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Hematological analysis of *Ctenopharyngodon idella*, *Megalobrama amblycephala* and *Pelteobagrus fulvidraco*: Morphology, ultrastructure, cytochemistry and quantification of peripheral blood cells

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ABSTRACT

The grass carp (*Ctenopharyngodon idella*), blunt snout bream (*Megalobrama amblycephala*) and yellow catfish (*Pelteobagrus fulvidraco*) are economically important fishes in China. Fish hematological features, especially the type and number of peripheral blood cells, are crucial for the evaluation of fish health and the diagnosis of fish diseases. Since the automatic blood cell count equipment for human is not suitable for fishes, the manual method is critical in the quantification of fish blood cells. To make sense of the comparison and interpretation of the blood cell count studies in different articles, the standardization of blood cell classification is necessary. In this study, erythrocytes (red blood cell, RBC), thrombocytes (TC) and leucocytes (i.e. white blood cells, WBC, including lymphocytes, neutrophils and monocytes) were well distinguished in blood smears with Giemsa staining and confirmed by transmission electron microscopy. RBC, TC and WBC were directly counted with an improved Neubauer counting chamber in a modified diluting solution. The differential leucocyte count (DLC) was carried out in blood smears. In view of the labeling characteristics of peroxidase (PO) positivity in neutrophils and non-specific esterase (α-ANAE) positivity in monocytes, PO positive cell percentage and α-ANAE positive cell percentage were also determined in cytochemistry staining smears. No difference was found for the percentages of neutrophils and monocytes between Giemsa staining and cytochemistry staining. The standardized classification, normal count ranges and sizes of the peripheral blood cells by the present systemic studies will provide useful references for monitoring the health status of grass carp, blunt snout bream and yellow catfish.

1. Introduction

The annual yield of aquaculture fish has been reached tens of millions tons in China since 2018 [1]. Fish in conditions of intensive farming must be able to cope with numerous stress factors that affect their basic physiologic function [2]. Hematological analysis is non-lethal and inexpensive is an important and powerful tool to monitor health and to diagnosis diseases [3]. Automatic instruments for mammal blood cell count have been developed and widely used for clinical practices. The automatic techniques for mammal blood cell counting based on the distinguishing feature that the mammalian platelets are cell fragments and the erythrocytes are non-nucleus cells [4]. Thus, white blood cells can be accurately counted after the lysing of the erythrocyte, which is overwhelming in number in the blood and cause difficulty for leucocyte counting. Since fish erythrocytes and thrombocytes, the counterpart of mammalian platelets, are all nucleated, it is hard to discriminate the lymphocytes from the bared nuclei after the erythrocyte lysing [4]. Thus automatic hematological instruments used in the field of medicine cannot be simply applied for fish hematological analysis. Although automatic blood cell counts for fish has been reported, no viable automatic system for fish blood cell count has been developed and the manual analysis method of blood cell count has reminded to play a critical role in fish hematology analysis [5]. The manual analysis method used is the visual counting of cells in an improved Neubauer counting chamber [5]. However, difficulty does arise due to the inability to lyse selectively the erythrocytes, leukocytes or thrombocytes; for this reason Hesser (1960) and Mulcahy (1970) adopted the avian diluting fluid of Shaw (1930), which consists of two vitally staining dyes enabling differentiation to be made between leukocytes, erythrocytes and thrombocytes [6]. The disadvantage of this stain is its instability. Hayems' fluid and 'r.b.c.' bulb diluting pipettes were used by Korzhuev (1964), and Hesser (1960) used these together

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with Gower's and Hendrick's diluting fluids, while Klontz & Smith (1968) found the Rees-Ecker diluting fluid and Shaw's diluting fluid were preferable [6].

There is a substantial amount of literature reported on types and structural characteristics of fish blood cells [7,8]. Different cell type has been defined not only structurally but also functionally. Quantification of fish blood cells has also been documented [9]. It is difficult to compare and interpret the results about the numbers of white blood cells. One primary reason is the classification of thrombocytes [7]. Although some authors acknowledge thrombocytes to be the counterpart of mammalian platelets, many others consider them to be a type of leukocytes [10]. It is generally accepted that fish blood cells follow the basic hematological pattern of higher vertebrates and constitute erythrocytes, thrombocytes and leucocytes which can be subdivided into lymphocytes, monocytes and granulocytes [11].

Grass carp (*Ctenopharyngodon idella*), blunt snout bream (*Megalobrama amblycephala*) and yellow catfish (*Pelteobagrus fulvidraco*) are economically important fish species in China [12]. However, the hematologic data available for grass carp, blunt snout bream and yellow catfish are fragmentary and their blood cells have not been systematically studied. The intensive farming of them requires efficient veterinary monitoring. Hematological analysis is a non-lethal and inexpensive mean for both the assessment of physiological status and the diagnosis of diseases of farmed fish [8]. Physiologic values for leukocytes, as well as morphologic, cytochemical, and ultrastructural analyses, have been determined for cultivated grass carp, blunt snout bream and yellow catfish [13,14]. However, there is some controversy regarding the thrombocytes of grass carp, blunt snout bream and yellow catfish, and reference intervals for red blood cell (RBC), white blood cell (WBC) and thrombocyte (TC) for these species have not been established. In addition, if the classification of fish peripheral blood cells is not standardized, especially thrombocyte, the future research on fish blood cells will still be in a chaotic stage.

The purpose of this study was to define reference intervals for RBC, WBC and TC, and thrombocyte was categorized as another group of cells besides erythrocyte and leucocyte. And then the morphology of these blood cells was characterized by Giemsa stained, cytochemical stained, and electron microscopy. This work will provide useful information for other researchers, besides, it could be used as a biomarker associated with stressor agents or an available tool to diagnose and monitor disease in these species. At the same time, this study provides a new approach to the research of peripheral blood cells by comparing the hematological parameters and leukocyte classification in the peripheral blood of fish with human peripheral blood.

