COVERING LETTER

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Title:

Histopathological Changes of Multiple Organs in Red-Footed Tortoise (*Chelonoidis carbonarius*) with Suspected Metabolic Bone Disease

Author(s) name:

Hani Plumeriastuti♥, Djoko Legowo, Annise Proboningrat, Gracia Angelina Hendarti, Bilqisthi Ari Putra

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Place and date:

Surabaya, November 11, 2022

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Hani Plumeriastuti

Histopathological Changes of Multiple Organs in the Red-Footed Tortoise (*Chelonoidis carbonarius*) with Suspected Metabolic Bone Disease

HANI PLUMERIASTUTI[1,♥], DJOKO LEGOWO[1], ANNISE PROBONINGRAT[1], GRACIA ANGELINA HENDARTI[2], BILQISTHI ARI PUTRA[1]

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Manuscript received: DD MM 2022 (Date of abstract/manuscript submission). Revision accepted: ..... 2022.

Abstract. Metabolic bone disease (MBD) is a disorder related to the mechanism of vitamin D and calcium metabolism, which generally occurs in reptiles, especially Chelonia and Lizards. A red-footed tortoise, which was clinically indicated to have a MBD, was necropsied in an effort to establish the diagnosis by histopathological examination. The purpose of this examination was to analyze the impact of the disease on various organs microscopically in patients with suspected MBD. The results showed that there was a decrease in the number of trabeculae and hematopoietic cells in the metatarsal bones, moderate myonecrotic and skeletal muscle atrophy, perineuritis, acute tubular necrosis and mild edema of the renal cortex, congestion and an increase in the number of melanomacrophages in the liver, as well as epicarditis and myocarditis in the heart. Several forms of histopathological changes seem to show pathophysiological relationships in multiple organs.

Key words: Hematoxylin-eosin, MBD, tortoise, septicemia.

Abbreviations: BMD (bone mineral density), MBD (metabolic bone disease), PTH (parathyroid hormone), UV (ultraviolet).

Running title: Metabolic bone disease in tortoise

INTRODUCTION

In the last decade, the trend of domesticating tortoises as pets in urban families is increasing. However, this can lead to many health problems (Patel and Patel, 2020). Management of nutrition, health, housing, and an inappropriate environment are predisposing factors to serious health issues in tortoises if not treated immediately. One of the problems that often affect tortoises is metabolic bone disease.

Metabolic bone disease (MBD) is a term for a collection of medical disorders commonly seen in captive reptiles, particularly in Chelonia (turtles, tortoises, and terrapins) and lizards, occasionally in snakes (Hedley, 2012). This complex disease typically affects the integrity and function of the skeletons (Doneley et al. 2017). MBD of various origins are the most common causes of muscle weakness and abnormalities of the skeleton and spine in reptile patients. MBD may manifest in a variety of pathological conditions, including nutritional secondary hyperparathyroidism, renal secondary hyperparathyroidism, osteoporosis, osteomalacia, osteopetrosis, fibrous osteodystrophy, pathological fractures, and more (Divers and Stahl, 2019).

Here, we present a case of a red-footed tortoise that died with some clinical symptoms that could be related to MBD. Our aim is to evaluate the histopathological findings of several organs associated with MBD in the animal.

CASE PRESENTATION

History

A 3-month-old dead red-footed tortoise (*Chelonoidis carbonarius*) weighing approximately 50 grams with a carapace length of 6 cm (Figure 1A-B) was sent to our laboratory. According to the owner's information, the tortoise had clinical symptoms of inappetence, anorexia, abnormal gait, and weakness. Previously, it had a history of shipping that was too long, about seven days, to finally die. Another tortoise, similar in age and weight, maintained together, had similar complaints.

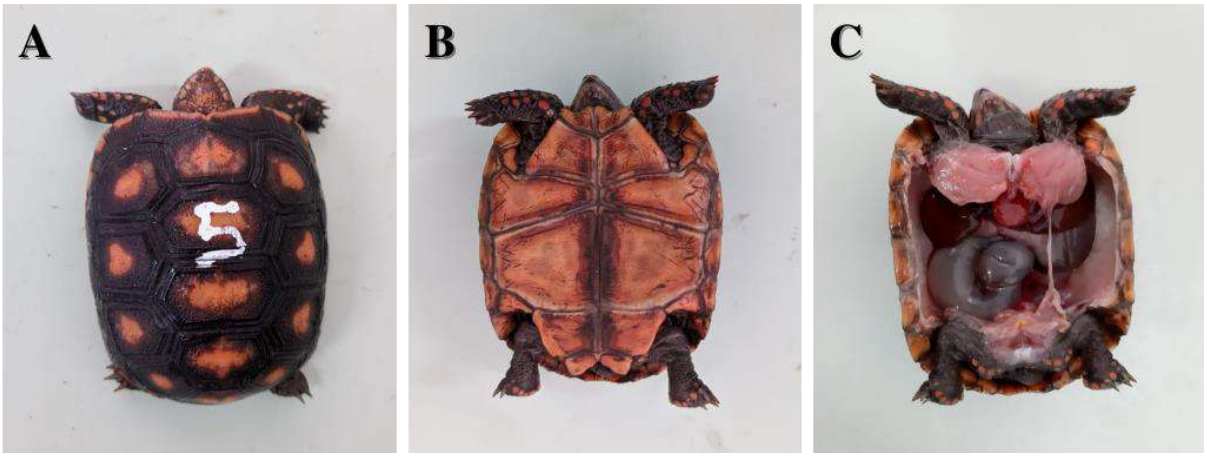

Figure 1. Gross examination of the tortoise body suspected of having MBD. A: the dorsal part. B: the ventral part. C: gross appearance of the internal organs.

Post-Mortem Examination

The necropsy we performed showed that the carapace and plastron were tender, but the size and consistency of the internal organs were normal and there was no accumulation of fluid in the abdominal cavity (Figure 1C). The kidney, liver, heart, leg muscles, and leg bones were collected for tissue processing and hematoxylin-eosin staining.

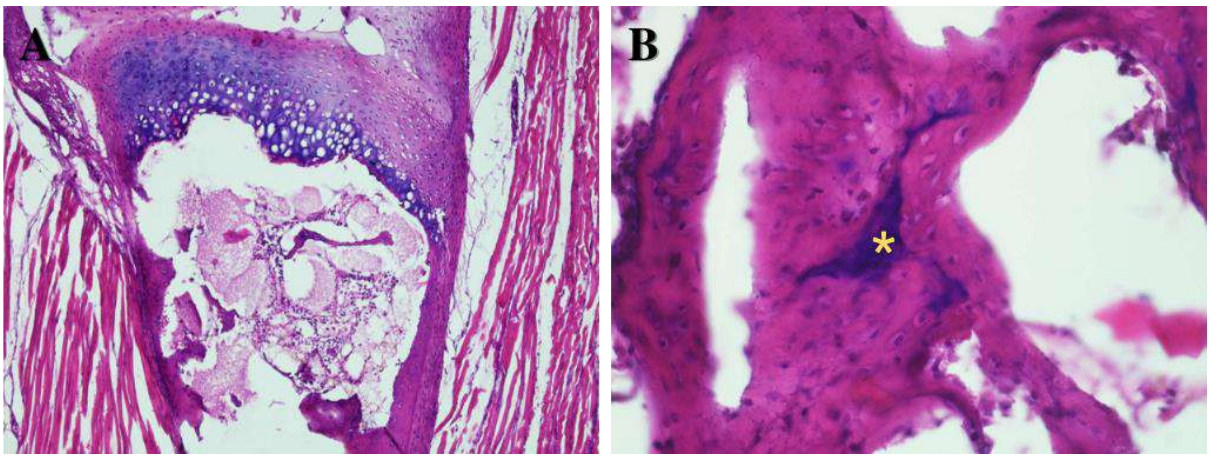

Figure 2. Histopathological appearance of the metatarsal bone of tortoise with suspected MBD (hematoxylin-eosin staining). A: metatarsal epiphyses with fewer trabeculae and loss of hematopoietic cell (100× maginification). B: hyaline cartilage (asterisk) within the trabecular matrix (400× magnification).

Hitopathological Examination

On the results of Hematoxylin and Eosin staining of tortoise metatarsal bones, there was a decrease in the number of trabecular bone in the epiphysis and hematopoietic cell loss (Figure 2A). Some sections also showed the presence of hyaline cartilage in the middle of the mature trabecular bone matrix (Figure 2B). In skeletal muscles, it was observed that many cells of the skeletal muscles were necrotic and some were atrophic (Figure 3A). Inflammatory cell infiltration also occurs between the striated muscle fibers accompanied by edema (Figure 4B). Peripheral nerve cells also underwent lysis and inflammation (Figure 3C).

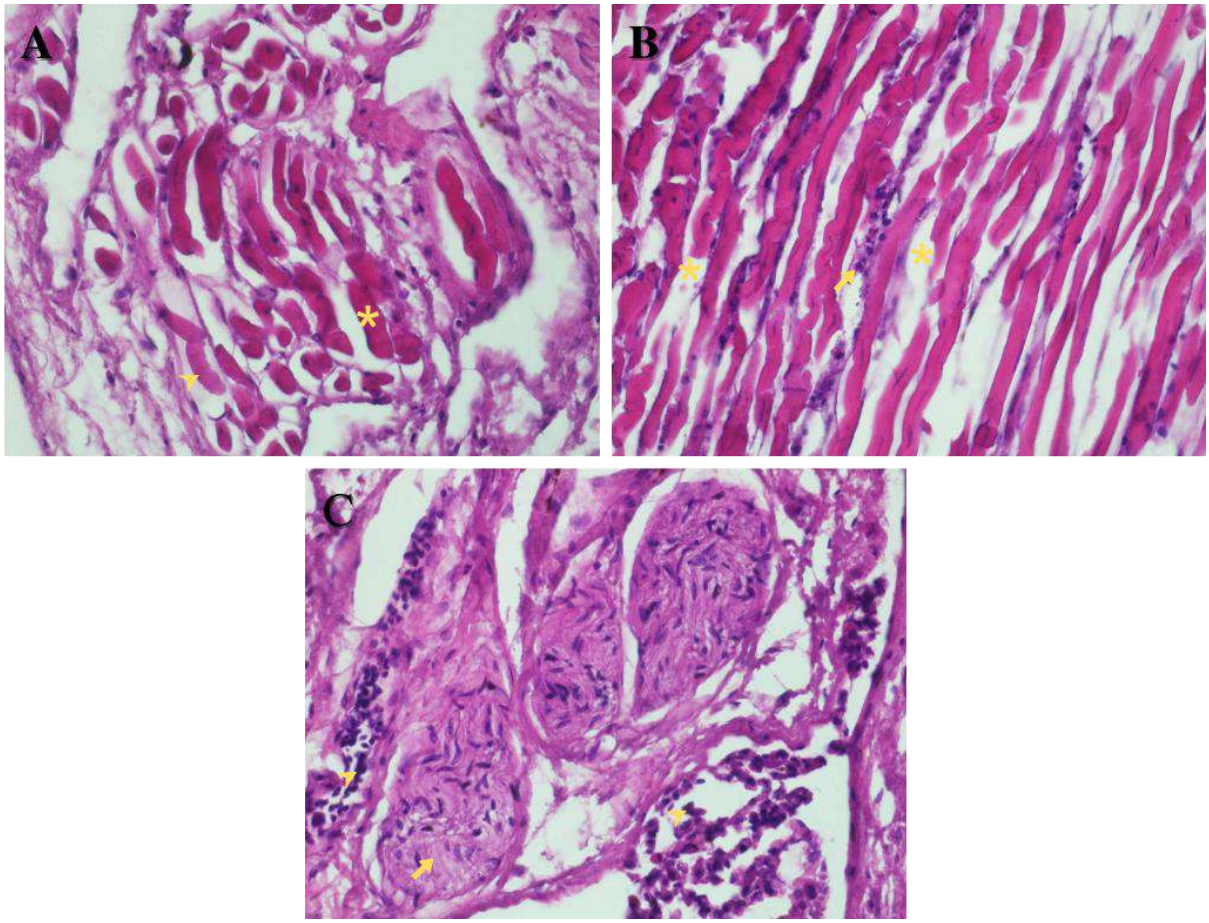

Figure 3. Histopathological appearance of tortoise skeletal muscles with suspected MBD (hematoxylin-eosin staining, 400× magnification). A: striated muscles with atrophic (asterisk) and necrotic (arrowhead). B: edema (asterisk) and infiltration (arrow) of inflammatory cells between muscle fibers. C: peripheral nerve cell lysis (arrow) and inflammatory cell (arrowhead) infiltration.

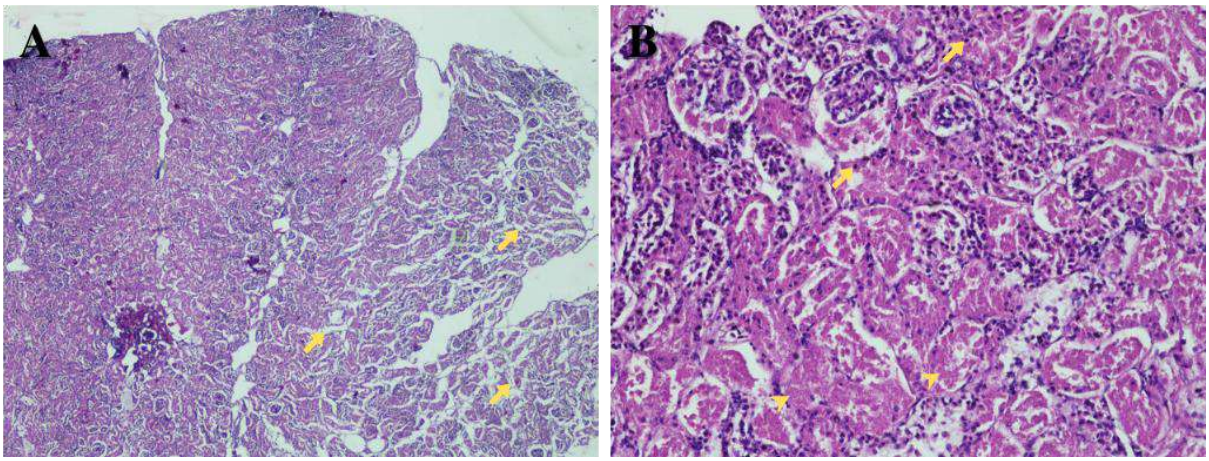

Figure 4. Histopathological appearance of the renal cortex of tortoise with suspected MBD (hematoxylin-eosin staining). A: edema (arrow) of the two right lobes of the kidney (40× maginification). B: pyknosis (arrow) and karyolysis (arrowhead) of convoluted tubule cells (200× magnification).

On microscopic examination of the kidney, edema appeared in two of the four lobes of the kidney (Figure 4A). The appearance of massive acute tubular necrosis was also clearly seen, characterized by convoluted proximal tubules that mostly underwent cell lysis and nuclear pyknosis (Figure 4B). Mild to moderate congestion was observed in the liver sinusoids (Figure 5A). An increase in the number of melanomacrophages has also occurred, and some have formed melanomacrophage centers (MMC). Additionally, there are few eosinophilic granular cells (EGCs) and some infiltrating

lymphocytes that aggregate to form lymphoid follicles (Figure 5B). Furthermore, microscopic observation also showed that the tortoise heart experienced epicarditis, myocarditis, and endocarditis, indicated by fairly massive lymphocytic infiltration in the epicardium, myocardium, and lumen of the endocardium, respectively (Figure 6A-B).

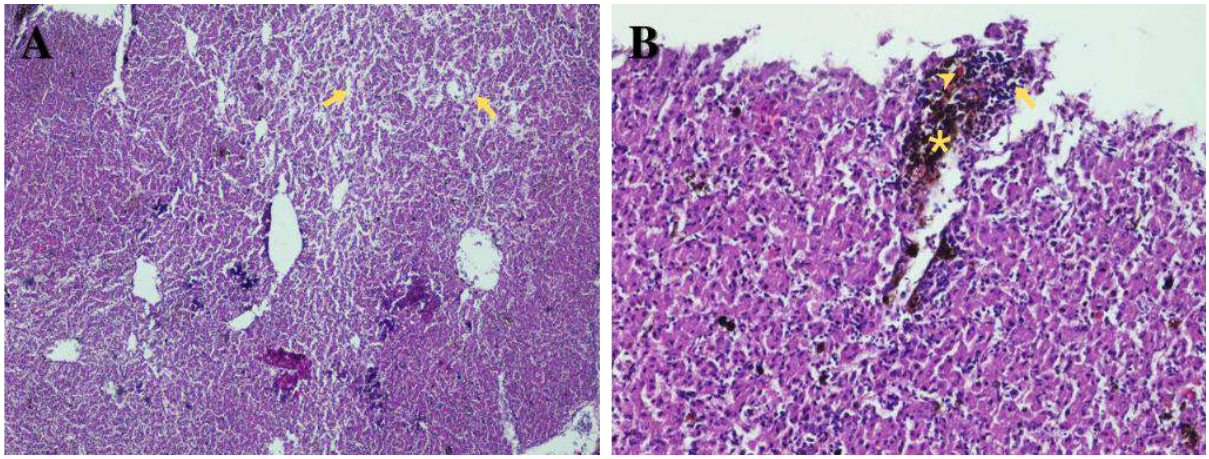

Figure 5. Histopathological appearance of the liver parenchyma of tortoise with suspected MBD (hematoxylin-eosin staining). A: some areas of the liver are congested (arrow) (40× maginification). B: infiltration of lymphocytes (arrow), eosinophilic granular cells (arrowhead), and melanomacrophage centers (asterisk) (200× magnification).

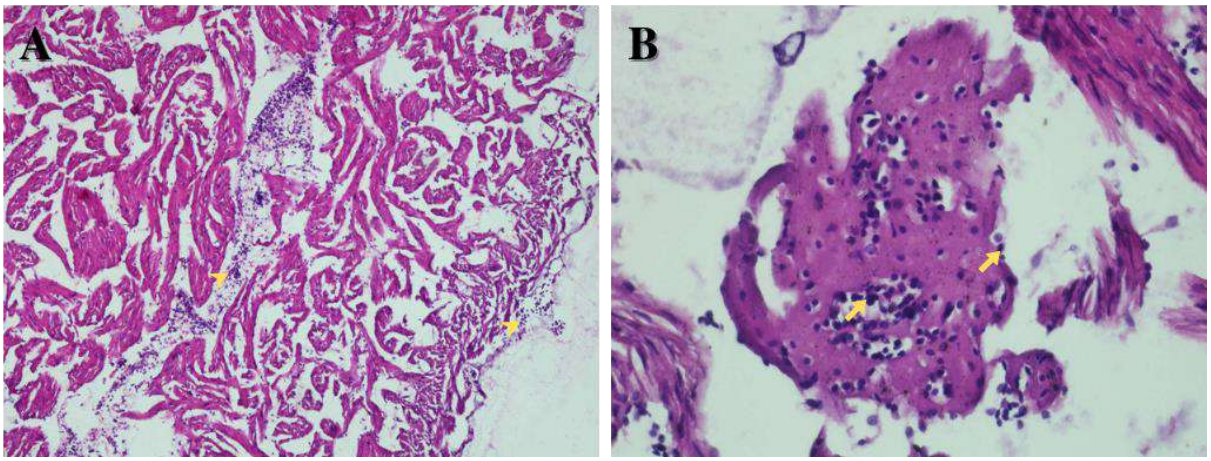

Figure 6. Histopathological appearance of the tortoise heart with suspected MBD (hematoxylin-eosin staining). A: infiltration of lymphocytic cells (arrowhead) in the epicardium and myocardium (100× maginification). B: infiltration of lymphocytic cells (arrow) in the lumen of the endocardium (400× magnification).

DISCUSSION

Based on clinical symptoms and the reported history, it was assumed that the tortoise that we had necropsy had MBD. One of the strong supporting reasons is hypocalcemia-induced vitamin D deficiency, which is related, first, to lack of UV light (due to long shipping journeys) and proper temperature, and/or inadequate and balanced nutritional intake. Inadequate exposure to UVB rays causes the epidermal cells of animal skin to be unable to produce vitamin D3 (cholecalciferol) which is the result of the conversion of pre-vitamin D and its precursor, pro-vitamin D (7-dehydrocholesterol). Deficiency of vitamin D3 in the blood circulation causes the liver to lack its capacity to produce calcidiol or 25-(OH)-vitamin D3 as the main storage form of vitamin D3. This continues to cause kidneys to fail to hydroxylate calcidiol to produce 1,25-(OH)2-vitamin D3 or calcitriol, which plays a vital role in the regulation of calcium and phosphorus balance (Diehl et al. 2018).

The endocrine hormone calcitriol is known to increase intestinal absorption of dietary calcium and phosphate, stimulate the storage of calcium and phosphate in the kidneys, and, together with parathyroid hormone (PTH), has a direct effect on bone by regulating calcium mobilization from bone. Lack of this hormone can cause disturbances in bone growth and development, as well as in maintaining mature bone tissue (Kumar et al. 2018).

Hypocalcemia due to hypovitaminosis D is usually compensated by increased secretion of PTH from hyperplastic parathyroid glands and subsequently hyperparathyroidism, leading to resorption of calcium from bone (Hall et al. 2020). Unfortunately, we did not observe the histopathological features of the parathyroids so we could not confirm hyperparathyroidism in this case.

Another possibility that can occur is low calcitriol so that the body cannot limit the occurrence of osteoclastogenesis and trigger bone resorption by osteoclasts, resulting in osteopenia (Li et al. 2017; Zachary, 2022). This may explain the loss of the large amount of trabecular bone in the metatarsal tortoise epiphyses that we observed. The loss of trabecular continuity leads to a reduction in the ability of the trabecular to withstand stress (Zachary, 2022); therefore, the tortoise appears to have an abnormal gait.

In this study, we also found the presence of cartilage within the trabecular matrix. This may be related to disturbances in endochondral ossification during the development of the young tortoise. Ostechondrosis is a disorder of chondrocyte maturation that results in delayed cartilage mineralization. In addition to calcitriol, 24,25-(OH)2-vitamin D3 produced by calcidiol hydroxylation in the proximal renal tubule also plays an important role in cartilage cell differentiation and matrix mineralization. This imbalance in plasma concentrations between vitamin D metabolites appears to be related to the disposition of osteochondrosis during the growth period of the animal (Zafalon et al. 2020).

Under normal conditions, bone marrow in newborns and very young animals is mainly composed of active hematopoietic tissue and has relatively few fats (Zachary, 2022). We found that the bone marrow within the epiphyseal metatarsal of the tortoise was hypocellular with a significantly reduced number of hemopoietic cells. It is still unclear how the pathophysiological relationship with the suspected MBD occurs. In another case, Turnbull et al. (2000) also reported bone marrow hypocellularity in hypothermic sea turtles. Bone marrow hypoplasia is commonly found in animals and humans with aplastic pancytopenia, a rare condition in which all hematopoietic lines in the bone marrow are aplastic or severely hypoplasiated, resulting in bone marrow failure. The cause is usually chemical agents that are cytotoxic to hematopoietic cells, or mutations or perturbations in hematopoietic cells and their environment caused by infectious agents (Zachary, 2022).

In this study, we found indications of infection and sepsis, possibly due to microbial flora, in the tortoise suspected of having MBD. The association between decreased bone mineral density (BMD) and the risk of infection and sepsis has recently been reported. Previous studies have shown that BMD is a prognostic factor for infections and sepsis in human patients. Schulze-Hagen et al. (2021) found that low BMD was closely related to high mortality rates in intensive care units, while patients with pulmonary infections had the lowest BMD. A recent study even demonstrated that low BMD is not only a potential predictor for patients with infections and sepsis, but also a new risk factor for infections and sepsis.

