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Thymoquinone ameliorated elevated inflammatory cytokines in testicular tissue and sex hormones imbalance induced by oral chronic toxicity with sodium nitrite

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ABSTRACT

Scientific evidence illustrated the health hazards of exposure to nitrites for prolonged time. Nitrites affected several body organs due to oxidative, inflammatory and apoptosis properties. Furthermore, thymoquinone (TQ) had curative effects against many diseases. We tried to discover the impact of both sodium nitrite and TQ on inflammatory cytokines contents in testicular tissues and hormonal balance both *in vivo* and *in vitro*. Fifty adult male SD rats received 80 mg/kg sodium nitrite and treated with either 25 or 50 mg/kg TQ daily by oral-gavage for twelve weeks. Testis were removed for sperms' count. Testicular tissue homogenates were used for assessment of protein and gene expression of IL-1β, IL-6, TNF-α, Nrf2 and caspase-3. Serum samples were used for measurement of testosterone, LH, FSH and prolactin. Moreover, all the parameters were measured in human normal testis cell-lines, CRL-7002. Sodium nitrite produced significant decrease in serum testosterone associated with raised FSH, LH and prolactin. Moreover, sodium nitrite significantly elevated TNF-α, IL-1β, IL-6, caspase-3 and reduced Nrf2. TQ significantly reversed all these effects both *in vivo* and *in vitro*. In conclusion, TQ ameliorated testicular tissue inflammation and restored the normal balance of sex hormones induced by sodium nitrite both *in vivo* and *in vitro*.

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1. Introduction

Sodium nitrite, important widely used food preservative, inhibited *Clostridium botulinum* spores growth in processed meat at a concentration 0.6% only [1]. Although sodium nitrite could inhibit the growth of bacteria, exposure to nitrites above health-based risk was declared to possess detrimental effects. It was accepted as weak carcinogen due to formation of nitrosamines in charred or overcooked meat or from the secondary amines inside the stomach [2]. In addition, chronic intake of sodium nitrite resulted in inflammation and apoptosis in many body organs [3–8].

Nigella sativa was reported to reduce body toxicity. One of the most important phytochemical bioactive ingredient that found in *N. sativa* was thymoquinone (TQ, 2-isopropyl-5-methyl-1,4-benzo quinone). TQ formed mainly 30–48% of *N. sativa* seeds [9]. TQ had wide spectrum of activity as antioxidant, anti-inflammatory, anticancer, antidiabetic and antibacterial [10–13].

We previously reported that TQ ameliorated the testicular tissue damage in rats produced by oral administration of sodium nitrite through several mechanisms like attenuation of oxidative stress and prevention of apoptosis [8]. However, we conducted this study to examine other mechanisms that were involved in the protective effects of TQ against sodium nitrite-induced testicular tissue inflammation and apoptosis. Moreover, the impact of sodium nitrite and TQ was explored on sex hormones balance in rats. All these effects was measured *in vitro* using human normal testis cell-lines, CRL-7002.

Abbreviations: FSH, follicle-stimulating hormone; IL-1β, interleukin-1β; IL-6, interleukin-6; LH, luteinizing hormone; Nrf2, nuclear factor erythroid 2-related factor-2; TNF-α, tumor necrosis factor-α; TQ, thymoquinone.

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2. Materials and methods

2.1. Animals

All animal procedures were accepted by "Ethical Committee" in Faculty of Pharmacy, University of Mansoura. Fifty adult male SD rats (170–180 g) were used. TQ and sodium nitrite was given daily to rats by oral gavage over a period of 12 weeks. Rats were classified into the following groups (10 rats each):

Control group. Normal saline was given daily to rats.
Control + TQ group. 50 mg/kg TQ were given daily to rats.
Sodium nitrite group. 80 mg/kg sodium nitrite were given to rats daily.
Sodium nitrite + TQ (25 mg/kg) group. 25 mg/kg TQ were given to rats accompanied by 80 mg/kg sodium nitrite daily.
Sodium nitrite + TQ (50 mg/kg) group. 50 mg/kg TQ were given to rats accompanied by 80 mg/kg sodium nitrite daily.

We used TQ and sodium nitrite in doses that were reported previously [8,9].

2.2. Animal scarification and sample collection

Rats were decapitated. Blood was collected from rats' trunk for preparation of serum, which was stored at −80 °C. The cauda epididymidis was separated and the adherent vessels, fat and tissues were removed. Gonado-somatic index was determined [14]:

$$\text{Gonado Somatic Index} = (\text{Gonad weight} \,/\, \text{body weight}) \times 100$$

Cauda epididymidis of one testis was cut longitudinally and compressed to release sperms. The sperm was counted as described previously [15]. One testis was homogenized in 10-fold of 0.01 M sodium potassium phosphate buffer (pH 7.4), cleared by centrifugation at 600*g* and kept at −80 °C. The other testis was directly stored at −80 °C for estimation of the gene and protein expression.

