Effects of sildenafil citrate on torsion/detorsion-induced changes in red blood cell and plasma lipid peroxidation, antioxidants, and blood hematology of male rats

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A B S T R A C T

Objective: The aim of this work was to determine effects of intrapertoneally-administered sildenafil citrate (SC) for prevention testicular injury after unilateral testicular torsion/detorsion (T/D) in rats on red blood cell (RBC) and plasma lipid peroxidation, antioxidants and blood hematology.

Study design: Thirty seven adult male wistar albino rats were divided into four groups: sham operated (group 1), T/D + saline (group 2), T/D + 0.7 mg SC (group 3) and T/D + 1.4 mg SC (group 4). Testicular torsion was created by rotating the right testis ‘720°’ in a clockwise direction for 2 h in all the groups, except for group 1.

Results: Our results showed that that testicular injury significantly induced erythrocyte reduced glutathion (GSH) (p < 0.05), malondialdehyde (MDA) in RBC (p < 0.01) and plasma (p < 0.05) and blood lymphocyte (p < 0.01) counts.

Administration of low dose SC led to significantly increase in the levels of RBC GSH (p < 0.05), plasma paraoxonase (PON1) (p < 0.01), nitric oxide (NO) (p < 0.01) and blood lymphocyte counts (p < 0.01), but to decreases in the levels of MDA in plasma and RBC, blood mean corpuscular volume (MCV) (p < 0.05) and eosinophil counts (p < 0.05). Treatment with high dose SC caused a significantly increase in PON1, vitamin E and β-carotene in plasma, levels of GSH in RBC and blood lymphocyte counts. On the other hand, results showed that high dose sildenafil significantly decreased plasma and RBC MDA levels. Total tissue damage scores of the group 2 were significantly higher than group 1 and 3.

Conclusion: Low dose SC appears to be beneficial in reducing the effects of injury to the testicular torsion.

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1. Introduction

Testicular torsion has been implicated in testicular injury and infertility. Testicular injury is proportional to the duration and degree of torsion. A possible cause of the testicular damage due to T/D is an ischemic/reperfusion (I/R) injury attributed to neutrophil infiltration and reactive oxygen species (ROS). Excess ROS and their toxic products cause DNA damage, lipid peroxidation in the cellular and mitochondrial membranes, resulting in cellular damage [1]. However, the protective role of enzymatic antioxidants such as catalase (CAT), and GPx and non-enzymatic antioxidants such as vitamin E, A and GSH against free radical attack is balanced under normal conditions [2]. It has been shown that sildenafil increases the activity of antioxidant enzymes, and decreases MDA level after spinal cord and testicular injury in rats [3–5]. Sildenafil may inhibit the production of lipid peroxidation via the activity and expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [5]. Some researchers reported that sildenafil and other phosphodiesterase inhibitors may have anti-inflammatory properties via inhibition of ROS, leukocyte infiltration [6] and inflammatory cytokines such as IL-1, IL-6 and TNF [7]. Nitric oxide (NO) causes an increase in cyclic guanosine monophosphate (cGMP), which results in relaxation of the smooth muscle, creating an increased blood flow [8]. It was shown that NO is also a potent inhibitor of NADPH oxidase expression, which in turn reduces superoxide formation [8,9]. Sildenafil is a PDE-5 inhibitor that augments the action of NO by preventing the hydrolysis of cGMP [10,11].

There is limited information about whether SC has any effects on MDA and antioxidant enzyme metabolism in plasma and RBC, although its effects on NO metabolism are well known. Therefore, in the present study, we investigated the protective effect of SC on the biochemical and hematological changes after testicular I/R injury.

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2. Materials and methods

The guidelines for the care and use of the animals approved by the local institution were followed. The local ethics committee approved this study. A total of 37 male Wistar Albino rats, weighing between 220 and 250 g were housed in a climate-controlled animal care facility, with a 12 h light/dark cycle. Before surgical procedures, the animals were provided with standard rat chow and water, ad libitum.

