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Original article

HAEMATOPOIETIC THROMBOCYTE PRECURSORS IN RAT FEMORAL AND STERNAL BONE MARROW

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Summary

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This research presents the first findings on thrombopoiesis for Wistar rats. Haemopoietic cells from the femur and the sternum were analysed by light microscopy in combination with infrared and near-ultraviolet light for fine cytoplasmic structure analysis. Five main types of thrombocyte precursor cells were identified in the bone marrow samples: megakaryoblast, promegakaryocyte and megakaryocyte (basophilic, acidophilic and thrombocytogenic). More intensive thrombopoiesis and morphologically differentiated cells were found in sternum samples.

Key words: bone marrow, megakaryocyte, platelets, thrombopoiesis, Wistar rat

INTRODUCTION

Bone marrow smears from rats usually contain a large number of megakaryocytes accounting for 0.40–0.77% of total nucleated cells in rat bone marrow (Saad *et al*., 2000). Density and distribution of megakaryocytes vary with technique and among smears; however, there are no published results regarding the precise number of megakaryocytes in rats. During late foetal and early postnatal life, the spleen is active in megakaryocytopoiesis. These cells are more abundant in the immature animal (neonates and juveniles), but were never found in the white pulp of spleen (Raval *et al*., 2014). This function is diminished as platelets production is shifted from the spleen to some other organ, like bone marrow (Bolliger, 2004).

Large megakaryocytes could be a product of an early thrombopoietic phase occurring in the first days of pregnancy as observed in the mammalian order of rodents (Rodentia) (Pacheco *et al*., 2002). The increase in the size of hepatic megakaryocytes might be a result of renal, hepatic and immunological development during foetal stages. This situation may also be the cause of a more increased and favoured production of humoral stimulation factors towards large cell oriented

megakaryocytopoiesis. In adult stages, proximity to sinusoids and microenvironment of the bone marrow itself have a greater role in megakaryocytopoiesis and cell differentiation in general (Pacheco *et al*., 2002).

As in other species, rat megakaryocytes are the largest haematopoietic cells. Mature megakaryocytes in rats are multinucleated, with nuclei often fused into a lobulated mass. Cytoplasm is abundant, light blue, and filled with fine eosinophilic granules (Bolliger, 2004). Younger megakaryocytes are smaller, with higher nuclear:cytoplasmic (N:C) ratios, more basophilic cytoplasm, and fewer nuclei. Maturation should be orderly, with a lower number of immature than mature forms. Megakaryocytes should not be confused with osteoclasts, which have separated nuclei and less abundant cytoplasm (Bolliger, 2004).

The ultrastructural observations showed that megakaryocytes in the bone marrow of rats are characterised by the absence of destruction of engulfed cells and phagosome formation in the megakaryocytes, which was defined as emperipolesis (Suljević *et al*., 2018). Morphologically, occasional megakaryocytic emperipolesis can be detected in the normal bone marrow. Emperipolesis was observed only in mature stage III megakaryocytes. The incidence of megakaryocyte emperipolesis was markedly increased in ageing rats that showed haematopoietic cell hyperplasia in the bone marrow (Bolliger, 2004). There is no evidence of cellular damage to the engulfed marrow cells or megakaryocytes. Possible explanation is that the megakaryocyte cytoplasm might provide a „secure place“ for normal granulocytes under an unfavourable bone marrow environment or that emperipolesis is attributed simply to a random movement and adherence of cells to the megakaryocytes in mice (Centurione *et al.*, 2004).

Megakaryocytes form platelets which play fundamental role in haemostasis and coagulation processes. Haematopoietic tissues, depending on the physiological anatomic characteristics, have a different rate of haematopoietic cell production. The aim of this study was to identify and to quantify haematopoietic precursors of megakaryocytes based on their morphology in the bone marrow of sternal and femoral bone in Wistar rats. Also, the rate of megakaryocytes production based on the number of less or more mature forms of megakaryocytic precursors was compared.

