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Research Article

Hemolymph analyses in medicinal leeches, *Hirudo verbana* and *H. sulukii* (Hirudinida: Hirudinidae)

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Abstract

The aim of this study was to determine the hemocyte type of medicinal leeches, *Hirudo sulukii* and *H. verbana*. It was also determined the fractions of hemolymph proteins in these leech species. According to the analyses, four types of hemocyte (prohemocyte, plasmatocyte, granulocyte, and eleocyte) were detected in two species. The prohemocytes have large nuclei, and their cytoplasm occupies a small area. The plasmotocytes are the most abundant hemocyte type. They are polymorphic cells and are found together as aggregations. The granulocytes are generally rounded in shape and typically have large granules in their cytoplasm. The eleocytes are the least found cells in hemolymph and have few granules in their cytoplasm. The areas of hemocyte types did not differ between the two species. Using polyacrilamid disc gel electrophoresis, it was determined that the hemolymph proteins of *H. verbana* were separated into 11 fractions or fraction groups. On the other hand, they were separated into 10 fractions or fraction groups in *H. sulukii*. In addition to qualitative differences, there were important quantitative differences between electropherograms of the two leech species. Therefore, these findings made a contribution to studies considering *Hirudo sulukii* as a new species different from *Hirudo verbana*.

Introduction

Invertebrates make up the largest group of animal groups and can be found almost anywhere. They can make self and non-self distinctions thanks to their innate immunity (Jiravanichpaisal *et al*., 2006; Aladailah *et al*., 2007; Melillo *et al*., 2018). Invertebrates have no antibodies and lymphocytes that form the basis of the humoral immune system. But invertebrates possess mechanisms that are seen as precursors of vertebrate immunity (Arıkan and Çakıcı, 2021). Innate immune system of invertebrates is made up of cellular and humoral parts working together to defend against unwanted organisms (Castellanos-Martinez *et al*., 2014). Cellular immunity including hemocyte or coelomocyte is responsible for phagocytosis, encapsulation, nodule formation and the production of reactive oxygen intermediates (Pipe, 1992; Hegaret *et al*., 2003; Canesi and Prochazkova, 2014). Hemocytes circulate in the hemolymph (Pham and Schneider, 2009) and have other important functions such as nutrient transport, digestion, excretion and wound repair (Cheng, 1975; Cheng, 1981; Chu, 2000; Franchini and Ottaviani, 2000; Mount *et al*., 2004). In invertebrates, different hemocyte types have been described (Buchmann, 2014). Humoral immunity contains antimicrobial peptides, phenoloxidase and lysozyme (Boulanger *et al*., 2006; Ovchinnikova *et al*., 2006). Prophenoloxidase system in some invertebrate phyla, including several insects, crabs, and worms, is an analogue to the complement system. This system is activated by a group of enzymes (Beck and Habicht, 1996; Atalayın *et al*., 2017).

Leeches have been widely used since ancient times to lower blood pressure (Gödekmerdan *et al*., 2011). In addition to their therapeutic effects on humans, leeches can also feed on vertebrates and invertebrates as parasites (Sağlam, 2012). Medicinal leeches of the genus Hirudo are used in traditional and complementary medicine practices (Singh, 2010). *Hirudo medicinalis* has been used in medicine since ancient times (Eldor *et al*., 1996; Orevi *et al*., 2000). The medicinal leech species identified so far in Turkey are *H. medicinalis*, *H. verbana* and *H. sulukii* (Karataş and Dernekbaşı, 2018). As we mentioned above, hemocytes have important functions in immune defense. On the other hand, there is no study about hemolymph analyses on *H. verbana* and *H. sulukii*. Therefore, we performed some analyses to determine whether there were differences in terms of hemocyte type, hemocyte area and hemolymph proteins in hemolymph of *H. verbana* and *H. sulukii*.

Material and methods

Hemocyte Preparation

Hemolymph specimens of leeches were collected into hematocrit tubes and then smears were prepared on clean slides. Air-dried hemolymph smears were stained with Wright's stain for one minute (Seiverd, 1972). The slides were rapidly washed with phosphate-buffered saline and then mounted with entellan. They were examined with a Zeiss Axioscope A1 light microscope and photographed using AxioCam Erc 5s digital camera.

