Kufa journal for Veterinary Medical Sciences Vol.(13) No.(2)

Kufa journal for Veterinary Medical Sciences

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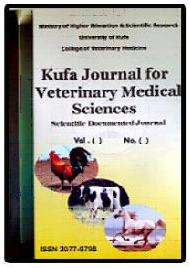


Study of Bone Marrow Cells During First Three Months Old Rats

Walaa Aladdin Mustafa Ali Saeed Alchalabi*

Department of physiology, Biochemistry & Pharmacology, College of Veterinary Medicine, University of Mosul, Mosul, Iraq

*Corresponding author: alchalabi@uomosul.edu.iq

Ali Alchalabi: https://orcid.org/0000-0003-0414-280X

Walaa Aladdin: https://orcid.org/0000-0001-6704-2481

Received date: 13 Jul. 2022 **Accepted:(** 486 **)** 8 **Sep.** 2022 **page: (** 23-13**) Published:**31 **Dec.** 2022

DOI: https://doi.org/10.36326/kjvs/2022/v13i23883

Abstract

Haematopoiesis is very complicated process of formation, development, and differentiation of bone marrow cells and its evaluation is very valuable diagnostic tool for the veterinary practitioner. So, the aim of study is to evaluate the healthy bone marrow cytology during first three months old. Ninety pups were involved throughout the study according to study design. Growing pups were kept with their moms till the weaning day, then divided into three main groups (1, 2, and 3 months old). After reaching the appropriate study age, bone marrow samples were obtained, and bone marrow smears were carried out and stained with MGGS. The results revealed presence of all types of haematopoietic cells and there are increase in different erythroid progenitor and myeloid progenitor with the age progress. Furthermore, the smears showed different cell line production with different differentiation stages of both erythroid and myeloid cells. In conclusion the bone marrow cells differentiation and proliferation are related to age progress independently. Haematopoietic cells differentiation and proliferation are regulated by bone marrow microenvironment. Myeloid cells are directly related to age progress.

Keywords: Bone marrow cytology, Erythroid progenitors, Haematopoiesis, Myeloid progenitor

Introduction

The process of formation, development and differentiation of bone marrow cells in the early stages of life is very complex. An investigation and evaluation of bone marrow (BM) cytology is an important way to evaluate hematopoietic system and is valuable in the diagnosis, prognosis, and monitoring of a variety of conditions and diseases [1].

The BM cytology undergoes different stages of proliferation and development [2], these stages result in formation and differentiation of multiple cell lines [3]. There are variant cell lines promotes from single stem cells known as pluripotent haematopoietic stem cell (PHSC), which is the mother cell of haematopoietic cells [4]. In rats, the erythroid cell line is characterized by first recognizable nucleated progenitor cell called rubriblasts or erythroblasts or normoblast, which earliest of four stages in development of the erythrocytes or rubricytes. In general, the majority of pharmaceutical/toxicological research studies use bone marrow assessment as the best way for detecting hemotoxicity to determine how the hematological system is affected by various new substances or compounds [3]. When the primary and secondary haematologic dysfunction or abnormalities cannot be interpreted by peripheral blood smear investigation, the bone marrow assessment is useful and advised [5]. Moreover, the BM appraisal is recommended for studying certain malignancies or investigating abnormalities in the haematopoietic system [6].

The significance of studying the bone marrow cytology is to figure out the basic

cytology of rat's bone marrow and its relation to age which play a crucial role in understanding the pathogenesis of haematopoietic disorders such as leukemia. So, the aim of the study is to study the bone marrow cytology during first three months old via evaluating different haematopoietic cells using special stain.

Materials and methods

Ethical approval

The study was performed at college of Veterinary Medicine of University of Mosul, laboratory animal house and research center under ethical committee approval letter with reference number UM.VET.2021.46.

