Impact of Sildenafil Citrate (Viagra) and Ethanol Interaction on Antioxidant Defense System in the Adult Male Albino Rats

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Abstract: The interactive effects of Sildenafil citrate (VIAGRA) and Ethanol Consumption on the antioxidant defense system in testis tissue of rats were studied in the present research work. Male Albino rats were divided into eight groups of six animals each. Control rats were administered normal saline orally. While experimental animals were fed Sildenafil citrate (VIAGRA) (1 μg gm⁻¹) and 18% ethanol (5 g kg⁻¹ Body weight) and sacrificed. A significant depletion of GSH content in testis was observed. This combination was found to be decreased Super Oxide Dismutase (SOD) activity in testis. Thio Barbiric Acid Reactive Substrate (TBARS) and Catalase (CAT) Activity were observed to be increased in testis. In contrast, Glutathione Peroxidase (GPx) activity was decreased in testis. The results are discussed in detail.

Key words: Sildenafil citrate (VIAGRA), ethanol, rat testis, antioxidant defense system

INTRODUCTION

Sildenafil citrate (VIAGRA), a specific phosphodiesterase-5 (PDE-5) inhibitor currently approved for the treatment of erectile dysfunction in men, has been shown to acutely enhance endothelium-dependent vasodilation in patients with heart failure (Katz, 2003). It is a selective PDE type5 (PDE5) inhibitor increasing the cGMP level in the target tissues and represents a powerful therapy for male erectile dysfunction (Milman and Amlod, 2002). Nitric oxide is a potent activator of soluble guanylate cyclase and causes cGMP formation in target cells (Garthwaite and Boulton, 1995). Phosphodiesterase type 5 (PDE-5) enzymes is highly specific for hydrolysis of cGMP and is involved in regulation of cGMP signaling (Kotera et al., 2000). Administration of an NO donor to rats with stroke significantly increase brain levels of cGMP, induces cell genesis and improves functional recovery (Zhang et al., 2001). Sildenafil citrate increase brain levels of cGMP, evokes neurogenesis and reduces neurological deficits when given to rats 2 or 24 h after stroke (Zhang et al., 2002).

Ethanol has shown beneficial effects of moderate drinking on the risk for cardio disease (Sillianakke, 2000). A study in American Indian men and women showed a significant inverse association of alcohol consumption with peripheral arterial disease (Fabsitz et al., 1999). Prolonged consumption of excessive amounts of alcohol increased medical risks for liver cirrhosis, several neuromuscular disorders and several types of cancer (Halsted, 1994). The interaction of ethanol and lipid metabolism was relevant to the effect of alcohol consumption on the pathogenesis of alcoholic fatty liver and hyperlipidemia and to atherosclerosis (Lawrence and Lieber, 1999).

In the 20th century, the poor dietary habits of alcoholics were widely accepted as explaining several obvious connections between heavy drinking and organ damage. Ethanol consumption appeared to induce oxidative stress in the liver and in extrahepatic tissues (Nordmann, 1994). Sensitivity to peroxidation was reported to be a function of the overall balance between pro-oxidants and antioxidants. Therefore, tissue antioxidants helped prevent the cellular damage caused by free radicals and free radical-mediated lipid peroxidation (Henning et al., 1991).

The formation of Reaction Oxygen Species (ROS) free radicals is a naturally occurring intracellular metabolic process. These oxidative damage to a number of molecules in cells including membrane lipids, proteins and nucleic acids (Ames, 1993; Mc Cord, 1993). The potential harmful effects of these species are controlled by the cellular antioxidant defense system (Bondy and Orozco, 1994). Reduced glutathione (GSH) is the predominant defense against ROS free radicals in testis tissues of the body (DeLeve and Kaplowitz, 1991). In addition,
antioxidant enzymes, such as Superoxide Dismutase (SOD), Catalase (CAT) and Glutathione Peroxidase (GPX) are essential in both scavenging ROS free radicals and maintaining cellular stability (Halliwell and Gutteridge, 1989; Somani, 1996). Under normal conditions, reductive and oxidation (Rubin, 1993). However, when the generation of ROS in cells impairs antioxidant defenses or exceeds the ability of the antioxidant defense system to eliminate them, leads to oxidative stress (Jenkins and Goldfarb, 1993). Ethanol is also oxidized by the microsomal ethanol oxidizing system to acetaldehyde and 1-hydroxymethyl radical by cytochrome P-450 IIIE1 (Anandatheerthavarada et al., 1993; Reinke et al., 1994). Therefore, it is important to investigate the responses of the ROS free radicals scavenging system in tissues of rats after chronic ingestion of Sildenafil citrate and Ethanol.

