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Bioaccumulation and cytological alteration of immune organs of chicken following inorganic arsenic exposure

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ABSTRACT

Arsenic is an ecotoxicant that has been found to affect both mammal and avian population. The present study deals with the arsenic deposition in different immune organs of arsenic exposed broiler chicken. Further, its effect on immune cell function and histological alteration was investigated. The study revealed that bursa and liver were the most arsenic deposition prone sites as compared to other immune organs. Histopathological study of the immune organs showed significant structural changes like increased bursal medullary region along with follicular atrophy and detachment of outer serosal layer from the muscularis layer in bursa, decrease in average diameter of white pulp in spleen, decreased cortical as well as medullary region along with less number of Hassall's corpuscle in thymus in the arsenic exposed birds. Arsenic induced apoptosis in peripheral blood mononuclear cells (PBMCs) was also detected and a positive correlation between apoptotic index and dose of arsenic was observed. It may be concluded that insult to avian immune organ by any toxic compound may threaten immune response and may lead to immunosuppression.

Keywords: Apoptosis, Bioaccumulation, Immune organs, Inorganic arsenic, Pathological changes

Arsenic toxicity causes skin, lung and bladder cancers in humans and is associated with diabetes and cardiovascular disease (Hughes *et al.* 2011). Ingestion of contaminated food and water is major source of arsenic toxicity. Inorganic arsenic (iAs) is readily absorbed after oral exposure and is usually cleared from blood within few hours (Hughes 2006). Long exposure of arsenic results in accumulation in various parts of the body and alters normal biological function of an organism (Tchounwou *et al.* 2012). Arsenic mediated apoptosis leads to diminished immune response and it also induces immunosuppression affecting lymphocytes, monocytes and macrophage activity (Wu *et al.* 2003).

In poultry, lymphoid tissue plays role in defence mechanism against diseases. Thymus, Bursa of Fabricius and bone marrow act as primary lymphoid organs whereas spleen, mucosal associated lymphoid tissues and germinal centers are secondary lymphoid organs. Maturation of T lymphocytes takes place in thymus, whereas Bursa of Fabricius is the site for B lymphocytes differentiation and maturation (Fellah *et al.* 2013). The thymus dependent components are mostly responsible for cell mediated immunity including immuno-surveillance, while bursa plays a major role in humoral immunity (Akter *et al.* 2006). Therefore, insult to thymus or bursa by any toxic compound could threaten immune system of poultry (Chaudhry *et al.* 2016). Spleen is an important site for antibody production and plays an important role in establishing the immune system (Bronte and Pittet 2013). Arsenicosisis due to its presence in soil, water, plant, animal and human continuum. As food is the most important sources of arsenic toxicity in human and chicken is a widely used as protein source in human diet, the bioaccumulation of arsenic in different organs of chicken merits in-depth study. Information on these aspects is very limited. Therefore, present study was designed to investigate the bioaccumulation of iAs in different immune organs of poultry following arsenic exposure and its effect on immune cell function and histological alteration was investigated.

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MATERIALS AND METHODS

Experimental birds and procedure: Thirty six newly hatched broiler chickens (Vencobb) were procured from Hi-Breed International Sales and Services, Kolkata, India and were kept in a brooder provided with *ad lib.* water and standard balanced feed. Chickens were randomly divided into 3 groups with 12 birds in each group; one group acted as control and the other two groups were exposed to 4 mg/L and 8 mg/L arsenic respectively through drinking water for 45 days.

Isolation of immune organs and histo-pathological study: The birds were sacrificed by cervical subluxation method and the organs like Bursa of Fabricius, spleen, thymus, dorsal part of the left lobe of liver were collected

