



Research Article

Protection Against Cadmium-Induced Abnormalities and Hepatotoxicity *in ovo* by *Allium sativum*

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Authors' Contributions

SA conceived the concept and executed the research. SS performed the experimental work. CA helped to study samples and wrote the manuscript.

Keywords

Cadmium chloride, *Allium sativum*, embryotoxicity, *Gallus domesticus*, hepatotoxicity

Abstract | Cadmium (Cd), a toxic heavy metal, is a potential carcinogen, mutagen and teratogen through bioaccumulation in tissues. Aim of present study was to analyze various developmental abnormalities by a sub-lethal dose of cadmium chloride and protective role of garlic (*Allium sativum*), to minimize the intensity of these toxicities. For this purpose, fertilized eggs of *Gallus domesticus* were randomly divided into four groups of forty eggs each. Control group was intact and untreated. Eggs of one group were injected with a sub-lethal dose of cadmium chloride (1.5 µg/egg) in albumin on 7th day of incubation. In another group, eggs were treated with cadmium chloride and fresh garlic juice (0.2 µg / egg) after a short interval of 10 minutes. Antidote group was injected with fresh garlic juice (0.2 µg / egg). Eggs of each group were incubated at 37 ± 0.5 °C and at 50-60% relative humidity till hatching. Natural hatching was obtained as 92.5, 32.5, 72.5, and 87.5% in control, dose, dose+antidote, and antidote groups respectively with delayed hatching in some cases in dose and dose+antidote groups. Chicks and dead embryos were studied for morphological and morphometric analysis. Liver tissues were dissected out from each group for histological analysis. Cadmium chloride induced significant developmental abnormalities such as reduced body weight and crown rump length, exencephaly, ablepharia, crossed beak, gastroschisis, crooked toes, non-alcoholic fatty liver and pyknosis. Fresh garlic juice has successfully attenuated these toxicities and increased the body weight, crown rump length. Results of present study determine that garlic is a potential antidote to ameliorate the cadmium induced teratogenic and hepatotoxic defects in developing *Gallus domesticus*.

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Introduction

Heavy metals including cadmium owing to human activities have become common environmental pollutants and bio accumulated in human through food chain (Jan *et al.*, 2015). Cadmium is known as group 1 carcinogen (Kim *et al.*, 2015). Prenatal exposure in mice has resulted into deleterious changes on the behavioral activities, neurotransmitters, oxidative stress, and brain neurons morphology (Allam *et al.*, 2016), and altered thymocyte

development in mice (Hanson *et al.*, 2010a). It is found to be renotoxic (Chen *et al.*, 2016), cytotoxic and embryotoxic (Rodriguez-Fragoso *et al.*, 2012; Witeska *et al.*, 2014; Zhao *et al.*, 2017; Memon and Pratten, 2013). It affects semen quality in human (Meeker *et al.*, 2008) and alters feeding pattern and urine volume in Wistar rat (Imafidon *et al.*, 2015). Interestingly, cadmium has been found to exert sex specific effects on birth size, DNA methylation (Kippler *et al.*, 2012; Kippler *et al.*, 2013) birth length and fetal growth (Romano *et al.*, 2016). It is reported as gametotoxic (Tualla and Bitacura, 2016), tumor angiogen (Wei *et al.*, 2017), neurotoxic (Gupta *et al.*, 2015), and osteotoxic (Ha *et al.*, 2016). Prenatal exposure and placental crossing

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over induces hepatotoxicity and DNA methylation (Castillo *et al.*, 2012; Sanders *et al.*, 2014; Vilahur *et al.*, 2015). Neurulation is found to be most vulnerable state (Robinson *et al.*, 2010) leading to neural tube defects in mice (Robinson *et al.*, 2011).

