Protective effect of aqueous jujube extract in Carbamazepine induced teratogenicity on Balb/c mice fetuses

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ABSTRACT

Aim: Carbamazepine (CBZ) is an anticonvulsant medication that can produce congenital anomalies. This study aimed to assess protective role of aqueous jujube extract (JE) on CBZ induced congenital anomalies in mice fetuses.

Methods: One hundred pregnant Balb/c mice were divided into 8 experimental (E) and 2 control (C) groups equally. The groups (E1, E5, E6) and (E2, E7, E8) received 50 and 100 mg/kg of CBZ, respectively IP, from GD 0 to GD15. Besides, groups (E5, E7) and (E6, E8) in addition to CBZ, were treated with 200 and 400 mg/kg JE, respectively from ten days prior to gestation, till GD15. The groups E3 and E4 received only 200 and 400 mg/kg of JE respectively. The control groups (C1, C2) received normal saline and tween-20 in turn. On GD18 dams cesarianed and their fetuses assessed for skeletal anomalies by using Alizarin red-alcian blue staining.

Results: CBZ induced various anomalies such as; limb defects, craniofacial malformations and etc in mice fetuses. However, these anomalies significantly decreased in groups which were co-administered with CBZ and JE.

Conclusion: Co-administration of JE and CBZ significantly decrease teratogenicity of CBZ. Therefore, JE may play a protective role against those properties of CBZ inducing teratogenicity.

Keywords: Carbamazepine, teratogenicity, malformation, jujube, protective

INTRODUCTION:

Epilepsy is a common disease that involves 0.5 to 1 percent of the community. In the community, there are women of childbearing age and almost one out of every 25 people is exposed to antiepileptic drugs (AEDs) [1]. Phenyoitoin, phenoobarbitone and CBZ are the first line drugs used for the management of epilepsy in clinics [2-3]. CBZ is one of the most widely-used antiepileptic drugs in clinics. It is highly effective for the treatment of partial onset and generalized
tonic-clonic seizures. It has also demonstrated good efficiency in the treatment of trigeminal and glossopharyngeal neuralgia and pains associated with neurological diseases like; multiple sclerosis and psychiatric disorders including; bipolar depression and even schizophrenia [4-6].

Chemically, CBZ is a neutral, lipid soluble compound that can easily cross the blood-brain barrier and other membranes in the body [7-8]. Comparative clinical trials have shown that although the effect of CBZ is equal to the other antiepileptic drugs such as; phenobarbital, phenytoin, pirimidine and valproic acid, its toxicity is less than theirs [9]. There are diverse opinions about teratogenic effects of CBZ, but majority of investigators all believe in the teratogenicity of AEDs [6, 10]. It has also been determined that consumption of AEDs like CBZ during pregnancy can produce some congenital anomalies such as; neural tube defects, cardiac, skeletal, and craniofacial abnormalities [6]. Furthermore, side effects like cognitive impairment associated with the chronic use of AEDs is another major concern [11-12]. Hence, herbal drugs with proven anticonvulsant efficacy could be used as an adjuvant to standard AEDs, which in turn could lower the side effects of AEDs [13].

Anomalies caused by CBZ can be generally divided into two groups: major abnormalities including; craniofacial defects, cardiovascular malformations and neural tube defects and minor anomalies such as; growth retardation, developmental delay, hypoplasia of the nail, or distal phalanges of the fingers [6, 14]. Antiepileptic treatment during pregnancy is associated with two to three fold increases in the rate of major congenital anomalies [7, 9].

Jujube, with scientific name (Ziziphus Jujube) (ZJ) is a thorny shrub with the average height of 8 meters. This plant is a native of tropic regions and its fruit has got high nutritional and medical properties. Thereby, it is widely used in traditional medicine [15]. Jujube was used in the past for a range of diseases such as; chronic fatigue, lack of appetite, cholera, pharyngitis, bronchitis, anemia, irritability and hysteria [16]. Moreover, some other properties such as; being analgesic, antidote, astringent, anticancer, diuretic, emollient, expectorant and sedative have also been taken into account [17]. Besides, sedative, tranquilizing, hypnotic , and antiepileptic effects of ZJ in experimental models in rats have also been reported [13].

