ORIGINAL ARTICLE

GMJ.2022;11:e2283
www.gmj.ir

Received 2021-09-20
Revised 2021-09-26
Accepted 2022-10-30

The Effect of Chlordiazepoxide Consumption on the Hippocampus of Neonatal Rats During Pregnancy

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Abstract

Background: Chlordiazepoxide is an anti-anxiety drug commonly used by young people and pregnant women to reduce anxiety. The adverse effects of this drug on cholinergic nervous system function have been demonstrated. Therefore, in this study, the effect of chlordiazepoxide consumption during pregnancy was evaluated on the rats infant hippocampus. **Materials and Methods:** Nine pregnant Wistar rats were randomly divided (n=3 per group) into control, experimental (daily intraperitoneal injection of chlordiazepoxide at a dose of 10 mg/kg for 21 days), and vehicle (same amount of normal saline) groups. Two weeks after birth, the neonate brains were removed from the skull and prepared for Nissl and TUNEL stainings. The expressions of pro- and anti-apoptotic genes were evaluated. **Results:** The number of healthy neurons in different areas of the neonatal hippocampus in the experimental group was significantly reduced compared to control and vehicle groups, and the number of apoptotic bodies was increased ($P<0.05$). However, there was no significant difference between the numbers of these cells in the control and vehicle groups. The expression of pro-apoptotic genes in the experimental group increased significantly compared to the control group instead of the expression of anti-apoptotic genes decreased significantly. **Conclusion:** Chlordiazepoxide during pregnancy can cause neuronal damage in the hippocampus of neonatal Wistar rats.

[GMJ.2022;11:e2282] **DOI:10.31661/gmj.v11i.2283**

Keywords: Apoptosis; Pregnancy; Chlordiazepoxide; Anti-Apoptotic; Pro-Apoptotic

Introduction

Benzodiazepines (BZDs) are one of the most widely used anxiolytics prescribed to pregnant women [1, 2]. Chlordiazepoxide is one of the most important BZDs, and its effect is long-lasting [3]. By acting on the central nervous system, it has calming, healing, reducing anxiety, anticonvulsant, and relaxing skeletal muscles effects. In the central nervous system, specific sites with high hypersensitivity to BZDs have been

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identified [4]. The important role of the hippocampus in transmitting information from short-term to long-term memory and spatial routing has been demonstrated.

The side effects of chlordiazepoxide have been shown to be related to its dose, and if the lowest effective dose and the shortest duration of treatment are prescribed, the side effects of the drug are significantly reduced [5]. BZDs secretion in the milk and its passage through the placenta has been observed, and its secretion has been reported to depend on drug properties, plasma binding protein, ionization, lipophilic degree, molecular weight, half-life, drug concentration in maternal blood, oral bioavailability, and pharmacokinetics [5]. The use of this group of drugs for pregnant women in the first 42 days of pregnancy is associated with many potential risks. The onset of teratogenic effects may be immediate or delayed, and symptoms may appear within a few days or three weeks after birth and last for several months. The greatest risk of abnormality is when the fetus is between two and eight weeks after fertilization [6, 7].

Although the main target of these drugs used during pregnancy is the mother, the fetus is an unwanted recipient, and the negative effects of some of these drugs on the fetus are the main problems of pregnancy [8]. There are few studies on neurological disorders caused by BZDs during pregnancy, and their effect on the growth and development of infants exposed to drugs during pregnancy, including chlordiazepoxide, remains unclear. However, animal studies have shown that if the fetus is exposed to BZDs, there is a possibility of long-term effects on the nervous system and its function [8]. Newly produced cells in the dentate gyrus can differentiate into functional hippocampal neurons. With age, this ability is diminished, and some believe that this is why cognitive capacity decreases with age [9]. Clinical and experimental studies suggest possible risks associated with the repeated administration of BZDs during the prenatal or early postnatal period on further development and behavior. Sparse developmental studies suggest that exposure of the immature brain to BZDs can result in cognitive alterations lasting long after the cessation of BZD exposure. However, the results of these studies are inconsistent [8].

