Research Article

Evaluation of Teratogenic Potential of Cyclobenzaprine in Pregnant Albino Rats and their Fetuses

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ABSTRACT

Muscle spasm is a painful involuntary contraction of muscles usually affecting leg muscles. They are among the common symptoms during pregnancy during the third trimester occurring to almost 50% of the pregnant women; it could be increased due the changes occurring during pregnancy in neuromuscular performance, joint laxity in the final stages of pregnancy and impaired blood supply to lower body organs and also due to the pressure on blood vessels and nerves resulting from the enlarged uterus, and insufficient minerals intake. Which lead to increasing glomerular filtration and greater needs of the fetus for receiving minerals from the mother reducing her serum calcium and magnesium levels; Cyclobenzaprine is a central nervous system muscle relaxant where it is intended for short-term use. It is well absorbed from the gastrointestinal (GI) tract, it relieves muscle spasms through a central action with no direct action on the neuromuscular junction, the current study investigates the teratogenic effect of cyclobenzaprine in detail using female rats (Rattusnorvegicus) during organogenesis stage. The pregnant rats were divided into two groups each of 10 rats, they were administrated orally from the 6th to 19th days of gestation. At 20th gestation day all females were weight then sacrificed by decapitation and their uteri were exposed under dissection. The rats were subjected to morphological analysis revealed non-significant reduction in weight of treated group and revealed embryo toxicity manifested in fetal growth retardation, external malformations manifested in subcutaneous hematoma at the: (tail, face, back, abdomen and fore, hind limbs) and clubfoot, open eyelid and open mouth, Skeletal examination displayed the absence of ossification of fetal bones with presence of wavy, curved and rudimentary ribs, and biochemical studies of placenta, maternal and fetal liver revealed a significant alteration in GSH, SOD, Catalase and MDA levels.

Keywords: Teratology, Muscle spasm, leg cramps, muscle relaxants, skeletal anomalies, liver, placenta.

INTRODUCTION

Teratology is the science which investigate the congenital malformation that occur at birth, where it is manifested as structural abnormalities due to faulty development, and are among the major causes of prenatal, perinatal and infant mortality and morbidity which may result in either the death of infants within weeks of its birth or long term disabilities, The incidence of the congenital malformations amounts to 2-3%, at birth, however by the elapse of the first neonatal year the incidence rises to about 5% [1]. The causes of congenital malformations may be divided into five broad groups: (a) single gene defects (mutant genes); (b) chromosome abnormalities; (c) multifactorial disorders which are the result of interaction between genetic predisposition and presumed environ- mental factors; (d) teratogenic factors; and (e) those with unknown cause; a teratogenic agent is responsible for producing such malformations when pregnant women are exposed to it where it causes anatomical defects in an embryo that was previously differentiating normally, Teratogenicity depends upon the ability of the agent to cross the placenta, as the placenta is a complex organ that acts as the interface between the mother and fetus where teratogens of large molecules with molecular weights greater than 1,000 do not easily cross the placenta into the embryonic-fetal bloodstream to exert potential teratogenic effect, the polarity, lipid solubility and presence of specific protein are from the factors that influence the rate and extent of placental transfer of xenobiotic. Rats and mice have a hemotrichorial and discoid type of placenta, eventually most of the drug will cross the placenta and reach the fetus mostly for beneficial use where the drugs may be steadily administered to the mother in order to treat specific fetal conditions. However, the trans-placental transfer of drugs may also have pernicious effects on the fetus, including teratogenicity or impairment of fetal growth and development which mostly occur during organogenesis stage. The effect of drugs may be direct or through alterations of utero placental blood flow.

Reactive Oxygen Species (ROS) are highly reactive molecules that are generated during normal metabolic process and cause an imbalance of pro-oxidants and antioxidants in the organism can cause oxidative stress and result in different disease such as liver, heart and brain tissue damage and cell death. Antioxidants such as ascorbic acid, alpha tocopherol, Superoxide Dismutase (SOD), catalase (CAT) and peroxidases can reduce pathological conditions and oxidative stress [2]. As the

potential toxicity of free radicals, they are usually inactivated by antioxidants before they can induce damage to lipids, proteins or nucleic acids. However, when free radicals are generated in excess or when the cellular antioxidant defense system is defective, they can stimulate chain reactions by interacting with proteins, lipids and nucleic acids causing cellular dysfunction and even death**.** The exposure of xenobiotic generating ROS can lead to oxidative stress during organogenesis stage which require low level of oxygen for rapid proliferation of cells as the exposure of mild oxidative tone may lead to cellular differentiation while the high oxidative tone can result in apoptosis or necrosis which as a result induce teratogenic effect.

