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The Morphological Characterization of the Blood Cells in the Central Asian Tortoise *(Testudo horsfieldii)*

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Abstract

In this study morphological characterization of leukocyte and erythrocyte were investigated in central Asian tortoise (*Testudo horsfieldii*) in Iran. 7 males and 7 females were used in this study. Erythrocyte and leukocyte morphology were described using Wright´s technique. The sizes of erythrocyte and their nuclei were measured using an ocular micrometer under objective lenses (100x). Results of this study were compared with previous work on the other reptile species. Because there were no significant differences between the erythrocyte size of female and male tortoise, the data from the females and males of individual species were pooled. The mean length of mature erythrocytes was 1.98 µm (± 0.16 SD, with a range 1.10 - 2.50 µm).The mean width of mature erythrocytes was 1.10 µm (± 0.14 SD, with a range of 0.80 - 1.90 µm).The mean length of nucleus was 0.71 µm (± 0.09 SD, with a range of 0.50 - 1.00 µm). The mean width of the nucleus was 0.52 µm (± 0.07 SD, with a range of 0.40 - 0.70µm). Five types of leukocytes were found in the tortoise blood namely basophiles, eosinophils, lymphocytes, heterophils and monocytes.

Key words: Erythrocyte, Leukocyte, Morphology, *Testudo horsfieldii*, Tortoise

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Introduction

In human and most mammals, erythrocytes are anucleated with the shape of a biconcave lens. Non-mammalian vertebrates particularly fish, reptile and birds however, possess oval to ellipsoidal erythrocytes with its nucleus retained. Fish erythrocytes have a wide range of sizes among different species.[1] Gulliver made an extensive study of the size of the red cells in vertebrate extending over a period of years. Cullen (1903) gives values for the skate and dogfish while Cleland and Johnson (1912) reported a considerable series, especially birds. Although Wintrobe (1961) tabulated the results of many investigators on red cell size, there have been relatively few reports on new red cell measurements and many species remain unreported.[2] Study of erythrocytes in many different species provides an interesting comparison of red cell size in relation to activities and habits.[3]

Jagoe and Welter reported the morphology of erythrocyte from 7 freshwater fish species namely largemouth bass (*Micropterus salmoides*), blue gill (*Lepomis macrochirus*), chain pickerel (*Esox niger*), yellow perch (*Perca flavescens*), mosquito fish (*Gambusia holbrooki*), red eye bass (*Micropterus coosae*) and rainbow trout (*Oncorhynchus mykiss*). They found that nuclear size and shape varied significantly among the species. Isolated nuclei were found to have conspicuous apertures or holes and the numbers as well as the sizes of these holes also varied significantly among species. However, variations in nuclear size and structure within species were smaller compared to interspecies differences.[1] The first studies on the blood of reptiles described the structures often comparing them with those of the other vertebrates. The literature on hematology of reptilian blood is on a few studies ,with most concerned with European species.[4] Today hematology no doubt plays a large part in the diagnosis of disease in amphibians and reptiles .It is clear that unless more basic work is done to establish normal hematological ranges, this future will always remain distant. While some workers have attempted to show changes in some parameters due to specific disease, others have concentrated more on establishing base lines for these values.[5] In mammals, leukocytes are easily identified on the basis of their morphological differences and the enzymes and other proteins stored in their cytoplasmic granules.[6] Neutrophils are highly specialized phagocytic cells involved in ingestion, death, and degradation of invading microorganisms. Eosinophils are cells that actively participate in the defense against parasitic infections, in the regulation of hypersensitivity reactions, and in the destruction of cancer cells.[6] Studies have been conducted on lower vertebrates in order to understand biological roles of leukocytes in defense mechanisms, and to establish phylogenetic studies and new experimental models. Some investigators have demonstrated the existence of 2 forms of eosinophils in the blood of turtles one of them a mature form and the other an immature form whereas others have stated that there are 2 distinct cell lineages, i.e. neutrophils and eosinophils. Because of the wide morphological variation of these cells in different animal species, it is impossible to characterize them solely on the basis of morphology. Veterinary hematology has relied on classical Romanowsky staining (e.g. Leishman, Wright and Giemsa) to identify erythrocytes, thrombocytes and reliable using classical staining methods. Neutrophils are present in some animals, but this cell types have been reported in a few species. Immature leukocytes also can be present in circulating blood. Thus, cytochemical staining of piscine leukocytes may be particularly useful for identification of cellular lineage and may suggest cell function. Apart from being useful for identifying cell types in blood and tissues, cytochemical staining is also

