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Original Article

Comparative Analysis of Heavy Metal Accumulation Pattern and Genotoxicity in Water Fowl

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ABSTRACT

Waterfowl spend their lives mostly on water bodies include ducks, geese, and swans, also include birds such as coots, grebes, moorhens, shorebirds and seabirds etc. Wetlands in Pakistan offer habitat to a variety of migratory birds. Bird's diversity facing problems due to loss of natural habitat and hunting causing serious issues to bird's territories. **Objective:** To investigate bioaccumulation of heavy metals and genotoxicity in waterfowl. **Methods:** This study investigated bioaccumulation of heavy metals and genotoxic effects that could result from exposure of waterfowl to heavy metals in the Chenab River, Punjab, Pakistan. The three different species, common teal (Anas crecca), little egret (Egretta garzetta) and mallard (Anas platyrhynchos) were obtained from head Marala, River Chenab. The liver, kidneys, heart, muscle, blood, and feathers of birds were collected for the purpose of determining the presence of heavy metals. The study investigated the most common heavy metals Pb, Cd, Ni, and Cr indicating higher concentrations of heavy metals in blood and feathers from the study site. At study sites Pb Cr, Ni concentrations were high ($P \leq 0.05$). **Results:** The results in the current study revealed metals concentration in different species trend as Anas crecca > Egretta garzetta > Anas platyrhynchos. The deposition of heavy metals in organ trends as Pb>Cr>Ni>Cd. The level of metals in blood trends as Pb>Cr>Ni>Cd. Metal concentrations in feathers trend as Pb>Cr>Ni>Cd. **Conclusions:** Expression analysis of anti-apoptosis Bcl-2 made for Egretta garzetta and genotoxicity results showed that the effect of metals in study groups found negative for the expression of the Bcl2 gene.

INTRODUCTION

Wetlands consolidate aquatic and terrestrial ecosystems and have both absolute and restricted capacities. Avifauna an important component of the wetland system occupies tropical pyramids in the wetland food web and nutrient cycles. Waterfowl spend their lives mostly on water bodies include ducks, geese, and swans, also including birds such as coots, grebes, moorhens, shorebirds, and seabirds etc. Wetlands in Pakistan offer habitat to a variety of migratory birds. Bird's diversity facing problems due to the loss of natural habitat and hunting causing serious issues to bird's territories [1]. The Chenab River has three main sub-regions (head marala, head khanki, head Trimmu). Head Marala is an important place for a variety of migratory waterfowl including Anatidea and Ardeidae [2]. The level of metals toxicity in the migratory birds depend on the food items, frequency, time of exposure and metals collect in their organs [3].

A high amount of heavy metals affects biological processes such as age, growth, feeding habits, and molting [4,5]. Waterfowl may suffer from physiological disturbance and even death due to the high concentration of heavy metals in organs and blood [6].

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Heavy metals induce genetic effects on waterfowl, metals have both indirect and direct effects (Eeva *et al*., 2005); indirectly increase the number of oxidative stress in species, and direct effects involve neurological or physiological changes [7].
Heavy metals induce aberrant gene expression and a high apoptosis rate. The accumulating tendency of heavy metals in the blood and feathers of waterfowls was shown to be statistically significant in this study. We investigated the effects of heavy metals toxicity on waterfowl by measuring the expression level of the anti-apoptosis *bcl-2* gene and found negligible effects on anti-apoptotic markers.

METHODS

To study the heavy metal concentration in waterfowl organs, Eighteen birds (n=18) of three waterfowl species including *Anas crecca*, *Egretta garzetta*, and *Anas platyrhynchos*, six (n=6) birds of each species were captured by hand in four trappings from the different regions of Chenab River at head Marala region. The average amount of dissolved oxygen from the starting point of sampling to the endpoint was found almost the same which was almost 7.
Both breast and tail feathers were taken from each species by cutting feathers at the distal part from random individuals, using stainless steel scissors to investigate trace metals in sediments on the sites of the river Chenab [8].

