Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2025.189

RESEARCH ARTICLE

Evaluation of Heavy Metal Accumulation, Oxidative Status, and Histopathological Changes in Brain, Liver, and Gonad Tissues of Bantam Chicken (*Gallus domesticus)* from Kui Buri District, Prachuap Khiri Khan Province, Thailand

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ARTICLE HISTORY (25-015)

Received: January 7, 2025
Revised: May 27, 2025
Accepted: June 3, 2025
Published online: June 29, 2025

Key words:
Gallus domesticus
Heavy metal toxicity
Hepatotoxicity
Neurotoxicity
Oxidative stress
Reproductive toxicity
Tissue bioaccumulation

A B S T R A C T

This study investigated the relationship between heavy metal accumulation and its impact on oxidative stress and histopathological alterations in bantam chickens (*Gallus domesticus*). The research focused on evaluating the extent of bioaccumulation and the associated oxidative and structural changes in critical organs, including the brain, liver, and gonads, to better understand the implications for animal health and physiological well-being. Thirty bantam chickens were collected from Kui Buri District, Prachuap Khiri Khan Province, Thailand. Brain, liver, and gonad tissues were dissected and used for the heavy metal analysis of cadmium (Cd), chromium (Cr), cobalt (Co), arsenic (As), and lead (Pb), oxidative status, and histopathology. Results indicated that the accumulation of heavy metals, including Cd and Co, was highest in the liver compared to the brain and gonads of bantam chickens. Tissue metal accumulation levels, such as Cd, Co, As, and Pb, did not exceed the recommended level, while all tissues exhibited similarly high and excessive levels of Cr accumulation. Evaluation of tissue oxidative status indicated that both lipid peroxidation (LPO) and superoxide dismutase (SOD) were higher in the brain than in the gonad and liver tissues, implying that the brain exhibited more stress than the gonad and liver, which indicated tissue-specific profiles of oxidative status in response to specific metal accumulation. Histological assessments indicated normal histology of brain neurons, i.e., cerebrum, midbrain, cerebellar Purkinje, granular and deep nuclei, and regular white matter, i.e., the optic tract and cerebellar mossy fiber. Normal histology was also represented in the liver tissue and gonads of the bantam chickens. Elevated levels of Cr in the brain exhibit a negative correlation with neuronal density and may adversely affect function.

To Cite This Article: Tanhan P, Imsilp K, Lansubsakul N and Thong-asa W 2025. Evaluation of heavy metal accumulation, oxidative status, and histopathological changes in brain, liver, and gonad tissues of bantam chicken (*gallus domesticus)* from kui buri district, prachuap khiri khan province, thailand. Pak Vet J, 45(2): 618-628. http://dx.doi.org/10.29261/pakvetj/2025.189

INTRODUCTION

Oxidative stress is characterized by an imbalance between the production of free radicals and the body's ability to neutralize them with antioxidants. This imbalance can cause cellular and tissue dysfunction and damage, potentially contributing to various diseases and aging. Multiple stressors trigger the production of reactive species, such as reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive chlorine species (RCS). Free radicals typically exist in the body, but their excessive production can overwhelm antioxidative defenses, potentially causing oxidative damage (Pizzino *et al.*, 2017). Oxidative stress also accounts for various pathological mechanisms, such as metabolic and degenerative human diseases (Jomova and Valko, 2011). In poultry, oxidative stress has been raised as a significant concern that impacts chickens' overall health in modern production systems with various stressors, i.e., technological, environmental, nutritional, and internal (Surai *et al.*, 2019). These stressors accumulate and result in oxidative stress, which leads to health alterations in chickens.

Certain nonessential elements, such as heavy metals like Cd, Pb, As, Cr, Co, Hg, and Ni, found in feed, soil, and water, may contribute as stressors. Furthermore, bioaccumulation is also found in poultry, fish, bivalves, crabs, and birds (Akan *et al.*, 2010; Imsilp *et al.*, 2024; Tanhan *et al.*, 2024). Extended exposure to heavy metals, even in low doses, can negatively affect human and animal health due to environmental accumulation (Mitra *et al.*, 2022). Even with exposure and accumulation, they can harm living organisms when specific concentrations are exceeded. In chickens, heavy metals accumulate in various organs, primarily the liver, brain, lungs, kidneys, and reproductive organs, due to continuous exposure. Previous studies have shown that heavy metal exposure can diminish feed conversion efficiency, egg production, growth, and reproductive ability (Aljohani, 2023). Chronic exposure to low doses of heavy metals can alter the microscopic structure of tissues, including the brain, liver, kidneys, and reproductive organs. Additionally, the increased accumulation of these heavy metals can lead to histopathological changes in the tissue of the chickens as well (Cheng *et al.*, 2016). The negative impact of heavy metal accumulation in tissues was also reflected in oxidative enzymatic activity, i.e., superoxide dismutase (SOD), catalase (CAT), and biochemical parameters such as the lipid peroxidation (LPO) indicated by malondialdehyde (MDA) that was increased (Tanhan *et al.*, 2023). Various concentrations of heavy metals accumulating in tissues can induce changes in oxidative response mechanisms. During both the adaptive and inhibitory stages, a greater number of adversities were observed in the latter stage (Imsilp *et al.*, 2024).

The present study focuses on the vital role of metal accumulation in tissue oxidative status and histopathological changes in bantam chickens, *Gallus domesticus*. We focus on bioaccumulation, oxidative responses, and histological changes concerning organ tissue for the well-being of the animals. This is because the bantam chickens in the sampling areas are essential to folk and local culture, and some serve as bantam breeders. They can be used commercially for various events, i.e., sports and shows. Mostly, they were allowed to freely live and feed in the open territories, which may have come in contact accidentally with contaminated hazards and waste, which are not reported in this area. These domestic fowls were from Kui Buri District, Prachuap Khiri Khan Province of Thailand, living around the Yang Chum Reservoir and plenty of agricultural regions and anthropogenic sources of contamination. There were no health and metal accumulation reports in the area. Additionally, the potential impact of heavy metals associated with oxidative stress in tissues can result in dysfunction and damage. Therefore, the present study aims to evaluate some metal accumulation, i.e., Cd, Pb, Cr, Co, and As, in the bantam chicken vital organs, such as the brain, liver, and gonad, according to evaluations of tissue oxidative status and histopathological changes.

