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Immunohistochemical Expression of Oxidative Stress Markers in Bovine Tissues Correlated to Cadmium Concentration

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ABSTRACT

A study was conducted to assess oxidative stress in bovine tissues specifically liver, kidney and lungs by using immunohistochemistry (IHC) and their correlation to cadmium (Cd) toxicity in bovine. Metallothionein and malondialdehyde are oxidative stress markers and their expression increases as cadmium concentration increases in tissues. In present study, a total of 62 bovine tissue samples were randomly collected from the animals found dead near industrial or expected polluted areas of Jabalpur city after detailed postmortem examination. These samples were processed for cadmium estimation and IHC staining in tissues. 200 mg of tissue samples were acid digested and cadmium concentration were estimated by using ICP-OES. In our study, cadmium concentration in tissues was ranged from 0.040 to 3.952 ppm in liver, 0.050 to 3.949 ppm in kidney and 0.020 to 3.134 ppm in lungs of bovine. These samples further grouped under three groups with cadmium level 0-1, 1-2 and > 2 ppm, according to Puls criteria. Majority of samples had cadmium level in the range of 0-1 ppm. Approximately 8, 13, 5% liver, kidney and lung tissues respectively had cadmium concentration > 2 ppm are considered under high risk. Formalin fixed and paraffin processed representative samples of liver, kidney and lungs were stained immunohistochemically by using commercially available antibodies for metallothionein and malondialdehyde protein. Tissues with high cadmium level showed increase in expression of metallothionein in nucleus and cytoplasm of the tissues along with increased cytoplasmic expression of malondialdehyde in liver, kidney and lung tissues as compared to low cadmium level.

Keywords: Bovine, Cadmium, Immunohistochemistry, Malondialdehyde, Metallothionein, Oxidative stress

Cadmium (Cd) is a heavy metal known to cause environmental and occupational hazards thus threatening to health of animal and public. Though, cadmium doesn't have known biological function neither in animals or humans but mimics the actions of other divalent metals that are essential to diverse biological functions (EFSA, 2009).

Cadmium is a widespread cause of toxicity in domestic animals all over the world. It affects all animals including bovine, horses, birds/poultry and dogs. Among them, cattle is most susceptible animal. Human activities spreading the cadmium widely throughout the environment. More than 90% of surface cadmium is due to use of phosphate fertilizers and use of sewage water in agriculture. These are potential source of toxicity in livestock. Cattle reared near the industrial area had approximately fivefold higher blood and tissue cadmium level. Cadmium concentration affects cattle of all ages and work as a multi systemic toxin thereby making kidney as a most vulnerable organ followed by organs of other systems like hepatic, respiratory and reproductive systems in the body (EFSA, 2009; Darwish *et al.,* 2015; Rana *et al.,* 2018).

Long term exposure of cadmium stimulates the oxidative stress in blood and various organs by generation of reactive oxygen species (ROS), alteration in antioxidant defense mechanism and increase in lipid peroxidation (Dhaliwal and Chhabra, 2016).

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The source of cadmium intake in bovine mostly is oral, and it mainly accumulates in the liver and kidney where it induces production of metallothionein (MT). Metallothionein is a low molecular–weight, cystein rich protein that binds cadmium with high affinity. This protein forms a complex with cadmium and decreases the free concentration of cadmium within the cell, but when the binding capacity of MT becomes saturated, the increased level of unbound cadmium ions initiates processes that can lead to liver and kidney injury (Buranasinsup *et al.,* 2011; Salinska *et al.*, 2013). It has been suggested that major pathophysiologic functions of metallothionein are to detoxify heavy metals, regulate heavy metal homeostasis, and scavenge reactive oxygen species. It is apparent that MT plays a critical role in protecting against heavy metals such as Cd (Klaassen *et al.,* 1999).

Another important protein malondialdehyde is a well known secondary product of lipid peroxidation, may be used as a biomarker of cell membrane injury. The monitoring of MDA levels in different biological systems can be used as an important indicator of lipid peroxidation both *in-vitro* and *in vivo* for various health disorders. The endogenous formation of MDA during intracellular oxidative stress and its reaction with DNA forms MDA DNA adducts which makes it an important biomarker of endogenous DNA damage (Dhaliwal and Chhabra, 2016; Zhang *et al.,* 2002).

