

RESEARCH ARTICLE

Expression Changes of Apoptotic Genes in Tissues from Mice Exposed to Nicotine

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Abstract

Objective: Smoking is the leading preventable cause of various diseases such as lung cancer, chronic obstructive pulmonary disease and cardiovascular disease. Nicotine, one of the major toxic components of tobacco, contributes to the pathogenesis of different diseases. **Methods:** Given the controversy about nicotine toxicity, the present study was conducted to determine apoptotic effects of nicotine on the heart, kidney, lung and liver of male mice. Real-time PCR was performed to identify mRNA expression changes in apoptotic-related genes between nicotine treated and control mice. **Result:** In the heart and lung, nicotine caused significant decrease in P53, Bax and Caspase-3 mRNA expression levels compared to the control group. However, in the kidney and liver, the result was significant increase in Bax, Caspase-2, Caspase-3 and a significant decrease in P53 mRNA expression ($p < 0.01$). DNA fragmentation assays indicated no fragmentation in the heart and lung, but in the kidney and liver of nicotine treated mice, isolated DNA was fragmented. **Conclusion:** Our study provided insight into the molecular mechanisms of nicotine anti-apoptotic effects on the heart and lung as well as pro-apoptotic effects on kidney and liver via a P53-independent pathway.

Keywords: Nicotine- apoptosis- liver- caspase-2- P53

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Introduction

Cigarette smoking is one of the most important cause of preventable morbidity and mortality in the world (Grundtvig et al., 2013; El-Kenawy et al., 2015). Before the popularity of smoking, the prevalence of non-communicable diseases such as cancers, cardiovascular diseases, diabetes and chronic respiratory diseases was low (Kassani et al., 2015). There is a close relationship between cigarette smoking and lung cancer also this cancer is one of the most common causes of cancer-related death in the world (Heusch and Maneckjee, 1998). Results from the study of smoking in Indonesian men showed that the highest rate of cancer is related to lung cancer, liver and nasopharynx cancer respectively (Kristina et al., 2015). Components of tobacco smoke can impact the cell cycle evolution, initiate tumor formation and promote tumor progression in different types of cancer through different molecular mechanisms (Schaal and Chellappan, 2014).

About 0.6–3.0 % of the dry weight of tobacco is nicotine, one of the major toxic components of tobacco, which is absorbed by the lungs and transported into the bloodstream (Argentin and Cicchetti, 2006) (Gritz et al., 2006). Nicotine half-life is 1-2 hours. It is lipid soluble; therefore, it quickly goes out of the plasma and moves into the intra-cellular space. Nicotine metabolization occurs

in the liver and its elimination takes place in the kidney (Lambers and Clark, 1996).

Nicotine blocks anti-oxidant enzymes, thereby increasing lipid peroxidation, followed by formation of reactive oxygen species (ROS). ROS or free radicals in the tissues are able to trigger cytoplasmic membrane damage and DNA fragmentation (Yang et al., 1998). Elevated ROS is a distinguished cause of the mitochondrial apoptotic pathway. When increased abnormally, it directly oxidizes DNA and triggers genotoxicity (Richter, 1998; Sastre et al., 2000).

Pro-apoptotic proteins (Bak, Bid, Bax) and anti-apoptotic proteins (Bcl-XL, Bcl-2), members of the Bcl-2 family, tightly control the balance between cell life and cell death (Adams and Cory, 2001; Martinou and Green, 2001). Bcl-2 protects the cells from apoptosis by binding to Bax, prevents them from oligomerization and translocation to mitochondria, stimulates permeability transition and activates caspase cascade subsequently (Gulbins et al., 2003; Green and Kroemer, 2004). P53, one of the most important intracellular sensors of stress and damage signals, can prevent uncontrolled cell growth (Vogelstein et al., 2000; Chipuk and Green, 2006). P53 can trigger apoptosis by activating the pro-apoptotic proteins of Bcl-2 family and consequently cause abnormal mitochondrial functioning: mitochondrial outer membrane permeabilisation (MOMP). In contrast, the

