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Cadmium induced Teratogenic Effects in Developing *Mus musculus*

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ABSTRACT

Cadmium, a heavy metal absorbed in human body via environment and cause severe toxicity. This study was designed to evaluate the teratogenic and embryotoxic effects of cadmium on developing mice. The pregnant mice were randomized into four treatment groups (3.13, 6.25, 12.5 and 25.0 µg/g B.W) and a control group and dose was given orally on days 6 to12 of gestation daily. Then mice were sacrificed and fetuses were recovered on day 18 of gestation. Recovered fetuses were fixed and analyzed on morphological, morphometric, histological and skeletal basis. Morphological studies showed the abnormalities such as distorted axis, cryptophthalmia, anophthalmia, open eyelids, micromelia, phocomelia, agnathia, hydrocephaly, angular tail and subcutaneous hemorrhages. Morphometric analysis indicated a significant ($p<0.05$ to $p<0.001$) reduction in fetal body weight, crown rump length, head circumference, eye circumference, forelimb and hindlimb lengths and tail size against controls. Histological observations showed brain defects, dilation in the 3rd and 4th ventricles (internal hydrocephaly), absence of 3rd and 4th ventricles (internal microcephaly) and under developed lateral part of cerebellum. Lungs defects included necrosis and underdeveloped lungs lobes while heart anomalies included microcardia, hypopericardium and necrosis in atrium and ventricles. Sections through liver shows necrosis and apoptosis and misshapen lobe. The transverse sections through kidney also showed necrosis and in some cases right kidney was missing. Skeleton studies showed partially and completely unossified bones as compare to control. It is concluded that Cadmium concentrations used in present study caused teratogenic and embryotoxic in mice fetuses.

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CA and A conceived the project and supervised its execution. SA and MT performed animal treatment, recovered samples from animals and wrote the article. ARK and MKAH prepared histological slides.

Key words
Cadmium, teratogenicity, embryotoxicity, mouse development.

INTRODUCTION

Metals are important components of our daily life. They have many categories depending on their properties (Abrikosov, 1988). Among all types of metals, heavy metals always remain a burning issue among the scientists. Excessive levels of heavy metals can be introduced into the environment in the form of industrial wastes or fertilizers, which enter into the food chain through plants or leaching into ground water and affects the living organisms (Bradl, 2005).

Cadmium is one of the toxic heavy metal that is nonessential transition metal (ATSDR, 2004). Human exposure to environmental cadmium is primarily, the result of fossil fuels combustion, phosphate fertilizers, natural sources, iron and steel production, cement production and related activities, nonferrous metals production, and municipal solid waste incineration. Bread, root crops, and vegetables also contribute to the cadmium in modern populations (Chang *et al*., 2012). Shell fishes such as the mussels, scallops and oysters are a major source of dietary cadmium and contain 100 to 1000 µg/kg. Meats and fruits contain 1 to 50 µg / kg (Petersson *et al*., 2004). Smoking is a major source of respiratory exposure to cadmium (Mortada *et al*., 2004). Wang and Du (2013) reported that the half life of the cadmium in the body is long and it take much time to excrete from the body therefore its long exposure leaves toxic effects on the tissues. Cadmium can easily accumulate in all body tissues but respiratory absorption of the cadmium is greater than the gastrointestinal absorption (Berglund *et al*., 1994). Women become more victim of cadmium absorption than the men (Vanderpool and Reeves, 2001). Iron deficiencies during pregnancy also lead to increased cadmium absorption (Agenta *et al*., 2004).

Cadmium causes fetal malformation and growth restriction by affecting the placenta (Wang *et al*., 2012). Even low concentration of cadmium may impair the physiological function of the placenta (Kuhnent *et al*., 1993). Cadmium causes rise in fetal death rate, the rate of the anomalies and decrease in fetal weight. Birth anomalies in developing embryos include hydrocephalus, urogenital abnormalities, cleft palate, micrognathia, clubfoot, small lungs and skeletal malformations (Holt and Webb, 1987). Cadmium exposure during neurulation induces a higher incidence of neural tube defects (Robenson *et al*., 2009). Padmanabham and Hameed in 1990, observed that by giving the different doses of

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cadmium limb malformations such as ectrodactyly, postaxial polydactyly, adactyly, phocomelia, meromelia and malrotation of the limbs were detected in significant number of fetuses. Cadmium may have a direct effect on bone mineralization, possibly related to calcium deficiency and an indirect effect on calcium absorption through vitamin D hydroxylation which may lead to osteomalacia (Goyer *et al*., 1994).

Cadmium has also been proven as a carcinogen by IARC *(*International Agency for Research on Cancer*)*. Cadmium produced lung, prostate and breast cancer in the experimental animal but recent studies on human do not convincingly support this stand point (Waalkes *et al*., 1999).

In view of above mentioned literature, it is unavoidable to evaluate embryotoxic and teratogenic effects of cadmium on mice fetuses with different doses and in different environment.

