Pakistan J. Zool., vol. 49(4), pp 1383-1389, 2017. **DOI**: http://dx.doi.org/10.17582/journal.pjz/2017.49.4.1383.1389

Exposure of Pregnant Mice to Hexavalent Chromium Causes Fetal Defects

Madeeha Arshad[1], Naveed Ahmad[2,*], Muhammad Khalid[3], Asmatullah[1], Mohammad Tahir[2], Khadija Naveed[1] and Asia Iqbal[4]

[1]Department of Zoology, University of the Punjab, Quaid-i-Azam Campus, Lahore, Pakistan
[2]Department of Environment Sciences, COMSATS Institute of Information Technology, Vehari Campus, Vehari, Pakistan
[3]Department of Veterinary Clinical Sciences, The Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, Hertfordshire, AL9 7TA, UK
[4]University of Veterinary and Animal Sciences, Pattoki Campus, Pattoki, Pakistan

ABSTRACT

The present study is aimed at determining the teratogenic effects of different doses of Cr VI, administered to pregnant mice. LD_{50} of Cr VI, was calculated as 88mg/kg of B.W in pregnant mice. Among 05 groups of pregnant females (10 females in each group), three were treated with 50%, 25% and 12.5% of the LD_{50} of Cr VI, respectively, with one control and one vehicle control group. Fetuses were recovered on 18th day of gestation and fixed for morphometric and morphological studies. Experimental groups showed variable degree of fetal abnormalities, along with reduction in body weights parallel to increase in dose concentration, as compared to control and vehicle control. Morphometric studies showed significant differences in nervous, integumentary and skeletal systems against control. Exencephaly, Omphalocoel, hygroma and limb abnormalities were recorded. It is shown that Cr VI causes teratogenic effects in mice embryos.

Article Information
Received 2 March 2016
Revised 8 June 2016
Accepted 27 November 2016
Available online 19 July 2017

Authors' Contribution
KN conceived and designed the study, AI performed photography, histology by MK, NA and MT performed experimental work. Asmatullah analyzed the data, MA wrote the article.

Key words
Hexavalent chromium [Cr (VI)], Teratogenic effects, Exencephaly, Omphalocoel, Hygroma.

INTRODUCTION

Atmospheric deposition of heavy metals in soil is a major source of their exposure to living organisms (Nicholson *et al*., 2003). Among these metals chromium (Cr) is being sixth most abundant in earth crust. According to WHO normal value of Cr VI in drinking water is 50 µg/L but in New Caledonia, California, Italy, Zimbabwe, Mexico and in Pakistan it is above the prescribed limit (73 µg Cr VI/L) (Oze *et al*., 2006), Cr level is shown to be high in different parts of Pakistan, specially, in big urban areas like Lahore and Gujranwala (Aftab *et al*., 2011; Bostan *et al*., 2007). Its level was much higher than set levels of FAO and EPA. Cr becomes part of ground water by leaching and used for drinking and other household jobs (Boncompagni *et al*., 2003).

Heavy metals including Cr are being involved in developmental toxicity besides general toxicity (Domingo, 1994). It is present in blood (4.58±0.62 ng/ml of serum) and can pass through placenta (Sundararaman *et al*., 2012). Its placental transfer was studied in rats using atomic absorption spectroscopy. Intragastric administration of ^{51}Cr, incorporated into brewer's yeast, to pregnant rats led to significant labeling of the newborn (Mertz *et al*., 1969). Reproductive physiology, of both males and females, is affected prior to effecting embryonic development *i.e.* damage to convoluted seminiferous tubules epithelium, reduction of spermatozoa formation and increase in prevalence of teratospermia, atrtic follicles congestion in stromal tissue, decrease in follicle and oocyte number, increased level of Cr(VI) in blood, increased duration of estrus cycle, disintegrated cell membranes of two layered follicular cells and altered villiform mitochondria in thecal cells (Li *et al*., 1999; Murthy *et al*., 1996; Elbetieha and Al-Hamood, 1997).

Certain exposure levels of Cr(VI) cause teratogenic effects in avian and mammalian models at various stages of embryonic development *e.g.* distorted embryos, reduction in the number of implantations and number of fetuses and reduced ossification (Erdélyi *et al*., 2006; Kanojia *et al*., 1996).

The present study describes the teratogenetic effects of Cr VI administered to pregnant mice.