Furthermore, this fruit is traditionally believed to have other properties such as; anti anxiety, appetite stimulating, narcotic and antiarrhythmic with low toxicity [18]. This feature may be because of triterpens that found in the plant seeds [18]. The antidepressant property of this fruit is demonstrated in Sharmuet.al. investigation, carried out on rat animal models. These investigators believed that this effect was due to the presence of flavonoids in jujube fruits [19]. In Acharya et.al study antianalgesic, anticonvulsant and anti inflammatory effects of ethanolic extract of jujube were presented [20]. Furthermore, it increases memory and learning span through elevation of estrogen level in the circulation and increases of nitric oxide and acetylcholine in the brain in rats [21]. In spite of extensive investigations that have been done about jujube fruit, unfortunately, there are few reports about its protective effects on drugs-induced malformations. Therefore, due to presence of polyphenols, some minerals and vitamins with antioxidative effects in jujube fruit, and the fact that so far no studies have been carried out on the protective effects of the plant on drug-induced teratogenicity, the present study aimed at determining the preventive effects of AJE on CBZ induced anomalies during organogenesis in Balb/c mice.

Materials and Methods:

**Animals and treatment:**

Adult mice of Balb/c weighing 25±3 gram (8 weeks old) were used in this study. The animals were kept in a climate--controlled temperature 20-22 C, relative humidity 50-55 percent and light /dark cycle (12 h). Dry food pellets and tap
water were provided ad libitum. After two weeks of accommodation to the environment, two virgin females were caged with a male of the same strain overnight and the presence of vaginal plug in the following morning was considered as gestation sign. This was designated as gestational day zero (GD0). One hundred pregnant mice were randomly divided into ten equal groups (n=10).

A: Control groups 1 and 2: Injected with normal saline and a solution of tween-20 respectively.

B: Experimental groups 1 and 2 (E1, E2): Injected with CBZ 50 and 100 mg/kg respectively.

C: Experimental groups 3 and 4 (E3, E4): Gavaged with AJE 200 and 400 mg/kg, respectively.

D: Experimental groups 5 and 6 (E5, E6): Injected with 50mg/kg CBZ and gavaged with 200 and 400 mg/kg JE, respectively.

E: Experimental groups 7 and 8 (E7, E8): Injected with 100 mg/kg CBZ and gavaged with 200 and 400 mg/kg JE, respectively.

The female mice were intraperitoneally injected with CBZ from GD0 to GD15. The pregnant mice were gavaged with 200 and 400 mg/kg of extract 10 day before mating that continued after pregnancy till GD15. On GD18, the pregnant mice were deeply anesthetized with ether and then cesareaned. Their uteruses were opened and the umbilical cords were cut close to the fetuses. The fetuses were assessed as either living or dead (to be done without). Then, the living fetuses were euthanized by means of hypothermia. Thereafter, each fetus was externally examined using research stereo-microscope (Olympus ZH Japan) to detect gross malformations. Finally, the fetuses with skeletal malformations were chosen and stained by alizarin red-s- alcian blue double staining according to a slightly modified Kimmel & Trammel technique [22].

**Drug and plant material:**

CBZ powder had been purchased from Mehrdarou, Pharmaceutical Company in Tehran, Iran and was dissolved in 0.1% Tween-20 (Merk Germany) in normal saline. Jujube fruit had been provided from Birjand local market, while its variety had been determined by a botanologist. The fruit was then washed and dried in shadow. Thereafter, they were powdered by means of a grinder. The obtained powder was dissolved in water and boiled for half of an hour. After cooling the mixture was centrifuged for 20 minutes at around 400 beats per minute. Then, its supernatant was collected and turned into powder under low temperature and pressure. The powder was stored at -70 C. The obtained powder was dissolved in normal saline and 200 and 400 mg/kg of the obtained solution were used for gavaging.

The ethical standard of the present study was approved by the Animal Care Committee of Birjand University of Medical Sciences, Iran.

**Statistics:**

The gathered data was analyzed by means of SPSS software (version 18), using Anova, X2 and Tukey tests. The unit for frequency analysis was fetus. Tukey test was applied after Anova between the control groups and each treated group. In regard to the frequency of the absorbed fetuses, external anomalies differences between the control groups and each treated group were tested with the X2 test when the frequency of each category was 5 or more, and with Fishers direct probability test in other cases. The differences were considered significant at P<0.05.

