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Effect of prenatal administration of therapeutic doses of topiramate on ossification of ribs and vertebrae in rat fetuses

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Abstract

There are few studies that have addressed the effects of prenatal exposure of topiramate on ossification of the bones derived from the paraxial mesoderm. This study aimed to evaluate skeletal ossification of ribs and vertebrae in 20-day-old rat fetuses after maternal exposure to two therapeutic doses of topiramate. Three groups of Sprague–Dawley pregnant rats were used: control, topiramate 50 mg/kg/day and topiramate 100 mg/kg/day treated groups. Topiramate was administered by gavage from day 6–19 of gestation. Fetuses were collected on day 20 by caesarian section. Fetal bones were stained with alizarin red and ossification was assessed. Results showed significant delayed ossification of ribs and vertebrae in topiramate-exposed fetuses at both doses and the effects were not dose dependent. In all examined groups, there was a direct correlation between the fetal weight and the number of complete ossified vertebral centers. Also, there were significant increases in skeletal abnormalities, particularly in ribs in both treated groups when compared to the control group. In conclusion, therapeutic doses of topiramate should be taken cautiously during pregnancy as they lead to fetal growth restriction and increases abnormalities of axial skeleton in rat fetuses.

***Keywords*:** topiramate, rat fetus, ossification, axial skeleton.

Introduction

Women with epilepsy are treated with antiepileptic drugs (AED) even during pregnancy and their developing fetuses may be adversely affected. Since epilepsy *per se* has been associated with an increased rate of malformations in offspring, it is difficult to attribute if the observed increase in frequency of malformations is a result of maternal epilepsy, drug treatment or a combination of both [1]. Many of the fetal syndromes associated with different classes of AEDs show similar features, suggesting that perhaps some of the teratogenic effects observed in neonates born to women exposed to AEDs during pregnancy are a result of the maternal epilepsy rather than the AED exposure [2].

Topiramate [2,3:4,5-bis-O-(1-methylethylidene)-β-D-fructopyranose sulphamate], a weak carbonic anhydrase inhibitor, is a second generation AED that is classified as 'Pregnancy Category-D drug' in terms of teratogenic risk [3]. There are no prospective clinical trials that have evaluated topiramate safety during pregnancy. Major congenital malformations such as oral clefts, hypospadius, skeletal anomalies and others were reported in neonates exposed to topiramate during pregnancy [4–6]. Low birth weight and spontaneous abortion were also identified [7]. Topiramate is used recently to produce weight loss in obese patients [8]

Research studies in animals have suggested the teratogenic potential of topiramate [9, 10]. In a preliminary study, the teratogenic potential of topiramate in mice at a very high doses 400–800 mg/kg, given intraperitoneally, has been demonstrated [11]. Previous studies demonstrated that topiramate, administered orally at 40, 100 and 200 mg/kg doses from days 9 to 12 of gestation produced skeletal anomalies in Charles Foster rats fetuses in a dose related manner but fetal weight was not reduced [10, 12].

Ossification of the different parts of the skeleton are regulated differently. The axial skeleton is routinely examined in standard developmental toxicity bioassays and has proven to be sensitive to a wide variety of chemical agents. Dysmorphogenesis of the vertebrae and ribs are induced due to exposure to a wide range of xenobiotics such as methanol, boric acid, valproic acid and others [13–15].

In this study, we report the effect of topiramate exposure in non-epileptic pregnant rats, at doses equivalent to human doses, on ossification of axial skeleton derived from the paraxial mesoderm, vertebrae and ribs, in 20-day-old preterm rat fetuses.

Materials and Methods

Virgin Sprague–Dawley rats weighing 150–250 g were kept at the animal house in an environmentally controlled room ambient temperature (25^0C, 12-hours light/dark cycles) in spacious wire mesh cages. Two weeks later one fertile male rat was placed into each cage with two females overnight. Pregnancy was detected by the presence of spermatozoa in the vaginal smear next morning and this was considered the first day of gestation (GD1).