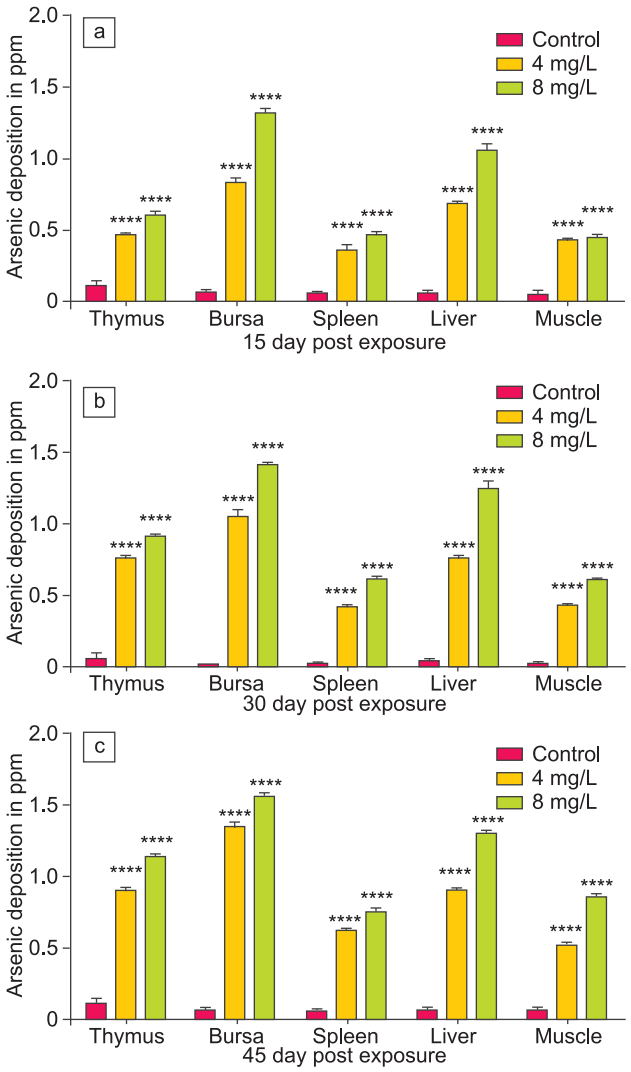

Fig. 1. Bioaccumulation of arsenic in thymus, bursa of fabricius, spleen, liver and muscle of control and arsenic exposed chicken in different time points post exposure. (a) 15 day post exposure, (b) 30 day post exposure and (c) 45 day post exposure. Significant differences with control group were designated as ****(P≤0.0001).

as per the standard procedure. Immune organs were weighed and index was determined by using the formula; Index = (immune organ weight × 1000)/ body weight of the chicken. Tissue samples were fixed in 10% formalin for further histological study. The samples were processed and permanent slides were prepared following haematoxylin-eosin staining with standard procedures.

Estimation of residual arsenic in vital organs: Tissue samples of immune organs including liver and muscle from healthy and arsenic treated chickens were digested as per standard methodology described by Le and Ma (1998) using triple acid mixture and arsenic content was estimated using an Atomic Absorption Spectrophotometer with Flow injection-Hydride generator (AAS-FIHG, GBC.932 B+) as previously described (Guha Mazumder *et al.* 2001).

Isolation of PBMC: Blood sample was collected in a heparinised vacutainer just prior to sacrifice of the birds. RBCs were lysed using RBC lysis buffer. After, blood was diluted in PBS and PBMCs were separated using Ficol-hypaque and re-suspended in RPMI 1640. The suspension was then layered over Ficol-hypaque (density 1.077) and was centrifuged at 0.4 × g for 30 min for separation of PBMCs. Cells were separated out at the interface, collected and washed thrice with PBS (*p*H–7.2). Then the cells were re-suspended in RPMI 1640 supplemented with L-glutamine (300 mg/mL), penicillin (100 IU/mL) and streptomycin (100 mg /mL). The isolated cells were further used for apoptosis study.

TUNEL Assay: Apoptosis was studied by TUNEL assay using a commercial kit (Dead End™ Colorimetric TUNEL System, Promega) as per the manufacturer's protocol. At least 10 randomly selected fields were counted. Apoptotic index was calculated using the following formula:

Apoptotic index = (Total number of apoptotic body × 100)/ Total number of cells.

Statistical analysis: Data were expressed as mean±SD. The data were analyzed using two-way analysis of variance with Bonferroni post tests using GraphPad (version 5, San Diego, California, USA).