Sepsis occurs after bacterial infections, leading to severe sepsis and septic shocks characterized by low blood pressure, ischemic, failure of multiple organs, and death (Drosatos et al. 2015). In this case, we observed inflammation of the liver, heart, muscles, and peripheral nerves, as well as renal tubular necrosis that can lead to acute renal failure. The decrease in the number and function of osteoblasts, associated with altered expression of IL-7 and lipocalin-2, may have a negative impact on human immunity and thus increase sensitivity to infections. Vitamin D may also explain the connection between BMD and infections and sepsis (Zhang et al. 2022). However, much remains to be done to confirm the factors and analyze the association between bone metabolism disorders and sepsis.

In conclusion, this study reports that the young tortoise we necropsied had a number of pathological conditions that led to suspicion of MBD. Inflammation of multiple organs due to sepsis that we found also seems to have a pathophysiological relationship with this disorder of bone metabolism. Further study is warranted to reach a final diagnosis and examine the relationship between MBD mineral and the risk of infection and sepsis in animals.

ACKNOWLEDGEMENTS

We thank the co-assistant students of the Division of Veterinary Pathology, Faculty of Veterinary Medicine, Universitas Airlangga, who assisted in finding the sample cases for this study.

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2. There is still a lack of information in the case history. Please provide more information, such as sex, anamnesis, and other details, such as the type of food the tortoises eat before they die and the shipping method, etc.

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4. Please use an arrow or mark with a dark color; in this picture, the yellow color is not clear or visible to describe the changes.

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COVERING LETTER

Dear **Editor-in-Chief,**

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Author(s) name:

Hani Plumeriastuti♥, Djoko Legowo, Annise Proboningrat, Gracia Angelina Hendarti, Bilqisthi Ari Putra

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Place and date:

Surabaya, November 11, 2022

Sincerely yours,

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Hani Plumeriastuti

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Manuscript received: DD MM 2022 (Date of abstract/manuscript submission). Revision accepted: ..... 2022.

Abstract. Exotic pet lovers' interest in keeping tortoises is increasing all over the world, including Indonesia. However, this trend cannot be separated from the potential emergence of various health problems in tortoises. One of the problems that often affects tortoises is metabolic bone disease. Metabolic bone disease (MBD) is a disorder related to the mechanisms of vitamin D and calcium metabolism, which generally occurs in reptiles, especially Chelonia and Lizards. A 3-month-old red-footed tortoise, which was clinically suspected to have a MBD, was necropsied as an effort to support the provisional diagnosis through histopathological evaluation. The purpose of this examination was to analyze the impact of the disease on various organs microscopically in patients with suspected MBD. The results showed a decrease in the number of trabeculae and hematopoietic cells in the metatarsal bones; moderate myonecrotic changes and atrophy in the skeletal muscle; inflammation of the perineuron; acute tubular necrosis and mild edema of the renal cortex; congestion and an increase in the number of melanomacrophages in the liver; as well as epicarditis and myocarditis in the heart. Several forms of the histopathological changes seem to indicate a pathophysiological relationship between the suspected metabolic bone dis ease and the multiple organs examined.

Key words: Hematoxylin-eosin, MBD, tortoise, septicemia.

Abbreviations: BMD (bone mineral density), MBD (metabolic bone disease), PTH (parathyroid hormone), UV (ultraviolet).

Running title: Metabolic bone disease in tortoise

INTRODUCTION

In the last decade, the trend of domesticating tortoises as pets in urban families is increasing and popular around the world (Patel and Patel, 2020). In Indonesia, the sulcata tortoise is one of the most popular tortoise pets because it is easy to find this captive breed in the reptile pet market (Raharjo et al. 2022). Another tortoise that is frequently kept as a traditional pet in houses is the red-footed tortoise (Mendoza et al. 2021). Their ease of care, low cost of ownership, and amazing coloration make them highly sought after by novice tortoise keepers.

The red-footed tortoise (*Chelonoidis carbonaria*) are members of the Anapsida subclass, Chelonia order, Cryptodira suborder, Testudines family, and Chelonoidis genus (da Silva et al. 2020). This species is native to South America and can be found from Panama to Paraguay, as well as parts of Bolivia, Brazil, Colombia, Ecuador, and Peru (Mendoza et al. 2022). *C. carbonaria* is a diurnal and terrestrial animal with a compact body and strong cylindrical limbs, ideal to support its heavy carapace and walk in rough terrain (da Silva et al. 2020; Mendoza et al. 2022). They are opportunistic omnivores in general, and their diet is heavily influenced by the seasonal availability of food (Mendoza et al. 2022). Their main food sources are leaves, grasses, flowers, fruits, carcasses, and other food found on the ground (da Silva et al. 2020).

The increasing trend of keeping tortoises among exotic animal enthusiasts also has the potential to lead to many health problems (Patel and Patel, 2020). *C. carbonaria* is the most common testudines kept as a pet in South America, which accounts for a large proportion of wildlife patients seen in veterinary practices (da Silva et al. 2020). In Indonesia, according to Raharjo et al. (2022), a study on the prevalence of disease in exotic pet patients at a clinic in Yogyakarta, Indonesia, during January-August 2020 showed that turtles and tortoises had the highest cases of 71.7%, compared to snakes (16.5%), iguanas (6.2%), lizards (4.1%), crocodiles (1%), and geckos (0.5%).

Management of nutrition, health, housing, and an inappropriate environment are predisposing factors to serious health issues in tortoises if not anticipated and treated immediately. Some of the health issues that tortoises in captivity can face include respiratory ailments caused by bacterial or viral infection (Papp et al. 2010; Gibbons and Steffes, 2013; Silveira et

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al. 2014; Ballourad et al. 2021), gastro-intestinal disease caused by parasite or viral infection (Gibbons and Steffes, 2013; Hallinger et al. 2018; Springer et al. 2020), and nutritional and metabolic disorders (Sari, 2020; Santos et al. 2022; Sartori et al. 2022).

Metabolic bone disease (MBD) is one of the metabolic disorders commonly seen in captive reptiles, particularly in Chelonia (turtles, tortoises, and terrapins) and lizards, occasionally in snakes (Hedley, 2012). In veterinary medicine, MBD refers to a group of pathological conditions that affect the integrity and function of multiple bones (Doneley et al. 2017). They are most generally caused by genetic, dietary, and/or hormonal disorders that impact bone growth and remodeling, typically through changes in calcium/phosphorus metabolism. MBD has traditionally been broken down as fibrous osteodystrophy, osteoporosis, and rickets/osteomalacia; however, many cases are difficult to specifically classify, particularly those caused by nutritional deficiencies, because multiple conditions may coexist. Therefore, cases reported in the literature should be scrutinized carefully, and confirmation by histopathological evaluation should be regarded as more definitive (Uhl, 2018).

Here, we present a case of a red-footed tortoise that died with some clinical symptoms that could be related to MBD. Our aim is to evaluate the histopathological findings of several organs associated with MBD in the animal.

CASE PRESENTATION

History

A 3-month-old dead red-footed tortoise (*Chelonoidis carbonarius*) weighing approximately 50 grams with a carapace length of 6 cm (Figure 1A-B) was sent to our laboratory. According to the owner's information, the tortoise had clinical symptoms of inappetence, anorexia, abnormal gait, and weakness. Previously, it had a history of shipping that was too long, about seven days, to finally die. Another tortoise, similar in age and weight, maintained together, had similar complaints.

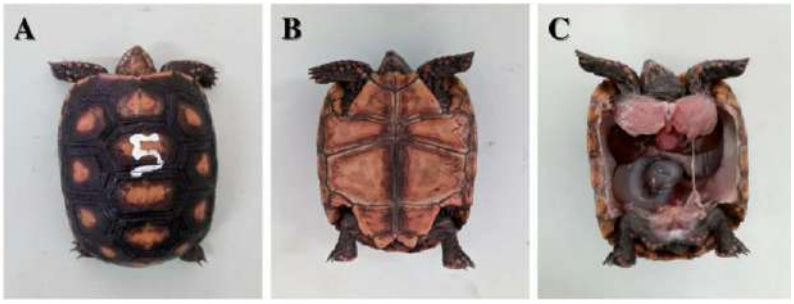

Figure 1. Gross examination of the tortoise body suspected of having MBD. A: the dorsal part. B: the ventral part. C: gross appearance of the internal organs.

Post-Mortem Examination

The necropsy we performed showed that the carapace and plastron were tender, but the size and consistency of the internal organs were normal and there was no accumulation of fluid in the abdominal cavity (Figure 1C). The kidney, liver, heart, leg muscles, and leg bones were collected for tissue processing and hematoxylin-eosin staining.

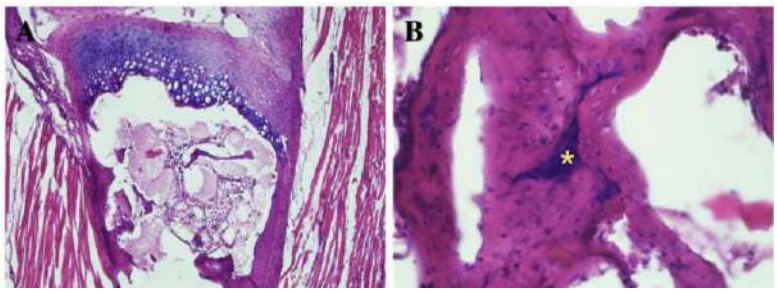

Figure 2. Histopathological appearance of the metatarsal bone of tortoise with suspected MBD (hematoxylin-eosin staining). A: metatarsal epiphyses with fewer trabeculae and loss of hematopoietic cell (100× maginification). B: hyaline cartilage (asterisk) within the trabecular matrix (400× magnification).

Hitopathological Examination

On the results of Hematoxylin and Eosin staining of tortoise metatarsal bones, there was a decrease in the number of trabecular bone in the epiphysis and hematopoietic cell loss (Figure 2A). Some sections also showed the presence of hyaline cartilage in the middle of the mature trabecular bone matrix (Figure 2B). In skeletal muscles, it was observed that many cells of the skeletal muscles were necrotic and some were atrophic (Figure 3A). Inflammatory cell infiltration also occurs between the striated muscle fibers accompanied by edema (Figure 4B). Peripheral nerve cells also underwent lysis and inflammation (Figure 3C).

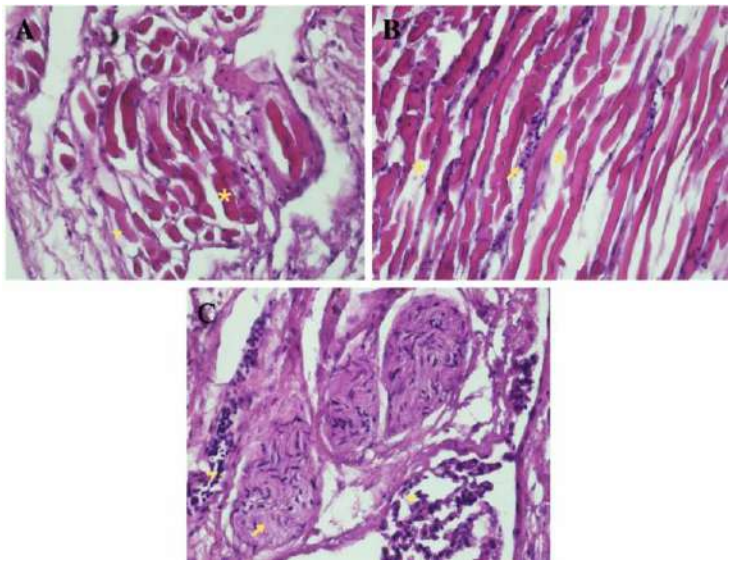

Figure 3. Histopathological appearance of tortoise skeletal muscles with suspected MBD (hematoxylin-eosin staining, 400× magnification). A: striated muscles with atrophic (asterisk) and necrotic (arrowhead). B: edema (asterisk) and infiltration (arrow) of inflammatory cells between muscle fibers. C: peripheral nerve cell lysis (arrow) and inflammatory cell (arrowhead) infiltration.

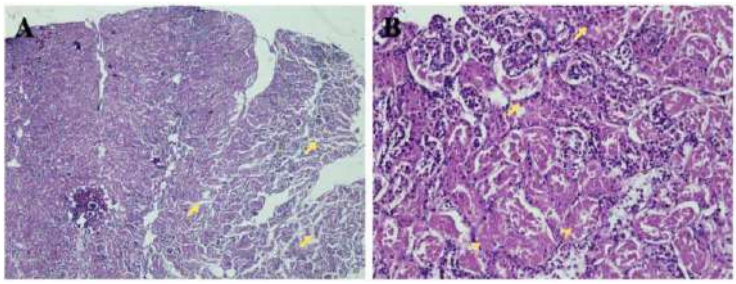

Figure 4. Histopathological appearance of the renal cortex of tortoise with suspected MBD (hematoxylin-eosin staining). A: edema (arrow) of the two right lobes of the kidney (40× maginification). B: pyknosis (arrow) and karyolysis (arrowhead) of convoluted tubule cells (200× magnification).

On microscopic examination of the kidney, edema appeared in two of the four lobes of the kidney (Figure 4A). The appearance of massive acute tubular necrosis was also clearly seen, characterized by convoluted proximal tubules that mostly underwent cell lysis and nuclear pyknosis (Figure 4B). Mild to moderate congestion was observed in the liver sinusoids (Figure 5A). An increase in the number of melanomacrophages has also occurred, and some have formed melanomacrophage centers (MMC). Additionally, there are few eosinophilic granular cells (EGCs) and some infiltrating lymphocytes that aggregate to form lymphoid follicles (Figure 5B). Furthermore, microscopic observation also showed that the tortoise heart experienced epicarditis, myocarditis, and endocarditis, indicated by fairly massive lymphocytic infiltration in the epicardium, myocardium, and lumen of the endocardium, respectively (Figure 6A-B).

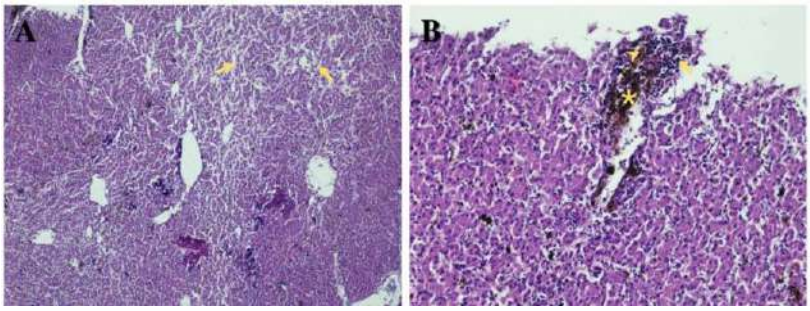

Figure 5. Histopathological appearance of the liver parenchyma of tortoise with suspected MBD (hematoxylin-eosin staining). A: some areas of the liver are congested (arrow) (40× maginification). B: infiltration of lymphocytes (arrow), eosinophilic granular cells (arrowhead), and melanomacrophage centers (asterisk) (200× magnification).

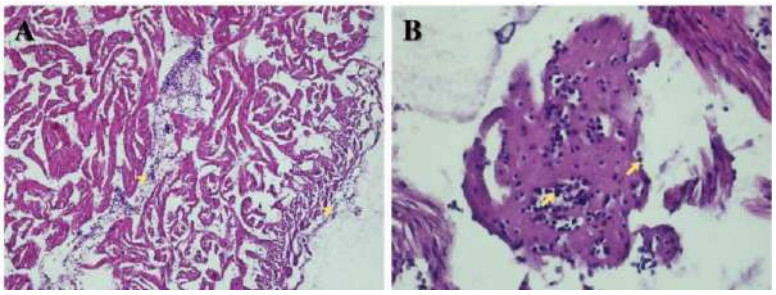

Figure 6. Histopathological appearance of the tortoise heart with suspected MBD (hematoxylin-eosin staining). A: infiltration of lymphocytic cells (arrowhead) in the epicardium and myocardium (100× maginification). B: infiltration of lymphocytic cells (arrow) in the lumen of the endocardium (400× magnification).

DISCUSSION

Based on clinical symptoms and the reported history, it was assumed that the tortoise that we had necropsy had MBD. One of the strong supporting reasons is hypocalcemia-induced vitamin D deficiency, which is related, first, to lack of UV light (due to long shipping journeys) and proper temperature, and/or inadequate and balanced nutritional intake. Inadequate exposure to UVB rays causes the epidermal cells of animal skin to be unable to produce vitamin D3 (cholecalciferol) which is the result of the conversion of pre-vitamin D and its precursor, pro-vitamin D (7-dehydrocholesterol). Deficiency of vitamin D3 in the blood circulation causes the liver to lack its capacity to produce calcidiol or 25-(OH)-vitamin D3 as the main storage form of vitamin D3. This continues to cause kidneys to fail to hydroxylate calcidiol to produce 1,25-(OH)2-vitamin D3 or calcitriol, which plays a vital role in the regulation of calcium and phosphorus balance (Diehl et al. 2018).

The endocrine hormone calcitriol is known to increase intestinal absorption of dietary calcium and phosphate, stimulate the storage of calcium and phosphate in the kidneys, and, together with parathyroid hormone (PTH), has a direct effect on bone by regulating calcium mobilization from bone. Lack of this hormone can cause disturbances in bone growth and development, as well as in maintaining mature bone tissue (Kumar et al. 2018).

Hypocalcemia due to hypovitaminosis D is usually compensated by increased secretion of PTH from hyperplastic parathyroid glands and subsequently hyperparathyroidism, leading to resorption of calcium from bone (Hall et al. 2020). Unfortunately, we did not observe the histopathological features of the parathyroids so we could not confirm hyperparathyroidism in this case.

Another possibility that can occur is low calcitriol so that the body cannot limit the occurrence of osteoclastogenesis and trigger bone resorption by osteoclasts, resulting in osteopenia (Li et al. 2017; Zachary, 2022). This may explain the loss of the large amount of trabecular bone in the metatarsal tortoise epiphyses that we observed. The loss of trabecular continuity leads to a reduction in the ability of the trabecular to withstand stress (Zachary, 2022); therefore, the tortoise appears to have an abnormal gait.

In this study, we also found the presence of cartilage within the trabecular matrix. This may be related to disturbances in endochondral ossification during the development of the young tortoise. Ostechondrosis is a disorder of chondrocyte maturation that results in delayed cartilage mineralization. In addition to calcitriol, 24,25-(OH)2-vitamin D3 produced by calcidiol hydroxylation in the proximal renal tubule also plays an important role in cartilage cell differentiation and matrix mineralization. This imbalance in plasma concentrations between vitamin D metabolites appears to be related to the disposition of osteochondrosis during the growth period of the animal (Zafalon et al. 2020).

Under normal conditions, bone marrow in newborns and very young animals is mainly composed of active hematopoietic tissue and has relatively few fats (Zachary, 2022). We found that the bone marrow within the epiphyseal metatarsal of the tortoise was hypocellular with a significantly reduced number of hemopoietic cells. It is still unclear how the pathophysiological relationship with the suspected MBD occurs. In another case, Turnbull et al. (2000) also reported bone marrow hypocellularity in hypothermic sea turtles. Bone marrow hypoplasia is commonly found in animals and humans with aplastic pancytopenia, a rare condition in which all hematopoietic lines in the bone marrow are aplastic or severely hypoplasiated, resulting in bone marrow failure. The cause is usually chemical agents that are cytotoxic to hematopoietic cells, or mutations or perturbations in hematopoietic cells and their environment caused by infectious agents (Zachary, 2022).

In this study, we found indications of infection and sepsis, possibly due to microbial flora, in the tortoise suspected of having MBD. The association between decreased bone mineral density (BMD) and the risk of infection and sepsis has recently been reported. Previous studies have shown that BMD is a prognostic factor for infections and sepsis in human patients. Schulze-Hagen et al. (2021) found that low BMD was closely related to high mortality rates in intensive care units, while patients with pulmonary infections had the lowest BMD. A recent study even demonstrated that low BMD is not only a potential predictor for patients with infections and sepsis, but also a new risk factor for infections and sepsis.

Sepsis occurs after bacterial infections, leading to severe sepsis and septic shocks characterized by low blood pressure, ischemic, failure of multiple organs, and death (Drosatos et al. 2015). In this case, we observed inflammation of the liver, heart, muscles, and peripheral nerves, as well as renal tubular necrosis that can lead to acute renal failure. The decrease in the number and function of osteoblasts, associated with altered expression of IL-7 and lipocalin-2, may have a negative impact on human immunity and thus increase sensitivity to infections. Vitamin D may also explain the connection between BMD and infections and sepsis (Zhang et al. 2022). However, much remains to be done to confirm the factors and analyze the association between bone metabolism disorders and sepsis.

In conclusion, this study reports that the young tortoise we necropsied had a number of pathological conditions that led to suspicion of MBD. Inflammation of multiple organs due to sepsis that we found also seems to have a pathophysiological relationship with this disorder of bone metabolism. Further study is warranted to reach a convincing confirmation and investigate the relationship between MBD and the risk of infection and sepsis in animals.

ACKNOWLEDGEMENTS

We thank the co-assistant students of the Division of Veterinary Pathology, Faculty of Veterinary Medicine, Universitas Airlangga, who assisted in finding the sample cases for this study.

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Histopathological changes of multiple organs in the Red-Footed Tortoise (*Chelonoidis carbonaria*) with suspected metabolic bone disease

Abstract. Exotic pet lovers' interest in keeping tortoises is increasing all over the world, including Indonesia. However, this trend cannot be separated from the potential emergence of various health problems in tortoises. One of the problems that often affects tortoises is metabolic bone disease. Metabolic bone disease (MBD) is a disorder related to the mechanisms of vitamin D and calcium metabolism, which generally occurs in reptiles, especially Chelonia and Lizards. A 3-month-old red-footed tortoise, which was clinically suspected to have a MBD, was necropsied as an effort to support the provisional diagnosis through histopathological evaluation. The purpose of this examination was to analyze the impact of the disease on various organs microscopically in patients with suspected MBD. The results showed a decrease in the number of trabeculae and hematopoietic cells in the metatarsal bones; moderate myonecrotic changes and atrophy in the skeletal muscle; inflammation of the perineuron; acute tubular necrosis and mild edema of the renal cortex; congestion and an increase in the number of melanomacrophages in the liver; as well as epicarditis and myocarditis in the heart. Several forms of the histopathological changes seem to indicate a pathophysiological relationship between the suspected metabolic bone disease and the multiple organs examined.

Key words: Hematoxylin-eosin, MBD, tortoise, septicemia.

Abbreviations: BMD (bone mineral density), MBD (metabolic bone disease), PTH (parathyroid hormone), UV (ultraviolet).

Running title: Metabolic bone disease in tortoise

INTRODUCTION[A1][A2]

In the last decade, the trend of domesticating tortoises as pets in urban families is increasing and popular around the world (Patel and Patel, 2020). In Indonesia, the sulcata tortoise is one of the most popular tortoise pets because it is easy to find this captive breed in the reptile pet market (Raharjo et al. 2022). Another tortoise that is frequently kept as a traditional pet in houses is the red-footed tortoise (Mendoza et al. 2021). Their ease of care, low cost of ownership, and amazing coloration make them highly sought after by novice tortoise keepers.

The red-footed tortoise (*Chelonoidis carbonaria*) are members of the Anapsida subclass, Chelonia order, Cryptodira suborder, Testudines family, and Chelonoidis genus (da Silva et al. 2020). This species is native to South America and can be found from Panama to Paraguay, as well as parts of Bolivia, Brazil, Colombia, Ecuador, and Peru (Mendoza et al. 2022). *C. carbonaria* is a diurnal and terrestrial animal with a compact body and strong cylindrical limbs, ideal to support its heavy carapace and walk in rough terrain (da Silva et al. 2020; Mendoza et al. 2022). They are opportunistic omnivores in general, and their diet is heavily influenced by the seasonal availability of food (Mendoza et al. 2022). Their main food sources are leaves, grasses, flowers, fruits, carcasses, and other food found on the ground (da Silva et al. 2020).

The increasing trend of keeping tortoises among exotic animal enthusiasts also has the potential to lead to many health problems (Patel and Patel, 2020). *C. carbonaria* is the most common testudines kept as a pet in South America, which accounts for a large proportion of wildlife patients seen in veterinary practices (da Silva et al. 2020). In Indonesia, according to Raharjo et al. (2022), a study on the prevalence of disease in exotic pet patients at a clinic in Yogyakarta, Indonesia, during January-August 2020 showed that turtles and tortoises had the highest cases of 71.7%, compared to snakes (16.5%), iguanas (6.2%), lizards (4.1%), crocodiles (1%), and geckos (0.5%).