MATERIALS AND METHODS

Experimental design: The experimental protocol was approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine, Kasetsart University (Approval No. ACKU66-VET-073). Per statistical power analysis and the 3Rs (Replacement, Reduction, and Refinement) ethical principles, 30 healthy bantam chickens (15 males and 15 females), aged 4–6 months, were selected. The birds were collected from five sampling locations(S1-S5): S1 (12°05’10"N 99°42’16"E), S2 (12°06’36"N 99°39’44"E), S3 (12°05’34"N 99°42’27"E), S4 (12°05’11"N 99°43’06"E), and S5 (12°05’03"N 99°42’08"E). These areas were around the Yang Chum Reservoir in Kui Buri District, Prachuap Khiri Khan Province, Thailand. The tissue samples were harvested from the domestic fowls that were euthanized by intravenous injection with barbiturate 300–600mg/kg. Tissue dissection was performed within 5–10 minutes after the barbiturate injection. The harvested tissues for oxidative analysis were washed in a cold normal saline solution, weighed, and stored in PBS (10% w/v). The tissues for metal analysis were washed with normal saline solution and weighed, then wrapped in foil, placed in a plastic bag, and stored in ice boxes. The tissue for histological analysis was washed in normal saline solution before being preserved in 10% neutral buffer formalin. All tissue samples were transported to the Department of Zoology, Faculty of Science, Kasetsart University, for further processing.

Heavy metal analysis: Heavy metal accumulations were analyzed in the Department of Pharmacology, Faculty of Veterinary Medicine, Kasetsart University. Tissue samples, i.e., brain, liver, and gonad, were oven-dried at 60°C and weighed thrice (0.5g). Heavy metal concentrations were examined within samples using inductively coupled plasma mass spectrometry (ICP-MS, PerkinElmer, NexION 2000, USA). The sample underwent a digestion procedure with 5mL of a reagent solution made of 69% nitric acid and 30% hydrogen peroxide in a 2:1 ratio. Upon achieving complete digestion, indicated by a pale yellow and transparent solution, the mixture was filtered using No. 4 Whatman filter paper. The volume of the resulting filtrate was then adjusted to 25mL with ultrapure water (18.2 mΩ/cm² Milli-Q water) in preparation for heavy metal analysis. Each metal’s concentration was determined using its corresponding calibration curve. The standard curve was established by injecting standard solutions (multielement standard for ICP-MS, PerkinElmer, USA) alongside blank solutions (1% nitric acid) into the ICP-MS. To ensure the precision and integrity of the heavy metal analysis, certified reference materials (EMR-CE278k) were used in the assessment. The percentage recoveries of the quantified elements within the certified materials exceeded 97.5% across all examined samples. Metal accumulation concentration was expressed as µg/kg of tissue dry weight (Imsilp *et al.*, 2024).

Oxidative status analysis: Biochemical reagents for this study were sourced from Chemical Express Co., Ltd. (Merck Millipore, Samutprakarn, Thailand). Tissues were washed with cold saline and homogenized in a 10% w/v phosphate-buffered saline (50mM, pH 7.4). The tissue homogenate was preserved for the measurement of MDA. At the same time, the supernatant harvested from a 10-minute centrifugation of the homogenate (at 10,000g, 4°C) was reserved for the assessment of total protein, SOD, and

CAT enzymatic activities. All oxidative parameters were evaluated using the colorimetric method (UV-5100B, Toption, Shaanxi, China).

Total protein: The supernatant (0.01mL) was mixed with RO water (0.79mL) and Bradford reagent (0.2mL) and incubated for 5minutes. Absorbance was read at 595nm. Protein concentration was calculated using the BSA (A2153, Sigma-Aldrich, Bangkok, Thailand) standard curve (0, 0.004, 0.008, 0.012, 0.016, 0.02mg/mL; y=33.043x+0.0356; R^2=0.9827), expressed as mg/mL (Kielkopf *et al.*, 2020).

Lipid peroxidation: Malondialdehyde (MDA) levels were determined by mixing 0.2mL of brain homogenate with 4% sodium dodecyl sulfate (817034, SAFC, Darmstadt, Germany), 1.5mL of 20% acetic acid (137130, SAFC, Darmstadt, Germany), and 1.5mL of 0.5% thiobarbituric acid (T5500, Sigma-Aldrich, Bangkok, Thailand). The mixture was heated at 95°C for 1 hour, centrifuged at 3,500rpm for 10 minutes, and then read at 532nm. Concentrations were calculated using an MDA (820756, Sigma-Aldrich, Bangkok, Thailand) standard curve (0, 3.73, 7.41, 11.04, 14.63, and 18.18µM; y=0.1461x+0.2072; R^2=0.969) and expressed as µM/mg of protein. (Sakamula and Thong-Asa, 2018).

Superoxide dismutase: Superoxide dismutase (SOD) activity was determined by combining 0.1mL of supernatant with 0.1mL of 0.0001M EDTA (30620, Sigma-Aldrich, Bangkok, Thailand), 0.5mL of carbonate buffer (pH 7.9), and 1mL of 0.0003M epinephrine (1236970, USP, Jakarta, Indonesia). The absorbance was measured at 480nm. The enzymatic activity of SOD is expressed as U/mg of protein, using a standard curve for SOD concentration represented by the equation y=0.0015x+0.0001 (R^2=0.998). The standard SOD activity used was 6,150U/mg, sourced from Merck, Darmstadt, Germany (Sakamula *et al.*, 2022).

Catalase: Catalase activity was measured using 50µL of supernatant diluted to 3mL with 0.05M PBS (pH 7.4) and 0.01M H_2O_2. Absorbance was read at 240nm, and CAT activity was calculated using the H_2O_2 extinction coefficient, reported as U/mg of protein (Dolrahman and Thong-Asa, 2024).

Histopathological analysis: Six chickens from each sampling area were sacrificed via intravenous injection with barbiturate 300–600mg/kg, and tissues, i.e., brain, liver, and gonad, were removed and fixed in 10% neutral buffer formalin (48 hours). Tissues were processed and embedded in paraffin blocks. Using a rotary microtome, they were cut into 5µm-thick sections. Five sections from each chicken were meticulously selected with 125µm intervals to guarantee sufficient distance and avoid assessing the same cell (Thong-Asa *et al.*, 2017).

Nissl staining: Brain sections were deparaffinized, rehydrated with xylene and ethanol, and rinsed in distilled water for 5 minutes. They were stained with 0.1% cresyl violet for 10 minutes, then dehydrated with ethanol, cleared with xylene, and mounted with a cover glass. Brain areas of interest, i.e., cerebrum, midbrain, cerebellar Purkinje, and deep nuclei, were captured at a magnification of x20, and the cerebellar granular cell layer was captured at a magnification of x40 using an Olympus BX51 microscope (Olympus, Kyoto, Japan). Six non-overlapping captured images were used for neuronal density analysis (% Nissl-positive cells) or counting cells/area using NIH Image J (Thong-Asa *et al.*, 2021).