RESULTS AND DISCUSSION

Bioaccumulation of arsenic in different organs like spleen, thymus, bursa of fabricius, liver and muscle of arsenic fed chickens (4 mg/L and 8 mg/L) and healthy control was estimated at different time intervals (15th, 30th and 45th day post exposure). Significant increase in residual arsenic deposition in all studied organs of the exposed than control group was observed at three time points (Fig.1). It was also found that bursa and liver were more prone to arsenic deposition. On 45th day post exposure, maximum arsenic deposition was observed in bursa of chicken exposed to 8 mg/L. Deposition of arsenic in chicken was positively correlated with arsenic concentration in drinking water and indicated that arsenic deposition was not only time dependent but also dose dependent.

Bursal and splenic index decreased significantly in 4 mg/L than in control group from 30th day post exposure onwards whereas in case of 8 mg/L group, both indexes decreased significantly from 15th day onwards. Whereas, significant decreases in thymic index of both the exposed groups were found from 15th day post exposure onwards as compared to those of control group (Fig. 2). The results of the experiment indicated that arsenic exposure caused marked atrophy of bursa, spleen and thymus in chicken.

Histopathological revealed that no changes were observed in histological structure of bursa up to day 30 of arsenic exposure but at day 45 post exposure; significant changes were found as compared with control. Significant decrease in cortex and medullar size was found in arsenic exposed than control group from day 30 of exposure onwards. Follicular atrophy and detachment of outer serosal layer from the muscularis layer were observed in 4 mg/L group on 45th day post exposure whereas in case of 8 mg/L group, same pathological changes were identified from 30th

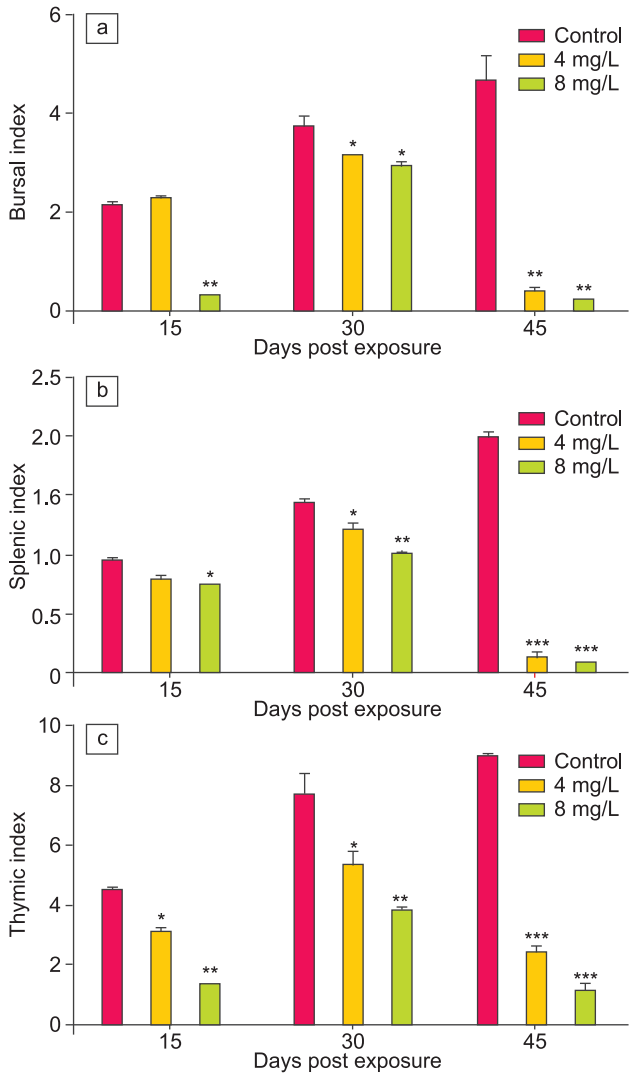

Fig. 2. Bursal index (a), splenic index (b) and thymic index (c) of healthy and arsenic fed chicken. Significant differences with control group were designated as *P≤0.05, **P≤0.01 and ***P≤0.001.