MATERIALS AND METHODS

This study was conducted at the Laboratory for Physiology, Faculty of Natural Sciences and Mathematics, University of Sarajevo, Bosnia and Herzegovina. All animals were well cared for according to the Animal Protection and Welfare Law of Bosnia and Herzegovina (“Službene Novine” 25/09).

Animals and breeding

Our main sample consisted of ten laboratory-bred adult Wistar rats (*Rattus norvegicus* s. Wistar, n=10), six males and four females. Sample animals were kept in a vivarium (individual medium plexiglass cages at 25 °C with 12/12 hours of light and dark cycle) and were fed pelleted food (Oxbow Essentials) with water *ad libitum*. At the time of biopsy, animals were approximately between 10 and 11 weeks old and at the same developmental stage. The weighing procedure was necessary for the anaesthetic dosage and euthanasia. The average body mass of rats

was 283.70±13.87 g. The largest number of individuals had body mass 260–280 g, and three individuals had an average weight of about 300 g.

Bone marrow extrapolation and staining

Selected animals were anaesthetised first by sufentanil/medetominide (subcutaneously – 50/150 μg/kg) and euthanised by doubling the dosage to comply with proper ethical procedures. Incisions by scalpel were made in the thoracic regions of the chest and hind legs to remove sternum and femurs. Tissue components were removed, and bones were thoroughly cleaned. Incision spots were marked before the cutting. To open the bone segments and avoid excessive damage to the bone marrow, surgical grade scalpels were used. Rectangular shaped cut (comprising of four precise cuts) was made on bone samples. Cut regions on femur were from most of the femoral body (shaft) area while sternum was cut from manubrium to xiphisternal joint. Access to bone marrow was achieved by lifting the rectangular cut-out of the bone. Bone marrow was removed physically by point gauge needle (21G/0.8×40 mm; Semikem) and placed on the microscopic slide. Using a small glass rod, the marrow sample was rolled over the slide in a slow paced and careful linear movement (touch technique). After the samples had dried at room temperature, they were stained by May-Grünwald-Giemsa stain (Semikem) and Leders stain (Semikem) for evaluation of peroxidase activity.

Table 1. Morphological characteristics of cells and nucleus of respective cell types

Shape		Staining property	
Cell	Nucleus	Cytoplasm	Nucleus
Megakaryoblast			
Oval with irregular outlines, the smallest cell in megacaryocytic cell line	Large nucleus with irregular structure, occupies 95% of cell	Basophilic	Basophilic
Promegakaryocyte			
Oval with irregular outlines, larger than nucleus in megakaryoblast	Consists of 4–5 nuclear segments	Basophilic	Basophilic
Basophilic megakaryocyte			
Irregular round shape, larger than promegakaryocyte	Several nuclear segments in group	Basophilic, light blue at the cell borders	Light blue
Acidophilic megakaryocyte			
Irregular elongated shape, larger than basophilic megakaryocyte	Large amount of separated nucleus segments, not grouped	Light blue around nucleus and light red in other parts	Acidophilic, non-homogenated
Thrombocytogenic megakaryocyte			
The largest cell in in megacaryocytic cell line, irregular „amoeboid“ shape	Fragmented and integral nuclei scattered in the cytoplasm	Bright acidophilic	Very bright acidophilic

Microscopic analysis

Main cell identification and analysis were performed on a light microscope (Olympus BX41) equipped with a digital camera (Olympus DP12). Image processing was done in a licensed software (Olympus DP12 Soft DP12-CB Ver.01.01.01.42.® Olympus Corp.). From each sternum and femur sample, 100 cells were included for identification and sequestial morphological analysis. Additional sample analysis was performed on a digital microscope (Bresser LCD Digital) with three light sources (white light, infrared at 850 nm and near ultraviolet at 365 nm).

Statistical analysis

Gathered data are represented as mean values with standard deviation (mean ± 1 SD) and coefficient of variation (CV in %). Students t-test was used to analyse the statistical distribution.