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anti-apoptotic proteins of Bcl-2 family (like Bcl-XL) can preclude MOMP (Larsen, 1993; van Loo et al., 2002) and cytochrome c (cyt c) release, which triggers Caspase-9 and induces the activation of caspase cascade. In a substitute pathway, Caspase-2 cleaves and activates BID, causing MOMP and cyt c release. Caspase-2 activation, possibly acts upstream of MOMP. Though, activated effector caspases (such as Caspase-3) also process and stimulate the Caspase-2 precursor. Loss or inhibition of Caspase-2 precludes P53-mediated apoptosis (Zhivotovsky and Kroemer, 2004).

Nicotine contributes to pathogenesis of diseases such as cancers, neurological diseases and lung diseases (Gritz et al., 2006). Different groups have studied the special apoptotic effects of nicotine, both in vitro and in vivo, and they have shown an association between nicotine exposure and apoptosis. Some studies have reported that nicotine triggers apoptosis (Yoshida et al., 1997; Arredondo et al., 2002; Wu et al., 2002; Galitovsky et al., 2004; Zhao and Reece, 2005), whereas others have shown a protective effect of nicotine through precluding apoptosis (Hejmadi et al., 2003; Suzuki et al., 2003; Utsumi et al., 2004; Wielgus et al., 2004; Copeland et al., 2005; Tizabi et al., 2005; Zhang et al., 2006). Considering the controversial results about the effects of nicotine toxicity, the present study was conducted to evaluate the apoptotic effect of intra-peritoneal nicotine injection on the liver, kidney, lung and heart of male mice.

Materials and methods

Materials

Twenty-four Balb/c male mice with a weight range of 28 ± 2 g were purchased from the Razi Institute, Tehran, Iran. Seven days before the experiment started, the mice were kept in the animal house in laboratory conditions, according to the institutional guidelines and the "National Institutes of Health Guide for the Care and Use of Laboratory Animals". This experimental study was administered under the permission of the Ethics Committee of Kermanshah University of Medical Sciences. (Certificate No. 93514). Nicotine solution (1-methyl-2-3-pyridyl-pirolidin; C10H14N2, Merck, Germany) was diluted with nicotine solvent (normal saline) (0.9%) for intraperitoneal injection, as an effective method to deliver nicotine based on other studies (Mohammadghasemi et al., 2012; Jalili et al., 2014). The LD50 value for nicotine in mice has been found to be 5.9 mg kg⁻¹ (Trochimowicz et al., 1994). Intraperitoneal (i.p.) injection of nicotine (2.5 mg/kg body weight/day) was done based on previous studies (Salahshoor et al., 2016a; Salahshoor et al., 2016b). The mice were randomly divided into two groups of three animals each: Group 1: Control (normal saline; 1 mL distilled water/daily) for 4 week. Group 2: nicotine treated (2.5 mg/kg body weight/day) for 4 week. Nicotine was injected i.p. once a day. At the end of the experimental period, all mice were quickly dissected under intraperitoneal ketamine / xylazine anesthesia, 24 h after the last dosing. The heart, liver, kidney and lung were removed, washed thoroughly with chilled normal saline, washed with ice cold DEPC (diethyl pyrocarbonate)

treated water to remove extraneous materials and stored at -80°C until use for RNA extraction and gene expression analysis.

