Assessment of thymoquinone effects on apoptotic and oxidative damage induced by morphine in mice heart

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Received January 16, 2018; Accepted June 26, 2018; Published June 30, 2018
Doi: http://dx.doi.org/10.14715/cmb/2018.64.9.5
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Abstract: Opioids bind to specific receptors that are located in the central nervous system (CNS) and many other organs such as cardiovascular tissue. Morphine binds to opioid receptors and can induce oxidative stress under some certain conditions. Thymoquinone (TQ) has shown many therapeutic effects such as anti-inflammatory, antioxidant and immunomodulatory ones. Considering the oxidative effects of morphine, antioxidant effects of TQ and effects of oxidative damage in various types of biomolecules, the present study was conducted to determine the effect of morphine plus TQ on the expression of apoptotic genes in the heart of male mice. Hence we used real-time PCR to identify alterations in mRNA expression of genes involved in apoptotic pathway, including p53, Bax and Bcl-2 between the morphine-treated and TQ plus morphine-treated mice. Serum nitric oxide (NO) (Griess assay) and total antioxidant capacity (TAC) were analyzed and compared. In the morphine group, compared to control group, a significant increase in P53 and Bax mRNA expression and a significant decrease in Bcl-2 mRNA expression were observed (p < 0.01). In TQ plus morphine groups, NO was decreased (P <0 .001) and TAC levels were increased significantly (P < .001). Interestingly, TQ (9 and 18 mg/kg) plus morphine caused a significant decrease in p53 and Bax and a significant increase in Bcl2 mRNA expression, compared to morphine-treated group (p < 0.01). Collectively, the results of this study indicated that TQ, as an antioxidant, can improve the apoptotic effects induced by morphine in the heart tissue of mice.

Key words: Morphine; Thymoquinone; Apoptosis; P53.

Introduction

Opioids bind to specific receptors that are located in the central nervous system (CNS) and many other organs such as cardiovascular tissue. They belong to the G protein-coupled receptors (1-3). Opioids can make significant changes in heart function, decrease sympathetic tone and lead to vasodilation (4). Morphine, an mu-opioid receptor agonist, was isolated by Friedrich Serturner in the 17th century. Morphine, due to its sedative and powerful analgesic effects, is often used for the treatment of intense pain; acute, chronic non-cancer pain, cancer-related pain as well as an initial anesthetic for cardiac surgery (5, 6). Released histamine under the influence of meperidine, hydrocodeone, hydromorphone and morphine can cause a significant reduction in blood pressure and systemic vascular resistance (4). The harmful cardiovascular-related events have been reported from long-term, open-label safety trials on long-acting morphine preparations (7). On the other hand, opioids can be effectively used for the treatment of chronic pains, so studying the risks associated with this type of drug is very important (4).

Morphine that binds to opioid receptors can induce oxidative stress under some certain conditions. It seems that oxidative stress plays an important role in progression of different pathological processes (8-11). Furthermore, it has been reported that some harmful physiological effects of morphine are related to the formation of reactive oxygen species (ROS) or reactive nitrogen species (RNS). Compared to other opioids, more attention has been paid to the relationship between morphine and oxidative stress. Morphine can promote the production of free radicals, decrease the activity of several components of antioxidant systems in target cells, combine these two ways and increase the oxidative stress (6). In addition, it directly increases the expression of nitric oxide. Morphine can increase the production of nitric oxide by regulating intracellular calcium and activating calcium/calmodulin-dependent NOS. Furthermore, morphine can increase the production of nitric oxide via naloxone-sensitive receptors (6, 7, 9) and regulate the function of vascular endothelial cells through autocrine/paracrine pathway. This pathway can regulate the expression of nitric oxide production. In fact, nitric oxide production signals have an impact on neuro-cardiovascular system via opioid receptors (12). Production of ROS under the influence of morphine and reduction of activity of antioxidant enzymes can lead to oxidative damage in various types of biomolecules, including DNA, lipids and proteins (6, 13, 14). So, DNA damage, protein oxidation and apoptotic induction are common serious events that are connected to this harmful process (6).

