Reproductive toxicity provoked by titanium dioxide nanoparticles and the ameliorative role of Tiron in adult male rats

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Abstract

Titanium dioxide nanoparticles (TDN) are widely used in paints, plastics, ceramics, cosmetics, printing ink, rubber and paper. Tiron is a water soluble metal chelator and antioxidant. This study was designed to investigate the reproductive toxicity of TDN in male albino rats and the ameliorative role of Tiron to minimize such toxic effects. Eighty adult male albino rats were assigned into 4 equal groups, group 1: control; group 2: received TDN at 100 mg/kg/day orally for 8 weeks; group 3: received Tiron at 470 mg/kg/day intraperitoneally for 2 weeks (the last 2 weeks of the experimental period); group 4: received both TDN and Tiron by the same previously mentioned dose, route and duration. The results revealed that TDN provoked reproductive toxicity which was proved by the deteriorated spermogram picture, high incidence of micronucleated RBCs, elevated oxidative stress parameters and up regulation of Testin gene. Whereas, Tiron co-treatment ameliorated most of these toxic alterations. Our findings highlighted the protective role of tiron against TDN intoxication.

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1. Introduction

Titanium dioxide nanoparticles (TDN) are among the most widely manufactured nanoparticles on a global scale [1]. Consequently, TDN are among the top five nanoparticles used in consumer products, paints, plastics, rubber and pharmaceutical preparations [2]. In addition, TDN are widely used in printing ink, paper, sunscreens, car materials and in water purification [3]. The dimensions of TDN are critical from the toxicity point of view, given that TDN have more prominent toxicity than conventional TiO2 particles [4]. The toxicity of TDN was much attributed to the generation and accumulation of reactive oxygen species (ROS) which give rise to an inflammatory response [5]; depletion of cellular antioxidant such as glutathione [6] and mitochondrial damage with prevention of ATP synthesis [7]. Some in vivo and in vitro studies confirmed the genotoxicity of TDN by micronucleus (MN) test, Ames test, mammalian cell gene mutation, DNA breaks and chromosomal alterations [8]. Titanium dioxide nanoparticles induced both DNA single and double-strand breaks and chromosomal damage [9]. They induced 8-hydroxydeoxyguanosine [10], γ-H2AX foci, micronuclei, and DNA deletions. The formation of γ-H2AX foci was indicative of DNA double-strand breaks and DNA damage [11]. Other reports suggested that TDN-induced genotoxicity is possibly caused by a secondary genotoxic mechanism associated with changes in gene expression of some related genes [12]. A recent study [13] found that oral administration of TDN significantly up-regulated testicular mRNA expression of IL-6 and TNF-α and down regulated Glutathione-S-Transferase (GST) and steroidogenesis related genes. TDN effectively activated caspase-3 and -9, decreased gene and protein levels of Bcl-2, Bax and cytochrome c, and promoted ROS accumulation and induced apoptosis [14]. Severe testicular pathological changes with decreased serum sex hormone levels and abnormal semen picture were observed in male mice exposed to TDN [15].

Tiron is 4,5-dihydroxy-1,3-benzene disulfonic acid. It is a water-soluble, a cell-permeable metal chelator and an antioxidant [16]. It has been found to be an efficient non-toxic chelator of various metals, such as uranium, arsenic, vanadium, and chromium [17]. In addition, Tiron is an effective antioxidant capable of scavenging a variety of free radicals and could also prevent the metal-catalyzed peroxidation of DNA or lipids [18]. There is still urgent need to provide a detailed overview on the current state knowledge about TDN-induced genotoxicity and the possible therapeutic role of chelators and antioxidants supplementation. Therefore, the present...
2. Materials and methods