2. Materials and methods

2.1. Fish

Grass carp, blunt snout bream and yellow catfish (weighing 30 g–50 g and lengthing 10 cm to 20 cm) were obtained from a fish farm in luhu (Hubei Province, China). The fish were kept in a recirculating freshwater system at 25–26 °C with a natural photoperiod, and they were fed twice a day with a commercial pellet diet (Haida, Hubei, China) at a rate of 2% body weight. All of the fish were acclimatized for 2 weeks before sampling. Neither visible lesions nor gross abnormalities were observed in any of the fish throughout the study.

2.2. Blood collection

The grass carp, blunt snout bream and yellow catfish (n = 50) were anesthetized with 3 - aminobenzoic acid ethyl ester methanesulfonate (MS222) (Sigma-Aldrich Co., St. Louis, MO, USA). Then the blood samples were collected by venipuncture from the caudal with a syringe of 1% heparin wetting, heparinized 5-mL collection tubes (endotoxin-free sodium heparin, Sigma, USA) were used for blood storage. When the blood smears were drawn, smears were prepared, air dried, and stained for the differential leukocyte count, cytochemical stained and morphologic examination. Total RBC, WBC and TC counts were performed within 2 h after blood collection.

2.3. Absolute number of RBC, WBC and TC counts

Fish use blood cell diluent (NaCl: 0.7 g, Neutral red: 3 mg, crystal violet: 1.5 mg, formaldehyde: 0.4 mL and distilled water: 100 mL) was used for counting total RBC, WBC and TC [7]. Total counts were measured manually by an improved Neubauer counting chamber as described by Hrubec and Smith [7]. Briefly, the RBC blood specimen was diluted 1 : 200 by using blood cell diluent, WBC and TC blood specimen were diluted 1 : 20. Erythrocytes were counted in 5 secondary squares of the center primary square. The number of RBC counted in the secondary squares (raw count) was multiplied by $200 \times 10 \times 10^6 \times (25/5)$ to calculate total RBC count per liter. Total WBC and TC counts were measured at the same time by counting all WBC and TC in the 4 corner primary squares. The total WBC and TC count per liter were calculated by multiplying the number of WBCs and TCs observed in the 4 primary squares times 5×10^7. To decrease analytical variability, cells were counted in both chambers of the improved Neubauer, and the number was averaged to produce the raw WBC, RBC or TC counts.

2.4. Preparation of morphological samples of peripheral blood cells

For light microscopy, blood smears, ten for each fish were confecioned. The blood smears form every fish were fixed in absolute methanol for 3 min at room temperature, and stained with Giemsa (Solarbio, China) for 15 min and finally rinsed with distilled water, air-dried, and sealed with neutral balsam (Sinopharm chemical Reagent Co., Ltd). The stained smears were studied and photos were taken under a Nikon Microphot FX, observing peripheral blood cells morphology.

For electron microscopy, blood was centrifuged at 3000 rpm and the serum was removed. Glutaraldehyde (2.5%) was added to the centrifuge tube on the surface of the deposited blood cells. A small (approx. 1 mm^3) clot was detached from the surface of the deposit and washed twice with phosphate buffer. The clot was then post-fixed in 1% OsO_4, dehydrated through a graded series to absolute ethanol and embedded in Epon 812. Ultrathin sections (50–80 nm) were cut with a Nova ultramicrotome (after semi-thin sectioning to find the best location) and stained with uranyl acetate and lead citrate before examination under a transmission electron microscope (Hitachi H-7000, Japan) [15].

2.5. Measurement of blood cell size and differential leukocyte count (DLC)

Under the oil microscopy, each blood smear was observed and analyzed by Nikon Microphot FX, and the field of visions with erythrocytes, leukocytes and thrombocytes were chosen for photography. Counting of different leukocytes was performed simultaneously and a total of 500 cells (lymphocytes, neutrophils and monocytes) were counted and expressing the result as a percentage. Two hundred cells of each type were measured with Photoshop CS5 analysis software, including the lengths and widths of the cells and nuclei. Data are presented as mean ± standard deviation.

2.6. Cytochemistry

Ten fish were used for cytochemical study. Cytochemical stained was performed by commercial kits (Solarbio, China). The following staining procedure were performed: peroxidase (DAB) and α - naphthyl acetate esterase (α - NAE, non - specific esterase; Solarbio). According to positive reaction, the neutrophils and monocytes in five hundred leukocytes were counted, and then analyzed to obtain the percentage of neutrophils and monocytes.

Table 1
The absolute number RBC, WBC and TC of peripheral blood cells in grass carp, blunt snout bream and yellow catfish.

	Grass carp	Blunt snout bream	Yellow catfish	Human *[43]*
RBC (10^4/μL)	175.95 ± 22.83	152.7 ± 11.77	140.65 ± 10.03	350–550
WBC (10^4/μL)	3.38 ± 0.10	1.82 ± 0.67	2.82 ± 0.74	0.4–1
TC (10^4/μL)	4.08 ± 0.12	3.06 ± 0.10	3.02 ± 1.14	10-30 ∗

Note: the ∗ indicates that the number of platelets in human peripheral blood.

3. Results

3.1. Absolute number of RBC, WBC and TC counts in peripheral blood of grass carp, blunt snout bream and yellow catfish

RBC, WBC and TC counts of heparinized blood in grass carp, blunt snout bream and yellow catfish were measured by modified Neubauer (Table 1). When RBCs, WBCs and TCs were counted with fish blood cell dilution solution, Under the 40 × microscope, RBCs were slightly yellow; WBCs were round and stained with neutral red; TCs were rod-shaped, smaller than red blood cells, and were transparent glass-like, and there was no obvious boundary between cytoplasm and nucleus (Fig. S1.). Erythrocytes were the dominant cell type in the blood of the fish and human, and thrombocytes were the most abundant blood cells after erythrocytes. However, the WBC counts were relatively small in peripheral blood cells.

3.2. Cell morphology in peripheral blood of grass carp, blunt snout bream and yellow catfish

Erythrocytes, three types of leucocytes: lymphocytes, monocytes and neutrophils and thrombocytes were distinguished and characterized by light and electron microscopy. The results showed that there were no significant differences of peripheral blood cell structure of grass carp, blunt snout bream and yellow catfish.

