Bulgarian Journal of Veterinary Medicine, 2021, **24**, No 1, 119–135
ISSN 1311-1477; DOI: 10.15547/bjvm.2019-0033

Original article

THE IMPACT OF ENVIRONMENTAL LEAD EXPOSURE ON WHOOPER SWAN (*CYGNUS CYGNUS*): PATHOLOGICAL AND IMMUNOHISTOCHEMICAL STUDIES

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Summary

Ahmed, M. S. & M. S. El-Neweshy, 2021. The impact of environmental lead exposure on Whooper swan (*Cygnus cygnus*): Pathological and immunohistochemical studies. *Bulg. J. Vet. Med.*, **24**, No 1, 119–135.

This study was carried out to investigate the pathology of environmental lead (Pb) poisoning in Whooper swans (*Cygnus cygnus*). A number of 12 out 54 swans (22.2%) randomly collected from Honshu, Japan from June 2005 to July 2007 were affected with Pb poisoning. Affected swans showed stained vent with greenish watery diarrhoea and impacted crop. The presence of Pb shots in the gizzard (50%) was confirmed by X-ray, and all cases showed a dark greenish coloured liver. Microscopically, the pathology of Pb poisoning in swans was multisystemic. The severity of the lesions was the highest in the CNS followed by the liver, kidney, spleen, lungs, gizzard, heart, bone marrow respectively and was the least in the peripheral nervous system. CNS lesions were cerebral haemorrhage, malacia, and spongiosis with astrocytic activation and increased neurofilaments accumulations. In addition, there were hepatic and renal hemosiderosis and apoptosis, hepatic granuloma, interstitial pneumonia, gizzard and myocardial necrosis and bone marrow hypoplasia. Chemical analysis of the Pb content in liver and kidneys ranged from 8.18 to 60.6 µg/g, respectively. The extent and severity of lesions varied among individuals and were mostly dose-dependent. Finally, these findings improved the diagnostic procedure of Pb poisoning in free-living Whooper swans.

Key words: immunohistochemistry, Japan, lead shots, lead poisoning, pathology, Whooper swan

INTRODUCTION

Lead (Pb) poisoning is still a noteworthy worldwide issue posing a public health problem of global dimensions (Patočka & Černý, 2003; Newth *et al.*, 2013). Pb is one of the most toxic metals known that affects all physiological systems in ani-

mals, its negative effects range from slight biochemical or physiological disorders to serious pathological damage of vital organs (EFSA, 2010; Franson & Pain, 2011).

Pb is a highly malleable and very dense metal. These two properties have encouraged its use for both fishing weights and ammunition. The popular sports of fishing and shooting have resulted over the years in the release of an enormous quantity of Pb into the environment, and large quantities of Pb have been deposited in water ways (Perrins *et al.*, 2003; Friend *et al.*, 2009). Pb poisoning has been widespread among waterfowl following accidental ingestion of Pb shots or fishing sinkers (Pain *et al.*, 2007; Martinez-Haro *et al.*, 2011) that dissolved by stomach acid and subsequently the toxic salt formed absorbed into the blood (Saito, 2009).

Acute Pb poisoning due to absorption of relative large amount of Pb with food within a short time causes mortality with subtle postmortem findings. Meanwhile, chronic Pb poisoned birds due to chronic, low Pb exposure become weak and usually die of starvation because the digestive system becomes paralysed (Scheuhammer, 1991; Vallverdú-Coll *et al.*, 2015). Pb is a neurotoxin that affects the muscle tone of the oesophagus, thus preventing ingested food from being moved along the digestive tract and increasing risk of starvation (Pattee & Pain, 2003; Franson & Russell, 2014).

Pb poisoning from ingested shots is thought to be a major cause of high mortality in waterfowls throughout the world. For this reason, the use of Pb-based ammunition has been subject to legislative issues and regulations in 30–35 countries over the past 50 years to protect waterbirds and their habitat (Pain, 1992; Mateo, 2009; Stroud, 2014; Kanstrup *et al.*, 2018).

There were few Japanese studies regarding Pb poisoning in waterfowl (Honda *et al.*, 1990; Ochiai *et al.*, 1992; 1993; Kim *et al.*, 1999; Ochiai *et al.*, 1999; Nakade *et al.*, 2005; Ishii *et al.*, 2017). Death of seventeen Whooper and eight Bewick's swans from Pb poisoning was recorded in winter of 1984–1987 at Niigata, Akita, Aomori and Hokkaido (Honda *et al.*, 1990). Pb poisoning in Whooper swans was reported in Lake Miyajima, on a small island located in Hokkaido Prefecture in 1989 (Ochiai *et al.*, 1992), deaths of geese and eight swans caused by Pb poisoning were also observed at the same location in 1990 and 1998, respectively (Ochiai *et al.*, 1993; Nakade *et al.*, 2005). High concentration of Pb were reported in tissues of four Steller's sea eagles and one white-tailed sea eagle in Hokkaido from 1986 to 1997 (Kim *et al.*, 1999). Moreover, Ishii *et al.* (2017) reported high hepatic and renal Pb concentration in 42% of Steller's sea eagles (18 of 43 cases) and 24% of white-tailed sea eagles (12 of 50 cases) that were found dead in Hokkaido (northern part), Honshu (the main island), and Shikoku (a southern island) of Japan from 1993 to 2015.