Management of nutrition, health, housing, and an inappropriate environment are predisposing factors to serious health issues in tortoises if not anticipated and treated immediately. Some of the health issues that tortoises in captivity can face include respiratory ailments caused by bacterial or viral infection (Papp et al. 2010; Gibbons and Steffes, 2013; Silveira et al. 2014; Ballourad et al. 2021), gastro-intestinal disease caused by parasite or viral infection (Gibbons and Steffes, 2013;

Hallinger et al. 2018; Springer et al. 2020), and nutritional and metabolic disorders (Sari, 2020; Santos et al. 2022; Sartori et al. 2022).

Metabolic bone disease (MBD) is one of the metabolic disorders commonly seen in captive reptiles, particularly in Chelonia (turtles, tortoises, and terrapins) and lizards, occasionally in snakes (Hedley, 2012). In veterinary medicine, MBD refers to a group of pathological conditions that affect the integrity and function of multiple bones (Doneley et al. 2017). They are most generally caused by genetic, dietary, and/or hormonal disorders that impact bone growth and remodeling, typically through changes in calcium/phosphorus metabolism. MBD has traditionally been broken down as fibrous osteodystrophy, osteoporosis, and rickets/osteomalacia; however, many cases are difficult to specifically classify, particularly those caused by nutritional deficiencies, because multiple conditions may coexist. Therefore, cases reported in the literature should be scrutinized carefully, and confirmation by histopathological evaluation should be regarded as more definitive (Uhl, 2018). The aim of this study was to report the occurrence of suspected MBD in red-footed tortoise (*C. carbonaria*) and describe the histopathological findings in several organs associated with the disease. This study also tried to describe the relationship between the suspected MBD and the histopathological changes that occur in several observed organs.

CASE PRESENTATION

History [A3][A4]

A 3-month-old dead red-footed tortoise (*Chelonoidis carbonarius*) weighing approximately 50 grams with a carapace length of 6 cm (Figure 1A-B) was sent to our laboratory. According to the owner's information, the tortoise had clinical symptoms of inappetence, anorexia, abnormal gait, and weakness. Previously, it had a history of shipping that was too long, about seven days, to finally die. Another tortoise, similar in age and weight, maintained together, had similar complaints.

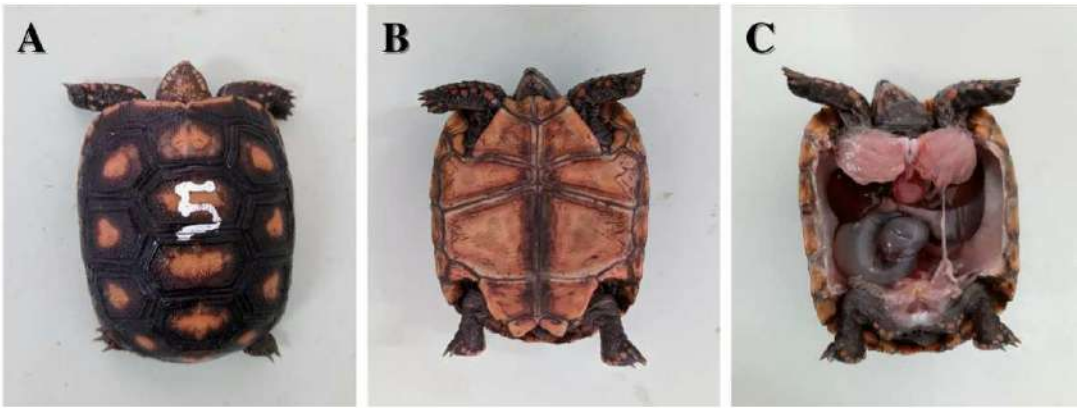

Figure 1. Gross examination of the tortoise body suspected of having MBD. A: the dorsal part. B: the ventral part. C: gross appearance of the internal organs.

Post-Mortem Examination

The necropsy that had been performed showed that the carapace and plastron were tender, but the size and consistency of the internal organs were normal, and there was no accumulation of fluid in the abdominal cavity (Figure 1C). The kidney, liver, heart, leg muscles, and leg bones were collected for tissue processing and hematoxylin-eosin staining.

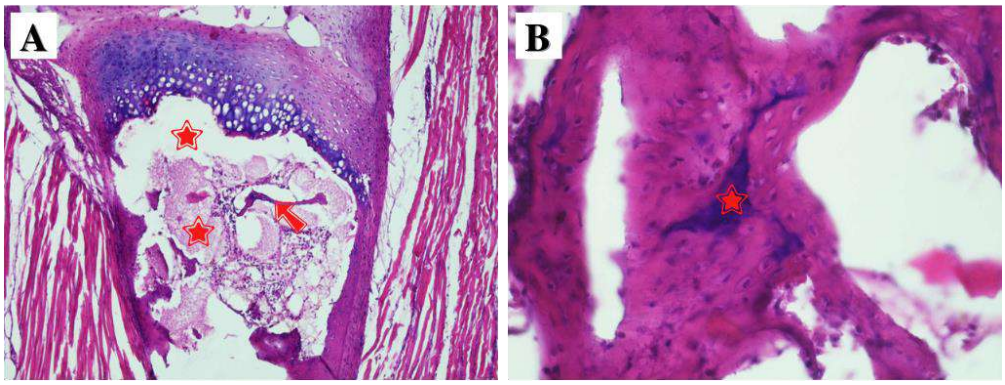

Figure 2. Histopathological appearance of the metatarsal bone of tortoise with suspected MBD (hematoxylin-eosin staining). A: metatarsal epiphyses with fewer trabeculae (arrow) and loss of hematopoietic cell (arrowhead) [A5][A6](100× maginification). B: hyaline cartilage (star) within the trabecular matrix (400× magnification).

Hitopathological Examination[A7][A8]

On the results of Hematoxylin and Eosin staining of tortoise metatarsal bones, there was a decrease in the number of trabecular bone in the epiphysis and hematopoietic cell loss (Figure 2A). Some sections also showed the presence of hyaline cartilage in the middle of the mature trabecular bone matrix (Figure 2B). In skeletal muscles, it was observed that many cells of the skeletal muscles were necrotic and some were atrophic (Figure 3A). Inflammatory cell infiltration also occurs between the striated muscle fibers accompanied by edema (Figure 4B). Peripheral nerve cells also underwent lysis and inflammation (Figure 3C).

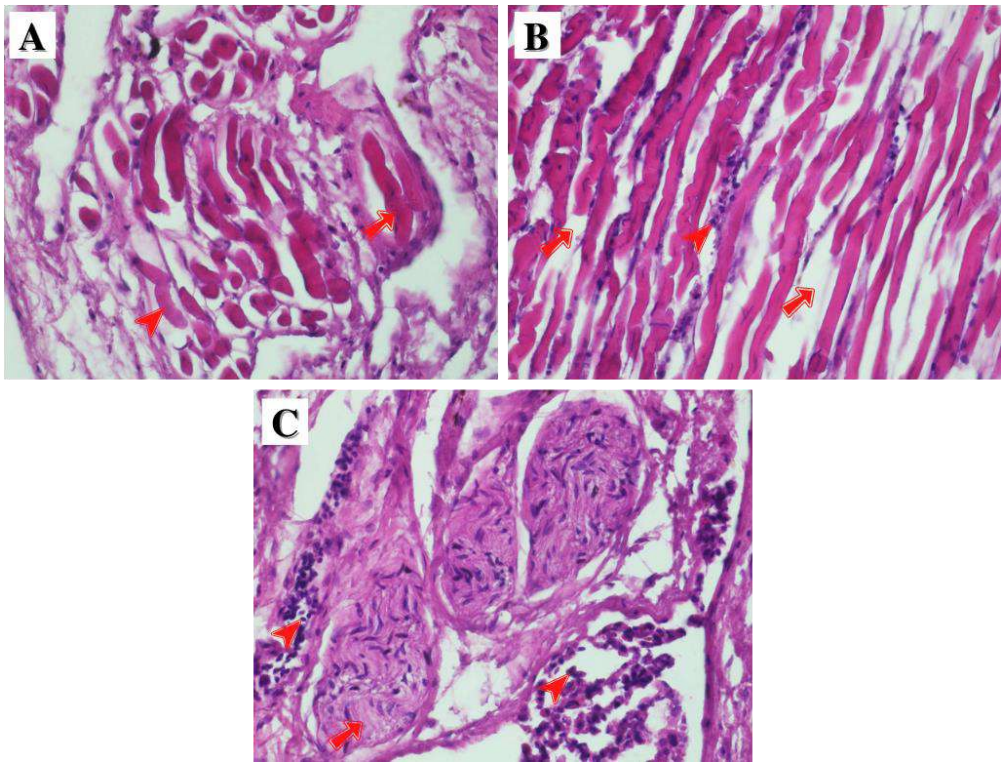

Figure 3. Histopathological appearance of tortoise skeletal muscles with suspected MBD (hematoxylin-eosin staining, 400× magnification). A: striated muscles with atrophic (arrowhead) and necrotic (arrow). B: edema (arrow) and infiltration of inflammatory cells (arrowhead) between muscle fibers. C: peripheral nerve cell lysis (arrow) and inflammatory cell (arrowhead) infiltration.

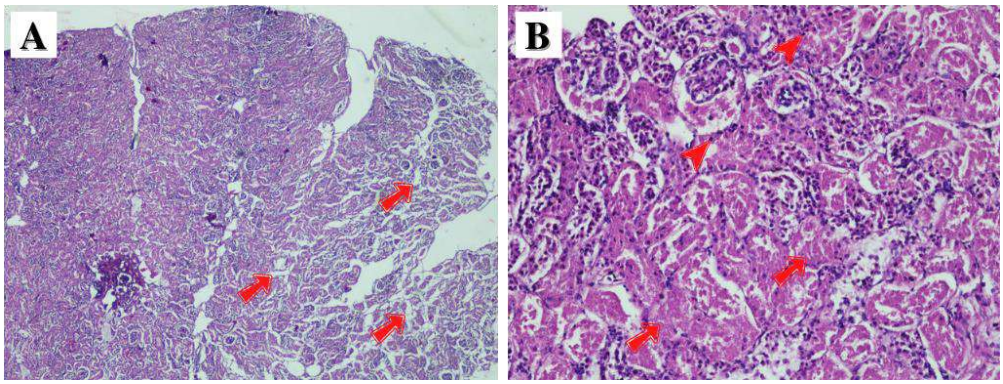

Figure 4. Histopathological appearance of the renal cortex of tortoise with suspected MBD (hematoxylin-eosin staining). A: edema (arrow) of the two right lobes of the kidney (40× maginification). B: pyknosis (arrowhead) and karyolysis (arrow) of convoluted tubule cells (200× magnification).

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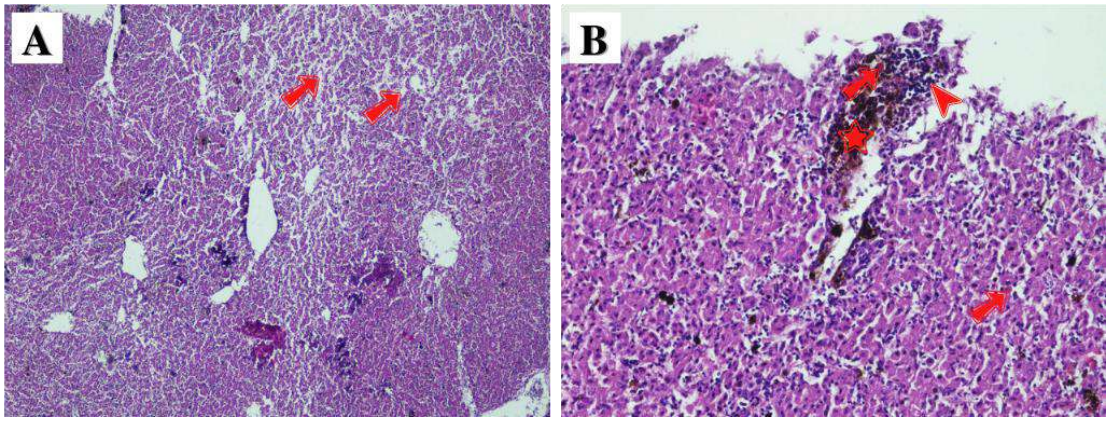

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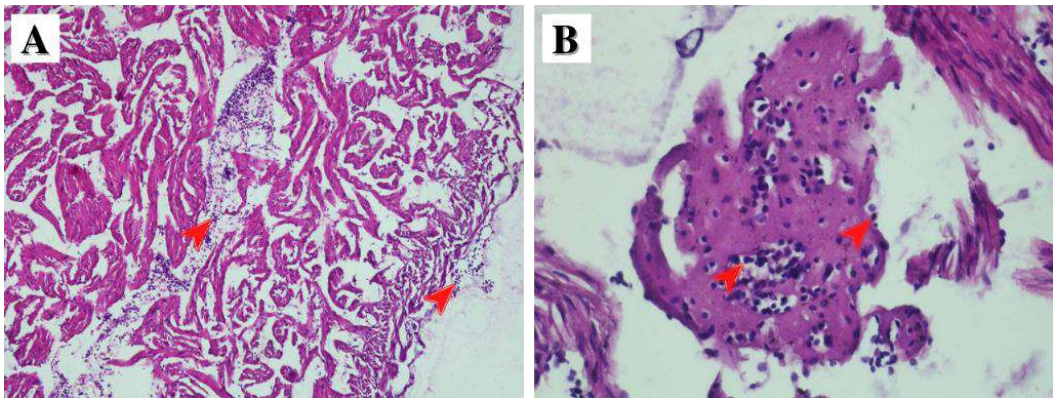

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DISCUSSION

Based on clinical symptoms and the reported history, it was assumed that the tortoise that had been necropsied had MBD. One of the strong supporting reasons is hypocalcemia-induced vitamin D deficiency, which is related, first, to lack of UV light (due to long shipping journeys) and proper temperature, and/or inadequate and balanced nutritional intake. Inadequate exposure to UVB rays causes the epidermal cells of animal skin to be unable to produce vitamin D3 (cholecalciferol) which is the result of the conversion of pre-vitamin D and its precursor, pro-vitamin D (7-dehydrocholesterol). Deficiency of vitamin D3 in the blood circulation causes the liver to lack its capacity to produce calcidiol or 25-(OH)-vitamin D3 as the main storage form of vitamin D3. This continues to cause kidneys to fail to hydroxylate calcidiol to produce 1,25-(OH)2-vitamin D3 or calcitriol, which plays a vital role in the regulation of calcium and phosphorus balance (Diehl et al. 2018).

The endocrine hormone calcitriol is known to increase intestinal absorption of dietary calcium and phosphate, stimulate the storage of calcium and phosphate in the kidneys, and, together with parathyroid hormone (PTH), has a direct effect on bone by regulating calcium mobilization from bone. Lack of this hormone can cause disturbances in bone growth and development, as well as in maintaining mature bone tissue (Kumar et al. 2018).

Hypocalcemia due to hypovitaminosis D is usually compensated by increased secretion of PTH from hyperplastic parathyroid glands and subsequently hyperparathyroidism, leading to resorption of calcium from bone (Hall et al. 2020). Unfortunately, this study did not observe the histopathological features of the parathyroids, so the hyperparathyroidism in this case could not be confirmed.

Another possibility that can occur is low calcitriol so that the body cannot limit the occurrence of osteoclastogenesis and trigger bone resorption by osteoclasts, resulting in osteopenia (Li et al. 2017; Zachary, 2022). This may explain the loss of the large amount of trabecular bone in the metatarsal tortoise epiphyses that we observed. The loss of trabecular continuity leads to a reduction in the ability of the trabecular to withstand stress (Zachary, 2022); therefore, the tortoise appears to have an abnormal gait.

In this study, it was also found that there is cartilage within the trabecular matrix. This may be related to disturbances in endochondral ossification during the development of the young tortoise. Ostechondrosis is a disorder of chondrocyte maturation that results in delayed cartilage mineralization. In addition to calcitriol, 24,25-(OH)2-vitamin D3 produced by calcidiol hydroxylation in the proximal renal tubule also plays an important role in cartilage cell differentiation and matrix mineralization. This imbalance in plasma concentrations between vitamin D metabolites appears to be related to the disposition of osteochondrosis during the growth period of the animal (Zafalon et al. 2020).

Under normal conditions, bone marrow in newborns and very young animals is mainly composed of active hematopoietic tissue and has relatively few fats (Zachary, 2022). This study found that the bone marrow within the epiphyseal metatarsal of the tortoise was hypocellular with a significantly reduced number of hemopoietic cells. It is still unclear how the pathophysiological relationship with the suspected MBD occurs. In another case, Turnbull et al. (2000) also reported bone marrow hypocellularity in hypothermic sea turtles. Bone marrow hypoplasia is commonly found in animals and humans with aplastic pancytopenia, a rare condition in which all hematopoietic lines in the bone marrow are aplastic or severely hypoplasiated, resulting in bone marrow failure. The cause is usually chemical agents that are cytotoxic to hematopoietic cells, or mutations or perturbations in hematopoietic cells and their environment caused by infectious agents (Zachary, 2022).

In this study, indications of infection and sepsis were also found, possibly due to microbial flora, in the tortoise suspected of having MBD. The association between decreased bone mineral density (BMD) and the risk of infection and sepsis has recently been reported. Previous studies have shown that BMD is a prognostic factor for infections and sepsis in human patients. Schulze-Hagen et al. (2021) found that low BMD was closely related to high mortality rates in intensive care units, while patients with pulmonary infections had the lowest BMD. A recent study even demonstrated that low BMD is not only a potential predictor for patients with infections and sepsis, but also a new risk factor for infections and sepsis.

Sepsis occurs after bacterial infections, leading to severe sepsis and septic shocks characterized by low blood pressure, ischemic, failure of multiple organs, and death (Drosatos et al. 2015). In this case, it was observed that there was inflammation of the liver, heart, muscles, and peripheral nerves, as well as renal tubular necrosis that can lead to acute renal failure. The decrease in the number and function of osteoblasts, associated with altered expression of IL-7 and lipocalin-2, may have a negative impact on human immunity and thus increase sensitivity to infections. Vitamin D may also explain the connection between BMD and infections and sepsis (Zhang et al. 2022). However, much remains to be done to confirm the factors and analyze the association between bone metabolism disorders and sepsis.

In conclusion, this study reports that the young tortoise necropsied had a number of pathological conditions that led to suspicion of MBD. Inflammation of multiple organs due to sepsis that we found also seems to have a pathophysiological

relationship with this disorder of bone metabolism. Further study is warranted to reach a convincing confirmation and investigate the relationship between MBD and the risk of infection and sepsis in animals.

ACKNOWLEDGEMENTS

The authors express their gratitude to Fajar Dany Prabayudha as a reptile expert and the co-assistant students of the Division of Veterinary Pathology, Faculty of Veterinary Medicine, Universitas Airlangga, who assisted in finding the sample cases for this study.

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Histopathological changes of multiple organs in the Red-Footed Tortoise (*Chelonoidis carbonaria*) with suspected metabolic bone disease

Abstract. Exotic pet lovers' interest in keeping tortoises is increasing all over the world, including Indonesia. However, this trend cannot be separated from the potential emergence of various health problems in tortoises. One of the problems that often affects tortoises is metabolic bone disease. Metabolic bone disease (MBD) is a disorder related to the mechanisms of vitamin D and calcium metabolism, which generally occurs in reptiles, especially Chelonia and Lizards. A 3-month-old red-footed tortoise, which was clinically suspected to have a MBD, was necropsied as an effort to support the provisional diagnosis through histopathological evaluation. The purpose of this examination was to analyze the impact of the disease on various organs microscopically in patients with suspected MBD. The results showed a decrease in the number of trabeculae and hematopoietic cells in the metatarsal bones; moderate myonecrotic changes and atrophy in the skeletal muscle; inflammation of the perineuron; acute tubular necrosis and mild edema of the renal cortex; congestion and an increase in the number of melanomacrophages in the liver; as well as epicarditis and myocarditis in the heart. Several forms of the histopathological changes seem to indicate a pathophysiological relationship between the suspected metabolic bone disease and the multiple organs examined.

Key words: Hematoxylin-eosin, MBD, tortoise, septicemia.

Abbreviations: BMD (bone mineral density), MBD (metabolic bone disease), PTH (parathyroid hormone), UV (ultraviolet).

Running title: Metabolic bone disease in tortoise

INTRODUCTION

In the last decade, the trend of domesticating tortoises as pets in urban families is increasing and popular around the world (Patel and Patel, 2020). In Indonesia, the sulcata tortoise is one of the most popular tortoise pets because it is easy to find this captive breed in the reptile pet market (Raharjo et al. 2022). Another tortoise that is frequently kept as a traditional pet in houses is the red-footed tortoise (Mendoza et al. 2021). Their ease of care, low cost of ownership, and amazing coloration make them highly sought after by novice tortoise keepers.

The red-footed tortoise (*Chelonoidis carbonaria*) are members of the Anapsida subclass, Chelonia order, Cryptodira suborder, Testudines family, and Chelonoidis genus (da Silva et al. 2020). This species is native to South America and can be found from Panama to Paraguay, as well as parts of Bolivia, Brazil, Colombia, Ecuador, and Peru (Mendoza et al. 2022). *C. carbonaria* is a diurnal and terrestrial animal with a compact body and strong cylindrical limbs, ideal to support its heavy carapace and walk in rough terrain (da Silva et al. 2020; Mendoza et al. 2022). They are opportunistic omnivores in general, and their diet is heavily influenced by the seasonal availability of food (Mendoza et al. 2022). Their main food sources are leaves, grasses, flowers, fruits, carcasses, and other food found on the ground (da Silva et al. 2020).

The increasing trend of keeping tortoises among exotic animal enthusiasts also has the potential to lead to many health problems (Patel and Patel, 2020). *C. carbonaria* is the most common testudines kept as a pet in South America, which accounts for a large proportion of wildlife patients seen in veterinary practices (da Silva et al. 2020). In Indonesia, according to Raharjo et al. (2022), a study on the prevalence of disease in exotic pet patients at a clinic in Yogyakarta, Indonesia, during January-August 2020 showed that turtles and tortoises had the highest cases of 71.7%, compared to snakes (16.5%), iguanas (6.2%), lizards (4.1%), crocodiles (1%), and geckos (0.5%).

Management of nutrition, health, housing, and an inappropriate environment are predisposing factors to serious health issues in tortoises if not anticipated and treated immediately. Some of the health issues that tortoises in captivity can face include respiratory ailments caused by bacterial or viral infection (Papp et al. 2010; Gibbons and Steffes, 2013; Silveira et al. 2014; Ballourad et al. 2021), gastro-intestinal disease caused by parasite or viral infection (Gibbons and Steffes, 2013;

Hallinger et al. 2018; Springer et al. 2020), and nutritional and metabolic disorders (Sari, 2020; Santos et al. 2022; Sartori et al. 2022).

Metabolic bone disease (MBD) is one of the metabolic disorders commonly seen in captive reptiles, particularly in Chelonia (turtles, tortoises, and terrapins) and lizards, occasionally in snakes (Hedley, 2012). In veterinary medicine, MBD refers to a group of pathological conditions that affect the integrity and function of multiple bones (Doneley et al. 2017). They are most generally caused by genetic, dietary, and/or hormonal disorders that impact bone growth and remodeling, typically through changes in calcium/phosphorus metabolism. MBD has traditionally been broken down as fibrous osteodystrophy, osteoporosis, and rickets/osteomalacia; however, many cases are difficult to specifically classify, particularly those caused by nutritional deficiencies, because multiple conditions may coexist. Therefore, cases reported in the literature should be scrutinized carefully, and confirmation by histopathological evaluation should be regarded as more definitive (Uhl, 2018). The aim of this study was to report the occurrence of suspected MBD in red-footed tortoise (*C. carbonaria*) and describe the histopathological findings in several organs associated with the disease. This study also tried to describe the relationship between the suspected MBD and the histopathological changes that occur in several observed organs.

