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Effect of Melamine Administration during Pregnancy on Foetal Bone Ossification

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Abstract

Aim: We aimed to study the effects of prenatal administration of two doses of melamine on foetal ossification centers in rats. **Methods**: Positively-mated, virgin, adult female Sprague-Dawley rats (n=24) were treated from day 6 to day 20 of gestation with solvent (control), melamine 300 mg/kg/day (group 1) or melamine 450 mg/kg/day (group 2). On day 21, half of the fetuses were examined for bone ossification abnormalities. **Results**: A total of 109 foetal skeletons were examined. The percentage of incomplete or absent bones in the entire skeleton was significantly less in group 1 and group 2 compared to control. These findings were more prominent in group 2 compared to group 1. Likewise, ossified centers were fewer in the sternum and metacarpal bones in group 1 and group 2 compared to control. No abnormal ossification was observed in metatarsal, skull, pubic or rib bones. Regarding the vertebral *centrae*, a significant increase in the number of absent or delayed bones was noticed only in group 2 compared to control. Specifically, the abnormalities were observed in the thoracic and sacral *centrae*. Similarly, group 2 was associated with fewer ossified centers in vertebral arch compared to control. The abnormal ossifications were observed in sacral and coccygeal bones. The only observed abnormality in vertebral ossification in group 1 was in coccygeal arch, compared to control. **Conclusions:** Prenatal administration of melamine caused dose-dependent retardation in bone ossification, which mainly affected the sternum, metacarpal, vertebral *centraee* and arch.

Keywords

Melamine, Gestational Exposure, Foetal Ossification, Teratogenicity, Rats.

1. Introduction

Melamine is a heterocyclic nitrogenous compound that is widely used in industry especially for manufacturing dining wares. It is also an ingredient in other essential products such as paints, coatings and glue. In food industry, due to its apparent high amino group content, some manufacturers intentionally add melamine to food products, such as pet food and powdered milk, in order to deceptively inflate their protein content aiming to generate more revenues (Tyan et al, 2009). The illegal addition of melamine to pet food resulted in a global outbreak of melamine poisoning in cats and dogs in several countries world-wide during 2004 and 2007 (Brown et al, 2007). A year later, a catastrophic melamine poisoning was reported in China were tens of thousands of infants and children were admitted to hospitals with acute renal failure and nephrolithiasis due to the ingestion of melamine-tainted infant formulae. Unfor-

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tunately, a few children lost their lives as a result of this incident (Chiu, 2008). This outbreak in humans dominated the food safety news for several years thereafter and attracted intense health concerns worldwide.

Previous animal studies have demonstrated that toxicity resulting from acute exposure to melamine is considered low at small doses (Zhang et al, 2012). Indeed, experiments on mice have revealed that melamine has a high lethal dose 50 (LD50) which ranged from 3.2 g/kg to 7.0 g/kg (Skinner et al, 2010). However, subacute and chronic exposure to melamine could lead to serious toxic effects which include infertility, urinary stone formation, hematuria, proteinuria, oliguria, renal failure, hepatic impairment, and transitional cell carcinoma in ureter and urinary bladder [Dai et al, 2015; Hau et al, 2009; Hu et al, 2012; Xu et al, 2013; An and Sun, 2017; Ogasawara, et al 1995; Reimschuessel et al, 2010). Acute renal failure and urinary stone formation remain the most serious toxicities resulting from exposure to melamine. Gao et al conducted a prospective cohort study on young children who had history of melamine poisoning as a result of ingestion of contaminated powdered milk. The authors reported that after 18 months of follow up, more than 90% of the participants spontaneously passed a stone and a significant proportion of the children had hematuria and proteinuria (Gao et al, 2016).

Data on the impact of melamine exposure during pregnancy on fetal ossification is scarce. As far as we know, only one study appeared in the literature which explored this topic. In this investigation, Kim and his colleagues reported a delay in fetal bone ossification at maternal doses beyond 400 mg/kg. Indeed, at 800 mg/kg dose, the investigators showed incomplete ossification in many bones including sternum, metacarpal, metatarsal, coccygeal and sacral vertebrae (Kim et al, 2011). Therefore, the main aim of this study was to extensively investigate the effect of exposure of pregnant dams to different doses of melamine on bone ossification in their offspring.

