Morphology and cytochemical patterns of peripheral blood cells of tiger frog (*Rana rugulosa*)

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Background.Tiger frog (*Rana rugulosa*) is a national second-class protected amphibian species in China with an important ecological and economic value. Blood cells play a vital role in gas transportation, immune defense and hemostasis. The morphology and number of different types of blood cells could reflect the health status of the animals. The objective of this study was to investigate the morphometry, microstructure and cytochemical patterns of peripheral blood cells in tiger frogs. Our results provided the hematologic basic data for clinical evaluation and pathological studies on tiger frogs.**Methods**. The number of blood cells in tiger frogs was counted on a blood count board, and the cell sizes were measured by a micrometer under light microscope. The morphology and classification of blood cells were studied by Wright-Giemsa staining, and the cytochemical pateerns was investigated by various cytochemical staining including periodic acid-Schiff (PAS), Sudan black B (SBB), peroxidase (POX), alkaline phosphatase (AKP), acid phosphatase (ACP), chloroacetic acid AS-D naphthol esterase (CAE) and α-naphthol acetate esterase (ANAE) staining. **Results**. Besides erythrocytes and thrombocytes, five types of leukocytes were identified in tiger frogs: neutrophils, eosinophils, basophils, lymphocytes and monocytes. The mean number of erythrocyte (RBC) was 1.33±0.15 millions/mm^3 in females and 1.12±0.08 millions/mm^3 in males. The mean leukocyte (WBC) count was $3.73±0.04×10^4$ mm^3 in females and $3.2±0.02×10^4$ mm^3 in males. The mean thrombocyte (TC) count was $1.7±0.01×10^4 mm^3$ in females and $1.9±0.04×10^4$ mm^3 in males. There were no statistically significant differences in the RBC, WBC and TC counts between female and male tiger frogs ($P > 0.05$). Small lymphocytes were the most abundant leukocytes, followed by large lymphocytes, Neutrophils, eosinophils and monocytes, basophils were the fewest. No significant differences in leukocytes percentage were found between two sexes. Monocytes were the largest, followed by neutrophils and eosinophils, large lymphocytes and basophils were smaller, small lymphocytes were the smallest. No statistically

significant differences in the size of various leukocytes between male and female frogs. Eosinophils were strongly positive for PAS, positive for SBB, POX, ACP, CAE, ANAE, while weakly positive for AKP staining; basophils were strongly positive for PAS, ACP, positive for SBB, CAE, weakly positive for ANAE, negative for AKP, POX staining; neutrophils were strongly positive for ACP, SBB, positive for PAS, POX, weakly positive for AKP, CAE and ANAE staining; monocytes were positive for PAS, SBB, ANAE, weakly positive for ACP, AKP, POX, CAE staining; large lymphocytes and thrombocytes were positive for PAS, ACP, weakly positive for ANAE, while negative for SBB, POX, AKP, CAE; small lymphocytes were similar to large lymphocytes, except for strongly positive for PAS and ACP staining.

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Abstract

Background.Tiger frog (*Rana rugulosa*) is a national second-class protected amphibian species in China with an important ecological and economic value. Blood cells play a vital role in gas transportation, immune defense and hemostasis. The morphology and number of different types of blood cells could reflect the health status of the animals. The objective of this study was to investigate the morphometry, microstructure and cytochemical patterns of peripheral blood cells in tiger frogs. Our results provided the hematologic basic data for clinical evaluation and pathological studies on tiger frogs.

Methods. The number of blood cells in tiger frogs was counted on a blood count board, and the cell sizes were measured by a micrometer under light microscope. The morphology and classification of blood cells were studied by Wright-Giemsa staining, and the cytochemical pateerns was investigated by various cytochemical staining including periodic acid-Schiff (PAS), Sudan black B (SBB), peroxidase (POX), alkaline phosphatase (AKP), acid phosphatase (ACP), chloroacetic acid AS-D naphthol esterase (CAE) and α-naphthol acetate esterase (ANAE) staining.

Results. Besides erythrocytes and thrombocytes, five types of leukocytes were identified in tiger frogs: neutrophils, eosinophils, basophils, lymphocytes and monocytes. The mean number of erythrocyte (RBC) was 1.33±0.15 millions/mm^3 in females and 1.12±0.08 millions/mm^3 in males. The mean leukocyte (WBC) count was 3.73±0.04×10^4 mm^3 in females and 3.2±0.02×10^4 mm^3 in

males. The mean thrombocyte (TC) count was $1.7\pm0.01\times10^4 mm^3$ in females and $1.9\pm0.04\times10^4$ mm^3 in males. There were no statistically significant differences in the RBC, WBC and TC counts between female and male tiger frogs ($P > 0.05$). Small lymphocytes were the most abundant leukocytes, followed by large lymphocytes, Neutrophils, eosinophils and monocytes, basophils were the fewest. No significant differences in leukocytes percentage were found between two sexes. Monocytes were the largest, followed by neutrophils and eosinophils, large lymphocytes and basophils were smaller, small lymphocytes were the smallest. No statistically significant differences in the size of various leukocytes between male and female frogs. Eosinophils were strongly positive for PAS, positive for SBB, POX, ACP, CAE, ANAE, while weakly positive for AKP staining; basophils were strongly positive for PAS, ACP, positive for SBB, CAE, weakly positive for ANAE, negative for AKP, POX staining; neutrophils were strongly positive for ACP, SBB, positive for PAS, POX, weakly positive for AKP, CAE and ANAE staining; monocytes were positive for PAS, SBB, ANAE, weakly positive for ACP, AKP, POX, CAE staining; large lymphocytes and thrombocytes were positive for PAS, ACP, weakly positive for ANAE, while negative for SBB, POX, AKP, CAE; small lymphocytes were similar to large lymphocytes, except for strongly positive for PAS and ACP staining.

Key words: *Rana rugulosa*. Blood cell. Hematological parameters. morphology. Cytochemistry

Introduction

Blood cells are vital to the animal body. The vertebrate blood cells can be grouped into

erythrocytes, leukocytes and thrombocytes. The erythrocytes serve as carriers for transporting oxygen and carbon dioxide (Fang et al., 2014; Yoshida, Prudent & Alessandro, 2019). The leukocytes are responsible to protect the body against both infectious disease and foreign invaders, while the thrombocytes play a major role in hemostasis and coagulation (Salakij et at., 2000; Arikan & Cicek, 2014; Peng et at., 2018). Blood cells are sensitive to changes in the animal internal physiological states and stimuli from the external environment, the morphology and number of different types of blood cells could reflect the health status of the animals, and their abnormal variations may be associated with inflammation, pathogenic microorganism infection or other diseases (Fang et al., 2014; Peng et al., 2018; Kehoe et al., 2020).

