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Light Microscopic and Ultrastructural Characteristics of Heterophil Toxicity and Left-shifting in Green Sea Turtles (*Chelonia mydas*) from Taiwan

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Heterophil toxic change (TC) and left-shifting (LS) are widely used as indicators of accelerated granulopoiesis. However, the ultrastructure of heterophil TC and LS in sea turtles remain poorly understood. This study aimed to describe the ultrastructural characteristics of sea turtle TC and LS heterophils, compare the staining quality of accessible staining methods, and provide a better understanding of the clinical applications and limitations of heterophil TC and LS examinations. Blood samples were collected from 21 rescued sea turtles from January 2017 to September 2018. Morphologic (n = 22) and ultrastructural (n = 15) examination of TC and LS heterophils were performed, and the qualities of three staining methods (Wright-Giemsa stain, Diff-Quik stain and Liu's stain) were analyzed to diagnose TC and LS heterophils. In addition, the diagnostic values of TC and LS heterophils were examined. Diff-Quik stain was significantly inferior in the assessment of heterophil TC and/or LS comparing to the Wright-Giemsa stain and Liu's stain (Mann-Whitney test, $P < 0.001$). Microscopic examinations of heterophil TC and/or LS were comparable to transmission electron microscopy examinations (Cohen's kappa coefficient, $\kappa = 1$). The correlation between the presence of heterophil TC and/or LS and clinical inflammatory state was weak (Spearman's rank correlation coefficient, $rs = 0.171$, $p = 0.445$). In conclusion, this is the first study to describe the ultrastructural characteristics of reptile TC and LS heterophils. Wright-Giemsa stain and Liu's stain were suitable staining methods for the microscopic observations of TC and LS heterophil in sea turtles. Given the poor correlation between TC and/or LS and clinical findings, TC and LS are not a suitable diagnostic indicator of green sea turtles' inflammation status.

Key words: Green sea turtle, Heterophil toxic change, Left shift, Staining methods, Ultrastructure.

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BACKGROUND

Reptile hematology is heterogeneous among species (Claver and Quaglia 2009); knowledge in this field is developing rapidly based on well-established general studies (Wilikinson 2004). However, insufficient species-specific data sometimes makes it difficult to interpret clinical information of rare species. Green sea turtle (*Chelonia mydas*) is on the International Union for Conservation of Nature (IUCN) Red List of Endangered Species (IUCN 2018); it is common in Taiwan waters (Cheng et al. 2019) and has been under increased threats in recent years (Cheng et al. 2019). Diseased individuals presented in rescue facilities required sophisticated medical care.

Most sea turtle blood cell morphologies were thoroughly described and presented using the Wright-Giemsa stain (Wood and Ebanks 1984; Cannon 1992; Bradley et al. 1998; Work et al. 1998; Casal and Orós 2007; Di Santi et al. 2013). In veterinary facilities or circumstances in which such stains are unavailable, veterinarians in Taiwan have relied on Diff-Quik or Liu's stain for blood cell differentiation and toxic heterophil examination. However, the staining quality of toxic change (TC) and left shifting (LS) heterophil among these staining methods had not been evaluated in sea turtles, therefore the uses of Diff-Quik and Liu's stains are greatly limited and doubted.

Heterophil TC and/or LS are widely used by veterinary clinicians as an indicator of accelerated granulopoiesis (Stacy and Innis 2017). Commonly described morphological alterations in reptile toxic heterophils include increased cytoplasm basophilia, degranulation, cytoplasmic vacuolation, foamy cytoplasm, nuclear pleomorphism, and abnormal cytoplasmic granulations (Campbell 2006; Strik et al. 2007; Stacy et al. 2011; Nardini et al. 2013; Campbell 2015a b; Stacy et al. 2017; Stacy and Innis 2017). Detailed descriptions and high quality micrographs of heterophil toxicity in sea turtle species have been published in recent years (Stacy and Innis 2017). Although hetrophil TC and LS both indicate active inflammation, purple cytoplasmic granules without morphological features of toxicity indicates LS, not TC (Stacy and Innis 2017). Morphological features of LS include the presence of retained primary granules, increased nuclear size, or increased cell size (Stacy and Innis 2017). However, no ultrastructural studies have been done on heterophil TC and LS of any reptile species, even though ultrastructural characteristics of blood cells have been described in some sea turtle species (Casal et al. 2007; Orós et al. 2010; Zhang et al. 2011).

This study aimed to expand our understanding of heterophil TC and LS by identifying ultrastructure characteristics, comparing the staining quality of accessible staining methods, and understanding the clinical application and limitations of heterophil TC and LS examination in green sea turtles.

MATERIALS AND METHODS

Animals and sample collection

The subjects of this study were sea turtles that were either stranded, rescued at sea, or victims of fishery bycatch and subsequently entered into the sea turtle rescue system under the authority of the Forestry Bureau, Council of Agriculture, Executive Yuan of Taiwan from January 2017 to September 2018. Sea turtles were treated, cared for and rehabilitated at the Sea Turtle Rescue Center of Northern Taiwan in the National Taiwan Ocean University or the National Museum of Marine Biology and Aquarium under the supervision of veterinarians until released or deceased. Samples for health examination were obtained from blood collections at National Taiwan University Veterinary Hospital or the rehabilitation centers. The animal use protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of National Taiwan University (IACUC Approval No: NTU106-EL-00170). Different sizes of sterile syringes (1 ml syringe with 26G needle, 3 ml syringe with 24G needle or 5 ml syringe with 23G needle, TERUMO MEDICAL CORPORATION, Tokyo, Japan) were selected based on the turtle's body size and used to locate the dorsal jugular vein. The 0.4–1 ml blood taken from each individual was sufficient for sample analysis. The volume of blood taken from each turtle followed rules for max 3 ml/kg in sea turtles (National Marine Fisheries Service Southeast Fisheries Science Center 2008).