Data Collection: Waterfowls were captured using big mesh nets with the assistance of local hunters for blood (3-5) sampling prior to release.

Digestion of feather samples for heavy metals analysis: To remove exterior contaminants for heavy metals concentration analysis, feather samples were washed three times with tap water, distilled water, and finally acetone. In an oven feathers samples dried at 20°C for 2h, crushed into small pieces, weighed and transferred into flask and added 1.0 mL of HNO3 and 0.25 mL of per-chloric acid for digestion. The digested samples were analyzed for heavy metals analysis by using atomic absorption spectrophotometer [9].

Digestion of blood samples for heavy metals analysis: Samples of blood 1 mL was collected in 100 mL digestive flasks after 10 mL concentrated nitric acid was added and the contents were stirred for 20 minutes [10]. After heating and drying the sample at room temperature, add 5 ml of chloric acid and mix vigorously until white vapors form and the sample volume is reduced to 2–3 ml. A final 50 mL of re-distilled water was added, and the level of heavy metals (Cr, Cd, Ni, Pb) in blood was measured using graphite furnace atomic absorption spectrometry [11].

Digestion of organ samples for heavy metals analysis: Heavy metals accumulation in the kidney, heart, liver, spleen, and muscle tissues assessed by 0.5 gram samples processed using a 20-ml mixture of 1:1 (volume/volume) 65 percent HNO_3: HCl for 30 minutes at 100°C (on a hotplate). Samples were then diluted to 5 ml volume using ultra-pure water for estimation of heavy metals.

Atomization atomic absorption spectrometry analysis: The concentrations of heavy metals Pb, Cr, Ni, and Cd in kidney, liver, heart, spleen, muscle tissue, blood, and feather were measured using an atomic absorption spectrometer (Hitachi Science and Technology Z 5000 Polarized Zeeman Flame/Graphite Furnace Atomic Absorption Spectrophotometer), as described by Andrade and colleagues (2014) [12].

***Bcl2* gene expression analysis:** *Bcl2* gene expression analysis from blood samples of waterfowl species *Egretta garzetta* made after collecting blood from bird's jugular vein in vacationers without anti-coagulant. For RNA extraction Trizol reagent (Invitrogen, USA) was utilized (37 ^{o}C for 10 minutes). Centrifugation was performed at 4oC for 15 minutes after the addition of 200 -µl chloroform for 5 minutes at room temperature, and the supernatant was transferred to a fresh micro centrifuge tube and incubated at room temperature for 10 minutes. 500 µl isopropanol was then added and centrifugation was made at 12000g at 4^{o}C for 10 minutes. RNA-pellet washed twice using 75% ethanol in diethylpyrocarbonate (DEPC; Sigma Aldrich, USA) water. Pellet was finally dried by adding RNase-free water to re-suspend the pellet. RNA quantification was finally made by spectrophotometer.

For cDNA synthesis, one microgram RNA employing Revert-Aid H Minus first strand-cDNA synthesis-kit (from Invitrogen, United States) followed according to the manual's instructions.

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Primer designing: For expression analysis of the *bcl2* gene in waterfowl species (*Egretta garzetta*) *Actb* genes as internal control primers are designed using the primer 3 website (www.primer3.cgi). Primer designed, synthesized and optimized (Table 1).

Gene	Forward Primer	Reverse Primer
Bcl2	CAG CCA GGA GAA ATC AAA CAG AGG	ATC GCC CTG TGG ATG ACT GAG
Actb	GTA GTT TCG TGG ATG CCA CA	TCC CTG GAG AAG AGC TAC G

Table 1: The sequence of primers used

Gene expression profiling and analysis: The quantitative real-time PCR analysis of waterfowl exposed to heavy metals made for anti-apoptotic Bcl_2 gene made using Maxima SYBR Green-qPCR master mix (Fermentas, USA. *Actb* is used as an internal control. Gene expression analysis was made by pico-Real software (Thermo Scientific, USA).