It is stated that the regulation of apoptosis in cells is made by members of the Bcl-2 family [9, 10], such as anti-apoptotic (Bcl-2, Bcl-xL, Mcl-1, Bcl-w, Bfl-1) and pro-apoptotic (Bax, Bak, Bok, Bcl-xs, Bad, Bid, Bik, Bim) proteins. These cell death-regulating proteins act with homodimeric and heterodimeric reactions and cause apoptosis by targeting mitochondrial outer membrane proteins [11]. However, due to the lack of a specific biochemical pathway for apoptosis, it has been stated that the balance between the expression levels of each protein is essential in sensitivity to apoptosis.

This study aimed to investigate the expression of pro- and anti-apoptotic genes following the administration of chlordiazepoxide during pregnancy in neonatal hippocampus rats.

Materials and Methods.

Animals

The current experimental study was performed on nine Wistar female rats (purchased from the Pasteur Research Institute, Tehran, Iran) at eight weeks of age, kept at 23-25 °C, 50% humidity, and 12-hours of the light-dark cycle. The rats were regularly examined by vaginal smear preparation and mating in separate cages in the estrous phase, and after observing vaginal plaque, the animals were kept for 21 days. The ethics committee of the Islamic Azad University of Marvdasht branch approved the protocol (approval code: IR.IAU.TMU.REC.1395.322), which conformed to the ethical guidelines of the 1975 Helsinki declaration.

Groups

The rats were randomly divided into groups as follows:

-Control group: Received no treatment.

-Experimental group: Chlordiazepoxide with the selected dose of 10 mg/kg was injected intraperitoneally every day during pregnancy (21 days).

-Vehicle group: Normal saline (1 cc) was injected daily during pregnancy.

Two weeks after born, the neonatal rats

were anesthetized and killed by sodium pentobarbital (40 mg/kg), and one neonatal hemisphere was stained by the Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and Nissl. The other hippocampus hemisphere was separated from other brain tissues for molecular studies. The samples were examined by an optical microscope (Biobase Co., China) with a magnification of 400×, and only pyramidal neurons with clear nuclei were considered as living and healthy cells. From each sample, eight photomicrographs were prepared, three photomicrographs with a minimum distance of 40 µm were randomly selected, and pyramidal neurons in the CA1 area of the hippocampus were counted by Image-Pro Plus (Leica DMLB, Germany).

Nissl Staining

This method was used to stain Nissl bodies in the neuronal cytoplasm. Coronal sections were cut posterior to Bregma fortune and were placed on albumin-coated slides. After clarification and filtration, the slides were stained using 1% crystal violet (Merck, Germany). Then slides were examined by microscope with a magnification of 400×. The cells with round and bright nuclei, with euchromatin appearance and containing a few nucleoli were considered as viable neurons, and cells whose nuclei were dense, multifaceted, and heterochromatin were considered as dead cells.

TUNEL Staining

Apoptosis was calculated by TUNEL assay. The TUNEL method, which detects the disintegration of DNA in the core during apoptotic cell death in situ, was employed using an apoptosis detection kit (Roche, In Situ Cell Death Detection Kit, Germany) as described previously [12]. Briefly, paraffin was removed by xylol, and 3µm thick tissue sections were digested with Proteinase K for 60 minutes. Then, samples were washed three times with anti-fluorescin-pod, each time for 30 seconds, as well as washed three times with fetal bovine serum (Thermo Fisher Co, USA), and kept at room temperature for 10 minutes using 50 µl of 3% H_2O_2-methanol solution to prevent endogenous peroxidase. After that, samples were placed in the ice bath (11% Triton-100) for five minutes and were incubated with TUNEL liquid (50 µl) for 60 minutes at 37 °C. Finally, samples were washed three times with phosphate-buffered saline (Bio Idea.Co, Iran) for five minutes.

RNA Extraction and cDNA Synthesis

RNA extraction kit (Denazist, Iran) was used to extract RNA from hippocampal tissue cells. The manufacturer's instructions were applied for RNA extraction. Agarose gel (Sigma Aldrich, USA) and nanodrop (Eppendorf Co., Germany) were used to determine the quality and quantity of extracted RNA, respectively.

The cDNA synthesis was performed using the cDNA Synthesis Kit (Easy cDNA Synthesis Kit, Denazist, Iran) and based on the manufacturer's instructions. Quantitative measurement of DNA was performed with nanodrop.

