Quinestrol Treatment Induced Testicular Damage via Oxidative Stress in Male Mongolian Gerbils (*Meriones unguiculatus*)

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Abstract: The hypothesis that quinestrol exerts testicular damage via oxidative stress was investigated in male gerbils using a daily oral gavage of 3.5 mg/kg body weight for 2 weeks (the multidose-treated group) or 35 mg/kg body weight (the single-dose-treated group). The testicular histological morphology, antioxidant capacity and malondialdehyde (MDA) concentration in testicular tissue and plasma were assessed at 15, 30, and 60 days following treatment. The results showed that the activity of the antioxidant enzymes, including superoxide dismutase (SOD) and glutathione peroxide (GSH-Px), and total antioxidant capacity (T-AOC), at 15 days after treatment in testicular tissue decreased, which led to the MDA concentration increasing while at the same time germ cells were rarefied and showed an irregular distribution in seminiferous tubules of quinestrol-treated gerbils. At 30 days, the testicular weight and antioxidant capacity continued to decrease, while the MDA concentration continued to increase, and testicular histopathological changes were more pronounced. Single-dose and multidose drug treatment had a similar effect on the antioxidant enzymes and MDA, but testicular damage was relatively severe at 15 and 30 days after multidose treatment. By 60 days of treatment withdrawal, however, the above parameters recovered to control levels. The results show that quinestrol causes reversible damage to gerbil testes that might be caused by the oxidative stress and that multidose treatment has more effects on testicular damage compared with one-dose treatment.

Key words: Mongolian gerbil (*Meriones unguiculatus*), oxidative damage, quinestrol, reversibility, testicular damage

Introduction

Levonorgestrel-Quinestrol (Code: EP-1) was first used as a new type of rodenticide by Zhibin Zhang of the Institute of Zoology, Chinese Academy of Sciences [31]. Subsequently, both laboratory and field experiments showed that EP-1 and its individual components, levonorgestrel and quinestrol, affected the structure of reproductive organs and exerted antifertility effects in many wild rodent species, such as the greater long-tailed...
hamster (*Tscherskia triton*) [32, 33], Mongolian gerbils (*Meriones unguiculatus*) [13, 14] and the Djungarian hamster (*Phodopus campbelli*) [30]. According to Meirong Zhao (2007), quinestrol is an effective inducer of EP-1 in male reproductive damage [34].

Quinestrol (17-alpha ethinylestradiol-3-cyclopentyl ether), a derivative of ethinylestradiol, is a long-acting synthetic estrogen. Its estrogenic activity is about 8 to 10 times that of its matrix, and it has strong antifertility effects (http://en.wikipedia.org/wiki/Quinestrol). Animal experiments show that after oral administration, quinestrol is absorbed by the gastrointestinal tract and stored in the adipose tissue; then it is gradually released into the bloodstream at low levels to maintain a long-lasting effective concentration [19, 20].

Quinestrol affects gonadotropin secretion via inhibition of the hypothalamus-pituitary-ovarian axis. Quinestrol-treated females are infertile with abnormal folliculogenesis. Recently, much attention has been focused on oxidative stress as a causal factor of male infertility [3]. Reactive oxygen species (ROS) are produced by aerobic cellular metabolism and can damage normal physiological functions of biological macromolecules such as the plasma membrane, carbohydrates and proteins. Different concentrations of ROS can induce apoptosis or cell necrosis [21]. The testicular tissue and sperm plasma membrane, which is rich in polyunsaturated fatty acids, are highly susceptible to ROS damage [6]. ROS and lipid peroxidation (LPO) are known to be the vital mediators in testis physiology [17, 29]. SOD and GSH-PX are the most important antioxidant enzymes to protect cells from oxidative stress [9]. Quinestrol is known to cause infertility in males by inducing testicular damage [13, 30–34]. However, as far as we know, few studies have examined whether quinestrol induces infertility involving oxidative stress damage and the reversibility of such damage. Thus, the aims of the present study were to test the hypothesis that quinestrol exerts its toxicity in the testis by causing oxidative damage and to test its reversibility.

