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Biochemical Profile, Micro-mineral Status and Metallothionein Expression in Abattoir Buffaloes Environmentally Exposed to Heavy Metals

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ABSTRACT

Background: The present study was planned to assess the biochemical and micro-minerals profile in blood and other tissues of buffaloes environmentally exposed to heavy metals.

Methods: Tissues (liver and kidney) and blood samples (n=50) were collected from local abattoir. Based on the level of heavy metals, animals were classified as exposed and control was found to have heavy metals in normal range. Blood and tissue sample from both groups were analyzed for micro-minerals, antioxidant status, metabolic profile and expression of metallothionein-2 (MT-2).

Result: Exposed group was found to have significantly ($p<0.05$) higher level of arsenic and chromium as compared to the control group. Level of Copper (Cu) and zinc (Zn) were observed to be significantly ($p<0.05$) higher in exposed animals as compared to control but their concentrations were below the permissible limit in both the groups. Cobalt (Co) and iron (Fe) level were normal in all tissues but Fe level was lower than permissible limit in blood. MalonIdialdehyde (MDA), the activities of superoxide dismutase (SOD) and catalase (CAT) was found to be significantly increased ($p<0.05$) in tissues and blood of exposed group. The exposed buffaloes were found to have significantly ($p<0.05$) increased glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatine kinase (CK), alkaline phosphatase (ALP), urea and creatinine level as compared to control group. Fold change expression of metallothionein (MT-2), had maximum in liver, followed by kidney and blood as compared to control group. The study concluded that heavy metals exposure and low concentration of micro-minerals in buffaloes could result in oxidative damage and alterations in the expression of metallothionein.

Key words: Buffalo, Cu, Co, Fe, LDH, Metallothionein.

INTRODUCTION

Modernization in the development of industries and agriculture is provoking the increase in environmental concentration of heavy metals in water, feed, air and soil (Hundal *et al.* 2006), missing from reference list. Farm animals, especially cattle and buffalo are very useful bio indicators of environmental pollution (FAO, 2019; Dhaliwal and Sushma 2016; Bhardwaj *et al.* 2021). Heavy metals like chromium (Cr) arsenic (As), cadmium (Cd) and lead (Pb) are potential bio accumulative toxins for the dairy production system (Li *et al.* 2005) and may cause deleterious effects on animal and human health.

Micro minerals are very essential part of animal ration. Their optimum quantity inside the body is essential for survival and proper functioning of the animal. As these are present in trace amount in the nature, their deficiency is usually frequented in animal. Since different tissues and blood of livestock are used in preparation of the different ration meals, therefore, it was of our interest to know about the status of these micro-minerals (Zn, Cu, Co and Fe) in tissues (liver, kidney) and blood of buffaloes exposed to heavy metals.

Heavy metals cause extensive tissue and cellular damage, which is largely due to imbalance between reactive oxygen species (ROS) production and defense presented by antioxidants. Heavy metal exposure causes alteration in the expression of antioxidant enzymes and cellular proteins. The intracellular low molecular weight cysteine-rich proteins having high affinity for heavy metals, including essential (Zn and Cu) and non-essential (Cd and Hg) trace elements (Kagi, 1991), metal binding proteins like metallothioneins (MTs) play important role in transporting and regulation of heavy metals (Margoshes and Vallce 1957; Masters *et al.* 1994).

The data regarding heavy metal exposure, micro-minerals and antioxidant levels in buffalo's tissue and blood

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in relation to MTs expression is scarcely available. Therefore, the present study was planned to assess the levels of heavy metals, micro-minerals, antioxidants, biochemical profile, and metallothionein (MT-2) expression in buffaloes slaughtered at abattoir.

MATERIALS AND METHODS

Ethical approval

Experimental protocols using buffaloes in this study have been approved by Institutional Animal Ethical Committee (IAEC) of Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India. All experiments with buffalo were carried out according to the guidelines of IAEC.

Location of sample sollection

Local abattoir of Murrah buffaloes (3-4 year of age) located in Ludhiana district, Punjab, India.