Primers

The primers for *Bax*, *Bcl-2*, *Bcl-x*, and *Bad* genes were designed using the Gene runner, Allel ID, and Primer express software V.3.0 (Applied Biosystems, USA). The characteristics of primers are given in Table-1.

Real-Time Polymerase Chain Reaction (PCR)

Real-time PCR ABI-7300 (Applied Science, Manheim, Germany) was done using a master mix and specific gene primers. Required components for real-time PCR are listed in Table-2.

The real-time PCR timing and temperature program started at 95 °C for 30 seconds for cDNA denaturation. In the next step, 40 cycles of 95 °C for 5 seconds and 60 °C for 31 minutes were performed. In the final step, the temperature cycle of 95 °C for 15 seconds, 60 °C for 30 seconds, and 95 °C for 15 seconds were used.

Statistical Analysis

All statistical analyses were performed using the SPSS statistical software package version 19 (IBM Corporation, Armonk, NY, USA). The results were shown as mean±standard deviation. To compare the significant

Table 1. The Sequence of Designed Primers

Genes	Sequence (3'-5')	Tm (°C)	Length (bp)	GC (%)
Bax	F: AGGGTGGCTGGGAAGGC	56.8	17	70.6
	R: TGAGCGAGGCGGTGAGG	57.7	17	70.6
Bcl-x	F: GCTGGTGGTTGACTTTCTCTCC	56.4	22	54.6
	R: GGCTTCAGTCCTGTTCTCTTCG	56.7	22	54.6
Bad	F: GGAGCATCGTTCAGCAGCAG	58	20	60
	R: CCATCCCTTCATCTTCCTCAGTC	57.6	23	52.2
Bcl-2	F: ATCGCTCTGTGGATGACTGAGTAC	56.1	24	50
	R: AGAGACAGCCAGGAGAAATCAAAC	56.7	24	45.8
GAPDH	F: AAGTTCAACGGCACAGTCAAGG	57.8	22	50
	R: CATACTCAGCACCAGCATCACC	56.7	22	54.6

Tm: Melting temperature

Table 2. Required Components for Real Time PCR

Materials	Concentration
SYBR premix EX Taq™ 11(2X)	25
PCR Forward primer (10 μM)	2
PCR Reverse primer (10 μM)	2
Rox Reference Dye or Dye 11 (50 X)	1
RT reaction solution (cDNA solution)	4
dH_2o (Sterilized distilled water)	16
Total	50

PCR: Polymerase chain reaction; **RT**: Reverse transcriptase

differences among the groups, one-way analysis of variance (ANOVA) and Tukey Post hoc tests were used. The probability level for the significant differences among the groups was considered $P<0.05$.

Results

Histopathological Findings

Examination of Nissl-stained control specimens showed that the neurons in the hippocampus were evenly and regular and that thin, rarely dark-colored cells with compact, dark nuclei were seen in the cells. The hippocampal tissue is uniform, and blood vessels are rarely seen around the tissue. In control samples stained with Nissl, it was observed that the neurons in the CA1 region of the hippocampus were uniformly arranged, and there were rarely dark cells with compact and dark nuclei indicating pyramidal neurons that go through their stages of degeneration. The observations in the vehicle group (normal saline) were similar to those in the control group (Figure-1).

In the experimental group, degenerated pyramidal cells with pyknotic nuclei in the CA1 region of the hippocampus were significantly higher than the control and vehicle groups (Figure-2).

TUNEL staining showed that apoptotic bodies were rarely seen in the control and vehicle groups, but these bodies were significantly higher in the experimental group compared with the other two groups (Figure-3).