Here, we evaluated the testicular toxicity of quinestrol in male gerbils by administration of two different doses. The dose levels of this experiment were based on the results of our preparative study in gerbils. Specifically, we examined (1) the effect of quinestrol on testicular weight as well as histological changes in testes and (2) the effect of quinestrol on the levels of MDA, SOD, GSH-Px, and T-AOC in vivo.

### Materials and Methods

#### Animals and quinestrol treatment

Forty-five adult male gerbils (4–6 months old, average weight 56.05 ± 7.94 g) captured from farmland in Kangbao County, Hebei Province, were used in this study. The animals were housed in individual polypropylene cages with a 12-h light: 12-h dark cycle at 20 to 21°C. All animals had *ad libitum* access to corn, sunflower seeds, and water in glass bottles with rubber stoppers and were provided with cabbage every three days. After 1 week of acclimation, they were randomly divided into 3 groups (15 animals per group), including the control group, the single-dose-treated group (the SDT group), and multi-dose-treated group (the MDT group). There was no significant difference in the body weights of the animals in each group ($F_{(2, 42)} = 0.087, P > 0.05$). The use of animals in this study was approved by the Chinese Association for Laboratory Animal Sciences. According to the results of our a preparative study, administration of single peanut oil or multiple of peanut oil has no effect on the body weight and reproductive organs of gerbils. So the control group was given 35 mg of peanut oil/kg body weight only once. The MDT group was given 3.5 mg of quinestrol/kg body weight for 5 consecutive days and then for another 5 days after a 2-day interval. The SDT group was given 35 mg of quinestrol/kg body weight only once. Five animals per group were collected for analysis at 15, 30, or 60 days after the first of treatment. The males were weighed, and blood samples were collected from the infraorbital venous plexus. After centrifugation, the serum was stored at −20°C until the antioxidant enzyme analysis. After sacrificing the animals by cervical dislocation, the right and left testes were immediately collected and weighed. Relative testicular weights were expressed as the weight of the testes (g)/body weight (kg). The right testis of each gerbil was immediately homogenized in ice-cold PBS (pH 7.4). The homogenate was centrifuged at 3,000 × g for 15 min, and the supernatant was collected to assay for antioxidant enzymes.
**Histology analysis**

The left testes were fixed in 2.5% glutaraldehyde-paraformaldehyde solution for 24 h. After fixing, the tissues were dehydrated in ethanol and embedded in paraffin. Five-micron sections were collected on glass slides, and routine hematoxylin and eosin (HE) staining was performed.

**Assessment of antioxidant activity**

GSH-Px activity was measured according to the method of Annamarie Drotar [6] using glutathione as the substrate. GSH-Px could promote the reaction of hydrogen peroxide (H$_2$O$_2$) and glutathione (GSH) to H$_2$O and oxidized glutathione (GSSG). The decrease rate of GSH could be used to express GSH-Px activity. GSH content was assayed according to the formation rate of 5-thio-2-nitrobenzoic acid (TNB) reduced from DTNB with 412-nm absorbance. The assays for total SOD were based on the ability to inhibit the oxidation of oxyamine by the xanthine–xanthine oxidase system [22]. The red product (nitrite) produced by the oxidation of oxyamine had absorbance at 550 nm. The lipid peroxidation products (measured as MDA) were measured by the thiobarbituric acid (TBA) colorimetric method [25], and optical density was measured at 532 nm. T-AOC levels were based on the ability to deoxidize Fe$^{3+}$ to Fe$^{2+}$, and optical density was measured at 520 nm. The protein concentration was determined using a standard commercial kit. Values were expressed as units (U) per mg protein for SOD, GSH-Px, T-AOC, and nmol/mg protein for MDA. Determinations and procedures were performed according to the recommended procedures provided by the commercial kits (Jiancheng Institute of Biotechnology, Nanjing, China).

