Involvement of Neurotransmitter and Nrf2 in Nicotine- and Cigarette Smoke-Induced Testicular Toxicity in Adult Rats

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ABSTRACT: Several organ systems can be affected by nicotine/cigarette smoking (CS); however, there is a gap of knowledge about the role of local neurotransmitter system, brain-derived neurotrophic factor (BDNF), and dopamine (DA) in testicular toxicity. Therefore, the aim of the present study is to explore the toxic impact of short- and long-term exposure to oral nicotine and passive CS on adult albino Wistar rats, using doses that closely mimic the human smoking scenario. Our results showed dose- and time-dependent loss of developing spermatogonia and spermatocytes of the seminiferous tubules, disruption of basement membrane, DNA damage, and high serum cotinine upon exposure to nicotine and CS, resulting in low sperm count as compared to control. Further, the results showed the upregulation of BDNF, DA, tyrosine hydroxylase (TH), and pro-oxidants, ie, reactive oxygen species (ROS) and inducible nitric oxide synthase (iNOS), in the exposed tests and downregulation of the antioxidants such as, ascorbate and Nrf2 when compared with the control. Thus, our results for the first time highlight a potential role of the local neurotransmitter system and antioxidant depletion (Nrf2) in nicotine/CS-induced testicular pathogenesis, which could underpin the development of therapeutic interventions targeted at oxidative stress-associated disorders, and probably establish a link with the brain system contributing to addiction.

KEYWORDS: nicotine, cigarette smoking, dopamine, BDNF, testis, Nrf2, oxidative stress, molecular mechanism

Introduction

Drug addiction is a chronically relapsing disorder characterized by compulsive drug seeking and abuse that affect neurocircuits involving cognitive and mood-associated behaviors, probably due to the pathological changes in different brain regions.1 As per the USDHHS,2 cigarette smoking (CS) is a powerful additive drug/psychostimulant and nicotine is one of the major pharmacologically active components of CS, which degenerates midbrain dopaminergic (DAergic) neurons via rapid activation of tyrosine hydroxylase (TH) and inhibition of monoamine oxidase B.3 Initial nicotine has a significant influence on DAergic function of central nervous system (CNS) and modulates DAergic system targeting dopamine D1 and D2 receptors (DA D1 and D2 Rs) in the caudate putamen of the adult rat brain.4,5 Acute doses of nicotine facilitate DA release, but chronic nicotine treatment decreases CNS DA turnover in the striatum.6 Further, like DA, brain-derived neurotrophic factor (BDNF) is another major neurochemicals that promotes cell survival, neural outgrowth, differentiation, plasticity and regeneration of damaged neurons sprouting through Trkβ R activation, and modulation of chemical synapses.7,8 Evidences suggest that nicotine induces BDNF and upregulates BDNF R (TrkβR) in rat brain.9

Acute Trkβ activation potentiates presynaptic DA release and transport in different brain regions.10 Moreover, high synaptic DA induces BDNF expression in adult midbrain to initiate addiction behavior such as, stress and depression via DA D1 R activation while using psychostimulant or substance abuse.11 Earlier studies indicate that nicotine binds to several proteins, including neurotransmitter (NT) Rs, plasma proteins, voltage-gated ion channels, enzymes, and hormones in different peripheral organs, including the testis.12,13 Rat adrenal pheochromocytoma cells exhibit reduced catecholamine (CA) release response upon nicotine tolerance.14 Nicotine/CS induces abnormal testis morphology and antioxidant depletion, and exerts negative impact on sperm production and fertility,15,16 epigenetic changes, and behavioral as well as cognitivé disorders.17,18 Although testicular binding sites for nicotine/CS have been demonstrated,19,20 the NT mechanism underlying nicotine/CS-induced testicular pathology is not fully reported. Different testicular actions of CA modulate testosterone production.21,22 TH, the rate-limiting enzyme of DA (CA) synthesis, is found in Leydig cells, seminal cells, neuron-like cells, and nerve fibers,23,24 and DA-R is located in neuron-like cells and nerve fibers in human testicular tissue,24 suggesting paracrine actions of DA in the spermatogenic process.25

COMPETING INTERESTS: Authors disclose no potential conflicts of interest.

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Further, testosterone plays a crucial role in regulation of neural structure and function, modulation of brain circuits at puberty, and regulation of social, risk-taking, and cognitive behavior.27 Interestingly, brain and testis exhibit maturation relation in terms of development of cerebral cortex and seminiferous epithelium.28 Cameron (2004) established that glial cell, CNS, testis, and uterus are immune-privileged organs, ie, barrier built-up between tissue and blood, and have common cell regulation.29 Testosterone and testis volume are also inversely related to the paternal behavior via ventral segmental area (VTA), the DA reward and motivation system; thereby, causing depressive attitude and care giving to the child.27 DA transporter blockade by psychostimulants also cause elevation of extracellular DA and DA R downregulation, resulting in addictive behavior.30,31 Interestingly, local effect of BDNF, like DA, is observed in several peripheral organs like testis, thyroid, and adrenal upon drug abuse.32,33 and activation of TrkB R releases intracellular calcium through phospholipase C, which subsequently potentiates BDNF release,34 but the interaction between testicular toxicity and local NT system, ie, DA and BDNF is yet to be understood.