Methods

RNA extraction

Total RNA was isolated using RNXTM PLUS buffer (CINNAGEN, Iran), according to the manufacturer's instructions. Briefly, 30-50mg of tissue were homogenized by mortar and pestle in liquid Nitrogen, and 1 ml ice cold RNXTM PLUS solution and 200 μl of Chloroform were added. The mixture was then centrifuged at 12000 rpm at 4°C for 15 min. After that, the aqueous phase was transferred to new RNase-free 1.5 ml tube. RNA was precipitated and washed with an equal volume of Isopropanol and 1 ml of 75% Ethanol, respectively. The pellet was dissolved in 50 μl of DEPC treated water. To remove genomic DNA from extracted RNA, DNaseI treatment was performed using DNaseI (CINNAGEN, Iran) according to the manufacturer's instruction. All RNA extracted samples were stored at -80°C for further experiments.

cDNA synthesis

Five hundred nanograms of extracted mRNA were reverse transcribed into cDNA using the cDNA synthesis kit (PrimeScript™ 1st strand cDNA Synthesis Kit, Takara) in 20 μl reaction mixture according to the manufacturer's instructions. The resulting cDNA kept at -20°C until use.

Real-Time PCR

The expression profile of P53, Bcl-2, Bax, Caspase-2 and 3 genes normalized using glyceraldehyde3-phosphatide dehydrogenase (GAPDH) as a housekeeping gene, were evaluated. The primer sequences of GAPDH, P53, Bcl2, Bax, Caspase-2 and 3 genes are shown in table 1. Real time PCR was carried out using Applied Biosystems™ Real-Time PCR instruments. 10 μl of SYBR Green PCR master mix, 2 μl of cDNA, and 200 nM primer set were used for amplification in 20 μl reaction mixture. All samples were amplified in triplicates in a 48-well plate and the cycling conditions were as follows: 10 second at 95°C , and 40 cycles at 95°C for 5 second and 60°C for 30 second. Relative quantification (RQ) = $2^{-\Delta\Delta\text{Ct}}$ formula was used for assessment of relative expression of genes.

DNA Fragmentation Assay

Genomic DNA was isolated from fresh tissue samples. Briefly, About 50 mg of fresh tissue was cut into smaller pieces and was suspended in 550 μl of Lysis buffer [2mM EDTA, 10 mM Tris-HCl (pH 8.0) and 400 mM NaCl], following the addition of Proteinase K and SDS (400ng and 0.6%, respectively). The solution was incubated at 55°C for 2h. Then 500 μl of DNA extraction buffer [4 mM Na2EDTA, 20 mM Tris-HCl, 100 mM NaCl (pH 7.4)] and 10% SDS was added and incubated in 70°C for 20min. Suspension centrifuged at 12000 rpm and upper phase was transferred in a new tube and 1 ml cold absolute ethanol was added. DNA was precipitated by centrifuge at 12,000 rpm and DNA pellet was re-suspended in 50 μl DNase free

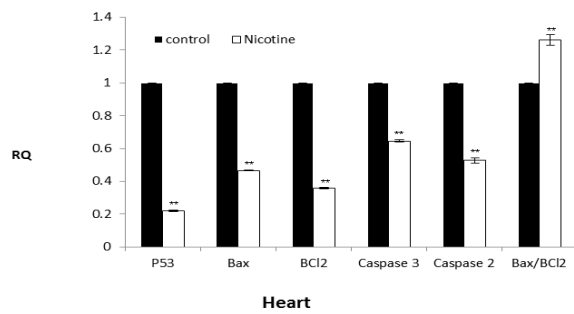


Figure 1. Changes in Apoptotic Gene Expression. The *P53*, *Bax*, *Caspase-2*, *Caspase-3* and *Bax/Bcl-2* ratio of mRNA Levels Comprised in Nicotine Treated Heart with Normal Saline Treated Control Mice. ** $p < 0.01$

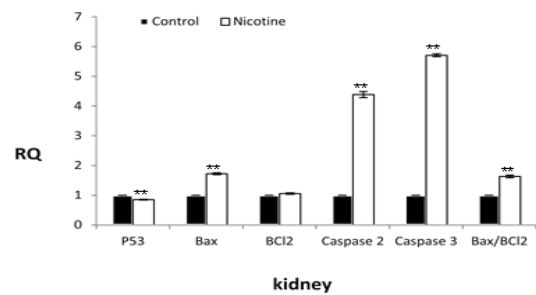


Figure 3. Changes in Apoptotic Gene Expression. The *P53*, *Bax*, *Caspase-2*, *Caspase-3* and *Bax/Bcl-2* Ratio of mRNA Levels in Nicotine Treated Kidney Comprised with Normal Saline Treated Control Mice. ** $p < 0.01$

