

Original Research Article

Thymoquinone enhances sperm DNA integrity in nicotine-induced infertile male rats

Farah Dayana Rosli¹, Noor Hashida Hashim^{2*}, Yusmin Mohd Yusuf³, Khairul Osman⁴, Siti Fatimah Ibrahim⁵, Nurul Kabir⁶

¹Institute of Graduate Studies, ²Biology Division, Center for Foundation Studies in Science, ³Centre for Research in Biotechnology for Agriculture, University of Malaya, 50603, ⁴Faculty of Allied Health Sciences, National University of Malaysia, 50586, ⁵Faculty of Medicine, National University of Malaysia, 56000, ⁶Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603, Kuala Lumpur, Malaysia

*For correspondence: **Email:** nhhpasum@um.edu.my; **Tel:** +60-3-79674629

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Abstract

Purpose: To assess the effects of thymoquinone (TQ) on the integrity of sperm DNA in nicotine-induced sperm impairment in rats.

Methods: Adult male Sprague Dawley rats were randomized into four equal groups: control group received normal saline orally for 60 days; nicotine group was subcutaneously injected with 5 mg/kg/day nicotine for 30 days and then given normal saline for the next 30 days; TQ group was given normal saline for 30 days followed by TQ at 5 mg/kg/day for 30 days; and nicotine-TQ group received 5 mg/kg of nicotine for 30 days and 5mg/kg of TQ for another 30 days. Sperm DNA breakages were evaluated using Comet assay. The expression levels of protamine 1 (PT1) and transition nuclear protein 2 (Tnp2) genes which are essential for the proper compaction of the sperm DNA were analyzed by quantitative polymerase chain reaction (qPCR).

Results: Thymoquinone significantly decreased DNA fragmentation in the sperm of nicotine-treated rats. However, there was no change in PT1 gene expression. Tnp2 was downregulated in the nicotine group and slightly upregulated in nicotine-TQ group ($p < 0.05$).

Conclusion: The results demonstrate the potential benefits of TQ in improving sperm DNA quality of nicotine-induced male infertility.

Keywords: Thymoquinone, Infertility, Nicotine, Sperm, DNA integrity, Protamine 1, Transition nuclear protein 2

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INTRODUCTION

Drugs and chemicals may adversely affect the male reproductive system. Smoking exerts injurious effects on sperm count, kinetic parameters, morphology and DNA integrity [1]. The adverse effects of cigarette smoking on

fertility could be attributed to the content of cigarettes which includes several toxic chemicals, mutagens and carcinogens. Nicotine represents 90 % of the total alkaloids in cigarettes [2] and mimics most of the deleterious effects of cigarette smoking [3]. A study by Mosadegh *et al* determined that nicotine caused

negative impacts by damaging DNA through apoptosis activation [4]. Increased levels of reactive oxygen species (ROS) by nicotine could also induce cellular oxidative injury that results in DNA breakage. Sperm with damaged DNA loses its ability to fertilize oocyte as sperm DNA integrity is an important factor for embryo development and offspring production [5].

DNA damage may also be a result of abnormal chromatin packaging during chromatin remodeling. The sperm chromatin is extremely compact and organized in a specific manner to provide a safe and secure transfer of paternal DNA [6]. During spermiogenesis, histones are replaced by transition nuclear proteins (Tnp). They are then replaced with protamines [7]. Disturbance of this could expose DNA to fragmentation and compromise fertility.

Medicinal herbs or plants have already been used in the treatment of various diseases. Among medicinal plants, *Nigella sativa* (NS) is a therapeutically-promising herb known for its pharmacological benefits. The benefits are linked to the main constituent of its volatile oil, the thymoquinone (TQ) [8]. The therapeutic potentials of Thymoquinone encompassed various areas, such as anti-bacterial activities and anxiety modulatory [9,10]. In the reproductive functions, TQ significantly improved semen quality and reproductive characteristics [11].

However, the effects of TQ on DNA integrity and genes responsible for chromatin packaging in the sperm cells of a nicotine-induced infertility model has not been studied so far. Therefore, the aim of the present study was to investigate the potential ameliorative role of TQ on infertility-induced sperm damage in rats.