Further, various psychostimulants/drugs such as, cocaine and caffeine induce oxidative stress by production of reactive oxygen species (ROS), depletion of cellular antioxidant defense, and subsequent cell death, thereby deteriorating seminiferous epithelium, which is associated with the upregulation of DA and DAergic marker, TH in Leydig cells and seminiferous tubules, and the downregulation of DA R mRNA expression; hence, indicating a link between redox-pathway, local NT function, and drug toxicity.35,36 ROS and reactive nitrogen oxide species (RNOS), depending on their levels in the cell, have been associated with a wide range of DNA-damaging events including base modification, strand breaking, and post-translational modifications of key proteins associated with development, NT signaling, maturation, and synaptogenesis. Further, both ROS and RNOS are regulated in the cells by upregulation of COX2 and inducible nitric oxide synthetase (iNOS), respectively, that ultimately increase the susceptibility to tissue damage, degenerative changes, or cell death, contributing several disorders. Moreover, nuclear factor-erythroid 2-related factor 2 (Nrf2) is the master regulator of cellular oxidative defense mechanism, which binds with antioxidant response element (ARE) in the promoter of several cellular oxidative defense mechanism, which binds with anti oxidant response element (ARE) in the promoter of several cellular oxidative defense mechanism, which binds with antioxidant defense, and subsequent cell death, thereby deteriorating seminiferous epithelium, which is associated with the upregulation of DA and DAergic marker, TH in Leydig cells and seminiferous tubules, and the downregulation of DA R mRNA expression; hence, indicating a link between redox-pathway, local NT function, and drug toxicity.35,36 ROS and reactive nitrogen oxide species (RNOS), depending on their levels in the cell, have been associated with a wide range of DNA-damaging events including base modification, strand breaking, and post-translational modifications of key proteins associated with development, NT signaling, maturation, and synaptogenesis. Further, both ROS and RNOS are regulated in the cells by upregulation of COX2 and inducible nitric oxide synthetase (iNOS), respectively, that ultimately increase the susceptibility to tissue damage, degenerative changes, or cell death, contributing several disorders. Moreover, nuclear factor-erythroid 2-related factor 2 (Nrf2) is the master regulator of cellular oxidative defense mechanism, which binds with antioxidant response element (ARE) in the promoter of several cytoprotective genes, including antioxidant and detoxifying enzymes, repair mediators, transcription factors of mitochondrial biogenesis, cell differentiation, and survival.37,38 But whether Nrf2 plays a critical role in testicular pathogenesis induced by oxyradical overload during exposure to nicotine/CS and underlying local NT mechanism as described in the present study is yet to be answered though there is a possibility of Nrf2 involvement.

Therefore, the present study aimed to evaluate the potential toxic consequences of short- and long-term exposure to nicotine/CS on testis of the adult rats with regard to the local NT mechanism. Here, we have investigated that nicotine- and CS-induced ROS and RNOS concentration (through iNOS expression) with concomitant lowering of ascorbate and Nrf2 expression in the adult rat testis leads to breakage of basement membrane, degeneration of developing spermatogonia (SG) and spermatocytes (SC) in the seminiferous tubules, resulting in lowering of sperm count. This is further associated with the upregulation of BDNF, DA, and DA marker, TH, at the translational level in the same area. Further, our result suggests colocalization of the NTs in the germinal cells of the seminiferous tubules upon exposure to nicotine/CS. To our knowledge, the results for the first time reveal the effect of nicotine/CS on the local NT expression, oxidative stress, and pathogenesis of sperm production cells of the testis, which may establish a probable link with the brain system.

Materials and Methods

Animals and grouping. The IAEC-NIOH Guidelines regarding the care and use of the animals were considered throughout the experiments in this study. Male albino Wistar rats (250–275 g BW) were housed in a temperature (23 ± 3°C) and humidity (55% ± 15%) controlled environment under 12-hour dark/light cycle with food and water ad libitum. After one week of acclimatization, the rats were divided into two major groups: the control group (n = 10) and the experimental group (n = 40). The experimental group was further divided into two subgroups: the CS group (n = 10) and the nicotine group (n = 30). In the CS group, two commercially available filtered cigarette (ITC, India) were passively inhaled for 1 hour in a tightly sealed whole-body inhalation chamber, and in the nicotine group, nicotine (Nic 3–9 mg/kg BW/day; dissolved in PBS) was administered orally, for 4 and 12 weeks as per our previous protocol. The doses closely mimic the amount of nicotine intake as with the CS in human;39 hence, the animal model used here may closely resemble the human situation. The control group was given PBS. The rats were anesthetized with an intraperitoneal injection of ketamine–xylazine after the indicated time of treatment as mentioned above, and the testis were removed. Tissue homogenates were prepared in PBS (0.02 M, pH 7.4) followed by ultracentrifugation (12,000 rpm, 10 minutes × 2), as described previously.5,40 Blood was collected from the hearts of the anesthetized rats through terminal bleeding procedure during euthanasia in serum separator tube (BD, USA) and, after 30 minutes, centrifuged at 1000 g for 10 minutes prior to making aliquots.41 Samples were cryo-preserved for further analysis, if required. Carcass was disposed by authorized private biomedical waste disposal agency (Pollucare Biomedical Management Pvt. Ltd., India).

