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Accumulation of some selected heavy metals in *Lepus nigricollis* from Pakistan

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ABSTRACT

Accumulation of heavy metals viz; Cd, Cr and Pb was investigated in Indian hares *(Lepus nigricollis)* collected from different areas of Pakistan. Three organs viz; kidney, liver and muscles of 15 female, 13 male and three juvenile individuals were studied to find out the level of Cd, Cr and Pb accumulation, and distribution among sex and age groups along with tissue. A significant variation in studied heavy metals among selected organs (kidney, liver and muscle) was recorded. Median concentration of Cd was highest in kidney (6.85µg/g) as compared to liver (6.00 µg/g) and muscle tissues (2.25µg/g). Concentration of Pb was highest in liver (9.32µg/g) as compared to kidney (8.42 µg/g) and muscles (4.12µg/g). In relation to age, Pb and Cr concentrations were much different while the concentration of Cd was significantly higher in kidney and liver of adults (7.98 µg/g and 7.43 µg/g) as compared to neonates (5.40 µg/g and 3.96 µg/g). In relation to sex, higher Cd concentrations were recorded in kidney of female individuals (7.5 µg/g) in comparison to males (6.75 µg/g) while Pb was higher in liver in males (9.43 µg/g) than in female Indian hares (7.27 µg/g). Level of Pb and Cd metals in *Lepus nigricollis* was higher than consumable limits of meat described by European Commission Regulation (2006) as: Cr-1.0µg/g, Cd-0.05 µg/g and Pb-0.1µg/g. Meat of *Lepus nigricollis* could not be consumed by human without health risk as it had a higher level of Cd and Pb concentrations.

KEY WORDS: Chromium, Cadmium, Lead, Accumulation, *Lepus nigricollis*, median.

INTRODUCTION

Heavy metals pollution is a serious problem at the global level whether it is from natural or anthropogenic source [1,2]. Rapid industrialization, mining, automobiles, agriculture and use of advance technology are the major sources of heavy metal pollution [3]. Heavy metal from fertilizers are continuously entering in trace amounts [4] and accumulated in soil, from where it accumulate in plants, then enter into the human food chain and cause a risk to human health [5,6]. Once these heavy metals enter into the food chain, it will ultimately transfer to human body directly or indirectly [7]. Heavy metals are toxic and cause risk to organisms [8] as well as human even in trace amounts [9]. Heavy metals in the food chain are more concerned at higher trophic levels [10,11] due to its health impacts on man [12].

Cadmium is a toxic element that occurs in almost all kind of surface waters, soils, and plants [13]. It is a major threat to wildlife and has been reported in different vertebrate populations but its toxicity in vertebrates is not well recognized [14,15]. However, it causes metabolic abnormalities and structural and functional disturbance in different organs such as visceral and reproductive organs [16] by changing the hormonal level [17] and decreasing fertility in male and female organisms [18]. Cadmium pollution is a serious problem and that causes toxicity in animals and human e.g. Itai-Itai disease [19].

Chromium is widely used in industries [20] and has two states Cr^{+3} and Cr^{+6} of which Cr^{+3} is an essential element for mammals to maintain protein, lipid and glucose metabolism but Cr^{+6} is carcinogenic in its impacts [21] may cause bronchial carcinomas. Most of Cr^{+6} produced as the result of emissions released by domestic and industrial activities [22]. Lead is not essential for growth of living organisms but has many adverse effects including disturbance of growth, metabolism and reproduction, neurobehavioral disorders, renal disruption and hypertension [23]. Sources of Pb contaminants include water, soil, paints, dust, petrol and brass plumbing fixtures [24].

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Wild hare (*Lepus nigricollis*) is a small game animal that occupies a variety of habitats ranging from deserts to thick forests. It includes agricultural fields, forest, crops, barren land and grasslands. Ideal habitat for this species includes large areas of scrub and uncultivated land, which merge with cultivated plains [25]. *Lepus nigricollis* is distributed throughout oriental region ranging from Pakistan to Indonesia and feed on grasses, forbs, crops and vegetables depending upon the availability of food type.

Lepus nigricollis is a threaten species due to hunting pressure, destruction of habitat, cutting of forests for agricultural purposes, forest fire, livestock competition [26] and environmental pollution. Environmental pollution due to industrialization cause metal toxicity. Objective of the present study was to assess the accumulation of heavy metals (Cd, Cr and Pb) in kidney, liver and muscles of *Lepus nigricollis* in relation to age and sex.