Animals

To produce the pups used in the study, fifteen healthy male and female rats were freely mated. First-time-pregnant rats were housed in terms of humidity, temperature, and light. According to the study design, a total ninety pups were involved throughout the study. The cages were labeled according the study design to one month, two months, and three months old (each large cage contain thirty weaning pups until the intended age is reached. tap water and standard rat pellets were provided ad libitum throughout the study. From weaned pups at day 28, one-month-old pups were selected, left for a further two days, and then had bone marrow samples taken [7]. Second group of thirty weaned pups left housed until reach two months old and their bone marrow was collected, same protocol of housing and sampling follow with third group of weaned pups until reached three months old after isolates the female from male rats to avoid pregnancy occur within studied rats.

Bone marrow collection

According to the specified study durations, when the age is completed, one month, two months, and three months, the studied rats were euthanized by physical method of euthanasia via cervical dislocation of unanesthetized animals **[**8]. Briefly, euthanized animal was back-side extending the hind limbs, shaved, aseptically prepared for skin incision on the lateral aspect of the thigh and both tibia and femur were exteriorized after removing the muscular and tendinous attachment. Femur and tibia separately of both the legs with a scissor the metaphyseal region of both bones was cut and inserting a needle into the medullary cavity, the bone marrow was flushed out using by 5 ml of PBS containing 3% EDTA in petri dish, transferred to centrifuge tube, then mixed by vortex, filtered by mesh and flushed out bone marrow was centrifuged in order to concentrate the cells for 10000 rpm/5 minutes. The supernatant was decanted and the sedimented cells were mixed well by pipetting and used for bone marrow smear.

Bone marrow smear

Briefly, after centrifuging the sample as mentioned above, a 200 µl of BM cell suspension was transferred to a clean microscope slide onto the center of the slide. By directly covering the first slide with a second one, we can let the bone marrow spread. Smoothly drag the tip slide off the bottom slide, lengthwise, without effort on the slide. A central, oval-shaped monolayer of BM cells is formed and typically is rich in different cells [9].

Staining protocol and cell counting

A modified staining protocol was followed in this study [2]. There is a protocol for staining and cell counting process [2]. 1:10 dilution of Giemsa stains with phosphate buffer pH 6.8 was used. While, the microscopes using Amscope camera (Amscope camera 18MP, USB 3.0 real time live video microscope digital camera, China) and Amscope Digital Camera Solution Disk software for analyzing the images. The counting of different BM cells and cytology evaluation was done by scanning the whole slides' fields by Amscope camera using its software and counting 200 different cells/ slide and then getting the percentages of each cell type per 3 slides.

Statistical analysis

Data analysis was conducted using IBM SPSS Statistics 22 (SPSS In. Chicago, IL., USA). Data for cytological evaluation and for were expressed as mean ± standard error (S.E) of cell type/ duration. The data was analysed by one-way analysis of variance (ANOVA) and. The differences between studied groups were performed with a probability value less than 0.05 determined as

statistically significant against the 1st month old rats.

Results

Cytologic evaluation of bone marrow during the 1st three months old

Evaluation and morphology of normal rat bone marrow

Bone marrow smear showed different shapes of PHSc that characterized by round, non-adherent, with a rounded nucleus and low cytoplasm-to-nucleus ratio, that give rise to other blood cells. Furthermore, the examination showed presence of haematopoietic cells which appear as a very beautiful picture that contains various blood cells of all kinds and forms, mature and immature, consisting of red and white blood cells precursors and megakaryocytes that form platelets, showing the stages of their development, growth and differentiation, as in the figures 1, 2, 3, and 4.

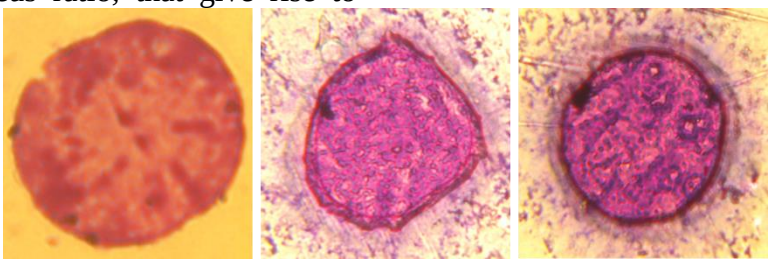


Figure 1. Pluripotent Stem cell of rats' bone marrow smear. Higher magnification reveals three different shapes of pluripotent stem cell. MGG stain. 100× objective magnification.