Chemicals and reagents. Most of the chemicals and reagents were procured from Sigma-Aldrich (USA) unless otherwise specified. Antibodies from Santa Cruz Biotechnology (USA), Sodium citrate, sulfuric acid and formaldehyde from Thermofisher Scientific (USA), and TEMED, SDS and tris hydrochloride from HiMedia (India) were purchased.
**Cotinine analysis.** Serum cotinine, a metabolite of nicotine, was analyzed as per the standard method provided with ELISA kit specific to mouse/rats from CalBiotech Inc. (USA). Samples were treated with cotinine HRP-enzyme conjugate and incubated for 60 minutes at 25°C in dark followed by addition of TMB substrate and stop solution as directed in the kit. Finally, cotinine concentration in the sample was measured at 450 nm, which was inversely proportional to the color intensity of the solution.

**Oxidative stress.** Total ROS was measured to detect the oxidative stress or damage, if any, upon exposure following the standardized protocol. Samples were treated with 2′,7′-dichlorofluorescein diacetate and incubated for 30 minutes at 37°C followed by washings to remove the excess dye. The reading was taken at \( \lambda_{ex} = 488 \text{ nm} / \lambda_{em} = 535 \text{ nm} \). Negative control (DMSO), positive control (potent oxidant, TPA) and vitamin C (antioxidant) treated control samples were run in parallel to validate the results. Absorbance (non-enzymatic antioxidant) in the samples reacted with 2,4-dini-trophenylhydrazine-thiourea to form osazone, which was dis-solved in sulfuric acid to give intense red color solution. The optical density was measured at 540 nm. Nrf2 and iNOS expressions were also detected in the pooled testis homogeneate by Western blotting as discussed below.

**Protein isolation and Western blotting.** Pooled testis homogenate was used to find out the expression of proteins (BDNF, TH, Nrf2, and iNOS) upon exposure through Western homogenate was used to find out the expression of proteins deparaffinization as per conventional histology technique, anti-whose gives additional support to our blotting data. After the preheated steam cooker at 90°C were immersed in sodium citrate buffer (pH 6.0) and kept in the preheated steam cooker at 90°C for 10 minutes followed by cooling at room temperature for 15 minutes. Slides were

**Immunohistochemistry.** To find out the localization of DA, TH, BDNF, and Nrf2 in the testicular tissue parallel to Western blotting, immunohistochemistry (IHC) was performed on the paraaffinized section as per mentioned previously, which gives additional support to our blotting data. After deparaffinization as per conventional histology technique, antigen retrieval was done on the sections. For this purpose, slides were immersed in sodium citrate buffer (pH 6.0) and kept in the preheated steam cooker at 90°C for 10 minutes followed by cooling at room temperature for 15 minutes. Slides were

**Comet assay.** To find out the oxidative stress-induced DNA damage, if any, after exposure, we followed the modified alkaline comet assay protocol in testis homogenate. Briefly, tissue homogenate was suspended on low-melting agarose and sandwiched between a layer of normal-melting agarose and a top layer of low-melting agarose on fully frosted slides, which was kept on ice during polymerization of each gel layer. Then, the slides were immersed in a lysis solution at 4°C for 1 hour followed by alkaline (pH 13.0) electrophoresis for 25 minutes at 300 mA and 0.75 v/cm. The slides were neutralized with Tris–HCl buffer (pH 7.5) and stained with ethidium bromide for 10 minutes. Tris buffer and methyl methanesulfonate were taken as negative and positive controls, respectively, to validate the results. A total of 100 cells were analyzed on each slide to determine the tail length (ie, indicator of DNA damage) and tail intensity (ie, percent genomic DNA migration toward tail) by QWin software (Leica, Germany) and CASP software (USA) using Leica DMI microscope attached with a computer. Olive tail moment (a factor combines tail length and percentage distribution (%) of DNA in tail) was also calculated.

**Tissue histology.** To find out the oxidative stress-induced tissue alteration, if any, after exposure, histology of testis was done on the paraffin-embedding sections by conventional eosin–hematoxylin staining. Slides were observed in bright field and photographed accordingly using Zeiss Axio Scope microscope having attached digital camera at different magnifications.

**Epididymal sperm count.** Adherent fat, blood vessels, and connective tissues were removed from cauda epididymis, and sperms were released by cutting the tissue longitudinally with scissors and pressing with forceps. Then, suspension was prepared using PBS containing collagenase (1:100) and pronase (1:1000) followed by incubation at 37°C for 30 minutes. Finally, sperms were counted in a Neubauer hemocytometer using WBC pipette by Zeiss Axio Scope microscope at 400× magnification. Both sides of the hemocytometer were counted and the average value was taken.

**Statistical analysis.** One-way analysis of variance followed by Tukey’s test for multiple comparisons were performed using VassarStat.Net (USA) to determine the significance of differences between the control and the experimental groups.
In every case, the acceptance level for statistical significance was $P < 0.05$. Data were presented as mean ± standard error of mean (SEM).

**Results**

The overall body weights of the nicotine- and CS-exposed adult male albino Wistar rats (327–350 g) and matched control rats (332–364 g) were not varied much throughout the study period, ie, up to 12 weeks though slightly reduced in the former, which is in corroboration with our earlier study. Individual organ weights, foods, and water consumption were also not varied among the different study groups (data not shown), as mentioned previously.