Sample preparation

For each individual, three blood smears were conducted immediately after sampling using the wedge technique with two microscope slides and left to air dry. No anticoagulant was used before making each blood film. Each blood film was stained with one of the three different staining methods. Wright-Giemsa stains were produced by an automatic slide stainer (HEMA-TEK 2000TM Slide Stainer, SIEMENS HEALTHCARE DIAGNOSTICS INC., Erlangen, Germany). The Diff-Quik stain (Diff-Quik, SYSMEX CORPORATION, Kobe, Japan) and Liu's stain (ASK® Liu's Stain A and B, TONYAR BIOTECH. INC., Taoyuan, Taiwan) were

manually prepared following user guides provided by the manufacturers. Liu's stain is a modified Romanowsky's stain composed of two dyes. Liu A, the anionic dye, contains eosin Y to stain cytoplasm and hemoglobin red. Liu B, on the other hand, is the cationic dye; it contains azur I and methylene azure, to stain the nucleus and basophilic granules blue. Stained blood smears were examined under a light microscope (CX22 LED Upright Microscope, OLYMPUS CORPORATION, Tokyo, Japan) at 1000× magnification with oil immersion to observe morphology. Intact, appropriately stained leukocytes in the monolayer part of the blood smear were identified, photographed and described.

The remaining blood samples were infused into lithium heparin anticoagulation tubes. Samples were stored at 4°C, and brought into the laboratory within 6 h. Heparinized whole blood was separated into two parts. One part was used for CBC and biochemistry analysis, and the other part was used for transmission electron microscope (TEM) sample processing. For CBC analysis, PCV was estimated through centrifugation (CN-830, HSIANGTAI MACHINERY INDUSTRY CO., LTD., New Taipei City, Taiwan) of capillary tubes (DIN ISO 12772, Assistent®, Germany), RBC counts and WBC counts were performed manually with a hematocytometer (Hemocytometer Reichert Bright-Line 1490, Hausser Scientific Horsham, USA) using Natt and Herrick's method; WBC differential counting was done by microscopic blood film examination described in the previous paragraph. For biochemistry analysis, aspartate aminotransferase, creatine kinase, calcium, total protein, blood urea nitrogen, glucose, creatinine, uric acid, phosphorus, alanine aminotransferase, and alkaline phosphatase were measured using an automated chemical analyzer (VITROS® 350 Chemistry System, John & Johnson company ortho-clinical diagnostic, Raritan, USA), and the remaining plasma was sent for protein electrophoresis (SPIFER® 3000 Electrophoresis Analyzer, HELENA LABORATORIES, Texas, USA) to measure albumin, alpha-1, alpha-2, beta, gamma globulins and the A/G ratio. The other part was used for TEM sample preparation by standard procedure as described in previous studies (Casal et al. 2007; Chansue et al. 2011; Zhang et al. 2011). Buffy coat was obtained by centrifugation (2200 G for 15 min), prefixed with 2.5% glutaraldehyde (Glutaraldehyde Solution 25%, MERCK, Darmstadt, Germany) and post-fixed with 1% osmium tetroxide (Osmium Tetroxide 4% Aqueous Solution, ELECTRON MICROSCOPY SCIENCES, Hatfield, USA). Fixed samples proceeded through serial dehydration with ethanol (Ethanol Absolute, MERK, Darmstadt, Germany) and serial infiltration with Spurr's resin (Low Viscosity Embedding Kit (By Dr. Spurr), ELECTRON MICROSCOPY SCIENCES). Infiltrated samples were embedded and placed through polymerization. An ultramicrotome (Ultramicrotome Leica EM UC7, LEICA, Wetzlar, Germany) with specialized diamond knives (DiATOME Diamond Knives Ultra 45°, DiATOME, Hatfield, USA) were used for ultramicrotomy of trimmed resin blocks. Ultrathin sections were double stained with uranyl acetate (Uranyl Acetate Crystals, MALLINCKRODT CHEMICAL WORKS, St. Louis, USA) and lead citrate (SPI-CHEM Lead Citrate, STRUCTURE PROBE, INC., West Chester, USA), and examined by TEM (JEM-1400 Electron Microscope, JEOL, Peabody, USA). Electoral micrographs were obtained with a CCD camera (ORIUSTM SC1000 CCD Camera, GATAN, INC., Pleasanton, USA). TC and LS heterophil ultrastructures were studied and described.

Study designs

The qualities of the Wright-Giemsa stain, Diff-Quik stain and Liu's stain were evaluated based on how reliably they detected heterophil TC and/or LS in sea turtles: 100 heterophils on each slide were examined and graded as excellent (95%–100% of the cell examined), good (80%–95%), borderline (65%–80%), poor (35%–65%), or undiagnostic (< 35%) depending on whether the applied stain demonstrated a result suitable for interpreting cellular changes. Staining features, including cytoplasmic granule integrity, nucleus appearance, proper staining and background artifacts, were taken into account.

The result of blood film examination using a light microscope to detect heterophil TC and LS during a 100-leukocyte count in sea turtles was correlated to the results of the ultrastructure based on the TEM examination. Blood samples were categorized as "TC and/or LS present" if TC and/or LS heterophils were seen during a 100-leukocyte count on a blood film, or if any abnormal heterophil ultrastructure was seen on TEM. "Toxic change absent" meant that no TC or LS heterophils were seen during a 100-leukocyte count on a blood film, or there was no difference in heterophil ultrastructure when compared with the normal ultrastructure described in the existing literature. The degree of toxic change was quantified as 1+/mild, 2+/moderate, or 3+/marked, while the quantity of cells affected was evaluated as 1+/few, 2+/many, and 3+/most.