**MATERIALS AND METHODS**

**Animals:** Male Albino rats, Wistar strain of body weight ranging 180-200 g bred in Central Animal House, Rajah Muthiah Medical College, Tamil Nadu, India, fed on standard pellet diet (Agro Corporation Private Limited, Bangalore, India) were used for the study and water was given ad libitum. The animals were housed in plastic cages under controlled Conditions of 12 h light/12 h dark cycle, 50% humidity and at 30±2°C. The animals used in the present study were care in accordance with the Ethical Committee for Animal Care of Annamalai University and the Indian National Law on Animal Care and use. (Register Number: 166/1999/CPCESA) (National Institute of Health, 1985).

This experimental study was conducted in the Department of Pharmacology, Biochemistry, Rajah Muthiah Medical College and Hospital, Annamalai University.

**Ethanol:** Absolute ethanol (AR) was obtained from Nellikuppam, Cuddalore District, South India.

**Drug:** Sildenafil citrate (VIAGRA) was purchased from Sigma-Aldrich, Inc. (USA).

All other chemicals and reagents used in the present study were of analytical grade and were obtained from Sigma Chemical Company, Saint Louis, USA and Hi media Laboratories, Mumbai, India.

**Experimental design:** Rats were divided into eight groups of six animals each and treated for 45 days as Follow:

- Control group of animals receive standard pellet diet and isocaloric glucose from a 40% glucose solution daily by intragastric intubation and served.

**Short term treatment**

- Albino rats with a single dosage of the drug (1 μg g⁻¹ body weight) along with 18% ethanol (3 g kg⁻¹ body weight) (Encmoto et al., 1999) and to be sacrificed after 1 h.

- Treated animals (Drug and ethanol) to be sacrificed after two and half h.

- Treated animals (Drug and ethanol) to be sacrificed after 4 h.

- Treated animals (Drug and ethanol) to be sacrificed after 24 h.

**Long term treatment**

- Treated animals (Drug and ethanol) with a single dosage daily for 15 days and to be sacrificed after 4 h of the last dosage.

- Treated animals (Drug and ethanol) with a single dosage daily for 30 days and to be sacrificed after 4 h of the last dosage.

- Treated animals (Drug and ethanol) with a single dosage daily for 45 days and to be sacrificed after 4 h of the last dosage.

Rats were maintained in isocalorific diet using glucose solution. (Total calories per day: 508 K Cal/kg body weight). At the end of the experimental period, the rats were killed by cervical decapitation and tissues (testis) were collected for various biochemical estimations.

**Preparation of tissue homogenate:** Known amount of tissue was weighed and homogenized in appropriate buffer for the estimation of lipid peroxidative indices and enzymatic and non-enzymatic antioxidants.

**Biochemical investigation**

**Estimation of lipid peroxidative indices:** Lipid peroxidation as evidenced by the formation of TBARS was measured by the method of (Niehaus and Samuelsson, 1968). In brief, 0.1 mL of tissue homogenate
(Tris-HCl buffer, pH 7.5) was treated with 2 mL of (1:1 ratio) TBA-TCA-HCl reagent (thiobarbituric acid 0.37%, 0.25N HCl and 15% TCA) and placed in water bath for 15 min, cooled and centrifuged at room temperature for 10 min at 1,000 rpm. The absorbance of clear supernatant was measured against reference blank at 535 nm with use of Spectrophotometer.