MATERIALS AND METHODS

Sexually mature Swiss Webster variety of albino mice *Mus musculus* (avg. b.w.25±2g) were used during this study which were purchased from Veterinary Research Institute. The animals were kept under conditions, which was according to the standard protocols of the approved animal treatment condition of medical ethics committee of Punjab University, Lahore, Pakistan. Standard conditions included 12 h dark and light cycle, housed in 12"x16" steel cages, under standard room temperature 27±2°C. Animals were fed with the National Chick Feed# 12 and had a free access to water via water bottles. Tags were utilized to determine the date of mating, dosing and dissection of animals. Females in proestrous stage were caged overnight with males of the same stock. The females were carefully examined next morning. Presence of vaginal plug was taken as day 0 of gestation. These females were separated. A total of 25 pregnant females were examined during this study. Which were then randomly divided into 5 groups, among them one group is specified as control group and other four groups were specified as dose groups.

Cadmium is available in powdered form under the trade mark of BDH Analar and is soluble in water. The doses were prepared by dissolving the weighed amount of metal in distal water in such a way that 0.1ml of solution contained desired concentration of cadmium. Doses used during this experiment were 3.12, 6.25, 12.5 and 25.0µg/g B.W. These doses were given to pregnant mice with the help of 1 ml plastic syringe. At the end of syringe, a stainless steel tube with blunt end covered by a rubber tube was attached which was specially prepared for oral feeding. Mice readily accepted the rubber tube. These doses (0.1ml of each dose group) were then pumped into the gullet on days 6-12 of gestation (organogenitic period) daily, once a day, which was readily engulfed by the mice. By this technique, there was minimum escape of dose occur.

On day 18 of gestation, the treated females were weighed and anesthetized using Ether as anesthetic. Mice were then sacrificed. Uteri of females were exposed by giving midline incision. The number of implantation sites was recorded. The fetuses were removed from uteri and weighed. Then these fetuses were fixed differently for different studies. For morphological, morphometric and histological studies fetuses were fixed in Bouin's fixative under the trade mark of BDH Analar for 48 hours. After 48 hours, fetuses were shifted to 70% ethanol.

The morphological studies were done to record anomalies of craniofacial region, trunk, limbs and tail region. The abnormal fetuses were macrophotographed by using microscope Labomed, CZM6, of Japan and camera, Panasonic TZ15.

The morphometric studies involved recording of fetal weight, crown rump length, head and eye circumferences, length of fore and hind limbs and tail length. All the measurements were made by analytical balance and digital vernier caliper. The head and eye circumference values ($p = mm^2$) were calculated for each fetus with the help of a computer based program the "Ellipse Circumference Calculator" (CSGN, 2006). The morphometric data were subjected to ANOVA by using SPSS software. The analysis was done on 95% confidence interval and at minimum levels of significance $p < 0.05$.

For histological observations selected fetuses from all groups were processed for paraffin sections. The fixed fetuses were routinely processed, embedded in wax and then 5µm thick transverse sections were stained with hematoxylin and eosin and studied under same microscope mentioned above (Spencer and Bancroft, 2008).

For skeletal preparations, the fetuses were preserved in 95% ethanol without using any fixative, they were eviscerated through a small abdominal incision and all thorasicoabdominal organs were removed. These eviscerated fetuses were placed in 2% KOH solution unless the flesh was almost completely removed and bones became visible. Alizarin Red S stain was used (1% aqueous solution made alkaline b 2 to 3 drops of 1% KOH) for 30 min. The deeply stained fetuses were placed in 1% KOH until the skeleton was clearly visible through remaining surrounding tissue. These stained skeletons were further cleared in 20% glycerinated and1% KOH (Kawamura *et al*., 1990). The stained specimens were preserved in 50% ethanolic glycerol for microscopic

studies and macrophotographed by using same model of microscope and camera as described for morphological studies.

RESULTS

In this study increase in percentage of malformed (having any kind of birth defect) and resorbed fetuses (dead embryo at any developmental stage after implantation) was observed in all dose groups as compared to control (Table I).

The morphometric observations during this study showed a significant ($p < 0.05$) reduction in mean body weight of the fetuses, head and eye circumferences, fore and hind limbs lengths and tail length as compared to control in dose group 3.125 µg/g B.W. In dose group 6.25 µg/g B.W and 12.5 µg/g B.W a significant ($p<0.01$) decrease was observed in mean fetal body weights and head circumference, CR length, eye circumference, fore and hind limbs lengths and tail lengths as compared to control. But in dose group 25.0 µg/g B.W., parameters such as CR length, eye circumference, fore and hind limbs lengths and tail lengths, mean fetal body weights and head circumference decreased at a level of significance ($p< 0.01$ - $p< 0.001$) as compared to control (Table II).