The relevance of using blood film examination of heterophil TC and/or LS on systemic inflammation in sea turtles was evaluated by comparing the heterophil TC and/or LS examination results with clinical and/or pathological diagnosis. A clinical and/or

pathological diagnosis was made using a combination of detailed history taking, physical examination, radiology, complete hematology exam including CBC and biochemistry, plus advanced imaging, microbiological cultures and/or histology, if warranted. The inflammatory state of each sea turtle was ranked as "inflammation absent (IA)" and "inflammation present (IP)". IP was assigned after a comprehensive analysis of the above-mentioned subjective, objective and definitive diagnosis procedures. Instances such as fish net tangling, long term stranded in history taking and traumatic injury during physical examination were found in subjective procedures. Objective evidence, for example, could be pneumonia, gastrointestinal bloating/obstruction, arthritis under radiology survey; leukocytosis, heterophilia and monocytosis upon complete blood cell count. In addition, any direct evidence of inflammation/infection in microbiological and pathological diagnosis could provide definitive diagnosis of inflammation. The end point of each case was defined as the time when the clinical diagnosis and inflammatory status were determined. In all cases, history taking, physical examination, blood work and image study were finished on the same day within one week of the turtles being rescued, while results of the microbiology analysis were returned within another week. In this study, microscopic examination of heterophil TC and/or LS on blood films were considered a reliable way to evaluate systemic inflammation if the presence of heterophil TC and/or LS on blood films strongly associated with a more severe clinical inflammatory state, and vice versa.

The efficacy of using blood film examination of TC and/or LS to predict treatment outcomes in sea turtles was also evaluated. The treatment outcomes of the sea turtles were classified into "released (R)," "treatment and/or rehabilitation have been continued for over 6 months at the time of data analysis or rehabilitation was continued after the end of the disease episode (T)" and "dead (D)". The final outcomes (R and D) of the individuals were represented whenever possible, regardless of the treatment time course.

Statistical analysis

Normality was assessed with Kolmogorov-Smirnov test and all variables were non-normally distributed. To evaluate the quality of the different staining methods, the Mann-Whitney test was applied to measure whether there was a significant difference (p-value < 0.05) among the three staining methods on the production of appropriate morphological and staining properties suitable for interpreting heterophil TC and/or LS. To evaluate the quality of blood film examination of heterophil TC and LS, Cohen's kappa coefficient (κ) was measured to access the degree of agreement between blood film examination and TEM examination for heterophil TC and LS detection. Spearman's rank correlation coefficient (rs) was used to measure the statistical dependence between the rankings of two variables, namely the strength of the association between the presence of TC and/or LS and the clinical inflammatory state, and the strength of the association between the presence of TC and/or LS and the treatment outcome.

RESULTS

Population

A total of 22 blood samples were collected from 21 green sea turtles (*Chelonia mydas*) (one hatchling, ten juveniles, eight subadults, and two adults). The results of a second blood sampling (six months apart) from one green sea turtle hatchling were included in this study because of a significant difference in body condition. Blood film was examined for every sample, but only 15 of them were sent for TEM examination

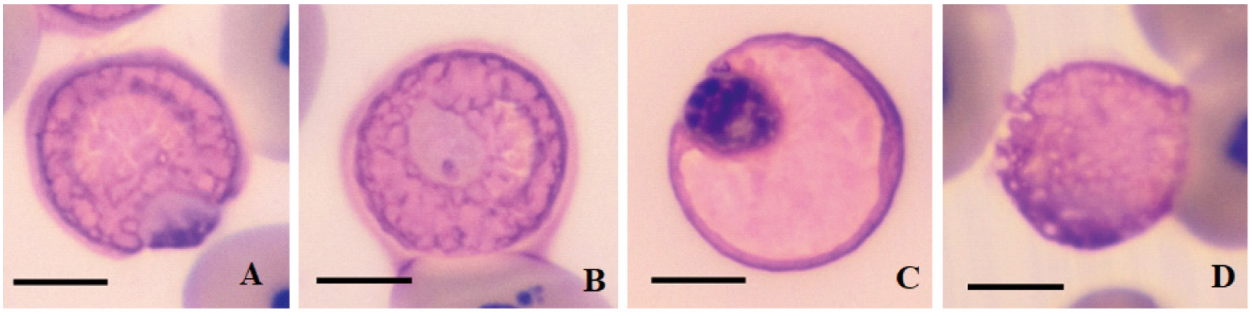

Fig. 1. Light microscope observation of heterophils from green sea turtles (*Chelonia myday*) studied in Taiwan. A: the eccentrically-located nucleus was pressed against the cellular membrane, Wright-Giemsa stain; B: a centrally located, palely stained nucleus, Wright-Giemsa stain; C: coalescing cytoplasmic granules were common in the Diff-Quik stain; D: the nucleus had an indistinct border and was partially obscured by cytoplasmic granules, Liu's stain. Scale bar = 10 μm.

owing to limited laboratory working hours and financial considerations.

Heterophil morphology and ultrastructure

Heterophils (Fig. 1) were similar in morphology and were the most commonly found granulocytes among the studied green sea turtles. TC and LS were generally rare—only seen on the blood films of two turtles. However, the degree of toxicity is marked with many or most of the cells affected in these two cases (Fig. 2). The most noticeable cellular changes included uneven staining of cytoplasmic granules, degranulation, increased cytoplasmic basophilia along with cytoplasmic changes, and the presence of basophilic cytoplasmic granules.

Ultrastructurally, heterophils (Fig. 3) were round in shape, with a few unremarkable small pseudopodia of the cellular membrane. The distinctive nucleus was eccentrically located and irregularly elliptical to elongated or bi-lobed in shape. A moderate amount of heterochromatin was seen scattered within the nucleus and aggregated around the nuclear membrane. Nucleoli were not prominent in most cells examined. The cytoplasm contained numerous round to elliptical or pleomorphic, moderately electron-dense granules, which were generally homogeneous in electron density, but could also be heterogeneous. Aside from the primary group of granules, a second type of granule was also evident that was significantly more electron-dense, more often rounded, and fewer in number. The two types of granules were comparable in size. Granules were distributed unevenly within the cytoplasm, resulting in only one type of granule being seen on some ultra-sections. Other cytoplasmic organelles were rare in heterophils of the sea turtles studied. Small numbers of mitochondria, rare single rows of rough-surfaced endoplasmic reticulum (RER) and small vesicles were occasionally found, while free ribosomes and polyribosomes were evenly dispersed within the cytoplasm. TC and/ or LS heterophils (Fig. 4) were extremely rare and difficult to find on ultra-sections in the sea turtles studied. The following are descriptions of 10–18 TC and/ or LS heterophils examined in two green sea turtles. Among the toxic heterophils examined, LS was commonly observed but not present in all cells.