Apoptosis or programmed cell death is a tightly organized process which is performed to keep the condi-
Thymoquinone against morphine apoptosis.

Morphine (C16H19NO3) and TQ (2-isopropyl-5-methylbenzo-1,4-quinone;C10H12O2) were obtained from Sigma Chemical (St Louis, USA) and were dissolved in their solvent (normal saline 0.9%) for administration.

The mice were randomly divided into six groups (n=6): Group 1: normal saline (0.4 ml/kg, once daily), Group 2: morphine (20 mg/kg, once daily within the first 5 days; twice per day within the next 5 days; and a dose of up to 30 mg/kg, twice per day from days 11 to 20), Group 3: TQ (9 mg/kg, once daily from days 1 to 20), Group 4: TQ (18 mg/kg, once daily from days 1 to 20) and Group 5: morphine plus TQ (9 mg/kg), Group 6: morphine plus TQ (18 mg/kg). Morphine and TQ were administered intraperitoneally.

At the end of the experiment and 24 h after the last dosing, the animals were quickly dissected under intraperitoneal ketamine / xylazine anesthesia. The heart was removed and washed with cold normal saline, extraneous materials were removed with ice cold DEPC (diethyl pyrocarbonate) treated water, and it was then stored at −80°C until use for RNA extraction and gene expression analysis.

RNA extraction

Isolation of total RNA was carried out by RNXTM PLUS buffer (CINNAGEN, Iran) based on the manufacturer’s instructions. Briefly, Using a mortar and pestle in liquid Nitrogen, 30-50 mg of the heart tissue were homogenized, and 1 ml ice cold RNXTM PLUS solution and 200 μl chloroform were added to it. The resulting mixture was then centrifuged at 12000 rpm at 4°C for 15 min. The aqueous phase was transferred to a new RNase-free 1.5 ml tube. After precipitation of RNA, it was washed with an equal volume of isopropanol and 1 ml 75% ethanol. The resulting pellet was dissolved in 50 μl DEPC-treated water. The genomic DNA from extracted RNA was removed by DNasel (CINNAGEN, Iran) based on the manufacturer’s instructions. RNA-extracted samples were stored at −80°C for further experiments.

cDNA synthesis

Reverse transcription of extracted mRNA was carried out by cdNA synthesis kit (PrimeScriptTM 1st strand cdNA Synthesis Kit, Takara). Then, 500 ng of mRNA were used in 20 μl reaction mixture based on the manufacturer’s instructions. The resulting cdNA was stored at −20°C until it was used for the other analysis.

Real-Time PCR

Glyceraldehyde-3-phosphatedehydrogenase (GAPDH) was used as a housekeeping gene to evaluate the relative expression profile of P53, Bcl-2 and Bax genes. The sequences of all real-time PCR primers (GAPDH, P53, Bcl2 and Bax genes) are shown in Table 1. Real time PCR was carried out using Applied Biosystems™ Real-Time PCR instruments. Next, 10 μl SYBR Green PCR master mix, 2 μl 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 seconds and 60°C for 30 seconds. Relative quantification (RQ)
increase in P53 and Bax mRNA expression and a significant decrease in Bcl-2 mRNA expression, compared to control group (p < 0.01). The significant increase in Bax/Bcl-2 ratio in the heart of morphine-treated mice also confirmed the induction of apoptosis in this tissue (p<0.01) Figure 1.

Effect of TQ on apoptotic gene expression of heart

Different doses of TQ caused a significant decrease in P53 and Bax and significant increase in Bcl-2 mRNA expression, compared to morphine-treated group (p < 0.01). On the other hand, Bax/Bcl-2 ratio had a significant decrease (p<0.01) (Figure 2).