In Japan, the use of Pb-based ammunition for hunting of large-sized animal species has been banned in Hokkaido since 2004, while in other areas, such as Honshu and Shikoku, there are few regulations regarding the use of Pb-based ammunition and the current situation of Pb poisoning is unknown (Ishii *et al.*, 2017) .

Over the past several years, there has been a consistent number of submissions during the winter months of wild swans that were found dead in Honshu Island, with no overt evidence of clinical symptoms. On gross examination, the birds were in good physical body condition and had adequate fat stores. The only gross

lesion was distended oesophagus with recently ingested feed; in the absence of other gross findings, this lesion raised a strong suspicion of Pb toxicity.

The current study aimed to clarify the environmental Pb-induced pathological and immunohistochemical changes and correlate these changes with the Pb concentration in soft tissues to improve the accuracy of this procedure as diagnostic tool of Pb toxicity in free-living Whooper swans (*Cygnus cygnus*).

MATERIALS AND METHODS

Animals and samples collection

The Whooper swans (*Cygnus cygnus*) were obtained from rescue/rehabilitation centre of Gifu University serving Gifu prefecture areas on Honshu – the main island of Japan during the period from June 2005 to July 2007. Nevertheless, a substantial number of these birds were rescued because of injuries unrelated to lead. The results were based on surveys of rescued swans and so were likely to be biased towards higher lead levels. However, almost all swans admitted to the rescue/rehabilitation centre hospitals were sampled unless they were considered healthy, and therefore ready for an immediate return to the wild. The samples were taken purely where lead poisoning was suspected (emaciation, greenish diarrhoea and impacted gizzard) then there would be an obvious bias towards high Pb levels.

One apparently normal Whooper swan was used as control for chemical analysis of Pb in soft tissues, histopathology and immunohistochemistry.

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Radiography and necropsy

Twelve suspected swans were examined grossly followed by radiography (soft X-ray apparatus Softex CMBW-2; Softex Co., Ltd, Tokyo, Japan). The carcasses were dissected and all gross lesions were recorded with special attention for the lesions associated with Pb poisoning.

Pb concentration in soft tissues (liver and kidney)

Liver and kidney samples from the necropsied swans were analysed in Gifu Wildlife Research Center with an atomic absorption spectrophotometer with a graphite furnace and an auto sampler following nitric acid digestion. Fifty microliter of the minced specimen was added to 200 µL of the diluent into a micro centrifuge tube. Then, 15 mL of the diluted sample was directly injected into the Z-8200. Determination of the Pb content from a standard curve was obtained by injecting 15 µL of the Pb working standards (Joselow & Bogden, 1972; Beyer *et al.*, 1998).

Histopathology

Specimens were collected from all organs of each carcass, fixed in 10% buffered formalin, dehydrated, cleared, embedded in paraffin wax, sectioned at 5 µm, stained with haematoxylin and eosin (H & E) then examined by light microscope. Also, consecutive sections were stained with Prussian blue stain for the identification of haemosiderin in liver and spleen and modified Fouchet stain for identification of bile pigment in the liver (Bancroft & Gamble, 2008).

Histopathological scores were + (mild), ++ (moderate) and +++ (severe).

Apoptosis detection

ApopTag® In Situ Apoptosis Detection Kit (Chemicon International Company, Temecula, CA) was used to detect apoptotic cells, or excessive DNA breakage in individual cells (Chapman *et al.*, 1995; Lozano *et al.*, 2009).

Immunohistochemistry

The immunohistochemical detection of astroglial cells, neurofilaments and macrophages was performed by the peroxidase-antiperoxidase (PAP) technique (Ashton-Key *et al.*, 1996; Frost *et al.*, 2000; Noreldin *et al.*, 2018) using glial fibrillary acidic protein (GFAP), neurofilaments antibody and lysozyme antibody (Dako, Hamburg, Germany); respectively. In brief, pretreatment with the antigen retrieval reagents (Epitope Retrieval) broke the protein cross-links formed by formalin fixation and thereby uncovered hidden antigenic sites. Endogenous peroxidase was deactivated by 3% H_2O_2 in methanol for 30 min for all antigens. For GFAP and lysozymes, sections were incubated at 37 °C for 20 minutes in enzyme solutions (trypsin) in the DakoCytomation Hybridizer apparatus then sections were allowed to cool for 20 minutes. Immunoenzyme staining was carried out using automatic immnostaining apparatus (Ventana, NE, ES-IHC, Hitashi). Sections were rinsed two times in phosphate buffered saline (PBS) for 2 minutes, followed by incubation with normal goat serum blocking solution at room temperature for 30 minutes. Sections were incubated with primary antibody at 4 °C overnight then were rinsed three times in PBS for 20 minutes. Peroxidase blocking achieved by incubation of sections in peroxidase blocking solution (3% H_2O_2 in PBS) for 10 minutes at room temperature then were rinsed three times in PBS for 20 minutes. Sections were incubated with secondary antibody for 30 minutes at room temperature (Anti-mouse for neurofilament, Anti-Rabbit for GFAP and Lysozyme) then were rinsed three times in PBS for 10 minutes. Sections were incubated with 3,3′-diaminobenzidine tetrahydrochloride (DAB)-H_2O_2 solution, pH 7.0, for 3 minutes then sections were rinsed three times in PBS for 2 minutes and finally counterstained with Mayer's haematoxylin.