The rats were anaesthetized with single intramuscular injection of 85 mg/kg ketamine hydrochloride (Parke-Davis, Ketalar, 50 mg/ml) and 6 mg/kg xylazine hydrochloride (Bayer, Rompun, 23.32 mg/ml). Following anesthesia, the skin of scrotal area was shaved and antisepsis was obtained by 10% povidone iodine solution.

The rats were distributed into 4 groups. The scrotum was entered through a midline incision. In the all groups except for the sham operated group, torsions were created by rotating the right testis 720° in clockwise direction for 2 h. The torsion was maintained by fixing the testis in the scrotum with a 4–0 silk suture, and the incision was closed. After a 2 h torsion period, the incision was entered, the suture was removed, and the right testis was detorted and replaced into the scrotum for 2 h. In the sham operated group (Group 1), the testis was taken out through the incision line and replaced and a silk suture was placed through the tunica albuginea.

Group 2 (T/D + saline, n = 10) was used as control group. Saline (2 ml of 0.9% NaCl) was injected intraperitoneally 1 h before detorsion.

In group 3 (T/D + 0.7 mg/kg low dose SC group, n = 10), treatment with intraperitoneal SC (Deva Holding A.S., Degra 50 mg), 2 ml from 0.7 mg/kg dissolved in 0.9% NaCl, was carried out 1 h before the detorsion.

In group 4 (T/D + 1.4 mg/kg high dose SC group, n = 10), treatment with intraperitoneal SC, 2 ml from 1.4 mg/kg dissolved in 0.9% NaCl, was carried out 1 h before the detorsion. The torsion lasted for 2 h followed by a detorsion period of 2 h.

2.1. Blood collection and preparation of blood samples

Just before the termination of the study, blood samples (4–6 ml) was taken from the vena porta puncture with a sterile injector with added EDTA, and placed into tubes, protected against light. Then bloods were separated into plasma and RBC by centrifugation at 1800 × g for 10 min.

2.2. Biochemical and hematology assay

Lipid peroxidation levels in plasma and haemolyzed RBC were measured with the thiobarbituric-acid reaction by the method of Placer et al. [12]. The methods of Goth [13] were used for the determination of CAT activities in haemolyzed RBC. The GSH content in RBC was measured at 412 nm on the spectrophotometer using the method of Sedlak and Lindsay [14]. GPx activity in RBC was measured at 37 °C and 412 nm according to Lawrence and Burk [15]. Total SOD activity was determined according to the method of Sun et al. [16]. The protein content in the plasma and haemolyzed RBC was measured by the method of Lowry et al. [17]. Vitamins A and E were determined in frozen plasma samples by a modification of the method described by method of Desia [18]. The levels of beta-carotene in plasma samples were determined according to the method of Suzuki and Katoh [19]. PON1 activity was measured using diethyl-p-nitrophenylphosphate as a substrate as previously described by Furlong et al. [20]. The nitric oxide content of the plasma was determined according to the method of Cortas and Wakid [21]. Hematological values were determined according to the standard procedures [22].

2.3. Histopathologic evaluation

Testicular tissues were fixed in Bouin’s solution and then embedded in paraffin. Serial sections were cut using a microtome at a thickness of 5 μm, and the sections were stained with

Fig. 1. (A) Normal testis morphology in group 1 (H&E, 400×); (B) severe capillary congestion, interstitial oedema and hemorrhage, and moderate degeneration in germinal cells in the testis of group 2 (H&E, 200×); (C) mild capillary congestion, interstitial oedema, hemorrhage and degeneration in germinal cells in the testis of group 3 (H&E, 200×); (D) severe capillary congestion, hemorrhage, and moderate interstitial oedema and degeneration in germinal cells in the testis of group 4 (H&E, 200×).
haematoxylin and eosin. The histopathologic sections were examined for the presence of degeneration in germinal cell, interstitial oedema, capillary congestion and hemorrhage under a microscope and were photographed (Fig. 1A–D). Five microscopy fields were used to determine the presence or severity of testicular tissue damage. Degeneration in germinal cell, capillary congestion, hemorrhage, and interstitial oedema were graded on a scale of mild (+), moderate (++) and severe (+++). Examination and scoring of the testis sections were performed in a blinded fashion by the same pathologist. The scores for each parameter were summed and the total tissue damage scores calculated.