Jabalpur region of Madhya Pradesh has a variety of defense activities like vehicle factory, Ordinance factory which are using the raw materials containing cadmium. Use of contaminated water with sewage water and phosphate mixed fertilizers also exist as consistent causes of cadmium intensification thereby making the city prone for cadmium pollution. Recent studies by the workers have reported increased level of heavy metals in different water bodies and bovine reared around the Jabalpur region (Singh *et al.,* 2013; Anil, 2017).

Keeping above facts on mind the present study was designed to assess the cadmium associated oxidative stress on major visceral organs of body i.e. liver, kidney and lungs.

MATERIALS AND METHODS

Time and Place of work

The study has been conducted for the period of nine months from July, 2017 to May, 2018 in the Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Jabalpur, with the collaboration of Directorate of Research Services, Nanaji Deshmukh Veterinary Science University (NDVSU), Jabalpur, Madhya Pradesh, India.

Sample collection

Collection of tissue samples

Total of 62 bovine, from the areas near high ways, industries and urban areas found dead during the study period were subjected for detailed post mortem examination and all the gross lesions were carefully recorded. Organs like liver, kidney and lungs were collected for estimation of cadmium concentration (at -20°C) and immune-histochemical studies (in 10% formalin).

Cadmium detection from tissue samples

Approximately 200 mg tissue samples (liver, kidney and lungs) separately were acid digested by adding nitric acid and hydrogen peroxide in a microwave digester for one hour and stored in -20°C till analysis of cadmium level (Welna *et al*., 2011). Cadmium level obtained by using extremely advance instrument called inductively coupled plasma optical emission spectroscopy (ICP-OES) (Thermo scientific; iCAP 7000 series). Calibration of instrument was achieved with 6 standards of known concentrations (5, 10, 25, 50, 75 and 100 ppb) prior to analysis of unknown sample. Concentrations of cadmium in the samples were obtained in ppb which further converted to ppm for data presentation

Immunohistochemistry

Oxidative stress in tissue samples were studied immunohistochemically by using commercially available antibody of metallothionein (MT) and malondialdehyde (MDA) in tissue sections of liver, kidney and lungs with reference to cadmium concentration.

Formalin fixed, paraffin processed tissue sections of liver, kidney and lungs were stained as per the method described by Bancroft (2008). The tissue sections were taken on histogrip (Life Technologies; 008050) treated slides and

deparaffinized by heat and xylene. Antigen retrieval was done in a Thermo PT module at 95ºC for 25 minutes in antigen retrieval solution.

After cooling the slides, endogenous peroxidase activity was blocked by applying 0.3% hydrogen peroxide (Fisher scientific, 18715) and non specific staining was prevented by using protein block, each for 10 minutes. The blow dried sections were incubated with primary antibody of Metallothionein (Novus biological; Anti-metallothionein (UC1MT, Lot-02011619) (1:200 dilution) and Malondialdehyde (Santacruz; F-25, sc-130087) (1:500 dilution) for 20 minutes and 60 minutes respectively.

After buffer washing the primary antibody amplifier and HRP polymer Quanto (Thermo Scientific; QPH 141222) were applied each for 15 minutes. The enzyme substrate reaction was visualized by applying DAB chromogen (Thermo Scientific; QHCX 141222) and counterstaining with hematoxylin. These stained slides were visualized under the light microscope and brown colored MDA and MT positive cells were observed.

Statistical analysis

Data gathered from the study were tabulated and analyzed using statistical one way analysis of variance (ANOVA) as described by Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

Cadmium concentration in tissues

Results of our study indicated that cadmium level noted in liver, kidney and lungs of all 62 post-mortem cases. The concentration of cadmium ranged from 0.040 to 3.952 ppm in liver, 0.050 to 3.949 ppm in kidney and 0.020 to 3.134 ppm in lungs. The mean cadmium level was 0.792±0.109 ppm in liver, 0.893±0.126 ppm in kidney and 0.622±0.087 in lung tissues. The highest cadmium concentration was recorded in kidney, followed by liver and lowest cadmium concentration noted in lung tissue.