MATERIALS AND METHODS

A total of 31 sampling sites were selected throughout the Pakistan including Sindh, Balochistan and Punjab provinces. Distribution of *Lepus nigricollis* in the world is given in Figure 1, while study area is given in Figure 2.

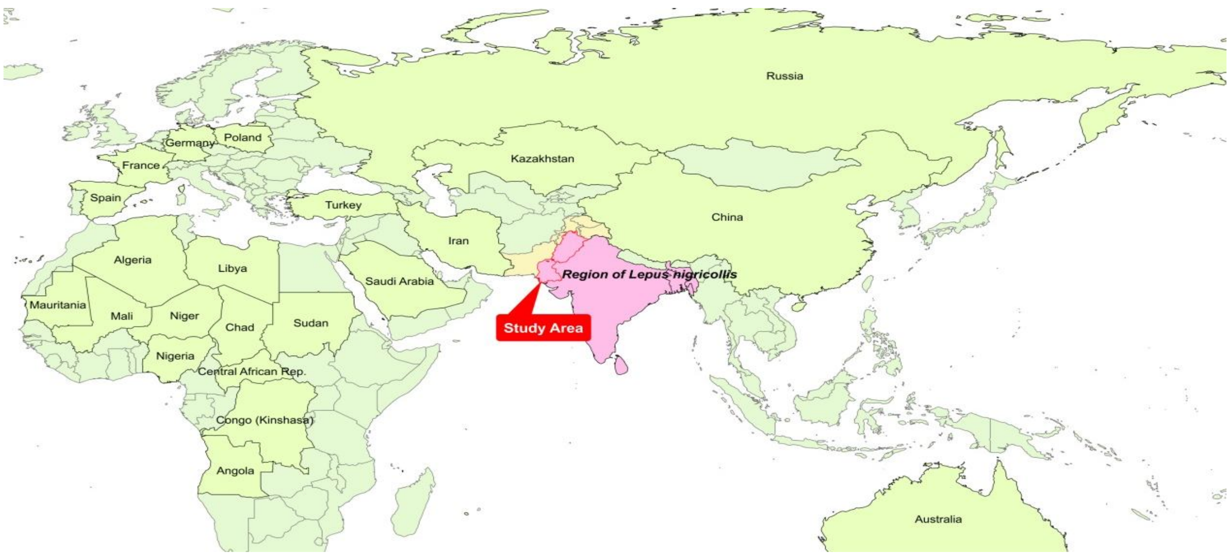

Figure 1: A modified map of *Lepus nigricollis* distribution in the world.

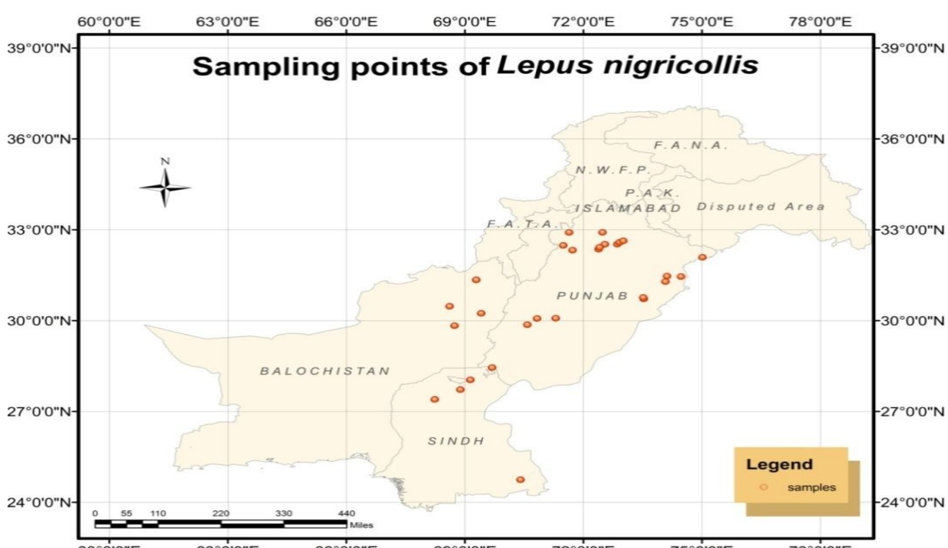

Figure 2: Map showing sampling sites from where *Lepus nigricollis* were collected.