Sample collection

(a) The liver and kidney tissue samples were collected on ice pack in tissue collection vials and blood samples were collected in heparinized vials.
(b) For RNA isolation buffalo's tissue samples were collected in RNALater® (Ambion, Inc., The RNA Company, Sigma-Aldrich, USA) and blood samples in Tri-Reagent BD (Sigma-Aldrich, USA) respectively from freshly slaughtered, apparently healthy animals and stored at -80°C until processing.

Processing of samples
Separation of plasma

Blood samples were centrifuged at 800 g, for 10 min at room temperature to separate plasma. The plasma samples were stored in aliquots at -20°C.

Preparation of 10% RBC hemolysate

After separation of plasma, sedimented cells were washed with 0.9% NaCl solution thrice and were hemolyzed with 9-fold volume of distilled water to prepare 10% hemolysate.

Preparation of tissue homogenate

A 10% tissue homogenate was prepared in 0.1 M Tris-HCl, pH-7.5 by using tissue homogenizer with Teflon pestle followed by sonication of the homogenized tissue. The homogenate obtained was centrifuged at 2500 g for 15 min. at 4°C in a refrigerated centrifuge. The supernatant was collected and immediately used for estimation of heavy metal and micro-minerals determination.

Tissue homogenate and hemolysate were used to analyze reduced blood glutathione, superoxide dismutase (SOD), lipid peroxidation and catalase.

Determination of heavy metals and micro-minerals in tissues homogenate and plasma

Glassware decontamination

All glassware used for heavy metals and micro-minerals estimation were washed in detergent, soaked overnight in chromic acid, rinsed several time with triple distilled water, and dried in hot air oven. Blood plasma and tissue homogenate were digested in a conical flask after adding 5 ml triple acid (HNO_3, 70% $HClO_4$ and H_2SO_4 in 10:3:1, v/v) (Ludmila, 1976). It was covered and kept overnight at room temperature. Then solution was heated on hot plate until it became clear and about 0.5-1 ml of solution is left. The resultant solution was diluted to 10 ml with triple distilled water and used for estimation of heavy metals and micro-minerals. The concentrations were estimated by inductively coupled plasma optical emission spectrometer (PerkinElmer, Optima 2100DV) by using standard operating conditions meant for specific heavy metals and minerals and as prescribed by the manufacturers. All the determinations were performed in duplicate. The animals with heavy metal levels within permissible range have been taken as control.

Antioxidants and biochemical parameters

Blood and tissues levels of erythrocytic malondialdehyde (MDA), superoxide dismutase (SOD) activity, the levels of reduced glutathione (GSH), glutathione peroxidase (GPx), catalase (CAT), vitamins C and E were analyzed as described by Bhardwaj *et al.* (2021). The concentration of glucose, total protein, albumin, creatinine, urea and the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) and creatine kinase (CK) in plasma were determined using Erba Manheim kits on the fully automatic biochemical analyzer (Global 240 BPC Biosed).

Isolation of RNA and real time PCR

Isolation of RNA and preparation of complementary DNA (cDNA) was carried out as per Bhardwaj *et al.* (2021). The cDNA was stored at -20°C for real-time Polymerase Chain Reaction (RT-PCR). Real time PCR (qPCR) was performed using aliquots of cDNA and KAPA SYBR FAST qPCR Master Mix (2X) kit (KAPA BIOSYSTEM, Sigma-Aldrich, USA). The oligonucleotide sequences for MT2 forward and reverse primers are 5′AAAGATTGCAAGTGCGCCTC3′ and 5′CACTTGTCCGAAGCCCCTTT3′ respectively. RPL4 (Ribosomal Protein L4) was used as the reference gene (Nygard *et al*. 2007) and forward and reverse primer sequence were 5′TTGGAAACATGTGTCGTGGG3′ and 5′GCAGATGGCGTATCGCTTCT3′ respectively. A melting curve analysis was performed for each primer pair.