Morphological analysis showed that the fetuses recovered from control group had normal size and well developed body organs (Fig. 1A). The fetuses from dose group 3.13 µg/g B.W showed morphological anomalies like open eyelids (13.30%) (Fig. 1B), forelimb displacement (21.00%) (Fig. 1B), micromelia (8.23%) (Fig. 1C), hydrocephaly (10.43%) (Fig. 1C), acheiria (Fig. 1C), cryptophthalmia (7.33%) (Fig. 1D). The fetuses from dose group 6.25 µg/g B.W showed morphological anomalies like distorted axis (10.01%) (Fig. 1E), hindlimb micromelia (14.08%) (Fig. 1E), wrinkled tail (16.67%) (Fig. 1E), forelimb micromelia (13.61%) (Fig. 1F), webbed feet (Fig. 1F), hydrocephaly (8.0%) (Fig. 1F), angular tail (23.0%) (Fig. 1F) and open eyelid (14.0%) (Fig. 1G).

The fetuses from dose group 12.5 µg/g B.W showed morphological anomalies like distorted axis (25.20%), hydrocephaly (9.43%) (Fig. 1H), meromelia (14.21) (Fig. 1H), angular tail (23.32) (Fig. 1H), agnathia (Fig. 1H), anophthalmia (14.0) (Fig. 1H), excencephaly (8.06) (Fig.1I), phocomelia (13.21) (Fig. 1I). The dose group 25.0 µg/g B.W included abnormalities like distorted axis (27.30%), exencephaly (10.90%), open eye lids (47.23%), anophthalmia (14.00%), fore limb displacement (40.10%), extension of fore limbs (14.21%), hind limb displacement (32.30%), phocomelia (10.11%), subcutaneous hemorrhages (57.70%) and angular tail (33.34%). Various anomalies and their percentages increased by increasing dose concentrations (Table III).

Histological studies through brain and abdominal regions were carried out to determine the anatomical defects. The selected brain sections from the control showed well developed lateral ventricles, spinal cord and diencephalons, third and fourth ventricles (Fig. 2A, B). Sections from dose group 12.5 µg/g B.W showed dilation of 3rd and 4th ventricles known as internal hydrocephaly (Fig, 2C, D). In dose group 25.0 µg/g B.W showed dilation of 3rd ventricle, underdeveloped lateral part of cerebellum and absence of 3rd and 4th ventricles (internal microcephaly) (Fig. 2E, F).

Sections through lungs and cardiac region in control showed well developed middle, cardiac and accessory lobes of lungs and chambers of heart (Fig. 3A). Fetuses of dose group 12.5 µg/g B.W showed small portion of middle lobe is missing, necrosis in accessory lobe of left lung, atrium is undeveloped and microcardia (Fig. 3B). In dose group 25.0 µg/g B.W underdeveloped middle lob, hydroparicardium and necrosis in ventricles were observed (Fig.3C,D).

The selected liver sections from the control showed well developed spinal cord, diaphragm, liver lobes, lobar interzones and hepatic veins (Fig. 4A). Sections from dose group 3.13 µg/g B.W showed necrosis in the liver (Fig. 4B). In dose group 6.25 µg/g B.W showed necrosis and small left liver lobe (Fig. 4C). The selected sections of kidney from the control showed well developed kidney, urinary bladder and spinal cord (Fig. 5A). Sections from dose group 3.13 µg/g B.W showed necrosis (Fig. 5B), and in dose group 6.25 µg/g B.W showed one kidney on right side is missing (Fig. 5C).

Skeleton preparation showed that well developed skeletal formation and calcification occur in control fetuses (Fig. 6A). Where as in dose group 3.13 µg/g B.W, fetuses showed slightly less calcification in limbs and tail region (Fig. 6B). In dose group 6.25 µg/g B.W fetuses with less calcification were observed (Fig. 6C). In dose group 12.5 µg/g B.W, fetuses showed slightly less calcification in limbs and tail region (Fig. 6D). In dose group 25.0 µg/g B.W almost all fetuses with unossified skeletons were observed (Fig. 6E).

DISCUSSION

Cadmium is one of the heavy metals. It is the soft, ductile, bluish white electropositive metal, which is resistant to corrosion (Sittig, 1985). The major source of exposure of cadmium to the persons of general population is food. Plants readily take up cadmium from

Table I.- Embryo toxic effects of cadmium on 18-day-old mouse fetuses recovered from pregnant mice exposed to different doses on days 6 to12 of gestation.

Dose groups (µg/g B.W)	No. of implantations (NI)	No. of fetuses Recovered (N)	Malformed fetuses (%)	Resorbed fetuses (%)
Control	50	50	0.00	0.00
3.13	49	40	65.22	34.78
6.25	41	32	71.52	28.48
12.5	36	28	71.88	28.12
25.0	28	20	60.85	39.15

Table II.- Morphometric analysis of 18-day-old mouse fetuses recovered from pregnant mice, administrated orally with different concentration of cadmium on day 6 to12 of gestation.