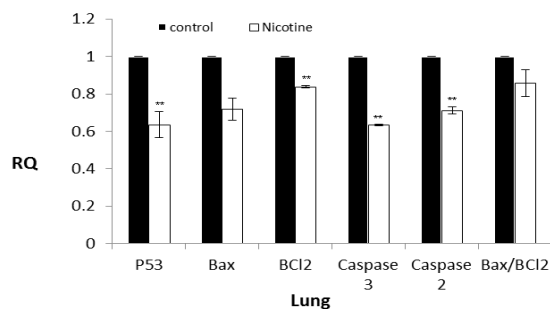


Figure 2. Changes in Apoptotic Gene Expression. The *P53*, *Bax*, *Caspase-2*, *Caspase-3* and *Bax/Bcl-2* Ratio of mRNA Levels in Nicotine treated Lung Comprised with Normal Saline Treated Control Mice. ** $p < 0.01$, * $p < 0.05$

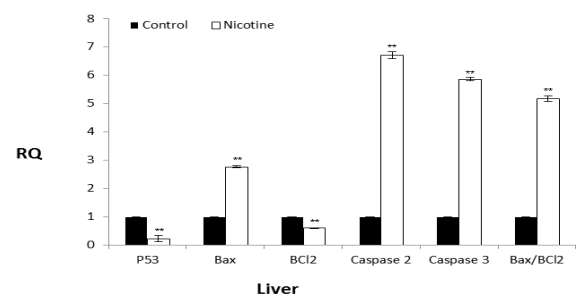


Figure 4. Changes in Apoptotic Gene Expression. The *P53*, *Bax*, *Caspase-2*, *Caspase-3* and *Bax/Bcl-2* Ratio of the mRNA Levels Comprised in Nicotine Treated Liver with Normal Saline Treated Control Mice. ** $p < 0.05$

water. Isolated DNA was observed by gel electrophoresis on 1.5 % agarose containing Gel Red (Biotium).

Statistical analysis

All data are presented as mean \pm SEM. Statistical differences among groups were carried out independent sample t-test. A two-tailed $p < 0.05$ was considered a statistically significant difference. Data were analyzed using SPSS 16.0.

Results

The effects of nicotine, on the *P53*, *Bax*, *Bcl2*,

Caspase-2 and *Caspase-3* mRNA expression levels in heart, lung, kidney, and liver tissues of male mice were studied. Despite increase in *Bax/Bcl-2* ratio, in heart of nicotine treated mice, significant decrease in *P53*, *Bcl2*, *Bax*, *Caspase-2* and *Caspase-3* mRNA expression levels were observed compared to control group ($p < 0.01$) (Figure 1). In the lung of nicotine treated mice significant decrease was observed in *P53*, *Bcl-2*, *Bax*, *Caspase-2*, *Caspase-3* mRNA expression levels ($p < 0.05$) and also, compared to control group *Bax/Bcl-2* ratio, didn't show significant differences ($P = 0.184$) (Figure 2). Gene expression analysis in the kidney of nicotine treated mice showed a significant increase in *Bax*, *Caspase-2*, *Caspase 3* and

