit supplied by Randox Laboratory, United Kingdom. SOD estimation is based on the generation of superoxide radicals produced by xanthine and xanthine oxidase, which react with nitroblue tetrazolium to form formazin dye. SOD activity is then measured at 560 nm by the degree of inhibition of this reaction and expressed as mmol/min per ml of plasma.

Catalase activity

Catalase activity was determined by the method by Beers and Sizer,34 which is based on decreasing absorbance of H2O2 solution decomposed by the enzyme, with a kit obtained from Randox Laboratory, United Kingdom. The quantity of H2O2 decomposed over a specified time is calculated from the molar absorbance coefficient. Absorbance is measured at 240 nm, and catalase activity is expressed as IU/l of plasma.

Total cholesterol level

Cholesterol was determined enzymatically with cholesterol esterase and cholesterol oxidase. Cholesterol esters are cleaved by the action of cholesterol esterase to yield free cholesterol and free fatty acids. The color intensity is directly proportional to the concentration of cholesterol and was determined spectrophotometrically. The full procedure of cholesterol determination was followed according to the instructions with the kit (Biosystems, Barcelona, Spain).

Lipid determinations

Briefly, HDL was measured in the supernatant after precipitation of apolipoprotein B-containing particles with dextran sulfate-MgCl2. For LDL, a specific detergent solubilizes the cholesterol from HDL, very low-density lipoproteins and chylophilicrons. The cholesterol esters are broken down by cholesterol esterase and cholesterol oxidase in a non-color-forming reaction. The second detergent (MES buffer, > 30 mmol/l, N,N-bis(4-sulfobutyl)-m-toluidine 1 mmol/l, detergent, pH 6.3) solubilizes cholesterol from LDL in the sample, and LDL cholesterol is then measured spectrophotometrically at 546 nm.

The method for determining triglycerides is based on use of a lipoprotein lipase for rapid, complete hydrolysis of triglycerides to yield glycerol, followed by oxidation to dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide produced reacts with 4-aminophenazone and 4-chlorophenol under the catalytic actions of peroxidase to form a red color, which is measured spectrophotometrically. The full procedure for determination of LDL, HDL and triglycerides was performed according to the manufacturer’s instructions (Biosystems, Barcelona, Spain).

Statistical analysis

Means and standard errors were calculated. Data were compared with Student’s t test. The level of significance for all experiments was set at p < 0.05.

Results

The results are shown in Table 1. Sildenafil and vardenafil increased the level of homocysteine in rat plasma by 93% and 67%, respectively, while tadalafil had no effect. Sildenafil and vardenafil also increased SOD activity by 35% and 46%, respectively; and sildenafil, vardenafil and tadalafil increased

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(Homocysteine and cardiovascular diseases)
the activity of catalase by 33%, 50% and 43%, respectively. The level of total cholesterol did not change after treatment of rats with any of the drugs, but sildenafil, vardenafil and tadalafil increased the levels of HDL by 25%, 41% and 25%, respectively.

Discussion

In this study, the level of homocysteine increased significantly in rats treated with sildenafil or vardenafil, but tadalafil had no significant effect, indicating that its mechanism of vasodilatory action is different than those of the other two drugs, perhaps associated with its prolonged effect. The study suggests, for the first time, that cardiovascular risk might be increased after prolonged use of sildenafil or vardenafil, due to increased homocysteine levels. In support of our suggestion, it has been shown that hyperhomocysteinaemia caused endothelial dysfunction in animal models and in cell culture. Homocysteine generates superoxide and hydrogen peroxide, both of which have been linked to damage of the endothelial lining of arterial vessels. Moreover, homocysteine changes the levels of coagulation factors, causing blood clot formation.

In rat aortic smooth muscle cells, homocysteine was shown to increase DNA synthesis and mRNA levels in cyclin D1 and cyclin A, which are important for the re-entry of these cells into the cell cycle. Furthermore, homocysteine promoted proliferation of the aortic smooth muscle cells. The growth-promoting effect of homocysteine on vascular smooth muscle cells, together with its inhibitory effect on endothelial cell growth, represent a possible mechanism for homocysteine-induced atherosclerosis.