2. Materials and Methods

2.1. Animals

Ethical approval was obtained from the Institutional Research and Ethics Committee at the Arabian Gulf University in Bahrain (Approval number 17-PI-12/2013). Nulliparous, virgin, female Sprague-Dawley rats (n=24) weighing 180–250grams were kept in separate cages in a pathogen-free environment. Prior to starting the experimental work, the rats were acclimated for one week in the animal facility under ambient temperature of 25C^{o}, 12-hour light-dark cycles, and *ad libitum* access to standard rodent chow and purified water. Following acclimatization, one fertile male rat was placed in each cage with two female rats for mating. Pregnancy was confirmed by microscopic examination of a smear taken from vaginal lavage every morning and the day on which the spermatozoa were detected was designated as the first day of gestation.

2.2. Experimental design

Starting from the 6th until the 20th day of gestation, pregnant dams were treated once daily by oral gavage and were randomly allocated into three experimen-

tal groups: Control (n=8) received the solvent 1% carboxymethylcellulose in water, group 1 (n=8) and group 2 (n=8) were administered melamine at doses of 300 mg/kg/day or 450 mg/kg/day, respectively.

On day 21 of pregnancy, the rats were euthanized by ether inhalation and laparotomy was performed. During this procedure, the fetuses were collected from the uterine horns, and immediately euthanized by ether. Then they were eviscerated and immersed for 30 seconds in hot water to facilitate skin removal. Approximately one half of the fetuses, randomly collected from each mother, were stained by Alizarin red to examine them for bone ossification abnormalities as previously described by our group (Fadel et al, 2012). Briefly, the specimens were initially kept in 95% ethanol for 2 to 3 days, after which they were washed with 1% KOH solution for a few days. Once the skeleton was distinctly visible, the specimens were transferred into a fresh KOH solution mixed with a few drops of Alizarin red stain. Following successful staining, the fetuses were kept in solutions containing 30%, 50% and 70% glycerin then finally in 100% glycerin solution with added thymol to prevent fungus growth.

Examination of bone ossification centers in the rat fetuses was systematically carried out by using a dissecting microscope. The chart which was used to record observations included the bones which are expected to be ossified in a rat fetus on day 20 of gestation which include skull, mandible, hyoid, sternum, ribs, forelimbs, hind limbs, vertebral column and hip as previously described (Nash et al, 1989). The examined bones were categorized into two groups: complete or absent, where the latter included bones which were either totally non-ossified or incompletely ossified.

2.3. Statistical analysis

The data were analyzed by using the Statistical Package for Social Sciences software (SPSS-23) using Pearson's Chi-Square test. Statistical significance was set at p value less than 0.05.

3. Results

A total number of 109 Alizarin red stained fetal skeletons were examined for bone ossification; control (n=39), group 1 (n=38) and group 2 (n=32). The following bones were evaluated during this study (Table 1): skull bones (frontal, parietal, interparietal, supraoccipital, exoccipital, basiocciptal, nasal, lacrimal, premaxilla, maxilla, zygoma, squamosal, tympanic bulla, basisphenoid, presphenoid and alisphenoid), mandible, hyoid body, hyoid arch, sternum, ribs, clavicle, scapula, humerus, radius, ulna, metacarpus, hand phalanges, femur, tibia, fibula, metatarsus, foot phalanges, ilium, ischium and pubis. Vertebral column examination included both vertebral *centrae* as well as vertebral arches. In both parts, we examined the cervical, thoracic, lumbar, sacral and coccygeal vertebrae.

3.1. Entire skeleton

The total number of bones (n=21,146) in the entire skeleton were examined. The number of incomplete or absent bones in group 1 was 707 (9.59%) which was sig-

Table 1. Effect of maternal melamine ingestion on bone ossification in 21-day rat fetuses.

