Erectile Dysfunction Drugs and Protein Expression of Glutathione S-transferase and Glutathione Peroxidase in the Liver of Male Rats

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Abstract: Erectile dysfunction (ED) drugs have been used for treatment of erectile dysfunction which affects the lives of approximately 300 million men worldwide. It is well known that the cytosolic glutathione S-transferase (GST) and glutathione metabolizing enzymes play an important role in the detoxification of many endogenous and exogenous compounds. Therefore, the present study aims at investigating the changes in activities and expression of glutathione S-transferase, and glutathione peroxidase (GPx) enzymes as well as antioxidant enzymes after treatment of male rats with a daily dose of sildenafil (1.48 mg/kg), tadalafil (0.285 mg/kg) and vardenafil (0.285 mg/kg) for three weeks. In addition, glutathione reductase (GR) activity, levels of both reduced glutathione and free radicals (measured as malondialdehyde, MDA), and histopathological examinations were assayed in the liver tissues of rats. The data of the present study showed that sildenafil, vardenafil, and tadalafil treatments significantly decreased the levels of glutathione, MDA and the activity of glutathione reductase in liver tissue of male rats. In addition, vardenafil and sildenafil increased superoxide dismutase and catalase activity. The total activity of GST was only decreased after treatment of rats with vardenafil, whereas the other two drugs did not change such activity. Interestingly, western immunoblot showed that protein expression of GST π isozyme was markedly reduced after treatment of rats with sildenafil. In addition, the activity and expression of glutathione peroxidase were significantly reduced after treatment of rats with either sildenafil or tadalafil, whereas vardenafil induced the activity and expression of this enzyme. It is concluded that ED drugs induced changes in the activity and protein expression of both GPx and GST π. In addition, these drugs have shown antioxidant activities which might be a new mechanism that can be added to the previous action of these drugs.

Key-words: Glutathione S-transferase, glutathione, free radicals, glutathione peroxidase, and glutathione reductase

1 Introduction

Erectile dysfunction (ED) is defined as the inability to maintain penile erection sufficient for satisfactory sexual performance. Phosphodiesterase type-5 (PDE-5) inhibitors have been developed for treatment of erectile dysfunction and act as a vasodilator drugs [1-3]. These drugs inhibit PDE-5 which is found in most vascular beds as well as cardiac myocytes [4-6]. PDE-5 inhibitors prevent the catabolism of nitric oxide, and therefore are potent vasodilators. The first PDE-5 inhibitor is sildenafil citrate (Viagra) which has been used for treatment of ED. Two additional drugs, tadalafil (Cialis) and vardenafil (Levitra) are also used in treatment of ED. Vardenafil has been shown in a rabbit model to increase intracavernosal pressure more quickly and to a greater extent than sildenafil [7, 8]. Vardenafil has a similar duration of action to sildenafil but is more potent and selective biochemically toward ED. Tadalafil is a long-acting PDE-5 inhibitor which is effective for up to 36 h in men [9, 10].