**Determination of non-enzymatic antioxidant status**

**Estimation of reduced glutathione:** Reduced glutathione (GSH) was determined by the method of Ellman (1959). To the homogenate added 10% TCA and centrifuged. 1.0 mL of supernatant was treated with 0.5 mL of Ellmans reagent (19.8 mg of 5, 5-dithiobisnitro benzoic acid (DTNB) in 100 mL of 0.1% sodium nitrate) and 3.0 mL of phosphate buffer (0.2M, pH 8.0). The absorbance was read at 412 nm.

**Determination of superoxide dismutase, catalase and glutathione peroxidase:** Superoxide Dismutase (SOD) was assayed utilizing the technique of Kakkas (1984). A single unit of enzyme was expressed as 50% inhibition of NBT (Nitroblue tetrazodium) reduction/min/mg protein.

Catalase (CAT) was assayed colorimetrically at 620 nm and expressed as nmols of H2O2 consumed/min/mg protein as described by Sinha (1972). The reaction mixture (1.5 mL) contained 1.0 mL of 0.01M pH 7.0 phosphate buffer, 0.1 mL of tissue homogenate and 0.4 mL of 2M H2O. The reaction was stopped by the addition of 2.0 mL of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio).

Glutathione Peroxidase (GPx) activity was measured by the method described by Ellman, (1959). Briefly, reaction mixture contained 0.2 mL of 0.4M phosphate buffer pH 7.0, 0.1 mL of 10 mM sodium azide, 0.2 mL of tissue homogenate (homogenised in 0.4M, phosphate buffer pH 7.0), 0.2 mL glutathione, 0.1 mL of 0.2 M hydrogen peroxide. The contents were incubated at 37°C for 10 min. The reaction was arrested by 0.4 mL of 10% TCA and centrifuged. Supernatant was assayed for glutathione content by using Ellmans reagent.

**Statistical analysis:** All the results obtained are expressed as Means±SD of six rats in each group. Statistical evaluation was done by using analysis of variance (ANOVA) followed by Duncan’s Multiple Range Test (DMRT). The statistical significance was at p<0.05 (Duncan, 1957).

**RESULTS**

There was no significant change in body weight of the rats observed, the productive role of Sildenafil citrate (VIAGRA) on oxidative stress induced by Ethanol.

**Lipid Peroxidation (LPO) levels of the testis of control and drug treated groups after short-term (1, 2, 4 and 24 h) and long-term periods (15, 30 and 45 days) showed the LPO levels were significantly (p<0.05) increased at 1 h, 2 h, 4 h and 24 h, respectively (Table 1). During long-term periods of treatments, the LPO levels were continuously increased in significant levels from 15 days to 45 days of treatment. The highest decline was 63.53% after 45 days of treatment. The increase in the mean value of TBARS after 15, 30 and 45 treatments were 20.59, 37.06 and 63.53%, respectively, over control values (Table 2).

Reduced glutathione (GSH) levels of the testis of control and drug plus ethanol treated groups after short-term (1, 2/3, 4 and 24 h) and long-term periods (15, 30 and 45 days). The GSH levels were significantly (p<0.05) decreased at 1 h, 2/3 h, 4 h and 24 h, respectively (Table 1). During long-term periods of treatments the GSH levels were continuously decreased in significant levels from 15 to 45 days treatment. The percentage decreased over the control was 12.43, 19.77 and 30.63%, respectively, after 15, 30 and 45 days of treatments (Table 2). The GSH levels were found to be significantly (p<0.05) decreased in the testis (30.63%) of control following 45 days treated rats.

<table>
<thead>
<tr>
<th>Groups (Treatment)</th>
<th>Parameters</th>
<th>LPO</th>
<th>GSH</th>
<th>SOD</th>
<th>CAT</th>
<th>GPx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>1.7±0.11</td>
<td>10.2±0.15</td>
<td>14.3±0.21</td>
<td>38.6±0.85</td>
<td>8.17±0.18</td>
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<tr>
<td>1 h</td>
<td></td>
<td>2.2±0.11</td>
<td>8.7±0.15</td>
<td>12.2±0.19</td>
<td>41.5±0.78</td>
<td>6.06±0.16</td>
</tr>
<tr>
<td>2/3 h</td>
<td></td>
<td>2.1±0.11</td>
<td>8.9±0.15</td>
<td>12.5±0.20</td>
<td>41.4±0.75</td>
<td>6.47±0.17</td>
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<tr>
<td>4 h</td>
<td></td>
<td>1.9±0.11</td>
<td>9.1±0.13</td>
<td>12.8±0.19</td>
<td>38.5±0.86</td>
<td>6.7±0.16</td>
</tr>
<tr>
<td>24 h</td>
<td></td>
<td>1.9±0.10</td>
<td>10.4±0.15</td>
<td>13.5±0.20</td>
<td>38.7±0.86</td>
<td>7.4±0.16</td>
</tr>
</tbody>
</table>

