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Nicotine induces apoptosis in TM3 mouse Leydig cells

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<table>
<thead>
<tr>
<th>CONTENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION..........................1</td>
</tr>
<tr>
<td>MATERIALS AND METHOD................3</td>
</tr>
<tr>
<td>1) DRUGS AND REAGENTS.............3</td>
</tr>
<tr>
<td>2) CELL CULTURE......................3</td>
</tr>
<tr>
<td>3) MTT CYTOTOXICITY ASSAY........3</td>
</tr>
<tr>
<td>4) TUNEL ASSAY.......................4</td>
</tr>
<tr>
<td>5) DAPI STAINING.....................4</td>
</tr>
<tr>
<td>6) DNA FRAGMENTATION...............5</td>
</tr>
<tr>
<td>7) RNA ISOLATION AND RT-PCR.......5</td>
</tr>
<tr>
<td>8) CASPASE ACTIVITY ASSAY.........7</td>
</tr>
<tr>
<td>9) WESTERN BLOT ANALYSIS..........7</td>
</tr>
<tr>
<td>10) STATISTICAL ANALYSES.........8</td>
</tr>
<tr>
<td>RESULTS..................................9</td>
</tr>
<tr>
<td>1) EFFECT OF NICOTINE ON VIABILITY OF TM3 CELLS..................9</td>
</tr>
<tr>
<td>2) MORPHOLOGICAL CHANGES INDUCED BY NICOTINE ADMINISTRATION..11</td>
</tr>
<tr>
<td>3) DNA FRAGMENTATION INDUCED BY NICOTINE.........................13</td>
</tr>
<tr>
<td>4) EFFECT OF NICOTINE ON mRNA LEVELS OF BAX AND BCL-2........15</td>
</tr>
<tr>
<td>5) CASPASE-3 ENZYME ACTIVITY ASSAY..........................17</td>
</tr>
<tr>
<td>6) WESTERN BLOT ANALYSIS OF CASPASE-3........................19</td>
</tr>
<tr>
<td>DISCUSSION............................21</td>
</tr>
<tr>
<td>REFERENCES............................25</td>
</tr>
<tr>
<td>KOREAN ABSTRACT.....................28</td>
</tr>
</tbody>
</table>
Introduction

The administration of nicotine, a key neuroactive component of cigarette smoke, induces apoptotic cell death in various organs including brain, spleen, and thymus (Berger et al., 1998; Gocze et al., 2000; Hakki et al., 2001). Nicotine has received much attention worldwide for its interference with normal endocrine functions, and it has been shown to cause testicular atrophy, gonadal dysfunction, erectile dysfunction, and male infertility by triggering testicular cytotoxicity (Gocze et al., 2000; Yeh et al., 1989; Wolf et al., 1996; Yamamoto et al., 1998; Kavitharaj et al., 1999). Leydig cells, situated in the interstitial component of the mammalian testis, are responsible for the bulk of testosterone production in males (Chen et al., 2001), and it has been reported that nicotine suppresses secretion of testosterone in adult male rats (Yeh et al., 1989; Kavitharaj et al., 1999).

Apoptosis, also known as programmed cell death, is a biological process that plays a crucial role in normal development and tissue homeostasis (Woodle et al., 1998). Apoptosis is characterized by morphological changes including progressive cell shrinkage with condensation, and fragmentation of nuclear chromatin and membrane blebbing (Kerr et al., 1997).

In numerous studies, the process of apoptosis has been shown to be regulated by the expression of several proteins. Two important proteins involved in apoptotic cell death are members of the bcl-2 family (Korsmeyer., 1999) and a class of cysteine proteases known as caspases (Cohen., 1997). The bcl-2 family can be separated into two functionally distinct groups: anti-apoptotic and pro-apoptotic. Bcl-2, an anti-apoptotic protein, is known to regulate
the apoptosis pathway and to prevent cell death, while bax, a pro-apoptotic protein of the family, is known to be expressed abundantly and selectively during apoptosis and to promote cell death (Oltavai et al., 1993). Bax has been shown to homodimerize, as well as to form heterodimers with bcl-2, and the ratio of bcl-2 to bax is thought to determine survival or death following apoptotic stimulation (Oltavai et al., 1993). Caspases are known to mediate a crucial stage of the apoptotic process and are expressed in many mammalian cells. Of particular interest is caspase-3, the most widely studied member of the caspase family and one of the key executioners of apoptosis, being responsible either partially or wholly for the proteolytic cleavage of many proteins (Cohen., 1997; Nagata., 1997).