Table 1. Primer Sequences Used for Real-Time PCR

Gene		Primer sequence (5' to 3')	Ta	Product size (bp)
GAPDH	F	AGAACATCATCCCTGCATCCAC	58	127
	R	GTCAGATCCACGACGGACACA		
P53	F	GTACCTTATGAGCCACCCGA	56.2	143
	R	AGAAGGTCCCCTGGAGTC		
Bax	F	CTCAAGGCCCTGTGCACTAA	58.4	120
	R	GAGGCCTCCCAGCCAC		
Bcl-2	F	CTCGTCGCTACCGTCGTGACTTCG	59.6	112
	R	ACCCCATCCCTGAAGAGTCC		
Caspase-2	F	ATGCTAACTGTCCAAGTCTA	53	167
	R	TCTCATCTTCATCAACTCC		
Caspase-3	F	TCTGACTGGAAAGCCGAAACTC	56	83
	R	TCCCACTGTCTGTCTCAATGCCAC		

a significant decrease in P53 mRNA expression levels ($p < 0.001$). However, Bcl-2 mRNA expression levels showed no significant differences in comparison to control group ($P = 0.189$), although Bax/Bcl-2 ratio differences were significant ($p < 0.01$) (Figure 3). Finally, nicotine treatment in liver revealed a significant increase in Bax, Caspase-2, Caspase-3, Bax/Bcl-2 ratio and a significant decrease in P53 and Bcl-2 mRNA expression levels compared to control group ($p < 0.01$) (Figure 4). Similar to control group, DNA Fragmentation Assay showed no fragmentation in the heart and lung tissue of nicotine treated mice; however, isolated DNA from the liver and kidney of nicotine treated mice had been fragmented (Figure 5).

Discussion

The present study was designed to investigate the effect of nicotine on apoptosis in heart, kidney, lung and liver tissues in vivo. The results of Real Time PCR showed that the toxic dose of nicotine inhibited heart and lung apoptosis, but it triggered liver and kidney apoptosis via Caspase-2, P53-independent and apoptotic pathway. In this process, activated Caspase-2 can trigger the mitochondrial apoptotic pathway. To the best of the researchers' knowledge, this is the first study to demonstrate nicotine activated P53-independent apoptosis of liver and kidney using gene expression analysis by Real-Time PCR method.

The pharmacokinetics knowledge about nicotine, as potential therapeutic agents, is under development. Our data can be expanded to similar human data. Nicotine, as a main ingredient of cigarette, is metabolized and eliminated in the liver and kidney, respectively (Lambers and Clark, 1996). There are contradictory reports about pro- or anti- apoptotic effects of nicotine; it induces apoptosis in the heart (Demiralay et al., 2007), kidney and bladder (Şener et al., 2005), lung (Demiralay et al., 2006) of rats and A549 lung epithelial cells (Ramage et al., 2006). On the other hand, others have proposed that nicotine has an anti-apoptotic effect on cardiac myocytes (Suzuki et al., 2003) and inhibits apoptosis in human lung adenocarcinoma- derived A549 cells (Nakada et al., 2012). These differences can be attributed to different mechanisms of apoptosis induction, the tissue where apoptosis occurs and different methods of apoptosis measurement.

Apoptosis is induced in the liver and kidney because the main path of nicotine metabolism takes place in the liver and elimination of the metabolites occurs in the kidney. Assuming that most of nicotine is metabolized by the liver, this means that about 70% of the drug is extracted from blood in each pass through the liver. The toxic effects of nicotine can be caused by itself or its metabolites such as cotinine (Benowitz et al., 2009; El-Sherbeeny et al., 2016). Different studies agree on a toxic dose of nicotine causing damage in liver (Bandyopadhyaya et al., 2008; Banerjee et al., 2012; Jalili et al., 2015). Our results, based on Real-Time PCR gene expression method, confirmed the findings of R. Ivey et al., (2014), based on Western blot and immunohistochemistry assay, which showed