Determination of Hemolymph Proteins by Electrophoresis

The hemolymph samples were centrifuged for 5 min at 600 ×g. The electrophoretic separation of hemolymph proteins was performed according to Arıkan *et al.* (2006), who slightly modified and applied the polyacrylamide disc gel electrophoresis method by Davis (1964). Electrophoretic separations were run using a Canalco Model 1200 electrophoresis apparatus (Canalco Inc., Rockville, Md., USA) at room temperature (20-25°C). Accordingly, a pH 6.7 stacking gel was layered above the pH 9 separation gel of 7.5% polyacrylamide, together with a pH 8.3 tris-glycine buffer system. 1000 µL of Brom Phenol Blue was added to the buffer solution at the top of the electrophoresis bath to monitor the separation. Gels containing separated proteins were stained with 0.5% Amido Black (Naphtol Blue Black 10- B), and the excess stain was passively discharged in 7% acetic acid baths. The densitometric curves of the separations were obtained at 500 nm by means of a Gelman ACD-15 39430 densitometer (Gelman Instrument Co., Ann Arbor, Mi., USA), and they were photographed. Qualitative evaluation of the gels was made directly from the electropherograms.

Statistical Analyses

For statistical analysis of hemocytes, a total of 100 hemocyte areas of each species were measured using ZEN 3.2 (blue edition) program. In a comparison of the two groups, an independent t-test was used for parametric groups. Statistical analyses were made by using IBM SPSS Statistics 25.0 (IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.) Data were presented as mean with standard deviation. We set the significance level at $p \leq 0.05$.

Results

Hemocyte types in H. verbana

Prohemocyte, plasmatocyte, granulocyte, and eleocyte hemocyte types were detected in hemolymph of *H. verbana*. Of these cells, prohemocytes have large nuclei and their cytoplasm occupies a small area (Fig. 1a). In plasmatocytes, the nucleus is located in the center and the cytoplasm occupies a larger area. However, plasmotocytes appear as polymorphic forms and are found together as aggregations. (Fig. 1b). Granulocytes are cells that are rarely found in hemolymph and have a few granules in their cytoplasm (Fig. 1d).

Hemocyte types in H. sulukii

Hemocyte types were determined as prohemocyte, plasmatocyte, granulocyte and eleocyte in hemolymph of *Hirudo sulukii*. The nuclei of prohemocytes are large enough to fill the entire cell, and the cytoplasm occupies a small area (Fig. 2a). In plasmatocytes, the nucleus is in the center part of the cell and the cytoplasm occupies a larger area (Fig. 2b). Granulocytes usually have large granules in their cytoplasm (Fig. 2c). Eleocytes are not frequently found cells, and have few granules in their cytoplasm (Fig. 2d).

Statistical Comparison of Hemocyte Types in H. verbana and H. sulukii

When areas of prohemocyte, plasmatocyte, granulocyte, and eleocyte cells belonging to

Hirudo verbana and *Hirudo sulukii* were compared, it was detected that there was no difference in hemocyte areas between leech species. In other words, there was no difference between the two types in terms of areas of prohemocyte, plasmatocyte, granulocyte and eleocyte hemocytes. These data were presented in detail in Table 1.

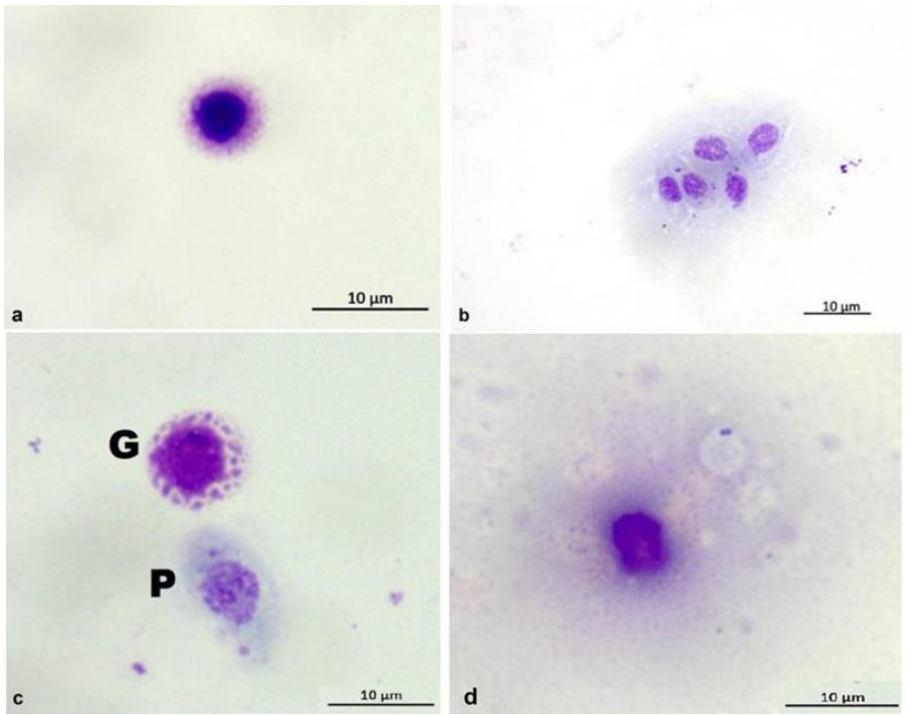

Figure 1: Hemocyte types of *Hirudo verbana* a) prohemocyte, b) plasmatocyte agregations, c) granulocyte (G) and plasmatocyte (P), d) eleocyte.