**Serum cotinine as marker of nicotine toxicity and CS.**

The aim of the present study was to investigate whether short-(4 weeks) and long- (12 weeks) term exposure to nicotine/CS imparts toxic outcome on testis (peripheral organ) of the adult rats and the underlying mechanism. As cotinine is the known valid marker of CS and/or nicotine exposure; therefore, dose- and time-dependent effects of oral nicotine and CS through passive inhalation on serum cotinine levels were measured. Cotinine was not detected in the serum of the control group, but as compared to the 3 mg/kg/day nicotine, serum values were significantly increased in the 6 and 9 mg/kg/day groups for both 4 and 12 weeks of exposure (Fig. 1A). Serum cotinine level in CS group was also maintained in the similar trend (Fig. 1B) though the extent of impact was slightly lower in the CS group than the nicotine groups.

**Nicotine- and CS-induced degenerative changes of seminiferous epithelium and DNA damage.**

Testis is structurally and functionally linked with both the spermatogenesis through germline cells in the seminiferous tubules and steroidogenesis involving interstitial cells of Leydig. Therefore, to demonstrate whether the exposure to nicotine/CS may exert any negative effects on the seminiferous tubular cells and Leydig cells, we first evaluated testis histology. As shown in Figure 2B–C, and E–F, eosin-hematoxylin staining revealed dose-dependent structural changes such as, basement membrane breakage along with degeneration of the developing SG in the seminiferous tubules of adult rats upon short-term exposure to nicotine/CS when compared with the control (Fig. 2A). Further, long-term exposure also showed similar degenerative changes even in SC (Fig. 2D–G), which was associated with the reduced sperm count under the same experimental conditions (Table 1). However, we could not find any variation of the Leydig cell histology upon exposure (Fig. 2H).

Further, to find out the nicotine’s DNA damaging potency, comet assay was performed for determining the double-stranded DNA (dsDNA) breaking in the testis (Table 2; Fig. 3A–C). The dose- and time-dependent increase in the percentage of comet tail DNA (ie, tail intensity) along with the tail length was observed in the present study after nicotine/CS of 4 and 12 weeks as compared to the control (Table 2). Further, higher DNA damage was noticed with high nicotine and CS for long-term exposure (12 weeks) than the low nicotine exposure for 4 weeks (Table 2). We also validated...
our data with the positive control (methyl methanesulfonate) and negative control (tris buffer). As expected, methyl methanesulfonate induced DNA damage, whereas no DNA damage was observed after treatment with tris buffer (Table 2).

**Nicotine and CS inhibited antioxidant defense and induced oxidative stress.** ROS and RNOS have both beneficial and harmful roles, depending on their concentrations and duration of exposure. Therefore, to assess whether nicotine/CS-induced testicular pathology is mediated through the alteration of cellular defense system and oxidative mechanism, total ROS, iNOS, Nrf2, and ascorbate were measured under different experimental conditions as stated above. Compared with the control, nicotine and CS dose dependently showed significantly high level of ROS in the testis upon 4- and 12-week treatment (Fig. 4A-B). Figure 4C-D revealed significant reduction of ascorbate concentration in the testis upon exposure to nicotine/CS for 4 and 12 weeks while comparing with the respective control. Further, iNOS expression in the pooled testis samples was upregulated remarkably with increased concentration of nicotine/CS at 4 and 12 weeks of exposure in adult Wistar rats (Fig. 4E-F). Nrf2, the marker of cellular defense system, showed significant dose- and time-dependent downregulation in the same tissue, whereas the control revealed reciprocal relations in both the cases (Fig. 4E-G). We further did IHC in support of our Western blot data. IHC result exhibited the similar trend of expression in the germinal cells of the seminiferous tubules among the exposed groups. Figure 4I indicated downregulation of Nrf2 in the developing SG and SC upon exposure as compared to the control (Fig. 4H); thereby establishing the dose- and time-dependent alterations of iNOS and Nrf2 protein expressions at the translational levels induced by nicotine/CS. Further, in the present study, upregulation of iNOS may be the causative factor of RNOS production in the testicular tissues upon exposure.

Moreover, we validated our data with the positive control (TPA), negative control (DMSO), and vitamin C treated control. As expected, TPA significantly induced, whereas vitamin C alone significantly reduced the ROS concentration and iNOS expression in the control testis, but the DMSO treated sample remained unchanged (data not shown). Interestingly, downregulation of the TPA-mediated oxidative stress in the control testis by vitamin C was also observed (data not shown). Taken together, our results indicated inhibition of antioxidant defense by lowering of ascorbate and Nrf2 expression with concomitant induction of oxidative stress by ROS and iNOS upon exposure to nicotine/CS.

**Nicotine and CS upregulated TH, DA, and BDNF expression.** Finally, in order to determine the possible local NT targets of nicotine/CS-induced degenerative changes and oxyradical overload of the adult rats, BDNF, DA, and TH, the rate-limiting enzyme of DA biosynthesis, were evaluated in the testis homogenates by Western blot and IHC. As shown

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**Table 1.** Effect of nicotine and CS on epididymal sperm count (10^6/mL) of the adult rats upon treatment with 3, 6, and 9 mg/kg BW/day nicotine (Nic) and 1 hour CS for 4 and 12 weeks as compared to the controls (Crt).