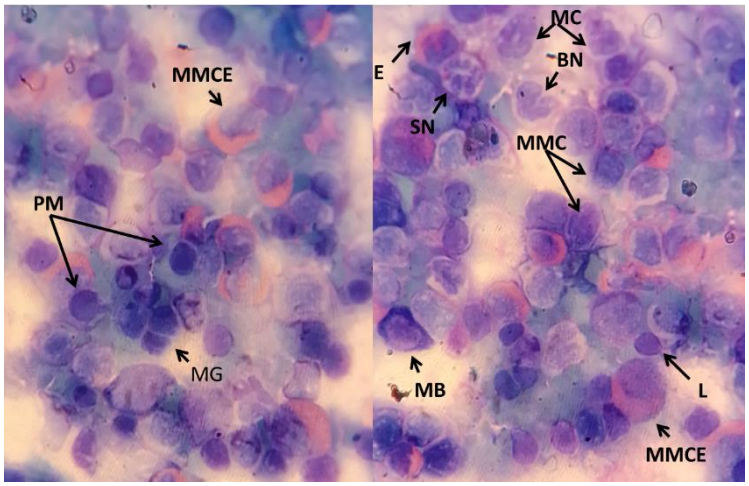


Figure 2. Different cells of rats' bone marrow smear. Higher magnification reveals myeloid precursors consisting of myeloblasts (MB), promyelocytes (PM), myelocytes (MC), metamyelocytes (MMC), metamyelocytes eosinophile (MMCE) band neutrophils (BN), and segmented neutrophils (SN), eosinophile (E) as well as megakaryocyte (MG). MGG stain.

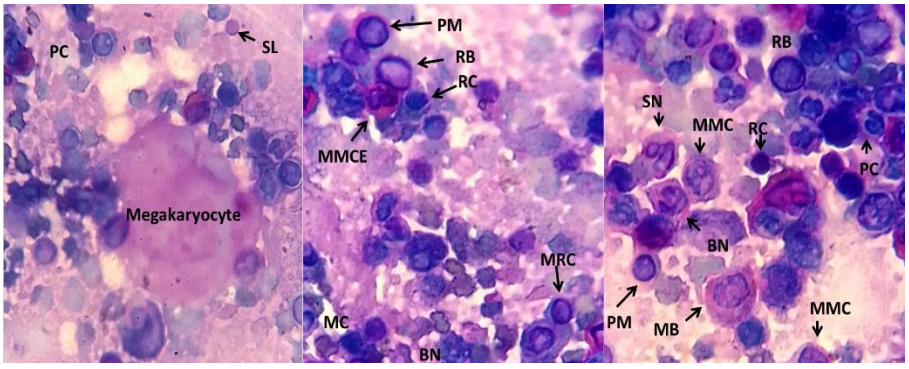


Figure 3. Bone Marrow Smear. Higher magnification reveals myeloid precursors consisting of myeloblasts (MB), promyelocytes (PM), myelocytes (MC), metamyelocytes (MMC), metamyelocytes eosinophile (MMCE) band neutrophils (BN), and segmented neutrophils (SN) as well as erythroid precursors consisting of rubriblasts (RB), prorubricytes (PR), rubricytes (RC), and metarubricytes (MRC) are identified. Polychromatophilic cells (PC). The myeloid to erythroid ratio is approximately 0.8. MGG stain.