Wright-Giemsa staining was widely used to observe the cell morphology and identify the blood cell types; meanwhile, various cytochemical stains were employed to detect the cellular chemical composition and recognize the functions of different blood cell types (Tavares-Dias & Marques-Barcellos, 2005; Kehoe et al., 2020; Oliveira et al., 2021). Intracellular glycogen and lipid can be displayed by Periodic acid-Schiff (PAS) and Sudan black B (SBB) staining, respectively, which may provide energy for phagocytosis (Ueda et al., 2001). Acid (ACP) and alkaline (AKP) phosphatases are lysosomal enzymes also related to the phagocytic process, being discharged into the phagocytic vacuoles of leukocytes that have ingested bacteria and other particles (Hirsch & Cohn, 1964). The peroxidase enzyme (POX) is a lysosomal enzyme which may play a significant role in the defense against bacterial infection (Bielek 1981; Dvorak, Estrella & Ishizaka, 1994). Chloroacetic acid AS-D naphthol esterase (CAE) is a specific granulocyte esterase, which may be responsible for cellular defense, facilitating diapedesis, toxic

product and microorganism inactivation, while α-naphthol acetate esterase (ANAE) is a non-specific esterases, which may play a crucial role in the processing and antigen-presenting of intracellular toxin and small molecules (Ueda et al., 2001; Azevedo & Lunardi, 2003).

At present, the classification, morphology and cytochemistry of peripheral blood cells have been generally investigated in various vertebrates, especially in human and mammals (Salakij et al., 2000; Techangamsuwan et al., 2010; Fang et al., 2014; Hernández et al., 2017). Amphibians are the transitional group of vertebrates from aquatic to terrestrial mode of life. In recent decades, a pandemic loss in amphibian biodiversity has occurred due to the destruction of ecological environment, climate change, and the spread of diseases (Zhou et al., 2004; Gutierre et al., 2008; Bricker, Raskin & Densmore, 2012). However, the immune system and the defence mechanisms of amphibians are poorly known, relatively few studies were reported on the morphology and especially the cytochemistry of amphibian blood cells. Madhusmita & Kumari (2014) have been only described the morphological features of dubois's tree frog (*Polypedates teraiensis*) peripheral blood cells; Gutierre et al. (2008) have been only detected the glycogen particles, lipids and peroxidase in the caecilian *Siphonops annulatus* granulocytes; Bricker, Raskin & Densmore (2012) compared the morphology and four cytochemical stains in the peripheral blood cells of American bullfrog (*Rana catesbeiana*) and African clawed frog (*Xenopus laevis*), finding erythrocytes and leukocytes of American bullfrog were larger than those of African clawed frog, as well as significant species specific differences in cell percentage and cytochemical patterns of the blood cells between these two amphibian species.

Tiger frog (*Rana rugulosa*), belonging to Amphibia, the Ranidae of Anura, is a species of

large edible frog widely distributed from southwest and south of china to south and southeast of Asia (Tian et al., 2011). The excellent flesh quality and high nutritional values make this species popular with consumers in China. In recent 20 years, due to excessive human hunting, pollution and habitat loss, the wild population of tiger frog has declined sharply in various places, and even become endangered in some areas (Tian et al., 2011; Li et al., 2014). To protect wildlife resources and meet the market demand, tiger frog has been listed as a national second-class protected species, and its artificial breeding has rapidly developed in China. Meanwhile, the basic research on tiger frog has been extensively carried out (Xie et al., 2009; Tian et al., 2011; Li et al., 2014), however, until now, little information is available concerning the morphology and especially the cytochemistry of its peripheral blood cells. In this study, we investigated the morphology and cytochemical patterns of the tiger frog blood cells by Wright-Giemsa staining and a range of cytochemical staining techniques. Our results could enrich the knowledge of peripheral blood cells in frogs, and provide reference data for the artificial culture and health examination of tiger frogs.

Materials & Methods

Animals and blood smears preparation

Fifteen male and fifteen female adult healthy tiger frogs (mean weight: 0.25-0.50 kg) were obtained from local frog farms in Wuhu City from June to September 2018; All frogs have normal appearance without obvious signs of disease. This work was approved by the ethics committee of Anhui normal university (Approval No. 201811).

126 Approximately 0.3 ml blood was taken from the ventral abdominal vein using a sterile 1 ml
127 syringe with 26g needles and placed quickly into a K_2-EDTA anticoagulant blood collection tube.
128 Blood smears were then immediately prepared, air-dried, and stored at 4℃ for further Wright-
129 Giemsa and various cytochemical staining.

130 **Wright-Giemsa staining**

131 Following methanol fixation for 15 min, the blood smears were treated with Wright-Giemsa
132 reagent according to the guidelines specified by the Biological Engineering Co. Ltd (Shanghai,
133 China). Briefly, blood smears were stained with Wright-Giemsa reagent for 1 min at room
134 temperature, then placed in a phosphate buffer(pH7.2) for 15 min. After 3 successive washings
135 in double distilled water, they were air-dried at room temperature. The stained blood smears
136 were examined under an Olympus DP71 digital camera coupled to a microcomputer system
137 (Tokyo, Japan).

138 **Cytochemical staining**

139 The cytochemical staining was performed according to the methods of Fang et al. (2014)
140 with minor modifications, which were briefly described below. The healthy human blood smears
141 were used as controls for cytochemical staining to ensure all staining worked as expected.

142 **PAS staining**

143 Blood smears were fixed with 95% ethanol solution for 10 min, and oxidized by 10 mg/ml
144 periodate solution for 15 min. After rinsed with double distilled water, they were stained with the
145 Schiff solution for 60 min. After 3 successive washings in double distilled water, they were
146 counterstained with 20 mg/ml methyl green solution for 15-20 min.

147 **SBB staining**

148 After fixed with formaldehyde vapor for 10 min, blood smears were stained with SBB
149 solution (300 mg SBB dissolved in 100 ml 70% ethanol) for 60 min. After 3 successive washings
150 in 70% ethanol and double distilled water, they were counterstained with Wright-Giemsa reagent
151 for 15-20 min.

152 **ACP staining**

153 Blood smears were stained with the incubation (12 ml pH 4.7 Acetic acid buffer mixed with
154 2 ml 20 mg/ml lead nitrate and 4 ml 32 mg/ml β-glycerin sodium phosphate and 74 ml double
155 distilled water) for 4 h at 37℃ after they were fixed with formaldehyde vapor for 10 min. After 3
156 successive washings in double distilled water, they were stained with 10% ammonium sulfide
157 solution for 30 min.