RESULTS

Out of 54 examined swans during the period 2005–2007, twelve birds were necropsied following Pb poisoning assumption based on the signs of emaciation, greenish diarrhoea and impacted gizzard. Radioopaque pictures (Fig. 1A) of Pb shots were seen inside the gizzard of four swans and inside gizzard and intestine of two swans. At necropsy, greenish diarrhoea tended to have stained the feathers surrounding the vent with impaction of the oesophagus, provenriculus and gizzard with food material. All necropsied swans exhibited hepatic atrophy with dark green pigmentation (Fig. 1B) with severely distended gall bladder. The gizzards of all necropsied swans showed greenish-stained koilin membrane with greenish coloured contents (Fig. 1C) admixed with Pb shots in six swans. The gizzard pads were usually roughened, thickened, and occasionally ulcerated (Fig. 1D).

The most prominent and obvious lesions in the brain of almost all birds were perivascular haemorrhages and oedema, cerebral spongiosis in the thalamic area and in the medullary white matter (Fig. 2A), features of focal early malacia in the cerebral cortex with neuronal ischemic injury, slight diffuse gliosis with increased number of glial cells. There was also focal

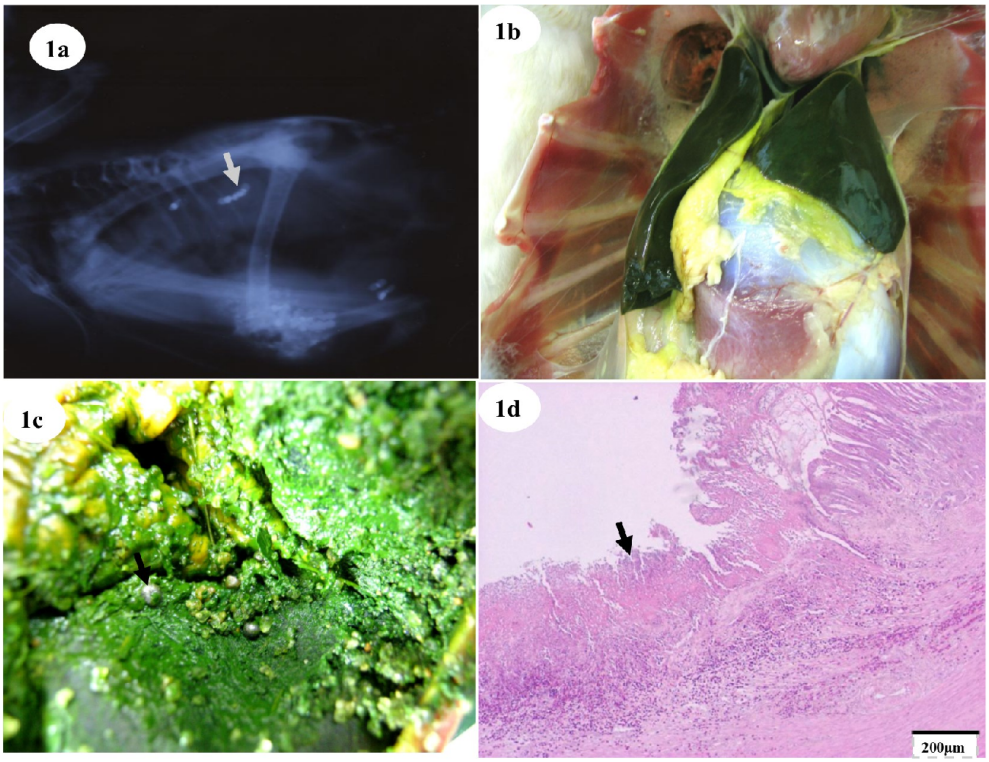

Fig. 1. Radiograph and postmortem findings of Pb-poisoned Whooper swans: A. presence of metallic lead pellets (arrow) in the intestine of lead poisoned birds. B. Green coloured atrophied liver; C. presence of lead shots (arrow) among the grit and greenish contents of the gizzard. D. Micrograph of gizzard tissue showing necrosis with complete erosion of the lining wall and massive infiltration of the inflammatory cells in the submucosa (H & E).

gliosis associated with prominent capillaries and disintegration of cerebral neuronal cells (Fig. 2B). Areas with more advanced malacia characterised by complete loss of neural structure and prominent astrocytosis shown by GFAP immunocytochemistry was also observed (Fig. 2C) together with increased accumulation of neurofilaments detected by using neurofilament antibodies (Fig. 2D). Moreover, the cerebellum revealed marked spongiosis of the white matter in seven swans. Examination of the spinal cord revealed slight spongiosis of dorsal columns in three swans and examination of sciatic nerve showed axonal swelling and distension of myelin sheaths in five swans.

The liver showed varying degrees of brown pigment deposition within Kupffer's cells as well as the cytoplasm of hepatocyte, which were determined to be haemosiderin pigment with positive Prussian blue staining. Clumps of bile pigments surrounded by hepatocytes forming a glandular like structure (Fig. 3A) which was thought to be haemosiderin or lipofuschin, but it exhibited emerald green colour as a positive result with Fouchet stain (Fig. 3B) and gave negative result with Schmorl's stain (a specific stain for

lipofuschin pigment). All swans showed granuloma like reaction with focal mononuclear cell infiltration in which the surrounding small hepatocytes contained intense eosinophilic cytoplasm and compressed the surrounding liver parenchyma (Fig. 3C).