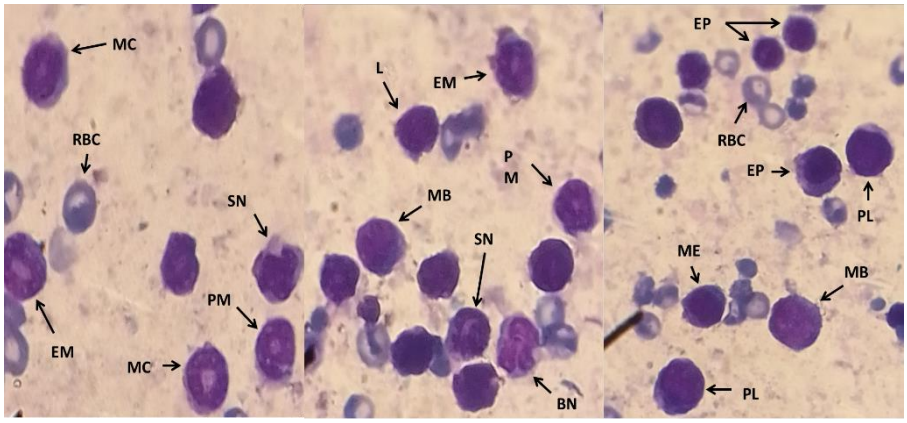


Figure 4. Granulocytic precursors and ring forms in bone marrow from a healthy rat. Higher magnification reveals myeloid precursors consisting of myeloblasts (MB), promyelocytes (PM), early myelocyte (EM), myelocytes (MC), band neutrophils (BN), and segmented neutrophils (SN) as well as erythroid precursors (EP), metarubricyte (ME), and prolymphocyte (PL) and lymphocyte (L) are identified. Polychromatophilic cells (PC). MGG stain.

Interestingly, the examination and evaluation of bone marrow smear revealed the three stages of megakaryocyte development and differentiation as well as platelets production. The megakaryocyte characterized by huge, multinucleated, granulated cytoplasm and irregular morphology cell. The megakaryoblast characterized by single, large, round nucleus; fine chromatin; pale nucleoli, scant deeply basophilic cytoplasm, few vacuoles, cytoplasmic blebs often present on the periphery of the cell. Promegakaryocyte appeared larger than the megakaryoblast, two to four distinct nuclei, more condensed chromatin, scant to moderate amounts of deeply basophilic cytoplasm, few vacuoles and cytoplasmic blebs. While the mature megakaryocyte appeared as large multilobulated nucleus with dense chromatin, scant to moderate basophilic cytoplasm, few vacuoles and cytoplasmic blebs with eosinophilic finely granular cytoplasm larger than promegakaryocyte. The platelet appeared as irregular eosinophilic fragments of cytoplasm, figure 5.

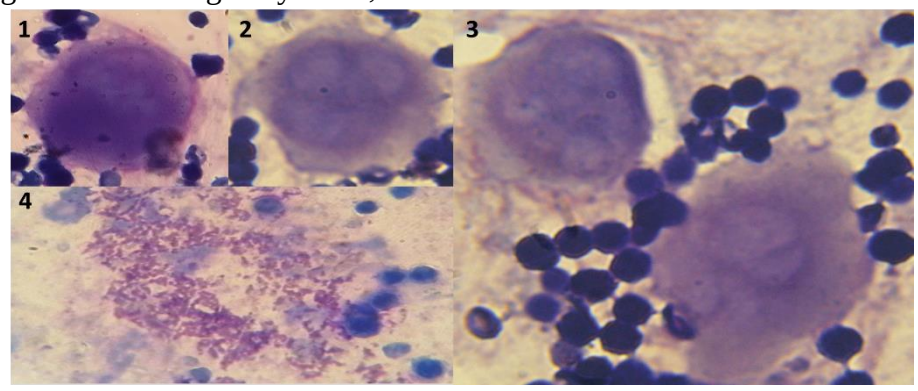


Figure 5. megakaryocyte precursors in bone marrow smear. Higher magnification reveals megakaryocyte precursors consisting of different cell types, (1) megakaryoblast, (2) promegakaryocyte, (3) megakaryocyte, and (4) platelets. MGG stain.

Figure 6, revealed a distinct form and shape of monoblast than those often seen, which display an abundance of dark to light blue cytoplasm with few to no fine azurophilic granules. The nucleoli are round, oval, or folded, and the nuclear chromatin is fine. There is often a single large nucleolus. Moreover, rats' bone smear revealed different lymphocytes with large nucleus, figure 7.