Parameters doses (µg/g B.W)	Body weight (mg±S.E.)	CR length (mm±S.E.)	Head circumference (mm^2 ±S.E.)	Eye circumference (mm^2 ±S.E.)	Forelimb length (mm±S.E.)	Hindlimb length (mm±S.E.)	Tail length (mm±S.E.)
Control	1334.81±24.36	27.79±0.85	38.35±0.69	3.54±0.16	6.68±0.21	7.29 ±0.19	8.88±0.46
3.13	677.46±87.37*	14.22±1.19*	23.09±3.57*	2.3 ±0.46*	4.76±0.81*	4.49 ±1.09*	6.78±1.16*
6.25	532.42±170.10**	10.37±3.17**	16.31±1.46**	1.79±0.38**	3.88±1.95**	3.7±2.39**	5.44±1.52**
12.5	588.18±92.26**	9.67±2.07**	14.64±6.08**	1.76±1.16**	2.85±1.43**	3.32±0.98**	4.16±0.85**
25.0	431.22±161.11***	8.06±2.10***	12.54±1.50***	1.68±0.24**	2.34±1.85**	3.02±3.29**	2.34±1.52**

Entries Key: parameter size ±Standard Error
Asterisks show significant differences compared to control mice: *=P< 0.05, **=P< 0.01, ***=P< 0.001

Table III.- Developmental anomalies induced by cadmium in 18-day-old mouse fetuses recovered from pregnant mice, administrated orally with different concentrations on days 6 to 12 of gestation.

Parameters dose (µg/g) B.W	Axis (%)	Brain (%)	Eyes (%)	Forlimbs (%)	Hindlimbs (%)	Skin (%)	Tail (%)
Control	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)
3.13	(0.00)	Hydrocephaly (10.43)	Open Eyelids (13.30) Cryptophthalmia (7.33)	Limb displacement (21.00) Micromelia (8.23)	Limb displacement (14.00)	(0.00)	Wrinkled tail (13.00)
6.25	Distorted axis (10.01)	Hydrocephaly (8.00)	Open eyelids (14.00)	Limb displacement (23.10) Micromelia (14.08)	Limb displacement (13.20)	Subcutaneous hemorrhage (23.57)	Angular tail (23.00) wrinkled tail (16.76)
12.5	Distorted axis (25.20)	Hydrocephaly (9.43) Excencephaly (8.06)	Open eyelids (43.30) Anophthalmia (14.0)	Limb displacement (34.10) Meromelia (14.21)	Limb displacement (25.00) Phocomelia (13.21)	Subcutaneous hemorrhage (45.20)	Angular tail (23.32)
25.0	Distorted axis (27.30	Exencephaly (10.90)	Open eyelids (47.23) Anophthalmia (14.50)	Limb displacement (40.10) Extension (14.21)	Limb displacement (32.00) Phocomelia (10.11)	Subcutaneous hemorrhage (57.70)	Angular tail (33.34)

