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Role of Thymoquinone in Attenuation of Nicotine Induced Apoptosis, Bax and Bcl-2 Expression in Male Mice Heart

Ahookhash Maryam*, Jalili Cyrus

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Abstract

Nicotine is a member of the main constituents of tobacco smoke and the main cause of dysfunction in the body's organs is considered. The effects of nicotine on the cardiovascular system are well recognized. Thymoquinone (TQ), one of the components of the herb black seed oil, has anti- inflammatory and anti-cancer effects. This study was conducted to assess the ability of TQ against induced apoptosis by nicotine in heart tissue. For this purpose, thirty six male mice Balb/c were randomly divided into six groups: control, nicotine (5 mg/kg), TQ (9 and 18 mg/kg), nicotine + TQ (9 mg/kg) and nicotine + TQ (18 mg/kg) group. *Bax* and *Bcl-2* gene expression in heart tissues were measured by quantitative real time PCR in all groups. Compare with control group, nicotine, TQ 9 and 18 mg/kg significantly increased the *Bax/Bcl-2* ratio expression. Nicotine +TQ 9mg/kg administration did not induce any significant changes in the *Bax/Bcl-2* ratio expression. In contrast, Nicotine +TQ 18mg/kg administration significantly decreased the *Bax/Bcl-2* ratio expression compared to control group. Treatment with nicotine, TQ 9 and 18mg/kg induced the apoptosis in cardiac tissue by increasing the *Bax/Bcl-2* ratio of expression. Pretreatment with TQ 18mg/kg to nicotine- treated mice inhibited the apoptosis in heart tissue and treatment of mice both dose 5 mg/kg nicotine and 9 mg/kg TQ did not lead to significant alterations in heart tissue. It can be concluded from this study that TQ exerts an anti apoptosis effect against nicotine- induced apoptosis.

Key words: Nicotine, Thymoquinone, Apoptosis, *Bax/Bcl-2*, Heart.

Introduction

Nicotine, which causes addiction, spread throughout the body by blood circulation. (Ruffle, 2014; Le Houezec, 2003) Nicotine is known as one of the main constituents of cigarette smoke. (Gallowitsch‐Puerta and Tracey, 2005) Tobacco smoke is a complex mixture that cannot be attributed its effects only to the ingredients because Its biological effects may change by concurrent consumption of other toxins and related to individual differences in metabolic activation. (Le Houezec, 2003; Hecht, 1999) The main role of smoking on coronary heart disease and damage to the vascular endothelium by cell toxicity has been proved that leads to endothelial cell dysfunction and beginning the pathogenesis of coronary atherosclerosis. (Ross, 1993; Pepine, 1998) Nicotine increases the rate and constricts the arteries contraction rate of the heart. (Wang and Wang, 1999) A lot of research has focused on the effects of nicotine on apoptosis and some of them have reported that nicotine increased cardiomyocyte apoptosis. (Zhou et al., 2010) Apoptosis is programmed death of cells, which activates a signaling cascade, leading to cell death. (Danial and Korsmeyer, 2004) One of the factors causing heart failure is death of heart cells by apoptosis. (Hirota et al., 1999) *Bcl-2* family proteins regulate the apoptosis mitochondrial pathway (Antonsson and Martinou, 2000) by changing mitochondrial membrane permeability that can lead to the release of cytochrome C. (Green, 2000) *Bcl-2* and *Bax* are members this family and analogue, which *Bax* promotes cell death while *Bcl-2* increases cell survival. (Reed, 1994) The *Bax/Bcl-2* ratio is extremely important for cells under the apoptosis for controlling cell death. (Sharpe et al., 2004) *Bcl-2* family proteins are expressed in heart tissue and have a key role to play in controlling of cardiac cell death. (Baldi et al., 2002) *Nigella sativa* (NS) (black seed) has been employed to treat diseases including high blood pressure, diabetes and inflammation as a medicinal herb. More biological activities of the NS are related to one of the components of its volatile oil of seeds of Thymoquinone, 2-isopropyl-5-methyl-1, 4-benzoquinone. (Ali and Blunden, 2003) It has been suggested that TQ may have a protective effect on heart tissue (Brown et al., 2014) and can help as a drug useful for protecting the heart cells against start and sustain damage. (Ojha et al., 2015) There are reports of apoptosis regulators ratio alteration by the TQ in several cells, namely HL-60 cells (El-Mahdy et al., 2005) and lung cancer cells (Ulasli

Ahookhash Maryam *
Students Research Committee, Kermanshah University of Medical Sciences, Kermanshah, Iran.