The pregnant rats were then randomly allocated into three groups of eight each. All groups received food and water *ad libitum*. Topiramate (Topiramax®, Janssen-Cilag, Switzerland) was dissolved in distilled water (10 mg/mL) and was administered intragastrically, from day 6 through day 19 of gestation at 9:00 a.m. (±30 minutes) to treatment groups: Group A received 50 mg/kg and Group B received 100 mg/kg topiramate daily. The control group (Group C) received corresponding volume of distilled water by intragastric route. The present study was done in healthy (non-epileptic) pregnant rats. The rats were weighed daily and the doses of topiramate were adjusted according to their bodyweight.

On day 20 of gestation, the rats were sacrificed with ether overdose. Fetuses recovered through caesarian section were removed from their membranes and were separated from their placentae. The fetal weights were recorded.

Approximately one half of the fetuses were collected randomly from each mother to be used for skeletal staining with Alizarin red [16]. The fetuses were killed with ether, then were immediately eviscerated and the skin was removed after immersing them for approximately 30 seconds in a water bath heated to 70^0C. Following this, the fetuses were placed in 95% ethanol for 2–3 days. The specimens were cleared in a solution of 1% potassium hydroxide for few days until the bones were clearly visible through the surrounding tissues. They were then transferred to a fresh solution of 1% potassium hydroxide, to which drops of Alizarin red stain were added. After staining, the specimens were transferred to solutions containing 30%, 50%, and 70% glycerin respectively and stored in 100% pure glycerin to which thymol was added to prevent fungal contamination. The other half of the fetuses were fixed in Bouin's solution to be used for a different study.

A skeletal scoring chart based on published literature [17] that indicates ossification centers expected to be present in the ribs and vertebrae on gestational day 20 rat fetus was designed. Examination of the ossification centers in each fetus (115 sites) (Table 1) was performed with the aid of a dissecting microscope and the results were recorded. Ossification was classified as either complete, delayed or absent. Abnormal ossification was also recorded.

Table 1 – ***Total number of examined sites of the ossified bony centers[a] in the control and topiramate treated groups in 20-day-old rat fetuses***

	Regions	No. of ossified bones / fetus	Total No. of ossified bones			Total[b]
			Control (45 fetuses)	*Group A (49 fetuses)*	*Group B (50 fetuses)*	
Ribs (right and left)	Ribs 1–13	26	1170	1274	1300	3744
Vertebral centra	Cervical C1–C7	7	0	0	0	7
	Thoracic T1–T13	13	585	637	650	1872
	Lumbar L1–L6	6	270	294	300	864
	Sacral S1–S4	4	180	196	200	576
	Coccyx Co1–Co2	2	90	98	100	288
Vertebral arches (right and left)	Cervical C1–C7	14	630	686	700	2016
	Thoracic T1–T13	26	1170	1274	1300	3744
	Lumbar L1–L6	12	540	588	600	1728
	Sacral S1–S4	8	360	392	400	1152
	Coccyx Co1–Co2	4	180	196	200	576
Total		122	5175	5635	5750	16567

Group A (topiramate 50 mg/kg) and Group B (topiramate 100 mg/kg); [a]Ossified centers that should appear in Sprague–Dawley rats fetus (Nash JE and Persaud TV, 1989 [17]). Note that cervical centra do not normally ossify at this stage of fetal development; [b]Total number of ossified vertebral centra that should appear in Sprague–Dawley rats fetuses in all groups.

The data were analyzed using *Chi*-square test and one-way analysis of variance (ANOVA) test. Statistical analysis was performed using the SPSS computer program (version 17).

The study was approved by the Research and Ethics Committee, College of Medicine & Medical Sciences, Arabian Gulf University, Bahrain. The universal ethical rules and regulations were strictly adhered during all the steps of animal experimentation.

Results

Maternal weight gain and fetal weight

The decrease in maternal weight gain [g] in both Group A (74.4±15.9) and Group B (79.4±9.3) when compared to the control group (80.6±19.2) was statistically not significant.

Fetal weight [g] was significantly reduced in Group A (2.06±0.20) and Group B (2.11±0.20) when compared

to the control group (2.50±0.28) (p<0.05). However, reduction in fetal weight between topiramate treated groups was not significant.