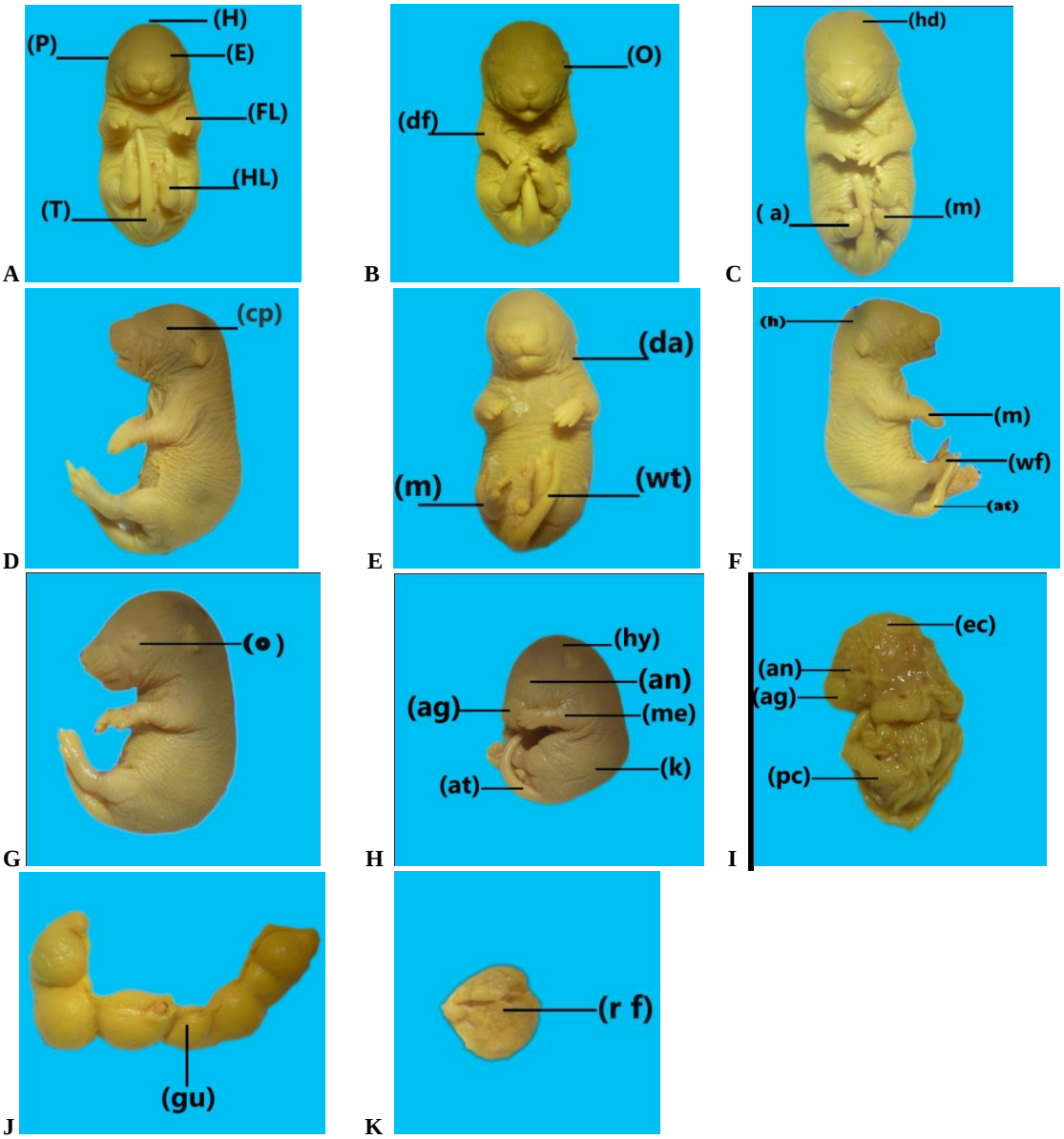

Fig. 1. Macrophotographs of 18-day-old mouse fetuses recovered from mothers exposed to different doses of cadmium on days 6 to12 of gestation. A, control; B, C and D, 3.13 µg/g B.W; E, Fand G, 6.25 µg/g B.W; H and I, 12.5 µg/g B.W and J and K, 25.0 µg/g B.W. Note: H, well developed head; E, well formed eyes with closed eyelids; FL, well developed forelimbs; P, well developed pina; HL, well developed hindlimbs; T, well developed tail; a, acheiria, ag, agnathia, an, anophthalmia; at, angular tail; cp, cryptophthalmia; da, distorted axis, df, distorted forelimbs; ec, exencephaly; gu, gravid uterus with resorbed fetuses; h, hemorrhages; hy, hydrocephali, k, kyphosis; k, kyphosis; m, meromelia, m, micromelia, me, meromelia, o, open eyelids; pc, phocomelia; rf, resorbed fetus; wf, webbed feet; wt, wrinkled tail.

the soil contaminated by the fall-out from the air, cadmium containing water used for irrigation and cadmium containing fertilizers are being utilized (Zhang *et al*., 2009).

During the present study the litter size was decreased and resorptions were increased by increasing the dose concentration (Table I). These findings are in accordance with the studies by Buchet *et al*. (1990). They

