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Morphological and cytochemical characterization of the sternal leukopoietic cell lineages in wistar rat

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Abstract
This research provides the first data on the morphological identification of the leukopoietic lineages (leukopoies) in the bone marrow of the Wistar rats. Seven types of cells were identified: myeloblasts, promyelocytes, myelocytes (neutrophils, eosinophils and basophils), metamyelocytes and band granulocytes. It was necessary to apply standard staining by using Pappenheim and cytochemical peroxidase method protocol for the proper cellular identification. Because of the consistency of the tissue, bone marrow isolation is not possible by the standard methods, it is only possible by the use of the touch technique. Mature neutrophils had high prevalence in the bone marrow, followed by the neutrophilic myelocytes. The largest among observed cells were the myeloblasts and promyelocytes. Identified cells showed great resemblance to human bone marrow cells, which is very important for any future experimental research.

Keywords: Bone marrow, leukopoiesis, myeloblasts, sternum, wistar rat

1. Introduction
Wistar rats are often used in toxicological studies, however manipulation with them did not produce any known haematological refence interval. Because of differences in hematology reference interval of Wistar rats hematological parameters, there is a need for evaluation of hematologic status untreated animals instead of using historical reference values and their significance in clinical trials [1]. Therefore, hemogram elements and their interpretation should be analysed carefully because there is existence of wide physiological variations range [2]. Due to the fact that unique hematologic referent interval was not established, it can be concluded that complete data about progenitor cells in bone marrow lack. Stem cell research is an important area of molecular and cell biology because of their strong proliferation and unlimited self renewal and multipotency [3].

The granulocytic lineages differentiate from common myeloid progenitor for all cells, followed by production of neutrophil progenitors, eosinophil progenitors, basophile progenitors and mast cells progenitors. Bone marrow contains two types of different cell. The blood cells are formed from hematopoietic stem cells, and the second type represent the mesenchymal stem cells that are included in the different cell lines such as formation of the osteoblasts [4]. Hematopoiesis can be considered as a system of differentiation resulting from pluripotent hematopoietic stem cells capable of differentiation and renewal [5]. Granulopoesis in adult mammals is carried in bone marrow, with expansion in the extramedullar tissues during disease of bone marrow [6]. Bone marrow stem cells culture obtained from rat is characterized by its morphological, biochemical and immunohistological properties [7].

The clinical research of bone marrow is recommended when there is a primary or secondary hematological disorder that can not be explained only by examination of peripheral blood [8]. As haematopoietic tissue, bone marrow provides a unique microenvironment for the proliferation, differentiation and release of erythrocytes, granulocytes, monocytes, lymphocytes, and thrombocytes [9]. Hematopoietic stem cells are in close connection with osteoblasts along endosteal border of bone marrow [10]. Therefore, the most intensive granulopoesis takes place in the vascular niches along the central bone marrow sinus [11]. The main aim of this research was isolation of the rat sternal bone marrow and analysis of hematopietic stem cells maturation stages.

2. Materials and Methods

2.1 Animal Breeding

Rats (*Rattus norvegicus* - Wistar strain; n=10), used in this experimental design, were bred at the vivarium, Laboratory for Physiology, Department of Biology, University of Sarajevo, Bosnia and Herzegovina. During the experimental stage, appropriate care, accommodation and animal handling were mandatory, thus preventing the abuse of animals in compliance with the ethical and law requirements. Handled animals were treated in accordance with the "Declaration on the Rights of Animals" (UNESCO, 1978), "Universal Declaration on Animal Welfare" (WSPA, 2000) and Animal Protection and Welfare Law of Bosnia and Herzegovina ("Službene Novine" 25/09). Selected rats were kept in the vivarium (individual combined cages at room temperature of 28 ^{0}C) and fed with the Oxbow Essentials (low-fat and nutritionally-balanced grade food) pellets for rats with water *ad libitum*.