**Results:**

**Review of fetal absorption in the studied groups:**

Findings upon cesarean section on GD18 are shown in table 1. Fetal resorption in experimental groups E1 (4.71%) and E2 (6.86%) were significant statically when compared with the control groups (C1, C2) which had no fetal resorption (p<0.05). Furthermore, both experimental groups E5 (3.66) and E6 (0.9) had some decrease in resorption in comparison with E1 (4.71) group, but this difference was significant only between E6 and E1 groups. Fetal resorption in both groups (E7 and E8) were
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significantly decreased when compared with the E2 group (Table1).

**Fetuses with ocular anomalies:**
Premature opening of one or both eyelids with mild to severe exophthalmia occurred in 3.92% and 8.42% of fetuses in experimental groups E1 and E2 respectively. These anomalies were often bilateral and approximately symmetrical. The percentage of this malformation decreased in experimental group E5 (1.9%) in comparison with E1 group (3.92 %) and this difference was not meaningful. But difference between E6 group (0.0 %) and E1 group (3.92%) was significant (p <0.05). The incidence of ocular anomalies reduced in the E7 (2.60 %) and E8 (0.0) groups as compared with E2 group (8.425), (p<0.05). The ocular abnormality was not observed in E3 and E4 groups who had received only JE at doses 200 and 400 mg/kg/respectively.

![Fig.1: photograph of mouse fetus with ocular defect (Open eyes) observed in E1 group.](image1)

**Fetuses with limb anomalies:**
Limb abnormalities were the other prevalent anomalies that observed in this study more frequently in the forms of micromelia and malrotation in both E1 (14.70%) and E2 (18.94%) groups and their differences were significant when compared with the control groups (p<0.05). The percentage of limb anomalies in E5 (6.66%) and E6 (4.54%) groups decreased significantly as compared with the experimental group E1 (p<0.05). Furthermore, the percentage of limb abnormalities in the E7 (7.82%) and E8 (5.12%) groups decreased significantly as compared with the experimental group E2 (p<0.05). the Experimental groups E3 and E4 which only received JE didn’t show limb anomalies (table1).

![Fig.2: photograph of mouse fetus with lower limb deformity (malrotation) observed in E1 group](image2)
Fetuses with vertebral column anomalies:
Vertebral column anomalies (more frequently observed in the form of scoliosis) occurred in 4.90% and 8.42% in the E1 and E2 groups respectively and their differences in comparison with the control groups (C1 and C2) were significant statically (p<0.05). The incidence of this anomaly was 3.8% and 3.66% in E5 and E6 groups respectively and they didn’t show significant differences when compared with E1 (4.9%) group. The comparison between incidence of this anomaly in the E7 (5.26%) and E8 (2.26%) groups wasn’t also significant when compared with E2 (8.42%) group.

Fig3: photograph of mouse fetus with vertebral column deformity (scoliosis) stained with double staining (Alizarin red -S and alcian blue) observed in E1 group

Fetuses with craniofacial anomalies:
These anomalies occurred more frequently in the forms of brachygnathia and calvaria in the experimental groups studied. The rate of this anomaly was 9.81% and 11.57% in the E1 and E2 groups respectively and it significantly increased as compared with the control groups (C1 and C2). Comparison between the percent of this anomaly in the E5 (8.56%) and E1 (9.8%) groups showed no significant difference, but that of the E6 (1.81%) significantly decreased as compared with the E1 group (p<0.05). Comparison between the rate of the E8 (1.70%) and the E2 groups didn’t reveal a significant difference, but the percentage of the E8 (1.70%) and the E2 was significantly different (p<0.05). These anomalies were not observed in the E3 and E4 which received only JE.
Fig4: photograph of mouse fetus with craniofacial deformity (Mandibular hypoplasia) stained with double staining (Alizarin red-S and alcian blue) observed in E1 group.

Table1. **External malformations in BALB/c mice treated with carbamazepine** (CBZ) and +jujube

Control groups 1 and 2: Injected with normal saline and a solution of tween-20 respectively.

Experimental groups 1 and 2 (E1, E2): Injected with CBZ 50 and 100 mg/kg respectively.

Experimental groups 3 and 4 (E3, E4): Gavaged with jujube extract 200 and 400 mg/kg, respectively.