EXPERIMENTAL

Animals

Male Sprague-Dawley rats (7 - 9 weeks), weighing 200 - 250 g were obtained from the University of Malaya Medical Centre (UMMC), University of Malaya. Prior to treatment, rats were acclimatized to the experimental conditions in the animal house for a week. The rats were placed in separate polypropylene cages. The animals were maintained at room temperature under standard conditions of 12:12 h light and dark cycles. Chow pellets and water were given *ad libitum* daily. The experiments were conducted in accordance with the guidelines provided by the Institute of Graduate Studies and the Institutional Animal Care and Use Committee

(IACUC) of University of Malaya {approval no. PASUM/30/12/2015/FDR(R)}, conforming to the guidelines by National Research Council, Guide for the Care and Use of Laboratory Animals [12].

Experimental design

Thirty-two rats were randomly divided into 4 groups (n = 8 per group): (1) Control group, force-fed with normal saline, per oral (p.o.) for 60 days; (2) nicotine group, subcutaneously (s.c.) injected with 5 mg/kg of nicotine for 30 days, followed by normal saline (p.o.) for 30 days; (3) TQ group, force-fed with normal saline (p.o.) for 30 days, followed by TQ at 5 mg/kg/day (p.o) for the next 30 days; (4) nicotine-TQ group, injected with 5 mg/kg/day of nicotine (s.c.) for 30 days, followed by force-feeding of TQ at 5 mg/kg/day for 30 days. Thymoquinone ($\geq 98\%$ pure) was purchased from Sigma-Aldrich (St. Louis, Missouri) while nicotine (L-nicotine, 99+% pure,) was purchased from Acros Organics (New Jersey, USA). All rats were euthanized on day 61 via ketamine-xylazine overdose (i.p.).

Sample collection

Upon euthanization, the cauda epididymis was excised from the rat testis, cut open and placed in a tube containing pre-warmed 1 mL phosphate buffered saline (PBS). The tube was placed in an incubator with 5 % CO₂, 37 °C for 30 min. Testes were decapsulated and cut into 50 – 100 mg pieces. For gene expression analysis, testes were placed in 1 mL Trizol and snapped-frozen in liquid nitrogen before storage in -80 °C.

Quantitative assessment of DNA damage (Comet assay)

The integrity of sperm DNA was assessed by performing the Comet assay with some modifications [13]. Frosted slides (StatLab Medical Product Inc., Texas) were covered with 200 μ L of 1 % (w/v) normal melting agar (NMA) and coverslips were placed promptly to allow it to solidify at 4 °C for 10 min. Following this, 100 μ L of sperm sample (1×10^5 sperm per mL) was added to 100 μ L of 0.5 % (w/v) low melting agarose (LMA) and 50 μ L of the suspension was pipetted onto the pre-coated NMA on the frosted slides. Fresh coverslips were placed and left at 4°C for 10 min. A second layer of 1 % LMA was pipetted and allowed to set. Following coverslips removal, the slides were placed into the first lysis buffer, at 4 °C for 1 h, then immersed in a second lysis buffer at 37 °C overnight.

After the lysis process, the slides were washed thrice with distilled water for 20 min to clear out

residual lysis buffer from the gels. The slides were then settled for 20 min in a horizontal electrophoresis tank (Sub-Cell Model 192 cell Horizontal Electrophoresis System, BioRad, Hercules, CA, USA) containing fresh electrophoresis buffer. Electrophoresis was conducted at 25V and 300 mA for 20 min in room temperature. The slides were then transferred in three change of neutralizing buffer for 20 min each. After neutralization, slides were left to air dry and stained with Sybr Green solution before coverslips were placed for image analysis. The fluorescent-labeled DNA was visualized using inverted a fluorescent microscope (Axiovert 200M, Carl Zeiss, Germany) at x200 magnification and captured using AxioCam MRm camera (Carl Zeiss, Germany).

Comet Assay Software Project (CASP) lab Version 1.2.3b1 (Free Software Foundation Inc., Boston, MA, USA) was used to analyze the comet images. Hundred comet images were randomly analyzed per slide and DNA damage profile (tail length (TL), tail moment (TM), olive tail moment (OTM), and % tail DNA) was computed.

RNA analysis

Trizol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to isolate total RNA from frozen rat testes according to the manufacturer's instructions. Essentially, the tissue was homogenized in Trizol (1 mL of Trizol per 50 – 100 mg of tissue) and incubated for 5 min at room temperature. An additional isolation step to remove insoluble materials from the homogenate was performed by centrifugation at 12000 x g for 10 min at 4 °C. The supernatant was carefully removed and placed in a new tube and mixed vigorously for 15 seconds following the addition of 200 µL of chloroform. The sample was incubated for 2 to 3 min at room temperature and centrifuged at 12000 x g, 4 °C for 15 min.

