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Effect of Repeated Injection of Cadmium on *Bax/Bcl-2* mRNA Level in Stomach of Rats

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Background and Aims: Cadmium is an important environmental pollutant and a potent toxicant to organisms. However, the toxicity of Cadmium and its influences on stomach is still unclear. We examined the effects of intraperitoneal injection of Cadmium on mRNA expression of *Bcl-2* and *Bax* genes in rat stomach.

Materials and Methods: Twenty eight male Wistar rats weighing 200 to 250 g were randomly divided into 4 groups. The control group received saline and the three other groups received Cadmium at doses of 1, 2 and 4 mg/kg for 15 successive days. One day after the last injection, the stomach was dissected and removed and then the expression of *Bax* and *Bcl-2* genes was evaluated using real time polymerase chain reaction.

Results: Cadmium exposure did not change on mRNA level of *Bax* at the doses of 1, 2 and 4 mg/kg in rat stomach cells. However, the mRNA level of *Bcl-2* gene decreased at doses of 1, 2 and 4 mg/kg (body weight) by 0.07, 0.03 and 0.01 times compared with the control cells ($p<0.001$).The ratio of *Bax/Bcl-2* increased significantly at the doses of 2 and 4 mg/kg ($p< 0.05$) compared to the control group.

Conclusions: This increased *Bax/Bcl-2* mRNA ratio induces cell apoptosisin rat stomach cells.

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Introduction

Cadmium is a toxic heavy metal that is emitted into the environment as a result of human activities including its use in industry. The major sources of Cadmium entry into the gastrointestinal tract are smoking and dietary intake [1]. Another source of Cadmium is inhalation of Cadmium-contaminated air [2]. Cadmium can be teratogenic and carcinogenic within different organs and tissues in human and animals [3, 4]. Furthermore, Cadmium is known as a category 1 carcinogenic substance by International Agency for Research on Cancer (IARC). Cadmium induces lung cancer and recent experimental studies have demonstrated its correlation with cancers of the bladder, pancreas, and stomach [5]. The carcinogenicity mechanisms of Cadmium could be related to the suppression of gene expression, inhibition of DNA damage repair, suppuration of apoptosis, and induction of oxidative stress, the formation of reactive oxygen species (ROS), interference with anti-oxidative enzymes, inhibition of DNA repair enzymes, deregulation of cell proliferation and suppressed apoptosis in body organs [3, 5]. Cadmium induces ROS generation and gastric mucosal and DNA lesions, alter gene regulation, signal transduction, gene abnormalities, and cell growth, ultimately leading to carcinogenesis. Cadmium also enhances gastric cell cancer [6]. In addition, Cadmium affects both gene transcription and translation and bears a role in apoptosis [7].

Messner et al. showed that Cadmium causes the activation of multiple death signals. Multi factors and genotype may determine the initiation and rate of death signals. Cadmium-induced death starts with an apoptosis-related mitochondrial membrane depolarization and a DNA damage response [8]. Cadmium induces apoptosis in renal tubular cells identified through *in vivo* studies [9]. One of the targets of Cadmium is also gastrointestinal tract [10]. Therefore, another result has indicated that exposure to Cadmium increases the risk of gastric cancer [6]. Long-term exposure to Cadmium enhances the mortality risk of several cancers including esophageal and gastric cancer [11].

In another study, it has been shown that the *Bcl-2* family regulates cell death of apoptosis in human and animal tissues. *Bcl-2* has anti-apoptotic properties in contrast to *Bax* having proapoptotic properties [12]. It has been known that lead can induce apoptosis and change the levels (imbalance) of *Bax*, *Bcl-2*, and mitochondrial dysfunction [13]. Previous studies have shown that alteration in the ratio of *Bax/Bcl-2* could be a determining factor for death or survival of the cells. An increase in this ratio induces apoptosis in brain cells [14].

However, little is known about the impact of molecular mechanism of Cadmium on stomach. Therefore, we have investigated a number of parameters inducing apoptosis-related gene expression and *Bax/Bcl-2* ratio on the stomach of rats.

Materials and Methods

Animals and experimental groups

This experiment was conducted on 28 male Wistar rats at 8 weeks of age, weighing 200-250 g procured from Veterinary Medicine of Tehran University (Iran). Animals were housed at 22±3°C and 12-hour light/dark cycle in the animal house of Parand Islamic Azad University and fed rodent chow and water. After 2 weeks of adaptation to the new environment, rats were randomly allocated into 4 groups (n=7): one control group and 3 experimental groups. All experiments conformed to the guidelines of the Ethical Committee of Parand Islamic Azad University.