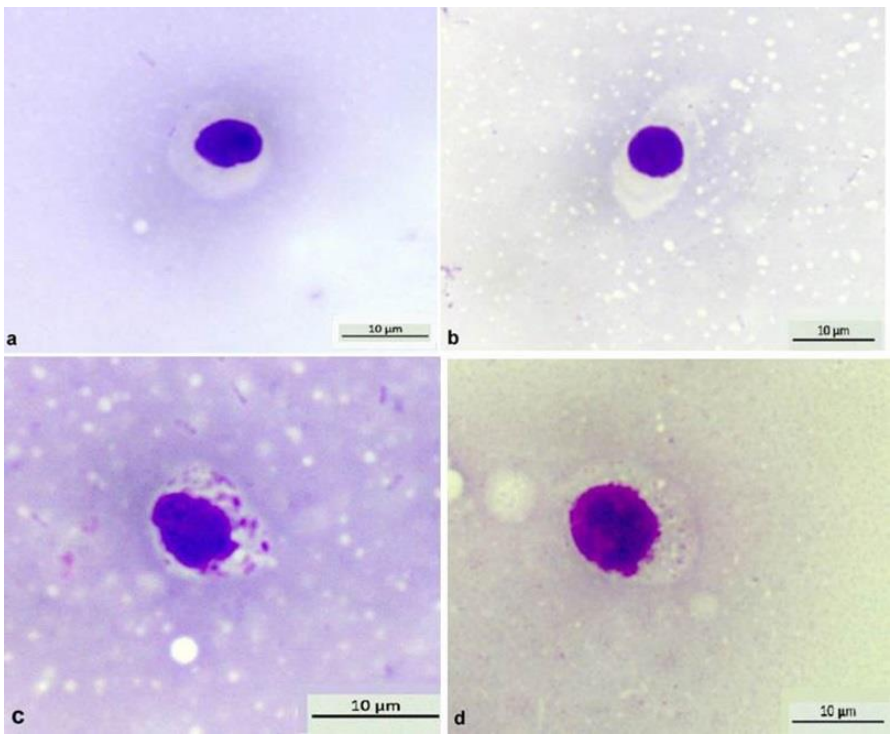

Figure 2: Hemocyte types of *Hirudo sulukii* a) prohemocyte, b) plasmatocyte, c) granulocyte, d) eleocyte.

Table 1: Comparison of the areas of *Hirudo verbana* and *Hirudo sulukii* hemocyte types (µm^2), (independent t-test).

Hemocyte Types	Species	Mean	SD	t-value	Sig.(2-tailed)
Prohemocyte	*H. verbana*	35.29	6.33	-0.946	0.350
	H. sulukii	37.05	5.44	-0.946	0.350
Plasmatocyte	*H. verbana*	48.62	11.77	0.691	0.491
	H. sulukii	47.05	8.13	0.691	0.491
Granulocyte	*H. verbana*	56.40	4.43	-0.629	0.352
	H. sulukii	57.14	4.74	-0.629	0.352
Eleocyte	*H. verbana*	80.54	8.70	-1.125	0.275
	H. sulukii	84.28	5.87	-1.125	0.275

Electrophoretic analysis of hemolymph proteins

According to electrophoretic and densitometric analyses, *H. verbana*'s hemolymph proteins were divided into 11 fractions or fraction groups (Fig. 3). In *H. sulukii*, hemolymph proteins were divided into 10 fractions or fraction groups (Fig. 4). In addition to qualitative differences, there were important quantitative differences between the electropherograms representing the two species (Figs. 3 and 4).

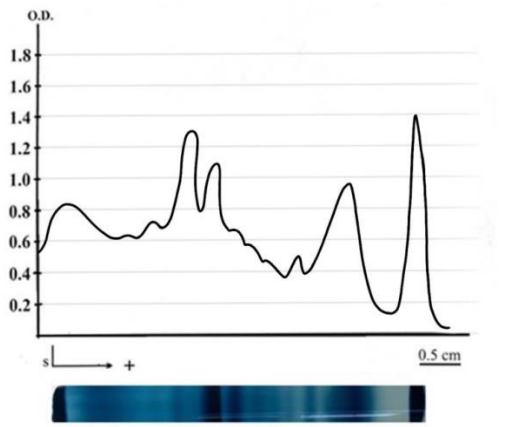

Figure 3: Densitometric tracing curve of a sample representing *Hirudo verbana* together with its gel photograph showing electrophoretic separation of hemolymph proteins. O.D: Optical density, S: Start (junction between stacking and separation gels).