158 **AKP staining**

159 Blood smears were fixed with 10% methanol formaldehyde solution (10 ml formaldehyde
160 mixed with 90 ml Methanol) for 1 min, and then stained with incubation solution (20 mg alpha-
161 phosphate naphthol sodium, 20 ml of 0.05 mol/L propylene glycol buffer, and 20 mg diazo fast
162 blue) for 45-60 min. After washing in double distilled water for 2 min, they were counterstained
163 with hematoxylin for 5-8 min.

164 **POX staining**

165 Each blood smear was stained with benzidine solution (3 ml of 0.1% tetramethylbenzidine
166 ethanol solution mixed with 30 μl of nitrosyl ferricyanide saturated solution) for 1 min at
167 room temperature, and oxidized by 0.7ml of 1% H_2O_2 for 6 min. After 3 successive washings in

168 double distilled water, they were counterstained with Wright-Giemsa reagent for 15-20 min.

169 **CAE staining**

170 Blood smears were fixed with 10% methanol formaldehyde solution (10 ml formaldehyde
171 mixed with 90ml methanol) for 30s, then incubated with staining solution (10 mg chloroacetic
172 AS-D naphthol dissolved in 0.5 ml acetone, 10mg of diazo fast blue dissolved in 5 ml double
173 distilled water, 5 ml veronal acetate buffer) for 30-40 min at 37℃. After 3 successive washings
174 in double distilled water, the smears were counterstained with 1mg/ml hematoxylin for 5-10 min.

175 **ANAE staining**

176 Blood smears were fixed in 10% formaldehyde saline for 3 min, rinsed in double distilled
177 water, and then stained with the incubation solution (400 mg of α-naphthol acetate dissolved in 2
178 ml of 50% acetone, 100 mg diazo fast blue dissolved in 100 ml of 0.067 mol/L phosphate buffer).
179 After 1h incubation at 37 °C and 3 successive washings in double distilled water, the smears
180 were counterstained with 10 g/L methyl green solution for 5-15 min.

181 **Evaluation of cytochemical staining intensity**

182 According to the evaluation methods described by Tavares-Dias & Marques-Barcellos
183 (2005), the results of cytochemical staining were expressed in terms of the intensity of the
184 cytochemical reactions: negative reaction (-), weakly positive reaction (+), positive reaction (+ +)
185 and strongly positive (+ + +).

186 **Statistical analysis**

187 The blood cells were observed and photographed with an Olympus BX61 microscope
188 equipped with an Olympus DP71 digital camera coupled to a microcomputer system (Tokyo,

Japan). The erythrocyte (RBC), leukocyte (WBC) and thrombocyte (TC) counts were determined manually with the Neubauer chamber as described previously (Wang et at. 2021). The cell sizes were manually measured using ocular micrometer. Twenty of each type of blood cells were randomly selected from each frog to measure size and 100 leukocytes were randomly selected from each frog to calculate the percentage of various leukocytes. Statistical comparisons of the differences in morphometric values among different cell types or between sexes were performed using one-way ANOVA analysis (SPSS 19.0, SPSS Inc, Chicago, USA). A p-value less than 0.05 was considered significant.

Results

In this study, erythrocytes, thrombocytes and five types of leukocytes: neutrophils, eosinophils, basophils, monocytes and lymphocytes were distinguished in the peripheral blood cells of tiger frog under light microscopy by Wright-Giemsa staining. The lymphocytes can be subdivided into large and small lymphocytes depending on the size of the nucleus and cytoplasm.

The size and morphology of tiger frog peripheral blood cells

Erythrocytes

The mature erythrocytes were oval and elliptical in shape with a large central, round or long elliptical, purple-stained nucleus and an abundant, dark red brown-stained cytoplasm free of granules (Fig. 1 A). Immature erythrocytes were typically smaller and rounder than mature ones with a round and relatively larger nucleus (Fig. 1 B, C), some of which appeared hypochromatic

and polychromatic (Fig. 1 C). The dividing erythrocytes were sporadically found in the peripheral blood of tiger frogs (Fig. 1 D). No statistically significant difference in the size of erythrocytes was found between male and female tiger frogs, while the erythrocyte nucleus of males was significantly larger than that of females ($P < 0.01$) (Table 1). There were no significant differences in the total number of erythrocytes between male and female frogs (Table 2).

Eosinophils

Eosinophils were round or oval, frequently irregularly outlined cells. They had an eccentric, round or sometimes bilobed nucleus. The purple-stained cytoplasm was filled with coarse, round or rod-shaped, orange acidophilic granules of different size that occasionally obscured the nucleus (Fig. 1E).

Basophils

Basophils were the smallest granulocyte in tiger frogs. They were difficult to find in the blood smears of tiger frogs. These cells were round with a central-to-eccentric rounded and dark purple-stained nucleus and serrated edges. The usually scarce cytoplasm contained numerous round and deep purple-stained basophilic granules, which often masked the nucleus (Fig. 1 F).

Neutrophils

Neutrophils were the largest granulocyte in tiger frogs. They were usually oval and irregular in shape. They had an eccentric, oval or round, dark violet-stained nucleus and abundant violet-stained cytoplasm with numerous blue-stained granules and several irregular blue cytoplasmic inclusions (Fig. 1 G). Some neutrophils had a lobed nucleus, usually 2-5 lobes connected by the

231 filaments (Fig. 1 H).

232 **Monocytes**

233 Monocytes were the largest leukocyte in tiger frogs. They were round, oval or irregular cells,
234 characterized by a prominent, eccentric, kidney-shaped and blue purple-stained nucleus. These
235 cells exhibit an agranular gray-blue cytoplasm, in which numerous round or oval vacuoles of
236 different sizes were usually observed (Fig. 1 I).

237 **Lymphocytes**

238 Lymphocytes were round or oval in shape and had a large, purple-stained nucleus; Large
239 and small lymphocytes were observed according to their diameters and relative amounts of
240 cytoplasm. Large lymphocytes had a bigger and lightly purple-stained nucleus with a greater
241 quantity of cytoplasm (Fig. 1 J), while small lymphocytes had a smaller but dark purple-stained
242 nucleus, and a rim of scant, light purple cytoplasm that was not always visible around the entire
243 nuclear margin (Fig. 1 K).

244 **Thrombocytes**

245 Thrombocytes were often rod-shaped or long spindle-shaped with an elongated spindle and
246 lightly purple-stained nucleus, and the cytoplasm was faint purple-stained with smooth or
247 irregular membranes. The scant cytoplasm was accumulated in the two poles when the
248 thrombocytes were long spindle-shaped (Fig. 1 L).