2.2 Experimental Procedures

Animals used for the study were between 13 and 16 weeks old, at the equal developmental stage with the mass between 247 and 311 g. Anesthesia was performed by the sufentanil/medetominide (50/150 µg/kg – administered by intramuscular injection) and euthanasia was performed by the doubling of the dosage. After animal euthanasia, primary incisions were made in the thoracic region of the chest and hind legs to expose the bone structures beneath. Secondary incisions were made to remove completely sternum and femurs, with the appropriate muscle, tendon and tissue residue cleansing. Four cuts in total were made on the femoral shaft while sternum was cut from manubrium to the xiphisternal joint. Afterwards, bone marrow was removed by a small gauge needle (21G; Semikem, Sarajevo, BiH) and placed on a clean microscopic slide. The bulk of the bone marrow was smeared gently (slight zig-zag movements with a pin) by the touch technique. Fresh smears were stained by May-Grünwald and Giemsa stains (Semikem, Sarajevo, BiH) followed by cytochemical staining done by Leders method (Semikem, Sarajevo, BiH) for proper evaluation of the peroxidase activity.

2.3 Microscopic Analysis

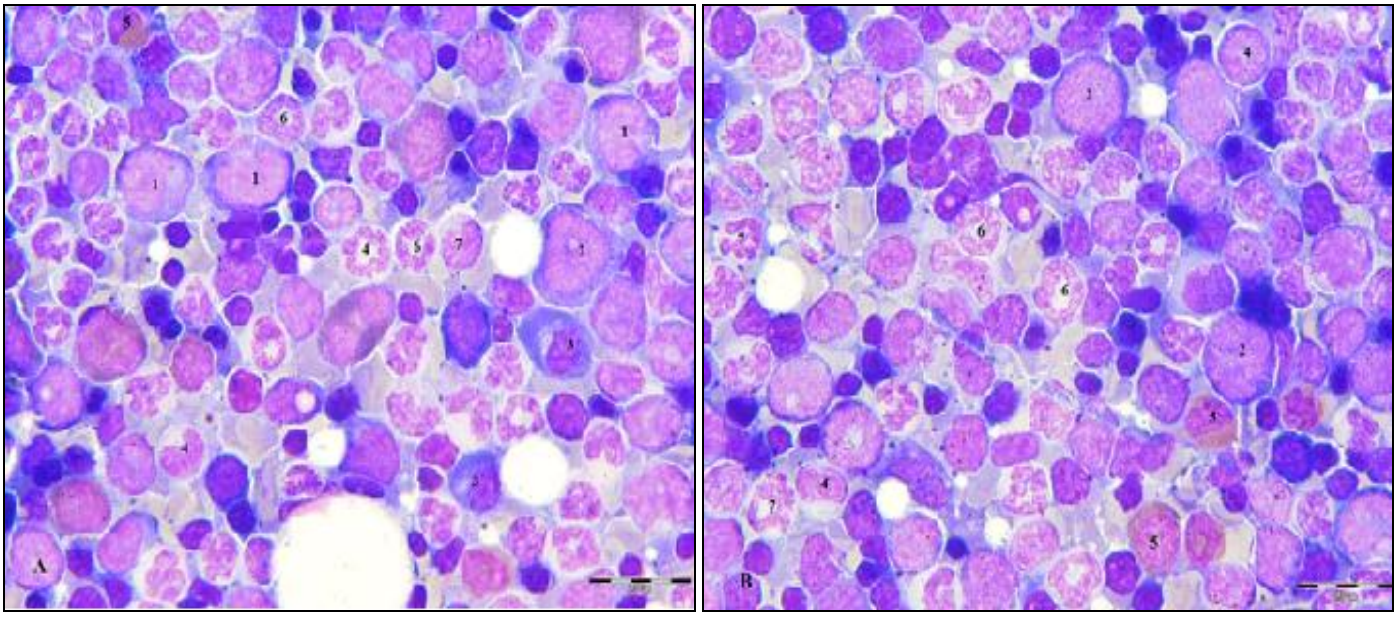
Cell identification and all morphometric parameters (total number, individual fractions in % and cell surface area) were performed on Olympus BX41 light microscope. Myeloid progenitor cells identification was achieved by utilization of Olympus DP12 camera. Image analysis and processing was done in the licensed software (Olympus DP12 Soft DP12-CB Ver.01.01.01.42. ® Olympus Corp.).