For hare collection, Commonwealth Scientific and Industrial Research Organization (C.S.I.R.O) modified mechanical traps were used [27]. Indian hares have a total length ranging from 40-70 cm and weight 1.35-7 kg.

They have long ears and large hind feet. Instead of mechanical traps, manual catching was also used for hare catching. Before the dissection process, age and sex of all specimens was determined. Crystalline dry weight was used [28] to find out the age of specimens. Morphometric criteria [28] were also used in identification method.

After examination of hare for the above said information, specimens were transferred to a lab for dissection. Dissection of specimens was carried out to take some selected internal organs/tissues (kidney, liver and muscles) for heavy metal analysis. After dissection selected organs of each specimen were sealed in separate lip lock plastic bags with all necessary information like specimen code, place, age, sex, and date of dissection, and then stored at - 20 °C before digestion process.

One gram wet sample of each organ was removed and digested with 1:4 mixtures of $HClO_4$ and HNO_3 [29] at 200 °C to 250 °C until the liquid became transparent. On being transparent volume was raised up to 50 ml with double distilled water. All digests were filtered through 0.42µm filter paper to remove impurities. These digests were transferred into polyethylene bottles that were rinsed with 5% HNO3. These bottles were at 4 oC to avoid contamination and chemical reactions.

According to Commission Regulation (EC) No 1881/2006, the maximum level for Cd and Pb in meat of bovine animals is 0.050 µg/g and 0.1 µg/g respectively. While permissible level for Chromium (Cr) is 1 µg/g.

Heavy metal analysis of samples was carried out by using Atomic Absorption Spectrometer (Varian FS 240AA) at some standard conditions discussed by Malik and Jadoon [30]. Analyses were done in the form of triplicate to increase the precision of results. To compare results One-way analysis of variance (ANOVA) and basic statistical analysis were performed. SPSS, and GenStat were used to find out the correlation and regression models to highlight the relationships among different variables (Cr, Cd, Pb, Male, Female, Neonate, Juveniles, Adults and sub adults).

RESULTS

A significant variation was found in accumulation of Cd, Cr and Pb among selected organs (kidney, liver and muscle) of all *Lepus nigricollis* that is given in table 1. The highest concentration of Cd metal was recorded in the kidney while the lowest was in muscle tissues. Cd concentration in kidney was in range of 2.50-17.10 µg/g. In liver and muscle Cd was in the range of 2.55-9.90 µg/g and 0.85-4.75 µg/g respectively. Concentration of Cr in kidney, liver and muscle was in the range of 0.15-2.40 µg/g, 0.70-3.40 µg/g and 0.05-1.25 µg/g respectively. Similarly, the concentration of Pb in different organs was in the range of: kidney (0.37-16.49 µg/g), liver (3.53-19.37 µg/g) and muscle (1.12-7.22 µg/g).

Table 1: Concentration (µg/g wet weight) of Cd, Cr and Pb in selected organs of Indian hares collected from different areas of Pakistan

	Kidney			Liver			Muscle		
	Cd	**Cr**	**Pb**	**Cd**	**Cr**	**Pb**	**Cd**	**Cr**	**Pb**
Max	17.10	2.40	16.49	9.90	3.40	19.37	4.75	1.25	7.22
Min	2.50	0.15	0.37	2.55	0.70	3.53	0.85	0.05	1.12
Mean	7.33	1.51	8.34	6.09	1.56	9.53	2.70	0.70	3.99
Med	6.85	1.60	8.42	6.00	1.50	9.32	2.25	0.70	4.12
SD	2.74	0.56	3.66	2.20	0.57	4.08	1.20	0.22	1.84

Max= Maximum, Min= Minimum, Mean= Mean, Med: Median, *SD*= Standard Deviation

In relation to sex and age, concentrations of selected heavy metals (Cd, Cr and Pb) in kidney, liver and muscle tissues are given in table 2-4. Range of Cd concentration in the kidney was 5.40-7.89 µg/g while in liver and muscle was 3.70-7.43 µg/g and 2.00-3.15 respectively. In comparison of sex, significantly higher (p<0.005) median concentration of Cd was reported in female kidney (7.5 µg/g) than in male kidney (6.75 µg/g). Whereas the median concentration of Cd was higher in kidney and liver of adult hares (7.98 µg/g and 7.43 µg/g) than in kidney and liver of sub-adult (6.70 µg/g and 6.67 µg/g) as well as neonate animals (5.40 µg/g and 3.70 µg/g). Therefore Cd concentration was increasing with increase in age of the specimen. The reported concentration of Cr was lower as compared to Cd and Pb in selected organs (kidney, liver and muscle). Range of Cr in kidney and liver was higher (1.30-1.68 µg/g and 1.40-1.65 µg/g) than in muscles (0.65-0.75 µg/g). Although the concentration of Cr was higher in kidney and liver of neonates (1.68 µg/g and 1.65 µg/g) but the difference was not statistically significant. Lead (Pb) was highly accumulated in kidney and liver. In comparison of sex, significantly higher median concentration of Pb was detected in the liver of male specimen (9.43 µg/g) than in female specimen (7.27 µg/g). In relation of age, the difference in Pb accumulation was not statistically significant.