2.4. Statistical analysis

Total tissue damage score data were analyzed using the Kruskal–Wallis test. Biochemical and hematological data were analyzed using one-way Analysis of Variance (ANOVA). The Duncan test was performed for multiple comparisons using the SPSS 11.0 for Windows. The data were expressed as means ± standard errors (SEM). Differences with p < 0.05 were considered statistically significant.

3. Results

3.1. Biochemical and hematological results

The levels of GSH in the RBC obtained from group 2 were significantly lower (p < 0.05) and the levels of MDA in RBC (p < 0.01) were significantly higher than those of group 1. Treatment with low and high dose SC significantly increased RBC GSH levels (p < 0.05), while treatment caused to decrease the levels of RBC MDA (p < 0.01) (Table 1).

Plasma NO levels obtained from group 2 were significantly lower (p < 0.05) and the levels of MDA in plasma (p < 0.05) were significantly higher than those of group 1. Plasma NO (p < 0.01) values, plasma PON1 activity (p < 0.01) in group 3 and 4 were significantly higher than those in group 1 and 2. Plasma vitamin E in group 4 were higher (p < 0.01) than those in group 1 and 2. Plasma β-carotene in group 4 were higher (p < 0.01) than those in group 1, 2 and 3. The plasma MDA (p < 0.05) in group 3 and 4 were significantly lower than those in group 2 (Table 2).

Lymphocyte counts in group 2 were significantly higher (p < 0.01) than those of group 1. Treatment with low dose SC significantly increased lymphocyte counts (p < 0.01), while treatment caused to decrease MCV values (p < 0.05) and eosinophil counts (p < 0.01). Lymphocyte counts (p < 0.01) in group 4 were higher than those in group 2 (p < 0.01).

### Table 1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group 1 (n = 7)</th>
<th>Group 2 (n = 10)</th>
<th>Group 3 (n = 10)</th>
<th>Group 4 (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT (k/g Hb)</td>
<td>81.21 ± 2.31</td>
<td>81.85 ± 3.44</td>
<td>83.39 ± 9.77</td>
<td>92.29 ± 13.03</td>
</tr>
<tr>
<td>GSH (µmol/ml)</td>
<td>1.17 ± 0.01a</td>
<td>1.04 ± 0.05</td>
<td>1.15 ± 0.03b</td>
<td>1.31 ± 0.01b</td>
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<tr>
<td>GPx (U/g protein)</td>
<td>77.60 ± 2.36</td>
<td>84.52 ± 3.29</td>
<td>86.07 ± 3.07</td>
<td>92.41 ± 3.52</td>
</tr>
<tr>
<td>MDA (nmol/ml)</td>
<td>10.76 ± 0.28c</td>
<td>13.66 ± 0.27</td>
<td>12.04 ± 0.12d</td>
<td>12.26 ± 0.07d</td>
</tr>
<tr>
<td>SOD (U/g Hb)</td>
<td>1091.97 ± 76.00</td>
<td>1077.77 ± 95.42</td>
<td>1049.87 ± 33.32</td>
<td>1180.07 ± 46.10</td>
</tr>
<tr>
<td>Total tissue damage score</td>
<td>0.29 ± 0.18e</td>
<td>2.60 ± 0.16</td>
<td>1.40 ± 0.13f</td>
<td>2.30 ± 0.15</td>
</tr>
</tbody>
</table>

* Statistically different from group 2, 3 and 4 (p < 0.05).
* Statistically different from group 1 and 2 (p < 0.05).
* Statistically different from group 2, 3 and 4 (p < 0.01).
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### Table 2