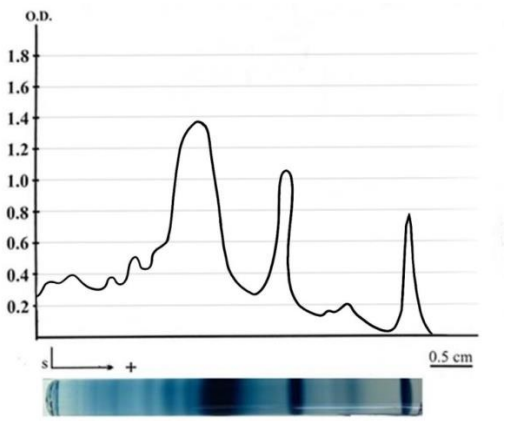

Figure 4: Densitometric tracing curve of a sample representing *Hirudo sulukii* together with its gel photograph showing electrophoretic separation of hemolymph proteins. O.D: Optical density, S: Start (junction between stacking and separation gels).

Discussion

Among invertebrates, annelids have a closed vascular system distinct from the coelomic fluid (Boidin-Wichlacz *et al*., 2012). When we examined *Hirudo verbana* and *Hirudo sulukii* species in terms of hemocyte types, it was found that prohemocytes, plasmatocytes, granulocytes, and eleocytes were present in both species. Prohemocytes are the smallest groups of precursor stem cells circulating in the hemolymph. They are characterized by having larger nuclei and a smaller cytoplasmic area within the hemolymph

(Öztürk *et al*., 2018). Plasmatocytes are the most abundant cell types in the hemolymph. In addition, these cells are seen in different forms in the hemolymph, that is, polymorphic (Yamashita and Iwabuchi, 2001). Plasmatocytes are considered as vertebrates' monocytes. These cells, like monocytes, have phagocytic activity (Evans *et al*., 2003; Hartenstein, 2006). Some plasmatocytes form aggregations. This is accepted as a defensive response (Ray *et al*., 2015; Atalayın *et al*., 2017). The second most abundant cell type in the hemolymph is granulocyte, which contains granules of various sizes in their cytoplasm (Larouche *et al*., 2019). Granulocytes resemble vertebrate cells such as basophils, neutrophils and eosinophils. These cells perform immune functions such as phagocytosis, encapsulation of pathogens, wound healing and blood coagulation. (Gupta 1991; Atalayın *et al*., 2017). In addition, some invertebrate species have another type of cell that contains a high percentage of granules in the hemolymph. These cells are called eleocyte. These hemocytes have vesicle-shaped structures in their cytoplasm (Roubalová *et al*., 2018).

In our study, areas of hemocyte types (prohemocyte, plasmatocyte, granulocyte, and eleocyte) were compared in two leech species, *Hirudo sulukii* and *Hirudo verbana*. It was determined that there was no difference between the areas of hemocyte cells of the two species. In addition, the same hemocyte types were detected in *H. medicinalis* (Atalayın *et al*., 2017). Because there are limited studies on leeches, we can compare our findings to some other invertebrate species. In a study performed on *Decticus verrucivorus*, *Eupholidoptera smyrnensis* and *Glyphotmethis*, hemocyte types were determined as prohemocyte, plasmatocyte, granulocyte, spherulocyte and oenocytoid (Öztürk *et al*., 2018). Spherulocytes and oenocytoids are present in these species, while eleocytes are absent.

Various researchers who work on blood-plasma proteins of amphibians and reptilians using biochemical methods (Chen, 1967; Ferguson, 1980; Arıkan *et al*. 1998, 1999; Arıkan and Çiçek, 2011; Afsar *et al*. 2014) found that age, gender, seasonal, physiological and environmental factors were effective on the number, concentration and speed of protein fractions in separation of plasma proteins. It was stated that among these factors, age, gender, seasonal, physiological and environmental factors caused quantitative differences, whereas hereditary variations led to qualitative differences. In the present study, differences were determined between electropherograms of *H. verbana* and *H. sulukii* species. Therefore, we can say that our electrophoretic analysis results supported the DNA studies in *H. verbana* and *H. sulukii* species made by Sağlam *et al*. (2016).

In this study, we found that both Hirudo species had similar hemocyte types and hemocyte areas. On the other hand, while hemolymph proteins of *H. verbana* were separated into 11 fractions or fraction groups, they were separated into 10 fractions or fraction groups in *H. sulukii*. In addition to qualitative differences, we detected important quantitative differences between electropherograms of two leech species. Therefore these findings

contributed to studies in which *Hirudo sulukii* was evaluated as a new species different from *Hirudo verbana*.

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Conflicts of interest

The authors declare that they have no competing interests.

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