the participation of Caspase-2 and iNOS –mediated apoptotic pathway in nicotine plus high fat diet-induced hepatocellular apoptosis. They indicated a significant increase in active Caspase-2 protein levels. El-Sherbeeny NA et al., (2016) showed that the liver cells treated by nicotine increased Caspase-3 activity in these cells and decreased nitric oxide (NO) levels. Also, oxidative stress caused by the presence of nicotine harmed the arteries and induced inflammation in the liver (El-Sherbeeny et al., 2016). Some studies have shown that nicotine and cotinine induce apoptosis in the liver directly or immunologically. Direct effects are associated with increased oxidative stress, lipid peroxidation, DNA damage and apoptosis (Husain et al., 2001). ROS production induced by nicotine can stimulate the activity of Caspase-2 as well as the production of nitric oxide (NO). On the other hand, NO production can be involved in cell damage and apoptosis (Tamm et al., 2008).

Renal tubular cells are directly exposed to nicotine through glomerular filtration, possibly causing toxic effects on the kidneys (Hukkanen et al., 2005). In a study conducted on the HK-2 kidney cells, nicotine induced oxidative stress and apoptosis (Kim et al., 2016). In another study, a significant increase in lipid peroxidation and DNA damage was observed in the mice treated with nicotine than in control group. Also, a significant increase was observed in mitochondrial DNA fragmentation of liver, kidney and spleen (Chakraborty et al., 2011).

Our results did not show a significant difference in Bax/Bcl2 ratio in the lung of nicotine treated mice; however, a significant decrease was observed in P53, Caspase-2 and Caspase-3 mRNA expression levels ($p < 0.05$). Xx et al showed that nicotine decreased apoptosis in the lung cancer cells through inactivating PP2A, Bax phosphorylation, and decreasing cytochrome c release (Xin and Deng, 2006). In normal cells, nicotine can stimulate the features consistent with cell transformation and the early stages of cancer formation such as increased cell proliferation, decreased cellular dependence on extracellular matrix for survival and decreased contact inhibition (Copeland et al., 2005). It was found that nicotine in the lung cancer cells can lead to increased expression of the bcl-2 protein and inhibits the apoptosis. Also, these effects can reduce the efficacy of chemotherapy in smokers (Cardinale et al., 2012).

Our study showed a significant increase in Bax/Bcl-2 ratio in the heart of nicotine treated mice, while there was a significant decrease in P53, Caspase-2 and Caspase-3 mRNA expression levels. The exact mechanism of apoptosis within the heart is not known. The process of apoptosis in the heart has a unique position within the domain of apoptosis, analogous to other ischemically challenged processes. Several studies have reported that nicotine exerts its protective effect via inhibiting apoptosis, while others have shown the opposite (Zhou et al., 2010). It seems that the zone of the heart in which apoptosis occurs is important, either ischemic zone or the ischemic border zone. It has been reported that nicotine increases susceptibility to ischemia/ reperfusion injury and aggravates heart damage via myocardial infarction (Hubbard et al., 2005; Miyauchi et al., 2005; Lawrence

et al., 2008). One of the conflicting areas in the study of apoptosis in the heart is the mechanism of induction. In some studies, apoptosis has been found to be induced by ischemia alone, and after ischemia followed by reperfusion, whereas in other studies ischemia alone is found not to be sufficient to trigger apoptosis. Lack of completion of the apoptotic process seems to be related to lack of ATP and / or conversion of apoptosis to necrosis by a secondary inflammatory response (Krijnen et al., 2002).

Nicotine through its genotoxic effects, also by facilitating tumor cell survival, metastasis, growth and resistance to chemotherapy can promote existing tumor that is initiated by other factors (Grando, 2014).

Therefore, nicotine can be either pro- or anti-apoptotic, which depends upon the concentration of the substance used, species-related variations in the metabolism of nicotine and the target cells.

In summary, our study provides an insight into the molecular mechanisms of nicotine anti- apoptotic effects on the heart and lung as well as pro-apoptotic effect on the liver and kidney via an increase in Caspase-2 expression. Therefore, targeting Caspase-2 may have a protective role in kidney and hepatic cells exposed to the toxic dose of nicotine.

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