RESULTS

Five different types of thrombopoietic cells were identified in the bone marrow of Wistar rats: megakaryoblast, promegakaryocytes and megakaryocytes (basophilic, acidophilic and thrombocytogenic). Table 1 presents the most important morphological characteristics of thrombopoietic cells including cell shape as well as cytoplasmic and nucleus colours.

Fig. 1 presents five identified and differentiated types of thrombopoietic cells including osteoclast and emperipolesis. All the cells are arranged in the "order" of their maturation. Identification was performed by light microscopy.

Fig. 2 shows megakaryocytes analysed by special microscopy using various types of light and wavelength. This analysis is used to detect "fine" cytoplasmic structure. The analysis showed that there were no specific characteristics in the cytoplasm that would require additional cell identification methods. This suggests that light microscopy is sufficient for the identification of megakaryocytes, but special microscopy is an excellent tool for visualising cytoplasmic maturation and fragmentation.

Table 2 shows the percentage of haematopoietic cells in the bone marrow of Wistar rats. Identification was analysed in the sternum and femur. The most numerous cells in both tissues were thrombocytogenic megakaryocytes. There was a greater number of basophilic megakaryocytes in the femur, while the other cells were more represented in the sternum. There was a significant difference in the number of cells between the sternum and the femur.

DISCUSSION

Bone marrow analysis and collection is an integral part of a vast number of studies in the field of haematology and immunology, predominantly from the femur (Soleimani & Nadri, 2009). The haematopoietic system serves as a paradigm for understanding much of adult stem cell biology (Challen *et al*., 2009). Therefore, much effort is directed at developing the *ex vivo* expansion of functional megakaryocytes for exploratory and potential therapeutic purposes. Immunofluorescence analysis of megakaryocyte distribution shows that these cells, although rare (less than 1%), are present in the bone marrow in the niche of diaphysis, epiphysis and metaphysis of the femoral sections, while a slight increase is present within the diaphysis of the femur (Malara *et al*., 2014).