MATERIALS AND METHODS

Six weeks old Swiss Webster albino male and female mice (Weight: 30±2) free from any viral, bacterial or

* Corresponding author: naveedahmad@ciitvehari.edu.pk
0030-9923/2017/0004-1383 $ 9.00/0

parasitic agent were purchased from Veterinary Research Institute, Lahore, Pakistan and were allowed to mate freely (1 male:2 female) in steel cages. Cages were sanitized prior to placing mice in them and were placed in well ventilated animal house with maintained temperature (20 ±02°C). Cages contained food pellets (National Feeds Ltd, Feed # 13, Crude Protein: 16.5±1%, Energy: 2700 Kcal/ Kg ±100) and water *ad libitum*. Safe and healthy condition was provided by continuous cleaning and maintaining temperature at 20±02°C.

Estimation of gestation period

Mated females were identified on the basis of deposition of semen at vaginal opening as sperm plug. It was considered as Day "0" of gestation. Females with sperm plug were separated from males and housed in different cages with minimum disturbance.

Determination of LD_{50} of $K_2Cr_2O_7$

LD_{50} was determined using Probit analysis (Weinberg *et al*., 1998). Sixty (60) pregnant females were divided into six groups, each with ten dams, were force fed orally via rubber tube cut from butterfly needle on 6th day of gestation. Mortality of only those females were considered, which died on or before 8th day of gestation. Potassium dichromate ($K_2Cr_2O_7$, BDH Prolabo Chemicals), was used as chemical source for Cr (VI). Six random doses used for all six groups of pregnant mice were prepared by dissolving 5, 25, 50, 100, 150 and 200 mg/kg of B.W. concentrations of $K_2Cr_2O_7$ in water. LD_{50} was calculated as 88 mg/kg of B.W.

Procedure adopted

In three experimental groups, females were force fed with 50% (44 µg/g B.W.), 25% (22 µg/g B.W.) and 12.5% of LD_{50} (11 µg/g B.W.) of $K_2Cr_2O_7$ dissolved in 0.1 ml of water on 6th day of gestation. Doses were given singly on GD6. In addition one control and one vehicle control groups were also maintained.

All dams were euthanized and given cesarean section on 18th day of gestation. Fetuses were recovered and fixed in Bouin's fluid for 48 h for easy morphometric and morphological studies (Yoritaka *et al*., 1996). Wet weight, crown rump length, head circumference, eye circumference, fore and hind limbs lengths and tail length was measured for morphometric analyses with digital balance and vernier caliper for each fetus recovered. For morphology, external structures were observed with the help of binoculars according to Makris *et al*. (2009).

Histological sections were proceeded and stained by the method of Khadija *et al*. (2011). Method of Kawamura *et al*. (1990) was used for fetal skeletal preparation.