day post exposure onwards. Increased numbers of bursal follicle with lymphocytic aggregation, macrophage and plasma cell infiltration were prominent at 45th day of arsenic exposure in both exposed groups (Table 1). Histopathological study on spleen revealed that the average diameter of white pulp was significantly decreased in both the arsenic exposed as compared to control group at both 30th and 45th day post exposure. Number of white pulp increased in both the groups as compared to control group at 45th day post exposure (Table 1). Similarly histological study on thymus revealed that there was decrease of cortex size from 15th day onwards whereas medullar size decreased from 30th day onwards in exposed groups. The reduction of size was dose and duration dependant. It was also noted that apparent number of Hassall's corpuscles increased in both arsenic fed as compared to those of control group (Table 1).

Apoptosis study revealed that in exposed groups, morphological changes in PBMCs were observed; cells with

Table 1. Micrometry of Bursal cortex, bursal medulla, spleenic white pulp, thymic cortex, thymic medulla cells from healthy and arsenic fed chicken at different time points post arsenic exposure

Days post exposure	Control	4 mg/L	8 mg/L
Bursal cortex (*μm*)			
15	2.91 ± 0.127	2.75 ± 0.187	2.66 ± 0.182
30	3.23 ± 0.152	2.16 ± 0.135*	2.10 ± 0.097**
45	3.27 ± 0.114	2.01 ± 0.087	1.83 ± 0.094
Bursal medulla (*μm*)			
15	2.33 ± 0.09	2.80 ± 0.115	2.56 ± 0.131
30	3.02 ± 0.117	2.11 ± 0.085*	2.07 ± 0.099*
45	3.19 ± 0.104	1.95 ± 0.122**	1.80 ± 0.109**
Spleenic white pulp (*μm*)			
15	0.96 ± 0.02	0.79 ±0.01	0.76 ± 0.005*
30	1.44 ± 0.01	1.215 ± 0.04*	1.01 ± 0.009**
45	3.99 ±0.02	0.130 ± 0.03***	0.082 ± 0.004***
Thymic cortex (*μm*)			
15	2.62 ± 0.065	2.15 ± 0.150*	2.11 ± 0.109*
30	2.67 ± 0.102	2.05 ± 0.142*	2.07 ± 0.089*
45	3.29 ± 0.168	1.89 ± 0.100**	1.79 ± 0.113**
Thymic medulla (*μm*)			
15	2.57 ± 0.064	2.34 ± 0.092	2.18 ± 0.096
30	2.62 ± 0.103	2.26 ± 0.113*	2.22 ± 0.062*
45	2.95 ± 0.11	2.07 ± 0.118**	2.04 ± 0.137**

Significant differences with control group were designated as *(p≤0.05), ** (pd"0.01) and ***(p≤0.001).

dark brown stain indicting DNA fragmentation were detected. Significant increase in apoptotic index were observed in 4 mg/L group on 45th day post exposure as compared to control whereas in case of 8 mg/L group, significant increase was detected from 15th day post exposure onwards. In case of 4 mg/L arsenic fed chicken, apoptotic index was 2.40±0.23 on 15th day and reached to 27.98±0.43 on 45th day. In case of 8 mg/L arsenic fed chicken, apoptotic index was 5.06±0.31 on 15th day and reached to 19.12±0.78 on 45th day post exposure.

Long-term exposure to arsenic has potential detrimental effect on human health; it may lead to cardiovascular disease, diabetes, cancer or even death. *In utero* and early childhood exposure has deleterious effect on cognitive development (WHO 2018). Both plants and animals can bio-accumulate arsenic from the environment within their tissues. When used as sources of food, these plants and animals can contribute to the ingestion of arsenic by humans. As chicken is one of the most widely used animal protein sources in human diet throughout the world, it is very important to understand the bioaccumulation of arsenic in different organs of chicken. In the present study, we estimated the bioaccumulation of arsenic in different organs of chicken and found that bursa and liver of chicken were more prone to arsenic deposition (Fig. 1). It was also observed that arsenic exposure lead to significant histological changes in the immune organs of chicken and

lead to apoptosis of PBMCs in a dose and duration dependent manner.