Erythrocytes were oval, long olive, in shape with a smooth surface and contained a round to oval, central, blue-purple nucleus and the cytoplasm was abundant, homogeneous and pale pink, pink, or pale purple (Fig. 1A). Occasionally, division of erythrocytes could be found in peripheral blood cells of grass carp (Fig. S2). And observed by electron microscopy, they had a long, oval nucleus, which were rich in heterochromatin. In addition, the cytoplasm was highly electron-dense and contained few organelles, only round vesicles could be observed. And there were a few mitochondria and profiles of endoplasmic reticulum presented in the cytoplasm of mature erythrocytes (Fig. 1B).

Thrombocytes present different morphology in blood smears, from fusiform to spindle or oval-shaped, with an oval and centrally located nucleus. They were single cells or groups of three to five cells distributed. And sometimes, it was hard to see the cytoplasmic boundary (Fig. 2A, B and C). Under electron microscopy, thrombocytes had elongated nucleus rich in heterochromatin. The cytoplasm contained numerous microtubules and large canaliculi that looked like clear vacuoles. Similarly, rare mitochondria, polyribosomes, and membrane-bound granules were observed in the cytoplasm. When cut in cross-section, it could be seen that thrombocytes had an eccentric, irregular nucleus and prominent canaliculi (Fig. 2A1, B1 and C1).

Lymphocytes were irregularly round, with finger-like protrusions on the surface contained a round to oval, central, blue nucleus which was rich in heterochromatin, and nucleoplasmic ratio was large, centered or tangential to the plasma membrane. The cytoplasm were homogeneous and surrounded by a thin rim of light blue peripheral cytoplasm (Fig. 3A, B and C). Ultrastructually, the lymphocyte exhibited fingerlike cell processes, large central nucleus contained dense patches of heterochromatin and one nucleolus, and the thin rim of cytoplasm contained numerous free ribosomes, mitochondria, rough endoplasmic reticulum and Golgi complex (Fig. 3A1, B1 and C1).

Monocytes characterized were round to oval cells with a smooth surface and contained a round to kidney shape, central, blue-purple nucleus and the cytoplasm was homogeneous and pale blue (Fig. 4A, B and C). Ultrastructually, their nucleus were comprised of a peripheral rim and central aggregates of heterochromatin with strands of euchromatin. The cytoplasm contained mitochondria, free ribosomes, numerous light vesicles, rough endoplasmic reticulum, Golgi complex, Golgi derived

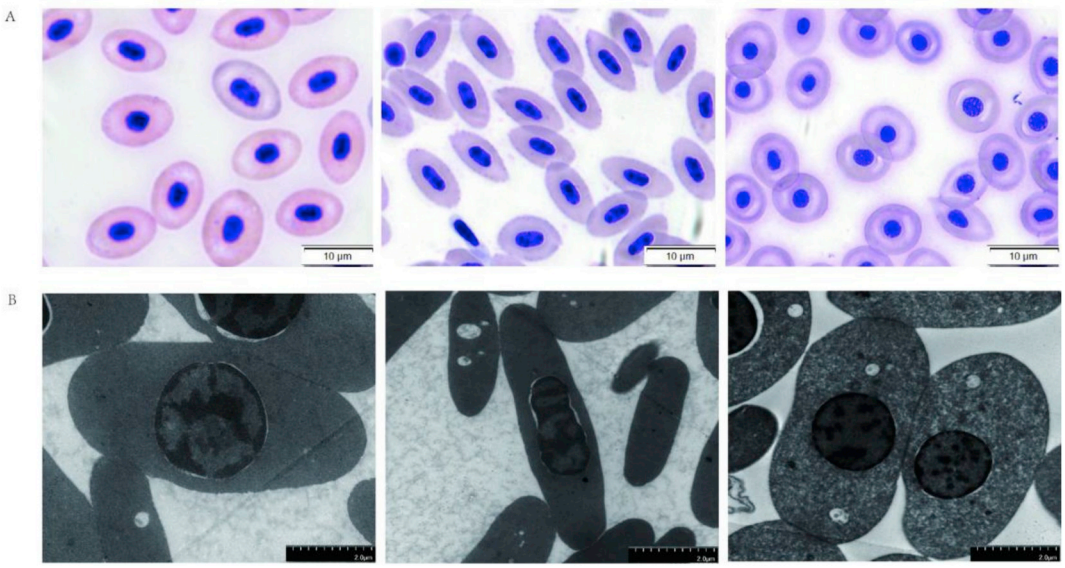

Fig. 1. Morphology of erythrocytes. A represented erythrocytes in grass carp, blunt snout bream and yellow catfish stained with Giemsa staining by light microscopy and the bar = 10 μm; B Morphology of erythrocytes by electronic microscopy. “a”, “b” and “c” represented erythrocytes in grass carp, blunt snout bream and yellow catfish and the bar = 2 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