Garlic is well known for its protective role against cancer and so many other diseases (Setiawan *et al.*, 2005) for its therapeutic and prophylactic effects (Ugwuja *et al.*, 2016) owing to biologically active antioxidant substances like alliinase, allicin, alliin, and S-allylcysteine (Cruz *et al.*, 2007; Majewski, 2014). Administration of garlic juice during pregnancy and lactation is known to protect from apoptosis in rat offspring's eye retina (Khordad *et al.*, 2013). Aged garlic extract (AGE), even can reduce side effects of anticancer drugs (Nasr, 2014). Keeping in view the potential ameliorative role of garlic, the present study is designed to find its protective activity in developing chicks against cadmium.

Materials and Methods

Chemical used and dose preparation

Cadmium chloride (SIGMA-ALDRICH), was used as source of cadmium in the study. A sublethal dose of cadmium (Džugan *et al.*, 2011) was prepared in sterilized 0.7% avian saline (Pawlak *et al.*, 2013) in such a way that each 0.05ml contained 1.5 µg of cadmium.

Preparation of antidote

Fresh garlic, Purple Glazer (*Allium sativum*) was used as antidote. Fresh garlic bulbs were purchased from local market in order to prepare fresh garlic juice. For this purpose, garlic cloves (2g) were peeled, washed, chopped and ground in pastel, mixed with distilled water and filtered afterwards and filtrate was used to prepare the desired concentration of 0.2 µg/0.05ml.

Experimental animal

Embryos of *Gallus domesticus* (domestic fowl, Comb Leghorn) were used as experimental animal. Fresh fertilized eggs (n=160) were purchased from a local hatchery at Pasrur road, Narowal. Eggs were cleaned, labelled and incubated horizontally at standard conditions (incubation days: D0-D21, temperature: 37 ± 0.5 °C, relative humidity: 50-60%) in a rolling egg incubator (24' × 30' × 17'). Humidity was maintained by keeping the water filled tray inside the incubator which was replaced after every 24 hours and its level was maintained to provide eggs with equal percentage of humidity throughout the incubation period. Eggs were observed through the candler on 4th day (D4) of incubation to check the embryonic development to remove the unfertilized eggs.

Experimental grouping and drug administration

On 7th day (D7) of incubation, all the fertilized eggs

were randomly divided into four groups of 40 eggs each as follows:

Control group: untreated

Dose group: CdCl₂ 1.5 µg /0.05 ml/egg

Dose + Antidote group: CdCl₂ 1.5µg/0.05ml/egg + Garlic juice 0.2 µg/0.05 ml/ egg (after a short interval of 10 minutes)

Antidote group: Garlic juice 0.2 µg/0.05ml/egg

Dose administration was carried out through a hole in the egg shell into albumen at blunt end using a sterile needle in sterilized environment of laminar air flow. Following injections, holes were sealed with molten paraffin wax immediately to avoid contamination. The remaining incubation period was continued safely until hatching.

Hatching of chicks

Fertilized eggs of each group were allowed to hatch naturally on 21st day of incubation. Immediately after hatching chicks were kept separately in an environmentally controlled room at 27-30 °C with a photocycle of 14 hours light and 10 hours dark for two days. Chicks were constantly supplied with pearl millet and fresh tap water for drinking during this time period.

Morphological analysis and macrophotography

Un-hatched embryos, hatched chicks of all groups were studied morphologically for developmental abnormalities of skull, beak, eyes, limbs, tail, vertebral column and abdomen.

Morphometric analysis

Body weight and crown rump length of each embryo was recorded for each group.

Histological preparation

Liver tissues were dissected out and subsequently chopped into small pieces with sharp cutter and preserved in Bouin's fluid for fixation for 48 hours and processed for histological analysis using paraffin wax and hematoxylin-eosin staining technique.

Microphotography and histological study

Histological sections of liver were observed for various anomalies using microscope SWIFT (M4000-D) and microphotographed with the help of digital camera BESTSCOPE (BUC2-500C).

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) Tukey test using GraphPad Prism (Version 5.01) to find out the significant difference ($p < 0.05$) among various groups.