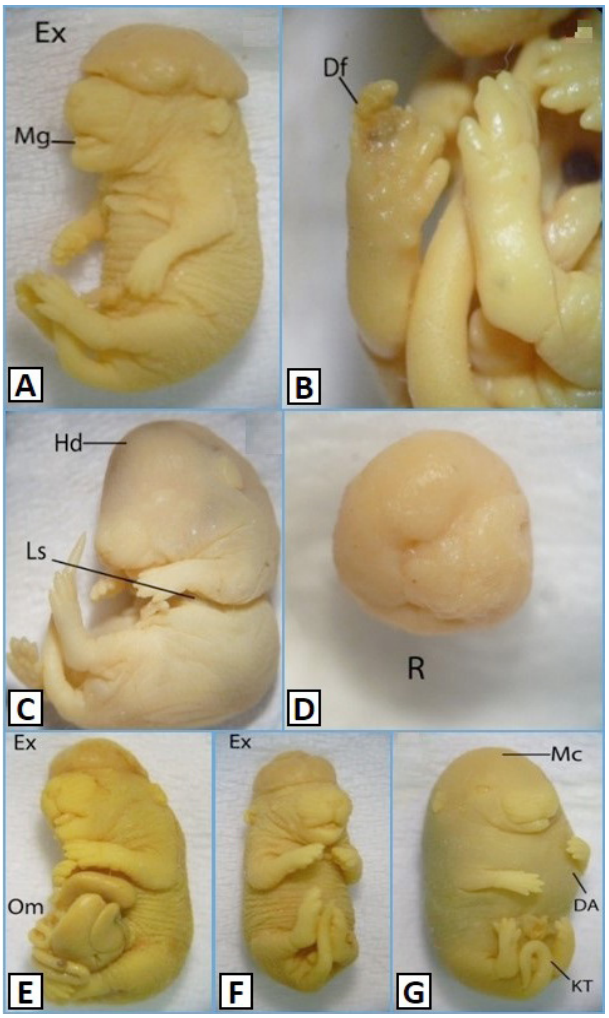

Fig. 1. Morphological features of 18 days oldfetuses of mice administration with $K_2Cr_2O_7$ at a dose of 22µg/g B.W. (A, B), and 11µg/g B.W. (C-G) Control, Vehicle control (VC); Df, deformed; Hd, hydrocephaly; Ls, laproschisis; Mg, macroglossia; R, resorption. DA, dilated abdomen; Ex, exencephaly; KT, kinked tail; Mc, Microcephaly; Om, Omphalocoel. Magnificaton: A=10X, B&D=25X, C, E&G=8X, F=9X.

Statistical analysis

One-way ANOVA was applied on means of all parameters in micrometery with 10 females in each group, further group wise comparison was done by applying Tukey's HSD (Honestly Significant Difference) test using IBM SPSS 20.

RESULTS

Morphological studies

Morphologically, fetuses in all experimental groups

showed variable degree of abnormalities, as compared to control and vehicle control (Table I). All experimental groups had fetuses having abnormalities like exencephaly, open eyes, sub cutaneous hemorrhages, macroglossia, hyperextension of limbs and runt fetuses. Fetuses from highest (44µg/g B.W.) and lowest (11µg/g B.W.) dose groups shared abnormalities like microcephaly, synotia, microtia, distended thoracic cavity, limb micromelia kinked and short tail. Hydrocephaly, resorptions and edema were found in highest (44µg/g B.W.) and middle (22µg/g B.W.) dose groups (Figs. 1, 2).

Abnormalities found exclusive to experimental groups are as follows: Craniostenosis, short snout, anophthalmia, microphthalmia, exophthalmia, palpabral coloboma, kyphosis, lordosis, spina bifida, prognathia, scoliosis, limb malrotation and deformation, clinodactyly, ectrodactyly, apodia, ankylodactyly, fused digits, flexed paw and hooked tail, at 44µg/g B.W.

Anotia, branchignathia, archinia, limb hyperflexion, laparoschisis, paw dysplasia, amelia and branched tail Omphalocoele at 22µg/g B.W. and 11µg/g B.W.

Table I.- Descriptive data of experiment, observed from 18 days old fetuses from different dose groups of hexavalent chromium. Litter size varied among groups, all fetuses were observed (normal and abnormal) including resorptions. Table also shows abnormalities present in each dose group.

Dose µg/g B.W.	Control	VC	11	22	44
No. of females	10	10	10	10	10
Litter size	102	88	83	50	74
Normal	102	88	72	30	38
Abnormal	0	0	6	4	11
Resorptions	0	0	2	6	12
No. of abnormalities					
Anophthalmia	-	-	-	-	1
Limb hyperextension	-		3	2	
Limb hyperflexion	-	-	-	-	2
Limb malrotation	-	-		2	
Limb micromelia	-	-	3	-	4
Spina bifida	-	-	-	-	4