The granulomas were composed mainly of macrophages that were pronounced by giving positive immunocytochemical lysosome reaction (Fig. 3D). Apoptosis characterised by highly condensed eosinophilic cytoplasm and karyorrhexis changes of the nucleus with remaining of small chromatin dots inside the affected cell giving positive brown colour with TUNEL method that was most prominent in the liver of affected swans (Fig. 3E). Seven swans showed fibrinoid degeneration of the blood vessel walls. Three swans showed frequent intra-nuclear inclusion bodies in the nuclei of the hepatocytes (Fig. 3F). Six swans exhibited bile duct proliferation with frequent papillary projections into the lumen.

Kidneys revealed deposition of haemosiderin pigment as well as bile pigment in the cytoplasm of the renal tubular lining epithelium in all swans. Granular cytoplasm and presence of sharply outlined

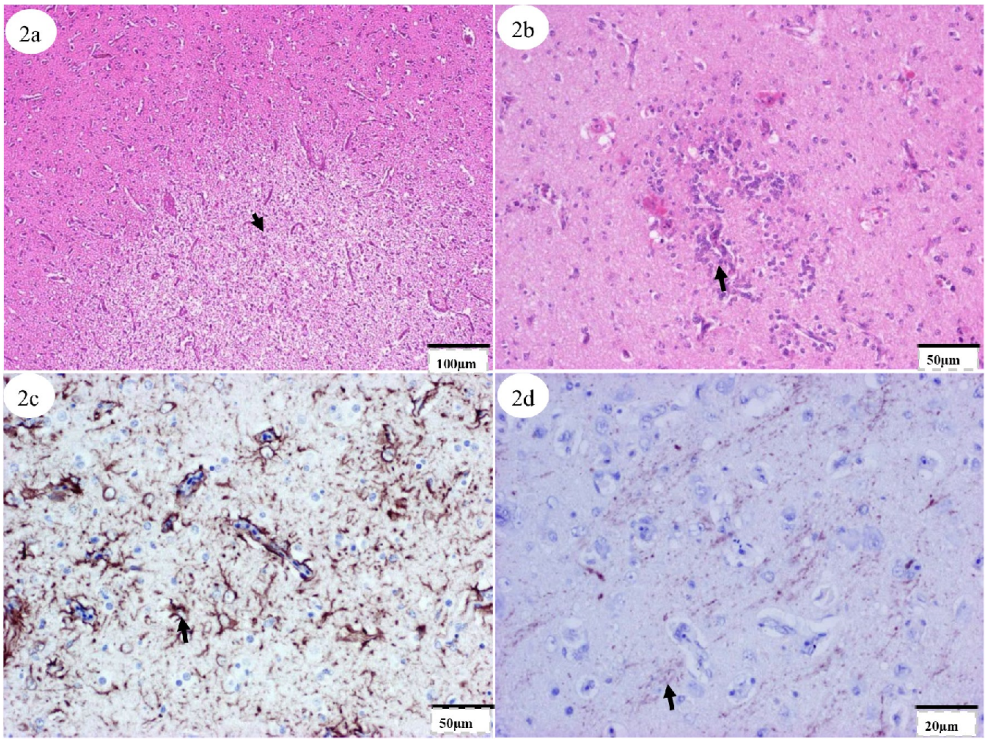

Fig. 2. Histopathological and immunohistochemical changes of brain in Pb-poisoned Whooper swans: A. Cerebrum with an area of advanced malacia (arrow) with prominent vascularisation (H & E); B. Cerebral focal gliosis (H & E); C. Cerebral astrocytosis: fibrillar astrocytes (arrow) were shown by immunohistochemical staining of GFAP (PAP method, DAB); D. Immunohistochemical detection of accumulated neurofilaments (arrow) in the area of cerebral malacia (PAP method, DAB).