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group 1 (n = 7)</th>
<th>Group 2 (n = 10)</th>
<th>Group 3 (n = 10)</th>
<th>Group 4 (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E vit (mmol/L)</td>
<td>6.68 ± 0.72</td>
<td>8.55 ± 0.75</td>
<td>9.40 ± 0.72</td>
<td>10.42 ± 0.66a</td>
</tr>
<tr>
<td>A vit (µmol/L)</td>
<td>0.83 ± 0.09</td>
<td>0.84 ± 0.10</td>
<td>0.88 ± 0.07</td>
<td>0.99 ± 0.17</td>
</tr>
<tr>
<td>β-carotene (µmol/ml)</td>
<td>40.13 ± 10.01</td>
<td>50.84 ± 10.20</td>
<td>40.49 ± 10.30</td>
<td>96.28 ± 9.69b</td>
</tr>
<tr>
<td>MDA (nmol/ml)</td>
<td>1.19 ± 0.01</td>
<td>1.78 ± 0.06c</td>
<td>1.06 ± 0.11</td>
<td>1.13 ± 0.04</td>
</tr>
<tr>
<td>NO (µmol/L)</td>
<td>30.08 ± 0.10d</td>
<td>32.05 ± 0.07</td>
<td>31.38 ± 0.12</td>
<td>31.38 ± 0.12</td>
</tr>
<tr>
<td>Paraoxonase (IU/L)</td>
<td>33.25 ± 5.16</td>
<td>38.17 ± 6.09</td>
<td>66.17 ± 7.06d</td>
<td>55.80 ± 2.00d</td>
</tr>
</tbody>
</table>

* Statistically different from group 1 and 2 (p < 0.01).
* Statistically different from group 1 and 2 (p < 0.01).
* Statistically different from group 1, 3 and 4 (p < 0.05).
* Statistically different from group 2, 3 and 4 (p < 0.01).
* Statistically different from group 3 and 4 (p < 0.01).
* Statistically different from group 1 and 2 (p < 0.01).
**Table 3**

Erythrocyte and total and differential leukocyte counts in all the groups (mean ± SE).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group 1 (n = 7)</th>
<th>Group 2 (n = 10)</th>
<th>Group 3 (n = 10)</th>
<th>Group 4 (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (10^6/μl)</td>
<td>7.59 ± 0.94</td>
<td>7.73 ± 0.33</td>
<td>7.25 ± 0.81</td>
<td>7.28 ± 0.7</td>
</tr>
<tr>
<td>WBC (10^3/μl)</td>
<td>6.31 ± 0.66</td>
<td>6.30 ± 0.46</td>
<td>6.28 ± 0.86</td>
<td>6.26 ± 0.76</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>13.58 ± 0.25</td>
<td>13.36 ± 0.10</td>
<td>13.96 ± 0.10</td>
<td>13.73 ± 0.12</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>39.83 ± 0.60</td>
<td>39.87 ± 0.66</td>
<td>39.20 ± 0.58</td>
<td>39.83 ± 0.70</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>57.80 ± 1.16</td>
<td>60.69 ± 3.97</td>
<td>54.07 ± 0.89</td>
<td>55.90 ± 1.32</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>19.70 ± 0.42</td>
<td>21.26 ± 1.79</td>
<td>19.25 ± 0.24</td>
<td>19.54 ± 0.22</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>34.14 ± 0.42</td>
<td>34.82 ± 0.57</td>
<td>35.63 ± 0.49</td>
<td>34.55 ± 0.90</td>
</tr>
<tr>
<td>Neu (%)</td>
<td>29.00 ± 0.51</td>
<td>28.37 ± 0.41</td>
<td>28.00 ± 0.54</td>
<td>27.66 ± 0.55</td>
</tr>
<tr>
<td>Eos (%)</td>
<td>3.16 ± 1.10</td>
<td>2.50 ± 0.18</td>
<td>1.80 ± 0.20</td>
<td>1.83 ± 0.30</td>
</tr>
<tr>
<td>Baso (%)</td>
<td>0.50 ± 0.22</td>
<td>0.62 ± 0.18</td>
<td>0.80 ± 0.22</td>
<td>0.50 ± 0.20</td>
</tr>
<tr>
<td>Lyn (%)</td>
<td>62.16 ± 0.98</td>
<td>65.87 ± 0.57</td>
<td>67.20 ± 0.20</td>
<td>67.66 ± 0.71</td>
</tr>
<tr>
<td>Mono (%)</td>
<td>2.64 ± 0.17</td>
<td>2.62 ± 0.26</td>
<td>2.40 ± 0.24</td>
<td>2.33 ± 0.33</td>
</tr>
</tbody>
</table>