No correlation was found between the decrease in maternal weight gain and the decrease in fetal weight in all groups, however a positive direct relation was found between fetal weight and ossification of the vertebrae. The ratio between the total number of complete ossified vertebral centers (centra and arches) and the fetal weight was found to be relatively constant in all groups (mean 30.7) and it was statistically not significant (p=0.432). This constant value indicates that the reduction of fetal weight is always accompanied by a parallel decrease in the number of complete ossified vertebral centers, or a parallel increase in the number of delayed or absent centers, in topiramate treated groups.

Ossification of ribs and vertebrae

A total of 144 fetal skeletons stained with Alizarin red were examined (Table 1). The effects of prenatal administration of two doses of topiramate on ossification of the axial skeleton, ribs and vertebrae, in 20-day-old rat fetuses were analyzed as follows:

Ribs

Twenty-six ribs were examined in each fetus. Control group showed 100% (n=1170) complete ossification of ribs (Table 1). Although no significant difference was apparent in ossification between groups, there was, however, an increase in the number of delayed/absent ribs (six ribs or 0.5%) and (31 ribs or 2.4%) in Group A and Group B, respectively, when compared to the control group (Figure 3, A and B). Supernumerary ribs were not included.

Vertebral column

Ossifications of the centra and arches of the vertebrae in the different regions are summarized in Tables 1–3.

Table 2 – ***Effects of maternal topiramate ingestion on vertebral centra ossified centers in 20-day-old rat fetuses***

Groups (No. of fetuses)	Thoracic (13)		Lumbar (6)		Sacral (4)		Coccygeal (2)	
	Complete ossification	*Delayed and absent ossification*	*Complete ossification*	*Delayed and absent ossification*	*Complete ossification*	*Delayed and absent ossification*	*Complete ossification*	*Delayed and absent ossification*
Control (45)	511 (87.4%)	74 (12.6%)	267 (98.9%)	3 (1.1%)	154 (85.6%)	26 (14.4%)	18 (20%)	72 (80%)
Group A (49)	337[a] (53%)	300 (47%)	258[b] (87.8%)	36 (12.2%)	86[a] (43.9%)	110 (56.1%)	0[a] (0%)	98 (100%)
Group B (50)	400[a] (61.5%)	250 (38.5%)	244[a] (81.3%)	56 (18.7%)	96[a] (48%)	104 (52%)	2[a] (2%)	98 (98%)

Group A (topiramate 50 mg/kg) and Group B (topiramate 100 mg/kg); [a]p<0.0001, compared to control; [b]p<0.001, compared to control; Note that the cervical centra do not ossify at this stage of fetal development.

Table 3 – ***Effects of maternal topiramate ingestion on vertebral arches ossified centers in 20-day-old rat fetuses***

Groups (No. of fetuses)	Cervical (7×2)		Thoracic (13×2)		Lumbar (6×2)		Sacral (4×2)	
	Complete ossification	*Delayed ossification*	*Complete ossification*	*Delayed and absent ossification*	*Complete ossification*	*Delayed and absent ossification*	*Complete ossification*	*Delayed and absent ossification*
Control (45)	596 (94.6%)	34 (5.4%)	1170 (100%)	0	540 (100%)	0	268 (74.4%)	92 (25.6%)
Group A (49)	330[a] (48.1%)	356 (51.9%)	1274 (100%)	0	572[b] (97.3%)	16 (2.7%)	168[a] (42.8%)	224 (57.2%)
Group B (50)	434[a] (62.0%)	266 (38.0%)	1296 (99.7%)	4 (0.3%)	594 (99.0%)	6 (1%)	172[a] (43.0%)	228 (57.0%)

Group A (topiramate 50 mg/kg) and Group B (topiramate 100 mg/kg); [a]p<0.0001, compared to control; [b]p<0.05, compared to control; Note that each vertebra has two arches and coccygeal vertebral arches showed no ossification in any of the study groups.