In the present study, it was investigated whether nicotine-induced testicular toxicity involves the induction of apoptosis in Leydig cells and whether it acts as an etiologic factor of male urogenital disorders. The effect of nicotine on the occurrence of apoptosis in cells of the TM3 cell line, derived from mouse Leydig cells, was investigated via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, 4,6-diamidino-2-phenylindole (DAPI) staining, terminal deoxynuclotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay, DNA fragmentation assay, reverse transcription-polymerase chain reaction (RT-PCR), caspase-3 enzyme assay, and Western blot analysis.
Materials and method

1) Drugs and reagents

Nicotine and DAPI were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The MTT assay kit and the TUNEL assay kit were purchased from Boehringer Mannheim GmBH (Mannheim, Germany). The DNA fragmentation assay kit was obtained from TaKaRa (Shiga, Japan), and the caspase-3 assay kit from CLONTECH (Palo Alto, CA, USA).

2) Cell culture

Mouse Leydig cells were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA) at 37°C in 5% CO₂, 95% O₂ in a humidified cell incubator, and the medium was changed every 2 days.

3) MTT cytotoxicity assay

Cell viability was determined using the MTT assay kit as per the manufacturer’s protocol. In order to determine the cytotoxicity of nicotine, cells were treated with nicotine at concentrations of 1 mM, 5 mM, 10 mM, 50 mM, and 100 mM for durations of 5 h and 24 h. Cultures of the control group were left untreated. Ten μl of the MTT labeling reagent was added to each well, and the plates were incubated for 4 h. 100 μl of the solubilization solution was then added to
each well, and the cells were incubated for another 12 h. The absorbance was then measured with a microtiter plate reader (Bio-Tek, Winooski, VT, USA) at a test wavelength of 595 nm and a reference wavelength of 690 nm. Optical density (O.D.) was calculated as the difference between the absorbance at the reference wavelength and that at the test wavelength. Percent viability was calculated as (O.D. of drug-treated sample/control O.D.) x 100.

4) **TUNEL assay**

For *in situ* detection of apoptotic cells, TUNEL assay was performed using ApoTag® peroxidase *in situ* apoptosis detection kit. TM3 cells were cultured on 4-chamber slides (Nalge Nunc International, Naperville, IL, USA) at a density of $2 \times 10^4$ cells/chamber. After 24 h exposure to nicotine at a concentration of 10 mM, the cells were washed with PBS and fixed by incubating in 4% PFA for 10 min at 4°C. The fixed cells were then incubated with digoxigenin-conjugated dUTP in a terminal deoxynucleotidyl transferase (TdT)-catalyzed reaction for 60 min at 37°C in a humidified atmosphere and were then immersed in stop/wash buffer for 10 min at room temperature. The cells were then incubated with anti-digoxigenin antibody conjugated with peroxidase for 30 min. DNA fragments were stained using 3,3’-diaminobenzidine (DAB) (Sigma Chemical Co., St. Louis, MO, USA) as the substrate for the peroxidase.

5) **DAPI staining**

In order to determine whether nicotine induces apoptosis, DAPI staining was performed
according to a previously described protocol (yim et al., 2000). Cells were first cultured on 4-
chamber slides. After treatment with nicotine at a concentration of 10 mM for 24 h, the cells
were washed twice with phosphate buffered saline (PBS) and fixed by incubation in 4%
paraformaldehyde (PFA) for 30 min. Following washing in PBS, the cells were incubated in 1
µ/ml DAPI solution for 30 min in the dark. The cells were then observed with a fluorescence
microscope (Zeiss, Oberköchen, Germany).