The aqueous phase was transferred to a new tube and 500 µl of isopropyl alcohol was added before centrifugation at 12000 x g, 4°C for 10 min. The RNA pellet was washed with 75 % ethanol and centrifuged again at 7500 x g for 5 min at 4 °C. The RNA pellet was then completely dissolved in 100 µL diethyl carbopyronate-treated water by incubating at 55 – 60 °C for 10 min. The concentration, purity and integrity of the RNA were determined by NanoDrop-1000 spectrophotometer (Thermo Scientific, Washington, USA) at 260/280 nm and by electrophoresis on a 1 % agarose gel, stained with GelStain (Transgen Biotech, Beijing, China).

Gene expression analysis by real-time PCR

cDNA was prepared from RNA by reverse transcription using the QuantiNova Reverse Transcription kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's protocol by utilizing 2 µg total RNA in a final volume of 20 µL. The obtained cDNA was stored at -20°C until analysis. The gene expression analysis of *PT1* and *Tnp2* genes were carried out using the QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Custom designed primers and probes as listed in Table 1 were synthesized by Integrated DNA Technologies. Primers were used at 250 nM and probes at 250 nM in a 20 µL reaction containing PrimeTime Gene Expression Master Mix (Integrated DNA Technologies, Coralville, IA, USA).

The thermal cycling conditions were set at initial denaturation of 3 min at 95 °C followed by 40 cycles of 95 °C for 5 sec and primer annealing/extension temperature at 60 °C for 30 sec. Amplification reactions were prepared in triplicate and no-template control was included. The relative expression levels of the target genes were determined by normalization to reference genes, Beta-actin (*Actb*) and Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Normalized expression level of the samples was calculated using the geometric mean of two reference gene quantities and factoring in the primer efficiency. The data was processed using qBase+ Version 3.0 (Biogazelle, Zwijnaarde, Belgium) for statistical analysis. Utilizing the "Stat Wizard" function, significant differences of expression between the groups were analyzed by one-way ANOVA test.

Statistical analysis

The data were processed by analysis of variance (ANOVA) followed Duncan post-hoc test. Significant level was set at $p < 0.05$. Data analysis was conducted with the aid of SPSS 23.0 software, except for the gene expression experiment.

RESULTS

DNA damage

The DNA damage caused by nicotine was evidenced by the increase in the DNA damage profile such as TL, TM, OTM and % tail DNA compared to the control and TQ group ($p < 0.05$; Table 2). DNA damage was reduced in the nicotine-TQ group compared to the nicotine group in all parameters.

Table 1: Sequence information for primers and probes used

Gene and accession nos.	Oligo	Sequences (5'-3')	Amplicon size (bp)
PT1 NM_001002850.1	PT1 Forward PT1 Reverse PT1 Probe	TGGCCAGATACCGATGCTG CCTAAAGGTGTATGAGCGGC CGAAGATGTCGCAGACGGAGGA	140
Tnp2 NM_017057.2	Tnp2 Forward Tnp2 Reverse Tnp2 Probe	GGAGAAGAGGAGGAGGAGGA ATTGGTGTGACTTTGAGGCC ATGGACACCAAGATGCAGAGCCTT	127
Actb NM_031144.2	Actb Forward Actb Reverse Actb Probe	TCTCCAGCCTTCCTTCCTG CACACAGAGTACTTGCGCTC AGGAGATTACTGCCCTGGCTCCTA	238
GAPDH NM_017008.4	GAPDH Forward GAPDH Reverse GAPDH Probe	GAGACAGCCGCATCTTCTTG TGACTGTGCCGTTGAACTTG AGATGGTGAAGGTCGGTGTGAACG	224

Table 2: Sperm DNA damage of rats treated with nicotine and TQ

Group	Tail length (arbitrary unit) (mean \pm SE)	Tail moment (arbitrary unit) (mean \pm SE)	Olive tail moment (arbitrary unit) (mean \pm SE)	Tail DNA % (mean \pm SE)
Control	18.98 \pm 0.28 ^a	1.45 \pm 0.09 ^a	1.52 \pm 0.09 ^a	1.17 \pm 0.07 ^a
Nicotine	31.24 \pm 1.12 ^c	4.73 \pm 0.20 ^c	5.21 \pm 0.26 ^c	7.06 \pm 0.36 ^c
Thymoquinone	20.46 \pm 0.31 ^a	1.57 \pm 0.10 ^a	1.72 \pm 0.10 ^a	1.23 \pm 0.08 ^a
Nicotine and thymoquinone	22.59 \pm 0.40 ^b	2.89 \pm 0.10 ^b	3.04 \pm 0.14 ^b	2.40 \pm 0.16 ^b