Based on data obtained from counting the number of apoptotic bodies in TUNEL staining based on DNA lesions induced by apoptosis, it was observed that the number of apoptotic bodies in the hippocampal CA1

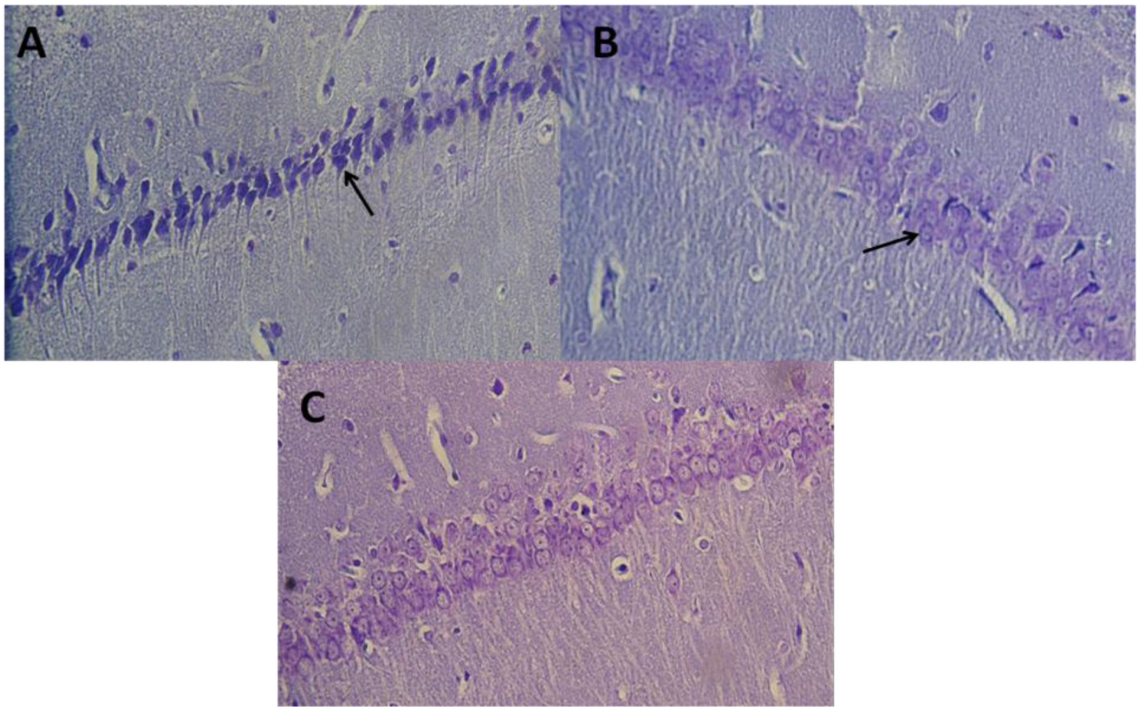

Figure 1. Nissl staining of hippocampal CA1 pyramidal cells neonates of rats. In the experimental group (**A**), degenerated cells with dark and pyknotic nuclei (arrow) were observed. In the control group (**B**), viable neurons (arrow) that are aligned regularly are similar to the vehicle group (**C**). Original magnification

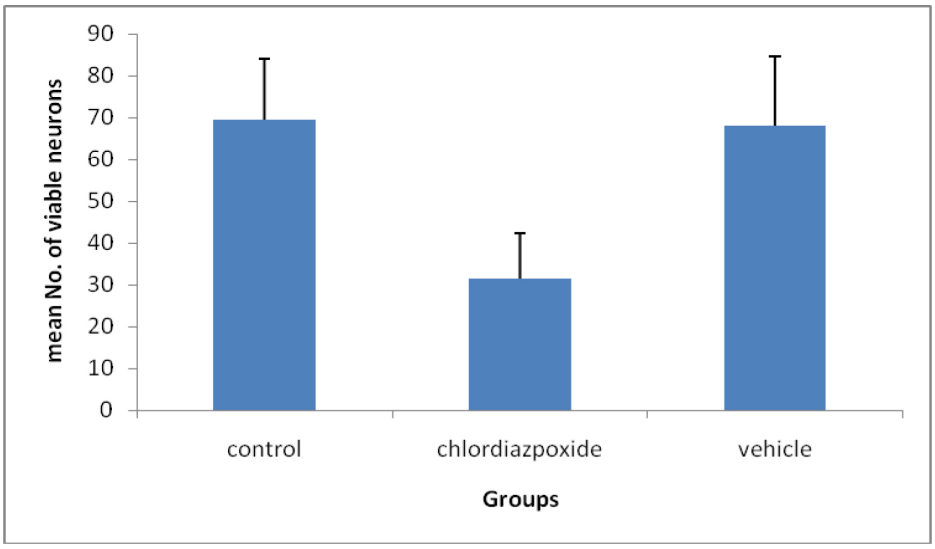

Figure 2. Comparison of viable pyramidal cells in the CA1 region of the hippocampus between groups. *P<0.05 vs. control