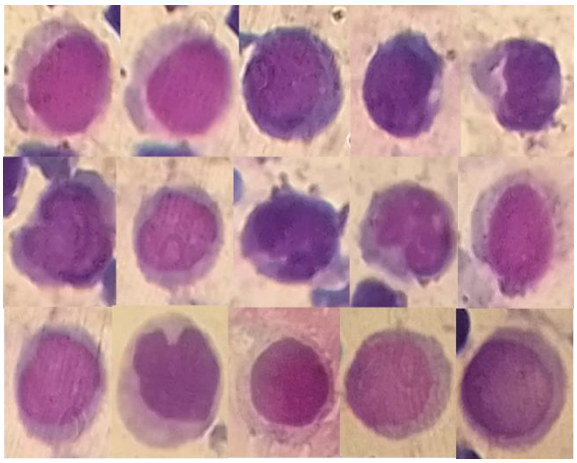


Figure 6. Monocyte precursors in bone marrow smear. Higher magnification reveals monocyte precursors consisting of different cell shapes of monoblast, high N/C ratio with lightly basophilic cytoplasm. MGG stain.

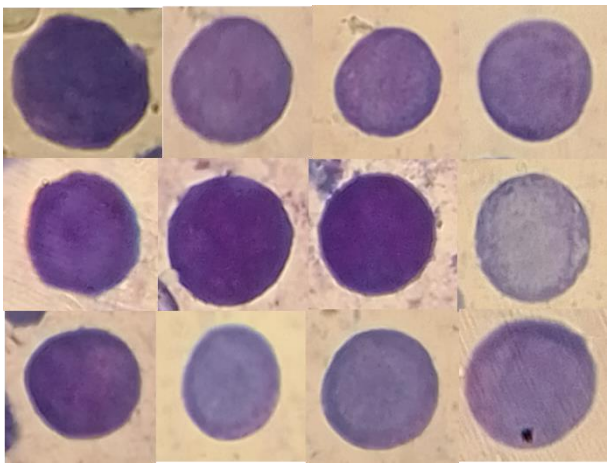
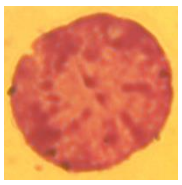
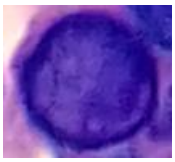
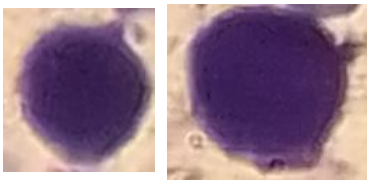


Figure 7. Lymphocytes in bone marrow smear. Higher magnification reveals different lymphocytes. MGG stain.

The haematopoietic cell study revealed different type of cells according to their morphological and staining feature as described in table 1 for erythroid cell line production. In this table the erythroid precursors revealed a series of proliferating cells which were consisting of rubriblasts, prorubricytes, and rubricytes and non-proliferating cells which consist of metarubricytes, and polychromatophilic erythrocytes, each cell type has own characteristic feature as listed in table 1.

Additionally, the bone marrow smear revealed the formation of cells from the granulocytic line, another cell line. Similar to how erythroid cell lines are produced, granulocyte cell lines also show mature and immature cells. Proliferating cells, such as myeloblasts and promyelocytes, are present in lower amounts than nonproliferating cells, such as metamyelocytes, nonsegmented granulocytes, and segmented granulocytes, which are typically found in rat bone marrow smears as in table 2.

Table 1: Erythroid cell line production in rat bone marrow

Cell type	Cell image	Cytological description
PHSC		Round, non-adherent, with a rounded nucleus and low cytoplasm-to-nucleus ratio, that give rise to other blood cells.
Rubriblast		An increased N/C ratio, round central nucleus, delicate chromatin, medium-sized pale nucleoli, narrow rim of intensely basophilic cytoplasm.
Prorubricyte		Smaller than the rubriblast, higher N/C ratio, round central nucleus, more open chromatin, nucleoli usually not visible, intensely basophilic cytoplasm
Rubricyte		Larger than prorubricytes but varies from small to intermediate; round central nucleus; coarsely clumped chromatin; basophilic cytoplasm. There is another type which smaller than prorubricyte.