**Statistics**

The differences between various groups were analyzed using one-way analysis of variance (ANOVA) by a multivariate comparison procedure using SPSS 12.0 software. The differences between two groups were analyzed using the Student’s t-test. Results were considered to be statistically significant at $P<0.05$. The data are expressed as means±SD.

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**Results**

**Gross changes in testes**

At 15 and 30 days after quinestrol treatment, the testes were significantly atrophic. The relative testicular weights of each gerbil of the SDT and MDT groups at 15 days were 6.704 ($P<0.001$) and 6.180 ($P<0.001$), respectively, compared with that of the control gerbils (17.011, Fig. 1, Table 1). At 30 days, the relative testicular weights were significantly different between the MDT (4.368) and SDT groups (8.815, $P<0.05$). At 60 days, the testicular morphology and size were restored such that they were similar to those of the control group (Fig. 1, Table 1). Therefore, quinestrol had a significant effect on testes, and its damage to the testes in the MDT group was more severe than that of the SDT group at 30 days. However, quinestrol-induced damage was reversed upon drug withdrawal.

**Histological changes in seminiferous tubules**

In the control group, the structure of the testis was

<table>
<thead>
<tr>
<th>Testicular weight (g)*</th>
<th>Days</th>
<th>15</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDT 0.418 ± 0.213a</td>
<td>0.520 ± 0.261d</td>
<td>0.863 ± 0.229b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDT 0.330 ± 0.268a</td>
<td>0.244 ± 0.061c</td>
<td>0.817 ± 0.168b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CK 0.940 ± 0.321b</td>
<td>0.924 ± 0.201b</td>
<td>0.961 ± 0.232b</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Relative testicular weight (g/kg body weight)*</th>
<th>Days</th>
<th>15</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDT 6.704 ± 3.154a</td>
<td>8.815 ± 2.586b</td>
<td>13.532 ± 2.431b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDT 6.180 ± 3.170a</td>
<td>4.368 ± 1.356c</td>
<td>14.194 ± 1.975b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CK 17.011 ± 3.045b</td>
<td>16.513 ± 3.045b</td>
<td>15.504 ± 2.241b</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Final body weight (g)*</th>
<th>Days</th>
<th>15</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDT 57.12 ± 4.15</td>
<td>57.83 ± 4.54</td>
<td>58.21 ± 5.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDT 57.25 ± 5.34</td>
<td>58.54 ± 5.27</td>
<td>56.95 ± 6.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CK 58.54 ± 5.04</td>
<td>57.14 ± 4.15</td>
<td>57.54 ± 5.43</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Results are expressed as means±SD. The mean differences between the values bearing different superscript letters within the same column or row are statistically significant (a, b, c, d, P<0.05).
intact. The seminiferous tubules were closely arranged. The testis structure was clear, and spermatogenic cells were tightly and orderly arranged in lumen. Sperm cells were dense, and a large number of sperm were in the lumen (Fig. 2D). At 15 days, only slight pathological changes were observed in the SDT group (Fig. 2A). The diameter of the seminiferous tubules was reduced. Some spermatogenic cells in the seminiferous tubules were loosened, and the sperm count was obviously reduced. At 30 days, the damage was increased (Fig. 2B). Many spermatogenetic cells had undergone necrosis, and the seminiferous epithelium was detached from the basement membrane. The proportions of spermatogonia, spermatocytes, and sperm cells were obviously different from those of the controls. In the MDT group, the pathological changes at 30 days were much more severe, and germ cell necrosis was obviously greater than that of the SDT group. Mature sperm were lacking or rarely pres-
ent in the lumen (Fig. 2F). At 60 days, the morphology and structure of the testes recovered such that it was similar to that of the control (Fig. 2C and 2G). Time-dependent damage was observed in the testes at 30 days, both in the SDT and MDT groups, and this damage was reversible.