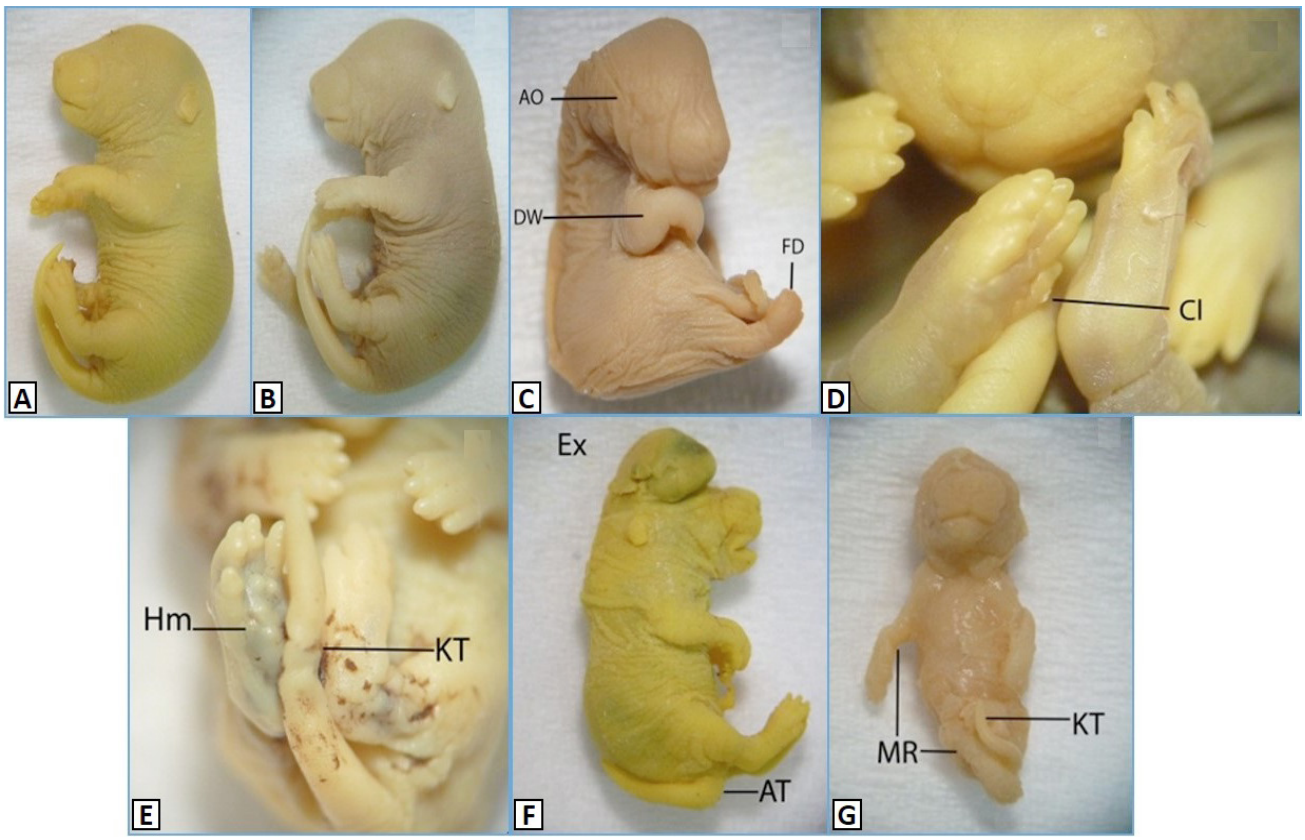

Fig. 2. Morphological features of 18 days old fetuses of mice, administered with $K_2Cr_2O_7$ at a dose of 44µg/g B.W. A, Control; B, VC, vehicle control; AO, anophthalmia; AT, abnormal tail; Cl, clinodactyly; DW, drooped wrist; Ex, exencephaly; FD, fused digits; Hm, hemorrhage; KT, kinked tail; MR, malrotation. Magnification: A=7X, B&C=8X, D=25X, E=20X, F=10X, F=9X.