Muscle cramps are painful, local, tangible, and involuntary skeletal muscular contractions that usually affect leg muscles. They are among the common symptoms occurring during pregnancy, especially during the third trimester upon which they are unidirectional; Cyclobenzaprine is a central nervous system (CNS) muscle relaxant for the treatment of muscle spasm, little is known about the toxic effect of cyclobenzaprine on pregnant female rats, however animal studies indicate that cyclobenzaprine does not act at the neuromuscular junction or directly on skeletal muscle, such studies show that cyclobenzaprine acts primarily within the central nervous system at brain stem as opposed to spinal cord levels, although its action on the latter may contribute to its overall skeletal muscle relaxant activity.

MATERIALS AND METHODS

Animals

The approval for the use of animals and for the procedures required for the experiments was obtained by the Cairo University, Faculty of Science Institutional Animal Care and Use Committee (IACUC) (Egypt), the present work was performed on healthful adult male and female rats (*Rattus norvegius*) of weight about 160-190 grams were obtained from the animal house of the Faculty of Veterinary, Cairo university- Egypt. The animals were reserve in suitable cage and maintained in 12 hours' light and dark cycle in temperature & humidity controlled environment. The rats were fed with standard food pellet and water ad libitum.

Mating procedure

After one week of acclimatization two female rats were housed overnight with a mature male rat, female rats were submitted to daily vaginal smear evaluations every morning for the microscopic detection of spermatozoa at the vaginal smear, which indicated mated rats and this was considered the day zero of gestation.

Experimental procedure

Organogenesis phase was the most critical period during gestation. Female rats were administrated orally (by gavage) once daily in the morning from 5_{th} day to 19_{th} day of gestation.

Drug

Multi relax (Cyclobenzaprine HCl 5 mg) was purchased from Apex pharma, the dose was 2.1 mg/kg, modified to suit the weight of rats.

Experimental Design

The pregnant rats were divided into two groups each of 10 rats. Rats were given cyclobenzaprine Hcl orally from the 6th to 19th days of gestation.

Group 1: Control – The pregnant rats received an equivalent volume of distilled water.

Group 2: The pregnant rats received cyclobenzaprine Hcl during gestational period (from gestation day 6 to 19).

Maternal-Fetal Investigation

At 20th gestation day all females were weight then sacrificed by decapitation and their uteri were exposed under dissection. The uteri horns were weight and opened the position and number of viable, resorbed, or dead fetuses were recorded. The placentas were checked carefully and their weights were recorded, where the corrected mother weight was 4.85 ± 2.03, percentage of pre and post implantation loss index was also recorded (10.43 ± 12.25, 12.04 ± 4.32) respectively**.** The fetal weighed was scored and examination for any external anomalies were performed under a dissecting microscope.

Skeletal examination

At the end of caesarian procedure, the fetuses were prepared for skeletal examination , both control and treated fetuses were fixed in 95% ethyl alcohol for 10 days for dehydration, after the dehydration is completed, they were cleared in acetone solution for 7–10 days to remove fats, lately fetuses were stained with alcian blue and alizarin red S stains for 4 days at 40º C , then washed carefully in running tap water for 2 hours then transferred into an aqueous solution of 1% KOH for clearing until the complete visibility of skeleton through the surrounding tissues. Cleared fetuses were placed successively into 50%, 80% and 100% glycerin solution, for 7 days each step. The cartilage parts appear blue while the bone ones appear red. Finally, the stained skeletons were examined under dissecting microscope [3].

Biochemical studies

Autopsy samples were taken from the liver, placenta of mother rats and fetuses in different groups were stored at -40◦C for oxidative stress investigation. Piece of each tissue were weighted and homogenized in 10 mmol/L phosphate buffer saline (PBS) as 10 % (W/V) at pH 7.4. The homogenates were centrifuged and the supernatants were taken for the estimation of: Glutathione Reduced (GSH), Catalase (CAT), Lipid Peroxidation (MDA) and (SOD) by the method of [4].