Cadmium nitrate administration

Cadmium (No3)2 (Cadmium nitrate) solution was purchased from kimia Pars, Inc (Merck, Germany). Whereas, Cadmium concentration in blood serves as a reliable indicator for a recent toxicity, injections were performed intraperitoneally with in a final volume of 1 ml for each dose. According to previous studies, the dose of Cadmium was chosen [15-18] and the targeted concentrations were prepared from Cadmium nitrate solution. The control group received saline (vehicle of Cadmium) and experimental groups were given Cadmium concentrations of 1, 2, 4 mg/kg body weight for 15 consecutive days. One day after the last injection, the rats were deeply anesthetized with chloroform and rapidly decapitalized. The stomach tissues was dissected off and were frozen in liquid nitrogen and stored at -80°C until further tests.

RNA extraction and CDNA synthesis

All the RNAs of stomach tissue were isolated using the RNX-TM plus (CinnaGen Inc., Tehran, Iran). The quantity and purity of extracted RNA was determined using a spectrophotometer (NanoDrop ND-2000, Wilmington, DE, USA), and only extracted RNAs with an A260/A280 ratio ranging from 1.8 to 2.0 were used for cDNA synthesis. Real time transcription was performed with 1 µg of RNA and a first strand cDNA synthesis kit (Fermentas, Thermo scientific, USA) according to manufacturer's instructions.

Real-time quantitative polymerase chain reaction (PCR) using SYBER green

Real-time PCR was used to evaluate the quantitative expression of mRNA for *Bcl-2*, *Bax* and *GAPDH* as the control. The relative quantification was performed in real time PCR by measuring increased fluorescence light as a result of SYBR Green bonding using an Illuminareal time PCR system (San Diego, CA 92122, USA). Amplification was performed in a final volume of 25 µl, which included 1 µl of cDNA, 12.5 µl of SYBR Green master mix (Master mix Green-No Rox, Ampliqon Denmark), 5 µmol of each complimentary primer in a volume of 0.5 µl, and 10.5 µl of deionized water. The selected primers were designed and underwent an extensive search using BLAST tool. The oligonucleotide sequences of *GAPDH*, Bcl-2 and *Bax* and primers annealing temperature used for real-time PCR are as follows: The oligonucleotide sequences of the primers as follows: Forward: 5′-TGCCACTCAGAAGACTGTGG -3′, and Reverse: 5′-GGATGCAGGGATGATGTTCT-

3′, for the rat *GAPDH* gene; Forward: 5'-GAGTACCTGAACCGGCATCT-3' and Reverse: 5'- GAAATCAAACAGAGGTCGCA-3' for the rat *Bcl-2* gene; Forward: 5'-TTGCTACAGGGTTTCATCCA-3' and Reverse: 5'-GAGTACCTGAACCGGCATCT-3' for the rat *Bax* gene. The amplification conditions were optimized as follows: pre denaturation: 94˚C for 5 min. followed by 35 cycles of denaturation: 94˚C for 1 min., annealing: 53˚C for 1 min. and extension: 72˚C for 5 min.

Quantitative gene expression was analyzed by comparative CT (ΔΔCT) method [19], using GAPDH as an internal control. The relative fold increase (RFI) was calculated using the following equation: $RFI=2^{-\Delta\Delta ct}$.

Statistical analysis

The data collected from the experiment was recorded and analyzed using SPSS 22 statistical software package. All the nominal data were expressed as the mean±SD. Statistical significance of differences throughout this study was performed using one way variance analysis and Tukey's test. A p-value of less than 0.05 was considered as statistically significant.

Results

Melting curve analysis for Real- time PCR products was obtained with the specific primer pairs for *Bcl-2, Bax* and *GAPDH* genes in rat stomach.

As figure 1 shows, Cadmium exposure did not significantly change the mRNA gene expression of *Bax* at the dose 1, 2 and 4 mg/kg in rat stomach. In stomach cells, the mRNA level of *Bax* gene was increased at doses of 1, 2 and 4 mg/kg (body weight) by 57, 249 and 249 times compared with control cells.

As figure 2 displays, Cadmium exposure significantly decreases the mRNA expression of *Bcl-2* gene at the dose 1, 2 and 4 mg/kg in rat stomach ($p<0.001$). In stomach cells, the mRNA level of *Bcl-2* gene decreased at doses of 1, 2 and 4 mg/kg (body weight) by 0.07, 0.03 and 0.01 times compared with the control cells. As illustrated in figure 3, in rat stomach, *Bax/Bcl-2* mRNA ratio significantly increases in doses of 2 and 4 mg/kg Cadmium body weight ($p<0.05$).