Luxol fast blue staining: The brain sections were initially deparaffinized and rehydrated using xylene and ethanol and incubated overnight at 56°C in a 0.1% Luxol fast blue (LFB) solution. After incubation, the sections were washed with 95% ethanol and distilled water. Next, they were differentiated in a lithium carbonate solution for 30 seconds and washed in 70% ethanol. Once again, the sections were rinsed with distilled water, dehydrated using ethanol and xylene, and finally mounted with a cover glass. White matter areas of interest, i.e., optic tract and cerebellar mossy fibers, were captured at a magnification of x20 using an Olympus BX51 microscope (Olympus, Kyoto, Japan). Six non-overlapping captured images were used to analyze the white matter density. The percentage of myelinated fiber was measured using NIH Image J (% LFB-positive fibers), and the data were represented as the percentage of white matter intact (Dolrahman and Thong-Asa, 2024).

Periodic acid Schiff method: The glycogen content in liver tissues was assessed using the periodic acid-Schiff (PAS) method. Five slides per chicken were deparaffinized, hydrated, and treated with periodic acid, then incubated in Schiff's reagent and washed. After rinsing in increasing alcohol concentrations and clearing in xylene, six images were captured at x20 magnification for each slide. The liver glycogen content was analyzed and represented as a % PAS-positive area using NIH Image J (Thong-asa *et al.*, 2019).

Hematoxylin and eosin staining: Liver and gonad tissue sections were examined using H&E routine methods. Briefly, deparaffinized and hydrated in distilled water. Then, the sections were stained in hematoxylin for 15 minutes, washed in running tap water, dipped in acid alcohol for a few seconds, and rinsed in running tap water. The sections were dipped in bluing solution for 2 minutes and washed in tap water. After that, they were counterstained in 1% eosin Y for 1 minute, washed in tap water for 5 minutes, dehydrated through increasing alcohol concentrations, cleared with xylene, and covered with a glass mounting. Overall histological observations of liver and gonad tissue were done under the microscope and NIH Image J (Imsilp *et al*., 2024).

Statistical analysis: Statistical analysis was performed with GraphPad Prism 8.0.1, presenting data as mean±standard deviation (SD). Normality and variance were assessed using Shapiro-Wilk and Levene's tests. One-way ANOVA with Tukey's post hoc test was used for comparisons, and Pearson's correlation evaluated relationships between metal concentrations and oxidative parameters, with a significance level set at $P<0.05$.

RESULTS

The present study evaluated metal accumulation in three tissues (brain, liver, and gonad) of bantam chicken. We found that Cd exhibited the highest accumulation in liver tissue (259.6µg/kg), followed by the gonad (10.23µg/kg) and brain (8.30µg/kg), Fig. 1a. The differences between the liver and the gonad ($P<0.0001$) and the brain ($P<0.0001$) were indicated. Cobalt also showed the highest accumulation in the liver (102.6µg/kg), followed by the brain (61.43µg/kg) and gonad (28.75µg/kg), Fig. 1c. A significant difference existed between the liver and gonad ($P<0.0001$) and brain ($P=0.0021$). Other metals, such as As and Pb, were also found to accumulate the most in the liver. At the same time, no significant difference was indicated when comparing the three types of tissues (Fig. 1d and 1e, respectively). Only Cr exhibited the highest accumulation in gonadal tissues; however, no significant difference was observed compared to other tissues (Fig. 1b).

Evaluation of oxidative status indicated the highest level of MDA in the brain, with significant differences from the liver ($P<0.0001$) and gonad ($P<0.0001$), (Fig. 1f). The present study also indicated that SOD level was higher in the brain than in the liver ($P=0.0103$) and gonad ($P=0.0174$), (Fig. 1h). Together with the enzymatic activity of CAT in gonads, which showed a significantly higher level than the liver ($P=0.0413$) but was not different from the brain.

Evaluation of metal accumulation, oxidative status, and histology in the brain: The evaluation of the brain's heavy metal accumulation, i.e., Cd, Cr, Co, As, and Pb, comparing sampling areas, showed no significant differences. Therefore, accumulation levels were interpreted in all sampling areas. They were Cd=8.30µg/kg, Cr=1,418µg/kg, Co=61.43 µg/kg, As=38.39µg/kg, and Pb=35.73µg/kg, in order of accumulation from the highest to the lowest was Cr>Co>As>Pb>Cd. Brain LPO and antioxidative enzymatic activities were also evaluated in the present study. The MDA level indicated lipid peroxidation, and its average value was 337.2µM/mg of protein. In contrast, antioxidant enzymatic activities such as SOD and CAT were 12.10 and 18.06U/mg of protein, respectively (Fig. 2i). No significant differences were found when comparing these parameters in each sampling area. Pearson's correlation analysis also showed no correlation between metal accumulation and oxidative status in the brain. These results were accompanied by neither histopathological change in the brain areas such as the cerebrum, midbrain, cerebellar Purkinje, deep nuclei, and granular cell layer nor white matter, i.e., the optic tract and cerebellar mossy fiber (Fig. 2a-2h). However, the accumulation of heavy metals in brain tissue does not lead to significant pathological changes in brain neurons and white matter, a moderate positive correlation was found between Cd accumulation and the enzymatic activity of CAT and SOD (Fig. 2j-2k).

Our study revealed minimal pathological changes in brain tissue but a weak negative correlation between Cr accumulation and cerebral neuronal density. (Fig. 2m). We found that Cr accumulation in the brain was higher than in other tested metals. A weak negative correlation of Cr was found with cerebral neuronal density but not the midbrain and cerebellar neurons, i.e., Purkinje, deep nuclei, granular cells, or white matter areas such as optic tract and cerebellar mossy fiber.

Evaluation of metal accumulation, oxidative status, and histology in the liver: Heavy metal accumulation ranged from high to low in the liver tissues of bantam chickens, ranging from Cr>Cd>Co>As>Pb (Fig. 2a). The comparison of accumulation in the liver did not differ in each sampling area, and the average value of metals from all sampling areas was Cd=259.60µg/kg, Pb=66.79µg/kg, Co=102.60µg/kg, and As=38.67µg/kg. Chromium was relatively high at 894.20µg/kg. Oxidative status indicated by MDA, CAT, and SOD showed no differences between sampling areas, and the average value from all regions was that MDA=131.7µM/mg of protein, CAT=14.77U/mg of protein, and SOD=7.80U/mg of protein (Fig. 3d).