Statistical analysis

The results are presented as means ± SEM for each group. Differences among groups were analyzed using the independent student T test. All statistical comparisons and tests were performed using Statistical Package for the Social Sciences (SPSS).

RESULTS

The teratogenic effect of cyclobenzaprine at the dose 2.1 mg/kg on the pregnant rats from 5th day to 19th day of gestation was estimated which recorded a significant effect on implantation site numbers, and resorption sites (Table 1), there was a non-significant reduction in the weight of treated mothers when compared to the control group. Cyclobenzaprine did not induce maternal toxicity but induced embryo toxicity which manifested in obvious fetal growth retardation which was indicated by the significant reduction of fetal body weight. There were not any dead litters recorded of neither the control nor the treated fetuses throughout the experiment, no macroscopic anomalies were observed in the control animals and it exhibited normal shape with normal length and corrected weight Fig.1. The fetuses of pregnant rats treated with 2.1 mg/kg of cyclobenzaprine showed several external malformation anomalies which manifested in subcutaneous hematoma (red patches at different parts of bodies) at the following regions: (tail, face, back, abdomen and fore, hind limbs) and clubfoot, open eyelid and open mouth as shown in Fig.2

Table 1: Showing pregnancy outcomes

Group	Control	Treated
No. of pregnant rats	10	10
No. of implantations	76	76
No. of resorptions	1	38
No. of corpus luteum	91	78
No. of viable fetuses	75	63
No. of dead fetuses	0	0
Fetal weight	2.97 ± 0.094	2.17 ± 0.104
Pre-implantation loss index %	27.75 ± 7.083	10.43 ± 12.25
Post implantation loss index %	1.25 ± 1.25	12.04 ± 4.32

Figure 1: A Photograph of untreated control fetus at 20th day of gestation showing: Normal morphology with normal weight and length

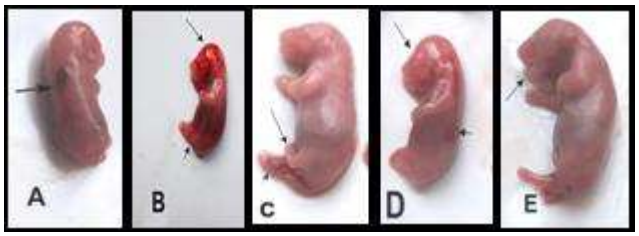

Figure 2: Showing: (A) Fetuses with back hematoma (Arrows), (B) Fetus with facial hematoma (long arrow), club foot (short arrow), and diminution in size, (C) Fetus with club foot (long arrow), hind limb hematoma (short arrow), (D) Fetus with open eyes (long arrow), back hematoma (short arrow), (E) Fetus with open mouth (Arrow)

Skeletal anomalies

The skeleton of rat fetuses consists of two main parts; the axial and the appendicular skeleton. The axial skeleton contains the bones of skull, vertebral column, ribs and sternum. The appendicular skeleton comprises the bones of pectoral, pelvic girdles and fore, hind limbs, the control fetuses showed normal ossification of the cranial bones which compromise the following bones; nasal, frontal, parietal, maxilla and mandible. The cartilage of sternum showed 6 ossified parts. Twenty-six ribs were examined in each fetus. In the thoracic region, (13 centra and 26 arches) and in the lumbar region, ossifications of six lumbar vertebrae (six centra and 12 arches) Fig.3

Fetuses maternally treated with cyclobenzaprine (2.1 mg/kg), showed incomplete ossification of cranial bones in 80.3 % nasal, 57.1 % frontal, 55.3 % parietal and 46.4 % in maxilla and mandible also showed impaired ossification of thoracic vertebrae in 50.7 % and lumbar vertebrae in 57.1 % and in 47.1 % of pectoral girdle (humerus, scapula, radius & ulna) and in 65 % at metacarpals and pelvic girdle showed incomplete ossification in 57.14 % of (femur, tibia, fibula and metatarsals), skeletal abnormalities was manifested in wavy ribs, curved ribs and rudimentary ribs as shown in Fig.4

