

Original Article

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

Parisa Alaei M.Sc., Parvin Khodarahmi* Ph.D., Masoud salehipour Ph.D.

Department of Biology, Branch of Parand, Islamic Azad University, Parand, Iran.

A B S T R A C T

Article history

Received 16 Dec 2017

Accepted 9 May 2018

Available online 13 Jun 2018

Key words*Bax**Bcl-2*

Cadmium

Rat

Stomach

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 apoptosis in rat stomach cells.

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, suppression 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 $22\pm 3^{\circ}\text{C}$ 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 (No_3)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 ($\Delta\Delta C_t$) method [19], using *GAPDH* as an internal control. The relative fold increase (RFI) was calculated using the following equation: $RFI = 2^{-\Delta\Delta C_t}$.

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 \pm 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 stomach of 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].

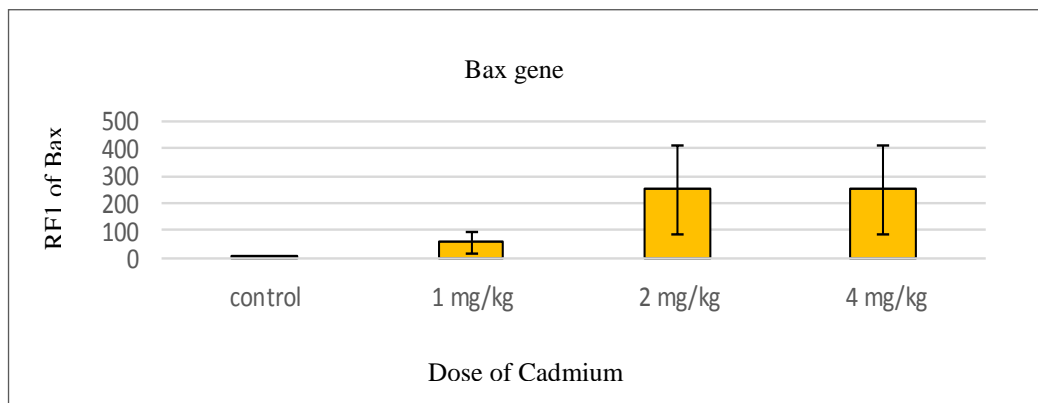


Fig. 1. Effects of Cadmium exposure on gene expression of *BAX* in the stomach of rats. The expression of gene *Bax* of control group was designated as 1, and the others were expressed as folds compared with the control. RFI= Relative fold increase.

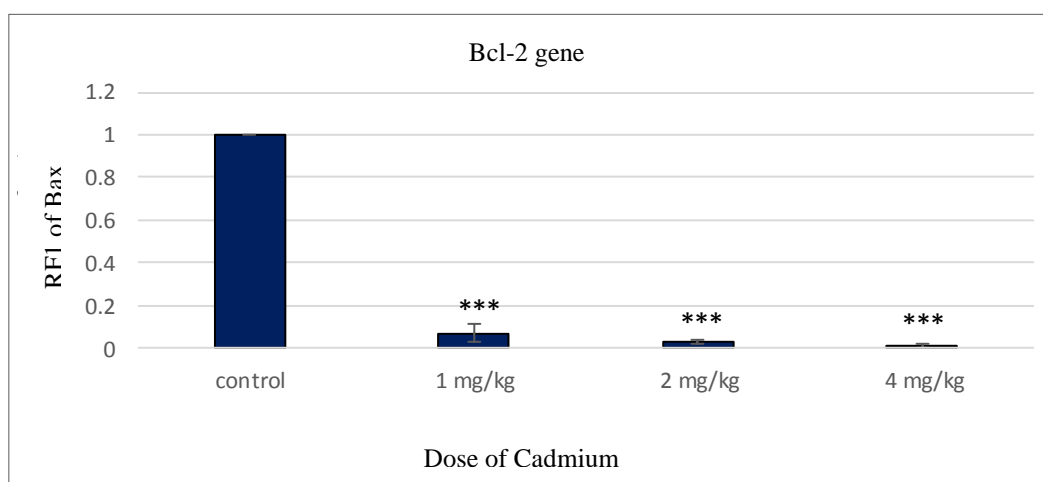


Fig. 2. Effects of Cadmium exposure on gene expression of *Bcl-2* in the stomach of rats. The expression of gene *Bcl-2* of control group was designated as 1, and the others were expressed as folds compared with the control. The mRNA level of *Bcl-2* gene was decreased at 1, 2 and 4 mg/kg of cadmium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