The liver histological assessment indicated normal hepatic lobules and healthy hepatocytes with a few steatoses (Fig. 3a). None of the degenerative forms were observed, i.e., inflammatory cell infiltration and liver tissue fragmentation. Normal hepatocytic histology was mostly present (Fig. 3a), and only a few necrotic cell deaths were observed. The percentage of hepatocytic areas related to the vacuolation area was not different (Fig. 3e). Hepatocytic density was about 51.08%, and the rate of vacuolation was 48.92%. Indeed, most vacuolations were fibroblast cells of connective tissue modified for fat accumulation or so-called adipocytes. Our present study did not use a specific dye to indicate fat droplets, such as oil red O. Therefore, we cannot indicate the premises of fat distribution in the liver.

The glycogen content in hepatocytic cells was evaluated using PAS staining (Fig. 3b). The results showed no difference between sampling areas, with an average value of 50.23% across all sampling areas (Fig. 3d). No correlation was observed between heavy metal accumulation and oxidative parameters or histopathological changes.

Evaluation of metal accumulation, oxidative status, and histology in gonads: The present study used the average data from both sexes to interpret metal bioaccumulation and oxidative status, as no significant differences were observed between the sexes. Heavy metal accumulation in the gonad (testis and ovary) of bantam chickens was Cr> Pb> As>Co and Cd (Fig. 4e). The comparison of accumulation levels in the gonads for each metal did not differ in each sampling area. The average value from all sampling areas was Cr=1,844µg/kg, Pb=60.91µg/kg, As=30.47µg/kg, Co=28.75µg/kg, and Cd=10.23µg/kg. These accumulation levels also exhibited high Cr accumulation in the gonad, like in the brain and liver. Oxidative status evaluation such as MDA, CAT, and SOD showed no differences between sampling areas, and the average value from all regions was that MDA=110.5µM/mg of protein, CAT=19.02U/mg of protein, and SOD=8.11U/mg of protein (Fig. 4f). Unlike in other tissues, Pearson's correlation indicated a moderate positive correlation between Pb accumulation and CAT activity in the gonad (Fig. 4g).

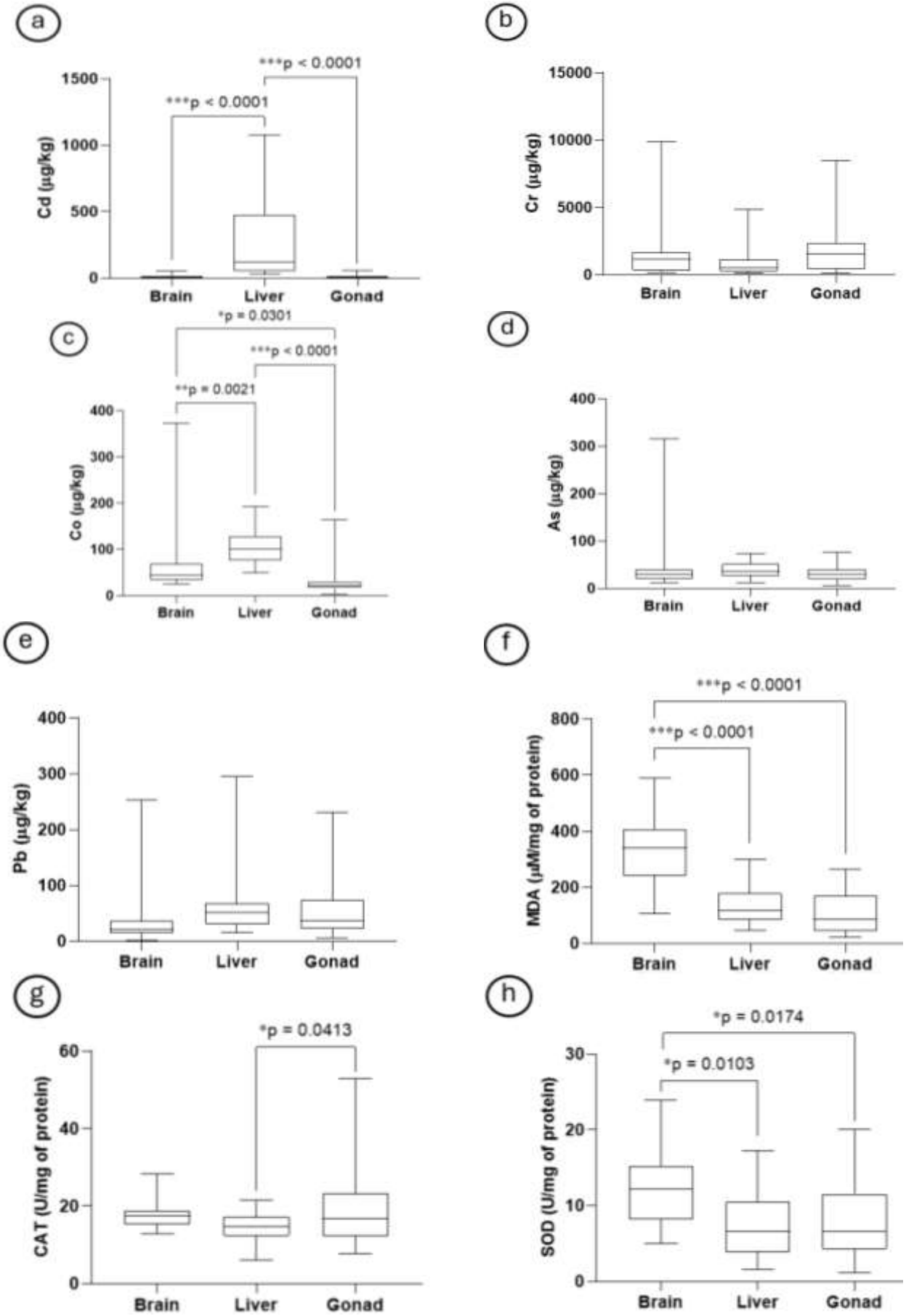

Fig. 1: Heavy metal accumulation levels, i.e., Cd (a), Cr (b), Co (c), As (d), and Pb (e) in the brain, liver, and gonad tissues of bantam chicken. Oxidative status in brain, liver, and gonad tissues, i.e., LPO level indicated by MDA (f) and enzymatic activity of CAT (g) and SOD (h). *indicate P<0.05; ** indicate P<0.005; *** indicate P<0.0005; Cd=cadmium; Cr=chromium; Co=cobalt; As=arsenic; Pb=lead; LPO=lipid peroxidation; MDA=malondialdehyde; CAT=catalase; SOD=superoxide dismutase.