Discussion

In this study, we examined the effects of Cadmium on ratio of *Bax/Bcl-2* in the stomach of rats exposed to Cadmium for 2 weeks. The gene expression of *Bax* in the stomachof rat did not significantly increased whereas the gene expression of *Bcl-2* in stomach of rat significantly decreased by Cadmium exposure (Figs. 1 and 2). The ratio of the *Bax/Bcl-2* to Cadmium exposure at doses of 2 and 4 mg/kg in the stomach of rats significantly increased. In a similar way of changes of gene expression of *Bax* and *Bcl-2*, lead exposure induced imbalance of *Bax/Bcl-2* [20].

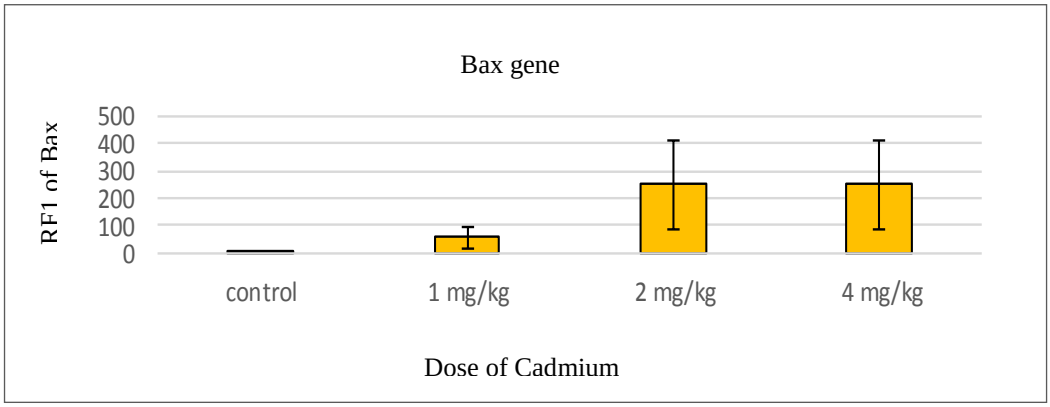

Fig. 1. Effects of Cadmium exposure on gene expression of *BAX* in the stomach of rats.
The expression of gene *Bax* of control group was designated as 1, and the others were expressed as folds compared with the control.
RFI= Relative fold increase.

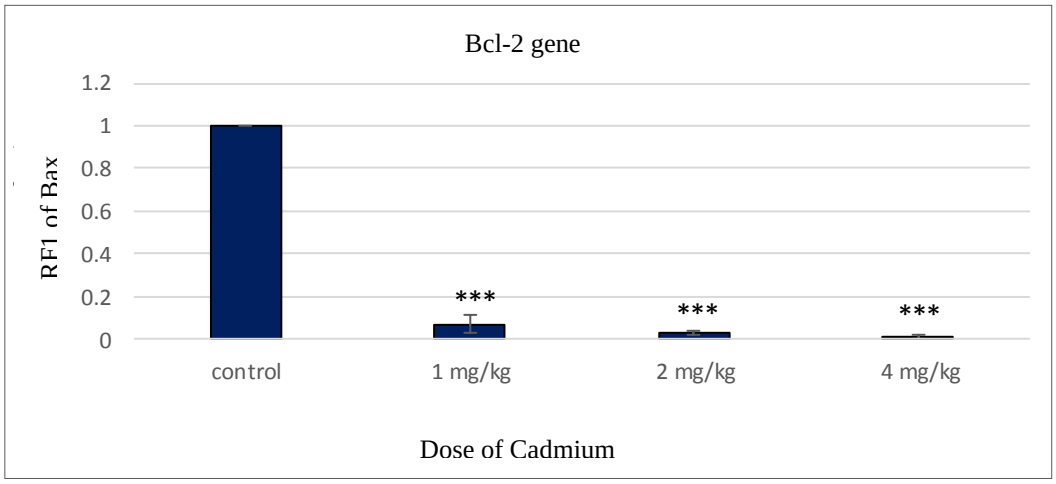

Fig. 2. Effects of Cadmium exposure on gene expression of Bcl-2 in the stomach of rats.
The expression of gene *Bcl-2* of control group was designated as 1, and the others were expressed as folds compared with the control.The mRNA level of *Bcl-2* gene was decreased at 1, 2 and 4 mg/kg of cadmium compared with control cells, ($p<0.001$). RFI= Relative fold increase