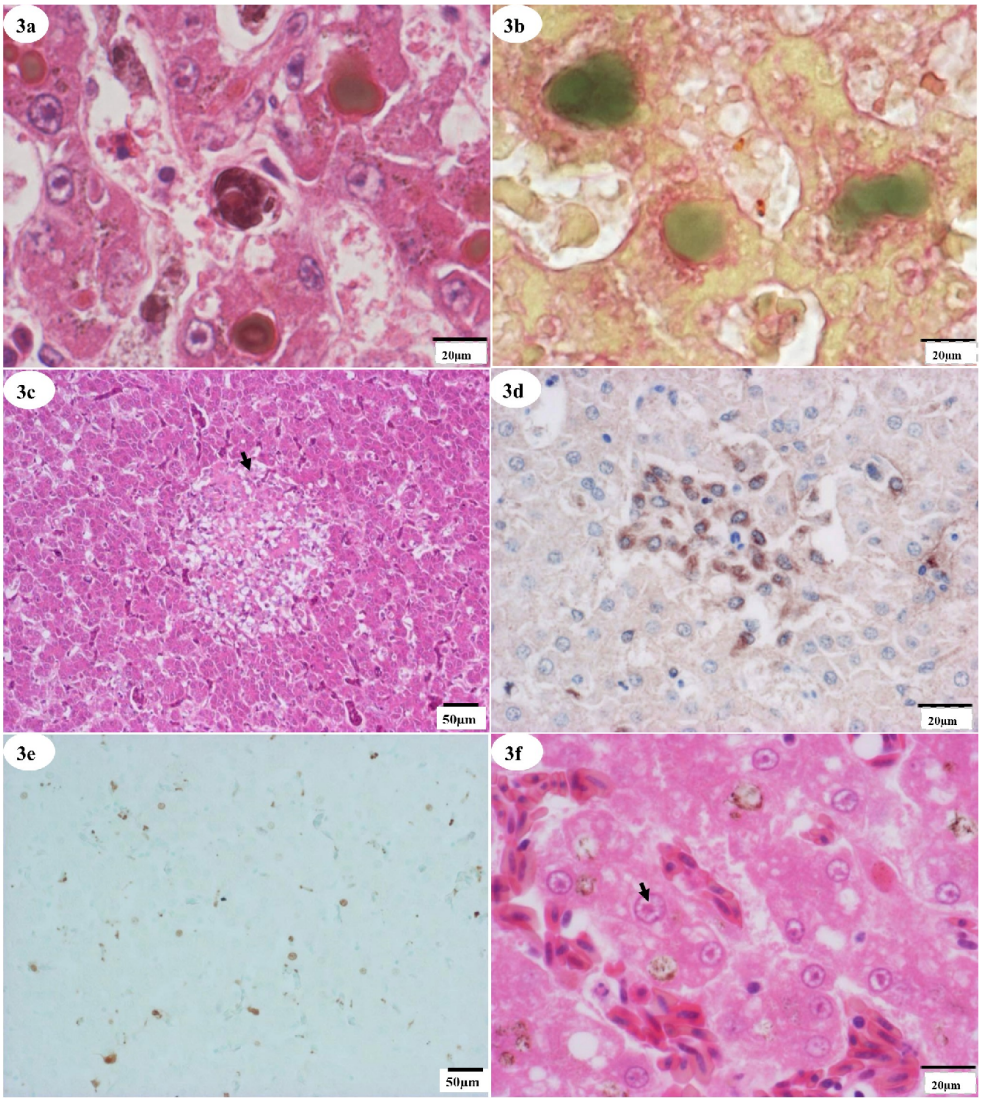

Fig. 3. Histopathological, immunohistochemical and apoptotic changes of liver in Pb-poisoned Whooper swans: A. clumps of bile pigments surrounded by hepatocytes forming a glandular like structure (H & E); B. Emerald green coloured clumps of bile pigment surrounded with hepatocytes (Fouchet stain); C. Focal hepatocellular necrosis (arrow) while most degenerated hepatocytes were replaced with large and foamy cells (H & E); D. Focal aggregates of macrophages giving brown coloured cytoplasmic granules as a positive immunohistochemical detection by lysozyme kits (PAP method, DAB); E. Positive apoptotic reaction in hepatic cells (ApopTag®); F. Hepatocytic intranuclear inclusions (arrow) characterised by eosinophilic droplets with remnant chromatin dots at the periphery of the nuclear membrane (H & E).

