**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 (Imafidion 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 angiogenic (Wei et al., 2017), neurotoxic (Gupta et al., 2015), and osteotoxic (Ha et al., 2016). Prenatal exposure and placental crossing

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**Citation**

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 (Dżugan 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: CdCl₂1.5 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.
June 2018 | Volume 33 | Issue 1 | Page 36

**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, gastroschisis, 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 1.5µg/0.05 ml/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).

**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.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total No. of eggs</th>
<th>% of natural hatching</th>
<th>% of deaths</th>
<th>% of malformation</th>
<th>% of resorption</th>
<th>CR length (mm Mean ± SEM)</th>
<th>Major abnormalities%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated)</td>
<td>40</td>
<td>92.5</td>
<td>-</td>
<td>-</td>
<td>7.5</td>
<td>101.20±2.311a</td>
<td>-</td>
</tr>
<tr>
<td>Dose (1.5µg/0.05 ml/egg)</td>
<td>40</td>
<td>32.5</td>
<td>48.14</td>
<td>42.5</td>
<td>35</td>
<td>78.80±8.157b</td>
<td>7.14 21.43 57.1 35.71</td>
</tr>
<tr>
<td>Dose (1.5µg/0.05 ml/egg) + Antidote (0.2µg/0.05 ml/egg)</td>
<td>40</td>
<td>72.5</td>
<td>18.18</td>
<td>15</td>
<td>22.5</td>
<td>96.20±4.042a</td>
<td>-</td>
</tr>
<tr>
<td>Antidote (0.2µg/0.05 ml/egg)</td>
<td>40</td>
<td>87.5</td>
<td>-</td>
<td>-</td>
<td>12.5</td>
<td>110.20±4.420a</td>
<td>-</td>
</tr>
</tbody>
</table>

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 1.5µg/0.05ml/egg of CdCl₂; D: Dose + antidote group treated with 1.5µg/0.05ml/egg of CdCl₂ and 0.2 µg/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: pyknosis

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
Cadmium induced teratogenicity and its attenuation in developing chick

in form of exencephaly, alephary 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).

Histogram 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 (Sandichler 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). Thiacremoneone, another constituent of garlic has potent anti-inflammatory and anti-arthritic properties through the inhibition of NF-κB (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.

References


June 2018 | Volume 33 | Issue 1 | Page 40