Bones	Group	Absent/delayed ossification	Complete ossification	Total number of bones
Entire skeleton	Control	646 (8.54%)	6920 (91.46%)	7566
	1	707 (9.59%)*	6665 (90.41%)	7372
	2	662 (10.66%)**#	5546 (89.34%)	6208
Sternum	Control	66 (33.85%)	129 (66.15%)	190
	1	47 (24.1%)*	148 (75.9%)	195
	2	44 (22.56%)*	151 (77.44%)	160
Metacarpal	Control	157 (40.26%)	233 (59.74%)	390
	1	185 (48.7%)*	195 (51.3%)	380
	2	154 (48.13%)*	166 (51.87%)	320
Metatarsal	Control	142 (36.41%)	248 (63.59%)	390
	1	153 (40.16%)	228 (59.84%)	381
	2	133 (41.43%)	188 (58.57%)	321
Skull bones	Control	20 (10.26%)	175 (89.74%)	195
	1	17 (14.78%)	98 (85.22%)	115
	2	24 (15%)	136 (85%)	160
Pubis	Control	16 (20.51%)	72 (92.31%)	78
	1	8 (10.53%)	68 (89.31%)	76
	2	0 (0%)	64 (100%)	64
Ribs	Control	0 (0%)	1014 (100%)	1014
	1	0 (0%)	988 (100%)	988
	2	0 (0%)	832 (100%)	832
Vertebral *centrae*	Control	99 (9.79%)	912 (90.21%)	1011
	1	105 (10.62%)	884 (89.38%)	989
	2	131 (15.75%)***#	701 (84.25%)	832
Thoracic *centrae*	Control	52 (10.26%)	455 (89.74%)	507
	1	54 (10.91%)	441 (89.09%)	495
	2	80 (19.23%)***##	336 (80.77%)	416
Lumbar *centrae*	Control	1 (0.43%)	233 (99.57%)	234
	1	2 (0.88%)	226 (99.12%)	228
	2	3 (1.56%)	189 (98.44%)	192
Sacral *centrae*	Control	1 (0.64%)	155 (99.36%)	156
	1	3 (1.97%)	149 (98.03%)	152
	2	7 (5.47%)*	121 (94.53%)	128
Coccygeal *centrae*	Control	46 (39.32%)	71 (60.68%)	117
	1	46 (40.35%)	88 (77.19%)	114
	2	41 (42.71%)	55 (57.29%)	96

Bones	Group	Absent/delayed ossification	Complete ossification	Total number of bones
Vertebral arch	Control	137 (6.76%)	1891 (93.24%)	2028
	1	140 (7.09%)	1836 (92.91%)	1976
	2	141 (8.47%)*	1523 (91.53%)	1664
Cervical arch	Control	19 (2.94%)	527 (81.58%)	646
	1	18 (3.38%)	514 (96.62%)	532
	2	12 (2.68%)	436 (97.32%)	448
Thoracic arch	Control	0 (0%)	1041 (100%)	1041
	1	0 (0%)	988 (100%)	988
	2	0 (0%)	832 (100%)	832
Lumbar arch	Control	0 (0%)	468 (100%)	468
	1	0 (0%)	456 (100%)	456
	2	2 (0.52%)	382 (99.48%)	384
Sacral arch	Control	13 (4.17%)	299 (95.83%)	312
	1	10 (3.29%)	294 (96.71%)	304
	2	31 (12.11%)***##	225 (87.89%)	256
Coccygeal arch	Control	100 (42.74%)	134 (57.26%)	234
	1	119 (52.19%)*	109 (47.81%)	228
	2	103 (53.65%)*	89 (46.35%)	192

Chi-Square, *p< 0.05, **p< 0.01, ***p< 0.001, compared to control, #p< 0.01, ##p< 0.001 compared to group 1. Group 1: melamine 300 mg/kg/day, group 2: melamine 450 mg/kg/day.

nificantly higher compared to control (p< 0.05). A similar observation was noticed in group 2 where the number of abnormally ossified bones reached 662 (10.66%) which was also statically significant compared to control (p< 0.01). More ossification abnormalities were detected in group 2 compared to group 1 (p< 0.05).

3.2. Sternum, forelimbs and hind limbs

A total of 585 sternum bones were analyzed. In comparison with control group, 47 bones (24.1%) were absent in group 1 compared to 44 bones in group 2 (22.56%). In both groups, the number of non-ossified bones was statistically significant compared to control (p< 0.05) but no difference was observed between melamine-exposed groups.

Regarding the forelimbs, melamine was found to affect metacarpal ossification. A total of 1092 metacarpal bones were examined. At lower dose of melamine used, 186 bones (48.82%) were absent (p< 0.05). Similarly, at the highest dose, 155 metacarpal bones were absent (48.29%), a finding that was statistically significant compared to the control group (p< 0.05). However, no difference was noticed between the two melamine-treated groups.

No significant decrease in the number of ossification centers was detected in metatarsal, skull, pubis or rib bones in all the groups.