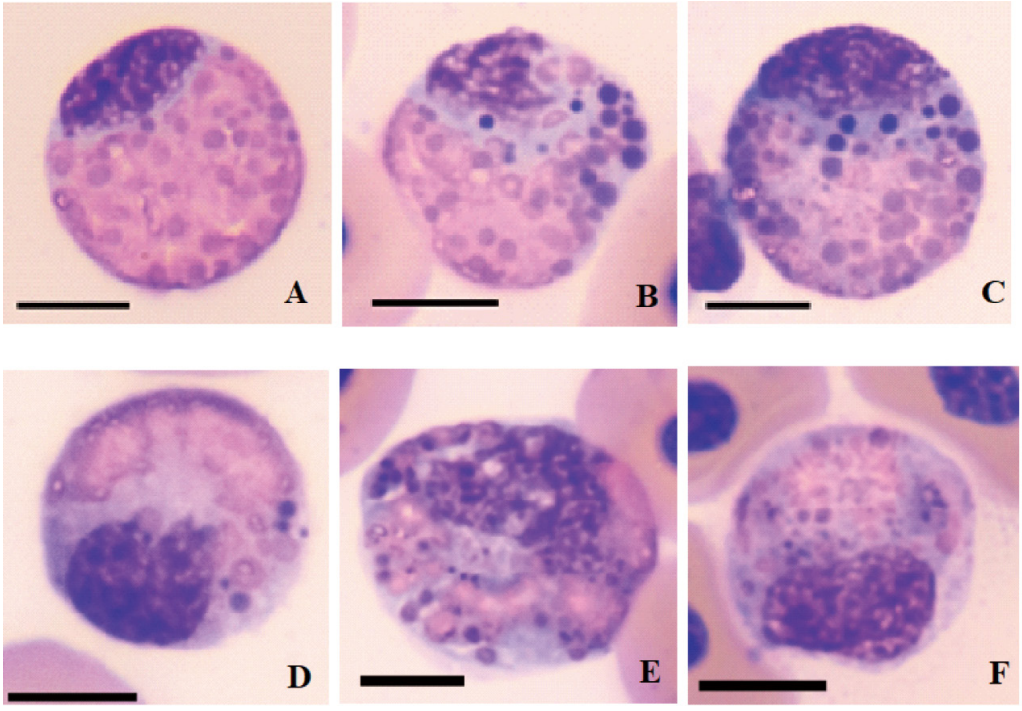

Fig. 2. Light microscope observation of toxic change and/or left-shifting of heterophils in green sea turtles in Taiwan. Wright-Giemsa stain. A: a left-shifted, non-toxic heterophil with primary and secondary granules; B: a left-shifted and mildly toxic heterophil with primary and secondary granules, increased cytoplasmic basophilia, and presence of basophilic granules; C: a left-shifted and mildly toxic heterophil with primary and secondary granules, more prominent cytoplasmic basophilia, and presence of basophilic granules; D: toxic heterophil with decreased number of cytoplasmic granules, marked cytoplasmic basophilia, and presence of basophilic granules; E: toxic heterophil with marked degranulation, marked cytoplasmic basophilia, and presence of more basophilic granules; F: toxic heterophil with marked degranulation and few eosinophilic granules remained, marked cytoplasmic basophilia, and presence of more basophilic granules. Scale bar = 10 μm.

The LS heterophil was characterized by a rounded and less indented nucleus, a higher N/C ratio, one or more prominent nucleoli, and a higher proportion of active euchromatin relative to the clumped and condensed, inactive heterochromatin. Compared with normal heterophils, toxic heterophils were rounded in shape and had a smooth cellular membrane. The number of cytoplasmic granules was significantly reduced. Granules generally became smaller or more variable in size, and while some granules remained rounded, others appeared more pleomorphic and had a less distinct membrane. As a result, the types of cytoplasmic granules could not be distinguished for certain. Some small, electron-dense granules were seen dispersed within the cytoplasm. Changes in electron density and structure within the primary granules were not apparent. The cytoplasm contained a much greater number of membranous organelles, such as mitochondria, Golgi apparatus, vesicles, and excessive RER (arranged dispersedly in the form of a single row, not in lamellar form). Clusters of dark polyribosomes were more numerous and clearly visible. No phagocytized materials were observed in the toxic heterophils examined.

Quality of different staining methods

The staining qualities of TC and/or LS heterophils were measured (Table 1). With the Wright-Giemsa stain, 85.7% of the slides were ranked diagnostic (above borderline) or better for producing appropriate morphological and staining properties were deemed suitable for interpreting heterophil TC and/or LS. With Liu's stain, 50% of the slides were diagnostic, but this was only 5% when using Diff-Quik stain. That is, over 95% of the slides that were Diff-Quik stained were ranked as poor or undiagnostic. No significant difference was found between Wright-Giemsa stain and Liu's stain (Mann-Whitney test, P-value = 0.060) in regards to the performance of heterophil TC and/or LS diagnosis. A significant difference was found between Diff-Quik stain and Wright-Giemsa stain (Mann-Whitney test, P-value < 0.001), and also between Diff-Quik stain and Liu's stain (Mann-Whitney test, P-value <0.001). Slides rendered poor or undiagnostic were due to significant drying artifact, coalescing of granules, or overstaining (Fig. 5).

Diagnostic value of heterophil TC and/or LS

The degree of agreement between the blood film and TEM examinations was measured for the 15 samples sent for TEM examination. Cohen's kappa coefficient (κ) = 1 indicated complete agreement between the two examinations (n = 15).