Statistical analysis

The data were analyzed using statistical package for social sciences (SPSS) software (ver. 16.0). Statistical comparison between means of different groups were carried out using independent t-tests. The result of real time PCR was then calculated using: ΔCt = Ct [Target]-Ct [Housekeeping] and $\Delta\Delta Ct$ = (ΔExp.) - (ΔControl). After using formula $2^{-\Delta\Delta Ct}$ the fold change expression of MT2 gene was calculated.

RESULTS AND DISCUSSION

Heave metals and micro-minerals levels

The data obtained from ICP-OES (Inductively coupled

plasma - optical emission spectrometry) analysis of tissue homogenate of liver, kidney and blood plasma has been categorized as heavy metal exposed and non-exposed groups (Fig 1). Initially, buffaloes having the levels of chromium and arsenic in liver, kidney and blood above the permissible limits were considered as heavy metals exposed group and other group as control group. The Cr concentration was found to be as high as 0.6323±0.09036 ppm in the liver tissue as low as 0.2408±0.02111 ppm in the blood of buffaloes. The As concentration was found as high as (0.7455±0.23163 ppm) in kidney followed by liver (0.705±0.20219 ppm) and blood (0.4718±0.03769 ppm) which are values higher than permissible limits (Puls, 1994; ANZFA, 2001).

The level of copper, zinc, cobalt and iron in exposed and control tissues (Liver, kidney) and blood has been presented in Fig 2. The concentration of copper in liver, kidney and blood was 2.3055±0.51262, 0.729±0.08597 and 0.6428± 0.06308 ppm respectively. When compared with the permissible limit of 200 ppm (ANZFA, 2001), the copper concentration was found to be very low in blood and tissues, which are exposed to heavy metals. Petra *et al*. (2011) have reported increase in blood Pb concentration in lead-exposed calves and decrease in blood Cu which was comparable to our study. On the contrary, Somasundaram *et al*. (2005) recorded higher Pb, Cd and Cu serum concentration in jersey crossbred cattle. The concentration of zinc in liver, kidney and blood was 6.2543±0.53465, 6.258±0.78513 and 2.7083±0.37866 ppm respectively. The level of zinc in all tissue and blood sample was found to be significantly lower as compared with permissible limits (150 ppm) set by ANZFA. The low concentration of zinc might be attributed to zinc deficient soils, consequently the fodder/cereals available to buffalo might be deficient of zinc. Perhaps, this was one of the reasons for low tissue content of zinc. Similar findings has been report by Ahmed *et al*. (2009) found low level of copper, zinc and iron level in buffalo-cow intoxicated with heavy metal. Contrarily, Skalicka *et al*. (2005) reported presence of higher Cu level in muscle, heart, liver and kidney of cows from a polluted area. Doyle and Spaudling (1978) found the concentration of Zn in liver of cattle in the range of 100-300 ppm. El-Salam *et al*. 2013 found the highest zinc concentration (41.85 mg/kg) in the meat of buffalo.

The mean levels of cobalt in liver, kidney and blood were found to be 0.0203±0.00331, 0.0225±0.004 and 0.0263± 0.00214 ppm respectively. Cobalt is required in the form of cobalt-containing vitamin B 12. Cobalt is widely distributed in the animal organs in relatively high concentrations in liver, kidney, bone, spleen and other glandular tissues. The analysis of tissues and blood of exposed slaughtered buffaloes had concentration of Co (Fig 2) which is within normal limits (<1 ppm) (McDowell 2012). The lowest (mean±S.E.) concentration of Fe (11.2005±1.43115 ppm) was found in blood (Fig 2) amongst