Our findings were in accordance with the findings of various researchers. Dogra *et al*. (1996) noted cadmium in the liver (0.27 ppm) and kidney (1.65 ppm) of naturally poisoned cases of cattle. Higher cadmium concentration in kidney followed by liver, muscle and blood of bovine was reported by scientist Lopez *et al.* (2000). Miranda *et al*. (2001) found 30.7 and 161 µg/kg cadmium in liver and kidney tissues of calves. Sedki *et al.* (2003) inspected the cadmium concentration in bovine grazing on the municipal wastewater spreading field in Morocco and found that these animals were seriously contaminated by toxic metal with specific target organ of liver and kidney, 5.1 µg/g and 10.3 µg/g respectively. Nriagu *et al*. (2009) reported 3.24 and 7.92 mg/kg Cd concentration in bovine livers and kidneys respectively. Waegeneers *et al*. (2009) observed 2.5 fold high cadmium concentration with mean of 2.862 mg/kg in bovine kidney by ICP-MS. Al-Zuhairi *et al*. (2015) found cadmium concentration in range of 0.12243 to 1.4750 ppm in bovine kidney. El-Wehedy *et al.*(2018) noticed cadmium concentration above maximum permissible limit in raw and cooked beef i.e. 0.089 and 0.115 mg/kg respectively in Egypt.

In present study, some of tissue samples had crossed the minimum tolerable dose of Cd i.e. >1.40 ppm, are considered as tissues under risk which further categorized as low and high risk based on obtained cadmium concentration and according to Puls criteria (Puls, 1994) (Table 1). More than 26 % of tissues were having cadmium concentration > 1 ppm. 8, 13 and 5% liver, kidney and lungs had Cd level >2 ppm. This indicates towards the bioaccumulative nature of Cadmium in liver, kidney and lung tissues.

Table 1: Cadmium concentration (ppm) in tissues of bovine (Mean ± SE)

Group	Tissue cadmium concentration (ppm)					
	Liver		Kidney		Lungs	
	N	Mean ± SE	N	Mean ± SE	N	Mean ± SE
Normal (0-1 ppm)	66%	0.280 ± 0.027 [a]	65%	0.286 ± 0.022 [a]	74%	0.281 ± 0.038 [a]
Low risk (1-2 ppm)	26%	1.423 ± 0.070 [b]	22%	1.565 ± 0.112 [b]	21%	1.370 ± 0.084 [b]
High risk (>2 ppm)	08%	2.963 ± 0.309 [c]	13%	3.082 ± 0.208 [c]	05%	2.616 ± 0.278 [c]

Means with different superscripts in column differed significantly ($p<0.01$).

Immunohistochemistry

Immunohistochemical analysis of oxidative stress marker

Metallothionein and Malondialdehyde were done in liver, kidney and lung tissues with low and high cadmium concentration and positive results of staining came out as brown color are represented in Fig. 1 to 6.

Metallothionein antigen is visualized by the anti-metallothionein antibody by peroxidase-DAB reaction, evident as brown color cytoplasmic as well as nuclear stain. There was increased expression of metallothionein observed in liver (Fig. 1), kidney (Fig. 2) and lung (Fig. 3) tissue with high cadmium concentration as compared to the tissues had low cadmium concentration.

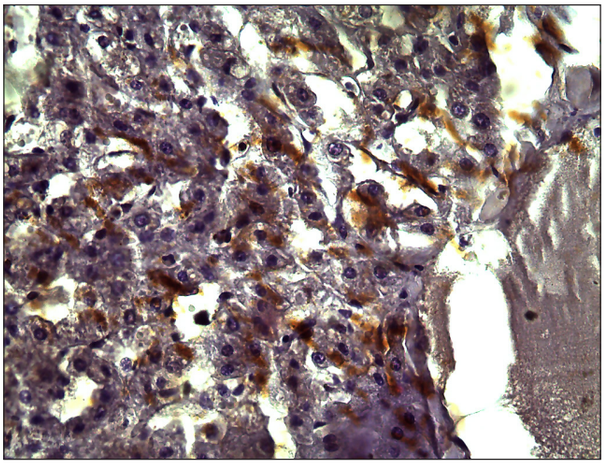

Fig. 1: Microscopic section of cattle liver showing expression of metallothionein (brown color) with high cadmium concentration (IHC 400×)