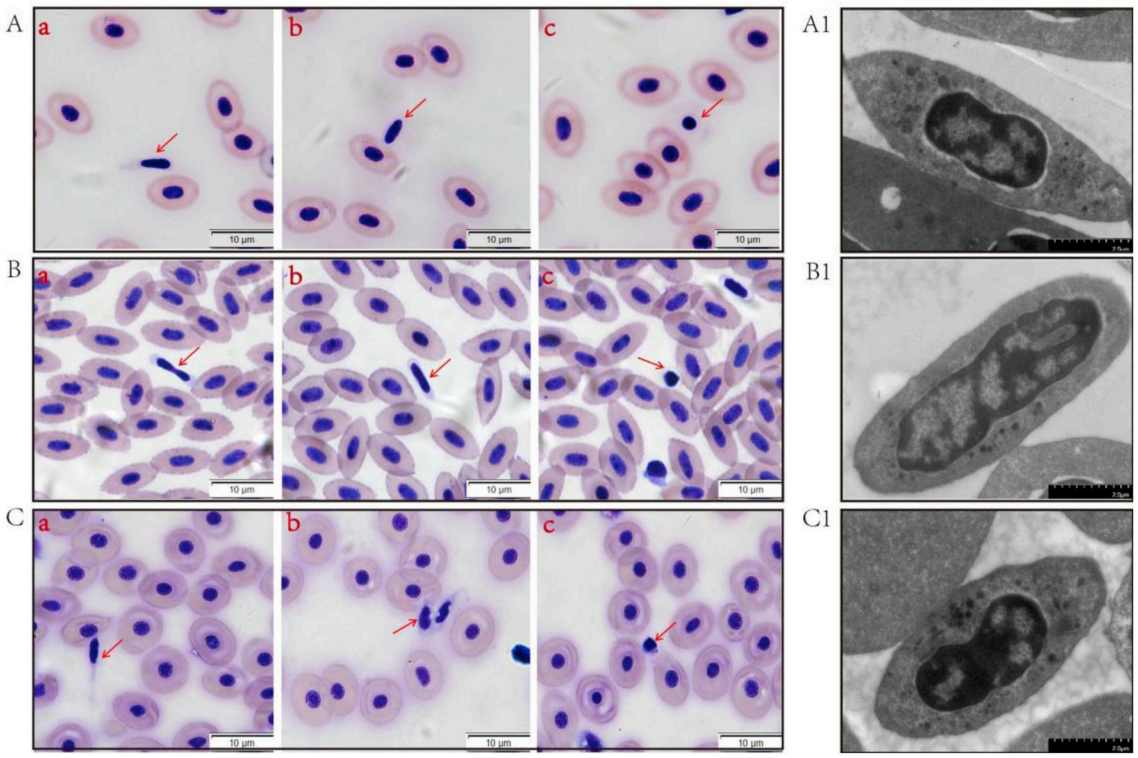

Fig. 2. Morphology of thrombocytes. A, B and C represented thrombocytes in grass carp, blunt snout bream and yellow catfish with Giemsa staining by light microscopy (the bar = 10 μm). A1, B1 and C1 represented thrombocytes in grass carp, blunt snout bream and yellow catfish by electron microscopy. "a", "b" and "c" represent different morphology of the thrombocytes, respectively. The red arrow indicates thrombocytes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

vesicles/granules. And cytoplasm was vacuolated and less dense than that of lymphocytes because fewer free ribosomes were present. And usually, pseudopodia were presented on the surface of the plasma membrane (Fig. 4A1, B1 and C1).

Neutrophils were round, ovoid, irregular, etc., and their nucleus were horseshoe-shaped and bilobed ribbon. Nucleus were frequently biased to one side of the cell, and they had a higher chromatin which were dyed purplish red or blue-purple. Their cytoplasm contained granules which were stained dark blue or stained small pink by Giemsa (Fig. 5A, B and C). Under electron microscopy, their heterochromatin were arranged peripherally in the nucleus, whereas strands of euchromatin were centrally oriented. The cytoplasm contained round to elongated granules, as well as

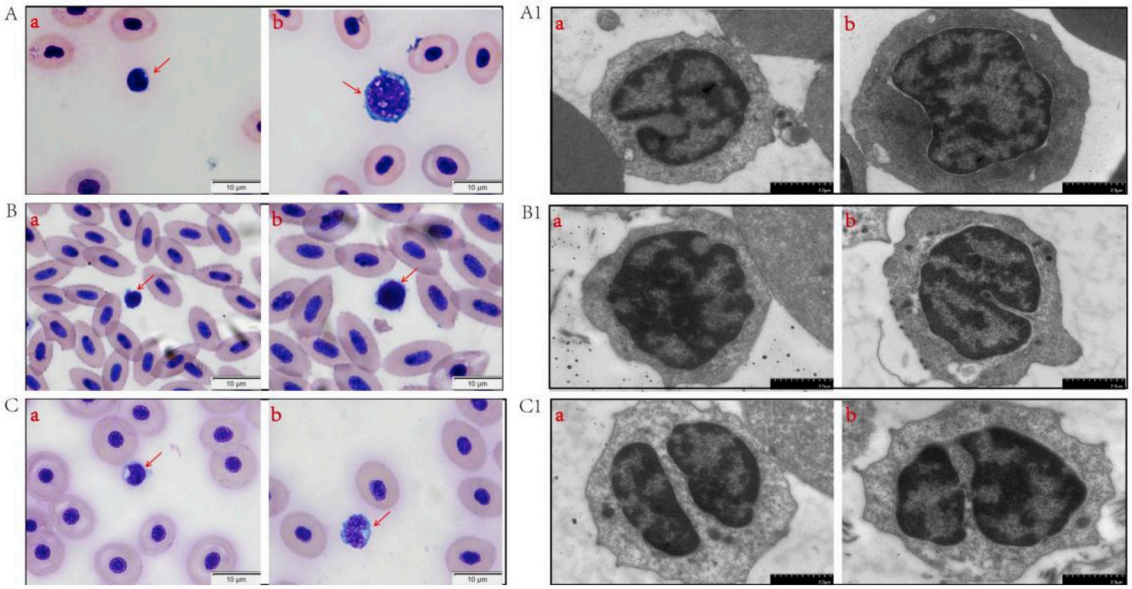

Fig. 3. Morphology of lymphocytes. A, B and C represented lymphocytes in grass carp, blunt snout bream and yellow catfish with Giemsa staining by light microscopy (the bar = 10 μm); A1, B1 and C1 represented lymphocytes in grass carp, blunt snout bream and yellow catfish by electron microscopy. "a" represented small lymphocytes and "b" represented large lymphocytes. The red arrow indicates lymphocytes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