Values are means±SD of six rats from each group. Values not sharing a common superscript letter differ significantly at p<0.05 (DMRT). p<0.05 (ANOVA) LPO-Lipid peroxidation (nmol 100 g wet tissue) GSH-Reduced Glutathione (mg g protein) SOD-Superoxide Dismutase (Enzyme concentration required for 50% inhibition of NBT reduction min mg protein)

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<td>8.17±0.18</td>
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<tr>
<td>15 D</td>
<td></td>
<td>2.0±0.10</td>
<td>8.6±0.13</td>
<td>11.8±0.17</td>
<td>41.7±0.82</td>
<td>5.89±0.17</td>
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<tr>
<td>30 D</td>
<td></td>
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<td>8.9±0.12</td>
<td>10.4±0.17</td>
<td>45.8±0.86</td>
<td>5.39±0.10</td>
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<tr>
<td>45 D</td>
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<td>9.7±0.16</td>
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<td>4.60±0.17</td>
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</table>

Values are means±SD of six rats from each group. Values not sharing a common superscript letter differ significantly at p<0.05 (DMRT).

**Table 1:** Short-term effects of a single dose of drug plus ethanol administration on lipid peroxidation, enzymes antioxidant and non-enzymes antioxidant in the testis of Albino rat

**Table 2:** Long-term effect of continuous dose of drug plus ethanol administration (single dose per day) on lipid peroxidation, enzymes antioxidant and non-enzyme antioxidant in the testis of Albino rat

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SOD levels of the testis during short-term period activity was decreased to 14,82, 12,46, 10,37 and 5,85%, respectively at 1, 2/, 4 and 24 h after treatment and in the same experiment, the SOD activity was reached to a control level (Table 1). Whereas, in long-term treatment, the SOD activity was declined significantly to (p<0.05) 17.33, 27.07 and 32.22%, respectively, after 15, 30 and 45 days of treatment (Table 2).

The treatment with drug plus ethanol caused a significant (p<0.05) increased in the activity of Catalase(CAT) in rat after short and long-term periods. During short-term period, it was increased to 7.90, 7.22 and 3.18% after 1, 2/ and 4 h, respectively. Whereas, after 24 h, the level was showed only minimal variation from control value (Table 1). The percentage increased after long-term treatment over the control group was found to be 8.00, 12.61 and 20.77 % for 15, 30 and 45 days, respectively (Table 2).

Glutathione Peroxidase (GPx) activity in testis of the control and drug plus Ethanol treated rat under short-term (1, 2/, 4 and 24 h) and long-term periods (15, 30 and 45 days) are shown in Table 1 and 2. GPx levels of the testis during short-term period showed decreased activity of about 26.56, 20.81, 17.50 and 9.30 %, respectively after 1, 2/, 4 and 24 h. Whereas in long-term treatment, the GPx activity was declined significantly to (p<0.05) 27.91, 34.03 and 43.70%, respectively, after 15, 30 and 45 days of treatment.