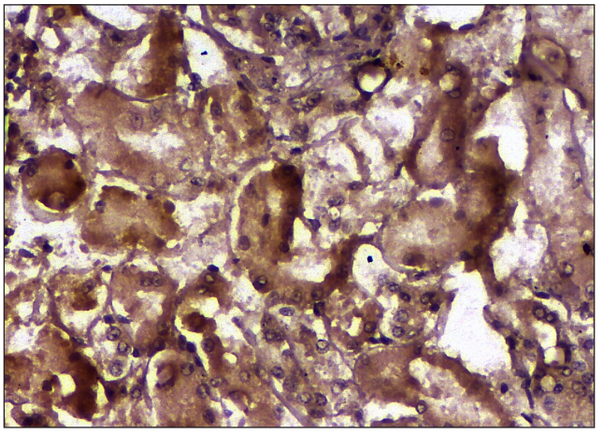

Fig. 2: Microscopic section of cattle kidney showing expression of metallothionein (brown color) with high cadmium concentration (IHC 400×)

In present study, immunoperoxidase staining of Malondialdehyde (MDA) antibody showed brown colored reaction which confined up to the cytoplasm of cells in the liver, kidney (Fig. 4) and lung tissues (Fig. 5). This reaction was quite prominent in tissues with high cadmium concentration as compared to the low cadmium concentration. Tissues had lowest cadmium concentration treated as control (Fig. 6).

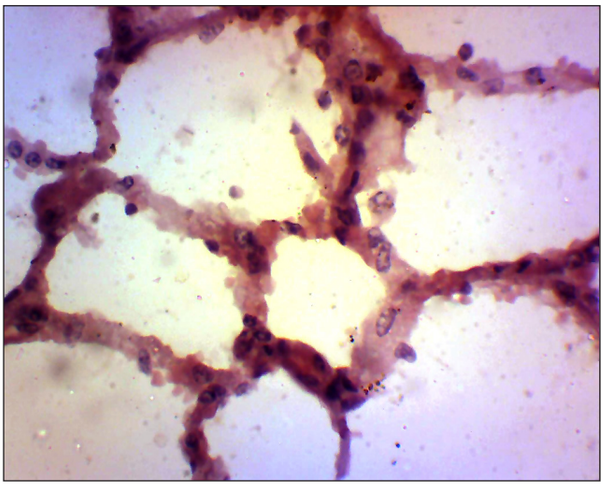

Fig. 3: Microscopic section of cattle lung showing expression of metallothionein (brown color) with high cadmium concentration (IHC 400×)

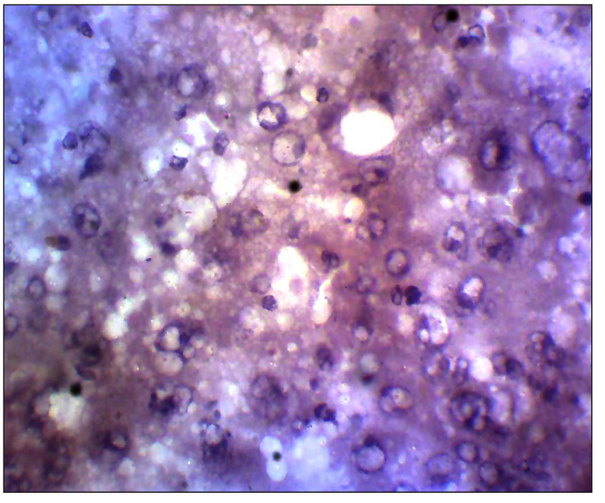

Fig. 4: Microscopic section of cattle liver showing expression of malondialdehyde (brown color) with high cadmium concentration along with moderate fatty changes (IHC 400×)

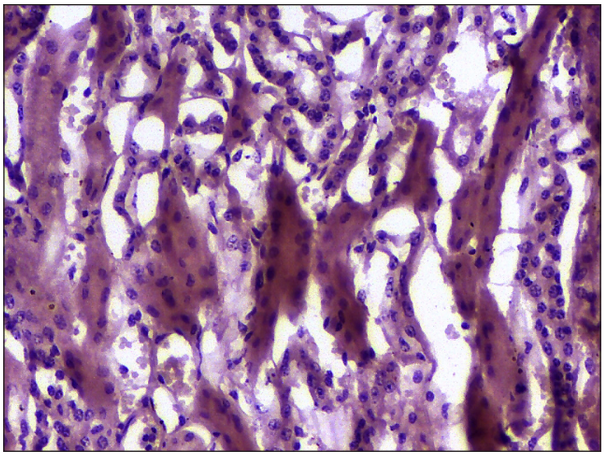

Fig. 5: Microscopic section of cattle kidney showing expression of malondialdehyde (brown color) with high cadmium concentration (IHC 400×)