Experimental groups 5 and 6 (E5, E6): Injected with 50 mg/kg CBZ and gavaged with 200 and 400 mg/kg jujube extract, respectively.

<table>
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<th>Parameters</th>
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<th>C1</th>
<th>C2</th>
<th>E1</th>
<th>E2</th>
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<td>10</td>
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<td>10</td>
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<tr>
<td>All fetuses N (%)</td>
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<td>121</td>
<td>106</td>
<td>102</td>
<td>114</td>
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<td>111</td>
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<td>117</td>
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<td>Absorbed fetuses N (%)</td>
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<td>(0)</td>
<td>(0.82)</td>
<td>(4.71)</td>
<td>(6.86)</td>
<td>(0.78)</td>
<td>(0.9)</td>
<td>(2.63)</td>
<td>(0.81)</td>
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<td>18*</td>
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<td>0</td>
<td>7*</td>
<td>18*</td>
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<tr>
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<td>8*</td>
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<tr>
<td>Craniofacial defects (%)</td>
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<td>11*</td>
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<td>0</td>
<td>2</td>
<td>2*</td>
<td>9</td>
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</table>

Experimental groups 3 and 4 (E3, E4): Gavaged with jujube extract 200 and 400 mg/kg, respectively.

Experimental groups 5 and 6 (E5, E6): Injected with 50 mg/kg CBZ and gavaged with 200 and 400 mg/kg jujube extract, respectively.
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Experimental groups 7 and 8 (E7, E8): Injected with 100 mg/kg CBZ and gavaged with 200 and 400 mg/kg jujube extract, respectively.
Groups E1 and E2 were compared with the control group.
Groups E5 and E6 were compared with the E1.
Groups E7 and E8 were compared with the E2.

*P < 0.05

DISCUSSION:
The findings of the present study showed that the use of CBZ during pregnancy at doses of 50 and 100 mg/kg caused fetal abnormalities such as; increasing fetal absorption, ocular defects, limb anomalies defectsof the vertebral column and craniofacial ones. Many studies regarding the use of CBZ that created anomalies have previously been done [23]. In a meta analysis study by Perez et al. on 1252 infants exposed to CBZ, it was discovered that the rate of congenital anomalies increased in the 2 to 3 fold in them in comparison with the control group [10]. In a research conducted by Vorhees et al., cases of fetal absorption were reported in rats exposed to CBZ [24], which is consistent with our finding. According to the present study the experimental groups which received CBZ experienced ocular defects such as; open eyes and exophthalmia which were significant when compared with the control groups (fige1), but these anomalies significantly decreased at dose dependent manner in groups that received CBZ and JE simultaneously. Sutcliff et al., observed four cases of congenital ocular anomalies in children whose mothers were treated with CBZ as monotherapy, at least during the first two months of pregnancy [25]. Ocular anomalylike exophthalmia were also reported in mice fetuses whose mothers consumed CBZ in Afshar et al., which is also consistent with our findings [23]. Fritz et al.[9], Jones et al. [26] and Orney and Chohen researches [27] on human samples, and studies of Azarbayjani and Danielsson[28] on mice have shown digital hypoplasia in infants whose mothers received CBZ during pregnancy. In the present study disturbances in the limbs development, which mostly presented as short fingers, was observed (fig2) in the experimental groups receiving only CBZ too. The current study demonstrated that these anomalies significantly reduced in dose dependent manner in the experimental groups which synchronously received CBZ and JE. On the basis of our study the experimental groups receiving the drug experienced some defects of their vertebral columns, such as; scoliosis (fig3). Moreover, the research conducted by Jone et al. [26], Azarbayjani and Danielsson[28], Artama et al. [29] and the epidemiological study of Holmes and colleagues [30], have all approved the occurrence of vertebral column defects, such as; scoliosis, spina bifida, kyphosis and lordosis following consumption of CBZ, which is consistent with our findings. Furthermore, these anomalies decreased in the experimental groups that simultaneously received CBZ and JE, but their differences were not significant when compared with the groups that only received CBZ.