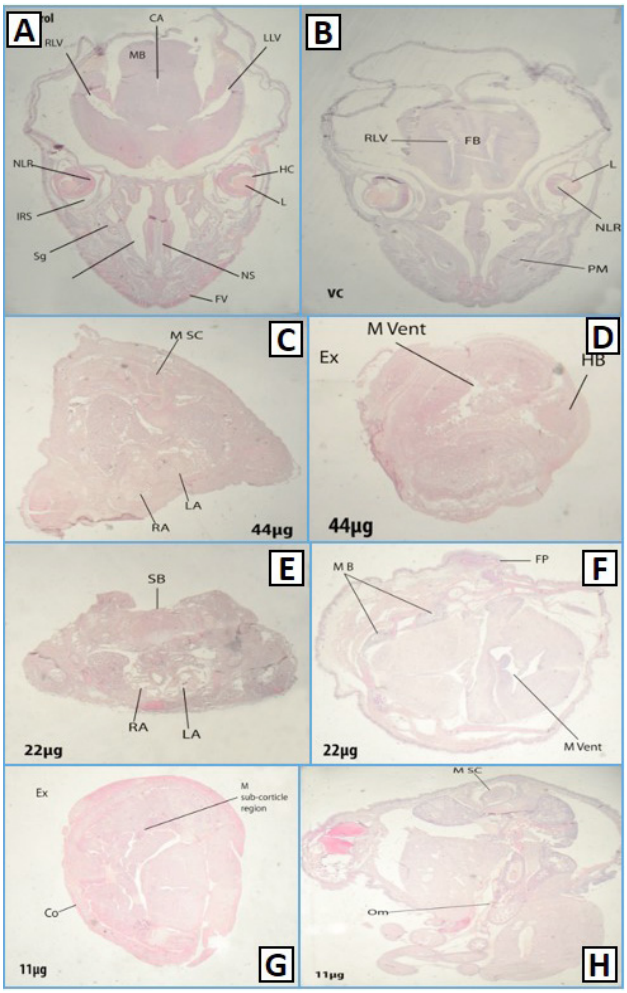

Fig. 3. Histological transverse sections of Control (A), Vehicle control (B) of different dose groups (44,22,11µg/g B.W) (C-H), 18 days old fetus from brain, Ex: exencephaly, M Vent: misshapen ventricle, HB: hind brain, MB, mandibular gland; FP, fused pinna, M sub corticle region: misshapen sub corticle region; Co, cortex; RA, right atrium; LA, left atrium; RA, right atrium; M Sc, misshapen spinal cord; SB, spina bifida; Om, Omphalocoel; NC, nasal cavity; Sg, serous gland; IRS, intra retinal space; RLV, right lateral ventricle, MB, mandibular gland; CA, cerebral aquaduct; LLV, left lateral ventricle; HC, hyaloid cavity; NS, nasal septum; FV, follicles of vibrissae; L, lens; FB, primordium of 1frontal bone; NLR, neural layer of retina; PM, pre molar.

Morphometric studies

All morphometric parameters, except eye circumference and forelimb length, in 44µg/g B.W. exposure group, including mean fetal weight and head circumference in 22µg/g B.W. dose group and hind limb length and tail length in both 22µg/g B.W. and 11µg/g B.W. dose groups were significantly ($p < 0.05$) different from controls. Vehicle control group had no difference from normal control group (Table II).

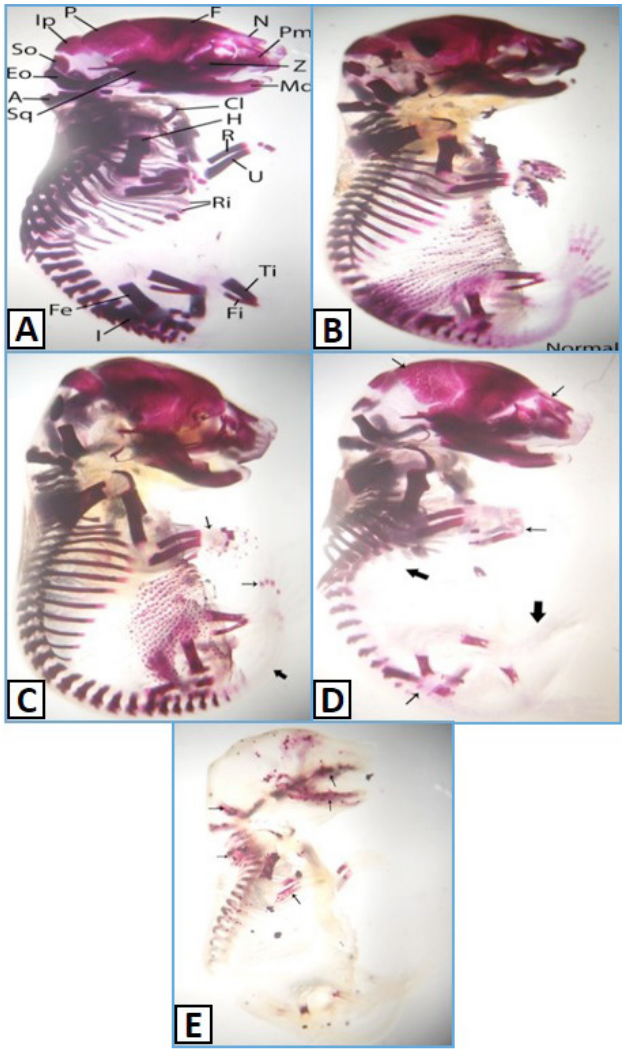

Fig. 4. Skeletons of control (A) and vehicle control (B), and selected sections from different dose groups (11,22,44µg/g B.W) (C-E). Simple arrows show less degree ossification while bold arrows show no ossification in three different dose groups. F, Frontal; N, Nasal; Pm, Pre Maxila; Z, Zygomatic; Md, Mandible; C, Clavicle; H, Humerus; R, Radius; U, Ulna; Ri, Ribs; Ti, Tibia; Fi, Fibula; I, Ilium; Fe, Femur; Sq, Squamosal; A, Atlas; Eo, Exocipital; So, Supraocipiltal; Ip, Interperitonial; P, Parietal.