Total vertebral centra

Thirty-two ossified vertebral centra were examined in each fetus (seven cervical, 13 thoracic, six lumbar, four sacral and two coccygeal). A total of 3600 ossified vertebral centra were examined in all groups (1125 in control, 1225 in Group A and 1250 in Group B). None of the cervical centra showed ossification in all examined groups, as cervical centra do not normally ossify at this stage of development.

Examination of the remaining vertebral centra showed a significant reduction of the number of complete ossified centra in Group A (55.65%) and Group B (59.4%) when compared to the control group (84.4%). No significant difference was found between treated groups (Figure 1).

Total vertebral arches

Sixty-four ossified vertebral arches (right and left for each vertebra) were examined in each fetus (14 cervical, 26 thoracic, 12 lumbar, eight sacral and four coccygeal). A total of 9216 vertebral arches were examined in all groups (2880 in control, 3136 in Group A and 3200 in Group B). There was a significant reduction of the number of complete ossified arches in Group A (74.7%) and Group B (78.0%) when compared to the control group (89.4%). Also, a significant difference was found between treated groups (Figure 1).

In the **cervical region**, ossifications of seven cervical vertebrae (seven centra and 14 arches) were evaluated in each fetus (Table 1). Ossifications of the cervical centra were not developed in any of the fetuses examined, while ossification of the cervical arches exhibited a highly significant decrease in the number of complete ossified centers in both topiramate treated groups when compared to the control group (Table 3). Delayed ossification appears as either non-fusion between the

anterior and posterior parts of the arch (Figure 2, A and B) or complete absence of the posterior part. The most common delayed cervical arches in both treated groups were in vertebrae 1 through 4 and were bilateral.

In the **thoracic region**, ossifications of 13 thoracic vertebrae (13 centra and 26 arches) were evaluated in each fetus (Tables 1 and 2; Figure 3, A–D). The number of complete ossified thoracic centra was significantly decreased in both treated groups when compared to the control group. No significant difference was noticed between the treated groups. However, the thoracic arches were almost completely ossified in all groups.

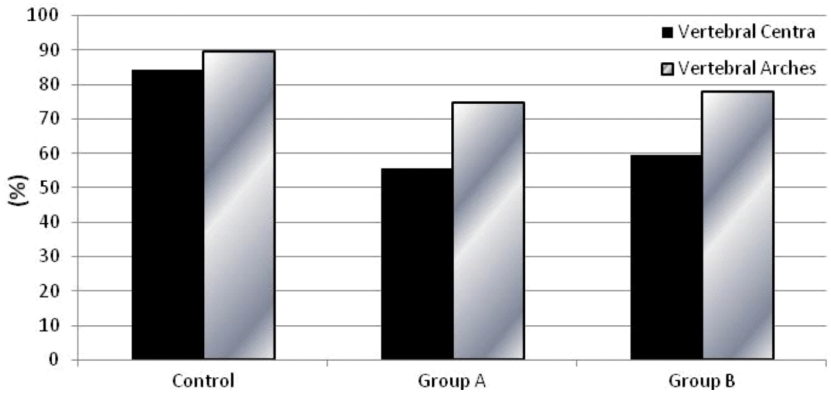

Figure 1 – ***A histogram showing the percentages of total ossified vertebral centra and arches in the control group, and topiramate treated Group A and Group B in 20-day-old rat fetuses.***