CASE PRESENTATION

History

A 3-month-old dead red-footed tortoise (*Chelonoidis carbonarius*) weighing approximately 50 grams with a carapace length of 6 cm (Figure 1A-B) was sent to our laboratory. According to the owner's information, the tortoise had clinical symptoms of inappetence, anorexia, abnormal gait, and weakness. Previously, it had a history of shipping that was too long, about seven days, to finally die. Another tortoise, similar in age and weight, maintained together, had similar complaints.

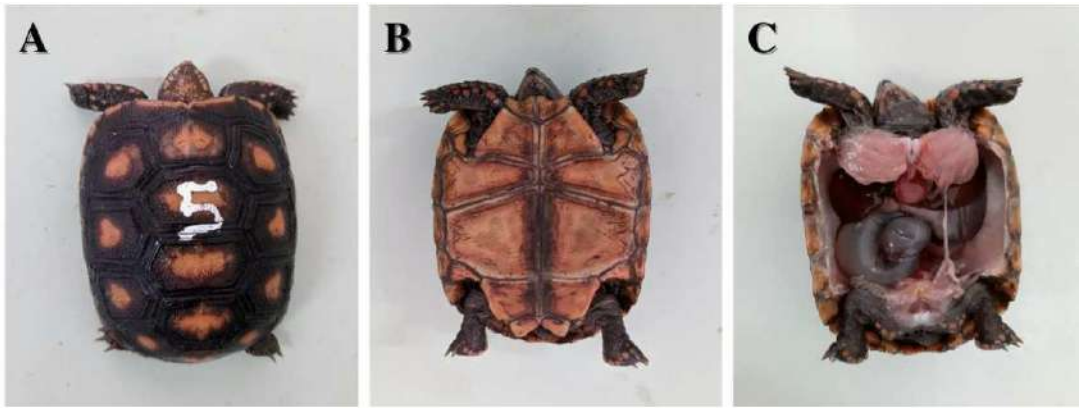

Figure 1. Gross examination of the tortoise body suspected of having MBD. A: the dorsal part. B: the ventral part. C: gross appearance of the internal organs.

Post-Mortem Examination

Prior to necropsy, external observations were made by examining the consistency of the carapace and plastron. Necropsy was performed by opening the abdominal area through the plastron section, then observations were made for the presence or absence of macroscopic changes in the internal organs and the accumulation of abnormal fluid in the abdomen of the tortoise.

The necropsy that had been performed showed that the carapace and plastron were tender, but the size and consistency of the internal organs were normal, and there was no accumulation of fluid in the abdominal cavity (Figure 1C).

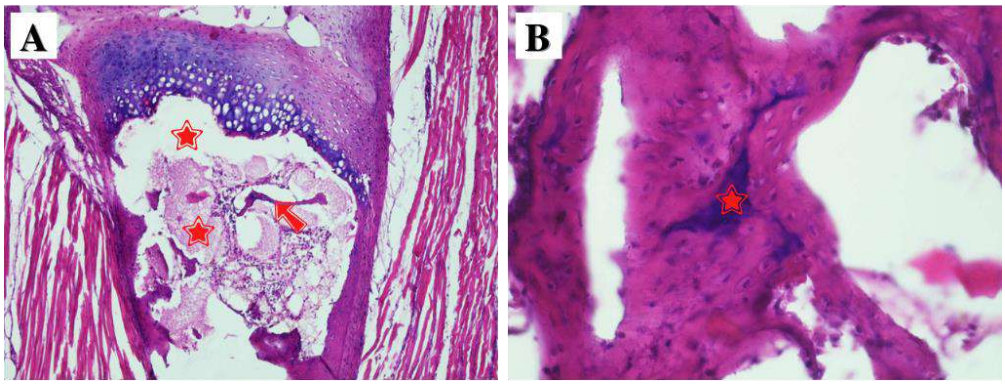

Figure 2. Histopathological appearance of the metatarsal bone of tortoise with suspected MBD (hematoxylin-eosin staining). A: metatarsal epiphyses with fewer trabeculae (arrow) and loss of hematopoietic cell (arrowhead) (100× magnification). B: hyaline cartilage (star) within the trabecular matrix (400× magnification).

Hitopathological Examination

The kidney, liver, heart, leg muscles, and leg bones were collected and fixed in 10% neutral buffered formalin. Organ samples were embedded in paraffin wax to form paraffin blocks, then 4 µm thick slices were cut and stained with Hematoxylin-Eosin. The histopathological slides were then observed microscopically with various magnifications of 40, 100, 200, and 400×.

On the results of the microscopical examination of tortoise metatarsal bones, there was a decrease in the number of trabecular bones in the epiphysis and hematopoietic cell loss (Figure 2A). Some sections also showed the presence of hyaline cartilage in the middle of the mature trabecular bone matrix (Figure 2B). In skeletal muscles, it was observed that many cells of the skeletal muscles were necrotic and some were atrophic (Figure 3A). Inflammatory cell infiltration also occurs between the striated muscle fibers accompanied by edema (Figure 4B). Peripheral nerve cells also underwent lysis and inflammation (Figure 3C).

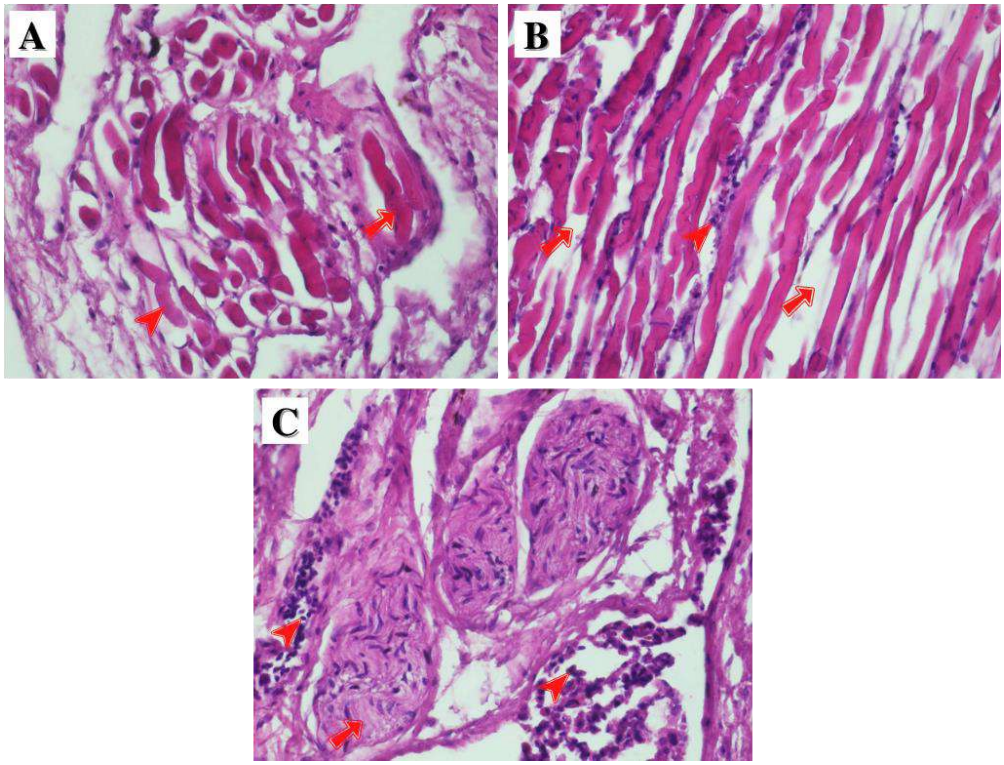

Figure 3. Histopathological appearance of tortoise skeletal muscles with suspected MBD (hematoxylin-eosin staining, 400× magnification). A: striated muscles with atrophic (arrowhead) and necrotic (arrow). B: edema (arrow) and infiltration of inflammatory cells (arrowhead) between muscle fibers. C: peripheral nerve cell lysis (arrow) and inflammatory cell (arrowhead) infiltration.

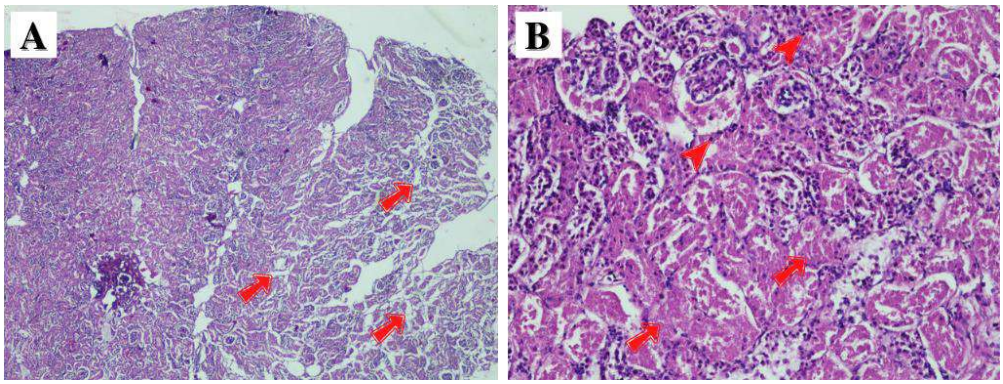

Figure 4. Histopathological appearance of the renal cortex of tortoise with suspected MBD (hematoxylin-eosin staining). A: edema (arrow) of the two right lobes of the kidney (40× maginification). B: pyknotic (arrowhead) and karyolitic (arrow) of convoluted tubule cells (200× magnification).

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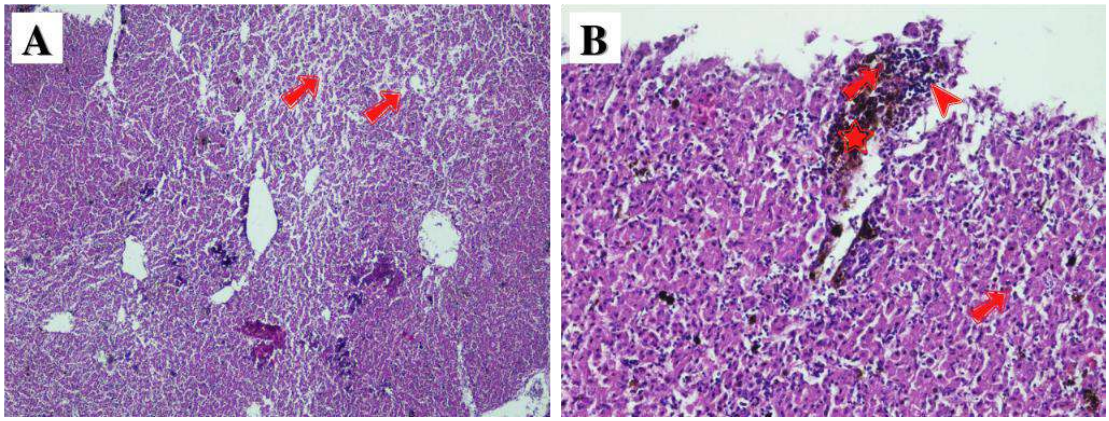

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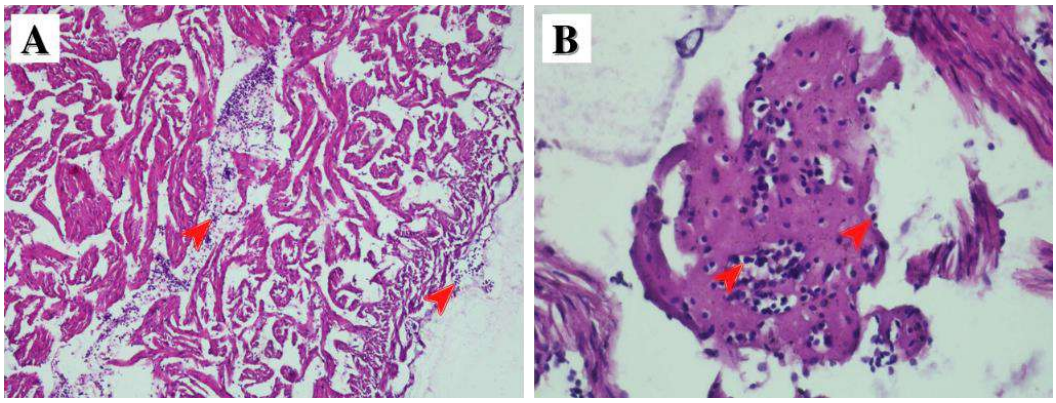

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DISCUSSION

Based on clinical symptoms and the reported history, it was assumed that the tortoise that had been necropsied had MBD. One of the strong supporting reasons is hypocalcemia-induced vitamin D deficiency, which is related, first, to lack of UV light (due to long shipping journeys) and proper temperature, and/or inadequate and balanced nutritional intake. Inadequate exposure to UVB rays causes the epidermal cells of animal skin to be unable to produce vitamin D3 (cholecalciferol) which is the result of the conversion of pre-vitamin D and its precursor, pro-vitamin D (7-dehydrocholesterol). Deficiency of vitamin D3 in the blood circulation causes the liver to lack its capacity to produce calcidiol or 25-(OH)-vitamin D3 as the main storage form of vitamin D3. This continues to cause kidneys to fail to hydroxylate calcidiol to produce 1,25-(OH)2-vitamin D3 or calcitriol, which plays a vital role in the regulation of calcium and phosphorus balance (Diehl et al. 2018).

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Another possibility that can occur is low calcitriol so that the body cannot limit the occurrence of osteoclastogenesis and trigger bone resorption by osteoclasts, resulting in osteopenia (Li et al. 2017; Zachary, 2022). This may explain the loss of the large amount of trabecular bone in the metatarsal tortoise epiphyses that we observed. The loss of trabecular continuity leads to a reduction in the ability of the trabecular to withstand stress (Zachary, 2022); therefore, the tortoise appears to have an abnormal gait.

In this study, it was also found that there is cartilage within the trabecular matrix. This may be related to disturbances in endochondral ossification during the development of the young tortoise. Ostechondrosis is a disorder of chondrocyte maturation that results in delayed cartilage mineralization. In addition to calcitriol, 24,25-(OH)2-vitamin D3 produced by calcidiol hydroxylation in the proximal renal tubule also plays an important role in cartilage cell differentiation and matrix mineralization. This imbalance in plasma concentrations between vitamin D metabolites appears to be related to the disposition of osteochondrosis during the growth period of the animal (Zafalon et al. 2020).

Under normal conditions, bone marrow in newborns and very young animals is mainly composed of active hematopoietic tissue and has relatively few fats (Zachary, 2022). This study found that the bone marrow within the epiphyseal metatarsal of the tortoise was hypocellular with a significantly reduced number of hemopoietic cells. It is still unclear how the pathophysiological relationship with the suspected MBD occurs. In another case, Turnbull et al. (2000) also reported bone marrow hypocellularity in hypothermic sea turtles. Bone marrow hypoplasia is commonly found in animals and humans with aplastic pancytopenia, a rare condition in which all hematopoietic lines in the bone marrow are aplastic or severely hypoplasiated, resulting in bone marrow failure. The cause is usually chemical agents that are cytotoxic to hematopoietic cells, or mutations or perturbations in hematopoietic cells and their environment caused by infectious agents (Zachary, 2022).

In this study, indications of infection and sepsis were also found, possibly due to microbial flora, in the tortoise suspected of having MBD. The association between decreased bone mineral density (BMD) and the risk of infection and sepsis has recently been reported. Previous studies have shown that BMD is a prognostic factor for infections and sepsis in human patients. Schulze-Hagen et al. (2021) found that low BMD was closely related to high mortality rates in intensive care units, while patients with pulmonary infections had the lowest BMD. A recent study even demonstrated that low BMD is not only a potential predictor for patients with infections and sepsis, but also a new risk factor for infections and sepsis.

Sepsis occurs after bacterial infections, leading to severe sepsis and septic shocks characterized by low blood pressure, ischemic, failure of multiple organs, and death (Drosatos et al. 2015). In this case, it was observed that there was inflammation of the liver, heart, muscles, and peripheral nerves, as well as renal tubular necrosis that can lead to acute renal failure. The decrease in the number and function of osteoblasts, associated with altered expression of IL-7 and lipocalin-2, may have a negative impact on human immunity and thus increase sensitivity to infections. Vitamin D may also explain the connection between BMD and infections and sepsis (Zhang et al. 2022). However, much remains to be done to confirm the factors and analyze the association between bone metabolism disorders and sepsis.

In conclusion, this study reports that the young tortoise necropsied had a number of pathological conditions that led to suspicion of MBD. Inflammation of multiple organs due to sepsis that we found also seems to have a pathophysiological

relationship with this disorder of bone metabolism. Further study is warranted to reach a convincing confirmation and investigate the relationship between MBD and the risk of infection and sepsis in animals.

ACKNOWLEDGEMENTS

The authors express their gratitude to Fajar Dany Prabayudha as a herpetologist and the co-assistant students of the Division of Veterinary Pathology, Faculty of Veterinary Medicine, Universitas Airlangga, who assisted in finding the sample cases for this study.

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Histopathological changes of multiple organs in the Red-Footed Tortoise (*Chelonoidis carbonaria*) with suspected metabolic bone disease

Abstract. Exotic pet lovers' interest in keeping tortoises is increasing all over the world, including Indonesia. However, this trend cannot be separated from the potential emergence of various health problems in tortoises. One of the problems that often affects tortoises is metabolic bone disease. Metabolic bone disease (MBD) is a disorder related to the mechanisms of vitamin D and calcium metabolism, which generally occurs in reptiles, especially Chelonia and Lizards. A 3-month-old red-footed tortoise, which was clinically suspected to have a MBD, was necropsied as an effort to support the provisional diagnosis through histopathological evaluation. The purpose of this examination was to analyze the impact of the disease on various organs microscopically in patients with suspected MBD. The results showed a decrease in the number of trabeculae and hematopoietic cells in the metatarsal bones; moderate myonecrotic changes and atrophy in the skeletal muscle; inflammation of the perineuron; acute tubular necrosis and mild edema of the renal cortex; congestion and an increase in the number of melanomacrophages in the liver; as well as epicarditis and myocarditis in the heart. Several forms of the histopathological changes seem to indicate a pathophysiological relationship between the suspected metabolic bone disease and the multiple organs examined.

Key words: Hematoxylin-eosin, MBD, tortoise, septicemia.

Abbreviations: BMD (bone mineral density), MBD (metabolic bone disease), PTH (parathyroid hormone), UV (ultraviolet).

Running title: Metabolic bone disease in tortoise

INTRODUCTION

In the last decade, the trend of domesticating tortoises as pets in urban families is increasing and popular around the world (Patel and Patel, 2020). In Indonesia, the sulcata tortoise is one of the most popular tortoise pets because it is easy to find this captive breed in the reptile pet market (Raharjo et al. 2022). Another tortoise that is frequently kept as a traditional pet in houses is the red-footed tortoise (Mendoza et al. 2021). Their ease of care, low cost of ownership, and amazing coloration make them highly sought after by novice tortoise keepers.

The red-footed tortoise (*Chelonoidis carbonaria*) are members of the Anapsida subclass, Chelonia order, Cryptodira suborder, Testudines family, and Chelonoidis genus (da Silva et al. 2020). This species is native to South America and can be found from Panama to Paraguay, as well as parts of Bolivia, Brazil, Colombia, Ecuador, and Peru (Mendoza et al. 2022). *C. carbonaria* is a diurnal and terrestrial animal with a compact body and strong cylindrical limbs, ideal to support its heavy carapace and walk in rough terrain (da Silva et al. 2020; Mendoza et al. 2022). They are opportunistic omnivores in general, and their diet is heavily influenced by the seasonal availability of food (Mendoza et al. 2022). Their main food sources are leaves, grasses, flowers, fruits, carcasses, and other food found on the ground (da Silva et al. 2020).

The increasing trend of keeping tortoises among exotic animal enthusiasts also has the potential to lead to many health problems (Patel and Patel, 2020). *C. carbonaria* is the most common testudines kept as a pet in South America, which accounts for a large proportion of wildlife patients seen in veterinary practices (da Silva et al. 2020). In Indonesia, according to Raharjo et al. (2022), a study on the prevalence of disease in exotic pet patients at a clinic in Yogyakarta, Indonesia, during January-August 2020 showed that turtles and tortoises had the highest cases of 71.7%, compared to snakes (16.5%), iguanas (6.2%), lizards (4.1%), crocodiles (1%), and geckos (0.5%).

Management of nutrition, health, housing, and an inappropriate environment are predisposing factors to serious health issues in tortoises if not anticipated and treated immediately. Some of the health issues that tortoises in captivity can face include respiratory ailments caused by bacterial or viral infection (Papp et al. 2010; Gibbons and Steffes, 2013; Silveira et al. 2014; Ballourad et al. 2021), gastro-intestinal disease caused by parasite or viral infection (Gibbons and Steffes, 2013;

Hallinger et al. 2018; Springer et al. 2020), and nutritional and metabolic disorders (Sari, 2020; Santos et al. 2022; Sartori et al. 2022).

Metabolic bone disease (MBD) is one of the metabolic disorders commonly seen in captive reptiles, particularly in Chelonia (turtles, tortoises, and terrapins) and lizards, occasionally in snakes (Hedley, 2012). In veterinary medicine, MBD refers to a group of pathological conditions that affect the integrity and function of multiple bones (Doneley et al. 2017). They are most generally caused by genetic, dietary, and/or hormonal disorders that impact bone growth and remodeling, typically through changes in calcium/phosphorus metabolism. MBD has traditionally been broken down as fibrous osteodystrophy, osteoporosis, and rickets/osteomalacia; however, many cases are difficult to specifically classify, particularly those caused by nutritional deficiencies, because multiple conditions may coexist. Therefore, cases reported in the literature should be scrutinized carefully, and confirmation by histopathological evaluation should be regarded as more definitive (Uhl, 2018). The aim of this study was to report the occurrence of suspected MBD in red-footed tortoise (*C. carbonaria*) and describe the histopathological findings in several organs associated with the disease. This study also tried to describe the relationship between the suspected MBD and the histopathological changes that occur in several observed organs.

CASE PRESENTATION

History

A 3-month-old dead red-footed tortoise (*Chelonoidis carbonarius*) weighing approximately 50 grams with a carapace length of 6 cm (Figure 1A-B) was sent to our laboratory. According to the information from the sender, the tortoise had clinical symptoms of inappetence, anorexia, an abnormal gait, and weakness. Previously, it had a history of shipping that was too long, about seven days, to finally die. Another tortoise, similar in age and weight, maintained together, had similar complaints.

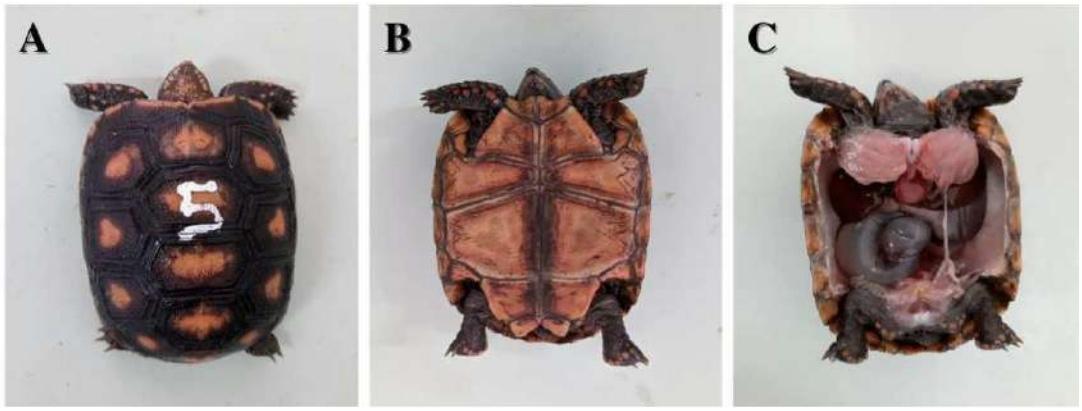

Figure 1. Gross examination of the tortoise body suspected of having MBD. A: the dorsal part. B: the ventral part. C: gross appearance of the internal organs.