<table>
<thead>
<tr>
<th>GROUP</th>
<th>4 WEEKS</th>
<th>12 WEEKS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crt</td>
<td>157.5 ± 5.42</td>
<td>152.5 ± 5.6</td>
</tr>
<tr>
<td>Nic 3</td>
<td>134.3 ± 3.62</td>
<td>111.1 ± 4.64</td>
</tr>
<tr>
<td>Nic 6</td>
<td>74.7 ± 1.79*</td>
<td>60.70 ± 6.75**</td>
</tr>
<tr>
<td>Nic 9</td>
<td>40.5 ± 1.55**</td>
<td>25.91 ± 3.62**</td>
</tr>
<tr>
<td>CS</td>
<td>48.1 ± 4.64**</td>
<td>30.30 ± 2.64**</td>
</tr>
</tbody>
</table>

**Notes:** Detail procedures are mentioned in the “Materials and methods” section. Results represent the mean ± SEM of three independent experiments. *P < 0.01; **P < 0.001 (vs. Crt).

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**Figure 2.** Representative photomicrograph of the adult rat testis showing nicotine- and CS-induced basement membrane disintegration, and loss of developing SG and SC (↑) in the seminiferous tubules upon treatment with 3, 6, and 9 mg/kg BW/day nicotine (Nic, B-F) and 1 hr CS (G) for 4 and 12 weeks as compared to the controls (Crt, A); while Leydig cell histology remain unaltered upon treatment (H). Detail procedures a mentioned in the “Materials and methods” section. A-B, G: ×100; C-F: ×200; H: ×1000.
Moreover, Seccareccia et al. reported that nicotine/CS-induced upregulation of the NTs at the translational levels occurs locally in the peripheral tissue upon exposure, which corroborates several previous observations. In the present study, we, for the first time, provide the evidence of significant testicular pathogenesis mediated by the local NT, DA, and BDNF, disruption of the redox homeostasis and the irreversible oxidative modification of protein and DNA upon short- (4 weeks) and long- (12 weeks) term exposure to psychostimulant nicotine and CS. Another issue relevant to this study is nicotine-, nornicotine- (produced by nicotine), and nicotine’s metabolic intermediate, cotinine-induced release of DA through activation of nACh Rs. We also explored a remarkable dose- and time-dependent shift of serum cotinine level in the experimental animals toward higher side without any detection in the controls as a result of the applied doses that resemble the human smoking scenario. Serum cotinine is not detected in the control animals due to its nonavailability of the substance in the serum throughout the study period, suggesting toxic impact of the studied doses upon exposure, which corroborates several previous observations. Moreover, Seccareccia et al. reported the positive association between self-reported nicotine exposure and serum cotinine levels among the smokers, nonsmokers reporting exposure to environmental tobacco smoke (ETS), and nonsmokers with no ETS exposure even after the adjustment of age and sociodemographic and behavioral factors. Nicotine exposure from the passive tobacco smoke can also endanger plasma nicotine concentration equivalent to the levels produced by the tobacco smoking and are associated with various health effects.

### Table 2. Effect of nicotine and CS on DNA comet in the testis of the adult rats upon treatment with 3, 6, and 9 mg/kg BW/day nicotine (Nic) and 1 hour CS for 4 and 12 weeks as compared to the controls (Crt).

<table>
<thead>
<tr>
<th>GROUP</th>
<th>TAIL LENGTH (μm)</th>
<th>TAIL INTENSITY (%)</th>
<th>OLIVE TAIL MOMENT (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 WEEKS</td>
<td>12 WEEKS</td>
<td>4 WEEKS</td>
</tr>
<tr>
<td>−ve control</td>
<td>0.0 ± 0.43</td>
<td>0.0 ± 0.52</td>
<td>0.0 ± 0.11</td>
</tr>
<tr>
<td>+ve control</td>
<td>4.04 ± 0.289</td>
<td>4.09 ± 0.366</td>
<td>6.30 ± 0.816</td>
</tr>
<tr>
<td>Crt</td>
<td>3.54 ± 0.245</td>
<td>3.71 ± 0.320</td>
<td>3.96 ± 1.416</td>
</tr>
<tr>
<td>Nic 3</td>
<td>3.76 ± 0.254</td>
<td>4.28 ± 0.393</td>
<td>5.21 ± 1.012</td>
</tr>
<tr>
<td>Nic 6</td>
<td>6.50 ± 2.000</td>
<td>7.17 ± 1.697</td>
<td>9.14 ± 2.960</td>
</tr>
<tr>
<td>Nic 9</td>
<td>9.74 ± 0.891†</td>
<td>12.28 ± 1.704**</td>
<td>24.27 ± 2.276**</td>
</tr>
<tr>
<td>CS</td>
<td>4.04 ± 0.456</td>
<td>11.1 ± 2.032**</td>
<td>10.36 ± 4.972</td>
</tr>
</tbody>
</table>

**Notes:** Detail procedures are mentioned in the “Materials and methods” section. Results represent the mean ± SEM of three independent experiments. *P < 0.05; †P < 0.01; **P < 0.001 (vs. Crt).