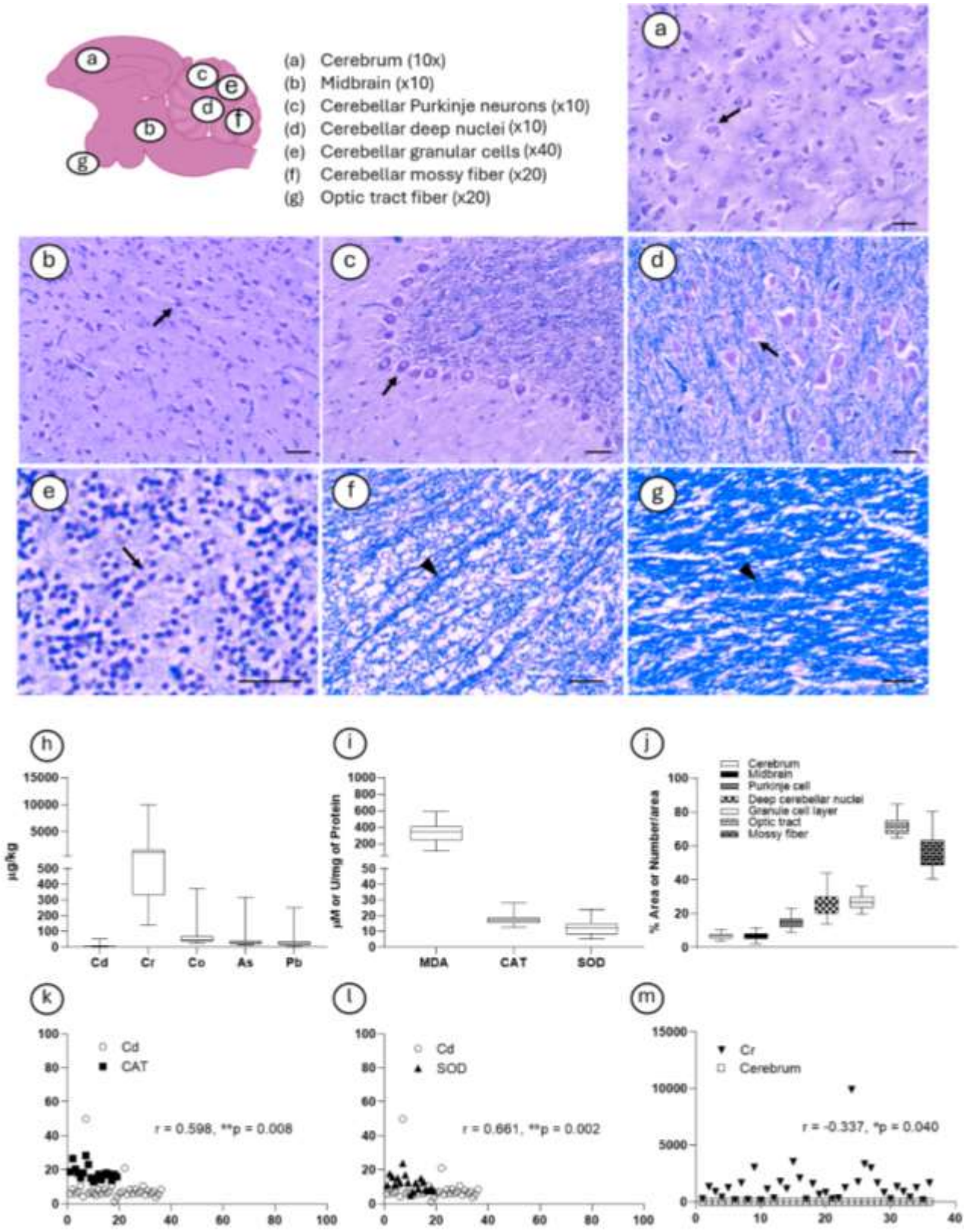

Fig. 2: Representative photomicrographs of the cerebrum (a), midbrain (b), cerebellar Purkinje neurons (c), deep nuclei (d), with Nissl stained, x10 of magnification, and granular cells (f) with Nissl stained, x40 of magnification, scale bar=50 µm (black arrows indicate neuronal cells). Representative photomicrographs of optic tract fiber (f) and cerebellar mossy fiber (h), in which myelinated axonal fibers are stained with luxol fast blue (LFB), indicated by the black arrowhead, x20 of magnification, scale bar=50 µm. The neuronal density represented by the percentage of Nissl-positive cells (in the cerebrum, midbrain, granular cells) and number/area (in the cerebellum, i.e., Purkinje and deep cerebellar nuclei), and white matter density (in the optic tract and cerebellar mossy fiber) was indicated by the percentage of LFB-positive fibers (h). The box and violin plots represented the accumulation levels of metals, i.e., Cd, Cr, Co, As, and Pb (h). Oxidative status of brain tissue indicated by MDA, CAT, and SOD (i). The box and violin plots of neuronal and white matter density in brain regions, i.e., cerebrum, midbrain, cerebellar Purkinje neurons, granular cells, deep cerebellar nuclei, optic tract, and cerebellar mossy fiber (j). Pearson's correlation analysis indicated the correlation between Cd and CAT (k), Cd and SOD (l), and cerebrum neuronal density and Cr (m). Cd=cadmium; Cr=chromium; Co=cobalt; As=arsenic; Pb=lead; MDA=malondialdehyde; CAT=catalase; SOD=superoxide dismutase. While * P<0.05 and **P<0.005.