Histological and skeletal studies

Anatomical sections of control and vehicle control mice fetuses showed normal left and right ventricles of brain, serous glands, intra-retinal space, lens, neural layer of retina, mandibular gland and cerebral aqua duct,

Table II.- Various morphometric parameters (mean±SEM) of 18 days old fetuses from different dose groups of hexavalent chromium. Litter size varied among groups, all fetuses were observed (normal and abnormal) excluding resorptions.

Dose µg/g B.W.	Weight (mg)	CR length (mm)	Head circumference (mm)	Eye circumference (mm)	Fore limb length (mm)	Hind limb length (mm)	Tail length (mm)
C	1342 ± 27	21.68 ± 0.28	25.93 ± 0.29	6.73 ± 0.16	7.15 ± 0.11	9.58 ± 0.24	11.19 ± 0.14
VC	1355 ± 19	21.41 ± 0.2	25.60 ± 0.18	6.66 ± 0.08	7.13 ± 0.16	9.28 ± 0.08	10.96 ± 0.13
11	1322 ± 58	21.32 ± 0.32	25.44 ± 0.43	6.47 ± 0.08	7.1 ± 0.03	8.94 ± 0.09*	10.38±0.08*
22	1117 ± 38*	21.14 ± 0.22	24.75± 0.23*	6.42 ± 0.01	6.96 ± 0.07	8.61 ± 0.11*	10.3 ± 0.19*
44	995 ± 26*	20.07 ± 0.26*	23.55*± 0.23	6.36 ± 0.1	6.96 ± 0.04	8.11 ± 0.09*	10.36±0.12*

* =p<0.05.

hyaloid cavity, nasal septum and nasal cavity, primordium of frontal bone, follicle of vibrissae (Fig. 3), while selected sections from dose group 44µg/g B.W exhibit misshapen spinal cord, left atrium, right atrium, exencephaly, misshapen ventricle and hind brain. Fetal sections from 22µg/g B.W revealed defects like spina bifida, right atrium, left atrium, mandibular gland and fused pinna. Defects like exencephaly, misshapen subcorticle region, misshapen spinal cord and omphalocoele were detected in sections of dose group 11µg/g B.W (Fig. 3).

Normal degree of ossification was observed in the skeleton of control and vehicle control mice fetuses (Fig. 4). However, the degree of ossification was decreased with increase in dose concentrations (Fig. 4).

DISCUSSION

This study is focused on developmental toxicity of Cr VI. Different dose groups were established with control and vehicle control group to check toxic effects of Cr VI on various anatomical parameters of fetuses.

Cr VI exposure can cause significant damage to pubertal development through alteration of antioxidants, anaemia, and altered hormone levels in utero through pubertal period. Induction of oxidative stress can be one of the mechanisms, causing Cr VI based cellular deteriorations. It was reported that Cr VI exposure adversely affects reproductive function by crossing through placenta barrier and even breast feeding. Higher levels of H_2O_2 and lipid peroxidation were seen with low levels of specific activities of antioxidant enzymes (Samuel *et al*., 2012). Cr VI causes oxidative stress and production of reactive oxygen species, which cause DNA damage, lipid peroxidation and alteration in calcium metabolism (Stohs and Bagchi, 1995; Valko *et al*., 2005).

It is now well established that oxyanions like Cr VI can pass through placenta as being in blood plasma may be with bounded form (Miyauchi *et al*., 2006). Three major signalling pathways were found to be active during Cr VI toxicity: i) activation of detoxification genes; ii) induction of signal transduction effectors; and iii) epigenetic modification of chromatin marks. Cr VI causes teratogenic effects in gross external morphology, by passing through placenta. Further, *in vitro* studies would be helpful for checking functions of oxidation scavengers during the embryo toxicity production of Cr VI (Fan *et al*., 2012).