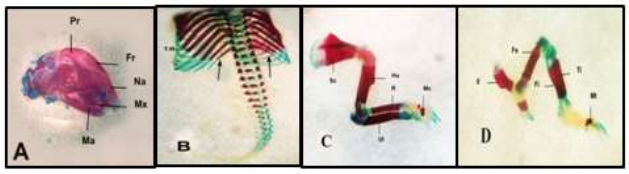

Figure 3: Photographs of the skeleton of fetus of control group showing: (A) Completely ossified Skull bones. (B) Normal ribs (Arrow) with completely ossified thoracic ribs, completely ossified thoracic vertebrae and completely ossified lumbar vertebrae. (C) Completely ossified pectoral girdle and forelimb. (D) Completely ossified pelvic girdle and hind limb

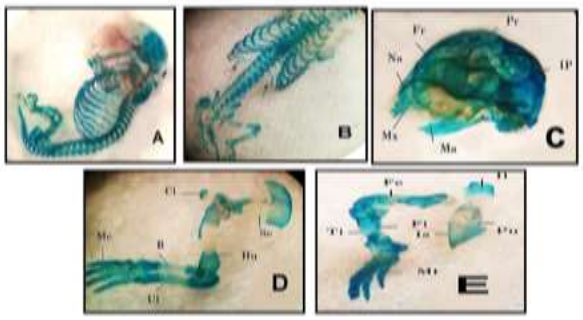

Figure 4: Photographs of the skeleton of fetus maternally treated with 2.1 mg/kg showing: (A) Completely unossified skeleton of fetus, (B) Wavy ribs (Arrows), (C) Complete unossification of skull bones, (D) Completely unossified pectoral girdle and fore limb, (E) Incomplete ossification of pectoral girdle and forelimb.

Fr= frontal, Pr= parietal, IP = Interparietal, N= nasal, Mx=maxilla, Ma= mandible. Sc= scapula, Hu=humerus, R= radius, Ul=ulna, MC= metacarpals. I= ilium, Fe= femur, Fi= fibila, Ti= tibia, MT= metatarsals.

Biochemical assays

The present study demonstrates the change of antioxidant defense system by administration of 2.1 mg/kg cyclobenzaprine to pregnant female rats, the data is presented in (Table 2) with Mean ± SEM.

GSH

There was a significant increase (P ≤ 0.05) in GSH level in group maternally treated with 2.1 mg/kg cyclobenzaprine in placenta and maternal liver (0.9313 ± 0.141) and (0.3732 ± 0.0379) respectively, while a non-significant increase in fetal liver (0.5146 ± 0.554), when compared to the control group.

Catalase

It was noted a significant decrease (P ≤ 0.05) in catalase activity levels in placenta (0.453 ± 0.0015) and fetal liver (0.0901 ± 0.0029) and significant increase (P ≤ 0.05) in maternal level (0.457 ± 0.0068) compared to the control one.

SOD

SOD showed non-significant reduction in its activity in placenta and fetal liver (14.87 ± 1.10), (36.12 ± 0.66) respectively and significant reduction in maternal liver (19.74 ± 3.55) in comparison with untreated group.

MDA

The treated group showed non-significant elevation in MDA level in placenta (13.23 ± 1.40) and fetal liver (5.35 ± 0.371) and significant elevation in maternal liver (7.96 ± 1.39) when compared to the control group.

Table 2: Showing effect of Cyclobenzaprine on placental, maternal & fetal liver tissue antioxidant (GSH, Catalase, SOD) and MDA of pregnant rats at 20th day of gestation

Tissue / Group	GSH C	GSH T	CAT C	CAT T	SOD C	SOD T	MDA C	MDA T
Placenta	0.403 ± 0.0740	0.9313 ± 0.141	0.528 ± 0.0025	0.453 ± 0.0015	42.28 ± 2.19	14.87 ± 1.10	0.321 ± 0.057	13.23 ± 1.40
Maternal liver	0.3732 ± 0.0379	0.7861 ± 0.0918	0.1573 ± 0.0173	0.457 ± 0.0068	113.05 ± 18.61	19.74 ± 3.55	1.27 ± 0.356	7.96 ± 1.39
Fetal liver	0.3861 ± 0.0865	0.5146 ± 0.554	0.1469 ± 0.012	0.0901 ± 0.0029	45.17 ± 0.82	36.12 ± 0.66	0.315 ± 0.066	5.35 ± 0.371