RESULTS

The heavy metals lead (Pb), chromium (Cr), nickel (Ni), and cadmium (Cd) were measured in blood, feather, and tissue samples of water birds from Head Marala in the Chenab River (AAS).

Anas crecca (common teal) study

Level of heavy metals observed in Feather Cr (31±5), Ni (29±5), Cd (5±1), Pb (88±44), whereas in blood Cr (31±8), Cd (9±2), Pb (156.0±54), Ni (33±5). However, in Liver Cr (57±9), Cd (7±3), Pb (186.0±50), Ni (41±8) observed. In Kidney Cr (40±9), Ni (31±12), Pb (170±50), Cd (5±3) estimated. Similarly, in Heart Cr (19±8), Ni (29±10), Cd (5±2), Pb (66±20). Whereas in Spleen Cr (15±8), Ni (25±10), Cd (4±2), Pb (56±30). In Muscle Cr (13±5), Ni (13±4), Cd (4±2), Pb (89±38).

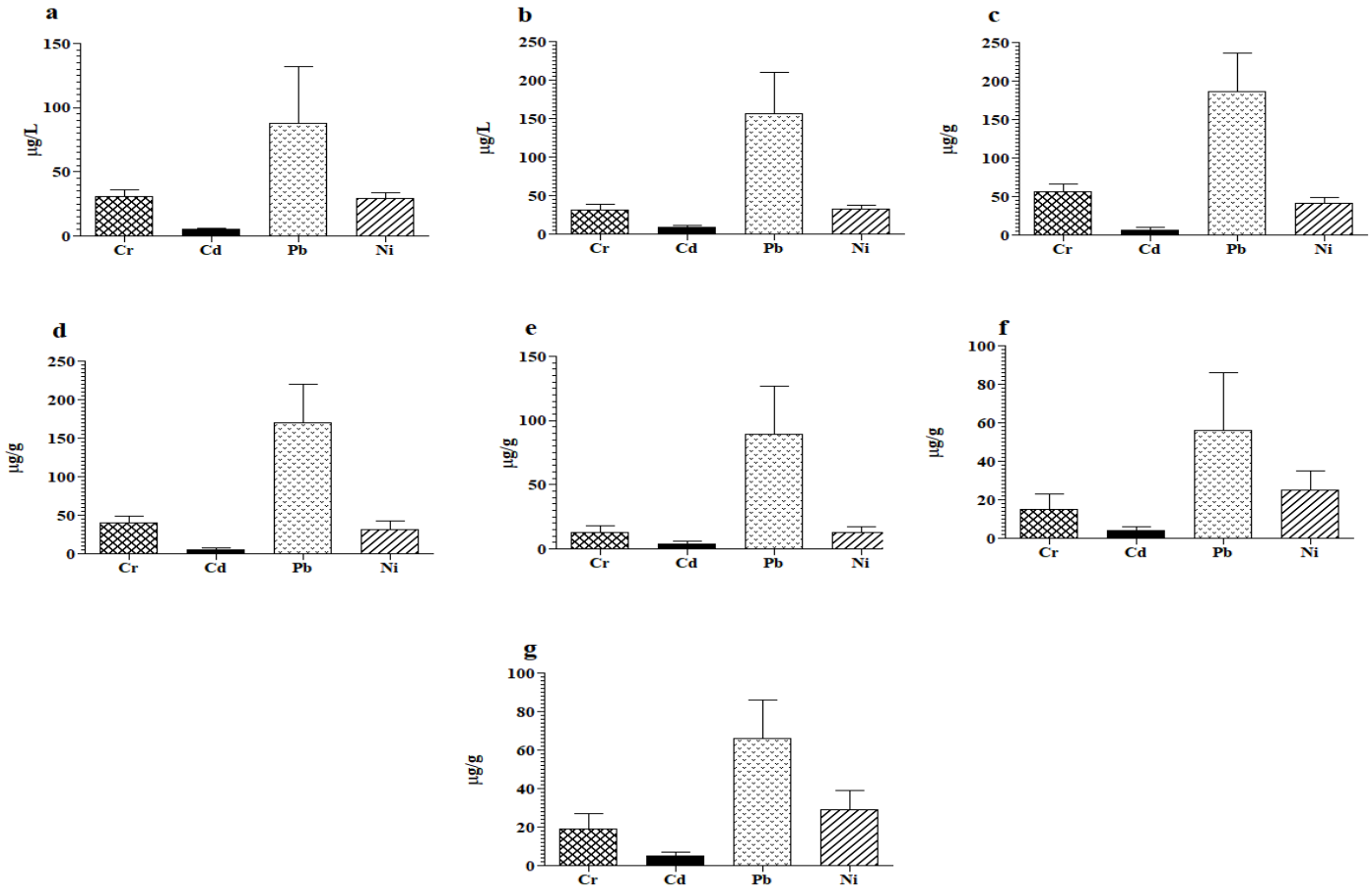

Figure 1: Metal concentration in analysis in common teal *Anas crecca* (a) feather (b) Blood (c) Liver (d) Kidney (e) Hearth (f) Spleen (g) Muscles. Bar representing in Mean and ±SD

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***Egretta garzetta* (Little egret) study**

In Feathers Cr (33±7), Ni (21±3), Cd (10±2), Pb (110±31) observed. Whereas in Blood Cr (38±8), Ni (24±3), Cd (5±2), Pb (127±25) estimated. However, in Liver, Ni (31±3), Cr (46±9), Cd (5±2), Pb (141±60) observed. The metal concentration in the Kidney is Cr (27±9), Cd (5±2.2), Pb (81±30), Ni (13±7) estimated. Also, in Heart Cr (23±7), Cd (5±1.9), Pb (70±27), Ni (17±4). In Spleen Cr (18±7), Cd (5±3.3), Pb (58±17), Ni (14±5). While in Muscle (18±5), Cd (5±2.4), Pb (41±28), Ni (14±4).