249 **The blood cell counts and percentage of leukocytes**

250 The number of erythrocytes, leukocytes and thrombocytes and percentages of various
251 leukocytes in tiger frogs were shown in Table 2. The mean number of erythrocyte (RBC) was

252 1.33±0.15 millions/mm^3 in females and 1.12±0.08 millions/mm^3 in males. The mean leukocyte
253 (WBC) count was 3.73±0.04×10^4 mm^3 in females and 3.2±0.02×10^4 mm^3 in males. The mean
254 thrombocyte (TC) count was 1.7±0.01×10^{4}mm^3 in females and 1.9±0.04×10^4 mm^3 in males.
255 There were no statistically significant differences in the RBC, WBC and TC counts between
256 female and male tiger frogs ($P > 0.05$). Small lymphocytes were the most abundant leukocytes,
257 followed by neutrophils and large lymphocytes, eosnophils and monocytes were fewer, basophils
258 were the fewest. There were no statistically significant differences in the total number of
259 leukocytes, the percentage of various leukocytes and the neutrophil to lymphocyte (N/L) ratio
260 between male and female frogs.

261 **The cytochemical staining features of peripheral blood cells in tiger frogs**

262 The cytochemical staining features of peripheral blood cells of tiger frogs were shown in
263 Fig. 2. The staining patterns of various types of cells was summerized in Table 3.

264 **PAS staining**

265 Eosinophils, basophils and small lymphocytes were strongly positive with a great number of
266 coarse deep purple red-stained granular deposits (Fig. 2 AA, AB, AF). Neutrophils, monocytes,
267 large lymphocytes and thrombocytes (Fig. 2 AC, AD, AE, AG) were all positive with a diffusely
268 or granular purple red-stained cytoplasm.

269 **SBB staining**

270 Neutrophils presented strongly positive for SBB staining, the cytoplasm was filled with a
271 large number of coarse and dark black-stained granules, which masked the nuclei (Fig. 2 BC);
272 eosinophils (Fig. 2 BA), basophils (Fig. 2 BB) and monocytes (Fig. 2 BD) exhibited positive

reactions with numerous dark black-stained granules; large lymphocytes (Fig. 2 BE), small lymphocytes (Fig. 2 BF) and thrombocytes (Fig. 2 BG) were negative with reddish cytoplasm.

ACP staining

Basophils, neutrophils and small lymphocytes were strongly positive with a great number of black brown granular or diffuse granules in the cytoplasm (Fig. 2 CA, CB, CC, CF); eosinophils,, large lymphocytes and thrombocytes were positive with numerous dark brown granular or diffuse granules in the cytoplasm (Fig. 2 CE, CG); monocytes were weakly positive for ACP staining with numerous brownish yellow diffuse granules (Fig. 2 CD).

AKP staining

Eosinophils (Fig. 2 DA), neutrophils (Fig. 2 DC) and monocytes (Fig. 2 DD) presented weakly positive for AKP with light gray diffuse staining in cytoplasm. Basophils (Fig. 2 DB), monocytes (Fig. 2 DD), large lymphocytes (Fig. 2 DE), small lymphocytes (Fig. 2 DF) and thrombocytes (Fig. 2 DG) were all negative for AKP staining with faint red or faint gray cytoplasm.

POX staining

Eosinophils and neutrophils (Fig. 2 EA, EC) showed positive reactions with coarse, black blue-stained granules in the cytoplasm, the gray blue-stained granules and orange granules were interlaced with each other; monocytes (Fig. 2 ED) presented weakly positive with a few blue-stained granules; basophils (Fig. 2 EB, EE, EF, EG), large lymphocytes, small lymphocytes and thrombocytes were all negative with light blue cytoplasm.

CAE staining

294 Eosinophils and basophils (Fig. 2 FA, FB) exhibited positive reactions with a diffusely or
295 granular ruby-colored cytoplasm; neutrophils and monocytes were weakly positive with light
296 ruby-colored staining (Fig. 2 FC, FD); large lymphocytes, small lymphocytes and thrombocytes
297 (Fig. 2 FE, FF FG) were negative with light red cytoplasm.

298 **ANAE staining**

299 Eosinophils and monocytes (Fig. 2 GA, GD) presented positive for ANAE staining with
300 numerous gray bule-stained granules in the cytoplasm. Basophils, neutrophils, large lymphocytes,
301 small lymphocytes and thrombocytes (Fig. 2GB, GC, GE, GF, GG) were all weakly positive for
302 ANAE staining with a diffusely or granular light gray bule-stained cytoplasm.

303

304 **Discussion**

305 **The morphological characteristics of erythrocytes**

306 Erythrocytes play a major role in transporting oxygen and carbon dioxide (Peng et al., 2018).
307 The mature erythrocytes of tiger frog presented elliptic or long oval in shape with an abundant
308 and faint red-stained cytoplasm free of granules, which were similar to those of non-mammalian
309 vertebrates (Bricker, Raskin & Densmore, 2012; Fang et al., 2014; Tang et al., 2015; Hernández
310 et al., 2017). Fang et al. (2014) have conferred that the erythrocyte size could reflect the position
311 of a species on the evolutionary scale: the erythrocyte size in lower vertebrates was larger than
312 that in higher vertebrates. However, in our study, the erythrocyte size in tiger frog
313 (14.04±1.26)×(10.00±0.89μm) was smaller than that in some reported reptiles, such as European
314 pond turtle (*Emys orbiculuris*) (20.58±1.80)×(12.09±1.27μm), Mediterranean pond turtle

(*Muarremys leprosu*) (20.53±1.36)×(11.01±0.94μm) and yellow-bellied slider (*Trachemys scripta scripta*) (18.42±1.53)×(11.46±0.95μm) (Perpiñán & Sánchez, 2009; Hernández et al., 2017), it was also smaller than that in American bullfrog (*Rana catesbeiana*) (23.21±1.43)×(13.53±1.32μm) and Chinese toad (*Bufo gargarizans*) (19.76±1.17)×(13.71±0.89μm) (Jin et al., 2015). Guo et al. (2002) have shown that the smaller the erythrocyte, the stronger of its capacity for oxygen transport. Therefore, the phenomenon that erythrocytes in tiger frog were relatively smaller may be related to the stronger activity of this frog. The size of erythrocytes in tiger frogs was not significantly different between sexes, which was inconsistent with that reported in dubois's tree frog whose erythrocytes in females were significantly larger than in males (Madhusmita & Kumari 2014). The dividing erythrocytes were found sporadically in tiger frog peripheral blood, which is in accord with toad (*Bufo gargarizans*), dubois's tree frog, Chinese sturgeon (*Acipenser sinensis*), sisorid catfish (*Glyptosternum maculatum*), piebald naked carp (*Gymnocypris eckloni*) and prenant's schizothoracin (*Schizothorax prenanti*), (Guo et al., 2002; Gao et al., 2007; Zhang et al., 2011; Fang et al., 2014; Tang et al., 2015), suggesting that besides the main haematogenic organs, erythrocytes could be produced from amitosis in tiger frog peripheral blood.