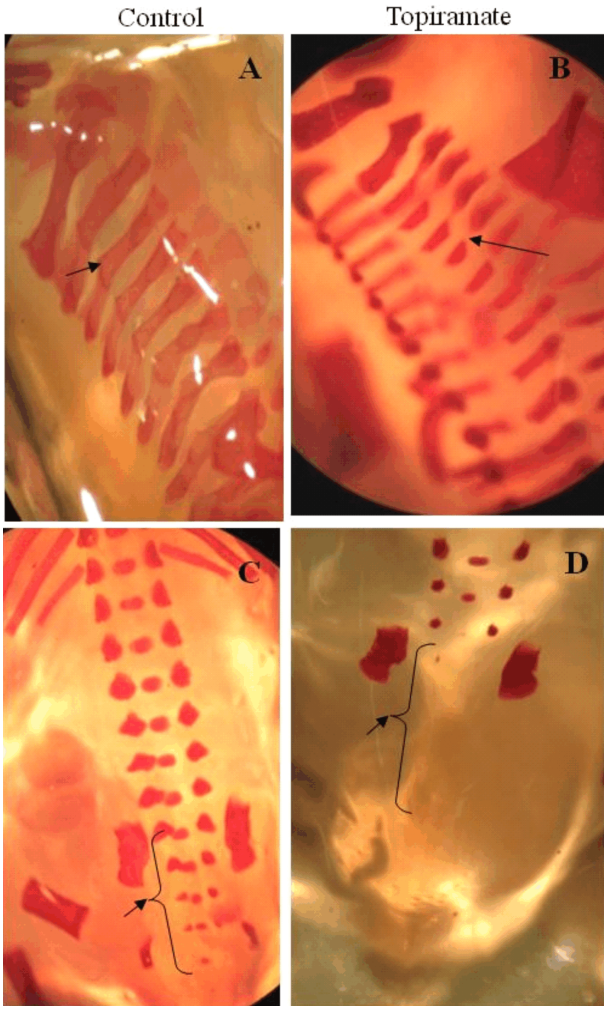

Figure 2 – ***Ossification of the cervical and sacro-coccygeal vertebrae of the 20-day-old rat fetuses in the control and topiramate treated groups: (A) Normal complete cervical arches (arrow); (B) Delayed ossification (non-fused) of cervical arches (arrow); (C) Normal ossification of the sacro-coccygeal vertebral centers (arrow); (D) Absent ossification of sacrococcygeal vertebral centers (arrow).***

In the **lumbar region**, ossifications of six lumbar vertebrae (six centra and 12 arches) were evaluated in each fetus (Table 1; Figure 2C; Figure 3, A, C and D). No significant difference was noticed in the ossified centra or arches between the topiramate treated groups. However, a highly significant reduction of the number of complete ossified centra was noticed in both treated groups when compared to control group (Table 2). Also, a significant decrease in the number of complete ossification of lumbar arches was noticed in Group A when compared to control (Table 3).

In the **sacral region**, ossifications of four sacral vertebrae (four centra and eight arches) were evaluated in each fetus (Table 1; Figure 2, C and D). A highly significant delay in ossification of the centra and arches was seen in both treated groups when compared to the control group (Tables 2 and 3). No significant difference was seen between both topiramate treated groups.

In the **caudal region**, ossification of two coccygeal vertebrae (two centra and four arches) were evaluated in each fetus (Table 1; Figure 2, C and D). The arches were not found ossified in any group. A highly significant decrease in the number of complete ossified centra was seen in the treated groups when compared to the control group (Table 2). No significant difference was noticed between treated groups.

Abnormal ossification

The abnormal ossification of the axial skeleton appeared in the form of abnormal vertebrae, abnormal curvature or dislocation of vertebral column, wavy ribs, rib agenesis and unilateral or bilateral development of 14^{th} supernumerary ribs (Figure 3, A–D).

The number of fetuses with abnormal ossification showed a significant increase in Group A and Group B when compared to the control group. No significant difference was seen between topiramate treated groups (Table 4). It was noticed that more than one abnormality could be found in the same fetus.