2.4 Statistical Analysis

Results were evaluated by the SPSS (Version 20.0, SPSS, Inc., Chicago, IL, USA) The results of the collected data were elaborated with the methods of the descriptive statististics (mean, standard deviation, range, coefficent of variation and Shapiro W test for analysing the normality of the data distribution). P values lower than 0,05 were considered significant

3. Results

Figure 1 (A and B) represents bone marrow smear isolated from sternum and stained by MGG.



(a) (b)

Fig 1(a & b): Sternal bone marrow smear of the Wistar rat (1. Myeloblast, 2. Promyelocyte, 3. Myelocyte, 4. Neutrophilic metamyelocyte, 5. Eosinophilic metamyelocyte, 6. Band neutrophile, 7. Segmented neutrophile

Figure 2 presents bone marrow smear isolated from sternum and stained by peroxidase.

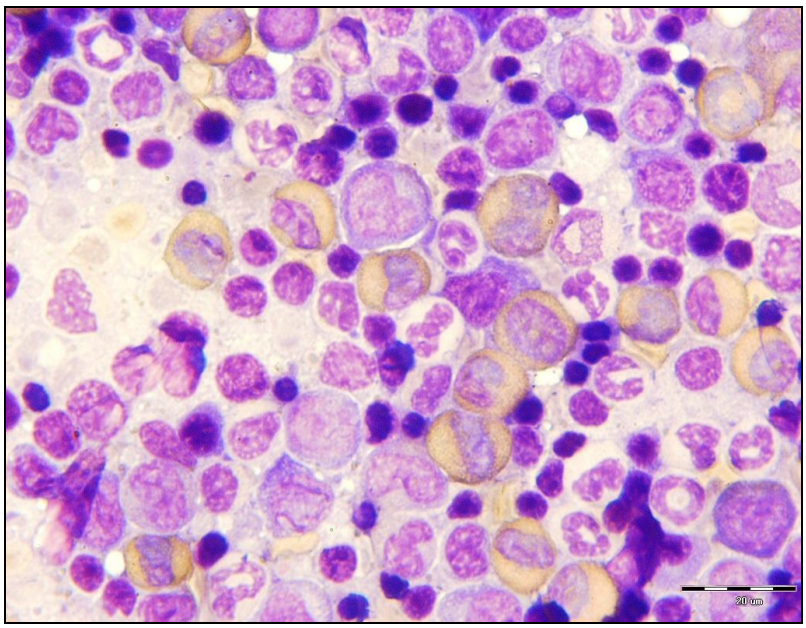


Fig 2: Sternal bone marrow smear of the Wistar rat -Myeloperoxidase activity (green cytoplasm is represented by neutrophilic myelocytes to neutrophilic granulocytes)

Table 1 presents percetage ration between the myeloid precursor cells including mature neutrophilic granulocytes in bone marrow. Mean and standard deviation, range, coefficient of variation and test of normality is presented for every type of the cells. From each sternum sample, 300 cells were included for identification and sequestial morphological analysis.

Table 1: Relation between myeloid precursor cells and mature neutrophils

Leukocytes lineage of cells	Mean±SD	Range	Cofficient of variation %	Shapiro-Wilk normality
Myeloblast	4.40±1.07	3.00-6.00	24.43	0.177
Promyelocyte	3.80±0.78	3.00-6.00	20.75	0.025*
Myelocyte	1.8±0.78	1.00-3.00	43.82	0.025*
Eosinophilic metamyelocyte	9.50±1.58	7.00-12.00	16.64	0.849
Basophilic metamyelocyte	3.60±1.26	2.00-6.00	35.13	0.445
Neutrophilic metamyelocyte	35.10±6.53	25.00-44.00	18.63	0.634
Band neutrophile	1.6±0.69	1.00-3.00	43.70	0.008*
Mature neutrophile granulocyte	40.20±5.94	31.00-48.00	14.77	0.654

* Statistical significant values at 0.05

Results showed that mature neutrophils take high percentage of total cells in bone marrow (40.20%). Neutrophilic myelocytes are the most abundant of all myeloid precursor cells. Shapiro Wilk W test of normality shows significant deviation of normal promyelocytes, band granulocytes and metamyelocytes distribution.