^{abc}Subscripts within the same column show a significant difference ($p < 0.05$); $n = 8$ rats per control and treatment groups

Gene expression of *PT1* and *Tnp2*

The expression of *PT1* was unaffected by nicotine and TQ as there was no significant change between the treatment groups and the control group (Figure 1). The *Tnp2* gene showed no expression change in the TQ group but was significantly downregulated in the nicotine group (0.047 ± 0.009). However, the expression of *Tnp2* in the nicotine-TQ group demonstrated a significant improvement compared to the nicotine group (0.111 ± 0.021) (Figure 2).

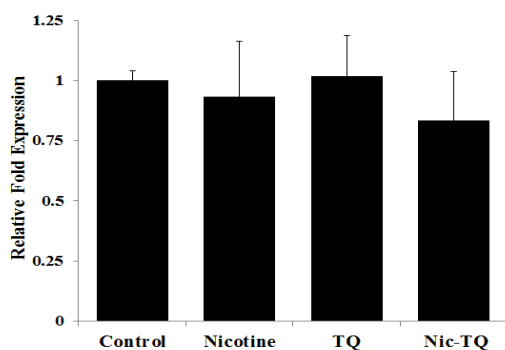


Figure 1: The effects of nicotine and thymoquinone on the expression of Protamine 1 (*PT1*). Results are expressed as normalized relative fold expression \pm SE

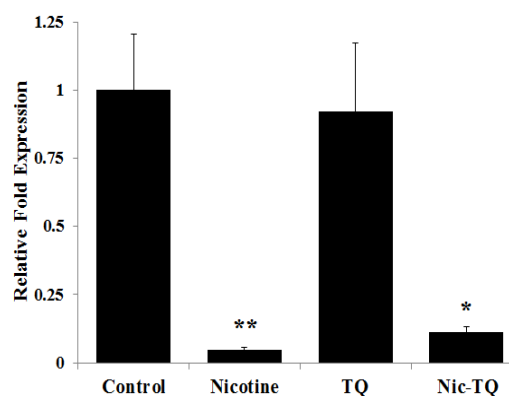


Figure 2: The effects of nicotine and thymoquinone on the expression of *Tnp2*. Results are expressed as normalized relative fold expression \pm SE. *Significantly different compared to the control group ($p < 0.05$). **Significantly different compared to control and nicotine-TQ group ($p < 0.05$)

DISCUSSION

Nicotine is a toxic substance which can be rapidly absorbed through the respiratory system, skin and oral mucosa. It is responsible for various health problems including the alteration of sperm characteristics [14].

In this study, a quantified assessment of sperm DNA damage was carried out using the Comet assay. The nicotine group showed the highest amount of DNA breakage in all parameters compared to the other groups. This concurred with a study by Arabi [15] which showed that nicotine promotes significant membrane impairments and DNA fragmentations in sperm. The damage of DNA may arise from three main mechanisms: Abnormal chromatin packaging, apoptosis and oxidative stress. Nicotine has been well documented to cause oxidative stress and oxidative damage to sperm DNA, rendering it to lose its ability to fertilize the oocyte. Reactive oxygen species might harm DNA by directly attacking the bases and the deoxyribose backbone. It also induces apoptosis through caspases activation by the release of cytochrome C protein from disrupted mitochondrial membranes [16]. This eventually causes DNA breakage.

In addition, the present study demonstrated significantly less DNA fragmentation in the nicotine-TQ group in all DNA damage profile compared to the nicotine group. This shows that TQ treatment reduced the deleterious effects of nicotine on DNA integrity. This is in agreement with a previous report which showed that TQ treatment caused a significant decrease in DNA damage in the cyclophosphamide-treated group [17]. The favorable effects of TQ could be implicated by its marked antioxidant capacity. Several studies have described TQ as an efficient scavenger of superoxide, hydroxyl radical and singlet molecular oxygen. The potent antioxidant potentials of TQ could reduce direct attacks of free radicals on the DNA molecule and mitochondria, thus reducing DNA fragmentation.

Apart from that, the expression levels of *PT1* and *Tnp2* genes between groups were determined. The product of those genes, PT1 and Tnp2 proteins are important for condensed chromatin packaging. Inefficient chromatin remodeling increases the susceptibility of sperm DNA to damages from stressors such as free radicals. In this study, there were no changes in *PT1* levels in all experimental groups compared to the control, indicating that nicotine and TQ have no influence on this particular gene. There was no prior study on the effects of nicotine on the protamination of sperm DNA. However, the result is in contrast to earlier findings whereby smoking was found to interfere with protamine expression at the mRNA level and these abnormalities could be attributed to smoking-related ROS [18].