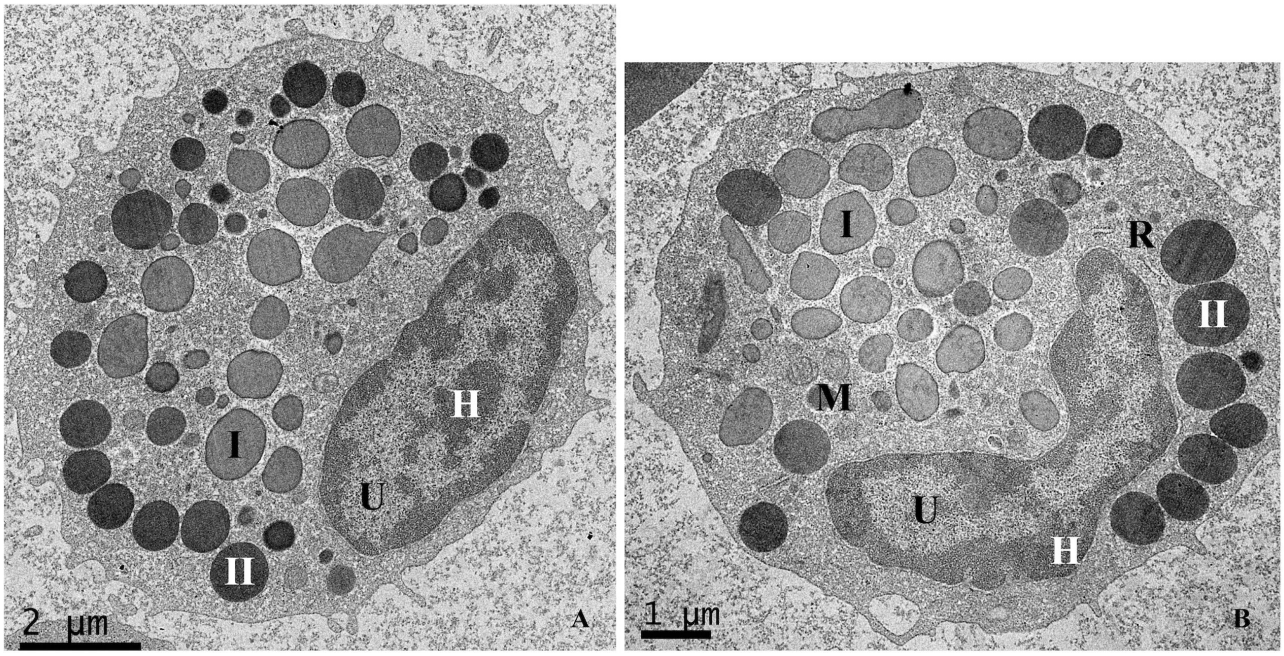

Fig. 3. Electron microscope observation of heterophil ultrastructure in green sea turtles (*Chelonia mydas*) studied in Taiwan. All cells were stained with uranyl acetate and lead citrate. H: heterochromatin; U: euchromatin; I: first type of granules; II: second type of granules; M: mitochondria; R: rough-surfaced endoplasmic reticulum (RER). A: the first type of granules were generally homogeneous in electron density, and a second type of granules were more electron-dense, fewer in number and comparable to the first type of granules in size, B: the two types of granules distributed unevenly within the cytoplasm.

Clinical and/or pathological diagnoses in the sea turtles were reported (Table 2). Also, to better comprehend the overall inflammatory conditions of the sea turtles, leukograms at absolute value were provided (Table 3). Five individuals were categorized in group IA, and 17 in group IP. Among them, heterophil TC and/or LS were present in the 100-leukocyte count blood film exams of two subadult green sea turtles. One had ileocolic intussusception and necrotic enteritis, and the other had complete carapace fracture, multiple full thickness plastron wounds, coelomitis and bacteremia; both were placed in the IP group. Heterophil TC and