Post-Mortem Examination

A post-mortem investigation was performed based on Flint et al. (2009). Prior to necropsy, an external examination was made by observing the dorsal (carapace) and ventral (plastron) surfaces, the dorso-ventral and rostro-caudal of the head, the forelegs and hind legs, and any external abnormalities (wounds or trauma, deformities, and missing body parts). The necropsy was attempted while the tortoise was in dorsal recumbency (plastron up). The plastron was separated from the carapace along the marginal bridge on both sides and from the skin at attachment areas. After that, the plastrons were removed from the carcass. Then an internal examination was made for the presence or absence of macroscopic pathological changes in the internal organs and the accumulation of abnormal fluid in the coelomic cavity of the tortoise. Furthermore, some organs, such as the kidney, liver, heart, hind leg musculature, and metatarsals, were collected for tissue processing.

The necropsy that had been performed showed that the carapace and plastron were tender. No abnormal conditions such as swelling, trauma, or injuries were found on the results of other external examinations. On internal examination, no significant macroscopic changes were found in the organs, such as inflammation, bleeding, enlargement or reduction in organ size, changes in color and consistency, and accumulation of fluid in the celomic cavity. Only one colon enlargement was found. Unfortunately, the tissue could not be collected for histopathological examination (Figure 1C).

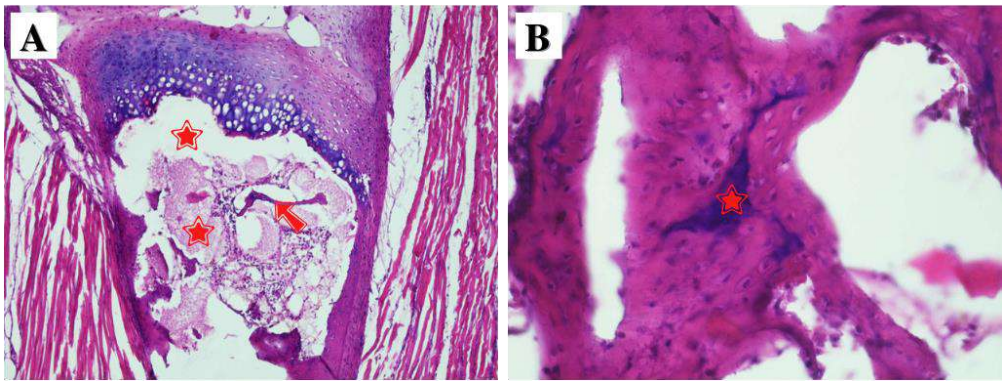

Figure 2. Histopathological appearance of the metatarsal bone of tortoise with suspected MBD (hematoxylin-eosin staining). A: metatarsal epiphyses with fewer trabeculae (arrow) and loss of hematopoietic cell (arrowhead) (100× magnification). B: hyaline cartilage (star) within the trabecular matrix (400× magnification).

Hitopathological Examination

The kidney, liver, heart, leg muscles, and leg bones were collected and fixed in 10% neutral buffered formalin. Organ samples were dehydrated in serial grades of alcohol: 60% alcohol (1 hour), 80% alcohol (2 hours), 90% alcohol (2 hours), 96% alcohol (2 hours), absolute alcohol (2 hours, three times), and xylol (2 hours, three times). Embedding was performed by putting the tissues into melted paraffin at 56–60 °C (2 hours, three times). The paraffin-filled tissues were then placed on a mold that had been filled with melted paraffin and left there until rigid. The tissue was thinly sliced at 3 micrometers. The sliced tissues were prepared on object glasses and stained with hematoxylin and eosin. The histopathological slides were then observed using microscope with various magnifications of 40, 100, 200, and 400×.

On the results of the microscopical examination of tortoise metatarsal bones, there was a decrease in the number of trabecular bones in the epiphysis and hematopoietic cell loss (Figure 2A). Some sections also showed the presence of hyaline cartilage in the middle of the mature trabecular bone matrix (Figure 2B). In skeletal muscles, it was observed that many cells of the skeletal muscles were necrotic and some were atrophic (Figure 3A). Inflammatory cell infiltration also occurs between the striated muscle fibers accompanied by edema (Figure 4B). Peripheral nerve cells also underwent lysis and inflammation (Figure 3C).

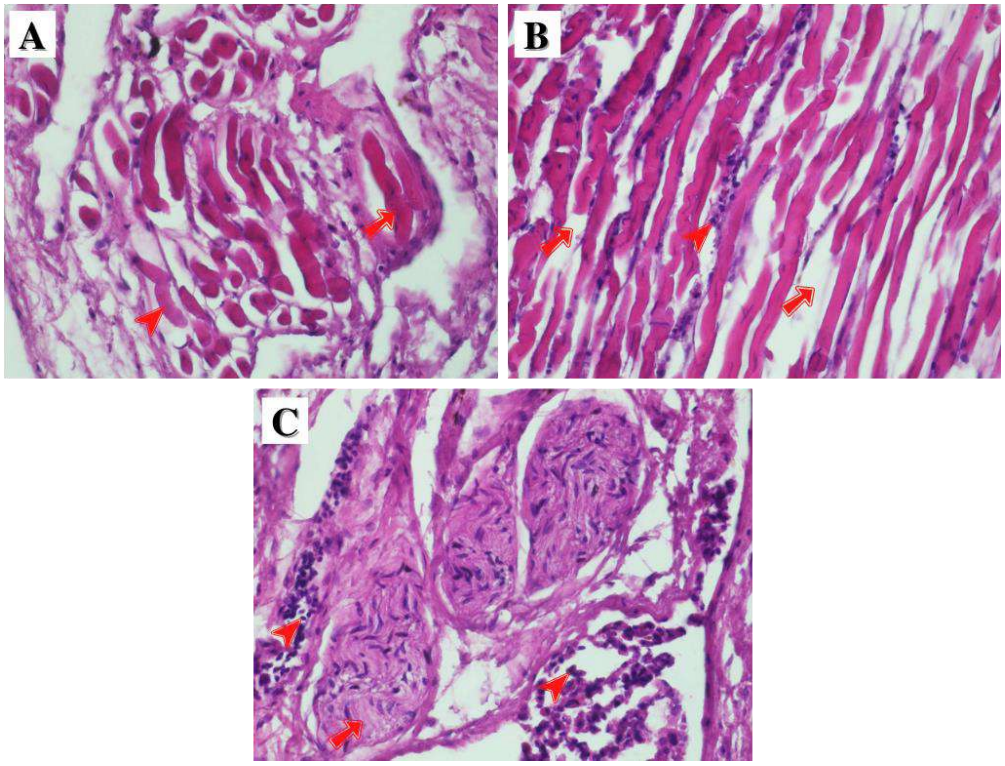

Figure 3. Histopathological appearance of tortoise skeletal muscles with suspected MBD (hematoxylin-eosin staining, 400× magnification). A: striated muscles with atrophic (arrowhead) and necrotic (arrow). B: edema (arrow) and infiltration of inflammatory cells (arrowhead) between muscle fibers. C: peripheral nerve cell lysis (arrow) and inflammatory cell (arrowhead) infiltration.

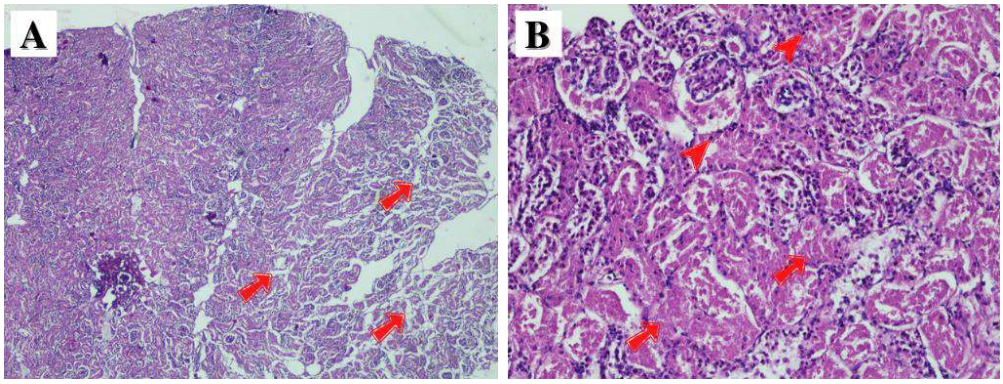

Figure 4. Histopathological appearance of the renal cortex of tortoise with suspected MBD (hematoxylin-eosin staining). A: edema (arrow) of the two right lobes of the kidney (40× maginification). B: pyknotic (arrowhead) and karyolitic (arrow) of convoluted tubule cells (200× magnification).

On microscopic examination of the kidney, edema appeared in two of the four lobes of the kidney (Figure 4A). The appearance of massive acute tubular necrosis was also clearly seen, characterized by convoluted proximal tubules that mostly underwent cell lysis and nuclear pyknosis (Figure 4B). Mild to moderate congestion was observed in the liver sinusoids (Figure 5A). An increase in the number of melanomacrophages has also occurred, and some have formed melanomacrophage centers (MMC). Additionally, there are few eosinophilic granular cells (EGCs) and some infiltrating lymphocytes that aggregate to form lymphoid follicles (Figure 5B). Furthermore, microscopic observation also showed that the tortoise heart experienced epicarditis, myocarditis, and endocarditis, indicated by fairly massive lymphocytic infiltration in the epicardium, myocardium, and lumen of the endocardium, respectively (Figure 6A-B).

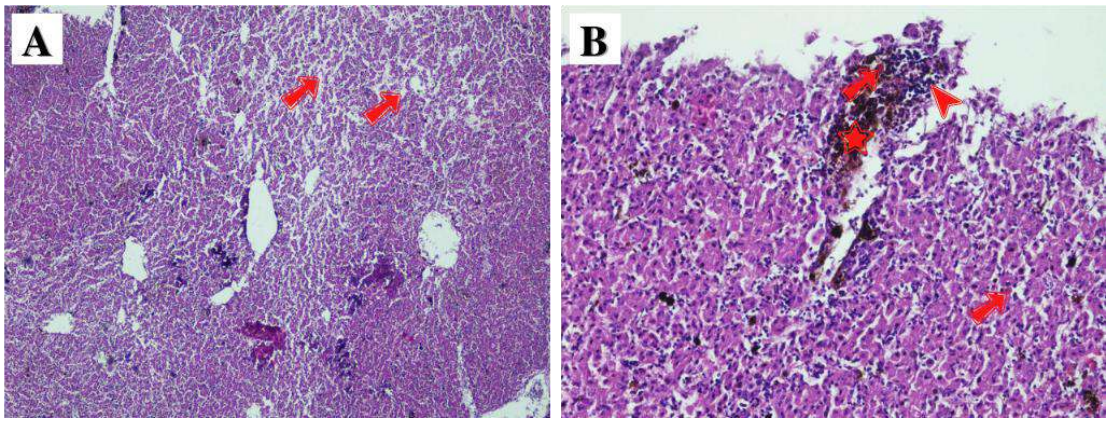

Figure 5. Histopathological appearance of the liver parenchyma of tortoise with suspected MBD (hematoxylin-eosin staining). A: some areas of the liver are congested (arrow) (40× maginification). B: infiltration of lymphocytes (arrowhead), eosinophilic granular cells (arrow), and melanomacrophage centers (star) (200× magnification).

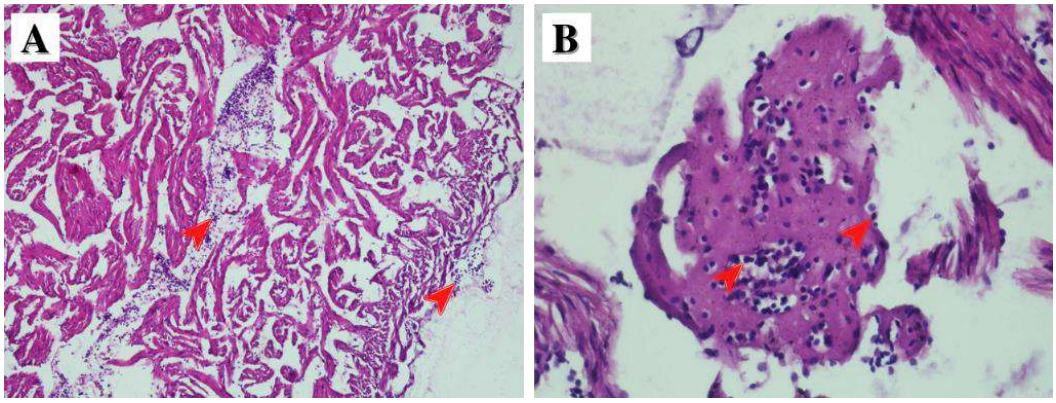

Figure 6. Histopathological appearance of the tortoise heart with suspected MBD (hematoxylin-eosin staining). A: infiltration of lymphocytic cells (arrowhead) in the epicardium and myocardium (100× maginification). B: infiltration of lymphocytic cells (arrowhead) in the lumen of the endocardium (400× magnification).

DISCUSSION

Based on clinical symptoms and the reported history, it was assumed that the tortoise that had been necropsied had MBD. One of the strong supporting reasons is hypocalcemia-induced vitamin D deficiency, which is related, first, to lack of UV light (due to long shipping journeys) and proper temperature, and/or inadequate and balanced nutritional intake. Inadequate exposure to UVB rays causes the epidermal cells of animal skin to be unable to produce vitamin D3 (cholecalciferol) which is the result of the conversion of pre-vitamin D and its precursor, pro-vitamin D (7-dehydrocholesterol). Deficiency of vitamin D3 in the blood circulation causes the liver to lack its capacity to produce calcidiol or 25-(OH)-vitamin D3 as the main storage form of vitamin D3. This continues to cause kidneys to fail to hydroxylate calcidiol to produce 1,25-(OH)2-vitamin D3 or calcitriol, which plays a vital role in the regulation of calcium and phosphorus balance (Diehl et al. 2018).

The endocrine hormone calcitriol is known to increase intestinal absorption of dietary calcium and phosphate, stimulate the storage of calcium and phosphate in the kidneys, and, together with parathyroid hormone (PTH), has a direct effect on bone by regulating calcium mobilization from bone. Lack of this hormone can cause disturbances in bone growth and development, as well as in maintaining mature bone tissue (Kumar et al. 2018).

Hypocalcemia due to hypovitaminosis D is usually compensated by increased secretion of PTH from hyperplastic parathyroid glands and subsequently hyperparathyroidism, leading to resorption of calcium from bone (Hall et al. 2020). Unfortunately, this study did not observe the histopathological features of the parathyroids, so the hyperparathyroidism in this case could not be confirmed.

Another possibility that can occur is low calcitriol so that the body cannot limit the occurrence of osteoclastogenesis and trigger bone resorption by osteoclasts, resulting in osteopenia (Li et al. 2017; Zachary, 2022). This may explain the loss of the large amount of trabecular bone in the metatarsal tortoise epiphyses that we observed. The loss of trabecular continuity leads to a reduction in the ability of the trabecular to withstand stress (Zachary, 2022); therefore, the tortoise appears to have an abnormal gait.

In this study, it was also found that there is cartilage within the trabecular matrix. This may be related to disturbances in endochondral ossification during the development of the young tortoise. Ostechondrosis is a disorder of chondrocyte maturation that results in delayed cartilage mineralization. In addition to calcitriol, 24,25-(OH)2-vitamin D3 produced by calcidiol hydroxylation in the proximal renal tubule also plays an important role in cartilage cell differentiation and matrix mineralization. This imbalance in plasma concentrations between vitamin D metabolites appears to be related to the disposition of osteochondrosis during the growth period of the animal (Zafalon et al. 2020).

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Sepsis occurs after bacterial infections, leading to severe sepsis and septic shocks characterized by low blood pressure, ischemic, failure of multiple organs, and death (Drosatos et al. 2015). In this case, it was observed that there was inflammation of the liver, heart, muscles, and peripheral nerves, as well as renal tubular necrosis that can lead to acute renal failure. The decrease in the number and function of osteoblasts, associated with altered expression of IL-7 and lipocalin-2, may have a negative impact on human immunity and thus increase sensitivity to infections. Vitamin D may also explain the connection between BMD and infections and sepsis (Zhang et al. 2022). However, much remains to be done to confirm the factors and analyze the association between bone metabolism disorders and sepsis.

In conclusion, this study reports that the young tortoise necropsied had a number of pathological conditions that led to suspicion of MBD. Inflammation of multiple organs due to sepsis that we found also seems to have a pathophysiological relationship with this disorder of bone metabolism. Further study is warranted to reach a convincing confirmation and investigate the relationship between MBD and the risk of infection and sepsis in animals.

ACKNOWLEDGEMENTS

The authors express their gratitude to Fajar Dany Prabayudha as a herpetologist and the co-assistant students of the Division of Veterinary Pathology, Faculty of Veterinary Medicine, Universitas Airlangga, who assisted in finding the sample cases for this study.

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Histopathological Perspectives of Multiple Organs in a Red-Footed Tortoise (*Chelonoidis carbonaria*) with Suspected Metabolic Bone Disease: A Case Report

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ABSTRACT

Introduction: Exotic pet lovers' interest in keeping tortoises is increasing all over the world, including Indonesia. However, this trend cannot be separated from the potential emergence of various health problems in tortoises. One of the problems that often affects tortoises is metabolic bone disease. Metabolic bone disease (MBD) is a disorder related to the mechanisms of vitamin D and calcium metabolism, which generally occurs in reptiles, especially Chelonia and Lizards. **Case Presentation:** A 3-month-old red-footed tortoise, which was clinically suspected to have a MBD, was necropsied as an effort to support the provisional diagnosis through histopathological evaluation. The purpose of this examination was to analyze the impact of the disease on various organs microscopically in patients with suspected MBD. The results showed a decrease in the number of trabeculae and hematopoietic cells in the metatarsal bones; moderate myonecrotic changes and atrophy in the skeletal muscle; inflammation of the perineuron; acute tubular necrosis and mild edema of the renal cortex; congestion and an increase in the number of melanomacrophages in the liver; as well as epicarditis and myocarditis in the heart. **Conclusion:** Several forms of the histopathological changes seem to indicate a pathophysiological relationship between the suspected metabolic bone disease and the multiple organs examined.

Keywords: Hematoxylin-eosin, MBD, Tortoise, Septicemia.

INTRODUCTION

In the last decade, the trend of domesticating tortoises as pets in urban families is increasing and popular around the world[1]. In Indonesia, the sulcata tortoise is one of the most popular tortoise pets because it is easy to find this captive breed in the reptile pet market[2]. Another tortoise that is frequently kept as a traditional pet in houses is the red-footed tortoise[3]. Their ease of care, low cost of ownership, and amazing coloration make them highly sought after by novice tortoise keepers.

The red-footed tortoise (*Chelonoidis carbonaria*) are members of the Anapsida subclass, Chelonia order, Cryptodira suborder, Testudines family, and Chelonoidis genus[4]. This species is native to South America and can be found from Panama to Paraguay, as well as parts of Bolivia, Brazil, Colombia, Ecuador, and Peru[5]. *C. carbonaria* is a diurnal and terrestrial animal with a compact body and strong cylindrical limbs, ideal to support its heavy carapace and walk in rough terrain[4,5]. They are opportunistic omnivores in general, and their diet is heavily influenced by the seasonal availability of food[6]. Their main food sources are leaves, grasses, flowers, fruits, carcasses, and other food found on the ground[4].

The increasing trend of keeping tortoises among exotic animal enthusiasts also has the potential to lead to many health problems[1]. *C. carbonaria* is the most common testudines kept as a pet in South America, which accounts for a large proportion of wildlife patients seen in veterinary practices[4]. In Indonesia, according to Raharjo *et al*.[2], a study on the prevalence of disease in exotic pet patients at a clinic in Yogyakarta, Indonesia, during January-August 2020 showed that turtles and tortoises had the highest cases of 71.7%, compared to snakes (16.5%), iguanas (6.2%), lizards (4.1%), crocodiles (1%), and geckos (0.5%).

Management of nutrition, health, housing, and an inappropriate environment are predisposing factors to serious health issues in tortoises if not anticipated and treated immediately. Some of the health issues that tortoises in captivity can face include respiratory ailments caused by bacterial or viral infection[7-10], gastro-intestinal disease caused by parasite or viral infection[8,11,12], and nutritional and metabolic disorders[13-15].

Metabolic bone disease (MBD) is one of the metabolic disorders commonly seen in captive reptiles, particularly in Chelonia (turtles, tortoises, and terrapins) and lizards, occasionally in snakes[16]. In veterinary medicine, MBD refers to a group of pathological conditions that affect the integrity and function of multiple bones[17]. They are most generally caused by genetic, dietary, and/or hormonal disorders that impact bone growth and remodeling, typically through changes in calcium/phosphorus metabolism. MBD has traditionally been broken down as fibrous osteodystrophy, osteoporosis, and rickets/osteomalacia; however, many cases are difficult to specifically classify, particularly those caused by nutritional deficiencies, because multiple conditions may coexist. Therefore, cases reported in the literature should be scrutinized carefully, and confirmation by histopathological evaluation should be regarded as more definitive[18]. The aim

of this study was to report the occurrence of suspected MBD in red-footed tortoise (*C. carbonaria*) and describe the histopathological findings in several organs associated with the disease. This study also tried to describe the relationship between the suspected MBD and the histopathological changes that occur in several observed organs.

CASE PRESENTATION

History

A 3-month-old dead red-footed tortoise (*Chelonoidis carbonarius*) weighing approximately 50 grams with a carapace length of 6 cm (Figure 1A-B) was sent to our laboratory. According to the information from the sender, the tortoise had clinical symptoms of inappetence, anorexia, an abnormal gait, and weakness. Previously, it had a history of shipping that was too long, about seven days, to finally die. Another tortoise, similar in age and weight, maintained together, had similar complaints.

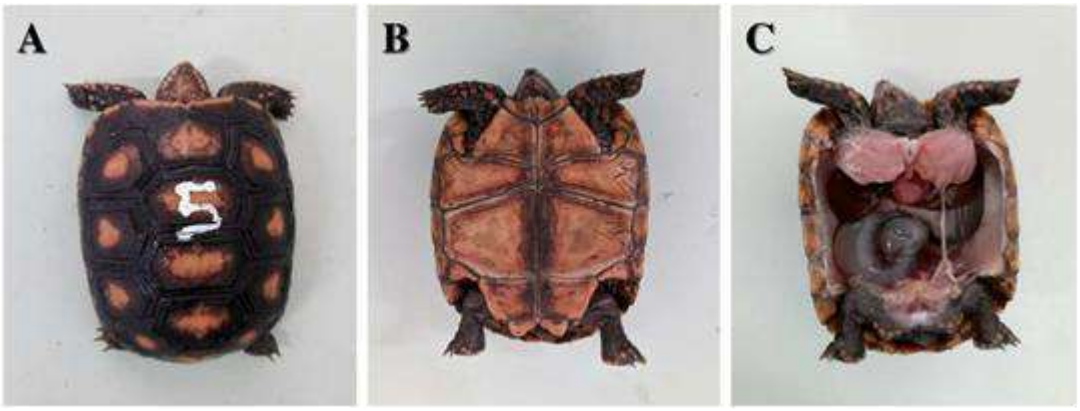

Figure 1 : Gross examination of the tortoise body suspected of having MBD. A: The dorsal view. B: The ventral view. C: Gross appearance of the internal organs.

Post-mortem findings

The post-mortem investigation that had been performed showed that the carapace and plastron were tender. No abnormal conditions such as swelling, trauma, or injuries were found on the results of other external examinations. On internal examination after necropsy, no significant macroscopic changes were found in the organs, such as inflammation, bleeding, enlargement or reduction in organ size, changes in color and consistency, and accumulation of fluid in the celomic cavity. Only

one colon enlargement was found. Some organs, such as the kidney, liver, heart, hind leg musculature, and metatarsals, were collected for tissue processing. Unfortunately, the intestine tissue could not be collected for histopathological examination (Figure 1C).