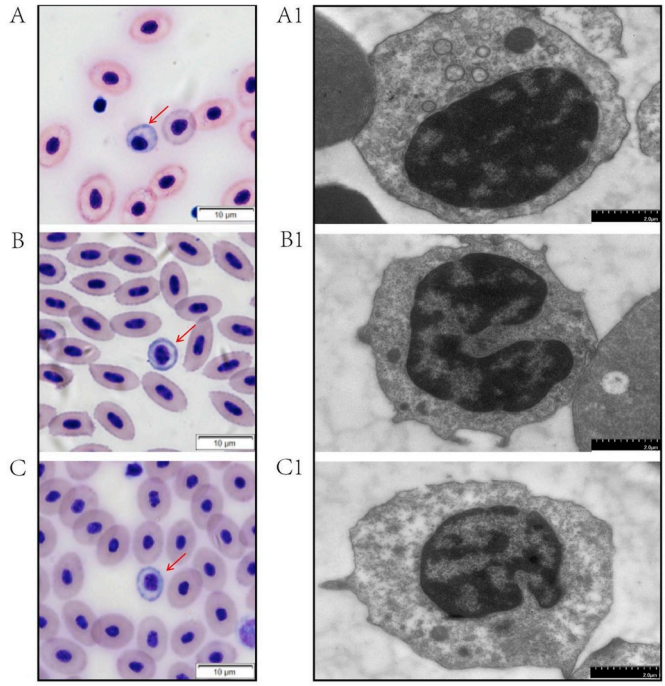

Fig. 4. Morphology of monocytes. A, B and C represented lymphocytes in grass carp, blunt snout bream and yellow catfish with Giemsa staining by light microscopy (the bar = 10 μm); A1, B1 and C1 represented monocytes in grass carp, blunt snout bream and yellow catfish by electron microscopy. The red arrow indicates monocytes. The red arrow indicates monocytes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

numerous light vesicles, some of which exhibited a content inside. And Golgi apparatus could be occasionally observed. Two different populations of granules, with distinctive ultrastructural aspect, were distinguished. Some of them were small, and rod shape with containing homogeneous electron-dense material. While the others were big and round, showing a heterogeneous electron-dense core and the interior of the particle contains a layer of filaments. In both type of granules, the content were surrounded by a light halo, and nucleus presented large peripheral heterochromatin blocks and a prominent nucleolus (Fig. 5A1, B1 and C1).

3.3. Differential leukocyte count (DLC) and cell size in peripheral blood of grass carp, blunt snout bream and yellow catfish

We established reference ranges for the DLC and cell sizes of peripheral blood in grass carp, blunt snout bream and yellow catfish. The results were shown in Table 2 and Table 3. In most grass carp, blunt snout bream and yellow catfish blood smears, the lymphocytes were the most common type of leukocytes and were accounting for about 81%, 76% and 78%. Small lymphocytes and large lymphocytes can be observed. In most cases, neutrophils were the more common and largest in all blood smears, but the small number of monocytes and no eosinophils or basophils were found. In human blood, lymphocytes account for only 20–40%. The neutrophils were the predominant leukocyte (accounting for up to 50–70% of all leukocytes) and the monocytes were rare. The eosinophils and Basophilias were very rare [16].

3.4. of neutrophils and monocytes after cytochemical stained in peripheral blood of grass carp, blunt snout bream and yellow catfish

The neutrophils were the only cells that were positive for peroxidase and the whole cytoplasm were stained light brown or brown with a blackish brown granule (Fig. 6B). The monocytes were the only cells which were positive for a-ANAE, and they were non-specific esterase positive, the whole cytoplasm of monocytes was stained red brown with a

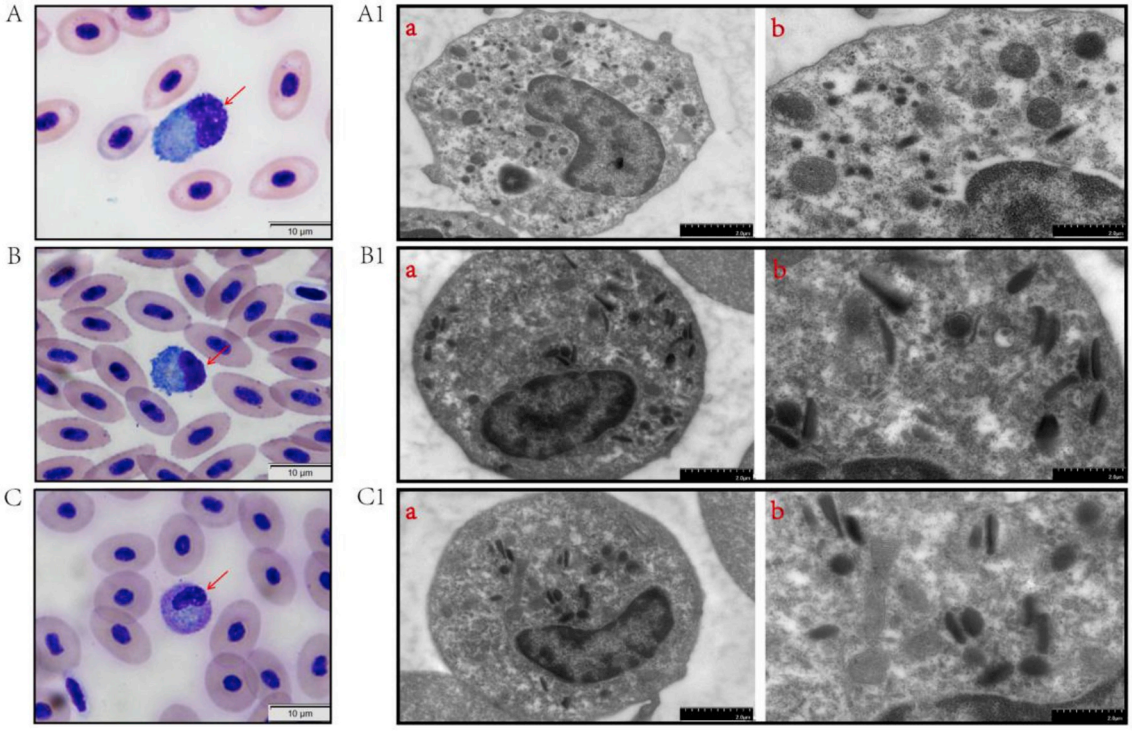

Fig. 5. Morphology of neutrophils. A, B and C represented neutrophils in grass carp, blunt snout bream and yellow catfish with Giemsa staining by light microscopy (the bar = 10 μm); A1,B1 and C1 represented neutrophils in grass carp, blunt snout bream and yellow catfish by electron microscopy and "a" represented neutrophils and "b" stands for a partial enlargement of the neutrophils. The red arrow indicates neutrophils. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Differential leukocyte count (DLC) of peripheral blood cells in grass carp, blunt snout bream and yellow catfish.

	grass carp	blunt snout bream	yellow catfish	*Human* [43]
Lymphocytes (%)	81.75 ± 5.74	79.25 ± 2.6	78.28 ± 1.6	20–40
Neutrophils (%)	14.25 ± 2.99	15.88 ± 3.52	16.14 ± 1.07	50–70
Monocytes (%)	4.5 ± 1.29	6.5 ± 1.2	6.57 ± 1.13	3–8
Eosinopenia (%)	-	-	-	0.5–5
Basophilia (%)	-	-	-	0–1

Note: "-" indicates that no such cells were found in the peripheral blood cells of grass carp, blunt snout bream and yellow catfish.