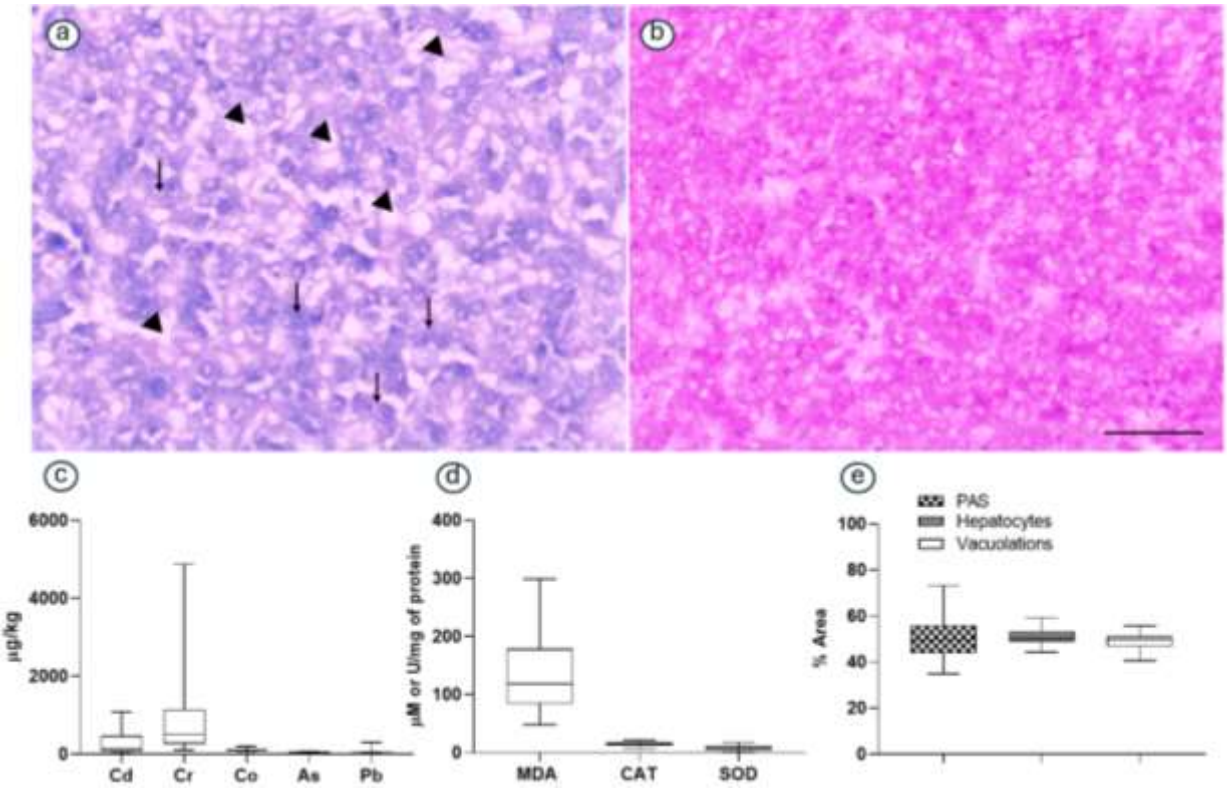

Fig. 3: Photomicrographs of liver tissue stained with H&E (a) and PAS (b), x20 magnification, scale bar=50 µm. The box and violin plots showed accumulation levels of Cd, Cr, Co, As, and Pb in liver tissues (c), oxidative status indicated by MDA, CAT, and SOD (d), and the percentage of PAS-positive cells, hepatocytes (black arrows), and vacuolations that resemble the adipocytes (black arrowheads) in liver tissue (e) of bantam chicken. Cd=cadmium; Cr=chromium; Co=cobalt; As=arsenic; Pb=lead; MDA=malondialdehyde; CAT=catalase; SOD=superoxide dismutase; PAS=periodic Schiff stain.

For histological observation, Bantam chicken testis exhibited a denser arrangement of the seminiferous tubules, which showed more spermatogenic activity. Tubules were slightly enlarged, representing the maturation, and displayed several blood vessels and the usual form of Leydig's cells (Fig. 4a). Seminiferous tubules were fully active and displayed all spermatogenic cells, i.e., spermatogonia, spermatocytes, and spermatozoa, differentiating and migrating toward the lumen. Some degenerating forms of seminiferous tubules were found, such as the space between the tubule and blood vessels (Fig. 4a). In the interior, close to the tubular wall (Fig. 4c), the counting of degenerative tubules in each sampling area was relatively low and exhibited about 2-3% relative to healthy ones. These results indicated that although some metals, such as Cr accumulation, were high, they rarely found testicular degeneration, which is referred to as dysfunction of the male reproductive organs in the bantam chicken in this region.

The bantam chicken ovary displayed many fast-developing follicles in an advanced yolk accumulation state (Fig. 4b, 4d). Some follicular atresia was found, but this is a normal physiological process in the ovary throughout the female reproductive life at all stages as follicles develop. Deformation of the ovarian structure was rarely observed in the ovary in the present study.

DISCUSSION

Metal accumulation, such as Co, Cd, Pb, and As, was slightly higher in the liver tissue than in the brain or gonads of bantam chicken. Although the highest accumulation was found in the liver, it did not exceed the recommended level (Mamun *et al.*, 2024). Typically, Co is required as cobalt-containing vitamin B12 (cobalamin) and is essential as a cofactor of many enzymes, which is unsurprising, as it was found in the highest amounts in the liver tissue (Osman *et al.*, 2021). Co is known to be widely distributed in animal organs, with relatively high concentrations found in the liver, kidneys, bones, spleen, and other glandular tissues (Akan *et al.*, 2010). As found in the present study, the accumulation levels of test metals were not at the toxic level. In the study region, Kui Buri District, potential contamination sources mainly come from agriculture, not industry, due to regional usage. Consequently, the contamination of heavy metals is unlikely to present a significant concern.

Evaluation of oxidative status, such as LPO level, as indicated by the MDA, exhibits a high level of LPO in the brain more than in the liver, indicating that the liver plays a vital role in forming antioxidant defense mechanisms (Balogh *et al.*, 2001). Our result also showed that SOD level was higher in the brain than in the liver. It has been reported that more SOD synthesis under stress conditions serves as an adaptive mechanism to reduce ROS formation, prevent oxidative stress, and maintain homeostasis (Surai *et al.*, 2019). It is considered a vital component of the cell's first level of antioxidant defense (Surai, 2018). Research also showed that the synthesis of SOD during stress acts as an adaptive mechanism to decrease ROS formation, lessen oxidative stress, and maintain homeostasis (Azadmanesh and Borgstahl, 2018). Together with the enzymatic activity