The results of our studies showed a high significance of exencephaly, anophthalmia, microcephaly, hydrocephaly, facial defects, and limbs defects omphalocoel intra growth retardation and resorptions. Marouani *et al*. (2010) performed experiments on rats and found similar results that are intrauterine growth retardation, facial defects, and resorptions. According to them the fetal defects may be caused by change in placental histology due to decidualcells atrophy and hypertrophy of blood lacunae. After administration of CrVI to the pregnant mice 6-12 days of gestation results were examined as there is incomplete or totally absent ossification in vertebral and facial as well as pelvic regions. Our findings are similar to those of Bailey *et al*. (2006), who observed cervical malformations after administration of Cr.

The difference in ossification of vertebrae may possibly be due the reduced enzymatic activity of alkaline phosphatase which alters calcium concentrations resulting in change in bone morphology and ossification (Sankaramanivel *et al*., 2006).

The present studies reveal that the administration of $K_2Cr_2O_7$ during organo-genetic period can damage the development of embryo. Further studies are of course required for understanding the mechanism of malformations.

ACKNOWLEDGEMENTS

This research was supported financially by Higher Education Commission (HEC) of Pakistan. We are highly

obliged for their support.

Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Aftab, T., Shafiq, T., Khan, B. and Chaudhry, M.N., 2011. Physicochemical properties, contamination and suitability of canal water for irrigation, Lahore Branch, Pakistan. *Pak. J. analyt. environ. Chem*., **12**: 88-94.

Bailey, M.M., Boohaker, J.G. and Sawyer, R.D., 2006. Exposure of pregnant mice to chromium picolinate results in skeletal defects in their offspring. *Birth Def. Res*., **77**: 244–249. https://doi.org/10.1002/bdrb.20081

Boncompagni, E., Muhammad, A., Jabeen, R., Orvini, E., Gandini, C. and Sanpera, C., 2003. Egrets as monitors of trace-metal contamination in wetlands of Pakistan. *Arch. environ. Contam. Toxicol*., **45**: 399-406. https://doi.org/10.1007/s00244-003-0198-y

Bostan, N., Ashraf, M., Mumtaz, A.S. and Ahmad, I., 2007. Diagnosis of heavy metal contamination in agro-ecology of Gujranwala, Pakistan using cattle egret (*Bubulcus ibis*) as bioindicator. *Ecotoxicology*, **16**: 247-251. https://doi.org/10.1007/s10646-006-0124-y

Domingo, J.L., 1994. Metal-induced developmental toxicity in mammals: A review. *J. Toxicol. environ. Hlth. Part A Curr. Iss*., **42**: 123-141.

Elbetieha, A. and Al-Hamood, M.H., 1997. Long-term exposure of male and female mice to trivalent and hexavalent chromium compounds: effect on fertility. *Toxicology*, **116**: 39-47. https://doi.org/10.1016/S0300-483X(96)03516-0

Erdélyi, M., Kertész, V., Balogh, K. and Mézes, M., 2006. *Effect of chromium on the development of the chicken embryo. Trace elements in the food chain.* Proceedings of an International Symposium on Trace Elements in the Food Chain, Budapest, Hungary, pp. 416-421. http://w3.chemres.hu/tefc2006/document/PB.pdf

Fan, Y., Ovesen, J.L. and Puga, A., 2012. Long-term exposure to hexavalent chromium inhibits expression of tumor suppressor genes in cultured cells and in mice. *J. Trace Elem. Med. Biol*., **26**: 188-191. https://doi.org/10.1016/j.jtemb.2012.04.009

Kanojia, R.K., Junaid, M. and Murthy, R.C., 1996. Chromium induced teratogenicity in female rat. *Toxicol. Lett*., **89**: 207-213. https://doi.org/10.1016/S0378-4274(96)03812-X

Kawamura, S., Kirohashi, A., Kato, T. and Yasuda, M., 1990. Cone-staining technique for fetal rat specimens without skinning and removing adipose tissue. *Congen. Anom.,* **30**: 93-95. https://doi.org/10.1111/j.1741-4520.1990.tb00498.x

Naeem, K., Ahmad, N. and Asmatullah, 2011. Embryotoxicity of orally administered misoprostol to pregnant mice. *Pakistan J. Zool*., **43**: 511-521.