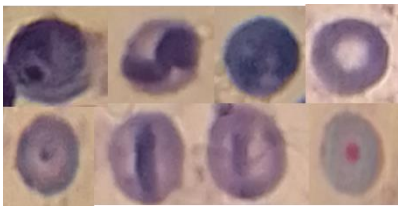
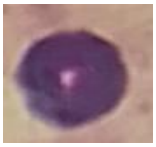
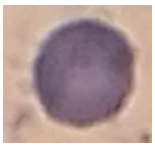
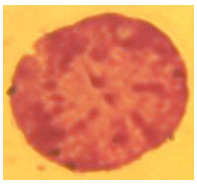
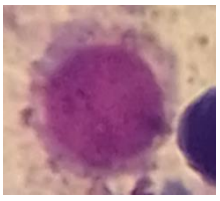
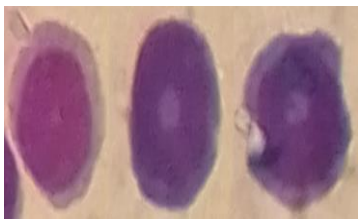
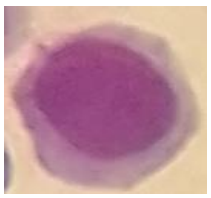
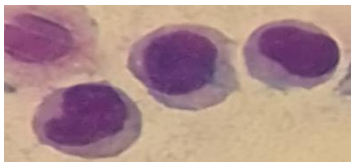
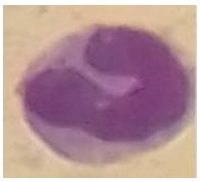
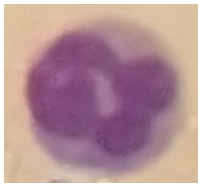
Metarubricyte (nucleated RBC)		larger size to polychromatophilic RBC; small, dark, homogenous, pyknotic nucleus; polychromatophilic or orthochromic cytoplasm
Polychromatophilic erythrocyte (reticulocyte)		Slightly larger than mature RBCs; nonnucleated, muddy blue (grayish-blue) cytoplasm (polychromatophilic).
Mature erythrocyte		Nonnucleated cell, cytoplasm stains orthochromic (red-orange)

Table 2. Myeloid cell line production in rat bone marrow

Cell type	**Cell image**	**Cytologic description**
PHSC		Round, non-adherent, with a rounded nucleus and low cytoplasm-to-nucleus ratio, that give rise to other blood cells.
Myeloblast		Same size or slightly smaller than promyelocyte, highest N/C ratio, round to oval nucleus, finely stippled chromatin pattern, one to several pale nucleoli, moderately basophilic cytoplasm with no or few primary granules
Promyelocyte		Larger or similar in size to myelocytes, higher N/C ratio, large round nucleus, lacy to coarse more open chromatin, too few indistinct nucleoli or nucleolar rings, light blue cytoplasm, no specific granules, scattered small reddish-purple (azurophilic) primary granules
Myelocyte		Larger or similar in size to metamyelocytes, higher N/C ratio, round to oval eccentric nucleus, more open chromatin, no nucleoli, cytoplasm and specific granules similar to more mature cells

Metamyelocyte		Larger or smaller than band cells, higher N/C ratio, bean-shaped nucleus, more open chromatin, no nucleoli, cytoplasm and specific granules similar to more mature stages
Band cell		Slightly larger than mature cells, curved nucleus with smooth parallel sides and no constrictions, chromatin less clumped, cytoplasm and specific granules similar to mature cells
Segmented granulocytes		Segmented nucleus, coarsely clumped chromatin, clear to lightly basophilic cytoplasm, specific granules present

Average number of differential cell count of healthy rat bone marrow during the 1st three months old