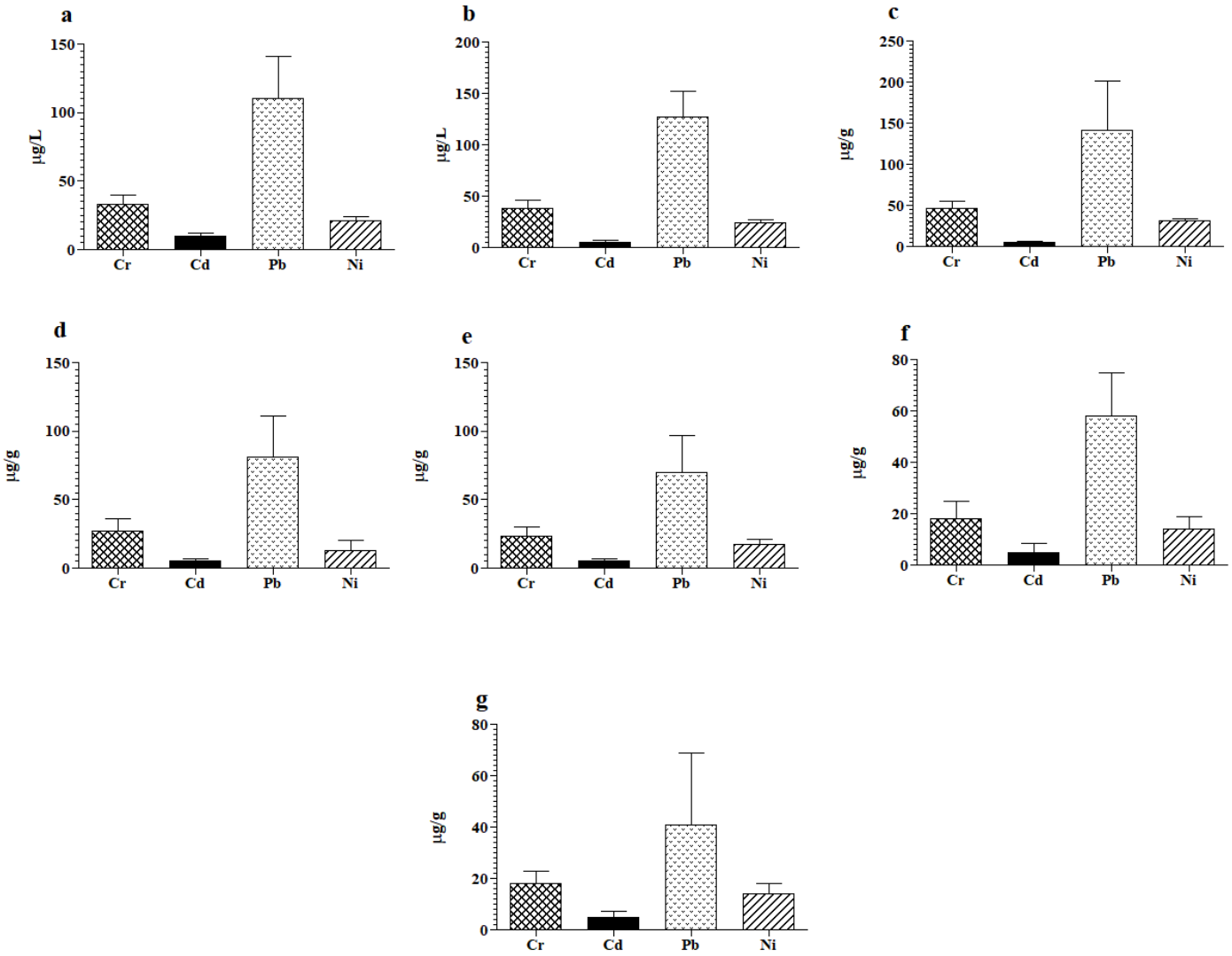

Figure 2: Metal concentration in little egret *Egretta garzetta* (a) feather (b) Blood (c) Liver (d) Kidney (e) Hearth (f) Spleen (g) Muscles. Bar representing in Mean and ±SD.

***Anas platyrhynchos* (*Mallard)* study**

While In feather Cr (19±6), Cd (5±3), Pb (74±26), Ni (35±10). However, In blood Cr (23±8), Cd (6±3), Pb (91±31), Ni (42±10). In Liver metals concentration as Cr (26±4), Cd (5.6±2.8), Pb (72±20), Ni (37±8). In Kidney Cr (26±4), Cd (5.6±2.8), Pb (72±20), Ni (37±8). Whereas in Heart Cr (14±3), Cd (4±2.3), Pb (62±20), Ni (22±5). In Spleen Cr (13±4), Cd (5±2), Pb (40±13), Ni (18±8). In Muscles Cr (13±4), Cd (3.8±1.9), Pb (33±10),Ni (15±8).