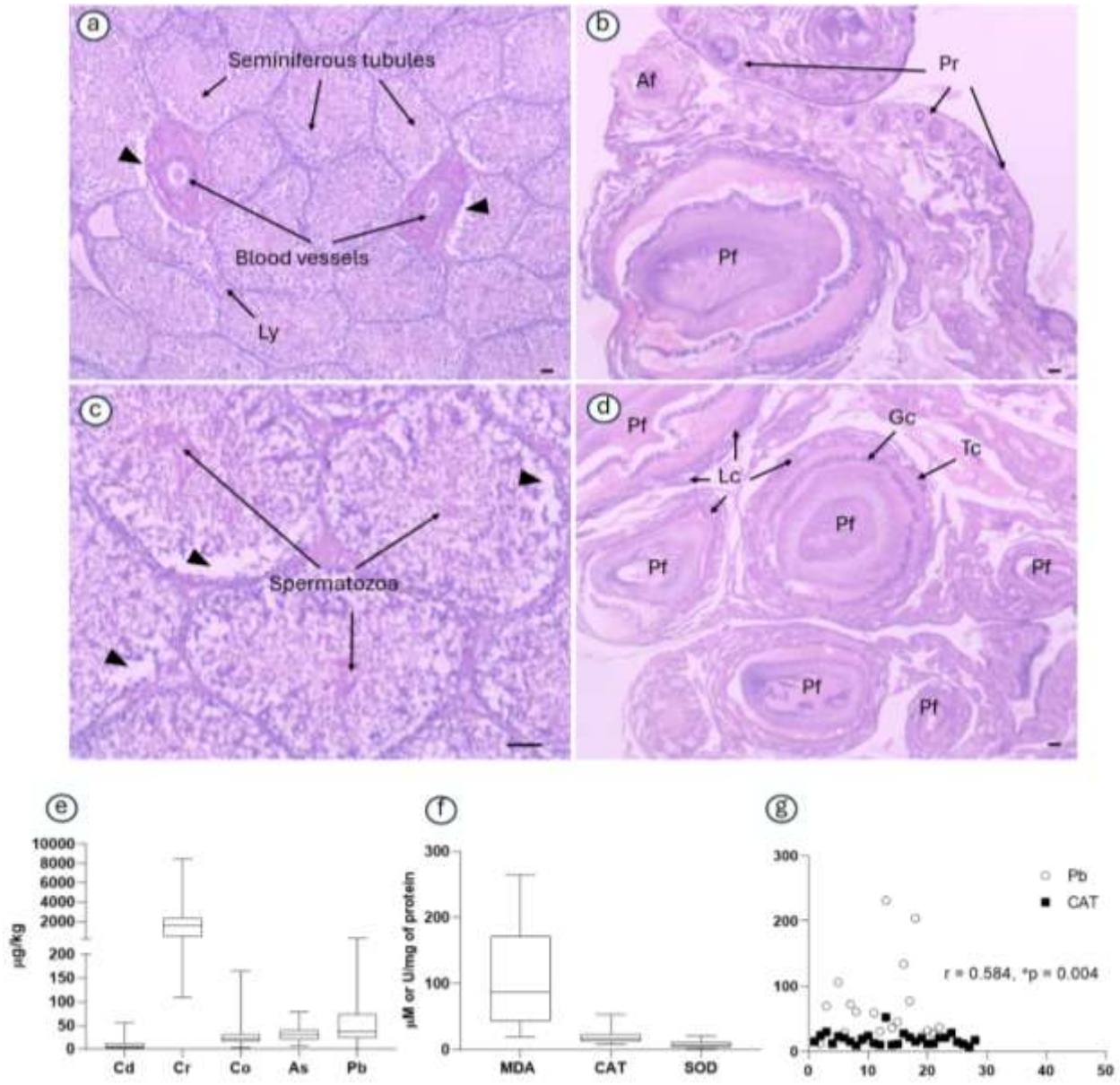

Fig. 4: Photomicrographs of gonad tissues, i.e., testis (a), ovary (b and d) with H&E staining, x4 magnification, and testis (c) at x20 magnification, scale bars=50 µm. The histograms showed Cd, Cr, Co, As, and Pb accumulation levels (e), oxidative status indicated by MDA, CAT, and SOD (f), and Pearson's analysis showed a moderate positive correlation between Pb and CAT (g). Arrowheads indicate that the testis' degenerative tissue is present around blood vessels and inside some seminiferous tubules. Af=arthritic follicle; Pr=primordial follicles; Pf=pre-ovulatory follicle; Th=theca layer; Gc=granulosa cell layer; Lc=Luteal cell clusters; Ly=Leydig's cells; Cd=cadmium; Cr=chromium; Co=cobalt; As=arsenic; Pb=lead; MDA=malondialdehyde; CAT=catalase; SOD=superoxide dismutase.

of CAT in gonads, which showed a significantly higher level than the liver and not different from the brain, it also implies the tissue-specific profiles of antioxidant enzymes. Tissue-specific profiles of antioxidant enzyme expression, including SOD, glutathione peroxidase (GSH-Px), and CAT, were observed during chick embryo development (Surai, 1999). However, in the adult chickens, as has been observed in the present study, we indicated that both LPO and SOD were higher in the brain than in other tissues, implying that the brain exhibited more stress than the gonad or liver. Previous research indicated that barbiturates specifically affect the brain, which may influence oxidative status more than other organs; this consideration is relevant to the present study. However, only one-time injection and this short-acting euthanasia may leave a minimal effect when assessing oxidative stress assessed within a week later (Dostalek *et. al*., 2007).

Heavy metal accumulation, i.e., Cd, Co, As, and Pb, in brain tissues were lower than usual and lower than the toxic levels reported in chicken tissues (Aljohani, 2023). Only Cr exhibited higher accumulation in all tissues than the recommended level (0.1mg/kg) in most food items (Kumpulainen, 1992). Brain lipid peroxidation indicated by MDA level, and enzymatic activities such as SOD and CAT did not difference as well as none of correlation between metal accumulation and oxidative status in the brain. These results accompanied neither histopathological change in the brain areas. Therefore, our results imply that heavy metal accumulation levels cannot induce significant oxidative stress and histopathological changes in the brains of bantam chickens. Despite significant metal accumulation, the lack of histopathological changes can be attributed to several biological factors. Organisms have mechanisms to store

and compartmentalize metals, which may reduce their immediate toxicity.

Furthermore, the body can initiate compensatory processes to lessen damage, and the specific metal, its concentration, and the duration of exposure all influence the degree of histopathological effects (Aljohani, 2023). In addition, a previous study has indicated toxic levels of some potential heavy metals to the brain, such as Cd=2.493 mg/kg, Pb=8.548mg/kg. It is reasonable that the present study result of the accumulation level is far lower than inducing toxicity and damage (Hossain *et. al*., 2022).

Although the accumulation of heavy metals in brain tissue does not lead to significant pathological changes in brain neurons and white matter, a moderate positive correlation was found between Cd accumulation and the enzymatic activity of CAT and SOD, this result indicates that Cd accumulation in the brain leads to an increase in antioxidant enzyme activity. Cadmium-induced oxidative stress is considered the critical factor. As a non-redox-active metal, Cd has been reported that it can alter the activity of antioxidant enzymes, including SOD, CAT, GSH-Px, glutathione reductase, and glutathione-S-transferase (Jomova and Valko, 2011). There were two stages of Cd-induced oxidative status change: the first was an adaptive stage, in which initial activation of antioxidative enzyme response occurred, and the second was the inhibitory stage, in which prolonged or high levels of Cd exposure induced a decrease in antioxidative enzyme activity and led to oxidative damage (Li *et al.*, 2012). In the present study, only the initial stage was affected by low levels of Cd accumulation, and Cd exhibited a more substantial effect on SOD rather than CAT, also mentioned (Imsilp *et al.*, 2024).