6) DNA fragmentation

For detection of apoptotic DNA cleavage, DNA fragmentation assay was performed using
ApopLadder EX™ DNA fragmentation assay kit. After treatment with nicotine at concentration
of 5 mM and 10 mM for 24 h, cells were lysed with 100 µl of lysis buffer. The lysate was then
incubated with 10 µl of a 10% SDS solution and 10 µl of Enzyme A at 56°C for 1 h and
then with 10 µl of Enzyme B at 37°C for another 1 h. This mixture was then centrifuged for
15 min after adding 70 µl of precipitant and 500 µl of ethanol. DNA was extracted by
washing the resulting pellet in ethanol and resuspending it in TE (Tris-EDTA) buffer. DNA
fragmentation was visualized by electrophoresis on a 2% agarose gel containing ethidium
bromide.

7) RNA isolation and RT-PCR

After treatment with nicotine at concentrations of 5 mM and 10 mM for 24 h, total RNA was
isolated from TM3 cells using RNAzol™B (TEL-TEST, Friendswood, TX, USA) as per the
manufacturer’s instruction. Two µl of RNA and 2 µl of random hexamers (Promega, Madison, WI, USA) were added together, and the mixture was heated at 65°C for 10 min. One µl of AMV reverse transcriptase (Promega, Madison, WI, USA), 5 µl of 10 mM dNTP (Promega, Madison, WI, USA), 1 µl of Rnasin (Promega, Madison, WI, USA), and 5 µl of 10 x AMV RT buffer (Promega, Madison, WI, USA) were then added to the mixture, and the final volume was brought up to 50 µl with dimethyl pyrocarbonate (DEPC)-treated water. The reaction mixture was then incubated at 42°C for 1 h.

PCR amplification was performed in a reaction volume of 40 µl containing 1 µl of the appropriate cDNA, 1 µl of each set of primers at concentration of 10 µM, 4 µl of 10 x reaction buffer, 1 µl of 2.5 mM dNTP, and 2 units of Taq DNA polymerase (TaKaRa, Shiga, Japan). For bax, the primer sequences were 5’-AGATGAACGTGGATGACATATGG-3’ (a 24-mer sense oligonucleotide starting at position 201) and 5’-CCACCTGGTTCTGGATCCAGACA-3’ (a 24-mer anti-sense oligonucleotide starting at position 446). For bcl-2, the primer sequences were 5’-TCCGTGCCTGACTTTAGCAAGCTG-3’ (a 24-mer sense oligonucleotide starting at position 741) and 5’-GGAATCCCAACCAGAGATCTCAA-3’ (a 24-mer anti-sense oligonucleotide starting at position 1051). For cyclophilin, the internal control used in the study, the primer sequences were 5’-ACCCACCGTGTCTCTCGAC-3’ (a 20-mer sense oligonucleotide starting at position 52) and 5’-CATTTGCCATGGACAGCTCAAC-3’ (a 20-mer anti-sense oligonucleotide starting at position 332). The expected sizes of the PCR products were 270 bp (for bax), 333 bp (for bcl-2) and 300 bp (for cyclophilin).

PCR procedure was carried out using a GeneAmp 9600 PCR system (Perkin Elmer, Norwalk,
CT, USA) under the following conditions: initial denaturation at 94°C for 5 min, followed by 30 amplification cycles, each consisting of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 30 sec, with an additional extension step at the end of the procedure at 72°C for 5 min. For cyclophilin, the PCR procedure was carried out under identical conditions except that 25 amplification cycles were executed. The final amount of RT-PCR product for each of the mRNA species was calculated densitometrically using Molecular Analyst™ version 1.4.1 (Bio-Rad, Hercules, CA, USA).