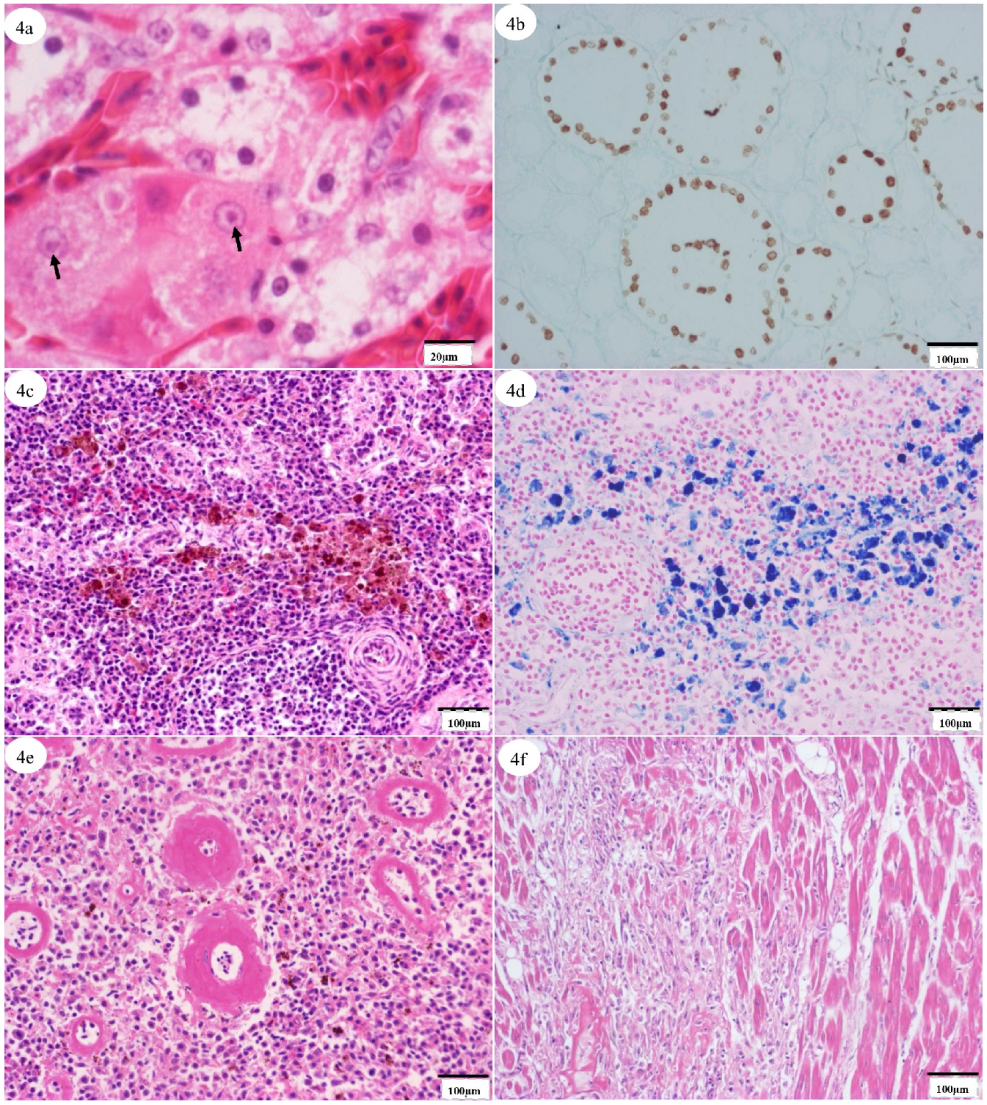

Fig. 4. Histopathological changes of kidney, spleen and heart in Pb-poisoned Whooper swans in addition to apoptotic changes of kidney: A. Eosinophilic intranuclear inclusions (arrows) with remnant chromatin dots at the periphery of the nuclear membrane of degenerated proximal tubular lining epithelium (H & E); B. Positive apoptotic reaction in the tubular lining epithelial cells (ApopTag®); C. Splenic haemosiderosis as macrophages laden with brown pigment (H & E); which became bluish pigment when stained with Prussian blue stain (D); E. Splenic fibrinoid degeneration featuring severely thickened blood vessel wall (H & E); F. Focal myocardial fibrosis where necrotic myocardial fibers were replaced with marked histiocytic and fibroplastic reaction (H & E).

clear vacuoles (fatty change) in the tubular lining epithelium and the presence of renal casts in the lumina of the renal tubules were evident degenerative changes. Eosinophilic intranuclear inclusions bodies were noticed in the lining epithelium of most proximal convoluted tubules (PCT) in six swans (Fig 4A). Five swans exhibited chronic inflammatory reaction in the form of sclerosis with interstitial fibrosis surrounding degenerated renal tubules with thickened capsules. In five swans, uric acid granulomas were present in the peri-renal adipose tissue with needle-shaped uric acid surrounded by mononuclear inflammatory cells, epithelioid cells and foreign body giant cells. Apoptosis was found in tubular epithelium of most PCT of the kidneys of all examined swans (Fig. 4B). The spleen showed a deposition of brown pigments within the parenchyma in all swans (Fig. 4C) which was confirmed to be haemosiderin by means of a positive reaction to Prussian blue stain (Fig. 4D). Fibrinoid degeneration was seen in the blood vessel walls in four swans (Fig. 4E). There was dense homogenous amorphous eosinophilic material in the red pulp of the spleen. Occasional focal necrosis of the spleen was also observed in five swans. The lungs showed severe congestion of inter- alveolar blood vessels, with interstitial edema. Chronic interstitial pneumonia with mononuclear cell infiltration was seen in nine swans. Occasional thrombosis was also observed in three swans.

Microscopically, necrosis in the lining wall of the gizzard with massive inflammatory cells infiltration were seen in seven swans. The heart showed haemosiderosis in the epicardial layer as well as in the adventitia of the aorta. Inflammatory changes in the form of interstitial myocarditis (Fig. 4F) with infiltration of inflammatory cells between the cardiac muscle were present in eight swans along with granulation tissue formation in the sub-epicardial layer. Necrosis with degenerated cardiac muscle fibres was seen in five swans. Bone marrow showed varying degrees of hyperplasia, with increased numbers of polychromatic erythroblasts in six swans. The microscopical examination of the intestine revealed haemosiderin pigment deposition in the intestinal crypts with infiltration of inflammatory cells in only three swans.

As shown in Table 1, the grades of the most prominent histopathological lesions evoked by Pb poisoning correlated to the concentration of Pb in the liver (8.18 to 60.6 µg/g wet weight) and the kidney (5.67 to 71.6 µg/g wet weight).