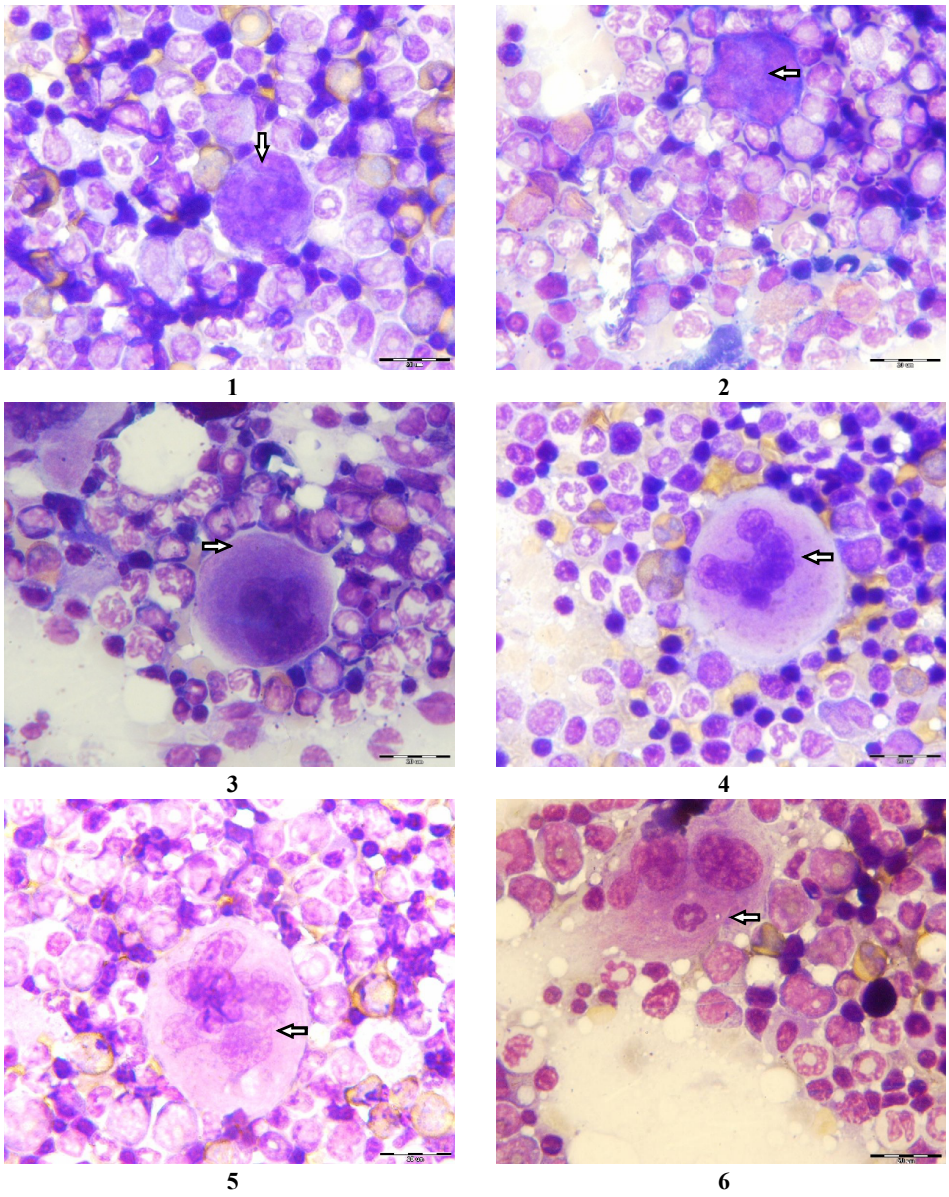
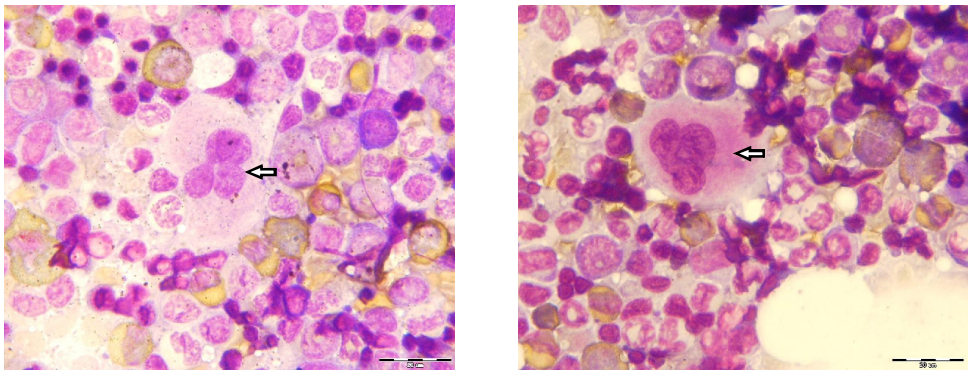


Fig. 1. Thrombopoietic lineage and osteoclast: 1. Megakaryoblast; 2. Promegakaryocyte; 3. Basofilic megakaryocyte; 4. Acidophilic megakaryocyte; 5. Thrombocytogenic megakaryocyte; 6. Emperipolesis with neutrophilic granulocyte;



7.1 7.2

Fig. 1 (cont'd). Thrombopoietic lineage and osteoclast. 7.1 and 7.2: Osteoclast.