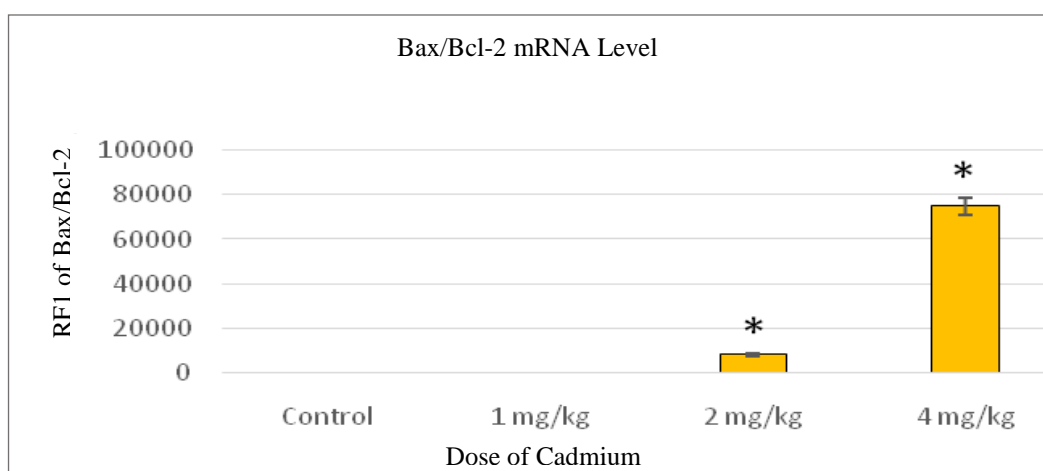


Fig. 3. Ratio of *Bax/Bcl-2* in rat Stomach
The *Bax/Bcl-2* mRNA level was increased at 2 and 4 mg/kg of cadmium compared with control cells, * $p < 0.05$

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 alters in gene expression and 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

translocation into 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 mitochondria into 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.

Acknowledgment

The results described in this paper were based on a student thesis thereby we thank her.

References

- [1]. Satarug S, Garrett SH, Sens MA, Sens DA. Cadmium, environmental exposure, and health outcomes. *Environ Health Perspect.* 2010; 118(2): 182-90.
- [2]. Seidal K, Jørgensen N, Elinder CG, Sjögren B, Vahter M. Fatal cadmium-induced pneumonitis. *Scand J Work Environ Health.* 1993; 19(6): 429-31.
- [3]. Hartwig A. Cadmium and cancer. *Met Ions Life Sci.* 2013; 11: 491-507.
- [4]. Asara Y, Marchal JA, Carrasco E, Boulaiz H, Solinas G, Bandiera P, et al. Cadmium modifies the cell cycle and apoptotic profiles of human breast cancer cells treated with 5-fluorouracil. *Int J Mol Sci.* 2013; 14(8): 16600-6616.
- [5]. Waalkes MP. Cadmium carcinogenesis. *Mutation Res.* 2003; 533(1-2): 107-120.
- [6]. Yuan W, Yang N, Li X. advances in understanding how heavy metal pollution triggers gastric cancer. *BioMed Res Int.* 2016; 2016: 7825432.
- [7]. Waisberg M, Joseph P, Hale B, Beyersmann D. Molecular and cellular mechanisms of cadmium carcinogenesis. *Toxicology* 2003; 192(2-3): 95-117.
- [8]. Messner B, Turkcan A, Ploner C, Laufer G, Bernhard D. Cadmium overkill: autophagy, apoptosis and necrosis signalling in endothelial cells exposed to cadmium. *Cell Mol Life Sci.* 2016; 73(8): 1699-713.
- [9]. Tokumoto M, Fujiwara Y, Shimada A, Hasegawa T, Seko Y, Nagase H, et al. Cadmium toxicity is caused by accumulation of p53 through the down-regulation of Ube2d family genes in vitro and in vivo. *J Toxicologic Sci.* 2011; 36(2): 191-200.
- [10]. Nair AR, DeGheselle O, Smeets K, Van Kerkhove E, Cuypers A. Cadmium-induced pathologies: where is the oxidative balance lost (or not)? *Int J Mol Sci.* 2013; 14(3): 6116-143.
- [11]. Wang M, Song H, Chen WQ, Lu C, Hu Q, Ren Z, et al. Cancer mortality in a Chinese population surrounding a multi-metal sulphide mine in Guangdong province: an ecologic study. *BMC Public Health* 2011; 11: 319.
- [12]. Akhtar RS, Ness JM, Roth KA. Bcl-2 family regulation of neuronal development and neurodegeneration. *Biochim Biophys Acta.* 2004; 1644(2-3): 189-203.
- [13]. Xu J, Ji LD, Xu LH. Lead-induced apoptosis in PC 12 cells: involvement of p53, Bcl-2 family and caspase-3. *Toxicol Lett.* 2006; 166(2): 160-67.
- [14]. Mahdavi S, Khodarahmi P, Roodbari NH. Effects of cadmium on Bcl-2/ Bax expression ratio in rat cortex brain and hippocampus. *Hum Exp Toxicol.* 2018; 37(3): 321-28.
- [15]. Ferrandino I, Favorito R, Annunziata M, Grimaldi MC. Cadmium induces apoptosis in the pituitary gland of *Podarcis sicula*. *Ann N Y Acad Sci.* 2009; 1163(1): 386-88.
- [16]. Haider S, Anis L, Batool Z, Sajid I, Naqvi F, Khaliq S, et al. Short term cadmium administration dose dependently elicits immediate biochemical, neurochemical and neurobehavioral dysfunction in male rats. *Metabol Brain Dis.* 2015; 30(1): 83-92.
- [17]. Unsal C, Kanter M, Aktas C, Erboğa M. Role of quercetin in cadmium-induced oxidative stress, neuronal damage, and apoptosis in rats. *Toxicol Indust Health.* 2015; 31(12): 1106-115.
- [18]. Kaur S, Sharma S. Evaluation of toxic effect of cadmium on sperm count, sperm motility and sperm abnormality in albino mice. *Int J Adv Res.* 2015; 3(3): 335-43.
- [19]. Asara Y, Marchal JA, Carrasco E, Boulaiz H, Solinas G, Bandiera P, et al. Cadmium Modifies the Cell Cycle and Apoptotic Profiles of Human Breast Cancer Cells Treated with 5-Fluorouracil. *Int J mol sci.* 2013; 14(8): 16600-6616.
- [20]. Xu J, Lian LJ, Wu C, Wang XF, Fu WY, Xu LH. Lead induces oxidative stress, DNA damage and alteration of p53, Bax and Bcl-2 expressions in mice. *Food Chem Toxicol.* 2008; 46(5): 1488-494.
- [21]. Templeton DM, Liu Y. Multiple roles of cadmium in cell death and survival. *Chem Biol Interact.* 2010; 188(2): 267-75.
- [22]. Hartwig A, Asmuss M, Ehleben I, Herzer U, Kostelac D, Pelzer A, et al. Interference by toxic metal ions with DNA repair processes and cell cycle control: molecular mechanisms. *Environ Health Perspect.* 2002; 110(S 5): 797-99.