The morphological characteristics of leukocytes

In this study, five types of leukocytes: neutrophils, eosinophils, basophils, monocytes and lymphocytes were observed respectively in tiger frog peripheral blood, which was in accord with those described in American bullfrog and African clawed frog (Bricker, Raskin & Densmore, 2012), while most of fish lack basophils (Fang et al., 2014; Zheng et al., 2015). The granules in

neutrophils, eosinophils and basophils of tiger frogs were usually round, which was consistent with those of other reported amphibians and mammals (Salakij et al., 2005; Prihirunkit et al., 2007; Gutierre et a., 2008; Bricker, Raskin & Densmore, 2012). Monocytes usually present a various and irregular appearance with some vacuoles in the cytoplasm, which was similar to those reported in the American bullfrog and African clawed frog (Bricker, Raskin & Densmore, 2012). Wang et al. (2001) have conferred that the vacuoles in monocytes may be related to phagocytosis.

The morphological characteristics of thrombocytes

The thrombocytes in lower vertebrates were equivalent to mammalian platelets in their function (Peng et al., 2018). However, there were great differences in their morphology. Mammalian platelets are enucleated and mostly round to oval disk-shaped (Salakij et al., 2000; Prihirunkit et al., 2007; Techangamsuwan et al., 2010), while thrombocytes in lower vertebrates mostly present round, teardrop, fusiformis or spindle in shape, with a nucleus (Salakij et al., 2002; Chansue et al., 2011; Bricker, Raskin & Densmore, 2012; Fang et al., 2014); The thrombocytes of the tiger frog mostly presented long rods or spindle in shape with clear edges and distinct boundaries between nucleoplasms, this morphological characteristics was in accord with that described in American bullfrog (Bricker, Raskin & Densmore, 2012) and king cobra (*Ophiophagus hannah*) (Salakij et al., 2002), but different from dubois's tree frog (Madhusmita & Kumari 2014), yellow-bellied slider (*Trachemys scripta scripta*) (Hernández et al., 2017) and captive bobtail lizard (*Tiliqua rugosa*) (Moller, Gaál & Mills, 2016), whose thrombocytes often presented round or oval in shape.

The blood cell counts and percentage of leukocytes

In this study, the RBC count was more in females (133,000,0/mm^3) than that in males (112,000,0/mm^3), which was consistent with that reported in Indian rhacophorid tree frog (*Polypedates maculatus*) (Mahapatra et al., 2012) and Dubois's Tree Frog (*Polypedates teraiensis*) (Madhusmita & Kumari, 2014), whose RBC counts were 570,000 and 620,000/mm^3 in females, 480,000 and 590,000/mm^3 in males, respectively. Glomski et al. (1997) reported that the RBC count of blood varies between 500,000 and 1,500,000/mm^3 on average in anurans. The RBC count of tiger frogs were within this range. Female tiger frogs tend to have more WBC than males, but the difference was not statistically significant. Similar reasults were reported by Mahapatra et al. (2012) in Indian rhacophorid tree frog, whose females showed higher WBC count (16,642/mm^3) than males (14,628/mm^3), likewise, the difference was not statistically significant.

The percentage of leukocytes were significantly different among different amphibian species. Lymphocytes were the most abundant in American bullfrog, followed by neutrophils and basophils, eosinophils and monocytes were the fewest (Bricker, Raskin & Densmore, 2012), while basophils were the most abundant leukocytes of African clawed frog, followed by lymphocytes and neutrophils, monocytes were significantly fewer, eosinophils were the fewest (Bricker, Raskin & Densmore, 2012); lymphocytes were the most abundant leukocytes of dubois's tree frog, followed by neutrophils and eosinophils, monocytes were significantly fewer, basophils were the fewest (Madhusmita & Kumari, 2014). In this study, small lymphocytes were the most abundant leukocytes, followed by large lymphocytes and neutrophils, the monocytes

and eosinophils were significantly fewer, basophils were the fewest. The neutrophil to lymphocyte (N/L) ratio has been known to reflect levels of stress hormones in vertebrates, and often been used by herpetologists to assess stress levels of amphibians (Davis & Maerz, 2008). The reference range of amphibian N/L ratio is between 0.01 to 0.67 (Davis 2009), the N/L ratio of tiger frog is within this range.

The cytochemical patterns of different leukocytes

Eosinophils are cells that play a role in phagocytosis and bactericidal effect, and actively participate in the defense against parasitic infections (Kay 1985). In this study, eosinophils were strongly positive for PAS and positive for SBB, POX, ACP, CAE, ANAE, weakly positive for AKP staining, which was different from those described in human whose eosinophils were negative for ACP, AKP and ANAE staining (Xu et al., 2003). This cytochemical pattern was also different from that of American bullfrog eosinophils which were positive for SBB, POX, and negative for AKP, CAE staining; and African clawed frog eosinophils which were weakly positive for POX, CAE, and negative for SBB, AKP staining (Bricker, Raskin & Densmore, 2012). The strongly positive reaction to PAS and positive to SBB suggested that tiger frog eosinophils contained a mount of glycogen and lipid, which are the important energy source of phagocytosis, moreover, the presence of ACP, AKP, POX, CAE in tiger frog eosinophils indicated that they may play a significant role in phagocytosis and bactericidal effect, but functional tests would be necessary to confirm. Additionally, the positive reaction for ANAE in tiger frog eosinophils indicated a difference from other species.

Human basophils were characterized by numerous coarse, blue-purple and unevenly

399 distributed basophilic granules, which mainly participate in allergic reactions with a relatively
400 weak phagocytic ability (Falcone, Zillikens & Gibbs, 2006). In this study, a number of basophilic
401 granules were also found in tiger frog basophils with strongly positive for PAS, ACP, and
402 positive for SBB, CAE, while negative for POX and AKP staining. This cytochemical pattern
403 was different from that of American bullfrog and African clawed frog, whose basophils were
404 negative for SBB staining (Bricker, Raskin & Densmore, 2012), while it was generally similar to
405 that described in human, except that the intensity in PAS, SBB and ACP staining of tiger frog
406 basophils was slightly stronger.