2.3. ELISA determination

ELISA kits were used to measure testicular contents of TNF-α, IL-1β and IL-6 (eBioscience Inc., San Diego, CA, USA). Moreover, ELISA kits were used to determine the serum levels of testosterone, FSH, LH and prolactin (MyBioSource, Inc., San Diego, CA, USA).

2.4. Real-time PCR

As we described previously [16], RNeasy Mini kit (Qiagen, Valencia, CA, USA) was used to isolate RNA from rat testis. Maxima SYBR Green/Fluorescein Master Mix (ThermoFisher, Grand Island, NY USA) was used to estimate the amount of RNA. QuantiTect Reverse Transcription Kit (Qiagen, USA) was used to reverse transcribe 1 μg of total RNA. Rotor-Gene Q (Qiagen, USA) was used to determine testicular tissues mRNA levels of TNF-α, IL-1β, IL-6, Nrf2 and caspase-3. For housekeeping gene, we used rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primer Express 3.0 (Applied Biosystems, USA) was used for designing primers (Table 1).

Table 1
The primer sets used.

Name	Sequence	Reference sequence
Rat GAPDH	5′-CCATCAACGACCCCTTCATT-3′ 5′-CACGACATACTCAGCACCAGC-3′	NM_017008.3
Rat Nrf2	5′-GAGACGGCCATGACTGAT-3′ 5′-GTGAGGGGATCGATGAGTAA-3′	NM_031789.1
Rat Caspase-3	5′-CGTCTGTGCTCCAGGCTTC-3′ 5′-TGTGAGTTCCTTCCTTTCTTTGTG-3′	NM_012922.2
Rat TNF-α	5′-AAATGGGCTCCCTCTCATCAGTTC-3′ 5′-TCTGCTTGGTGGTTTGCTACGAC-3′	X66539
Rat IL-1β	5′-CACCTCTCAAGCAGAGCACAG-3′ 5′-GGGTTCCATGGTGAAGTCAAC-3′	M98820
Rat IL-6	5′-TCCTACCCCAACTTCCAATGCTC-3′ 5′-TTGGATGGTCTTGGTCCTTAGCC-3′	M26745

2.5. Western blot

RIPA buffer was used for homogenization of tissues (Millipore, Temecula, CA, USA). Protein assay kit was used for estimation of protein quantity (Bio-Rad Laboratories Inc., Hercules, CA, USA). 30 μg samples were separated by SDS-PAGE followed by electroblotting into nitrocellulose membrane. Polyclonal antibodies (1:500 dilutions) for Nrf2 and cleaved caspase-3 (Abcam Co., Cambridge, MA, USA) were added and kept overnight at 4 °C. β-actin (1:2000, Sigma Aldrich Chemicals Co.) was used for reprobing membranes. Sheep anti-rabbit antibodies conjugated with horseradish peroxidase (1:5000 dilutions) was used with the aid of chemiluminescence (GE Healthcare, Piscataway, NJ, USA). Relative optical density (ROD) was used to express the data.

2.6. Tissue culture

2.6.1. Cell lines

The CRL-7002 cell line (ATCC, Manassas, VA, US) was used. CRL-7002 cells were cultured in DMEM medium complemented with fetal bovine serum (10%) and a mixture of streptomycin and penicillin (1%). CRL-7002 was incubated at 37 °C for 24 h in carbon dioxide incubator.

2.6.2. MTT assay

Cells were switched into serum-free medium. Sodium nitrite (250, 500, 750 and 1000 μM) and/or TQ (10, 50 and 100 μM) were added to the plates. The plates were incubated into humidified carbon dioxide incubator for another 48 h. MTT assay was used for determination of cell viability [17].

2.6.3. LDH cytotoxicity

The protocol was described previously by our group [17]. LDH reaction mixture (Sigma Aldrich Chemicals Co., St. Louis, MO, USA) was used. Triton-X 100 (1%) was used as positive control for normalizing the results.

2.7. Statistical analysis

Mean ± standard error or standard deviation were used to express the quantitative variables. Kolmogorov–Smirnov test was employed to check normal distribution of samples. ANOVA was used for comparison of means between groups followed by post hoc Bonferroni correction tests. SPSS version 20 was employed for calculation of significance at $P < 0.05$ (Chicago, IL, USA).

3. Results

3.1. TQ ameliorated sodium nitrite-induced changes in testis weight and sperm count

Sodium nitrite caused 48.3% reduction in sperm count in comparison with the control group. Moreover, sodium nitrite gave rise to 1.72- and 1.92-fold in testis weight as well as Gonado Somatic Index, respectively when compared with controls (Fig. 1).

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