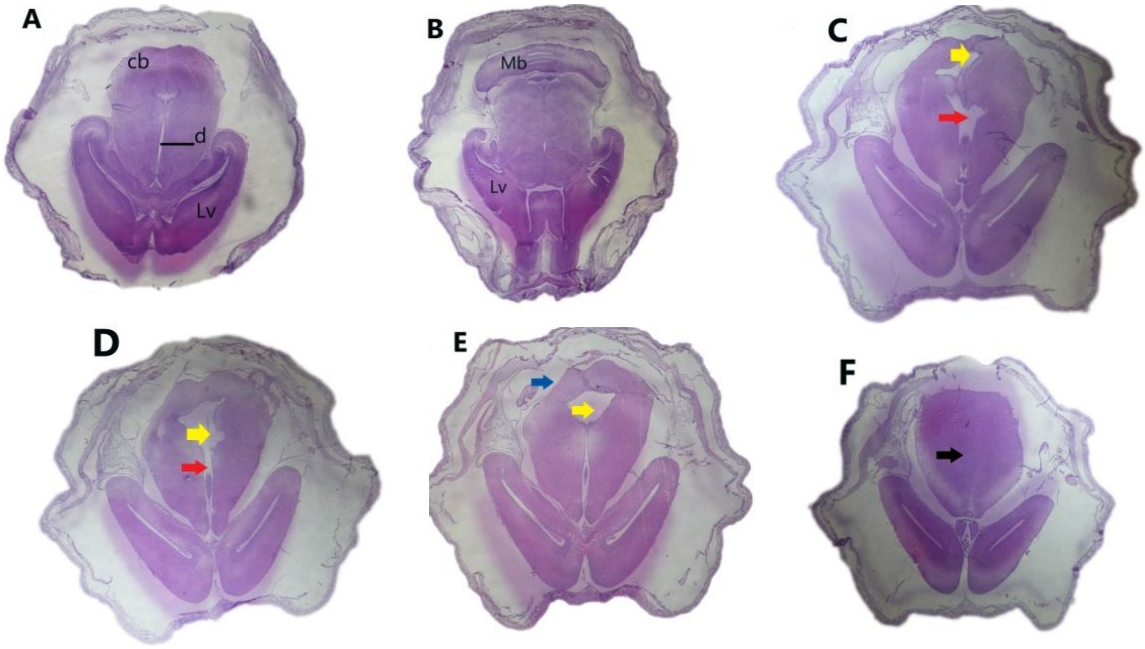

Fig. 2. Microphotographs of sections through brain region of 18-day-old mouse fetuses recovered from mothers exposed to different doses of cadmium on days 6 to 12 of gestation. A, control; B, 6.25 µg/g B.W; C and D, 12.5 µg/g B.W; E and F, 25.0 µg/g B.W. Note: Mb, midbrain; Cb, cerebellum; d, diencephalon; Lv, lateral ventricles; yellow arrows: dilation of 4th ventrical; red arrows, dilation of 3rd ventrical (internal hydrocephaly); blue arrow, underdeveloped lateral part of cerebellum; black arrow, absence of 3rd and 4th ventricles (internal microcephaly).

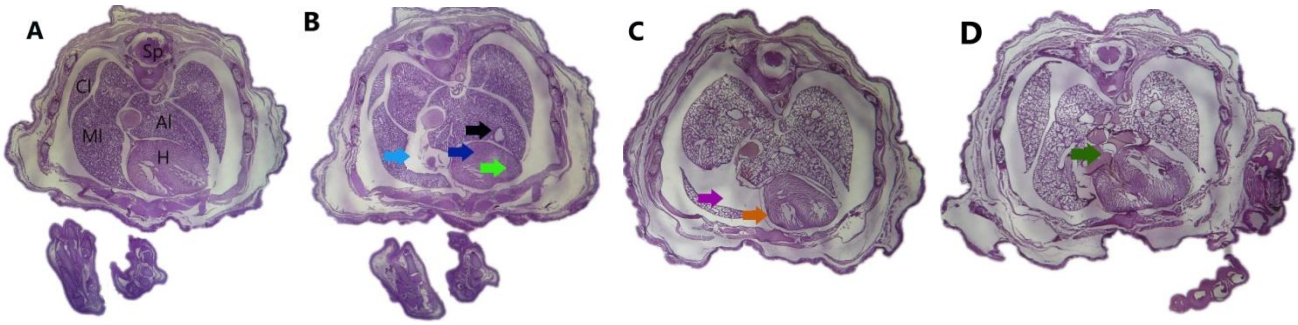

Fig. 3. Microphotographs of sections through lungs and heart region of 18-day-old mouse fetuses recovered from mothers exposed to different doses of cadmium on days 6 to 12 of gestation. A, control; B, 12.5 µg/g B.W; C and D, 25.0 µg/g B.W. Note: Sp, spinal cord; Ml, middle lobe of right lung; Cl, caudal lob; Al: accessory lobe; H, heart; cyan blue arrow: small portion of middle lobe is missing; parrot green arrow, left ventrical is underdeveloped; purple arrow, underdeveloped middle lobe; orange arrow, hydroparicardium; pea green arrow: necrosis in atrium; dark blue arrow, microcardia; black arrow: necrosis in accessory lobe of lung.

studied that the injection of higher cadmium dose to female mice caused ethiothelial changes in the blood vessels and low birth weights.

Limb defects observed in this study were limb displacement, meromelia, phocomelia and extension of limbs which were parallel to the study of Memon and Pratten (2013). They concluded from their studies that cadmium caused limb defects by injection of cadmium to the pregnant mice on day 8, 9, 10 of gestation. Subcutaneous hemorrhages were also observed in this study which were supported by the study of Anthony *et al.* (1978). He claimed that cadmium in body bind to the red blood cells that causes blocking of the blood vessels which lead to hemorrhages.