The cytosolic glutathione S-transferases (GST) and glutathione (GSH) play an important role in the detoxification of many environmental chemicals including mutagens and carcinogens [11-13]. GSH is one of the most abundant intracellular thiols and protects hepatic cells from the lethal effects of toxic and carcinogenic compounds [14,15]. In addition, GSH maintains functional form and enhances
metabolic clearance of dietary lipid peroxides [12,15]. Also, GSH is an important determinant of cellular sensitivity to a wide variety of drugs and other cytotoxic compounds [16,17]. Conjugations of GSH with toxic compounds may eventually result in GSH depletion which can be compensated by glutathione reductase (GR) activity [13,18]. GSH depletion is often associated with cytotoxicity, and there are some indications that conjugation reactions can be more detrimental to the cell rather than redox cycles [19, 20]. It thus seems possible that GSH depletion may promote tumor development through a mechanism that involves cytotoxicity and other different ways [21]. Previous studies have demonstrated that GSH and GSTs reduced the covalent binding of epoxides of well-known chemical carcinogens, e.g. aflatoxin B1, and benzo[a]pyrene, with DNA and other macromolecules and this reduction in DNA binding was found to be effective in decreasing the hepatocarcinogenesis induced by these compounds [22-26]. There is a growing interest among researchers regarding the role of oxidative stress in the pathophysiological mechanism of ED [27]. Oxidative stress occurs when there is an imbalance between prooxidants and the ability of the antioxidants to scavenge excess reactive oxygen species [28]. The role of oxidative stress and reactive oxygen species has been extensively evaluated in the pathophysiological mechanisms of male and female infertility [27]. However, its role in ED has not been investigated comprehensively. Both in vitro and in vivo studies have shown a significant association between the production of reactive oxygen species and erectile dysfunction, especially in diabetic animal models [29]. Oxidative stress is demonstrated to be involved in the pathophysiological mechanism of erectile dysfunction (ED) [30]. Superoxide dismutase (SOD), an antioxidant enzyme catalyzing the conversion of superoxide anion (O•2 to hydrogen peroxide (H2O2) and molecular oxygen (O2), is a promising therapeutic target for ED [30]. In vivo gene therapy and adult stem cell-based ex vivo gene therapy are two attractive current gene therapies for the treatment of ED [30].

A large body of evidence showed that PDE-5 inhibitors are generally safe, being used by millions of patients around the world with the exception for the marked hypotensive effect of these drugs in men when combined with nitrates and α-blockers used to treat essential hypertension [11, 12]. It is generally accepted that reduced GSH and GSH-metabolizing enzymes have an important role in the protection of mammalian cells against oxidative stress and alkylating agents [31]. To the best our knowledge, no previous studies have been conducted on the role of sildenafil, tadalafil, and vardenafil in the alteration of the activities and the expression of both glutathione S-transferase and glutathione peroxidase, and also on the levels of both glutathione and free radicals in rat livers. Therefore, this study was carried out to show the changes in these biochemical parameters since such changes in the activities of these enzymes might change the liver's capacity of drug detoxification especially after long-term use of these drugs.

2. Materials and Methods:

2.1. Chemicals

Sildenafil, tadalafil, vardenafil pills manufactured by Pfizer Pharmaceutical Company, Lilly Corporation, Schering-Plough Corporation, USA, respectively were obtained from local drug stores at Egypt. Sulfosalicylic acid, bis-(3-carboxy-4-nitrophenyl)-disulfide, 1-chloro-2, 4-dinitrobenzene and all other chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA. Monoclonal antibodies of glutathione S-transferase and glutathione peroxidase were obtained from ABCAM, UK.

2.2. Animals

Fifty male Sprague–Dawely rats (weighing 100 – 120 g) were obtained from the animal house of Faculty of Medicine, Alexandria University, Alexandria, Egypt. The rats were housed in standard cages where food and water were provided ad libitum. After a period of acclimation, animals were divided into four groups. The first group (11 rats) was used as control (C) and received ddH2O as vehicle. Thirteen rats in each group of the second, third, and fourth groups received a daily dose of sildenafil (1.48 mg/kg), tadalafil (0.285 mg/kg) and vardenafil (0.285 mg/kg) respectively for three weeks. At the end of the experimental period, rats were sacrificed by cervical decapitation and fasting blood samples were collected in heparinized tubes. Plasma samples were obtained by centrifugation at 4000 rpm for 20 min and stored at –80°C until use.