2.1. Animals

The Local Research Ethical Committee of the Faculty of Veterinary Medicine, Cairo University, Egypt approved the design of the experiments (Approval No.: CUFVM/F/Tm./2014/37) and the protocol conforms to the guidelines of the National Institutes of Health (NIH). A total of 80 adult male albino rats (180–200 g) were randomly divided into 4 equal groups as follows: The first (Control) group was ip injected with normal saline daily during the last 2 weeks of experimental period. The second group received TDN daily at 100 mg/kg by oral intubation [19] for 8 weeks. The same rats were ip injected with normal saline daily during the last 2 weeks of experimental period. The third (Tiron) group received Tiron daily during the last 2 weeks of experimental period by ip injection at 470 mg/kg [20]. The fourth (TDN + Tiron) group received both TDN and Tiron by the same previously mentioned dose, route and duration of exposure for each. At the end of the experiment, the rats were euthanized; blood, testes and epididymis were collected for subsequent investigation.

2.2. Spermogram picture

The spermatozoa were collected from the tail of epididymis and vasa deferentia and examined for mass motility, live and dead sperms, sperm abnormalities and sperm cell concentration [21].

2.3. Measurement of serum testosterone level

Serum testosterone level was measured in the collected serum samples using Calbiotech Inc. testosterone ELISA kit.

2.4. Biochemical investigations

Assessment of oxidative stress via determination of lipid peroxidation “malondialdehyde” (MDA), reduced glutathione (GSH) levels and catalase (CAT) activity were done on testes homogenate using Diagnostics kits.

2.5. Micronucleus test (for peripheral blood)

The frequency of micronuclei in erythrocytes100 cells/rat was analyzed and total 500 erythrocytes/group were detected under a binocular microscope using a 1000X oil-immersion lens [22].

2.6. Total RNA extraction

Total RNA was isolated from rat testes by Total RNA Purification Kit (Jena Bioscience, Cat. No. PP-2105) according to the manufacturer instructions.

2.7. Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR was done by using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Cat. No. #K1622).

2.8. Real time PCR (qPCR) for testin gene

Real time PCR (qPCR) was done by Luminaris Color HiGreen Low ROX qPCR Master kit (Thermo Scientific, Cat. No. #K0371). Two pairs of oligonucleotide primers were designed using primer3 software, based on the testin gene sequence of rat (accession number; NM_173132.2); forward primer (AAATCCCCAGGAGC- GAGGAA) and the reverse one (GCTGTTCTTTAGGAGCCAAA). Each assay included triplicate samples for each tested cDNAs and no-template negative control. Data were normalized by using GAPDH gene as an internal control [23]. Two-steps cycling protocol was adjusted as follows; UDG pre-treatment at 50 °C for 2 min, Initial denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 60 s. Fluorescent data were acquired during each extension phase. The fold change over control samples were calculated using CT, ΔCT, ΔΔCT by Mxpro software Stratagene.

2.9. Immunohistochemical investigation

Paraffin sections prepared from testes of control and treated groups were used for immunohistochemical detection of apoptosis by Caspase-3 technique [24].

2.10. Histopathological examination

The obtained testes were subjected to histopathological examination [25].

3. Results

3.1. Sperms’ viability and morphological abnormalities

Oral administration of TDN resulted in a significant decrease in sperms’ viability percentage with a significant increase in the incidences of morphological abnormalities compared to the control (Table 1). The recorded sperm’s abnormalities in TDN group included deformed and detached heads; curved and coiled tails. Oral exposure to TDN plus Tiron resulted in a significant increase in sperm’s viability percentage and a significant decrease in the incidences of morphological abnormalities compared to TDN group.

3.2. Results of serum testosterone

Oral administration of TDN resulted in a significant decrease in serum testosterone level compared to the control. While exposure to TDN plus Tiron induced a significant increase in serum testosterone level compared to the TDN group (Table 2).