Table 2 shows the mean values of the cell surface of myeloid precursor cells and mature neutrophils.

Table 2: Mean values of the myeloid precursor cells and mature neutrophils surface

Leukocytes lineage of cells	Mean±SD	Range	Cofficient of variation %	Shapiro-Wilk Normality
Myeloblast	185.66±4.32	179.58-192.28	2.32	0.559
Promyelocyte	243.08±7.77	229.57-253.23	3.20	0.621
Myelocyte	107.42±7.97	92.17-119.48	7.42	0.864
Eosinophilic metamyelocyte	116.45±15.67	88.95-135.27	13.45	0.482
Basophilic metamyelocyte	148.77±23.94	117.23-176.94	16.09	0.124
Neutrophilic metamyelocyte	132.46±17.68	98.06-158.67	13.34	0.950
Band neutrophile	95.72±7.70	85.74-107.76	8.04	0.411
Mature neutrophile granulocyte	112.36±5.84	103.17-123.95	5.20	0.645

Promyelocytes have the largest surface, followed by myeloblasts. Among the myelocytes, the highest variation in the cell surface values was observed. Significant deviations from the normal distribution were not found for any observed cell type.

Figure 3 shows single cells of myleoid line and mature neutrophils

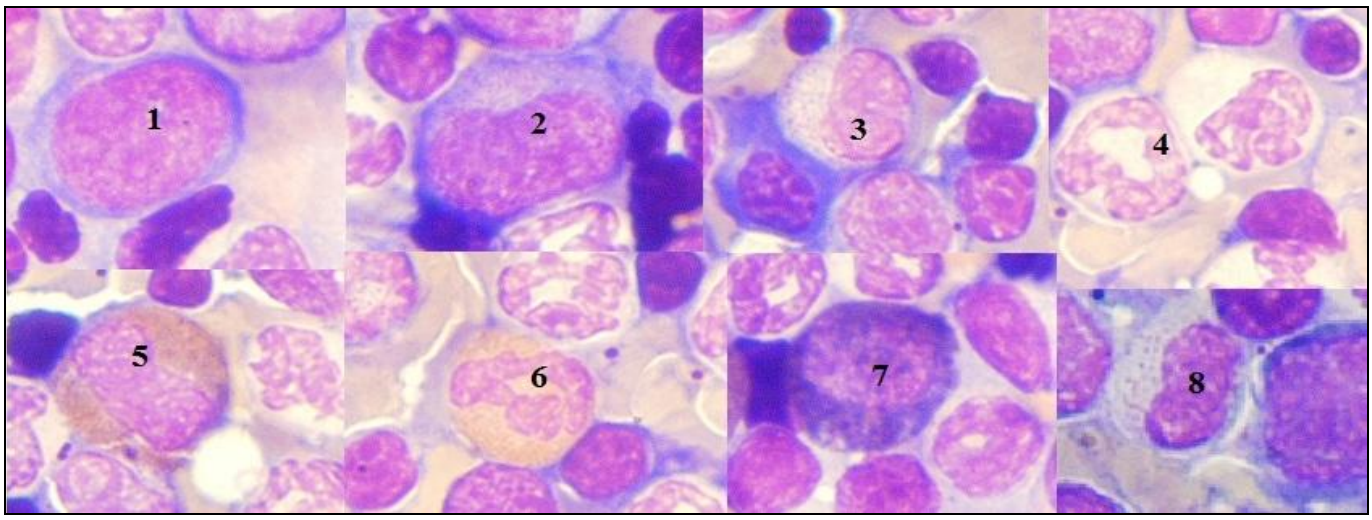


Fig 3: Single myeloid cells and mature neutrophils (1. Myeloblast, 2. Promyelocyte, 3. Myelocyte, 4. Segmented neutrophile, 5. Eosinophilic metamyelocyte, 6. Eosinophilic granulocyte, 7. Basophilic metamyelocyte, 8. Metamyelocyte

4. Discussion

We have identified seven distinct myeloid cell types from the sternal bone marrow. Terminal cell maturation stages were also evident, beside myeloid precursor cells. During the hematopoiesis, it is possible to notice high level diversity of precursore cell types in the different stages of the maturation. For proper identification and the count of the observed cell types, morphological features, such as cell size, nucleus position and size as well as granulation profiles must be determined. Due to the several cell shapes (from round to irregular), cell surface parameters should be included, to ascertain the complete morphology of the leukopoietic cells.