Table 2: Concentration of cadmium in Indian hare in relation to sex and age

	Cd in Kidney					Cd in Liver					Cd in Muscle				
Parameters	**Med**	**Mean**	**Min**	**Max**	***SD***	**Med**	**Mean**	**Min**	**Max**	***SD***	**Med**	**Mean**	**Min**	**Max**	***SD***
Male	6.75	7.08	4.60	10.45	1.89	6.90	6.27	2.90	9.90	2.09	2.25	2.88	1.50	4.70	1.06
Female	7.50	7.82	2.85	17.10	3.30	7.20	6.36	2.55	9.55	2.35	2.00	2.47	0.85	4.75	1.34
Adults	7.98	9.38	7.20	17.10	3.29	7.43	7.13	2.55	9.15	1.96	2.35	2.57	0.90	4.45	1.22
Sub-adult	6.70	7.15	2.85	11.65	2.20	6.90	6.67	3.05	9.90	2.15	3.15	2.91	0.85	4.75	1.34
Neonate	5.40	5.60	2.50	8.80	1.83	3.70	3.96	2.90	4.95	0.72	2.03	2.44	1.25	4.25	0.97

Max= Maximum, Min= Minimum, Mean= Mean, Med: Median, *SD*= Standard Deviation

Table 3: Concentration of Chromium in Indian hare in relation to sex and age

	Cr in Kidney					Cr in Liver					Cr in Muscle				
Parameters	**Med**	**Mean**	**Min**	**Max**	***SD***	**Med**	**Mean**	**Min**	**Max**	***SD***	**Med**	**Mean**	**Min**	**Max**	***SD***
Male	1.60	1.53	0.80	2.40	0.52	1.45	1.52	0.70	3.40	0.67	0.75	0.70	0.05	1.10	0.26
Female	1.40	1.39	0.15	2.30	0.59	1.40	1.55	0.70	2.65	0.54	0.70	0.71	0.35	1.25	0.21
Adults	1.30	1.30	0.65	2.20	0.44	1.43	1.47	0.80	2.65	0.59	0.65	0.63	0.35	0.80	0.17
Subadult	1.60	1.53	0.15	2.40	0.62	1.45	1.43	0.70	2.15	0.44	0.75	0.72	0.05	1.25	0.26
Neonate	1.68	1.69	0.55	2.30	0.55	1.65	1.89	1.25	3.40	0.69	0.73	0.75	0.60	1.10	0.16

Max= Maximum, Min= Minimum, Mean= Mean, Med: Median, *SD*= Standard Deviation

Table 4: Concentration of Lead in Indian hares in relation to sex and age

	Pb in Kidney					Pb in Liver					Pb in Muscle				
Parameters	**Med**	**Mean**	**Min**	**Max**	***SD***	**Med**	**Mean**	**Min**	**Max**	***SD***	**Med**	**Mean**	**Min**	**Max**	***SD***
Male	8.08	7.09	0.37	14.32	3.94	9.43	9.21	3.53	17.77	3.88	3.87	3.56	1.12	6.92	1.80
Female	8.68	8.62	4.62	12.74	2.62	7.27	8.77	4.84	13.72	3.46	4.12	4.42	1.81	7.22	1.89
Adults	9.48	8.35	2.12	12.74	4.23	10.06	9.49	3.53	13.72	3.88	3.89	3.95	1.34	6.84	2.05
Subadult	8.39	7.46	0.37	11.17	2.84	7.27	8.82	4.84	17.77	3.68	4.49	4.51	1.59	7.22	1.66
Neonate	8.27	9.96	4.03	16.49	4.33	10.44	10.90	4.31	19.37	5.09	2.27	3.06	1.12	5.83	1.80

Max= Maximum, Min= Minimum, Mean= Mean, Med: Median, *SD*= Standard Deviation

Correlation analysis (Table 5) showed significant ($p < 0.05$) relationships between metal concentrations accumulated in different hare organs. Cd concentration accumulated in kidney had a positive relationship with Cd accumulation in the liver, Pb in liver and muscle whereas negative relationship with Cr in kidney and muscle. Cd in liver showed a negative relationship with Cr in kidney and liver while positive relation with Pb in muscles. Cr in liver had a relationship with Pb in liver. Pb Concentration in liver had a significant positive relationship with Pb accumulation in kidney. Similarly Pb concentration accumulated in the muscles also had a significant positive relationship with a Pb concentration in liver.