Histopathological findings

On the results of the microscopical examination of tortoise metatarsal bones that had been stained with hematoxylin and eosin, there was a decrease in the number of trabecular bones in the epiphysis and hematopoietic cell loss (Figure 2A). Some sections also showed the presence of hyaline cartilage in the middle of the mature trabecular bone matrix (Figure 2B). In skeletal muscles, it was observed that many cells of the skeletal muscles were necrotic and some were atrophic (Figure 3A). Inflammatory cell infiltration also occurs between the striated muscle fibers accompanied by edema (Figure 4B). Peripheral nerve cells also underwent lysis and inflammation (Figure 3C).

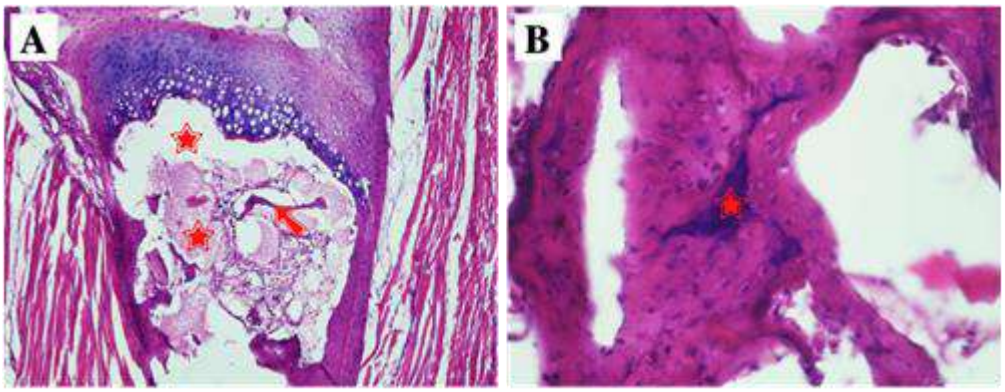

Figure 2 : Histopathological appearance of the metatarsal bone of tortoise with suspected MBD (hematoxylin-eosin staining). A: metatarsal epiphyses with fewer trabeculae (arrow) and loss of hematopoietic cell (arrowhead) (100× magnification). B: hyaline cartilage (star) within the trabecular matrix (400× magnification).

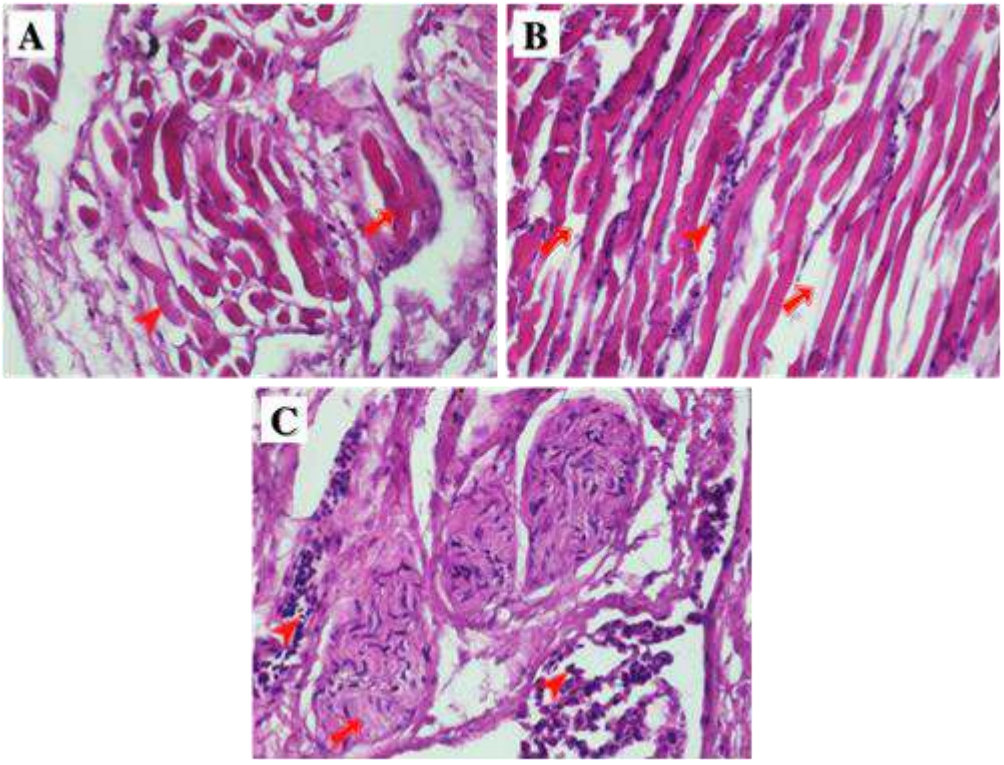

Figure 3 : Histopathological appearance of tortoise skeletal muscles with suspected MBD (hematoxylin-eosin staining, 400× magnification). A: striated muscles with atrophic (arrowhead) and necrotic (arrow). B: edema (arrow) and infiltration of inflammatory cells (arrowhead) between muscle fibers. C: peripheral nerve cell lysis (arrow) and inflammatory cell (arrowhead) infiltration.

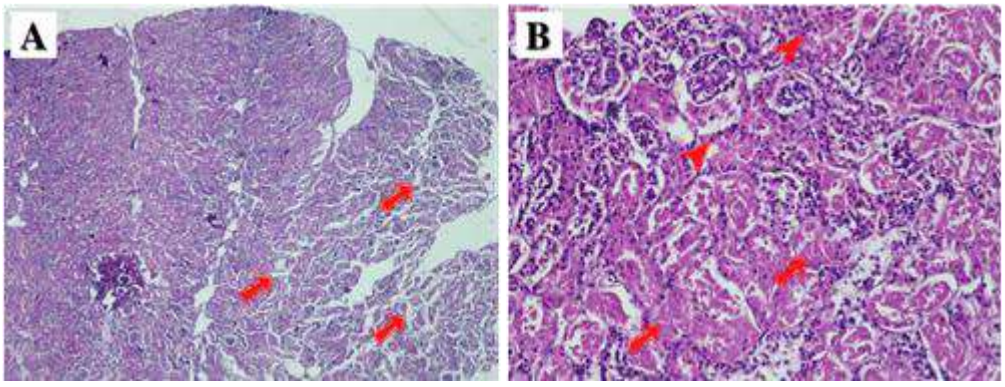

Figure 4 : Histopathological appearance of the renal cortex of tortoise with suspected MBD (hematoxylin-eosin staining). A: edema (arrow) of the two right lobes of the kidney (40× maginification). B: pyknotic (arrowhead) and karyolitic (arrow) of convoluted tubule cells (200× magnification).

On microscopic examination of the kidney, edema appeared in two of the four lobes of the kidney (Figure 4A). The appearance of massive acute tubular necrosis was also clearly seen, characterized by convoluted proximal tubules that mostly underwent cell lysis and nuclear pyknosis (Figure 4B). Mild to moderate congestion was observed in the liver sinusoids (Figure 5A). An increase in the number of melanomacrophages has also occurred, and some have formed melanomacrophage centers (MMC). Additionally, there are few eosinophilic granular cells (EGCs) and some infiltrating lymphocytes that aggregate to form lymphoid follicles (Figure 5B). Furthermore, microscopic observation also showed that the tortoise heart experienced epicarditis, myocarditis, and endocarditis, indicated by fairly massive lymphocytic infiltration in the epicardium, myocardium, and lumen of the endocardium, respectively (Figure 6A-B).

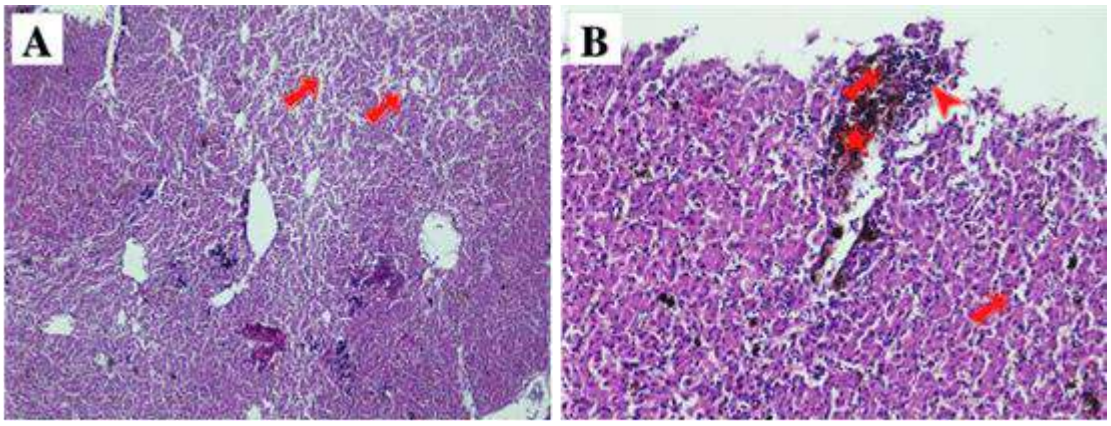

Figure 5 : Histopathological appearance of the liver parenchyma of tortoise with suspected MBD (hematoxylin-eosin staining). A: some areas of the liver are congested (arrow) (40× maginification). B: infiltration of lymphocytes (arrowhead), eosinophilic granular cells (arrow), and melanomacrophage centers (star) (200× magnification).

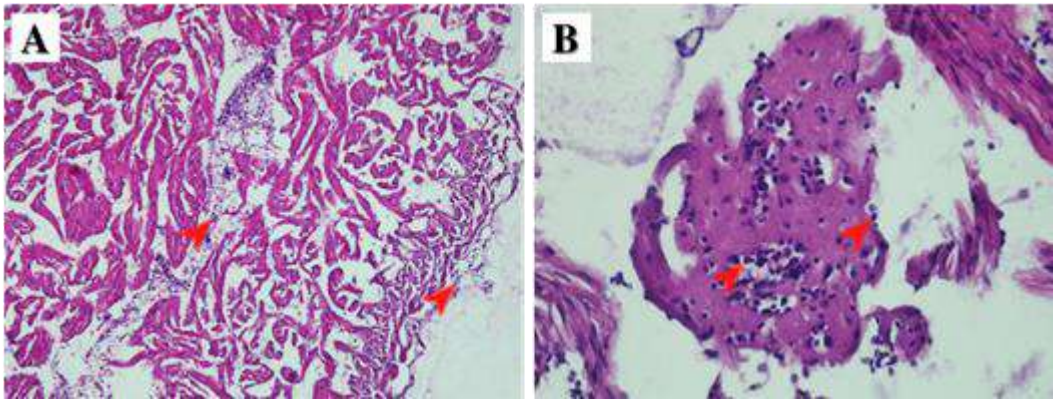

Figure 6 : Histopathological appearance of the tortoise heart with suspected MBD (hematoxylin-eosin staining). A: infiltration of lymphocytic cells (arrowhead) in the epicardium and myocardium (100× maginification). B: infiltration of lymphocytic cells (arrowhead) in the lumen of the endocardium (400× magnification).

DISCUSSION

Based on clinical symptoms and the reported history, it was assumed that the tortoise that had been necropsied had MBD. One of the strong supporting reasons is hypocalcemia-induced vitamin D deficiency, which is related, first, to lack of UV light (due to long shipping journeys) and proper temperature, and/or inadequate and balanced nutritional intake. Inadequate exposure to UVB rays causes the epidermal cells of animal skin to be unable to produce vitamin D3 (cholecalciferol) which is the result of the conversion of pre-vitamin D and its precursor, pro-vitamin D (7-dehydrocholesterol). Deficiency of vitamin D3 in the blood circulation causes the liver to lack its capacity to produce calcidiol or 25-(OH)-vitamin D3 as the main storage form of vitamin D3. This continues to cause kidneys to fail to hydroxylate calcidiol to produce 1,25-(OH)2-vitamin D3 or calcitriol, which plays a vital role in the regulation of calcium and phosphorus balance[19].

The endocrine hormone calcitriol is known to increase intestinal absorption of dietary calcium and phosphate, stimulate the storage of calcium and phosphate in the kidneys, and, together with parathyroid hormone (PTH), has a direct effect on bone by regulating calcium mobilization from bone. Lack of this hormone can cause disturbances in bone growth and development, as well as in maintaining mature bone tissue[20].

Hypocalcemia due to hypovitaminosis D is usually compensated by increased secretion of PTH from hyperplastic parathyroid glands and subsequently hyperparathyroidism, leading to resorption of calcium from bone[21]. Unfortunately, this study did not observe the histopathological features of the parathyroids, so the hyperparathyroidism in this case could not be confirmed.

Another possibility that can occur is low calcitriol so that the body cannot limit the occurrence of osteoclastogenesis and trigger bone resorption by osteoclasts, resulting in osteopenia[22,23]. This may explain the loss of the large amount of trabecular bone in the metatarsal tortoise epiphyses that we observed. The loss of trabecular continuity leads to a reduction in the ability of the trabecular to withstand stress[23]; therefore, the tortoise appears to have an abnormal gait.

In this study, it was also found that there is cartilage within the trabecular matrix. This may be related to disturbances in endochondral ossification during the development of the young tortoise. Ostechondrosis is a disorder of chondrocyte maturation that results in delayed cartilage mineralization. In addition to calcitriol, 24,25-(OH)2-vitamin D3 produced by calcidiol hydroxylation in the proximal renal tubule also plays an important role in cartilage cell differentiation and matrix mineralization. This imbalance in plasma concentrations between vitamin D metabolites appears to be related to the disposition of osteochondrosis during the growth period of the animal[24].

Under normal conditions, bone marrow in newborns and very young animals is mainly composed of active hematopoietic tissue and has relatively few fats[23]. This study found that the bone marrow within the epiphyseal metatarsal of the tortoise was hypocellular with a significantly reduced number of hemopoietic cells. It is still unclear how the pathophysiological relationship with the suspected MBD occurs. In another case, Turnbull et al.[25] also reported bone marrow hypocellularity in hypothermic sea turtles. Bone marrow hypoplasia is commonly found in animals and humans with aplastic pancytopenia, a rare condition in which all hematopoietic lines in the bone marrow are aplastic or severely hypoplasiated, resulting in bone marrow failure. The cause is usually chemical agents that are cytotoxic to hematopoietic cells, or mutations or perturbations in hematopoietic cells and their environment caused by infectious agents[23].

In this study, indications of infection and sepsis were also found, possibly due to microbial flora, in the tortoise suspected of having MBD. The association between decreased bone mineral density (BMD) and the risk of infection and sepsis has recently been reported. Previous studies have shown that BMD is a prognostic factor for infections and sepsis in human patients. Schulze-Hagen et al.[26] found that low BMD was closely related to high mortality rates in intensive care units, while patients with pulmonary infections had the lowest BMD. A recent study even demonstrated that low BMD is not only a potential predictor for patients with infections and sepsis, but also a new risk factor for infections and sepsis.

Sepsis occurs after bacterial infections, leading to severe sepsis and septic shocks characterized by low blood pressure, ischemic, failure of multiple organs, and death[27]. In this case, it was observed that there was inflammation of the liver, heart, muscles, and peripheral nerves, as well as renal tubular necrosis that can lead to acute renal failure. The decrease in the number and function of

osteoblasts, associated with altered expression of IL-7 and lipocalin-2, may have a negative impact on human immunity and thus increase sensitivity to infections. Vitamin D may also explain the connection between BMD and infections and sepsis[28]. However, much remains to be done to confirm the factors and analyze the association between bone metabolism disorders and sepsis.

CONCLUSION

In conclusion, this study reports that the young tortoise necropsied had a number of pathological conditions that led to suspicion of MBD. Inflammation of multiple organs due to sepsis that we found also seems to have a pathophysiological relationship with this disorder of bone metabolism. Further study is warranted to reach a convincing confirmation by further laboratory diagnostics (blood calcium, parathyroid hormone, etc.) and investigate the relationship between MBD and the risk of infection and sepsis in animals.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

The authors express their gratitude to Fajar Dany Prabayudha as a herpetologist and the co-assistant students of the Division of Veterinary Pathology, Faculty of Veterinary Medicine, Universitas Airlangga, who assisted in finding the sample cases for this study.

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Pharmacogn J. 2022; 14(6) Suppl:
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
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Case Report

Histopathological Perspectives of Multiple Organs in a Red-Footed Tortoise (*Chelonoidis carbonaria*) with Suspected Metabolic Bone Disease: A Case Report

Hani Plumeriastuti[1,*], Annise Proboningrat[1], Djoko Legowo[1], Bilqisthi Ari Putra[1], Gracia Angelina Hendarti[2]

ABSTRACT

Introduction: Exotic pet lovers' interest in keeping tortoises is increasing all over the world, including Indonesia. However, this trend cannot be separated from the potential emergence of various health problems in tortoises. One of the problems that often affects tortoises is metabolic bone disease. Metabolic bone disease (MBD) is a disorder related to the mechanisms of vitamin D and calcium metabolism, which generally occurs in reptiles, especially Chelonia and Lizards. Case Presentation: A 3-month-old red-footed tortoise, which was clinically suspected to have a MBD, was necropsied as an effort to support the provisional diagnosis through histopathological evaluation. The purpose of this examination was to analyze the impact of the disease on various organs microscopically in patients with suspected MBD. The results showed a decrease in the number of trabeculae and hematopoietic cells in the metatarsal bones; moderate myonecrotic changes and atrophy in the skeletal muscle; inflammation of the perineuron; acute tubular necrosis and mild edema of the renal cortex; congestion and an increase in the number of melanomacrophages in the liver; as well as epicarditis and myocarditis in the heart. Conclusion: Several forms of the histopathological changes seem to indicate a pathophysiological relationship between the suspected metabolic bone disease and the multiple organs examined.
Key words: Hematoxylin-eosin, MBD, Tortoise, Septicemia.

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History

- Submission Date: 13-11-2022;
- Review completed: 19-12-2022;
- Accepted Date: 21-12-2022.

DOI : 10.5530/pj.2022.14.

Article Available online

http://www.phcogj.com/v14/i6

INTRODUCTION

In the last decade, the trend of domesticating tortoises as pets in urban families is increasing and popular around the world.[1] In Indonesia, the sulcata tortoise is one of the most popular tortoise pets because it is easy to find this captive breed in the reptile pet market.[2] Another tortoise that is frequently kept as a traditional pet in houses is the red-footed tortoise.[3] Their ease of care, low cost of ownership, and amazing coloration make them highly sought after by novice tortoise keepers.

The red-footed tortoise (*Chelonoidis carbonaria*) are members of the Anapsida subclass, Chelonia order, Cryptodira suborder, Testudines family, and Chelonoidis genus.[4] This species is native to South America and can be found from Panama to Paraguay, as well as parts of Bolivia, Brazil, Colombia, Ecuador, and Peru.[5] *C. carbonaria* is a diurnal and terrestrial animal with a compact body and strong cylindrical limbs, ideal to support its heavy carapace and walk in rough terrain.[4,5] They are opportunistic omnivores in general, and their diet is heavily influenced by the seasonal availability of food.[6] Their main food sources are leaves, grasses, flowers, fruits, carcasses, and other food found on the ground.[4]

The increasing trend of keeping tortoises among exotic animal enthusiasts also has the potential to lead to many health problems.[1] *C. carbonaria* is the most common testudines kept as a pet in South America, which accounts for a large proportion of wildlife patients seen in veterinary practices.[4] In Indonesia, according to Raharjo *et al.*,[2] a study on the prevalence of disease in exotic pet patients at a clinic in Yogyakarta, Indonesia, during January-August 2020 showed that turtles and tortoises had the highest cases of 71.7%, compared to snakes (16.5%), iguanas (6.2%), lizards (4.1%), crocodiles (1%), and geckos (0.5%).

Management of nutrition, health, housing, and an inappropriate environment are predisposing factors to serious health issues in tortoises if not anticipated and treated immediately. Some of the health issues that tortoises in captivity can face include respiratory ailments caused by bacterial or viral infection,[7-10] gastro-intestinal disease caused by parasite or viral infection,[8,11,12] and nutritional and metabolic disorders.[13-15]

Metabolic bone disease (MBD) is one of the metabolic disorders commonly seen in captive reptiles, particularly in Chelonia (turtles, tortoises, and terrapins) and lizards, occasionally in snakes.[16] In veterinary medicine, MBD refers to a group of pathological conditions that affect the integrity and function of multiple bones.[17] They are most generally caused by genetic, dietary, and/or hormonal disorders that impact bone growth and remodeling, typically through changes in calcium/phosphorus metabolism. MBD has traditionally been broken down as fibrous osteodystrophy, osteoporosis, and rickets/osteomalacia; however, many cases are difficult to specifically classify, particularly those caused by nutritional deficiencies, because multiple conditions may coexist. Therefore, cases reported in the literature should be scrutinized carefully, and

Cite this article: Plumeriastuti H, Proboningrat A, Legowo D, Putra BA, Hendarti GA. Histopathological Perspectives of Multiple Organs in a Red-Footed Tortoise (*Chelonoidis carbonaria*) with Suspected Metabolic Bone Disease: A Case Report. Pharmacogn J. 2022;14(6) Suppl:.

confirmation by histopathological evaluation should be regarded as more definitive.[18] The aim of this study was to report the occurrence of suspected MBD in red-footed tortoise (*C. carbonaria*) and describe the histopathological findings in several organs associated with the disease. This study also tried to describe the relationship between the suspected MBD and the histopathological changes that occur in several observed organs.

CASE PRESENTATION

History

A 3-month-old dead red-footed tortoise (*Chelonoidis carbonarius*) weighing approximately 50 grams with a carapace length of 6 cm (Figure 1A-B) was sent to our laboratory. According to the information from the sender, the tortoise had clinical symptoms of inappetence, anorexia, an abnormal gait, and weakness. Previously, it had a history of shipping that was too long, about seven days, to finally die. Another tortoise, similar in age and weight, maintained together, had similar complaints.

Post-mortem findings

The post-mortem investigation that had been performed showed that the carapace and plastron were tender. No abnormal conditions such as swelling, trauma, or injuries were found on the results of other external examinations. On internal examination after necropsy, no significant macroscopic changes were found in the organs, such as inflammation, bleeding, enlargement or reduction in organ size, changes in color and consistency, and accumulation of fluid in the celomic cavity. Only one colon enlargement was found. Some organs, such as the kidney, liver, heart, hind leg musculature, and metatarsals, were collected for tissue processing. Unfortunately, the intestine tissue could not be collected for histopathological examination (Figure 1C).

Histopathological findings

On the results of the microscopical examination of tortoise metatarsal bones that had been stained with hematoxylin and eosin, there was a decrease in the number of trabecular bones in the epiphysis and hematopoietic cell loss (Figure 2A). Some sections also showed the presence of hyaline cartilage in the middle of the mature trabecular bone matrix (Figure 2B). In skeletal muscles, it was observed that many cells of the skeletal muscles were necrotic and some were atrophic (Figure 3A). Inflammatory cell infiltration also occurs between the striated muscle fibers accompanied by edema (Figure 4B). Peripheral nerve cells also underwent lysis and inflammation (Figure 3C).

On microscopic examination of the kidney, edema appeared in two of the four lobes of the kidney (Figure 4A). The appearance of massive acute tubular necrosis was also clearly seen, characterized by convoluted proximal tubules that mostly underwent cell lysis and nuclear pyknosis (Figure 4B). Mild to moderate congestion was observed in the liver sinusoids (Figure 5A). An increase in the number of melanomacrophages has also occurred, and some have formed melanomacrophage centers (MMC). Additionally, there are few eosinophilic granular cells (EGCs) and some infiltrating lymphocytes that aggregate to form lymphoid follicles (Figure 5B). Furthermore, microscopic observation also showed that the tortoise heart experienced epicarditis, myocarditis, and endocarditis, indicated by fairly massive lymphocytic infiltration in the epicardium, myocardium, and lumen of the endocardium, respectively (Figure 6A-B).