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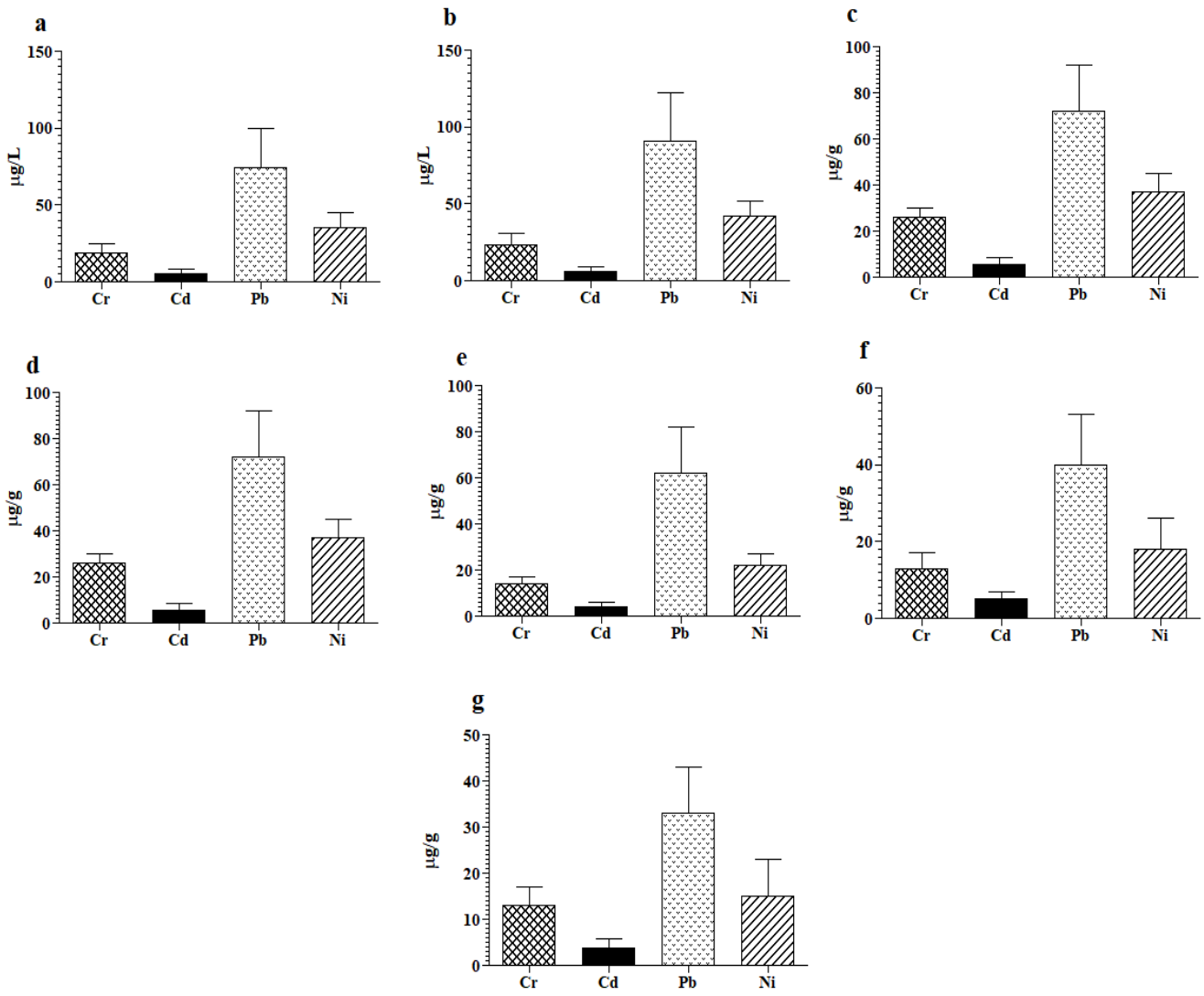

Figure 3: Metal concentration in *Mallard* (*Anas platyrhynchos*) (a) Feather (b) Blood (c) Liver (d) Kidney (e) Hearth (f) Spleen (g) Muscles. Bar representing in Mean and ±SD.

Real-Time PCR Analysis: The effect of heavy metals (Cd) exposure on the anti-apoptotic marker Bcl2 gene in Little egrets (Egretta garzetta) was investigated by Real-Time PCR technique. To see if the heavy metal has any effect on birds, RNA was isolated and cDNA was prepared for qRT-PCR investigation. Metals (Cd, Cr, Pb, Ni) had no effect on the expression of the anti-apoptotic gene Bcl2 in the study groups.