critical for identifying immunological cell types associated with developmental and pathological processes. The need to identify these features in leukocytes encouraged numerous studies on turtles by Pitol *et al.* (2007).[6] The size and shape of red blood cell and their nuclei in *Testudo greca* and *Testudo hermanni* have been studied by Tosunoglu *et al.* in turkey. In this study they analyzed the hematological parameters of two Testudo species native in Turkey in an attempt to establish their normal reference intervals (2005).[5] By the fact that the *Testudo horsfieldii* species has its characterization not well defined in the literature. In Iran, up till now, there have been no studies on erythrocyte and its nucleus size and leukocyte in circulating blood in healthy central Asian tortoise (*Testudo horsfieldii*). This information would be valuable to determine the health status of the *Testudo horsfieldii*. Therefore, the study was designed to determine the size of erythrocytes and its nucleus and the shape and type of leukocyte in central Asian tortoise *(Testudo horsfieldii*) in Iran.

Materials and Methods

In this study 14 adult specimens (7 males and 7 females) were collected from the region of Zayanderod River in Esfahan and were brought to the laboratory. The blood samples of the live specimens were obtained in the laboratory within three days of their capture. The necessary blood samples were taken from cardiac puncture via syringe. Blood smears were prepared immediately after blood was obtained in heparinized capillary tubes. Three or five blood smears were prepared per individual. The smears were air-dried and stained with wright´s stain. Twelve drops of wright´s stain were dropped to the slides and allowed to stand for 10 min at room temperature, and then they were washed with distilled water and allowed to dry. On each slide 50 mature erythrocyte and their nuclei were measured by means of an ocular micrometer under objective lenses (100x). By this technique 50 erythrocyte sizes were calculated from these measurements. Erythrocyte and nuclear sizes were calculated to the formula respectively: ES = [(EL x EW x π/ 4] and NS = [(NL x NW x π)/ 4]; ES = erythrocyte size; EL = erythrocyte length; EW = erythrocyte width; NS = nucleus size; NL = nucleus length; NW = nucleus width.[1] The leukocyte identification was based on staining parameters and the morphology seen under light microscopy analysis.

Results

The mean straight carapace lengths (SCL) of Asian tortoise (Fig. 1) were measured 20.00 ± 1.27 cm and mean body weights 1440 ± 114.01 g. The blood cell morphology of erythrocyte (red blood cell) in *Testudo horsfieldii* are nucleated, oval cells and their nuclei are also oval and located centrally (Fig. 2). The cytoplasm of mature erythrocytes appear both light and dark pink and was homogeneous under wright´s stain. The nuclei of mature erythrocytes are chromophilic. Because there were no significant differences between the erythrocyte size of female and male tortoise, the data from the females and males of individual species were pooled. The mean length of mature erythrocytes was 1.98 µm (± 0.16 SD, with a range 1.10 - 2.50 µm). The mean width of mature erythrocytes was 1.10 µm (± 0.14 SD with a range of 0.80 - 1.90 µm).The mean length of nucleus was 0.71 µm (± 0.09 SD, with a range of 0.50 - 1.00 µm). The mean width of the nucleus was 0.52 µm (± 0.07 SD, with a range of 0.40 - 0.70 µm) and also erythrocyte and nucleus sizes and length/width ratios of *Testudo horsfieldii* are given in Table1.

Table1. Erythrocyte dimensions of central Asian Tortoise *(Testudo horsfieldii*) in both sexes.