Table 3
Cell size of peripheral blood cells in grass carp, blunt snout bream and yellow catfish.

Cell size (μm)		Erythrocytes	Large lymphocytes	Small lymphocytes	Neutrophils	Monocytes	Thrombocytes	
							long	short
grass carp	Cell length	12.31 ± 0.78	8.90 ± 1.65	5.30 ± 0.80	10.74 ± 2.06	8.95 ± 0.92	8.56 ± 1.15	4.34 ± 0.29
	Cell width	8.27 ± 0.72	7.82 ± 1.47	4.84 ± 0.82	9.19 ± 1.56	8.06 ± 0.74	3.58 ± 0.34	3.88 ± 0.34
	Nucleus length	5.17 ± 0.47	7.03 ± 1.08	4.31 ± 0.56	7.30 ± 1.64	5.21 ± 0.49	5.96 ± 0.62	3.59 ± 0.32
	Nucleus width	3.59 ± 0.28	6.25 ± 1.32	3.78 ± 0.74	5.27 ± 0.93	4.36 ± 0.46	2.75 ± 0.30	3.28 ± 0.38
blunt snout bream	Cell length	13.61 ± 0.85	8.68 ± 1.22	6.45 ± 0.85	10.72 ± 0.91	9.28 ± 1.02	10.48 ± 1.45	3.94 ± 0.49
	Cell width	7.47 ± 0.55	7.57 ± 1.03	5.37 ± 0.59	9.97 ± 0.99	8.34 ± 0.57	3.77 ± 0.56	3.58 ± 0.51
	Nucleus length	6.38 ± 0.42	6.94 ± 1.07	5.32 ± 0.12	6.46 ± 1.07	5.68 ± 0.42	6.98 ± 0.74	3.32 ± 0.29
	Nucleus width	3.63 ± 0.35	6.29 ± 0.66	4.84 ± 0.76	4.70 ± 0.93	4.41 ± 0.03	2.46 ± 0.33	2.98 ± 0.32
yellow catfish	Cell length	12.22 ± 0.92	8.79 ± 1.52	6.37 ± 0.78	10.97 ± 1.43	9.80 ± 0.81	10.89 ± 1.21	4.34 ± 0.52
	Cell width	9.98 ± 0.83	6.99 ± 1.01	6.03 ± 0.41	9.24 ± 1.26	8.18 ± 0.99	4.56 ± 0.45	3.87 ± 0.56
	Nucleus length	4.59 ± 0.42	6.31 ± 0.57	5.08 ± 0.67	6.97 ± 0.90	5.76 ± 0.72	8.07 ± 0.56	3.73 ± 0.47
	Nucleus width	3.68 ± 0.50	5.67 ± 0.75	4.54 ± 0.60	5.35 ± 0.80	4.51 ± 0.24	2.98 ± 0.50	3.53 ± 0.38

blackish brown granule seen with few cells (Fig. 6D). According to the characteristics of the positive reaction, the neutrophils and monocytes in five hundred leukocytes were counted, and then analyzed to obtain the percentage of neutrophils and monocytes. No difference was found for the percentage of neutrophils and monocytes between the results of Giemsa staining and cytochemistry staining (Fig. 6A and C).