DISCUSSION

Although plumbism involves various clinical symptoms, researchers cannot always be certain that a bird has died from this cause. Many symptoms can only be observed through necropsy findings (Day *et al.*, 2003). A major problem with all studies of toxicity, including Pb toxicity in swans, is that there is no clear point at which one can say that a bird is healthy or sick. Some swans with relatively low levels of Pb in their blood exhibit severe signs of Pb poisoning, while others with higher blood Pb levels can appear unaffected (De Francisco *et al.*, 2003)

The current investigation was based mainly on gross and microscopic findings, as well as chemical analysis of the liver and kidney to evaluate Pb poisoning in swans. The most characteristic symptom of Pb poisoning in swans was the presence of greenish diarrhoea with stained feathers surrounding the vent; this was consistent with earlier findings in Whooper swans,

Table 1. Summary of lead concentration in soft tissues, radiographic, pathological, apoptosis and IHC findings in twelve lead-poisoned Whooper swans

	C	1	2	3	4	5	6	7	8	9	10	11	12
Sex/Age	F/Y	F/A	F/A	F/A	M/A	F/A	F/A	M/A	F/A	ND/Y	F/A	F/Y	F/ND
Lead concentration (µg/g)													
• Liver	0.151	12.5	29.4	NA	17.2	60.6	13.1	15.1	NA	8.18	20.7	37.9	29.4
• kidney	0.186	59.2	57.3	NA	25.4	39.7	46.3	12.6	NA	5.67	25.1	25.3	71.6
Greenish watery diarrhoea	–	++	+++	++	+	+++	+	+	++	++	++	+++	++
Radiograph	–	–	+	+	–	–	–	+	+	+	-	-	+
Greenish liver with distended gall bladder	–	++	+	+	++	+++	+	+	++	++	+	++	++
Impacted gizzard with greenish content	–	++	+++	++	++	++	+	++	++	++	+	++	+++
Liver													
• Haemosiderosis	–	+	+	+++	+	++	++	++	+	+	+	++	+
• Granuloma	–	++	+++	++	+	+	+	+++	+	+	+	++	++
• Fibrinoid degeneration	–	+	–	–	+	+	–	+	-	–	-	+	+
• Intranuclear inclusions	–	–	+	+	–	+	–	-	-	–	-	+	–
• Bile duct proliferation	–	–	++	+	–	++	++	+	-	–	-	–	++
• Apoptosis	–	+	+	–	+	+++	+	+	–	-	+	++	+
Kidney													
• Haemosiderosis	–	+	+	–	++	+	++	–	+	+	+	–	+
• Uric acid granuloma	–	–	++	–	+	–	–	+	–	+	–	–	+
• Interstitial fibrosis	–	++	+	–	–	–	–	++	+	–	+	–	–
• Intranuclear inclusions	–	++	++	+	–	+	–	–	+	–	–	–	+
• Apoptosis	–	++	+	+	+	+	+	++	++	+	+	+	+

Table 1 (cont'd). Summary of lead concentration in soft tissues, radiographic, pathological, apoptosis and IHC findings in twelve lead-poisoned Whooper swans

	C	1	2	3	4	5	6	7	8	9	10	11	12
Central nervous system													
• Cerebral spongiosis	–	++	+	+	++	+	+	++	++	+	+	+	++
• Cerebral malacia	–	+	+	+	++	+	++	++	+	+	+	++	+
• Astrocytosis	–	++	++	+	+	+	+	++	+	+	+	+	++
• Neurofilaments accumulation	–	+++	+++	+	+	+++	++	+	+	+	+	++	+++
• Cerebellar spongiosis	–	++	+	–	+	–	–	+	–	+	+	+	–
• Spinal cord spongiosis	–	+	–	+	-	–	–	–	–	–	–	–	+
Peripheral nervous system													
• Sciatic nerve: Axonal swelling	–	+	+	–	–	+	+	–	–	–	–	–	+
Gizzard													
• Necrosis	–	+	–	++	+	++	–	–	+	–	++	–	+++
Lung													
• Chronic interstitial pneumonia	–	++	+	+	+	++	+	–	–	–	++	+	+
Spleen													
• Hemosiderosis	–	++	+	++	+	+	+	+	+	+	++	+	+
• Fibrinoid degeneration	–	+	–	–	+	–	–	–	+	–	+	–	–
• necrosis	–	+	-	+	+	–	–	–	–	–	+	–	+
Heart													
• Necrosis	–	++	+	–	–	++	–	–	–	–	–	+	+
Bone marrow													
• Hyperplasia	–	+	–	+	–	–	++	–	–	–	++	–	++

C: control; F: female; M: male; A: adult; Y: young; NA: not analysed; ND: not determined; – absent; + mild; ++ moderate; +++ severe.

other waterfowls, raptors (Jacobson *et al.*, 1977; Friend, 1985; Ochiai *et al.*, 1992).

In the current study, Pb shots were found in the gizzard contents of 50% of the total number of affected swans which is higher than that reported in other birds. Mateo *et al.* (2003) reported that the frequency of appearance of shots in the gizzards of waterfowl was 10% in geese, 25% in mallards and 11% in gat well. However, the absence of Pb shots in the gizzard of swans does not necessarily indicate that the bird is not affected. Several swans showed Pb shots in their gizzards with varying size in different stages of dissolution. Shots lodged in the lumen of the gizzard may be worn by the acidic conditions and the grinding action that dissolves the Pb so that ions can be absorbed or even regurgitated by the bird (Warner *et al.*, 2014) or expelled anally (Anderson, 1975).

The most consistent gross lesion is the evidence of bile stasis; the gall bladder was engorged with viscous, dark-green bile. The biliary tree within the liver and the liver itself may be distinctly green. Inflammatory reactions in the liver such as the granuloma-like lesions indicating an accompanying infection which may be attributed to the Pb-induced immunosuppression effect. Furthermore, our results showed that anaemia was a constant gross lesion in all examined swans which may be explained by the production of damaged and defective red blood corpuscles and associated with haemosiderosis in the liver, kidneys, and spleen of the poisoned birds (Wilson *et al.*, 2009; Franson & Russell, 2014).