	N	Range	Minimum	Maximum	Mean	SD	SE	Variance
EL(µm)	700	1.40	1.10	2.50	1.98	0.16	0.010	0.026
EW(µm)	700	1.10	0.80	1.90	1.10	0.14	0.008	0.18
NL(µm)	700	0.50	0.50	1.00	0.71	0.09	0.006	0.009
NW(µm)	700	0.30	0.40	0.70	0.52	0.07	0.004	0.005
ES(µm)^2	700	2.70	0.93	3.63	1.76	0.39	0.025	0.15
NS(µm)^2	700	0.34	0.16	0.50	0.30	0.07	0.004	0.005
EL/EW	700	116.82	0.18	117	2.23	7.30	0.46	53.22
NL/NW	700	0.80	1.00	1.80	1.39	0.18	0.11	0.032
NS/ES	700	0.76	0.06	0.82	0.17	0.06	0.003	0.003

EL: Erythrocyte length; **EW**: Erythrocyte width; **NL**: Nucleus length; **NW**: Nucleus width; **ES**: Erythrocyte size**; NS**: Nucleus size

Five types of leukocytes were found in the tortoise blood namely basophiles, eosinophils, lymphocytes, heterophils and monocytes. Basophiles presented spherical conformation with segmented nucleus, spherical granules in cytoplasm (Fig. 3). Eosinophils were defined as spherical shape and peripheral nucleus, with cytoplasm filled by oval granules (Fig. 4). Small and spherical lymphocytes, with eccentric nucleus, were observed in almost all cytoplasm (Fig. 5). Monocytes were found in circulating blood characterized by oval and peripheral nucleus and abundant cytoplasm (Fig. 6). Heterophil is the most abundant leukocyte found in health. Heterophils contain large, eosinophilic, ovoid, cytoplasmic granules. The cytoplasm, which can be difficult to visualize, is light blue or clear (Fig. 7). The nucleus is frequently displaced toward the edge of the cell. It was basophilic with dense chromatin. Thrombocytes showed elliptic conformation, with a little cytoplasm and nucleus with elliptic shape too (Fig. 7).

Fig 1. Dorsal view of Asian central Tortoise

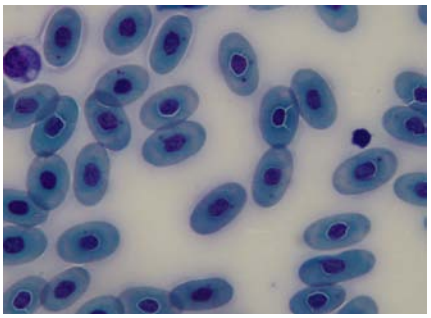

Fig 2. The blood smear of Asian tortoise showing erythrocyte with nucleus. (Wright stain, 100x)

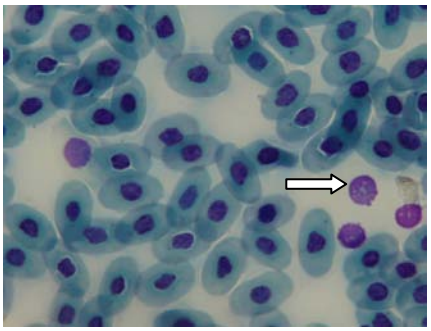

Fig 3. Basophil (white arrow) showing spherical shape with dark purple-gray cytoplasmic granules (Wright stain, 100x)

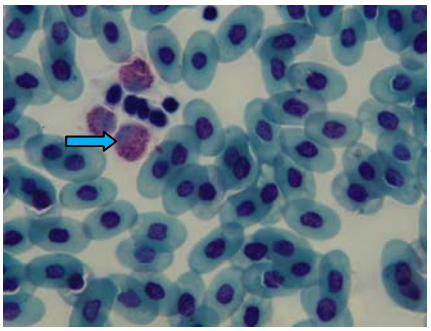

Fig 4. Eosinophil of central Asian tortoise (blue arrow) containing an oval nucleus with large round red cytoplasmic granules (Wright stain, 100x)

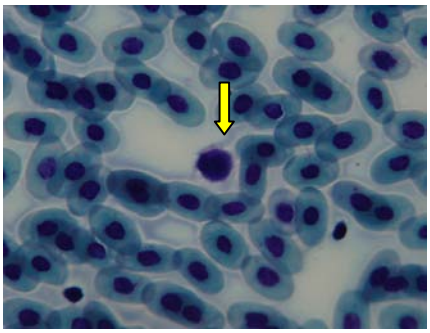

Fig 5. Lymphocyte from central Asian tortoise (yellow arrow). The nucleus is round and slightly indented. A thin rim of light blue cytoplasm is apparent (Wright stain, 100x).