DISCUSSION

Sildenafil citrate has been used for the treatment of erectile dysfunction by many patients. The cardiovascular disease has resulted due to cardiovascular properties of the drug (Gillies et al., 2002). Ethanol abuse is a major cause of health problem and a public health issue (Choi et al., 1998). Several studies have shown that the incidence of alcoholism and the mortality of patients with alcoholic liver disease have been increasing in Korea (Kweon et al., 1998). It has been observed from that present study that chronic ingestion of Sildenafil citrate plus Ethanol were significantly depleted LPO levels in testis of rat. The LPO is determined by the balance between the production of oxidants and the removal and scavenging of those oxidants by antioxidants (Filio, 1996; Halliwell, 1987; Lopez Torres et al., 1993). The malondialdehyde (MDA) content, a measure of lipid peroxidation, was assayed in the form of Thiobarbituric Acid Reacting Substances (TBARS) by method of (Okhawa et al., 1979). The resulting free radicals damaged the testis through a peroxidative mechanism. Earlier studies in this lab have demonstrated a defective metabolism of lipid peroxides in other tissues of diabetic animals (Prince et al., 1998; Venkateswaran and Pari, 2002). It is evident from the present study that the chronic ingestion of Sildenafil plus Ethanol were significantly increased MDA levels (an end-product of lipid peroxidation) in testis. Lipid peroxidation can be used as an index for measuring the damage that occurs in membranes of tissue as a result of free radical generation (Dianzani, 1985; Husain and Somani, 1997). The enhanced lipid peroxidation in the kidney after ingestion of ethanol consistent with findings of other who has repeated that increased MDA levels (Orellana et al., 1998).

Further, it has been observed from the present study that the chronic ingestion of Sildenafil plus Ethanol has significantly decreased the GSH contents in the testis of Albino rat, the GSH is highly prone to oxidative damage caused by enhanced ROS free radicals and GSH depletion (DeLeve and Kalpolwitz, 1991). Marselos et al. (1991) has reported that the effect of nicotine on the body can influence the enzymes of ethanol metabolism. It is quite possible that the combination of drug plus ethanol induced excessive generation of ROS free radicals to a greater extent than that of ingestion of ethanol or administration of drug alone.

It has been shown that the chronic ingestion of Sildenafil plus Ethanol has significantly decreased SOD activity in the testis tissue of treated rat. Generally, Superoxide dismutase catalyzed to scavenge excess superoxide anions and convert them to H_2O_2 (Husain and Somani, 1997). Biphasic fluxes of SOD activities are common and an increase or decrease may relate to the presence of excess superoxides (Bondy, 1992). Inhibition of SOD activity in the testis tissues may be a consequence of decreased de novo synthesis of SOD protein or irreversible inactivation of enzyme proteins from increased free radical production resulting from ethanol metabolism (Santiard et al., 1995). Jenkins and Goldfarb (1993) have reported that decreased SOD activity reflects oxidative stress. Similar results have been reported from the present study that Sildenafil citrate plus Ethanol treated rat than control rat.

In contrast, the chronic ingestion of Sildenafil plus Ethanol has significantly increased CAT activity in the testis of treated rat. The primary role of CAT is to scavenge H_2O_2 that has been generated by free radicals or by SOD in removal of superoxide anions and to convert it to water (Ribiere et al., 1992). Catalase also has a secondary role in the metabolism of ethanol (Rubin, 1993). Therefore, it is inferred from the present study that the increase in testis CAT activity may be related to excess H_2O_2 production resulting from ethanol metabolism or SOD inhibition. An increased in CAT activity in the testis.
would indicate enhanced ethanol tolerance by these particular tissues. However, increased CAT activity in testis after chronic exposure to Sildenafil plus Ethanol are indicative of an efficient elimination of toxic H$_2$O$_2$ from the respective tissue.

In addition, it has been observed from the chronic ingestion of Sildenafil plus Ethanol from the present study that the GPx activity was significantly decreased in the testis of treated rat than control rat. Glutathione peroxidase works nonspecifically to scavenge and decompose excess hydroperoxides, including H$_2$O$_2$, which may be prevalent under oxidative stress (Chen et al., 1995; Nordmann and Rouach, 1996; Somani, 1996). The decreased GPx activity seems to indicate the susceptibility of testis to Sildenafil plus Ethanol induced oxidative stress.

It may be inferred from the present finding that the combination of chronic doses of Sildenafil plus Ethanol exerted similar, and in some cases additive, effects on the antioxidant defense system and caused oxidative tissue injury, which may be associated with an increased in flux of ROS free radicals after metabolism of Sildenafil and Ethanol.

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