area of rat pups whom their mothers were given chlordiazepoxide during pregnancy was significantly different from the control group (P=0.045). Also, this difference was significant between the experimental and the vehicle groups (P=0.001), while this difference was not significant between the control and vehicle groups (P=0.968). Evaluation of these data showed that chlordiazepoxide administration during pregnancy increased the number of apoptotic bodies in the CA1 region of the neonatal hippocampus.

Genes Expressions

The electrophoresis of PCR products for each gene was done, and the results confirmed the

specific replication of the genes (Figure-4). Our findings showed that taking chlordiazepoxide during pregnancy increases the expression of the *Bax* gene. Significant differences in the expression of *Bax* were observed between the experimental group compared to vehicle and control groups ($P<0.05$). However, there was no significant difference in the expression of *Bax* between the vehicle and control groups. On the other hand, the results showed no significant difference between the groups in terms of the expression of *Bad* gene.

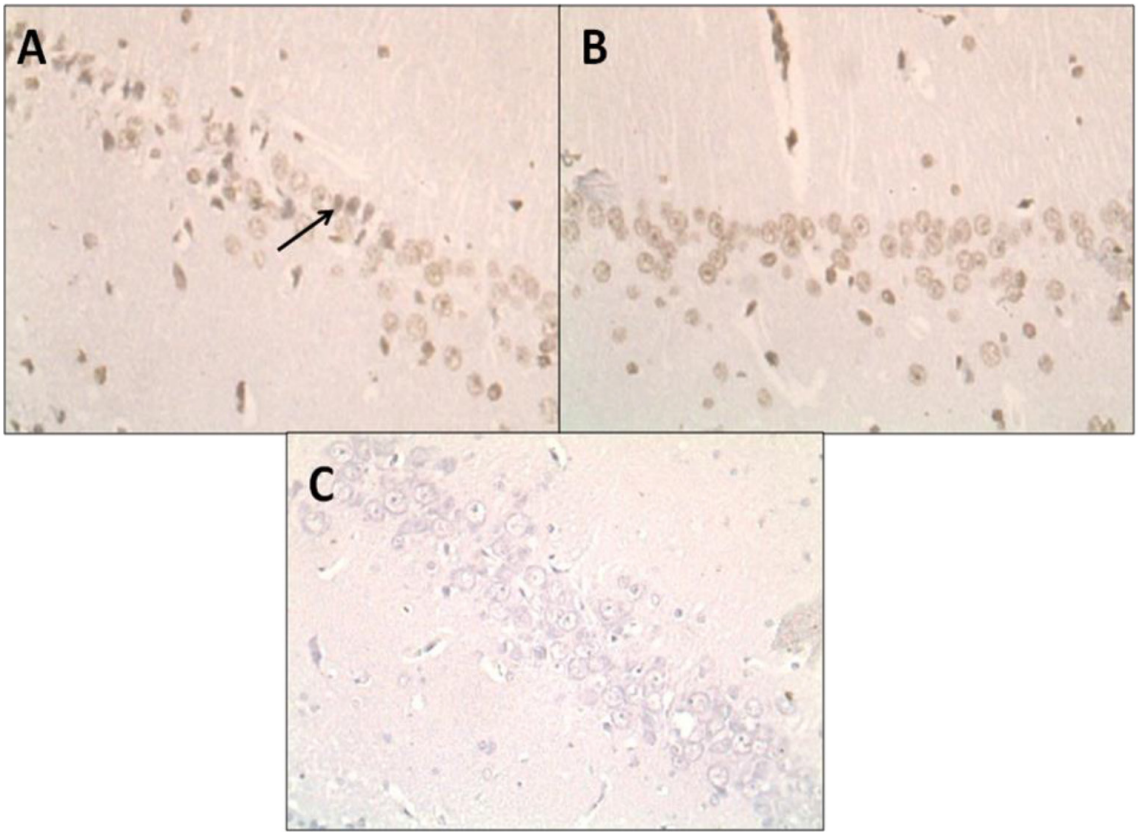

Figure 3. TUNEL staining of hippocampal CA1 pyramidal cells revealed numerous apoptotic bodies (arrow) in the experimental group (**A**). However, the control group (**B**) has viable neurons similar to the vehicle group (**C**).