Effect of TQ plus morphine on heart apoptotic gene expression

Interestingly, TQ (9 and 18 mg/kg) plus morphine caused a significant decrease in Bax and P53 and significant increase in Bcl-2 mRNA expression, compared to

### Table 1. Primer sequences used for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5' to 3')</th>
<th>Ta</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>F AGAACATCATTCCCTGACCATCCAC</td>
<td>58</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>R GTCAGATCAGCGACGGGACACA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P53</td>
<td>F GTACCTTATGAGCCCAACCCGA</td>
<td>56.2</td>
<td>143</td>
</tr>
<tr>
<td></td>
<td>R AGAAGGTTTCCAAGCCGMTGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bax</td>
<td>F CTCAAGGCCCCTGACTAAT</td>
<td>58.4</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>R GAGGCTTCCAAGCCAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl-2</td>
<td>F ACCCATCCTGAGAGTTC</td>
<td>59.6</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>R ACCCATCCTGGAGAGTTC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

= 2−ΔΔCt formula was used for assessment of relative expression of genes.

### Griess assay

Measurement of Nitric oxide (NO) was done based on Griess colorimetric assay. So, NEED (N-(1-naphthyl) ethylenediamine dihydrochloride), sulfonamide solutions and nitrite standards were prepared. To measure nitrite concentration in serum, 100 μl of the samples were deproteinized by zinc sulfate and then transferred to the wells. Then, 100 μl chloride vanadium, 50 μl sulfonamide and 50 μl NEED solutions were added afterward. Incubation of the cells was done at the temperature of 30 °C in darkness. The optical density (OD) of samples was measured by ELISA reader at the wavelength of 540 nm.

### Total antioxidant capacity test

TAC was measured by TAS randox kit, and 1.30 - 1.77 mmol/l of Plasma was used to do it.

### Statistical analysis

All data are presented as mean± SEM. Statistical differences between two groups were determined by independent two-tailed sample t-test and among 3 groups or more were assessed by one-way ANOVA test. Results were considered statistically significant when p <0.05. Data were analyzed by SPSS 16.0.

### Results

#### Effect of morphine on heart apoptotic gene expression

As it is shown in Figure 1, morphine induced apoptosis in the heart which is evidenced by a significant

### Figure 1. Effect of morphine on heart apoptotic gene expression. The p53, Bax, Bcl2 and Bax/Bcl2 ratio of the mRNA levels morphine treated heart in comparison with normal saline treated control mice. ** p<0.01 (mean±SEM).

#### Figure 2. Effect of TQ on heart apoptotic gene expression. The p53, Bax, Bcl2 and Bax/Bcl2 ratio of the mRNA levels comprised in TQ treated heart in comparison with morphine treated mice. ** p<0.01 (mean±SEM).

#### Figure 3. Effect of TQ on apoptotic effects of morphine. The p53, Bax, Bcl2 and Bax/Bcl2 ratio of the mRNA levels comprised in TQ treated heart in comparison with morphine treated mice. ** p<0.01 (mean±SEM).
Chronic morphine administration can significantly increase apoptosis in the heart tissue of mice. It has been indicated that vulnerability to apoptotic effects of morphine in this tissue. Previous studies have demonstrated that TQ can reduce the apoptotic effects of morphine in the heart tissue of mice (39). The present study for the first time demonstrated that TQ can reduce the apoptotic effects of morphine in the heart tissue of mice (39).

In recent years, the effect of morphine on oxidative stress, cell viability and apoptosis has been considered in many studies. Some of them have reported that morphine can induce apoptosis in microglia and neurons (40) and macrophages (41) or SH-SY5Y (42). On the other hand, the protective effects of morphine have been reported in macrophages (43) or heart (44). In some studies, it has been shown that morphine can improve the formation of ROS in SH-SY5Y (42) and macrophages (41). So, it can be concluded that the effect of morphine on apoptosis depends on the cell type or experimental context. In this study, the protective effects of TQ on the morphine-induced disorders were assessed in terms of nitric oxide secretion and total antioxidant capacity. Opioids that have oxidative properties can increase apoptosis by producing free radicals in many cells (36). The physiological effects of morphine can induce apoptosis in the body cells as well as production of nitrogen species and reactive oxygen substances (45). Morphine increases the production of free radicals by activating lipid peroxidation, which blocks the antioxidant enzymes. This process can cause the formation of reactive oxygen species or free radicals. Free radicals can lead to cell membrane destruction and DNA segmentation (46).