8) Caspase activity assay

Caspase enzyme activity was measured using ApoAlert® caspase-3 assay kit according to the manufacturer’s protocol. In brief, after treatment with nicotine at concentrations of 5 mM and 10 mM for 24 h, cells were lysed with 50 µl of chilled Cell Lysis Buffer. 50 µl of 2 x reaction buffer (containing DTT) and 5 µl of the appropriate conjugated substrate at a concentration of 1 mM were added to each lysate. The mixture was incubated in a water bath at 37°C for 1 h, and the absorbance was measured with a microtiter plate reader at a test wavelength of 405 nm.

9) Western blot analysis

After treatment with 5 mM and 10 mM nicotine for 24 h, cells were collected, and protein was isolated using Pro-prep protein extraction solution (Intron Biotech. Seoul, Korea). The protein content was measured using the colormetric Bio-Rad protein assay kit (Bio-Rad,
of protein was electrophoresed on sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred onto a nitrocellulose membrane (Schleicher & Schuell GmbH, Postfach, Germany). Mouse caspase-3 antibody (Santa Cruz Lab., Santa Cruz, CA, USA) was used as the primary antibody. Horseradish peroxidase-conjugated anti-mouse antibody. (Amersham, Quebec, Canada) was used as the secondary antibody. Detection of bands was performed using the ECL system (Amersham, Quebec, Canada).

10) Statistical analyses

Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Scheffe’s post-hoc test, and results are expressed as mean ± standard error mean (SEM). Differences were considered significant for \( P < 0.05 \).
Results

1) Effect of nicotine on viability of TM3 cells

In order to assess the cytotoxic effect of nicotine on mouse Leydig cells, TM3 cells were cultured with nicotine at final concentrations of 1 mM, 5 mM, 10 mM, 50 mM, and 100 mM and MTT assay was carried out, with cells cultured in nicotine-free media as the control. The viabilities of cells incubated with nicotine at concentrations of 1 mM, 5 mM, 10 mM, 50 mM, and 100 mM for 5 h were 96.92 ± 2.68%, 75.89 ± 1.06%, 67.43 ± 1.23%, 22.75 ± 0.84%, and 14.32 ± 0.27% of the control value, respectively. The viabilities of cells incubated with nicotine at concentrations of 1 mM, 5 mM, 10 mM, 50 mM, and 100 mM for 24 h were 91.56 ± 1.87%, 69.20 ± 0.86%, 26.20 ± 0.16%, 22.10 ± 0.65%, and 12.86 ± 0.04% of the control value, respectively. The present results show that nicotine exerts cytotoxic effect on TM3 cells in a concentration- and time-dependent manner.
Fig. 1. Effect of nicotine on cell viability. Viability was determined via MTT assay.
Results are presented as mean ± standard error mean. * represents $P < 0.05$ compared to the control group.
2) *Morphological changes induced by nicotine administration*

To characterize nicotine-induced changes in cell morphology, cells were examined by phase-contrast microscopy. As shown in Fig. 2A, cells treated with 10 mM nicotine for 24 h were seen to have detached from the dish, with cell rounding, cytoplasmic blebbing, and irregularity in shape.

In DAPI assay, cells were observed *via* fluorescence microscopy following treatment with DAPI, which specifically stains the nuclei. The assay has revealed the presence of nuclear condensation, DNA fragmentation, and perinuclear apoptotic bodies upon nicotine treatment at a concentration of 10 mM for 24 h (Fig. 2B).

To further confirm the induction of apoptosis by nicotine in TM3 cells, nicotine-treated cells were analyzed *via* TUNEL assay. As shown in Fig. 2C, TUNEL-positive cells were shown to be stained dark brown under the light microscope, and nuclear condensation was observed.
Fig. 2. Characterization of nicotine-induced death in TM3 cells. In nicotine-treated group, 5 mM of nicotine was incubated for 24 h. A, Photomicrograph of phase-contrast microscope. Cell shrinkage, irregularity in cellular shape, and cellular detachment were seen in the nicotine-treated cultures. B, TM3 cells stained with DAPI. White arrows indicate condensed nuclei. C, TM3 cells stained via TUNEL assay. Black arrows indicate where condensed and marginated chromatin have been labeled. All scale bars represent 100 μm.
3) DNA fragmentation induced by nicotine