Jalili Cyrus
Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran.

***Email:** m.ahookhash57@gmail.com

et al., 2013) which TQ induced the apoptosis by changing the ratio of *Bax/Bcl-2*. Much research on nicotine as a component of cigarette smoke have been carried out, and is now, nicotine, one of the factors contributing to the promotion of heart diseases has been introduced. (Helen et al., 2000) The heart tissue is composed of cells that are differentiated and these cells will not be renewed after the injury. Therefore, the heart function reduced due to loss of a number of their cells. There are reports about the involvement of apoptosis in reducing myocyte in many heart diseases. (Olivetti et al., 1996; Guerra et al., 1999) TQ has an apoptosis function in cancer cells, such as neuroblastoma cells, lung and breast cancer cells. (Ulasli et al., 2013; Paramasivam et al., 2012; Rajput et al., 2013) So far, few studies have been carried out in relation to the anti-apoptosis effect of TQ on other cellular damages except cancer. Here, we hypothesize TQ has an anti apoptosis effect and designed our study aimed to explore the effects of TQ protection against induced apoptosis by nicotine. Also we investigated TQ effect on apoptosis regulator proteins and hope which plant compounds affecting apoptosis help in the treatment of heart disease.

Materials and Methods

Chemicals

Nicotine free base was purchased from Merck (Germany) and diluted with saline. TQ (Sigma Aldrich, china) was prepared as a 1 M stock solution in hot saline (50°C). *Bax* and *Bcl-2* primers for quantitative real-time RT-PCR were purchased from the Cinnacollon Company (Iran).

Animal treatment

Thirty six male mice Balb/c with a weight range of 27-30 grams were purchased from Razi Institute of Iran and all animals were housed in plastic cages, kept in a conditioned atmosphere at 20±2 °C with 12-h light/dark cycles. The mice had unrestricted access to food and water. All the mice were randomly divided into six groups (6mice /group). Group 1 (Control group) received normal saline solution. Group 2 (positive control) received 5mg/kg of nicotine. (Cho Ping et al., 2014) Group 3 received 9 mg/kg of TQ. Group 4 received 18 mg/kg of TQ (Nili-Ahmadabadi et al., 2011) and groups 5, 6 received nicotine following pretreatment with 9, 18 mg/kg of TQ, respectively. All animals received the drugs by intraperitoneal injection daily in the morning for 4weeks. 24 hours after the last injection, mice were necropsy while they were in deep anesthesia by ether. Initially, blood of hearts was evacuated and the hearts were removed from the chest. The whole hearts were sectioned into small pieces and heart tissues were stored at –70°C for analysis. All the surgical and experimental procedures were in accordance with institutional animal care guidelines.

Isolation of total RNA from heart tissue

The heart tissue samples were demolished in liquid nitrogen and total RNA was isolated using RNx-plus synthesis kit (Cinna Gen. Iran) according to the manufacturer's instructions. RNase-free DNase I (o. 5 µl) and 10× reaction buffer with $mgcl_2$ (1µl) and DEPC-treated water were added to the total RNA (1µg) and incubated for 30 min at 37°C to digest and remove genomic DNA. The reaction was stopped by adding EDTA and incubates at 65°C for 10 min finally the integrity of the total RNA sample was verified using red gel electrophoresis.

Reverse transcription PCR (RT-PCR)

The mRNA fraction of total cellular RNA from each sample was converted to cDNA by reverse transcription (RT) using the cDNA synthesis kit (Tacara. Japan) according to the manufacturer's instructions with random 6 mers (0.5µl) and oligo-dT (0.5µl) as primers in a 10 µl reaction containing 1 µl of total RNA, polymerase enzyme (0.5µl), 2µl of buffer and nucleotide-free water at 37°C for 15s followed by 85°C for 5s in the thermocycler (Eppendorf, USA). The resulting cDNA kept at −20°C until use.