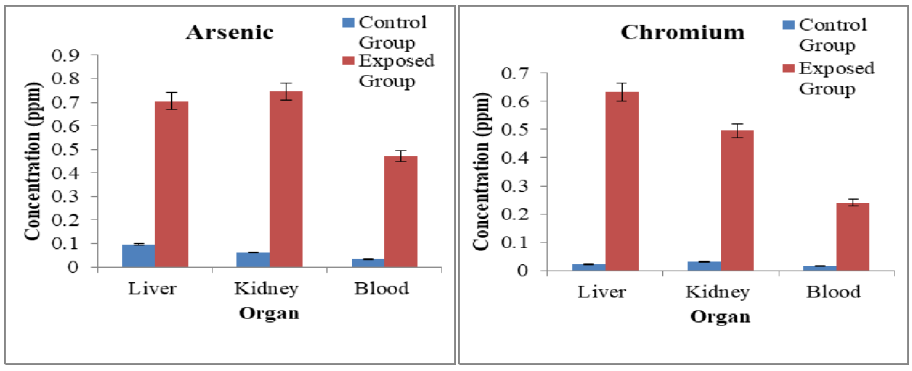

Fig 1: Heavy metal concentration (ppm) in tissues and blood sample of slaughtered buffaloes (Mean±S.E.).

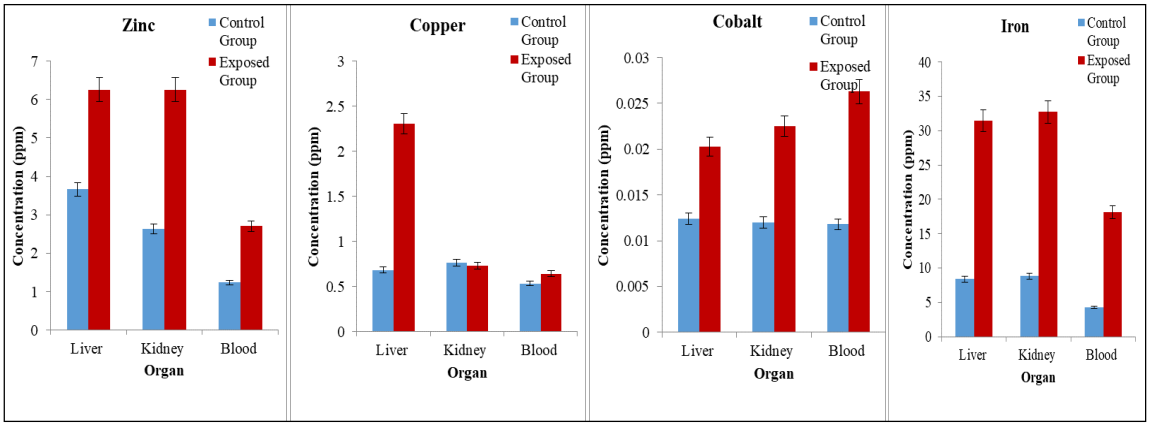

Fig 2: Micro mineral concentration (ppm) in tissues and blood sample of slaughtered buffaloes (Mean±S.E.).

all the tissues analyzed. The permissible limit of iron in food is generally 30-150 mg/kg (Demirezen and Urec 2006). Heavy metal exposed group were found to be carrying lower level of micro minerals like zinc, copper, cobalt and iron as compared to control group in the present study.

Oxidative stress markers

In the current study, the overall Mean±S.E value of MDA level (Fig 3) was found to be significantly ($p<0.05$) increased in all tissues and blood of heavy metals exposed group in comparison to the control group. Increased level of tissue and erythrocytic MDA might be due to over production of reactive oxygen species (ROS) (Sinha *et al*. 2008; Rana *et al*. 2010). The increased erythrocytic malondialdehyde levels observed in buffaloes having both arsenic and chromium levels above the permissible limit, in this study are in agreement to Rana *et al*. (2010a) who observed elevated lipid peroxides in red blood cells of naturally arsenic exposed cattle. Other authors have reported similar findings in blood (Rana *et al.* 2010; Dhaliwal and Sushma 2016; Yeotikar *et al*. 2018) and tissue (Bhardwaj *et al*. 2021) of heavy metal exposed buffaloes.