In order to ascertain the induction of apoptosis by nicotine, DNA fragmentation, reflecting the endonuclease activity characteristic of apoptosis, was performed. As seen in Fig. 3, nicotine treatment at a concentration of 10 mM for 24 h resulted in the formation of definite fragments, which could be seen via electrophoresis as a characteristic ladder pattern, and 5 mM nicotine treatment for 24 h showed weak intensity of DNA laddering.
Fig. 3. Electrophoretic examination of the effect of nicotine on the genomic DNA of TM3 cells. Genomic DNA was extracted and analyzed via electrophoresis on 2% agarose gels containing ethidium bromide. A, control group; B, 5 mM nicotine-treated group; C, 10 mM nicotine-treated group.
4) Effect of nicotine on mRNA levels of bax and bcl-2

RT-PCR analyses of the mRNA levels of bax and bcl-2 were performed in order to provide an estimation of the relative levels of expression of these genes. In the present study, with the mRNA levels of bax and bcl-2 in the control cells set at 1.00, the level of bax mRNA was markedly increased, to 4.64 ± 0.46 following treatment with 5 mM nicotine and to 7.27 ± 0.40 following treatment with 10 mM nicotine for 24 h (Fig. 4 Above). The level of bcl-2 mRNA was decreased, to 0.64 ± 0.08 following treatment with 5 mM nicotine and to 0.42 ± 0.05 following treatment with 10 mM nicotine for 24 h (Fig 4 Below).
Fig. 4. Results of RT-PCR analysis of the mRNA levels of \textit{bax} and \textit{bcl-2}. \textit{Cyclophilin} mRNA was also reverse–transcribed and amplified as the internal control. Results are presented as mean ± standard error mean. * represents $P < 0.05$ compared to the control group. A, control group; B, 5 mM nicotine-treated group; C, 10 mM nicotine-treated group.
5) Caspase-3 enzyme activity assay

Caspase-3 enzyme activity was measured using DEVD peptide-nitroanilide (pNA). After 24 h of exposure to nicotine at concentrations of 5 mM and 10 mM, the product of DEVD-pNA cleavage was increased from 7.38 ± 0.56 pM to 19.54 ± 1.52 pM and 28.43 ± 2.72 pM, respectively (Fig 5).
Fig. 5. Result of caspase-3 enzyme assay. Nicotine increased caspase-3 enzyme activity. Results are presented as mean ± standard error mean. * represents $P < 0.05$ compared to the control group. The rate of DEVD-pNA cleavage was measured at 405 nm. DEVD-fmk is a caspase inhibitor.
6) Western blot analysis of caspase-3

Caspase-3 is synthesized as precursor molecules, and is approximately 32 kDa in size. Immunoblotting analysis revealed that the activated form of caspase-3 was increased in cells treated with 5 mM and 10 mM nicotine for 24 h as dose-dependent manner; a new band, corresponding to p17 of caspase-3, was detected in nicotine-treated cells.
Fig. 6. Result of Western blot analysis of caspase-3 protein level. β-actin, the internal control, was detected at the band corresponding to 46 kDa. A, control group; B, 5 mM nicotine-treated group; C, 10 mM nicotine-treated group.
Discussion

The aim of the present study was to investigate whether nicotine induces apoptosis in testicular Leydig cells. It has been demonstrated that nicotine is associated with damage in various types of tissue (Berger et al., 1998; Gocze et al., 2000; Hakki et al., 2001), especially reproductive organs (Gocze et al., 2000; Wolf et al., 1996). In recent years, apoptotic cell death in testicular cells has been studied extensively, and apoptosis has been observed in testicular cells exposed to various pathologic factors (Kumi-Diaka et al., 2000; Zhu et al., 2000). Yamamoto et al. (1998) reported that nicotine administration induces changes in gonadal functions and deficiency in sperm maturation and spermatogenesis and has a detrimental effect on the sperm-fertilizing potential of male rats. In the present study, it has been demonstrated that nicotine induces apoptosis in Leydig cells.