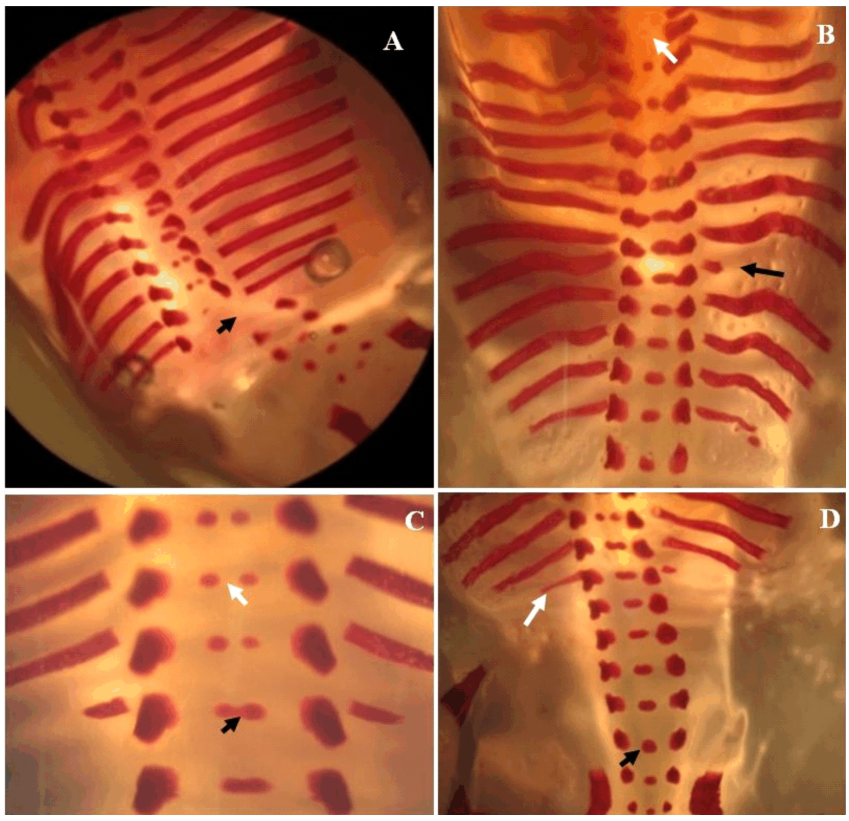

Figure 3 – ***Abnormal ossification in the axial skeleton of the 20-day-old rat fetuses treated with topiramate: (A) Vertebral dislocation (arrow) due to absence and delayed ossification centers of thoracic and lumbar vertebrae; (B) Wavy ribs and incomplete rib ossification (black arrow) and absence of 1st and 2nd thoracic centra ossification (white arrow); (C) Delayed centra, non-fused (white arrow) and dumbel shaped (black arrow); (D) Supernumerary rib (white arrow) and complete ossified lumbar centrum (black arrow).***

Table 4 – ***Maternal topiramate ingestion and abnormal ossification of the axial skeleton in 20-day-old rat fetus***

Groups (No. of fetuses)	No. of fetuses with abnormal ossified bones	Abnormal vertebrae		Abnormal ribs	
		Number	*Shape*[b]	*Supernumerary rib (14th)*	*Wavy*
Control (45)	5 (11.1%)	0	0	2	4
Group A (49)	15 (30.6%)[a]	5	2	11	3
Group B (50)	15 (30%)[a]	1	4	14	1

Group A (topiramate 50 mg/kg) and Group B (topiramate 100 mg/kg); [a]Chi-square test compared to control (p<0.05); [b]Abnormal curvature; N.B. More than one abnormality could be found in the same fetus.

Discussion

Epilepsy in general has been associated with an increased rate of malformations in offspring. The present study was conducted in a non-epileptic pregnant rat to determine the effect of topiramate *per se* on pregnancy outcome. During development, the factors affecting the pattern formation of the axial skeleton remain poorly understood. Genetic variations, either resulting from spontaneous mutations or because of xenobiotic exposure may disrupt this patterning and lead to a wide variety of skeletal alterations [13, 14]. The present study was designed to evaluate ossifications of ribs and vertebrae in preterm rat fetuses following maternal exposure to two different doses of AED topiramate, equivalent to therapeutic doses in humans [18–20]. For this purpose the pattern of ossification, i.e., complete, delayed or absent is studied [21–23].

According to the compensatory mechanism phenomena, it has been suggested that a missed ossification centre during fetal development could be repaired and reappeared in postnatal period. This mechanism could explain the absence or the presence of skeletal malformations when antiepileptic drugs such as diazepam and valproic acid were used [15, 24–26]. Failure of this mechanism may result in permanent skeletal abnormalities postnatally such as spina bifida [27]. In the present study, delayed ossification and abnormalities of the ribs and vertebrae were seen in both groups treated with topiramate (50 mg/kg and 100 mg/kg), in accordance with the results obtained by Singh M and Mishra A [10], and Padmanabhan BR [11]. On the other hand, it was noticed that there were no statistical differences between the effects of the two therapeutic doses of topiramate, used in the present study, that conclude that these effects are not dose related which disagree with the conclusion declared by Singh M and Mishra A [10].