The level of oxidative stress in animal body can be assessed by free radical scavenging enzymes such as SOD, CAT and GST (endogenous antioxidants) in blood (Roy *et al*. 2013). The overall level of superoxide dismutase and catalase activity in heavy metal exposed groups was found to be significantly ($p<0.05$) increased in exposed group as compared to control group (Fig 3). The increase in tissue and erythrocytic SOD and CAT activity might be a cellular protection mechanism against enhanced production of superoxide radicals during heavy metal exposure (Yamanaka *et al.* 1991). As SOD and CAT have a protective role against oxygen free radical-induced damage, their induction can be understood as an adaptive response to oxidative stress (Bhardwaj *et al*. 2021). Since SOD catalyzes the dismutation of superoxide anion to H_2O_2, which is in turn the substrate of CAT, this fact could explain the observed increment of the two enzyme activities. Contrary to the current findings, few other studies reported significant inhibition of the antioxidant enzyme activities in blood of heavy metal exposed buffalo and cattle (Dhaliwal *et al.* 2016; Dash *et al.* 2019; Yeotikar *et al.* 2018; Bhardwaj *et al*. 2021).

Level of blood GSH was found to be significantly ($p<0.05$) lower in exposed group as compared to control group buffaloes. The reduction in GSH makes cells more prone to oxidative injuries (Kumar and Padhy 2013). The decrease in level of GSH and increase in level of MDA is in agreement with previous studies in cattle and buffalo blood (Dhaliwal *et al.* 2016; Bhardwaj *et al*. 2021). Gurer *et al*. (2000) reported decrease in GSH level in rat chronically exposed to different heavy metals. Ahmed *et al.* (2009) showed that hypocupremic cow had increased MDA level, decreased total antioxidant activity, GSH-R and ascorbic acid, which causes oxidative stress to buffaloes and cows resulting in cessation of ovarian activity.

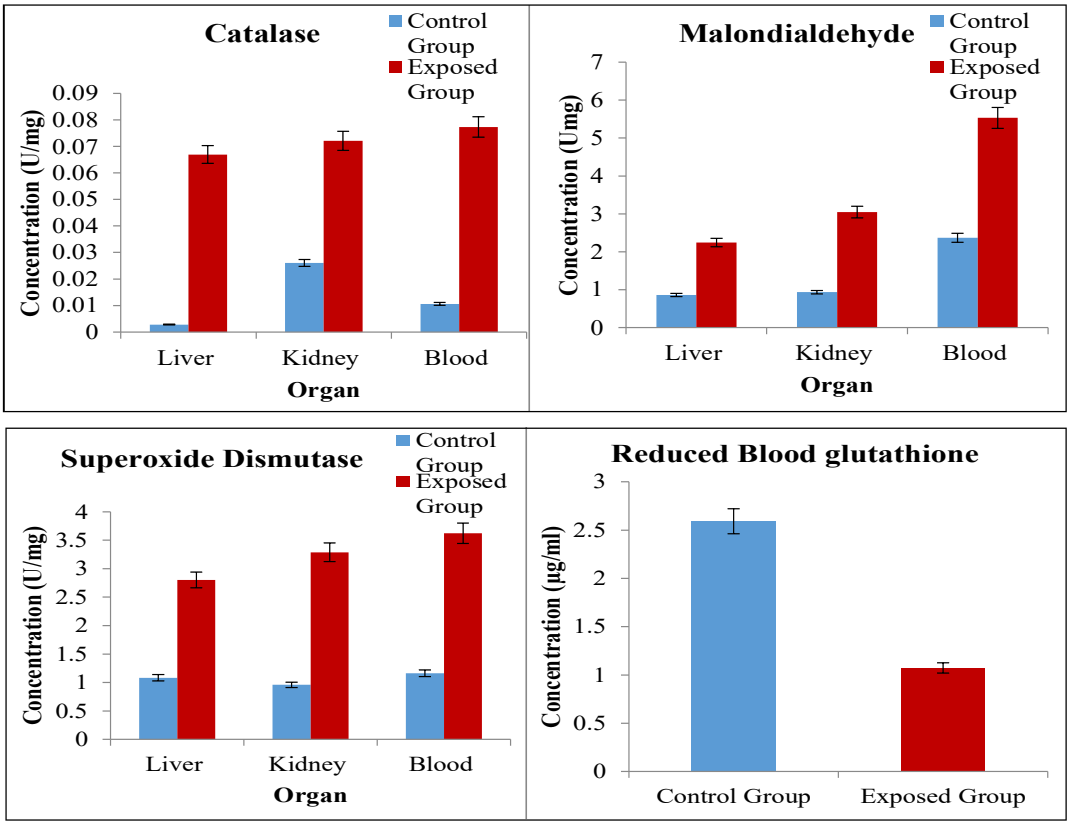

Fig 3: Oxidative stress markers in vital organs of buffalo environmentally exposed to heavy metals.