Assessment of cell viability made in the present study via MTT assay showed that nicotine exerts a cytotoxic effect on TM3 cells in a dose- and time-dependent manner. Nicotine was also shown to cause characteristic changes in the morphology of TM3 cells. The apoptotic nature of nicotine-induced cell death has been confirmed in multiple ways in this study. Under the phase-contrast microscope, nicotine-treated cells were seen to undergo cell shrinkage, cytoplasmic condensation, and irregularity in shape. Apoptotic bodies, the presence of which is a stringent morphological criteria of apoptosis, were characteristically present in nicotine-treated cells stained with DAPI. It has also been reported that cells undergoing apoptosis exhibit cytoplasmic blebbing, nuclear shrinkage, chromatin condensation, irregularity in shape, and retraction of processes (Cohen., 1993). Furthermore, it has been shown that DNA strand breaks occur during
The process of apoptosis, and it is known that the nicks in the DNA molecules can be detected via TUNEL assay (Berger et al., 1998). In the present study, typical TUNEL-positive cells were observed in nicotine-treated cells. It is also known that apoptosis involves the activation of endonucleases and that this activation results in the cleavage of genomic DNA into well-defined fragments, which appear as a characteristic ladder pattern upon agarose gel electrophoresis (Eastman et al., 1992). To provide evidence supporting the involvement of apoptosis in nicotine-induced cytotoxicity, DNA fragmentation assay was performed. The nicotine-treated cells revealed the distinctive ladder pattern characteristic of apoptosis, which is consistent with the results presented by Berger et al. (1998) in their study involving rat hippocampal progenitor cells.

The level of bcl-2, an anti-apoptotic protein, was also examined in the present study. Members of the bcl-2 family of proteins are characterized by their ability to form a complex combination of heterodimers with bax and homodimers with itself (Reed., 1997). When bax, the first pro-apoptotic homologue to be identified, is overexpressed in cells, apoptotic death in response to a death signal is accelerated; this has resulted in its designation as a death agonist. When bcl-2 is overexpressed, it heterodimerizes with bax, and cell death is repressed. Presumably, the ratio of bax to bcl-2 serves to determine the susceptibility of cells to apoptosis (Korsmeyer., 1999). In the present study, nicotine treatment resulted in an increase in bax expression and a decrease in bcl-2 expression. The mechanisms underlying the pro-survival activity of bcl-2 have been shown to include its activity as an antioxidant, preserving the mitochondrial membrane potential and blocking the release of cytochrome c, thus keeping caspases in their inactive forms (Hockenbery et al., 1993; Cai et al., 1998).
Caspases, a family of cysteine proteases, are integral parts of the apoptotic pathway; caspase-3 in particular, when activated, has many cellular targets that, when severed and/or activated, produce the morphologic features of apoptosis. The present data has revealed increased expression of the activated form of caspase-3, and up-regulation of caspase-3 enzyme activity in cells exposed to nicotine (Cohen., 1997).

Based on the results of the present study, nicotine appears to activate specific intracellular death-related pathways, probably bax-dependant activation of caspase-3, inducing apoptosis in Leydig cells. It can thus be suggested that nicotine-induced apoptosis of Leydig cells is an important mechanism behind nicotine-related urogenital disorders in men.
Conclusions

To investigate whether nicotine induces apoptosis in TM3 mouse Leydig cells, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay, 4,6-diamidino-2-phenylindole (DAPI) staining, DNA fragmentation assay, reverse transcription-polymerase chain reaction (RT-PCR), caspase-3 enzyme assay, and Western blot analysis were performed in the present study. Through morphological and biochemical analyses, it was demonstrated that TM3 cells treated with nicotine exhibit several features of apoptosis. It was also shown that nicotine increases the mRNA level of \textit{bax} and decreases that of \textit{bcl-2}. In addition, nicotine enhanced the expression of the activated form of caspase-3 and caspase-3 enzyme activity. Based on the results, it appears that nicotine activates specific intracellular death-related pathways, probably involving bax-dependent activation of caspase-3 in mouse Leydig cells.
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