Real-time PCR

The real time RT-PCR primers of selected genes were designed by using of the National Center for Biotechnology Information (NCBI) and computer software oligo. The expression fluctuations of *Bax* and *Bcl-2* genes coding for proteins implicated in the induction or inhibition of apoptosis normalized using glyceraldehydes3-phosphate dehydrogenase (GAPDH) as a housekeeping gene was evaluated (Table 1). Real time PCR was carried out using an AB system (Applied Biosystems. USA) and Tacara kit (Japan) according to the manufacturer's instructions. 5µl of SYBR Green PCR master mix, 0.3µl of Rox, 1µl of cDNA, and 0.2µl primer set were used for amplification in 10µl reaction mixture. The solution was subjected to heat at 95°C for 10s, followed by 40 cycles of 95°C for 5s, 60°C for 30s and 72°C for 45s according to the expression level of the target gene. Computations were done by Counting on the values of cycle threshold (Ct) by normalizing the average Ct value of each treatment compared to its opposite endogenous control (GAPDH).

Table 1: Real-time RT-PCR primers sequences, temperature melting and PCR products lengths.

Gene	Sequence	TM(°c)	bp
BCL-2.F	5′-CTCGTCGCTACCGTCGTGACTTCG-3′	66.1	112
BCL-2.R	3′-ACCCCATCCCTGAAGAGTTCC-5′	59.8	
BAX.F	5′-CTCAAGGCCCTGTGCACTAA-3′	57.6	120
BAX.R	3′-GAGGCCTTCCCAGCCAC-5′	58.4	
GAPDH.F	5′-AGAACATCATCCCTGCATCCA-3′	59.6	127
GAPDH.R	3′-GTCAGATCCACGACGGACACA-5′	61.1	

Statistical analysis

Quantitative data were expressed as mean ± SD. Differences between groups were determined with a one-way ANOVA followed by a Tukey test. A value of P <0.05 was considered to denote statistical significance.

Results

Effects of TQ and nicotine on expression of Bax and Bcl 2 genes

The effects of TQ and nicotine on the *Bcl-2* (Figure1) and *Bax* (Figure2) mRNA expression levels in heart tissue were studied. Interestingly, TQ resulted in a significant decrease in *Bax* and in *Bcl2* mRNA expression levels compared to control group in a dose dependent manner ($p = 0$). Administration of nicotine at a dose of 5 mg/kg significantly reduced the *Bax* and *Bcl-2* mRNA expression levels compared to the control group ($p = 0$). Treatment of mice both doses 5 mg/kg nicotine and TQ (9, 18 mg/kg) caused a significant decrease in the expression of *Bax* compared to the control group in a dose dependent manner ($p = 0$). Treatment of mice both dose 5 mg/kg nicotine and TQ (9, 18 mg/kg) caused a significant decrease in the expression of *Bcl-2* compared to the control group which dose 18 mg/kg TQ decreased more *Bcl-2* mRNA expression levels than the dose 9 mg/kg TQ ($p = 0$).

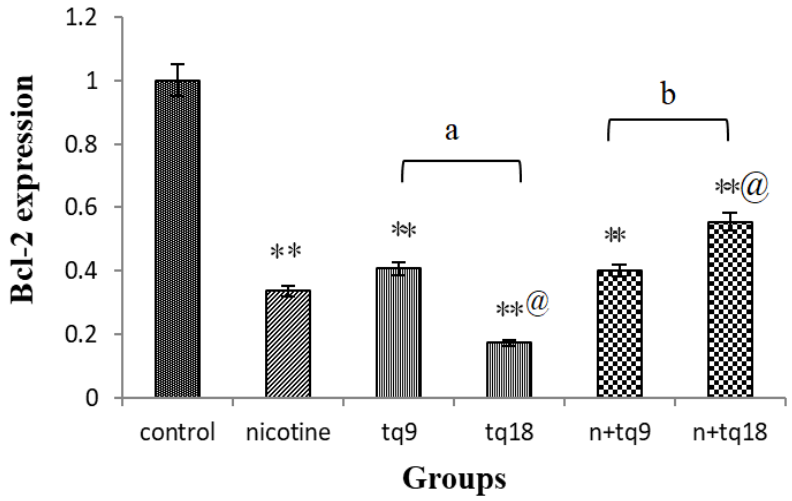

Figure 1. Effects of TQ and nicotine on the *Bcl-2* gene expression in samples, ** (p = 0) versus experimental groups to control group, a (p = 0) versus tq9 to tq18 and b (p = 0) versus n (nicotine) + tq9 to n (nicotine) + tq18, @ (p = 0) versus tq18 and n (nicotine) + tq18 to nicotine group.