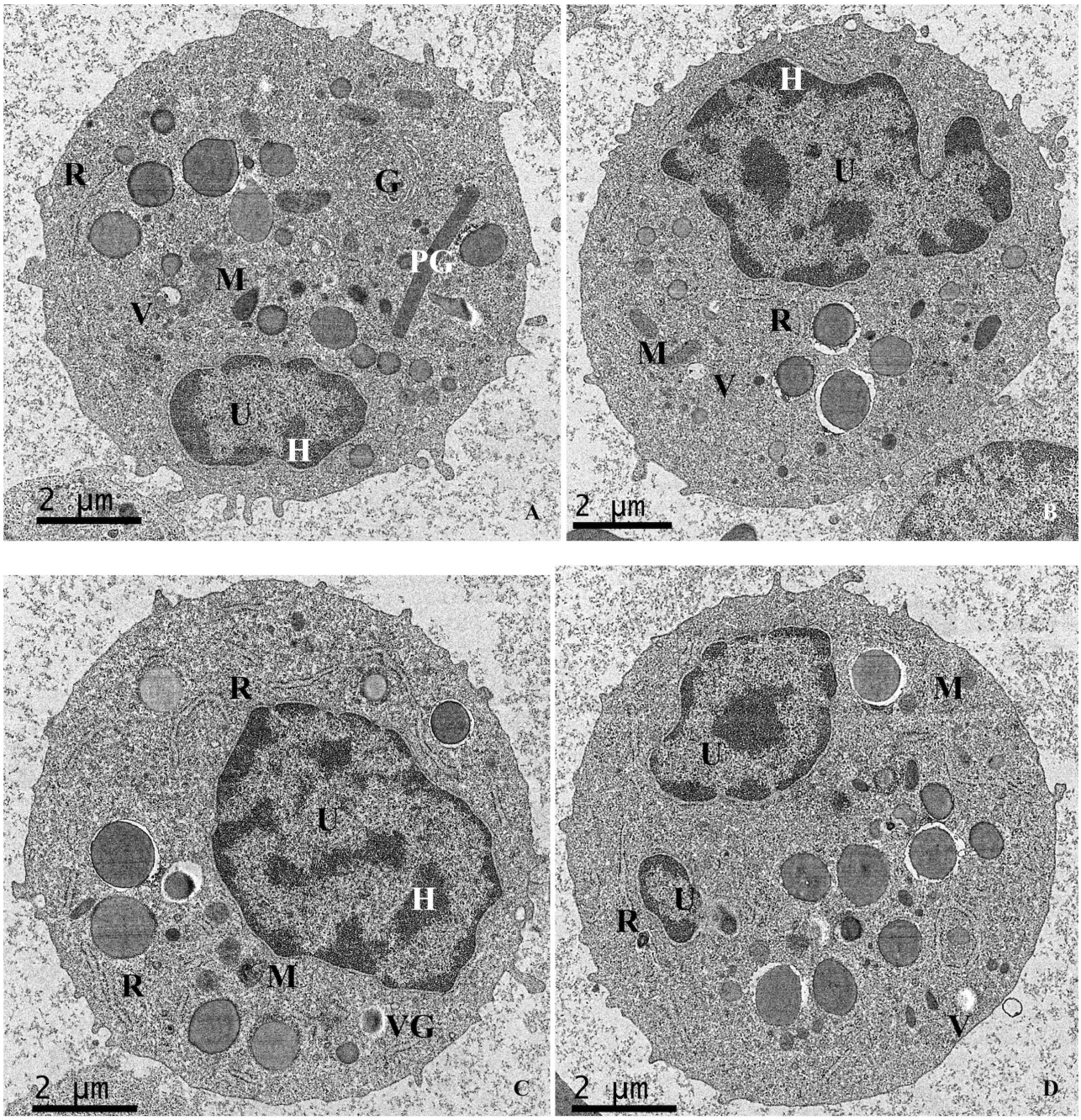

Fig. 4. Electron microscope observation of toxic heterophil ultrastructure in green sea turtles in Taiwan. All stained with uranyl acetate and lead citrate. H: heterochromatin; U: euchromatin; M: mitochondria; G: Golgi apparatus; V: vesicles; R: endoplasmic reticulum (ER); PG: pleomorphic granules. A: reduced number of cytoplasmic granules, granules became smaller or more variable in size, pleomorphic granules, prominent Golgi apparatus; B: higher N/C ratio and significantly reduced granules; C: reduced granules, abundant RER arranged dispersedly in the form of a single row; D: reduced granules, granules became smaller or more variable in size, prominent RER arranged dispersedly in the form of a single row.

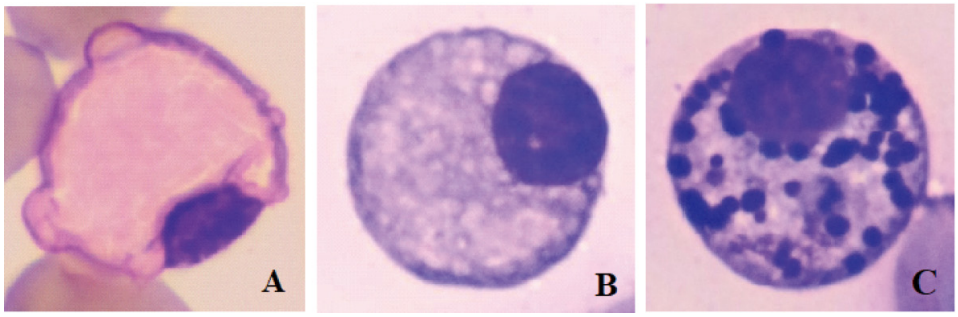

Fig. 5. Light microscope observation of leukocytes from sea turtles studied in Taiwan. Examples of staining properties unsuitable for heterophil toxic change diagnosis. A–C: Examples of failure to demonstrate appropriate staining properties for reliable interpretation of cellular changes. During the 100-heterophil count, the higher the percentage of these kinds of cell seen on a stained slide, the less diagnostic the slide; A: granule coalescence caused the loss of cytoplasmic detail, therefore changes in the granule number/staining properties/size and shape could not be reliably assessed, Diff-Quik stain; B: overstaining caused abnormal coloration of the cell, therefore cytoplasmic basophilia and changes of the granule staining properties could not be reliably assessed, Diff-Quik stain; C: overstaining caused abnormal coloration of the granules, therefore changes in the granule staining properties could not be reliably assessed, Liu's stain.

Table 1. Frequency of three staining methods in cytologic diagnosis of heterophil toxic change: 85.71% of the slides were ranked diagnostic (above borderline) or better in producing a reliable diagnosis of heterophil toxic change with the Wright-Giemsa stain and 50% with Liu's stain; over 96% of the slides were ranked as poor or undiagnostic using Diff-Quik stain. No significant difference was found between the Wright-Giemsa stain and Liu's stain (Mann-Whitney test, P-value = 0.060) in regard to the performance of heterophil toxic change diagnosis. A significant difference was found between the Diff-Quik stain and Wright-Giemsa stain (Mann-Whitney test, P-value < 0.001), and between Diff-Quik stain and Liu's stain (Mann-Whitney test, P-value < 0.001)

100 heterophils per slide	Wright-Giemsa stain	Diff-Quik stain*	Liu's stain
Excellent (95–100)	5/21 (23.81%)	0/21 (0%)	2/21 (10.00%)
Good (80–95)	5/21 (23.81%)	0/21 (0%)	5/21 (25%)
Borderline (65–80)	8/21 (38.10%)	1/21 (5.00%)	3/21 (15.00%)
Poor (35–65)	2/21 (9.52%)	1/21 (5.00%)	6/21 (30.00%)
Undiagnostic (< 35)	1/21 (4.67%)	18/21 (90.00%)	4/21 (20.00%)

Table 2. To find out the clinical application of heterophil toxicity in green sea turtle, 21 rescued green sea turtles (*Chelonia mydas*) were assigned to groups of presence or absence of inflammation based on history, physical exam, leukogram findings, imaging study and microbiology, pathology result if applicable. Results of light microscopic and transmission electron microscopic examination were correlated with clinical inflammatory state, and treatment outcome of the studied sea turtles. Cut-off values and reference for blood work interpretations with absolute numbers are listed in table 3

ID	Age group	Clinical diagnosis	Inflammation (Present/ Absent)	Findings consistent with inflammation (Y/N)								Heterophil toxic change		Outcome
				History	Physical exam	Leuko-cytosis	Hetero-philia/ increased het%	Mono-cytosis/ increased mono%	Imaging	Micro-biology	Patho-logy	LM	TEM	
1063973	Hatchling	Healthy	A	N	N	N	N/Y	N/N	N	-	-	N	N	T
1063973-0621	Hatchling	Necrotic renal granuloma	P	N	N	N	N/Y	N/N	-	-	Y	N	-	D
1054660	Juvenile	Necrotizing enteritis, pneumonia, systemic spirorchidiasis, chronic nephritis	P	Y	Y	N	Y/Y	N/N	Y	Y	Y	N	N	D