DISCUSSION

There are a few studies published for assessing the safety of the drug during human pregnancies thus the data from the animal teratogenicity studies are very warden. Animal experiment is the first scientific preference to determine the teratogenic effect of a substance whether it is teratogenic or not, for the estimation of potential teratogenic effect of any given drug on human health. In relation to that, this study documented the teratogenic effect of cyclobenzaprine on the rat fetuses, it has been indicated that occurrence and intensity of this effect depend on the dose, administration way of the drug, and the gestation stage. Muscles are involved in every movement you make, they get longer and shorter to move to hold the body where the human body contains approximately 600 skeletal muscles; skeletal muscle is voluntary, where it contracts at a person's volition. Though a muscle spasm is a sudden violent involuntary contraction of a muscle or group of muscles, it can also occur due to hypocalcemia (low level of calcium in blood), hypokalemia and hyperkalemia (low and high level of potassium respectively) which is very important for muscle cell functioning, when a muscle undergoes spasm it continuously contracts rather than relaxing and contraction in quick succession. Spasm can be linked to the damage that muscle spasm cause to the nerve fibers in the brain and spinal cord (central nervous system). Leg cramps are reported to occur in 5% to 30% of all pregnant women, most often during the later months of pregnancy and without relationship to other complications or to unfavorable fetal outcome [5]. Cyclobenzaprine is a central nervous system (CNS) muscle relaxant intended for short-term use, it is used to manage muscle spasms associated with acute musculoskeletal disorders, such as low back strain, muscle tenderness, or movement restriction, it does not have activity directly on muscle tissue, with animal data suggesting that this agent acts primarily in the brainstem. Similar to this study, [6] administration of

guaifenesin; an expectorant with muscle relaxant activities at the following doses 250 mg/kg, 350 mg/kg, 500 mg/kg and 600 mg/kg neither find any mortality nor any signs of intoxication in pregnant rats during the study period , in the current study, there was non-significant reduction in placental, corrected maternal body weight and uterus weight of orally administrated 2.1 mg/kg cyclobenzaprine when compared to the control group during gestation period, there were no signs of toxicity which manifest in vaginal bleeding or abortion or miscarriage related to cyclobenzaprine treatment on the pregnant rats. In addition, there has been an increase in post implantation loss index and decrease in pre implantation loss index which is a sign of fetal toxicity due to increased resorption rates that may be due to the passage of cyclobenzaprine or its metabolite throughout the placental barriers and having a toxic effect on the fetuses. Significant decrease in number of fetus/mother might be attributed to the lack of oval production or of the basic cell constituent as a result of drug administration which may be attributed to the direct toxic action of the cyclobenzaprine on the early developed fertilized ovum. Opposing to our study, Skarpa and colleagues [7] administrated non-depolarizing muscle relaxant atracium subcutaneously at daily doses 0.10 or 0.15 mg/kg (twice) to pregnant rabbits on day 6 and 18 of gestation and found no teratogenic effect of the compound while the administration of cyclobenzaprine to female pregnant rats during the period of organogenesis produced significant decrease in fetal weight which may be due to the potential effect of cyclobenzaprine on embryos and fetal tissues. Our study showed some morphological changes that manifested in subcutaneous hematoma (red patches at different parts of bodies) which may be due to the anticoagulant activity of cyclobenzaprine at the following regions: (tail, face, back, abdomen and fore, hind limbs), correlating with Shabbir [6], who also found several hemorrhagic spots at different regions, such as brain, abdominal, hind limb, and subcutaneous regions as compared with control group when administrated guafenesin at the following doses 250 mg/kg, 350 mg/kg, 500 mg/kg and 600 mg/kg. Previous studies have shown that different drugs that exhibit anti-coagulant activity also cause developmental toxicity [6], it also showed clubfoot, open eyelid, and open mouth which indicate the direct effect of multi relaxant drug on embryo development. Maintenance of skeletal integrity involves a dynamic biological equilibrium between osteoclast-induced bone resorption and osteoblast-mediated bone formation. This study exhibited increased incidence of skeletal abnormalities which summarized in retardation or absence of ossification of cranial bones at (nasal, frontal, parietal, maxilla and mandible) regions, complete unossification of sternum, vertebral column (thoracic and lumbar vertebrae) with presence of fused shape vertebrae, and lack of ossification of fore limb (pectoral girdle region), hind limb (pelvic girdle region) especially at metacarpals and metatarsals, where decreased ossification resulted from a reduction of calcium contents of embryonic bone tissues. Ossification defects may be attributed either to disruption of either the Ca_2 supply or the bone maturation itself [8], or decreased placental transport capacity [9]. The ossification process in the fetus itself was deficient, as indicated by decreased osteocalcin levels [10] Retarded both bone growth [11]. In previous study by Abdelwahab and his team revealed that oral treatment of pregnant rats with myolgin during the gestation with dose 500 mg/kg showed no cases of external malformations but caused skeletal abnormalities as un-ossified skull bones and weak ossification of phalanges [12]. In our study, the ribs appeared to exhibit incomplete and complete unossification and also exhibited gross malformations as wavy ribs (also called “knobby” or “angulated”). According to Sterz [13] they are a reversible condition which can be the combined result of decreased mineralization due to the activation of osteoclasts, and increased intrauterine pressure resulting from the stimulation of prostaglandin synthesis. And curved, rudimentary ribs were also observed. Oxidative stress is the imbalance between antioxidants and pro-oxidants in favor of antioxidants where it plays a major role in protecting against molecular oxidative damage, indeed pregnancy exposes too many complications that can be related to an alteration of oxidative stress. Oxidative stress is considered a risk factor during pregnancy [14]. GSH is the major non-enzymatic antioxidant in cells that guards against oxidative injury by reducing hydrogen peroxide (H_2O_2) and scavenging ROS and nitrogen species [15], profound GSH depletion, especially in the mitochondria, can sensitize to further stress on mitochondria resulting in oxidative tissue injury. SOD is one of the important enzymatic antioxidants in cells and is considered the first line of defense against oxygen free radicals and catalase protects the cells from the accumulation of H_2O_2 by dismu tating it to form H_2O_2 and O_2 [16]. MDA is a product of lipid peroxidation, the increase in lipid peroxidation is an indication of decline in defense mechanisms of enzymatic and non-enzymatic antioxidants [17]. The placenta is a temporary and critical organ for proper fetal development, it is the main communication between the mother and its fetus, and the placenta not only provides a link between the circulation of two distinct individuals (maternal and fetal) but also acts as a barrier to protect the fetus from xenobiotics in the maternal blood. In the placental tissue, the current results revealed increase in GSH level, non-significant elevation of MDA level, decrease in catalase and non-significant reduction in SOD level when compared with control one which induced oxidative stress damage by the administration of 2.1 mg /kg cyclobenzaprine to the pregnant rat during gestation period. In maternal liver tissue, the level of GSH is increased under the treatment with cyclobenzaprine, significant rise in hepatic CAT and significant decrease in SOD