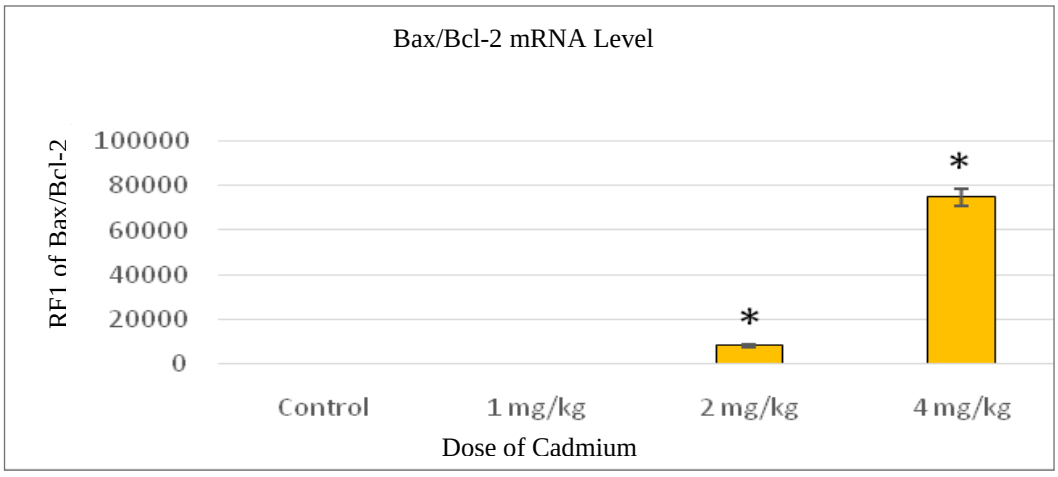

Fig. 3. Ratio of *Bax/Bcl-2* in rat Stomach
The *Bax/Bcl-2* mRNA level was increased at 2 and 4 mg/kg of cadmium compared with control cells, *p<0. 05

Cadmium can be inhibiting apoptosis and DNA repair, stimulating cell proliferation and promoting cancer in a number of tissues [21]. At the cellular level, Cadmium exposure causes inducing damage to DNA and cell membranes by inhibiting different types of DNA repair, and by inducing apoptosis in mammalian cells [22]. The results reveals that Cadmium exposure induces clear genotoxic activities on both the upper and distal parts of the gastrointestinal tract [23].

One of the products of normal cellular metabolism is Reactive oxygen species (ROS). Low and moderate concentration of ROS are helpful [24]. In contrast, at high concentrations, ROS induces damage to cell structures [25] and neurotoxicity by altering the expression of the oxidative stress-related genes [26]. Cadmium can induce ROS generation [25]. ROS generation causes gastric mucosal damage, as well as various gastrointestinal (GI) diseases including peptic ulcers, GI cancers and altersin gene expressionand signal transduction [24].

Furthermore, research indicates that catalase is involved in antioxidant defense mechanisms and prevent excessive levels from ROS at the cellular. Cadmium exposure can increase catalase activity by generating high ROS levels in gastric cancer [27].

The *Bcl-2* family regulates mitochondrial membrane permeability through a family of proto-oncogenes. The *Bcl-2* family is anti-apoptotic (*Bcl-2*) or pro-apoptotic *(Bax*) [28]. *Bax* is in the cytosol, under physiological conditions. An apoptotic trigger leads to translocationinto the mitochondrial membrane. *Bax* can homodimerize or heterodimerize with pro-apoptotic members, thus forming mitochondrial pore and increasing membrane permeability, thereby apoptogenic factors such as cytochrome c release, and induces initiation of apoptosis [29, 30]. The anti-apoptotic protein, *Bcl-2* inhibits the ability of *Bax* to increase membrane potential [31] and antagonizing the apoptotic cascade by a direct interaction [32] and cell fate may be determined by balance of these proteins.

In this study, we demonstrated that Cadmium decreases the expression of *Bcl-2* genes in rat stomach. This is in line with other studies that have shown modulation of the same genes in apoptosis [14, 33-35]. In this study we found out that alteration in the ratio of *Bax*: *Bcl-2* can be a key determining factor in the release of apoptotic factors from mitochondriainto the cytosol. Apoptotic factors such as cytochrome c promotes caspases activation and initiation of apoptosis [36, 37]. An increase in this ratio may induce apoptosis, and a decrease in this ratio may have deleterious effect of cytotoxic stimuli [34, 35]. The critical role of the Bax/Bcl-2 ratio in cytochrome c release and initiation of apoptosis mediated through the mitochondrial pathway has also been determined [37].

Conclusions

The results of the current study showed that intraperitoneal administration of Cadmium decreases of anti-apoptotic *Bcl-2* genes expression in rat stomach. Due to an increment

in the *Bax/Bcl-2* ratio,it is likely that cadmium-induced apoptosis in the rat stomach is dependent on the mitochondrial pathway.

Conflict of Interest

All of the authors declare that they have no conflict of interest.

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