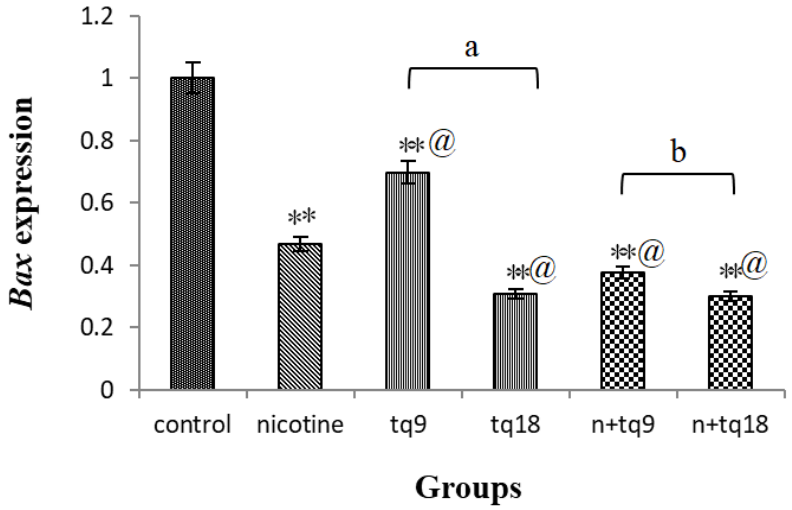

Figure 2. Effects of TQ and nicotine on the *Bax* gene expression in samples, ** (p = 0) versus experimental group to control group, a (p = 0) versus tq9 to tq18 and b (p = 0) versus n (nicotine) + tq9 to n (nicotine) + tq18, @ (p = 0) versus tq9 and 18 and n (nicotine) + tq 9 and 18 to nicotine group.

Effects of nicotine and TQ on Bax/Bcl 2 ratio

As showed in Figure 3, administration of nicotine significantly increased the *Bax/Bcl-2* ratio compared to the control group ($p = 0$) and in groups receiving TQ at doses of 9 and 18 mg/kg, equally, increased *Bax/ Bcl*-2 ratio ($p = 0$). Also, administration doses of 9 and 18 mg/kg of TQ significantly increased the *Bax/Bcl*-2 ratio compared to the nicotine group ($p = 0$). In nicotine + TQ9 group no significant change in *Bax/ Bcl*-2 ratio was observed ($p = 0$) and in the nicotine + TQ18 group *Bax/ Bcl*-2 ratio significantly decreased ($p = 0$) compared to the control group. In the nicotine + TQ (9&18 mg/kg) groups significantly decreased *Bax/ Bcl*-2 ratio compared to the nicotine group which synergistic nicotine + TQ18 decreased more *Bax/ Bcl*-2 ratio than synergistic nicotine + TQ9 ($p = 0$).

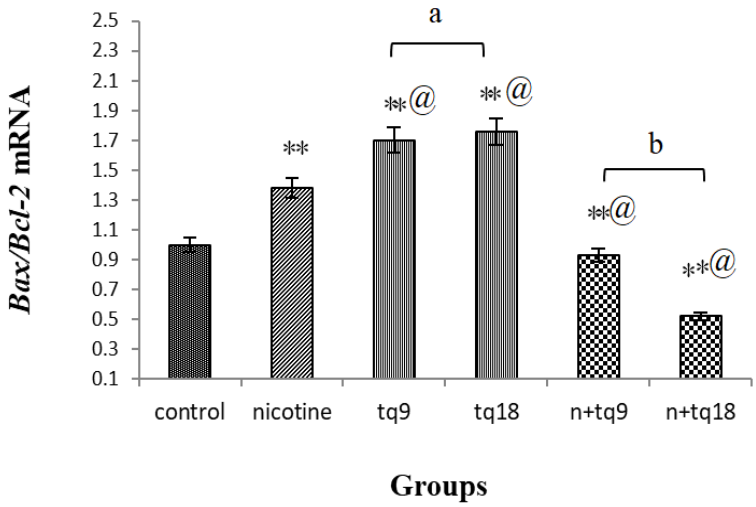

Figure 3. Effects of TQ and nicotine on the ratio of *Bax/Bcl-2* in samples, ** (p = 0) versus control group, a (p = 0) versus tq9 to tq18 and b (p = 0) versus n (nicotine) + tq9 to n (nicotine) + tq18, @ (p = 0) versus tq9 and 18 and n (nicotine)+ tq 9 and 18 to nicotine group.