activities. This suggests that cyclobenzaprinehas increase d the ROS generated that may elevate the oxidative dama ge to the hepatocytes and which may improve the activiti

es of the liver antioxidant enzymes. Also there is a significant increase in MDA level was also observed compared to the untreated group, which suggest enhancement of lipid peroxidation which lead to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radica ls. During organogenesis period, the embryo is more susceptible to oxidative stress with the delicate balance between oxidants and antioxidants that can be disrupted by exogenous agents that induce ROS production and lead to oxidative stress, many teratogens can modulate redox and impact on the fetus at key periods in development [18]. The current study showed non-significant elevation in GSH and MDA levels, significant reduction in CAT activities and non-significant reduction in SOD activities in fetal liver of maternally administrated 2.1 mg/kg cyclobenzaprine when compared to the control group. Previous studies by Lecutier and Taussig [19] showed that thalidomide is associated with multiple birth defects, including phocomelia, with experiments using limb bud cells removed from rat and rabbit embryos, evaluation of oxidative stress was demonstrated with various thalidomide concentrations. This appears to be regulated through redox shift resulting from depletion of GSH and increased GSSG in the nucleus, and this may imply that various transcription factors are affected by thalidomide through redox regulation [20]. Previous studies demonstrated that excessive oxidative stress during organogenesis modifies lipids, proteins and DNA leading to placental endothelial dysfunction, ischemia-reperfusion injury, interrupted nutrient (calcium) transfer to fetus, altered osteogenic gene transcription causing skeletal malformations and fetal mal-development of functional organs thus impacting the fetus development.

CONCLUSION

Cyclobenzaprine showed significant developmental toxicity and teratogenic effects on maternally administrated fetuses at selected test dose (2.1 mg/kg) so careful use is suggested during pregnancy

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