3.3. Results of the biochemical tests

Oral administration of TDN resulted in a significant increase in testicular MDA level; a significant decrease in its GSH content; however it has a non-significant effect on CAT activity compared to the control. Tiron treatment induced a non-significant decrease in testicular MDA level; a significant increase in GSH content and a non-significant increase in CAT activity compared to the control. Oral exposure to TDN plus Tiron showed a significant decrease in testicular MDA level; a significant increase in its GSH content and a non-significant increase in CAT activity compared to the TDN group. At the same time, the MDA level was significantly higher in the TDN + Tiron-treated rats than that of control (Table 3).

3.4. The incidence of micronucleated RBCs among TDN and/or Tiron treated rats

Results revealed that oral administration of TDN resulted in a significant elevated incidence of micronucleated RBCs % compared to the control. The micronuclei were of variable sizes (small;
Table 1
Sperm cell’s viability and morphological abnormalities in the exposed rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Live sperms (%)</th>
<th>Morphological abnormalities (%)</th>
<th>Tail abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Head abnormalities</td>
<td>Curved tail</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Detached head</td>
<td>Coiled tail</td>
</tr>
<tr>
<td>Control</td>
<td>90.80 ± 1.2</td>
<td>1.60 ± 0.40</td>
<td>2.60 ± 0.40</td>
</tr>
<tr>
<td>TDN</td>
<td>68.60 ± 2.94</td>
<td>9.20 ± 0.49</td>
<td>11.20 ± 0.73</td>
</tr>
<tr>
<td>Tiron</td>
<td>91.00 ± 0.7</td>
<td>1.00 ± 0.32</td>
<td>1.80 ± 0.58</td>
</tr>
<tr>
<td>TDN + Tiron</td>
<td>84.50 ± 2.5</td>
<td>4.00 ± 0.32</td>
<td>5.80 ± 0.37</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SE (n = 10 rats/group).

3.5. Immunohistochemical results

Immunohistochemical examination by caspase-3 technique for detection of apoptosis revealed normal cells without apoptosis in the testis of control; Tiron and TDN + Tiron - treated rat groups, while the testis of TDN exposed rats showed marked apoptosis (Fig. 2).

3.6. The histopathological results

No pathological changes could be seen in the testis of the control group. Also, the testes of Tiron-treated rats showed normal seminiferous tubules without any pathological alterations. However, the obtained testis sections from TDN- exposed rats showed interstitial edema and sloughing of its germinal epithelium with different apoptotic changes such as pyknosis, karyolysis and karyoschisis. Whereas, the daily Tiron treatment of rats intoxicated by the TDN ameliorated most of these alterations where the testis showed only mild degree of sloughing and congestion (Fig. 3).

3.7. Results of real time PCR of Testin gene

Oral administration of TDN alone resulted in a marked increase in the level of Testin gene expression (27.47 folds) compared to the control. However, exposure to TDN plus Tiron induced marked decrease in the level of Testin gene expression (6.23 folds) compared to the TDN exposed group. Tiron group showed no significant difference in testin gene expression compared to control (Table 5).

4. Discussion

This study was set out to assess the protective impact of Tiron as an antioxidant and a metal chelator against the TDN-induced reproductive and genotoxic effects in male rats. Our results revealed that TDN induced a significant decrease in sperm motility, viability with an increased incidence of sperm abnormalities. While, the Tiron administration increased the viability percentage with a significant decrease in the recorded morphological abnormalities percentages compared to the TDN group. The decrease in sperm count and motility are valid indices of male infertility in laboratory animals. TDN demonstrated a significant dose-dependent increase in sperm abnormalities percentage and a significant decrease in sperm cell concentration [26], a significant decrease in sperm motility and an increase in the incidence of sperm abnormalities [27]. In contrast to our results, no significant difference in the sperm numbers among the TDN group was mentioned [26]. Accumulated data showed that ROS-mediated oxidative stress alters testicular cells structure and function ending by its damage [28]. This may be attributed to the fact that testes are rich in polyunsaturated fatty acids which are the source of RO. The TDN as ROS generator induced spermatogenic damage. This could be confirmed by the recorded results of TDN which induced a significant increase in testicular MDA level and a
significant decrease in GSH content and CAT activity. However, the co-administration of tiron ameliorated the TDN toxic effect such that a significant increase in GSH level and a significant decrease in MDA level compared to the TDN-exposed group were detected. In the same context, authors mentioned that the activity of antioxidative enzymes were decreased in a dose-dependent manner following TDN exposure of rats. They attributed the reduction in these enzymatic activities to the inhibition of the mRNA expression of these enzymes by TDN [27]. Others recorded that co-administration of Tiron, as an antioxidant, ameliorated beryllium [30] or aluminium [20] oxidative stress parameters in rats.