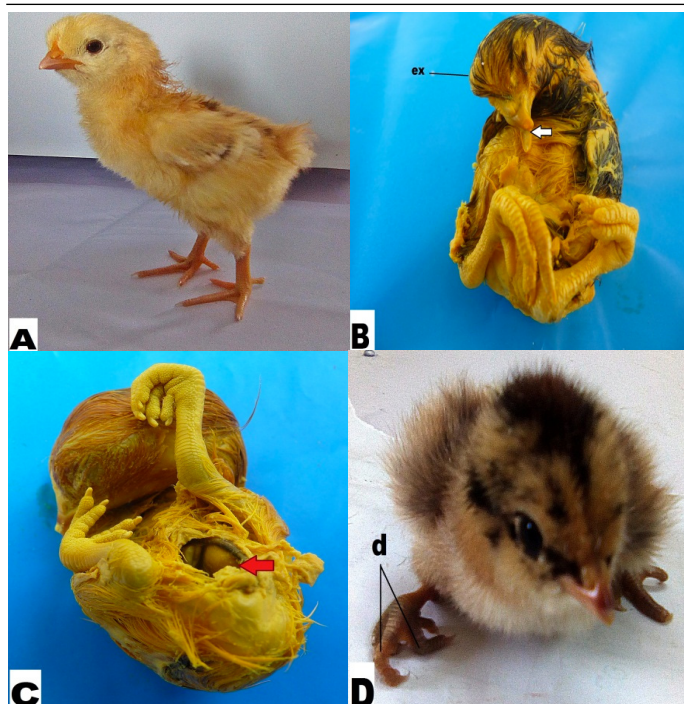


Figure 1: A macrophotograph of chicks of different dose groups.

A: Control group (untreated); B & C: Dose group treated with $1.5\mu\text{g}/0.05\text{ml}/\text{egg}$ of CdCl_2 ; D: Dose+antidote group treated with $1.5\mu\text{g}/0.05\text{ml}/\text{egg}$ of CdCl_2 and $0.2\mu\text{g}/0.05\text{ml}/\text{egg}$ of garlic juice. Note: (ex: exencephaly; Arrow white: crossed beak; Arrow red: gastrochisis; d: crooked toes).

Results

Morphological analysis

Chick hatchlings as well as embryos of control group were quite healthy and uniform in appearance and in other anatomical details. The body was well differentiated into head, neck and trunk regions. They had completely developed morphological features, including head crown, beak, eyes, fore limbs, hind limbs and digits. In dose group, 32.5% of embryos hatched naturally at day 21. These embryos showed minor morphological abnormalities, retarded growth and weight loss as compared to control group. Among rest in this group, 48.14% dead embryos were recovered on 23rd day. Embryos recovered on D23 showed various anomalies. Exencephaly, ablepharia, crossed beak, gastrochisis, and crooked toes was observed in 7.14, 21.43, 57.1 and 35.71 of embryos respectively in dose group, while crooking of toes was also observed in 21.43% of chicks in dose +antidote group (Table 1 and Figure 1 A-D). Hence, overall percentage of resorbed embryos in dose group was 35% and malformed embryos was 42.5%.

In dose+antidote group, 72.5% embryos hatched naturally at 21st day of incubation. A total of 10% delayed hatching was recorded in dose and dose+antidote group on 23rd day of incubation. All the hatchlings were subjected to morphological studies (Table 1). With the exception of some malformed hatchlings, all were quite healthy. They

had completely developed morphological features, including head crown, beak, eyes, fore limbs and hind limbs. In this experimental group, 15% were malformed while 22.5% embryos were resorbed during incubation. A total of 87.5% naturally hatched chicks of antidote group were similar to control group with only 12.5% embryonic resorption (Table 1).

Morphometric analysis

Significant difference in birth weight was observed among all groups except control and antidote as well as dose+antidote and antidote group (Figure 2).

Histological analysis

Histological analysis of liver of chicks from control group appeared with intact and normal association of sinusoids and hepatic cords. A cross section of liver of chick embryo treated with $1.5\mu\text{g}/0.05\text{ml}/\text{egg}$ cadmium chloride showed various abnormalities including steatosis leading to pyknosis. Section through liver of chicks treated with dose plus antidote shows increased number of dividing cells indicating recovery from toxicity of cadmium. Antidote group showed normal association of sinusoids and hepatocytes similar to control group (Figure 3A-D).