SOD and GSH-Px activities and T-AOC in plasma

At 15 and 30 days after treatment, the SOD and GSH-Px activities and the T-AOC levels of the treated groups were less than those of the control groups (Table 2). At 15 days, quinestrol treatment significantly decreased the SOD (86.5, 95.3, and 99.0 U/ml) and GSH-Px activities (268.6, 303.8, and 365.6 U/ml) and T-AOC (2.13, 3.54, and 6.33 U/ml) both in the MDT and SDT groups compared with the control groups. At 30 days, antioxidant parameters including the SOD activities (84.7, 88.3, and 100.3 U/ml), GSH-Px activities (234.6, 250.4, and 371.6 U/ml), and T-AOC (2.97, 3.22, and 6.67 U/ml) were still lower both in the MDT and SDT groups compared with the control groups. At 60 days after treatment, the above parameters were significantly increased. Quinestrol significantly decreased the activity of the antioxidant enzymes, including SOD and GSH-Px in plasma. But at 60 days after treatment, all of these parameters were restored.

SOD and GSH-Px activities and T-AOC in testes

The changes in the measured parameters for each group in the testes followed the same trend as in the plasma (Table 3). At 15 days, the proportion of the SOD activities to that of the control group in MDT group is higher than that of the SDT group to control group (82.6 and 69.6%, respectively), but at 30 days, proportion of the MDT and SDT groups to control group were similar (64.4 and 63.8%). At 15 days, the GSH-Px activity of the control group was 2.57 and 1.89 times those of the MDT and SDT groups, while at 30 days, the activity of control group was 2.5 times those of both treated groups. At 15 days, the testicular T-AOC proportions of the MDT and SDT groups to the control group were similar (76.5 and 82.3%, respectively), but at 30 days, the proportions decreased to 51.3 and 47.3%. At 60 days, all of the indices were significantly increased in the treated gerbils. Quinestrol decreased the total antioxidant activity by decreasing the antioxidant enzyme activity, and at 60 days following treatment, the damage to the antioxidant system was alleviated.

The Protein concentration in the testis and MDA concentration in the plasma and testes

The MDA and protein concentrations of the different groups are shown in Table 4. At 15 and 30 days, the protein and MDA concentrations in the testes were significantly higher than those of the controls. At 15 and 30 days, the protein concentrations of the treated groups were nearly twice as high as that of the control group. At 15 and 30 days, the testicular MDA concentration of the MDT group was twice as high as that of the control group while of the SDT group was only 1.7 times than that of the control. At 60 days, all of these measurements returned to normal levels. At 15, 30, and 60 days, the change in the MDA levels was not significant in plasma. The data indicated that the testicular damage was reversible, although the damage induced by quinestrol was significant.

Discussion

In this study, the relationship between reproductive toxicity and oxidative damage induced by quinestrol was studied in male gerbils using daily oral gavage. The results showed that the testes in the MDT group animals were significantly atrophic after 15 and 30 days following quinestrol treatment. Our previous study showed that male gerbils exposed to quinestrol showed a significant decrease in epididymides, seminal vesicles, semen quality, and reproductive rates (data not published). The present study shows that the seminiferous tubules were severely damaged and degenerated, that a large number of germ cells underwent pyknosis or necrosis and that almost no mature sperm were in the lumen. The relative testicular weights of the single-dose group were significantly lower than those of the control group, but they were significantly higher than those of the multidose group. Interestingly, the pathological changes were mild compared with the multidose group, and residual normal tubules could be found. Quinestrol can cause testicular atrophy, sperm cell death, and testis damage in varying degrees depending on the exposure method. In addition,
Table 2. Comparison of the SOD and GSH-Px activities, and the T-AOC in plasma at 15, 30, and 60 days after treatment with quinestrol