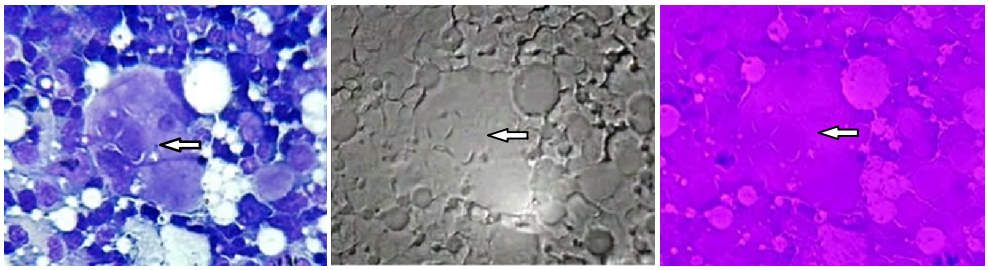


Fig. 2. Megakaryocyte under different light sources. Left: white light; middle: infrared (850 nm); right: near-ultraviolet (365 nm).

Table 2. Percentage of hematopoietic cells (mean ± SD) in sternum and femur of bone marrow.

Cells				
Megakaryoblast	Promega-karyocyte	Basophilic megakaryocyte	Acidophilic megakaryocyte	Thrombocytogenic megakaryocyte
Femur				
5.00±1.15 CV=23.09	18.10±1.91 CV=10.56	18.00±2.36 CV=13.09	11.00±1.89 CV=17.14	47.90±3.57 CV=7.46
Sternum				
10.08±1.75 CV=16.21	7.6*±1.51 CV=19.81	10.09*±1.91 CV=17.54	19.20*±3.05 CV=15.87	51.50*±2.64 CV=5.12

CV – coefficient of variation; * $P<0.05$ vs femur.

This study also encompassed megakaryocyte analysis within the diaphysis of femur and sternum cavity. The maturity of megakaryocyte and their size depend on their position in the vascular space. However, mature and larger megakaryocytes are always present near the sinusoids. This tissue localisation is considered to be appropriate for mature megakaryocytes, as they elongate their cytoplasmic processes

through endothelial fenestrae and release thrombocytes into circulation – vascular space (Junt *et al*., 2007). Besides the bone marrow biopsy of the femur, for thrombopoietic cells, sternum bone marrow was also analysed. The analysed cell types have shown the significant differences between femoral and sternal tissues. More megakaryoblasts were identified in the sternum, suggesting increased haematopoietic intensity. Therefore, more mature megakaryocytes were found such as the acidophilic and the thrombocytogenic types. According to the previous studies (Junt *et al*., 2007; Malara *et al.*, 2014), this could be due to the presence of a greater number of sinuses and the proximity of megakaryocytes to sinusoids in sternal bone marrow (Thon *et al*., 2010).

Active haematopoiesis in different bones of rat bone marrow could be the result of a small number of mature platelets, creating the largest megakaryocytes in the animal world. Five different haematopoietic cells appear to take part in the formation of finite platelets as our study shows. So far, there is no classification of these thrombopoietic cells in this rat strain, but the identified cells indicate the same similarities as in human thrombopoiesis (McCarthy, 2003). In rats, several studies suggest megakaryocytes presence but without specific differentiation (Soleimani & Nadri, 2009; Smailagić *et al.*, 2013). By comparing the sizes of these cells to human counterparts, we observed larger rat cell dimensions. This certainly could be the result of a different microenvironment of bone marrow tissue, the lesser number of sinusoids, the size of the animal and its life expectancy respectively (Kowata *et al*., 2014).

The size of the cell as well as the correlation between the cytoplasmic and nuclear ratio of hepatic megakaryocytes in white rabbit from New Zealand show certain differences as early as 22 days of intrauterine life. This supports morpho-functional mechanisms of hepatic megakaryocytosis expressed on the fifteenth day of intrauterine life (Pacheco *et al*., 2002).

Large liver megakaryocytes were observed during the twelfth day of foetal development in mice, with significant variations in size between twelfth and fifteenth days of pregnancy. During the postnatal development, hepatic megakaryocytes show in their size, volume and N/C ratio several similarities with adult animal bone marrow megakaryocytes (Pacheco *et al*., 2002).