2.3. Enzyme Assessments

At the designated time point, the thoracic cavity of rats was opened for whole body. Liver tissues were vigorously washed in an iced solution of 0.25 M sucrose, which contained 0.001 M EDTA, to avoid
contamination from erythrocyte-containing enzymes. Liver tissues were homogenized in 3 volumes (w/v) 0.1 M phosphate buffer, (pH 7.4) and centrifuged at 12000 xg for 20 min at 4°C. Reduced glutathione level was estimated in the supernatant of liver tissue homogenate according to the method of Mitchell et al. [32], using sulfosalicylic acid for protein precipitation and bis-(3-caboxy-4-nitrophenyl)-disulfide for color development. Glutathione reductase activity was assayed by monitoring the oxidation of NADPH at 340 nm. A unit of enzyme activity represents 1 nmole of NADPH oxidized/min/mg protein. GST activity was determined according to the method of Chi-Yu et al. [34]. The conjugate of GSH with 1-chloro-2, 4-dinitrobenzene (CDNB) was measured at 340 nm using a double beam spectrophotometer. A unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 mmol of CDNB conjugate/mg protein/min under the assay conditions. Calculations were made using a molar extinction coefficient of 9.6 mM$^{-1}$ cm$^{-1}$.

2.3.1. Glutathione Peroxidase
Glutathione peroxidase enzyme activity (GPx; EC. 1.11.1.9) was assayed according to the method of Chiu et al., 1976 [36]. The reaction mixture (1 mL) containing 0.05mL of the enzyme source, 0.05MTris- HCl buffer (pH 7.6), 1.5mMGSH, and cumenehydroperoxide was incubated for 5 min at 37°C. In another tube, the control sample was prepared without cumenehydroperoxides and incubated for 5min at 37°C. To both control and test samples, 1.0 mL of TCA (15%) was added, while 0.1mL cumenehydroperoxide was added to the control only. Both tubes were incubated for 10 min at 37°C and centrifuged at 3000 rpm for 20 min. Tris-HCl buffer (pH 8.9) and 1.5mM DTNB were added to 1mL supernatant for both sample and control. The optical density of the yellow color obtained was measured at 412 nm within 5min. Result was expressed as U/gm tissue.

2.3.2 Superoxide dismutase activity
A measurement of SOD was performed according to the method of Sun et al., [37]. SOD estimation was based on the generation of superoxide radicals produced by xanthine and xanthine oxidase, which react with nitrobluetetrazolium (NTB) to form formazan dye. SOD activity was then measured at 560 nm by the degree of inhibition of this reaction and was expressed as nmol/min/ml plasma.

2.3.3. Catalase Assay
Catalase activity was determined following the method by Beers and Sizer based on the decreasing in the absorbance of H2O2 solution decomposed by the enzyme [38]. The quantity of H2O2 decomposed over specified time was calculated using the molar absorbance coefficient. Absorbance was measured at wavelength 240 nm and catalase activity was expressed in IU/L plasma.

2.3.4. Western Immuno-blotting Technique
From pooled sample of each treatment, 20 µg of S-9 liver supernatant proteins were prepared and subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transblotted to Hybond-C nitrocellulose membrane (Amersham, UK). Both GST π and glutathione peroxidase isozymes were visualized after binding with their specific monoclonal antibodies. Chemiluminescence signals were detected according to the manufacturer’s instructions (Abcam, UK).

2.4. Liver tissues preparation and histopathological examination
The liver tissues were fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer for 24 h. Blocks of liver tissue were taken from the median lobe and prepared for embedding in glycol methacrylate (Historesin, Leica) using standard techniques. Subsequently, sections of 5 µm thicknesses were obtained and stained with Eosin and Hematoxylin dyes for microscopic assessment. The histological sections were examined under a light microscope using a 121 intersection grid placed at the ocular lens, coupled to a 100X objective. The hepatic parenchyma was classified as one of the following: hepatocyte cytoplasm or degenerative hepatocytes, central vein, portal space, and inflammatory infiltrate.

2.5. Statistical analysis
Each enzyme assay was performed and means and standard errors were calculated. Data were compared by a Student’s t-test and the level of significance for all experiments was set at P<0.05.