407 Neutrophils are important phagocytes and they are also vitally important in the immune
408 system (Roos et al., 1983). In this study, neutrophils were strongly positive for ACP, SBB, and
409 positive for PAS and POX staining, this cytochemical pattern was similar to that described in
410 human, some reptiles and amphibians (Xu et al., 2003; Casal & Orós., 2007; Chansue et al., 2011;
411 Cooper-Bailey et al., 2011; Bricker, Raskin & Densmore, 2012; Hernández et al., 2017). The
412 strongly positive for ACP, SBB, and positive for PAS and POX staining indicated that tiger frog
413 neutrophils may have a strong capability of phagocytosis and bactericidal effect. A small mount
414 of ANAE was found in tiger frog neutrophils, similar characteristics were also reported in the
415 American bullfrog and toad neutrophils (Bricker, Raskin & Densmore, 2012; Jin et al., 2015),
416 while human neutrophils were not stained with ANAE, suggesting that amphibian neutrophils
417 have different enzyme content compared with mammals.

418 Monocytes in human mainly play a dual role of phagocytosis and antigenic processing
419 (Azevedo & Lunardi, 2003; Shigdar, Harford & Ward, 2009)). In this study, monocytes were

positive for PAS, SBB and ANAE, and weakly positive for ACP, AKP, POX and CAE staining, which was similar to those described in human except for AKP (negative in human monocytes); while different from those described in American bullfrog and African clawed frog, whose monocytes were positive for CAE, negative for SBB, POX or ACP (Bricker, Raskin & Densmore, 2012). The positive reaction to PAS, SBB, ANAE and weakly positive reaction to ACP, AKP, POX, CAE of tiger frog monocytes indicated that they may have different enzyme content to other amphibians.

Lymphocytes belong to agranulocytes and play a significant role in both innate and acquired immunity (Shigdar, Harford & Ward, 2009). In this study, large lymphocytes and small lymphocytes were observed, and their cytochemical pattern was generally similar, except that the intensity in PAS and ACP staining of small lymphocytes was slightly stronger than that of large lymphocytes. The cytochemical pattern of tiger frog lymphocytes was generally similar to that reported in American bullfrog, African clawed frog and toad except for CAE and ANAE staining (Bricker, Raskin & Densmore, 2012; Jin et al., 2015). The presence of glycogen, ACP and ANAE in tiger frog lymphocytes may suggest functional differences.

The cytochemical pattern of thrombocytes

Thrombocytes are known to play a significant role in hemostasis and coagulation (Peng et al., 2018). In this study, thrombocytes were positive for PAS and ACP, and weakly positive for ANAE, while negative for SBB, POX, AKP and CAE staining. This cytochemical pattern was similar to that described in human platelets except for the PAS staining (Xu et al., 2003), while different from that of thrombocytes from toad which were only strongly positive for ACP

441 staining (Jin et al., 2015). Some vacuoles have been reported to be present in the cytoplasm of
442 thrombocytes in fish species (Gao et al., 2007; Fang et al., 2014). The positive reaction for PAS
443 and ACP and the negative reaction for SBB, POX, AKP and CAE in tiger frog thrombocytes is
444 similar to other species.
445
446 **Conclusions**
447 In conclusion, this study presented the first comprehensive investigation on the
448 morphological features and cytochemical patterns of tiger frog peripheral blood cells. The blood
449 cell morphology was generally similar between tiger frog and other reported amphibian species,
450 while their cytochemical patterns had some notable species specificity. For example, the tiger
451 frog eosinophils showed positive for almost all the cytochemical stains; ACP is ubiquitous in
452 leukocytes and thrombocytes; basophils were positive for SBB staining; monocytes were weakly
453 positive for CAE staining. Our study could enrich the knowledge of peripheral blood cell
454 morphology and chemistry in frogs, and provide baseline data for health condition evaluation
455 and disease diagnosis of tiger frogs.
456
457 **Acknowledgments**
458
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461

Declarations

Conflict of interest No potential conflict of interest was reported by the author(s).

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Ethics approval This work was approved by the ethics committee of Anhui normal university (Approval No. 201811). All the handling and sampling were performed in compliance with standard vertebrate protocols and veterinary practices, and in accordance with national and provincial guidelines.

Data availability All data generated or analysed during this study are included in this published article and its supplementary information files.

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Table 1(on next page)

The morphological parameters of peripheral blood cells in tiger frogs

Note: ** indicates that the difference between males and females is extremely significant ($P < 0.01$), different superscript letters (a, b, c, d, e, f and g) suggest significant difference among different cells in the same column.

Table 1. The morphological parameters of peripheral blood cells in tiger frogs

(Mean±SD，μm, N=30)

Cell types	Females		Males	
	Cell length	Cell width	Cell length	Cell width
Erythrocytes	15.09±1.26^{d}	10.60±0.87^{d}	14.61±1.47^{d}	9.83±1.10^{d}
(nuclei)	(5.38±0.46)^{a}	(4.42±0.48)^{a}	(8.56±1.21)^{b}**	(5.56±0.37)^{ab}**
Eosnophils	13.76±0.55^{d}	11.26±0.75^{e}	13.92±1.94^{d}	11.25±0.76^{e}
Basophils	9.28±1.00^{c}	7.99±0.91^{c}	8.74±1.27^{b}	7.69±1.15^{c}
Neutrophils	15.93±2.68^{e}	12.59±1.99^{f}	14.51±2.68^{d}	12.03±3.32^{e}
Monocytes	22.60±2.36^{g}	17.70±1.43^{g}	23.03±2.11^{f}	17.70±1.68^{f}
Large lymphocytes	10.52±1.25^{c}	9.20±1.25^{d}	10.00±1.07^{c}	8.79±0.8^{d}
Small lymphocytes	7.18±0.81^{b}	6.20±0.89^{b}	7.33±1.08^{a}	6.42±0.94^{b}
Thrombocytes	17.83±1.94^{f}	4.23±0.43^{a}	17.36±1.89^{e}	4.73±0.52^{a}

Note: ** indicates that the difference between males and females is extremely significant ($P < 0.01$), different superscript letters (a, b, c, d, e, f and g) suggest significant difference among different cells in the same column.

Table 2(on next page)

The blood cell counts and leukocytes percentage of peripheral blood cells in tiger frogs (Mean±SD, μm, N=30)

Different superscript letters (a, b, c, d, e, f) suggest significant difference among different leukocytes.