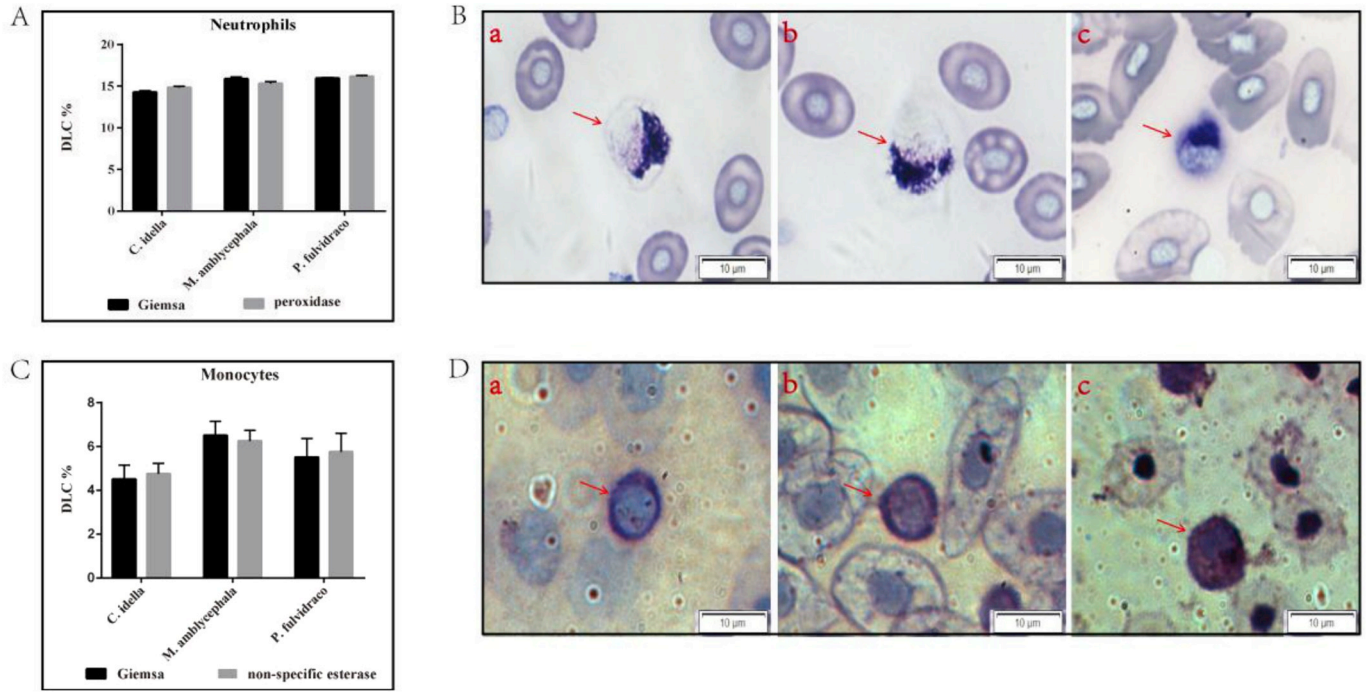

Fig. 6. Cytochemistry staining of monocytes and neutrophils. A. After peroxidase staining and Giemsa staining, the comparison of the percentage of neutrophil in the leukocyte in the grass carp, blunt snout bream and yellow catfish; B. a, b and c represented peroxidase reactivity in neutrophil granules in grass carp, blunt snout bream and yellow catfish. C. After non-specific esterase staining and Giemsa staining, the comparison of the percentage of monocytes in the leukocyte in the grass carp, blunt snout bream and yellow catfish. D. a, b and c represented diffuse staining in the cytoplasm of a monocyte showing a-naphthyl butyrate esterase activity in grass carp, blunt snout bream and yellow catfish. The red arrow indicates neutrophil of peroxidase positive reaction and monocytes of non-specific esterase positive reaction. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

There is growing interest in the study of hematological parameters and structural features of fish blood cells which are important for aquaculture purposes. In the present study, hematologic reference intervals were established by a defined population of healthy grass carp, blunt snout bream and yellow catfish. We first used the improved Neubauer counting chamber to count the RBC, WBC and TC. Then, standard light and electron microscopy techniques are used to tentatively identify the blood cell lineages on the basis of cell morphology. Finally, we used peroxidase stained and non-specific esterase stained to characterize peripheral blood cells, and obtain the percentage of neutrophils and monocytes.

RBC, WBC and TC counts are an important parameter for assessing the health and immune system of fish [7]. The presence of nucleated erythrocytes and thrombocyte in fish blood precludes the application of leucocyte counting methods used in mammals, and manual analysis is currently the only reliable and routine method for fish blood cell count. Mammalian cells commonly by indirect staining method in the determination of fish hematology parameters is not feasible. Therefore, the blood cell count of fish must be changed to the direct staining method. The erythrocytes were counted using a Neubauer haemocytometer [7]. Standard Hayem's or Natt and Herrick's (1952) solution was most used as a dilution solution for erythrocytes. The results are expressed as the number of erythrocytes in 1ul of blood. However, the total leukocyte and thrombocyte count acquisition for fish was precluded by several factors [17]. The leukocytes were generally well counted by using the method of Jerrett and Mays (1973), a modified application of Blain's method, or Natt and Herrick's (1952) and with a Neubauer haemocytometer [18]. According to Jerrett & Mays (1973), neutral red, diluted with 0.007 of physiological water at a ratio of 1:5000, and 12% formalin mixture that was prepared again with 0.007 of physiological water were mixed at a ratio of 1:1 [19]. Natt & Herrick solution (1952) contain 3.88 g of sodium chloride, 2.50 g of sodium sulfate, 1.74 g of sodium phosphate, 0.25 g of potassium phosphate, 7.5 ml of formalin (37%), 0.10 g of methyl violet [19]. The mixture dilutes to 1000 ml and filter. The diluent for leukocytes count described by Natt and Herrick (1952) and generally it is suitable for all non-mammalian vertebrates [18]. However, the absolute count of thrombocyte was not clearly counted in Jerrett and Mays (1973), a modified application of Blain's method, or Natt and Herrick's. In addition, the thrombocyte count and size were calculated from the blood smears prepared with Wright's or Giemsa's stain by most authors [7].

In this study, we systematically established the reference intervals for the RBC, WBC and TC counts in the peripheral blood of grass carp, blunt snout bream and yellow catfish, and compared to the values previously reported of them. However, the total number of RBC, WBC and TC counts varies use to the fish species, physiologic age, sex, season, feeding and nutrition [20]. Our data showed the number of human RBC is 2–4 times higher than that of fish [16], this may be due to the number, size and morphology of animal cells are related to the evolution and ecological adaptation of animals [21]. Mammalian red blood cells are smaller and more numerous, so that the volume of red blood cells is reduced and the cell surface area is increased, which plays a role in improving respiratory function [22]. In addition, the disappearance of mammalian erythrocyte nucleus reduces the respiratory capacity of erythrocyte itself, which are more conducive to the completion of its transport function [23]. Red blood cells of fish have large nuclei, which reflects the low degree of evolution of fish. The reference intervals for total and differential WBC counts of grass carp, blunt snout bream and yellow catfish obtained here were higher than those reported for these species by human. And thrombocytes have been divided into the same counts as leukocytes, a procedure that precludes comparison with the results obtained here. In peripheral blood cells of human, platelets are the formed elements of the blood, which are small and colourless, no nucleated and moderately refractive bodies [24]. However, the thrombocytes of fish have nucleus and the same coagulation function as the platelet of mammals. And some studies have shown that the thrombocyte of fish has the function of phagocytosis [25].

Erythrocytes are the dominant cell type in the blood of the vast majority of fish species and most other vertebrates [26,27]. In the present study, grass carp, blunt snout bream and yellow catfish blood cells were characterized microscopically and hematological indices were analyzed. The mature erythrocytes in grass carp, blunt snout bream and yellow catfish have shown an average size and ultrastructural features similar to those described for mature erythrocytes of other fish species and like in all the species examined so far, they are the predominant cell type found in the blood [28]. At the same time, the peripheral blood in grass carp can also be distinguished from a small number of dividing red blood cells under light microscope (Fig. S2), which is similar to the *Acipenser sinensis* [26], *Siniperca chuatsi* [8] and *Schizothorax (schizothorax.) prenanti* [29], and so on. This indicates that the erythrocytes in grass carp can be directly divided and proliferated from the peripheral blood. Under the electron microscope, the cytoplasm of erythrocytes in the peripheral blood of grass carp, blunt snout bream and yellow catfish contains mitochondria and other structures, indicating that the metabolism of these three kinds of fish is relatively vigorous, which is consistent with the results reported on several other kinds of fish [26,29]. Currently, it is generally believed that whether mitochondria can be observed in fish erythrocyte samples by electron microscopy may be related to the difference between species, age or the development status of red blood cells in fish. And there are also vacuoles in the erythrocyte, which has been rarely reported in fish, study has reported that vacuoles in erythrocyte of fish are related to their phagocytic function [30].