In the bone marrow smears of the rats collected over the study period, the differential count of hematopoietic cells revealed a small change in the average amount of these cell types. And relating those averages to age, we found that these statistics varied little with age in comparison to the age of one month as a reference. Based on cell morphological characteristics recorded on the basis of three slides/animals/ages evaluated, the total number of examined cells and their average numbers were determined. There were no differences in the average number of both rubriblasts and prorubricytes during the first three months of life, indicating that age has no effect on erythrocyte precursors. When compared to one month old, the averages for both rubricyte and metarubricyte revealed significant changes with time (P 0.05). A significant increase in the average number of reticulocytes was also seen in the bone marrow smear of three-month-old rats compared to one-month-old and two-month-old rats at P 0.05. Leukocyte progenitor demonstrated a significant increase in the average numbers of all myelocyte precursors as well as differentiated myelocytes from myeloblast to band cells. This increase was clearly seen in rats' smears at two and three months old compared to one month old as a reference for the effect of age progress on the average myeloid cell count, and it was noted at P 0.05. Additionally, in rat smears from two and three months old, the average number of monocyte precursors and monocytes showed a considerable drop. Comparing one-month-old rats to those that were two or three months old, lymphoid cells revealed a non-significant drop in their average number at P 0.05. table 3.

Table 3. The average count of hematopoietic cells in the healthy rats' bone marrow smears

Hematopoietic cells	**One month old**	**Two months old**	**Three months old**	***P* value**
Rubriblast	14.16 ± 0.097	13.83 ± 0.136	13.86 ± 0.137	0.119
Prorubricyte	13.74 ± 0.110	13.54 ± 0.130	13.49 ± 0.107	0.270
Rubricyte	20.09 ± 0.145	20.29 ± 0.198	**21.78 ± 0.145*a**	0.000
Metarubricyte	20.56 ± 0.155	**21.48 ± 0.183***	**20.31 ± 0.164 a**	0.000
Reticulocyte	20.09 ± 0.176	**20.74 ± 0.130***	**21.69 ± 0.154*a**	0.000
Myeloblast	14.83 ± 0.158	**17.21 ± 0.203***	**18.35 ± 0.191*a**	0.000
Promyelocyte	19.80 ± 0.143	**20.49 ± 0.140***	**20.72 ± 0.100***	0.000
Myelocyte	19.54 ± 0.129	**20.19 ± 0.110***	**20.80 ± 0.129*a**	0.000
Metamyelocyte	19.64 ± 0.136	**21.34 ± 0.186***	**21.64 ± 0.156*a**	0.005
Band cell	20.67 ± 0.102	**21.02 ± 0.125***	**21.87 ± 0.134*a**	0.000
Segmented granulocytes	20.49 ± 0.123	20.54 ± 0.132	**21.00 ± 0.124*a**	0.007
Monocytes	10.60 ± 0.104	**10.00 ± 0.094***	**9.93 ± 0.081*a**	0.000
Lymphoid cells	13.76 ± 0.118	13.66 ± 0.135	13.61 ± 0.140	0.690

Discussion

In order to sustain healthy hematopoiesis and a quiescent state with the required rate of self-renewal, the bone marrow stem and self-renewal cells' activity is mostly tied to their bone marrow microenvironment. The primary goal of the current study was to assess how haematopoiesis changed with age.

The results were exhibited no variations noticed in number of both rubriblasts and prorubricyte during the study period, while other erythroid progenitor cells were showed marked increase in their number with age. At the same time, an increase in leukocytes progenitor was also observed with age, including all types of leukocytes except for lymphocytes and their progenitor cells during the study period which showed no alteration in their number with age. In general, the multipotent stem cells are group of heterogeneous haematopoietic stem cells which have the capacity to self-renew and discern into the functional blood cell types of the body [10]. Parmar et al., 2007, the BM, where a small number of quiescent stem cells give rise to a large number of committed progenitors, is where established mammalian haematopoiesis originates. Our results support these claims [11] who explain how the committed cells are refill all of the blood cell lineages throughout the lifetime of the animal. Additionally, because hematopoietic stem cells (HSCs) have the unique capacity to support themselves through self-renewal, their proliferative potential is also notable [12].