Table 2. The blood cell counts and leukocytes percentage of peripheral blood cells in tiger frogs (Mean±SD, μm, N=30)

	Females	Range	Males	Range
Erythrocyte count($\times10^6$/mm^3)	1.33±0.15	1.12-1.47	1.12±0.08	1.01-1.19
Leukocyte count($\times10^4$/mm^3)	3.73±0.04	3.19-4.12	3.2±0.02	2.86-3.48
Thrombocyte count($\times10^4$/mm^3)	1.7±0.01	1.59-1.78	1.9±0.04	1.52-2.40
Eosnophils(%)	7.82±0.67^b	7.08-8.39	10.21±1.98^b	7.96-11.72
Basophils(%)	2.59±0.42^a	2.15-2.98	1.86±0.36^a	1.63-2.28
Neutrophils(%)	17.22.±1.90^d	15.97-19.40	20.62±1.23^c	19.66-22.01
Monocytes(%)	12.55±2.10^c	10.94-14.92	16.08±1,61^{cd}	14.88-17.91
Large lymphocytes(%)	27.85±2.36^e	25.75-30.41	23.97±2.21^d	20.5-24.75
Small lymphocytes(%)	31.69±1.78^f	29.85-33.40	28.12±4.85^e	22.8-32.30
Neutrophil/lymphocyte ratio	0.36±0.04	0.38-0.41	0.41±0.03	0.37-0.44

Different superscript letters (a, b, c, d, e, f) suggest significant difference among different leukocytes.

Table 3(on next page)

The cytochemical staining patterns of leukocytes in tiger frogs blood cells

Note: "-" negative; "+" weakly positive; "+ +" positive; "+ + +" strong positive

Tab 3. The cytochemical staining patterns of leukocytes in tiger frogs blood cells

Cell types	PAS	SBB	ACP	AKP	POX	AS-D	ANAE
Eosinophils	+++	++	++	+	++	++	++
Basophils	+++	++	+++	-	-	++	+
Neutrophils	++	+++	+++	+	++	+	+
Monocytes	++	++	+	+	+	+	++
Large lymphocytes	++	-	++	-	-	-	+
Small lymphocytes	+++	-	+++	-	-	-	+
Thrombocytes	++	-	++	-	-	-	+

Note: "-" negative; "+" weakly positive; "+ +" positive; "+ + +" strong positive

Figure 1

Wright-Giemsa staining of tiger frogs blood cells

A. erythrocyte, ellipsoid shape with light red brown-stained cytoplasm free of granules; B. immature erythrocyte; C. immature erythrocyte with hypochromasia and polychromasia; D. dividing erythrocyte; E. eosinophil, round cell with an eccentric nucleus, and the cytoplasm was filled with coarse, round and orange acidophilic granules of different size; F. basophil, round cell with a central-to-eccentric rounded and dark purple-stained nucleus; G. neutrophil, spindle-shaped cells with numerous purple-stained granules; H. oval neutrophil with a lobed nucleus; I. monocyte, oval cell with a prominent, eccentric, kidney-shaped nucleus; J. large lymphocyte, round cell with a bigger and lightly purple-stained nucleus; K. small lymphocyte, round cell with a smaller but dark purple-stained nucleus; L. thrombocyte, spindle-shaped cell with an elongated spindle and lightly purple-stained nucleus; Bar=10μm.