<table>
<thead>
<tr>
<th>Days</th>
<th>SOD (U/ml)*</th>
<th>GSH-Px (U/ml)*</th>
<th>T-AOC (U/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SDT</td>
<td>MDT</td>
<td>CK</td>
</tr>
<tr>
<td>30</td>
<td>88.346 ± 15.587a</td>
<td>84.657 ± 13.659b</td>
<td>100.296 ± 16.040a</td>
</tr>
<tr>
<td>60</td>
<td>100.337 ± 18.396a</td>
<td>105.539 ± 17.883b</td>
<td>102.716 ± 9.845b</td>
</tr>
</tbody>
</table>

*Results are expressed as means ± SD. The mean differences between the values bearing different superscript letters within the same column or row are statistically significant (a), (b), (c), and (d); P<0.05.

Table 3. Comparison of the SOD, and GSH-Px activities and the T-AOC in the testes at 15, 30, and 60 days after treatment with quinestrol

<table>
<thead>
<tr>
<th>Days</th>
<th>SOD (U/mg prot)*</th>
<th>GSH-Px (U/mg prot)*</th>
<th>T-AOC (U/mg prot)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SDT</td>
<td>MDT</td>
<td>CK</td>
</tr>
<tr>
<td>15</td>
<td>159.388 ± 27.301c</td>
<td>133.923 ± 5.746a</td>
<td>192.369 ± 29.415b</td>
</tr>
<tr>
<td>30</td>
<td>125.466 ± 20.562a</td>
<td>126.551 ± 9.181b</td>
<td>196.625 ± 33.448b</td>
</tr>
<tr>
<td>60</td>
<td>192.646 ± 42.559b</td>
<td>195.123 ± 38.902b</td>
<td>192.393 ± 17.682b</td>
</tr>
</tbody>
</table>

*Results are expressed as means ± SD. The mean differences between the values bearing different superscript letters within the same column or row are statistically significant (a), (b), and (c); P<0.05.

Table 4. Comparison of the protein and MDA concentrations in testes and the MDA concentration in plasma at 15, 30, and 60 days after treatment with quinestrol

<table>
<thead>
<tr>
<th>Days</th>
<th>Protein concentration in testicular tissue (g/l)*</th>
<th>MDA concentration in testicular tissue (nmol/mg prot)*</th>
<th>MDA concentration in plasma (nmol/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SDT</td>
<td>MDT</td>
<td>CK</td>
</tr>
<tr>
<td>15</td>
<td>2.261 ± 0.146a</td>
<td>2.362 ± 0.252a</td>
<td>1.224 ± 0.091b</td>
</tr>
<tr>
<td>30</td>
<td>2.307 ± 0.241a</td>
<td>2.284 ± 0.192a</td>
<td>1.211 ± 0.088b</td>
</tr>
<tr>
<td>60</td>
<td>1.184 ± 0.149b</td>
<td>1.124 ± 0.210a</td>
<td>1.167 ± 0.104b</td>
</tr>
</tbody>
</table>

*Results are expressed as means ± SD. The mean differences between the values bearing different superscript letters within the same column or row are statistically significant (a), (b), and (c); P<0.05.
after 60 days following treatment, the absolute and relative testicular weights of each group returned to normal levels, and damage to the testis was recovered, which suggests that damage to the testes of male gerbils induced by quinestrol is reversible.