Further, the effects of TQ on the heart tissue of mice were studied and compared with the morphine-treated tissue. The results showed that TQ could have anti-apoptotic effects in this tissue. Previous studies have indicated that TQ can induce apoptosis via p53-independent (27) and p53-dependent (23) pathways. The apoptotic effect of TQ has been reported in cancer cells, but its apoptotic or antiapoptotic role has not been documented in other cellular damages yet. The results of El-Ghany et al indicated that TQ has an anti-apoptotic effect against hepatic ischemia reperfusion injury so that it targets the apoptotic regulators of the Bcl-2 family proteins (30). Unlike cancerous cells, primary mouse keratinocytes and normal cells have shown resistance to the apoptotic effects of TQ (47).

Finally, the apoptotic effects of simultaneous administration of TQ and morphine on the heart tissue of mice were studied. The results of Real Time PCR showed that expression of apoptotic genes, compared to morphine-treated tissue, was reduced significantly. This study for the first time demonstrated that TQ can reduce the apoptotic effects of morphine in the heart tissue of mice.

It has been reported that TQ is an opioid receptor stimulating compound with medicinal potential that can be effective in the treatment of opioid dependence (48). TQ can reduce the toxic effects of drug metabolism and decrease the oxidative stress (26). This natural antioxidant might be useful to decrease the oxidative stress induced by chronic morphine administration.

**Discussion**

Morphine is one of the opioids that is used as an analgesic. It can be addictive and cause physiological dependence (31). Morphine in high doses can cause respiratory, cardiovascular, gastrointestinal or psychiatric problems (32-35). Apoptosis can be induced by opioids in some cells (36). In the present study, the effects of morphine on the expression of apoptotic genes in the heart tissue of mice were investigated. In comparison with control group, the expressions of p53 and Bax increased markedly, and Bcl-2 expression reduced significantly (P < 0.001) (Figure 4).

The total antioxidant capacity in serum decreased significantly in the morphine group compared to the control group (P < 0.05). Also, TAC levels increased significantly in TQ plus morphine groups (18mg/kg) compared to control and morphine groups (P < .001) (Figure 5).

**Total Antioxidant Capacity**

Figure 4. Nitric oxide levels in the different groups of mice receiving morphine and TQ compared with control group. (** p<0.001 Control), (& p<0.001 Morphine), (mean±SEM).

Figure 5. Serum Total Antioxidant Capacity levels in the different groups of mice receiving morphine and thymoquinone compared with control group. (* p<0.05 ** p<0.001 Control), (& p<0.001 Morphine), (mean±SEM).
by morphine in the heart of mice. Previous studies have shown that treatment with TQ can have a preventive effect on the toxicity of morphine in the kidneys through various ways such as regulation of metabolic activities of kidney, reduction of oxidative stress and acting as an anti-apoptotic factor (49). In this regard, Mahmoud et al indicated that treatment with TQ, through expression of Bcl-2 as an anti-apoptotic factor, could induce protective effects on the kidneys (50). It can improve the oxidative stress status in the renal fibrosis too (TQ protects the rat kidneys against renal fibrosis). TQ, by antioxidant defense mechanisms that affect lipid peroxidation, can reduce the antioxidant markers of MDA and NO and increase SOD, CAT and GPx levels (51). TQ has different effects on various cells in different conditions. Further investigations are needed to determine its apoptotic effects and mechanisms.

Generally, the results of this study indicated that TQ, as an antioxidant, can improve the apoptotic effects induced by morphine in the heart tissue of mice. All told, it seems thymoquinone, as an antioxidant, recovering the apoptotic effects induced by morphine in the heart tissue of mice.

Acknowledgements
The authors wish to acknowledge the financial support provided (Grant number, 95634) by Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran.

Interest conflict
The authors declare that they have no competing interest.

Author’s contribution

References
2. Insel P, Sneed A, Murray F et al. GPCR expression in tissues and cells: are the optimal receptors being used as drug targets? British journal of pharmacology 2012; 165(6): 1613-1616.