The results of the current study further contradicted a study by Hammadeh *et al* who

revealed that oxidative stress induced by cigarette smoking causes poor protamine replacement process [19]. The researchers also revealed that smoking affected *PT1/Protamine 2 (PT2)* expression ratio by the under-expression of *PT2*, suggesting that smoking affects *PT2* greater than *PT1*. The genetic mechanism underlying smoking-induced protamine abnormality is still unclear.

Only *PT1* was examined in the analysis of protamine expression in this current study given that rats, the model animal for the present study, do not express *PT2* [20]. It is unclear whether the unaltered expression of *PT1* in the current study was due to insubstantial nicotine inducement or nicotine having no effect on *PT1*. Consequently, the ameliorative role of TQ on *PT1* gene was not able to be determined. Nevertheless, it can be safely assumed that the DNA damage observed in this study was not a result of abnormal *PT1* expression.

Interestingly, *Tnp2* was significantly down-regulated in the nicotine group and showed improvements in the nicotine-TQ group. To the best of found knowledge, the effects of nicotine on *Tnp2* expression level have not been studied, much less by TQ. Transition nuclear proteins (Tnp) participate in the initial condensation of the chromatin, where it transiently replaces the testis-specific histone variants before being replaced by protamines. This suggests that the disruption in *Tnp* expression may also impair DNA integrity. Some studies reported distinct roles for Tnp1 and Tnp2 proteins and proposed that Tnp1 is involved in histone removal whereas Tnp2 is involved in chromatin condensation [21]. Transition nuclear protein 2 (Tnp2) also affects DNA integrity through its roles in DNA nick repair during spermiogenesis and the ultimate degree of chromatin condensation [22]. Temporary nicks during histone-protamine replacement need to be fixed before they persist and evolve into DNA fragmentation in mature sperms.

The molecular mechanism responsible for nicotine-induced Tnp2 abnormality is still unclear. From the present study, it is possible to deduce that the alteration of *Tnp2* expression level in the nicotine group may be caused by oxidative stress through the attack of ROS towards the mRNAs. As an antioxidant agent, TQ shows the ability to slightly improve *Tnp2* expression in the nicotine-TQ group. Hormone inhibition by nicotine might also provide a possible explanation for Tnp2 dysregulation.

Reports have indicated that nicotine could inhibit follicular stimulating hormone (FSH) and

luteinizing hormone (LH) release from the pituitary gland. The inhibition of these hormones could affect *Tnp2* regulation as deprivation of FSH signaling was found to greatly decrease *Tnp* and protamine levels [23]. Furthermore, Cacciola *et al* found that downregulation of GnRH occurred with *Tnp2* mRNA [24]. However, there is no conclusive evidence of *Tnp2* being involved in the direct pathway of the hypothalamic-pituitary-testicular axis.

Nevertheless, it appears that the relationship between *Tnp2* expression and DNA damage in this study has yet to be ascertained. A review paper by Leduc *et al* reported that DNA fragmentation in double *Tnp* knockout models did not persist and the DNA condensing impairment might be compensated by protamines [25]. Therefore, further investigation of the effect of nicotine and TQ on *Tnp2* should be explored in the future.

CONCLUSION

The findings of this study show that administration of nicotine has harmful effects on sperm DNA quality and integrity in rats. However, administration of TQ improves the integrity of sperm DNA. Apart from that, in the analysis of genes responsible for sperm chromatin condensation, nicotine and TQ treatment demonstrated no effect on *PT1* gene. This suggests that *PT1* gene does not influence sperm DNA damage in this study. Conversely, *Tnp2* was significantly down-regulated in the nicotine group and showed improvement in nicotine-TQ group. Dysregulation of *Tnp2* may have affected condensation and caused fragmentation of the DNA. Nevertheless, further studies are required to confirm these findings and to explore the effects of nicotine and TQ at the molecular level.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. FDR was involved in carrying out the lab work, interpretation of data and preparation of the manuscript. NHH was involved in the grant application, study design and conduction, data analyses and manuscript revision. YMY designed experiment and participated in gene expression analysis. KO and SFI participated in the study design, and provided theoretical and technical guidance. NK was involved in study design and writing of the manuscript. All authors read and approved the final manuscript for publication.

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