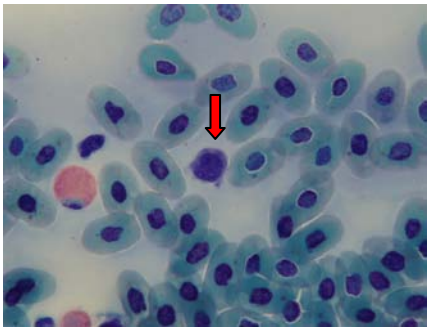

Fig 6. Monocytes from central Asian tortoise (red arrow). The nucleus is bean or heart shaped with moderately granular chromatin. The cytoplasm may range from blue to gray (Wright stain, 100x).

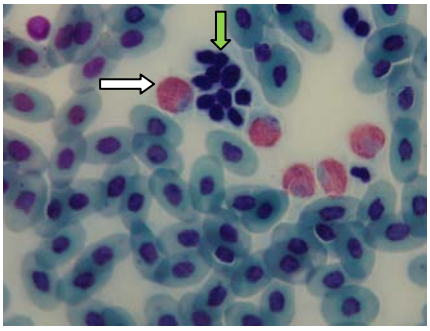

Fig 7. Heterophils with numerous red granules (white arrow); and thrombocytes (green arrow) (Wright stain, 100x)

Discussion

One of the most important functions of erythrocytes is to carry oxygen and carbon dioxide, and its surface area to size ratio is also a determining factor in the tissues. Thus, a small erythrocyte offers the possibility of a higher rate of exchange than a larger one.[3,7] The erythrocyte size reflects the position of a species on the evolutionary scale: in lower vertebrates and those with a not-so successful evolutionary past, in cyclostomes, elasmobranches and urodeles, the erythrocytes are large, but in higher vertebrates (mammals) the same cells are smaller and do not contain nuclei.[2] Erythrocytes are morphologically similar among various species of reptiles Saint Girons in 1970[17] In reptiles sizes vary greatly Sevinç *et al.* in 2000.[7] The erythrocytes of Russian tortoises, *grionemys horsfieldi*, were reported to be long or irregular in shape by Knotková *et al.*(2002).[8] Uğurtaş *et al.*(2003) pointed out a positive correlation between erythrocyte size and nucleus size for individuals belonging to families Testudinidae and Emydidae (Testudinidae: r = 0.494, $P < 0.001$; Emydidae: r = 0.668, $P < 0.001$).[9] Similar results were obtained for individuals belonging to family Scincidae (r = 0.302, $P < 0.01$) (Atatür *et al.* 2001).[10] Metin *et al.*(2008) also found positive correlation (r = 0.61 $P < 0.05$) by pooling *M. caspica* and *M. rivulata* together as Bataguridae family. Nucleus in mature erythrocyte is round in green turtles (*Chelonia mydas*).[11,12] The differences in erythrocyte morphometric may be caused by dissolved gases. Erythrocytes are highly specialized for transporting oxygen. In general; erythrocytes are longer in size in the lower orders. For example, reptiles have larger erythrocyte than birds and birds have larger ones than mammals. However, erythrocyte size also varies greatly within orders. For example, in mammals the smallest erythrocyte occurs in deer, sheep and goats while the largest erythrocyte occurs in elephant and some large rodent.[1] Investigation carried out by various authors reported that the sizes of erythrocyte vary in members of the 4 orders of reptiles.[3,4,7] Within the class Reptiliathe largest erythrocytes are seen in *Sphenedon punctatus* ,turtle, and corocodilians.[3,4] The smallest erythrocytes