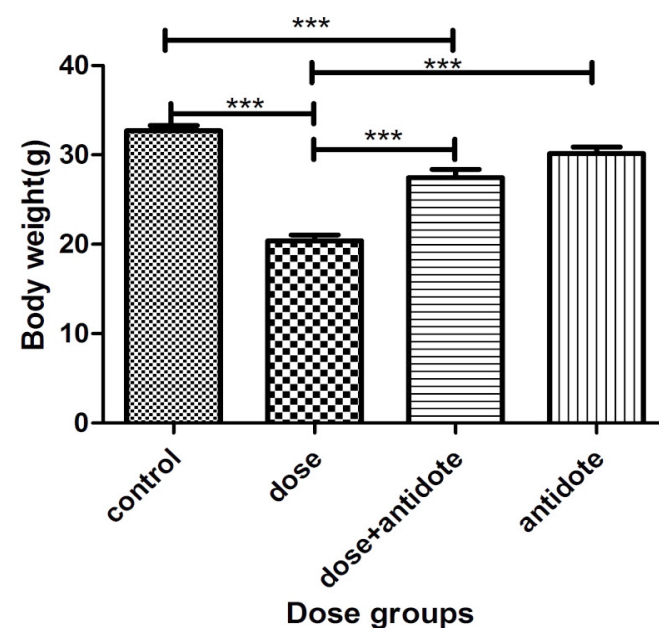


Figure 2: A bar graph showing body weight of chicks of different dose groups in ovo exposure to cadmium at 7th day of incubation.

Control: untreated ; Dose group: treated with $1.5\mu\text{g}/0.05\text{ml}/\text{egg}$ of CdCl_2 ; Dose+antidote group: treated with $1.5\mu\text{g}/0.05\text{ml}/\text{egg}$ of CdCl_2 and $0.2\mu\text{g}/0.05\text{ml}/\text{egg}$ of garlic juice; antidote group: treated with $0.2\mu\text{g}/0.05\text{ml}/\text{egg}$ of garlic juice (***) indicate significant difference at $p < 0.05$).

Discussion

Cadmium is notorious for its toxicity in adults as well as in prenatal exposure (Jacobo-Estrada et al., 2017). In this study it has been found to reduce body weight (Figure 2)

Table I: Ameliorative effects of fresh garlic juice against cadmium induced teratogenicities in chick embryos against control.

Group	Total No. of eggs	% of natural hatching	% of deaths	% of malformation	% of resorption	CR length mm Mean \pm SEM	Major abnormalities%				
							Exen-cephaly	Ableph-aria	Crossed beak	Gastro-schisis	Crooked toes
Control (untreated)	40	92.5	-	-	7.5	101.20 \pm 2.311a	-	-	-	-	-
Dose (1.5 μ g/0.05 ml/egg)	40	32.5	48.14	42.5	35	78.80 \pm 8.157b	7.14	21.43	57.1	35.71	35.71
Dose(1.5 μ g/0.05 ml/egg) +Antidote(0.2 μ g/0.05 ml/egg)	40	72.5	18.18	15	22.5	96.20 \pm 4.042a	-	-	-	-	21.43
Antidote (0.2 μ g/0.05 ml/egg)	40	87.5	-	-	12.5	110.20 \pm 4.420a	-	-	-	-	-