DISCUSSION

Trace metal contamination from the environment may be due to the excretion of contaminants from human activities or the release of waste material [13]. The origin of heavy metals might either be due to bedrock weathering or contaminated sediments from mine. Heavy metal contamination in water birds may be due to polluted water. The non-essential heavy metal cadmium may cause harmful effects by binding with building networks of proteins when concentrated in the body through absorption either by the respiratory or digestive system [14]. The results in the current study revealed high metals concentrations in different species trends as *Anas crecca* > *Egretta garzetta* > *Anas platyrhynchos*. Metals deposition in blood and organ tissues trends Pb>Cr>Ni>Cd. The metal concentrations in organs are in the following order: Liver>Kidney>heart>spleen > muscle tissues. Gender did not contribute significantly to the accumulation of trace metals [15]. The study site had a significantly highest value of Pb than other metals is in consistent with (Burger and Gochfeld,

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1991) that interspecific differences were significant for metal accumulation trends [16]. The feathers of three studied species had the significantly elevated concentration for Pb is in agreement with the two studies [16,17].

Heavy metal affects birds' egg size, and egg thickness and reduces eggshell and DNA in birds [18]. In the current analysis, significantly higher values of (Cr) Chromium were observed in organ tissue and blood samples among all species.

The results of the present study had lower cadmium deposition in blood, feather, and organ tissues than the threshold concentration and it may be due to low cadmium contamination at the study site. Cadmium accumulation was lower than the background levels and the results are in consistence with other research [19].

A variety of hormones require Ni as a cofactor. Excessive nickel ingestion, on the other hand, can cause cell damage, altered hormonal and enzymatic function, oxidative stress, and neurotoxicity [20]. The mean Ni concentrations found in blood serum, feathers, and organ samples in this study were higher than the threshold levels.

The current study indicated high heavy metal levels that may be a consequence of long-term use of fodder crops with high metals contents. Agricultural procedures, crops, and fertilizers that enter water bodies consumed by water birds can be attributed to the high percentages of heavy metals in the blood and tissues of birds.

Expression analysis of *bcl-2* genes in waterfowl blood: The *bcl-2* anti-apoptosis genes family regulates mitochondrial membrane permeability including *bcl-2* apoptotic gene triggers the apoptotic cascade [21], and cell fate may be determined by the balance of *bcl-2* proteins. Studies reported heavy metals to decrease expression levels of anti-apoptotic *bcl-2* genes in different animal studies [22]. Similar studies demonstrated modulation of *bcl-2* gene in apoptosis pathways [23]. In the current study, genotoxic effects of heavy metal exposure evaluated by *bcl-2* gene expression analysis, however, the effect of heavy metals was observed negligible in a study group (little egret (*Egretta garzetta*) for expression of *bcl2* anti-apoptotic gene. However, further studies are needed to explore any role of heavy metals in causing genotoxicity in waterfowl.

CONCLUSIONS

Due to industrialization methods and anthropogenic activities, heavy metals directly enter the water bodies resulting in elevated levels of metals in water. The results revealed that higher HMs concentration in numerous concentrations as *Anas crecca* > *Egretta garzetta* > *Anas platyrhynchos* with metal deposition trends Pb>Cr>Ni>Cd. The metal concentrations in organs within the following order: Liver>Kidney>heart>spleen>muscle tissues, equally metals deposition high in blood compared with a feather. Biological watching is believed to be satisfactory for quantifying serious heavy metal abundance and bioavailability. This study found the upper level of serious heavy metals at the head Marala, River Chenab. Meanwhile, we tend to overlook the fact that birds largely pay their lives in water or close to water. However, our study evaluated that heavy metals exposure in waterfowl induces no effects on cell apoptosis estimated by a negative effect on *bcl-2* gene expression. In conclusion, special attention is needed to the security of water birds. However, more studies square measures are needed to evaluate the danger to water birds' lives.

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