The TDN administration resulted in a significant decrease in serum testosterone level compared to the control. The reduction in the hormone production may be exerted by the detrimental effects of TDN on Leydig cells [31]. Moreover serum testosterone level of the TDN exposed group significantly decreased with increasing the dosages of TDN compared to control [15]. Some researchers reported that there was a significant decrease in both serum and testicular testosterone concentrations indicating that TDN affects Leydig cell function [32]. The other hypothesis is that nanoparticle exposure may decrease the steroidogenic acute regulatory star gene expression. This leads to a reduction in testosterone production [33].

The current study recorded that TDN induced apoptosis. This was proved by a significant activation in caspase-3 among TDN exposed rats. The intravenous injection of TDN resulted in a statistically significant elevation of caspase-3, indicating that TDN induced apoptosis in germ cells [27]. Our results proved that oral administration of TDN resulted in a significant increase in micronucleated RBCs % compared to the control. This is in agreement with the in vivo results that stated a positive result of micronuclei assay after administration of TDN [3]. In contrast to our results, some reports stated negative results of micronuclei assay after TDN exposure [34]. TDN exposure impaired the cell’s ability to repair DNA by deactivation of both nucleotide excision repair (NER) and base excision repair (BER) pathways [35]. Testicular dysfunctions following exposure to TDN may be related to a significant alterations in the expression of genes involved in spermatogenesis, as well as steroid hormone metabolic processes [15].
Testin, a Sertoli cell secretory protein whose mRNA is predominantly expressed in the testis, was shown to become tightly associated with Sertoli cell membrane upon its secretion. The concentration of Testin secreted by Sertoli cells in vitro or in vivo is inversely correlated to the presence of intact testicular cell junctions. Both in vitro and in vivo studies demonstrated that disruption of testicular cell junctions can lead to surge in the concentration of Testin mRNA level [36]. TDN causes the production of free radicals and can lead to damage of DNA that may be a predisposing factor for cancer and tumors formation [37]. Our genotoxicity results revealed also that oral administration of TDN resulted in marked up-regulation of Testin gene compared to control. However, oral exposure to TDN plus Tiron induced a sharp down regulation in this gene compared to the TDN-alone-exposed group. In coincidences with our genotoxicity results, a study showed that 254 genes were obviously altered in the TDN-exposed testicular tissue compared to control. Of the altered genes, 153 were up-regulated and 101 down-regulated [15]. The TDN exposure markedly changed the mRNA level of several genes involved in cholesterol transport and testosterone biosynthesis [26]. Expression of testin gene was used as a sensitive marker to indicate the direct damage of spermiogenesis [36]. Other steroidogenesis related genes [Androgen Binding Protein (ABR), 17β-Hydroxysteroid Dehydrogenase (17β-HSD), cytochrome P450 17A (CYP17a) and aromatase] showed down regulation in TiO2 administered group [13]. The current study was carried out to investigate the reproductive and genotoxicity of TDN and the ability of Tiron to ameliorate such adverse effects. Our findings proved that Tiron could minimize the oxidative stress and genotoxicity provoked by the TDN exposure in rat testes model. Thus, our data proved the effectiveness of tiron as an antioxidant against the TDN toxicity.

**Conflict of interest**

The authors declare that there’s no conflict of interest regarding the publication of this paper.

**References**