Discussion

In the present study, we, for the first time, provide the evidence of significant testicular pathogenesis mediated by the local NT, DA, and BDNF, disruption of the redox homeostasis and the irreversible oxidative modification of protein and DNA upon short- (4 weeks) and long- (12 weeks) term exposure to psychostimulant nicotine and CS.
Figure 4. (Continued)
Figure 4. Effects of nicotine and CS on oxidative stress and antioxidant defense of the adult rats upon treatment with 3, 6, and 9 mg/kg BW/day nicotine (Nic; A, C, E-G, I) and 1 hour CS (B, D, E-G) for 4 and 12 weeks as compared to the controls (Crt; A-H). Detail procedures are mentioned in the "Materials and methods" section. Results represent the mean ± SEM of the three independent experiments. Testis homogenates are prepared for measurement of ROS by DCF-fluorescence (A, B) and ascorbate in mg/ml (C, D). $^\text{*}P < 0.05; ^\text{**}P < 0.01; ^\text{***}P < 0.001$ (vs. Crt). iNOS and Nrf2 protein expression (E) in pooled testis homogenates are assessed by Western blot where β-actin is taken as loading control. Densitometric evaluations of iNOS (F) and Nrf2 (G) protein bands are done by GelQuant.Net software. $^\text{*}P < 0.05$; $^\text{**}P < 0.01$ (vs. Crt). Representative photomicrographs of Nrf2 protein expression (brown) in the developing SG and SC of the seminiferous tubule by IHC (H, I). H, I: x400.
Nicotine/smoke modulate local neurotransmitters and testis toxicity

with the nicotine-induced changes in human behavior. All together, evidences suggest that serum cotinine may play a key role in promoting tobacco smoking linked with psychiatric conditions; thus representing a new potential therapeutic agent against stress disorder.

CS and/or nicotine that generates during CS cause health hazard, exerting diverse physiological and biochemical effects. As seminiferous tubules are the main buffer system to regulate sperm production, we hypothesized that exposure to nicotine/CS like other drugs/psychostimulants may negatively modulate spermatogenic process. In the present study, as compared to the controls, we found that oral nicotine and passive CS manifested dose- and time-dependent histological and functional alteration of the testis, including loss of

**Figure 5.** Effects of nicotine and CS on testicular NT expression of the adult rats upon treatment with 3, 6, and 9 mg/kg BW/day nicotine (Nic, A-C, E) and 1 hour CS (A-C, F) for 4 and 12 weeks as compared to the controls (Crt, A-D). Detail procedures are mentioned in the “Materials and methods” section. Results represent the mean ± SEM of the three independent experiments. BDNF and TH (marker of DA) proteins expression (A) in pooled testis homogenates are assessed by Western blot where β-actin is taken as loading control. Densitometric evaluations of TH (B) and BDNF (C) protein bands are done by GelQuant.Net software. *P < 0.05; **P < 0.01 (vs. Crt). Representative photomicrographs showed colocalization of BDNF (black), DA (brown), and TH (black) protein expression in the basement membrane and developing SG of the seminiferous tubules by IHC (D-F). D: ×200; E: ×400.
developing SG, SC, and breakage of basement membrane, resulting in deterioration of sperm count. Our results are in corroboration with the several previous observations showing psychostimulant or drug abuse that can induce germinal cell loss including SC and spermatids, resulting in disruption of spermatogenesis.\textsuperscript{17–19,53,54} Rajpurkar et al\textsuperscript{55} claimed that CS reduces the number of germinal cells, height of germinal epithelium, tubular diameter and induces apoptosis in the testis due to the presence of many toxic substances in cigarettes.\textsuperscript{56} However, Leydig cell histology that remains unaltered upon exposure in the present study may indicate that the current doses may not be able to initiate any change in the testicular steroidogenic process; the discrepancy of which with the previous reports\textsuperscript{57,58} may be due to the differences in the experimental design, species, strains, etc.

CS is also considered as an environmental aging accelerator\textsuperscript{59} partly because it induces pathological changes on the testis structure and functions due to exacerbation of oxidative stress, ie, ROS generation and repression of antioxidant defense.\textsuperscript{36,53,60} CS further significantly increases the level of ROS in the seminal vesicles,\textsuperscript{61} degrades phospholipids,\textsuperscript{54} induces sperm cell injury through several pathways, and finally impacts sperm structure, quality, and functions.\textsuperscript{62–64} Kuladip et al\textsuperscript{53} showed a significant decrease in testis histology due to the overproduction of ROS, resulting in oxidative membrane lipid degradation followed by structure–function alteration upon nicotine treatment. Mutations in mitochondrial genome may also occur after exposure that disturbs the formation of morphologically and functionally mature spermatozoa, leading to male infertility due to the burden of ROS-induced oxidative stress.\textsuperscript{65} Particularly, lipid peroxidation is considered as the main mechanism by which ROS-induced impairment of testicular function is observed.\textsuperscript{66} In the present study, the dose- and time-dependent testicular (seminiferous tubule) degenerative changes and DNA damage (dsDNA breaking detected by DNA comet) induced by oxidative stress (ie, high total ROS level and iNOS expression) and antioxidant depletion (ie, low ascorbate concentration and Nrf2 expression) upon nicotine/CS exposure suggested that oxidative burden is the most stringent criteria for maintaining tissue integrity during exposure.\textsuperscript{67,68} Further, the dose-dependent DNA strand break due to the formation of peroxynitrite indicates that nitric oxide (NO)-mediated pathogenesis may play a vital role in tobacco-associated conditions.\textsuperscript{69} Nicotine-induced DNA strand breakage is pH dependent; thus, alkaline saliva generated by the chewing betel quid causes DNA damage, where high ROS contributes to oxidative stress-associated pathogenesis.\textsuperscript{70} Overall, higher DNA as well as seminiferous tubule damage was noticed in the present exposed groups, which exhibited high oxyradical overload and antioxidant depletion.