Values not sharing common letters are significantly different from each other

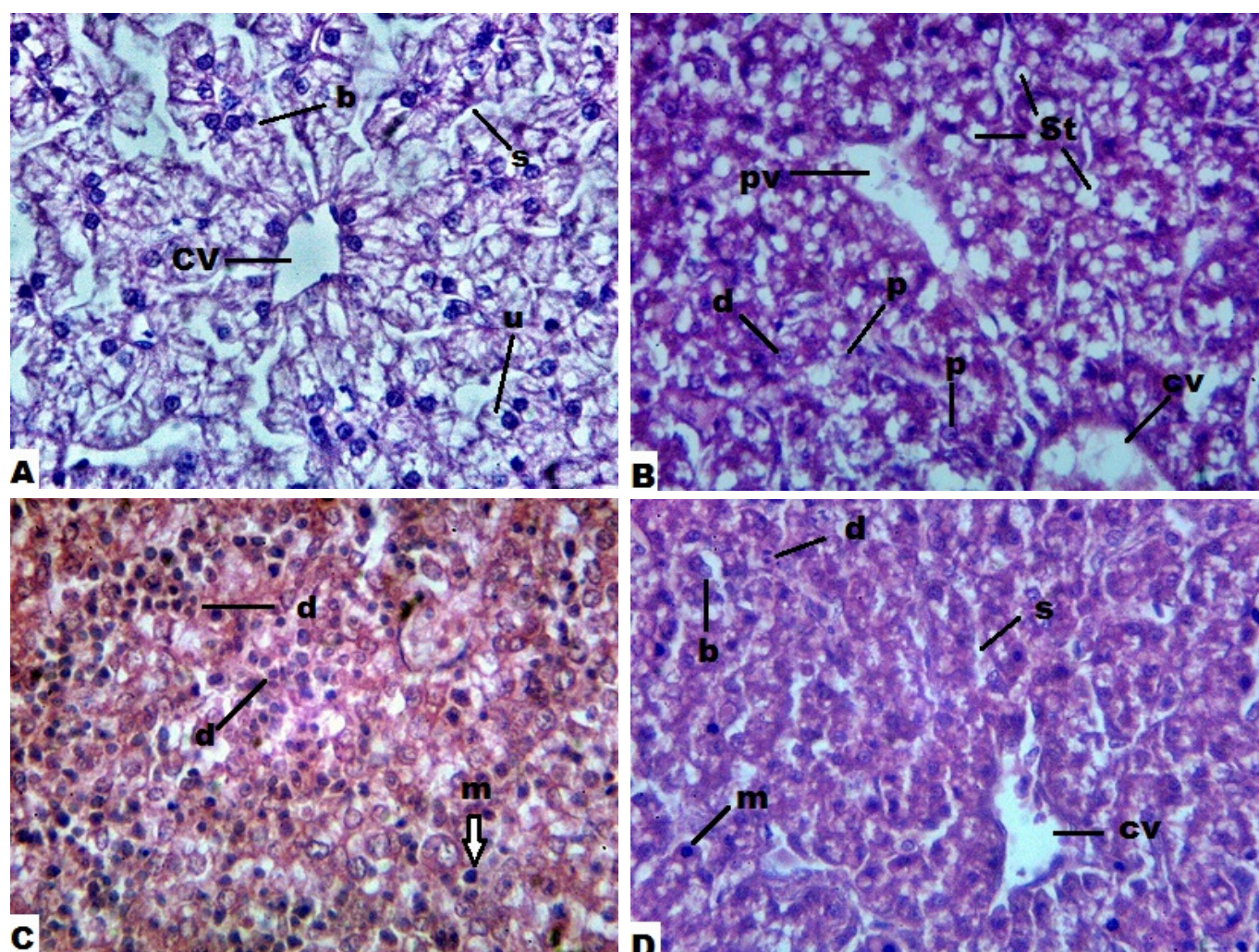


Figure 3: Microphotographs through histological sections of liver of chicks of different dose groups in ovo exposure to cadmium at 7th day of incubation (H&E 400X).