are found in the *Lacertidae* family.[3,4,7] In the exchange of oxygen and carbon dioxide in the tissues. Thus, a small erythrocyte in *Testudo horsfieldii* offers a possibility of greater rate of exchange than a larger one. Likewise an elliptical body is more efficient than a spherical one of the same volume. Longer nuclei could offer a somewhat greater surface for exchange with the cytoplasm. The role of the nucleus in red cell function of many species with nucleated erythrocytes has never been clarified. By the fact that the *Testudo horsfieldii* specie has its characterization not well defined in the literature, it was proposed in this study the leukocyte characterization of the blood, stained by wright and analyzed under light microscope Studies related to leukocytes characterization in tortoise do not present a consensus. The classification of reptilian leukocytes posses many problems since these cells show morphological variation within the class and several different nomenclatures have been used to describe them.[8] For example, light microscopy Taylor & Kaplan (1961) divided leukocytes into neutrophils, basophils, eosinophils, lymphocytes and monocyteson in turtles.[13] Saint Girons (1970) reported the presence of eosinophils, azurophils, neutrophils and plasma cells in reptiles.[4] Sypek and Borysenko (1998) described eosinophils and heterophils in reptilian blood.[14] Cannon *et al* 1996 divided granulocytes into basophils and eosinophils on phase-contrast microscopy.[15] Wood and Ebanks (1984) described eosinophils and neutrophils.[16] Widely accepted opinion is that reptilian (Montali. 1988) and avian heterophils (Brooks *et al.* 1996) have functions similar to mammal neutrophils.[17,18] According to Canfield (1998) the mammalian neutrophil is equivalent to the non-mammalian heterophil.[19] The heterophil, excluding mammals has coarse, red to brown, speculated to irregular granules of variable size and either a bilobed (birds and some lizards) or unlobed nucleus (most reptiles and amphibians). Azevedo and Lunardi's (2003) observations showed that 2 types of eosinophilic granulocytes were present in blood of *Chrysemys dorbigni.* Eosinophils and neutrophils are granulocytic leukocytes present in the blood of most vertebrates.[20] The existence of these two cell types in reptiles is a matter of controversy. To avoid confusion, some researchers suggest that the term neutrophils be restricted to mammals and the term heterophil to non-mammals.[21,22] In this study, it appears that on the basis of light microscopic findings there are three main types of granulocytes (heterophils, eosinophils, basophils) and two main types of agranulocytes (lymphocytes and monocytes) in *Testudo horsfieldii.* We identified heterophils as having an eccentrically placed nucleus and being round-oval or mostly bi-lobed in shape. The cytoplasm was filled with numerous elongated granules. Lymphocytes may be small, medium or large. Canfield (1998) stated that cytoplasm may contain small vacuoles and azurophilic granules.[19] In the present study, the nucleus of lymphocytes almost filled the cytoplasm of the cell. The amount of cytoplasm was lower and of light blue color. Monocytes are large cells with unlobed or lobed nuclei and a large amount of lightly basophilic cytoplasm. The similarity of thrombocytes and lymphocytes in reptiles is known.[4] Canfield & Shea (1988) reported that thrombocyte morphology was influenced by the degree of aggregation.[23] Saint Girons (1970) reported that thrombocytes were small, oval cells characterized by elongate, centrally located, highly chromophilic nuclei.[4] Knotková *et al.* (2002) identified two types of thrombocytes in Russian tortoises, *Agrionemys horsfieldi*: oval with a good visible membrane, a faintly stained cytoplasm and rectangular with small projections of lightly basophilic cytoplasm.[8] They attributed this variability to ageing, function and artifact. The

similarity of thrombocytes and lymphocytes in reptiles is known.[24] The present study reports that thrombocytes are formed in cell groups, with centrally located dark-stained nuclei and their cytoplasm is difficult to see at the light microscopic level. The result of this study add new information to our knowledge of *Testudo horsfieldii* and provide an important database for veterinarians, scientists, and biologists assessing tortoise medicine, ecology and survival.

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