3. Results and Discussion
Mammalian GSTs are presently grouped into four classes on the basis of their immunological properties and related primary structure [39-42]. Of
Role of oxidative stress and reactive oxygen species in the pathophysiological mechanisms has been extensively evaluated [48, 49]. Both in vitro and in vivo studies have shown a significant association between the production of reactive oxygen species and liver damage in rats [50]. Sildenafil reduced the levels of malondialdehyde (MDA) in human and increased the activity of antioxidant enzymes after testicular injury in rats [51]. In agreement with this finding, levels of MDA were significantly decreased by 28, 36 and 23% after treatment of rats with tadalafil, vardenafil, and sildenafil respectively [Table 1]. This decrease in MDA might be due to the induction of superoxide dismutase and catalase after treatment of rats with these agents since these enzymes play an important role in scavenging of free radicals [52-54]. The present study showed that vardenafil and sildenafil significantly increased the activities of both superoxide dismutase by 35, 17% and catalase by 56, 32% respectively. In agreement with our present study, it has been found that sildenafil improved the antioxidants concentrations [catalase (CAT), superoxide dismutase (SOD)], and improved oxidative stress in patients [55]. High levels of superoxide anion and hydrogen peroxide cause an elevation of oxidative stress [56]. It is possible that increased SOD and CAT activities are a response to the increased superoxide anion generation and probably represent a defense mechanism to protect the cell against an oxidative insult. Quenching of oxidative stress by sildenafil, vardenafil and sildenafil could sustain the bioavailability of NO for vasodilatation, and this could be another new possible mechanism of actions of ED medications since most cases of erectile dysfunction (ED) are associated with oxidative stress [57,58]. Supporting our new observation, sildenafil depresses hydrogen peroxide generation by acting as a superoxide dismutase (SOD)-mimetic by preventing reactive oxygen species (ROS) generation [59, 60].

Glutathione peroxidase, glutathione reductase and glutathione are playing an important role in scavenging free radicals [12, 15]. In the present study, the activity of GR was markedly decreased in liver tissues of male rats after treatment with sildenafil, vardenafil and tadalafil by 38, 51 and 22% respectively (Table 1). In addition, the level of GSH was significantly decreased after treatment of rats with sildenafil, vardenafil and tadalafil by 36, 51, 23% respectively. In accordance with the present study, it has been established that treatment of male rats with sildenafil was found to reduce the level of GSH in both prostate and brain tissues [61,62]. It has been proposed that the total level of GSH is partially dependent on the reduction of the oxidized form of glutathione (GSSG) to the reduced form of GSH by glutathione reductase. It seems that there are different sources of GSH synthesis such as GSH synthase and γ-glutamyltranspeptidase because the activity of glutathione reductase is markedly inhibited after the treatment of rats with ED drugs. In a previous study, there was no statistical difference in human erythrocyte glutathione peroxidase activity between the placebo and sildenafil citrate-treated groups [63]. It seems that changes in GPx activity is dependent on animal species since glutathione peroxidase activity did not change in human erythrocyte [63], whereas such activity and the expression of glutathione peroxidase were inhibited after treatment of rats with tadalafil and/or sildenafil [Table 1 and Figure 1B]. Therefore, inhibition of glutathione peroxidase, glutathione reductase activities, and depletion of glutathione levels may enhance liver toxicity. Supporting this finding, the histopathological examination showed that sildenafil citrate and vardenafil caused inflammation and fibrosis in rat liver cells [Figure 2D], whereas tadalafil did not cause any changes [Figure 2 B&C]. It is concluded that ED drugs changed the activities and expression of phase II drug metabolizing enzymes also, they decreased free radical levels and increased the antioxidant properties by increasing SOD and CAT level, which can attenuate the oxidative stress resulted from many endogenous sources.
Fig. 1: Western immunoblot analysis shows the protein expression of A) Glutathione S-transferase π isozyme, B) Glutathione peroxidase. Lanes 1, 2, 3 and 4 represent the pooled proteins of matched control groups, vardenafil, tadalafil, and sildenafil respectively.