- a Statistically different from group 1 and 2 (p < 0.05).
- b Statistically different from group 1 and 2 (p < 0.05).
- c Statistically different from group 2, 3 and 4 (p < 0.01).
- d Statistically different from group 3 and 4 (p < 0.05).

4. Comments

MDA is a secondary product of oxidative stress formed during lipid peroxidation and is significantly increased by testicular I/R damage [3]. Perk et al. [23] have found that SC significantly increased the level of GSH and activities of SOD and CAT and slightly decreased level of MDA at 6 and 24 h in the blood of healthy men, but the activity of GPx did not change. They suggested that the antioxidant effect of SC may be via inhibition of phosphodiesterase. The increase in red blood cell CAT values after SC treatments has been attributed to the increase of free radicals and lipid peroxidation [6]. It has been shown that sildenafil and vardenafil increases SOD, GSH-Px, and CAT activities and decreases MDA level in testicular T/D injury in rats [3,4]. Another PDE-5 inhibitor, tadalafil, increases activities of the antioxidant enzymes, such as SOD, CAT and GSH-Px, and decreases MDA level after spinal cord injury in rats [5] and ovaries I/R injury [10]. It has been suggested that the level of GSH in erythrocyte at postoperative day 3 in the ischemic anastomosis rats was significantly elevated by SC therapy [8]. In our study, RBC and plasma MDA levels were significantly higher and RBC GSH level was significantly lower in group 2 compared with the other groups. Intraperitoneal administration of low and high SC after ischemia increased the level of RBC GSH significantly, increased RBC CAT and GPx activities slightly, but insignificantly, and decreased the level of RBC and plasma MDA significantly when compared with group 2. This decrease in group 2 may have occurred as a result of enzyme consumption by ROS during oxidative stress. The decrease in MDA level and increase in these enzymes after SC administration suggest a protective effect of SC on testicular tissue owing to its antioxidant effects. SC appear to have inhibitory effects on superoxide radical production in mitochondria by enhancing cGMP level and by inhibiting the expression of nicotinamide adenine dinucleotide phosphate NADPH oxidase [6]. If the activity and expression of NADPH oxidase are inhibited, then the formation of ROS will decrease and the amount of antioxidant enzymes will increase [23].

Previous studies [3,4] have shown that administration of sildenafil and vardenafil during perifusion reduced apoptotic cells and testicular necrosis. In another study, SC treatment prevented deterioration of renal function, reduced histological damage, inflammation and apoptosis, and preserved renal capillary integrity in a renal model [24]. Histopathological examination revealed increased degeneration in germinal cells, capillary congestion and interstitial edema, and hemorrhage in group 2, which were ameliorated by low dose SC (Fig. 1C). These findings show that low dose SC reduced total tissue damage score in the testis of group 3. This results is in agreement with those of du Toit et al. [25] indicating that the protective properties of SC at a low dose are due to cGMP-elevating and cAMP suppressing effects in the ischaemic hearts of rats. However, in the present study, administration of high dose SC to rats in group 4 did not have a protective effect on testicular I/R injury when compared with group 2. Similarly, in a study [26], no beneficial actions on left ventricular function were evident after a high dose of SC (1.4 mg/ kg) administration 30 min before myocardial ischemia to rats. In addition, Reffelman and Klener [27] have failed to show a decrease in myocardial necrosis following I/R in the rabbit using a dose of 1.45 mg/kg of SC. The ineffectiveness of high dose SC on histopathological changes may be related to their cAMP elevating effects in the ischaemic testis. Another possibility is that the high dose SC led to exacerbation of I/R injury, as suggested by the poorer reperfusion mechanical function [25]. Consistently, Elrod et al. [28] presented evidence that low dose sildenafil was most effective, reducing infarct by 50% compared with vehicle-treated animals, high dose sildenafil was ineffective.