The testes were atrophic, their structures were damaged, and the antioxidant system was also significantly changed in the treated groups. The activities of SOD and GSH-Px and the T-AOC were significantly less than those of the controls at 15 and 30 days following quinestrol treatment (Table 2). The SOD activity was significantly different between the SDT and MDT groups after 15 days of treatment. These data indicated that the impact of multiple doses on SOD was more severe. In addition, the protein concentration was increased (Table 4), so the decrease in the SOD and GSH-Px activities and the T-AOC were not due to changes in protein content. SOD, which catalyses the dismutation of superoxide radicals to H$_2$O$_2$ and molecular oxygen [8], is one of the most important enzymes for scavenging oxygen free radicals [9]. A decrease in SOD activity that resulted in ROS generated by testicular tissue that cannot be eliminated over time affects normal germ cell function, leading to germ cell death. The germ cell membranes are rich in unsaturated fatty acids that readily undergo lipid peroxidation [6]. GSH-Px scavenges alkyl (R'), RO', and ROO' radicals that may be formed from oxidized membrane components, and it uses GSH as a substrate [1, 2]. Thus, it is possible that the depletion of testicular GSH-Px levels by quinestrol may ultimately make the testicular cells even more sensitive to oxidative stress, resulting in damage to the sperm cells. Data from the T-AOC assay showed that the total antioxidant capacity in vivo was significantly decreased in response to quinestrol treatment. This result further confirmed that the testicular damage induced by quinestrol was due to reduced levels of antioxidant enzymes. After 60 days following the first treatment, the absolute and relative testicular weights were restored, and the antioxidant enzyme (SOD and GSH-Px) activities and T-AOC returned to normal.

Lipid peroxidation of unsaturated fatty acids in sperm membranes is one of the most important effects of ROS-induced cell damage [12]. Malondialdehyde (MDA) is an end-product of lipid peroxidation that indicates the level of lipid peroxidation [12, 18]. Lipid peroxidation (LPO) plays an important role in testicular toxicity and carcinogenicity. An increase in lipid peroxidation may alter the cellular membrane structure and then block cellular metabolism [7]. At 15 and 30 days after the treatment with quinestrol, the increase in MDA concentration in the testes indicated the testicular tissue was sensitive to quinestrol. The antioxidant enzyme activities were decreased, and at the same time, lipid peroxides were generated in the testes, which aggravated sperm cell damage. The MDA concentration in the single-dose group was significantly less than that of the multidose group (Table 4), which suggested that testicular germ cell damage caused by the multidose treatment was more severe than that of the single-dose treatment. However, after 60 days, the MDA concentration returned to normal, and the testicular damage was reversed. These data further confirmed that the level of MDA reflected the degree of germ cell damage.

The SOD and GSH-Px activities and the T-AOC in plasma of the quinestrol-treated gerbils were significantly decreased compared with those in the control (Table 3). However, the MDA concentration was not significantly different compared with the control (Table 4). This result suggests that other antioxidants participate in clearing ROS in the plasma, thereby reducing the accumulation of lipid peroxides. Quinestrol mainly induces reproductive toxicity, while the effect on the rest of the body is weaker.

Our data indicate that quinestrol treatment causes an imbalance in the oxidation and antioxidant system in blood and testicular tissue. Quinestrol induces ROS and simultaneously reduces antioxidant enzyme activity, leaving germ cells in their oxidation state, which results in spermatogenic cell damage. Mitochondrial permeability changes at low concentrations of ROS, which triggers the release of cytochrome C and apoptosis factor (AIF) activated caspase-9 and initiates the apoptosis cascade [15, 26, 28]. High concentrations of ROS directly damage the electron transfer chain and reduce the capacity of mitochondrial ATP synthesis significantly [5, 16]. The cells are necrotic because of a lack of energy [5, 16]. Because ATP is essential for the apoptosis of complex components, the loss of ATP will prolong activation of caspase [27]. Reactive oxygen species also can affect
biochemical reactions or biological structures, which can in turn affect certain cell signaling cascades. They also oxidize thioredoxin and indirect active apoptosis signal kinase (ASK-1), which leads to apoptosis [11], and induce expression of the transcription factor NF-κB, which upregulates the NF-κB target gene FasL and increases apoptotic sensitivity [4, 10]. Additionally, they increase the cytoplasmic Ca^{2+} concentration, activate endogenous calcium-dependent nucleic acid enzymes and lead to shearing of DNA, chromatin condensation, and the initiation of apoptosis [23].

Acknowledgments

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References