In 1989, for the first time, facial dysmorphism was introduced by Jones et al. regarding the infants who were exposed to CBZ in uterine [26]. Subsequently, a collection of craniofacial anomalies was introduced as CBZ fetal syndrome [31]. Ornoy and Cohen also reported facial anomalies in infants whose mother were taking CBZ during pregnancy [27]. Besides, Moor et al., in a clinical investigation observed cases of minor anomalies including; epicantic fold, upward rimapalpebrae, short nose etc, following the administration of CBZ [32].The relationship between the use of CBZ and jaw abnormalities, most of which in humans and animal experimental models, was cleft palate have been reported so far [9]. Fitz and colleagues showed these defects in mice fetuses exposed to CBZ [9]. Azarbayjani and Danielson reported similar findings, too [28]. Matalon et al., [33], Wide et
Introducing a management of CBZ [34].
In this research, similar to what was mentioned, cases of craniofacial abnormalities such as; calvaria deformity and mandibular hypoplasia, were observed in the experimental groups, treated with CBZ, whose differences with the control groups were significant. But these anomalies significantly decreased in dose dependent manner in the groups that had simultaneously received CBZ drug and JE, as compared with the groups that received the only CBZ. Our searching in the electronic resources about protective effects of JE on CBZ or other drugs inducing various abnormalities did not yield anything to be compared with our findings. Therefore, our study is the first and a unique one. Regarding the possible mechanism of teratogenic decisive effects of CBZ on the occurrence of congenital anomalies and the protective role of the JE in reducing them, the following hypothesis can be proposed:

Various studies show that CBZ causes teratogenic effects on fetuses through three possible mechanisms:
1-Increasing of oxidative stress and reactive oxygen species (ROS).
2-Acting as folate (salt of folic acid) antagonist
3-Increasing of homocysteine level in the serum

CBZ increases oxidative activity and causes loss of antioxidant defense system and the production of free radicals in the body. Free radicals within cells and tissues can cause irreversible oxidation of DNA, proteins and lipids. As a result, many enzymes become inactive and this leads to cells death. For example, it is proved that lack of both catalase and superoxide dismutase enzymes, which have the effect of destroying free radicals, can damage the cells of neural crest. These cells play an essential role in the formation of craniofacial skeleton. Therefore, damage of these cells cause skeletal defects of the head and the face [35]. In addition to macromolecular damage, oxidative stress can also affect the regulation of gene expression [36]. Due to weak antioxidant defense system of a developing fetus, especially during organogenesis period, its life is threatened by free radicals [37].

Antiepileptic drugs such as; CBZ and valproic acid act as folate antagonist. They accelerate the degradation and disturbance of folate absorption [38]. According to the role of folate in DNA synthesis, especially during embryonic period, with very fast cell proliferation, request for this substance increases. The presence of folate antagonists such as; CBZ and complication of folate deficiency occurs in the forms of fetal anomalies such as; neural tube defect, skeletal abnormalities in the face and the palate and also in the limbs [39-41].

Investigations have shown that the level of homocysteine amino- acid increases in people with CBZ administration, and hyperhomocysteinemia leads to reduced level of activity of antioxidants such as; E, B and A vitamins in tissues, thereby, free radicals increase. Increasing of produced free radicals lead to disturbance in the normal functioning of tissues and occurrence of congenital malformations [42].

Existence of several phenolic compounds in jujube fruit, bring about its antioxidant properties. Due to containing triterpens, flavonoid, abundant C vitamin and the others vitamins such as; B group and A, jujube fruit is considered as a valuable source of nutrition. Some mineral elements including calcium, potassium, bromine and rubidium also exist in the jujube fruit [43]. Ascorbic acid or vitamin C is the major water soluble antioxidant and its regenerative power isused for rehabilitation manifested in radical / non-radical reactions [44].

The revival of tocopherol radical caused by ascorbic acid is alpha-tocopherol. This reaction is very essential in preventing the progress of lipid peroxidation in plasma membranes, lipoprotein etc. Some studies have also shown that ascorbic acid can directly and significantly neutralize different radicals and active oxygen species such as; superoxide, hydroxyl, peroxyl, hydrogenperoxide, hypochlorite or oxygen [44].
CONCLUSION:
According to our findings, it seems that JE can probably inhibit CBZ teratogenicity on fetuses to a large extent. It is unknown how much other probable teratogenic effects of CBZ can be inhibited by JE. Furthermore, the exact mechanism or mechanisms involved in this phenomenon is/are not yet clear, which requires further investigations.

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Conflict of interest
The authors have declared that there is no conflict of interests.

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