The anomalies related to CNS were included microcephaly, hydrocephaly and exencephaly. Veeriah *et*

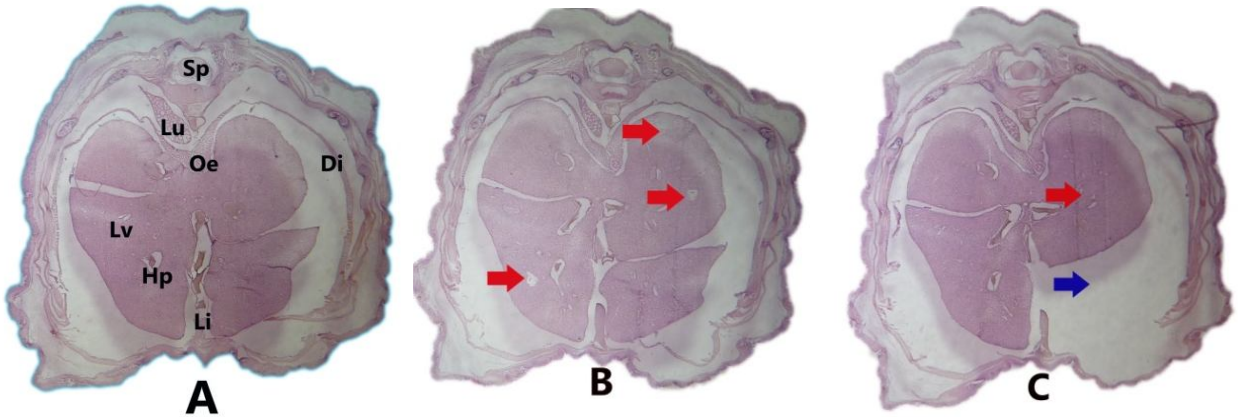

Fig. 4. Microphotographs of transverse sections of 18-day-old mouse through liver region fetuses recovered from mothers exposed to different doses of cadmium on days 6 to 12 of gestation. A, control; B, 3.13 µg/g B.W; C, 6.25 µg/g B.W. Note: Di, diaphram; Hp: hepatic vein; Li, lobar interzone; red arrows: necrosis; blue star: misshapen left lobe of liver; Lu, lung; Lv, liver; Oe, oesophagus; Sp, Spinal cord.

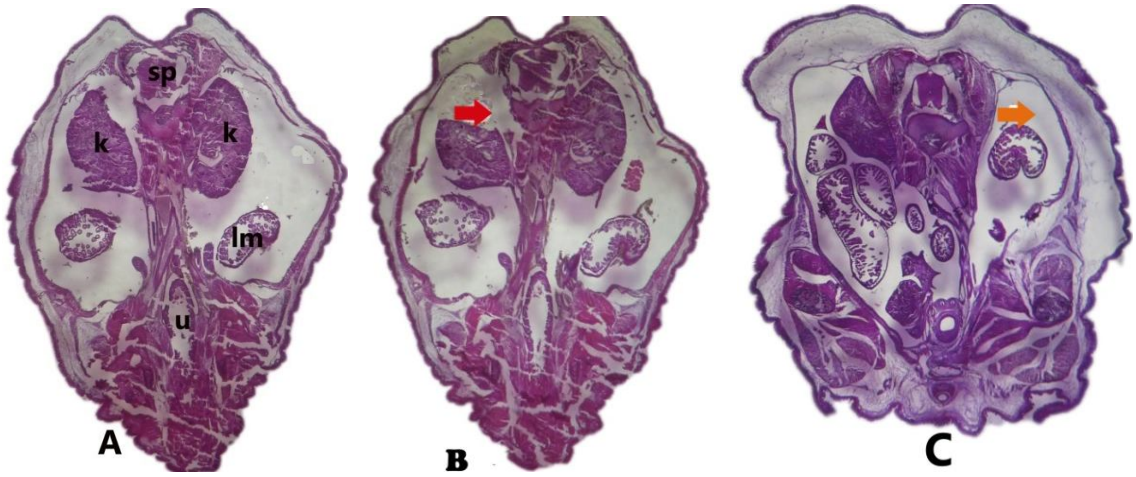

Fig. 5: Microphotographs of transverse sections of 18-day-old mouse through kidney region fetuses recovered from mothers exposed to different doses of cadmium on days 6 to 12 of gestation. A, control; B, 3.13 µg/g B.W; C, 6.25 µg/g B.W. Note: k, kidney; lm, medullary region of left testes; red arrow, necrosis; orange arrow, right kidney missing; Sp, spinal cord; u, urinary bladder.

al. (2014) reported the association of CNS malformation and cadmium.

Furthermore skeletal preparation showed reduced calcification and unossification in this study which were in accordance to study of Sughis *et al*. (2001). They found that cadmium in bone may interfere with calcification, decalcification and bone remodeling.