Table 2. (Continued)

ID	Age group	Clinical diagnosis	Inflammation (Present/ Absent)	Findings consistent with inflammation (Y/N)								Heterophil toxic change		Outcome
				History	Physical exam	Leuko-cytosis	Hetero-philia/ increased het%	Mono-cytosis/ increased mono%	Imaging	Micro-biology	Patho-logy	LM	TEM	
1064184	Juvenile	Healthy	A	N	N	N	Y/Y	N/N	N	-	-	N	N	R
1070022	Juvenile	Healthy	A	N	N	N	N/Y	N/N	N	-	-	N	N	R
1070856	Juvenile	Open fracture, osteomyelitis	P	Y	Y	N	N/Y	N/N	Y	-	-	N	N	R
1071086	Juvenile	Corneal ulceration	A	N	N	N	N/N	N/N	N	-	-	N	N	R
1071085	Juvenile	Pneumonia	P	N	N	N	N/Y	N/N	Y	-	-	N	-	R
1071150	Juvenile	Osteomyelitis	P	Y	Y	N	Y/Y	N/N	Y	Y	-	N	-	R
1071443	Juvenile	Gastrointestinal foreign body	P	Y	Y	N	Y/Y	N/N	Y	-	-	N	-	R
PH2337	Juvenile	Neuroblastoma, periophthalmitis, systemic spirorchidiasis, enteritis	P	Y	Y	N	Y/Y	N/N	-	-	Y	N	-	D
1072633	Juvenile	Gastrointestinal foreign body	P	N	N	N	Y/Y	N/N	Y	-	-	N	-	R
G9	Subadult	Trauma	P	Y	Y	N	Y/Y	N/N	-	-	-	N	N	R
G46	Subadult	Herpes infection, papilloma	P	Y	Y	N	N/N	N/N	-	-	-	N	N	T
G47	Subadult	Herpes infection, papilloma	P	Y	Y	N	N/N	N/N	-	-	-	N	N	T
G48	Subadult	Capture myopathy, wounds from web trapping	P	Y	Y	N	N/N	N/N	-	-	-	N	N	R
G49	Subadult	Superficial wounds, malnutrition	A	N	N	N	N/N	N/N	-	-	-	N	N	R
1061896	Subadult	Ileocolic intussusception, ulcerative and necrotizing enteritis, spirorchidiasis	P	N	N	N	Y/Y	N/N	Y	-	Y	Y*	Y	D
1070170	Subadult	Pneumonia	P	N	N	N	N/Y	N/N	Y	-	-	N	-	R
1072678	Subadult	Complete carapace fractures, full thickness plastron wounds, osteomyelitis, coelomitis, bacteremia	P	Y	Y	N	N/Y	N/N	Y	Y	Y	Y**	Y	D
1070557	Adult	CNS spirochid infection (meningoencephalitis and ventriculitis, spinal cord meningitis), aortitis, hepatitis	P	N	N	Y	Y/Y	N/N	N	-	Y	N	N	D
268307111	Adult	Enteritis	P	N	N	-	-/-	-/-	-	-	Y	N	N	D

LM, light microscopy exam of blood films; TEM, transmission electron microscopy exam; Y, specific finding consistent with inflammation; N, specific finding does not consistent with inflammation; P, classified as presence of inflammation clinically; A, classified as absence of inflammation clinically; R, released; T, treatment and/or rehabilitation have been continued for over 6 months at the time of data analysis or at the end of the disease episode; D, dead; CNS, central nervous system. * Most of the heterophils were presented as marked toxic change under light microscopy examination. ** Many of the heterophils were presented as marked toxic change under light microscopy examination; –, not evaluated.

LS were absent in all other samples, regardless of the clinical inflammatory state. The correlation between the presence of heterophil TC and/or LS and the clinical inflammatory state of the sea turtles was measured. Spearman's rank correlation coefficient (*rs*) was 0.171, indicating a very weak positive (*rs* < 0.20 by definition) and insignificant (p = 0.445) correlation between the two (N = 22).

The treatment outcomes for all the sea turtles were documented and ranked on the prognosis from "released (R)" to "dead (D)." Twelve sea turtles were categorized in group R, and three were categorized in group T; in all of them, toxic change was absent from the 100-leukocyte count. Seven individuals were categorized in group D, including one hatchling sea turtle that was sampled twice due to different disease episodes; among them TC and/or LS were present in a subadult green sea turtle with ileocolic intussusception and necrotic enteritis, but was absent in all others. TC and/or LS were present in the subadult green sea turtle with the complete carapace fracture, multiple full thickness plastron wounds, coelomitis and bacteremia, but absent from the other three. The correlation between the presence of toxic change and the outcome of the sea turtle treatment was measured. Spearman's rank correlation coefficient (*rs*) was -0.392, indicating a weak negative (*rs* = -0.20 to -0.39 by definition) and insignificant (p = 0.071) correlation between the two (n = 22).

DISCUSSION

This study aimed to expand our understanding of heterophil TC and LS. To our knowledge, this is the first study describing reptile TC and LS ultrastructures and demonstrate staining differences.

TC and LS are well-recognized indicators of

Table 3. Leukograms of 21 rescued green sea turtles (*Chelonia mydas*); the information provides a further understanding of the inflammatory status of studied individuals, which are correlated with the results of light microscopic and transmission electron microscopic examination of toxic heterophil