Fig. 2: Photomicrographs of histopathological examination of liver tissues of A) control B) tadalafil C) vardenafil, and D) sildenafil-treated rats
Table 1 showed changes in the activity of phase II drug-metabolizing and antioxidant enzymes after treatment of rats with a daily dose of tadalafil, vardenafil and/or sildenafil for consecutive three weeks.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Control</th>
<th>Tadalafil</th>
<th>Vardenafil</th>
<th>Sildenafil</th>
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<tbody>
<tr>
<td>GST (Unit/mg protein)</td>
<td>0.79±0.015</td>
<td>0.79±0.02</td>
<td>0.60±0.017</td>
<td>0.74±0.013</td>
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<tr>
<td></td>
<td>(NS)</td>
<td></td>
<td>(-25%, P&lt;0.05)*</td>
<td>(NS)</td>
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<tr>
<td>Glutathione Reductase (nmole oxidized NADPH/mg protein/min)</td>
<td>95.6±2.67</td>
<td>75.0±3.36</td>
<td>46.0±2.1</td>
<td>59.7±1.57</td>
</tr>
<tr>
<td></td>
<td>(-22%, P&lt;0.001)*</td>
<td>(-51%, P&lt;0.001)*</td>
<td></td>
<td>(-38%, P&lt;0.001)*</td>
</tr>
<tr>
<td>Glutathione peroxidase (IU/gm Wet tissue)</td>
<td>34.2±2.2</td>
<td>21.9±3.2</td>
<td>42.7±3.0</td>
<td>24.6±3.1</td>
</tr>
<tr>
<td></td>
<td>(-36%, P&lt;0.05)**</td>
<td>(+25%, P&lt;0.05)**</td>
<td></td>
<td>(-28%, P&lt;0.05)**</td>
</tr>
<tr>
<td>Glutathione (µmole GSH/g liver)</td>
<td>2.1±0.13</td>
<td>1.65±0.09</td>
<td>1.03±0.04</td>
<td>1.36±0.02</td>
</tr>
<tr>
<td></td>
<td>(-23%, P&lt;0.001)*</td>
<td>(-51%, P&lt;0.001)*</td>
<td></td>
<td>(-36%, P&lt;0.001)*</td>
</tr>
<tr>
<td>TBARS (µmoles/g liver)</td>
<td>16.1±0.48</td>
<td>11.6±0.29</td>
<td>10.3±0.38</td>
<td>12.36±0.11</td>
</tr>
<tr>
<td></td>
<td>(-28%, P&lt;0.001)*</td>
<td>(-36%, P&lt;0.001)*</td>
<td></td>
<td>(-23%, P&lt;0.001)*</td>
</tr>
<tr>
<td>Catalase activity (IU/m/L)</td>
<td>167.8±0.79</td>
<td>246.5±4.35</td>
<td>261.1±3.83</td>
<td>221.5±1.89</td>
</tr>
<tr>
<td></td>
<td>(+47%, p&lt;0.001)*</td>
<td>(+56%, p&lt;0.001)*</td>
<td></td>
<td>(+32%, p&lt;0.001)*</td>
</tr>
<tr>
<td>SOD activity (mmol/min/mL)</td>
<td>2.44±0.04</td>
<td>2.62±0.20</td>
<td>3.29±0.19</td>
<td>2.87±0.07</td>
</tr>
<tr>
<td></td>
<td>(NS)</td>
<td></td>
<td>(+35%, P&lt;0.001)*</td>
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</table>

Values are expressed as Mean ± SEM.
NS: values are not significant statistically compared to the control group where P > 0.05.
* Values are significantly different compared to control group where P < 0.05.
** Values are significantly different compared to control group where P < 0.001.
Conflict of Interests
The authors declare that there is no conflict of interests regarding the publication of this paper.

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