It has been emphasized that one of the main mechanisms of teratogenic action which affects ossification is oxidative stress [28, 29]. Topiramate was found to be a free radical scavenger [30] and its role as antioxidant indicates that its effect on ossification detected in the present study is not due to the presence of free radicals.

Previous studies demonstrated that maternal body weight gain was reduced significantly following administration of different doses of antiepileptic drugs in experimental animals [15, 31]. In the contrary, maternal weight gain in the present study showed no significant difference between the different groups. This result indicates that the reduction in fetal weight in the

present study is not related to the maternal nutritional status. On the other hand, treatment with topiramate in previous studies was associated with metabolic acidosis due to its inhibitory effect on carbonic anhydrase, which lead to renal bicarbonate loss [32, 33]. This mechanism may affect the process of ossification in the present study.

In the present study, topiramate was given to pregnant rats throughout day 6 to 19 of gestation. The administration of topiramate during the embryonic and fetal growth periods, explains the reduction in bone ossification and in fetal weight. On the contrary, Singh M and Mishra A [10] did not found reduction in fetal weight in spite of using topiramate at doses of 40, 100 and 200 mg/kg body weight, which may be attributed to the timing of maternal administration of the drug in the early embryonic period only through days 9 to 12 of gestation. Sucheston ME *et al.* [34] found a positive correlation between the measurement of ossified long bones and fetal weight in mice. Also, in the present study, reduction of fetal weight in topiramate treated groups was accompanied by a parallel decrease in the number of complete ossified vertebral centers. The results of the present study suggests that topiramate produce prenatal growth restriction in accordance with Ariyuki F *et al.* [22] who concluded that a low body weight accompanied by a reduced ossification is a good indicator for prenatal growth retardation.

Bone development depends upon many factors including nutrition, hormones, genes, environment and physical activity [35]. One of the mechanisms involved in bone development was based on Frost's mechanostat theory [36], where bone cell activity is coordinated by the mechanical requirements of the bone. When the mechanical challenge exceeds an acceptable threshold, the mechanostat set point, bone tissue is added at the location where it is mechanically necessary [36]. During pregnancy, the physical activity of the fetus is achieved through regular fetal kicks against the uterine wall [37]. The hypothesis of mechanostat-controlled fetal bone development is supported by axial skeletal developmental defects observed in many other conditions such as neuromuscular disorders [38–40] and pharmacological interference of fetal muscle contractions [41] where low bone mass at birth and decreased periosteal expansion are observed. Since topiramate crosses the placenta and accumulates in fetal tissue [42], it is plausible that a decrease in fetal physical activity may cause delayed ossification and bone formation. It would be interesting to study effect of topiramate on fetal activity to confirm this hypothetical explanation linking fetal activity with axial skeleton development.

Skeletal variations are caused by genetic as well as environmental factors. It is important to consider developmental variations in skeletal number, shape or size [43]. Chondrogenesis, the initial step in embryonic skeletal development, is associated with signaling events that ultimately result in the regulation of gene transcription and function. The interconnected nature of these pathways underscores the effect of genetic and teratogenic perturbations that result in skeletal birth defects [44]. It has been suggested that COX-2 may induce apoptosis in the sclerotome of transgenic rat embryos via the up-regulation of p53 [45], which may result in skeletal birth variations. Thus, it may be important to study the effect of topiramate on similar signaling pathways that are involved in axial bone development.

Conclusions

Therapeutic doses of topiramate should be taken cautiously during pregnancy as they lead to fetal growth restriction and increases abnormalities of the axial skeleton in rat fetuses. Different mechanisms could be involved to produce such effects. Future studies should address the underlying molecular mechanisms and implications of the observed prenatal effects of topiramate.

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