NO has an important role in modulating tissue injury and blood flow in normal and several pathological conditions. Altered NO levels in spinal cord and ovary tissue injuries have also been reported [5,10]. Our results indicated that plasma NO level after testicular T/D was significantly decreased in group 2 compared with group 1. A decrease in plasma NO level after T/D might be due to consumption of NO by superoxide anion. Superoxide anion reacts with NO to form peroxynitrite, which is known to cause tissue injury and alterations in vascular tone [6]. Sildenafil has a potent inhibitory effect on the formation of superoxide anion through a reduction of both NADPH oxidase activity and expression. The resultant decrease in endogenous superoxide anion would in turn increase the bioavailability of NO [6,11]. Several studies have shown that PDE-5 inhibitors (sildenafil, vardenafil and tadalafil) could increase tissue and blood NO levels in rats with ovary injury [10] and spinal cord injury [5]. In our study, plasma NO levels in group 3 and group 4 were increased after low and high dose SC when compared with group 2. This effect of PDE-5 inhibitors augment the action of NO by preventing the hydrolysis of cGMP, and NO inhibits the expression of NADPH oxidase, which in turn reduces the I/R injury in the testis [6,11].

PON-1 is a high-density lipoprotein-associated antioxidant enzyme with paraoxonase, arylesterase and diaxozanone activities. Also, human serum paraoxonase activity is a strong predictor of coronary artery disease and it is reported to have a significant effect on the occurrence of myocardial infarction [29]. Verit et al. [30] reported that administration of tadalafil citrate to patients with erectile dysfunction exerts a beneficial acute effect on the cardiovascular system by reducing serum levels of oxidative stress and increasing serum levels of total antioxidants and PON1. In the
current study, in group 3 and 4 treatment with the low and high dose SC before detorsion increased plasma PON1 levels when compared with group 1 and 2. These findings show that increased plasma PON1 activity in rats after SC administration is associated with decreased oxidative stress, suggesting a protective role for PON1 against oxidative stress in testicular I/R injury rats. Rozenberg et al. [31] suggested a protective role for the antioxidant enzyme PON1 against streptozotocin-induced diabetes development.

The plasma vitamin E and β-carotene levels were significantly higher in group 4 than those in groups 1 and 2. There is a synergistic effect between vitamin E and SC. Vitamin E enhanced the therapeutic effect of the PDE5 inhibitor. Vitamin E combined with SC enhanced erectile function better than either vitamin E or SC alone in diabetic patients [32]. In the literature, there were no reports about the effects of SC on the plasma levels of vitamin A, E and β-carotene. Therefore, these parameters were not a matter of dispute. However, the increased plasma vitamin E and β-carotene levels in the group 4 may have occurred as a result of antioxidant effect of SC on free radical inhibition.

In the present study, low dose SC administered in group 3 resulted in decline of eosinophil counts and MCV values when compared with group 2, and no any changes other blood parameters. In the rats treated with high dose SC the lymphocyte counts were significantly higher in comparison to groups 1 and 2, while other blood parameters were not significantly different. Treatment with SC in patients with diabetes did not have any effects on hemoglobin, hematocrit values and eosinophil and leucocyte counts [33]. Little et al. [34] reported that total hemoglobin (Hb), mean corpuscular volume (MCV), white blood cell count (WBC), mean corpuscular hemoglobin concentration (MCHC) did not change in adults with sickle cell disease after sildenafil treatment.

In conclusion, the results of the present study suggest that the effects of SC on histopathological recovery in the tests after I/R injury depended on the dose. Beneficial effects were evident after treatment with low dosage, but were lost after doubling the dose. Whereas, the beneficial effects of low and high dose SC treatments were demonstrated by the changes of biochemical parameters.

References