Metabolic profile (mean±S.E.) in plasma of abattoir buffaloes

The present study also explored the influence of high heavy metals and low level of micro-minerals on some biochemical markers in buffalo (Fig 4). Presence of toxic heavy metals in blood and low level of Cu, Zn and Fe than permissible level, leads to significant increase in glucose level in blood. In addition, total protein and albumin level in plasma (Fig 4) were observed to be decreased significantly ($p<0.05$). The glucocorticoid system might have been disturbed thus leading to increase in glucose and decrease in total protein and albumin level in metal exposed group (Kaltreider *et al.* 2001). Increased glucose has been reported due to high exposure of different heavy metals like arsenic and lead in ruminant and mice (El-Nekeety *et al.* 2009; Rana *et al.* 2010; Mohajeri *et al.* 2014; Dash *et al.* 2016; Bhardwaj *et al.* 2021). Significant ($p<0.05$) decrease in total protein levels in heavy metal exposed buffaloes might be due to toxic effects of these heavy metals on protein synthesis. The reduced levels of albumin reported in the present study might be due to liver dysfunction caused by heavy metals (As, Cr). The reduced levels of total protein and albumin have been reported in heavy metal exposed fish (Panigrahi *et al.* 2016; Javed *et al.* 2017). The elevated level of creatinine and urea in heavy metal exposed buffaloes might be due to nephrotoxic metals like As, Pb, Cr. (Bhardwaj *et al.* 2021).

Liver and kidney are considered principal target organs for various heavy metals like arsenic lead and chromium. It has been found that level of AST, ALT, ALP show significant ($p<0.05$) increase (Fig 4) revealing that there is damage to liver tissue due to increase in concentration of heavy metals. Since the liver tends to accumulate heavy metals, hepatic involvement is reported most commonly as a complication of chronic exposure (Winski and Carter, 1998). Liver dysfunction is accompanied by elevated level of serum hepatic marker enzymes, which are indicative of cellular leakage and loss of functional integrity of cell membrane in the liver. High levels of plasma ALT and AST are sensitive indicators of hepatic damage (Kaneko *et al*. 1997). The membrane bound enzyme ALP activity is also related to status and function of hepatic cells. Increased plasma ALP can be due to increased synthesis in the presence of increased biliary pressure. Level of serum urea and creatinine showed significant ($p<0.05$) increase in concentration explaining heavy metal effects on kidney (Bhardwaj *et al.* 2021).

Plasma enzymes LDH and CK were found to be significantly ($p<0.05$) higher (Fig 4) showing anaerobic changes in body at the time of slaughtering and muscular injury to animal body. LDH, CK activity is found to be highest in skeletal muscle. Various toxic agents including metals can cause myonecrosis that in turn results in elevated plasma CK activity (Kaneko *et al*. 1997). In the present study, increased level of plasma hepatic and muscle function enzymes in buffaloes might be due to extensive injury of liver and skeletal muscles due to chronic heavy metal exposure and also due to stress in animal at time of stress. Several reports explained the elevated levels of plasma