3.3. Vertebral *centrae*

In the vertebral *centrae*, we evaluated a total of 2832 bones (13 thoracic, 6 lumbar, 4 sacral and 2 coccygeal in each skeleton). Overall, no decrease in the number of ossified centers was observed in group 1. However, a significant decrease was observed in group 2 (131, 15.75%) compared to control ($p< 0.001$) and group 1 ($p< 0.01$). In the thoracic *centrae*, 1418 bones were analyzed. Defective ossification was observed in 80 bones (19.23%) which was significantly more than control ($p< 0.001$) and group 1 ($p< 0.001$). For the sacral *centrae*, 436 bones were examined. Likewise, group 2 showed a higher rate of missing bones (7, 5.47%) which was remarkably higher compared to control ($p< 0.05$) but not different than group 1. For the lumbar and coccygeal *centrae*, no notable findings were observed.

3.4. Vertebral arch

A total of 5668 vertebral arch bones were examined (14 cervical, 26 thoracic, 12 lumbar, 8 sacral, and 4 coccygeal). Overall, the total number of non-ossified bones in the entire vertebral arch in group 2 was 141 (47%) which was significantly more than the control group ($p< 0.05$). Nevertheless, no notable difference between group 2 and group 1 was reported. In the sacral arch, we examined a total of 872 bones. The number of ossification centers was fewer in group 2 compared to control ($p< 0.001$) and group 1 ($p< 0.001$). Similarly, in the coccygeal bones, we examined 654 bones. Less ossification was recorded both in group 1 ($p< 0.05$) and group 2 ($p< 0.05$) compared to control. However, no difference was observed between the two melamine-treated groups. For the thoracic, lumbar and cervical parts of the vertebral arch, no positive findings were reported.

4. Discussion

Most research on melamine focused on renal stone formation and nephrotoxicity since they are the major causes of mortality and morbidity (Hau et al, 2009; Lam et al, 2009). However, only one study has addressed the effects of melamine on fetal bone ossification (14). In that particular study, Sprague-Dawley dams were treated with 200, 400 or 800 mg/kg/day and bone ossification in their fetuses was examined. The outcomes of that study reported notable results at 800 mg/kg/day only. In a recent study, our group has demonstrated that melamine negatively affected intrauterine growth in Sprague-Dawley fetuses when pregnant rats were exposed to 300 and 400 mg/kg/day (unpublished data). In the current study, we observed that melamine resulted in a significant decrease in the number of ossified centers in certain parts of the skeleton at lower doses than previously reported (Kim et al, 2011).

Analysis of the data on the entire skeleton revealed that the number of incomplete or absent bones was statistically significant in group 1 and group 2 compared to the control fetuses. In addition, we reported more ossification abnormalities in group 2

compared to group 1, indicating a dose-dependent pattern. These findings suggest that both melamine doses resulted in detrimental effects on the number of ossified centers- a finding which contradicted the reports of Kim and co investigators who concluded that no adverse effects on ossification were observed at dose of 400 mg/kg/day.

Our findings on individual bone groups showed that the number of ossification centers was significantly fewer in fetal sternum and metacarpal bones at both melamine doses studied. Although Kim et al showed that melamine remarkably affected sternum and metacarpal ossification, these changes were detected only at a dose of 800 mg/kg/day while no significant changes were observed at doses of 200 and 400 mg/kg. In addition, Kim and colleagues reported that metatarsal bone ossification was adversely affected, a finding that was not reported in our study.

Regarding the data on vertebral *centrae*, the negative impact of melamine was noticed at 450 mg/kg dose and changes were observed in thoracic and sacral regions. Since the cervical *centrae* are not normally ossified at this gestational age, none of the bones in this part were appreciated in all the groups. In the vertebral arch, however, changes were observed at both doses of melamine. Specifically, at 300 mg/kg, less ossification was observed in the coccygeal arch. However, at 400 mg/kg, the number of ossified bones was significantly less in the coccygeal and sacral regions. Compared to our findings, Kim and co investigators reported that changes in vertebral ossification were seen at 800 mg/kg melamine dose and that the changes were noticed in the sacral and coccygeal parts only. Again, that group reported no ossification defects at melamine doses less than 400 mg/kg/day.

In conclusion, Maternal exposure to melamine during pregnancy delayed fetal bone ossification. Although some of these adverse consequences were observed at 300 mg/kg, they were more prominent at 450 mg/kg/day. More specifically, the sternum, metacarpal, and parts of the vertebral *centrae* and arches were markedly affected.

5. Acknowledgments

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