Our study revealed minimal pathological changes in brain tissue with a weak negative correlation between Cr accumulation and cerebral neuronal density. A study has shown that Cr accumulation is also higher in the chicken brain compared to the liver (Hossen *et al.*, 2022), and we also found that Cr accumulation in the brain was higher than in other tested metals. A weak negative correlation of Cr was found with cerebral neuronal density but not the midbrain and cerebellar neurons, i.e., Purkinje, deep nuclei, granular cells, or white matter areas such as optic tract and cerebellar mossy fiber. This may involve the susceptibility differences in specific brain areas in response to metal accumulation, and our result revealed that cerebral neurons may be the most susceptible to Cr accumulation. Different brain regions are unique in functions and are composed of different or similar types of cells; for example, cerebral neurons may be formed of granular and pyramidal cells and are responsible for many functions, such as sensory, association, and motor control (Kuenzel, 2018). The Purkinje has a multipolar neuronal shape, is located in the cerebellum, and works with round-shaped granular and various deep cerebellar nuclei for motor coordination (Shehan, 2012). Indeed, the present study reported only cellular density indicated by the percentage of Nissl-positive neuronal cells, which is reduced when Cr increases; if this happens more, it may lead to hypo- or dysfunction of the cerebrum. However, further studies are needed to characterize more specific pathologies according to functional changes and the precise accumulation, which can be defined as toxic levels of the metals.

Heavy metal accumulation in the liver tissues of bantam chickens, i.e., Cd, Pb, As, and Co, did not exceed the recommended level (Akan *et al.*, 2010), while Cr was relatively high and exceeded the recommended level (Kumpulainen, 1992). In addition, none of the degenerative forms of liver tissue were observed, i.e., inflammatory cell infiltration and liver tissue fragmentation. Normal hepatocytic histology, and only a few necrotic cell deaths were observed. The percentage of hepatocytic areas related to the vacuolation area was not different. Indeed, most vacuolations were fibroblast cells of connective tissue modified for fat accumulation or so-called adipocytes. Typically, the fat and protein content of the chicken liver was 35 and 65%, and the fat content of chicken liver ranged from 2.65 to 10.07g/100g depending on the breed (Cieslik *et al.*, 2011). Our present study did not use a specific dye to indicate fat droplets, such as oil red O. Therefore, we cannot indicate the premises of fat distribution in the liver. Glycogen content in hepatocytic cells indicated by PAS staining was used to detect liver cells' metabolism and synthesis function (Hui *et al.*, 2017), which showed that none differed among sampling areas. We indicated no correlation between heavy metal accumulation and oxidative parameters or histopathological changes in the liver. Even though Cr exhibited the highest accumulation level compared to other metals, its concentration was not high enough to induce histopathological alterations. Previous findings indicated that a Cr concentration of approximately 3 mg/kg can cause changes in the microscopic structures of chicken liver tissue (Ognik *et al.*, 2020). Therefore, our results for metal accumulation related to oxidative status and histology of the liver of the bantam chicken imply a confident, safe metal level for oxidative status, morphology, and function in this study area.

The accumulation levels of heavy metals such as Pb, Cd, Co, and As in the gonad did not exceed the recommended level (Akan *et al.*, 2010). Chromium accumulation in the gonad was high, similar to that in the brain and liver, which exceeded the recommended level (Kumpulainen, 1992). Unlike in other tissues, Pearson's correlation analysis indicated a moderate positive correlation between Pb accumulation and CAT activity in the gonad, suggesting a heavy metal effect on antioxidant enzymatic activity. Previous research has shown that Pb exposure significantly decreases the activities of CAT in the chicken ovary (Ma *et al.*, 2020). In the present study, the increase in Pb accumulation appeared to enhance CAT activity, indicating an adaptive rather than inhibitory stage, as previously stated (Imsilp *et al.*, 2024).

In histological observations, the bantam chicken testis showed a more compact arrangement of seminiferous tubules, suggesting increased spermatogenic activity. The tubules appeared slightly enlarged, indicating maturation, and contained numerous blood vessels and the typical form of Leydig's cells. The seminiferous tubules were fully functional, exhibiting all stages of spermatogenic cells, spermatogonia, spermatocytes, and spermatozoa, differentiating and moving towards the lumen. Some seminiferous tubules were degenerating, particularly in spaces between the tubules and blood vessels and near the tubular walls. These findings suggest that testicular degeneration was infrequently observed despite elevated levels of certain metals like Cr, indicating a lack of

dysfunction in the bantam chicken's male reproductive organs in this area.

The bantam chicken ovary exhibited numerous rapidly developing follicles in a late yolk accumulation phase. While some follicular atresia was noted, this is a typical physiological process that occurs at all stages of female reproductive life as follicles progress. This phenomenon has been extensively studied, predominantly in chickens with sexual maturity (Mfoundou *et al*., 2021). Ovarian structural deformation was infrequently observed in the current study. These findings suggest that ovarian degeneration, an indication of dysfunction in the female reproductive organs of bantam chickens in this area, was rarely detected.

Conclusions: The accumulation of heavy metals, specifically Cd and Co, was highest in the liver of bantam chickens compared to the brain and gonads. Most accumulation levels did not exceed the recommended limits. Tissue oxidative status revealed that both LPO and SOD levels were elevated in the brain compared to the gonad and liver tissues, suggesting that the brain experienced greater stress than these other organs. There were Dostalek tissue-specific profiles of oxidative status in response to specific metal accumulation. Histological assessments indicated normal histology of brain neurons, i.e., cerebrum, midbrain, cerebellar Purkinje, granular and deep nuclei, and white matter, i.e., the optic tract and cerebellar mossy fiber. Normal histology was also observed in the liver tissue and gonads of the Bantam chickens. The accumulation level of potential heavy metals in these vital organs was far lower than the toxic concentration. The present study indicates natural bioaccumulation in key organs of bantam chickens, correlating with oxidative status and histological changes. Therefore, the study on control experiments, reproducible effects on specific tissue confirmation, and research on expression and molecular studies also need to emphasize the precise link between heavy metal exposure and accumulation-induced pathological cascades. Future research also focuses on the environmental and human health risks associated with raising bantam chickens for public consumption.

Acknowledgments: This research was financial support from Kasetsart Veterinary Development Funds (VET.KU2023-KPVRF02) and the International Science Branding (ISB), Faculty of Science at Kasetsart University, Thailand.

Authors contribution: WT conceived and designed the research, biochemical, and histological analysis, analyzed all data and artworks, and wrote the manuscript. PT conceived and designed the experiments, contributed reagents, materials, and sample collection, and performed experiments. NL collected samples and performed experiments. KI conceived and designed the experiments and sample collection.

Funding: The authors declare there is individual funding.

Data availability: Data is available on reasonable request.

Ethics approval: This study was approved by the Animal Ethics Committee, Faculty of Veterinary Science, Kasetsart University (ID#ACKU66-VET-073).

Disclosure statement: The authors declare no competing interests.

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