Table 5: Correlation between heavy metal concentration in selected organs (kidney, liver and muscles) of *Lepus nigricollis*

	Cd-K	**Cd-L**	**Cd-M**	**Cr-K**	**Cr-L**	**Cr-M**	**Pb-K**	**Pb-L**	**Pb-M**
Cd-K	1								
Cd-L	0.489*	1							
Cd-M	-0.091	0.094	1						
Cr-K	-0.315*	-0.437*	-0.003	1					
Cr-L	-0.098	-0.308*	-0.144	0.059	1				
Cr-M	-0.312*	0.108	0.139	0.009	-0.102	1			
Pb-K	-0.011	-0.14	-0.236*	0.134	0.195	-0.069	1		
Pb-L	0.230*	0.115	0.001	0.073	0.284*	-0.199	0.500*	1	
Pb-M	0.276*	0.271*	-0.051	0.203	0.051	-0.242*	0.073	0.431*	1

Note: Marked correlations are significant at $p < 0.05$.
Cd-K= Cadmium in kidney, Cd-L= Cadmium in liver, Cd-M= Cadmium in body muscle,
Cr-K= Chromium in kidney, Cr-L= Chromium in liver, Cr-M= Chromium in body muscle, Pb-K= Lead in kidney, Pb-L= Lead in liver, Pb-M= Lead in body muscle.

DISCUSSION

Generally, heavy metals are absorbed by plants from the soil and their sources either natural sources viz; parent rock material, erosion and weathering of ores or human activities, which include energy and production of fuels, transmission of power, use of waste water for irrigation purposes and agricultural activities [31,32]. An increase in industrialization is responsible for increasing the concentration of heavy metals in atmosphere [3]. In agricultural areas with heavy metals can be originated from many different sources, including fertilizers, manures, agrochemicals, sewage, liming materials, irrigation water and atmospheric deposition. Atmospheric deposition is an anthropogenic source of heavy metal contamination and metal deposition on vegetation as well as on soil. Generally it causes higher concentrations of heavy metals than other sources.

In Indian hares (*Lepus nigricollis)* oral route is the major path of metal uptake. Feeding of metal contaminated vegetation causes the accumulation of heavy metals in hare and disrupts integrity of the food chain. Concentrations of these metals present in an organism can disturb the biological functioning [24]. There are many different biotic and a biotic variables that affect the uptake of the pollutant from the source to an organism [33,34,35,36]. After uptake, it interacts with metabolism of biological individual on four sites including metabolism, excretion, storage and action sites [36,37,38].

There was a significant variation of heavy metal accumulation among different hare organs (kidney, liver and muscles). Toxic metals (Pb, Cd and Cr) showed a different accumulation pattern with high values in kidney and liver while low values in skeletal muscles. Pb was the heavy metal that recorded with the highest value of accumulation while Cr was least accumulated heavy metal.

Generally mammals uptake heavy metals through ingestion and inhalation [39] then into different organs of the body (kidney, liver and muscles). Therefore major path of heavy metal exposure in animals is oral consumption [40]. Age of organism and tissue specificity both are important biological factors that influence the level of metal accumulation [39]. The higher concentration of heavy metal accumulates in soft tissues of mammals such as kidney and liver [41]. According to Kolesarova et al. [24], targets of heavy metals are the heart, liver, kidney and nervous system but kidney is the most affected body organ in mammals. Accumulation in these organs varies depending upon different factors like time of exposure, form of heavy metals and heavy metal concentration in contact. Body muscles of hares are being used as meat by human. Hares are at second trophic level food web so study of metal accumulation in selected body organs (kidney, liver, muscle) was carried out to estimate the impact in hares as well as in other mammals.

Cadmium is a toxic heavy metal [13] with ability to cause different biological disorders in mammals. Its major accumulation takes place in liver and kidney of wild [42] and farm animals [43]. Many authors [44,42] declared that Cd was much accumulated in kidney as compare to other body organs.