As the MGG staining is capable to mark the main cell compartments and target identification features (abovementioned nuclei size, color and granulation profiles), in this study we were more oriented toward additional staining and comparative analysis. As confirmed by the different sources [12, 13], additional granular profiling and stain combination may yield better identification opportunities in myeloid cell types.

In comparison to human, guinea pig and rabbit counterparts, rat bone marrow myeloid precursors show similarities to human counterparts the most [14]. As guinea pigs and rabbits have heterophils, rats possess differentiated cells such as neutrophils (with three types respectively). Granules present in heterophils are brighter and more robust, while in eosinophils they are numerous and more round in shape (which concurs with rat eosinophils) [15, 16, 17, 18]. A very interesting fact is that rabbits possess an increased number in basophils (15-30%), in contrast to rats, guinea pigs and humans [19]. In a similar work [20] more details were focused on rat granulocytopoiesis. It was determined that the first identifiable cell of granulocyte line was myeloblast. This cell was identified by its fine chromatin structure and several nucleoli in the nucleus, as well as basophilic granules in its cytoplasm (aided by cells larger size as well). From promyelocyte over metamyelocyte line (basophil, acidophil and neutrophil subtypes) to mature cells, maturation process was linked to the granule type in cytoplasm, nucleus chromatin structure and texture as well as cell morphometric characteristics (nucleoli presence was mostly characteristic in the younger precursore cells).

Peroxidase (or in case of myeloid cells – myeloperoxidase) presence or activity in different cell types could be used as either identification marker or elimination tool. As it is present in cell lineages starting with promyelocyte to segmented neutrophil [21] it represents an edge towards better determination of several cell types without adding confusion. This enzyme is mostly located in rough endoplasmic reticulum (RER), Golgi cisternae and notably in primary granules (azurophilic) [22]. Stage and cell precursor type determine the presence of this enzyme. As we used the combined stain, bone marrow cells were more effectively identified, due to the additional confirmation of peroxidase activity and presence. Different peroxidase color precipitations were observed during the comparative study. Red and brown hues were noted in neutrophils and eosinophils (promyelocyte - myelocyte and metamyelocyte included) and finely grained texture was observed in monocytes while negative reactions were seen in early myeloblasts, basophils, lymphocytes and thrombocyte lineage (since the study was myeloid type oriented, other cell types were observed but excluded) [23]. This stain combined with MGG stain, helps us to identify late stages of certain cell maturation processes due to its association with the specific cytoplasmic granules.

Younger cell precursors (mostly blasts) were more easily identified after combining MGG and peroxidase staining, as it could be associated with the early peroxidase synthesis and primary granules residues during the maturation process.

The largest cells were first precursors such as myeloblasts and promyelocytes, alike in their human homologues. By measuring cell size, aforementioned cell types were also the biggest in the terms of size. Tendency to decrease in size and increase in cellular compacting is a maturing process trait. In our work it could be observed by combining peroxidase and MGG staininig. This leads to additional reassurance that targeted cells indeed were decreasing in the size as we could observe the subtle changes in the cytoplasm and nucleus.

Matured granulocytes account for a large portion of the total cell count in the bone marrow of rats. This is a result of intensified maturation process of myeloid precursors (neutrophil myelocytes were highest in the number) in rat bone marrow. Peripheral blood smear analysis accounts for

this statement as the most numerous cells are neutrophil granulocytes (segmented and non segmented).

5. Conclusions

This study presents the first data on the morphometrical differentiation of the stem cells and the identification of leukopoietic lineage cells in the sternum of rats. It is necessary to develop new techniques and methods for easier bone marrow isolation due to its structure and consistency. It is also necessary to combine other cytochemical methods that would allow the identification of the first cell precursor. The presence of all types of leukocyte precursors in bone marrow in rats, as in humans, is important for experimental research, especially in the field of the molecular biology, hematology and oncobiology, and for the treatment of the certain diseases in the different organisms, including humans.

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