- [23].Breton J, Le Clere K, Daniel C, Sauty M, Nakab L, Chassat T, et al. Chronic ingestion of cadmium and lead alters the bioavailability of essential and heavy metals, gene expression pathways and genotoxicity in mouse intestine. *Archiv Toxicol.* 2013; 87(10): 1787-795.
- [24].Bhattacharyya A, Chattopadhyay R, Mitra S, Crowe SE. Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. *Physiol Rev.* 2014; 94(2): 329-54.
- [25].Valko M, Rhodes C, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem Biol Int.* 2006; 160(1): 1-40.
- [26].Rahman MF, Wang J, Patterson TA, Saini UT, Robinson BL, Newport GD, et al. Expression of genes related to oxidative stress in the mouse brain after exposure to silver-25 nanoparticles. *Toxicol Lett.* 2009; 187(1): 15-21.
- [27].Mateo MM, Martin B, Beneit MS, Rabadan J. Catalase activity in erythrocytes from colon and gastric cancer patients. Influence of nickel, lead, mercury, and cadmium. *Biol Trace Element Res.* 1997; 57(1): 79-90.
- [28].Tsujimoto Y, Shimizu S. Bcl-2 family: life-or-death switch. *FEBS lett.* 2000; 466(1): 6-10.
- [29].Eskes R, Antonsson B, Osen-Sand A, Montessuit S, Richter C, Sadoul R, et al. Bax-induced cytochrome C release from mitochondria is independent of the permeability transition pore but highly dependent on Mg²⁺ ions. *J Cell Bio.* 1998; 143(1): 217-24.
- [30].Vyssokikh MY, Zorova L, Zorov D, Heimlich G, Jürgensmeier JM, Brdiczka D. Bax releases cytochrome c preferentially from a complex between porin and adenine nucleotide translocator. Hexokinase activity suppresses this effect. *Mol Biol Rep.* 2002; 29(1-2): 93-196.
- [31].Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. *Gen Dev.* 1999; 13(15): 1899-911.
- [32].Yang E, Zha J, Jockel J, Boise LH, Thompson CB, Korsmeyer SJ. Bad, a heterodimeric partner for Bcl-x L and Bcl-2, displaces Bax and promotes cell death. *Cell* 1995; 80(2): 285-291.
- [33].Zhou T, Jia X, Chapin RE, Maronpot RR, Harris MW, Liu J, et al. Cadmium at a non-toxic dose alters gene expression in mouse testes. *Toxicol lett.* 2004; 154(3): 191-200.
- [34].Eleawa SM, Alkhateeb MA, Alhashem FH, Bin-Jaliah I, Sakr HF, Elrefaey HM, et al. Resveratrol reverses cadmium chloride-induced testicular damage and subfertility by down-regulating p53 and Bax and upregulating gonadotropins and Bcl-2 gene expression. *J Reproduc Dev.* 2014; 60(2): 115-27.
- [35].Zongping L. Oxidative stress and mitogen-activated protein kinase pathways involved in cadmium-induced BRL 3A cell apoptosis. *Oxid med cell longev.* 2013; 2013: 516051.
- [36].Czabotar PE, Lessene G, Strasser A, Adams JM. Control of apoptosis by the Bcl-2 protein family: implications for physiology and therapy. *Nature Reviews: Mol Cell Biol.* 2014; 15(1): 49-63.
- [37].Zhu L, Han MB, Gao Y, Wang H, Dai L, Wen Y, et al. Curcumin triggers apoptosis via upregulation of Bax/Bcl-2 ratio and caspase activation in SW872 human adipocytes. *Mol Med Rep.* 2015; 12(1): 1151-156.