A: Control group (untreated); B & C: Dose group treated with 1.5 μ g/0.05ml/egg of CdCl₂; D: Dose+antidote group treated with 1.5 μ g/0.05ml/egg of CdCl₂ and 0.2 μ g/0.05ml/egg of garlic juice. Note: b: bi-nucleated hepatocyte; s: sinusoid; cv: central vein; u,m: uni-nucleated hepatocyte; pv: portal vein; st: steatosis; d: dividing hepatocyte; p: pyknotic

and successful natural hatching, induce simultaneously the malformations, resorption and death as compared to control. Among deformities, crossed beak was found to be the most prevalent. Gastroschisis, the most lethal one

was second major anomaly obtained. Crooked toes and ablepharia were third abundant malformation, while exencephaly was encountered with least incidences. Such craniofacial and skeletal malformations and retarded growth

in form of exencephaly, ablephary have also been recorded in mice and in vitro studies (Paniagua-Castro *et al.*, 2008; Arguelles-Velazquez *et al.*, 2013). Inhalation of cadmium even is found toxic and causes cataractogenesis in chronic smokers (Ramakrishnan *et al.*, 1995). Crooking of toes may result from decreased bone mineral density, as it is known to cause bone brittleness for such reason in rat (Bhattacharyya, 2009). In chick embryos, such anomalies have dose relationship (Rodriguez-Fragoso *et al.*, 2012). Among 11 differentially expressed genes (DEGs) in chicken, which are linked with beak deformity in addition to biosynthesis of unsaturated fatty acids and glycerolipid metabolism (Bai *et al.*, 2014), over-expression of LOC426217 in the beak is thought to be the actual responsible (Bai *et al.*, 2016). Cadmium is found to induce delayed hatching in this study as also reported by Dzugan and Lis (2016).

Histological analysis in current study revealed non-alcoholic fatty liver (steatosis) leading to pyknotic cells with shrunk nuclei. Such results are also evidenced in human (Hyder *et al.*, 2013; Go *et al.*, 2015) that may be due to altered gene expression in human hepatocellular carcinoma (HepG2) cells (Cartularo *et al.*, 2015), or decrease in hepatic enzymatic and non-enzymatic antioxidants reduced glutathione, catalase, superoxide dismutase (Oyinloye *et al.*, 2016).

Mechanism of toxicity of cadmium, like other metals is oxidative stress, DNA damage, ER stress lipoperoxidation, mitochondrial fragmentation, and cell death (Xu *et al.*, 2013; Chen *et al.*, 2015; Nair *et al.*, 2013; Wei *et al.*, 2014; Jamakala and Rani, 2015; Kim *et al.*, 2015; Veeriah *et al.*, 2015; Ruiter *et al.*, 2016; Xu *et al.*, 2017) through either MAPK pathways (Yiran *et al.*, 2013), or by decreasing expression and activity of SIRT3 protein and promotes the acetylation of superoxide dismutase 2 (Guo *et al.*, 2014; Pi *et al.*, 2015).

In the group, co-treated with garlic along with cadmium, increased body weight, least malformations and recovery of fatty liver with increased number of normal and dividing cells, possibly due to its anti-Cd properties, radical scavenging assay, ferric reducing ability power assay, chelating activities, superoxide, and hydroxyl scavenging assay (Poljsak and Fink, 2014; Boonpeng *et al.*, 2014), complexation of Cd to glutathione (GSH) and metallothionein (MT), prevention of endoplasmic reticulum (ER) stress, mitophagy and metabolic stress, as well as expression of chaperones (Sandbichler and Hackner, 2016). Aged garlic extract (AGE) contains S-allylcysteine (SAC) activates Nrf2 factor and inhibits prooxidant enzymes, and chelating effects (Cola-N-Gonzalez *et al.*, 2012). Thiocremone, another constituent of garlic has potent anti-inflammatory and anti-arthritis properties through the inhibition of NF- κ B (Ban *et al.*, 2009).

Cadmium toxicity is suggested to overcome by high water intake, chemical antidotes (Rafati *et al.*, 2017), however, dietary strategies including plants is likely to be more cheaper to encounter such unseen hazards (Zhai *et al.*, 2015), as the use of fresh garlic along with high-fat diet keeps safe from its hepatotoxicity (Qamar *et al.*, 2016). Results of present study authenticate the potent ameliorative nature of garlic against cadmium and suggest its use in any way at regular basis to withstand the harmfulness of unseen toxins like cadmium through food and water.

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