The body's need for these multilineage haematopoietic cells and their function in bodily defense mechanisms, particularly the immune system, may be the cause of the dramatic increase in myeloid cells with age. The low count of stem cells and progenitors such as rubriblasts, prorubricytes, myeloblasts, promyelocytes, and megakaryoblasts was recorded in current study

findings with their range ~ 15 cell/ animal's bone marrow sample could be due to the fact that these types of cells are the basic component forming different cell lineages, and also due to their capacity to self-renewal and restriction of multipotency in distinct degree to differentiate into various cell types. This opinion is consistent with what the other researchers pointed out [13], who indicated that stem cells and precursors are present in minor population due to their ability to self-renewal and limited multipotency to proliferating and forming other cell lines. The importance of these cells lies in their high ability to form two important lineages of vital cells in the cellular formation of the bone marrow. The importance of these cells lies in their high ability to form two important lineages of vital cells in the cellular formation of the bone marrow, such as hematopoietic, and mesenchymal due to its pluripotency characterization which under certain circumstances these cells could undergo the trans-differentiation and generate nonrelevant cell types [14]. Furthermore, because of the progenitor cells have the ability to migrate and diffuse into other tissues [4] this plays a key role in their distribution within the bone marrow tissue.

According to study findings, the age plays another role in maintain self-renewal and differentiation potential over time via its effect on many of the intrinsic aspects of HSCs by affecting on their potential, and independently of their microenvironment [15]. The results of this study are in agreement with the findings of [16], who revealed that the human bone marrow hematopoietic stem cells are increased in frequency and myeloid-biased with age via an increased differentiation potential to the myeloid lineage and a decrease in the potential to differentiate into the lymphoid lineage. Interestingly, this dramatic elevation in myeloid progenitors could be due to HSCs differentiation potential which is not homogenous especially myeloid-restricted repopulating progenitors which considered as young HSCs. [17], indicates that the myeloid-restricted repopulating progenitors subpopulation increases dramatically with age with the limited increase in the number of multipotent HSCs [16]. Moreover, the study results are in agreement with the findings of **[**17], who found that as mice age, their HSCs exhibit a marked decrease in lymphopoiesis and increase in myelopoiesis. In general, increase in myelopoiesis, and decrease in lymphopoiesis during aging could be affected by different cellular pathways within the microenvironment as an interaction between these cells and their aging niche, for instance, in young and aged mice there is evidence that the HSC populations respond differently to cytokines such as IL-7 and TGF-β [18]. It is likely that the cause of potential variation within HSCs population during aging is for two main reasons, the first is might be due to changing of all myeloid progenitors from myeloid-lymphoid potential to myeloid-partisan only with age progress and the second reason is most myeloid-biased progenitors outperforms the equilibrium of cells during aging. This opinion is quit supported by [19, 20], few myeloid- partisan cells can be presents with balanced-potential HSC in young two-months old mice suggesting that the young mouse HSC pool may contain lineage-biased clones that compete for niches signals. This is why the majority of myeloid progenitors are myeloid with age progress rather than balanced in lymphopoiesis and myelopoiesis. Bone marrow cell activity, differentiation and development operate within a very complex internal microenvironment regulated by many internal factors. Among these factors is what is related to the natural oxidative stress resulting from the formation and production of free radicals as a product related to the levels of hypoxia within this microenvironment [20, 21]. One of the obvious and well-known matters in the case of oxidative stress is a balance between free radicals and antioxidants, both enzymatic and non-enzymatic, to reduce the negative effect of these radicals and prevent or reduce their attack on proteins, fats and nucleic acids in cells causing unwanted effects at the cellular level [22].

Conclusion

The results of the current study show that differentiation and proliferation of bone marrow cells are connected to aging

independently. The bone marrow microenvironment controls the development and proliferation of hematopoietic cells. Age progression is directly correlated with myeloid cells. The morphological analysis revealed various forms of identical myeloid progenitor cells at the same age and stage of differentiation and proliferation.

Acknowledgment

I would like to express my gratitude to the College of Veterinary Medicine, University of Mosul for giving me this opportunity to get degree of MSc Physiology. Also, I would like to thanks everybody support me during this work.

Conflict of interest

The authors have no conflict of interest.

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