Thrombocytes are the most abundant blood cells except erythrocytes. Platelets count as 10–30% of blood cells in human, while thrombocytes count as 3–4% of blood cells in fish [16]. This may be because platelets are small pieces of cytoplasm liberated from the cytoplasm of mature megakaryocytes in the bone marrow [30]. However, thrombocytes in fish are blood cells with nuclei. The current study shows that thrombocytes of lower vertebrates are armed with full phagocytic functions, in addition to their previously described ability for the production of cytokines that control immune responses [25]. In this study, we have recognized two types of thrombocytes under the light microscopy, both may occur in the same preparation: one elongate/fusiform cell with cytoplasm extending from one or both poles of the oval nucleus and the other round, with a very small amount of ragged cytoplasm surrounding a rounded nucleus. The latter may easily be confused with a small lymphocyte in light microscopy, but the ragged pale cytoplasm is a diagnostic characteristic of thrombocytes [26]. So thrombocytes can be easily recognized with respect to lymphocytes according to morphological features and size, as summarized in Table3 and fig (2 and 3). In contrast, other authors failed to distinguish thrombocytes from lymphocytes according to size and morphology [31]. In teleost thrombocytes, only a single population of granules has been reported under electron microscope and these have a clear space between the granule content and the bounding membrane, which is characteristic of lysosomes. In some studies of fish, thrombocytes have been included in the same counts as leukocytes, a procedure that precludes comparison with the results obtained here [32].

Lymphocytes are usually the most common leucocyte type present in the blood of some fish, accounting for as much as 65–80% of the total leucocyte population. And high nucleoplasmic ratio is the structural characteristic of lymphocytes in fish and all other vertebrates [7]. In some work the lymphocytes have been classified into “large” or “small” groups, however, there is also study remarked that lymphocyte size changed continuously and there was no evidence that the function of a lymphocyte was related to its size [33].

According to the definition of the mononuclear phagocyte system, which is comprised of cells related developmentally and functionally to

each other, the monocyte is a partly differentiated cell with moderate phagocytic properties and is generally found in the blood [34]. Monocytes have been reported to be present in many fish species, it has been also reported that monocytes are absent from the blood of brown trout *Salmo trutta* and goldfish *Carassius auratus* [34,35]. In this study, under the light and electron microscopy, we found the presence of monocytes in peripheral blood cells of grass carp, blunt snout bream and yellow catfish.

It is assumed that the three types of granulocyte described in higher vertebrates (heterophils/neutrophils, eosinophils and basophisl) are also present in fish, although sometimes more than three, or only one or two circulating types of granulocyte have been described for some fish species [36]. In our study, we find only one kind of granulocyte-neutrophils. Cell like basophils and eosinophils can not be observed. Neutrophils inform and shape immune responses, contribute to the repair of tissue as well as its breakdown, use killing mechanisms that enrich our concepts of specificity, and offer exciting opportunities for the treatment of neoplastic, auto inflammatory and autoimmune disorders [37].

In the study of peripheral blood cells in grass carp, blunt snout bream and yellow catfish, leukocyte have been classified as lymphocytes, neutrophils, monocytes. In differential leucocyte count (DLC), neutrophils of human are the most common type while lymphocytes of fish were the most common type. After observed the peripheral blood of a variety of fish [38], it was found that the proportion of neutrophils was higher in the late and young stages, lower in the rod-shaped stage, and lower in the lobulated stage. In previous studies, researchers have suggested that neutrophil nuclear lobulation was a sign of cell aging, but the reason of its formation may be related to the degree of species evolution [39].

The measurement results of peripheral blood cells in grass carp, blunt bream and yellow catfish showed that neutrophils and monocytes were the largest individuals with similar size. Next was the large lymphocyte. And the thrombocyte and small lymphocyte were the smallest. The blood cells of grass carp, blunt snout bream and yellow catfish were significantly smaller than those of sturgeon such as Chinese sturgeon, but similar in size to those of other teleosts [7,35]. The size of blood cells in vertebrates is consistent with their evolutionary level. It is generally believed that the higher the evolutionary status of animals, the smaller the red blood cells, the more the number of red blood cells, because the shrinkage of red blood cell volume will bring about an increase in surface area, play a role in improving respiratory function [40]. This study showed that the cell density of three species of fish is higher than that of most fish and some sturgeon, which indicates that their evolution degree is higher than that of other sturgeon.

Positive peroxidase staining in neutrophils of fish confirmed the observations on other fish species and displayed cytochemical staining properties very similar to that of mammalian neutrophils [41]. The peroxidase is an enzyme characteristic of mammal neutrophils and eosinophils and participates in the defence mechanism against bacterial infection. Alpha-naphthyl acetate esterase is a non-specific esterase for mono-nuclear cells, which can be used for distinguishing monocytic cell types from granulocytic cells [42]. In our study, peroxidase and specific esterase were used to stain the peripheral blood cells in grass carp, blunt snout bream and yellow catfish to obtain the percentage of neutrophils and monocytes, which was not significantly different from the percentage of neutrophils and monocytes after Giemsa staining.

In conclusion, we first established a reference interval for the absolute number of RBC, WBC and TC using an improved blood count plate. And then the peripheral blood cells were stained by Giemsa staining, and the erythrocytes, thrombocytes, lymphocytes, neutrophils and monocytes could be distinguished under light microscope. These cell types were confirmed under the electron microscope. As for the peripheral blood differential leukocyte count (DLC) and size, we established the reference value of the relative number of lymphocytes, neutrophils and monocytes. Finally, No significant difference was found between Giemsa staining and cytochemistry staining on the percentage of neutrophils and monocytes.

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Appendix A. Supplementary data

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