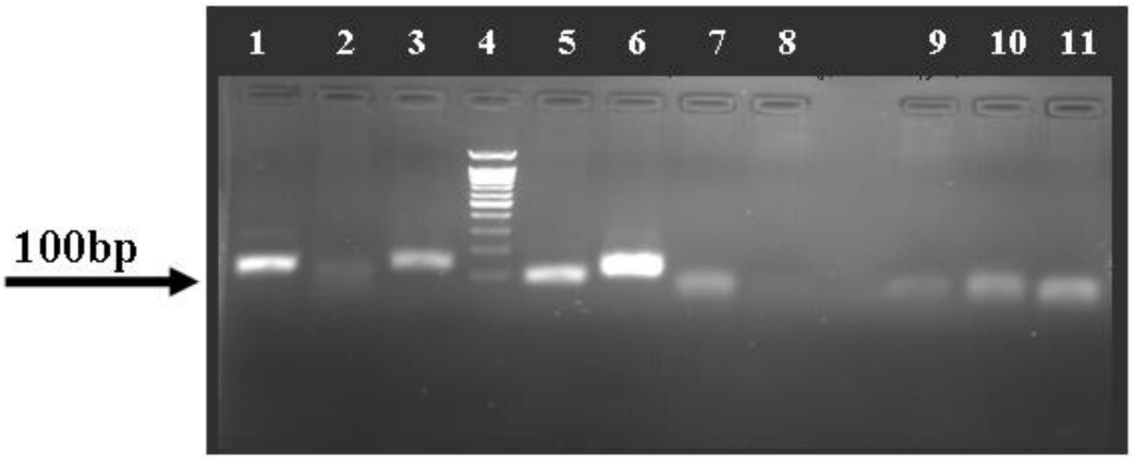

Figure 4. Real-Time PCR product electrophoresis from normal samples on 1.5% agarose gel. 1: Bad, 2:Bax, 3:Bcl-2, 4: Size marker (100bp), 5:Bcl-x, 6:GAPDH, 7: NTC_{bad}, 8: NTC_{bax}, 9:NTC_{Bcl2}, 10: NTC_{Bclx}, and 11: NTC_{GAPDH}

However, in terms of *Bcl-2* and *Bclx* gene expression, there were significant differences in the rats' group that received chlordiazepoxide compared to the control and vehicle groups, and the reduction of expression of this gene in the group that received chlordiazepoxide was confirmed. These results indicate an increase in the expression of pro-apoptotic genes and a decrease in the expression of anti-apoptosis genes due to the use of chlordiazepoxide during pregnancy in the hippocampus of neonatal rats (Table-3).

Discussion

In the present study, 10 mg/kg daily of chlordiazepoxide was given to rats throughout their pregnancies, and the findings showed a sharp decrease in the number of pyramidal cells in the hippocampus of these infants relative to the control and vehicle groups. As a result, taking this drug during pregnancy can cause neurological damage in infants.

According to the American College of Obstetricians and Gynecologists guidelines, there is a possibility of congenital malformations with the use of chlordiazepoxide, and because the use of this drug is rarely emergency and vital, it should almost not be administered during pregnancy [13].

Chlordiazepoxide is structurally very similar to diazepam, and according to diazepam and oxazepam studies, it causes anomalies in mice [14]. N-Desmethyldiazepam is a metabolite of chlordiazepoxide and diazepam that could be detected in the fetus from the mother until delivery [14]. One common response to stress is the release of ACTH from the pituitary gland by acting on the hypothalamus. ACTH releases corticosteroids from the adrenal glands [15]. The pituitary-hypothalamic-adrenal axis is one of the axes that seems to be affected by BZDs during the developmental stage of the nervous system [15].

According to neurochemical and behavioral observations, the most sensitive time for long-term effects of the BZDs on the brain of newborn rats is in the last week of pregnancy [16]. The use of post-natal BZDs in mice has had a different effect on drug use during pre-natal periods, and according to studies in humans, the second trimester of pregnancy is the most sensitive period for the long-term effects of BZDs [17].