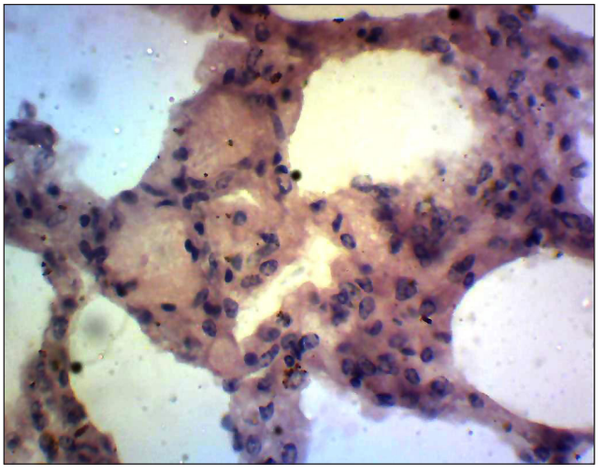

Fig. 6: Microscopic section of cattle lungs showing expression of malondialdehyde (brown color) with high cadmium concentration (IHC 1000×)

Similar to our observations various workers reported the increased oxidative stress in field and experimental cadmium toxicity studies. Banerjee *et al.* (1982) studied immunohistochemical localization of metallothionein in liver and kidney of rats when injected $CdCl_2$ (0.6 mg/kg) I.P. for 2 weeks. Nordberg *et al.* (1994) found cadmium-metallothionein complex with severe tubular injury in mice when injected with cadmium in high concentration. Oxidative stress was demonstrated by increased MDA level in blood, serum, liver and kidney tissues in rats administered with single dose of $CdCl_2$ by Kara *et al.* (2005). Wang *et al.* (2009) administered cadmium chloride (50 mg/l) in drinking water for 8 weeks. They reported immunohistochemical expression of metallothionein I gene by PCR in rat kidney. Buranasinsup *et al.* (2005) noticed increased expression of metallothionein (MT) by immunofluorescence at 0.05 ppm of cadmium in cattle. Salinska *et al.* (2013) reported increased metallothionein in tissues when exposed with 60 µg/g dry weight cadmium in wild and laboratory-bred bank voles.

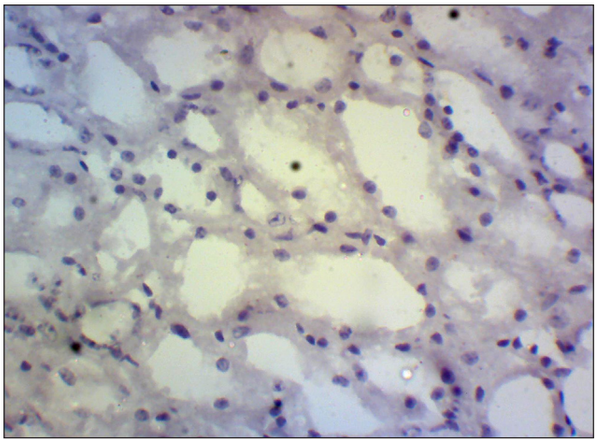

Fig. 7: Microscopic section of control cattle kidney (IHC 400×)

CONCLUSION

Present study pointed the fact that cadmium concentration was obvious in all examined tissue samples of bovine. This is a frightening situation, as quite high proportion of tissues crossed the minimal tolerable dose of cadmium. Over expression of oxidative stress markers indicated that these bovine were under stress. Cells were undergoing degeneration, as noted with increased malondialdehyde, in liver, kidney and lung tissues with high cadmium concentration. Similarly, metallothionein expression pointed the increased overload of cadmium in these tissues, especially in kidney. This oxidative stress may affect the health and productivity of the animals in future. As cadmium is slowly accumulating in the tissues, signifying continuous source of cadmium pollution in food chain of animals, which may further enhance to human beings. It is suggested to prevent the leaching of cadmium in the environment right now before the situation is getting worse.

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