Additionally, there is evidence that ROS and RNOS are partly because it induces pathological changes on the testis structure and functions due to exacerbation of oxidative stress, ie, ROS generation and repression of antioxidant defense.\textsuperscript{36,53,60} CS further significantly increases the level of ROS in the seminal vesicles,\textsuperscript{61} degrades phospholipids,\textsuperscript{54} induces sperm cell injury through several pathways, and finally impacts sperm structure, quality, and functions.\textsuperscript{62–64} Kuladip et al\textsuperscript{53} showed a significant decrease in testis histology due to the overproduction of ROS, resulting in oxidative membrane lipid degradation followed by structure–function alteration upon nicotine treatment. Mutations in mitochondrial genome may also occur after exposure that disturbs the formation of morphologically and functionally mature spermatozoa, leading to male infertility due to the burden of ROS-induced oxidative stress.\textsuperscript{65} Particularly, lipid peroxidation is considered as the main mechanism by which ROS-induced impairment of testicular function is observed.\textsuperscript{66} In the present study, the dose- and time-dependent testicular (seminiferous tubule) degenerative changes and DNA damage (dsDNA breaking detected by DNA comet) induced by oxidative stress (ie, high total ROS level and iNOS expression) and antioxidant depletion (ie, low ascorbate concentration and Nrf2 expression) upon nicotine/CS exposure suggested that oxidative burden is the most stringent criteria for maintaining tissue integrity during exposure.\textsuperscript{67,68} Further, the dose-dependent DNA strand break due to the formation of peroxynitrite indicates that nitric oxide (NO)-mediated pathogenesis may play a vital role in tobacco-associated conditions.\textsuperscript{69} Nicotine-induced DNA strand breakage is pH dependent; thus, alkaline saliva generated by the chewing betel quid causes DNA damage, where high ROS contributes to oxidative stress-associated pathogenesis.\textsuperscript{70} Overall, higher DNA as well as seminiferous tubule damage was noticed in the present exposed groups, which exhibited high oxyradical overload and antioxidant depletion.

Additionally, there is evidence that ROS and RNOS are regulated in the cells by the upregulation of COX, and iNOS, respectively, which ultimately increases the susceptibility to tissue damage, degeneration, and cell death, leading to different pathological conditions and disorders. Like ROS, NO concentration and NOS expression have dual role in the various cellular processes, depending on the activity and localization of the isoforms in the cells.\textsuperscript{71,72} The enhanced expression of iNOS in the tobacco habituets compared to the non-habituets indicates the possible influence of tobacco on iNOS expression and NO production.\textsuperscript{69} Mahmoud and Amer\textsuperscript{73} showed prophylactic effect of ascorbate against nicotine-induced oxidative damage of the peripheral organs. Ascorbate also acts as a vitamin and neuronal modulator involving DAergic system.\textsuperscript{74} Moreover, Nrf2 signaling pathway plays a central role in the regulation of cellular antioxidant mechanism on exposure to environmental/oxidative stress, CS, nicotine, infection, or inflammation.\textsuperscript{75,76} On exposure to oxidants, Nrf2 activates the transcription of its target genes via ARE by disrupting the Nrf2–Keap1 interaction.\textsuperscript{77} Studies also suggested that drugs that can activate Nrf2 are protective; hence, overcoming the negative impact of coffee, tobacco, and other psychostimulants.\textsuperscript{78,79} The upregulation of a subset of putative Nrf2 target genes that regulate glutathione metabolism can also protect DA neurons.\textsuperscript{78} Ramsey et al\textsuperscript{80} proved that oxidative stress could be an initiator or mediator of motor neuron death in diseases where nuclear translocation of Nrf2 is impaired, leading to dysfunction of the Nrf2 pathway resulting in decrease cellular defense system against oxyradical overload. Further, disruption of Nrf2 impairs induction of Nrf2/ARE pathway, leading to exacerbation of oxidative stress, inflammation, and cellular dysfunction.\textsuperscript{81} Therefore, combining data from our laboratory and elsewhere as mentioned above, suggest that (1) testicular RNOS production may be stimulated by the upregulation of iNOS expression upon exposure to nicotine/CS and (2) both the high concentrations of RNOS and ROS along with the downregulation of Nrf2 in SG and SC disbalance the redox homeostasis in the testicular microenvironment. Moreover, consistent with its potent antioxidant activity, vitamin C alone antagonizes high ROS and RNOS concentrations in the testis, revealing vitamin C-induced blocking of the oxidative stress during data validation. Finally, degenerative changes of the basement membrane and the seminiferous tubular cells mentioned above, mediated through the upregulation of ROS and RNOS (by iNOS) with concomitant depletion of the antioxidant defense (ie, ascorbate, Nrf2) in the present study, is triggered by the dose- and time-dependent exposure of oral nicotine and passive CS to the adult Wistar rats.