Circulating levels of thrombopoietin (TPO) induce concentration-dependent proliferation and maturation of megakaryocyte progenitors by binding to the c-Mpl receptor (Deutsch & Tomer, 2006). Mpl activity is regulated by a complex cascade of signalling molecules that induce the action of specific transcription factors to drive megakaryocyte proliferation and maturation (Liu *et al*., 2011; Potts *et al*., 2015). In situations where decrease in circulating platelet numbers is evident, the resulting rise in TPO levels allows a higher proportion of progenitors to commit to and/or complete megakaryocyte development in bone marrow (Plutero & Kahr, 2016). Machlus *et al.* (2016) reported a potential positive feedback mechanism whereby the platelet-borne inflammatory cytokine chemokine ligand 5 (CCL5) can stimulate megakaryocytes to produce platelets.

Novel research demonstrated that IL-21-mediated enhanced megakaryopoiesis mainly occurs in the bone marrow via IL-

21R. IL-21 stimulatesf the proliferation of megakaryocyte progenitors via JAK3/ STAT3 pathway (Benbarche *et al*., 2017). T cells mainly produce IL-21, indicating the regulation of megakaryopoiesis through adaptive immunity**.**

It appears that IL-6 is a multifunctional interleukin and a strong promoter of megakaryocyte maturation process. This was observed (by our special microscopic methods) as increased size of megakaryocytes as well as the increase in thrombocyte number. Additionally, erythropoietin along with IL contributes to increased stimulation of processes in the cytoplasm of megakaryocytes, leading to the final formation of thrombocytes (Lane *et al.*, 2000; Thon & Italiano, 2010). In summary, taking above-mentioned studies into account, these large thrombopoietic cells along with cell, cytoplasm and nuclei size and ratio, are a consequence of early factor activation. These factors include erythropoietin, thrombopoietin and IL-6 during intrauterine development. This early activation of factors mentioned above results in very large rat megakaryocytes formation at a very early stage of rat hematopoiesis. This also shows that factors present in intrauterine haematopoiesis could be differently expressed in adult bone marrow cells, along with IL-6 .

Subcutaneous application of IL-6 (10 µg/day) causes cytoplasmic microtubule bundle formations which proportionally lead towards thrombocyte formation (Kaser *et al*., 2001). This suggests IL-6 as a megakaryocyte forming and thrombocytogenic megakaryocyte fragmenting factor which increases mature thrombocytes number. As for thrombopoietin, it appears to be responsible for complete megakaryocyte maturation through granule formation, membrane demarcation and cytoplasmic fragmentation into mature thrombocytes respectively (McCarthy, 2003). When we observe cell membrane shape in early development stages, we can recognise clear cell contours and the usual cell attributes. Different cell shapes and structure are a product of their maturation process which conditions drastic changes in size and N/C ratio. Dynamic pathway and sequence maturation of hepatic megakaryocytopoiesis in rabbits, explains their different cell shapes. This process of full maturation takes three days to complete (Pacheco *et al*., 2002).

Additionally, the nuclear membrane also possesses irregular structures. Intranuclear compartments observed in this study were usually with oval and reniform shape with irregular lobular structure. The shape of these components clearly deviates from the standard circular shape. Indeed, these irregular nuclear structures and contours are a unique trait of acidophilic and thrombocytogenic megakaryocytes (Centurione *et al*., 2004).

In conclusion, we identified five types of haematopoietic thrombocyte precursors in rat bone marrow. In contrast to femoral diaphysis, increased haematopoietic cell production and increased mature megakaryocytes number were observed in sternal bone marrow samples. Thrombocyte precursor maturation process encompasses an increase in overall cell dimensions, producing a very large terminal mature cell, so in our research, megakaryocytes represent the largest haematopoietic cells in Wistar rat haematopoiesis. A smaller number of thrombocyte precursors and the terminal large mature cell could be the result of several factors such as number and the close proximity of bone marrow sinusoids, and life longevity of rats as one of the critical factors for intensified haematopoiesis.

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