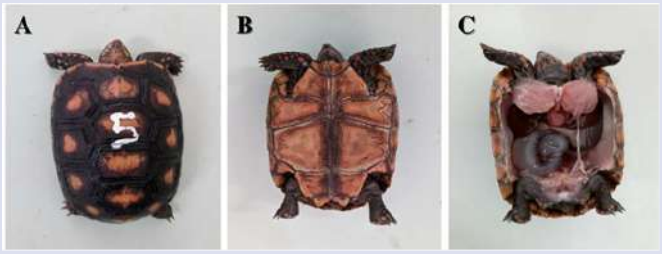

Figure 1: Gross examination of the tortoise body suspected of having MBD. A: The dorsal view. B: The ventral view. C: Gross appearance of the internal organs.

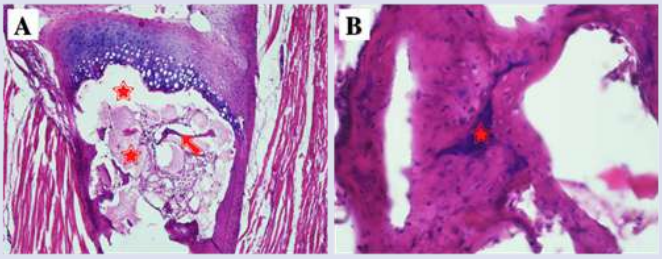

Figure 2: Histopathological appearance of the metatarsal bone of tortoise with suspected MBD (hematoxylin-eosin staining). A: metatarsal epiphyses with fewer trabeculae (arrow) and loss of hematopoietic cell (arrowhead) (100× magnification). B: hyaline cartilage (star) within the trabecular matrix (400× magnification).

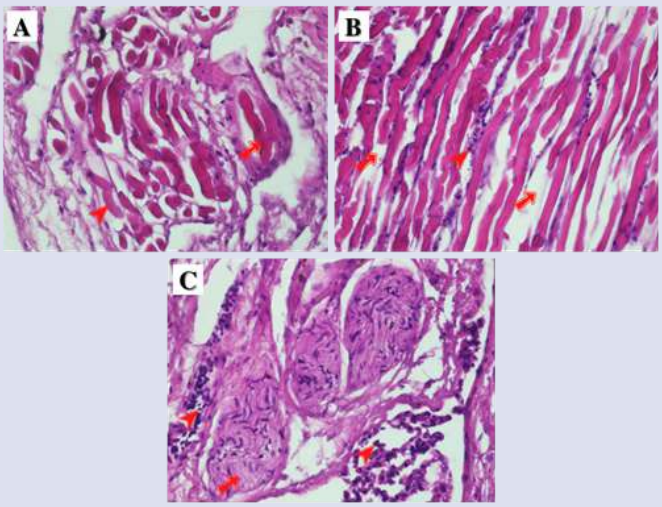

Figure 3: Histopathological appearance of tortoise skeletal muscles with suspected MBD (hematoxylin-eosin staining, 400× magnification). A: striated muscles with atrophic (arrowhead) and necrotic (arrow). B: edema (arrow) and infiltration of inflammatory cells (arrowhead) between muscle fibers. C: peripheral nerve cell lysis (arrow) and inflammatory cell (arrowhead) infiltration.

DISCUSSION

Based on clinical symptoms and the reported history, it was assumed that the tortoise that had been necropsied had MBD. One of the strong supporting reasons is hypocalcemia-induced vitamin D deficiency, which is related, first, to lack of UV light (due to long shipping journeys) and proper temperature, and/or inadequate and balanced nutritional intake. Inadequate exposure to UVB rays causes the epidermal cells of animal skin to be unable to produce vitamin D3 (cholecalciferol) which is the result of the conversion of pre-vitamin D and its precursor, pro-vitamin D (7-dehydrocholesterol). Deficiency of vitamin D3 in the blood circulation causes the liver to lack its capacity to produce calcidiol or 25-(OH)-vitamin D3 as the main storage form of vitamin D3. This continues to cause kidneys to fail to hydroxylate calcidiol to produce 1,25-(OH)2-vitamin D3 or calcitriol, which plays a vital role in the regulation of calcium and phosphorus balance.[19]

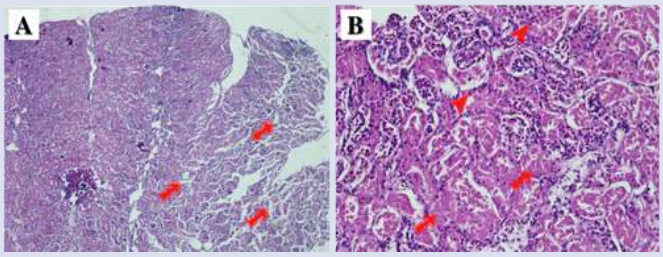

Figure 4: Histopathological appearance of the renal cortex of tortoise with suspected MBD (hematoxylin-eosin staining). A: edema (arrow) of the two right lobes of the kidney (40× maginification). B: pyknotic (arrowhead) and karyolitic (arrow) of convoluted tubule cells (200× magnification).

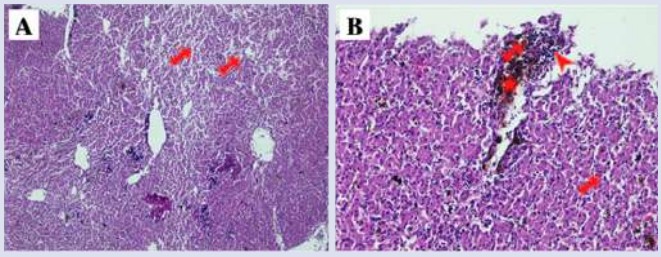

Figure 5: Histopathological appearance of the liver parenchyma of tortoise with suspected MBD (hematoxylin-eosin staining). A: some areas of the liver are congested (arrow) (40× maginification). B: infiltration of lymphocytes (arrowhead), eosinophilic granular cells (arrow), and melanomacrophage centers (star) (200× magnification).

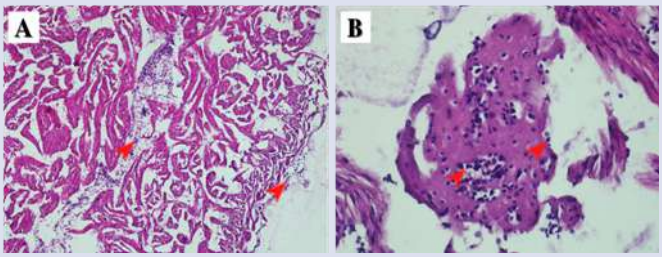

Figure 6: Histopathological appearance of the tortoise heart with suspected MBD (hematoxylin-eosin staining). A: infiltration of lymphocytic cells (arrowhead) in the epicardium and myocardium (100× maginification). B: infiltration of lymphocytic cells (arrowhead) in the lumen of the endocardium (400× magnification).

The endocrine hormone calcitriol is known to increase intestinal absorption of dietary calcium and phosphate, stimulate the storage of calcium and phosphate in the kidneys, and, together with parathyroid hormone (PTH), has a direct effect on bone by regulating calcium mobilization from bone. Lack of this hormone can cause disturbances in bone growth and development, as well as in maintaining mature bone tissue.[20]

Hypocalcemia due to hypovitaminosis D is usually compensated by increased secretion of PTH from hyperplastic parathyroid glands and subsequently hyperparathyroidism, leading to resorption of calcium from bone.[21] Unfortunately, this study did not observe the histopathological features of the parathyroids, so the hyperparathyroidism in this case could not be confirmed.

Another possibility that can occur is low calcitriol so that the body cannot limit the occurrence of osteoclastogenesis and trigger bone resorption by osteoclasts, resulting in osteopenia.[22,23] This may explain the loss of the large amount of trabecular bone in the metatarsal tortoise epiphyses that we observed. The loss of trabecular continuity leads to a reduction in the ability of the trabecular to withstand stress;[23] therefore, the tortoise appears to have an abnormal gait.

In this study, it was also found that there is cartilage within the trabecular matrix. This may be related to disturbances in endochondral ossification during the development of the young tortoise. Ostechondrosis is a disorder of chondrocyte maturation that results in delayed cartilage mineralization. In addition to calcitriol, 24,25-(OH)2-vitamin D3 produced by calcidiol hydroxylation in the proximal renal tubule also plays an important role in cartilage cell differentiation and matrix mineralization. This imbalance in plasma concentrations between vitamin D metabolites appears to be related to the disposition of osteochondrosis during the growth period of the animal.[24]

Under normal conditions, bone marrow in newborns and very young animals is mainly composed of active hematopoietic tissue and has relatively few fats.[23] This study found that the bone marrow within the epiphyseal metatarsal of the tortoise was hypocellular with a significantly reduced number of hemopoietic cells. It is still unclear how the pathophysiological relationship with the suspected MBD occurs. In another case, Turnbull *et al.*[25] also reported bone marrow hypocellularity in hypothermic sea turtles. Bone marrow hypoplasia is commonly found in animals and humans with aplastic pancytopenia, a rare condition in which all hematopoietic lines in the bone marrow are aplastic or severely hypoplasiated, resulting in bone marrow failure. The cause is usually chemical agents that are cytotoxic to hematopoietic cells, or mutations or perturbations in hematopoietic cells and their environment caused by infectious agents.[23]

In this study, indications of infection and sepsis were also found, possibly due to microbial flora, in the tortoise suspected of having MBD. The association between decreased bone mineral density (BMD) and the risk of infection and sepsis has recently been reported. Previous studies have shown that BMD is a prognostic factor for infections and sepsis in human patients. Schulze-Hagen *et al.*[26] found that low BMD was closely related to high mortality rates in intensive care units, while patients with pulmonary infections had the lowest BMD. A recent study even demonstrated that low BMD is not only a potential predictor for patients with infections and sepsis, but also a new risk factor for infections and sepsis.

Sepsis occurs after bacterial infections, leading to severe sepsis and septic shocks characterized by low blood pressure, ischemic, failure of multiple organs, and death.[27] In this case, it was observed that there was inflammation of the liver, heart, muscles, and peripheral nerves, as well as renal tubular necrosis that can lead to acute renal failure. The decrease in the number and function of osteoblasts, associated with altered expression of IL-7 and lipocalin-2, may have a negative impact on human immunity and thus increase sensitivity to infections. Vitamin D may also explain the connection between BMD and infections and sepsis.[28] However, much remains to be done to confirm the factors and analyze the association between bone metabolism disorders and sepsis.

CONCLUSION

In conclusion, this study reports that the young tortoise necropsied had a number of pathological conditions that led to suspicion of MBD. Inflammation of multiple organs due to sepsis that we found also seems to have a pathophysiological relationship with this disorder of bone metabolism. Further study is warranted to reach a convincing confirmation by further laboratory diagnostics (blood calcium, parathyroid hormone, etc.) and investigate the relationship between MBD and the risk of infection and sepsis in animals.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGEMENTS

The authors express their gratitude to Fajar Dany Prabayudha as a herpetologist and the co-assistant students of the Division of Veterinary Pathology, Faculty of Veterinary Medicine, Universitas Airlangga, who assisted in finding the sample cases for this study.

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Cite this article: Plumeriastuti H, Proboningrat A, Legowo D, Putra BA, Hendarti GA. Histopathological Perspectives of Multiple Organs in a Red-Footed Tortoise (*Chelonoidis carbonaria*) with Suspected Metabolic Bone Disease: A Case Report. Pharmacogn J. 2022;14(6)Suppl:.

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History

- Submission Date: 13-11-2022;
- Review completed: 19-12-2022;
- Accepted Date: 21-12-2022.

DOI : 10.5530/pj.2022.14.

Article Available online

http://www.phcogj.com/v14/i6

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The increasing trend of keeping tortoises among exotic animal enthusiasts also has the potential to lead to many health problems.[1] *C. carbonaria* is the most common testudines kept as a pet in South America, which accounts for a large proportion of wildlife patients seen in veterinary practices.[4] In Indonesia, according to Raharjo *et al.*,[2] a study on the prevalence of disease in exotic pet patients at a clinic in Yogyakarta, Indonesia, during January-August 2020 showed that turtles and tortoises had the highest cases of 71.7%, compared to snakes (16.5%), iguanas (6.2%), lizards (4.1%), crocodiles (1%), and geckos (0.5%).

Management of nutrition, health, housing, and an inappropriate environment are predisposing factors to serious health issues in tortoises if not anticipated and treated immediately. Some of the health issues that tortoises in captivity can face include respiratory ailments caused by bacterial or viral infection,[7-10] gastro-intestinal disease caused by parasite or viral infection,[8,11,12] and nutritional and metabolic disorders.[13-15]

Metabolic bone disease (MBD) is one of the metabolic disorders commonly seen in captive reptiles, particularly in Chelonia (turtles, tortoises, and terrapins) and lizards, occasionally in snakes.[16] In veterinary medicine, MBD refers to a group of pathological conditions that affect the integrity and function of multiple bones.[17] They are most generally caused by genetic, dietary, and/or hormonal disorders that impact bone growth and remodeling, typically through changes in calcium/phosphorus metabolism. MBD has traditionally been broken down as fibrous osteodystrophy, osteoporosis, and rickets/osteomalacia; however, many cases are difficult to specifically classify, particularly those caused by nutritional deficiencies, because multiple conditions may coexist. Therefore, cases reported in the literature should be scrutinized carefully, and

Cite this article: Plumeriastuti H, Proboningrat A, Legowo D, Putra BA, Hendarti GA. Histopathological Perspectives of Multiple Organs in a Red-Footed Tortoise (*Chelonoidis carbonaria*) with Suspected Metabolic Bone Disease: A Case Report. Pharmacogn J. 2022;14(6) Suppl:.

confirmation by histopathological evaluation should be regarded as more definitive.[18] The aim of this study was to report the occurrence of suspected MBD in red-footed tortoise (*C. carbonaria*) and describe the histopathological findings in several organs associated with the disease. This study also tried to describe the relationship between the suspected MBD and the histopathological changes that occur in several observed organs.

CASE PRESENTATION

History

A 3-month-old dead red-footed tortoise (*Chelonoidis carbonarius*) weighing approximately 50 grams with a carapace length of 6 cm (Figure 1A-B) was sent to our laboratory. According to the information from the sender, the tortoise had clinical symptoms of inappetence, anorexia, an abnormal gait, and weakness. Previously, it had a history of shipping that was too long, about seven days, to finally die. Another tortoise, similar in age and weight, maintained together, had similar complaints.

Post-mortem findings

The post-mortem investigation that had been performed showed that the carapace and plastron were tender. No abnormal conditions such as swelling, trauma, or injuries were found on the results of other external examinations. On internal examination after necropsy, no significant macroscopic changes were found in the organs, such as inflammation, bleeding, enlargement or reduction in organ size, changes in color and consistency, and accumulation of fluid in the celomic cavity. Only one colon enlargement was found. Some organs, such as the kidney, liver, heart, hind leg musculature, and metatarsals, were collected for tissue processing. Unfortunately, the intestine tissue could not be collected for histopathological examination (Figure 1C).

Histopathological findings

On the results of the microscopical examination of tortoise metatarsal bones that had been stained with hematoxylin and eosin, there was a decrease in the number of trabecular bones in the epiphysis and hematopoietic cell loss (Figure 2A). Some sections also showed the presence of hyaline cartilage in the middle of the mature trabecular bone matrix (Figure 2B). In skeletal muscles, it was observed that many cells of the skeletal muscles were necrotic and some were atrophic (Figure 3A). Inflammatory cell infiltration also occurs between the striated muscle fibers accompanied by edema (Figure 4B). Peripheral nerve cells also underwent lysis and inflammation (Figure 3C).

On microscopic examination of the kidney, edema appeared in two of the four lobes of the kidney (Figure 4A). The appearance of massive acute tubular necrosis was also clearly seen, characterized by convoluted proximal tubules that mostly underwent cell lysis and nuclear pyknosis (Figure 4B). Mild to moderate congestion was observed in the liver sinusoids (Figure 5A). An increase in the number of melanomacrophages has also occurred, and some have formed melanomacrophage centers (MMC). Additionally, there are few eosinophilic granular cells (EGCs) and some infiltrating lymphocytes that aggregate to form lymphoid follicles (Figure 5B). Furthermore, microscopic observation also showed that the tortoise heart experienced epicarditis, myocarditis, and endocarditis, indicated by fairly massive lymphocytic infiltration in the epicardium, myocardium, and lumen of the endocardium, respectively (Figure 6A-B).

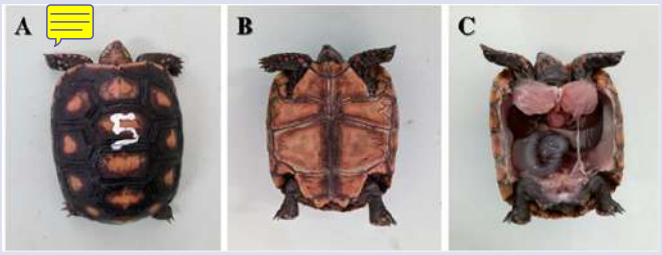

Figure 1: Gross examination of the tortoise body suspected of having MBD. A: The dorsal view. B: The ventral view. C: Gross appearance of the internal organs.

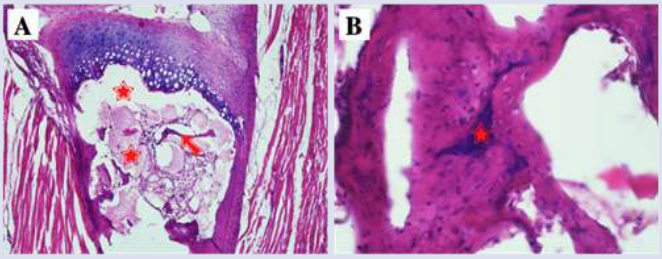

Figure 2: Histopathological appearance of the metatarsal bone of tortoise with suspected MBD (hematoxylin-eosin staining). A: metatarsal epiphyses with fewer trabeculae (arrow) and loss of hematopoietic cell (arrowhead) (100× magnification). B: hyaline cartilage (star) within the trabecular matrix (400× magnification).

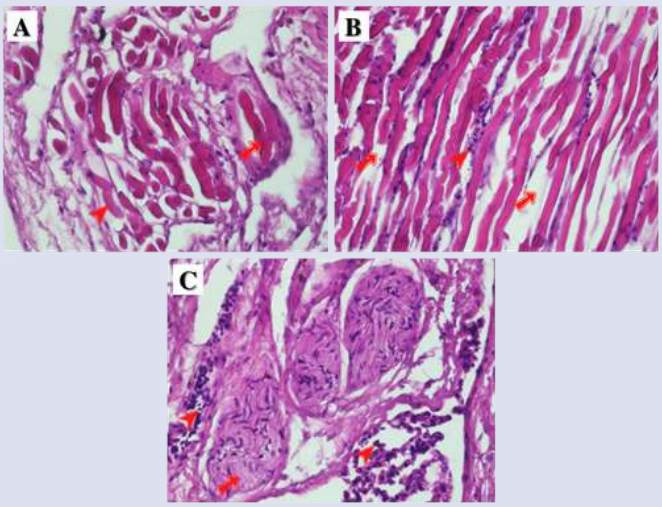

Figure 3: Histopathological appearance of tortoise skeletal muscles with suspected MBD (hematoxylin-eosin staining, 400× magnification). A: striated muscles with atrophic (arrowhead) and necrotic (arrow). B: edema (arrow) and infiltration of inflammatory cells (arrowhead) between muscle fibers. C: peripheral nerve cell lysis (arrow) and inflammatory cell (arrowhead) infiltration.

DISCUSSION

Based on clinical symptoms and the reported history, it was assumed that the tortoise that had been necropsied had MBD. One of the strong supporting reasons is hypocalcemia-induced vitamin D deficiency, which is related, first, to lack of UV light (due to long shipping journeys) and proper temperature, and/or inadequate and balanced nutritional intake. Inadequate exposure to UVB rays causes the epidermal cells of animal skin to be unable to produce vitamin D3 (cholecalciferol) which is the result of the conversion of pre-vitamin D and its precursor, pro-vitamin D (7-dehydrocholesterol). Deficiency of vitamin D3 in the blood circulation causes the liver to lack its capacity to produce calcidiol or 25-(OH)-vitamin D3 as the main storage form of vitamin D3. This continues to cause kidneys to fail to hydroxylate calcidiol to produce 1,25-(OH)2-vitamin D3 or calcitriol, which plays a vital role in the regulation of calcium and phosphorus balance.[19]

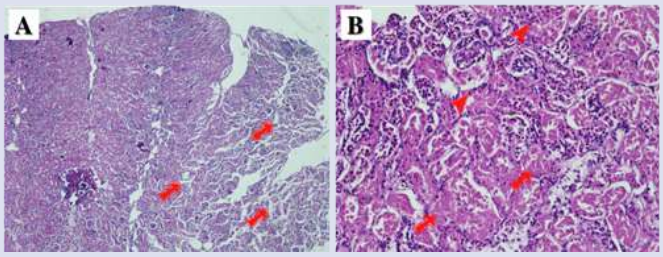

Figure 4: Histopathological appearance of the renal cortex of tortoise with suspected MBD (hematoxylin-eosin staining). A: edema (arrow) of the two right lobes of the kidney (40× maginification). B: pyknotic (arrowhead) and karyolitic (arrow) of convoluted tubule cells (200× magnification).

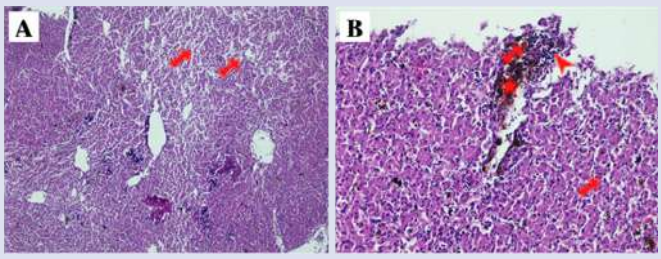

Figure 5: Histopathological appearance of the liver parenchyma of tortoise with suspected MBD (hematoxylin-eosin staining). A: some areas of the liver are congested (arrow) (40× maginification). B: infiltration of lymphocytes (arrowhead), eosinophilic granular cells (arrow), and melanomacrophage centers (star) (200× magnification).

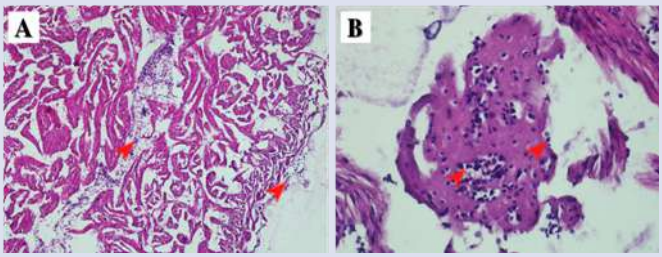

Figure 6: Histopathological appearance of the tortoise heart with suspected MBD (hematoxylin-eosin staining). A: infiltration of lymphocytic cells (arrowhead) in the epicardium and myocardium (100× maginification). B: infiltration of lymphocytic cells (arrowhead) in the lumen of the endocardium (400× magnification).

The endocrine hormone calcitriol is known to increase intestinal absorption of dietary calcium and phosphate, stimulate the storage of calcium and phosphate in the kidneys, and, together with parathyroid hormone (PTH), has a direct effect on bone by regulating calcium mobilization from bone. Lack of this hormone can cause disturbances in bone growth and development, as well as in maintaining mature bone tissue.[20]

Hypocalcemia due to hypovitaminosis D is usually compensated by increased secretion of PTH from hyperplastic parathyroid glands and subsequently hyperparathyroidism, leading to resorption of calcium from bone.[21] Unfortunately, this study did not observe the histopathological features of the parathyroids, so the hyperparathyroidism in this case could not be confirmed.

Another possibility that can occur is low calcitriol so that the body cannot limit the occurrence of osteoclastogenesis and trigger bone resorption by osteoclasts, resulting in osteopenia.[22,23] This may explain the loss of the large amount of trabecular bone in the metatarsal tortoise epiphyses that we observed. The loss of trabecular continuity leads to a reduction in the ability of the trabecular to withstand stress;[23] therefore, the tortoise appears to have an abnormal gait.