Discussion

Nicotine administration at a dose of 5 mg/kg induced the apoptosis in cardiac tissue by increasing the *Bax/Bcl-2* ratio of expression. In connection with the effects of nicotine on the tissues have been conducting many studies with contradictory results such as study Demiralay et al. (2006) which investigated effects of nicotine in the female rats lung and observed the apoptosis index raised to 84% in nicotine-treated animals. Also, our result of nicotine treatment on apoptosis is in good agreement with study Yang et al. (2004) who

investigated the effects of nicotine on cultured human umbilical vein endothelial cells (HUVEC) and reported that nicotine by increasing *Bax/Bcl-2* ratio induced apoptosis and the excessive apoptosis in endothelial cells induced by nicotine may predispose smokers to cardiovascular complications. However, our findings were disagreeing with the report's Suzuki et al. (2003) they investigated the hypothesis of apoptosis inhibition in heart by nicotine and demonstrated that nicotine caused a 50% reduction in apoptosis induced by lipopolysaccharide (LPS) in rat cardiac myocyte. By the same token Aali et al. (2015) reported that nicotine decreased *Bax/Bcl-2* ratio in the breast cancer cells line.

Interest in herbal medicines has increased their use. Black seed has been applied to improvement diseases such as hypertension, diabetes and inflammation as a medicinal herb. (Entok et al., 2014) cDNA analysis by Real time PCR technique showed that TQ (9&18 mg/kg) induced apoptosis in heart tissue by a significant increase in the *Bax/Bcl-2* ratio of expression although TQ decreased the genes expression of both *Bax* and *Bcl-2*. Many studies have examined TQ apoptosis effects which associated with increasing *Bax/Bcl-2* ratio, namely Gali study on mice keratinocyte cancer cells and study of Paramasivam on TQ apoptosis effects in mouse neuroblastoma cells (Paramasivam et al., 2012; Gali-Muhtasib et al., 2004) which support the results of this study. Nevertheless, many probes have proven that the apoptosis effects of TQ are relevant to cancer cells. There is a study that investigated TQ apoptosis effects in another damage than cancer. In contrast to our results El-Ghany et al. (2009) reported that 20 and 50 mg/kg/day TQ doses caused a significant decrease *Bax/Bcl-2* ratio and exerted an anti apoptosis effect in a model of hepatic ischemia- reperfusion injury. In this study pretreatment with TQ 18 mg/kg to nicotine treated mice inhibited apoptosis in heart tissue. Given that TQ and nicotine induced apoptosis and TQ more *Bax/Bcl*-2 ratio than the nicotine was expected that synergistic of nicotine and TQ increased apoptosis index in cardiac tissue which not only apoptosis index not increased but also this index fell. It is sensible to accept that changes in apoptosis following TQ administration could be the effect of altered gene expression. Also in this present study pretreatment with TQ at a dose of 9 mg/kg for 28 days before administration of nicotine did not cause significant alterations in heart tissue. So far, most studies have centered on the apoptosis performance of TQ in cancer cells and have introduced the TQ as a potent inducer of apoptosis. It may be identified this feature of TQ cause is which anti-apoptosis effects of TQ almost is not contemplated. Incidentally the study, which simultaneously be examined the effects of nicotine and TQ on apoptosis not found.

How TQ as a potent inducer of apoptosis in cancer and normal cells plays an apoptosis role in some conditions (single administration) and anti-apoptosis in others (synergistic with nicotine) requires further investigation. It can be concluded from this study that TQ exerts an anti apoptosis effect against nicotine- induced apoptosis through reducing *Bax/Bcl-2* ratio in a dose dependent manner.

Conflict of Interests

All authors have no commercial or financial conflict of interests in the products described in this research paper.

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