Cell death contributes to many neurodegenerative diseases due to its importance in developing the nervous system. Many neurological diseases have been caused by the gradual decline of a specific group of neurons and disorders in the movement and function of the central nervous system, such as Parkinson's and Alzheimer's [8]. Many factors, such as oxidative stress, improper homeostasis of Ca^{+2}, mitochondrial dysfunction, and the lack or deficiency of interactions between neurotrophic factors and their target tissues, or a combination of these factors, can contribute to these diseases [8]. Apoptosis is a physiological cell death that naturally causes the removal of old, damaged, extra, and harmful cells and is essential for the development of tissue homeostasis. Apoptosis is involved in tissue repair and regeneration of the self-reactive T cells [18]. Any disruption in the process of apoptosis could lead to a disease that may be due to reduced cell death as well as the growth and development of cancer cells or autoimmune disorders [18].

Conversely, an abnormal increase in cell death is also seen in diseases such as

Table 3. The Expression of *Bax*, *Bcl-2*, *Bad*, and *Bcl-x* in Neonatal Rats

Genes	Groups			P-value
	Control	Vehicle	Chlordiazepoxide	
Bcl-x	1	0.84±0.2	0.49±0.06	<0.05
Bcl-2	1	0.78±0.22	0.49±0.3	<0.05
Bad	1	1.2±0.44	1.24±0.56	<0.05
Bax	1	1.12±0.77	4.14±1.89	<0.05

neurodegenerative disorders and acquired immunodeficiency syndrome [19]. Apoptosis has two main extrinsic and intrinsic pathways. The intrinsic or mitochondrial pathways are involved in both cell' life and death. In response to cell death signals, the permeability of mitochondrial outer membranes causes different expressions of *Bcl-2* family proteins and release of pro-apoptotic molecules such as apoptotic inductive factor, cytotropic C, Smac/DIABHT, cytochrome C, and endonuclease G from the space between the two mitochondrial membranes to the cytoplasm [20].

BZDs, including chlordiazepoxide, bind to the molecular components of the GABAA receptor present in the neuronal membranes of the central nervous system, and this receptor acts as an ion channel and is activated by the GABA neurotransmitter [21]. According to electrophysiological studies, BZDs enhance inhibition by GABA at all levels of the neural axis, including the spinal cord, hypothalamus, hippocampus, black body, cerebral cortex, and cerebral cortex [22]. Studies have shown that activation of the GABA receptor plays a role in the development of apoptosis [3]. Thus, it can be concluded that the activation of GABA by BZDs causes neuronal damage by apoptosis in neural cells of the neonatal brain [23]. Studies showed that BZDs use in infants causes myoclonus, epilepsy, and abnormal movements [2]. Hartz *et al*. revealed that from 50282 pregnant mothers, 257 women intake chlordiazepoxide during the first four months of pregnancy and 483 mothers in the fifth and subsequent months of pregnancy [24]. The results of this study showed that the risk of malformation, stillbirth, and death were not increased in infants up to four years of age, and there were no brain injuries according to the scores from the motor status (at eight months) and intelligence quotient tests (at four years) [24]. However, in our study, the negative effect of chlordiazepoxide on the brain of neonatal rats was observed.

The *Bcl-2* protein family is an important regulator in the apoptotic process, which itself consists of two groups of anti- and pro-apoptotic proteins. Among the members of this family, *Bcl-2* and *Bcl-x* are anti-apoptotic, and *Bad* is pro-apoptotic, which plays an important role in the apoptosis process [25].

Conclusion

The results of the current study showed that daily injection of chlordiazepoxide at the dose of 10 mg/kg during pregnancy by increasing the expression of *Bax* and *Bad* genes and reducing the expression of *Bcl-2* and *Bcl-x* genes could cause neuronal damage in the neonatal hippocampus that plays an important role in cognitive and behavioral programming.

Acknowledgments

All the colleagues of the Faculty of Medical Sciences of Tehran Azad University are sincerely thanked for their cooperation in this project.

Conflict of Interest

The authors declare no conflict of interest in this study.

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