Along with a number of fetal morphological defects, histopathological anomalies of brain found during this study were dilations and absence of ventricles and underdeveloped cerebellum which were supported by Sun *et al*. (2005) study. They reported the cadmium caused neuronal cell apoptosis and necrosis. Sections through the cardiac region showed the abnormalities microcardia, necrosis in atrium, underdeveloped ventricles, hydropericardium and lungs abnormalities including underdeveloped lobes of lungs, which were also observed by Yang *et al*. (1997). They described cadmium induced oxidative cellular damage in fetal lungs cells. Histopathological anomalies of liver found in this study were necrosis and misshapen lobes which were supported by Hideaki *et al.* (2004). They studied that cadmium is accumulated in the liver and causes necrosis and hepatotoxicity. Sections through kidney region showed necrosis and missing of left kidney that is linked to the study of Chen *et al.* (2013). They concluded by

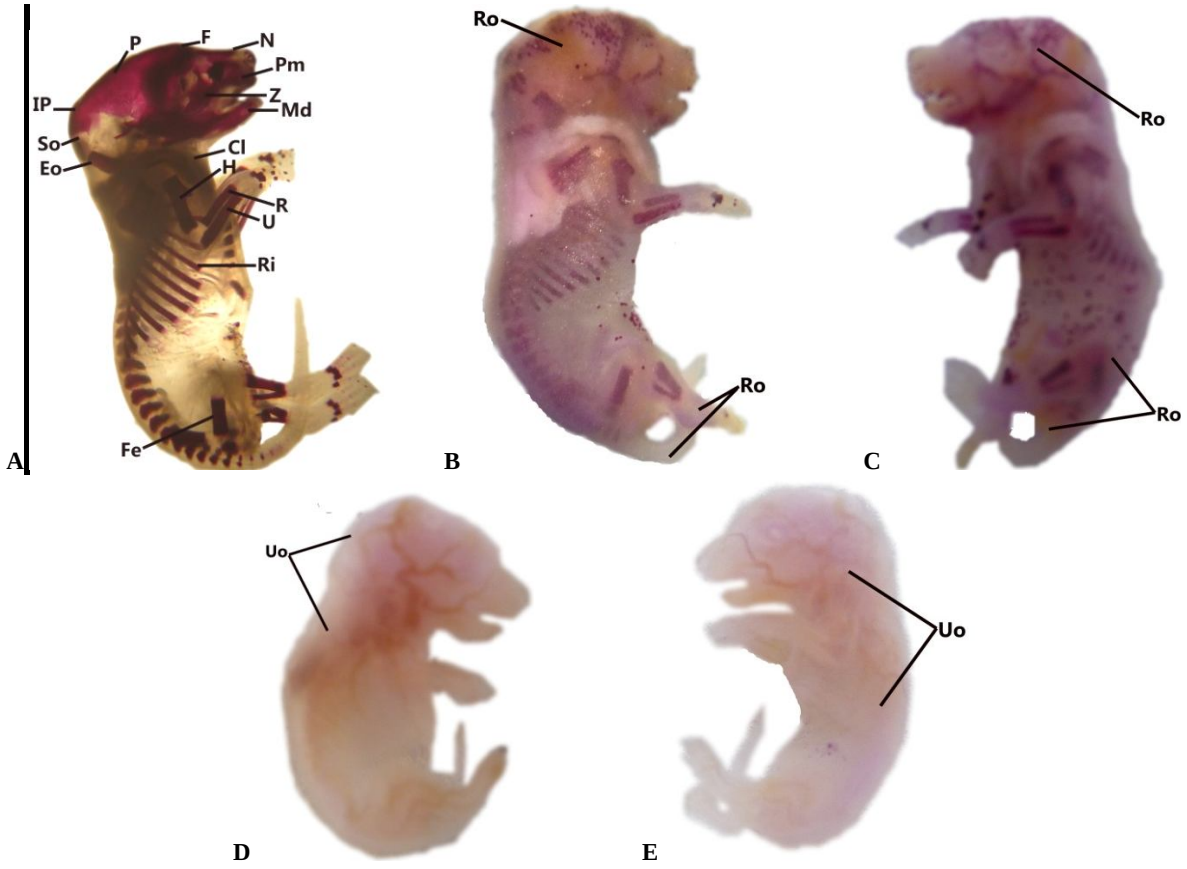

Fig. 6. Macrophotographs of Fetal skeletal preparations of 18-day-old mouse fetuses recovered from mothers exposed to different doses of cadmium on days 6 to 12 of gestation.Note: A, control skeleton showing well ossified skeleton; B, skeleton from 3.13 µg/g BW; C, skeleton from 6.25 µg/g BW; D, from 12.5 µg/g BW; E, from 25.0 µg/g BW. **Note:** Cl, clavical; Eo, exoccipital; F, frontal; Fe, femur; Fi, fibula; H, humerus; Ip, interparital; N, nasal; P, parietal; Pm, premaxilla; R, radius; Ri, ribs; Ro, reduced ossification; So, supraoccipital; U, ulna; Uo, unossified; Z, zygomatic.

their study that different doses of cadmium cause renal hypertrophy and degenerative changes in tubules and glomeruli were evident.

CONCLUSION

On the basis of this study it is concluded that cadmium showed teratogenic effects in developing mice fetuses. It caused a vast range of morphological, morphometric, histological and skeletal abnormalities in mice. This study will provide awareness about the toxic effects of this metal particularly from the stand point of view for its teratogenic and embryotoxic effects. In the light of the above mentioned findings, it is suggested that females should take care and avoid exposure of cadmium during critical stages of pregnancy.

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