The brain is a highly susceptible organ to Pb toxicity, because it contains relatively low levels of the anti-oxidative stress enzymes (Savolainen, 1978; Ahamed & Siddiqui, 2007), and in part because of its high myelin-associated content, which makes it vulnerable to the propagation of peroxidative process. Pb exposure results in a reduction in the accumulation of myelin in brain (Toews *et al.*, 1983; Brubaker *et al.*, 2009), and it often causes focal or diffuse myelin sheath fragmentation and reactive astrocytosis in the white matter (Malandrini *et al.*, 2001; Jwad, 2012). In the current study the brains of examined swans showed spongiosis of the cerebral and cerebellar white matter and ischemic neuronal injury in cerebral cortex and cerebellar Purkinje cells and also, mild spongy changes in the spinal cord and sciatic nerve. The patterns of such microscopic changes have been reported previously (Hunter & Wobeser, 1980; Goetz & Washburn, 1999; Gurer & Ercal, 2000).

Astrocytic activation plays a major role in the homeostatic maintenance of CNS in response to neuronal impairment. The GFAP is an intermediate filament protein that is expressed by numerous cell types of CNS including astrocytes. Accumulation of GFAP in brain tissue used as indicator of reactive astrocytosis, which is a characteristic neuropathological finding of ischemic brain injury (Malandrini *et al.*, 2001; Müller *et al.*, 2012).

It was clear that the reactivity of astrocytes with the GFAP antibody was high in the affected birds which means that glial and neuronal functions were altered with chronic Pb exposure. The relation between GFAP and ischaemia was proved by Kindy *et al.* (1992) who examined injury-induced changes in GFAP mRNA and protein in a focal hypoxic-ischaemic injury in rodent brain and they found regionally specific increases in GFAP mRNA expression and GFAP immunoreactivity in the first 2 weeks after hypoxic-ischaemic injury of the cereberal neurons.

In contrast, Stoltenburg-Didinger *et al.* (1996) reported that chronic Pb exposure did not affect glial and neuronal functions. The mechanism leading to this differential induction and their physiological and functional significance are not clear at present.

Intra-nuclear electron-dense granules were observed in the hepatocytes of three swans. This finding has been considered as one of the strongest presumptive evidence of Pb intoxication in birds (Bagley & Locke, 1967). These Pb–protein complexes are observed as typical intracellular inclusions (Clemens *et al.*, 1975; Mateo *et al.*, 1997).

Immunohistochemical staining for apoptotic cells revealed strong immunoreactivity in kidney and liver tissues of birds with higher level of Pb concentration in their soft tissues. This result was coincided with that of Özcan *et al.* (2007) who observed severe degeneration, tissue damage and apoptotic cells in renal tubular epithelium and liver tissues during Pb intoxication in geese.

In the current investigation, intranuclear electron-dense granules in epithelium of PCT were reported in six swans with high Pb concentration. This is one of the strongest presumptive evidence of lead intoxication in birds (Goyer *et al.*, 1970). This might be due to the direct toxic effect of Pb on the tubular epithelium. Pb is absorbed by the proximal tubular cells, where it binds to specific lead-binding protein. These Pb-protein complexes are observed as typical intra-cellular inclusions (Mateo *et al.*, 2003). It has been observed that Pb nephropathy in swans is associated with gout; which may be attributed to Pb-induced hyperuricaemia and decreased renal excretion of uric acid.

The Pb-induced cardiac damage, including myocarditis, myocardial necrosis and fibrinoid necrosis of arteries has been reported previously (Beyer *et al.*, 1998; Henny *et al.*, 2000).

No difference in Pb content was found between male and female, these results were consistent with those described previously (Helander *et al.*, 2009; Warner *et al.*, 2014) but were inconsistent with Eskildsen & Grandjean (1984), who observed that female swans demonstrated slightly higher tendency to suffer Pb poisoning than did males. The mechanisms causing this difference have not yet been established.

Regarding to Pb concentrations in soft tissues in poisoned swans, chemical analysis showed that the Pb concentrations in the liver and the kidneys were 8.18–60.6 µg/g and 5.67–71.6 µg/g wet weight, respectively indicated Pb exposure and poisoning. Previous reports concluded that hepatic Pb concentrations exceeding 6–8 µg/g in mallards (Sass *et al.*, 1991), 5 µg/g in geese (Beyer *et al.*, 1998) indicate toxic exposure; and that 10 µg/g wet weight is a reliable threshold concentration for diagnosing Pb poisoning in waterfowls even in the absence of the pathological information. Therefore, reports of Pb concentrations in the liver and kidneys by other researchers were consistent with, but not quite as severe as that of our findings. The upper-limit values for Pb in the liver are highly variable, although values of 8 µg/g wet weight and higher can be taken as being clearly indicative of Pb poisoning in swans (Friend, 1985; Warner *et al.*, 2014).

The present study revealed that the extent and severity of lesions of Pb poisoning varied among individuals and was mostly dose-dependent. The pathology of Pb poisoning in swans was multisystemic. The severity of the lesions was the highest in the CNS followed by the liver, kidney,

spleen, lungs, gizzard, heart and bone marrow respectively and were least in the peripheral nervous system.

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Paper received 06.03.2019; accepted for publication 16.06.2019

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