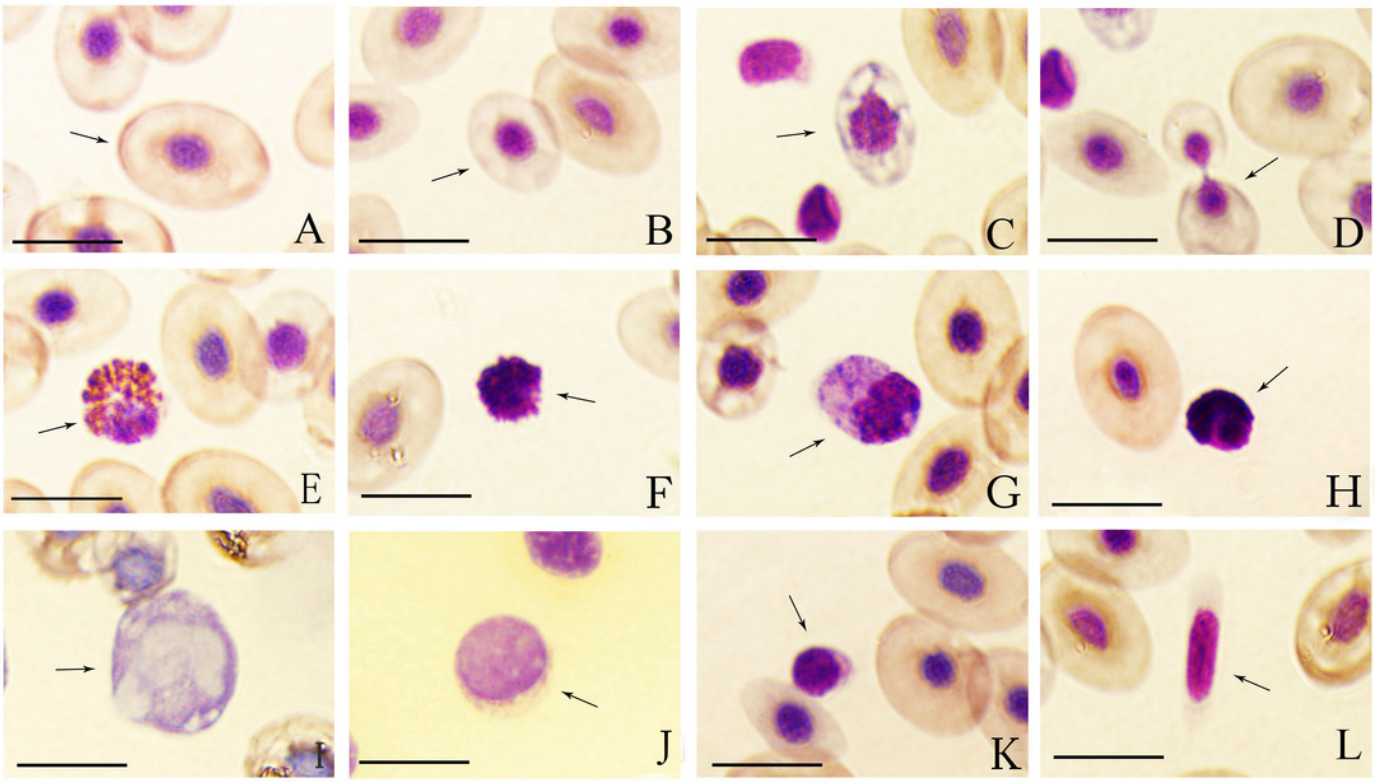
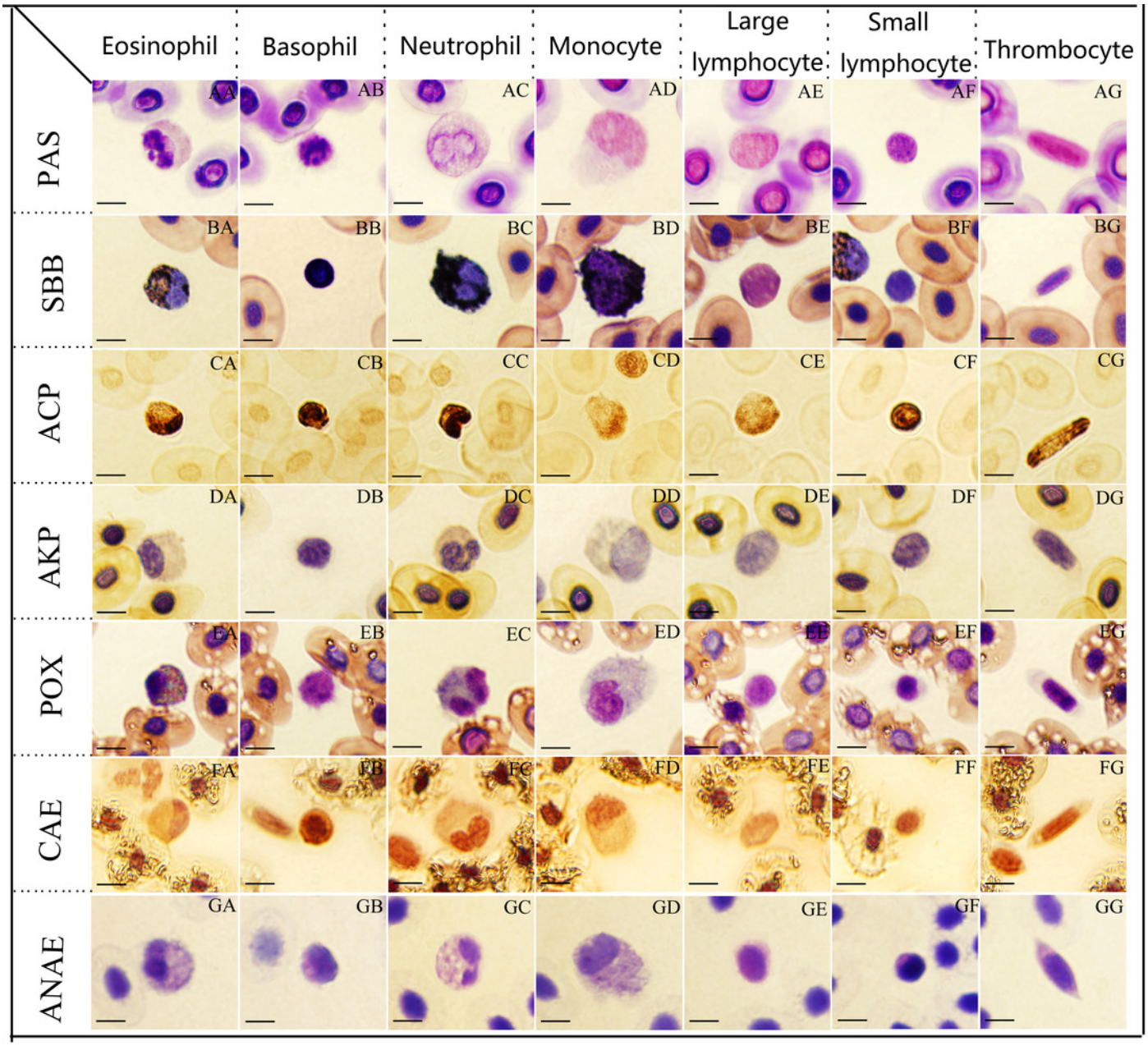


Figure 2

The cytochemical staining patterns of tiger frogs blood cells.

Eosinophil (AA-GG): (AA) strongly positive reaction with a great number of coarse deep purple red-stained granular deposits, PAS. (BA) positive with numerous dark black-stained granules, SBB. (CA) positive with a large amounts of dark brown granular or diffuse granules, ACP. (DA) weakly positive with light gray diffuse staining in cytoplasm, AKP. (EA) positive with coarse, black blue-stained granules, POX. (FA) positive with a diffusely or granular ruby-colored cytoplasm, CAE. (GA) weakly positive with numerous gray bule-stained granules in the cytoplasm, ANAE. Basophil (AB-GB): (AB) strongly positive for PAS. (BB) positive for SBB, (CB) strongly positive with a great number of black brown granular or diffuse granules, ACP. (DB) negative for AKP. (EB) negative for POX. (FB) positive for CAE. (GB) weakly positive with a diffusely or granular light gray bule-stained cytoplasm, ANAE. Neutrophil (AC-GC): (AC) positive with a diffusely or granular purple red-stained cytoplasm, PAS. (BC, CC, EC) strongly positive with a large number of coarse and dark black-stained granules, which masked the nucleus, SBB. (CC) strongly positive for ACP, (EC) positive for POX, respectively. (DC, GC) weakly positive for AKP and ANAE. (FC) weakly positive with light ruby-colored staining, CAE. Monocyte (AD-GD): (AD, BD,GD) positive for PAS, SBB, ANAE. (CD) weakly positive with a amount of brownish yellow diffuse granules, ACP. (DD) weakly positive for AKP. (ED,FD) weakly positive for POX and CAE. Large lymphocytes (AE-GE): (AE, CE) positive for PAS and ACP. (BE) negative for SBB. (DE, EE, FE) negative for AKP, POX and CAE. (GE) weakly positive for ANAE. Small lymphocytes (AF-GF): (AF,CF) strongly positive for PAS and ACP. (BF) negative for SBB. (DF, EF, FF) negative for AKP, POX, CAE. (GF) weakly positive for ANAE. Thrombocyte (AG-GG): (AG, CG) positive for PAS and ACP. (BG) negative for SBB. (DG, EG, FG) negative for AKP, POX, CAE. (GG) weakly positive for ANAE. (Bar=10μm)


Eosinophil
Basophil
Neutrophil
Monocyte
Large lymphocyte
Small lymphocyte
Thrombocyte
PAS
SBB
ACP
AKP
POX
CAE
ANAE
AA
AB
AC
AD
AE
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