In this study, it was also found that there is cartilage within the trabecular matrix. This may be related to disturbances in endochondral ossification during the development of the young tortoise. Ostechondrosis is a disorder of chondrocyte maturation that results in delayed cartilage mineralization. In addition to calcitriol, 24,25-(OH)2-vitamin D3 produced by calcidiol hydroxylation in the proximal renal tubule also plays an important role in cartilage cell differentiation and matrix mineralization. This imbalance in plasma concentrations between vitamin D metabolites appears to be related to the disposition of osteochondrosis during the growth period of the animal.[24]

Under normal conditions, bone marrow in newborns and very young animals is mainly composed of active hematopoietic tissue and has relatively few fats.[23] This study found that the bone marrow within the epiphyseal metatarsal of the tortoise was hypocellular with a significantly reduced number of hemopoietic cells. It is still unclear how the pathophysiological relationship with the suspected MBD occurs. In another case, Turnbull *et al.*[25] also reported bone marrow hypocellularity in hypothermic sea turtles. Bone marrow hypoplasia is commonly found in animals and humans with aplastic pancytopenia, a rare condition in which all hematopoietic lines in the bone marrow are aplastic or severely hypoplasiated, resulting in bone marrow failure. The cause is usually chemical agents that are cytotoxic to hematopoietic cells, or mutations or perturbations in hematopoietic cells and their environment caused by infectious agents.[23]

In this study, indications of infection and sepsis were also found, possibly due to microbial flora, in the tortoise suspected of having MBD. The association between decreased bone mineral density (BMD) and the risk of infection and sepsis has recently been reported. Previous studies have shown that BMD is a prognostic factor for infections and sepsis in human patients. Schulze-Hagen *et al.*[26] found that low BMD was closely related to high mortality rates in intensive care units, while patients with pulmonary infections had the lowest BMD. A recent study even demonstrated that low BMD is not only a potential predictor for patients with infections and sepsis, but also a new risk factor for infections and sepsis.

Sepsis occurs after bacterial infections, leading to severe sepsis and septic shocks characterized by low blood pressure, ischemic, failure of multiple organs, and death.[27] In this case, it was observed that there was inflammation of the liver, heart, muscles, and peripheral nerves, as well as renal tubular necrosis that can lead to acute renal failure. The decrease in the number and function of osteoblasts, associated with altered expression of IL-7 and lipocalin-2, may have a negative impact on hum[illegible]mmunity and thus increase sensitivity to infections. Vitamin D may also explain the connection between BMD and infections and sepsis.[28] However, much remains to be done to confirm the factors and analyze the association between bone metabolism disorders and sepsis.

CONCLUSION

In conclusion, this study reports that the young tortoise necropsied had a number of pathological conditions that led to suspicion of MBD. Inflammation of multiple organs due to sepsis that we found also seems to have a pathophysiological relationship with this disorder of bone metabolism. Further study is warranted to reach a convincing confirmation by further laboratory diagnostics (blood calcium, parathyroid hormone, etc.) and investigate the relationship between MBD and the risk of infection and sepsis in animals.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGEMENTS

The authors express their gratitude to Fajar Dany Prabayudha as a herpetologist and the co-assistant students of the Division of Veterinary Pathology, Faculty of Veterinary Medicine, Universitas Airlangga, who assisted in finding the sample cases for this study.

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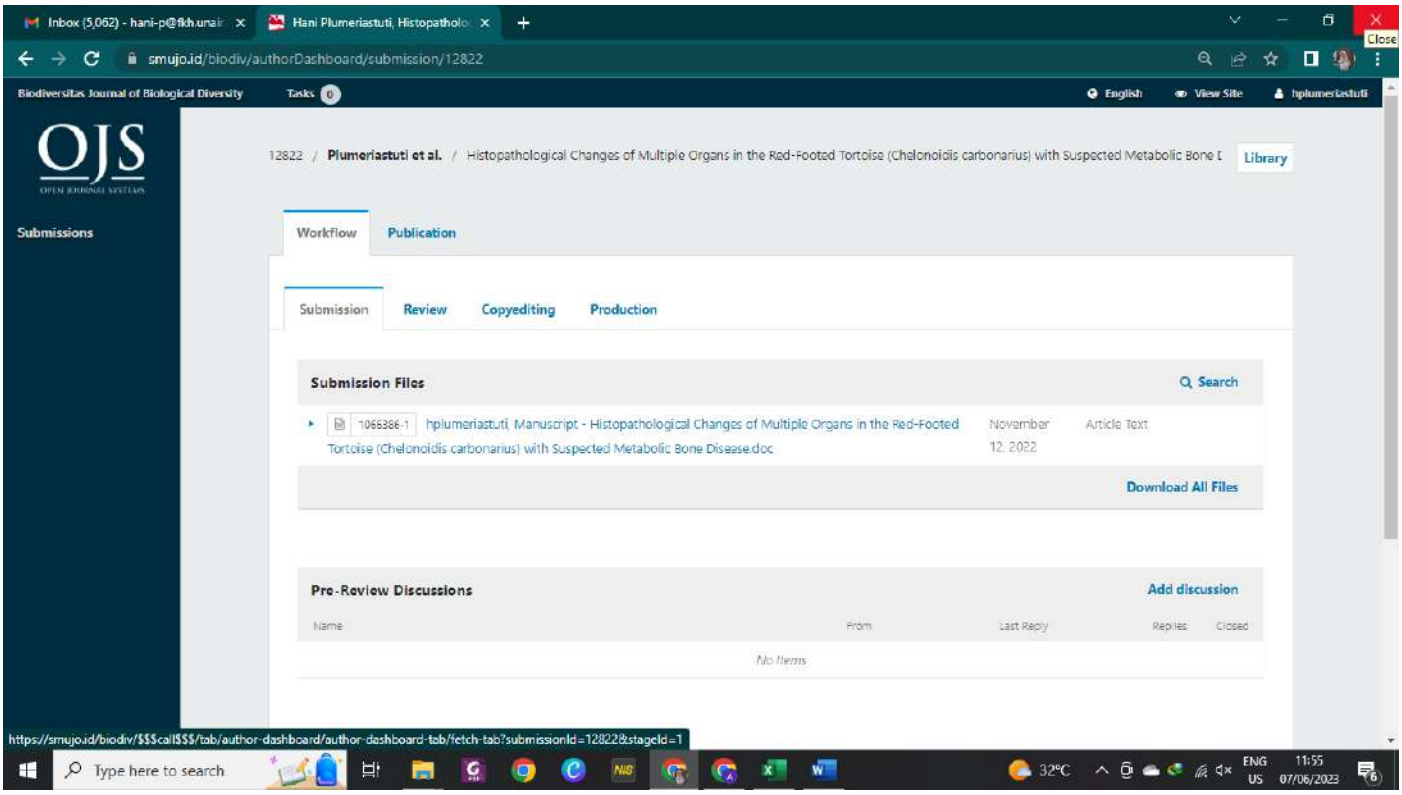
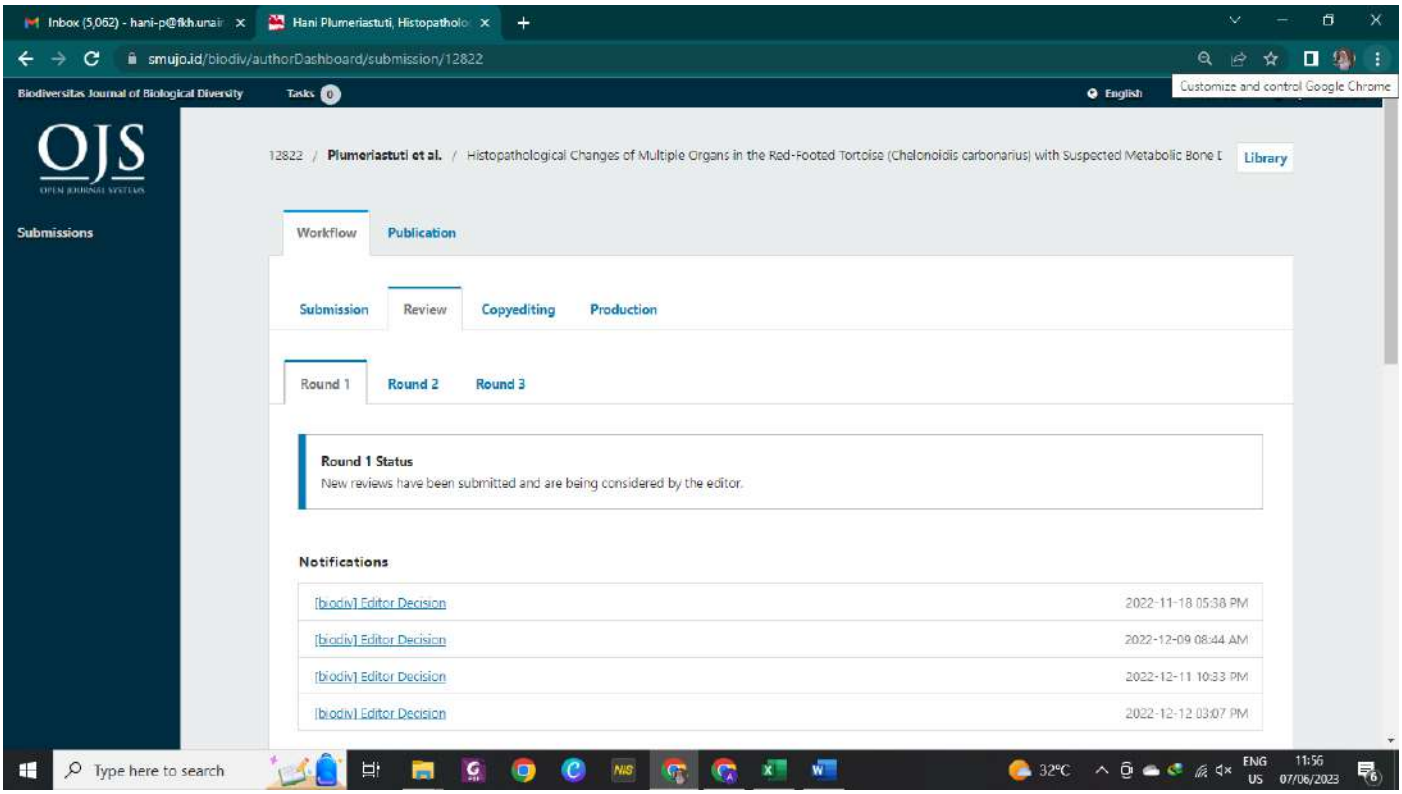
Cite this article: Plumeriastuti H, Proboningrat A, Legowo D, Putra BA, Hendarti GA. Histopathological Perspectives of Multiple Organs in a Red-Footed Tortoise (*Chelonoidis carbonaria*) with Suspected Metabolic Bone Disease: A Case Report. Pharmacogn J. 2022;14(6)Suppl:.

1. Article Submission to Biodiversitas Journal of Biological Diversity



2. Article Review



First Revision

Notifications

[biodiv] Editor Decision

2022-11-18 05:38 PM

Hani Plumeriastuti, Djoko Legowo, Annise Proboningrat, Gracia Angelina Hendarti, Bilqisthi Ari Putra:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Histopathological Changes of Multiple Organs in the Red-Footed Tortoise (Chelonoidis carbonarius) with Suspected Metabolic Bone Disease".

Our decision is: Revisions Required

Biodiversitas Journal of Biological Diversity

Second Revision

Notifications

[biodiv] Editor Decision

2022-12-09 08:44 AM

Hani Plumeriastuti, Djoko Legowo, Annise Proboningrat, Gracia Angelina Hendarti, Bilqisthi Ari Putra:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Histopathological Changes of Multiple Organs in the Red-Footed Tortoise (Chelonoidis carbonarius) with Suspected Metabolic Bone Disease".

Our decision is: Revisions Required

Reviewer C:

1. The author does not explain "why this case is important" in the introduction, and I cannot see why the author wrote the case. Please explain that for a comprehensive introduction.
2. There is still a lack of information on the case history. Please provide more information, such as sex, anamnesis, and other details, such as the type of food the tortoises eat before they die, the shipping method, etc.
3. You must indicate where the changes are described in the image with a pointer, mark, or arrow.
4. Please use an arrow or mark with a dark color; in this picture, the yellow color is not clear or visible to describe the changes.
5. Please don't use "we" in the discussion. Instead, use a passive sentence to explain what you found.

Recommendation: Revisions Required

[biodiv]

Participants

Smujo Editors (editors)

Hani Plumeriastuti (hplumeriastuti)

Messages

Note	From
Kindly send your revised paper. _____ Biodiversitas Journal of Biological Diversity	editors 2022-12-09 02:10 PM
Thank you very much. I have submitted the revised paper at the Revision menu	hplumeriastuti 2022-12-10 02:40 AM
You have not, pls, check it again.	editors 2022-12-10 03:00 AM
I am sorry because I uploaded the wrong file. I have re-uploaded the revised article. Thank you for reminding me. hplumeriastuti, C-rev.1biodiversitas - Revised Article.doc	hplumeriastuti 2022-12-11 04:41 PM

Add Message

Third Revision

Notifications

[biodiv] Editor Decision

2022-12-11 10:33 PM

Hani Plumeriastuti, Djoko Legowo, Annise Proboningrat, Gracia Angelina Hendarti, Bilqisthi Ari Putra:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Histopathological Changes of Multiple Organs in the Red-Footed Tortoise (Chelonoidis carbonarius) with Suspected Metabolic Bone Disease".

Our decision is: Revisions Required

Pls, add detailed "Materials and Methods"

Reviewer A:
Recommendation: Revisions Required

Response for Reviewer Comment

Participants Edit

Smujo Editors (editors)

Ayu Astuti (ayu)

Hani Plumeriastuti (hplumeriastuti)

Messages

Note	From
Thanks for the valuable advice. We have added some of the methods in lines 76–79 and 90–93. We also made changes to some of the words or phrases in lines 86, 94, 112, and 194 as highlighted in the manuscript.	hplumeriastuti 2022-12-12 05:34 AM
Dear Author(s), Kindly adjust your paper and refer to Guidance for Author, see here: https://smujo.id/biodiv/guidance-for-author To inform you, for Original research paper, your paper should contain. - Your own original data (in part or in whole) from your field or laboratory observations. - about 3000-6000 words from introduction to conclusion, tables and figures are excluded. - about 20 references need to be cited. - at least 80% of the References must come from scientific journals published in the last 10 years. - Only 10% of the References can use local language (not English). - A Certificate of Proofreadinng from USA, UK, Canada or Australia is needed (still optional). The above provisions are excluded for taxonomic-themed papers.	editors 2022-12-12 09:37 AM

3. Rejection

Notifications

[biodiv] Editor Decision

2022-12-12 03:07 PM

Hani Plumeriastuti, Djoko Legowo, Annise Proboningrat, Gracia Angelina Hendarti, Bilqisthi Ari Putra:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Histopathological Changes of Multiple Organs in the Red-Footed Tortoise (Chelonoidis carbonarius) with Suspected Metabolic Bone Disease".

Our decision is to: Decline Submission
Note: Although we are interested in publishing your paper, we have to decline it, because your paper does not have "Materials and Methods" nor is it a review type paper.

4. Article Submission to Pharmacognosy Journal

Manuscript Received & Peer Review Acceptance -PJ-22-1078 External Inbox ×

Pharmacognosy Journal <editor.phcogi@gmail.com> Thu, Dec 15, 2022, 5:24 PM
to me, annise.p, djokolegowosaid, beeza06, grace.anatomifkh

Dear Dr. Hani Plumeriastuti**,**

Thank you very much for your submission to the Pharmacognosy Journal. We have received your article entitled "Histopathological Perspectives of Multiple Organs in a Red-Footed Tortoise (Chelonoidis carbonaria) with Suspected Metabolic Bone Disease: A Case Report". The article is now under our PRELIMINARY EVALUATION and we will keep you updated on the article status accordingly.

As we are an open-access non-profit organization, we require publication charges to recover our editorial and production expenses. They cover the range of publishing services we provide which includes the provision of online tools for editors and authors, article production and hosting, liaison with abstracting and indexing services, and customer services. The publication charges are 1249 GBP for the accepted article. You are required to process publication charges upon acceptance of your article (after peer-review). Kindly confirm your response to proceed with the review process.

We thank you for submitting your valuable work to the Pharmacognosy Journal.

Kindly get back to us for further queries.

Have a great day.!

hani plumeriastuti <hani-p@fkh.unair.ac.id> Dec 16, 2022, 11:41 AM
to Pharmacognosy

Dear Editor,

Thank you for the information.

Could I get 650 GBP in publication charges for this December issue?

I do not have a budget for 1249 GBP.

Thank you very much.

Best regards,

Hani Plumeriastuti

Pharmacognosy Journal <editor.phcogi@gmail.com> Dec 16, 2022, 12:58 PM
to me

Dear Hani,

Thanks for your email. You are requested to pay 750 GBP upon the article acceptance.

Kindly confirm to initiate the review process.

Regards,
Emily Flora

•••

hani plumeriastuti <hani-p@fkh.unair.ac.id> Dec 16, 2022, 4:32 PM
to Pharmacognosy

Dear Editor,

We confirm our intention to pay 750 GBP.

Please send me the invoice as soon as possible to publish in the December issue.

Thank you very much.

Best regards,

Hani Plumeriastuti

Pharmacognosy Journal <editor.phcogi@gmail.com> Dec 17, 2022, 6:28 PM
to me

Dear Hani,

Thank you for your kind response and confirming the charges. Soon, we will get back to you with the review comments.
Will let you know the issue once the article is accepted.

Regards,
Emily Flora

•••

Manuscript Accepted for Publication-PJ-22-1078 External Inbox ×

Pharmacognosy Journal <editor.phcogi@gmail.com> Wed, Dec 21, 2022, 1:49 PM
to me

Hani Plumeriastuti, Annise Proboningrat, Djoko Legowo, Bilqisthi Ari Putra, Gracia Angelina Hendarti:

We have reached a decision regarding your submission to Pharmacognosy Journal, "Histopathological Perspectives of Multiple Organs in a Red-Footed Tortoise (Chelonoidis carbonaria) with Suspected Metabolic Bone Disease: A Case Report".

Our decision is to: Accept Submission

I truly appreciate all your hard work, dedication and commitments towards this work. This paper is well described clearly and it is accepted to publish without any revisions.
Kindly acknowledge, so that we can send you the credit card invoice (750 GBP) accordingly.
Awaiting your response.

Regards,
Emily Flora

hani plumeriastuti <hani-p@fkh.unair.ac.id> Wed, Dec 21, 2022, 1:57 PM
to Pharmacognosy

Dear Editor,

Thank you very much for the good news.

I intend to make a bank transfer payment. Can you inform me of the mechanism, please?

Thank you.

Best regards,

Hani Plumeriastuti

Pharmacognosy Journal <editor.phcogi@gmail.com>
to me

Fri, Dec 23, 2022, 4:07 PM

Dear Hani,

Thank you for your kind response. Please find the attached wire transfer invoice. Kindly make the payment and send us the receipt at the earliest.
Regards,
Emily Flora

•••

One attachment • Scanned by Gmail

PJ-22-1078 Wire T...

Pharmacognosy Journal <editor.phcogi@gmail.com>
to me

Mon, Dec 26, 2022, 2:49 PM

Dear Hani,

Could you please let me know the current status of the payment?
Awaiting your reply.
Regards,
Emily Flora

•••

hani plumeriastuti <hani-p@fkh.unair.ac.id>
to Pharmacognosy

Tue, Dec 27, 2022, 3:19 PM

Dear Editor,

I would like to apologize for my belated reply.
I just made a payment by wire transfer today.
Here is the proof of payment.

•••

One attachment • Scanned by Gmail

Payment Proof_H...

Pharmacognosy Journal <editor.phcogi@gmail.com> Dec 27, 2022, 7:42 PM

to me

Dear Hani,

Thank you for making the payment. Soon, we will send you the galley proof.

regards,
Emily Flora

•••

hani plumeriastuti <hani-p@fkh.unair.ac.id> Dec 28, 2022, 7:39 AM

to Pharmacognosy

Dear Editor,

Thank you very much.
But we would like to apologize because we forgot to include the invoice number on the proof of payment.

Best regards,
Hani Plumeriastuti

•••

Pharmacognosy Journal <editor.phcogi@gmail.com> Dec 28, 2022, 12:13 PM

to me

No Issues.. we will look into this.

•••

Pharmacognosy Journal <editor.phcogi@gmail.com> Dec 29, 2022, 11:26 AM

to me

Dear Hani,

Please find the attached galley proof. Let me know if any changes are needed within 24hrs.

Regards,
Emily Flora

•••

One attachment • Scanned by Gmail

PJ-14-6s-1078.pdf

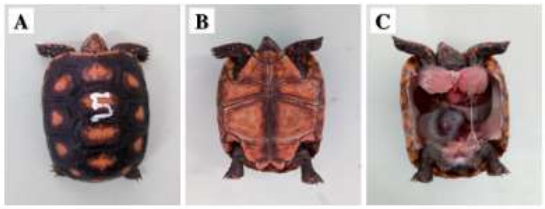
hani plumeriastuti <hani-p@fkh.unair.ac.id> Dec 29, 2022, 6:41PM

to Pharmacognosy

Dear Editor,

Thank you for sending the galley proof.
There are a few changes or additions that I would like to propose to this paper:

1. I would like to change the image in **Figure 1** to have a transparent border. A revised figure is attached.



2. I would like to change the last paragraph of the **Discussion** in the sentence "**The decrease in the number and function of osteoblasts, associated with altered expression of IL-7 and lipocalin-2, may have a negative impact on human immunity and thus increase sensitivity to infections**" to become "**The decrease in the number and function of osteoblasts, associated with altered expression of IL-7 and lipocalin-2, may have a negative impact on the body's immunity and thus increase sensitivity to infections**".

3. I would like to change the **Acknowledgment** into this: "**The authors express their gratitude to Fajar Dany Prabayudha as a herpetologist, Arif Nur Muhammad Ansori for helping improve the final writing of this manuscript, and the co-assistant students of the Division of Veterinary Pathology (Faculty of Veterinary Medicine, Universitas Airlangga) who assisted in finding the sample cases for this study**.".

Thank you for the help.

Pharmacognosy Journal <editor.phcogi@gmail.com> Dec 30, 2022, 4:41PM

to me

Dear Hani,

Kindly highlight the changes in the galley proof (not in word document) on a sticky note and send us back, so that our production team will make the changes accordingly.

regards,
Emily Flora

•••

hani plumeriastuti <hani-p@fkh.unair.ac.id> Dec 30, 2022, 9:39PM

to Pharmacognosy

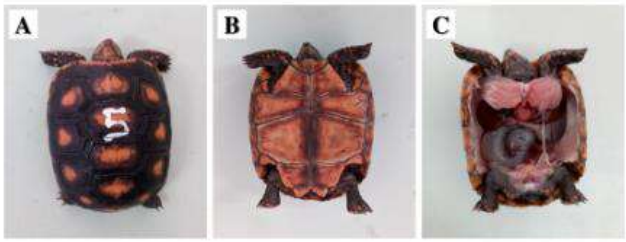
Dear Editor,

I have provided highlights and comments for some of the words and sentences in the attached galley proof.

Thank you very much.

Best regards,

Hani Plumeriastuti



Pharmacognosy Journal <editor.phcogi@gmail.com> Jan 2, 2023, 1:19 PM

to me

Dear Hani,
Please find the attached corrected galley proof.
Regards,
Emily Flora

•••

One attachment • Scanned by Gmail

PJ-14-6s-1078.pdf

hani plumeriastuti <hani-p@fkh.unair.ac.id> Jan 2, 2023, 9:27 PM

to Pharmacognosy

Dear Editor,

It looks perfect.
Thank you very much for your help and cooperation.

Best regards,
Hani Plumeriastuti

•••

Pharmacognosy Journal <editor.phcogi@gmail.com> Jan 2, 2023, 11:58 PM

to me

You are welcome.

•••