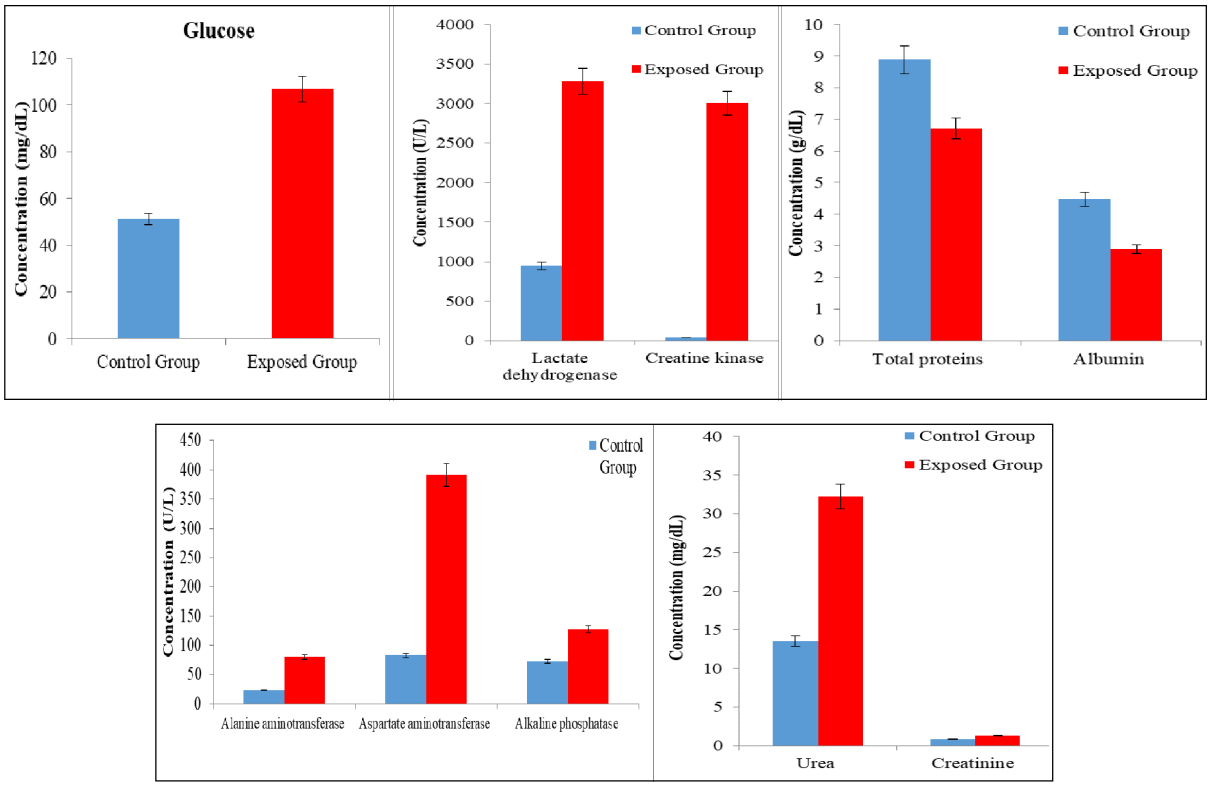

Fig 4: Metabolic profile (mean±S.E.) of buffaloes environmentally exposed to heavy metals.

hepatic and muscle function enzymes due to heavy metal in cattle and mice (El-Nekeety *et al.* 2009; Rana *et al.* 2010; Mohajeri *et al.* 2014; Dash *et al.* 2016; Bhardwaj *et al.* 2021). Increased levels of plasma urea and creatinine with decreased plasma total proteins and albumin levels observed in the present study may indicate protein catabolism, degenerative changes and hypo function of kidneys and liver (Bhardwaj *et al.* 2021). These results clearly showed that heavy metal exposure to buffalo have adverse effect on hepatic, renal and muscle tissue which is in accordance with the previous reports (El-Nekeety *et al.* 2009, Rana *et al.* 2010; Mohajeri *et al.* 2014).

Expression of metallothionein (MT-2)