The present study showed that sildenafil and vardenafil significantly increase the activities of both SOD and catalase. Other studies also found that sildenafil raised the concentrations of these antioxidants and reduced oxidative stress. High levels of superoxide anion and hydrogen peroxide increase oxidative stress, and it is possible that increased SOD and catalase activities are a response to the increased superoxide anion generated as a result of increased homocysteine and probably represent a defense mechanism activated to protect the cell against an oxidative insult. Quenching of oxidative stress by sildenafil and vardenafil could sustain the bioavailability of nitric oxide for vasodilation, which may be another mechanism of action of ED drugs, as most cases of ED are associated with oxidative stress. In support of our new observation, hyperhomocysteinaemia was found to increase oxidative stress and was blocked by induction of SOD by sildenafil. Moreover, sildenafil depresses hydrogen peroxide generation by mimicking SOD and preventing generation of reactive oxygen species.

It is becoming widely accepted that hypercholesterolaemia is associated with endothelial dysfunction due to an increase in superoxide anion production. In the present study, however, treatment of rats with ED drugs did not change the levels of total cholesterol and LDL significantly; however, the level of HDL increased after treatment with sildenafil or vardenafil, with no change after treatment with tadalafil. The level of triglycerides was decreased only by treatment with sildenafil, vardenafil, with no change after treatment with tadalafil. The level of HDL increased after treatment with sildenafil or vardenafil and consequently increased the levels of oxidative stress. Other studies have also found that enhanced HDL levels are due mainly to induction of SOD and catalase.

In summary, this study demonstrates that sildenafil and vardenafil increase the levels of homocysteine, indicating a possible mechanism for the endothelial dysfunction associated with hyperhomocysteinaemia. Another important finding of this study is that ED drugs increase antioxidant properties by increasing SOD and catalase levels, which can attenuate the oxidative stress resulting from hyperhomocysteinaemia.

Funding

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<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>Treatments</th>
<th>Sildenafil (Viagra)</th>
<th>Tadalafil (Cialis)</th>
<th>Vardenafil (Levitra)</th>
</tr>
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<tbody>
<tr>
<td>Homocysteine level [μM/L]</td>
<td>7.75 ± 0.79</td>
<td>12.95 ± 1.77∗ [+67%, p &lt; 0.05]</td>
<td>14.92 ± 1.788 [+93%, p &lt; 0.05]</td>
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<tr>
<td>SOD activity [mmol/min/mL]</td>
<td>2.264 ± 0.19</td>
<td>3.20 ± 0.179∗ [+42%, p &lt; 0.05]</td>
<td>2.98 ± 0.133∗ [+32%, p &lt; 0.05]</td>
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<td>Catalase activity [Unit/L]</td>
<td>168.0 ± 12.9</td>
<td>252.7 ± 21.9∗ [+50%, p &lt; 0.05]</td>
<td>224.0 ± 16.0∗ [+33%, p &lt; 0.05]</td>
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<tr>
<td>Total cholesterol level [mg/DL]</td>
<td>91.7 ± 5.0</td>
<td>113.9 ± 6.4 [NS, p &gt; 0.05]</td>
<td>106.2 ± 7.12 [NS, p &gt; 0.05]</td>
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<tr>
<td>HDL [mg/DL]</td>
<td>36.2 ± 2.0</td>
<td>51.0 ± 2.1∗ [+42%, p &lt; 0.05]</td>
<td>44.0 ± 2.18∗ [+23%, p &lt; 0.05]</td>
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<tr>
<td>LDL [mg/DL]</td>
<td>69.0 ± 6.8</td>
<td>71.8 ± 5.6 [NS, p &gt; 0.05]</td>
<td>71.0 ± 6.9 [NS, p &gt; 0.05]</td>
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<tr>
<td>Triglycerides [mg/DL]</td>
<td>103.0 ± 14.4</td>
<td>94.0 ± 5.9 [NS, p &gt; 0.05]</td>
<td>86.0 ± 5.65∗ [−17%, p &lt; 0.05]</td>
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Values are expressed as means ± SEM.

NS: Values are not significantly different from the corresponding control value.

* Values are significantly different from the corresponding control value at p < 0.05.

** NS, Values are not significantly different from the corresponding control value at p > 0.05.
References