In the present study, we further explore the underlying local NT mechanism in oxidative stress-induced degenerative changes of the testis, and the potential role of nicotine/CS doses in this regard. CA, especially DA, and TH, the DA marker, are found to be important modulators of testis volume, seminiferous epithelium maturation, and spermatogenesis as well as testosterone production.\textsuperscript{23,26} DA, BDNF, and oxidative stress also have a potential role in structure–function impairment
and total antioxidant depletion in the testicular tissues.\textsuperscript{36,82,83} Nicotine further binds with the Rs, hormones, enzymes, etc. and exerts negative impact on sperm production and male fertility, and behavioral disorders.\textsuperscript{15,14,16–19} Testosterone, on the other hand, regulates cognitive behavior by modulating brain circuits and neuronal maturation involving DAergic midbrain reward system that originate in VTA, project to nucleus accumbens (NAc), amygdale, hippocampus, and cerebral cortex.\textsuperscript{27} Thus, in NAcc and striatum, the reinforcing effect of nicotine and other psychostimulants is characterized by increasing DA transmission that binds to the DA Rs, which can further induce DA R downregulation.\textsuperscript{6,7,30,31} James et al.\textsuperscript{23} proved the existence of direct brain–testis pathway that controls testicular function using retroviral viral tracing from the testicular TH expressing nerve fibers to the midbrain DAergic area. The presence of DA and DAergic marker, TH, in different testicular tissues further establish the paracrine action of DA in spermatogenesis.\textsuperscript{22,24–26} Several previous observations have also shown the positive evidence of brain–testis relation.\textsuperscript{27–29}

Further, regulation of the DAergic development is mediated by other neurotrophic factors, including BDNF,\textsuperscript{11} which promotes sprouting of DAergic axons and regulates plasticity of the DAergic neurons.\textsuperscript{84} Studies also showed that psychostimulant-induced enhancement of DA transmission\textsuperscript{55} and TH at the DAergic terminals mediated through NOS gene.\textsuperscript{85} Moreover, NT-induced cell degeneration is thought to be involved in NT autoxidation and disruption of the cellular redox system.\textsuperscript{35,86} BDNF also increases in the serum of the smokers with mental disorders than the nonsmokers\textsuperscript{87,88} linked to increase in age or weight, where peripheral BDNF levels may comprise BDNF that originates in the CNS neurons.\textsuperscript{89} These observations suggest that nicotine-induced high level of the peripheral BDNF may contribute to the synaptic rearrangements involving development and maintenance of the smoking habit as well as local activity in the peripheral organ (testis) through its Rs.\textsuperscript{32,31} Several studies further indicated nicotine-induced upregulation of BDNF and BDNF Rs in the midbrain DAergic system,\textsuperscript{10,90} but downregulation of the hippocampal 5-hydroxytryptamine (5-HT) overflow.\textsuperscript{91} It is possible, therefore, that a decrease in 5-HT R activation may be one mechanism by which long-term nicotine treatment increases BDNF expression in the peripheral organ too.\textsuperscript{92}

In consistent with the previous observations as discussed above, our data exhibit colocalization of BDNF, DA, and DAergic component, TH, in the basement membrane and developing SG of the seminiferous tubules upon exposure to nicotine/CS. Moreover, upregulation of these molecules at the translational level in the exposed rat testis indicated a possible secondary effect of nicotine/CS on spermatogenic modulation through interaction between these two NT systems parallel with high serum cotinine, intense oxyradical overload, cell degeneration, DNA damage, and antioxidant depletion. Therefore, we prove the hypothesis that nicotine/CS treatment may interfere with the pro- (ROS, iNOS, RNOS) and anti- (ascorbate, Nrf2) oxidative elements in the testicular milieu, which influence the local NT action (BDNF, DA) in regulating and maintaining the homeostasis; and thus, may establishing a link with the brain system contributing to addiction.

**Conclusion**

To our knowledge, this is the first study reporting the specific action of nicotine and CS on the local NTs, BDNF, and DA in the spermatogenic modulation where pro-oxidants centrally control a series of downstream signaling that highlights the NT interventions as a possible mechanism of pro-oxidative stress in testicular pathogenesis including DNA damage, germinal cell loss, and basement membrane disruption with consequent downregulation of the cellular antioxidant defense system, especially Nrf2. However, further mechanism-based studies on the activity of the NT R blockers in the animal models are warranted in order to elucidate BDNF-DAergic-CAergic system and Nrf2 interaction with one another during development of nicotine/CS-stress based on nicotine-dependent tobacco abuse.

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**Author Contributions**

Study design, Western blotting, IHC, comet, cotinine, oxidative stress, and data analysis: NN. Histology: NN and AKG. Donated rats for study: SP. Animal dosing, literature survey, and manuscript preparation: NN and DNG. All the authors have also read and approved the final manuscript version.

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Nicotine/smoke modulate local neurotransmitters and testis toxicity