Metallothionein-2 was studied to check its proportionate fold change expression resultant to heavy metal exposure in blood and tissues (liver and kidney). Amplification of RPL4 and MT2 gene with NTC was done using real time PCR (Fig 5). The melting curve analysis showed that all amplicons produced single-peak melting curves at the expected temperature, implying specific amplification (Fig 6). Fold change expression of MT-2 in environmentally heavy metal exposed samples have been presented in (Fig 7). Mean Ct value for Liver-MT, Kidney-MT and Blood-MT were 20.0344±0.661, 19.7225±0.953 and 21.5923±0.953 respectively. This was compared with endogenous gene RPL4 whose mean Ct values in liver, kidney and blood exposed to heavy metals were 22.9529±0.887, 22.0075±1.486 and 24.5466±0.647 respectively. It has been found that fold change expression ($2^{\wedge-\Delta\Delta ct}$) of metallothionein-2 (MT-2) in liver (8.162±1.595) showed up-regulated expression followed by kidney (5.257±1.351) and blood (3.0096±0.952) compared to control group. In recent study, it has been observed that in low trace minerals and heavy metals exposed group MT-2 level is up regulated. It might be because of MTs having high affinity for essential heavy metals (Zn and Cu) and non-essential (Cd and Hg) elements (Kagi, 1991). Primarily, metallothionein binds tightly to Cu and Zn. However, in presence of high levels of heavy metals, trace-minerals will be replaced by heavy metals (Shaw *et al.* 1991). Bhardwaj *et al.* (2021) reported maximum expression of MT-2 in liver followed by kidney and blood in both separate chromium and arsenic exposed group in buffalo. Apart from chelating heavy metals, MT-2 also has antioxidant property. Up-regulation of MT-2 expression might protect animal body from oxidative stress by scavenging the free radicals, generated due to increased level of heavy metals in body (Ruttkay-Nedecky *et al.* 2013; Liu *et al.* 2007). Marked increase in metallothionein was also reported in liver of lead and nickel heavy metal injected mice (Šveikauskaitė *et al.* 2014) which is in line with the present study. Increased expression of MT-2 could be a cellular defense mechanism, which either prevents the damage caused due to heavy metals by chelating them or lessen their effect by acting as cellular antioxidant.

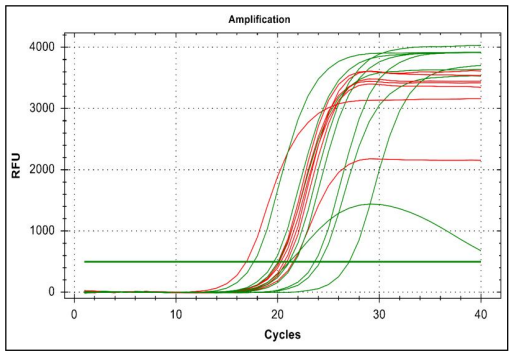

Fig 5: Amplification curve of RPL4 (green) and MT2 (red).

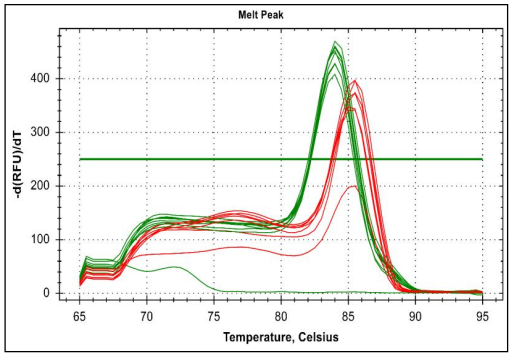

Fig 6: Melt peak curve of RPL4 (green) and MT2 (red) along with one NTC (no amplification).

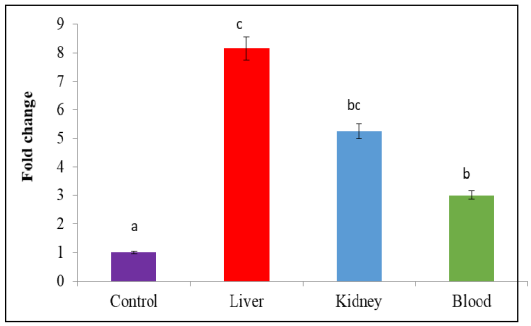

Fig 7: Fold change expression of metallothionein (MT-2) in tissue and blood of exposed buffalo (Different superscripts are significantly different ($p<0.05$).

CONCLUSION

The data obtained from blood and tissue of abattoir buffaloes, which were exposed to heavy metals have lower level of micro-minerals and altered metabolic and antioxidative status along with change in the expression of metallothionein.

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