The insult to thymus by any toxic compound could threaten immune function (Ahmed *et al.* 2012). Histopathological changes in lymphoid organs are associated with arsenic exposure was investigated to know the extent of damage. In our experiment, histological changes were observed in bursa of fabricius following exposure to arsenic. Marked reduction in follicular size and detachment of muscularis layer in bursa was detected. In thymus, a dose and duration dependent reduction in size of cortex and medulla was also observed. The other primary lymphoid organ showed changes in term of and number of Hassall's corpuscles. The changes indicated thymus had less counted number of Hassall's corpuscles when chicken was provided with water having sodium arsenite. Decreased number of Hassall's corpuscles indicated the altered response of humoral immunity. Hassall's corpuscles have a critical role in dendritic cell mediated secondary positive selection of medium to high affinity self reactive T-cells, leading to the generation of $CD4^+$ $CD5^+$ regulatory T cells within the thymus (Watanabe *et al.* 2005). In primary organ, the size of the medulla and cortex has been decreased significantly which is visible by the size of the lymphocyte in medulla and cortex which indicates altered response of immunity as medulla is the site for maturing lymphocytes and cortex is the site for matured lymphocytes. In case of secondary lymphoid organ (spleen), there was a decrease in the size of white pulp which indicated lesser number of lymphocytes. The decrease in lymphocyte content may directly affect the immune system (Alberts *et al.* 2002). Martin-Chouly *et al.* (2011) reported that arsenic could act as an immunosuppressive agent and hinder the function of several key immune cells. In the present study, changes in relative organ weights varied with the different treatment groups. Atrophy was characterized by lymphoid depletion and thinning of follicular cortices or depletion of lymphocytes in the medulla. Similar report was observed by Nandi *et al.* (2006). In the present study, it was found that bursa and liver of chicken were more prone to arsenic deposition. Similar observation was reported in rat where ten times more deposition of arsenic in hepatic tissue than in muscle was reported (Lasky *et al.* 2004). Bioaccumulation of arsenic in liver was also reported by Singh *et al.* (2014).

In general, apoptosis is a homeostatic mechanism of the body to maintain cell populations in tissues and occurs during development and aging. When cells are damaged by disease or harmful agents, they also undergo apoptosis (Norbury and Hickson 2001). Arsenic caused elevation of TUNEL positive nuclei that trigged apoptosis and cell death. Our findings suggested that arsenic induced apoptosis of chicken PBMCs and corroborated with the findings of Chen *et al.* (2000), who reported that inorganic arsenic triggered cells to undergo apoptosis through downregulation of bcl-2 and upregulation of bax. Our findings also suggested that the apoptosis induced by arsenic was concentration and duration dependent. It was found that with increased concentration and duration of exposure, the apoptotic index increased. This work simulated the work of Chen *et al.* (1998). In a previous work, apoptotic property of arsenic on hepatocytes was reported in a rodent model (Rana *et al.* 2010). Arsenic induced apoptosis had also been reported in different cell lines like HepG2 (Roy *et al.* 2016) and human Leukemia (HL-60) cells (Yedjou *et al.* 2010). It has been postulated that arsenic induces apoptosis through a c-Jun NH2-Terminal Kinase-dependent, p53-independent pathway (Huang *et al.* 1999). Arsenic has been reported to be associated with apoptotic-inducing effect on immune cells (Slukvin and Jerrells 1995). Arsenic is more toxic than heavy metals in inducing apoptosis of lymphoid cells which further suggest that this phenomenon may contribute to some immunotoxic effect *in-vivo* (de la Fuente *et al.* 2002). de la Fuente *et al.* (2002) also reported that arsenic was toxic at concentrations as low as 1.0 µM and showed a significant pre-apoptotic effect.

Present study is the first documentation of *in vitro* and *in vivo* apoptosis of PBMCs in poultry birds following exposure to different concentrations of arsenic. Histopathological changes established the detrimental role of sodium arsenite on immune organs. These structural changes could lead to improper functioning of immune cells which could further hamper the normal functioning of immune system.

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