ID	Age group	Leukogram					
		WBC (x 10^9/L)	Heterophil (x 10^9/L); Heterophil(%)	Lymphocytes (x 10^9/L); Lymphocytes(%)	Monocytes (x 10^9/L); Monocytes(%)	Eosinophil (x 10^9/L); Eosinophil(%)	Basophil (x 10^9/L); Basophil(%)
Reference (Lewbart et al. 2014)	23 immature 5 mature	6.58 (1.76–22.4)	1.06 (0.23–2.59); 16.4(8–35)	3.42 (range error); 50.5(33–67)	0.79 (0.18–2.92); 12(6–32)	1.41 (0.37–5.16); 20.8(5–31)	0.02 (0–0.13); 0.36(0–2)
1063973	Hatchling	2.886	2.136; 73	0.345; 12	0.260; 9	0; 0	0.144; 5
1063973-0621	Hatchling	3.330	1.365; 41	1.199; 36	0.766; 23	0; 0	0; 0
1054660	Juvenile	5.550	4.052; 73	1.443; 26	0; 0	0.056; 1	0; 0
1064184	Juvenile	9.546	4.773; 50	3.627; 38	0.955; 10	0.191; 2	0; 0
1070022	Juvenile	6.216	2.238; 36	3.232; 52	0.684; 11	0.062; 1	0; 0
1070856	Juvenile	7.770	3.885; 50	3.108; 40	0.777; 10	0; 0	0; 0
1071086	Juvenile	3.774	0.830; 22	2.491; 66	0.415; 11	0.038; 1	0; 0
1071085	Juvenile	3.108	1.336; 43	1.368; 44	0.404; 13	0; 0	0; 0
1071150	Juvenile	7.104	3.836; 54	2.771; 39	0.497; 7	0; 0	0; 0
1071443	Juvenile	7.548	5.057; 67	2.189; 29	0.302; 4	0; 0	0; 0
PH2337	Juvenile	4.662	3.263; 70	1.305; 28	0; 0	0.093; 2	0; 0
1072633	Juvenile	9.324	5.874; 63	2.145; 23	1.305; 14	0; 0	0; 0
G9	Subadult	5.106	1.838; 36	2.757; 54	0.306; 6	0.204; 4	0; 0
G46	Subadult	4.662	0.559; 12	3.357; 72	0.326; 7	0.420; 9	0; 0
G47	Subadult	7.770	0.932; 12	5.750; 74	1.088; 14	0; 0	0; 0
G48	Subadult	6.438	0.644; 10	0.521; 81	0.515; 8	0.644; 1	0; 0
G49	Subadult	4.662	0.793; 17	2.983; 64	0.606; 13	0.280; 6	0; 0
1061896	Subadult	6.216	2.984; 48	1.492; 24	1.430; 23	0.186; 3	0.124; 2
1070170	Subadult	3.108	1.273; 64	0.870; 28	0.249; 8	0; 0	0; 0
1072678	Subadult	4.218	2.404; 57	0.464; 11	1.350; 32	0; 0	0; 0
1070557	Adult	26.418	19.285; 73	3.434; 13	2.113; 8	1.585; 6	0; 0
268307111	Adult	-	-	-	-	-	-

WBC: white blood cell. -: not evaluated.

accelerated granulopoiesis in inflammatory diseases. It is known from the studies of human neutrophils that increases in polyribosomes and RER indicate increased protein synthesis, while primary granulations are present in a more primary state of heterophil development, and result from an altered affinity for Romanowsky stain stain that is possibly due to alterations in the membrane composition, but no ultrastructural differences were found when compared with normal granules (McCall et al. 1969; Schofield et al. 1983; Maxwell and Robertson 1998). Similar cellular changes are presumed in TC and/or LS heterophils of reptiles and birds. This study confirmed that increased cytoplasmic basophilia resulted from increased clusters of polyribosomes and excessive RER (arranged dispersedly in the form of a single row, not in lamellar form), and while a reduced number of granules and some changes in shape were observed, no significant ultrastructural differences were seen between granules of normal and TC and/or LS heterophils. However, the extensive membrane ruffling and pseudopod formation, enlarged ellipsoidal rod-shaped dense granules and vacuolation observed ultrastructurally in chickens' toxic heterophils were not seen in sea turtles (Latimer et al. 1988). As in mammals, cytochemical studies on TC and LS heterophils and studies on bone marrows will be essential in the future to understand the comparative aspects between species.

To answer the question of whether TC and/or LS are sensitive and reliable indicators of systemic inflammation in sea turtles, this study presents the correlations among each individual's clinical inflammatory state, whether TC and/or LS were present on blood film examination, and the treatment outcome. The results suggest that TC and/or LS are not as sensitive or reliable in evaluating a sea turtle's inflammatory/infectious state or prognosis as they are in many other species, but the presence of TC and/or LS did truly represent severe infection and the poor outcome of two individuals. According to the results of the current study, clinicians should take other clinical findings such as disease history, results of a physical exam, absolute leukocyte count, absolute heterophil count, heterophil percentage and other diagnostic results into consideration instead of only heterophil TC and/or LS when evaluating the inflammatory state of sea turtles (Table 2).

A great challenge that this study faced was the difficulty in obtaining electron micrographs of targeted cell types of sufficient quality and quantity. Blood cells were separated by centrifugation according to cell densities. However, unlike the clearly layered mammalian blood cells in the buffy coat, the separation of layers is much less evident and ineffective in reptiles. Heterophils, for instance, are present among erythrocytes and mononuclear cells, while erythrocytes are normally present in the buffy coat but contaminate all layers (Fontes et al. 2018). It is therefore nearly impossible to target a specific cell type prior to examining the sections. The complicated TEM sample processing added more risk of creating artifacts or damaging the samples. For example, the repeated rinsing necessary to stain with uranyl acetate and lead citrate often leads to creases on the ultrathin sections; in the worst case, loss of sections can occur. Finally, the morphology and ultrastructure of TC and/or LS described in the current study were based on two green sea turtles, and discrepancy may remain, even though the authors tried to present as many examples as possible.

CONCLUSIONS

This is the first study to describe the ultrastructural characteristics of TC and LS heterophils, and found that the Wright-Giemsa stain and Liu's stain were suitable staining method for observing TC and LS heterophils under a light microscope in green sea turtles. Given the poor correlation between TC and/or LS and clinical findings, TC and LS are not suitable diagnostic indicators in green sea turtles. These clinical applications will be beneficial in the medical care, and consequently conservation, of endangered sea turtle species.

List of abbreviations

TC, toxic change.
LS, left-shifting.

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Ethics approval consent to participate: Our study was performed according to the regulations from the Institutional Animal Care and Use Committee of National Taiwan University IACUC Approval No: NTU106-EL-00170. Furthermore, our study was performed according to the international, national and institutional rules on animal experimentation.

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