Li, H., Chen, Q., Li, S., Xu, Y., Yao, W. and Chen, C., 1999. Studies on male reproductive toxicity caused by hexavalent chromium. *Chinese J. Preven. Med*., **33**: 351-353.

Makris, S.L., Solomon, H.M., Clark, R., Shiota, K., Barbellion, S. and Buschmann, J., 2009. Terminology of developmental abnormalities in common laboratory mammals (version 2). *Congr. Anom*., **49**: 123-246. https://doi.org/10.1111/j.1741-4520.2009.00239.x

Marouani, N., Tebourbi, O., Mokni, M., Yacoubi, T.M., Sakly, M., Benkhalifa, M. and Rhouma, B.K., 2010. Embryotoxicity and fetotoxicity following intraperitoneal administrations of hexavalent chromium to pregnant rats. *Zygote*, **19**: 229-235. https://doi.org/10.1017/S0967199410000274

Mertz, W., Roginski, E.E., Feldman, F.J. and Thurman, D.E., 1969. Dependence of Chromium transfer into the rat embryo on the chemical form. *J. Nutr*., **99**: 363-367.

Miyauchi, S, Srinivas, S.R., Fei, Y.J., Gopal, E., Umapathy, N.S., Wang, H., Conway, S.J., Ganapathy, V. and Prasad, P.D., 2006. Functional characteristics of NaS2, a placenta-specific Na⁺-coupled transporter for sulfate and oxyanions of the micronutrients selenium and Chromium. *Placenta*, **27**: 550-559. https://doi.org/10.1016/j.placenta.2005.05.004

Murthy, R.C., Junaid, M. and Saxena, D.K., 1996. Ovarian dysfunction in mice following chromium (VI) exposure. *Toxicol. Lett*., **89**: 147-154. https://doi.org/10.1016/S0378-4274(96)03803-9

Nicholson, F.A., Smith, S.R., Alloway, B.J., Carlton-Smith, C. and Chambers, B.J., 2003. An inventory of heavy metals inputs to agricultural soils in England and Wales. *Sci. Total Environ*., **311**: 205-219. https://doi.org/10.1016/S0048-9697(03)00139-6

Oze, C., Bird, D.K. and Fendorf, S., 2006. Non-anthropogenic hexavalent chromium genesis in soil and groundwater. *Geol. Soc. Am. Abs. Prog*., **38**: 438.

Samuel, J.B., Stanley, J.A., Sekar, P., Princess, R.A., Sebastian, M.S. and Aruldhas, M.M., 2012.

Persistent hexavalent Chromium exposure impaired the pubertal development and ovarian histoarchitecture in wistar rat offspring. *Environ. Toxicol*., https://doi.org/10.1002/tox.21810

Sankaramanivel, S., Jeyapriya, R. and Hemalatha, D., 2006. Effect of chromium on vertebrae, femur and calvaria of adult male rats. *Human exp. Toxicol*., **25**: 311–318. https://doi.org/10.1191/0960327105ht627oa

Stohs, S.J. and Bagchi, D., 1995. Mechanisms in the toxicity of metal ions. *Free Rad. Biol. Med.,* **18**: 321-336. https://doi.org/10.1016/0891-5849(94)00159-H

Sundararaman, P.G., Sridhar, G.R., Sujatha, V. and Anita, V., 2012. Serum chromium levels in gestational diabetes mellitus. *Ind. J. End. Met*., **16**: S70-S73. https://doi.org/10.4103/2230-8210.94266

Valko, M., Morris, H. and Cronin, M.T.D., 2005. Metals, toxicity and oxidative stress. *Curr. Med. Chem*., **12**: 1161-1208. https://doi.org/10.2174/0929867053764635

Weinberg, G.L., Vadeboncouer, T., Ramaraju, G.A., Garcia-Amaro, M.F. and Cwik, M.J., 1998. Pretreatment or resuscitation with a lipid infusion shifts the dose-response to bupivacaine-induced asytole in rats. *Anesthesiology*, **88**: 1071-1075. https://doi.org/10.1097/00000542-199804000-00028

Yoritaka,A.,Hattori,N.,Uchida,A.,Tanaka,M.,Stadtman, E.R. and Mizuno, Y., 1996. Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson disease. *Proc. natl. Acad. Sci*., **93**:2696-2701. https://doi.org/10.1073/pnas.93.7.2696