During the present study, the higher Cd concentrations were recorded in the kidney than liver and muscles of *Lepus nigricollis*. Higher concentration of Cd in kidney (1.57 mg/g) than in liver (0.16 mg/g) of brown hare was also recorded by Kramárová et al. [13]. They also studied Cd concentration in different game animals and found higher concentrations in kidney (0.213 to 2.387 mg/kg) than in liver (0.06 – 0.48 mg/kg). Results of Massanyi et al. [40] also showed higher Cd in kidney of adult hares (1.521mg/g) than in liver (0.154 mg/g). Similarly Toman et al. [43] reported higher Cd contents in kidney of pheasants than in liver. Results of the present study were also supported by Langgemach [45] who found higher concentrations of Cd in kidney than in liver and muscles of wild hares.

In the current study, Cd concentration showed a significant relation with sex as it was higher in female kidney (7.5 µg/g) than in males (6.75 µg/g). Findings of Massanyi et al. [40] were similar to the current study as authors reported higher Cd concentrations in female kidney (1.464 mg/kg) than in males (1.384 mg/kg). Lutz and Slamečka [46] also reported higher concentrations of Cd in female kidney than in males that is supporting the results of current study. Falandysz et al. [47] studied Cd concentration in red deer and reported higher concentrations of Cd in kidney than liver and muscle which is similar as recorded in the present study. A significant variation in Cd concentration was also recorded among different age groups. Cd concentrations were higher in kidney of adult hares than in sub-adults and neonates [48]. Its higher concentration in adults was due to the large time of exposure with contamination. The same trend was recorded by Škrivanko et al. [49] as in adults was (0.783 mg/kg) higher than in younger ones (0.028 mg/kg). Massanyi et al. [40]also showed the same trend as in his study.

Chromium is an essential element in humans to metabolize glucose [22]. In mammals it plays an important role to metabolize carbohydrates. Its accumulation in an organism depends upon the rout of entry and chemical status [50]. In comparison of sex, the concentration of Cr was higher in kidney of male specimen (1.60 µg/g) than in female (1.40 µg/g) while with respect to age, neonates showed Cr concentrations (1.68 µg/g) in their kidney higher than sub-adults (1.30 µg/g) and adults (1.60 µg/g).

Higher concentrations of Pb were recorded in neonate in present study (10.44 µg/g) of *Lepus nigricollis* but difference was not statistically significant. Similar results were reported by Massanyi et al. [40]. Lutz &Slamek [46] also recorded higher level of Pb in juveniles. Venäläinen et al. [51] also recorded higher concentration of Pb in kidney (1.38mg/kg) than in liver (0.864mg/kg) while Cd was many times higher in kidney (12.42mg/kg) than in liver (0.864mg/kg). Pedersen and Lierhagen [52] recorded higher Pb content in kidney of juveniles (0.137-1.247 mg/kg) than in adults (0.122-0.413mg/kg). Cd was higher in kidney (55.2-219.9mg/kg) than in liver (1.68-

10.90mg/kg). The trend of accumulation was similar as observed in the present study. In comparison to results of current study, Cd was many times higher but Pb was lower in their results. On the other hand, Tataruch [53]and Kleiminger& Holm [54] reported higher concentrations of Pb in adult's liver than juveniles. On the comparison of sex, current study showed higher Pb content in liver of male hares than in female. Results of Massanyi et al. [40] were also same in line with the current study.

CONCLUSIONS

In present study concentration of selected heavy metals (Cd, Cr and Pb) accumulated in different hare organs were higher than the permissible level in meat that can be used by humans without the fear of health risk. Only Cr concentration (mean=0.75 µg/g) in muscle tissues was below the maximum limit. Other selected metals Cd and Pb were in the ranges from 0.85-17.10µg/g and 3.53-19.37 µg/g respectively while maximum permissible limits of heavy metals in mammalian meat are: Cr-1.0µg/g, Cd-0.05 µg/g and Pb-0.1µg/g [55,56]. Therefore, according to current regulations, it can be emphasized that the meat of Indian hares *(Lepus nigricollis)* is contaminated. Though average consumption of hares in Pakistan is very low and only a small number of people eat hare as food. It was concluded that meat of hare was not suitable for human consumption because of high metal concentration in their bodies as other authors [57,58] also recommended that internal organs of game animals should not be consumed by human.

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The authors declare that they have no conflicts of interest in the research.

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